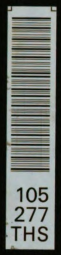
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STRUCTURE OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE: PARTIAL SEQUENCE OF AN ANOMOLOUS TRYPTIC PEPTIDE OF 20 AMINO ACIDS

presented by

DOUGLAS IVAN COOK

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# STRUCTURE OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE: PARTIAL SEQUENCE OF AN ANOMOLOUS TRYPTIC PEPTIDE OF 20 AMINO ACIDS

by

Douglas Ivan Cook

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ABSTRACT

STRUCTURE OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE: PARTIAL SEQUENCE OF AN ANOMOLOUS TRYPTIC PEPTIDE OF 20 AMINO ACIDS

By

Douglas Ivan Cook

Even with the high resolution X-ray crystallographic data being attained for 2-keto-3-deoxy-6-phosphogluconate aldolase from Pseudomonas putida, it is necessary to know the primary sequence of the polypeptide chain in order to determine the overall three-dimensional structure.

Initial work on the sequence determination involved studies on the active site region, as a sixteen residue tryptic peptide and as a fifty residue cyanogen bromide peptide.

As a strategy for extension of the known primary sequence the $^{14}C$-pyruvate-labelled, reduced and $^{14}C$-carboxymethylated enzyme was cleaved with trypsin after blockage of the lysine residues, with citraconic anhydride, and a set of 16 plus peptides was fractionated by column chromatography. A 20 residue peptide containing $^{14}C$-carboxymethylcysteine, was isolated. This peptide was

further cleaved with thermolysin and the resulting fragments separated by column chromatography.

From these fragments plus the major peptide the sequence of the major peptide was determined to be: Ile-Thr-Glu-Val-Gly-Ala-Leu-[Ser, Asx, (SCm)Cys-Gly]-Glx-Arg-Pro-Glu-Thr-Gly-Ala-Val-Leu.

To My Parents

## ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to Dr. W. A. Wood for his encouragement and support throughout the course of this research. Thanks are also due to Mr. Paul Kuipers for his excellent technical assistance throughout this work.

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## ABBREVIATIONS

The abbreviations used in the text are: KDPG, 2-keto-3-deoxy-6-phosphogluconate; PTH, phenyl thiohydantion; TPCK, L-(1-tosylamido-2-phenyl) ethylchloromethyl ketone; SCm, S-carboxymethyl; ANS, amino napthalene disulfonic acid; and EDC, ethyl-dimethylaminopropyl carbodiimide.

The three-letter symbols for the amino acid residues are those recommended by the International Union of Biochemistry, as published in the Biochemical Journal, 102, 23 (1967). They are: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, trypthophan; Tyr, tyrosine; Val, valine; Asx, aspartic acid or asparagine; and Glx, glutamic acid or glutamine.

# INTRODUCTION

In 1952, an alternative to the so-called hexose monophosphate shunt was first discovered in Pseudomonas saccharophila by Entner and Douderoff (1). They demonstrated, using, cell suspensions that anaerobic decomposition of 1-$^{14}$C-glucose led to almost quantitative recovery of the label as $CO_2$. They also demonstrated that the $CO_2$ was released by a mechanism other than primary decarboxylation of a $C_6$ skeleton to yield a pentose. They showed further that the mechanism involved a cleavage of 6-phosphogluconic acid, or some derivative of this, to yield pyruvate and 3-phosphoglyceraldehyde.

The pathway was later (2-4), shown to involve two enzymes: 6-phosphogluconate dehydrase (EC-4.2.1.12) which converted 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (KDPG), and KDPG aldolase (EC 4.2.1.14) which cleaved KDPG to yield pyruvate and D-glyceraldehyde-3-phosphate. It has since been shown that this gluconate pathway functions exclusively among eubacteria, in which it plays a major catabolic role in the utilization of hexoses (1-5), hexonates (6-9) and hexuronates (10-13).

KDPG aldolase has been shown to react mechanistically, at least in part, as a Class 1 FDP aldolase, as it is

inactivated by treatment with borohydride in the presence of pyruvate (14) producing 6-N-(2-n-hydroxypropyl) lysine (15); while in the presence or borohydride alone or borohydride plus glyceraldehyde-3-phosphate no effect was seen (14). This inactivation or absence of inactivation is identical with that seen for Class 1 aldolases which react with a Schiff base mechanism.

A similar inactivation of KDPG aldolase is seen when the enzyme is incubated with sodium borohydride plus a number of analogs of KDPG, including 5-keto-4-deoxyglucarate, 2-keto-4-hydroxyglucarate, 2-keto-3-deoxygluconate, 2-keto-3-deoxygalactonate, and 2-keto-3-deoxy-6-phosphogalactonate; none of these compounds undergo cleavage to three carbon atom compounds as with KDPG (15). These results indicate that Schiff base formation is not very specific and is not a sufficient driving force for this aldolytic cleavage to occur. Later work by Roseman (18), using piperidine-2-carboxylic acid as a Schiff base model compound confirmed this observation that the Schiff base formation was not a sufficient driving force for cleavage to occur. It therefore seems necessary that besides the azomethine-forming lysine residue an additional nucleophile may be required to assist in the overall catalytic process (16,19).

Ingram and Wood (15), showed that fluorodinitrobenzene (FDNB), reacted with lysyl residues, distinct from the azomethine forming lysine, of KDPG aldolase with resultant

inactivation of the enzyme. The reaction of the lysyl residues with FDNB could be prevented by KDPG, glyceraldehyde-3-phosphate, and phosphate but not by pyruvate. From this they concluded that the dinitrophenylated residues functioned in binding the phosphate moiety of KDPG. However, work by Barran and Wood (20), showed that the decrease in activity was due to conformational changes in the enzyme, as the dinitrophenylated enzyme, which had residual activity, had nearly the same binding affinity for substrate as did the native enzyme. Therefore, the derivitized lysyl residues were probably in the active site region, but were not altering the binding as postulated, but rather were changing the conformation of the enzyme so as to destroy its catalytic activity.

The histidine residues have also been studied for possible involvement in catalytic activity (21). Destruction of the single histidine residue of the above led to inactivation of the proton exchange ability of the enzyme however the Schiff base formation was retained, indicating that at most the histidine was partially responsible for the secondary driving force of the overall reaction.

Meloche (22), has shown that depending on the conformational state of the enzyme, inactivation with bromopyruvate, a substrate analog, led to either esterfication of a carboxylate group or alkylation of a mercaptyl ion. From this it was proposed that each of these bases may play a role in the action of the enzyme, one being involved in

activating the pyruvate proton exchange reaction and the other functioning in the cleavage or condensation reaction. From later work Meloche (23), has concluded that the glutamate and not the mercaptyl ion is important in both reactions.

Recently Meloche et al. (24), have shown from stereochemical and kinetic studies involving enzyme catalyzed tritium exchange from water into bromopyruvate that the bromopyruvate did occupy the binding site normally used by pyruvate.

Physically, KDPG aldolase is also a very interesting protein. Hammerstedt et al. (25), have shown, by centrifugation studies, that the native enzyme has a molecular weight of 73,000±2,000 daltons and a subunit molecular weight of 23,000±2,000 daltons. These data are in close agreement with those obtained both by disc gel electrophoresis and amino acid analysis (26).

From this physical analysis there is an indication that KDPG aldolase is a trimeric enzyme consisting of three identical or nearly identical subunits.

Further substantiation of the trimeric structure was obtained from the following observations: (a) the native enzyme contains 12 cysteine residues, but no cystine. After carboxymethylation with $^{14}$C-iodoacetic acid and subsequent tryptic digestion, 4 radioactive peptides were isolated; (b) the native enzyme contains 66 lysine and arginine residues; following tryptic digestion and peptide

mapping, the numbers of peptides as detected with specific reagents for histidine, tyrosine, cysteine, tryptophan and arginine were consistent with three identical subunits; (c) three moles of $^{14}$C-pyruvate per mole of enzyme were covalently bound by borohydride reduction; (d) three moles of carboxy terminal asparagine were released by digestion of S-carboxy-methylated enzyme with carboxypeptidase A; (e) hybridization using native and malelylated enzyme indicated 4 hybrid species as detected by disc gel electrophoresis (26).

X-ray crystallographic data showed that the crystal of KDPG aldolase existed in the cubic system with a symmetry space group of $P2_13$ with twelve equivalent positions (27). This suggested that the molecules of KDPG aldolase are assemblages of trimers in the crystal with the subunits related by a three fold rotational axis.

More recent X-ray crystallographic work (28), using three heavy metal derivatives of the enzyme yielded an X-ray diffraction map of 3.5 A resolution. This image has shown that the subunits of the aldolase are definitely arranged in a trimeric structure.

Although it has now been proven that KDPG aldolase is a trimer (19,28), there is still a great deal to be learned about the structure and function of this enzyme, of major importance is the identification of the structure that provides the driving force for cleavage, and the detailed secondary structure of the active site.

To aid in this work, experiments are now underway to extend the resolution of the X-ray data beyond the 3.5 A level now attained to about 2.0 A. However the detailed structure cannot be assigned to the electron density data without knowledge of the total primary sequence.

Initial work on the determination of the primary sequence was done by Robertson et al. (29), who making use of the Schiff base forming ability of the enzyme, used radioactive pyruvate and cyanoborohydride reduction to identify the active site peptide. Using the radioactive label they isolated a hexadecapeptide containing the azomethine forming lysine, and found the sequence to be Phe-6-N-(1-carboxy-ethyl)-Lys-Phe-Pro-Ala-Glu-Ile-Ser-Gly-Gly-Val-Ala-Ala-Ile-Lys.

More recently the sequence around the active site lysine has been extended by Tsay (30) who, using the same radioactive label, isolated a fifty residue cyanogen bromide peptide containing the active site tryptic peptide of Robertson, and determined the sequence of this peptide to be Gly-Tyr-Ala-Leu-Gly-Tyr-Arg-Arg-Phe-Lys*-Leu-Phe-Pro-Ala-Glu-Ile-Ser-Gly-Gly-Val-Ala-Ala-Ile-Lys-Ala-Phe-Gly-Gly-Gly-Pro-Phe-Asn-Ile-Arg-Phe-Cys(SCm)-Pro-Thr-Gly-Asx-Gly-Val-Ala-Pro-Asn-Val-Arg-Tyr-Asn-Met. The asterisk indicates the active site lysine residue.

