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Arabidopsis thaliana ACYL-CoA-BINDING PROTEIN: STRUCTURE, FUNCTIONS, GENETICS

presented by

Raymond S. Pacovsky

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John B. Ohlrogge
Major professor

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Arabidopsis thaliana ACYL-CoA-BINDING PROTEIN: STRUCTURE, FUNCTIONS, GENETICS

By

Raymond S. Pacovsky

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AN ABSTRACT OF A DISSERTATION

Arabidopsis thaliana ACYL-CoA-BINDING PROTEIN: STRUCTURE, FUNCTIONS, GENETICS

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A cDNA clone from *Arabidopsis thaliana* encoding a protein with 92 amino acid residues waS identified as the gene that encoded an acyl-CoA-binding protein (ACBP). Coding sequence of this clone was expressed in *E. coli*, and the gene product (10.4 kDa) was purified. Recombinant *A. thaliana* ACBP (rAthACBP) bound acyl-CoAs selectively and protected these esters from microsomal acyl-hydrolases. ACBP was shown by Western blotting to be expressed in all tissues of *Arabidopsis* and *Brassica napus*, and ACBP was markedly up regulated during storage lipid biosynthesis. Therefore, we conclude that ACBP is a housekeeping protein in all cells involved in acyl-CoA utilization.

The thermodynamics of binding for a number of different acyl-CoA esters to rAthACBP was analyzed using titration microcalorimetry and van't Hoff analysis. Binding affinity between acyl-CoA and rAthACBP increased as acyl-chain increased. The overriding contribution to free

energy was enthalpy, rather than entropy, at 27° C. Unsaturated or mono-hydroxylated C_{18}-CoAs bound to rAthACBP with similar binding constants. van't Hoff analysis indicated that the optimal temperature of binding was 15° C, and this set point was determined more by temperature-dependent entropy than enthalpy. A marked entropy-enthalpy compensation existed so that over a moderate range of temperature or cellular salt concentrations the binding of a particular acyl-CoA to rAthACBP would change only slightly. Although binding was driven by enthalpy, release of ligand was entropy dependent. To our knowledge, this is the first report to suggest ligand release is entropy driven.

Southern analysis of *A. thaliana* and other species indicated that the gene encoding ACBP (designated *acb*) is part of a small multi-gene family. Northern hybridization to *A. thaliana* RNA suggested that all tissues express low to moderate *acb* levels. In developing oilseeds and germinating seedlings, *acb* was strongly expressed indicating an involvement not only in triacylglycerol biosynthesis, but also fatty acid utilization for ß-oxidation or the glyoxylate/gluconeogenesis cycles. The *acb* gene appears transcriptionally regulated and may be a target for alteration of gene expression in oilseeds to alter oil composition or in germinating seedlings, to alter carbon utilization patterns.

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CHAPTER 1

ACYL-CoA-BINDING PROTEINS (ACBP); STRUCTURE AND FUNCTION

One function common to all living organisms is the biogenesis of a membrane that serves to separate self from environment. Biological processes such as cell cycle, cellular differentiation, and embryonal development often involve the biosynthesis of new cellular membranes, which is controlled by complex molecular mechanisms. One of the molecules involved in this rich drama is the acyl-CoA-binding protein (ACBP). Here is it's story.

A. Acyl-CoA-binding protein: Biochemistry

1. Sources of ACBP

ACBP has been found in all cells of higher eukaryotes, usually as a "housekeeping" protein, but in some tissues, it has also been recruited for specialized functions (Alho et al., 1988). Free fatty acids (FAs) or free acyl-CoA esters can be toxic to cells as a result of their ability to act as detergents that disrupt protein folding or membrane integrity. Earlier work in animal systems was initiated to find proteins that bind to acyl-CoAs or FAs, thereby protecting the cell against damage that would otherwise result when FAs are mobilized for cellular processes. It was initially believed that the fatty acid-binding protein (FABP) and acyl-CoA-binding protein (ACBP) were identical (Haunerland et al., 1984) because a partially purified protein extract from cow liver was capable of binding both FAs and acyl-CoAs.

However, Knudsen and colleagues demonstrated and confirmed the

presence of ACBP in a preparation of bovine liver FABP because of the ability of ACBP to cause termination of fatty acid synthetase (FAS) at medium-chain lengths (C_8-C_{12}) *in vitro* (Hansen et al., 1984). This premature termination at less than C_{16} or C_{18} required the presence of an acyl-CoA-utilizing system, in the form of either a binding protein (for example, bovine serum albumin or ACBP), an alpha-cyclodextran, or a microsomal TAG-synthesizing system (Knudsen and Grunnet, 1982).

Using a number of approaches, some of which utilize other properties of ACBP that will be discussed later, ACBP or the cDNA encoding this protein have now been isolated from a number of animal species, such as human (Marquardt et al., 1986), rat (Mocchetti et al., 1986; Knudsen et al., 1989), mouse (Owens et al., 1989), bovine (Mikkelsen et al., 1987), pig (Chen et al., 1988), ox (Webb et al., 1987), duck (Todaro et al., 1991), insects (Synder and Feyereisen, 1993; Kolmer et al., 1994) and yeast (Rose et al., 1992; Knudsen et al., 1993). An alignment of the polypeptide sequences or the derived amino acid sequence from the various animal cDNAs demonstrates a high degree of homology within these ACBPs (Figure 1).

In plants, a cooperative pathway exists between the endoplasmic reticulum and the plastid for the biosynthesis of galactolipids containing polyunsaturated FAs (PUFA). Export of acyl chains from the chloroplast (mainly in the form of oleoyl-CoA) towards the endoplasmic reticulum results in a return of diacylglycerol moieties, perhaps in the form of phosphatidylchloine (PC) containing two di- or triunsaturated acyl chains which will be used for the biogenesis of thylakoid galacto-glycerol lipids (Arondel and Kader, 1990). Transfer of oleoyl-CoA from plastid to ER is likely regulated by ACBP, whereas movement of glycero-

Figure 1. Animal ACBPs aligned to show a comparison of amino acid residues. Residues that are in contact with the adenosine-3'-phosphate or the pantotheine group have a "b" above that column. Residues that are invariant in all ACBPs have a "+" sign above that column. Residues that are conserved in all animal ACBPs have a "^" above that column. KQAT box (acyl-tail binding) and DAWN box (CoA binding) are underlined.

```
                        b       b                       b
          ^   +   ^ ^ ^ ^   ^     ^ ^     ^   ^   ^ ^ +     +   ^
Man   S Q A E F E K A A E E V R H L K T K P S D E E M L F I Y G H   30
Pig   S Q A E F E K A A E E V K N L K T K P A D D E M L F I Y S H   30
Ox    S Q A E F D K A A E E V K H L K T K P A D E E M L F I Y S H   30
Dog   S Q A E F D K A A E D V K H L K T K P A D D E M L Y I Y S H   30
Rat   S Q A D F D K A A E E V K R L K T Q P T D E E M L F I Y S H   30
Mouse S Q A E F D K A A E E V K R L K T Q P T D E E M L F I Y S H   30
Tort. S Q A E F D K A A E E V K Q L K S Q P T D E E M L Y I Y S H   30
Duck  A E A A F Q K A A E E V K Q L K S Q P S D Q E M L D V Y S H   30
Chick S E A A F Q K A A E E V K E L K S Q P T D Q E M L D V Y S H   30

        b                                               b
  + + + ^ ^ + ^   ^ ^   ^ + +   ^ ^     ^ ^ ^ + ^ + +
Man   Y K Q A T V G D I N T E R P G M L D F T G K A K W D A W N E   60
Pig   Y K Q A T V G D I N T E R P G I L D L K G K A K W D A W N G   60
Ox    Y K Q A T V G D I N T E R P G M L D F K G K A K W D A W N E   60
Dog   Y K Q A T V G D I N T E R P G L L D L R G K A K W D A W N Q   60
Rat   F K Q A T V G D V N T D R P G L L D L K G K A K W D S W N K   60
Mouse F K Q A T V G D V N T D R P G L L D L K G K A K W D S W N K   60
Tort. F K Q A T V G D I N T E R P G F L D F K G K A K W D A W D A   60
Duck  Y K Q A T V G D V N T D R P G F L D F K G K A K W D A W N A   60
Chick Y K Q A T V G D V N T D R P G M L D F K G K A K W D A W N A   60

                        b
  ^ ^ ^     ^ ^   + ^ ^   +     ^ ^     ^ ^   ^ ^ ^ ^
Man   L K G T S K E D A M K A Y I N K V E E L K K K Y G I         86
Pig   L K G T S K E D A M K A Y I N K V E E L K K K Y G I         86
Ox    L K G T S K E D A M K A Y I D K V E E L K K K Y G I         86
Dog   L K G T S K E D A M K A Y V N K V E D L K K K Y G I         86
Rat   L K G T S K E N A M K T Y V E K V E E L K K K Y G I         86
Mouse L K G T S K E N A M K T Y V E K V D E L K K K Y G I         86
Tort. L K G M A K E E A M K A Y I A K V E E L K G K Y G I         86
Duck  L K G M S K E D A M K A Y V A K V E E L K G K Y G I         86
Chick L K G M S K E D A M K A Y V A K V E E L K G K Y G I         86
```

* *Abbreviations:* Tort., tortoise

lipids to the plastid may be due to a presumed class I lipid transfer protein (LTP). Intracellular phospholipid transport (class I activity) must be distinguished from the extracellular transport facilitated by other LTPs that have been isolated and whose cDNA was cloned from plants (Bernhard et al., 1991).

The information presented in this thesis represents the first characterization of the polypeptide encoded by any plant ACBP gene (*acb*). Prior to this work, the plant protein had not been characterized, although subsequently, putative cDNAs have been isolated from a number of different sources. Hills et al. (1993) used a polymerase-chain reaction (PCR) approach to isolate a presumptive ACBP cDNA from rapeseed (*Brassica napus*) and provided evidence that there are multiple loci for the ACBP gene in this plant, a result similar to that of acyl-carrier protein (ACP) in *B. napus* (Safford et al., 1988). As a result of genome sequencing projects, cDNAs have been isolated from rice (*Oryza sativa*; Uchimiya et al., 1992), corn (*Zea mays*; Keith et al., 1993), *Arabidopsis thaliana* (Newman et al., 1994) and castor bean (*Ricinus communis*; van de Loo et al., 1995) that have high sequence identity to the *A. thaliana* ACBP (Chapter 2). With the exception of the rapeseed and *Arabidopsis* experiments, none of these randomly selected cDNA sequences have been further investigated.

2. Functions of acyl-CoA-binding protein/diazepam-binding inhibitor

Acyl-CoA-binding protein has multiple names and multiple functions. Historically, the ACBP polypeptide was first purified from rat brain on the basis of its ability to displace diazepam (Marquardt et al., 1986) from the gamma-aminobutyric acid receptor type A ($GABA_A$)

receptor (Stephenson, 1995), and it was, therefore, given the name diazepam-binding inhibitor or DBI by Guidotti et al. (1983). ACBP or DBI is also known as endogenous benzodiazepine or endozepine. ACBP does not appear to be the direct neurotransmitter in the brain. Rather, two different peptide fragments, resulting from endoprotease digestion, appear to serve that role (Ferrero et al., 1986).

In addition, DBI has been purified from a number of peripheral organs, such as pig intestine (Chen et al., 1988), rat liver (Knudsen et al., 1989) and bovine liver (Mogensen et al., 1987). DBI has been shown to be expressed in all rat tissues, with the highest expression in adrenal gland, liver, and the somatic tissue of the testes (Bovolin et al., 1990), and the lowest expression was found in spleen, lung, and muscle (Knudsen et al., 1989). Endozepine-like peptides have also been found enriched in the epithelium of the rat gastrointestinal tract (Steyaert et al., 1991).

In contrast to these GABA-ergic receptors, often regarded as the "central" type of benzodiazepine receptor, there is the "peripheral" type of benzodiazepine receptor, which is located on mitochondria and regulates steroidogenesis. DBI influences steroid biosynthesis (Papadoupoulos et al., 1991) by stimulating cholesterol delivery to the inner mitochondrial membrane (Yangibashi et al., 1988). Recently, antisense oligonucleotides for DBI were shown to inhibit hormone-stimulated steroid production in Leydig cells (Boujrad et al., 1993).

It is still uncertain to what extent ACBP/DBI is a true multifunctional protein. In plants and yeast, there is no nervous sytem (so no GABA-ergic pathways) and no glucose-induced insulin secretion. Clearly, there are fewer possibilities for ACBP to have specialized functions in yeast or plants. A unicellular organism like yeast

performs all of the basic metabolic functions and does not require complex intercellular signaling and interactions necessary in a multicellular organism (Mandrup et al., 1991). The highly conserved nature of ACBP through evolution suggests that ACBP performs a similar function in all these organisms (Knudsen et al., 1993). Research into the plant ACBP should address the question: Can this housekeeping protein have specialized functions in certain cell types or during certain states of ontogeny?

3. Acyl-CoA-binding protein: Structure and mutant studies

There has been interest in the physical properties and structure of this protein since it was suggested that ACBP is an acyl-CoA carrier, or that it regulates the size of the acyl-CoA pool (Knudsen et.al., 1989). The first three-dimensional structures for ACBP (bovine) were determined by one-dimensional (Andersen et al., 1991) and two-dimensional nuclear magnetic resonance spectroscopy (Andersen and Poulsen, 1992). Later, high resolution nuclear Overhauser enhancement spectroscopy (NOESY) produced structures to a resolution of 1.4 Å for both the *apo*-enzyme (Andersen and Kragelund, 1992) and the *holo*-enzyme (Kragelund et al., 1993).

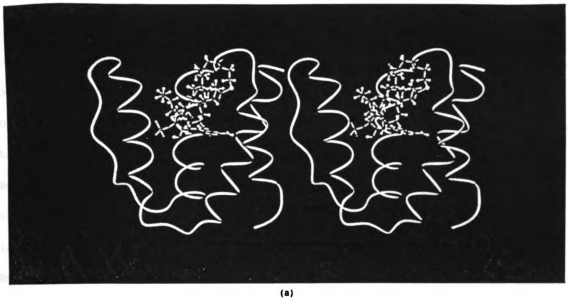
The animal homologue is a highly conserved 10 kDa protein containing 86 amino acid residues arranged into 4 helical regions (Andersen et al., 1991). These helices span residues 1-15, 20-36, 51-60 and 65-85. All of the ACBPs isolated to date contain two highly conserved regions. The first, in the second helical region, is five to seven amino acids in length (underlined in Figure 1, above) and was shown to be the hydrophobic binding site for the fatty-acyl chain by photoaffinity labelling (Hach et al., 1990). The second, in the loop

between the second and third helix (underlined in Figure 1), has high affinity for the CoA portion of the molecule, although there are other amino acid residues outside of this region that make contact with the adenosine-3'-phosphate or the pantotheine portion (marked with a "b" in Figure 1) of the CoA head group (Knudsen, 1991).

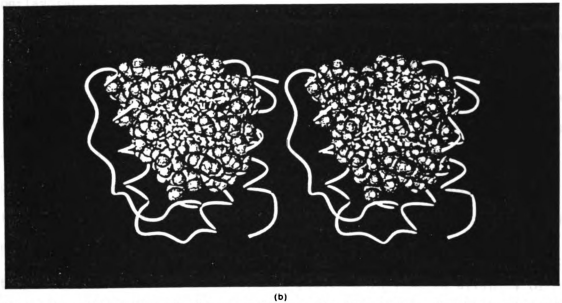
The overall structure of bovine ACBP has been described as a shallow bowl which has a rim and exterior sides that are highly hydrophilic but contains an interior and an exterior base that are predominantly non-polar (Andersen and Poulsen, 1992). Helical regions A1 and A4, then A2 and A3 run in parallel pairs with two lysines (Lys_{13} and Lys_{32}) at the inside bottom of the bowl (Figure 2). An earlier alternative description for the conformation of this protein likens it to a boomerang with helix 1 and 2 in anti-parallel arrangement in one arm and helix 3 and a portion of helix 4 forming the other arm of the boomerang (Knudsen, 1991). The hydrophobic binding site lies in the angle connecting the two arms.

The adenosine-3'-phosphate binds near residues of each of the four helices so that this group lies over the opening to this shallow bowl (like a hat) and allows water to be excluded from the hydrophobic pocket (Kragelund et al., 1993). The polar part of the pantetheine and the pyrophosphate are structured in the bound ligand to form an interface with solvent. The ligand also forms a set of contacts where the adenine, pantetheine, and the palmitoyl-chain are associated. Therefore, overall the structure of the ACBP/ligand complex is organized to protect the lipophilic portions of the acyl-CoA molecule from the aqueous medium (Kragelund et al., 1993).

The individual binding of either free CoA or free long-chain FAs



(a)



(b)

Figure 2. Structure of the recombinant bovine ACBP. Stereo views of the lowest energy structure of the bovine ACBP-palmitoyl CoA complex showing the (a) fold of the palmitoyl-CoA in the ACBP binding site. The ligand is shown as a ball and stick model and the protein as a smooth ribbon through the peptide backbone. (b) Close-up of ligand-protein interactions with only interacting residues of the protein shown. (after Kragelund et al., 1993. **J. Mol. Biol.** *230,* 1260-1277).

to rACBP has been reported to be significantly weaker than the binding of intact ligand (Rasmussen et al., 1990; Rosendahl et al., 1993). The binding site is organized so that only long-chain acyl-CoA esters will bind strongly, and neither of the two main ligand constituents alone has the necessary determinants to bind strongly. Docking of short chain acyl-CoAs in the binding site shows that the omega-end of the chain cannot reach the lipophilic cavity in the bottom of the binding site (Andersen and Poulsen, 1992). Very long acyl-CoAs will probably extend beyond the lipophilic pocket and bring the aliphatic omega-end into a non-favorable contact with solvent.

Ongoing work by Knudsen and his Danish colleagues has resulted in the characterization of the structure and ligand binding of bovine ACBP variants that were generated by site-directed mutagenesis (Roepstorff et al., 1995; Poulsen, personal communication). Twelve different oligonucleotide-directed mutants were screened for altered ligand binding and specific residues were identified that are critical for acyl-CoA binding. Salt bridges or/and hydrogen bonds were formed between ligand and Ala_9, Tyr_{28}, Lys_{32}, Lys_{54} and Tyr_{73} (Figure 1). Substitutions of other amino acids in the mutant ACBPs resulted in lowered affinity or specificity for the acyl-CoA tail in ligand (Roepstorff et al., 1995). An overall comparison of the structures between the *apo-* and *holo-* forms of the protein suggests that ligand binding was not associated with major conformational changes.

4. Plant acyl-CoA-binding proteins

Prior to the initiation of this work, nothing was known about plant ACBPs. The first indication that a plant ACBP had been isolated

and cloned was reported in December 1992 by the *Arabidopsis* genome sequencing project at Michigan State University (Newman et al., 1994).

The cDNA clone (pSCC12T7P) containing 464 bp and an open reading frame encoding a polypeptide with 92 amino acids had 57% sequence identity to bovine ACBP (Chapter 2). While this work was in progress, an ACBP cDNA was derived from *B. napus* (Hills et al., 1994). Using degenerate oligonucleotide primers synthesized to encode those amino acids that were highly conserved and form either the acyl-chain binding pocket (KQAT box) or the contact points with the CoA head group (DAWK box), a PCR amplification product was cloned and subsequently used as a probe to clone the full-length cDNA. The Hills et al. (1994) clone encoded a 92 amino acid clone with a 76% sequence identity to the *A. thaliana* clone (pSCC12T7P). All the residues that bind the adenosine-3'-phosphate of the CoA moiety (Ala_1, Tyr_{30}, Lys_{34}, Lys_{56}, Tyr_{74}) were present with the same relation found in ACBPs from other species.

The *Brassica* and *Arabidopsis* ACBPs both appeared to be cytosolic proteins since these clones lacked a signal peptide (Figure 3). Expression patterns in *Brassica* (Hills et al., 1994) and *Arabidopsis* (Chapter 2) indicated that ACBPs were present in all plant tissues. In particular, the ACBP mRNA was highly expressed during seed mid-maturity or seedling germination when TAG biosynthesis or ß-oxidation activities were significant (Chapter 4).

Additional cDNAs for ACBP were isolated serendipitously as part of the Japanese rice cDNA sequencing project (Uchimiya et al., 1992) and the corn EST sequencing project (Keith et al., 1993). In addition, Turner and Somerville found a castor bean ACBP cDNA while screening mid-maturity castor seed cDNA for the castor hydroxylase gene (van de Loo et

Figure 3. Fungal, insect and plant ACBP sequences aligned so that a comparison of amino acid residues can be made. Residues that are invariant in all ACBPs have a "+" sign above that column. Residues that are conserved in all fungal, insect or plant ACBPs have a "^" above that column. The KQAT box (residues bind acyl tail) and the DAWK box (residues bind CoA head group) are underlined.

```
                           b       b                             b
               ^   +       ^           ^         ^         +   ^ +
S.c.     V S Q - L F E E K A K A V N E L P T K P S T D E L L E L Y A   29
S.m.     V S Q - L F E E K A K A V N E L P T K P S T D E L L E L Y G   29

M.s.   M S L Q E Q F D Q A A S N V R N L K S L P S D N D L L E L Y A   31
D.m.   M V S E - Q F N A A A E K V K S L T K R P S D D E F L Q L Y A   30

Ath    M G L K E E F E E H A E K V N T L T E L P S N E D L L I L Y G   31
Bna    M G L K E D F E E H A E K V K K L T A S P S N E D L L I L Y G   31
Ghi    M G L K E E F E E H A E K V K T L P A A P S N D D M L I L Y G   31
Osa    M G L Q E D F E A V R R K G K T L P E S T S N E N K L I L Y G   ND
Rco    M G L K E D F E E H A E K A K T L P E N T T N E N K L I L Y G   31
Zma    M G L Q E E F X E H X E K A K T L P E S T S N E N K L I L Y G   31

       /--------- helix 1 -----------/         /----- helix 2 -----
```

```
          b                                             b
      ^   + + +     +               + +             ^   +     + +
S.c.  L Y K Q A T V G D N D - K E K P G I F N M K D R Y K W E A W E   59
S.m.  L Y K Q A T V G D N D - K E K P G I F N M K D R Y K W E A W E   59

M.s.  L F K Q A S A G D A D P A N R P G L L D L K G K A K F D A W H   62
D.m.  L F K Q A S V G D N D T A - K P G L L D L K G K A K W E A W N   60

Ath   L Y K Q A K F G P V D T S - R P G M F S M K E R A K W D A W K   61
Bna   L Y K Q A T V G P V T T S - R P G M F S M K E R A K W D A W K   61
Ghi   L Y K Q A T V G P V N T S - R P G M F N M R E K Y K W D A W K   61
Osa   L Y K Q A T V G D V N T A - R P G I F A Q R D R A K W D A W K   ND
Rco   L Y K Q A T V G P V N T S - R P G M F N M A D R A K W D A W K   61
Zma   L Y K Q A T V G D V N T D - R P G I F Y Q K D R A K W D A W K   61

     ---- helix 2 ---/                      /----- helix 3 ------
```

```
                                b
            ^           +       + ^     ^     ^
S.c.  N L K G K S Q E D A E K E Y I A L V D Q L I A K Y S S *           86
S.m.  D L K G K S Q A D A E K E Y I A Y V D N L I A K Y S S *           86

M.s.  K K A G L S K E D A Q K A Y I A K V E S L I A S L G L Q *         90
D.m.  N K Q K G K S S E A A Q E Y I T F V E G L V A K Y A  *            86

Ath   A V E G K S S E E A M N D Y I T K V K Q L L E V A A S K A S T *   92
Bna   A V E G K S T D E A M S D Y I T K V K Q L L E A E A S S A S A *   92
Ghi   A V E G K S K E E A M G D Y I T K V K Q L F E A A G S S *         89
Osa   A V E G K S K E E A M S D Y I T K V K Q L L E E A A A S A A S *   92
Rco   A V E G K S K E E A M S D Y I T K V K Q L L E E A A A S A *       90
Zma   L F E G K S K D E A M N D Y I T K V K Q L Q E E A A A S *         89

- helix 3 --/       /------------- helix 4 ------------/
```

Abbreviations: fungal: *S.c.*, *Saccharomyces cerevisiae*; *S.m.*, *Saccharomyces monacensis*; insect: *M.s.*, *Manduca sexta*; *D.m.*, *Drosophila melanogaster*; plant: *Ath*, *Arabidopsis thaliana*; *Bna*, *Brassica napus*; *Ghi*, *Gossypium hirsutum*; *Osa*, *Oryza sativa*; *Rco*, *Ricinus communis*; and *Zma*, *Zea mays*.

al., 1995), while Reddy et al. (1996) isolated a putative ACBP from cotton. The *Oryza sativa* (D28303), *Gossypium hirsutum* (U35015), *Zea mays* (T25215), and *Ricinus communis* clones are aligned with the *Arabiopsis thaliana* (T04081) and *Brassica napus* (X77134) sequences (Figure 3; Genebank accession numbers in parenthesis). In addition, sequences of fungal origin (the yeasts: *Saccharomyces cerevisiae* and *Saccharomyces monacensis*) or insect origin (*Drosophila melanogaster* and *Manduca sexta*) are also aligned with the plant-derived ACBPs (Figure 3).

A comparison between the appropriate designators and a range of physical properties (molecular weight, extinction coefficient and absorption coefficient) for the purified recombinant proteins is listed below (Table 1). These values have been derived by the laboratory of Jens Knudsen, University of Odense, Denmark (personal communication).

B. Acyl-CoA-binding protein: Physiology

1. Animal versus plants: Lipid biochemistry

Long-chain fatty acyl-CoA is the metabolically active form of FAs in both animal, fungal and plant cells. Various enzymatic conversions of the FA substrate occur as an acyl-CoAs including certain desaturations, chain elongations, glycerolipid esterifications, and oxidation reactions in all higher organisms. Besides being a very important acyl-donor, acyl-CoA is a metabolic intermediate that acts as a modulator of various enzymes and specialized cells (Taupin et al., 1993). In contrast to animal cells, where ACBP has been processed and recruited for neuropeptide and endocrine functions, plant ACBP has as its sole function the sequestration and transport of acyl-CoA to various sites within the cell (Figures 4 and 5).

The ability of ACBP to bind acyl-CoAs was discovered by Mogensen

Table 1 Physical characteristics of a collection of ACBPs.

organism	designator	MW (daltons)	extinction coeff. $mM^{-1}\ cm^{-1}$	absorption coeff. $A^{1mg/ml}$ (210 nm)
human	humACBP	9950*	16.50	1.657
dog	dogACBP	9989*	17.78	1.798
rat	ratACBP	9938*	15.22	1.531
bovine	bovACBP	9955*	16.50	1.657
mouse	musACBP	9938*	15.22	1.531
duck	dckACBP	9656*	16.50	1.709
tortoise	torACBP	9834*	16.50	1.678
chicken	chkACBP	9687*	16.50	1.703
pig	pigACBP	9807*	16.50	1.682
yeast	SceACBP	9930	17.78	1.791
S. mona.	SmoACBP	9953	19.06	1.915
A.thaliana	AthACBP	10386†	15.22	1.465

* includes N-terminal acetyl group

† includes N-terminal methionine

et al. (1987). Knudsen's group utilized protein fluorescence or electromagnetic spin resonance (ESR) spectroscopy to demonstrate that acyl chains of 8 to 16 carbons bound to ACBP to the same extent (Mikkelsen and Knudsen, 1987). Acetyl-CoA and butyrol-CoA bound only slightly (Knudsen et al., 1989). In the ESR binding experiments, acyl-CoAs with 14 to 22 carbons in the chain displaced 12-doxyl-stearoyl-CoA from bovine ACBP with high affinity (Rosendahl and Knudsen, 1992). However, the presence of 1, 2, or 3 double bonds in the chain did not affect binding significantly.

Apparent K_ds for palmitoyl-CoA and oleoyl-CoA were determined to be 0.22 µM and 0.14 µM respectively (Rasmussen et al., 1990) using a Lipidex assay that subsequently has been shown to give false binding values. Reports indicated that long-chain acyl-CoAs have several regulatory functions (Woldegiorgis et al., 1982; Knudsen, 1990), such as in moderating the activity of acetyl-CoA carboxylase (Nikawa et al., 1979), the mitochondrial adenine nucleotide translocase, and interacting with hormone receptors such as the nuclear triiodothryronine receptor (Li et al., 1990). Similarly, extramito-chondrial ß-oxidation is regulated by the size of the acyl-CoA and FA pool (Kaikaus et al., 1993).

However, the actual physiological effect of acyl-CoAs in the cell is obtained in the low-to-mid n*M* range. In contrast, animal acyl-CoA concentrations are reported to be from 22 to 83 nmoles/gram tissue (Rasmussen et al., 1993). This corresponds to between 240 µg ACBP/ml (or 25 µM ACBP) and 850 µg ACBP/ml (or 87 µM ACBP). If long-chain acyl-CoAs were found free in the cytosol, both FA synthesis and ATP biogenesis would be completely inhibited in the liver (approximately 50

nmoles/g tissue) where high rates of both these processes occur. Both acetyl CoA carboxylase and the mitochon- drial nucleotide translocase were protected against acyl-CoA inhibition in the presence of ACBP (Rasmussen et al., 1993).

Compare these values with the ACBP concentrations from *Arabidopsis* seed (140 μg ACBP g^{-1} FW or 45 μM ACBP) or mid-maturity *Brassica* seed (205 μg ACBP g^{-1} FW or 65 μM ACBP) where rapid TAG biogenesis is occurs (Chapter 2). Fully mature vegetative tissue like root or leaf have levels of ACBP from 5 to 30 μg ACBP g^{-1} FW (or 0.7 to 5 μM) indicating a significantly smaller acyl-CoA pool in plant cells compared to well-fed animal cells (Chapter 2, thesis). Unfortunately, it is not possible to compare ACBP levels with the concentration of endogenous acyl-CoA esters in plants since measurement of acyl-CoA in plants is difficult (Kopka et al., 1995).

Acyl-CoAs can be removed from the mitochondrial acyl-CoA synthetase (Rasmussen et al., 1994). Overexpression of the recombinant ACBP in transgenic yeast significantly increased the acyl-CoA pool size (Mandrup et al., 1993b) demonstrating that ACBP *in vivo* can act to increase the acyl-CoA pool.

Cells must continuously utilize non-esterified FAs released by the action of acyl-CoA hydrolases (ACH) found in the endoplasmic reticulum, vacuolar membrane, plasma membrane and in the cytoplasm. Within cells, the free FAs are converted to acyl-CoA esters with the aid of ATP and the action of a fatty acid:CoA ligase, more commonly referred to as an acyl-CoA synthetase (ACS). This reesterification of certain FAs (for example, highly unsaturated FAs) provides special acyl-CoAs (Thompson et al., 1993) that can be reincorporated into glycerolipids without loss of unique FAs (Figure 4). ACS, then, allows the cell to handle a large

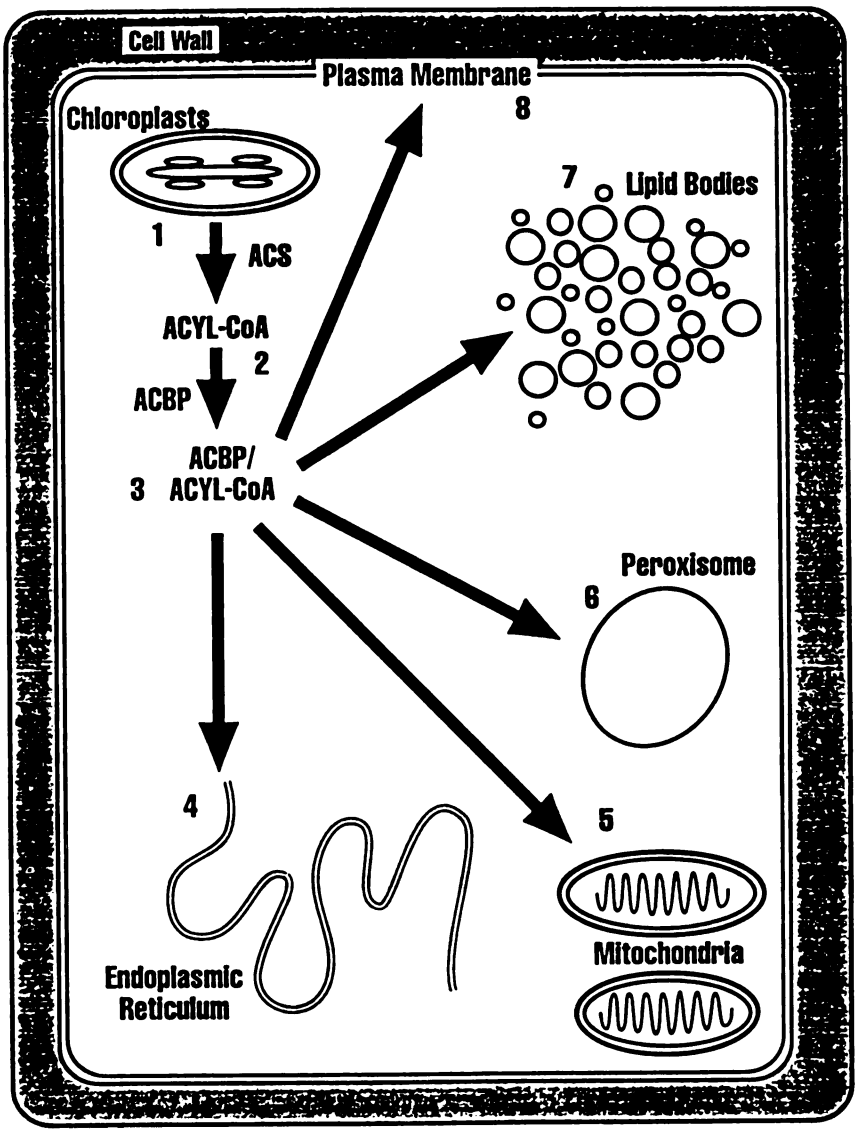


Figure 4. Involvement of acyl-CoA binding protein (ACBP) in lipid biogenesis and turnover. Fatty acid biosynthesis (1) produces FAs for the chloroplast acyl-CoA synthetase (2) which generates the substrate for ACBP (3). Acyl-CoAs must be transported to endoplasmic reticulum (4) for phospholipid biosynthesis and fatty acyl desaturation, mitochondria (5) or peroxisome (6) for ß-oxidation, lipid bodies (7) for TAG biosynthesis, plasma membrane (8) for retailoring reactions, and perhaps the cell wall (9) for wax (cutin and suberin) biosynthesis.

flux of non-esterified FAs, a process more prevalent in animals than in plants.

In vivo FA biosynthesis occurs principally in plastids (Ohlrogge et al., 1979), while enzymes involved in the synthesis of non-plastidal membrane lipids and TAG are localized in microsomes (Mudd, 1980). Therefore, an intracellular transport molecule may be required to mediate transport of acyl-CoAs from plastids to the endoplasmic reticulum (Figure 4). Similarly, use of energy stored in TAG by germinating seedlings or the turnover of membrane phospholipids requires transport of liberated FAs, in the form of acyl-CoAs, to glyoxysomes for the glyoxylate cycle (Figure 5) or mitochondria for subsequent ß-oxidation (Harwood, 1988).

