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SUBSTRATE SPECIFICITY AND SPECTROSCOPIC PROPERTIES OF 2,4-DICHLOROPHENOXYACETIC ACID/α-KETOGLUTARATE DIOXYGENASE

presented by

Ruth E. Saari

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Major professor

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SUBSTRATE SPECIFICITY AND SPECTROSCOPIC PROPERTIES OF 2,4-DICHLOROPHENOXYACETIC ACID/α-KETOGLUTARATE DIOXYGENASE

By

Ruth Elizabeth Saari

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ABSTRACT

SUBSTRATE SPECIFICITY AND SPECTROSCOPIC PROPERTIES OF 2,4-DICHLOROPHENOXYACETIC ACID/α-KETOGLUTARATE DIOXYGENASE

By

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2,4-Dichlorophenoxyacetic acid (2,4-D) was one of the first herbicides used which act by mimicking a plant growth hormone; it and related phenoxy herbicides are still in wide use today. It is not highly persistent in soil, due to microbial biodegradation. In the soil isolate *Ralstonia eutropha* JMP134, the first step in catabolism of 2,4-D is catalyzed by 2,4-D/α-ketoglutarate (α-KG)-dioxygenase (TfdA). This O_2 and Fe(II) dependent enzyme couples the oxidative decarboxylation of α-KG to the oxidation of 2,4-D producing 2,4-dichlorophenol and glyoxylate. TfdA is a useful model protein for studying α-KG-dependent dioxygenases because it is readily purified and assayed.

TfdA was shown to utilize thiophenoxyacetic acid (TPAA) to produce thiophenol, allowing the development of a continuous spectrophotometric assay for the enzyme using the thiol-reactive reagent 4,4'-dithiodipyridine. In contrast to the reaction with 2,4-D, the kinetics of TPAA oxidation were non-linear and ascorbic acid was found to be required for and consumed during TPAA oxidation. The ascorbic acid was needed to reduce an oxidized inactive state of the enzyme formed in the absence of substrate or the presence of TPAA prior to turnover. Evidence also was obtained for the generation of an irreversibly inactivated enzyme species by an oxidative reaction. Based on initial rate

studies at optimal ascorbate concentrations, the k_{cat} and K_m values for TPAA were estimated to be 20-fold lower and 80-fold higher than the corresponding values for 2,4-D.

TfdA hydroxylates at C-2 of 2,4-D to produce an unstable hemiacetal. Dichlorprop, an analog of 2,4-D containing a methyl group at C-2, was used as a substrate to gain insight into the stereochemistry of this hydroxylation. TfdA from JMP134 was shown to use the (S)-enantiomer of dichlorprop, indicating that it likely hydroxylates 2,4-D to give the (R)-hemiacetal. The same stereospecificity was observed with *Burkholderia cepacia* RASC, another 2,4-D degrading strain which possesses a closely related TfdA. By contrast, a strain of *Alcaligenes denitrificans* which grows on the phenoxy herbicide mecoprop degraded (R)-dichlorprop. Despite the opposite stereospecificity of *A. denitrificans* cell extract, the dichlorprop disappearance was catalyzed by an α-KG dioxygenase, and genomic DNA from this isolate hybridized to $tfdA_{RASC}$.

The inactive copper form of TfdA was studied by electron paramagnetic resonance (EPR), electron spin echo envelope modulation (ESEEM), and x-ray extended absorption fine structure (EXAFS) spectroscopies. Cu-TfdA possesses at two equatorial histidines and two other N/O ligands in the presence or absence of α-KG, one of which disappeared upon addition of 2,4-D. EXAFS of the active Fe-TfdA showed 5-6 N/O ligands, including ~2 histidines, one of which was displaced upon binding of 2,4-D, whereas little change was observed upon binding of α-KG.

To the Creator behind the irreducible complexity of biochemistry

ACKNOWLEDGEMENTS

I thank the researchers from other labs who have collaborated directly on this project, as detailed in the acknowledgment sections of individual chapters in this dissertation. I also thank Olga Maltseva and Kirsti Ritalahti of MSU's Center for Microbial Ecology for useful discussions on 2,4-D biodegradation and assistance with HPLC maintenance. As well, I thank Center for Microbial Ecology for funding most of my stay here at MSU as a graduate students. The secretarial staffs of the Center, and well as of the Biochemistry and Microbiology Departments, are thanked for courteous assistance with such matters as ordering supplies, enrollment, and getting my paycheck to the right place.

I would like to thank members of the lab for assistance and advice related to this project. I particularly thank Debbie Hogan who did the hybridization studies of *Alcaligenes denitrificans,* and also engaged in many fruitful discussions on the TfdA/TauD project. Julie Dunning and Raghavakaimal Padmakumar are more recent members on the TfdA/TauD project, and also provided valuable discussion on the project. As well, I would like to thank Gerry Colpas, Raghavakaimal Padmakumar, and Kazuya Yamaguchi for advice on spectroscopy, and Vladimir Romanov and Ruth Schaller for their work on keeping the HPLC and FPLC equipment running. And I would like to thank the aforementioned lab members, along with Tim Brayman, Mary-Beth Moncrief, Anatoly Slepenkin, and Aileen Soriano for making the lab a pleasant place to work.

Bob Hausinger, my advisor, did a great job. He is keenly interested in the science, and leads the people in his lab by drawing them into the same enthusiasm rather than by pushing them. He gave guidance for the direction of the work and set up collaborations with other labs, but also encouraged my initiative in designing experiments. He helped me greatly during the writing of the thesis by returned draft versions of the thesis promptly with suggestions that made the work more focused and clarified the flow of thought. Any remaining obscurities are my fault.

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LIST OF ABBREVIATIONS

AA	L-δ-(α-aminoadipoyl)
ACC	1-aminocyclopropane-1-carboxylate
ACV	L-α-amino-δ-adipoyl-L-cysteinyl-D-valine
α-KG	α-ketoglutaric acid
2,4-D	2,4-dichlorophenoxyacetic acid
DTDP	4,4'-dithiodipyridine
DEPC	diethylpyrocarbonate
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
ESEEM	electron spin echo envelope modulation
EXAFS	extended X-ray absorbance fine structure
fwhm	full width at half-maximum
HPLC	high pressure liquid chromatography
HPPD	hydroxyphenylpyruvate dioxygenase
IPNS	isopenicillin N-synthase
LMCT	ligand to metal charge transfer
MLCT	metal to ligand charge transfer
MCPA	2-methyl-4-chlorophenoxyacetic acid

MMO	minimal medium
MSU	Michigan State University
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TfdA	2,4-D/α-KG dioxygenase
TPAA	thiophenoxyacetic acid
TrpSoy	Trypticase Soy broth
UV	ultraviolet
XAS	X-ray absorbance spectroscopy

CHAPTER 1

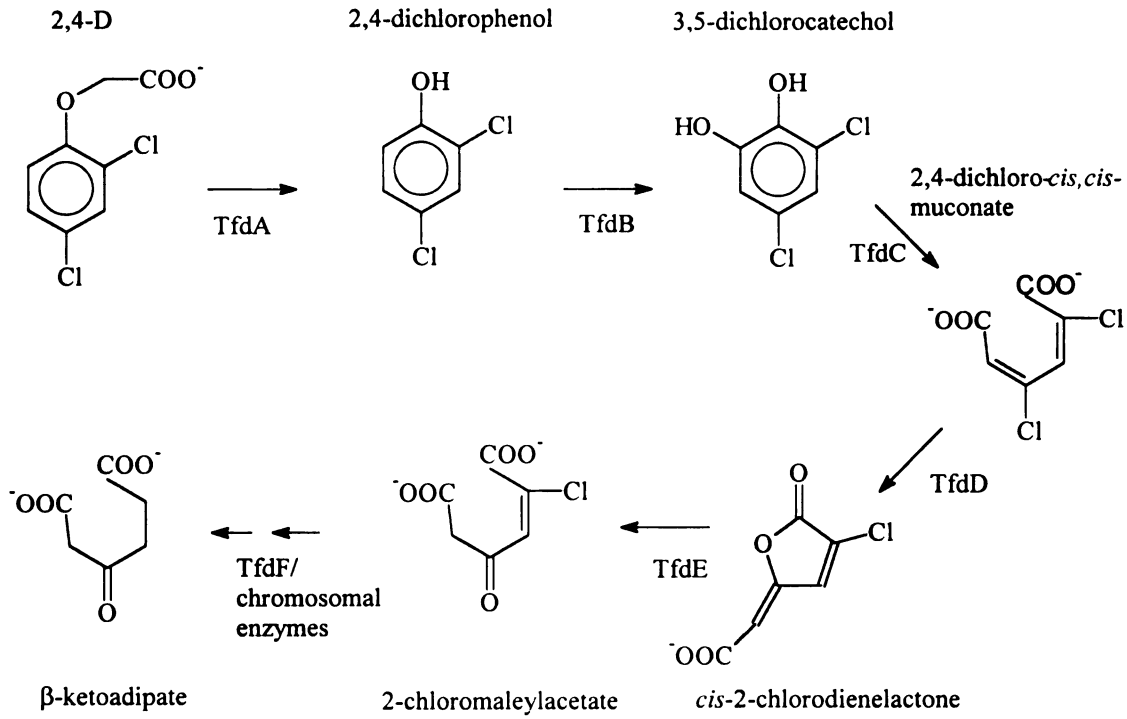
INTRODUCTION

The section in chapter 1 describing 2,4-D catabolism by *Ralstonia eutropha* JMP134 is based on a review article for which I was one of the authors: Hausinger, R. P., Fukumori, F., Hogan, D. A., Sassanella, T. M., Kamagata, Y., Takami, H., and Wallace, R. E. (1996). "Biochemistry of 2,4-D degradation: evolutionary implications." Microbial Diversity and Genetics of Biodegradation, K. Horikoshi, M. Fukuda, and T. Kudo, eds., Japan Scientific Press, Tokyo.

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used broadleaf herbicide that is rapidly biodegraded in soil (reviewed by e.g. (Loos 1969; Sinton et al. 1986)). Several bacteria capable of degrading 2,4-D have been isolated, of which the best characterized is *Ralstonia eutropha* JMP134 (Don and Pemberton 1981; Pemberton et al. 1979). The enzymes that catalyze the upper portion of the degradative pathway in this bacterium are encoded by the *tfd* genes on the pJP4 plasmid (Don et al. 1985) and include the main focus of this thesis, TfdA. This introduction will describe the pathway of 2,4-D metabolism in *R. eutropha* JMP134, discuss the environmental and evolutionary significance of TfdA, examine the biochemical and genetic relationships between TfdA and other α-ketoglutarate (α-KG)-dependent dioxygenases, and provide an outline of the remainder of the thesis.

2,4-D catabolism by *R. eutropha* JMP134

The overall pathway for metabolism of 2,4-D in *R. eutropha* (formerly *Alcaligenes eutrophus* (Yabuuchi et al. 1995)) JMP134 is shown in Scheme 1. The biochemistry of catabolism in this bacterium was recently reviewed and compared to variant 2,4-D degradation pathways in other microorganisms (Hausinger et al. 1996). Below, I detail aspects of the first step in the reaction and provide an overview of the remaining steps.

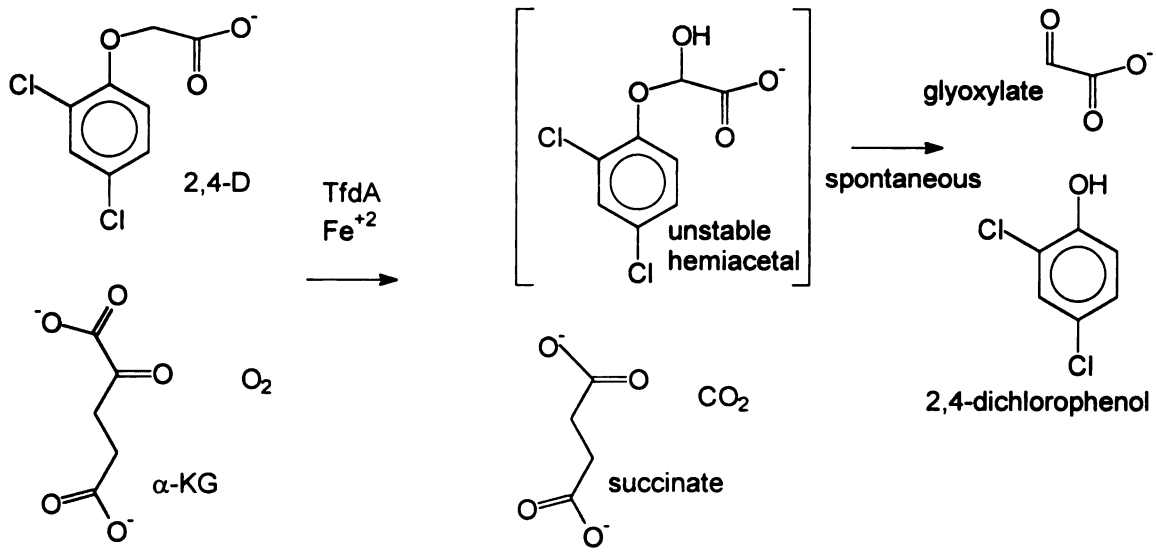


Scheme 1. Pathway for 2,4-D degradation in *R. eutropha* JMP134.

TfdA, 2,4-D/α-KG-dependent dioxygenase; TfdB, 2,4-dichlorophenol hydroxylase; TfdC, chlorocatechol dioxygenase; TfdD, chloromuconate cycloisomerase; TfdE, dienelactone hydrolase; TfdF, maleylacetate reductase

Ether bond cleavage as the first step of 2,4-D degradation by R. eutropha *JMP134*

TfdA, the first enzyme in the 2,4-D catabolic pathway encoded on pJP4, catalyzes the oxidative removal of the acetate moiety from the ring of this herbicide, releasing 2,4-dichlorophenol (Scheme 1). As early as 1957 certain microorganisms were known to degrade 2,4-D via a pathway containing a phenolic intermediate (Steenson and Walker 1957). From studies carried out with a 2,4-D degrading strain of *Arthrobacter*, the reaction was suggested to proceed via hydroxylation of the methylene carbon to give a hemiacetal, which is unstable and spontaneously decomposes to form the corresponding phenol and glyoxylate (Helling et al. 1968; Tiedje and Alexander 1969). This proposal was based on studies with ^{18}O ether-labeled 2,4-D showing that all the ^{18}O oxygen remains in the phenol, and on the identification of glyoxylate as one of the products. The *R. eutropha* enzyme was initially assumed to be a monooxygenase, e.g. (Don et al. 1985; Streber et al. 1987), but was later shown to be an α-KG dependent dioxygenase (Fukumori and Hausinger 1993a). The enzyme catalyzes the decarboxylation of α-KG to form succinate and CO_2 and couples this reaction to hydroxylation of substrate (Scheme 2). TfdA was the first example of an α-ketoglutarate-dependent dioxygenase involved in xenobiotic metabolism; by contrast, the remaining enzymes in the pathway showed similarities to previously described enzymes in chloroaromatic metabolism, as outlined in the following section. The work described in this thesis focuses on further characterization of the kinetics and metallocenter of the 2,4-D/α-KG dioxygenase, and on investigations of the diversity of ether-cleavage enzymes among 2,4-D degraders.