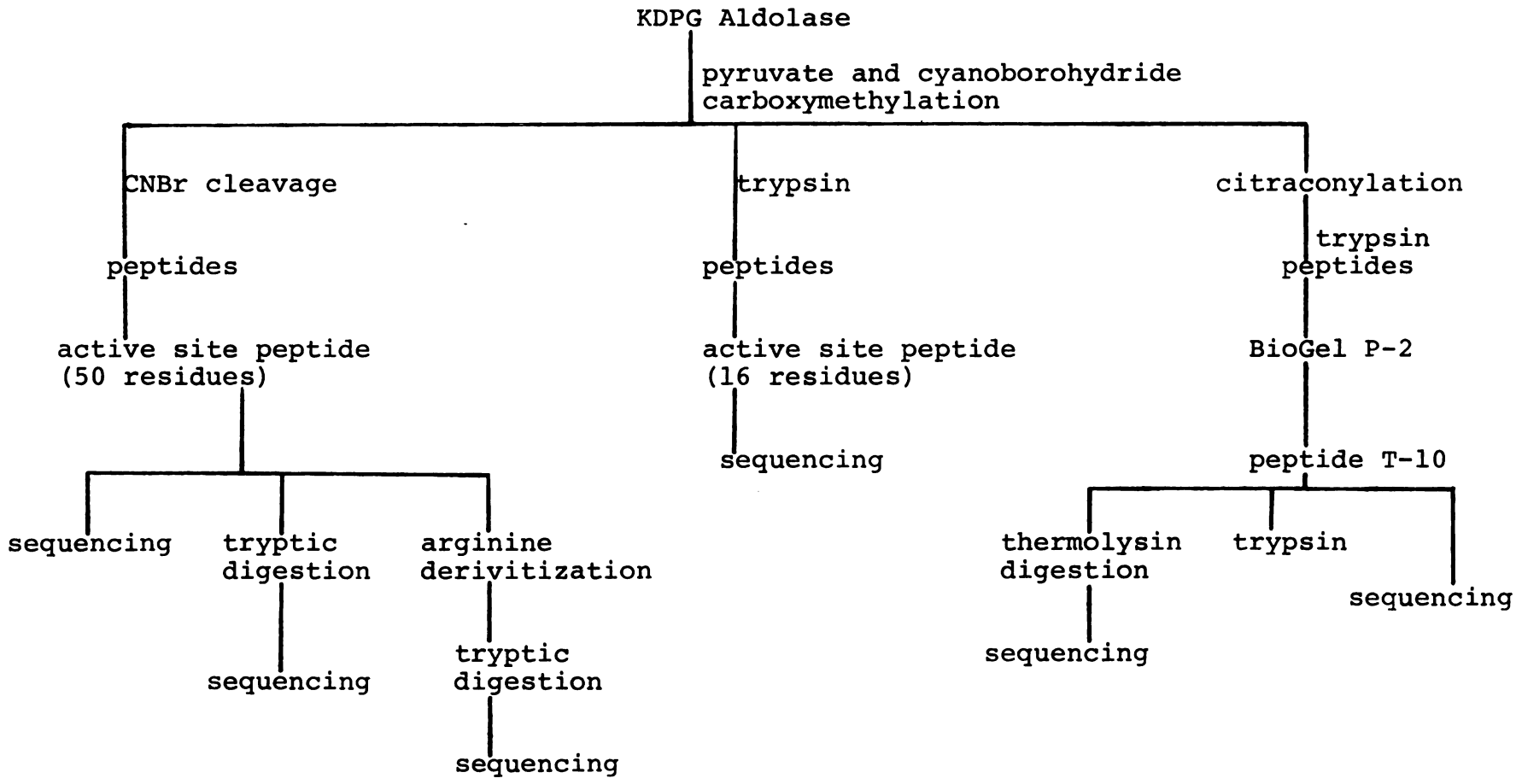
Determination of the sequence of the remaining 80% of the protein subunit is now underway making use of the plan

shown in Figure 1. The plan consists of three phases. (1) Cleavage of the derivitized aldolase with cyanogen bromide and sequencing of the resulting fragments, including the 50 residue active site peptide. (2) Cleavage with trypsin and sequencing of the hexadecapeptide from the active site. (3) Blockage of the lysine residues in the derivitized aldolase followed by trypsin digestion and sequencing the fragments, to obtain overlaps of the cyanogen bromide fragments.

In the present work one peptide isolated in the third strategy is reported. Specifically $^{14}$C-pyruvate-labelled, reduced and carboxymethylated aldolase was cleaved with trypsin, after blockage of the lysine residues with citraconic anhydride. A radioactive peptide, other than the active site peptide, containing 20 amino acid residues was isolated and its primary sequence partially determined using a combination of peptidase and Edman degradations.

Figure 1

Sequencing strategy for KDPG Aldolase



# EXPERIMENTAL PROCEDURE

## MATERIALS

Trypsin (TRTPCK, treated with L-(tosylamido-2-phenyl)-ethylchloromethyl ketone) and carboxypeptidase A (COADFP, bovine pancreas, treated with DFP and recrystallized) were obtained from Worthington Biochemical Corp. Leucine amino peptidase, amino peptidase M, lactic dehydrogenase (Type III), deoxyribonuclease and carboxypeptidase B (DFP, hog pancreas) were purchased from Sigma Chemical Co. Iodoacetic acid was from Sigma Chemical Co. and was recrystallized from carbon tetrachloride:benzene; 1:2, b/b., before use. Iodoacetic acid-$^{14}$C, 0.05 mCi per 0.00376 mM, and pyruvate-3-$^{14}$C, 8.7 mCi per mM were obtained from New England Nuclear Corp. Sephadex gels were purchased from Pharmacia Fine Chemicals. Dowex 50-X2, Dowex 50-X8 and the P-gels were purchased from BioRad Laboratories. Sodium cyano-borohydride was obtained from Ventron Corporation, Alfa Products, and was purified by the procedure of Borch et al. (31). Calcium phosphate gel was prepared by the method of Colowick (32). Potassium gluconate was a product of Charles Pfizer and Company. 2-Amino-1,5-napthalene-disulfonic acid (ANS) was a product of Aldrich Chemical Company. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

HCl was purchased from BioRad Laboratories. Fluorescamine Cheng-Chin polyamide plates, PTH-amino acid standards, and 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidazole were purchased from Pierce Chemical Company, chemicals for the Edman degradation, including phenyl isothiocyanate, benzene, trifluroacetic acid, pyridine and ethylene dichloride were all Sequanal grade and obtained from Pierce Chemical Company. Pyridine and N-ethylmorpholine used were commercial grade and were redistilled from ninhydrin before use. All other chemicals were the best commercial grade available.

## METHODS

Growth of bacteria: Pseudomonas putida strain A 3.12 (ATCC 12633) was cultured according to the method of Hammerstedt et al. (25). This involved growth of the organisms in a liquid medium containing $MgSO_4 \cdot 7H_2O$, citric acid, $(NH_4)_2PO_4$, $FeCl_3$, and potassium gluconate. Large scale culturing was carried out using a 130 liter New Brunswick Scientific Fermentor containing 100 liters of the above gluconate medium. Cells were harvested after reaching stationary phase (about ten hours), with an average yield of around 1700 g, wet weight, cell paste per culture.

Enzyme purification: KDPG aldolase was purified and crystallized using the method of Hammerstedt et al. (25) with slight modifications. The cell paste was suspended in cold water (1 ml per gram paste). To this DNAse was added, and the suspension passed through a precooled Manton-Gaulin homogenizer. The resulting homogenate was centrifuged and the precipitate discarded. HCl was added dropwise, with stirring, to the supernatant, to a final pH of about 1.5 and the resulting precipitate was removed by centrifugation and discarded. Following this three ammonium

sulfate fractions were obtained; the aldolase activity precipitated at 1.9 M ammonium sulfate. The precipitate containing the aldolase activity was resuspended in 0.01 M phosphate, adjusted to pH 6.0. The resulting suspension was dialyzed against the same buffer to remove residual ammonium sulfate. After dialysis, the enzyme was adsorbed to calcium phosphate gel and the gel was washed with water and 0.01 M phosphate buffer to remove unbound activity. The enzyme was eluted from the gel by washing with 0.1 M phosphate and powdered ammonium sulfate was added to the eluate to give a final concentration of 2.5 M. The resulting precipitate was suspended in 0.01 M phosphate pH 6.0, to a protein concentration of 12 mg per ml. The enzyme was then crystallized using saturated ammonium sulfate, with the resulting crystals stored at 4°C in 2.5 M ammonium sulfate until use.

Enzyme assay: The aldolase assay is that of Hammerstedt et al (25), using lactic dehydrogenase and NADH and following optical density decrease at 340 nm. One unit of activity was defined as the amount of aldolase which, under the conditions of the assay, catalyzed an absorbance change of 1.0 A units per minute in a microcuvette of 1 cm pathlength.

Protein determination: Protein concentration was determined either by the method of Lowry et al. (33) using crystalline bovine serum albumin as the standard, or by the 280/260

spectrophotometric method using the following relationship: Protein (mg/ml)= $1.55A_{280}-0.76A_{260}$ (34).

Amino acid analysis-Ninhydrin system: The composition of KDPG aldolase, its derivatives, or peptides derived from it were determined by using a noncommercial amino acid analyzer (35). Appropriate amounts of salt free peptide or protein solution (3-10 nmoles) were transferred to the hydrolysis vial and then dried on a rotary evaporator. After addition of 0.5 ml of constant boiling HCl and 10 mg of crystalline phenol, (when required) the vial was degassed and sealed in a vacuum below 50 microns. The hydrolysis was performed at 110°C for twenty-four hours. The hydrolysate was then dried with a rotary evaporator, and redissolved in an appropriate volume of 0.2 M citrate buffer pH 2.0. An aliquot of the hydrolysate containing, up to an equivalent of ten nmoles of peptide or protein was analyzed in the amino acid analyzer, with norleucine as an internal standard. Cysteine was determined as S-carboxymethylcysteine for the iodoacetate-treated protein or as cystine for the native protein using an air oxidation procedure of Moore and Stein (36). Corrections for hydrolytic loss were generally made for threonine and serine (5 and 10% respectively). For detection of free amino acid residues released by chemical or enzymatic cleavage of peptides, the reaction mixture was acidified to pH 2.0, and then applied directly to the amino acid analyzer without prior acid hydrolysis.

Radioactivity measurements: Radioactivity from $^{14}C$-pyruvate or $^{14}C$-iodoacetate was measured on a Packard Tri-Carb. liquid scintillation spectrometer (Model 3324). Brays scintillation coctail was used (38). In cases where accurate counting was required corrections were made for quenching by the use of internal standards.

Assay of peptides: Peptide monitoring was carried out using a fluorometric assay. Samples containing from 0.05 to 2 nmoles were placed in a 10 by 75 mm glass tube and dried under reduced pressure. The residue was dissolved in 1.25 ml of sodium borate buffer, pH 9.0. This was then placed on a vortex mixer and 0.4 ml of 0.03% fluorescamine in dry acetonitrile was added with stirring. The fluorescence was measured within fifteen minutes in a filter fluorimeter (American Instruments Co.) using a 390 nmeter excitation filter and a 475-490 nmeter emission filter. L-leucyl-alanine was used as a standard.

$^{14}C$-Pyruvate labelling of KDPG aldolase: KDPG aldolase (400 mg, 5.56 μM) in 79 ml of 0.1 M potassium phosphate, pH 6.0, was incubated at 4°C with pyruvate-3-$^{14}C$ (49.77 μM, 89.3 μCi). After thirty minutes of incubation with gentle stirring, $NaBH_3CN$ (21.81 μM in 1 ml) was added to reduce the intermediate enzyme-substrate complex to the 6-N-(2-n-hydroxypropyl) lysine form. The reaction was allowed to proceed for two hours and the enzyme assayed for activity. At the end of the two hour period 99.9% of

the enzyme activity was destroyed and the reaction was terminated. The labelled enzyme was precipitated by adding ammonium sulfate to a final concentration of 2.5 M, and centrifuged. The pellet was resuspended in 1.98 M Tris-HCl buffer, pH 8.6, containing 6.98% EDTA, and dialyzed against the same buffer, at 4°C.