Activation of long-chain FAs, catalyzed by long-chain ACS, is a central reaction in the metabolism and utilization of fatty acids. In animal cells, it is the first step of ß-oxidation, while in plant cells it is involved at many points: lipid biogenesis (substrate formation) and the glyoxylate cycle (substrate activation), in addition to ß-oxidation (Figure 5). Fatty acids are synthesized as acyl-acyl carrier proteins (acyl-ACP) by a dissociable type-II fatty acid synthetase (FAS) complex within plastids (Ohlrogge and Browse, 1995), and free FAs are released from the FAS complex by the action of thioesterases (Harwood, 1988). These FAs can then diffuse across the outer chloroplast envelope, where they will encounter ACS and become esterified to Coenzyme A. Subsequently, they will participate in the anabolic and catabolic reactions within a cell that result in either membrane or neutral lipid biogenesis (Thompson, 1993), energy generation, or the glyoxylate cycle and gluconeogenesis (Ohlrogge and Browse, 1994).

In plants very-long chain FAs (VLCFA) are required for wax

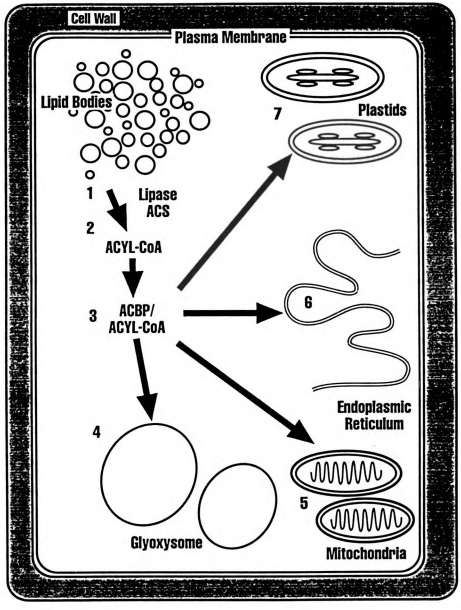


Figure 5. Involvement of acyl-CoA binding protein in triacylglycerol utilization during seed germination. Fatty acids released from TAG in seed lipid bodies (1) by lipases. These FAs are converted to acyl-CoAs through the action of acyl-CoA synthetase (2). These acyl esters must be transported by ACBP (3) to the glyoxysome (4) for use in the glyoxylate cycle and gluconeogenesis or for ß-oxidation. The mitochondria (5) can also produce energy for the cell through ß-oxidation.

biosynthesis (Kolattakudy, 1987), and in the case of some oilseeds, the VLCFAs can be found in storage TAG. In the high erucic acid lines of rapeseed, $C_{20:1}$ and $C_{22:1}$ FAs make up to 55% of seed TAG (Sebedio and Akman, 1981), while *Arabidopsis* TAG contains at least 10% C_{20} and C_{22} FAs in the seed oil (Voelker et al., 1992).

In animal cells, VLCFAs are continuously provided by circulating albumin complexes in the blood or lymph. In some cases, extracellular hydrolases will liberate non-esterified FAs from phospholipids near the cell surface. Within the cells, these FAs are converted to acyl-CoAs that can then be used for energy generation, or in the case of arachidonic and eicosapentenoic acids, the corresponding acyl-CoA forms are substrates for the lipoxygenase reactions that result in the formation of the prostaglandins and other eicosanoids (Papadoupoulos et al., 1991). Liver and adipose contain high levels of non-selective ACS providing these tissues with the ability to handle large amounts of various free FAs, converting them into glycerolipids for storage or export (Mangroo and Gerber, 1992).

2. ACBP and DBI: Physiology

In animals and yeast, long-chain acyl-CoAs play a central role in regulation of metabolism as allosteric regulators of several enzymes and translocators including protein kinase *c*, glucokinase, acetyl-CoA carboxylase (Nikawa et al. 1979), citrate synthetase, and mitochondrial ATP/ADP translocase (Woldegiorgis et al. 1982). Therefore, the relative amounts and types of acyl-CoAs within a cell represent a dynamic balance between synthetic and hydrolytic processes.

Initially, binding specificities for bovine and rat ACBP for

acyl-CoAs of different chain lengths were determined by fluorescence quenching of hydrophobic tyrosines in ACBP (Mikkelsen and Knudsen, 1987). Acyl-CoAs having a chain length of 10 to 20 were bound with increasing affinity, with C_{18}- and C_{20}-CoAs showing the tightest binding (Rosendahl et al., 1993). The binding affinity and stoichiometry of 1 mol acyl-CoA/mol ACBP was confirmed by Rasmussen et al. (1990). Only recently has work been done on the C_{22}- and C_{24}- acyl-CoAs (Rasmussen et al., 1994), which may be important in plants that contain VLCFA in storage TAG (Taylor et al., 1991) or use VLCFA to produce long-chain fatty alcohols (von Wettstein-Knowles, 1993) for surface waxes (Kolattakudy, 1987).

Although once used with great regularity (Li et al., 1993), the binding assays based on displacement or the Lipidex-1000 procedure have come under increasing criticism (Vork et al., 1990). Apparently the Lipidex-1000 assay can significantly under report the maximum acyl-CoA binding. For this reason, a non-radioactive, and yet direct, technique was sought for both the binding of acyl-CoAs to ACBP or free FAs to FABP. Titration microcalorimetry is a suitable alternative to the Lipidex assay, with a large number of additional benefits (Miller and Cistola, 1993) that will be discussed in Chapter 3 (thesis).

ACBP has been found in all animal tissues examined as a cytosolic protein (Knudsen et al., 1989). The highest ACBP concentration was found in liver (0.3-0.6% total soluble protein) which amounted to 400 nmol/g protein (Knudsen, 1990). This is two- to four-fold the reported concentration of acyl-CoA (108-250 nmol/g protein) reported by Woldegiorgis et al. (1985), but recent acyl-CoA measurements (360 nmol/g protein) indicate that these earlier values were significantly low

(Rosendahl and Knudsen, 1992).

Therefore, sufficient ACBP may exist in animals to bind the entire acyl-CoA pool. The cellular concentration of ACBP and long-chain acyl-CoA in rat livers was approximately equimolar 25 to 35 nmol/g tissue, with about 20% of the acyl-CoA located in the mitochondria. ACBP is not found within the mitochondria. Acyl-CoAs within mitochondria are bound to acyl-CoA utilizing enzymes, predominantly those of the ß-oxidation cycle (Knudsen, 1990). Occasionally, concentrations of up to 50 nmol/g tissue were found (Rasmussen et al., 1993). If the liver ACBP concentration is 30 µM, the cytosolic acyl-CoA/ACBP ratio is 0.8, and the average K_d for ligand binding is 4.5 X 10^{-14} M, then the free cytosolic acyl-CoA concentration will be 0.2 pM (Rasmussen et al., 1994).

The very high binding affinity and low free acyl-CoA concentration immediately raises the question as to which pathways can ACBP donate acyl-CoAs. Using multilamellar phosphatidylcholine liposomes immobilized on nitrocellulose-containing ^{14}C-hexadecanoyl-CoA, bovine ACBP was capable of selectively extracting radiolabelled acyl-CoAs and delivering them to either carnitine palmitoyl-transferase of intact mitochondria or to microsomes for phosphatidate biosynthesis (Rasmussen et al., 1994).

On the basis of the presence of ACBP in *S. cerevisiae*, a unicellular organism expressing mainly metabolic functions (Rose et al., 1992) and extreme structural conservation through phylogeny, ACBP could be classified as a housekeeping protein (Mandrup et al., 1992).

3. Comparison of ACBPs with fatty-acid binding proteins

FABP has been suggested by a number of workers to be involved in intracellular storage and transport of free FAs. Since FABP can also

bind acyl-CoAs (Bass, 1988), the question of which protein, ACBP or FABP, is involved in acyl-CoA metabolism has arisen. The concentration of FABP in rat liver was about five-fold greater than the concentration of ACBP (Knudsen et al., 1989). Therefore, ACBP must exhibit a much greater affinity for acyl-CoAs than FABP in order to play the dominant role in the metabolism of acyl-CoA.

ACBP was able to give practically complete protection against microsomal ACH (Chapter 2), whereas high FABP concentrations relative to the acyl-CoA concentration gave only slight protection (Rasmussen et al., 1990). Interestingly, it has been shown that ACS was stimulated by the addition of FABPs (Burrier et al., 1987). Some of the previous reports of FABP interacting with acyl-CoAs was due to ACBP impurities in FABP preparations (Knudsen, 1990), a situation that has been recognized before (Börchers et al., 1990) and was noted after the very first FABP was purified (Haunerland et al., 1984). Similarly, a new study using FABP indicated oleoyl-CoA bound at two sites with a moderate affinity (K_d = 3 to 14 μM) using the Lipidex-1000 assay (Hubbell et al., 1994), a value that is 10 to 100 times larger (lower affinity) than oleoyl-CoA binding to ACBP.

Of even greater physiological significance, two FABPs were unable to extract significant amounts of acyl-CoAs from multi-lamellar liposomes (Rasmussen et al., 1990), while ACBP was able to extract acyl-CoA efficiently under similar conditions. These results suggest that ACBP could protect membranes and enzymes against the damaging detergent effects of acyl-CoAs (Mandrup et al., 1992). In addition, ACBP, but not FABP, was capable of protecting acyl-CoA from hydrolysis by microsomal thioesterases (Rasmussen et al., 1990).

4. ACBPs and cellular metabolism

Apparently, ACBP plays a significant role in the lipogenic process and may alter both the quality and quantity of the lipids produced. These experiments on ACBP affinity for acyl-CoAs and the tissue concentration of ACBP (two- to four-fold excess compared to acyl-CoA) clearly demonstrate that acyl-CoAs are bound to ACBP and not to FABP *in vivo*. ACBP, then, was intimately, if not exclusively, involved with the metabolism and transport of acyl-CoAs in cells (Knudsen, 1990).

If ACBP is directly involved in acyl-CoA metabolism within cells, one would expect the level of ACBP to vary with developmental status of a given tissue. When 3T3-L1 fibroblasts were induced to differentiate into adipocytes, which synthesize and store large quantities of TAG, the level of ACBP as measured by ELISA increased three- to ten-fold while ACBP mRNA increased two- to six-fold (Hansen et al., 1991).

In our experiments with measuring ACBP concentration directly using antibodies raised to the recombinant protein, we found an 8-fold increase in *Brassica napus* seeds during mid-maturity, a time when there was rapid TAG production (Chapter 2, thesis). Due to the difficulty of measuring *in vivo* acyl-CoA concentrations (Post-Beittenmiller et al., 1992), it was not possible to compare acyl-CoA and ACBP levels to one another. In addition, Mogensen et al. (1987) clearly demonstrated that the presence of ACBP could cause FAS to prematurely release medium-chain FAs *in vitro*. Given these results, ACBP was proposed to act as an acyl-CoA pool former and/or transporter of acyl-CoA (Knudsen et al., 1993).

C. ACBP genes (*acp*): Structure and expression

1. Genome organization for *acb* genes

Southern blot analysis of rat liver DNA using a 458 bp-*Eco*R I ACBP

fragment as a probe indicated that there were six hybridizing fragments seen in each lane (Hansen et al., 1991). Since none of the restriction enzymes cut within the ACBP exons, each fragment would correspond to at least one exon in the rat genome. Given the small size of the coding region for the expressed ACBP, it was likely that the probe hybridized either to the functional ACBP genes, possible isoforms, or to one or more related pseudogenes (Hansen et al., 1991).

The rat ACBP gene was cloned by Mandrup et al. (1992) and was used to examine the ACBP gene family in the rat. There were one expressed gene and four processed pseudogenes, one of which was shown to exist in two allelic forms. In Southern blot analysis, ACBP cDNA probes hybridized to several fragments in genomic DNA of rat (Mocchetti et al., 1986), human (Gray, 1987) and mouse (Owens et al., 1989) indicating that ACBP was encoded by a small multi-gene family. A human variant has also been cloned from a human hypothalamic cDNA library suggesting that the ACBP pre-mRNA might be subject to alternative splicing.

The expressed ACBP gene in rat was organized into four exons and three introns. There was a remarkable correspondence between the ends od each exon in *acb* and the ends of each of the four helices of the protein (Mandrup et al., 1992). In both rat brain and liver, transcription was initiated from either two major or a number of minor initiation sites. The promoter region of the ACBP gene was located in a CpG island and lacked a canonical TATA box, thus exhibiting all the hallmarks of a typical housekeeping gene (Mandrup et al., 1992). No evidence was found in this 1992 study for alternative splicing.

2. ACBP gene expression in heterologous systems

Expression of a synthetic gene for bovine recombinant ACBP (rACBP)

in *E. coli* resulted in 12-15% of total protein as ACBP (Mandrup et al., 1991). This ACBP migrates in SDS-PAGE in an anomalous fashion, as shown before (Mogensen et al., 1987). It has an apparent MW of 7.2 kDa instead of the actual MW of 10 kDa. No attempt was made to determine if overexpression of the rACBP altered *E. coli* lipid pools, acyl-CoA concentration, or total FA composition. However, considering that it is acyl-ACP and not acyl-CoAs that have a central role in lipid biosynthesis in prokaryotes, one may not see a difference.

The same synthetic rACBP was placed under an inducible yeast galactose promoter, and heterologous overexpression was followed in transgenic yeast (Mandrup et al., 1993b). Expression of rACBP resulted in a significant increase in the cellular acyl-CoA pool, which was correlated with ACBP levels. Palmitoyl-CoA also increased significantly while steroyl-CoA and oleoyl-CoA decreased. This suggests that ACBP, by removing acyl-CoA from the yeast FAS-ACS complex, may cause an early termination of chain elongation (Mandrup et al., 1993b). Similarly, this work indicates that ACBP not only transports acyl-CoAs, but it also determines the size and composition of the acyl-CoA pool.

Expression of ACBP mRNA was maximal in rat liver postnatally at day 1, whereas it increased linearly in kidney and heart from day 1 to adulthood (Mandrup et al., 1992). The biosynthesis of ACBP was up-regulated in the cerebellum and cerebral cortex of rats made tolerant to benzodiazepines (Mochetti et al., 1991). This up-regulation in ACBP biosynthesis indicates that there was a feedback signal that compensates for the probable desensitization or down-regulation of the benzodiazepine receptor acquired after chronic exposure to the drug.

The transcript for ACBP is highly enriched in brain (Taupin et al., 1993), in adipocytes synthesizing TAG (Hansen et al., 1991) and in

liver cells of rats that have been fed a high-fat diet (Rosendahl and Knudsen, 1992). In brain cells desensitized to alcohol, ACBP was up-regulated since it is the precursor for two neuropeptides which bind to the $GABA_A$ receptor (Guarneri et al., 1990).

3. Plant isoforms of ACBP

Southern blot analysis of *Brassica napus* indicated that there were six ACBP genes (or genes and pseudogenes) present (Hills et al., 1994), although only 5 bands were visible following Southern hybridization. *B. napus* is an amphidiploid species derived from the diploid species *B. rapa* and *B. oleracea*. Three of these loci have been mapped to chromosomes 5, 8 and 9, while a fourth locus maps to chromosome 15. Two of the loci have yet to be identified with a linkage group (Hills et al., 1994). No work was performed to determine how many of the putative genomic sequences were expressed in rapeseed. This work suggests that there three *acb* genes in the diploid crucifers. In the tetraploid *B. napus* one would expect six copies of the gene.

One might expect that there would be at least two isoforms in plants, one "housekeeping" version expressed in all cells and then a second specialized isoform with differences in the C-terminal portion of the protein. The various isofoms might be expected to dock with different acyl-utilizing enzymes. Since the specificity for this interaction would be built into helix 4 of ACBP (Chapter 2), we might expect that the C-terminal portions of the different *acb* isoforms would display the greatest sequence divergence. The expression pattern for the single isoform isolated from *B. napus* suggested that more than one isoform may function in any cell type (Hills et al., 1994).

D. Conclusions

The acyl-CoA-binding protein described here has some unique properties in terms of lipid-protein and membrane-protein interactions. At the molecular level, two-dimensional nuclear magnetic resonance spectroscopic analysis of both the protein and protein-substrate complex have revealed several important structural features which suggest function. The concentration of ACBP increases or decreases along with changes acyl-CoA pool size.

On the basis of the broad range of distribution within an individual organism, its biochemical properties, gene structure and mode of acyl-CoA binding, we conclued that ACBP is a housekeeping protein. Also, it is clear that in the conservation of amino acid sequence over three biological kingdoms, ACBP can certainly be considered a housekeeping protein. However, cell-type specific expression of ACBP (usually referred to in these studies by its other label, DBI) in several animal tissues suggests the existence of specific regulatory mechanisms for DBI/ACBP gene expression. ACBP/DBI, then, has a dual nature: it served as a housekeeping protein in certain cell types, but it has been recruited for additional specific, physiological tasks in specialized cell types or under certain environmental conditions. Alho's group has recently proposed that ACBP/DBI was significantly upregulated in secretory tissues with high energy consumption (Malpighian tubes, adipose tissue, adrenal glands, gametes, and larval, but not the adult brain, of *Drosophila melanogaster*), suggesting that ACBP was involved with energy metabolism in certain cell types that depended on substrate (FA versus monosaccharides) for energy production (Kolmer et al., 1994).

Despite an extensive body of literature, it remains to be unequivocally established what physiological function ACBP fulfills. A number of secondary effects have been attributed to this protein or to its peptide fragments (neurotransmitter inhibition, steroidogenesis, insulin secretion, antimicrobial effects). However, none of these secondary effects have been convincingly demonstrated.

Postscript

and yet relation appears
A small relation expanding like the shade
of a cloud on sand
a shape on the side of a hill.
- Wallace Stevens

a shape inside of a cell.
an idea, a force, a gale.
- Raymond Pacovsky

Acknowledgments

I wish to thank Anne Plovanich-Jones, Robert Bandurski and Marcia Wood for their critical reading of this chapter. In addition, the following insight by John Von Neumann was appreciated as I put this chapter together: "The sciences do not try to explain, they hardly try to interpret, they mainly make models. A model is a mathematical construct which with the addition of certain verbal interpretations, describes observed phenomena. The justification of such a mathematical construct is solely and precisely that it is expected to work."

CHAPTER 2

CHARACTERIZATION OF AN ACYL-CoA-BINDING PROTEIN FROM *Arabidopsis thaliana*

Abstract

A cDNA clone was obtained from *Arabidopsis thaliana* that encodes a protein containing 92 amino acid residues with high sequence identity (57%) to bovine acyl-CoA-binding protein (ACBP). The coding sequence of this clone was expressed in *E. coli* and the gene product (10.4 kDa) was purified. The recombinant *A. thaliana* ACBP (rAthACBP) was shown to bind acyl-CoA esters and protected acyl-CoAs from degradation by microsomal acyl-hydrolases. Antibodies that were raised to rAthACBP recognized the native *Arabidopsis* ACBP and also cross-reacted with a number of other plant ACBPs, including rapeseed (*Brassica napus*) ACBP. The pattern of expression and level of the gene product were examined in various tissues of *Arabidopsis* and *Brassica* using Western blotting. *A. thaliana* tissues contained between 3 and 143 μg AthACBP g^{-1} FW depending on the tissue (0.4 to 14 nmol g^{-1} FW). Developing *B. napus* seeds underwent a 12-fold increase in ACBP levels during seed maturation and triacylglycerol accumulation (20 to 250 μg ACBP g^{-1} FW), with the highest concentration corresponding to 26 nmol g^{-1} FW.

INTRODUCTION

Acyl-CoA-binding proteins (ACBP) have been found in all animal tissues examined (Knudsen et al. 1993). In yeast, the genomic DNA for

ACBP has been cloned (Rose et al. 1992). In insects, ACBP cDNAs have been isolated (Snyder and Feyereisen, 1993; Kolmer et al. 1994), and in *Brassica napus* (Hills et al., 1993) and *Ricinus communis* (van de Loo et al., 1995) cDNAs have been cloned. These low molecular weight (approximately 10 kDa) cytosolic proteins were normally found in concentrations that are sufficient to account for binding of the entire acyl-CoA pool. The human, bovine, rat, and porcine ACBPs were highly conserved proteins and demonstrated approximately 56% sequence identity (Knudsen, 1993). More importantly, there was nearly 78% identity in the deduced amino acid sequence of the two conserved regions that define the hydrophobic pocket and the loop that binds the acyl-CoA head group.

Although it has been demonstrated that ACBP from rat and bovine sources binds long-chain acyl-CoA esters with high affinity (Rasmussen et al., 1990; Rosendahl et al., 1993), the actual biological function of ACBP is not known. Overexpression in yeast resulted in an increase in cytosolic acyl-CoA concentrations (Mandrup et al., 1992). These and other observations have led to the suggestion that ACBPs are involved in the intracellular transport and storage of acyl-CoAs (Knudsen, 1991). The ability to sequester long-chain acyl-CoA esters may prevent allosteric inhibition of specific enzymes (for example, acetyl-CoA carboxylase, glucokinase, citrate synthetase, protein kinase *c*; Rosendahl and Knudsen, 1992) and may protect cells from the adverse effects of high concentrations of acyl-CoA on membrane integrity (Mandrup et al., 1992).

It has been established, in rat and bovine systems, that ACBP is a housekeeping protein based on its gene structure and expression characteristics (Mandrup et al., 1992). Rasmussen et al. (1993) speculated that this housekeeping protein was involved in acyl-CoA metabolism but

was not a protein that had specialized functions correlated with tissue specificity.

De novo FA biosynthesis occurs primarily in the plastid (Ohlrogge et al., 1979). FAs are only converted to acyl-CoAs upon export to the cytoplasm. The route of transport from plastid to incorporation into glycerolipids by acyltransferases of the ER is unclear. Because of the importance of acyl-CoAs as substrates for all of the non-plastidial acyltransferases, this project was initiated to assess the role of this protein in plant lipid metabolism. A recent report described a putative ACBP cDNA from rapeseed (Hills et al., 1994), but to date, no work has been reported that characterizes the plant protein. In this study, we describe ACBP from *Arabidopsis thaliana*, demonstrate its ability to bind acyl-CoAs, and characterize tissue specificity and temporal differences in ACBP expression in developing *B. napus* seeds.

MATERIALS AND METHODS

Bacterial strains and plant material. The *Escherichia coli* strains used were DH5alpha (Bethesda Research Laboratories) and BL21(DE3) (Studier and Moffatt, 1986). *Arabidopsis thaliana* ecotype Columbia was grown in a growth chamber (16 hr photoperiod, 27°/23° C, 450 µmol m^{-2} sec^{-1}). Fully expanded leaves and mid-maturity siliques were harvested, frozen in liquid nitrogen, and then transferred to a -80° C freezer. After the entire shoot had been removed and the soil washed away, roots were patted dry and similarly frozen and stored. Dry *A. thaliana* seeds were used directly for protein extracts. *Brassica napus* cv. Bridger, *Cuphea lanceolata* cv. Ait, *Carthamus tinctorius* cv. S - 400, *Nicotiana tabaccum* cv. SR1) and *Coriandrum sativum* were grown in a greenhouse under natural conditions (15 to 16 hr photoperiod, 32°/23°

C, maximum PPFD 920 μmol m^{-2} sec^{-1}). Fully expanded leaves, early maturing silique, staged seed derived from inflorescences tagged at the point of flowering then removed from the silique and labeled according to days after flowering (DAF), and young flowers and roots of *Brassica napus* were collected. Similarly mid-maturity seed and fully expanded leaves of *Cuphea*, safflower, tobacco, and coriander were harvested. All of this tissue was frozen in liquid nitrogen within 10 min of being collected and was stored at -80° C. Mid-maturity seed and fully expanded leaves of *Glycine max* cv. Dare were harvested from a field at Michigan State University, were frozen on dry ice (-56° C) and were subsequently stored at -80° C.

cDNA clone. A 464 bp cDNA that contained an ORF which encoded a polypeptide (Figure 6) with a high degree of sequence similarity to bovine ACBP (57% identity) or the human ACBP (49% identity, Figure 1) was obtained from the *Arabidopsis thaliana* genome project (Newman et al. 1994). The original clone (EST 20896, Genbank Accession Number T04081) was derived from a cDNA library that had been constructed from poly(A)$^+$ RNA isolated from all tissues of *Arabidopsis thaliana* (Newman et al. 1994) in a lambda ZipLox (Palazzolo et al. 1990) vector system. The plasmid (pSCC12T7P), a pZL1 derivative (Gibco BRL, Gaithersburg, MD), was obtained by *in vivo* excision using the phage P1 *cre*.

Protein overexpression. Cloning sites at the 5' and 3' end of the cDNA coding sequence were added so that the *Arabidopsis* DNA could be placed in-frame into an *E. coli* expression vector. The cDNA insert, originally contained in plasmid pSCCT7 (a pZL1 derivative) was amplified by polymerase chain reaction (PCR) using PCR primers that generated an in-frame *Nde* I site at the 5' end (ccatATGGGTTTGAAGGAGGAA) and a *Bam*H I site proximal to the tandem stop codon at the 3' end (ggatccTCAGGTTGAAG-

Figure 6. The nucleotide sequence and derived amino acid sequence of *Arabidopsis thaliana* ACBP. Start Methionine is at position number 1 and stop codons are underlined.

```
-60   TTCTCCGTCTTACACTCATTTAATTCTCCT
-30   ACCAATCTCAACTTCCGACGTCTATTCATC
  1   ATGGGTTTGAAGGAGGAATTTGAGGAGCAC
      M  G  L  K  E  E  F  E  E  H
 31   GCTGAGAAAGTGAATACGCTCACGGAGTTG
      A  E  K  V  N  T  L  T  E  L
 61   CCATCCAACGAGGATTTGCTCATTCTCTAC
      P  S  N  E  D  L  L  I  L  Y
 91   GGACTCTACAAGCAAGCCAAGTTTGGGCCT
      G  L  Y  K  Q  A  K  F  G  P
121   GTGGACACCAGTCGTCCTGGAATGTTCAGC
      V  D  T  S  R  P  G  M  F  S
151   ATGAAGGAGAGAGCCAAGTGGGATGCTTGG
      M  K  E  R  A  K  W  D  A  W
181   AAGGCTGTTGAAGGGAAATCATCGGAAGAA
      K  A  V  E  G  K  S  S  E  E
211   GCCATGAATGACTATATCACTAAGGTCAAG
      A  M  N  D  Y  I  T  K  V  K
241   CAACTCTTGGAAGTTGCTGCTTCCAAGGCT
      Q  L  L  E  V  A  A  S  K  A
271   TCAACCTGATGAATCAAATCCTCATCTGCA
      S  T  *  *
301   GTAACTTTATCTTAAGCATCAAAATAACAT
331   TGCATAAGACTTGTTCTTTGCTCTTGTGTT
361   TCTATCATATTTAAGCTATCTTNCTTTGTC
```

CCTTGGA) of the gene.

The *acb*-1 start and stop codon are underlined above. Mutagenized bases used to facilitate cloning at the 5' end (*Nde* I site) or at the 3' end (*Bam*H I site) are in lower case. Heat denatured pSCC12T7P (50 ng) plasmid DNA, 25 pmol of each of the two primers, 20 nmol of dNTPs and 2.5 U of *Taq* polymerase in 100 µl 1 x *Taq* polymerase buffer were polymerized for 30 cycles of 1 min at 92° C, 1.5 min at 55° C and 1.5 min at 72° C. The amplification product was gel purified, blunt-ended using Klenow fragment and ligated into the *Eco*R V site of pBluescript using T4 DNA ligase and polyethylene glycol 4000 (15%) to enhance blunt-ended ligation.

After selection in DH5alpha and isolation of the correct recombinant plasmid, a *Nde* I - *Bam*H I digest was performed, and the insert bearing ACBP was gel purified. The fragment was then subcloned into either pET3a or pET15b that had also been digested with *Nde* I and *Bam*H I and was then transformed into *E. coli* BL21(DE3). Following verification of the correct recombinant plasmid (pRO412 or pRO414) in BL21(DE3), ten ml cultures (2 x YT media) were grown and induced with IPTG. Cells were lysed with 0.5% SDS, the extracts were clarified by centrifugation (10,000 x *g*, 10 min, 4° C) and soluble proteins were examined on SDS-PAGE mini-gels (15% polyacrylamide).

There was a prominent band at approximately 8 kDa in the case of cells carrying pRO412 (pET3a derivative) or pRO414 (pET15b derivative), whereas no band was visible in extracts from BL21(DE3) alone or cells carrying pET3a or pET15b. One L cultures were inoculated with cells carrying either pRO412 or pRO414, and at an OD_{600} of 0.5, the cultures were induced with IPTG. After 4 additional hours of growth (final OD_{600}

= 1.2), cells were spun down and resuspended in distilled water. Soluble proteins were obtained after repeated flash-freeze/thaw cycles. The recombinant ACBP (rACBP) represented approximately 30% of total soluble protein for a total yield of 40 mg ACBP/L culture.

Antibody preparation. The rACBP was partially purified using an initial ammonium sulfate (60%) precipitation (ACBP remains soluble) followed by a preparative polyacrylamide gel purification (16 cm x 20 cm gel, 16% polyacrylamide, 8.5 mg total protein loaded). Proteins separated by electrophoresis were transferred to nitrocellulose paper (0.2 µm pore size), then stained with 0.05% Ponceau Red. The readily visualized ACBP band was cut out. The yield in the entire 15-cm wide band was approximately 800 µg of ACBP (1.0 mg appeared to remain in the gel and 1.0 mg migrated through the first nitrocellulose filter onto a backup nitrocellulose filter).

Two 1.5-cm sections of the nitrocellulose (each containing 80 µg ACBP) were separately dissolved in 0.5 ml of methyl sulfoxide. Each one was either mixed with 0.5 ml of Hunter's TitreMax or 0.5 ml of complete Freund's adjuvant. These preparations were then injected subcutaneously into two separate rabbits, 0.1 ml at each site of injection, by the University Laboratory Animal Research (ULAR) Facility at Michigan State University. A booster shot containing an additional 40 µg was applied after 25 days. Antisera were collected at two-week intervals.

Antibody titre was determined using immunodot blot. An extract of BL21(DE3)/pRO412, induced to produce the recombinant ACBP, was diluted to 100 ng ACBP μl^{-1}; 1.0 µl then was spotted onto a nitrocellulose square (1.0 cm^2). Proteins transferred onto these squares were then fixed with 15% formaldehyde and blocked with 1 x blotto. Antisera were diluted from between 1:250 and 1:3000 with 1 x blotto. Two nitro-

cellulose squares were incubated with each primary antisera dilution. Secondary antisera consisted of goat anti-rabbit antisera conjugated to alkaline phosphatase, diluted 1:2000 in 1 x blotto.

To determine the sensitivity of the anti-ACBP antibodies, an extract of the recombinant *A. thaliana* ACBP that had been raised in *E. coli* was diluted from between 100 to 0.1 ng ACBP μl^{-1} and 1, 3 or 5 µl of each of these preparations was spotted on a strip of nitrocellulose filter paper, along with an extract of BL21(DE3) that did not contain pRO412 or pRO414 and a spot of distilled water (buffer control). Proteins were fixed and filters were blocked. These filters were incubated with primary antisera (1:1000 dilution) and secondary antisera (1:2000) as described above.

Protein purification. *E. coli* cells (BL21(DE3)/pRO412) were grown in 2 x 1 L flasks of 2 X YT media (containing ampicillin at 200 µg ml^{-1}) to an OD_{600} of 0.5 and then were induced with IPTG. After approximately 7 hours of additional growth, cells were pelleted (11,750 *g* for 10 min). Pellets were resuspended in distilled water to a total volume of 25 to 30 ml. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.8 m*M*. Cells were lysed using four freeze-thaw cycles with vortexing in between cycles. The cell lysate was frozen in liquid nitrogen until solid a final time, thawed quickly, and then heated to 55-60° C in a water bath.

Additional water was added (175 ml) and the cell debris was homogenized with a homogenizer (Brinkmann Instruments, Westbury, NY). Then, TCA was added with continuous homogenization to a final concentration of 5%. The mixture was left on ice for one hour and then was centrifuged at 16,300 *g* for 12 min at 4° C. The TCA pellet was

resuspended in 100 ml of 30 mM Tris-HCl, pH 9.0. A second TCA precipitation was performed, and the resulting pellet was resuspended in 30 m*M* Tris-HCl, pH 9.0. The pH was adjusted to 9.0 with 10 M NaOH before loading the solution onto a Q-sepharose fast-flow anion exchange column. The column was eluted with a linear gradient of 0-0.5 M NaCl in 30 m*M* Tris-HCl, pH 9.0. Fractions (10 ml each) were collected and analyzed for the recombinant ACBP by SDS-PAGE. Fractions with highly purified ACBP were pooled.

Binding Assay. The Lipidex-1000 binding assay is one means to demonstrate high affinity acyl-CoA binding (Rasmussen et al., 1990). The indicated amount of [1- ^{14}C]hexadecanoyl-CoA (specific activity 1.9 x 10^{12} Bq/mol or 50 mCi/mmol) was diluted to a final volume of 150 µl of binding buffer (10 m*M* potassium phosphate buffer, pH 7.4). Either ACBP, bovine serum albumin (BSA), or acyl carrier protein (ACP) was dissolved in 50 µl of binding buffer to a concentration of 0.8 µ*M*. ^{14}C-palmitoyl-CoA and protein samples were mixed (final protein concentration was 0.2 µ*M* while acyl-CoA concentrations were 0.1, 0.2, 0.3, 0.4, and 0.5 µ*M*), incubated at 37° C for 30 min, then chilled on ice for 10 min. Samples were mixed with 400 µl of ice-cold 50% slurry of Lipidex-1000 in binding buffer. After 20 min on ice, the samples were centrifuged (12,000 *g*) for 5 min at 4° C. A 200 µl aliquot of the supernatant was removed and counted for radioactivity. Replicas and controls were according to Rasmussen et al. (1990).

We also devised a novel binding assay using native 10% PAGE to test partially purified extracts for binding of [^{14}C]-oleoyl CoA. Exactly 0.5 nmol of rAthACBP was incubated with 0.5 nmol of [^{14}C]-oleoyl CoA (specific activity 1.9 x 10^{12} Bq/mol or 55,000 dpm total) in a volume of 20 µl for 30 min at 4° C. Immediately after incubation, 8 µl

of 4 x native PAGE loading buffer was added to each sample, and the entire mixture was loaded onto a native discontinuous polyacrylamide (10%) gel. The proteins were separated, the gel was dried and autoradiography was performed.

Acyl-CoA hydrolysis protection assay. Hydrolysis of acyl-CoA was measured according to the following protocol. Incubation of 20 µ*M* [^{14}C]-oleoyl-CoA (50m Ci/mmol; 2000 pmol) was carried out with safflower microsomes (176 µg total protein) in 0.1 *M* phosphate buffer, pH 7.2, in a total volume of 100 µl for 3 or 6 min at 27° C. BSA or ACBP were at a final concentration to 20 µM. Reaction was terminated by addition of 500 µl of chloroform:methanol:water (50:50:1) and lipid extraction (Bligh and Dyer, 1951) was performed. Fifty µl of the total lipid extract was spotted on a silica gel TLC plate, along with free fatty acid standards. The TLC plate was developed in chloroform:methanol:acetic acid:water (85:15:10:3.5) and visualized with iodine. Free fatty acid spots were scraped and then counted using liquid scintillation.

Protein extracts. Extracts were prepared from various tissues of *Arabidopsis thaliana*, canola, safflower, soybean, tobacco, coriander, and *Cuphea* using an extraction buffer that contained 50 m*M* Tris-HCl, pH 7.0, and 1.0 m*M* phenylmethylsulfonyl fluoride. Protein concentration was determined by Bradford (1976) assay. Immature rapeseed embryos at specific developmental stages were collected (2 to 45 days after flowering, DAF). Extracts were prepared and protein was quantified. ACBP levels were estimated on Western blots where either 90, 45, 30 or 15 µg of total protein had been added to each lane.