Scheme 2. Reaction catalyzed by TfdA.

Active TfdA is a homodimer with subunits of M_r 32,000 containing one ferrous ion per subunit (Fukumori and Hausinger 1993b). Ascorbic acid, while not required for the reaction, increases the rate somewhat and improves the linearity of the reaction (*ibid.*). α-KG as the cosubstrate gave the best activity, but the enzyme had some activity with analogs that lacked the distal (non-keto) carboxyl group (pyruvate, α-ketobutyrate, α-ketovalerate, α-ketocaproate, α-ketoisovalerate; k_{cat}/K_m < 1% of that for 2,4-D, *ibid.*), or in which the chain was lengthened by one methylene unit (α-ketoadipate; k_{cat}/K_m = 7% of that for 2,4-D). TfdA showed no activity towards analogs in which the chain was shortened by one methylene group (oxaloacetate) or when the α-keto group was missing (β-ketoglutarate, malonate, succinate, glutarate). The dioxygenase showed relatively

broad specificity towards the aromatic substituent, accepting various methyl- and chloro-substituted aryl rings including the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), as well as the derivative in which a methyl group added at the acetate side chain (i.e., the herbicide dichlorprop; 2-(2,4-dichlorophenoxy)propionate).

Remaining steps in 2,4-D degradation by* R. eutropha *JMP134

The remaining enzymes in the 2,4-D degradation pathway convert 2,4-dichlorophenol to TCA-cycle intermediates (Scheme 1). The second enzyme in the pathway, TfdB, is 2,4-dichlorophenol hydroxylase and catalyses aryl hydroxylation of 2,4-dichlorophenol to give dichlorocatechol. The enzyme contains flavin, uses NAD(P)H as a cosubstrate and shows sequence similarity to enzymes involved in hydroxylation of salicylate and other phenols (Farhana and New 1997; Perkins et al. 1990). The 3,5-dichlorocatechol undergoes *ortho* cleavage, catalyzed by the dioxygenase TfdC, to yield 2,4-dichloro-*cis,cis*-muconate, which is the inducing intermediate for the 2,4-D pathway (Filer and Harker 1997). The dioxygenase has not been purified, but an apparently identical enzyme has been purified from *Burkholderia cepacia* CSV90 (Bhat et al. 1993), and shown to possess a ferric metallocenter. The TfdC sequence is similar to other chlorocatechol dioxygenases, including those encoded by *clcA* (*clc* genes carry out 3-chlorobenzoate degradation) and *tcbC* (*tcb* genes are involved in chlorobenzene metabolism) (van der Meer et al. 1992). The muconate is converted to 2-chlorodienelactone with elimination of chloride anion by the enzyme chloromuconate cycloisomerase or TfdD (Kuhm et al. 1990; Perkins et al. 1990). This cycloisomerase shows sequence similarity with that encoded by *clcB* (Perkins et al. 1990).The ring of this lactone is then opened by the action of dienelactone hydrolase or TfdE (*ibid.*) to form 2-

chloromaleylacetate. TfdE is clearly related in sequence to other dienelactone hydrolases such as those encoded by *clcD* and *tcbE* (van der Meer et al. 1992). A chromosomally-encoded maleylacetate reductase has been purified from JMP134/pJP4 and shown to convert 2-chloromaleylacetate to 3-oxoadipate via maleylacetate (Seibert et al. 1993). The amino terminal sequence of the chromosomally-encoded enzyme is similar to that encoded by the plasmid-borne *tfdF* gene, raising the possibility that TfdF carries out the same type of reaction. Further evidence for this supposition comes from a purified maleylacetate reductase involved in 3-chlorobenzoate degradation by a *Pseudomonas* sp. (this enzyme shows around 80% homology to TfdF and to TftE, a maleylacetate reductase involved in degradation of 2,4,5-T by *Burkholderia cepacia* AC1100 (Kasberg et al. 1995; Kasberg et al. 1997)). The 3-oxoadipate produced by this two-step reaction can be further degraded by chromosomally-encoded enzymes to form succinate and acetyl coenzyme A, which feed into the TCA cycle. By the combined action of all of the above enzymes, *R. eutropha* JMP134 is capable of growth on 2,4-D as a sole carbon source.

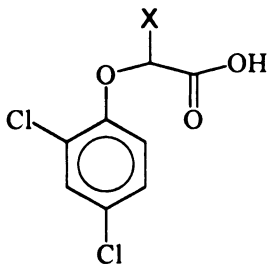
Environmental/Evolutionary Significance of TfdA

2,4-D is a member of the phenoxy-type herbicides, which are selective towards broad-leaf plants. Investigations into the herbicidal properties of phenoxy acids and related plant-growth regulators started in the early 1940's (Slade et al. 1945; Templeman and Marmoy 1940), and these chemicals came into widespread use in the USA in 1947. Current application rates are 55 million pounds per annum in the U.S. (Szmedra 1997). The high usage rate of these herbicides makes it important to characterize their fate in the

environment. 2,4-D biodegrades readily in aerobic soil, with a half life in the range of 3 to 42 days (reviewed in (Loos 1969; Sandmann et al. 1988)), depending on the soil type. Under anaerobic conditions (flooded soil) or in dry soil, it takes longer (Han and New 1994; Ou 1984; Parker and Doxtader 1983; Sattar and Paasivirta 1980; Yoshida and Castro 1975). Other phenoxy herbicides (Scheme 3) are also generally readily degraded, with half-lives in soil within the same range as 2,4-D: 3-7 days for 2-chloro-4-methylphenoxyacetic acid (MCPA) (Buser and Müller 1997; Vink and van der Zee 1997), 4 to 10 days for mecoprop (Müller and Buser 1997; Vink and van der Zee 1997), 4 to 22 days for dichlorprop (Buser and Müller 1997; Garrison et al. 1996), and 5 to 42 days for fenoxaprop (Smith and Aubin 1990). Quite variable half life times have been reported for fluazifop, between 11 days to about 12 weeks (Smith 1987) and references therein). For diclofop-methyl, 50% disappearance required 12-18 days in one report (Martens 1978) and up to 3 to 5 weeks in another (Smith 1977). In side-by-side comparisons of 2,4-D and 2,4,5-T, the more highly chlorinated 2,4,5-T generally takes longer to degrade (Alexander 1965; McCall et al. 1981; Yoshida and Castro 1975). The study by McCall in five different soil types showed 12 days on average to allow 50% decomposition of 2,4,5-T compared to 4 days for 2,4-D in the same soil. Degradation of 2,4-D and other phenoxy herbicides was shown to be biologically mediated, since little or no disappearance was seen in sterilized soil (Brown and Mitchell 1948; Hernandez and Warren 1950; Nègre et al. 1988; Yoshida and Castro 1975). While the focus of this thesis is on cleavage of the ether bond of 2,4-D, some useful information may be obtained from studies of the degradation of other phenoxy herbicides, since they contain the same

ether link connected to an aryl ring. Notably, some isolates or consortia tested are able to grow on multiple phenoxy compounds (e.g., (Ehrig et al. 1997; Golovleva et al. 1990; Hallberg et al. 1991; Hoffmann et al. 1996; Kilpi et al. 1980; Nickel et al. 1997)). The ether bond in these herbicides is also susceptible to cleavage, demonstrated by the formation of the corresponding phenols from 2,4,5-T (McCall et al. 1981; Rosenberg and Alexander 1980), fenoxaprop (Smith 1985b; Smith and Aubin 1990) dichlorprop (Ludwig et al. 1992), mecoprop (Smith 1985a), diclofop (Martens 1978; Smith 1977) and fluazifop (Nègre et al. 1993), in soil or by soil-derived mixed cultures.

The numbers of 2,4-D degrading microorganism in soils not previously treated with 2,4-D have been reported with a range over several orders of magnitude, between of 1.2/g soil to 8.8 x 10^8/g soil, and were usually lower than those in the same soil type treated with 2,4-D (Sandmann et al. 1988). Many bacteria capable of catalyzing 2,4-D degradation have been isolated, including *Achromobacter* sp, *Alcaligenes paradoxus, A. xylosoxidans, Azotobacter chroococum, Arthrobacter* sp., *Bordetella* sp., *Burkholderia cepacia*, *Flavobacterium* sp., *F. peregrinium, Mycoplana* sp., *Nocardiodes simplex*, *Nitrobacter winogradski, Pseudomonas pickettii, P. putida, P. testosteronii, Rhodoferax* and *Sphingomonas paucimobilis* (Amy et al. 1985; Balajee and Mahadevan 1990; Bollag et al. 1967; Chaudhry and Huang 1988; Fulthorpe et al. 1995; Greer et al. 1992; Gunalan and Fournier 1993; Ka et al. 1994a; Kozyreva et al. 1993; Mäe et al. 1993; Sinton et al. 1986; Smith and Aubin 1991; Steenson and Walker 1956; Walker and Newman 1956). As well, bacteria have been isolated which are capable of degrading the

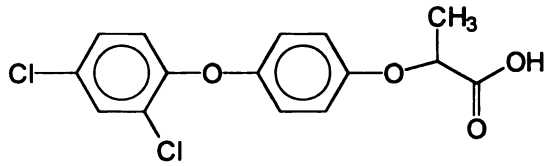


X= H 2,4-dichlorophenoxyacetic acid

2,4-D

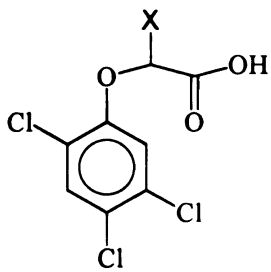
X=CH_3 2-(2,4-dichlorophenoxy)propionic acid

Dichlorprop



2-[4-(2,4-dichlorophenoxy)phenoxy]-propionic acid

Diclofop

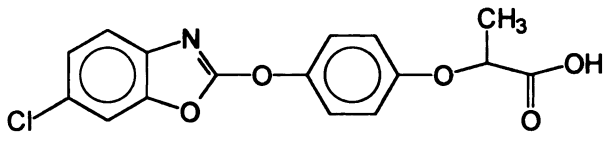


X=H 2,4,5-trichlorophenoxyacetic acid

2,4,5-T

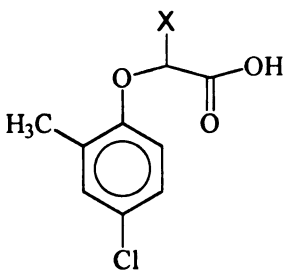
X=CH_3 2-(2,4,5-trichlorophenoxy)propionic acid

Silvex



2-[4-[(6-chloro-2-benzoxazolyl)oxy]-phenoxy)propionic acid

Fenoxaprop

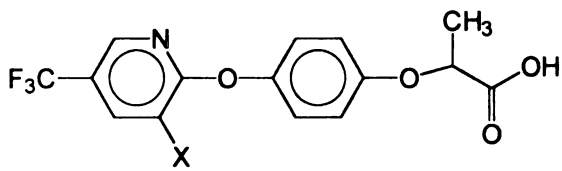


X=H 2-methyl-4-chlorophenoxyacetic acid

MCPA

X= CH_3 2-(2-methyl-4-chlorophenoxy)propionic acid

Mecoprop



X=H 2-[4-[(5-trifluoromethyl)-2-pyridinyl]oxy]-phenoxy]propionic acid

Fluazifop

X=Cl 2-[4-[(5-trifluoromethyl)-6-chloro-2-pyridinyl]oxy]-phenoxy]propionic acid

Haloxyfop

Scheme 3. Phenoxyacetic acid and phenoxypropionic acid herbicides.

methyl ester of diclofop (*Sphingomonas paucimobilis, Acinetobacter baumannii, Chryseomonas luteola, P. aureofaciens, P. cepacia* and *P. fluorescens*) (Smith-Grenier and Adkins 1996), dichlorprop (*Rhodoferax fermentans*) (Ehrig et al. 1997) and mecoprop (*Alcaligenes denitrificans* and *Sphingomonas herbicidovorans)* (Tett et al. 1994; Zipper et al. 1996).

More recently, studies have been carried out to determine the diversity of strains and pathways for 2,4-D degradation at the community level, using multiple isolates or bulk soil DNA. Taxonomic characterization of a collection of 46 isolates capable of degrading 2,4-D grouped them into five classes according to membrane composition (fatty acid methyl ester "FAME" analysis) and carbon source oxidation (BIOLOG) (Tonso et al. 1995). Hybridization of the *tfdA, tfdB* and *tfdC* genes with restriction-digested genomic DNA from 32 2,4-D degraders (mostly overlapping with the strains used in the taxonomic study above) showed a "mosaic" pattern of hybridization- that is, some hybridized to *tfdA* and *tfdB* but not *tfdC*, some to all three, some to only *tfdB*, and so on (Fulthorpe et al. 1995). The hybidizations were carried out at three different stringencies, and varying degrees of hybridization were observed to *tfdA,* including six isolates which did not hybridize at all (corresponding to less than 60% sequence identity); the non-hybridizing isolates may contain distantly related TfdA's or may use a completely different type of enzyme (i.e., not an α-KG-dependent dioxygenase). The different hybridization patterns and the variety of species among the 2,4-D degraders suggest that this metabolic capability has evolved through extensive interspecies transfer of homologous degradative genes. In support of this hypothesis, transfer of diverse plasmids

carrying genes for 2,4-D degradation has been observed from soil bacteria to a recipient strain of *R. eutropha* JMP134 which had been cured of pJP4 (Top et al. 1996). Additional evidence for non-TfdA like enzymes in 2,4-D degradation comes from studies of 2,4-D degraders in soil aggregates; the investigators obtained 68 isolates of which 25% were not stable (Vallaeys et al. 1997). The unstable 2,4-D^+ phenotype in these cases may reflect loss of the genes responsible or lack of gene expression (if the encoded enzymes have some other natural substrate and fortuitously degrade 2,4-D but are not induced by the herbicide). Of the cultures which had stable expression of the 2,4-D degradative capability, only two contained a gene similar to *tfdA* from JMP134. In another study comparing the diversity of 2,4-D degraders obtained by direct plating compared to selection in 2,4-D containing broth, 30% of the isolates from direct plating did not hybridize to the *tfdA* or *tfdB* genes of JMP134 or RASC (Dunbar et al. 1997), compared to only 2% when the isolates were obtained by enrichment. The low percentage of non-*tfd*-like pathways among the isolates obtained by enrichment suggests that this method for obtaining 2,4-D degraders (which is the technique traditionally used) underestimates their diversity.