Carboxymethylation of the labelled enzyme: The $^{14}C$-pyruvate-labelled enzyme was converted to the S-carboxymethyl derivative according to the procedure of Crestfield (39). To the dialyzed enzyme, 26.4 g of recrystallized urea was added. This gave a final concentration of 8 M urea in 1.44 M Tris-HCl with 5% EDTA. After stirring for approximately one hour to dissolve the enzyme, the solution was flushed with nitrogen. The reaction was initiated, by the addition of 0.632 ml mercaptoethanol, and the mixture was stirred in the dark, under nitrogen, for three hours. After three hours 1.738 g iodoacetate (specific activity 2.99 μCi/mM), was added and the solution was incubated at room temperature for one hour. Excess reagents were removed by dialysis, in the dark at 4°C, against 5 liters of 50 mM N-ethylmorpholine acetate, pH 8.2, containing 2.5% EDTA. The dialyzed enzyme solution was partially dried by lyophylization, and the extent of carboxymethylation determined by amino acid analysis of the acid hydrolyzed enzyme. Either by (1) the absence of cystine in air oxidized samples, or (2) the presence of S-carboxymethylcysteine.

Blockage of lysine residues by citraconic anhydride: The citraconylation, to prevent trypsin cleavage of lysine residues, was carried out according to the procedure of Gibbons et al. (40). The $^{14}$C-pyruvate-labelled, carboxymethylated enzyme, in 50 mM N-ethylmorpholine acetate buffer, pH 8.2, containing 5% EDTA, was treated at 0°C with a one-hundred fold molar excess of citraconic anhydride, with continuous stirring. The reagent was initially insoluble but went into solution as the reaction proceeded. The pH was maintained at 8.2 by the addition of N NaOH; the reaction was continued until base was no longer consumed. After two hours, consumption of base ceased and the reaction of the protein with fluorescamine was less than 1% of the initial value. At this time the reaction was terminated by dialysis, at 4°C against 0.1 M ammonium bicarbonate, pH 7.9, to remove excess reagents.

Tryptic digestion of $^{14}$C-pyruvate-labelled, reduced and carboxymethylated and citraconylated KDPG aldolase: Prior to use L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK)-treated trypsin was dissolved in 0.01 N HCl to a concentration of 0.5% and incubated for two hours at 4°C (41). Tryptic digestion of the triply derivitized aldolase (400 mg in 0.1 N $NH_4HCO_3$) was initiated by the addition of 4 mg of the acid-treated TPCK-trypsin. The digestion was allowed to proceed at 37°C, while the pH was maintained at 7.9 by the addition of 1 N HoAC. After

three hours TPCK-trypsin was added to give a final enzyme to substrate ratio of 1:80, and the incubation continued until the consumption of acid ceased. At this point the digestion was terminated by applying the digestion mixture to a Sephadex G-75 column, (70 x 3.5 cm), the peptides were then eluted with 10 mM $NH_4OH$. The column was monitored for both protein and radioactivity. Apprioriate fractions were pooled and dried.

Separation of tryptic peptides on Sephadex G-25: Pooled fractions from the Sephadex G-75 column were applied to a Sephadex G-25 column (170 x 4 cm). And the peptides were eluted with 10 mM $NH_4OH$. Fractions were assayed for protein and radioactivity as previously. Those fractions comprising a distinct peak were pooled and dried for future use.

Deblocking of lysine residues: The deblocking procedure used was essentially that of Gibbons and Perham (40). Dried and pooled fractions, from the Sephadex G-25 column, were resuspended in 10 mM HCl and incubated for six hours at room temperature. Following this the samples were dried and stored for later use.

Separation of the tryptic peptides on Dowex 50: Separation of the peptides with Dowex was essentially according to the procedure of Schroeder et al. (42), with one major modification. The resin was suspended in 4 M pyridine

acetate buffer pH 5.3, and packed in the column in the same buffer. The column (100 x 4 cm), packed by the above method was then equilibrated with two column volumes of 0.2 M pyridine acetate buffer pH 3.1. The tryptic peptides, derived by pooling of peak 1 (fractions 20-225) from the Sephadex G-25 column, were resuspended in a minimal volume of 50% formic acid and diluted to 50 ml with 0.2 M pyridine acetate buffer pH 3.1. Development of the chromatogram was with a buffer system containing 4.5 column volumes of 0.2 M pyridine acetate buffer pH 3.1, in the mixing chamber and 9 column volumes of 2 M pyridine acetate buffer pH 5.0, in the reservoir chamber. This gradient was terminated when it was 60% complete, and the buffer in the reservoir replaced with 8.5 M pyridine acetate buffer pH 5.6. The peptide elution profile was established by assaying every third fraction for protein and radioactivity, as described in the preceeding sections. Fractions comprising a distinct peak were pooled, and the volatile buffers removed in a rotary evaporator.

Naming of tryptic peptides: Naming of the tryptic peptides was on an arbitrary basis of the order of elution from the Dowex 50 column.

Purification of peptide T-10: The T-10 peptide (fractions 284-322 from the Dowex 50 column) was redissolved in 1% ammonium hydroxide, and was applied to an 80 x 0.9 cm column of BioGel P-2 (minus 400 mesh) previously equilibrated

with the same buffer. The peptides were eluted with 1% ammonium hydroxide, and alternate fractions were assayed for peptide and radioactivity. Those fractions comprising a major peak were pooled and dried.

Edman degradation: The peptide (50-200 nmoles) in an appropriate salt free buffer was transferred to a 2 ml conical tube and lyophylized. It was resuspended in 0.1 ml of freshly prepared 50% pyridine, plus 0.05 ml of 25% triethylamine containing 1% ethanolamine, and thoroughly degassed in a moderate stream of nitrogen through a capillary tube emmersed in the solution. 0.005 ml of phenyl isothiocyanate was added and the nitrogen bubbling was continued for another minute. The tube was capped with parafilm, and the coupling reaction was allowed to proceed at 37°C for two hours. Excess reagents were extracted three times with benzene and the remaining reaction mixture was taken to dryness under a vacuum. Cyclization was performed by the addition of 0.1 ml of trifluoroacetic acid and incubating for 0.5 hours at 37°C. The trifluoroacetic acid was removed in vacuo and the resulting residue extracted three times with ethylene dichloride. The resulting thiazolinone was then converted to its corresponding phenyl thiohydantion (PTH), by the method of Guyer (43). The method involved heating the dried thiazolinone, under a nitrogen atmosphere, at 80°C for thirty minutes. Alternatively, the phenyl thiohydantion was formed by the method of Tarr (44), which

involved heating of the dried thiazolinone at 50°C for ten minutes in a mixture of methanol: concentrated HCl; 10:1 and then adding water and heating for a further five minutes. The peptide residue was resuspended in 50% pyridine and an aliquot removed for amino acid analysis. The volume was restored to 0.1 ml by the addition of 50% pyridine and the sample was subjected to another degradative cycle. In some cases the PTH amino acid produced was examined by methods described in the following sections.

Direct identification of phenyl thiohydantion amino acids on polyamide sheets: The thin layer chromatographic method of Summers et al. (45), was used to identify the PTH amino acids at sub-nmole levels. About 0.2-0.3 nmoles of the unknown PTH amino acid was dissolved in ethyl acetate, and spotted on the left-hand corner of a 5 x 5 cm polyamide sheet (4 mm from the bottom and left side). A mixture of authentic PTH amino acids (corresponding to the composition of the peptide) was applied to the other side of the sheet in a position located exactly coincident with the above spot. The sheet was developed in the first solvent system (toluene: n-pentane: glacial acetic acid, 60:30:35 v/v) containing 0.025% of 2-(4-t-butylphenyl)-5-(4-biphenyl)-1,3,4-oxidazole as a fluorescent indicator. When the solvent front had reached the top edge, the sheet was removed and dried in a stream of hot air. Chromatography in the second dimension was conducted in 35% acetic acid.

Again when the solvent reached the top edge of the sheet, the chromatogram was removed and dried in a stream of hot air. The chromatogram was inspected under short wavelength ultraviolet light, and spots where fluorescence was quenched on the sheet were circled. The unknown PTH amino acid was identified by matching the spots with the standards on the reverse side of the sheet.

Gas chromatographic identification of phenyl thiohydantion amino acids: The gas chromatographic identification of PTH amino acids was carried out by a slight modification of the procedure of Pissano et al. (45). The column adsorbant consisted of equal volumes of 7.33% SP-400, 5.33% OV-210, and 0.66% OV-225 on Chromosorb W-HP 80-100 mesh. Injector temperature was 275°C, detector temperature was 300°C. After injection the sample was held at 180°C for ten minutes and then programmed at 7.5°C per minute to 285°C where it was held for fifteen minutes. 0.001 ml of ethyl acetate solution of the PTH amino acid (about 3-5 nmoles) was injected on the column. Identification was on the basis of retention times relative to standards. At any time when there was a possible doubt as to the identity of the PTH amino acid, the unknown PTH was coinjected with the standard of the same identity and the presence of only one peak was taken as proof of the identity of the unknown PTH amino acid.

Dansylation of peptides: The dansylation procedure used was that of Gros and Labouesse (46), for peptides. An aliquot of peptide (about one nmole) was dried in an hydrolysis vial. To this was added 0.001 ml $H_2O$, 0.02 ml 50 mM bicarbonate buffer, pH 8.3, and 0.03 ml of 10 mM dansyl chloride in acetone. The reaction was allowed to proceed at room temperature for 30 minutes at which time it was terminated by the addition of 0.001 ml N NaOH. The mixture was lyophylized and 0.1 ml of constant boiling HCl was added for hydrolysis. Hydrolysis was carried out for 4 hours at 110°C in a sealed vial. After hydrolysis the acid was removed on a rotary evaporator and the residue taken up in 0.2 ml of water, extracted three times with 0.2 ml portions of ether and the two phases chromatographed separately.

Chromatography of dansyl amino acids on polyamide sheets: The chromatography method for the dansyl amino acids was that of Woods and Wang (47). The system was the same as that for PTH amino acids with the following exceptions:
(1) solvent 1, water: 90% formic acid, 200:3 v/v;
(2) solvent 2, benzene: glacial acetic acid, 9:1 v/v;
(3) the dansyl amino acids are fluorescent and are visible directly under a short wavelength ultraviolet lamp.

Digestion of peptides with carboxypeptidase A and B: A suspension of carboxypeptidase A (10 nmoles) was washed three times with 1 ml of cold water, and dissolved in 10%

$LiCl_2$; carboxypeptidase B was used directly without further purification. No free amino acids were detected when 5 nmoles of either enzyme preparation was examined in the amino acid analyzer. Both carboxypeptidase A and B were added to the reaction mixture, A in a 1:500 enzyme to substrate ratio, and B in a 1:50 enzyme to substrate ratio. Hydrolysis was performed at 37°C, in 0.2 M N-ethylmorpholine acetate buffer pH 8.5 (unless otherwise stated for a given experiment). Appropriate aliquots of peptide were withdrawn from the reaction mixture at each time interval and the reaction terminated by freezing. The samples were diluted with 0.2 M citrate buffer, pH 2.0, and applied to the amino acid analyzer without further treatment.