Western blot analysis. Protein (15 to 90 µg total) was denatured in SDS/ß-mercaptoethanol and was applied to a 16% or 20%

SDS-polyacrylamide gel. Following semi-dry transfer to nitrocellulose, bands were visualized using a 1:1400 or 1:1800 dilution of the primary antisera and a 1:2000 dilution of the alkaline phosphatase-conjugated goat anti-rabbit antiserum. ACBP band intensities of plant extracts were compared to *E. coli* recombinant ACBP standards.

RESULTS

The *A. thaliana* cDNA contains only one open reading frame that can encode a polypeptide of no more than 18 amino acids. This clone contains appropriate 5' sequences for ribosome binding (Figure 6) and efficient translation (Kozak, 1989) in plants (A at -3 and G at +4).

At -40 bp, there is an in-frame stop-codon (underlined, Figure 6), indicating that this cDNA sequence could not encode an acyl-CoA-binding domain of a larger polypeptide. There is also a tandem stop-codon at base pairs 277 to 282. Alignment of the bovine and human ACBP amino acid sequences with the derived amino acid sequence from the *A. thaliana* ACBP, indicates the sequence identity between *Arabidopsis* ACBP and bovine or human ACBP was 57% or 49%, respectively (Figure 1).

The coding sequence of pSSC12T7P was subcloned into *E. coli* expression vectors pET3a and pET15b. After induction with IPTG, a major protein band was detected at 8 kDa. Based on the cDNA sequence, the *A. thaliana* ACBP contains 92 amino acids (Figure 6), encodes a polypeptide of 10,385.75 kDa (including the N-terminal methionine), but migrates in SDS-PAGE with an apparent MW of only 8.2 kDa. This anomalous migration of ACBPs in SDS-PAGE was observed first for bovine ACBP by Mogensen et al. (1987). The mature plant protein is likely to contain only 92 amino acids, which would have a molecular weight of 10,236 kDa since the N-terminal methionine is probably removed, as is the case in all animal

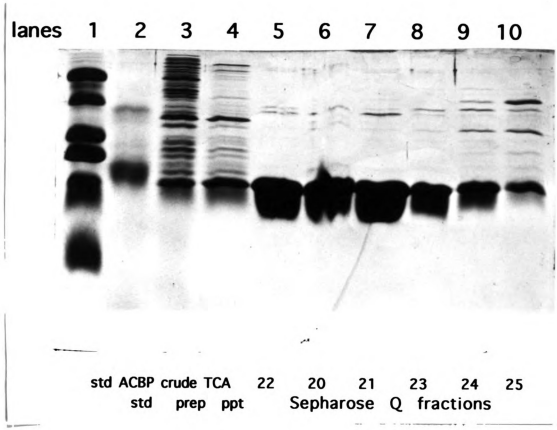


Figure 7. SDS-PAGE of proteins from ACBP purification steps. Lane 1, low molecular weight standards; lane 2, 200 ng ACBP standard; lane 3, initial supernate from BL21(DE3) debris; lane 4, protein following two TCA precipitations; lane 5, Q-sepharose fraction 22; lane 6, Q-sepharose fraction 20; lane 7, Q-sepharose fraction 21; lane 8, Q-sepharose fraction 23; lane 9, Q-sepharose fraction 24; lane 10, Q-sepharose, fraction 25.

(Knudsen, 1992) and yeast (Rose et al., 1992) ACBPs. The estimated isoelectric point from the derived amino acid sequence is 5.04.

Sequence comparisons suggested clone pSSC12T7P encoded a plant ACBP. To obtain more direct evidence for this assertion, several methods were used to examine the ability of the protein encoded by pSSC12T7P to bind acyl-CoA.

ACBP purification. The putative rAthACBP was purified to near homogeneity. Two successive TCA precipitations significantly enriched the rACBP from BL21(DE3) cells that expressed the protein (Figure 7, lanes 3 and 4), while the *Arabidopsis* ACBP in fractions 20 through 23 from Q-sepharose anion exchange chromatography were over 95% pure (Figure 7, lanes 6 through 8).

Mobility assay. Binding activity of rAthACBP (pET3a-rACBP) or rAthACBP-polyHis (rAthACBP fused to a poly-His tract; pET15b-rACBP) was examined by native PAGE (Figure 8). Labeled [^{14}C]-oleoyl-CoA remained bound to rAthACBP or rAthACBP-polyHis during and after native gel electrophoresis. The mobility of the radioactive bands in native (10%) PAGE matches that of the rAthACBP and rAthACBP-polyHis as visualized by Coomassie-blue staining (data not shown).

The rAthACBP-poly-His fusion protein was longer by six His residues (98 amino acid residues rather than 92 residues) and contained six or more positive charges more than rAthACBP. The shift towards a more basic protein and the greater MW (7.5% of total) explained the difference between the original rAthACBP and the fusion protein (Figure 8).

Lipidex-1000 binding. The ability of the purified rACBP (fraction 22, Figure 7, lane 5) to bind acyl-CoA was demonstrated with the Lipidex-1000 assay. For comparison, bovine serum albumin and acyl-carrier protein (ACP) were also tested. BSA has a demonstrated

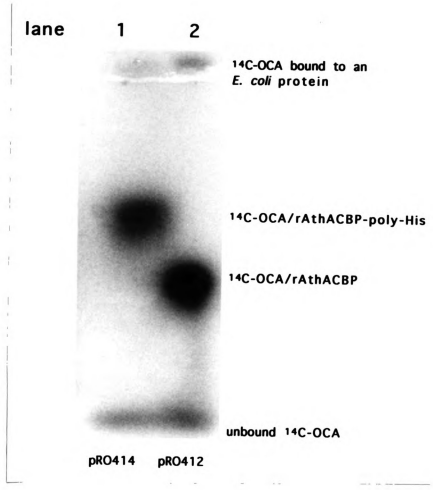


Figure 8. The activity of the rAthACBP in a crude protein extract from *E. coli* BL21(DE3) cells is illustrated in this autoradiogram of a native gel (10% PAGE) where a crude protein extract containing rAthACBP protein capable of binding [^{14}C]-oleoyl-CoA. Lane 1, rAthACBP-polyHis (from BL21/pRO414); lane 2, rAthACBP (from BL21/pRO412).

Hexadecanoyl-CoA bound (mol/mol)

Figure

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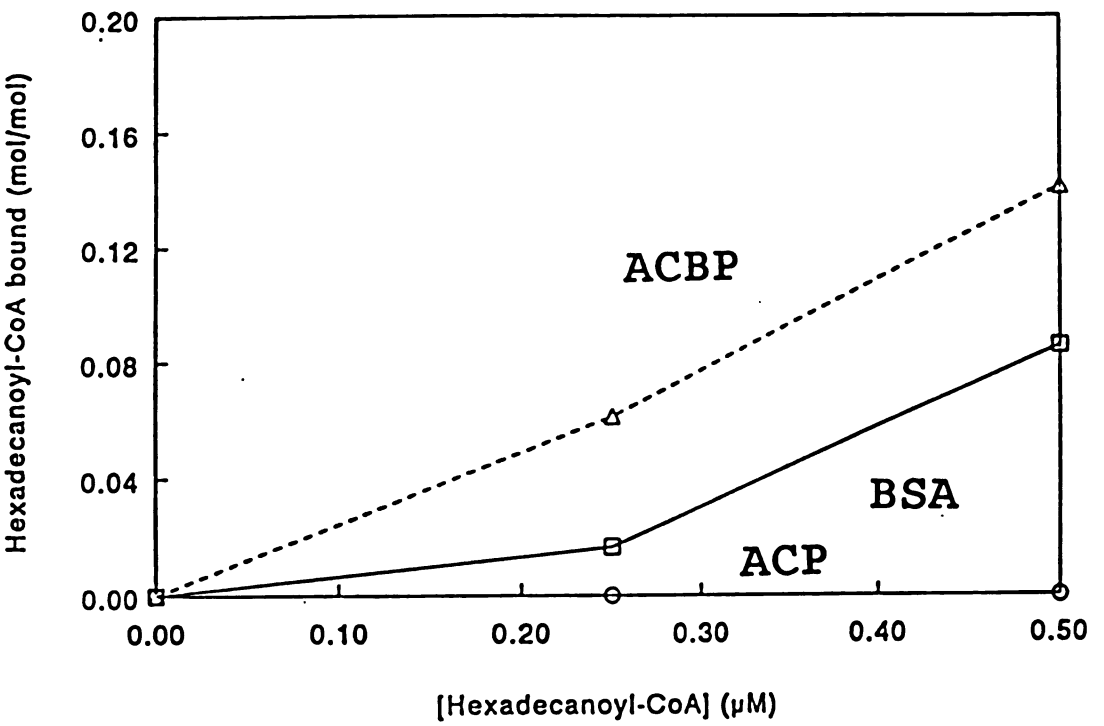

Figure 9. Demonstration of comparative binding of oleoyl-CoA esters to rAthACBP (triangles), bovine serum albumin (boxes) and acyl-carrier protein (circles) by the Lipidex-1000 assay. Results represent the means of triplicate analyses.

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ability to bind acyl-CoA (Mogensen et al., 1987), thus serving as a positive control. ACP however does not bind acyl-CoA and thus was included as a measure of non-specific binding. Both ACBP and BSA were effective at binding [^{14}C]-palmitoyl-CoA at low μM concentration ACP did not bind palmitoyl-CoA to any appreciable extent (Figure 9). There was an increase in amount of ligand bound as [^{14}C]-palmitoyl-CoA concentration increased for both the ACBP and BSA, as one might expect. Lipidex data are presented here (Figure 9) in order to demonstrate binding of acyl-CoA by ACBP. Because of the competitive binding between ACBP and Lipidex for acyl-CoAs (i.e., ACBP can extract bound acyl-CoA from Lipidex at 0° C) interpretation of Lipidex binding data is complex (Miller and Cistola, 1993), yet can be a guide for assessing relative binding (Rosendahl et al., 1993). Future experiments using microcalorimetry will be designed to accurately determine binding affinities.

Acyl-CoA hydrolysis protection. Acyl-CoA hydrolysis assays were conducted using safflower microsomal preparations. There is usually enough ACBP present in cells to bind the acyl-CoA present and in animal systems, ACBP has been found to have direct impact on formation and maintenance of acyl CoA pools> For these reasons, it has been proposed that one function of ACBP is to protect against acyl-CoA hydrolysis by the hydrolytically active microsomal membranes (Rasmussen et al., 1993). The effectiveness of rAthACBP on protection of acyl-CoAs from hydrolysis was compared to that of BSA in the safflower microsomal system. Purified rAthACBP protein and BSA were both effective at protecting acyl-CoA from hydrolysis (Figure 10). ACBP had a slightly higher protective effect than BSA (approximately 30% less oleoyl-CoA hydrolysis). ACBP and BSA decreased acyl-CoA hydrolysis by over 75%.

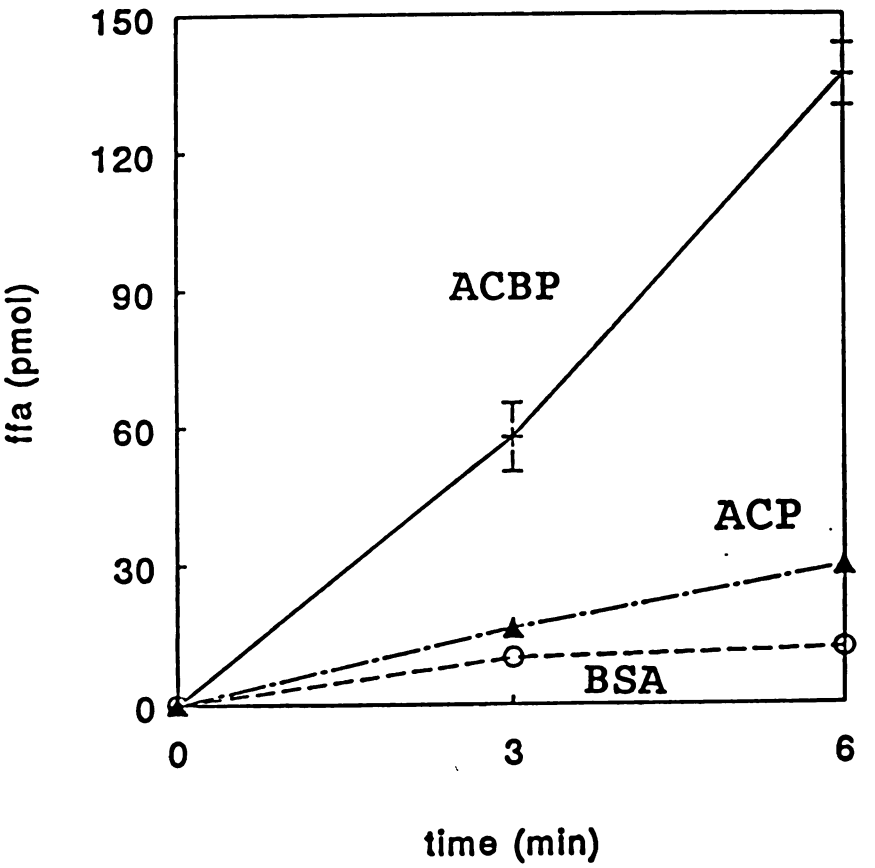

Figure 10. Acyl-CoA acyl-hydrolase protection results. Incubation of ^{14}C-oleoyl-CoA with microsomal preparations either containing no protein (crosses), BSA (triangles) or ACBP (circles).

Free acyl-CoAs can incorporate into membranes, the result of which may be quite deleterious to membrane-associated enzymatic activities and other membrane functions (Figure 10). These *in vitro* assays demonstrate the effectiveness of recombinant plant ACBP at preventing acyl-CoA hydrolysis as had been demonstrated previously for recombinant bovine ACBP (Rasmussen et al., 1993). By analogy, another ACBP function may be to protect membranes against acyl-CoA incorporation.

Tissue specificity. Anti-ACBP antibodies were used to examine the distribution of ACBP in plant tissues. The titre and specificity of the anti-ACBP antibodies were determined using immunodot blots and Western blotting. A 1:2000 dilution of the anti-ACBP antiserum resulted in visualization of 4 to 5 ng of the recombinant ACBP, whereas a dilution of 1:1200 allowed the visualization of as little as 1.0 ng of ACBP (data not shown).

Extracts of *A. thaliana, B. napus*, safflower, soybean and tobacco (mostly from seed or silique) were separated by SDS-PAGE and transferred to nitrocellulose. Anti-ACBP antibodies were used to develop this blot. Extracts of *A. thaliana, B. napus*, and safflower seed contained cross-reacting bands with mobilities similar to the *Arabidopsis* ACBP standard (data not shown). To a lesser extent, coriander and tobacco proteins with the correct MW were able to cross-react with the anti-ACBP antibodies. The intensity of the signal for extracts from *Arabidopsis* was estimated to represent between 0.18 to 1.25 μg (mg protein)$^{-1}$ or 3.1 to 142.5 μg g^{-1} FW (Table 2). A rough estimate of ACBP concentration in *A. thaliana* is between 0.02 - 0.13% of soluble protein, or 0.30 to 13.9 nmol g^{-1} FW. Taking into account the moisture content of these tissues, this was a cellular concentration of 0.36 to 46.4 μ*M* AthACBP (Table 2).

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Table 2. Acyl-CoA-binding protein (ACBP) concentrations in various tissues of *Arabidopsis thaliana* or *Brassica napus* from Western blots using anti-*A. thaliana* ACBP antibodies.

plant/tissue	ACBP conc [ng (mg prot)$^{-1}$]	tissue ACBP levels [µg (g FW)$^{-1}$]	cellular ACBP conc [nmol (g FW)$^{-1}$]	cellular ACBP conc [µM]*
Arabidopsis thaliana				
root	220	3.1	0.36	0.31
leaf	180	5.6	0.54	0.68
silique	540	29.7	2.93	3.54
seed	1250	142.5	13.9	46.4
Brassica napus				
root	150	2.6	0.25	0.29
leaf	240	8.3	0.81	1.03
flower	390	17.2	1.69	1.91
silique	510	31.6	3.08	3.74
seed	1620	205.2	20.03	65.72

*Cellular concentrations based on the water content of the various tissues. It was assumed tht 100% of cellular water was in the cytoplasm, an obvious overestimation. The actual cellular ACBP concentration is likely to be higher than the values presented here.

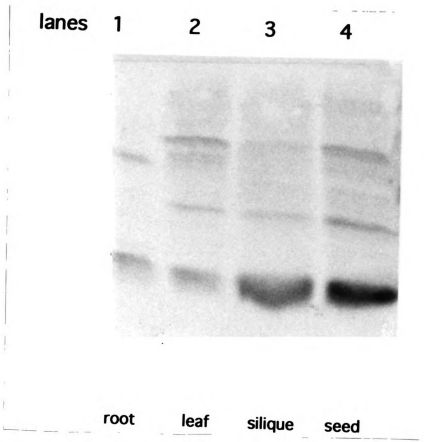

Figure 11. Tissue specificity of ACBP in *Brassica napus* (visualized by Westerns). Lane 1, *B. napus* (root); lane 2, *B. napus* (leaf); lane 3, *B. napus* (flower); lane 4, *B. napus* (silique).

Extracts were examined from root, leaf, flower, silique and seed tissues from *B. napus* (Figure 14). ACBP was detected in all tissues of *B. napus*, as was the case for *A. thaliana*. *Brassica* root tissue had the lowest ACBP concentration (2.6 μg g^{-1} FW or 0.25 nmol g^{-1} FW), while leaf, flower and silique contained concentrations of ACBP estimated to be between 8.3 to 205 μg g^{-1} FW or from 0.81 to 20 nmol g^{-1} FW, which amount to cellular concentrations between 1 and 66 μ*M* (Table 2). The concentrations that are reported here for *B. napus* represent the lowest possible concentrations since the anti-*Arabidopsis* ACBP antibodies will not cross-react with as much intensity with the *Brassica* ACBP.

Rapeseed ACBP levels varied from 20 to 250 μg g^{-1} FW depending on the developmental stage (Figure 15). Although fully mature rapeseed contained only 132 μg ACBP g^{-1} FW (or 14 nmol g^{-1} FW), mid- to late-maturity rapeseed had 250 μg g^{-1} FW (or 26 nmol g^{-1} FW), the highest concentration in any plant tissue measured so far (approximately 65 μ*M* ACBP or 0.23% of total protein). This concentration of ACBP corresponded to that in animal liver (rat liver 24-40 nmol g^{-1} FW), the richest source of ACBP in animals (Knudsen et al., 1993). The increase in ACBP levels preceded the general increase in protein levels (Figure 15) that is seen during seed maturation (Crouch and Sussex, 1981). Due to the difficulties involved in measuring acyl-CoA concentration, these ACBP concentrations (8 to 60 μ*M*) can only be compared to acetyl-CoA levels in the chloroplast (30 to 50 μ*M*; Post-Beittenmiller at al., 1992).

DISCUSSION

In vivo fatty acid biosynthesis occurs principally in the plastids of plant cells (Ohlrogge et al., 1979), while the enzymes

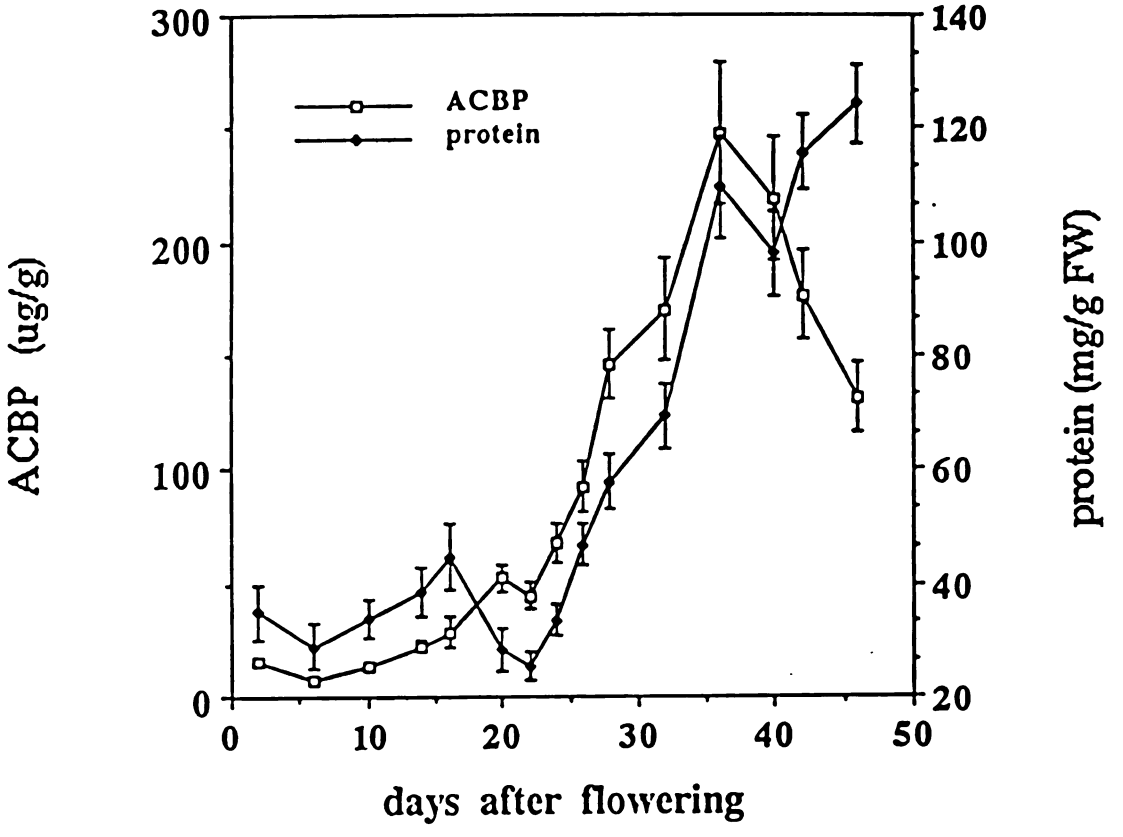

Figure 12. Ontogeny of protein content (mg protein/g FW) and ACBP content (µg ACBP/g FW) in developing *Brassica* embryos. At 2 DAF and at 10 DAF developing seeds could not be separated from flower or silique tissue.

involved in the synthesis of non-plastidial membrane lipids and triacylglycerol (TAG) are localized in the endoplasmic reticulum. Therefore, an intracellular lipid transfer molecule may be required to mediate the transport of acyl-CoAs from the plastid to the endoplasmic reticulum.

The *A. thaliana* ACBP characterized in the present paper may be such a molecule. This protein, bound acyl-CoA (Figure 8), was found in all plant tissues examined (Figure 11). This would be expected of a housekeeping protein, as would be its wide distribution in nature. Furthermore, the increase in expression that was seen in developing *B. napus* seeds (Figure 12) was approximately coincident with TAG accumulation (Taylor *et al.*, 1991) suggesting that this molecule may be involved in providing acyl-CoA substrates for storage lipid synthesis. Cell-type specific expression of ACBP in developing oilseeds suggests that ACBP has a dual nature: it serves as a housekeeping protein in all cell types, but it has also been recruited for additional functions in those cells where lipid biogenesis or utilization is extreme. It would be useful to compare ACBP content (16-170 pmol ACBP mg^{-1} protein) to the size of the acyl-CoA pool. However, measurements of plant acyl-CoA levels are unreliable and new methodologies for acyl-CoA quantification have yet to be developed (Kopka et al, 1995). Perhaps a more definitive demonstration of the role of this protein in lipid biosynthesis awaits overexpression and antisense studies in transgenic plants.

After searching the Genbank database using the TBLASTN program, additional presumptive plant ACBPs were found (Figure 3). The five potential ACBP sequences can be obtained from Genbank directly using the following accession numbers: thale cress (*Arabidopsis thaliana*) T04081, rapeseed (*Brassica napus*) X77134, cotton (*Gossypium hirsutum*) U35015,

rice (*Oryza sativa*) D28303, castorb bean (*Rinculus communis*) T24230 and maize (*Zea mays*) T25215. Of these five sequences, only that of rapeseed was deliberately sought; the *Arabidopsis* (Newman et al., 1994), rice (Uchimiya et al., 1993) and maize (Keith et al., 1992) mRNA were reported after sequencing random cDNAs as part of genome projects. These ACBP cDNAs were found within the first 2000 clones selected at random, which is consistent with an ACBP mRNA abundance greater than that of 0.05%.

Alignment of the plant ACBPs indicated a high degree of homology (Figure 3). No insertions were required to align the different plant ACBP which strongly suggests that the secondary structural elements and the overall fold of this protein were strongly conserved among the different plant species. There was 59% sequence identity among the plant ACBP sequences, which was very similar to the conservation among the animal homologues (Knudsen 1993). Kragelund et al. (1993) characterized the three-dimensional structure of the complex between the bovine ACBP and palmitoyl-CoA. ACBP has been characterized as a 4-helix protein. Interestingly, the plant sequences display more sequence identity in the C-terminal portion of the protein (helix 4) than the corresponding animal sequences, where there is higher sequence identity in the N-terminal portion (helix 1).

All four residues identified by Kragelund et al. (1993) as involved in hydrogen bonds or strong electrostatic interactions with the 3' phosphate of CoA (Tyr28, Lys32 and Lys54; numbered here as Y_{30}, K_{34}, and K_{56}) or participating in the coordination to adenine (Tyr-73, numbered Y_{75} in plant ACBP) were conserved (Figure 3). When acyl-CoA was bound to ACBP, the omega-end of the acyl chain lay between helix 2

and 3 and was protected from solvent (Figure 2) by the phenyl ring of Phe-49 (Kragelund et al., 1993), a function that F_{49} likely serves in the plant ACBPs. Similarly, there was an invariant L_{17} followed by proline or threonine (P_{18} or T_{18}), which appeared to correspond to Lys-18 and Pro-19 in the animal ACBPs. In the bovine ACBP, these residues were perturbed by the binding of the thioester carbonyl in acyl-CoA.

In animal ACBP (Figure 2), three residues form H bonds either to adenine (Ala-9), pantotheine (Lys-18) or ribose (Lys-32). K_{17} and K_{34} are conserved in the all plant ACBPs, but in only 3 of 5 plant ACBPs was there A_{11}. Of interest is the strong conservation of amino acid residues in helix 4 (D_{74}YITKVKQL_{82}), the helix that has the least number of contacts to acyl-CoA. Only Tyr75, which moves between helix 1 and 2, is involved in substrate binding (Kragelund et al., 1993). This tight conservation of sequence in the plant ACBPs may imply that this motif is involved in protein-protein interactions *in situ*, such as when ACBP "docks" with an acyl-CoA synthetase or an acyl transferase. Helix 4 undergoes the greatest amount of conformational shift upon substrate binding, suggesting that the area of molecular recognition between proteins and ACBP is remarkably dependent on the absence or presence of substrate.

CONCLUSIONS

The putative *A. thaliana* ACBP shows sequence homology to the animal ACBPs and binds acyl-CoA. Antibodies raised against the *Arabidopsis* cDNA gene product recognize polypeptides in all the *Arabidopsis* tissues examined and in a number of other plant species as well. ACBP increases markedly in developing *B. napus* seeds at a stage

of ontogeny that is coincident with TAG biosynthesis. From these results, we can infer that ACBP is capable of mediating acyl-CoA availability within plant cells, and it likely is involved in intermembrane transport, glycerolipid biosynthesis, and ß-oxidation, as in animal cells (Rasmussen et al., 1994). A comparison of the thermodynamic properties of the rAthACBP and a comparison with bovine ACBP is currently in progress.

Acknowledgments

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In addition, the following insight by Wilhelm Reich was appreciated as I put this chapter together. "Functional thinking does not tolerate static conditions. For it, all natural processes are in motion. Nature, too, flows in every single one of its diverse functions as well as in its totality."

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CHAPTER 3

INTERACTIONS BETWEEN THE *Arabidopsis thaliana* ACYL-COENZYME A-BINDING PROTEIN AND ACYL-CoAs: BINDING AND THERMODYNAMIC PROPERTIES

Abstract

The thermodynamics of a number of different acyl-CoA esters binding to recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP) were analyzed using titration microcalorimetry and van't Hoff analysis. The binding affinity of acyl-CoAs for rAthACBP increased with acyl chain length with C_{20}-CoA having the tightest binding, although the differences between the binding of acyl chains differing by 4 carbon atoms or less was not substantial. The overriding contribution to the free energy (ΔG^o) decrease upon binding of ligand to rAthACBP was enthalpic (ΔH^o) rather than entropic ($T\Delta S^o$). Compared to bovine or yeast ACBP, the plant ACBP had a lower affinity for acyl-CoAs of the same chain length at 27° C. Unsaturated or monohydroxylated acyl-CoAs bound to the rAthACBP with similar binding constants. van't Hoff analysis using octoyl-CoA, lauroyl-CoA and oleoyl-CoA indicated that the optimum binding temperature was approximately 15° C. Binding of acyl-CoA esters decreased as the temperature was raised, mainly by decreasing the temperature-dependant entropic contribution. Gibbs free energy change for ligand binding was favorable at all temperatures studied and was only weakly temperature dependent. The change in the specific heat capacity (ΔC_p) was relatively large (-948 J mol^{-1} K^{-1}), probably due to

a preponderance of hydrophobic interactions, which leads to a rather strong enthalpy-entropy compensation. We propose that the binding of acyl-CoA to ACBP was driven by enthalpy, but the release of acyl-CoA from the complex was driven by entropy. These data would indicate that the *Arabidopsis* ACBP does not display a marked discrimination or a clear preference for any C_{16}- to C_{20}-acyl-CoA. To my knowledge, this is the first time that the force behind the release of substrate has been attributed to entropy.

INTRODUCTION

In animal systems there is good evidence that the protein that transports and can donate acyl-CoAs for both ß-oxidation and glycerolipid biosynthesis is the acyl-CoA-binding protein (ACBP; Rasmussen et al., 1994). ACBP has been found in all eukaryotic cells examined (Knudsen et al., 1993). The murine ACBP gene has all of the hallmarks of a housekeeping gene (Mandrup et al., 1992).

Many animal cells use circulating triglycerides as an energy source. Through the action of lipases, cells must be prepared to cope with large influxes of free FAs, usually completely saturated with 16 to 20 carbon atoms in the acyl chain (Knudsen et al., 1993). Free FAs were quickly converted to acyl-CoAs. Typical acyl-CoA concentrations were reported between 25 and 90 μM, and the corresponding ACBP concentrations ranged between 20 and 100 μM (Rasmussen et al., 1993).

The situation is markedly different in plants. As long as photosynthesis continues, ß-oxidation is negligible and unnecessary, even in non-photosynthetic tissues, such as the root. The carbon

source, most abundant in the plant vasculature, is sucrose (Nolte and Koch, 1993). Use of FAs for ß-oxidation, and more importantly, the glyoxylate cycle and gluconeogenesis usually has been limited to seedling germination and senescing tissues (Harwood, 1988). Therefore, mature plant vegetative tissue maintains a low free FA or free acyl-CoA concentration. The levels of these metabolites is based largely on slow fatty acid turnover. In transgenic yeast overexpressing bovine ACBP, there was a significant increase in acyl-CoA pool size (Mandrup et al., 1993). By comparison, long-chain acyl-CoA levels are difficult to measure in plants (Kopka et al., 1995), but if we assume that the concentrations of acyl-CoAs are similar to cellular ACBP concentration, then the acyl-CoA concentration in vegetative tissues may range between 0.7 and 5 μM, while developing mid-maturity oilseeds may contain up to 50 μM acyl-CoA (Chapter 2, thesis).

Titration calorimetry has been used to obtain binding constants and thermodynamic parameters for FABP, other lipid binding proteins (Miller and Cistola, 1993), and ACBP (Rasmussen et al., 1994). Earlier studies used Lipidex-1000 to measure acyl-CoA binding to ACBP, but this method has been shown to give only relative values for binding (Volk et al., 1990). An important feature of this method is its ability to determine binding constants in a matter that does not perturb the system's equilibrium. Another unique feature of the assay is its ability to quantitate both enthalpic and entropic contributions to the binding reaction (Jin et al., 1993). Similarly, it is possible to distinguish the stoichiometry of binding, the classes of binding sites and certain binding interactions, such as coopertivity using titration microcalorimetry (Miller and Cistola, 1993).

The unique tertiary structures of proteins reflect the strong

stabilizing influence of hydrophobic interactions (Stowell and Rees, 1995). Similarly, we expect that hydrophobic interactions will influence the binding of a ligand with a strongly hydrophobic epitope such as acyl-CoA and will provide a large contribution to the stability of the ligand-protein complex (Mizutani et al., 1994). Sources of such stability are still uncertain: is the ligand-free state unstable due to partial denaturation, a more relaxed secondary structure, solvent effects or packing interactions (Murphy and Freire, 1992)?

In *Arabidopsis thaliana*, a cDNA that encoded a putative ACBP was isolated containing an open reading frame capable of generating a cytosolic protein consisting of 92 amino acid residues (Chapter 2, thesis). Expression of this gene in *E. coli* produced a protein that was capable of specifically binding acyl-CoA and protecting this ligand from thioesterase hydrolysis. This protein, a recombinant *A. thaliana* ACBP (rAthACBP) was purified by ion exchange and HPLC, and subsequently used in these titration microcalorimetery experiments.

Our objective was to examine the interaction between the recombinant *A. thaliana* acyl-CoA-binding protein and acyl-CoA esters by measuring the binding constants and thermodynamic properties using microcalorimetry.

MATERIALS AND METHODS

Bacterial strain. The *Escherichia coli* strain used to produce the recombinant *Arabidopsis* ACBP was BL21(DE3) using the pET3a (Studier and Moffatt, 1986) expression system (Chapter 2, thesis).

Chemicals. Non-esterified fatty acids were from Sigma Chemical Co. (St. Louis MO, U.S.A.) or Larodan Fine Chemicals (Malmø, Sweden). Coenzyme A was from Pharmacia (Uppsala, Sweden). Ammonium acetate for

preparation of the buffer and other chemicals used in acyl-CoA biosynthesis were from Merck (Darmstadt, Germany). Solvents were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland) and were HPLC grade.

Preparation of recombinant *Arabidopsis* ACBP. Recombinant *A. thaliana* ACBP was produced in BE21(DE3) cells harboring pRO412 as described previously (Chapter 2, thesis). Fermentation of the bacteria and induction of the recombinant *acb* gene was according to Sambrook et al. (1989). Purification of the protein was carried out according to Mandrup et al. (1991). The *Arabidopsis* ACBP was first redissolved in distilled H_2O, and later the concentration was adjusted to 0.025 m*M* in 25 m*M* NH_4OAc, pH 6.0. Protein concentration was determined by reading the absorbance at 280 nm and using an extinction coefficient of 15.520 M^{-1} cm^{-1}.

Synthesis of acyl-CoA esters. Short-chain acyl-CoAs (C_2-C_6) were synthesized and purified from commercially available acyl anhydrides (Stadtman, 1957). Acetic, butyric or caproic anhydride (43 µmol) were each dissolved in *tert*-butanol (0.5 mL). Next, 0.5 mL of a solution of the lithium salt of coenzyme A (CoA-S^- Li^+, 6.2 µmol, 5.0 mg) in 0.2 *M* $KHCO_3$ or 0.2 *M* $NaHCO_3$ were added. After 30 min at room temperature, 2 *M* HCL was added to the mixture until pH was between 4 and 5. Acetyl- , butyryl-, or hexanoyl-CoA were separated from either lithium acetate, lithium butyrate or lithium caproate and subsequently purified by HPLC (solution A: 80% acetonitrile and 20% 25 m*M* NH_4OAc, pH 5.3 and solution B: 20% acetonitrile and 80% 25 m*M* NH_4OAc, pH 5.3 using a gradient of 10 to 70% solution A over 40 minutes. Products and reactants were monitored using an ultraviolet detector (260 nm).