Isolates with a fairly different *tfdA* gene from that of JMP134 can still catalyze similar reactions. *Burkholderia cepacia* RASC, an isolate identified as having a *tfdA* sequence with moderate identity (60-75%) to the corresponding gene in *R. eutropha* JMP134, has been cloned and the TfdA purified and characterized (Suwa et al. 1996). The enzyme from RASC is slightly longer (10 amino acids) than that from JMP134; however, in the overlapping region they are 80% identical in sequence. The two enzymes

are similar in requiring Fe^{2+} and α-KG, their specific activities with 2,4-D, their preferred substrates (2,4-D) and substrate range. A more extreme example is seen with *Sphingomonas paucimobilis* 1443; although this strain uses an α-KG-dependent dioxygenase to cleave the phenoxy ether bond as shown by requirement for α-KG and ferrous ion for activity in cell extracts (Sassanella et al. 1997) but is not closely genetically related as shown by lack of hybridization to *tfdA* or *clcA* even at low stringency (Fulthorpe et al. 1995; Ka et al. 1994a) and by generation of a PCR amplification product (with *tfdA*-specific primers) which cannot be aligned with *tfdA* (McGowan 1995). A gene probe for this organism (called *Spa,* to an uncharacterized target) was prepared and used on total soil DNA to show that in one of two soils tested *S. paucimobilis* or related organisms became the dominant 2,4-D degraders upon repeated applications of 2,4-D (*tfdA*-containing organisms dominated in the other soil). By contrast, at least one case is known of a completely different class of oxygenase that catalyzes formation of phenols from phenoxyacetic acid derivatives; namely, a monooxygenase is used in the degradation pathway of *B. cepacia* AC1100 (formerly *Pseudomonas cepacia*) (Kilbane et al. 1982). This organism was isolated for its ability to degrade 2,4,5-T, but resting cells and cell extracts can also degrade 2,4-D and phenoxyacetic acid (*ibid*). 2,4,5-T is converted to 2,4,5-trichlorophenol and glyoxylate by a two-component monooxygenase consisting of a reductase, specific for NADH, and a terminal oxygenase (Xun and Wagnon 1995). The latter component has two types of subunits (calculated molecular masses 51 kDa and 18 kDa) in an $\alpha_2\beta_2$ stoichiometry, with one non-heme iron and one [2Fe-2S] cluster per αβ heterodimer (Danganan et al. 1994;

Dikanov et al. 1996; Xun and Wagnon 1995). The genes encoding these subunits (termed *tftA* and *tftB*) have been cloned (Danganan et al. 1994) and the predicted amino acid sequences were found to have high homology to BenA and BenB from the benzoate 1,2-dioxygenase system of *Acinetobacter calcoaceticus,* as well as to XylX and XylY from the toluate 1,2-dioxygenase system of *P. putida.* Resting cells of *P. aeruginosa* containing the cloned *tftAB* genes were able to degrade a variety of phenoxyacetic acids to the corresponding phenols, including 2,4-D, 2,4,5-T and 4-chlorophenoxypropionate (Danganan et al. 1995). The rates of 2,4-D and 2,4,5-T conversion to the corresponding phenols by the purified enzymes was about 1000-fold slower than by the 2,4-D/α-KG dioxygenase, TfdA. Thus far, no studies have reported use of the *tft* genes to probe phenoxy herbicide degraders, so it is unclear whether "non-*tfdA*" microorganisms use this pathway or if there is yet more uncharacterized diversity in the pathway.

The evolution of 2,4-D catabolic capability is of interest as a basic research question in microbial ecology, and also for potential applications in the bioremediation of chlorinated aromatics. One of the questions relates to whether the *tfdA* gene has evolved new substrate specificity from an ancestral enzyme, which now only degrades 2,4-D and closely related phenoxy compounds, or whether it is an enzyme with a broad substrate range that acts on both naturally occurring and xenobiotic compounds *in situ.* While no native substrate has been found at present, some lines of evidence point towards TfdA having a ubiquitous natural substrate. Even in soils with no known history of 2,4-D application, enrichments capable of 2,4-D degradation have been found (Fulthorpe et al. 1996), and *tfdA* gene probes hybridize to total community DNA, albeit at low levels (Xia

et al. 1995). A study of bacterial isolates obtained from soil on non-selective (heterotrophic) medium found that 37% of them contain *tfdA*-like genes as shown by PCR amplification and hybridizaton, but none of the them were capable of 2,4-D degradation (Hogan et al. 1997), suggesting that the product of the *tfdA*-like sequence was involved in metabolizing something other than 2,4-D. One possibility is that the substrates are aromatic compounds, chlorinated or not, present naturally in the environment, such as halogenated metabolites formed by fungi and bacteria (Gribble 1992; Siuda and deBernardis 1973) or aryl-ether compounds released during fungal degradation of lignin (Ribbons 1987). Alternatively, the substrate used by microbes containing these *tfdA*-like sequences may have little structural resemblance to the phenoxy herbicides.

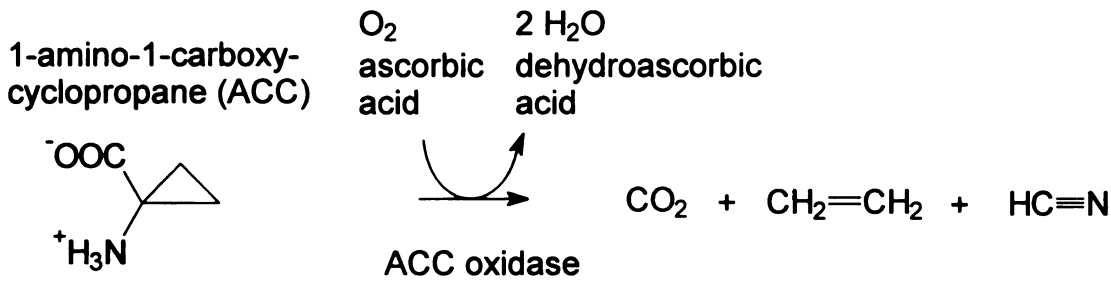
Other organisms besides bacteria are reported to metabolize 2,4-D and related herbicides, including fungi, plants, and animals. Some of the organisms carry out cleavage of the phenoxy ether bond, and knowledge of the eukaryotic enzymes involved may provide clues for the diversity of bacterial enzymes capable of this cleavage reaction. Fungi in soil are capable of 2,4-D and 2,4,5-T mineralization (i.e. conversion of the carbon in these compounds to CO_2) (Entry et al. 1993; Ryan and Bumpus 1989). The fungi *Dichomitus squalens* (Reddy et al. 1997) and *Aspergillus niger* (Shailubhai et al. 1983) metabolize phenoxyacetic acids to the corresponding phenols as the first step in degradation of these compounds; however, the enzymes carrying out these transformations have not been identified. Several vegetables, trees and weeds can mineralize the acetic acid side chain of 2,4-D or other phenoxy herbicides; however, the

rate in most plants was low (1-2% of the dose per day) and not the major metabolic pathway except in apple, garden lilac, red currant and strawberry (reviewed in (Loos 1969)). Phenol production from phenoxy herbicides has been demonstrated for strawberry (Luckwill and Lloyd-Jones 1960), bean, sunflower, corn, barley (Chaudhry and Huang 1988), sweetgum and southern red oak (Fitzgerald et al. 1967) and tulip bulbs (Topal et al. 1993). The enzyme responsible for phenol production from 2,4-D in tulip bulbs was isolated from microsomes and shown to be a cytochrome P-450. The same class of oxygenases can carry out this reaction in humans; a cloned hepatic P450 (isozyme 3A4) has also been shown to be capable of removing the side chain of 2,4-D, releasing 2,4-dichlorophenol (Mehmood et al. 1996). However, O-dealkylation is not the major metabolic route in animals; most studies of phenoxy herbicides have shown that they are excreted unchanged near quantitatively in the urine (reviewed in (Loos 1969)). In summary, in two non-bacterial organisms, the enzymes involved in phenol production from phenoxy herbicides were shown to be P-450 monooxygenases; thus this class of oxygenase is a candidate for the enzymes involved in non-*tfdA*-like phenoxy degradative pathways in bacteria. As the enzymes in other organisms are elucidated, it may provide additional candidates.

Relationship of TfdA to other α-KG-dependent dioxygenases

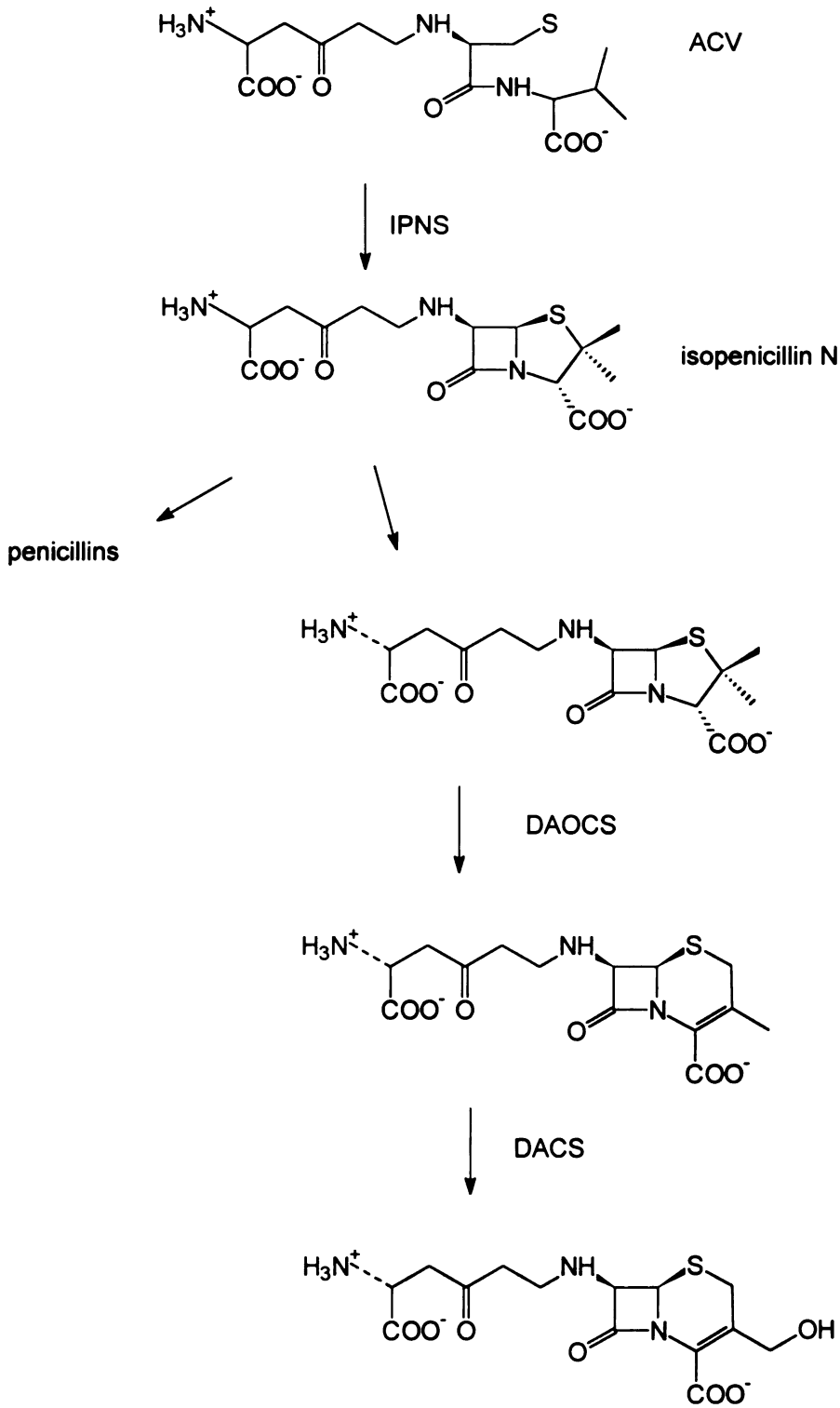
TfdA is one of a large group of enzymes sharing the requirement for dioxygen, ferrous ion, and α-KG as a cosubstrate. Activity of these enzymes is often stimulated by ascorbic acid. They act on a diverse set of substrates that includes proteins, intermediates in the synthesis of plant hormones and pigments, and precursors of certain antibiotics (for

recent reviews, see (De Carolis and De Luca 1994; Prescott 1993; Prescott and John 1996)). Most of the reactions generate a hydroxylated product, but some carry out reactions such as sequential oxidations of a methyl to a carboxyl group. The first α-KG dioxygenase identified was prolyl 4-hydroxylase, discovered in 1967 (Hutton Jr. et al.), and is often considered to be the prototype enzyme for this class. It catalyzes the hydroxylation of proline residues in procollagen to generate mature collagen. Other α-KG-dependent dioxygenases that act on amino acids, either free or in proteins, include the prolyl 3-, proline 4-, proline 3-, lysyl and aspartyl/asparaginyl β–hydroxylases (Kivirikko and Myllylä 1985; Mori et al. 1997; Onishi et al. 1984; Risteli et al. 1977; Wang et al. 1991). Additional α-KG-dependent dioxygenases act instead on nucleotides: thymine 7-hydroxylase and thymidine 2'-hydroxylase (Bankel et al. 1972; Liu et al. 1973). A number of α-KG-dependent dioxygenases are involved in synthesis of plant metabolites and hormones, including gibberelin 20-hydroxylase, hyoscyamine 6-hydroxylase, flavanone 3β-hydroxylase, desacetoxyvindoline 7-hydroxylase and flavanone synthase (Britsch 1990; Britsch et al. 1981; De Carolis et al. 1990; Hashimoto and Yamada 1987; Lange and Graebe 1989). Also in plants is 1-aminocyclopropane-1-carboxylate (ACC) oxidase, involved in producing ethylene for plant ripening (Scheme 4). While this enzyme does not use α-KG, it shares sequence similarities with several α-KG-dependent dioxygenases including flavanone 6-hydroxylase (Hamilton et al. 1990), and it requires ascorbate as a substrate (Dong et al. 1992; Smith and John 1993). α-KG-dependent dioxygenases and related ferrous non-heme enzymes are also involved in the biosynthesis



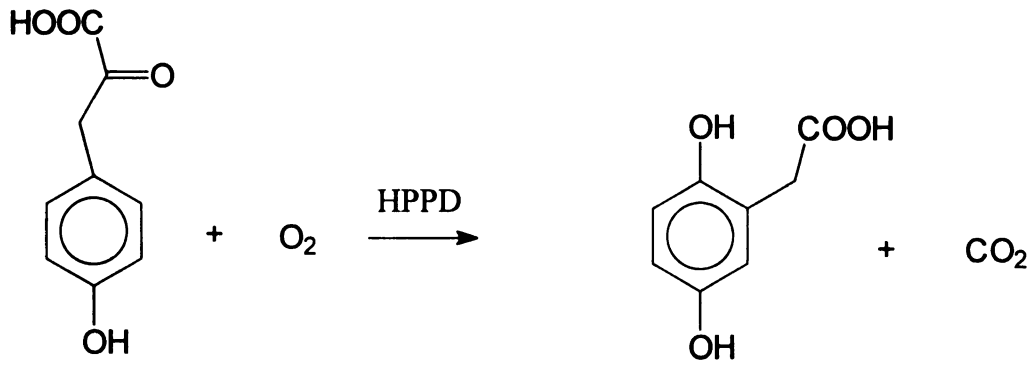
Scheme 4. Reaction catalyzed by ACC oxidase.
(Dong et al. 1992)

of bacterial antibiotic cephamycins, such as isopenicillin N synthase (IPNS), cephalosporin 7α-hydroxylase (Xiao et al. 1991), deacetylcephalosporin C synthase (DACS) (Turner et al. 1978) and deacetoxycephalosporin C synthetase (DAOCS) (Baldwin and Abraham 1988). DAOCS requires α-KG, Fe^{2+}, O_2 and a reducing agent, but does not catalyze net substrate oxygenation, rather it participates in oxidative cyclization and desaturation reactions (Scheme 5), but is usually classified together with the α-KG dependent dioxygenases. IPNS is another enzyme which does not require α-KG for activity, nor does it hydroxylate its substrate, but does require iron, O_2, and a reducing agent, and shows amino acid sequence similarity to DACS and DAOCS (Scheme 5) (Kovacevic and Miller 1991; Kovacevic et al. 1989; Perry et al. 1988). Finally, 4-hydroxyphenylpyruvate dioxygenase (HPPD) carries out a reaction formally similar to the α-KG dioxygenases in that it decarboxylates an α-keto acid and hydroxylates its substrate, in a reaction that consumes oxygen (Scheme 6; (Lindblad et al. 1970)). However, in this case, the α-keto acid is part of the substrate, rather than a separate cofactor.