Digestion with leucine amino peptidase: Prior to use leucine amino peptidase was activated by the procedure of Mitz and Schleuter (48). The procedure involved the incubation of the enzyme in the following solution; 2.0 ml $H_2O$, 0.1 ml $MnCl_2$, 0.1 ml 0.5 M Tris-HCl buffer, pH 8.5, for a minimum of two hours at 37°C. Digestion was performed on the peptide which was resuspended in 0.035 M Tris-HCl buffer, pH 8.5, containing 0.01 M $MgCl_2$. Incubations were carried out at 37°C, for the times noted in a given experiment and at an enzyme to substrate ratio of 1:5000. After appropriate time intervals samples were removed and treated as in the case of the carboxypeptidase digestions.

Modification of peptide T-10 with 2-amino-1,5-napthalene disulfonic acid (ANS): Modification of the peptide with ANS was done according to the procedure of Foster et al. (49). One equivalent of lyophylized peptide (about 200 nmoles) was reacted with eight equivalents each of N-ethyl,N-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and ANS in 0.2 ml of water previously adjusted to pH 4.0 with 0.01 N HCl and the reaction mixture was stirred for four hours at room temperature. The modified peptide was lyophylized and subjected to Edman degradation as previously outlined for the unmodified peptide.

Thermolysin digestion of peptide T-10: Thermolysin digestion of peptide T-10 (300 nmoles in 0.2 ml water adjusted to pH 8.0 by the addition of 0.05 N NaOH) was initiated by the addition of 0.001 mg of thermolysin. The hydrolysis was conducted at 25°C, and the pH maintained at 8.0 by the addition of 0.05 N NaOH. After one hour 0.002 mg of thermolysin was added, and the incubation allowed to proceed for a further three hours, at the end of this time all consumption of base had ceased and the reaction was assumed to be complete. The material from the digestion was lyophylized and then chromatographed as described in the following section.

Purification of the thermolysin peptides of T-10: The lyophylized thermolysin digest of peptide T-10 was redissolved in 0.05 M pyridine acetate buffer, pH 2.4, and the pH

adjusted to 1.5 with HCl. This material was centrifuged to remove an insoluble precipitate. The portion of the digest that remained in solution was applied to a Dowex 50-X2 column (50 x 0.6 cm), previously equilibrated with the pH 2.4 buffer. Development of the chromatogram was at 50°C, with a buffer flow rate of 10 ml per hour, and a gradient consisting of 100 ml of 0.05 M pyridine acetate buffer, pH 2.4 and 100 ml of 0.5 M pyridine acetate buffer, pH 3.75, the second portion of the gradient consisted of 75 ml of the 0.5 M pyridine acetate buffer, pH 3.75, and an equal volume of 2.0 M pyridine acetate buffer pH 5.0. Every third fraction was assayed for fluorescence and radioactivity and appropriate fractions pooled for later work.

Purification of peptide T-10-Th-1: Fractions 43-49 from the Dowex 50 chromatography of the thermolysin digest of peptide T-10 were subjected to further purification by chromatography on a BioGel P-2 column (130 x 0.6 cm) equilibrated with 20% pyridine. The peptide was resuspended in this buffer and applied to the column which was then developed in the same buffer. Each fraction was assayed for fluorescence and radioactivity and appropriate fractions were pooled and dried. All values for fluorescence were rounded to the nearest integral value.

Digestion of peptide T-10 with trypsin: Trypsin was pretreated as outlined in a previous section, peptide T-10 (10 nmoles) was digested with the pretreated trypsin at a 1:50 enzyme to substrate ratio, for two hours at 37°C. At the end of the digestion time the reaction was terminated by applying the digest to a Sephadex G-75 column. Each fraction was assayed for radioactivity and the radioactive fractions pooled and dried. These were then split into two lots with one-half hydrolyzed and applied to the amino acid analyzer and the other half subjected to Edman degradation.

# RESULTS

Preparation of crystalline KDPG aldolase: For sequence work it is necessary to attain protein of high purity. Table 1, summarizes the purification of 1.2 kg of Pseudomonas putida paste to yield on an average from 40-50 mg of crystalline KDPG aldolase. For this work ten preparations were carried out yielding 425 mg of aldolase. The aldolase was assumed to be homogenous from the following criterion: (a) the specific activity of the enzyme (10,482 units per mg of protein), this was somewhat lower than the 13,350 units per mg of protein published by Meloche and Wood (17). However the specific activity does not change appreciably on recrystallization which indicates that the protein is homogeneous; (b) polyacrylamide disc gel electrophoresis of the crystalline enzyme indicated a single component; (c) as shown in Table 2, the amino acid composition (columns II and III) of derivitized enzyme is in agreement, within experimental error, of the data reported by Tsay (29) for homogenous enzyme.

Purification of peptide T-10: The aldolase was treated with $^{14}$C-pyruvate and cyanoborohydride, carboxymethylated, citraconylated and then cleaved with trypsin as described

Table 1. Purification of KDPG Aldolase from Pseudomonas putida.

The data presented are based on 1.2 kg of cell paste. The purification procedure and assay are those of Hammerstedt et al. (24), as outlined in the methods section.

| Step | Volume | Total activity | Specific activity | Purification | Recovery |
|---|---|---|---|---|---|
| | ml | units ($\times 10^5$) | units/mg protein | fold | % |
| Crude extract | 3690 | 26.57 | 6.9 | ------ | 100 |
| Acid treatment | 2580 | 19.09 | 47.7 | 6.9 | 72 |
| Ammonium sulfate precipitate | 58 | 16.4 | 1287 | 186.5 | 62 |
| Calcium phosphate gel elution | 202 | 11.4 | 3375 | 489 | 43 |
| Crystallization | | | | | |
| first crystal | 8 | 7.91 | 10181 | 1476 | 29.77 |
| second crystal | 7.5 | 4.84 | 10482 | 1519 | 18.22 |

Table 2. Amino acid composition of KDPG aldolase and its derivatives.

An appropriate amount of KDPG aldolase was hydrolyzed at 110°C for 24 hours with 0.5 ml constant boiling HCl as detailed in the Methods section. The hydrolysates were examined by an ultrasensitive amino acid analyzer. Cysteine was determined as SCm-cysteine (columns II and III) or cystine (column I) and expressed as cysteine. Corrections for hydrolytic losses were made for threonine (5%) and serine (10%). The presence of N-(1-carboxyethyl)-lysine was indicated in columns II and III but not quantitated. The percentage yield is expressed as the sum of the weights of recovered amino acids, after correction for water addition on hydrolysis, divided by the weight of the sample before hydrolysis. The data were obtained from an average of at least three analyses. Numbers represent residues based on 9 residues of tyrosine per mole of enzyme.

Table 2.

| Amino Acid | Native | $^{14}$C-pyruvate-labelled, reduced and carboxymethylated enzyme. | |
|---|---|---|---|
| | | Cook | Tsay (29) |
| Cysteine | 12.67 | 11.04 | 11.62 |
| Aspartic | 50.32 | 53.81 | 50.80 |
| Threonine | 30.98 | 35.54 | 29.17 |
| Serine | 27.06 | 30.93 | 26.43 |
| Glutamic | 59.21 | 57.11 | 58.78 |
| Proline | 45.14 | n.d. | n.d. |
| Glycine | 62.34 | 58.64 | 58.98 |
| Alanine | 91.45 | 89.61 | 88.56 |
| Valine | 40.31 | 39.69 | 40.61 |
| Methionine | 19.80 | 17.82 | 21.99 |
| Isoleucine | 52.24 | 57.02 | 53.09 |
| Leucine | 59.73 | 61.41 | 60.41 |
| Tyrosine | 9.00 | 9.00 | 9.00 |
| Phenylalanine | 20.74 | 19.76 | 19.66 |
| Tryptophan | 11.56 | n.d. | 11.78 |
| Histidine | 2.88 | 3.36 | 3.04 |
| Arginine | 41.95 | 39.77 | 42.89 |
| Lysine | 20.14 | 19.23 | 18.18 |
| N-(carboxyethyl-lysine | | + | + |
| $NH_3$ | 10 | n.d. | n.d. |
| Yield % | 99.28 | 95.6 | 95.29 |

in the Methods section. The tryptic peptides were first fractionated on a Sephadex G-75 column as shown in Figure 2-A. Two peaks were separated: peak 1, fractions 25-45, contained trypsin, undigested KDPG aldolase, and possibly a tryptic digest core: peak 2, fractions 65-120, contained the tryptic peptides. Peak 2, contained 87% of the radioactivity applied to the column. The second peak was further purified while the peak containing the tryptic core was dried and kept for later investigation.

The elution diagram from the chromatography, of peak 2, on Sephadex G-25, is shown in Figure 2-B. Again the peptides separated into two peaks; peak 2-1, fractions 120-225, contained over 95% of the applied radioactivity, while peak 2-2, fractions 250-295, contained less than 5% of the radioactivity and had very little protein as based on fluorescence.

The tryptic peptides contained in peak 2-1 were further purified by fractionation on Dowdex 50-X2 as shown in Figure 2-C. For the present work peptide T-10 was further purified, while the other pooled fractions were dried and kept for future work.

Figure 2-D shows the purification of peptide T-10 on BioGel P-2. The peak containing the radioactivity (from S-carbosymethylcysteine) was not totally symmetrical which indicated some contamination on the trailing edge of the peak, the last portion of the peak was not pooled to reduce contamination.

Figure 2. Purification of peptide T-10 from KDPG aldolase.

A) Chromatography of tryptic peptides on Sephadex G-75.

The $^{14}$C-pyruvate-labelled, reduced, carboxymethylated, and citraconylated KDPG aldolase was cleaved with trypsin and the resulting peptide mixture was applied to a Sephadex G-75 column (70 x 3.5 cm) as described in the Methods section. The column had been previously equilibrated with 10 mM $NH_4OH$ and was eluted with the same buffer. Fractions of 7.4 ml were collected and every fifth fraction was assayed for protein and radioactivity. Appropriate fractions were pooled and designated as follows: peak 1, fractions 25-45, trypsin and undigested KDPG aldolase; peak 2, fractions 65-120, tryptic peptides.

B) Chromatography on Sephadex G-25.

Tryptic peptides from peak 2, of the Sephadex G-75 column were run on a Sephadex G-25 column (170 x 4 cm) as described in the Methods section. The peptides were eluted with 10 mM $NH_4OH$. Fractions of 8.5 ml were collected and every third fraction was assayed for protein and radioactivity. Fractions 120-225, peak 2-1, and fractions 250-295, peak 2-2 were pooled for further work.

C) Chromatography of tryptic peptides on Dowex 50-X2.