Medium (C_8-C_{14})- and long (C_{16} -C_{20})-chain acyl-CoAs were produced and purified from mixed anhydrides according to Rosendahl *et al.* (1993). Briefly, 200 µmol of the appropriate acid was dissolved in methylene chloride (1.0 mL) and triethylamine (28 µl, 200 µmol) was slowed added while stirring for 30 min at room temperature. The solution was then cooled to 0° C and kept at this temperature for 60 min. Methylene chloride was removed using dry N_2 and the crude product was dissolved in 3.0 mL of *tert*-butanol. Half of this solution (1.5 mL) was added to 1.5 ml solution of Coenzyme A lithium salt (30 µmol, 24.3 mg) in 0.27 *M* $KHCO_3$. The reaction mixture was stirred for 30 min at room temperature and then 2.0 *M* HCl was added until the pH was equal to 4.0. Crude product was separated from the reactants and purified by HPLC using the conditions stated above.

Very long-chain acyl-CoA esters (C_{22}-C_{24}) and highly unsaturated acyl-CoAs were produced using the technique of Kamijo *et al.* (1984). Ricinoleoyl-CoA was synthesized using the Stobart and Stymne method (Smith et al., 1992). The concentration of the various CoA esters was determined spectrophotometrically by measuring the absorption at 260 nm and using an extinction coefficient of 14.700 M^{-1} cm^{-1}.

Microcalorimetry. An Omega titration microcalorimeter (MicroCal Inc., Northampton, MA, U.S.A.) which was routinely calibrated using standard electrical pulses was used to measure the binding of ligand to ACBP. Both the ligand and the ACBP were dissolved in 25 m*M* NH_4OAc, pH 6.0, a buffer appropriate to ACBP-binding studies (Færgeman et al., 1996). The protein solution, at a concentration of *c.* 0.025 m*M* (or 0.25 mg ACBP/ml) was placed in the sample cell (1.68 mL) at 27° C (300 K) and was stirred at 400 rpm until thermal equilibration with the

reference cell (0.02% sodium azide) was reached. The baseline was monitored for 10 min and then 4 μL of ligand solution (usually 0.5 m*M*) was added every 2.5 min for the next 75 min (30 injections total). There were at least 3 to 4 replicate measurements for the binding of each acyl-CoA to rAthACBP at each temperature, and over 40 mg ACBP were used to complete the work reported here.

Thermogram data were analyzed using methods developed by Wiseman et al. (1989) after peak integration using Origin software (MicroCal, Inc.). This provided binding constants and enthalpy information. Isotherms were also fit to appropriate models to obtain other thermodynamic quantities (in the differential mode) using software originally developed by the National Research Council of Canada and the Carlsberg Research Laboratories (Sigurskjold et al., 1991).

Temperature effects. For purposes of comparison of the rAthACBP with bovine rACBP, much of the calorimetry was performed at 27° C, a temperature that is higher than that normally encountered by the early spring weed *Arabidopsis thaliana*. Three van't Hoff series were carried out by examining the binding of $C_{8:0}$-CoA, C_{12}-CoA or $C_{18:1}$-CoA to rAthACBP in 5 degree intervals between 285 and 305 K. (12° to 32° C). The slope of the dependence of ΔH^{o} on T provides a convenient mechanism for estimating ΔC_p for ligand binding.

The temperature dependence of ΔH^{o} yielded the heat capacity change for the reaction, ΔC_p, and this was calculated for each of the three ligands used. Many proteins display an interesting property as the ambient temperature is raised. With increases in temperature, the contribution of enthalpy (ΔH^{o}) to the free energy change increases, while the entropy ($T\Delta S^{o}$) decreases in a near one-to-one ratio. This

property, known as an entropy/enthalpy compensation (Varadarajan et al., 1992) was measured for the binding of rAthACBP with $C_{18:1}$-CoA, $C_{12:0}$-CoA and $C_{8:0}$-CoA.

The van't Hoff equation is usually integrated to give a linear form, and if enthalpy depends linearly on temperature while the change in heat capacity is temperature-independent, then the van't Hoff equation can be written in the form:

$$\ln K = \ln K_r + \Delta C_p [-(1 + \ln T_r) + T_r(1/T) - \ln(1/T)],$$

where K_r is the binding constant at T_r. (Note the reference temperature has been chosen so that $\Delta H_r = 0$.) Average association constants were plotted as a function of temperature, and ΔH^o and ΔC_p were determined from the following equations:

$$\ln K = -\Delta H^o/RT + \Delta S^o/R \text{ ; and}$$

$$\ln K = (\Delta C_p/R) [T_H/T - \ln(T_S/T) - 1],$$

where T_H and T_S are the temperatures at which ΔH^o and ΔS^o are zero, respectively (Ha *et al.*, 1989).

RESULTS

Saturated acyl-CoA binding series. Raw data from a typical microcalorimetry run (Figure 13A) and the integrated heat per injection (Figure 13B) were used to determine the thermodynamic quantities that are reported. Curve fitting of results in this format are used to calculate the values for ΔH^o, ΔG^o, and $T\Delta S^o$. From these quantities, K (the association constant) can be calculated from ΔG^o by using the Nernst equation and ΔS^o can be obtained from $T\Delta S^o$.

Calorimetry will measure all of the enthalpy changes that

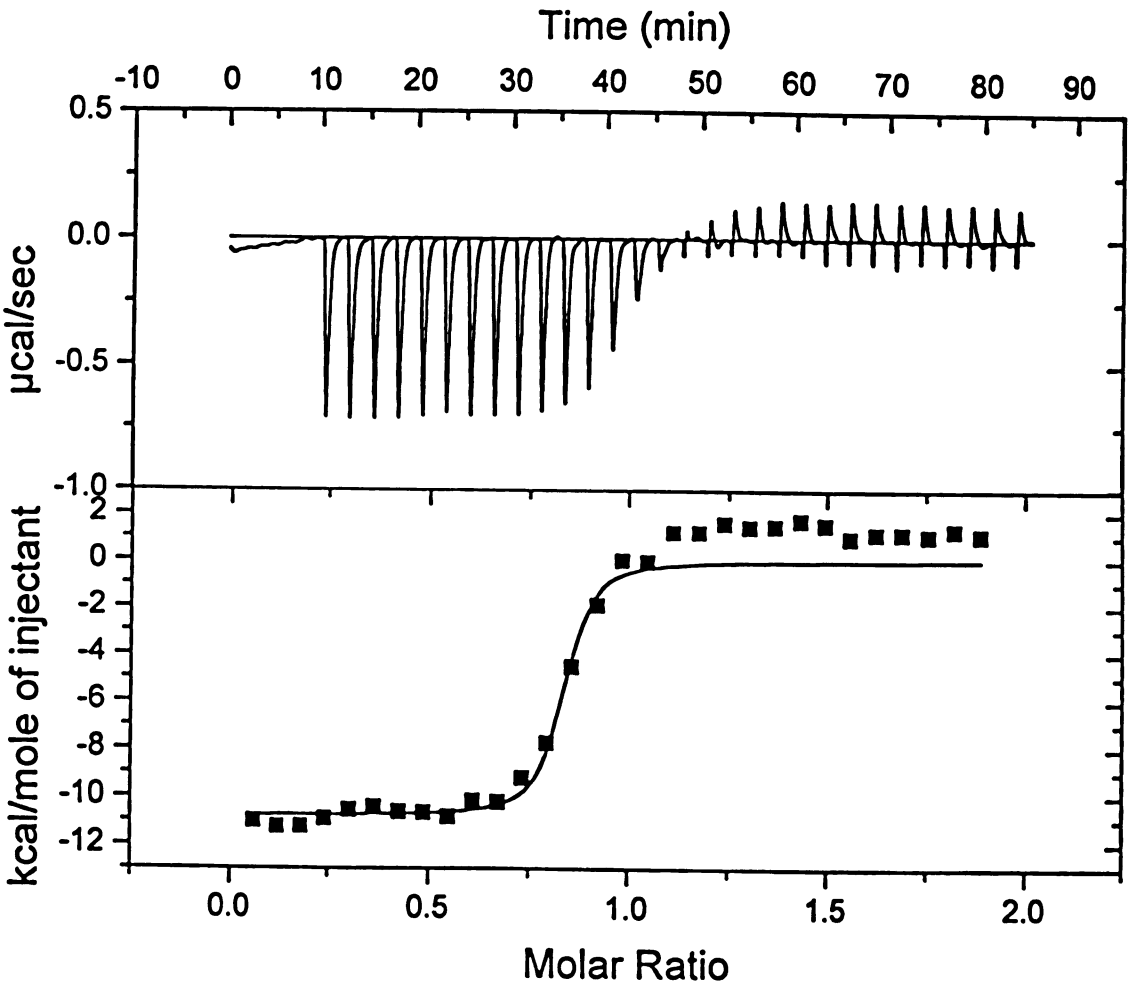

Figure 13. Microcalorimetry run for the binding of oleoyl-CoA to *Arabidopsis* ACBP (**13A**) and data fitting from the raw values (**13B**). Note the positive heat of dilution (right of midpoint in **Figure 13A**) results in curve fitting with less than a good fit after the mid-point in the titration (**Figure 13B**). These data required additional curve fitting subtracting heat of dilution (Sigurskjold et al., 1991. **Eur. J. Biochem.** *197,* 239-246).

accompany a binding reaction: ΔH_{int}, the intrinsic enthalpy change due to the binding reaction, and ΔH_{con}, all of the possible concomitant reactions that may be present but do not influence the binding constant, *K*. Such reactions could, for example, involve changes in protonation, hydration and conformation.

Enthalpy changes associated with events other than binding (i.e., phase changes from monolayer to bilayer) were accounted for in the "heat of dilution" which was subtracted from the total heat measured. In the study of binding proteins with highly insoluble ligands, the current problems pertain to slow or incomplete equilibration between protein and substrate (Miller and Cistola, 1993).

There was more than 20 kJ mol^{-1} difference in the free energy of binding (Table 3) between rAtACBP/acetyl-CoA (-24 kJ mol^{-1}) and rAtACBP/$C_{20:0}$-CoA (-48.5 kJ mol^{-1}). In general, as the acyl-CoA chain increases in length, more contacts were made in the hydrophobic binding pocket of rAthACBP. Consequently there was a steady ΔH^o decrease as the fatty acyl chain grows from four carbons to 18 carbons. The enhanced binding of C_{20}-CoA compared to C_{18}-CoA was due to an unfavorable entropy contribution with steroyl-CoA binding (Table 3).

The binding constants (*K*) for saturated acyl-CoA esters ranges between 44.6 μ*M* for acetyl-CoA and 35 n*M* for dodeceoyl-CoA (Table 4). If these binding constants are used to calculate dissociation constants (K_d) using the relationship ($K = 1/K_d$), then these dissociation constants will be equal in magnitude to the amount of free ligand at half-saturation. By this criteria, there is no physiologically significant binding of acetyl-CoA to rAthACBP. By contrast, steroyl-CoA binds to rAthACBP in the low n*M* range (Table 4).

Table 3. Thermodynamic properties for the binding of a range of saturated acyl-CoA esters with recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP).

chain length *n*	$\Delta G°$ *kJ mol^{-1}*	$\Delta H°$ *kJ mol^{-1}*	$-T\Delta S°$ *kJ mol^{-1}*	$\Delta S°$ *J mol^{-1} K^{-1}*
2:0	-24.5 ± 3.5	-18.8 ± 7.6	-5.7 ± 8.4	19.2 ± 9.0
4:0	-26.5 ± 1.6	-16.9 ± 2.0	-9.7 ± 5.9	32.5 ± 11.3
6:0	-31.8 ± 3.1	-20.9 ± 5.3	-10.9 ± 5.8	36.5 ± 18.9
8:0	-32.7 ± 2.8	-29.5 ± 7.8	-3.2 ± 6.3	11.9 ± 10.3
12:0	-41.0 ± 1.2	-31.9 ± 1.0	-9.1 ± 1.8	30.5 ± 5.4
14:0	-43.2 ± 1.3	-38.2 ± 1.7	-5.0 ± 1.9	17.0 ± 6.1
16:0	-43.2 ± 2.9	-40.7 ± 5.9	-2.5 ± 2.6	8.4 ± 8.1
18:0	-46.0 ± 3.9	-50.5 ± 6.1	+4.6 ± 4.5	-15.1 ± 9.5
20:0	-48.5 ± 2.1	-45.9 ± 5.1	-2.6 ± 10.5	8.8 ± 10.9
22:0	-43.4 ± 2.9	-28.4 ± 8.3	-15.0 ± 8.0	50.7 ± 18.5

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

Table 4. Binding constants of a range of saturated acyl-CoA esters interacting with recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP).

chain length	K	K_d
n	M^{-1}	nM
2:0	$2.24 \pm 0.34 \times 10^4$	44 600 ± 6 800
4:0	$3.57 \pm 0.44 \times 10^5$	2 800 ± 350
6:0	$4.17 \pm 0.59 \times 10^5$	2 400 ± 330
8:0	$6.80 \pm 0.11 \times 10^5$	1 470 ± 24
12:0	$1.76 \pm 0.44 \times 10^7$	57.0 ± 15
14:0	$4.10 \pm 0.40 \times 10^7$	24.0 ± 2.3
16:0	$6.29 \pm 0.11 \times 10^7$	16.0 ± 2.8
18:0	$2.62 \pm 0.67 \times 10^8$	3.8 ± 0.9
20:0	$3.56 \pm 0.89 \times 10^8$	2.8 ± 0.7
22:0	$5.01 \pm 0.67 \times 10^7$	19.0 ± 2.6

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

For the same quantity of palmitoyl-CoA to bind to rAthACBP as for steroyl-CoA to bind, there would have to be four times more $C_{16:0}$-CoA as there was $C_{18:0}$-CoA (Table 4). For comparison, with equal concentrations of $C_{20:0}$-CoA and $C_{18:0}$-CoA and rAthACBP, the competitive binding (which could lead to displacement of one ligand by another) would allow similar binding (57% $C_{20:0}$-CoA and 43% $C:_{18:0}$-CoA).

Acyl-chain length dramatically affected the interaction between the acyl-CoA ligand and the plant ACBP (Figure 4). Free energy and enthalpy tended to decrease linearly from acetyl-CoA until an acyl chain length of 20 had been reached. Interestingly, the entropy contribution diminished as the acyl-chain grows to C_{18}-CoA. The marked increase in ΔH^o as one compared the binding of C_{18}-CoA, C_{20}-CoA and C_{22}-CoA to rAthACBP indicated that these longer chains were promoting fewer contacts between the acyl-CoA and the binding protein. The turnaround in the entropy of binding observed with longer acyl-CoA esters (Figure 4) indicated a significant change in the conformation of the ligand-protein complex.

Slopes in the linear region of the curve (up to C_{18}-CoA, see Figure 5) were equivalent to the thermodynamic contributions per methylene group: $\Delta\Delta H^o$ = - 4705 ± 138 J mol^{-1}, $\Delta\Delta G^o$ = - 3292 ± 97 J mol^{-1}, and $T\Delta\Delta S^o$ = + 1419 ± 196 J mol^{-1}.

Free energy decreases related to increases in chain length were largely due to the decrease in enthalpy. Except for steroyl-CoA, all of the ligands tested in the saturated acyl-CoA series had large favorable free energy contributions from changes in enthalpy and significantly smaller favorable entropy changes.

The intercepts for the curves generated for the change in a

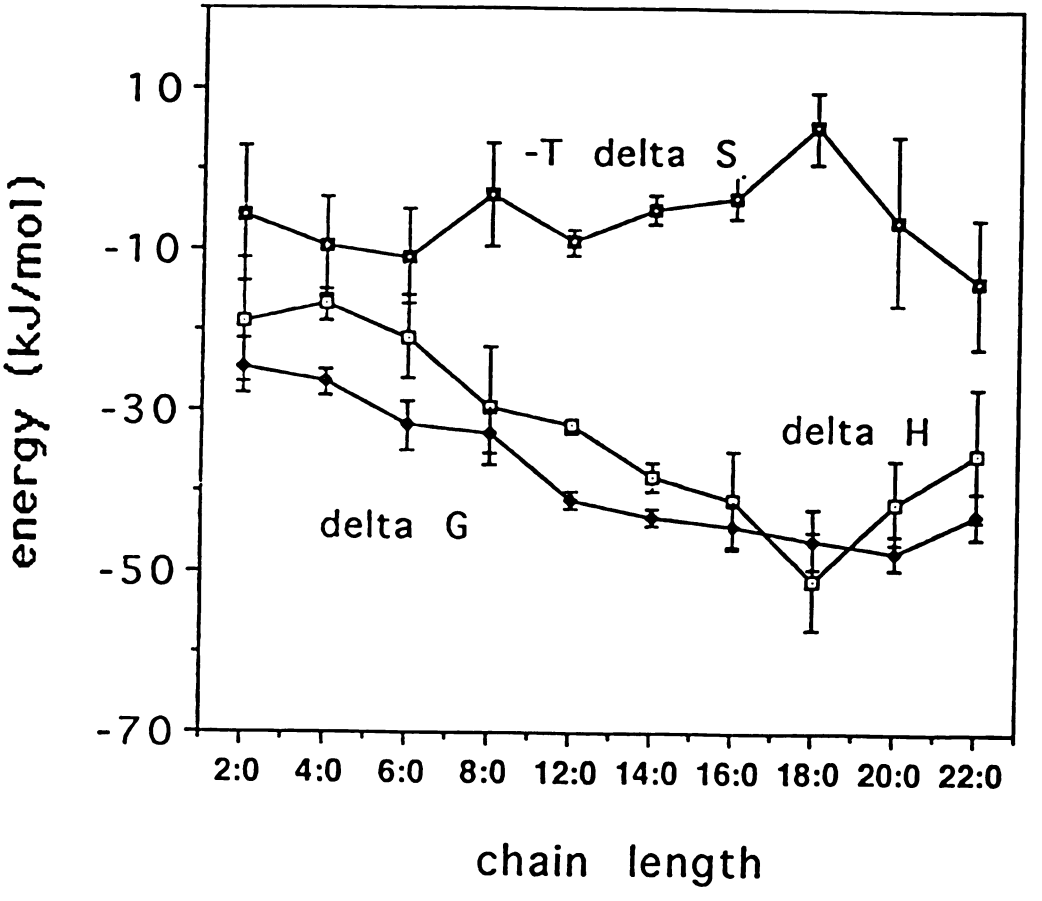

Figure 14. Thermodynamic quantities for acyl-CoA binding to *Arabidopsis* ACBP as effected by increasing acyl-chain length. Free energy (ΔG^{o}), enthalpy (ΔH^{o}) and temperature-dependent ($T\Delta S^{o}$) entropy changed as acyl-CoA chain length increased.

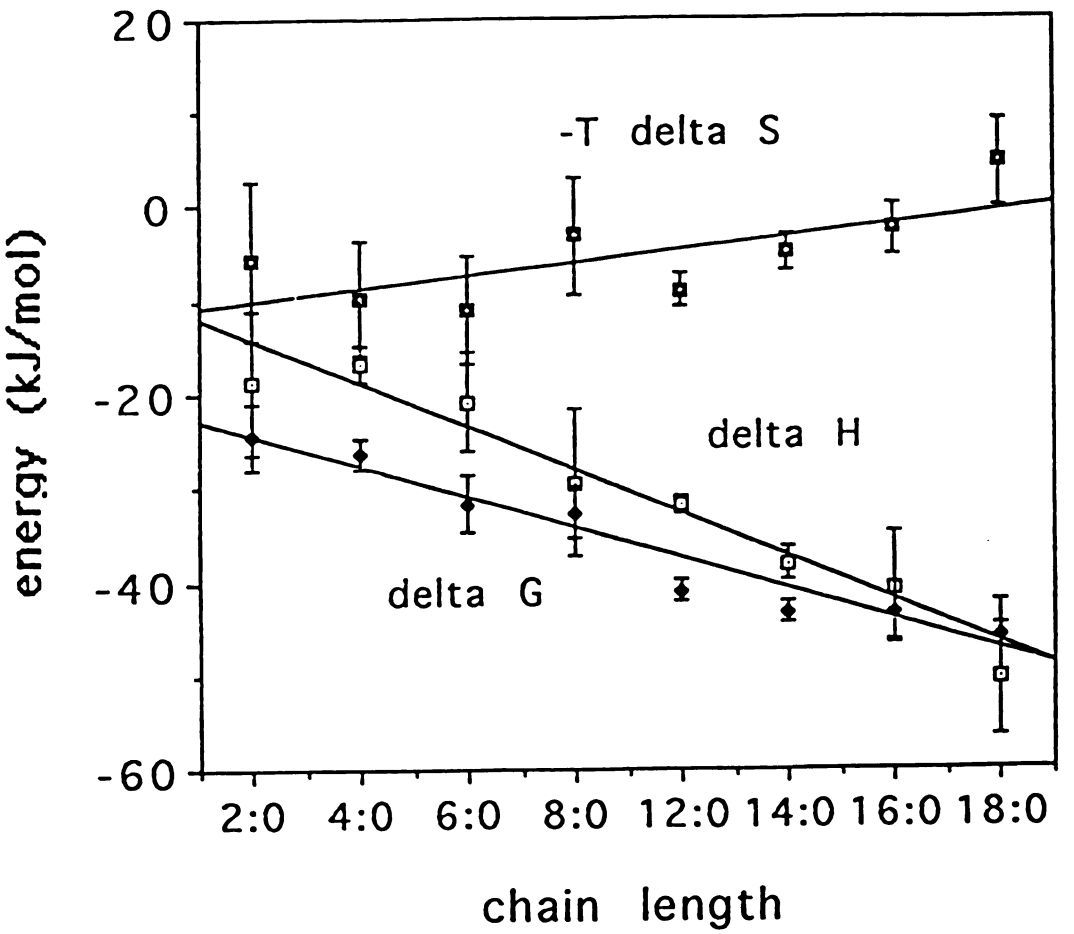

Figure 15. Slopes for the thermodynamic quantities resulting from the binding of acyl-CoA to *Arabidopsis* ACBP as acyl-chain length varies.

thermodynamic quantity with chain length would represent the binding of free Coenzyme A to rAthACBP (Figure 5). These values are equal to: ΔG^{o} = - 21.3 kJ mol^{-1}, ΔH^{o} = - 11.6 kJ mol^{-1}, and $-T\Delta S^{o}$ = - 9.7 kJ mol^{-1}, which would yield a binding constant of 5.8 x 10^{3}. (Actually this will be even less when the equations take into account the intercept at zero and not at 2:0; see Figure 19). Such a low binding constant would indicate that there was only the weakest interaction between rAthACBP and free CoA, and there would have to be over 170 μM of free CoA within a cell before a significant amount would be tied up by ACBP. Apparently acetyl-CoA has been measured in plant chloroplasts at 30 to 50 μM (Post-Beittenmiller at al., 1992), and so it is unlikely that this activity would ever occur at physiologically relevant conditions.

Unsaturated and hydroxylated acyl-CoA series. The free energy of binding for acyl-CoA and rAthACBP was very similar over the range of unsaturates from 18:1 to 18:3 (Table 5). The binding of the fully saturated steroyl-CoA was energetically favored over the unsaturated acyl-CoAs, perhaps due to the lowered flexibility of the unsaturated acyl chains. There was an easily recognizable enthalpy-entropy compensation as the acyl chain became more unsaturated. The ΔH^{o} decreased and the $T\Delta S^{o}$ increased as the number of double bonds in the 18-carbon acyl chain increased (Table 5). This indicated that although there was an increase in contacts between ligand and binding protein (- ΔH^{o}) there were less structural looseness and vibrational modes possible for the ligand-protein complex (+ $T\Delta S^{o}$).

The binding of ricinoleoyl-CoA to rAthACBP provided an interesting contrast to the binding of oleoyl-CoA to rAthACBP. Both oleoyl-CoA and ricinoleoyl-CoA bind to *A. thaliana* ACBP with about -42 kJ mol^{-1}, (Table 5), but both achieve this value in different ways. The contacts made

Table 5. Thermodynamic properties for the binding of a range of hydroxylated or unsaturated C_{18}-CoA esters with recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP).

degree of saturation	ΔG°	ΔH°	$-T\Delta S^{\circ}$	ΔS°
n	*kJ mol^{-1}*	*kJ mol^{-1}*	*kJ mol^{-1}*	*J mol^{-1} K^{-1}*
18:0	-46.0 ± 3.9	-50.5 ± 6.1	4.5 ± 4.6	-15.1 ± 9.5
18:1	-41.8 ± 1.2	-52.2 ± 2.8	10.4 ± 3.2	-34.8 ± 10.1
18:2	-43.3 ± 6.2	-52.4 ± 3.5	9.1 ± 3.0	-31.4 ± 9.9
18:3	-44.3 ± 0.9	-57.7 ± 1.8	13.4 ± 1.9	-43.7 ± 6.2
-OH 18:1	-42.5 ± 4.1	-38.8 ± 5.6	-3.7 ± 7.1	+12.6 ± 23.6

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

between oleoyl-CoA and ACBP generate -52 kJ mol^{-1} of energy, while the loss of entropy decreases free energy by +10 kJ mol^{-1}. Ricinoleoyl-CoA generates significantly fewer contacts with ACBP (-39 kJ mol^{-1}), but the $T\Delta S^{o}$ So term was negative due to an increase in entropy (Table 5), rather than a decrease in entropy as with oleoyl-CoA binding.

A comparison of binding constants for the unsaturated and hydroxylated C18-CoA esters gave an idea of the success of different acyl-CoAs in binding to ACBP (Table 6). Under conditions of competitive binding and with equal concentrations of oleoyl-CoA, linoleoyl-CoA and ACBP, 40% of the bound acyl-CoA would be $C_{18:3}$-CoA, 37% would be $C_{18:2}$-CoA, and 23% would be $C_{18:1}$-CoA. Steroyl-CoA has a three-to-four-fold higher dissociation constant with rAthACBP than does any of the C_{18}-unsaturated acyl-CoAs (Table 6).

It was interesting that the easiest of the C_{18}-CoAs to be released was oleoyl-CoA and ricinoleoyl-CoA. The concentration of oleoyl-CoA or ricinoleoyl-CoA would reach over 36 n*M* before significant quantities would be sequestered by ACBP. For steroyl-CoA or linoleoyl-CoA, significant amounts of each ligand would bind to ACBP after 4 n*M* or 15 n*M*, respectively, was reached (Table 6).

In examining the thermodynamic quantities for binding of the C_{18} unsaturates to rAthACBP many trends become obvious (Figure 16). Free energy values for the binding of various unsaturated acyl-CoAs to rAthACBP varied only slightly with few statistically significant differences as the number of double bonds in the acyl chain increased. Enthalpy steadily decreased while temperature-dependent entropy steadily increased in what appeared to be a strict entropy-enthalpy effect (Figure 16). Entrophic factors, then, drive the decreased binding of

Table 6. Binding constants based on a range of unsaturated or hydroxylated C_{18}-CoA esters that bound to the recombinant *Arabidopsis thaliana* acyl-CoA-binding protein.

degree of saturation	K	K_d
n	M^{-1}	nM
18:0	2.62 ± 0.67 x 10^8	3.82 ± 0.98
18:1	2.77 ± 0.35 x 10^7	36.1 ± 4.56
18:2	5.95 ± 0.30 x 10^7	16.8 ± 0.85
18:3	7.00 ± 0.20 x 10^7	14.3 ± 0.41
-OH 18:1	3.06 ± 0.39 x 10^7	36.7 ± 4.68

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

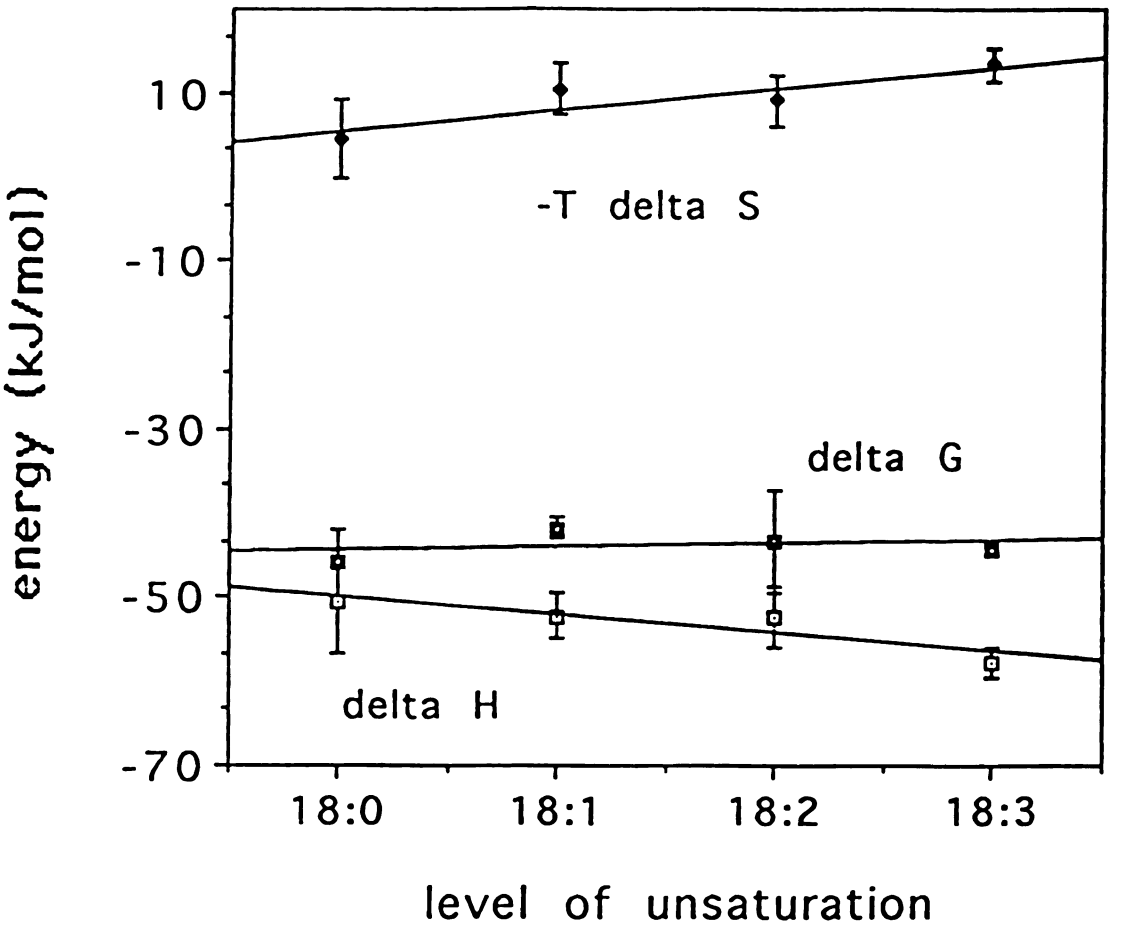

Figure 16. Thermodynamic quantities for the binding of unsaturated acyl-CoA esters to *Arabidopsis* ACBP as the number of double bonds increase for these C_{18}-CoAs. Parameters for ΔG^{o}, ΔH^{o} and $-T\Delta S^{o}$ change with increasing double bonds

polyunsaturates to rAthACBP. The trend for an increasing enthalpic contribution with an increased number of double bond amounted to - 2180 ± 192 J mol^{-1} per double bond, while the decreasing entropic contribution is equal to + 2540 ± 288 J mol^{-1} per double bond (Figure 16).

van't Hoff series. The heat capacity change (ΔC_p^o) was constant over the range of temperature used as evidenced by the linearity of ΔH^o against temperature (Figure 17). The fact that the slope of these lines was very close to 1.0 indicated that with temperature there would likely be a strong entropy-enthalpy compensation with regards to Gibbs free energy changes. Values of ΔH^o at different temperatures were fit to straight lines for each ligand (Figures 17 a,b,c) by least-squares fitting (r^2 = 0.993 for oleoyl-CoA; r^2 = 0.751 for lauroyl-CoA; r^2 = 0.933 for octoyl-CoA), and the slope yielded the standard heat capacity change for the binding of 1 mol of ligand to 1 mole of rAthACBP (ΔC_p = - 1012 ± 11 J mol^{-1} K^{-1} for oleoyl-CoA; - 846 ± 68 J mol^{-1} K^{-1} for lauroyl-CoA; - 954 ± 34 J mol^{-1} K^{-1} for octoyl-CoA). A weighted-average of these results yields - 945.2 ± 36 J mol^{-1} K^{-1}.

The thermodynamic parameters for the temperature series indicated that the ligand binding to rAthACBP increased as the temperature decreased until 290 *K* (17° C) was reached (Table 7). Although at 300 K (27° C), the binding of the acyl-CoA esters was enthalpy driven ($\Delta H^o > T\Delta S^o$), at 290 *K* (17° C) the ligand-protein interaction was entropy driven ($\Delta H^o < T\Delta S^o$). Earlier it was pointed out that the binding of oleoyl-CoA to rAthACBP produced a positive $-T\Delta S^o$ term at 300 *K*, which was unlike the $-T\Delta S^o$ term for the binding of rAthACBP to any other ligand. Examining the decreasing $-T\Delta S^o$ contribution to Gibbs free energy as temperature increased, it was not surprising that around 22 to

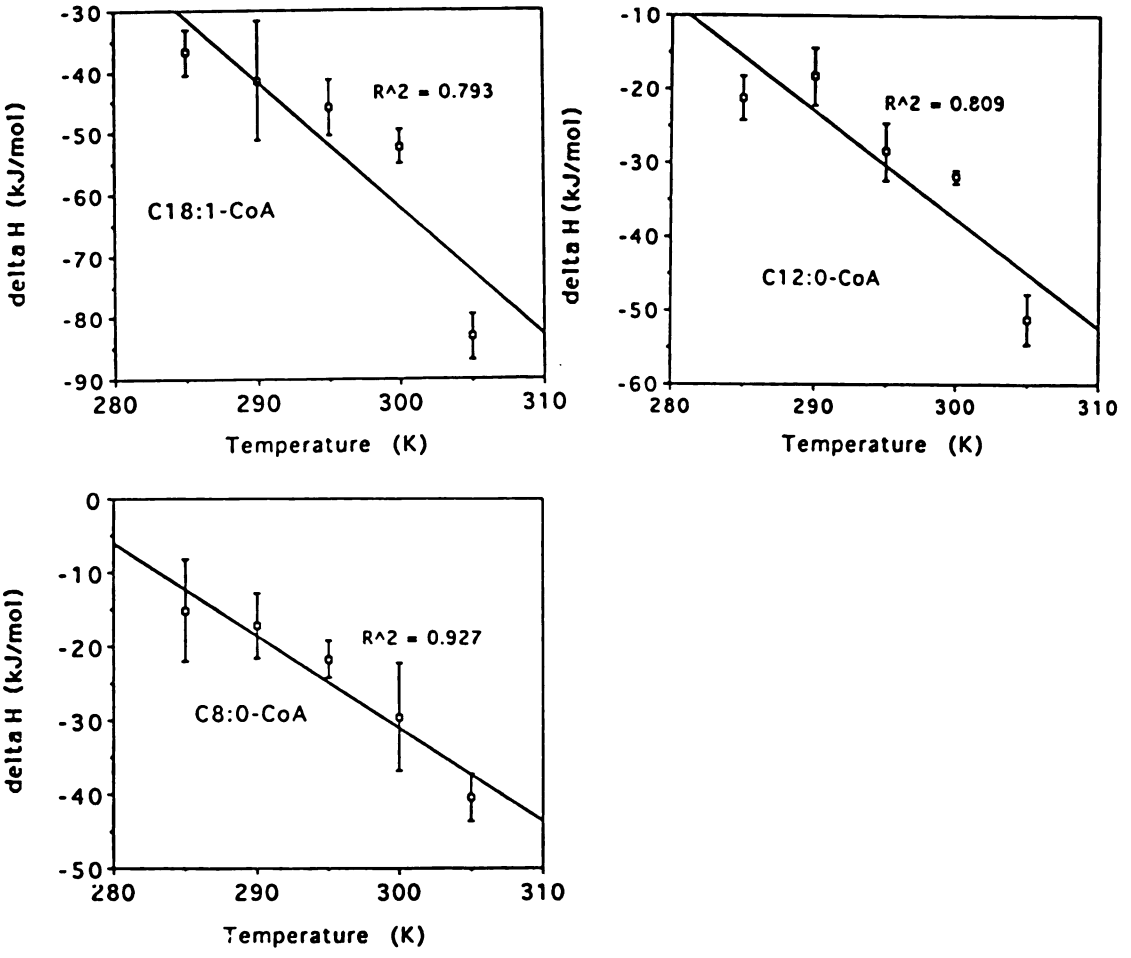

Figure 17. Dependence of enthalpy on temperature for the binding of *Arabidopsis* ACBP to: **17A)** oleoyl-CoA, **17b)** lauroyl-CoA, and **17c)** octoyl-CoA. The slope is the specific heat capacity (ΔC_p) for ligand binding.