Scheme 5. Reactions catalyzed by IPNS, DAOCS and DACS.

after (Prescott 1993)

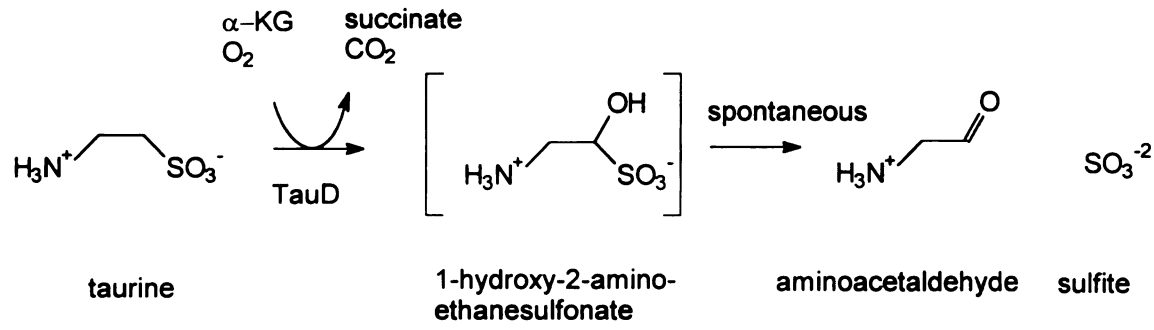


4-hydroxyphenylpyruvate homogentisate

Scheme 6. Reaction catalyzed by HPPD.
(Lindblad et al. 1977; Lindstedt et al. 1977)

The only known a-KG-dependent dioxygenase with clear sequence identity (30%) to TfdA is TauD, (van der Ploeg et al. 1996). The gene was identified on the basis of its role in taurine (2-aminoethanesulfonate) utilization as a sulfur source during sulfur starvation (ibid). The enzyme is a homodimer with subunits of 32.4 kDa (calculated), and catalyzes the cleavage of taurine to form aminoacetaldehyde and sulfite, likely through an unstable hydroxylated intermediate analogous to that in the TfdA reaction (Eichhorn et al. 1997) (Scheme 7). Two other DNA sequences have been reported that encode putative polypeptides with about 30% amino acid identity to TfdA. These open reading frames from Saccharomyces cerevisiae (SwissProt accession # Q50719) and Mycobacterium tuberculosis (SwissProt accession # Q12353) presumably also encode a-KG-dependent dioxygenases, but the gene products have not been characterized. Other than these three

known/putative a-KG-dependent dioxygenases, no reported enzymes share overall sequence similarity to TfdA.



Scheme 7. Reaction catalyzed by TauD.
(Eichhorn et al. 1997)

The lack of close sequence relationship between TfdA and other members of the α-KG-dependent dioxygenase family is not very surprising as these enzymes as a group do not display much overall sequence identity, even amongst dioxygenases acting on related substrates (Prescott 1993). For instance, within the dioxygenases working on amino acids, bovine aspartyl (asparaginyl) β-hydroxylase shows no overall significant homologies to prolyl or lysyl hydroxylase (Jia et al. 1992). Similarly, lysyl hydroxylase (an α_2 dimer) shows less than 20% overall amino acid sequence identity to prolyl 4-hydroxylase α or β subunits (Myllylä et al. 1991). As well, γ-butyrobetaine shows only 10-15% amino acid sequence identity to the subunits of lysyl 5-hydroxylase or the α and β subunits of prolyl 4-hydroxylase or aspartyl β-hydroxylase (Rüetschi et al. 1993a). Finally, comparison of the *Pseudomonas* HPPD sequence to the protein data bank showed no close relationship to characterized proteins (Rüetschi et al. 1993b). Despite this lack of overall sequence homology, selected motifs related to binding of the metallocenter can

be identified. For example, amino acid sequence comparison of 10 IPNS and 42 α-KG-dependent dioxygenases revealed the presence of a common motif, H-X-D-X_{53-57}-H (Borovok et al. 1996). Another comparison (Myllylä et al. 1992) of 10 α-KG-dependent dioxygenases and related non-heme ferrous dioxygenases revealed two short segments, His 1 and His 2, of ~9 and 25 amino acids, respectively, that appear to be conserved and provide a H-X-D-X_{54-76}-H signature (the H-X-D is part of His 1 and the lone H is the first residue of His 2). Sequences that resemble these signature motifs are also present in TfdA. An analogous motif may also be present in vertebrate α-KG-dependent enzymes (Jia et al. 1994).

TfdA is a useful model protein to study the metallocenter and mechanism for this class of enzymes. For one thing, TfdA is small and readily isolated, since it has been cloned and overexpressed in *Escherichia coli* and the purification only requires two chromatographic steps (Fukumori and Hausinger 1993a). In addition, the substrates are non-polymeric commercially available molecules. Lastly, there are several assays developed, including rapid colorimetric methods (King et al. 1991; Sassanella et al. 1997). As a prelude to my studies involving the TfdA metallocenter and mechanism, the following sections describe what is known or hypothesized about the active sites and reaction mechanism of α-KG dioxygenases.

Order of substrate binding and product release

α-KG-dependent dioxygenases and related enzymes show ordered mechanisms; i.e. substrates add sequentially to form a central complex, turnover occurs, and then products are released sequentially. Equilibrium kinetics for prolyl 4-hydroxylase are

consistent with binding of Fe^{2+}, α-KG, O_2, and the polypeptide substrate to the enzyme in this order, the binding of Fe^{2+} being at thermodynamic equilibrium (Myllylä et al. 1977). The products are released only after hydroxylation, in the order hydroxylated polypeptide, CO_2 and succinate. In the absence of peptide substrate for prolyl 4-hydroxylase, the decarboxylation of α-KG can still proceed, albeit at a lower rate, but the K_m for oxygen is an order of magnitude higher in this reaction (De Jong and Kemp 1984), suggesting that in the normal reaction, the peptide substrate binds before oxygen. Desacetoxyvindoline 4-hydroxylase has an ordered mechanism where α-KG binds first, followed by O_2 and desacetoxyvindoline (De Carolis and De Luca 1993) and then products are released in the order deacetylvindoline, CO_2, and lastly succinate. An analogous order is observed for lysyl hydroxylase, with binding of α-KG, O_2, then peptide substrate, and release of hydroxylated peptide, CO_2, and succinate in that order, although the order of hydroxylated peptide and CO_2 is not certain (Puistola et al. 1980a; Puistola et al. 1980b). In contrast, oxygen is the last substrate to bind for thymine 7-hydroxylase, after α-KG and then thymine, and the products are released in the order: CO_2, succinate and 5-hydroxymethyluracil (Holme 1975). Fe^{2+} is bound first and released last, and need not be released each catalytic cycle. Steady state kinetics of HPPD demonstrate an ordered bi bi kinetic mechanism (recall that HPPD has an internal keto-acid so it doesn't need α-KG), in which the 4-hydroxyphenylpyruvate adds prior to oxygen, and CO_2 is released prior to homogentisate (Rundgren 1977), although the data also permitted a Theorell-Chance mechanism.

Uncoupled reactions and the role of ascorbic acid

Several α-KG dioxygenases exhibit so called "uncoupled" reactions, in which α-KG is decarboxylated, but the substrate is not hydroxylated. These reactions typically occur in the absence of substrate or in the presence of an analog of the hydroxylated substrate. Lysyl and prolyl hydroxylases from chicken catalyze uncoupled decarboxylation in the absence of peptide substrate at a rate of ~4% and 1% of the coupled reaction, respectively (Puistola et al. 1980a; Tuderman et al. 1977). For algal prolyl hydroxylase, the rate is 10% (Kaska et al. 1987). The uncoupling increases in the presence of certain non-substrate polypeptides for prolyl hydroxylase (Counts et al. 1978; Rao and Adams 1978). Roughly 2/3 of the reactions of rat liver γ-butyrobetaine dioxygenase are uncoupled unless potassium ions are present, which improves the coupling so that only ~5% of the reactions are uncoupled (Wehbie et al. 1988); the mechanism of this effect is unknown and is not likely relevant for TfdA since this enzyme shows no uncoupling during 2,4-D turnover in a reaction mix that contains no potassium (Fukumori and Hausinger 1993b). When the poor substrate 5,5-dimethylhexanoic acid (hydroxylation rate 0.1% of natural substrate) was used with this enzyme, almost six decarboxylations occurred for each hydroxylation (even though potassium was present) (Ziering and Pascal Jr. 1990). By contrast, less than 1% of the uncoupled reaction was observed in the absence of substrate for γ-butyrobetaine hydroxylase from human or *Pseudomonad* sources (Holme et al. 1982). However, in the presence of product (L-carnitine), or its enantiomer (D-carnitine), the uncoupled reaction (in the absence of substrate) proceeded at 5 to 36% of the rate of decarboxylation during substrate

hydroxylation (depending on the enantiomer and the enzyme source). Uncoupled turnover is not observed for proline 4-hydroxylase in the presence of proline, but does occur in its absence at about 1% of the rate for the coupled reaction (Lawrence et al. 1996). Oddly enough, L-ascorbic acid was inhibitory to the coupled reaction at 0.5-1 mM, attributed to possible competition for the α-KG binding site and to peroxide generation (*ibid.*). The decarboxylation of α-KG is tightly coupled to substrate hydroxylation in both TfdA and TauD (Eichhorn et al. 1997; Fukumori and Hausinger 1993b).

The role of ascorbic acid in stimulating activity of these enzymes requires further comment. Initially, ascorbate was thought to be required for the prolyl hydroxylase reaction, albeit not consumed stoichiometrically (Nietfeld and Kemp 1981; Tuderman et al. 1977). The enzyme was later shown to catalyze the coupled reaction at a high rate in the absence of ascorbate, but only for a short time (>90% inactivation in one minute); activity can be partly restored by addition of ascorbate, but not of Fe^{2+} (De Jong et al. 1982; Myllylä et al. 1978). De Jong *et al* (1982) monitored the oxidation state of the enzyme using electron paramagnetic resonance (EPR) spectroscopy (Fe^{2+} is EPR silent; mononuclear Fe^{3+} is EPR active) and concluded that the iron is rapidly oxidized to the ferric state upon binding of α-KG. Furthermore, ascorbic acid was shown to reduce the iron of prolyl hydroxylase to the ferrous state. However, another study indicated that inactivation of prolyl 4-hydroxylase incubated with oxygen and α-KG could only be prevented by ascorbate, not reversed (Nietfeld and Kemp 1981). In the uncoupled reactions of prolyl and lysyl hydroxylase in the absence of peptide, ascorbic acid is consumed stoichiometrically (De Jong and Kemp 1984; Myllylä et al. 1984). Thus, the

role of ascorbic acid seems to be reduction of the oxidized (Fe^{3+} containing) enzyme during the uncoupled reaction cycles. For TfdA, proline 3-hydroxylase, and hyoscyamine 6β-hydroxylase, activity does not require ascorbic acid, although each of these enzymes is stimulated by this reductant (Fukumori and Hausinger 1993b; Hashimoto and Yamada 1987; Mori et al. 1996).

Crystallography, chemical modification and mutagenesis studies to identify key residues in catalysis

Strong evidence for the identity of likely metallocenter ligands in the α-KG-dependent dioxygenases comes from the crystal structures of IPNS. The structure of Mn(II)-IPNS (Roach et al. 1995) shows an enzyme core buried within a jelly roll motif formed by eight beta strands (Figure 1). The metal ligands are His 214, Asp 216, His 270 (note the H-X-D-X_{53}-H motif), Gln 330 and two water molecules, the latter oriented towards a hydrophobic cavity within the protein. The structure of substrate-bound Fe(II)-IPNS obtained under anaerobic conditions (Roach et al. 1997) indicates that upon binding of the substrate δ-(L-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to the enzyme, Gln 330 is replaced by the thiol group of ACV, and one of the waters is displaced by a methyl group of the substrate, leaving the iron five-coordinate. Upon addition of nitric oxide (NO, a dioxygen analog), this diatomic molecule binds to the iron (Fe-N-O angle of 120) *trans* to the sulfur-ACV ligand. Some differences from IPNS must exist in the active sites of α-KG-dependent dioxygenases because most of their substrates don't contain sulfur and they carry out a different reaction (hydroxylation rather than oxidative cyclization). No crystal structure of an α-KG-dependent dioxygenase has been reported,

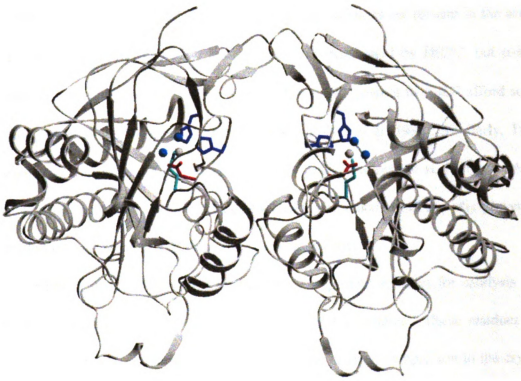


Figure 1: Ribbon structure of Mn-IPNS

Prepared using coordinates deposited to the Brookhaven Protein Data Bank (accession code 1IPS). The two His, one Asp, and one Glu ligands (stick figures) of the manganese (sphere) are shown, along with the two water ligands (spheres).

but crystals have been generated for proline 4-hydroxylase, DAOCS, and TauD (pers. comm., Peter Roach, University of Oxford, UK).

Preliminary evidence for the identity of residues involved in catalysis has come from studies with amino acid modifying reagents. Studies with the histidine-selective reagent diethylpyrocarbonate (DEPC) show inactivation of several α-KG-dependent dioxygenases, and this loss of activity can usually be prevented or slowed by the presence of substrates or competitive inhibitors, suggesting that histidines are present in the active site. In particular, prolyl and lysyl hydroxylases are inactivated by DEPC, but α-KG, ascorbate, or a reversible inhibitor that is competitive with respect to α-KG afford some protection, whereas the peptide substrates do not (Myllylä et al. 1992). Similarly, TfdA (Fukumori and Hausinger 1993b), proline 4-hydroxylase (Lawrence et al. 1996) and ACC oxidase (Zhang et al. 1995) are inactivated by DEPC and inactivation is partly prevented by inclusion of their respective substrates in the reaction mix.