Those tryptic peptides found in peak 2-1 from the Sephadex G-25 column were applied to a Dowex 50-X2 column

Figure 2 (cont'd)

(100 x 4 cm) as described in the Methods section. The column was developed with a concave gradient of pyridine acetate buffer. Gradient system 1 consisted of 4.5 column volumes of pyridine acetate buffer 0.2 M, pH 3.1, and 9 column volumes of pyridine acetate, 2 M, pH 5.0. When this gradient system was 60% completed it was replaced by a second gradient system consisting of the buffer in the mixing chamber remaining as it was with the buffer in the reservoir chamber being replaced with pyridine acetate, 8 M, pH 5.6. Every third fraction was assayed for protein and radioactivity. Appropriate peaks were pooled and designated as follows: T-1, fractions 98-114; T-2, fr. 115-125; T-3, fr. 126-146; T-4, fr. 147-174; T-5, fr. 175-193; T-6, fr. 194-201; T-7, fr. 202-230; T-8, fr. 231-260; T-9, fr. 261-283; T-10, fr. 284-322; T-11, fr. 346-381; T-12, fr. 390-443; T-13, fr. 443-502; T-14, fr. 506-550; T-15, fr. 622-674.

D) Purification of peptide T-10 on BioGel P-2.

The fractions representing peptide T-10 from the Dowex 50-X2 column were dried and applied to a BioGel P-2 column (80 x 0.9 cm). The column had been previously equilibrated with 1% $NH_4OH$ and was developed in the same buffer. Alternate fractions were assayed and appropriate fractions were pooled and designated as follows: fractions 20-27, peptide T-10 and fractions 33-37, peptide T-10-1.

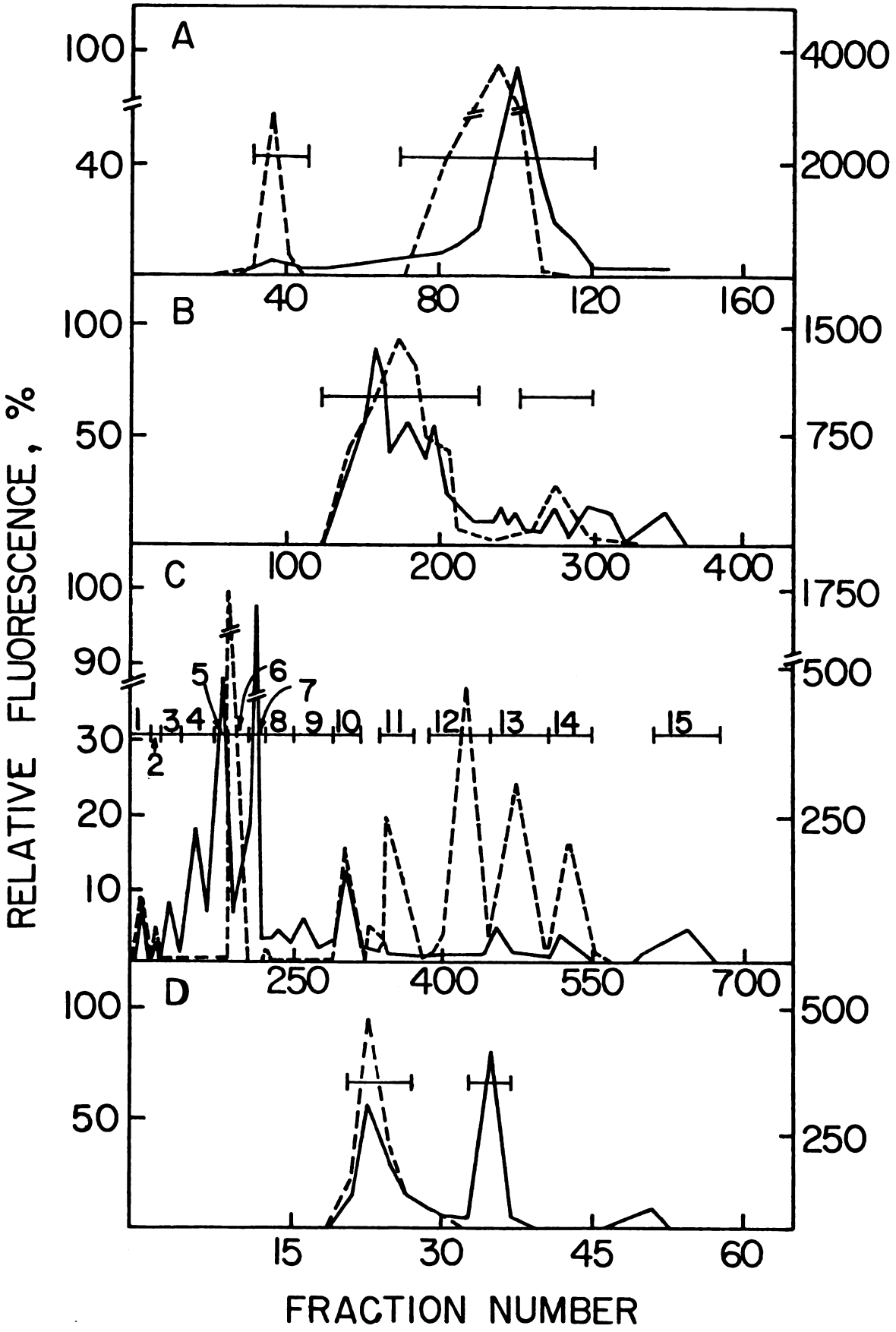

A
B
C
D
100
40
4000
2000
40
80
120
160
100
50
1500
750
100
200
300
400
100
90
30
20
10
1750
500
250
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
250
400
550
700
100
50
500
250
15
30
45
60
RELATIVE FLUORESCENCE, %
FRACTION NUMBER

Figure 2

The amino acid composition of the major peak is given in Table 3, the number of each residue per mole of peptide is close to integral, based on this as well as analysis of the N-terminal amino acid (presented in a later section), it was concluded that the peptide was in a homogeneous state. At this point the recovery of peptide T-10 was 9.49% or 1583 nmoles from a starting quantity of 16,680 nmoles of KDPG aldolase subunit. Possible reasons for the low yield will be dealt with in a later section. As such a small amount of peptide was available for sequencing, it was necessary to greatly alter the normal sequencing procedures both because of the much higher sensitivity required and because of problems introduced by the more sensitive procedures (this will also be dealt with in a later section).

Amino-terminal and carboxy-terminal analysis of peptide T-10: The amino terminus of peptide T-10 was established by a single step of Edman degradation, followed by analysis of the PTH derivative. Gas-liquid chromatography of the PTH derivative showed a single peak corresponding to PTH isoleucine standard. From this plus subtractive Edman analysis in which isoleucine disappeared, isoleucine was identified as the amino terminal residue. The first five cycles of Edman degradation are summarized in Table 4, and show that the N-terminal sequence of peptide T-10 is:

Ile-Thr-X ...

Table 3. Amino acid composition of peptide T-10 and its thermolysin fragments.

Peptide samples (3-10 nmoles) were hydrolyzed at 110°C for 24 hours with constant boiling HCl as detailed in the Methods section. The composition of each peptide is expressed in terms of the molar ratio of the constituent amino acids based on: for T-10, Th-0, Th-4, and Th-5 arginine; Th-2 an average of isoleucine and leucine; Th-3b an average of leucine and arginine; Th-3a leucine; Th-1 alanine. The integral values in parenthesis were assumed residue values. Threonine and serine were corrected for the 5 and 10% hydrolytic destruction respectively. Any residue present at a level of less than 10% of the residue on which the peptide ratios were based was taken as being 0.

Table 3.

| Amino Acid | T-10 | Th-0 | Th-1 | Th-2 | Th-3a | Th-3b | Th-4 | Th-5 |
|---|---|---|---|---|---|---|---|---|
| Aspartic | 0.8(1) | 1.43(1) | 0.41 | 0.32(0) | 0 | 1.09(1) | 0.93 | 0 |
| SCm-cysteine | 0.93(1) | + | 0.73 | 0 | 0 | 0 | 0 | 0 |
| Threonine | 1.74(2) | 1.08(1) | 1.4 | 1.2(1) | 0 | 0.67(1) | 0 | 0 |
| Serine | 0.6(1) | 0.94(1) | 0.51 | 0.34(0) | 0 | 1.04(1) | 1.27 | 0 |
| Glutamic | 3.33(3) | 2.01(2) | 0.54 | 1.4(1) | 0 | 2.39(2) | 1.71 | 2.34(2) |
| Proline | 1.18(1) | 0 | 0 | 0 | 0 | 0 | 0 | 1.00(1) |
| Glycine | 2.72(3) | 2.05(2) | 1.88 | 1.29(1) | 0 | 1.68(2) | 1.32 | 0 |
| Alanine | 1.65(2) | 1.13(1) | 1(1) | 1.03(1) | 0 | 1.54 | 0 | 0 |
| Valine | 2.02(2) | 0.97(1) | 0.73 | 0.74(1) | 0.84(1) | 1.04(1) | 0 | 0 |
| Isoleucine | 0.99(1) | 1.69(1) | 0.52 | 0.92(1) | 0 | 0.71(1) | 0.46 | 0 |
| Leucine | 2.34(2) | 0.95(1) | 0.61 | 1.08(1) | 1(1) | 1.03(1) | 0.71 | 0 |
| Arginine | 1(1) | 1(1) | 0 | 0 | 0 | 0.98(1) | 1(1) | 1(1) |
| Total residues | 20 | 13 | ----- | 7 | 2 | 13 | ----- | 4 |

As is shown in Table 4, the next three cycles of Edman degradation had no effect on the composition, indicating that blockage of the N-terminal of the peptide to Edman degradation had occurred. This problem will be dealt with in more detail later.

The partial sequence for the carboxy-terminal end of peptide T-10 was obtained, by carboxypeptidase digestion as shown in Table 5. Digestion of peptide T-10 for up to three hours with carboxypeptidase A or A and B gave the following order of amino acids:

$NH_2$.... X-Thr-Gly-Ala-Val-Leu-COOH.

Further digestion of peptide T-10 with the same levels of carboxypeptidase A and B, up to eight hours, released one further residue in a significant amount giving a sequence of:

$NH_2$.....X-Glu-Thr-Gly-Ala-Val-Leu-COOH.

Even with this prolonged digestion period there was not complete release of all the amino acids and no trace of extra amino acids other than glutamic acid, which indicates that one of the next two amino acids is resistant to carboxypeptidase digestion.

Digestion of peptide T-10 with thermolysin: Because of the problems with Edman degradation of peptide T-10, a limited amount of information could be attained from direct sequencing of this peptide. As a result it was necessary to carry out

Table 4. Partial sequence determination of peptide T-10 by subtractive Edman degradation.

A 200 nmole sample of Peptide T-10 was subjected to Edman degradation as detailed in the Methods section. The acid hydrolysate (3-5 nmoles) from each degradation cycle was examined on an amino acid analyzer for its constituents. Cycles 1-5 are on native Peptide T-10. Cycle 4-Modified was the fourth cycle run on Peptide T-10 which had been previously reacted with 2-amino-1,5 napthalene disulfonic acid (ANS), to block the side chain carboxyl groups.