Table 7. Thermodynamic properties for the binding of $C_{8:0}$-CoA, $C_{12:0}$-CoA, and $C_{18:1}$-CoA esters with recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP) from 285 *K* to 305 *K*.

T	ΔG^{o}	ΔH^{o}	$-T\Delta S^{o}$	ΔS^{o}
K	*kJ mol*$^{-1}$	*kJ mol*$^{-1}$	*kJ mol*$^{-1}$	*J mol*$^{-1}$ *K*$^{-1}$
$C_{8:0}$-CoA				
285	-32.5 ± 3.8	-15.1 ± 6.9	-17.4 ± 6.9	61.1 ± 23.8
290	-36.2 ± 1.9	-17.2 ± 4.4	-19.0 ± 4.2	66.1 ± 12.1
295	-34.8 ± 0.9	-21.7 ± 2.5	-13.1 ± 2.2	44.5 ± 7.1
300	-32.7 ± 2.8	-29.5 ± 7.4	-3.2 ± 6.3	10.8 ± 10.2
305	-33.5 ± 1.7	-40.4 ± 3.2	+6.8 ± 3.6	-22.4 ± 9.3
$C_{12:0}$-CoA				
285	-39.5 ± 1.7	-21.2 ± 3.0	-18.3 ± 9.0	64.2 ± 13.3
290	-47.0 ± 1.5	-18.3 ± 4.0	-26.3 ± 3.9	90.8 ± 3.8
295	-44.3 ± 2.8	-28.5 ± 3.8	-15.8 ± 4.0	53.7 ± 13.1
300	-41.0 ± 1.2	-31.9 ± 1.0	-9.1 ± 1.8	30.5 ± 5.4
305	-42.4 ± 1.4	-51.2 ± 3.4	+8.8 ± 3.5	-28.9 ± 10.8
$C_{18:1}$-CoA				
285	-41.5 ± 2.0	-36.8 ± 3.7	-4.7 ± 3.2	16.6 ± 11.2
290	-48.1 ± 2.6	-41.5 ± 9.7	-6.6 ± 9.4	22.9 ± 13.9
295	-43.7 ± 2.0	-45.9 ± 4.6	+2.2 ± 2.1	-7.6 ± 7.2
300	-41.8 ± 1.2	-52.2 ± 2.9	+10.4 ± 3.2	-34.8 ± 10.1
305	-41.2 ± 1.8	-83.1 ± 3.7	+42.5 ± 3.7	-139.4 ± 11.0

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

25° C there would be a change of sign for the entropy term (Table 7). At all the temperatures tested, the net free energy for binding contained a favorable contribution from enthalpy changes and either a moderate-to-small favorable contribution from entropy for lauroyl-CoA and octoyl-CoA or a small negative contribution to a moderate favorable contribution from entropy for oleoyl-CoA (Table 7).

The factor that decided the optimum size of ΔG° was temperature-dependent entropy (Figure 18). Enthalpy decreases with temperature, and so does the $-T\Delta S^{\circ}$ contribution until 290 *K*, after which the continued decrease in ΔH° was counteracted by an increase in $-T\Delta S^{\circ}$. The minimum value for the temperature-dependent entropy thus determined the minimum ΔG° value and the temperature at which ligand-protein interactions were optimal was 17° C (290K, see Figure 18).

The influence of temperature on the binding constant for acyl-CoA/rAthACBP binding follows the trend for changes in Gibbs free energy with temperature (Table 8). The highest binding constants were measured at 290 K (17° C), and in general, there was between a 2.5 to five-fold increase in ligand binding at 17° C compared to 27° C (temperature chosen to determine the binding of the saturated CoA and unsaturated CoA esters to ACBP). Taking into account this difference in the values for binding constants (Table 8), the physiologically relevant values for the dissociation of palmitoyl-CoA or stearoyl-CoA from the plant ACBP (K_d) was determined to be between 1 and 4 n*M*.

As temperature varied, entropy increased as enthalpy decreased for the binding of acyl-CoA to rAthACBP (Figure 19). This entropy-enthalpy compensation was strongly linear for oleoyl-CoA (Figure 19a), lauroyl CoA (Figure 19b) or octoyl-CoA (Figure 19c). Entropy was inversely

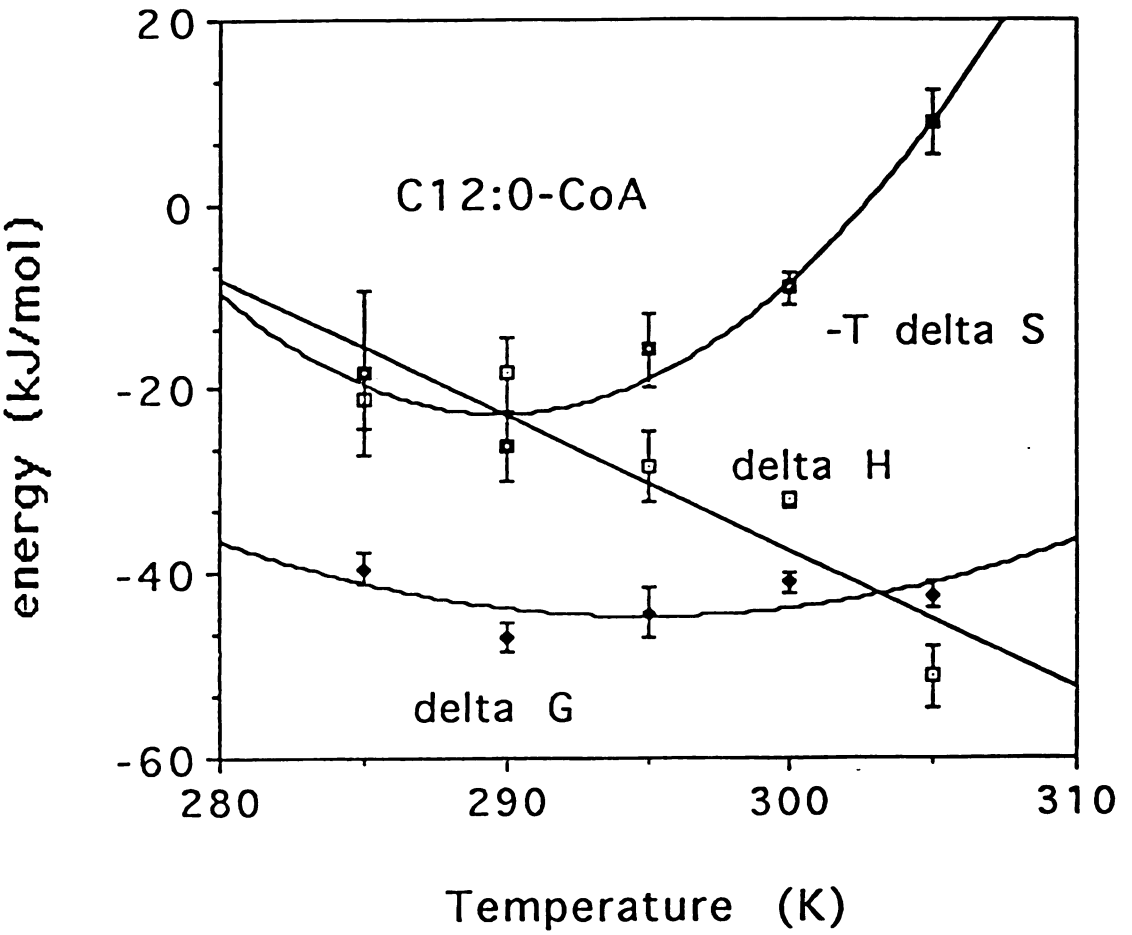

Figure 18. Changes in free energy (ΔG°), enthalpy (ΔH°) and temperature-dependent entropy ($-T\Delta S^{\circ}$) for the binding of lauroyl-CoA with *Arabidopsis* ACBP as temperature increases.

Table 8. Binding constants for $C_{18:1}$-CoA, $C_{12:0}$-CoA and $C_{8:0}$-CoA with recombinant *Arabidopsis thaliana* acyl-CoA-binding protein (rAthACBP) from 285 *K* to 305 *K*.

T	K $C_{8:0}$-CoA	K $C_{12:0}$-CoA	K $C_{18:1}$-CoA
K	M^{-1}	M^{-1}	M^{-1}
285	$1.04 \pm 0.13 \times 10^6$	$1.92 \pm 0.19 \times 10^7$	$4.21 \pm 0.59 \times 10^7$
290	$3.90 \pm 0.22 \times 10^6$	$9.89 \pm 0.26 \times 10^7$	$6.53 \pm 0.96 \times 10^8$
295	$1.53 \pm 0.12 \times 10^6$	$5.67 \pm 0.19 \times 10^7$	$6.16 \pm 0.69 \times 10^7$
300	$6.80 \pm 0.11 \times 10^6$	$1.76 \pm 0.44 \times 10^7$	$2.77 \pm 0.35 \times 10^7$
305	$1.16 \pm 0.51 \times 10^6$	$1.47 \pm 0.15 \times 10^7$	$1.21 \pm 0.34 \times 10^7$

Standard deviations are based both on the arithmetic deviation from the mean and the uncertainty generated from curve-fitting.

proportional to enthalpy for oleoyl-CoA (r^2 = 0.97), lauroyl-CoA (r^2 = 0.97) or octoyl-CoA (r^2 = 0.98). van't Hoff analysis indicated that the ΔH^o for rAthACBP binding to oleoyl-CoA, lauroyl-CoA and octoyl-CoA was equivalent to -43.7 ± 4.2 (Figure 20a), or -30.3 ± 2.8 (Figure 20b), or -25.6 ± 1.5 (Figure 20c), respectively.

Plotting the natural logarithm of the binding constant as a function of temperature is yet another way to determine certain thermodynamic parameters (such as ΔH^o) as well as estimating the optimal binding temperature. This type of van't Hoff analysis indicated that the optimal binding temperature for the three different ligand-ACBP combinations was approximately 290 *K* (Figure 20).

DISCUSSION

While examining the binding of different acyl-CoAs to the recombinant bovine ACBP by microcalorimetry (Færgeman et al., 1996) an unexpected result was obtained. The binding of acyl-CoAs with 16 or more carbon atoms in the acyl chain was significantly more favorable energetically than the binding of acyl-CoAs with acyl chains containing less than 14 carbon atoms. This specificity of the animal ACBP for C16- to C20-CoA was not apparent in the fluorescent quenching, electric spin resonance spectroscopy, or Lipidex assays performed previously (Færgeman et al., 1996) and was not a feature of either the yeast or plant ACBP. The plant ACBP does not show the highly significant preference for C_{18}- and longer acyl chains demonstrated by bovine ACBP. In light of the pronounced efflux of FAs from serum albumins in animal cells, this preference for longer acyl-CoA esters has a protective effect against the FAs whose import is likely.

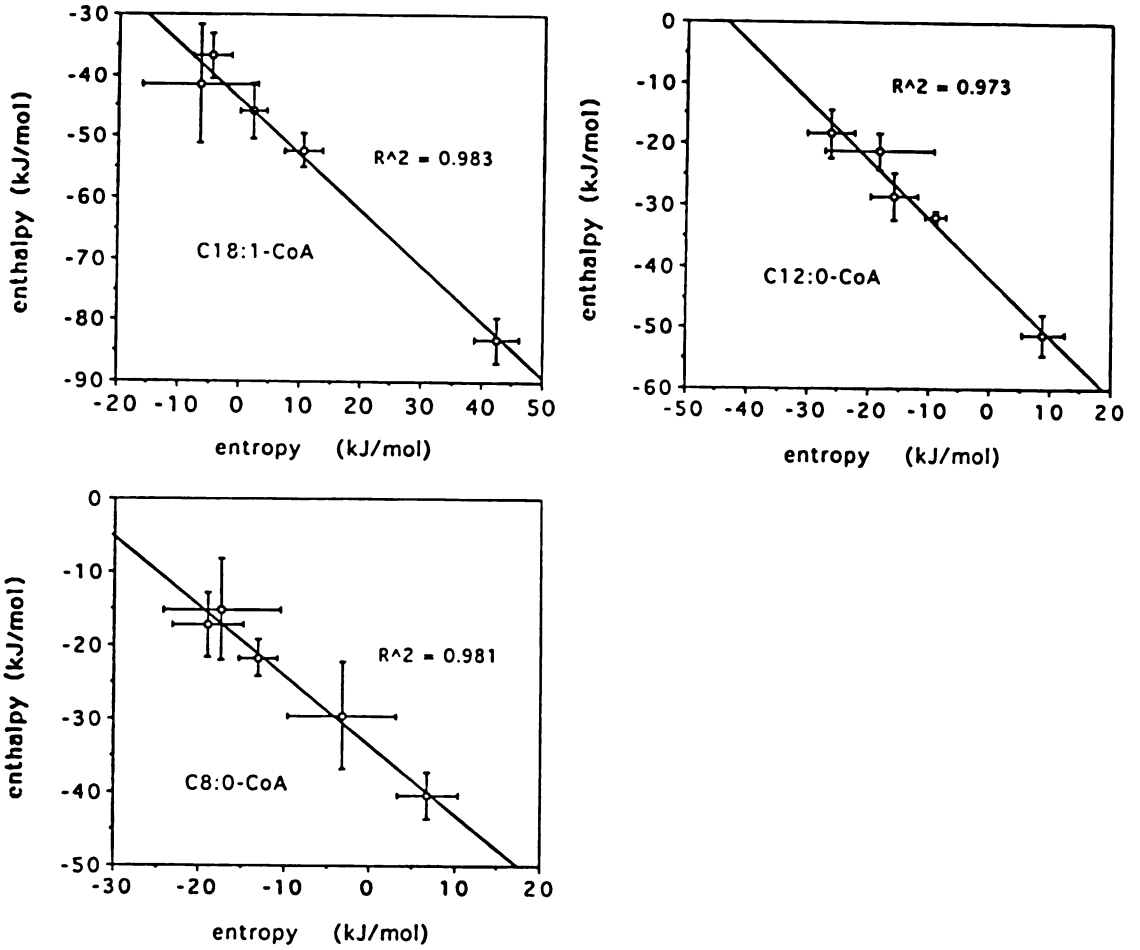

Figure 19. Entropy-enthalpy compensation (relative adjustments of ΔH^{o} and $T\Delta S^{o}$ as temperature varies) for the binding of *Arabidopsis* ACBP to: **19a**) oleoyl-CoA, **19b**) lauroyl-CoA, or **19c**) octoyl-CoA.

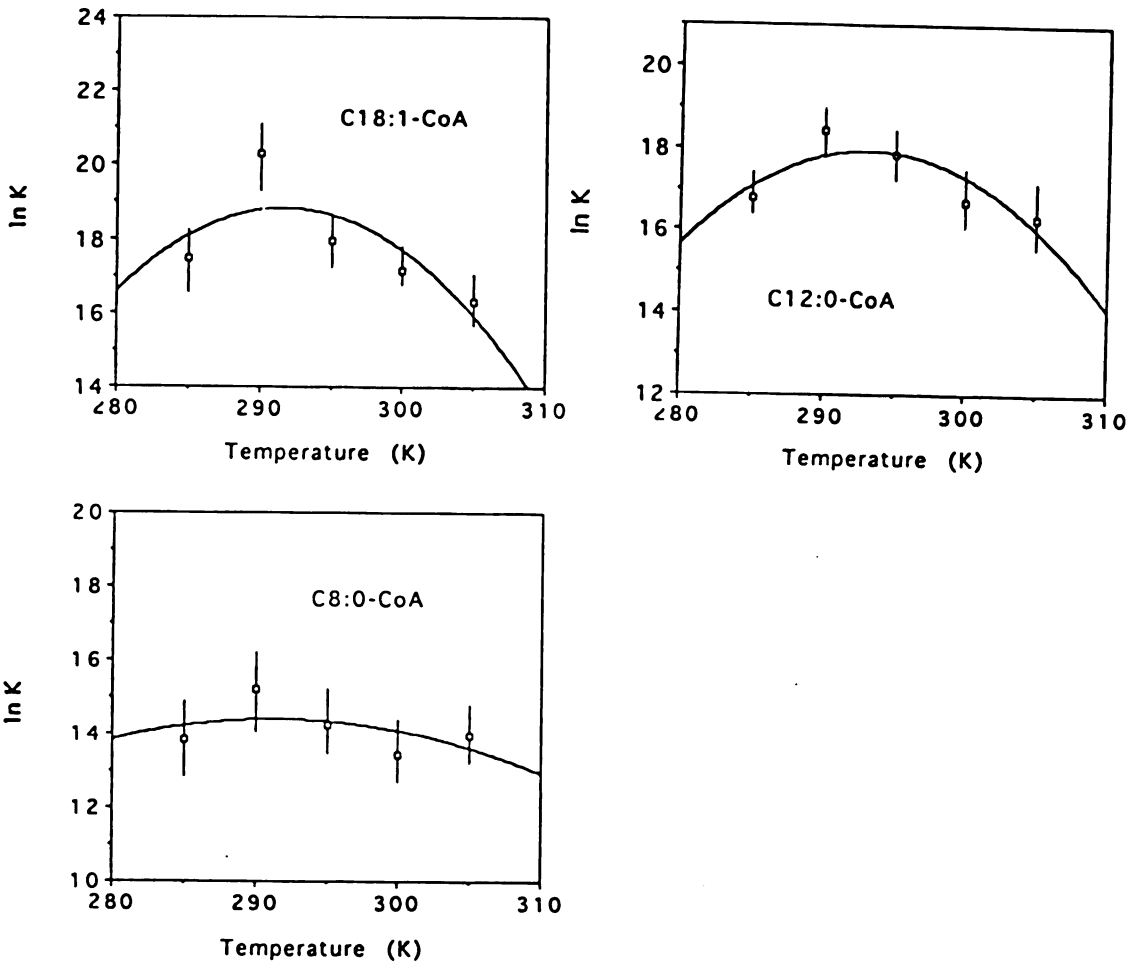

Figure 20. van't Hoff binding curves for the temperature dependence of *Arabidopsis* ACBP binding to: **20A)** oleoyl-CoA, **20b),** lauroyl-CoA, and **20c)** octoyl-CoA. Curve fitting is used to calculate enthalpy of ligand binding (ΔH^{o}) for an independent measurement of enthalpy.

Many thermodynamic quantities are influenced by the addition of the hydroxyl side chain to transform oleoyl-CoA into ricinoleoyl-CoA (Figure 21). In many respects, ricinoleoyl-CoA behaves as it if were actually a shorter acyl-chain with regards to the heat of dilution (Figures 13 and 21) and enthalpy of binding (Table 5). Apparently ricinoleoyl-CoA does not make as many contacts in the hydrophobic acyl-chain binding pocket (greater ΔH^{o}), but the ligand-ACBP complex displayed greater conformational freedom (lowered $-T\Delta S^{o}$). We would predict that release of ricinoleoyl-CoA was facilitated by the apparent lack of conformational rigidity in the ligand-protein complex (positive entropy relative to other C_{18}-CoA esters, Table 5).

The binding of ricinoleoyl-CoA to ACBP indicates that some of the enhanced contacts when ACBP binds oleoyl-CoA involves intramolecular contacts along the hydrocarbon chain (a foldback effect). We arrive at this conclusion based on the large difference between ΔH^{o} of binding for oleoyl-CoA and ricinoleoyl-CoA (12 kJ mol^{-1}). Such a difference could not be due to a limited number of contacts between ACBP amino acid residues and the acyl-CoA backbone, rather apparently intra- molecular contacts must also have been disrupted.

The change in the heat capacity (ΔC_p) involving protein solutions is determined by the hydrophobic and hydrophilic interactions both for protein folding and protein-ligand interactions (Livingstone et al., 1991; Privalov and Makhatadze, 1992). A simpler relation than this one was obtained by Livingstone et al. (1991), who related heat capacity change only to ΔA_n (surface area of contact) and found that:

$$\Delta C_p/\Delta A_n = -1.05 \text{ J mol}^{-1} \text{ K}^{-1} \text{ Å}^{-2}.$$

For rAthACBP binding to $C_{18:1}$-CoA, $C_{12:0}$-CoA or $C_{8:0}$-CoA this would

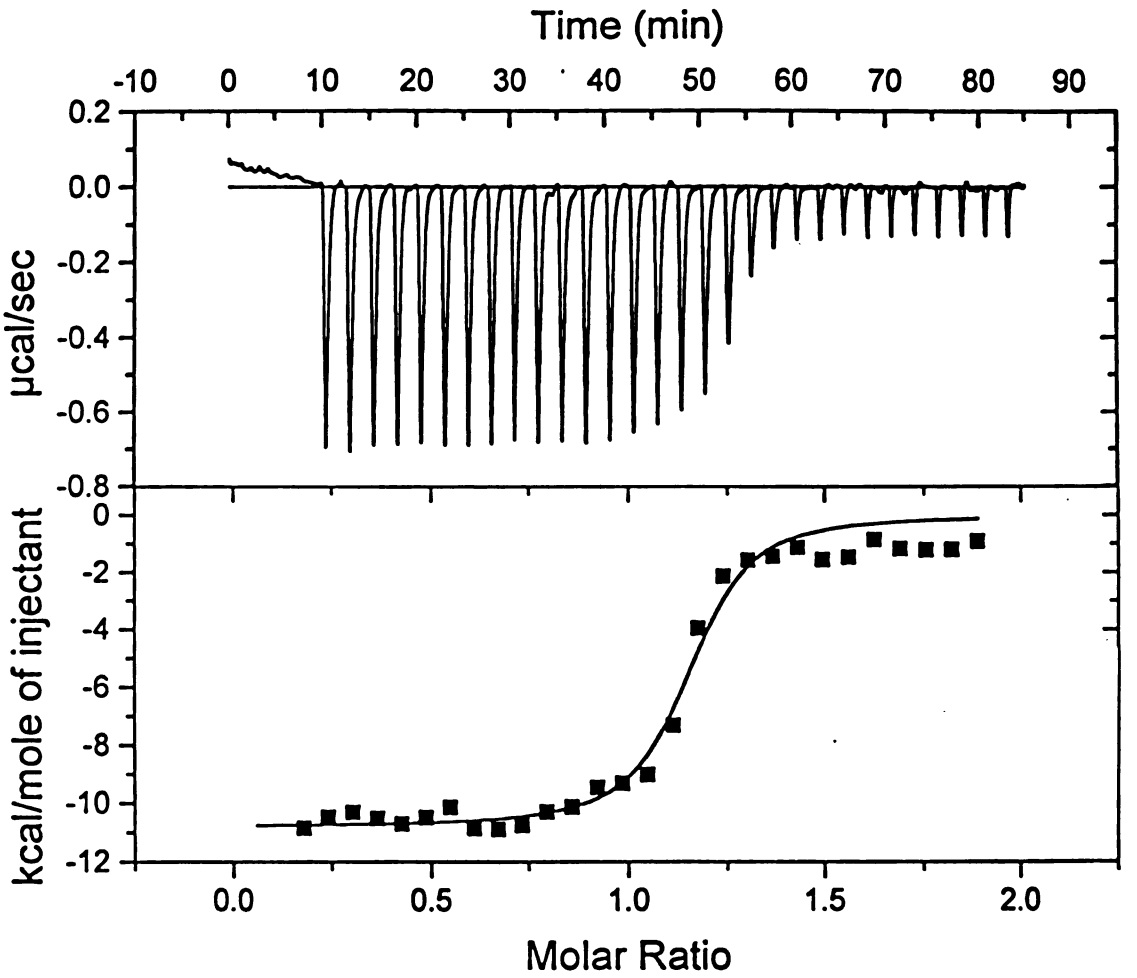

Figure 21. Microcalorimetry run (21A) and fitted data (21B) for the binding of ricinoleoyl-CoA to *Arabidosis* ACBP. Note the heat of dilution was negative, unlike that for oleoyl-CoA binding (see Figure 17). Curve fitting was direct and no specialized methods were required (Sigurskjold et al., 1991. **Eur. J. Biochem.** *197*, 239-246).

amount to a ΔA_n = 1968 Å^2, or 1403 Å^2, or 1275 Å^2, respectively. The area of contact for each methylene carbon in the aliphatic acyl chain was equivalent to 109 Å^2, 117 Å^2 or 159 Å for $C_{18:1}$-CoA, $C_{12:0}$-CoA or $C_{8:0}$-CoA, respectively. Interestingly, without stearic constraint, $C_{8:0}$-CoA may make more contact with the protein per methylene group than does $C_{18:1}$-CoA or $C_{12:0}$-CoA where there may be more intra-acyl chain contacts.

Energetically, the stabilizing and destabilizing interactions involved with ligand binding were rather closely balanced with a net difference of less than 10 kJmol^{-1} for a given acyl-CoA over a 20° C range (Table 7). In general, values for ligand binding can be greater for enzyme cofactors (Mizutani et al., 1994), but this was well in the range for substrate binding to enzymes (Ladbury et al., 1994). This level of free energy stabilization was minor compared to the free energy decrease upon folding (approximately 60 kJ mol^{-1}) based on lysozyme (Privalov and Makhatadze, 1992)

There were very few conformational changes for ACBP following acyl-CoA binding (Kragelund et al., 1993). Since ACBP does not foster an enzymatic alteration of the acyl-CoA held in the active site, concomitant reactions to be at a minimum. The enthalpy gained in ligand binding can be assumed to be due to the number of contacts made between acyl-CoA and the protein. Polar interaction between the CoA headgroup and charged amino acid residues in ACBP and non-polar interactions between the fatty acyl chain and the non-polar binding pocket in ACBP contributed to the final favorable enthalpy.

It may be that the ACBP-acyl-CoA conformation was very stable in an aqueous environment since the interaction was markedly stabilized by hydrophobic effects. Once ACBP begins to interact with the phospholipid

environment in which the integral acyl-CoA utilizing enzymes are found, the stable tertiary structure for the complex would begin to relax. If the base of the ACBP molecule were to exist in a primarily apolar environment, the contribution of the hydrophobic effect to the binding stability would be greatly diminished (Stowell and Rees, 1995).

Entropy for protein folding and ligand binding is assumed to consist of three contributions (Spolar and Record, 1994):

$$\Delta S_{\Diamond} = \Delta S_{HE} + \Delta S_{rt} + \Delta S_{conf}$$

where ΔS_{HE} is the contribution from the hydrophobic effect, ΔS_{rt} is the contribution from the rotational and transitional changes, and ΔS_{conf} is the contribution from the conformational changes. Vibrational entropy changes were ignored in this analysis. The first term is expected to be positive, whereas the last two terms are both negative (Ross and Subramanian, 1981).

For acyl-CoA binding to rAthACBP, ΔS_{conf} will vary only a little with ligand bound since conformational changes upon binding are insignificant (Kragelund et al., 1993). Apparently, for both folding and acyl-CoA binding, ΔS_{HE} predominated.

The hydrophobic contribution, as a function of temperature, can be estimated (Spolar and Record, 1994) from:

$$\Delta S_{HE} = -1.35\ \Delta C_p \ln(T/386)$$

This relationship certainly held after 17° C when ΔS decreased as temperature increased (leading to a positive $T\Delta S^{\circ}$) offsetting the increasing contribution of ΔH° to free energy (Figure 6). Under these conditions, $\Delta S_{he} > \Delta S_{rt}$. Before 17° C $\Delta S_{HE} < \Delta S_{rt}$, which led to a positive $T\Delta S^{\circ}$ as temperature decreased. These two competing

contributions to entropy largely set the optimum temperature for a minimum ΔG° (Spolar and Record, 1994).

Binding of the acyl-CoA ester to rAthACBP becomes easy to explain: Enthalpy drives the formation of the acyl-CoA/ACBP complex, as with antibody-antigen interactions (Ramen et al., 1995). Release of the acyl-CoA then becomes problematic. If the difference in ΔH° alone were to explain the loss of acyl-CoA from the complex, the acyl-CoA utilizing enzymes receiving an acyl-CoA from ACBP would have to bind acyl-CoA with an affinity (K) in excess of $10^8\ M^{-1}$. The other explanation, which is more likely, release of the acyl-CoA ester was driven primarily by temperature-dependent entropy.

The data from the van't Hoff analysis suggest that at higher temperatures there was more order in the acyl-CoA/rAthACBP complex than free ACBP resulting in a positive $-T\Delta S^{\circ}$ term. If ΔH° (release) for acyl-CoA from the ACBP/acyl-CoA complex were about as large as ΔH° (bind) for the formation of the complex, then release of ligand depends on entropy only.

Release of ligand has largely been ignored by thermodynamic studies of protein-substrate interactions. This has occurred because many binding studies have involved enzymes (Varadarajan et al., 1992), repressor-DNA interactions (Sigurskjold et al., 1991; Sigurskjold and Bundle, 1992) or antibody-antigen binding (Raman et al., 1995). It has been assumed that an enzyme will release product since product is structurally different from substrate, which the enzyme binds with high affinity (Honig and Yang, 1995). The binding of DNA or antigen has been dissected, but the release of the promoter or antigen has largely been ignored (Ladbury et al., 1994; Jin et al., 1993).

The temperature dependence of both enthalpy and entropy (Figure

22) emphasizes that near the optimum binding temperature, Gibbs free energy is most sensitive to the contribution from entropy. It may be that docking with the appropriate acyl-CoA utilizing enzyme causes a relaxing of the ligand-protein complex. The hydrophobic patch on the exterior base of the protein may interact with a phospholipid membrane, while the lip of the ACBP bowl transfers the adenosine portion of the CoA head group to the active site of the appropriate enzyme, such as an acyltransferase. At the molecular level, two-dimensional nuclear magnetic resonance-nuclear overhauser effect spectroscopic analysis of both the protein and protein-substrate complex have revealed a number of important structural features which have provided insight into the mode of acyl-CoA binding (Kragelund et al., 1993).

The very high binding affinity and low free acyl-CoA concentration immediately raised the question as to which pathways ACBP can donate acyl-CoAs. Using multilamellar phosphatidylcholine liposomes immobilized on nitocellulose containing ^{14}C-hexadecanoyl-CoA, recombinant bovine ACBP was capable of selectively extracting the radiolabeled CoA and delivering them to either the carnitine palmitoyl-transferase of intact mitochondria or to microsomes for phosphatidate biosynthesis (Rasmussen et al., 1994).

Acknowledgments

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CHAPTER 4

MOLECULAR ANALYSIS OF THE ACYL-CoA BINDING PROTEIN GENE *acb*-1 FROM *Arabidopsis thaliana* AND OTHER DICOTS

ABSTRACT

In this work we demonstrate, for the first time, the presence of genes encoding acyl-CoA-binding proteins (ACBP) in *Arabidopsis,* safflower, soybean, clover and sunflower by Southern hybridization. Three, or possibly four, putative ACBP genes (*acb*-1 homologues) were identified in *Arabidopsis thaliana*. There were multiple forms of *acb* in *Brassica napus*, *Carthamus tinctorius*, *Glycine max*, *Helianthus annus* and *Trifolium repens*. These results suggest that the *acb* gene is part of a small multi-gene family in a number of different plant genera. Northern analysis indicated that *acb* is expressed in all tissues of *A. thaliana,* and its expression is markedly elevated in seeds accumulating triacylglycerol or seedlings utilizing oil storage reserves.

INTRODUCTION

Acyl-CoA-binding proteins (ACBP) and their genes (*acb*; Rose et al, 1992) have been isolated from a number of sources. The first information on amino acid sequence was from cow (Mikkelsen et al., 1987). The first *acb* nucleotide sequence was derived from a cDNA clone isolated from human brain mRNA. This sequence encoded an endogenous ligand for the $GABA_A$ receptor, and was referred to as the diazepam binding inhibitor (DBI; Gray et al., 1986). Knudsen et al. (1989) later demonstrated that DBI and ACBP were identical.

Labeled ACBP cDNA hybridized with several genomic DNA restriction fragments in rat (Mocchetti et al., 1986), human (Gray, 1987) and mouse (Owens et al., 1989), indicating that in animals ACB was encoded by a small multigene family. For rat, six hybridizing fragments were seen in each lane (Hansen et al., 1991). The *acb* gene was cloned and used to examine the rat ACBP gene family where one expressed gene and four processed pseudogenes were found (Mandrup et al, 1992). Variants of human ACBP were cloned from a hypothalmic cDNA library suggesting that the transcript may undergo alternative splicing (Owens et al., 1989).

The ACBP promoter region was located in a CpG island and lacks a canonical TATA box, exhibiting all the hallmarks of a traditional housekeeping gene (Mandrup et al., 1993). A number of potential *cis*-acting element binding sites involved with regulation of *in vivo* ACBP levels have been identified. In animal or insect cells, ACBP expression was high in cells involved in secretion or various transport processes (Kolmer et al., 1994). A potential C/ERB-alpha-binding sequence in the ACBP promoter suggests ACBP levels may be transcriptionally regulated in liver cells and adipocytes (Owens et al., 1989). Copies of the hexanucleotide response element for peroxisome proliferators (Tugwood et al., 1992) involved with ß-oxidation were found in the rat ACBP promoter (Mandrup et al., 1991).

The endogenous cellular peroxisome proliferator may respond to a common intermediate in fatty acid metabolism (Göttlicher et al., 1992) and, following ligand binding, become an active transcriptional activation factor. In the rat ACBP promoter, there were a large number of distinct response elements less than 1 kb from the transcriptional start sites that were likely involved with tissue specificity or temporal regulation (Mandrup et al., 1993).

In the case of plants, the *Brassica napus* acyl-carrier protein (ACP) promoter (da Silva et al, 1992) and the *Arabidopsis thaliana* oleosin promoter (Plant et al., 1994) both contain response elements conferring stage- or tissue-specificity. As ACP and oleosin levels increased during seed maturation, a concomitant rise in ACBP was anticipated as well (Chapter 2). Therefore, we expected that the *acb-1* promoter would contain response elements similar to those found in the *Brassica* ACP promoter or the *Arabidopsis* oleosin promoter.

Our objectives were to determine the number of *acb* genes in different economically important oilseeds and to isolate and characterize at least one *acb*-1 genomic clone from *A. thaliana*.

MATERIALS AND METHODS

Bacterial strains and plant material. The strain of *Escherichia coli* used to propagate the lambda GEM11 genomic library of *A. thaliana* was KW251 (Sambrook et al., 1989).