Further evidence related to identification of important residues for catalysis and metallocenter binding arise from site-directed mutagenesis studies. These residues are generally chosen based on the amino acid modification studies, comparison to the crystal structure of IPNS, or by alignment of sequences for the same enzyme from different species or related enzymes. In particular, the previously mentioned H-X-D-X_{53-57}-H motif common to several α-KG-dependent dioxygenases and related enzymes has been a target for a number of studies. As an illustration of this approach, His 675 of bovine aspartyl (asparaginyl) β-hydroxylase, in a region of local homology to other α-KG-dependent dioxygenases, was selected for mutagenic studies. Mutation of this residue to Ala abolishes activity; mutation to some other residues allows some activity to remain,

the level of which is correlated with the metal-binding ability of the replacements (Jia et al. 1994; McGinnis et al. 1996). Furthermore, of 12 His, five Asp, and four Asn mutated in human lysyl hydroxylase, three His mutations and one Asp mutation inactivated the enzyme (Pirskanen et al. 1996). Three of the mutations which abolish activity alter a H-X-D-X_{49}-H sequence, and thus these three residues are thought to be involved in Fe^{2+} binding. The role of the remaining His (close to the C-terminal His of the motif) is unclear. Five His and three Cys that are conserved among prolyl hydroxylases from different species were mutated to Ser in the human enzyme (Lamberg et al. 1995); three of the His mutations abolished activity and were postulated to function as metal ligands, while two mutations of Cys abolished activity by preventing proper subunit assembly but were not thought to be part of the catalytic site. Site-directed mutagenesis of human prolyl 4-hydroxylase revealed the importance of the three key residues in a His-X-Asp-X_{58}-His motif to catalysis, attributed to their role in iron binding (Myllyharju and Kivirikko 1997). A Lys and a His ~20 amino acids towards the C-terminal from this motif were also important to activity, attributed to their function of binding the C-5 carboxyl group of α-KG and perhaps also its C-1 carboxyl (*ibid.*). Prior to crystallographic determination of IPNS, two Cys (255 and 106) were mutated in this enzyme, causing decreases but not complete loss of activity (Kriauciunas et al. 1991). In addition, mutation of His 272 causes loss of activity in IPNS (Tiow-Suan and Tan 1994), later revealed to arise from its role as a metal ligand (the numbering for the *Cephalosporium acremonium* enzyme used here correlates to His 270 in the *Aspergillus nidulans* enzyme that was crystallized). Of six histidine residues mutated in ACC

oxidase, four mutant proteins were catalytically active, whereas several amino acid replacements at two histidines (177, 234) and an aspartate (179) in the His-X-Asp-X_n-His motif resulted in inactive or low activity proteins (Zhang et al. 1997), consistent with a role of these ligands in metallocenter binding. As mentioned earlier, this His-X-Asp-X_{53-57}-His motif is seen in TfdA as well (His 113, Asp 115, His 167), and work is in progress by Deborah Hogan in the Hausinger lab to mutagenize these three presumed metallocenter ligands.

Metallocenter spectroscopy of α-KG-dependent dioxygenases and related enzymes

Only very limited spectroscopic characterization has been performed for α-KG-dependent dioxygenases. In early work, prolyl 4-hydroxylase was reported to have a small EPR signal (due to partial oxidation of Fe^{2+} to Fe^{3+}) typical of high-spin d5 ion in a rhombic environment (De Jong et al. 1982). Quite recently the Fe^{2+} center of clavaminate synthase was examined by UV/Vis, circular dichroism (CD) and magnetic CD spectroscopies (Pavel et al. 1998). The resting enzyme had a 6-coordinate ferrous center. Addition of α-KG under anaerobic conditions gives rise to altered 6-coordinate geometry and a pink color, attributed to a metal ligand charge- transfer from the iron to α-KG. Detailed analysis and comparison to models suggests that α-KG binds in a bidentate fashion (through the α-keto group and one of the carboxyl oxygens) to the metallocenter.

In contrast to the paucity of information about the metal coordination environments of the α-KG-dependent dioxygenase, several spectroscopic techniques have been used to characterize IPNS. Mössbauer spectroscopy indicates the presence of high spin Fe^{2+} that is perturbed upon anaerobic binding of the substrate ACV, consistent with

increased covalency of the coordination environment (Chen et al. 1989). The oxygen analog NO binds the EPR-silent active site to give an EPR-active species similar to those seen for the nitrosyl adducts of other Fe^{2+}-containing proteins. The signal remains but becomes more rhombic when ACV binds, indicating that it perturbs the geometry of the active site but does not displace the NO (*ibid.*). The enzyme·ACV·NO complex is pink, and by similarity to rubredoxin (a ferric enzyme with thiol ligands) the absorbance is attributed to a metal charge-transfer interaction between the thiol moiety of ACV and the iron. Cobalt- or copper-substituted IPNS shows optical spectra that are characteristic of five- or six-coordinate divalent metallocenters (Ming et al. 1990). The EPR spectrum of the copper-substituted enzyme indicates tetragonally distorted copper binding site (*ibid.*). The visible and EPR spectra show changes upon binding of ACV that are consistent with thiolate binding to the copper in Cu(II)-IPNS (*ibid.*). Proton nuclear magnetic resonance (NMR) of Fe-IPNS suggests that three imidazole ligands bind to the metal, and resonances from the metal ligands are perturbed upon addition of ACV (Ming et al. 1991). X-ray absorption spectroscopy (XAS) studies of Fe(II)-IPNS confirm that a sulfur is present close to the iron upon binding of ACV (Randall et al. 1993; Scott et al. 1992) , along with several N/O atoms. Site-directed mutagenesis of IPNS to remove both of its cysteines confirms that the sulfur ligand to the metallocenter is derived from ACV rather than an endogenous thiol (Orville et al. 1992). Electron spin echo envelope modulation (ESEEM) spectroscopy of Cu(II)-IPNS shows two equatorially bound histidines, and comparison of the spectra in D_2O and H_2O suggests that one ligand is a water which is displaced upon ACV binding (Jiang et al. 1991). Additionally, NMR studies suggest an

Asp residue is coordinated to the metal in Fe(II)-IPNS·NO (Ming et al. 1991). Finally, X-ray crystallography of IPNS showed octahedral coordination of the metallocenter by His 214, Asp 216, His 270 and Gln 330 and by two water molecules, which changed to square pyramidal geometry upon binding of ACV (anaerobically) and returned to octahedral when NO (oxygen analog) was also bound (Roach et al. 1995; Roach et al. 1997).

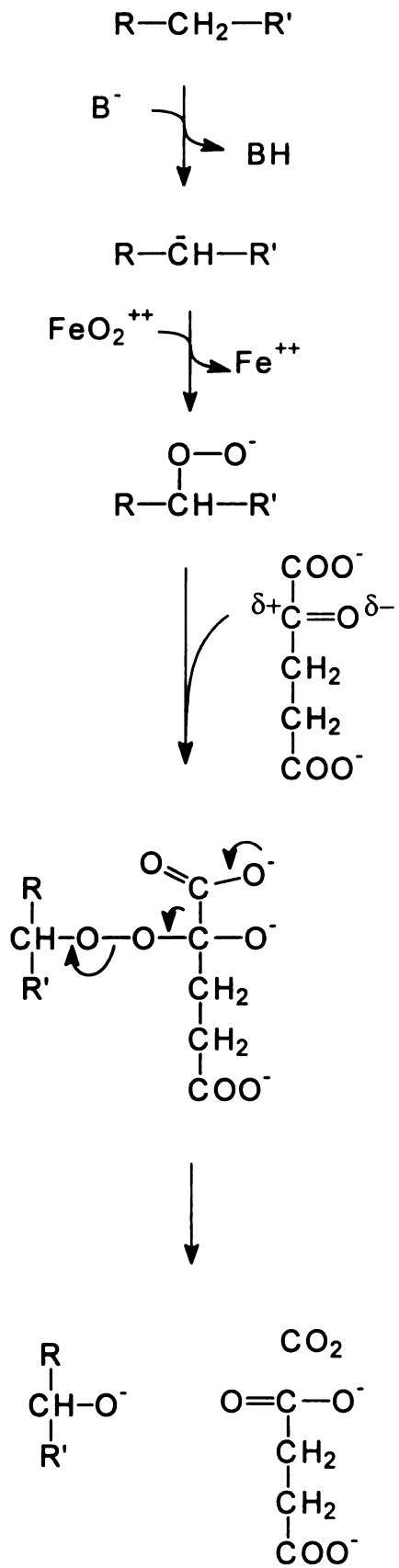
Nature of the reactive iron and oxygen-containing intermediate(s)

In the reaction catalyzed by α–KG-dependent dioxygenases, one atom of diatomic oxygen is transferred to the substrate and the other is transferred to α-KG and forms part of the product succinate. The sites of hydroxylation are typically unactivated carbons, which are not readily susceptible to electrophilic or nucleophilic attack; thus, activation of the oxygen is required. A variety of different mechanisms have been proposed for the activation of oxygen to react with the substrate and cosubstrate, and to explain the role of the iron. These can be grouped as proposals in which oxygen attacks the substrate first, or the cosubstrate first, or concerted mechanisms. Mechanistic insights for the α-KG-dependent dioxygenases and the related enzymes HPPD, ACC, and IPNS, are described in this section.

Hydroperoxy mechanism

Early workers proposed mechanisms (for γ-butyrobetaine hydroxylase and HPPD) in which an enzymatic base removes a proton from the substrate to form a carbanion, which then attacks $Fe^{2+}\cdot\cdot O{=}O$ to yield the corresponding peroxide substrate (Cardinale et al. 1971; Holme et al. 1968; Holme et al. 1982; Lindblad et al. 1969; Lindstedt and

Lindstedt 1970). In the case of α-KG-dependent dioxygenases, the peroxide would nucleophilically attack the α-keto group of α-KG, forming a peroxo bridge (Scheme 8). In this proposal, decarboxylation of α-KG is coupled to cleavage of the O-O bridge, yielding hydroxylated substrate, CO_2 and succinate. An analogous reaction was proposed for HPPD, except that the α-ketoacid forms part of the hydroxylated substrate rather than being a separate molecule (Daly and Witkop 1963). Mechanisms involving initial oxygenation of the substrate are inconsistent with the observations that α-KG can be decarboxylated by many α-KG dioxygenases in the absence of substrate, whereas substrate cannot be hydroxylated in the absence of α–KG (e.g. (Counts et al. 1978; De Jong and Kemp 1984; Holme et al. 1982; Rao and Adams 1978)). Furthermore, methylene hydrogens are not very acidic, and so it is unlikely that the enzyme would have a base sufficiently strong to remove a proton from the hydroxylatable substrate (Blanchard et al. 1982; Hamilton 1971). As well, the fact that selected HPPD substrates lacking the 4-hydroxyl group (phenylpyruvate and 4-fluorophenylpyruvate; (Taniguchi et al. 1964)) are substrates for HPPD has been used to argue against this mechanism, because these substrate cannot derive resonance stabilization from the presence of the *para* hydroxyl group. However, this last argument based on HPPD substrates may not be valid, as noted by Crouch et al. (1997) because phenylpyruvate is only a poor substrate for HPPD, and the fluoro group in 4-fluorophenylpyruvate may in fact be capable of resonance stabilization. On balance, this mechanism is not currently favored by most researchers in the field.



Scheme 8. Hydroperoxy mechanism for α-KG-dependent dioxygenases.

(Cardinale et al. 1971; Daly and Witkop 1963; Holme et al. 1968; Holme et al. 1982; Lindblad et al. 1969; Lindstedt and Lindstedt 1970)

Oxyferryl mechanism

The mechanisms cited most commonly at present involve oxidative decarboxylation of the α-keto acid first to generate an iron-bound oxygen atom by one of several possible schemes (Blanchard and Englard 1983; Crouch et al. 1997; Hanauske-Abel and Günzler 1982; Siegel 1979). For simplicity, only one of the most recent mechanisms, backed up by model compound studies (Chiou and Que 1995), is shown in Scheme 9. The oxy-ferryl species abstracts a hydrogen atom from the substrate, yielding a ferric-hydroxyl complex and a carbon-centered radical. These undergo recombination to give the hydroxylated product and the regenerated ferrous metallocenter. This mechanism is consistent with the uncoupled decarboxylation reactions noted for many of these enzymes where ascorbic acid is required to regenerate the ferrous form of the enzyme from an oxidized intermediate (De Jong et al. 1982). It is also agrees with kinetic isotope effects in thymine 7-hydroxylase which indicate that α-KG is undergoes and irreversible reaction prior to proton abstraction from the substrate (Holme 1982).

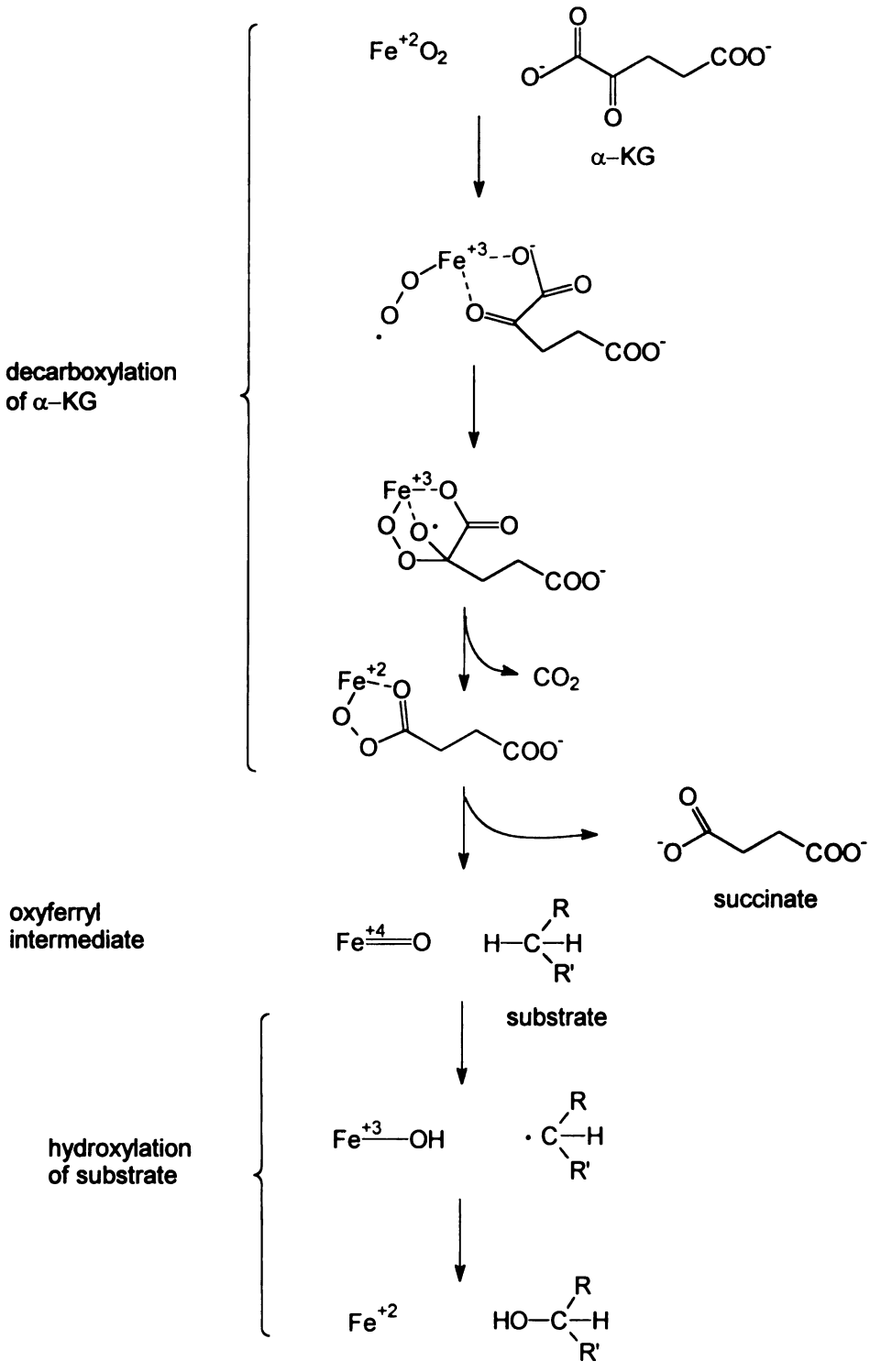
Evidences for the oxy-ferryl intermediate come from model chemistry for the hydroxylation at unactivated carbon. In the presence of iron and hydrogen peroxide, cyclohexanol and 9-hydroxynornabornane were hydroxylated, and the regio- and stereo-specificity of the products were more consistent with Fe(IV)=O being the oxidizing agent than free hydroxyl radical (Groves and Van Der Puy 1976). In a similar system using an aromatic peroxyacid plus iron to hydroxylate cyclohexanol, the stereochemistry of hydroxylation was again consistent with intermediacy of a metal oxo species involved in

"hydrogen abstraction [from the cyclohexanol], electron transfer, and nucleophilic capture of the incipient carbonium ion" (Groves and McClusky 1976).