Table 4.

| Amino Acid | T-10 | 1 | 2 | 3 | 4 | 5 | 4-Modified |
|---|---|---|---|---|---|---|---|
| Aspartic | 0.8(1) | 0.67 | 0.62 | 0.62 | 0.59 | 0.63 | 0.68 |
| SCm-cysteine | 0.93(1) | 0.88 | 0.78 | 0.71 | 0.83 | 0.90 | 0.75 |
| Threonine | 1.74(2) | 1.74 | 1.09 | 1.13 | 1.01 | 0.99 | 1.28 |
| Serine | 0.60(1) | 0.66 | 0.59 | 0.53 | 0.51 | 0.51 | 0.50 |
| Glutamic | 3.33(3) | 3.46 | 3.33 | 3.13 | 3.09 | 2.92 | 3.08 |
| Proline | 1.18(1) | 1.34 | n.d. | n.d. | 0.98 | n.d. | 1.40 |
| Glycine | 2.72(3) | 2.64 | 2.27 | 2.54 | 2.57 | 2.61 | 2.52 |
| Alanine | 1.65(2) | 1.79 | 1.75 | 1.81 | 1.82 | 1.87 | 1.73 |
| Valine | 2.02(2) | 2.13 | 2.16 | 2.34 | 2.32 | 2.17 | 2.06 |
| Isoleucine | 0.99(1) | 0.36 | 0.27 | 0.33 | 0.32 | 0.32 | 0.37 |
| Leucine | 2.34(2) | 2.28 | 1.96 | 2.04 | 2.11 | 2.19 | 2.29 |
| Arginine | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Residue assigned | | | Isoleucine-Threonine------ | | | ---- | ---- |

Table 5. Determination of the Carboxy-terminal sequence of peptide T-10 with carboxypeptidase A and B.

Digestion of peptide T-10 was conducted at 37°C in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5. Digestion was carried out for the time intervals indicated with a mixture of carboxypeptidase A and B at an enzyme to substrate ratio of 1:100 and 1:500 respectively. The amount of amino acid released is expressed as nmoles per three nmoles of peptide as determined by amino acid analysis of free amino acids released by the digestion.



| Time | Amino Acid released | | | | | |
|---|---|---|---|---|---|---|
| min | Leucine | Valine | Alanine | Glycine | Threonine | Glutamic |
| 20 | 2.78 | 1.45 | 0.51 | 0.20 | ----- | ---- |
| 30 | 3.06 | 1.63 | 1.09 | 0.43 | ----- | ---- |
| 60 | 2.86 | 1.80 | 1.23 | 0.55 | 0.50 | ---- |
| 180 | 2.89 | 2.12 | 1.77 | 1.03 | 0.94 | ---- |
| 480* | 3.00 | 2.57 | 1.68 | 1.64 | 1.16 | 0.43 |

* Values at 480 minutes normalized to 3 nmoles of leucine.

fractionation of T-10 in order to obtain peptides from which the sequence could be determined. The cleavage opportunities with this peptide were limited as it contained no methionine, or lysine. Although it contained an internal arginine it was demonstrated that this residue was resistant to tryptic cleavage. As a consequence it was necessary to utilize a less specific cleavage method with the hope of isolating useful peptides. It was determined that thermolysin was the most viable alternative cleavage method as it is generally fairly specific in that it cleaves on the N-terminal side of residues with a large aliphatic side chain such as leucine, valine, etc. As a result peptide T-10 was digested with thermolysin.

Purification of thermolysin peptides of T-10: The thermolysin digest of peptide T-10 was first fractionated on a Dowex 50-X8 column as shown in Figure 3. The fractions as marked, which made up a distinct peak were pooled and submitted to amino acid analysis to determine if further purification was necessary. Amino acid analysis of the thermolysin peptides are summarized in Table 3; recoveries of the peptides are shown in Table 6. Of these peptides, Th-3b, and Th-4 were recovered in too low a quantity to work with, while peptides Th-0, Th-2, Th-3a and Th-5 were sufficiently pure for use without further purification. An attempt to purify peptide Th-1 on BioGel P-2 resulted in splitting of this fraction into numerous small peaks all of

Figure 3. Separation of Thermolysin fragments of Peptide T-10 on Dowex 50-X8.

300 nmoles of Peptide T-10 was digested for four hours with thermolysin as described in the Methods section. The digest was chromatographed on Dowex 50-X8 using a gradient consisting of 0.05 M pyridine acetate, pH 2.4, and an equal amount of 0.5 M pyridine acetate, pH 3.75. The second portion of the gradient consisted of equal volumes of 0.5 M pyridine acetate, pH 3.75 and 2.0 M pyridine acetate, pH 5.0. Every third fraction was assayed and the appropriate fractions were pooled for later work. These peptides were designated as: T-10-Th-1, fractions 43-49; T-10-Th-2, fr. 50-61; T-10-Th-3a, fr. 64-71; T-10-Th-3b, fr. 72-79; T-10-Th-4, fr. 91-100; T-10-Th-5, fr. 103-112. An insoluble precipitate removed prior to application of the sample to the column was designated as T-10-Th-0.

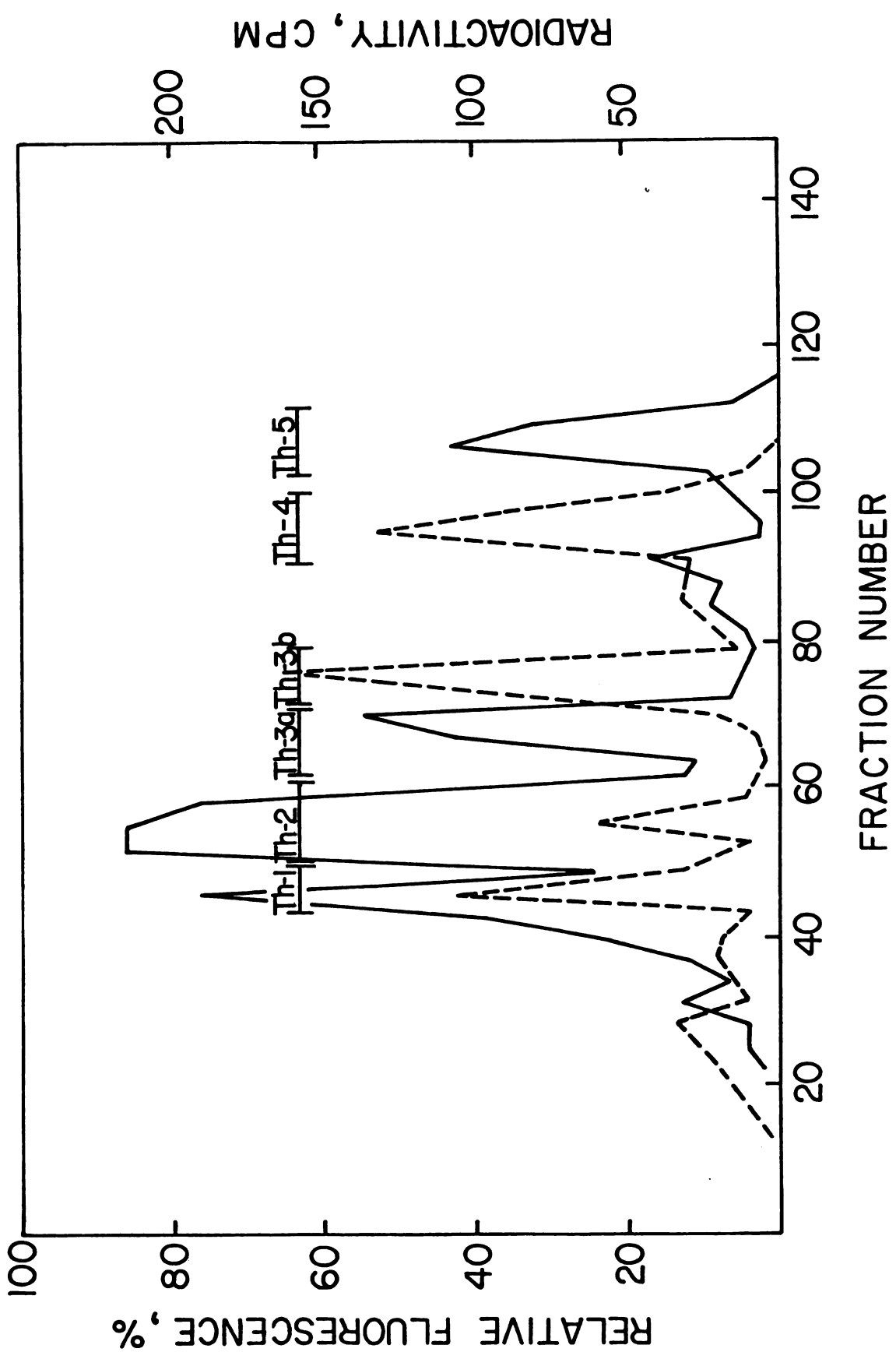

RADIOACTIVITY, CPM
200
150
100
50
RELATIVE FLUORESCENCE, %
100
80
60
40
20
FRACTION NUMBER
20
40
60
80
100
120
140
Th-1
Th-2
Th-3a
Th-3b
Th-4
Th-5

Figure 3

Table 6. Recovery of peptide T-10 and the thermolysin peptides derived from it.

The amount of each peptide recovered was calculated from amino acid analysis of the final purified product. The percentage recoveries were based on the amount of starting material.

| Peptide | nmoles recovered | % recovery |
|---|---|---|
| T-10 | 1583 | 9.49 |
| Th-0 | 36 | 12 |
| Th-1 | 42 | 14 |
| Th-2 | 126 | 48 |
| Th-3a | 105 | 35 |
| Th-3b | 12 | 4 |
| Th-4 | 12 | 4 |
| Th-5 | 90 | 30 |

which were too small to work with. These peaks represented at most 15 nmoles and in the case of the largest peak, it was still made up of at least two components, the smaller peaks which appeared to be in a pure state were present in approximately 3-5 nmole quantities.

Sequence studies on peptide Th-0: Two cycles of Edman degradation were carried out on this peptide with the resultant PTH amino acids being identified by comparison to standard PTH amino acids by polyamide chromatography. The first cycle released only PTH isoleucine thereby establishing that the peptide was pure and also that this 13 residue peptide was derived from the amino terminus of peptide T-10 which contained only one isoleucine, at the N-terminus. The second cycle released threonine in agreement with that found from the Edman degradations of peptide T-10.

Pilot digestion of the peptide with carboxypeptidase A released no amino acids, while digestion with carboxypeptidase A and B released a small amount of arginine only.

Sequence studies on peptide Th-2: From compositional analysis peptide Th-2, which also contained an isoleucine, was assumed to be a fragment derived from the N-terminal region of the 13 residue peptide Th-0. This was confirmed by one Edman cycle from which PTH isoleucine was released and identified by polyamide chromatography.

Carboxypeptidase digestion of this peptide released leucine, alanine and glycine in that order, as shown in Table 7. From this result the partial sequence of the peptide was determined to be:

$NH_2$-Ile-Thr-[Glx-Val]-Gly-Ala-Leu-COOH.

Total digestion of this peptide with leucine aminopeptidase gave the composition shown in Table 8, showing the Glx to be a glutamic acid.