The *Arabidopsis thaliana* ecotype Columbia was grown in a growth chamber (16 hr photoperiod, 27°/23° C, 450 umol m^{-2} sec^{-1}) and fully expanded leaves, roots, siliques and mid-maturity seeds (including silique material) were harvested, frozen in liquid nitrogen and then transferred to -80° C. Approximately 200 mg of *A. thaliana* seed was aseptically germinated for 2 days at 27° C under a 14-hour photoperiod. The seedlings were collected, frozen in liquid nitrogen and then stored -80° C. *Brassica napus* cv. Bridger, *Helianthus annus* cv. RW635 and *Carthamus tinctorius* cv. S-400 were grown in a greenhouse under natural conditions (approximately 14 to 15 hour photoperiod, 30°/24° C, maximum PPFD 850 umol m^{-2} sec^{-1}). Two fully expanded leaves were taken from

each plant for DNA extraction, frozen in liquid nitrogen and transferred to a -80° C freezer. *Trifolium repens* cv. Ladino and *Glycine max* cv. Dare were grown in the field at Michigan State University; leaves were harvested in August 1992, placed on dry ice (-56° C) and stored at -80° C.

Southern blotting. DNA was extracted from leaves of 6 plant species: *A. thaliana*, rapeseed (*B. napus*), safflower (*C. tinctorius*), soybean (*G. max*), clover (*T. repens*) and sunflower (*H. annus*) using a modified procedure based on Dellaporte et al. (1983). The quality of the undigested, freshly-extracted DNA was verified using agarose gel electrophoresis, and small-scale digests (1.0 µg) were carried out for 3 hours to determine a suitable ratio of restriction endonuclease-to-DNA for 100% digestion (Sambrook et al., 1989).

Based on these small-scale digests, 25 µg of DNA was digested using *Eco*R I, *Pst* I, *Bam*H I, *Hin*d III, *Pvu* II and *Bgl* II. The various restriction endonucleases were selected to provide a useful range of fragments. A small aliquot, representing approximately 4.0 µg of digested DNA was removed, electrophoresed in a 0.75% agarose gel, and visualized with ethidium bromide to insure complete digestion.

Ten to 11 µg of DNA from the six digests for each plant species were separated in a 0.75% agarose gel lacking ethidium bromide for a 12-hour overnight run. Following electrophoresis, DNA was transferred to nitrocellulose using the capillary transfer method of Southern (1975), using acid depurination of the DNA (0.2 *M* HCl for only 10 min). Subsequent ethidium bromide visualization of the dehydrated agarose gels indicated that over 95% of the 21.2 kb *Hind* III/*Eco*R I-fragment of lambda DNA or the 23.3 kb *Hind* III-fragment of lambda DNA (MW markers) was transferred to the nitrocellulose.

To eliminate background, prehybridization was carried out for 12 hours (Southern) in 6 x SSPE, 0.5% dry milk, 0.5% SDS. This buffer avoids Denhart's solution. *Arabidopsis* and *Brassica* DNA were hybridized under more stringent conditions (65° C), while hybridization of safflower, soybean, clover and sunflower DNA was under moderately stringent conditions (54° C).

Probe labeling. The entire *A. thaliana* ACBP cDNA (464 bp) from the original plasmid pSCC12T7P, a pZL1 derivative (Newman et al., 1994) was labeled with alpha-^{32}P-dCTP using either single-stranded or double-stranded PCR. The PCR primer for the 5' terminus (CCATATGGGTTTGAAGGAGGAA) and the primer for the 3' terminus (GGATCCTCAGGTTGAAGCCTTGGA) were used either singly or in combination. Heat-denatured pSCC12T7P DNA (50 mg), 25 pmol of a single primer (one-stranded PCR) or 25 pmol each of the two primers (two-stranded PCR) 20 nmol each of dATP, dGTP and dTTP, 5 nmol of dCTP, 50 µCi of alpha-^{32}P-dCTP and 2.5 U of *Taq* polymerase in 100 µl of 1 x *Taq* polymerase buffer were mixed together. Amplification occurred during 30 cycles of 1 min at 92° C, 1.5 min at 55° C and 1.5 min at 72° C.

Northern and RNA dot blotting. Frozen flowers and siliques of *A. thaliana* (kindly provided by P. Quail) were sorted using a size classification into an approximate developmental series, consisting of flowers, small (< 0.5 cm), mid-sized (0.5 to 1.5 cm) and large (> 1.5 cm) siliques. RNA was isolated by phenol-SDS extraction and LiCl precipitation. Briefly between 0.1 and 0.2 g of tissue was ground in 0.5 ml of extraction buffer (Nagy et al., 1988) in a microcentrifuge tube. The suspension was then extracted twice with phenol:chloroform: isoamylalcohol (25:24:1) and once with chloroform:isoamylalcohol (24:1). RNA was then precipitated with one-half volume 8 *M* LiCl. For Northern

analysis, either 5 µg or 10 µg of total RNA was electrophoresed in formaldehyde agarose gels and then transferred to nylon membranes (BioTrans, Irvine, CA) in 8 x SSC (Sambrook et al., 1989).

For slot-blot analysis, dilutions (3.0, 1.0, 0.3, and 0.1 µg) of total RNA were applied to nylon membranes using a vacuum manifold (Schleicher and Schuell) according to the manufacturer's instructions. RNA was bound to the membranes with ultraviolet light (Stratalinker, Stratagene). A *Eco*R I-*Hin*d III restriction fragment of *acb*-1 was heat denatured and following serial dilution was applied to a set of wells in concentrations from 50 ng to 2 pg to serve as a positive internal control for the quantification of the RNA. Signal levels for dsDNA was expected to be twice that for ssRNA so a correction factor of 0.5 was applied. mRNA abundance was determined visually, and estimates were made on three separate samples per tissue.

An *Eco*R I-*Pvu* II fragment from plasmid pR0400, containing only the coding sequence of *acb*-1, was labeled with a dU-digoxigenin analogue (Genius Kit, Boeringer-Mannheim) by the random primer method using the Klenow fragment of DNA polymerase I (Feinberg and Vogelstein, 1984). Prehybridization, hybridization and wash conditions, as well as colorimetric development of the chromophore, were according to the manufacturer's instructions (Boeringer-Mannheim).

Genomic and cDNA library screening. A genomic library of *A. thaliana* ecotype Columbia and cDNA libraries of tobacco and soybean were screened using the AthACBP sequence as a probe. The characteristics of each library used appears in Table 9. Prehybridization and hybridization of filters were performed at 65° C for the *Arabidopsis* library and at 54° C for the cDNA libraries. Following the washes and air-drying, autoradiography was performed (3-day and 7-day exposure).

Table 9. Characteristics of the genomic and cDNA libraries screened with the *Arabidopsis thaliana acb* probe.

Characteristic	*Arabidopsis* genomic library	*Nicotiana* cDNA library	*Glycine* cDNA library
lambda phage	GEM 11	zap II	zap II
host	KW 251	XL1 Blue	XL1 Blue
primary clones	200,000	100,000	150,000
avg. insert size (kb)	20	2.0	2.2
titre (PFU/ml)	5.8×10^7	4.3×10^8	3.0×10^6
plaques/plate	46,000	43,000	45,000
plaques screened	276,000	184,000	225,000
oversampling/plate	*c.* 4	*c.* 2.2	*c.* 2.4
probe sp act (dpm/µg)	4.8×10^8	2.1×10^8	7.7×10^7
dpm/filter	8.6×10^6	5.3×10^6	5.8×10^6
hybrid temp (° C)	65	55	53
presumptive clones	16	6	11
confirmed positives	6	5	N D
library frequency	$24/1 \times 10^6$	$37/1 \times 10^6$	$44/1 \times 10^6$

Autoradiographs were aligned with specific petri plates containing one of the lambda libraries, and small sectors (10 mm^2) were picked from the top agarose to generate an *Arabidopsis* sub-libraries highly enriched in a presumptive ACBP clone.

RESULTS

Gene number.

When the *Arabidopsis* DNA was freshly extracted and digested, a clearer hybridization pattern was obtained (Figure 22). A *Hind* III digest displayed four bands, three of which had been visualized on the earlier blot (6.3 kb, 2.3 kb, and 1.8 kb) and a fourth band at 3.9 kb. The *Bgl* II digest had two bands (11.0 kb and 10.0 kb). The *Pvu* II digest had one high molecular weight band (12.7 kb), and the *Bam*H I digest revealed four bands hybridized to the probe. The *Eco*R I digest showed four prominent bands (6.5, 4.3, 1.6 and 0.6 kb) with partially digested fragment at 4.2 kb and 0.1 kb that likely arise from the 4.3 kb band (Figure 22).

All the other plant species have multiple copies. On the basis of the Southern blot between the *A. thaliana acb*-1 probe and rapeseed (*B. napus)* DNA, rapeseed has at least three and perhaps four ACBP genes (Figure 23). Both *Bam*H I digests and *Eco*R I digests of *B. napus* DNA contained four DNA restriction fragments that hybridized to *acb*. There were only three positive fragments in the *Hind* III-digested DNA (4.2 kb, 2.2 kb and 1.3 kb). There was no hybridization to *Bgl* II-, *Pvu* II- or *Pst* I-digested *Brassica napus* DNA. Other digests indicate that the *Pst* I reaction was contaminated with non-specific DNAses and probably the *Bgl* II and *Pvu* II reactions as well (Figure 23).

Gene expression. Total RNA was extracted from various tissue

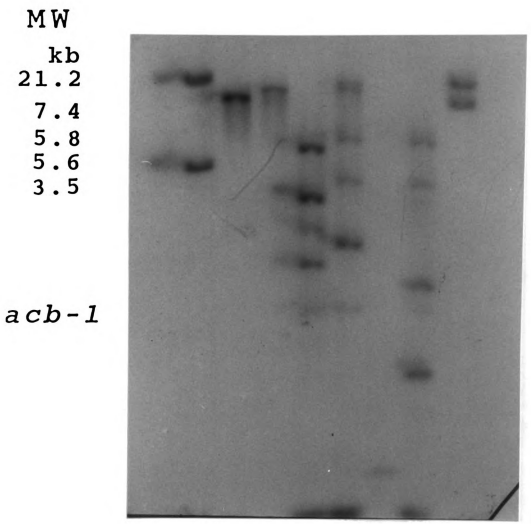

Figure 22. Southern blot analysis of genomic DNA from *A. thaliana* using the *acb*-1 probe. Ten µg of digested DNA loaded in each lane (*Bgl* II, lane 3; *Pvu* II, lane 4; *Hin*d III, lane 5; *Bam*H I, lane 6, *Pst* I, lane 7; *Eco*R I, lane 8). Molecular weight markers in outside lanes (lambda *Eco*R I-digested DNA, lanes 1 and 2; lambda *Hin*d III-digested DNA, lane 9).

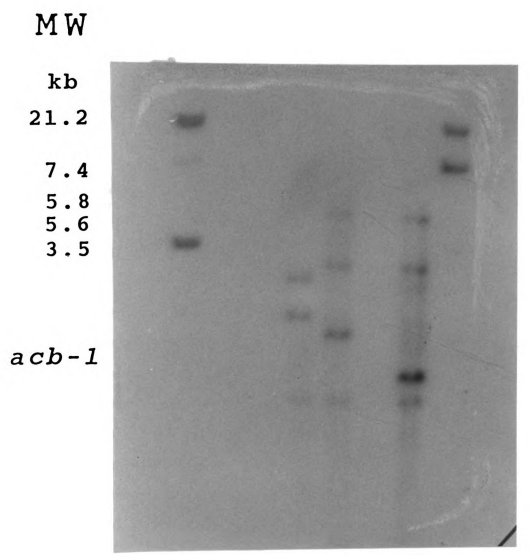

Figure 23. Hybridization of *acb*-1 to *Brassica napus* genomic DNA. Ten µg of digested DNA loaded into each lane (*Bgl* II, lane 2; *Pvu* II, lane 3; *Hin*d III, lane 4; *Bam*H I, lane 5; *Pst* I, lane 6; *Eco*R I, lane 7). Molecular weight markers found in lanes 1 and 8 and MW's provided.

samples from *A. thaliana* and 10 µg of denatured RNA was used for northern hybridization (Figure 24). Both mid-maturity seed (silique also present) and germinating seedling (cotyledon primary source) had the highest level of *acb* expression (lanes 3 and 5). Fully mature seed carried a significant level of the *acb* transcript stored and intact in the dormant cells (lane 4). Vegetative tissue expressed lower levels of *acb* with root (lane 2) showing the lowest concentration of ACBP mRNA (Figure 24). The approximate MW of the *A. thaliana acb* transcript was 1600 bp. It appears that there was hybridization to only one band for each of the RNA samples probed.

Flowers and siliques of *A. thaliana* were separated into an approximate developmental series based on size, and the RNA samples were hybridized to *acb*-1 (Figure 24). Flower RNA weakly bound *acb*-1 (lane 2) while all the silique samples hybridized with moderate strength to our probe (lanes 3 through 5). The *acb* transcript was highest in germinating *Arabidopsis* cotyledons (lane 6) used as our internal positive control. No noticeable differences were observed among the *acb* transcript level found in siliques carrying immature, mid-maturity or fully mature seeds, but this may not be surprising since seed tissue represents only a small proportion of the silique in *Arabidopsis*.

To quantify the levels of *acb* mRNA, a series of dot blots were made (Table 10). Expression of the *acb* gene was reported relative to a specific quantity of total RNA (1.0 µg) and to the proportion of total RNA (%). We did not attempt to quantify the hybridization signal in terms of pg μg^{-1} total RNA.

Leaf mRNA levels for ACBP gene expression was three-fold higher than in roots, although the protein concentration was similar (Table 10). Flowers contained an *acb* mRNA concentration between that of root

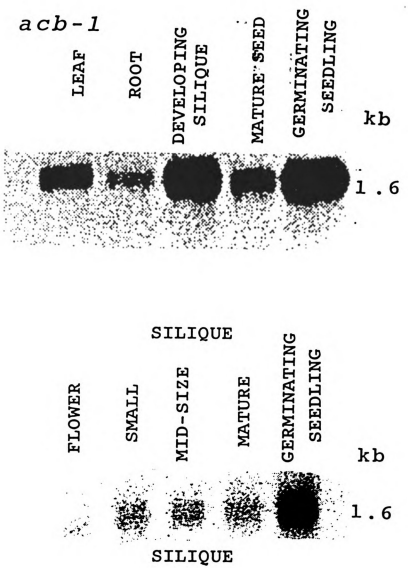

Figure 24. Northern blot of *Arabidopsis* DNA using *acb*-1 as a probe. Tissue specificity (**24A**): Ten µg of total RNA extracted from *A. thaliana* and transferred to nylon (leaf, lane 1; root, lane 2; developing seed, lane 3; mature seed, lane 4; germinating seedling, lane 5). Transcript size for the *acb*-1 gene shown at right. Temporal specificity (**24B**): total RNA from *A. thaliana* (10 µg) flower (lane 1), small silique (lane 2), mid-maturity silique (lane 3), fully mature silique (lane 4) and germinating seedling (lane 5) compared.

Table 10. Steady state *acb* mRNA concentrations as compared to ACBP protein concentrations in various *A. thaliana* tissues as determined by dot blotting.

tissue	relative *acb* mRNA levels (1 = root *acb* conc)	proportion % total mRNA	ACBP levels ng (mg protein)$^{-1}$
Leaf	3.0	0.017	180
Root	1.0	0.005	220
Flower	2.0	0.010	n.d.
Developing silique	11.0	0.056	1250
Fully mature silique	4.0	0.020	540
Germinating seedling	12.0	0.060	n.d.

Values are means based on three replicate determinations. ACBP levels from Chapter 2, thesis. n.d. = not determined.

and leaf. There was a five-fold increase in ACBP transcript levels in mid-maturity silique compared with flower and approximately a eight-fold difference in the levels of ACBP, as measured by western blotting (Table 10). Such a close linkage between polypeptide and transcript levels suggests transcription regulation as the primary control for gene expression.

Germinating seedlings and mid-maturity *Arabidopsis* siliques contained the highest *acb* transcript level, *c.* 23.0 pg (µg total RNA)$^{-1}$. This amounts to *c.* 0.06% of all cellular RNA, or somewhat less than 1% of all transcripts in the cell.

Genomic library screening. Presumptive clones were identified after comparing primary and replica filters. Apparently these clones contained a genuine *acb* sequence since the hybridization was done under high stringency. Frequencies of the putative *acb* genes are given in Table 9.

cDNA sequences. The plant cDNA sequences for ACBP genes revealed some interesting similarities (Figure 25). In general, the 5' end of most of these *acb* homologues was AT rich. The proximal promoter elements contained necessary ribosome binding and efficient translation start sequences (Kozak, 1989). Very little sequence identity between clones existed upstream of the start ATG (Figure 25). None of these cDNA sequences appeared to encode an acyl-CoA binding domain of a larger polypeptide. The *O. sativa* and *Z. mays* sequences diverge most from the *A. thaliana* DNA, but within the 5' coding region of *acb*, there was approximately 72% sequence identity.

The *acb* coding sequences began to diverge markedly after nucleotide 250. The last 30 nucleotides before the stop codon showed significant divergence among plant *acb* sequences (Figure 25). Coding

Figure 25. Alignment of ACBP cDNA sequences from five different plant *acb*-1 genes. Sequences derive from *Arabidopsis* (*Ath*), rapeseed (*Bna*), cotton (*Ghi*), rice (*Osa*), castor bean (*Rco*) or maize (*Zma*). The full sequence has been broken into 5' non-coding sequences, coding sequences, and 3' non-coding sequences. Codons responsible for amino acid residues in the four helices are shown, and those italicized form contact points between the polypeptide chain and the Coenzyme A head group. The KQAT box, LILY box and DAWK box is indicated. Stop codons are underlined. Within the coding sequence invariant nucleotide positions have an astrick above, while conserved positions (in term of coding for a specific amino acid) have a circumflex above.

5' non-coding sequence

```
AthACBP                                          (-71)    >TGTCCTCGTC TTCTC -56
GhiACBP                                          (-70)     >GAGCTGTTG TTCTC -56
RcoACBP                                          (-73) >CG GTGCGGCCGC TCTAG -56


AthACBP CGTCTTACAC TGATTTAATT CTCCTACCAA TCTCAACTTC CGACGTCTAT TCATC  -1
BnaACBP               >GATCT GCGCAATTTA TCTCAACTTC TGACGTCTAA TCATC  -1
GhiACBP TCTCTCTCTC TTTCCCATAG TTTCACCCAT CTTTTAATCC ATCGCTCACA AAATT  -1
OsaACBP                   >AA GAAGAAGAGA AGAATCTGAA AAACTTTAAG CCGCT  -1
RcoACBP AACTAGTGGA TCCCCCGGGC TGCAGGAATT CGGCACGAGG NTCACACTCT CTACC  -1
ZmaACBP          > CCGAGAAGAG ACCAGGCCGA AGGAAAACAA TCCTGAAACC AAGCC  -1
```

coding sequence

```
          *** ***  **  ** **  **  ***  ** *^^         ^^^ **^ *   **^
AthACBP   ATG GGT TTG AAG GAG GAA TTT GAG GAG CAC GCT GAG AAA GTG AAT  45
BnaACBP   ATG GGT TTG AAG GAC GAC TTT GAG GAG CAC GCT GAG AAA GTG AAG  45
GhiACBP   ATG GGT TTG AAG GAG GAA TTC GAG GAG CAT GCC GAG AAA GTT AAG  45
OsaACBP   ATG GGT CTG CAG GAG GAT TTT GAG GCA GTA CGC CGA AAG GGG AAG  45
RcoACBP   ATG GGT TTG AAG GAG GAT TTT GAG GAG CAC GCA GAG AAA GCT AAG  45
ZmaACBP   ATG GGT CTG CAG GAG GAA TTT NAG GAG CAT NCT GAG AAG GCA AAG  45
        / -------- helix 1 ---------------------- helix 1 --------- /
```

Figure 25. (continued). **coding sequence**. (continued).

```
                                                                  LILY  BOX
          *^   *   *  *        *      **^ ***  *^   ^ **  **^ ^*^ **^
AthACBP   ACG CTC ACG GAG TTG CCA TCC AAC GAG GAT TTG CTC ATT CTC TAC  90
BnaACBP   AAG CTC ACC GCG AGC CCA TCT AAC GAG GAC TTG CTC ATC CTC TAC  90
GhiACBP   ACC CTC CCG GCC GCC CCT TCG AAT GAT GAC ATG CTC ATT CTC TAT  90
OsaACBP   ACC TTG CCG GAG AGC ACT AGC AAC GAG AAC AAG CTT ATC CTC TAT  90
RcoACBP   ACC CTT CCA GAA AAT ACT ACG AAC GAG AAC AAA CTA ATT TTG TAT  90
ZmaACBP   ACC TTG CCT GAG AGC ACG AGC AAT GAG AAC AAG CTG ATC CTC TAC  90
                                  / ---------- helix 2 -----------

          ACYL TAIL  BOX
          **^ **^ *** *** **  *** *^  ^** **^     **  ^^  **^     ***
AthACBP   GGA CTC TAC AAG CAA GCC AAG TTT GGG CCT GTG GAC ACC AGT CGT 135
BnaACBP   GGT CTC TAC AAG CAA GCC ACC GTT GGG CCA GTG ACC ACC AGT CGT 135
GhiACBP   GGA CTG TAC AAG CAA GCT ACT GTT GGA CCA GTG AAT ACC AGC CGT 135
OsaACBP   GGA CTC TAC AAG CAG GCC ACC GTT GGA GAT GTC AAT ACT GCT CGT 135
RcoACBP   GGC CTT TAC AAG CAA GCC ACC GTT GGA CCA GTC AAT ACC AGC CGT 135
ZmaACBP   GGG CTC TAC AAG CAA GCC ACG GTT GGA GAT GTG AAT ACC GAT CGT 135
          ------------ helix 2 ------------ /

                                                      CoA BINDING BOX
          **  **  **^ ***   *     *^^ **  *^^ ^^  **^ *** *** **^ *^*
AthACBP   CCT GGA ATG TTC AGC ATG AAG GAG AGA GCC AAG TGG GAT GCT TTG 180
BnaACBP   CCT GGG ATG TTC AGC ATG AAG GAA AGA GCC AAG TGG GAC GCT TGG 180
GhiACBP   CCT GGA ATG TTC AAC ATG AGG GAA AAG TAC AAG TGG GAT GCA TGG 180
OsaACBP   CCT GGC ATA TTC GCC CAG AGG GAC AGG GCG AAA TGG GAT GCA TGG 180
RcoACBP   CCT GGA ATG TTC AAC ATG AGA GAC AGA GCA AAG TGG GAC GCA TGG 180
ZmaACBP   CCG GGC ATA TTC TAC CAA AAG GAC AGG GCC AAG TGG GAT GCC TGG 180
                              / --------------- helix 3 ----------

          CB BOX
          **^ **^ *** *** **^ *** **    * **  **^ **  *** ^   **^ **
AthACBP   AAG GCT GTT GAA GGG AAA TCA TCG GAA GAA GCC ATG AAT GAC TAT 225
BnaACBP   AAG GCC GTT GAA GGG AAA TCA ACG GAC GAA GCC ATG AGT GAC TAC 225
GhiACBP   AAG GCT GTT GAA GGG AAA TCA AAG GAG GAA GCA ATG GGG GAT TAT 225
OsaACBP   AAA GCT GTT GAA GGC AAA TCG AAG GAG GAA GCA ATG AGC GAC TAC 225
RcoACBP   AAA GCC GTT GAA GGG AAA TCT AAG GAG GAA GCA ATG AGT GAC TAT 225
ZmaACBP   AAA GCT GTT GAA GGC AAA TCG AAG GAT GAG GCG ATG AAT GAC TAC 225
          --------- helix 3 --------/              / --- helix 4 --
```

Figure 25. (continued). **coding sequence** (continued)

```
         *** **  **  **^ **  **  **      **  *   *   *^   ^      ^
AthACBP  ATC ACT AAG GTC AAG CAA CTC TTG GAA GTT GCT GCT TCC AAG GCT 270
BnaACBP  ATC ACT AAG GTG AAG CAA CTC CTT GAA GCA GAG GCT TCC TCC GCT 270
GhiACBP  ATC ACC AAA GTA AAA CAA CTG TTT GAG GCT GCT GAA AGT TCT TGA 270
OsaACBP  ATC ACC AAG GTG AAG CAG CTC CTG GAG GAG GCT GCT GCT TCT GCA 270
RcoACBP  ATC ACT AAA GTC AAA CAG CTG CTG GAG GAA GCT GCT GCT TCT GCT 270
ZmaACBP  ATC ACC AAG GTG AAG CAG CTC CAG GAG GAG GCT GCC GCC TCT TAA 270
         -------- helix 4 ----------------- helix 4 ------ /
```

3' non-coding sequence

```
AthACBP  TCA ACC TGA TGAATCAAAT CCTCATCTGC AGTAACTTTA TCTTAAGCAT CAA 322
BnaACBP  TCA GCT TGA TGGTGATCTA TTAGTGCTCT TATGCAGTAA TTCTATCTAA GCT 322
GhiACBP  TGT GTG TGT GTATGACCAA TGAAACTGGT GTAATTTCAA GCCCTTGTCG GTT 322
OsaACBP  GCT TCT TAA GAAGTTATTG AACTCTATGC TACATGGCTT CTTGTCAGCT TGA 322
RcoACBP  TAG TGT ATC GACCATTTAT CCTGTTGCTC CATTGTGTTT GCAAATGTTA AAA 322
ZmaACBP  AAG TCT CTT AATAGTGTTG CCAAACAGCT ATTTGTCAGA TGAGCTTTGT GCC 322

AthACBP  AATAACATTG CATAAGACTT GTTCTTTTGC TCTTGTGTTT CTATCATATT TAAG 376
BnaACBP  TCAAATAAGT TTAGCATAAG AGCTTGTTCT TTTACTTCTT G<              363
GhiACBP  ACTTGGGGTT TTCATTACTA TGAATAAATT ATGTACTCTT TTTCTATTGC TATT 376
OsaACBP  ACAACTCTAT GCTAATCTTG GTTTGCTTGA CAACTTACCA TTCTATTCAA TTCG 376
RcoACBP  TCAGTTTTAA TAAATAATTC CGTGGAGTGT AATTGTGNAT GGTACCTTGT GGTT 376
ZmaACBP  ATTTCCCTCA TCATTTAGAA GTATATCATT CGATCCATTT GGGGATCTTG TTAA<376

AthACBP  AGCTATCTTN CTTTGTNNNC ATGGTGTGAT CTCNTAAAAA TNCTTGATAT TGGT 430
GhiACBP  ATGTTATTTA CAGTAG<                                        392
OsaACBP  CGGGATGT<                                                 384
RcoACBP  TTTCTNCTAA AATAAATTCC AATCCTTTGT GGNACCTAAA TTAGGTTCTA AAAA 430

AthACBP   TAAACCAGNG GATCATGNA AACTNAA<                            454
RcoACBP   AAAAAAAAAA A<                                            441
```

exon lengths were similar for *Arabidopsis, Brassica* and rice (279 bp), while castor bean was 273 bp and maize was 270 bp. The 3' untranslated region of the *acb* homologues became increasingly AT rich with strings of As or Ts of four or more nucleotides repeating until the sequence ended (Figure 25). Such sequences are known to be putative sites for the initiation of the poly-$(A)^+$ tail biosynthesis.

DISCUSSION

Gene number. Our data suggest that there were three or possibly four ACBP genes (*acb* homologues) in *Arabidopsis*, and it appears that at least one if not two of those isoforms may be expressed in *Arabidopsis*. It was uncertain whether these various bands represent three identical copies of the *acb*-1 gene (unlikely), different isoforms of the *acb*-1 gene (more likely), or various alleles of *acb*-1 (unlikely since a large amount of polymorphism would be required for such markedly divergent patterns). Therefore, the *acb*-1 gene was actually one member of a small multi-gene family.

Since none of the restriction enzymes cut within the known exons for *acb*-1, each fragment should correspond to at least one *acb*-1 exon in the *Arabidopsis* genome. Given the small size of the coding region for the expressed ACBP, it was likely that the probe hybridized to the intact functional ACBP gene, any possible isoforms and to one or more related pseudogenes.

Southern blot analysis of *Brassica napus* indicated that there were six *acb* genes present (Hills et al., 1994), although only 5 bands were visible following Southern hybridization. *B. napus* is an amphidiploid species derived from the diploid species *B. rapa* and *B. oleracea* (Hosaka et al., 1990). Six ACBP genes were found in the *B. napus* genome. Three

of these loci have been mapped to chromosomes 5, 8 and 9, while a fourth locus maps to chromosome 15. Two of the loci have yet to be identified with a linkage group (Hills et al., 1994). No work was performed to determine how many of the putative genomic sequences were expressed in rapeseed. This suggests that there are three *acb* genes in the diploid crucifers, and in the tetraploid *B. napus,* six copies of the gene.

In rapeseed and related *Brassica* species most genomic sequences have been shown to be highly polymorphic (Figdore et al., 1988). Therefore, it was not surprising that there were as many as six *acb* loci (Hills et al., 1994) considering *B. napus* is amphidiploid (Hosaka et al., 1990). Therefore, three *acb* loci in the n = 9 diploids that gave rise to rapeseed is reasonable.

How many of these different *acb* sequences are expressed? If there are as many pseudogenes in plants as in animals, then there may be only one isoform that undergoes complete transcription and translation and functions in the cell. One might speculate that there could be at least two ACBP isoforms in plants, one "housekeeping" version expressed in all cells and then a second specialized isoform with differences in the C-terminal portion of the protein. Since helix 4 is involved with protein-protein interactions (Kragelund et al., 1993) the specificity would likely be built into the carboxyl-end of the protein. We might expect that the 3' coding sequences of the different *acb* genes within a given species would display the greatest sequence divergence since the various isoforms might be expected to dock with different acyl-utilizing enzymes. The expression pattern for the single isoform isolated from *Brassica napus* suggests that more than one isoform may function in any cell type (Hills et al., 1994).

Gene expression. Northern hybridization indicated that the

mature *A. thaliana* mRNA transcript was approximately 1600, which included 276 bp of coding sequence. From these data it is impossible to determine the number and size of introns present in the *acb*-1 gene. If we assume that there are three introns (as with the mammalian *acb* gene) we might expect the genomic *acb* sequence to be at least 2800 bp (not including the promoter).

In Northern blots one mRNA band hybridized to the *acb* probe suggesting either a single functional gene or functional *acb*-1 isoforms with transcripts of similar sizes. In rat, there are multiple *acb* genes, but some are processed pseudogenes and only one *acb* gene is actually expressed (Mandrup et al., 1992). It may be that the *acb* isoforms are all approximately the same size, so only one size class of mRNA shows up after denaturing electrophoresis. However, within the population of 1.6 kb transcripts there may have been more than one ACBP isoform expressed in *Arabidopsis*.

During conversion of animals (3T3) cells into adipocytes, a number of transcripts, including ACBP, were expressed (Stephens et al., 1993). Transcripts for steryol-CoA desaturase, lipid binding protein (aP2) and the insulin-responsive glucose transporter were also up-regulated. As the transcriptional activation factor C/EBP was involved in *trans*-activation of these genes (Owens et al., 1989), we might expect that a C/EBP-like transcriptional factor could enhance the expression of *acb* in lipogenic or lipid-utilizing tissue. Promoters for the *acb* gene should be examined to see if C/EBP-response elements exist, as has been done for mouse promoters (Li et al., 1990).

In plant cells, the highest ACBP expression appeared in developing seed or in the cotyledon of a germinating seedling. These cells were involved with TAG biosynthesis and lipid storage or TAG catabolism and ß-

oxidation or glyoxylate cycle and gluconeogenesis. High *acb* expression in the rat also occurred in the liver and in adipose tissue. However, in animals there was also elevated *acb* expression in secretory cells, intestine and brain (Owens et al., 1989) indicating the specialized function for ACBP at these sites. In general, there was a reasonable agreement between the ACBP protein concentration (Chapter 2, thesis) and *acb* mRNA (Table 9) suggesting that ACBP abundance was correlated with *acb* expression.

Library screening. The fact that the *Arabidopsis* probe hybridized so well to soybean, clover and safflower DNA indicates that this cDNA has a high degree of sequence identity with other dicot ACBP homologues. As such, this ACBP sequence can be used to find, isolate and purify other ACBP homologues and isoforms from a cDNA library from any of these species. Of the 6 separate, independently-isolated clones from the *A. thaliana* genomic library, all may contain the exact same insert, contain the same gene (*acb*-1) but on an overlapping piece of DNA (different amounts of 5' and 3' flanking regions) or may contain more than one genomic isoform. Yet one other possibility exists: one of these genomic clones may encode a regulatory protein that has an ACBP-like domain with other functional domains part of the protein. For example, this bifunctional protein could contain a DNA-binding domain, as in the case of a nuclear-localized transcriptional activating factor (Hawkins and Lamb, 1995).

DNA sequences. In rat, the four *acb* exons of the ACBP gene and the four helices of the ACBP polypeptide correspond strongly with one another (Hansen et al., 1991). If the same conservation between exons and helices exists with the *Arabidopsis acb* gene and AthACBP, then exon-intron boundaries can be established. The helices for bovine ACBP span

residues 1-15, 20-36, 51-60 and 65-85. (Andersen et al., 1991). The corresponding helices in the *A. thaliana* ACBP span residues 1-17, 22-39, 52-65 and 70-88 (see Figure 16, chapter 2, p. 67). The first codon encoding the loops in between helices in *Arabidopsis* ACBP were either CTG, GTT, GCT whereas the last codon in these loops was AGC, ATG or AAA (Figure 35).

At the 5' end, most of these cDNA sequences terminate before -35. Some features of this region can be compared. *Arabidopsis* and castor bean 5'-untranslated sequences are both GC-rich, as with the expressed rat gene (Hansen et al., 1991). *Arabidopsis*, *Brassica* and *Oryza* all contain ATTTA boxes within the first 30 nucleotides upstream of the start ATG (Figure 35). *Rinculus* contains a CCGGGC box, which is a well-characterized response element for GCF (granulocyte colony factor). Transcriptional start sites for expressed *acb* in rat were -60 and -64 bp with the canonical TATA box at -93 bp (Mandrup et al., 1992).

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CHAPTER 5

SUMMARY AND PERSPECTIVES

I. Acyl-CoA-binding protein

My research at Michigan State University always proceeded from the same assumptions concerning experimental design, rigorousness in strategy employed, and determination of success or failure. This approach, also known as the scientific method, has been the principle guiding science since the seventeenth century. A consideration of newer concepts regarding analysis of complex systems is provided later in this chapter.

A. Structure

The *Arabidopsis thaliana* cDNA clone displayed a high degree of sequence homology to animal ACBPs that were found in the database in 1992 (Ebeling and Jimenez-Montano, 1990). Overexpression of this cDNA in *E. coli* produced a protein that could selectively bind acyl-CoA. Antibodies raised to the recombinant protein bound to an 10.4 kDa gene product in all *Arabidopsis* and *Brassica napus* organs examined indicating that ACBP is a "housekeeping" protein required by all cells. Marked increase in ACBP concentration during oil biogenesis in *B. napus* and deposition of ACBP in mature canola oilseeds argued that ACBP serves a specialized function in lipid metabolism.

Microcalorimetry has been used to examine substrate-enzyme or antigen-antibody interactions for a number of years (Biltonen and Langerman, 1979) and recently acyl-CoA and bovine ACBP (Rasmussen et

al., 1994) or *Arabidopsis* ACBP (Chapter 3, thesis) have also been measured. An analysis of the five plant ACBPs sequenced to date reveals that helix 4, the portion of the protein that displays the greatest conformational shift upon substrate binding (Kragelund et al., 1993), is likely to be the domain involved in protein-protein interactions.