A complex mechanism for IPNS, again involving an oxyferryl intermediate, has been proposed on the basis of studies with various substrate analogs (Blackburn et al. 1995) and further refined based on the spectroscopic evidence and crystal structure of the enzyme with and without substrate (ACV) (Roach et al. 1995; Roach et al. 1997). Because there is so much known about this enzyme and it may serve as a model for thinking about α-KG-dependent dioxygenases, the current mechanism for IPNS is illustrated in Scheme 10. An oxyferryl intermediate was also proposed recently for the mechanism of ACC oxidase (Zhang et al. 1995), but differs from other mechanisms in that one of the iron ligands is proposed to be bicarbonate. This anion was included in the mechanism because CO_2 is a required activator for the enzyme (Dong et al. 1992; Fernandez-Maculet et al. 1993; Poneleit and Dilley 1993; Smith and John 1993). Since the a-KG-dependent dioxygenases release CO2 from a-KG, they would potentially be able to generate a bicarbonate ligand.

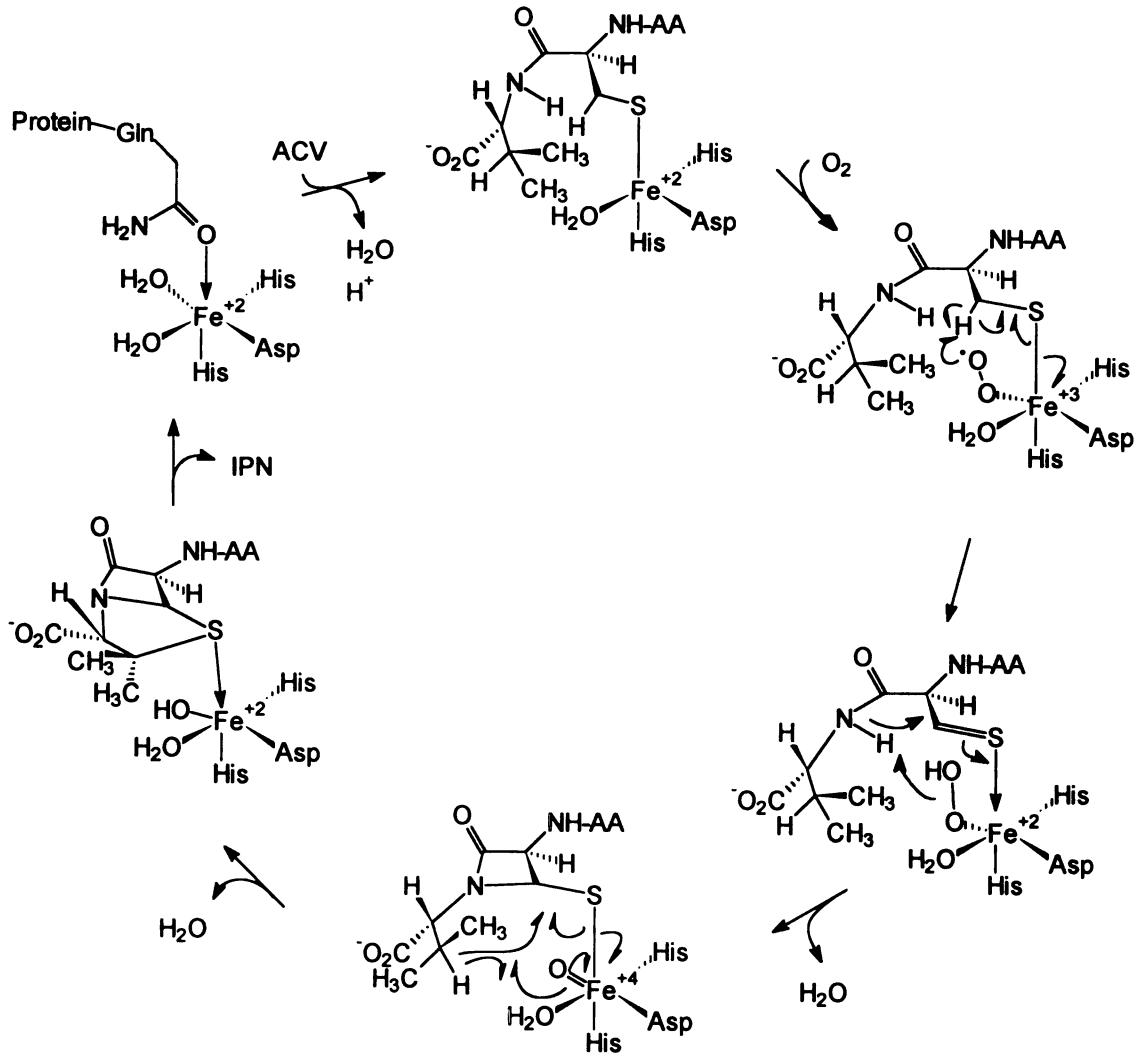
Other mechanisms

Several other mechanisms for the α-KG-dependent dioxygenases have been proposed over the years. A so-called oxenoid mechanism (Hamilton 1971) has not stood up to experimental tests that examined for its postulated peroxyacid intermediate (Cardinale and Udenfriend 1974; Counts et al. 1978; Jefford and Cadby 1981a; Tuderman et al. 1977). Another mechanism proposing iron-carbon bond formation at the α-ketoacid



Scheme 9. Oxyferryl mechanism for α-KG-dependent dioxygenases.

Based on mechanisms proposed by (Blanchard and Englard 1983; Hanauske-Abel and Günzler 1982),



Scheme 10. Oxyferryl mechanism for IPNS.

AA= L-δ-(α-aminoadipoyl) (after (Roach et al. 1997))

with subsequent insertion of dioxygen into this bond was suggested by Jefford (1992). No models for this type of chemistry are known. Finally, based on quantum considerations, Hobza (1973) proposed a mechanism that involves dioxygen coordinated side-on to iron, a geometry which is noted to be without chemical precedent by Hanauske-Abel and Günzler (1982), based on their review of extensive studies of iron-dioxygen complexes. Further, this mechanism predicts oxidation of the metallocenter to the Fe^{3+} state, necessitating stoichiometric ascorbic acid to reduce the iron, a prediction not in agreement with the observed stoichiometry of ascorbate utilization (Myllylä et al. 1978; Tuderman et al. 1977). In summary, the available evidence for this family of enzymes is most consistent with the mechanism shown in Scheme 9.

Outline of thesis

The remainder of this thesis describes my work to study the metallocenter and mechanism of TfdA, a ferrous ion- and α-KG-dependent dioxygenase that degrades the herbicide 2,4-D. Chapter 2 describes studies of TfdA activity towards an analog of 2,4-D containing sulfur in place of the ether oxygen. The initial rationale was to develop a continuous spectrophotometric assay for TfdA by reacting the released thiophenol with a thiol-reactive reagent. In addition, I considered the possibility that the sulfur might be oxidized, as has been seen with HPPD acting on a sulfur analog of its substrate (Pascal Jr. et al. 1985), and might serve as a probe of enzyme catalytic specificity. As well, I thought that the substrate or product might interact with the iron metallocenter, giving rise to a spectroscopic signal as has been seen with ACV bound to IPNS (Randall et al.

1993; Scott et al. 1992). The sulfur-containing analog was found to be a substrate for TfdA, but resulted in increased rates of inactivation of the enzyme. Of greater interest, turnover exhibited an absolute requirement for ascorbic acid, which led to further characterization of the ascorbate dependency and inactivation processes. In Chapter 3, the stereochemistry of hydroxylation of purified TfdA and of cell extracts from JMP134 and other 2,4-D degraders is determined by using high pressure liquid chromatography (HPLC) and gas chromatograph (GC) methods. For these studies, I used an analog of 2,4-D containing a methyl group to replace one of the C-2 hydrogens on the side chain, and identified which was degraded. This compound, dichlorprop, is a herbicide, and thus identification of bacterial enantiospecifity is of interest from an environmental point of view with respect to the influence of the microbial community at a particular site on the persistence of each enantiomer. Chapter 4 describes my collaborative spectroscopic characterization of TfdA by XAS and optical, EPR and ESEEM spectroscopies. These studies have provided insights into the coordination geometry and ligand identities of the metallocenter, and the changes that occur upon binding substrate and cofactor. In the last chapter I present a model of TfdA reactivity and provide some perspective on future studies.

CHAPTER 2

ASCORBIC ACID-DEPENDENT TURNOVER AND REACTIVATION OF 2,4-DICHLOROPHENOXYACETIC ACID/α-KETOGLUTARATE DIOXYGENASE USING THIOPHENOXYACETIC ACID

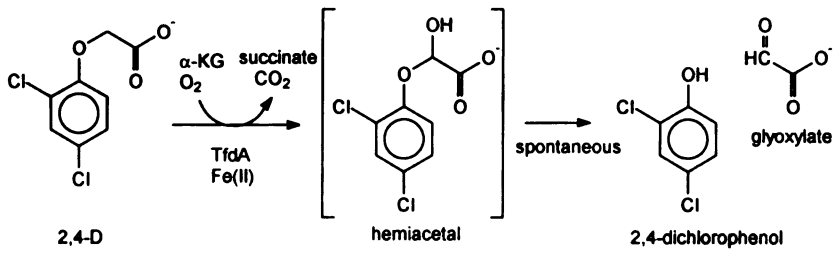
This chapter is based almost entirely on a published article of the same title: Saari, R. E., and Hausinger, R. P. (1998). " Ascorbic acid-dependent turnover and reactivation of 2,4-dichlorophenoxyacetic acid α-ketoglutarate dioxygenase using thiophenoxyacetic acid." *Biochemistry*, 37(9), 3035-3042.

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is a broad leaf herbicide that is rapidly degraded in the environment. The soil isolate *Ralstonia eutropha* (formerly *Alcaligenes eutrophus;* (Yabuuchi et al. 1995)) JMP134 carries the pJP4 plasmid which encodes a series of enzymes in the pathway for 2,4-D mineralization (Don et al. 1985). The first enzyme in the biodegradative pathway is 2,4-D/α-ketoglutarate (α-KG) dioxygenase (Fukumori and Hausinger 1993a; Fukumori and Hausinger 1993b) or TfdA (from the *tfdA* gene designation; (Streber et al. 1987)). This ferrous ion-dependent enzyme catalyzes the oxidation of 2,4-D to form 2,4-dichlorophenol and glyoxylic acid coupled with oxidative decarboxylation of α-KG to form CO_2 and succinate, as illustrated in Scheme 11. The reaction is thought to proceed via hydroxylation at the 2,4-D methylene group followed by spontaneous breakdown of the resulting hemiacetal.

Mechanistic details of the TfdA-catalyzed reaction are unknown, although there is information regarding the order of substrate and cofactor binding, the identity of some of the metallocenter ligands and the identity of additional amino acids required for activity. Electron paramagnetic resonance and electron spin echo envelope modulation studies of inactive copper-substituted TfdA provide evidence for the binding of α-KG prior to 2,4-D (Whiting et al. 1997). These spectroscopic studies also demonstrate the presence of two histidyl groups binding the metallocenter. Additional, non-ligand histidines are likely to be essential for catalysis based on enzyme inactivation studies with the histidine-reactive reagent diethylpyrocarbonate that revealed protection only by the simultaneous presence

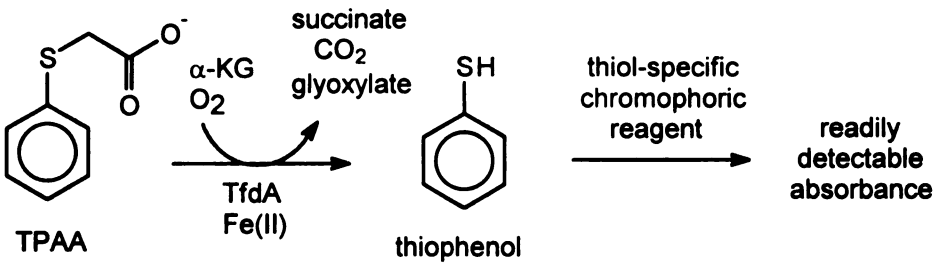
of Fe(II), α-KG, and 2,4-D (Fukumori and Hausinger 1993b). Cysteinyl residues do not participate in catalysis since thiol-reactive reagents do not inhibit TfdA. Based on the chemical modification studies, a model of the active site has been proposed involving histidine residues coordinating the iron atom and stabilizing binding of substrate and cofactor (Fukumori and Hausinger 1993b).