Sequence studies on the Dipeptide Th-3a: The sequence of the dipeptide was established by one dansylation reaction. The dansyl amino acid released was compared to standard dansyl amino acids by polyamide chromatography and shown to be valine. Making the sequence of the dipeptide

$NH_2$-Val-Leu-COOH.

identical with the order observed from the carboxypeptidase digestion of peptide T-10, for the two C-terminal residues of peptide T-10.

Sequence studies on the tetrapeptide Th-5: Two cycles of Edman degradation were run on peptide Th-5, there was no disappearance of any amino acids as determined by amino acid analysis of the residual peptide. When the peptide was dansylated, a small amount of glutamyl residue was released indicating that the N-terminal residue was either

Table 7. Determination of the carboxy-terminal sequence of peptide Th-2 with carboxypeptidase.

The digestion conditions for peptide Th-2 were as given in Table 5.

| Time | Amino acid released | | |
|---|---|---|---|
| min | Leucine | Alanine | Glycine |
| 25 | 2.28 | 0.57 | 0.19 |
| 45 | 2.36 | 0.67 | 0.31 |
| 90 | 2.50 | 0.76 | 0.43 |
| 120 | 2.65 | 0.94 | 0.63 |

Table 8. Compositional analysis of peptide Th-2.

5 nmoles of peptide Th-2 was hydrolyzed at 110°C for 24 hours with constant boiling HCl and subsequently analyzed for amino acid constituents as outlined in the Methods section (column 1).

A second 5 nmole aliquot of Th-2 was digested with leucine aminopeptidase and analyzed as outlined in the Methods section (column 2).

| Amino Acid | Acid hydrolysis | Enzymatic digestion |
|---|---|---|
| Threonine | 1.2(1) | 1.13(1) |
| Glutamyl | 1.4(1) | ------ |
| Glutamic acid | ----- | 1.03(1) |
| Glycine | 1.29(1) | 0.99(1) |
| Alanine | 1.03(1) | 0.86(1) |
| Valine | 0.74(1) | 0.83(1) |
| Isoleucine | 0.92(1) | 1.07(1) |
| Leucine | 1.08(1) | 1.00(1) |

glutamic acid of glutamine. No amino acids were released on prolonged digestion with carboxypeptidase A and B.

Digestion of peptide T-10 with trypsin: It is known that trypsin will not cleave a bond which has a proline on its C-terminal side (49,50,51). To establish that this was the case for peptide T-10, it was digested with trypsin and the digest chromatographed on Sephadex G-75. Figure 4, shows that trypsin digested T-10 elutes as a symmetrical peak. The amino acid analysis of this fraction showed the identical composition to peptide T-10 (Table 9). One cycle of Edman degradation released only PTH isoleucine as detected by polyamide chromatography showing that there was only one peptide present. Hence one must assume the presence of the arginine-proline bond, as trypsin would cleave other arginine-X bonds.

Construction of the sequence of peptide T-10: As previously presented, the T-10 peptide contains an amino and carboxy terminal sequence of:

$NH_2$Ile-Thr-X.....  (Table 4)  and

$NH_2$ .....X-Glu-Thr-Gly-Ala-Val-Leu-COOH  (Table 5).

Further digestion of peptide T-10 by thermolysin resulted in 7 small fragments (of which 4 were used in subsequent sequencing work). On the basis of the data obtained from the whole peptide it was possible to place these fragments. Th-0 and Th-2 both contained an isoleucine N-terminal and

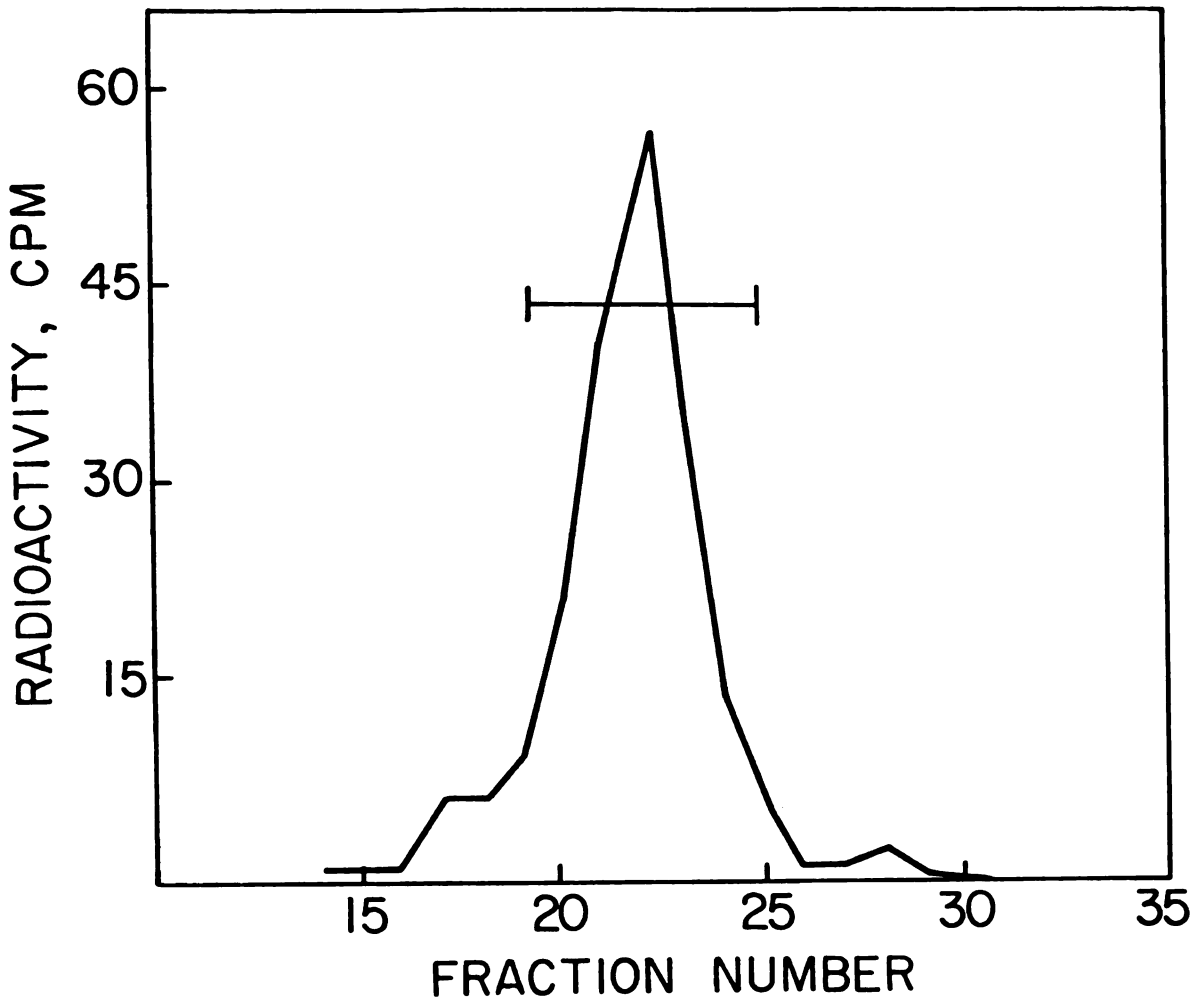



Figure 4

Gel filtration on Sephadex G-75 of products resulting from tryptic cleavage of peptide T-10.

Peptide T-10 was digested with trypsin and the resulting products were applied to a Sephadex G-75 column (60 x 0.6 cm) as described in the Methods section. Each fraction was assayed for radioactivity and fractions 19-25 were pooled for later work.

Table 9. Compositional Analysis of the major peak from tryptic hydrolysis of peptide T-10.

Peptide T-10 was digested with trypsin, two hours at 37°C, at an enzyme to substrate ratio of 1:50, and chromatographed on Sephadex G-75. A portion of the major radioactive peak was hydrolyzed for 24 hours at 110°C with constant boiling HCl.

| Amino Acid | Peptide T-10 | Tryptic digest of T-10 |
|---|---|---|
| Aspartic | 0.80(1) | 0.73(1) |
| SCm-cysteine | 0.93(1) | 0.81(1) |
| Threonine | 1.74(2) | 1.73(2) |
| Serine | 0.60(1) | 0.51(1) |
| Glutamic | 3.33(3) | 3.42(3) |
| Proline | 1.18(1) | 1.43(1) |
| Glycine | 2.72(3) | 2.45(3) |
| Alanine | 1.65(2) | 1.59(2) |
| Valine | 2.02(2) | 1.62(2) |
| Isoleucine | 0.99(1) | 0.88(1) |
| Leucine | 2.34(2) | 2.29(2) |
| Arginine | 1 (1) | 1 (1) |
| Total | 20 | 20 |

as peptide T-10 contained only one isoleucine which was shown to be N-terminal, these two peptides represented different degrees of digestion of the N-terminal region of peptide T-10. The Th-0 peptide had isoleucine and threonine at its N-terminal as expected and contained an arginine at its C-terminal. The heptapeptide, Th-2, had isoleucine at the N-terminal and Gly-Ala-Leu at the C-terminal. The dipeptide, Th-3a, Val-Leu consisted of two residues released from the C-terminal of peptide T-10. The tetrapeptide, Th-5, which contained an arginine, had a Glx N-terminal. Since the single arginine is at residue 13 of Th-0, and Glx is N-terminal, and since the third glutamyl residue of T-10 has been shown to be at position 15, Th-5 must occupy residues 12-15. From this evidence the thermolysin peptides could be arranged in the sequential order as:

$$[\text{---}\underline{\text{Th-2}}\text{-----}]_{7} \qquad {}_{11}[\text{-}\underline{\text{Th-5}}\text{-----}]_{15}$$

$$[\text{----}\underline{\text{Th-0}}\text{----------}]_{13} \qquad {}_{18}[\text{-}\underline{\text{Th-3a}}\text{--}]_{20}$$

Placement of additional residues by indirect evidence: Peptide Th-2 was known to contain the following amino acid sequence:

$$NH_2\text{-Ile-Thr-[Glu,Val]-Gly-Ala-Leu-COOH}$$

From data obtained on Edman degradation or enzymatic digestion, the order of residues 3 and 4 could not be obtained. However it is known that N-terminal glutamic acid residues can cyclize spontaneously to form pyrrolidone carboxylic acid (51). An

N-terminal blocked in this manner would no longer react with N-terminal reagents such as phenyl isothiocyanate.

Amino napthalene disulfonic acid in a reaction similar to peptide bond synthesis will react with, carbodiimide activated, carboxyl groups of acidic residues in a peptide, to derivitize these residues (49). As a consequence the derivitization would prevent the postulated cyclization of the glutamic acid residue in question. Therefore an attempt was made to show that pyrrolidone carboxylic acid formation had occurred by reaction of the putative blocked peptide with ANS. After reaction with this reagent, four Edman cycles with the treated T-10 were unsuccessful in releasing any amino acids past threonine, as shown in Table 4. However, it was felt that on the basis of the reactivity of glutamic acid as such that position 3 is probably Glu. From this valine must then occupy position 4, making the sequence of peptide Th-2:

$NH_2$-Ile-Thr-Glu-Val-Gly-Ala-Leu-COOH.