This immediately suggests two characteristics not reported elsewhere: recognition between ACBP and the proteins which act as acyl-CoA donors or acceptors depends on whether ligand is bound or not, and tertiary structure perturbations induced during protein docking may alter the binding affinity between ACBP and its ligand. In other words, the uptake and release of ACBP may be controlled by the formation of protein complexes (Yangibashi et al., 1988), which is influenced by the ligand-binding status of any lipid-transfer protein.

Interestingly, there is now new evidence that the activation of acyl-CoA synthetase (ACS), an enzyme with which ACBP can be expected to interact, is via reversible membrane association, a situation similar to the activation of protein kinase *c,* glycogen synthetase and cytidylyl-transferase (Mangroo and Gerber, 1992). This would suggest that ACBP may also interact directly with phospholipid moieties in the membrane. Since the exterior surface of the protein is hydrophobic (Kragelund et al., 1993), it may float on a lipid bilayer of acyl chains partially submerged in the membrane with which it is docking.

It may be that the delivery of acyl-CoA to an acyl-CoA utilizing complex or transporter (e.g., palmitoyl-carnitine transferase) was governed by an interaction similar to that between a high density lipoprotein (HDL) cholesterol complex and the receptor SR-BI (Acton et al., 1996). This system, where there is selective lipid transfer without protein turnover, which is saturable and operates without

receptor-mediated endocytosis has many features similar to acyl-CoA transfer mediated by ACBP. Additional molecular, physiological and genetic studies of ACBP function should help to further our understanding of the mechanism by which ACBP donates acyl-CoA esters to acyl-CoA utilizing systems and the role that ACBP plays in acyl-CoA transport.

Another polypeptide that has been purified and studied in animals is the microsomal triglyceride transfer protein (MTP) (Wetterau and Zilversmit, 1984). To date a plant homologue has not yet been discovered. Apparently MTP catalyzes the transport of TAG and cholesteroyl ester (CE) between membranes. The exact physiological function of MTP is not known since it is highly enriched in the liver and intestinal mucosa. Due to its ability to transfer triglycerides between membranes and its presence in the ER, it may play a role in the biogenesis of triglyceride-rich plasma lipoproteins, very low-density lipoproteins in the liver or intestinal chylomicrons (Wetterau et al., 1991). Perhaps both ACBP and MTP interact with diacyl- glycerol:acyl-CoA acyltransferase. Apparently, MTP is involved with assembly of the plasma lipoproteins which transport TAG. Similarly, it would be interesting to compare the structural motifs in the oleosins (Cummins et al., 1993) to those of MTP since the olesins do a similar function by stabilizing the lipid body.

B. Function

The recombinant *A. thaliana* ACBP shows a differential preference for the binding of different acyl-CoA substrates, but does not display a marked discrimination or a clear preference for any C_{16}- to C_{20}-acyl-CoAs. While the animal ACBP homologue prefers acyl-CoAs with chain

lengths between C16 and C20 (Rasmussen et al., 1994), the plant ACBP lacks this specificity.

The change in the specific heat capacity (ΔC_p) was relatively large (-948 J mol^{-1} K^{-1}), probably due to a preponderance of hydrophobic interactions, which leads to a rather strong enthalpy-entropy compensation. We propose that the binding of acyl-CoA to ACBP is driven by enthalpy, but the release of acyl-CoA from the complex is driven by entropy. Both enthalpy and entropy were temperature dependent with the optimum binding temperature most sensitive to entropy rather than enthalpy. Formation of a docking protein-ACBP/acyl-CoA complex may promote relaxation of the ACBP-ligand contacts and facilitate acyl-CoA release. To our knowledge, this is the first time that the force behind the release of substrate has been attributed to entropy.

The acyl-CoA-binding protein described here has some unique properties in terms of lipid-protein and membrane-protein interactions (Stowell and Rees, 1995). Binding of saturated or unsaturated acyl-CoAs was driven by enthalpy, and the release of ligand from binding protein was most likely entropy driven. Very little difference existed between the binding of unsaturated acyl-CoAs and saturated ligands of the same chain length. At the optimum temperature of binding (17° C), the contribution of entropy to the favorable free energy was nearly as strong as that of enthalpy.

Rasmussen et al. (1990) compared the affinities of bovine hepatic (hFABP) and cardiac FABP (cFABP) with ACBP for the binding of acyl-CoAs using a number of assays. All of these experiments showed that ACBP has a much higher affinity for acyl-CoAs than does FABP. The binding of acyl-CoAs to the two FABPs in the Lipidex-1000 assay was so insignifi-

cant that no meaningful K_d values could be calculated, whereas ACBP effectively bound acyl-CoA esters with a K_d of 0.1-0.2 μM.

Recent theoretical and experimental advances have allowed a better understanding of the regulation of some biochemical pathways (Cao and Hatch, 1995). Unfortunately, not enough data exist for this type of metabolic flux analysis in lipid biosynthetic pathways, but work is underway (Post-Beittenmiller at al., 1991 and 1992). *In vivo* measurements of glycogen synthetase activity indicated that the changes in flux through this enzyme were related to the activation of the enzyme, not to the level of substrate (Shulman et al., 1995). Allosteric effects made the greatest impact on glycogen accumulation, but these changes occurred not with GS, but with the glucose transporter. Activation of GS matched the turnover of the enzyme with the flux of glucose into the cell (Shulman et al., 1995). If a similar mechanism worked for the acyl transferases, then enzyme activity adjusts to the rate at which fatty acids are synthesized by FAS or acyl-CoAs are transported by ACBP.

We have seen that malonyl-CoA and long-chain acyl-CoAs serve as metabolic coupling factors (Prentki et al., 1992), and ACBP appears to protect a number of enzymes from the feedback inhibition of free acyl-CoAs (Chapter 2, thesis). This raises a question: is the primary function of ACBP transport or does it act as a sensor monitoring acyl-CoA concentration? If ACBP does act as a sensor, then the amount of ACBP binding ligand could regulate specific cellular processes.

A key regulatory point in the assembly of glycerolipids is the acyl-CoA utilizing acyltransferases, enzymes that are membrane associated and highly enriched in the ER (Thompson, 1993), as well as thylakoid

and inner mitochondrial matrix membranes. Similarly, the biosynthesis of cuticular waxes starts with acyl-CoA substrates for the generation of 16- and 18-carbon hydroxy fatty acids (von Wettstein-Knowles, 1993), and these substrates must make their way to the various cellular sites. ACBP, as well as other lipid transfer proteins, may play a role in monitoring the concentration of specific lipid moieties, and these proteins may be able to modulate acyltransferase activities directly or stimulate G proteins or phospholipase activities and thus influence *de novo* lipid biosynthesis (Munnik *et al.*, 1995).

C. Genetics

Southern hybridization indicated that there were three, or possibly four, ACBP genes (*acb*-1 homologues) in *A. thaliana,* surprising for a plant with such a small genome (Goodman et al., 1995). A number of other plant species contained genomic DNA sequences that were capable of hybridizing with the *A. thaliana acb*-1 probe. Multiple forms of *acb* were observed in rapeseed, safflower, soybean, sunflower, and clover. Northern analysis indicated *acb* was expressed in all tissues of *A. thaliana*, and its expression was markedly elevated in seeds accumulating TAG or seedlings utilizing oil-storage reserves.

In general, there was a reasonable agreeement between the cellular concentration of ACBP (Chapter 2, thesis) and *acb* mRNA concentration (Table 9) suggesting that ACBP abundance was correlated with *acb* expression. In plant cells, ACBP expression appeared in developing seed or cotyledon of germinating seed- lings. These cells were involved with TAG biosynthesis and storage or TAG catabolism and ß-oxidation or glyoxylate cycle and gluconeogenesis, a situation analogous to that seen in animal cells (Hansen et al., 1991). High *acb* expression in the rat

also occurred in the liver and in adipose tissue. Additionally, *acb* expression was elevated in secretory cells, intestine and brain (Owens et al., 1989).

Certainly the *acb* transcript level was controlled in various plant tissues (Chapter 4, thesis) suggesting that it was likely that transcriptional regulation was yet another way in which the cell modulated ACBP concentrations. Polyunsaturated fatty acids (PUFA) regulate the transcription of a number of important lipogenic genes (e.g., FA synthetase, steroyl-CoA desaturase) and glycolytic genes (e.g., glucose transporter, glucose-6-P dehydrogenase) in animal liver (Clark and Jump, 1996). Suppression of the expression of these genes involves nuclear factors and is independent of eicosanoid biosynthesis (Amri et al., 1995). It was likely that this interaction between PUFA and gene expression was through a nuclear-localized factor which blocked transcription by binding either free PUFA or a PUFA-CoA ester and a specific response element in the promoters of the appropriate lipogenic or glycolytic genes. We expect that the domain which binds PUFA in this transcriptional factor would have similar structural motifs as FABP (Veerkamp and Maatman, 1995) or ACBP (Knudsen et al., 1993).

Such coordinate regulation of genes encoding glycolytic or lipogenic enzymes suggests FAs or their acyl-CoA derivatives play a very important role in mediating primary metabolism within cells (Jump et al., 1994). In rat, coordinate induction of ACBP, FABP and peroxisomal ß-oxidation by peroxisome proliferators has been observed (Vanden Heuvel et al., 1993) suggesting lipid catabolic pathways were modulated transcriptionally in response to an increases in the intracellular pool of fatty acids of acyl-CoA esters.

We did not find a clone hybridizing to the *A. thaliana acp* gene

that encoded a polypeptide longer than ACBP (Chapter 4, thesis). Speculation exists that an important regulatory protein could contain an ACBP-like acyl-CoA-binding domain and also contain a DNA-binding domain that would interact with response elements in promoters proximal to acyl-CoA utilizing enzymes. Such a multifunctional gene, encoding a multi-functional, multi-domain polypeptide (Hawkins and Lamb, 1995) was not found.

II. PERSPECTIVES

A. Purpose and puzzles

My intent in coming to Michigan State University was to study the genetics and molecular biology of plant lipid biosynthesis. During the last five years I have largely met this goal by learning how to apply the scientific method to the analysis of biochemical systems. Early on in my studies I was confounded by an obvious fact: apparently there were not enough genes in a single plant to account for all of the structural and cellular diversity seen. This question was first raised after examining a small piece of paper plastered to Dr. Ohlrogge's file cabinet and found in other locations throughout his lab (Table 10).

Based on this information (confirmed in Goodman et al., 1995), we can conclude that even the most complex plants contain **no more than** 60,000 genes arranged in no more than *circa* 10^{10} base pairs. This compares with *c.* 80,000 genes in 1-2 x 10^9 base pairs (Guyer and Collins, 1995) in higher animals. There are at least 200 different cell types in a common plant (Esau, 1966) with a multitude of organelles, metabolons, non-coding RNA species, enzymes and structural proteins in each cell type. The numbers of cells in a single plant is beyond counting; the number of structural elements in a leaf exceeds the total

Table 10. The information about the genome structure of *Arabidopsis thaliana* posted in the laboratory of J. B. Ohlrogge.

The *Arabidopsis* genome

70,000 kb total	avg. gene is 3 kb
63,000 kb coding sequences	avg. intron is 215 bp
20,000 genes estimated	avg. gene has 3.13 introns
avg. 5' untranslated in cDNA = 161 bp	
avg. primary transcript is 2.4	
avg. 3' untranslated in cDNA = 232 bp	

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number of genes in the plant, to say nothing of the informational complexity found in macromolecules (Ebeling and Jimenez-Montano, 1990). Consequently, genes, the instruction set, must command the growth of cells and structures most parsimoniously, and in the case of a long-lived species, such as *Sequoia sempervirens*, the organism must end up with functioning structures that will last centuries. The riddle: how is this accomplished?

Some of the most important paradigms in biology arise from the theory of evolution. Therefore, to a great extent much of the thinking in biology has been influenced by Charles Darwin: the biological world fulfills a design shaped by natural selection. This selection pressure operates not on genes or embryos directly, but on the final product. We explain the shape of a leaf or the activation of a biochemical pathway based on its cause-effect relation. We think only of what purpose it serves, and we link the structure to the function and the genetics to the function. We tend to forget that there are forces governing growth and development that have more to do with forms in motion than with natural selection.

The research I performed always proceeded from the same assumptions concerning experimental design, rigorousness in the strategy employed, model building, the testing of hypotheses, the determination of success or failure. All of these arise from the scientific method, the principle guiding science since the early seventeenth century.

B. New insights

One alternative to our present approach to research is to study the behavior of complex biological systems using the tools of chaos

theory, recently renamed complexity theory, which uses non-linear dynamics for modeling highly interactive systems. The central tenet of chaos theory is that the complexity inherent in most biological systems conceals an underlying order which has previously eluded characterization, and the only way to analyze a system where there are multiple interactions between individual system components is by using non-linear dynamics (Gleick, 1987). Chaos is a poor descriptor term since in its normal usage chaos means disordered. Here the term describes the deterministic random fluctuations of a given behavior in a system that are so complex as to appear random. That is, the apparent disorder actually masks an underlying pattern of relatively sophisticated ordering (Capra, 1982). If there is anything characteristic of biological signals, it is that they fluctuate, and yet, there is direction and correlation, pattern and eventually, an underlying order. Now, the presence of unexpected fluctuations or oscillations could not simply be ignored. Where chaos begins, classical science stops (Meakin, 1986).

The advantage of introducing the concepts used in fractal geometry and chaos theory into the experimental approaches or the current paradigms used to model biological systems is to change our mind set. The application of fractal rules to biological systems is a new tool whose use has generated unexpected results. These concepts serve to motivate us to consider alternative approaches, and perhaps, to develop different hypotheses that can be tested along side those based on a more reductionist approach derived from Bacon's scientific method (Platt, 1964).

All our concepts, paradigms, and theories are limited and approximate (Gleick, 1987). Modeling and theoretically guided experimentation are required to better understand the processes that occur within cells, organisms or entire ecosystems, and the detailed analysis

of single components within these systems will not shed more light on the "self-organizing" (neither planned nor directed) behaviors or the patterns that arise within these complex systems (Grubb, 1992). Lindenmayer's (1968) pioneering work on plants has been extended so effectively that one has difficulty distinguishing computer-generated plants from photographs (Prusinkiewicz et al., 1990).

The mathematics used to describe these complex systems requires the use of non-linear dynamics, whose differential equations must often be solved using iteration (computationally) rather than by classical calculus or algebraic methods (May, 1976). By analogy, classical Euclidian geometry is not powerful enough to cope with pattern formation in complex systems, and so, fractal geometry, which is closer to nature, is more complex, and augments the stylish simplicity of Euclid, has been adopted instead. It is no surprise that it was not possible to make any progress in the field prior to the advent of powerful computers.

It was apparent by the early-1980's that many complex systems displayed certain common behaviors that appeared to be characteristic of all complex systems. In fact, one of the greatest contributions of complexity theory has been the identification of certain behaviors that appear universal in complex systems (Glass and Malta, 1990). Fractals, the minimum unit found in any chaotic system, from tornadoes to sunflower whorls to flux across a boundary, have the characteristic of being highly random and yet highly coherent at the same time (Gleick, 1987). One of the early tenets of chaos theory was that the behaviors of complex systems cannot be explained by analyzing single components of a system (Mandelbrot and van Ness, 1968). The reductionist scientific approach would not explain interesting or unexpected behaviors that arose from the spontaneous interaction of the components, referred to as

"emergent properties" (Yorke and Li, 1975). The very property that appealed to some scientists caused other researchers to spurn complexity theory. To some, chaos is a science of process, rather than one of stasis, a science of becoming rather than of being (Gleick, 1987).

C. Complexity theory and biological systems

The first principle is that disorder exists. Physicists and biologists most often want to discover regularities and consistencies. Of what use, then, is disorder? We must recognize disorder, as well as order, and we should not be confounded about complexity. Rather, we should attune ourselves to it (Yorke and Li, 1975). In the past, people have seen chaotic behavior in innumerable circumstances. They were running a biological experiment, and the experiment behaved in an erratic manner. Then, they tried to "fix it" or they gave up. They explained the erratic behavior by saying that there was a lot of noise or the experiment was bad, and in some cases, this was true.

However, a growing body of theoretical and experimental evidence suggests that most biological systems operate within or will regularly enter domains of chaotic behavior. At the molecular level, glycolytic metabolism (Markus and Hess, 1984), enzyme cascades (Welch, 1986), and cyclic nucleotide signaling in *Dictyostelium* (Martiel and Goldbeter, 1985) displayed features predicted by complexity theory. Similarly the flux of metabolites across the plasma membrane has been correctly modeled using complexity theory (Mangroo et al., 1995), as have specific cellular growth processes (Witten and Cates, 1986), or electrophysiological phenomena (Berridge, 1995). Indeed, one of the best descriptions of an "informational measure" for biological macromolecules came from chaos game representations (Dutta and Das, 1992; Goldman, 1993).

At the cellular level, ion channel kinetics (Liebovitch and Tőth, 1991), cardiac cell behavior (Guevara et al., 1981) and dendritic growth (Prochiantz, 1985) have been successfully modeled using chaos theory and fractal geometry. At the level of whole organisms, stomatal conductance and leaf photosynthesis were not linked by simple relationships, but were described by non-linear dynamics (Baldocchi, 1994), as in the function of neural systems (Hayashi et al., 1983; Rapp et al., 1985). Many light-dependent processes in plants follow oscillations predicted by chaos theory (Haglund et al., 1992). In a similar way, transitions in the state of the brain as demonstrated by electroencephalography were fractal in nature (Xu and Xu, 1988). Similarly, many clear examples exist of complexity theory applied to ecosystem dynamics (Grubb, 1992). Much of the early work on chaos in biological systems concentrated on population growth in discreet generations (Guckenheimer et al., 1976; May, 1976), predator-prey relations (Schaffer, 1986), competition in a perennial grassland (Tilman and Wedin, 1991), the physiology of blood production (Mackey and Glass, 1977), and the dynamics of biochemical reactions (Olsen and Degn, 1977). Indeed, the apparent randomness, which is actually ordered in a complicated way, makes us rethink the very idea of scientific analysis in a complex biological system.

One approach to the problem of complexity: DNA surely cannot specify the vast number of spatial structures that result in a flower or a tree, but the DNA can specify the repeating process of bifurcation and development. Such processes suit nature's purposes. Fractal scaling appears universal in morphogenesis (Gleick, 1987).

D. Metabolic control theory

Biochemists had to start somewhere when they began by analyzing

cellular metabolism, and over 70 years ago, they started at the level of individual enzymes. From there, they progressed to studying control mechanisms, dissecting transport using "model systems" (Yangibashi et al., 1988) or examining the flux of metabolites through multi-enzyme pathways. However, do any of the models produced then or more recently accurately describe the flux through these pathways *in vivo*? Later, when the tools became available, transcriptional, RNA processing, translational and post-translational control points in the expression of genes were identified. Although we know so much about these processes (Hawkins and Lamb, 1995), has a *single* control point been found in these genetic control mechanisms for any specific gene? Can we really make a sensible, accurate prediction regarding the control of any given multi-step enzymatic pathway in any given cell or organism?

Despite the appeal of metabolic control theory (Kacser and Porteous, 1987), experimental applications have lagged because of the limited availability of *in vivo* data. The v_{max} for GS was highly sensitive to glucose-6-phosphate concentration *in vivo*, which suggested that proximal steps have the highest flux control coefficients for the pathway, and the activities of glucose transport and hexokinase activity must be insensitive to G6P concentration (Shulman et al., 1995).

Just as in the physical sciences, these results have raised questions concerning the reliable quantitative characterization of chaotic behavior. It is becoming increasingly obvious that measurement of these dynamic behaviors are required. Both Li et al. (1992) and Lüttge and Beck (1992) successfully explained the periodic oscillations in specific biochemical pathways in plants using chaos theory. After all, with a great number of potential interactions among the elements of

complex systems, the behavior of *in vivo* biological systems and the emergent properties capable in these systems is not likely to be understood by dissecting and analyzing the individual parts. Pattern formation in certain cells can also be precisely modeled and predicted using complexity theory and fractal analysis (Meakin, 1986).

The potential for chaotic behavior in biochemical systems would seem to be immense. Not only are such systems non-linear, but there are sequences of reactions lying between major control points in most metabolic pathway (Markus et al., 1984). In the case where the flux through biochemical pathways, such as FA or lipid biosynthesis (Post-Beittenmiller at al., 1991; Ohlrogge et al., 1993), can change very rapidly in response to internal cellular or environmental conditions, non-linear dynamics can assist us in understanding regulatory points in the pathway, and more importantly, the multiple interactions that this pathway may have with the rest of cellular metabolism. Mangroo and Gerber (1992) set out to study FA uptake by *E. coli* and found a number of new, interesting observations regarding the recruitment of proteins to the inner membrane, an example of new accomplishments using a novel approach.

The first studies using fractal analysis to model a biochemical pathway were those of Markus and his group (Markus and Hess, 1984; Markus et al., 1984; Hess and Markus, 1987) who looked at glycolysis in yeast. Feedback regulation exists in this pathway so that elevated ADP concentrations were capable of stimulating phosphofructokinase (PFK), whereas elevated phosphoenolpyruvate (PEP) concentrations inhibited PFK. Elevated levels of ATP, on the other hand, stimulated pyruvate kinase. This pattern of inhibition and stimulation is deterministic and highly non-linear. The prediction was that even with a steady glucose influx, the output of ATP would be periodic (low input flux) or chaotic (high

input flux), depending on the concentration and interval for glucose addition, and this is what was found (Hess and Markus, 1987).

Concentrations of the product (ATP) appeared to fluctuate in a seemingly random way (Markus et al., 1984). In an earlier era, if you tried to publish such measurements, you might have been heavily criticized. Perhaps the reviewers would have said that such fluctuations were due to an inability to conduct the experiment properly: there was too much noise, too much variation, experimental errors were too pronounced for the data to be published. In this case, the commonly held belief is that if experimental conditions were held constant, then output should either be constant or periodic. Obviously, this is not true for systems that display chaotic behaviors.

Similarly, autocatalysis is an intriguing area where regulation occurs by complex mechanisms. A recent research result has interesting consequences for the ACBP results presented in this thesis. Yamashita et al. (1995) found that the activation of a UDP-glucuronosyltransferase occurred only after the enzyme acylated *itself*. Protein acylation has been reported previously and in some cases has a regulatory role (Knoll and Gordon, 1993), but this is the first report of a multi-functional enzyme activating itself using acyltransferase activity. The substrate for this cell-wall bound enzyme: acyl-CoA. The question: how does the acyl-CoA traverse the membrane and enter the intercellular space? The finding that approximately 10% of total protein found in phloem sap was ACBP may give us an insight into this question. Even with all the molecular details about ACBP (Kragelund et al., 1993) we still cannot answer this question. More recently, Shulman et al. (1995) has cast doubt on many of the experiments done with flux control to date. It was widely believed that GS was regulated by covalent phosphorylation, but

using *in vivo* ^{13}C-NMR measurements, these authors concluded that glucose transport/hexokinase controls glycogen synthesis flux, and the role of covalent phosphorylation of GS was to adapt GS activity to the flux (not *vice versa*). In addition, the authors suggested that further advances in ^{13}C-NMR and ^{31}P-NMR would allow the accurate measure of the glucose-6-phosphate and the rate of glycogen synthesis *in vivo*. Shulman et al. (1995) suggested that the role of enzymatic phosphorylation was to control the concentration of the intermediates, rather than the fluxes, calling into question the contemporary paradigm that kinase/phosphatase activities regulate flux control.

It has long been understood that non-linearity in feedback processes serves to regulate and control (Welch, 1986; Hess and Markus, 1987). Simply put, a linear process, given a slight nudge, tends to remain slightly offtrack, whereas a non-linear process, given the same nudge, tends to return to its starting point. With all such control phenomena, a critical issue is robustness: How well can a system withstand small jolts? Equally critical in biology is flexibility; organisms must respond to circumstances that vary rapidly and unpredictably.

Although Bacon's scientific method was a valuable tool for analyzing single components in a system, the elucidation and characterization of single components rarely gives us an insight into the vast repertoire of behaviors that come from system interaction (Grubb, 1992). New non-destructive approaches are required to assess processes that occur *in vivo* so that we may increase our sophistication and understanding.

E. Conclusions

Now, where do we go from here? Is molecular biology the culmination of biochemistry and cellular biology? Let us take a look at

where the new and challenging frontiers lie.

I suggest that newer directions may come from complexity theory. Just as genetic engineering provided tools so that biochemists and cell biologists could study development and differentiation, the new disciplines of artificial intelligence and virtual reality, non-linear dynamics and fractal geometry will provide us with the means to measure the processes in and the functions of complicated systems. If we do not appreciate these new tools, then we may believe that we ***understand*** and ***can control*** the biosphere around us. We may attempt to perform a feat of genetic engineering because the underlying idea is conceptually intriguing (Van Rooisen and Moloney, 1995), without considering all the ramifications of our acts. We may undertake a project because we **can** do it, not because we **should** do it. Unfortunately, this is a perfect prognosis for a catastrophe (Vitousek, 1995).

III. ACBP: Conclusions

Immunological analysis of the AthACBP protein (Chapter 2, thesis) microcalorimetric measurement of protein-substrate complexes (Chapter 3, thesis), Southern analysis of the gene *(acb-1)* and northern quantification of gene expression (Chapter 4, thesis) reveal a number of important structural features that have provided insight into the mode of acyl-CoA binding and regulation. The concentration of ACBP or its mRNA increases or decreases with acyl-CoA utilization. AthACBP appears involved with trafficking of fatty-acyl substrates between various organelles and in certain cell types (mid-maturity seed, germinating cotyledon) where acyl-CoA metabolism was extreme. There was a pronounced up-regulation of cellular ACBP (Chapter 4, thesis) in acyl-CoA-utilizing cells.

One can imagine what would happen *in vivo* if the bovine recombinant ACBP were to be overexpressed in a plant. Bovine ACBP would out-compete a plant ACBP for substrate, and if the bovine ACBP were incapable of docking with any of the appropriate acyl-CoA utilizing enzymes, then a concomitant rise in the acyl-CoA pool might be expected. These acyl-CoAs, however, would not be readily available to the plant cell, so although the pool size would be greater, utilization of those acyl-CoA esters would be more difficult for the plant cell.

Molecular genetics has provided scientists with powerful tools for the study of complex metabolic pathways (Szerszen et al., 1994) and for the production of novel crop varieties with economically valuable food and fiber uses (Töpfer et al., 1995). The typical modular organization of the ACBP gene (Chapter 4, thesis) where each exon encodes a well-defined structural element (Mandrup et al., 1992) suggests that alternatively spliced transcripts could encode functional proteins. Different fusion proteins carrying an acyl-CoA-binding domain could acquire catalytic properties (Okuley et al., 1994), regulatory functions (Yamishita et al., 1988) or even be a target for biotechnological manipulation (Van Rooijen and Moloney, 1995).

However, further progress depends on the elucidation of the complex regulatory mechanisms that limit lipid biosynthesis as well as understanding the partitioning of photosynthate into precursors of protein, carbohydrate or lipid moieties. Most of our knowledge about lipid binding and transfer by proteins or lipoproteins comes from *in vitro* experiments (Tall, 1995). Although it has been assumed that these proteins simply increase the mobility of certain lipids, it may be that they monitor the level of a specific lipid and have regulatory properties as well (Skinner et al., 1995). It will be interesting to

see if ACBP or an ACBP-like domain will be found that serves such a regulatory function. Clearly, FAs are not only utilized as building blocks for membrane and storage lipid biosynthesis, providing structural components and fuel for cells, but they also serve as important mediators of gene expression.

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In addition, the following insight by Max Plank (1915) was appreciated as I put this chapter together. "It is our habit to approach the explanation of a physical process by splitting this process into elements, but this procedure presupposes that the splitting of the whole does not affect the character of the whole...One cannot understand irreversible processes, such as those displayed by complicated systems, on the assumption that all properties of the whole may be approached by a study of its parts."

BIBLIOGRAPHY

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., and Krieger, M. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. **Science** *271,* 518-520.

Agerberth, B., Boman, A., Jörnvall, H., Mutt, V., and Boman, H.G. 1993. Isolation of three antibacterial peptides from pig intestine: Gastric inhibitory polypeptide (7-42), diazepam-binding inhibitor (32-86) and a novel factor, peptide 3910. **Eur. J. Biochem.** *216,* 623-629.

Alho, H., Fremeau, R.T., Jr., Tiedge, H., Wilcox, J., Bovolin, P., Brosius, J., Roberts, J.L., and Costa, E. 1988. Diazepam binding inhibitor gene expression: Localization in brain and peripheral tissues in rat. **Proc. Natl. Acad. Sci. (USA)** *85,* 7018-7022.

Amri, E.Z., Bonino, F., Alihaud, G., Abumrad, N., and Grimaldi, P.A. 1995. Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. **J. Biol. Chem.** *270,* 2367-2371.

Andersen, K.V., Ludvigsen, S., Mandrup, S., Knudsen, J., and Poulsen, F. M. 1991. The secondary structure in solution of acyl-Coenzyme A binding protein from bovine liver using ^{1}H nuclear magnetic resonance spectroscopy. **Biochem.** *30,* 10654-10663.

Andersen, K.V., and Poulsen, F.M. 1992. Three dimensional structure of acyl-Coenzyme A binding protein from bovine liver. **J. Mol. Biol.** *226,* 1131-1141.

Andrews, J., and Keegstra, K. 1983. Acyl-CoA synthetase is located in the outer membrane and acyl-CoA thioesterase in the inner membrane of pea chloroplast envelopes. **Plant Physiol.** *72,* 735-740.

Arondel, V., and Kader, J.C. 1990. Lipid transfer in plants. **Experimentia** *46,* 579-585.

Bafor, M., Stobart, A.K., and Stymne, S. 1990. Properties of the glycerol-acylating enzymes in microsomal preparations from the developing seeds of safflower (*Carthamus tinctorius*) and turnip rape (*Brassica campestris*) and their ability to assemble cocoa-butter type fats. **J. Amer. Oil Chem. Soc.** *67,* 217-225.

Baldocchi, D. 1994. An analytical solution for coupled leaf photosynthesis and stomatal conductance models. **Tree Physiol.** *14,* 1069-1079.

Bass, N.M. 1988. The cellular fatty acid binding proteins: Aspects of structure, regulation, and function. **Intern. Rev. Cytol.** *111,* 143-184.

Berge, R.K., Flatmark, T., Osmundsen, H. 1984. Enhancement of long chain acyl-CoA hydrolase activity in peroxisomes and mitrochondria of rat liver by peroxisome proliferators. **Eur. J. Biochem.** *141,* 673-644.

Bernatzky, R., and Tanksley, S.D. 1986. Genetics of actin-related sequences in tomato. **Theor. Appl. Genet.** *72,* 314-321.

Bernhard, W.R., Thoma, S., Botella, J., and Somerville, C.R. 1991. Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. **Plant Physiol.** *95,* 164-170.

Berridge, M.J. 1995. Capacitive calcium entry. **Biochem. J.** *312,* 1-11.

Bethesda Research Laboratories. 1986. BRL pUC host: *E. coli* DH5∂™ competent cells. **Focus** (Bethesda Res. Labs.) *8,* 9-12.

Biltonen, R.L., and Langerman, N. 1979. Microcalorimetry for biological chemistry: Experimental design, data analysis, and interpretation. **Methods Enzymol.** *61,* 287-318.

Bligh, F.G., and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. **Can. J. Biochem. Physiol.** *37,* 911-917.

Borboni, P., Condorelli, L., de Stefanis, P., Sesti, G., and Lauro, R. 1991. Modulation of insulin secretion by diazepam binding inhibitor and its processing products. **Neurophamacol.** *30,* 1399-1403.

Boujrad, N., Hudson, J.R., and Papadoupoulos, V. 1993. Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothiolate oligodeoxynucleotide antisense to diazepam-binding inhibitor. **Proc. Natl. Acad. Sci. (USA)** *90,* 5728-5731.

Bovolin, P., Schlichting, J., Miyata, M., Ferrarese, C., Guidotti, A., and Alho, H. 1990. Distribution and characterization of diazepam binding inhibitor (DBI) in peripheral tissues of rat. **Regul. Pept.** *29,* 267-281.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** *72,* 248-254.

Browse, J., and Somerville, C. 1991. Glycerolipid biosynthesis: Biochemistry and regulation. **Ann. Rev. Plant Physiol. Plant Molec. Biol.** *42,* 467-506.

Burrier, R.E., Manson, C.R., Brecher, P. 1987. Binding of acyl-CoA to liver fatty acid binding protein: Effect on acyl-CoA synthesis. **Biochem. Biophys. Acta.** *919,* 212-230.

Börchers, T., Højrup, P., Nielsen, S.U., Roepstorff, Spener F., and Knudsen, J. 1990. Revision of the amino acid sequence of human heart fatty acid-binding protein. **Mol. Cell. Biochem.** *98,* 127-133.

Cao, S.G., and Hatch, G.M. 1995. Oleate stimulation of incorporation of exogenous glycerol into cardiolipin in isolated perfused rat heart does not involve direct activation of the CDP-DG pathway. **Biochem. Cell. Biol.** *73,* 299-305.

Capra, F. 1982. *The turning point*. 464 p. Simon and Schuster: New York.

Chen, Z.-W., Agerberth, B., Gell, K., Andersson, M., Mutt, V., Östenson, C.-G., Efendic, S., Barros-Söderling, J., Persson, B., and Jörnvall, H. 1988. Isolation and characterization of porcine diazepam-binding inhibitor, a polypeptide not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin release. **Eur. J. Biochem.** *174,* 239-245.

Clark, S.D., and Jump, D.B. 1996. Polyunsaturated fatty acid regulation of hepatic gene transcription. **Lipids** *31,* 7-11.

Crouch, M.L., and Sussex, I.M. 1981. Development and storage-protein synthesis in *Brassica napus* L. embryos *in vivo* and *in vitro*. **Planta** *153,* 64-74.

Cummins, I., Hills, M.J., Ross, J.H.E., Hobbs, D.H., Watson, M.D., and Murphy, D.J. 1993. Differential, temporal and spatial expression of genes involved in storage oil and oleosin accumulation in developing rapeseed embryos. **Plant Molec. Biol.** *23,* 1015-1027.

da Silva, J., Robinson, S.J., and Safford, R. 1992. The isolation and functional characterization of a *B. napus* acyl carrier protein 5' flanking region involved in the regulation of seed storage lipid synthesis. **Plant Molec. Biol.** *18,* 1163-1172.

Dellaporte, S.L., Wood, J., and Hicks, J.B. 1983. A plant DNA minipreparation: Version II. **Plant Mol. Biol. Rep.** *1,* 19-21.

Dutta, C., and Das, J. 1992. Mathematical characterization of chaos

game representation: New algorithms for nucleotide sequence analysis. **J. Mol. Biol.** *228,* 715-719.

Ebeling, W., and Jimenez-Montano, M.A. 1990. On grammars, complexity and information measures of biological macromolecules. **Math. Biosci.** *52,* 53-71.

Feinberg, A.P, and Vogelstein, B. 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. **Anal. Biochem.** *137,* 266-267.

Ferrero, P., Santi, M., Conti-Tronconi, B., Costa, E., and Guidotti, A. 1986. Study of an octadecaneneuropeptide derived from diazepam binding inhibitor (DBI): biological activity and presence in rat brain. **Proc. Natl. Acad. Sci. (USA)** *83,* 827-831.

Figdore, S.S., Kennard, W.C., Song, K.M., Slocum, M.K., and Osborn, T.C. 1988. Assessment of the degree of restriction fragment length polymorphism in *Brassica*. **Theor. Appl. Genet.** *75,* 833-840.

Glass, L., and Malta, C.P. 1990. Chaos in multi-looped negative feedback systems. **J. Theor. Biol.** *145,* 217-223.