Scheme 11. Reaction catalyzed by TfdA

The experiments reported here focus on the interaction of TfdA with thiophenoxyacetic acid (TPAA). These studies were undertaken in an attempt to develop an alternate assay for TfdA and as a means to explore specific aspects of the enzyme mechanism. Assuming that TPAA is a substrate for TfdA, the released thiophenol could be reacted with any of a number of thiol-specific chemical reagents to provide a simple, continuous, colorimetric assay for the enzyme (Scheme 12). Such an assay may be more useful than the commonly used discontinuous colorimetric (King et al. 1991) and HPLC (Fukumori and Hausinger 1993a) assays, and may complement the recently devised continuous spectrophotometric assay for TfdA based on 4-nitrophenoxyacetic acid (Sassanella et al. 1997). The latter assay is limited in usefulness because many TfdAs, including that from *R. eutropha*, exhibit a high K_M for this substrate and it is not utilized by all TfdA-like activities. In addition to allowing the possible development of a new

assay, I reasoned that studies of thioether-substituted substrates may provide insight into the mechanism of substrate binding and hydroxylation. For example, a postulated interaction between the ether heteroatom and the metal center (Fukumori and Hausinger 1993b) may be more readily detected by selected spectroscopic methods when using the large, soft, thioether group of TPAA than for the hard, small, oxygen ether linkage found in 2,4-D. Additionally, TPAA may be a useful mechanism-based inhibitor of TfdA if the thiophenol generated by turnover were to interact tightly with the metallocenter. Finally, the regiospecificity of hydroxylation for TfdA could be examined by using the thioether substrate; i.e., in addition to hydroxylation at the methylene carbon, the enzyme could reasonably oxidize the sulfur heteroatom to the sulfone or sulfoxide. Precedent for such a change in regiospecificity is available in peptidylglycine α-amidating monooxygenase, for which substitution of a thioether for the oxyether in the substrate [(4-nitrobenzyl)-oxy]acetic acid changes the reaction from O-dealkylation to sulfoxidation (Katopodis and May 1990). As well, an analog of 4-hydroxyphenylpyruvate in which the acetate side chain was lengthened by insertion of a sulfur (thioether) bond was oxidized on the sulfur rather than on the carbon by hydroxyphenylpyruvate dioxygenase (Pascal Jr. et al. 1985).



Scheme 12. Assay of TfdA using TPAA as a substrate

I report that TPAA is a substrate for TfdA, that the thiol released from this substrate can be used in a qualitative assay for TfdA, and that the reaction mechanism observed with TPAA is distinct from that seen with 2,4-D in its requirement for ascorbic acid. Studies to characterize the differences in mechanism for the different substrates provide important new insight into the mechanism and inactivation reactions of the enzyme.

Experimental procedures

Chemicals.

Phenoxy and thiophenoxy compounds were obtained from Aldrich. α-[1-^{14}C]KG was obtained from DuPont NEN. Bovine liver catalase and bovine erythrocyte superoxide dismutase were purchased from Sigma. Other chemicals were reagent grade.

Purification of TfdA.

TfdA was purified from *Escherichia coli* DH5α (pUS311), carrying the *tfdA* gene of *R. eutrophus* JMP134, as previously described (Fukumori and Hausinger 1993a). The protein was stable for months when stored at 4°C in the buffer used in the final chromatography step (20 mM Tris, 1 mM EDTA, ~60 mM NaCl, pH 7.2). In indicated cases, the EDTA concentration was reduced by overnight dialysis of the protein (12-14,000 molecular weight cutoff, Spectra/Por, VWR Scientific) against 10 mM imidazole buffer, pH 6.75. Protein concentrations were determined by using the Bio-Rad protein assay with bovine serum albumin as the standard.

Assays of TfdA activity.

Typical assay mixes contained 50 μM $(NH_4)_2Fe(SO_4)_2$, 50 μM ascorbic acid, 1 mM α-KG, 10 mM imidazole (pH 6.75), the indicated amount of thiophenoxyacetic or phenoxyacetic acid substrate, and TfdA. Reactions were performed at 30°C. For 2,4-D colorimetric assays, reactions were quenched with EDTA (5 mM final concentration) and the levels of 2,4-dichlorophenol were determined by using the 4-aminoantipyrene method (King et al. 1991) based on a measured extinction coefficient of 15.7 $mM^{-1}cm^{-1}$. For TPAA spectroscopic assays, thiophenol levels were determined by reaction with 4,4'-dithiodipyridine (DTDP) (200 μM final concentration) to release thiopyridine which was monitored at 324 nm, using a measured extinction coefficient of 21.2 $mM^{-1}cm^{-1}$. The assay was used both for fixed time assays, in which the DTDP was added after terminating TfdA turnover by adding EDTA, and for continuous assays, in which the DTDP was added before starting turnover. In the former case, it is essential to correct for a slow, time-dependent rise in absorbance at 324 that likely arises from ascorbic acid-dependent reduction of DTDP. Alternatively, TPAA and thiophenol concentrations were determined by HPLC using a RP-18 LiChrosorb column (E.M. Separations) with a mobile phase of phosphoric acid (0.1% in water) and methanol in varying proportions, with detection at 250 nm. TfdA activity was also measured by release of $^{14}CO_2$ from α-[1-^{14}C]KG (specific activity of 6000 cpm/nmol, concentration 1 mM). One milliliter reactions in sealed vials were terminated by addition of 0.25 ml 50% trichloroacetic acid, and the released CO_2 was trapped in plastic wells (Kontes) with 0.2 ml 1 N NaOH, and counted (Beckman LS 7500). These data were corrected for 70% recovery of counts.

In selected studies, TfdA assay reactions were terminated with an equal volume of stop solution [metaphosphoric acid (10%, w/v), thiourea (1 mM), and EDTA (1 mM)] and analyzed by HPLC for remaining ascorbic acid concentrations. Samples were chromatographed on an RP-18 column (E.M. Separations or Waters Delta Pak) with a mobile phase of 50 mM potassium phosphate buffer (pH 3), and the eluent was monitored by electrochemical (Model 5200 Coulochem II, ESA Inc.) or 266 nm detection (Series 1050, Hewlett Packard).

To assess the effect of anaerobic conditions on enzyme stability, incubation mixes (1 ml in serum bottles with 9.7 ml capacity) were prepared in an anaerobic chamber and sealed with silicon/PTFE septa and aluminum crimp caps (Sun International Trading). To some of the samples, 3 ml of oxygen was added by syringe and the bottles were shaken 15 min to equilibrate. TfdA (160 μg, EDTA removed) was added by syringe and aliquots were removed at various times for enzyme assay using 2,4-D. TfdA can adsorb to the septa; thus, the bottles were not inverted while removing the TfdA samples.

To assess the effect of superoxide dismutase and catalase, various amount of these enzymes were added to TPAA turnover mixes (prior to TfdA addition). Superoxide dismutase activity was measured by inhibition of hematoxylin autooxidation (Martin Jr et al. 1987). Catalase activity was determined by following the disappearance of hydrogen peroxide at 240 nm, as described (Lück 1963) except that imidazole buffer (pH 6.75, 10 mM) was used rather than phosphate buffer for the reaction.

Synthesis of dehydroascorbic acid

Dehydroascorbic acid was prepared by oxidation of 20 mM ascorbic acid in water with bromine, followed by purging with nitrogen gas (Wells et al. 1995). The concentration of dehydroascorbic acid was verified by reaction of an aliquot (~0.1 mM final concentration) with dithiothreitol (10 mM) in phosphate buffer (pH 7, 25 mM), and observing the absorbance at 266 nm, which arises from production of ascorbic acid.

Synthesis of phenylsulfinylacetic acid and phenylsulfonylacetic acid.

Phenylsulfinylacetic acid was synthesized by oxidation of TPAA with a slight molar excess of aqueous sodium metaperiodate at 0 °C and recrystallized from ethyl acetate (Leonard and Johnson 1962). Phenylsulfonylacetic acid was synthesized by oxidation of TPAA with hydrogen peroxide at 50 °C (Srinivasan et al. 1989). Product identities were confirmed by ^{1}H-NMR in $CDCl_3$. TPAA: δ 3.7 (s, 2H); δ 7.2-7.5 (m, 5H); δ 11.3 (s, 1H). Phenylsulfinylacetic acid: δ3.8 (s, 2H); δ 7.4-7.7 (m, 5H); δ 8.5 (s, 1H). Phenylsulfonylacetic acid: δ 3.8 (d, 1 H) δ 4.0 (d, 1 H) δ 7.5-7.7 (m, 5 H), δ 13.2 (broad, s, 1 H)

Analysis for proteolysis.

TfdA (150 μg) was incubated at 30°C for 18 minutes with TPAA (10 mM) in assay mix containing 400 μM ascorbic acid. Aliquots (0.6 μg protein) were compared to samples incubated at 4°C or 30°C with imidazole buffer (10 mM) by analysis on a 12% denaturing SDS-polyacrylamide gel (Laemmli 1970).

Numerical computations

Michaelis constants were calculated by the method of Cornish-Bowden (Cornish-Bowden 1995) or by non-linear regression using KaleidaGraph (Synergy Software), using values based on initial rates (V_o). For enzyme samples that lost activity over time, the rate of product formation with TPAA as a substrate appeared to be consistent with a first order rate inactivation process, as shown in equation 1, where k_{app} is the apparent rate constant of inactivation, P_t is the product at time t, and V_t is the rate of product formation at time t.

$$dP_t/dt=V_t=V_o\, e^{-kappt} \qquad \text{(Equation 1)}$$

Integration of this equation, given the boundary condition that there is no product present at the start of the reactions, yields equation 2:

$$P_t = V_o\,(1-e^{-kappt})\,k_{app}^{-1} \qquad \text{(Equation 2)}$$

Progress curves of thiophenol production versus time were fitted to equation 2 by non-linear regression using KaleidaGraph.

Results

Demonstration that TPAA is a substrate for TfdA.

Using standard assay conditions that were optimized for 2,4-D degradation (Fukumori and Hausinger 1993b), TfdA was shown by HPLC and spectrophotometric methods to form thiophenol from TPAA. As illustrated in Figure 2, however, the concentrations of thiophenol generated from various initial concentrations of TPAA leveled off by approximately 10 min and accounted for, at most, 3% of the added

substrate. The decreases in rates of thiophenol formation as a function of time could be fit by assuming a first-order inactivation process, with inactivation rate constants of 0.22 min^{-1} for samples containing 0.1 or 1 mM TPAA, and 0.59 min^{-1} for samples containing 10 mM TPAA (however, see further discussion below). These results contrast with the linear production of 2,4-dichlorophenol from 2,4-D over this time span.

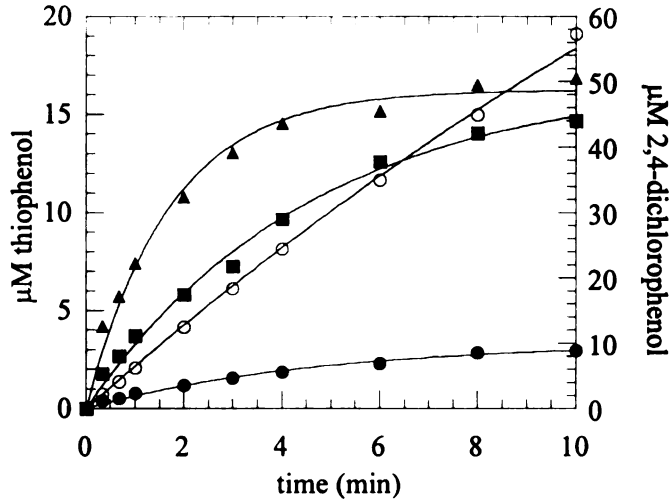


Figure 2. TfdA-catalyzed production of thiophenol from TPAA.

Thiophenol production from TPAA by TfdA (21 µg/ml) was examined in the presence of 0.1 (●), 1 (■) or 10 mM (▲) substrate using standard assay buffer conditions as described in the text. The reactions were stopped at the indicated times with EDTA, and the amounts of product thiophenol were determined after reaction with DTDP. The data were fit to equation 2 as described in the methods, and gave apparent inactivation rate constants of 0.22 ± 0.02 min^{-1}, 0.22 ± 0.02 min^{-1}, and 0.59 ± 0.02 min^{-1} respectively. For comparison, 2,4-dichlorophenol production from TfdA (0.42 µg/ml) was examined in the presence of 0.3 mM 2,4-D (○). The reactions were stopped at the indicated times with EDTA, and the levels of product 2,4-dichlorophenol were determined after reaction with 4-aminoantipyrene. Each point represents the average of three determinations.

The inactivation observed during thiophenol production was not due to product inhibition. For example, no significant inhibition of TPAA decomposition was observed

when the reaction was examined in the presence of 30 μM thiophenol. Furthermore, the possibility was discounted that alternate oxidation products, such as the phenylsulfinylacetic acid or phenylsulfonylacetic acid, were formed and subsequently acted as inhibitors. In particular, alternative reaction products with absorbance at 250 nm were not detected during HPLC analyses and authentic samples of the sulfur-oxidized compounds failed to act as effective inhibitors of the reaction (data not shown).

Thiophenol production from TPAA requires and consumes ascorbic acid.

Since the linearity of 2,4-D degradation by TfdA previously was shown to be somewhat dependent on the presence of ascorbic acid (Fukumori and Hausinger 1993b), the ascorbic acid dependency of TPAA utilization was examined. As illustrated in Figure 3A, TfdA-catalyzed formation of thiophenol from TPAA exhibited an absolute dependence on the presence of ascorbic acid. This result contrasts with conversion of 2,4-D to 2,4-dichlorophenol where ascorbic acid is not required for activity. Thiophenol formation by TfdA exhibited an optimal ascorbate concentration of ~ 200 μM, well above the 50 μM concentration used in the routine assay for 2,4-D. For all ascorbic acid concentrations, however, the activity decayed in a pseudo-first-order process (Figure 3B). At ascorbate concentrations below ~100 μM, the apparent inactivation rate was elevated and variable, while higher concentrations yielded a more uniform values of approximately 0.16 min^{-1}. Initial rates of thiophenol production appeared to reach a plateau between 200 to 800 μM ascorbic acid, with half-maximal rates observed at about 50 μM levels (Figure 3C).

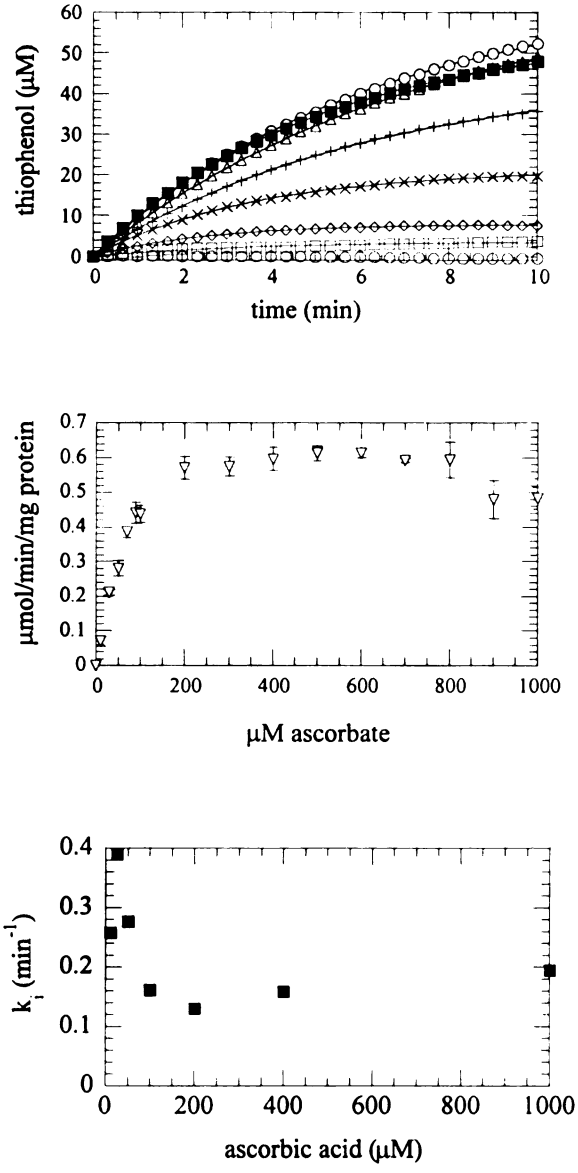


Figure 3. Effect of ascorbic acid concentration on the production of thiophenol from TPAA by TfdA.