Peptide Th-5. From the standard procedures little was learned of this peptide, other than its composition as shown in Table 3, and that it contained a glutamyl residue at its amino terminal. From the overall composition of peptide T-10 and the following considerations the peptide was placed between residues 12 and 15 of peptide T-10. (1) the only arginine in peptide T-10 is at position 13. (2) since the N-terminal of Th-5 is a glutamyl residue, by difference this

fact requires that residue 12 of T-10 is also a glutamyl residue and that residues 14 and 15 be proline and glutamic acid respectively. (3) it was shown previously from proteolytic digestion of T-10 that residue 15 was glutamic acid. (4) trypsin did not cleave the arginine-X bond which would indicate an arginine-proline bond. This would make the sequence of peptide Th-5:

$$NH_2\text{-Glx-Arg-Pro-Glu-COOH.}$$

Figure 5, summarizes the overall sequence of peptide T-10 as established in this work.

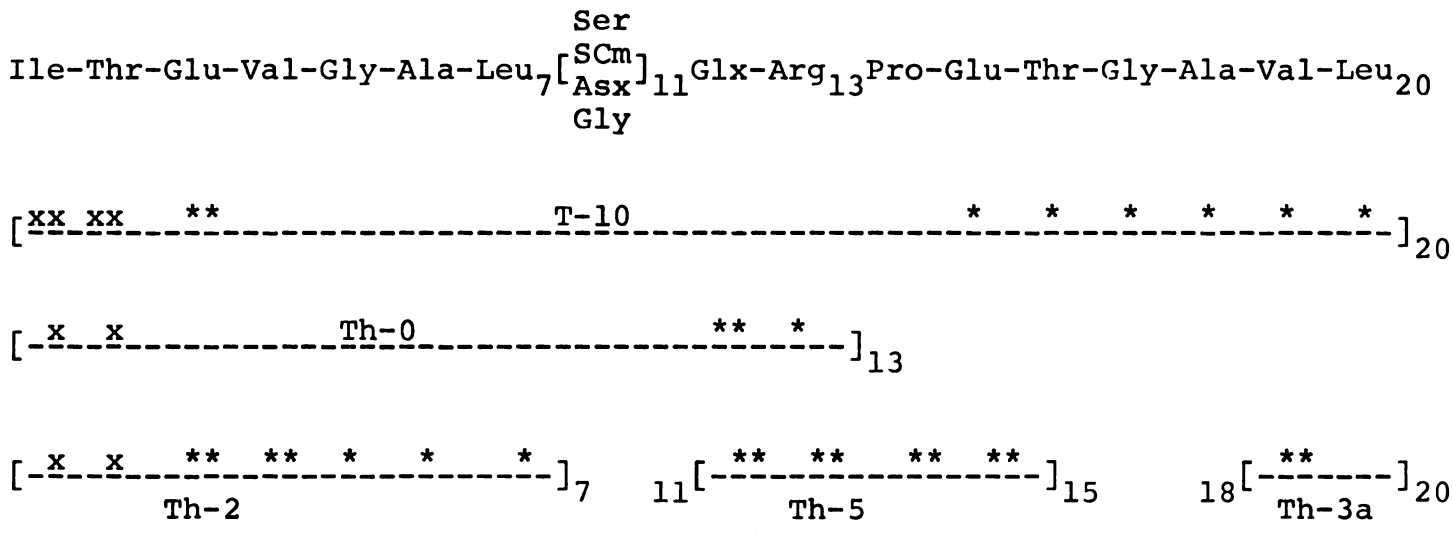
The data cited herein do not permit assignment of residues 8-11. This region contains Ser, Scm, Asp, and Gly. Additional experiments as yet to be performed, on Th-0 (using a fluorescence amino acid analyzer under development) should establish the order in this region.

Figure 5. Summary of the sequence analysis of peptide T-10

Various symbols are adopted to denote the methods employed to elucidate the sequences. They are as follows: **xx**, subtractive Edman degradation; **x**, those residues identified by thin layer chromatography of their PTH derivatives; *, those residues released by carboxypeptidase digestion; **, residues determined by some alternative means.

Figure 5

Sequence of peptide Trypsin-10-KDPG Aldolase



# DISCUSSION

The chromatogram of the tryptic fragments of KDPG aldolase demonstrates that more than the expected number of fragments were obtained from the digestion of the lysine blocked subunit of the aldolase. This may have been due to at least two factors: 1) not all of the lysine residues reacted with the citraconic anhydride and as a result were still available for cleavage with trypsin; or 2) some nonspecific cleavage was catalyzed by trypsin. Normal trypsin is known to show some chymotryptic activity (53), and also trypsin undergoes autolysis, to yield a number of poorly characterized proteolytic derivatives, the specificity of which is unknown. In a number of cases this nonspecific cleavage has been reported to be as high as 30-40% of the normal sensitive bond cleavage (54,55,56).

The second possibility has been shown to be the case (at least in part) in this situation, as peptide T-10 was shown to have a leucine C-terminal, with recovery of the peptide (10%) in the range of that observed by others in this situation.

On initial observation it may seem possible that this peptide is the C-terminal from the aldolase subunit rather than a nonspecific cleavage product. However from work by

Robertson et al. (26), it was demonstrated that the C-terminal region of the KDPG aldolase subunit contained asparagine, isoleucine and lysine all close to its C-terminal. As lysine is not even found in this peptide and the asparaginyl and isoleucine residues in the peptide (T-10), are both at a considerable distance from the C-terminal, it can be stated with total confidence that peptide T-10 is not the C-terminal of the aldolase subunit, but rather a nonspecific cleavage product.

Because of the low yield of peptide T-10, the possibility has been considered that the peptide was released from trypsin by autodigestion. As shown in Table 6, recovery of peptide T-10 was 9.49% or 1583 nmoles. For digestion with trypsin 5 mg of trypsin was added to the reaction mixture, which represents a maximum of 210 nmoles of a given peptide (assuming a 100% yield). As this is only 13% of the quantity of peptide T-10 recovered the likelihood that the peptide came from the trypsin is for all practical purposes nonexistent.

Similarly it can be shown for the thermolysin peptides used in the sequencing that, of the peptides used Th-0, for which 36 nmoles were recovered, was in the lowest yield. This represented over 1000 times the amount of peptide which could be attributed to the thermolysin. In all other cases yields were higher, making it even more difficult for one to assume that there is any chance of contamination.

Another possible source of contamination, which could have resulted in production of peptide T-10, is in the KDPG aldolase itself. However as previously stated disc gel electrophoresis of the purified aldolase gave only one discrete band, and the amino acid composition of this enzyme preparation is in agreement with that of Tsay (30), who had protein of higher specific activity.

Also as the peptide was a nonspecific cleavage product it would be in low yield from any source, and as a result would require that the quantity of contamination by a second protein be high enough that it could be detected by one of the above criterion.

A second anomoly occurred in peptide T-10 in that, it was found to contain an uncleaved internal arginine bond after tryptic digestion. This can be explained by the fact that the arginine has been demonstrated to be linked to a proline on its C-terminal side. It is known that trypsin cannot cleave this bond (49,50,51).

A great deal of difficulty was encountered in attempting Edman degradations on peptide T-10 and the two thermolysin peptides, Th-0 and Th-2, which were derived from its N-terminal. Due to the presence of a glutamic acid residue near the N-terminal, it was possible to complete only two successful Edman cycles on any of these peptides. From peptide Th-2, it was shown that the third residue was either glutamic acid or valine. It was postulated that the third residue was indeed glutamic acid which had undergone

a spontaneous cyclization, during the Edman degradations, to form pyrrolidone carboxylic acid which as it has no free amino terminal, would then be blocked to further Edman degradation. In an attempt to prove this, peptide T-10 was derivitized with amino napthalene disulfonic acid, after activation with a water soluble carbodiimide. And Edman degradations were run on the derivitized peptide. As ANS reacts with all free carboxyl groups (both side chain and terminal) it should no longer have been possible for the side chain of the glutamic acid to cyclize. However no appreciable difference was seen with this peptide after four Edman cycles. The reason for this is unknown. Laursen (58) states that they have found that coupling yields with the carbodiimide reaction are quite variable (10-80%), and totally unpredictable. If this is the case it is possible that the glutamic acid residue in question remained underivitized and as a result could undergo cyclization as before.

As the order of four of the amino acids (#8-11), could not be established, it is impossible to speculate on the three-dimensional structure of the peptide. The only thing that can be said as to the nature of the peptide, is that it has a net negative charge of at least (-1), from the arginine and two glutamic acid residues. It may be more negative than this as the third glutamyl residue and the asparaginyl residue may also later be shown to be acids as

well. If this is the case it will give a net negative charge of possibly as high as (-3).

There were a great many problems encountered in this work with recovery of the peptides. The formation of either a digestion resistant tryptic core of the aldolase subunit, or association of larger peptide material to form an insoluble precipitate led to the loss of approximately 13% of the radioactivity and 18% of the peptides from the initial digest.

Two aspects could be studied to possibly eliminate this problem. One is the efficiency of carboxymethylation in opening up the protein and making it available for digestion. If the reaction is not complete it may be possible that a portion of the subunit never becomes available for digestion, and if this is the case it may be worthwhile to determine if other derivatives are more efficient in opening up the protein for digestion. The second possibility mentioned above is that larger peptides are associating and forming an insoluble precipitate. This could be eliminated or at least reduced to a large degree by carrying out the digestion with trypsin in 2-3 M urea. Urea has no appreciable effect on tryptic activity, over short periods of time (when kept at low concentrations), and would aid in elimination of aggregation of peptides as well as making the subunit more available to proteolytic digestion.

Another area in which yields were low was from the ion exchange chromatography on Dowex 50, both X-2 and X-8. As was shown in Table 6, yields varied from less than 5% to in the vicinity of 50% for some peptides. Even the yield of 50% is not satisfactory and it is felt that, in future work all possible attempts should be made to eliminate this type of purification procedure in favor of alternatives from which reasonable recovery may be anticipated.

The poor yields of T-10 are due to (1) peptide T-10 is a nonspecific cleavage product, and (2) the losses in column chromatography are high. Thus the quantity of peptide T-10 available for sequencing was less than 10% of that initially anticipated. This made it much more difficult to carry out the sequence analysis, as it was necessary to alter all of the normal sequencing procedures such that they were much more sensitive. This alteration required working with extremely minute quantities in an attempt to maintain optimum levels of reactants in both chemical and enzymatic reactions. Also with the small quantities used contamination by reagents, even the so called Sequanal grade reagents, was a major problem. Such reagents had to be further purified in order to eliminate free amino acid contamination.

Another factor which became limiting in the work was the sensitivity of the amino acid analyzers. The practical limit of sensitivity with the ninhydrin analyzers available, was in the range of 2-3 nmoles per amino acid. Work

has been in progress to develop a usable fluorescence analyzer with a limit of sensitivity in the range of 100 pmoles. When this developmental work is completed and the analyzer is working satisfactorily, it is anticipated that yet to be established sequences of peptide T-10 will be completed.

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