Gleick, J. 1987. *Chaos: Making a new science*. 352 p. Viking Penguin: New York.

Goldman, N. 1993. Nucleotide, dinucleotide, and trinucleotide frequencies explain patterns observed in chaos game representations of

DNA sequences. **Nuc. Acids Res.** *21,* 2487-2491.

Goodman, H.M., Ecker, J.R., and Dean, C. 1995. The genome of *Arabidopsis thaliana*. **Proc. Nat. Acad. Sci. (USA)** *92,* 10831-10835.

Gray, P.W. 1987. Molecular biology of diazepam binding inhibitor. **Neuropharmacol**. *26,* 863-865.

Grosbois, M., Guerbette, F., and Kader, J.-C. 1989. Changes in level and activity of phospholipid transfer protein during maturation and germination of maize seeds. **Plant Physiol.** *90,* 1560-1564.

Grubb, P.J. 1992. A positive distrust in simplicity -- lessons from plant defenses and from competition among plants and among animals. **J. Ecol.** *80,* 585-610.

Grunstein, M. and Hogness, D. 1975. Colony hybridization: A method for the isolation of cloned cDNAs that contain a specific gene. **Proc. Natl. Acad. Sci. USA** *72,* 3961-3965.

Guarneri, P., Berkovich, A., Guidotti, A., and Costa, E. 1990. A study of the diazepam binding inhibitor (DBI) processing products in human cerebrospinal fluid and in postmortem human brain. **Neuropharmacol.** *29,* 419-428.

Guidotti, A., Forchetti, C., Corda, M., Konkel, D., Bennett, C., and Costa, E. 1983. Isolation, characterization and purification to

homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. **Proc. Natl. Acad. Sci. (USA)** *80,* 3531-3535.

Guevara, M.R., Glass, L., and Shrier, A. 1981. Phase locking, period doubling bifurcations, and irregular dynamics in periodically stimulated cardiac cells. **Science** *214,* 1350-1353.

Guyer, M.S., and Collins, F.S. 1995. How is the human genome project doing and what have we learned so far? **Proc. Natl. Acad. Sci. (USA)** *92,* 10841-10848.

Göttlicher, M., Widmark, E., Li, Q., and Gustafsson, J.-A. 1992. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. **Proc. Natl. Acad. Sci. (USA)** *89,* 4653-4657.

Ha, J.-H., Spolar, R.S., and Record, M.T. 1989. Role of the hydrophobic effect in stability of site specific protein-DNA complexes. **J. Mol. Biol.** *209,* 801-816.

Hach, M., Pedersen, S.N., Börchers, T., Højrup, P., and Knudsen, J. 1990. Determination by photoaffinity labeling of the hydrophobic part of the binding site for acyl-CoA esters on acyl-CoA-binding protein from bovine liver. **Biochem J.** *271,* 231-236.

Haglund, K., Ramazanov, Z., Mtolera, M., and Pedersén, M. 1992. Role of external carbonic anhydrase in light-dependent alkalinization by

Fucus serratus L. and *Laminaria saccharina* (L.) Lamour (Phaeophyta). **Planta** *188,* 1-6.

Hansen, H.O., Andreasen, P.H., Mandrup, S., Kristiansen, K. and Knudsen, J. 1991. Induction of acyl-CoA-binding protein and its mRNA in 3T3-L1 cells by insulin during preadipocyte-to-adipocyte differentiation. **Biochem. J.** *277,* 341-344.

Hansen, H.O., Grunnet, I., and Kundsen, J. 1984. Triacylglycerol synthesis in goat mammary gland. The effect of ATP, Mg^{2+} and glycerol-3-phosphate on the esterification of fatty acids synthesized *de novo*. **Biochem. J.** *220,* 513-519.

Harwood, J.L. 1988. Fatty acid metabolism. **Ann. Rev. Plant Physiol. Plant Mol. Biol.** *39,* 101-138.

Haunerland, N., Jagschies, G., Schulenberg, H., and Spener, F. 1984. Occurrence of two fatty acid-binding proteins in bovine liver cytosol and their binding of fatty acids, cholesterol, and other lipophilic ligands. **Hoppe-Seyler's Z. Physiol. Chem.** *365,* 365-376.

Hawkins, A.R., and Lamb, H.K. 1995. The molecular biology of multidomain proteins: Selected examples. **Eur. J. Biochem.** *232,* 7-18.

Helentjaris, T., King, G. Slocum, M.K. Sidenstrang, C., and Wegman, S. 1985. Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. **Plant Mol. Biol**. *5,* 109-118.

Hess, B., and Markus, M. 1987. Order and chaos in biochemistry. **Trends Biochem. Sci.** *12,* 45-48.

Hills, M.J., Dann, R., Lydiate, D., and Sharpe, A. 1994. Molecular cloning of a cDNA from *Brassica napus* L. for a homologue of acyl-CoA-binding protein. **Plant Mol. Biol.** *25,* 917-920.

Honig, B., and Yang, A.-S. 1995. Free energy balance in protein folding. **Adv. Protein Chem.** *46,* 27-58.

Hosaka, K., Kainian, S.F., McGrath, J.M., and Ouiros, C.F. 1990. Development and chromosomal localization of genome-specific DNA markers of *Brassica* and the evolution of amphidiploids and n=9 diploid species. **Genome** *33,* 131-142.

Hubbell, T., Behnke, W.D., Woodford, J.K., and Schroeder, F. 1994. Recombinant liver fatty acid binding protein interacts with fatty acyl-Coenzyme A. **Biochem.** *33,* 3327-3334.

Jin, L., Yang, J., and Casey, J. 1993. Thermodynamics of ligand binding to *trp* repressor. **Biochem.** *32,* 7320-7309.

Judson, H.F. 1979. *The eighth day of creation.* Simon and Schuster: New York.

Jump, D.B., Clarke, S.D., Thelen, A., and Liimatta, N. 1994. Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids. **J. Lipid Res.** *35,* 1076-1084.

Kader, J.C. 1985. Lipid binding proteins in plants. **Chem. Phys. Lipids** *38,* 51-62.

Kader, J.C. 1990. Intracellular transfer of phospholipids, galactolipids and fatty acids in plant cells. **Subcell. Biochem.** *16,* 69-111.

Kaikaus, R.M., Sui, Z., Lysenko, N., Wu, N.Y., Ortiz de Montellano, P.R., Ockner, R.K., and Bass, N.M. 1993. Regulation of pathways of extramitochondrial fatty acid oxidation and liver fatty acid-binding protein by long-chain monocarboxylic fatty acids in hepatocytes. **J. Biol. Chem.** *268,* 26866-26871.

Kacser, H., and Porteous, J.W. 1987. Metabolic control theory. **Trends Biochem. Sci.**, *12,* 5-14.

Keith, C.S., Hoang, D.O., Barret, B.M., Feigelman, B., Nelson, M.C., Thai, H., and Baydorfer, C. 1993. Partial sequence analysis of 130 randomly selected maize cDNA clones. **Plant Physiol.** *101,* 329-332.

Kinlaw, C.S., Gerttula, S.M., and Carter, M.C. 1994. Lipid transfer protein genes of loblolly pine are members of a complex gene family. **Plant Mol. Biol.** *26,* 1213-1216.

Knoll, L.J., and Gordon, J.I. 1993. Use of *Escherichia coli* strains containing *fad* mutations plus a triple plasmid expression system to study the import of myristate, its activation of *Saccharomyces cerevisiae* acyl-CoA synthetase, and its utilization by *S. cerevisiae*

myristoyl-CoA: protein N-myristoyltransferase. **J. Biol. Chem.** *268*, 4281-4290.

Knudsen, J. 1991. Acyl-CoA binding and transport, an alternative function for diazepam binding inhibitor (DBI), which is identical with acyl-CoA-binding protein. **Neuropharmacol.** *30*, 1405-1410.

Knudsen, J. 1990. Acyl-CoA-binding protein (ACBP) and its relation to fatty acid-binding protein (FABP): An overview. **Molec. Cell. Biochem.** *98*, 217-223.

Knudsen, J., and Grunnet, I. 1982. Transacylation as a chain termination mechanism in fatty acid synthesis by mammalian fatty acid synthetase. **Biochem. J.** *202*, 139-143.

Knudsen, J., Højrup, P., Hansen, H.O., Hansen, H.F., and Roepstorff, P. 1989. Acyl-CoA-binding protein in the rat. Purification, binding characteristics, tissue concentrations and amino acid sequence. **Biochem. J.** *262*, 513-519.

Knudsen, J., Mandrup, S., Rasmussen, J.T., Andreasen, P.H., Poulsen, F., and Kristiansen, K. 1993. The function of acyl-CoA-binding protein (ACBP)/diazepam binding inhibitor (DBI). **Molec. Cell. Biochem.** *123*, 129-138.

Kolattakudy, P.E. 1981. Structure, biosynthesis and degradation of cutin and suberin. **Ann. Rev. Plant Physiol.** *32*, 539-567.

Kolattakudy, P.E. 1987. Lipid-derived defense polymers and waxes and their role in plant-microbe interaction. *In:* Stumpf, P.K. and Conn, E.E. (eds.) *The Biochemistry of Plants*, v. *9*, pp. 291-314. Academic Press: New York.

Kolmer, M., Ross, C., Tirronen, M., Myöhänen, S., and Alho, H. 1994. Tissue-specific expression of the diazepam-binding inhibitor in *Drosophila melanogaster*: Cloning, structure, and localization of the gene. **Molec. Cell. Biol.** *14*, 6983-6995.

Kopka, J., Ohlrogge, J.B., and Jaworski, J.G. 1995. Analysis of in vivo levels of acyl-thioeseters with gas chromatography/mass spectrometry of butylamide derivatives. **Anal. Biochem.** *224*, 51-60.

Kozak, M. 1989. The scanning model for translation: an update. **J. Cell. Biol.** *108*, 229-235.

Kragelund, B.B., Vilbour, K., Madsen, J.C., Knudsen, J., and Poulsen, F.M. 1993. Three-dimensional structure of the complex between acyl-CoA binding protein and palmitoyl-Coenzyme A. **J. Mol. Biol.** *230*, 1260-1277.

Ladbury, J.E., Wright, J.G., Sturtevant, J.M., and Sigler, P.B. 1994. A thermodynamic study of the *trp* repressor-operator interaction. **J. Mol. Biol.** *238*, 669-681.

Li, Q., Yamamoto, N., Inoue, A., and Morisawa, S. 1990. Fatty acyl-CoAs are potent inhibitors of the nuclear thyroid hormone receptor *in*

vitro. **J. Biochem.** *197*, 699-702.

Li, Q., Yamamoto, N., Morrisawa, S., and Inoue, A. 1993. Fatty acyl-CoA binding activity of the nuclear thyroid hormone receptor. **J. Cell. Biochem.** *51*, 458-464.

Li, Y.X., Halloy, J., Martiel, J.L., Wurster, B., and Goldbeter, A. 1992. Suppression of chaos by periodic oscillations in a model for cyclic AMP signaling in *Dictyostelium* cells. **Experimentia** *48*, 603-606.

Li, X., Huang, J.H., Rienhoff, H.Y., and Liao, W.S.L. 1990. Two adjacent C/ERB-binding sequences that participate in the cell-specific expression of the mouse serum amyloid A3 gene. **Mol. Cell. Biol.** *10*, 6624-6631.

Lindenmayer, A. 1968. Mathematical models for cellular interactions in development. II. Simple and branching filaments with two-sided inputs. **J. Theor. Biol.** *18*, 300-315.

Livingstone, J.R., Spolar, R.S., and Record, M.T., Jr. 1991. Contribution to the thermodynamics of protein folding from the reduction in water-accessible nonpolar surface area. **Biochem.** *30*, 4237-4244.

Lüttge, U., and Beck, F. 1992. Endogenous rhythms and chaos in crassulacean acid metabolism. **Planta** *188*, 28-38.

Mackey, M.C., and Glass, L. 1977. Oscillations and chaos in physiological control systems. **Science** *197*, 287-289.

Mandelbrot, B.B., and van Ness, J.W. 1968. Fractal brownian motions, fractional noises and applications. **SIAM Rev.** *10,* 422-437.

Mandrup, S., Andreasen, P.H., Knudsen, J., and Kristiansen, K. 1993a. Genome organization and expression of the rat ACBP gene family. **Mol. Cell. Biochem.** *123,* 55-61.

Mandrup, S., Hummel, R., Ravn, S., Jensen, G., Andreasen, P.H., Gregersen, N., Knudsen, J., and Kristiansen, K. 1992. Acyl-CoA-binding protein/diazepam binding inhibitor gene and pseudogenes. **J. Mol. Biol.** *228,* 1011-1022.

Mandrup, S., Højrup, P., Kristiansen, K., and Knudsen, J. 1991. Gene synthesis, expression in *Escherichia coli,* purification and characterization of the recombinant bovine acyl-CoA-binding protein. **Biochem. J.** *276,* 817-823.

Mandrup, S., Jepsen, R., Skott, H., Rosendahl, J., Højrup, P., Kristiansen, K., and Knudsen, J. 1993b. Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. **Biochem. J.** *290,* 369-374.

Mangroo, D., and Gerber, G.E. 1992. Fatty acid uptake in *E. coli*: Regulation by recruitment of fatty acyl-CoA synthetase to the membrane. **Biochem. Cell. Biol.** *71,* 51-56.

Mangroo, D., Trigatti, B.L., and Gerber, G.E. 1995. Membrane permeation and intracellular trafficking of long chain fatty acids:

Insights from *Escherichia coli* and 3T3-L1 adipocytes. **Biochem. Cell. Biol.** *73,* 223-234.

Markus, M., and Hess, B. 1984. Transitions between oscillatory modes in a glycolytic model system. **Proc. Natl. Acad. Sci. (USA)** 1984. *81,* 4394-4398.

Markus, M., Kuschmitz, D., and Hess, B. 1984. Chaotic dynamics in yeast glycolysis under periodic substrate input flux. **FEBS Lett.** *172,* 235-238.

Marquardt, H., Todaro, G., and Shoyab, M. 1986. Complete amino acid sequences of bovine and human endozepines. **J. Biol. Chem.** *261,* 9727-9731.

May, R.M. 1976. Simple mathematical models with very complicated dynamics. **Nature** *261,* 459-467.

Meakin, P. 1986. A new model for biological pattern formation. **J. Theor. Biol.** *118,* 101-113.

Meijer, E.A., de Vries, S.C., Sterk, P., Gadella, D.W.J., Wirtz, K.W.A., and Hendriks, T. 1993. Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota* L.). **Molec. Cell. Biochem.** *123,* 159-166.

Mikkelsen, J., Højrup, P., Nielsen, P.F., Roepstorff, P., and Knudsen, J. 1987. Amino acid sequence of acyl-CoA-binding protein from cow

liver. **Biochem. J.** *245,* 857-861.

Mikkelsen, J., and Knudsen, J. 1987. Acyl-CoA-binding protein from cow. Binding characteristics and cellular and tissue distribution. **Biochem. J.** *248,* 709-714.

Miller, K.R., and Cistola, D.P. 1993. Titration calorimetry as a binding assay for lipid-binding proteins. **Mol. Cell. Biochem.** *123,* 29-37.

Mizutani, M.Y., Tomioka, N., and Itai, A. 1994. Rational automatic search method for stable docking models of protein and ligand. **J. Mol. Biol**. *243,* 310-326.

Mocchetti, I., Einstein, R., and Brosius, J. 1986. Putative diazepam binding inhibitor peptide: cDNA clones from rat. **Proc. Natl. Acad. Sci. (USA)** *83,* 7221-7225.

Mogensen, I.B., Schulenberg, H., Hansen, H.O., Spener, F., and Knudsen, J. 1987. A novel acyl-CoA-binding protein from bovine liver. Effect on fatty acid biosynthesis. **Biochem. J.** *241,* 189-192.

Mudd, J.B. 1980. Phospholipid biosynthesis. *In:* Stumpf, P.K. and Conn, E.E. (eds.) *The Biochemistry of Plants*. v. *4,* pp. 250-282. Academic Press: New York.

Munnik, T., Arisz, S.A., de Vrise, T., and Musgrave, A. 1995. G protein activation stimulates phospholipase D signaling in plants.

Plant Cell *7,* 2197-2210.

Murphy, K.P., and Gill, S.J. 1991. Solid model compounds and the thermodynamics of protein unfolding. **J. Mol. Biol.** *222,* 699-709.

Murphy, K.P., and Freire, E. 1992. Thermodynamics of structural stability and cooperative folding behavior in proteins. **Adv. Protein Chem.** *43,* 313-361.

Murphy, K.P., Xie, D., Garcia, K.C., Amzel, L.M., and Freire, E. 1993. **Proteins: Struct. Funct. Genet.** *15,* 113-120.

Nagy, F., Kay, S.A. and Chua, N.-H. 1988. A circadian clock regulates transcription of the wheat *Cab*-1 gene. **Genes Develop.** *2,* 376-382.

Newman, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikel, N., Somerville, S., Thomashow, M., Retzel, E., and Somerville, C. 1994. Genes galore: A summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. **Plant Physiol.** *106,* 1241-1255.

Nikawa, J., Tanabe, T., Ogiwara, H., Shiba, T., and Numa, S. 1979. Inhibitory effects of long-chain acyl-Coenzyme A analogues on rat liver acetyl-Coenzyme A carboxylase. **FEBS Lett.** *102,* 223-226.

Nolte, K.D., and Koch, K.E. 1993. Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. **Plant Physiol.** *101,* 899-905.

Ohlrogge, J., Jaworski, J., Post-Beittenmiller, D., Roughan, G., Roessler, P., and Nakahira, K. 1993. Regulation of flux through the fatty acid biosynthesis pathway . In: *Biochemistry and molecular biology of membrane and storage lipid biosynthesis in plants.* N. Murata and C. Somerville (Eds.) Amer. Soc. Plant. Physiol.: Rockville, MD.

Ohlrogge, J.B., Kuhn, D.N., and Stumpf, P.K. 1979. Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. **Proc. Natl. Acad. Sci. (USA)** *76,* 1194-1198.

Ohlrogge, J., and Browse, J. 1995. Lipid biosynthesis. **Plant Cell** *7,* 957-970.

Okuley, J. Lightner, J., Feldmann, K., Yadav, N., Lark, E. and Browse, J. 1994. *Arabidopsis FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. **Plant Cell** *6,* 147-158.

Olsen, F.L., and Degn, H. 1977. Chaos in an enzyme reaction. **Nature** *267,* 177-178.

Owens, G., Sinha, A., Sikela, J., and Hahn, W. 1989. Sequence and expression of the murine diazepam binding inhibitor. **Mol. Brain Res.** *6,* 101-108.

Palazzolo, M.J., Hamilton B.A., Ding, D., Martin, C.H., Mead, D.A., Mierendorf, R.C., Raghavan, K.V., Meyerowitz, E.M., and Lipshitz, H.D. 1990. Phage lambda cDNA cloning vectors for subtractive hybridization, fusion-protein synthesis and Cre-*loxP* automatic plasmid subcloning.

Gene *88,* 25-36.

Papadoupoulos, V., Berkovich, A., and Krueger, K.E. 1991. The role of diazepam binding inhibitor and its processing products at mitochondrial benzodiazepine receptors: regulation of steroid biosynthesis. **Neuropharmacol.** *30,* 1417-1423.

Papadoupoulos, V. Guarneri, P., Krueger, K.E., Guidotti, A., and Costa, E. 1992. Pregnenolone biosynthesis in C6-2B glioma cell mitochondria: Regulation by a mitochondrial diazepam binding inhibitor receptor. **Proc. Natl. Acad. Sci. (USA)** *89,* 5113-5117.

Plant, A.L., van Rooijen, G.J.H., Anderson, C.P., and Maloney, M.M. 1994. Regulation of an *Arabidopsis* oleosin gene promoter in transgenic *Brassica napus*. **Plant Molec. Biol.** *25,* 193-205.

Platt, J.R. 1964. Strong inference. **Science** *146,* 347-353.

Post-Beittenmiller, D., Roughan, P.G., and Ohlrogge, J.B. 1992. Regulation of plant fatty acid biosynthesis: Analysis of acyl-CoA and acyl-ACP substrate pools in spinach and pea chloroplasts. **Plant Physiol.** *100,* 923-930.

Post-Beittenmiller, D. Jaworski, J.G., and Ohlrogge, J.B. 1991. *In vivo* pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. **J. Biol. Chem.** 266, 1858-1865.

Pretki, M., Vischer, S., Glennon, M.C., Regazzi, R., Deeney, J.T., and Corkey, B.E. 1992. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. **J. Biol. Chem.** *267,* 5802-5810.

Price, H.M., Ryan, R.O., and Haunerland, N.H. 1992. Primary structure of locust flight muscle fatty acid-binding protein. **Arch. Biochem. Biophys.** *297,* 285-290.

Privalov, P.L., and Makhatadze, G.I. 1992. Contribution of hydration and non-covalent interactions to the heat capacity effect on protein unfolding. **J. Mol. Biol.** *224,* 715-723.

Prochiantz, A. 1985. Neuronal growth and shape. **Dev. Neurosci.** *7,* 189-198.

Prusinkiewicz, P., Lindenmayer, A., Hanan, J.S., Fracchia, F.D., Fowler, D.R., de Boer, M.J.M., and Mercer, L. 1990. *The algorithmic beauty of plants.* Springer-Verlag: New York.

Ramen, C.S., Allen, M.J., and Nall, B.T. 1995. Enthalpy of antibody-cytochrome C binding. **Biochem.** *34,* 5831-5838.

Rasmussen, J.T., Börchers, T., and Knudsen, J. 1990. Comparison of the binding affinities of acyl-CoA-binding protein (ACBP) and fatty acid binding protein (FABP) for long-chain acyl-CoA esters. **Biochem. J.** *265,* 849-855.

Rasmussen, J.T., Færgeman, N.J., Kristiansen, K., and Knudsen, J. 1994. Acyl-CoA-binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for B-oxidation and glycerolipid synthesis. **Biochem. J.** *299,* 165-170.

Rasmussen, J.T., Rosendahl, J., and Knudsen, J. 1993. Interactions of Acyl-CoA binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor. **Biochem. J.** *292,* 907-913.

Reddy, A.S., Ranganathan, B., Haisler, R.M., and Swize, M.A. 1996. A cDNA encoding acyl-CoA-binding protein from cotton. **Plant Physiol.** (in press)

Roepstorff, P., Schram, K.H., Andersen, J.S., Rafn, K., Baldursson, T., Krøll, J., Poulsen, K., Knudsen, J., and Kristiansen, K. 1995. Evaluation of mass spectrometric techniques for characterization of engineered proteins. **Molec. Biotechnol.** *4,* 1-12.

Rose, T.M., Schultz, E.R., and Todaro, G.J. 1992. Molecular cloning of the gene for the yeast homolog (*ACB*) of the diazepam binding inhibitor/endozepine/acyl-CoA binding protein. **Proc. Natl. Acad. Sci. (USA)** *89,* 11287-11291.

Rosendahl, J., Ertbjerg, P., and Knudsen, J. 1993. Characterization of ligand binding to acyl-CoA-binding protein. **Biochem. J.** *290,* 321-326.

Rosendahl, J., and Knudsen, J. 1992. A fast and versatile method for

extraction and quantification of long-chain acyl-CoA esters from tissue: content of individual long-chain acyl-CoA esters in various tissues from fed rat. **Anal. Biochem.** *207,* 63-67.

Roy, A., DeJong, J., Lamparski, D., George, T., and Linnoila, M. 1991. Depression among alcoholics -- relationship to clinical and cerebro-spinal fluid variables. **Arch. Gen. Psychiatry** *48,* 428-432.

Safford, R., Windust, J.H.C., Lucas, C. Da Silva, J., James, C.M., Hellyer, A., Smith, C.G., Slabas, A.R., and Hughes, S.G. 1988. Plastid-localized seed acyl carrier protein of *Brassica napus* is encoded by a distinct, nuclear multigene family. **Eur. J. Biochem.** *174,* 287-295.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Press: Cold Spring Harbor, N.Y.

Schaffer, W.M., and Kot, M. 1986. Chaos in ecological systems: The coals that Newcastle forgot. **Trends Ecol. Evol.** *1,* 58-63.

Sebedio, J.L., and Ackman, R.G. 1981. Fatty acids of *Brassica campestris* var candle seed and oils at various stages of refining. **J. Amer. Oil Chem. Soc.** *58,* 972-976.

Shulman, R.G., Bloch, G., and Rothman., D.L. 1995. *In vivo* regulation of glycogen synthase and the control of glycogen synthesis. **Proc. Natl. Acad. Sci. (USA)** *92,* 8535-8542.

Shoyab, M., Gentry, L., Marquardt, H., and Todaro, J. 1986. Isolation

and characterization of a putative endogenous benzodiazepine (endozepine) from bovine and human brain. **J. Biol. Chem.** *261,* 11968-11973.

Sigurskjold, B.W., Altman, E., and Bundle, D.R. 1991. Sensitive titration microcalorimetric study of the binding of *Salmonella* O-antigenic oligosaccharides by a monoclonal antibody. **Eur. J. Biochem.** *197,* 239-246.

Sigurskjold, B.W., and Bundle, D.R. 1992. Thermodynamics of oligosaccharide binding to a monoclonal antibody specific for a *Salmonella* O-antigen point to hydrophobic interactions in the binding site. **J. Biol. Chem.** *267,* 8371-8376.

Simon, T.C., Roth, K.A., and Gordon, J.I. 1993. Use of transgenic mice to map *cis*-acting elements in the liver fatty acid-binding protein gene (*Fabpl*) that regulate its cell lineage-specific, differentiation-dependent, and spatial patterns of expression in the gut epithelium and in the liver acinus. **J. Biol. Chem.** *268,* 18345-18358.

Singh, S.S., Nee, T.Y., and Pollard, M.R. 1986. Acetate and mevalonate labeling studies with developing *Cuphea lutea* seeds. **Lipids** *21,* 143-149.

Skinner, H.B., McGee, T.P., McMaster, C.R., Fry, M.R., Bell, R.M., and Bankaitis, V.A. 1995. The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. **Proc. Natl. Acad. Sci. (USA)** *92,* 112-116.

Smith, M.A., Jonsson, L., Stymne, S., and Stobart, K. 1992. Evidence for cytochrome b_5 as an electron donor in ricinoleic acid biosynthesis in microsomal preparations from developing castor bean (*Ricinus communis* L.). **Biochem. J.** *287,* 141-144.

Snyder, M.J., and Feyereisen, R. 1993. A diazepam-binding inhibitor (DBI) homolog from the tobacco hornworm *Manduca sexta*. **Mol. Cell. Endocrinol.** *94,* R1-R4.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **J. Mol. Biol.** *98,* 503-517.

Spolar, R.S., Livingstone, J.R., and Record, M.T., Jr. 1992. Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. **Biochem.** *31,* 3947-3955.

Spolar, R.S., and Record, M.T., Jr. 1994. Coupling of local folding to site-specific binding of proteins to DNA. **Science** *263,* 777-784.

Stadtman, E.R. 1957. Preparation and assay of acyl-Coenzyme A and other thiol esters. **Methods Enzymol.** *3,* 931-941.

Stephens, J.M., Butts, M., Stone, R., Pekala, P.H., and Bernlohr, D.A. 1993. Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipoctye differentiation by antagonists of adipogenesis. **Molec. Cell. Biochem.** *123,* 63-71.

Stephenson, F.A. 1995. The $GABA_A$ receptors. **Biochem. J.** *310,* 1-9.

Steyaert, H., Tonon, M.C., Tong, Y.A., Smihrouet, F., Testart, J., Pelletier, G., and Vandry, H. 1991. Distribution and characterization of endogenous benzodiazepine receptor ligand (endozepine)-like peptides in the rat gastrointestinal tract. **Endocrinol.** *129,* 2101-2109.

Stowell, M.H.B., and Rees, D.C. 1995. Structure and stability of membrane proteins. **Adv. Protein Chem.**, *46,* 279-311.

Studier, F.M., and Moffatt, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-expression of cloned genes. **J. Mol. Biol.** *189,* 113-130.

Szerszen, J.B., Szczyglowski, K., and Bandurski, R.S. 1994. *iaglu,* a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. **Science** *265,* 1699-1701.

Tai, S.P., and Kaplan, S. 1985. Phospholipid transfer proteins in microorganisms. **Chem. Phys. Lipids** *38,* 41-50.

Tall, A. 1995. Plamsa lipid transfer proteins. **Ann. Rev. Biochem.** *64*, 235-257.

Taupin, V. Gogusev, J., Descamps-Latscha, B., and Zavala, F. 1993. Modulation of tumor-necrosis factor-alpha, interleukin-1B, interleukin-6, interleukin-8, and granulocyte/macrophage colony stimulating factor

expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetraneuropeptide. **Mol. Pharmacol.** *43*, 64-69.

Taylor, D.C., Weber, N., Barton, D.L., Underhill, E.W., Hogge, L.R., Weselake, R.J., and Pomeroy, M.K. 1991. Triacylglycerol bioassembly in microspore-derived embryos of *Brassica napus* L. cv Reston. **Plant Physiol.** *97*, 65-79.

Thompson, G.A., 1993. Response of lipid metabolism to developmental change and environmental perturbation. In: *Lipid metabolism in plants*, T.S. Moore, ed., pp. 591-619. CRC Press: Boca Raton, FL.

Tilman, D., and Wedin, D. 1991. Oscillations and chaos in the dynamics of a perennial grassland. **Nature** *353*, 653-655.

Todaro, G.J., Rose, T.M., and Shoyab, M. 1991. Human DBI (endozepine): relationship to a homologous membrane associated protein (MA-DBI). **Neuropharmacol.** *30*, 1373-1380.

Tong, Y., Toranzo, D., and Pelletier, G. 1991. Localization of diazepam-binding inhibitor (DBI) mRNA in rat brain by high resolution *in situ* hybridization. **Neuropeptides** *20*, 33-40.

Tsuboi, S., Osafune, T., Tsugeki, R., Nishimura, M., and Yamada, M. 1992. Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and possible role in lipid metabolism. **J. Biochem.** *111*, 500-508.

Tugwood, J.D., Issemann, I., Anderson, R.G., Bundell, K. R., McPheat, W.L., and Green, S. 1992. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl-CoA oxidase gene. **EMBO J.** *11,* 433-439.

Töpfer, R., Martini, N., and Schell, J. 1995. Modification of plant lipid synthesis. **Science** *268,* 681-686.

Uchimiya, H., Kidou, S., Shimazaki, T., Aotsuka, S., Takamatsu, S., Nishi, R., Hashimoto, H., Matsubayashi, Y., Kidou, N., Umeda, M., and Kato, A. 1992. Random sequencing of cDNA libraries reveals a variety of expressed genes in cultured cells of rice (*Oryza sativa* L.). **Plant J.** *2,* 1005-1009.

van de Loo, F.J., Turner, S., and Somerville, C. 1995. Expressed sequence tags from developing castor seeds. **Plant Physiol.** *108,* 1141-1150.

Vanden Heuvel, J.P., Sterchele, P.F., Nesbit, D.J., and Peterson, R.E. 1993. Coordinate induction of acyl-CoA binding protein, fatty acid binding protein and peroxisomal ß-oxidation by peroxisome proliferators. **Biochim. Biophys. Acta** *1177,* 183-190.

Van Meer, G. 1989. Lipid traffic in animal cells. **Ann. Rev. Cell. Biol.** *5,* 247-275.

van Rooijen, G.J.H., and Moloney, M.M. 1995. Plant seed oil-bodies as carriers for foreign proteins. **Bio/Technol.** *13,* 72-77.

Varadarajan, R., Connelly, P.R., Sturtevant, J.M., and Richards, F.M. 1992. Heat capacity changes for protein-peptide interactions in the ribonuclease S system. **Biochem.** *31,* 1421-1426.

Veerkamp, J.H., and Maatman, R.G.H.J. 1995. Cytoplasmic fatty acid-binding proteins: Their structure and genes. **Prog. Lipid Res.** *34,* 17-52.

Vergonolle, C. Arondel, V., Tchang, F., Grosbois, M., and Guerbette, F., Jolliot, A., and Kader, J.C. 1988. Synthesis of phospholipid transfer proteins from maize seedlings. **Biochem. Biophys. Res. Commun.** *157,* 37-41.

Vitousek, P.M. 1994. Beyond global warming: Ecology and global change. **Ecol.** *75,* 1861-1876.

Voelker, T.A., Worrell, A.C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E., and Davies, H.M. 1992. Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. **Science** *257,* 72-74.

von Wettstein-Knowles, P.M. 1993. Waxes, cutin and suberin. In: *Lipid metabolism in plants.* T.S. Moore, ed., pp. 127-166. CRC Press: Boca Raton, FL.

Vork, M.M., Glatz, J.F.C., Surtel, D.A.M., van der Vusse, G.J. 1990. Assay of the binding of fatty acids by proteins: evaluation of the Lipidex 1000 procedure. **Mol. Cell. Biochem.** *98,* 111-117.

Webb, N.R., Rose, T.M., Malik, N., Marquardt, H., Shoyab, M., and Todaro, G.J. 1987 Bovine and human cDNA sequences encoding a putative benzodiazepine receptor ligand. **DNA** *67,* 71-79.

Welch, G.R. 1986. *The fluctuating enzyme.* John Wiley & Sons: New York.

Wetternau, J.R., Aggerbeck, L.P., Laplaud, P.M., and McLean, L.R. 1991. Structural properties of the microsomal triglyceride-transfer protein complex. **Biochem.** *30,* 4406-4412.

Wetterau, J.R., and Zilversmit, D.B. 1984. A triglyceride and cholesterol ester transfer protein associated with liver microsomes. **J. Biol. Chem.** *259,* 10863-10866.

Wirtz, K.W.A., and Zilversmit, D.B. 1968. Exchange of phospholipids between liver mitochondria and microsomes *in vitro.* **J. Biol. Chem.** *243,* 3596-3602.

Wiseman, T., Williston, S., Brandts, J.F., and Lin, L.-N. 1989. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. **Anal. Biochem.** *17,* 131-137.

Witten, T.A., and Cates, M.E. 1986. Tenuous structures from disorderly growth processes. **Science** *232,* 1607-1612.

Woldegiorgis, G., Yousufzai, S.Y.K., and Shrago, E. 1982. Studies on the interaction of palmitoyl-Coenzyme A with the adenine nucleotide translocase. **J. Biol. Chem.** *257,* 14783-14787.

Woldegiorgis, G., Spennetta, T., Corkey, B.E., Williamson, J.R., and Shrago, E. 1985. Extraction of tissue long-chain acyl-CoA esters and measurement by reverse-phase high-performance liquid chromatography. **Anal. Biochem.** *150,* 8-12.

Wootan, M.G., Bernlohr, D.A., and Storch, J. 1993. Mechanism of fluorescent fatty acid transfer from adipocyte fatty acid binding proteins to membranes. **Biochem.** *32,* 8622-8627.

Xu, N., and Xu, J. 1988. The fractal dimension of EEG as a physical measure of conscious brain activity. **Bull. Math. Biol.** *50,* 559-565.

Yamashita, A., Watanabe, M., Tonegawa, T., Sugiura, T., and Waku, K. 1995. Acyl-CoA binding and acylation of UDP-glucuronosyltransferase isoforms of rat liver: their effect on enzyme activity. **Biochem. J.** *312,* 301-308.

Yangibashi, K., Ohno, Y., Kawamura, M., and Hall, P.F. 1988. The regulation of intracellular transport of cholesterol in bovine adrenal cells: Purification of a novel protein. **Endocrinol.** *123,* 2075-2082.

Yorke, J.A., and Li, T.-Y. 1975. Period three implies chaos. **Amer. Math. Monthly** *82,* 985-992.