A, Progress curves for thiophenol production for varied ascorbic acid levels. TfdA (36 μg/ml) was incubated in the presence of 10 mM TPAA and assay buffer containing ascorbic acid at 0 (○), 10 (□), 25 (◇), 50 (×), 100 (+), 200 (Δ), 400 (●) and 1000 μM (■) concentrations. Thiophenol production was monitored continuously at 324 nm, and fit to equation 2. B, Effect of ascorbic acid concentration on the apparent inactivation rates of TfdA during turnover of TPAA. C, Effect of ascorbic acid concentration on the initial rate of thiophenol production from TPAA (10 mM) by TfdA (20 μg ml^{-1}). The data represent the average of two determinations.

HPLC methods were used to demonstrate that ascorbic acid was consumed during the TfdA-catalyzed reaction with TPAA. For example, comparison of ascorbic acid consumption with thiophenol production at selected timepoints during reactions using a wide range of reductant concentrations indicated that 1.55 ± 0.09 moles of ascorbic acid were used per mole of product generated (Figure 4). Under TPAA turnover conditions (i.e., the presence of enzyme, Fe(II), a-KG, and this aromatic substrate) over 60% of the reductant (initially 200 μM) was lost during a 10 min time interval (see Table 1). For comparison, a decrease of only about 10% of the ascorbic acid was observed under conditions in which 2,4-D was used as a substrate. The extent of ascorbic acid loss during 2,4-D turnover was less than that observed when the enzyme was incubated in the presence of Fe(II) alone, Fe(II) plus a-KG, or Fe(II) plus TPAA (~30% consumption in each case).

In light of the demonstration that ascorbate is consumed during the reaction, portions of the data shown in Figure 2 and Figure 3 may be reinterpreted. The apparent inactivation rates calculated for the 10 mM TPAA sample in Figure 2 and the < 100 μM data in Figure 3B may represent a summation of the actual inactivation rate (0.12 to 0.22 min^{-1}) and activity loss due to ascorbate depletion. Restated, it was reasonable to suspect that low concentrations of ascorbic acid may limit the extent of the reaction. Evidence supporting this conjecture was derived from continuous spectrophotometric assay supplementation studies. Using a variation of the experiment shown in Figure 3A, TfdA was reacted with TPAA in the presence of Fe(II), α-KG, and buffer containing 50 or 400 μM ascorbic acid, and at selected timepoints the mixtures were supplemented with

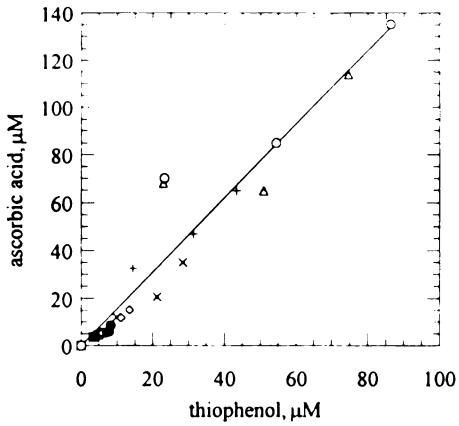


Figure 4. Stoichiometry of thiophenol production and ascorbate consumption during TPAA turnover.

TfdA (40 μg/ml) was incubated in 10 mM TPAA assay mix with, 10 (□), 15 (○), 25 (◇), 50 (×), 100 (+), 200 (Δ) or 400 (●) μM ascorbic acid and the reactions were monitored continuously at 324 nm. Replicate aliquots were stopped at various times and the levels of ascorbate consumed were calculated based on the concentrations of ascorbic acid remaining as assayed by HPLC. The Fe(II) concentration was lowered to 10 μM to reduce background consumption of ascorbic acid, and the data were corrected to account for non-enzymatic losses.

Table 1. Consumption of ascorbic acid by TfdA.

Reaction Components[a]	Ascorbic acid consumption[b]	
	μM	%
blank	-16 ± 25	-8
Fe	63 ± 8	32
Fe + α-KG	57 ± 15	28
Fe + TPAA	60 ± 14	30
Fe + α-KG + TPAA (turnover)	127 ± 10	64
Fe + α-KG + 2,4-D (turnover)	21 ± 16	10

[a] All mixes had imidazole (10 mM, pH 6.75) and 200 μM ascorbic acid. When present, the other concentrations were : Fe(II), 50 μM; TPAA, 10 mM; α-KG, 1 mM; 2,4-D, 1 mM, TfdA 36 μg/ml

[b]The levels of ascorbic acid remaining after 10 min reaction were measured by HPLC. The differences from the initial concentration were calculated and corrected for the losses in the absence of TfdA, which averaged 7 μM . The values shown represent mean ± standard deviation for triplicate samples.

400 μM additional ascorbic acid (Figure 5). The supplementation led to a burst of product formation at each time point in samples that originally contained 50 μM ascorbic acid; however, no effect was noted for samples that initially possessed 400 μM reductant. The rates observed immediately after adding supplemental ascorbate to the 50 μM samples appeared to parallel the rates at that timepoint for sample to which 400 μM reductant was added at the start of the reaction. Further studies were performed to distinguish whether the burst of activity upon supplementation was due to re-activation of TfdA or to some effect of ascorbate that was specific to TPAA turnover; i.e. the effect of ascorbic acid supplementation was examined using the substrate 2,4-D. Aliquots of enzyme from the TPAA turnover mix with 50 μM ascorbic acid were assayed for their activity towards 2,4-D mix immediately prior to or after supplementation of the TPAA mix by 400 μM ascorbic acid. The observed ~ 3-fold enhancement in activity towards 2,4-D upon adding supplemental ascorbic acid (data not shown) was consistent with the presence of an ascorbate-reversible inactivation process.

Because ascorbic acid was consumed in the TfdA-catalyzed decomposition of TPAA, it was important to test whether the ascorbate-derived product had an effect on this reaction. Ascorbic acid is known to be oxidized to ascorbyl radical, which disproportionates to ascorbate and dehydroascorbic acid (Bielski et al. 1975). Based on its ability to react with thiols such as glutathione (Winkler 1987), dehydroascorbic acid could reasonably react with thiophenol to interfere with the spectrophotometric assay. This concern was negated by the demonstration that addition of authentic dehydroascorbic acid in concentrations up to 400 μM did not affect the thiophenol

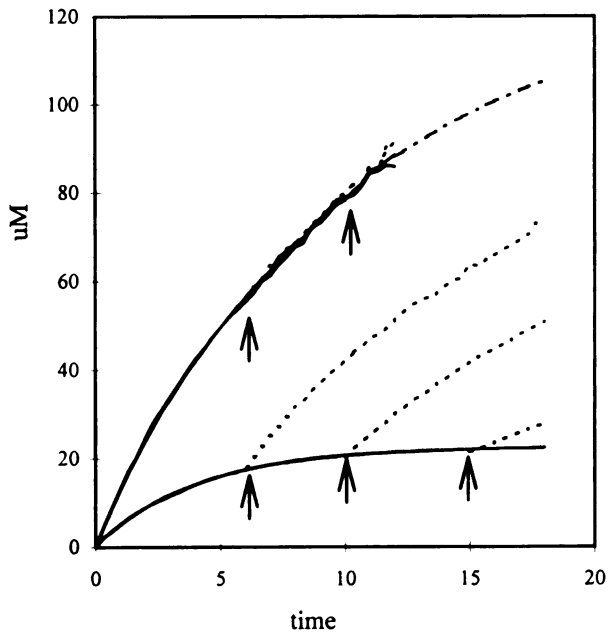


Figure 5. Effect of supplemental ascorbic acid on progress curves for the TfdA-catalyzed decomposition of TPAA.

TfdA (40 μg/ml) was reacted with 10 mM TPAA in the presence of 50 μM (lower curves) or 400 μM (upper curves) ascorbic acid initially, and at the times indicated by the arrows, the reaction mixtures were supplemented with an additional 400 μM ascorbic acid (dashed lines). The reactions were monitored continuously at 324 nm. Three independent time courses were averaged for each experiment and normalized to their rate at 5 min. The curve for 400 μM ascorbic acid was extrapolated from 13 to 18 minutes (dot-dash line) because the absorbance readings were above the linear range.

standard curve at the pH of the assay. Another potential complication from dehydroascorbic acid is that it might react with arginine residues in the protein, since α,β-diketones such as glyoxal and 2,3-butanediones are known to form a complex with this amino acid residue (Yankeelov Jr 1972). Interactions between dehydroascorbic acid and TfdA do not appear to be a major source of inactivation, since dehydroascorbic acid (400 μM) was only slightly inhibitory to TfdA turnover of TPAA (7 ± 1 % rate decrease).

α-KG decomposition is not uncoupled from substrate hydroxylation.

The demonstration that ascorbic acid was consumed during the TfdA-catalyzed hydroxylation of TPAA raised the possibility that α-KG decomposition may be partially uncoupled from substrate hydroxylation. Such a situation is known to occur for several other α-KG-dependent dioxygenases, especially when using poor substrates. For example, in addition to carrying out its normal reaction prolyl hydroxylase catalyzes the oxidative conversion of α-KG to CO_2 plus succinate in the absence of substrate hydroxylation (Counts et al. 1978; De Jong and Kemp 1984; Myllylä et al. 1984; Rao and Adams 1978; Tuderman et al. 1977). This uncoupled reaction in prolyl hydroxylase leaves the enzyme metallocenter in an oxidized state that requires the presence of ascorbic acid to restore the catalytically active species. The consumption of ascorbic acid in the reaction of TfdA with TPAA could plausibly arise from a similar uncoupling process.

To assess the extent of uncoupling of α-KG decomposition from substrate hydroxylation in the TfdA-catalyzed reaction with TPAA, the levels of CO_2 produced or

α-KG consumed were compared to the amount of thiophenol generated. Using ^{14}C-labeled cofactor in the assay and monitoring the formation of both ^{14}C-CO_2 and thiophenol, the ratio of CO_2 released to thiophenol produced by TfdA (54 μg/ml) was shown to be 1.1 ± 0.3 for a range of ascorbic acid concentrations (10 to 400 μM). To compare product formation with α-KG consumption, reactions were run with limiting concentrations of α-KG and plentiful ascorbic acid (400 μM) while measuring the total amount of thiol released; a ratio of 1.07 ± 0.03 moles of α-KG consumed per mole of thiophenol produced was established. These data are inconsistent with the presence of extensive uncoupling of α-KG decomposition from TPAA hydroxylation.

Oxidative inactivation of TfdA.

The non-linear reaction kinetics observed when using TPAA led us to carry out a detailed analysis of TfdA inactivation rates under a variety of conditions. When the enzyme was incubated in assay buffer (including 400 μM ascorbate) that lacked TPAA, the enzyme was found to lose activity with pseudo-first-order kinetics at a rate of 0.50 ± 0.03 min^{-1} (determined by measuring initial rates of thiophenol production upon addition of TPAA to the incubation mixtures at varying times). Analysis of the rate of inactivation for another aliquot of the same TfdA sample incubated in the same buffer conditions, but with 10 mM TPAA included at the start of the reaction (i.e., during turnover conditions), yielded a value of 0.42 ± 0.03 min^{-1}. (The exact inactivation rates were found to depend on the TfdA preparation, with values for other protein samples ranging from 0.2 to 0.4 min^{-1} during TPAA turnover). Thus, TPAA offered little to no protection from enzyme inactivation, and increasing the TPAA concentration to as high

as 32 mM did not offer improved protection from inactivation. By contrast, inclusion of 1 mM 2,4-D in the buffer gave rise to negligible rates of enzyme inactivation (Fig. 1; k_i = 0.04 ± 0.01 min^{-1} when the 2,4-D data were fit to Equation 2). These data provide evidence for the presence of an apparently irreversible enzyme inactivation process for incubation mixtures containing enzyme or enzyme plus TPAA, but not for enzyme plus 2,4-D. Further results demonstrated that oxygen was involved in the inactivation process. Specifically, TfdA inactivation rates were compared for samples that were incubated in anaerobic and aerobic reaction mixes and assayed for activity towards 2,4-D. After a 20 min incubation period oxygenated samples containing and lacking TPAA possessed only 4% and 21%, respectively, of the activity of the corresponding samples incubated anaerobically.

The oxidative inactivation process was more fully characterized to examine the identity of the oxygen species that was responsible. Inclusion of catalase at 200 μg ml^{-1} in the assay (capable of removing 90 μmol H_2O_2/min) failed to protect the enzyme against inactivation during TPAA turnover (data not shown). These results suggested that free hydrogen peroxide was unlikely to be the agent responsible for inactivation, although addition of 30 mM H_2O_2 to the assay mixture abolished TfdA activity. Inclusion of superoxide dismutase (100 or 370 U) also failed to protect the enzyme against inactivation. An alternative active oxygen species that could play a role in TfdA inactivation was the hydroxyl radical. Addition of hydroxyl radical scavengers (DMSO, formate, glucose, mannitol, thiourea; 0.1 to 10 mM) failed to abolish or significantly diminish the inactivation process (data not shown). The results preclude the involvement

of free hydroxyl radicals, but do not rule out the possibility of a protein-associated species.

Because hydroxyl radicals are known to be formed from Fe-EDTA complexes in the presence of ascorbic acid (Udenfriend et al. 1954), I tested whether the EDTA concentration in the assay mixture had an effect on the inactivation rate. Enzyme samples were rigorously purified of the trace levels of EDTA present from enzyme storage, and the inactivation rates were determined for varied concentrations of Fe(II) and EDTA (Figure 6). The rates of inactivation increased from a value of 0.1 min^{-1} in the absence of EDTA to double this value as the EDTA concentration approached that of Fe(II). When the EDTA concentration exceeded the concentration of Fe(II), no activity was detected due to the complete sequestration of the metal ion. The enzyme was stable under high EDTA conditions, as shown by restoration of activity after removal of EDTA. These results are consistent with the participation of hydroxyl radicals during EDTA-dependent inactivation, perhaps involving a TfdA·Fe(II)·EDTA complex.

Protein fragmentation has been associated with oxidative inactivation of other enzymes such as aminocyclopropane carboxylate oxidase (Barlow et al. 1997); thus, the stability of TfdA during inactivation was assessed. Following 18 min of incubation under conditions of TPAA turnover, or control experiments, TfdA samples were examined for fragmentation by denaturing polyacrylamide gel electrophoresis. The turnover conditions were conservatively estimated to lead to at least 80% loss of activity towards TPAA; however, no significant decreases in the band on the gel for TfdA and no significant increases in other bands were observed (data not shown).

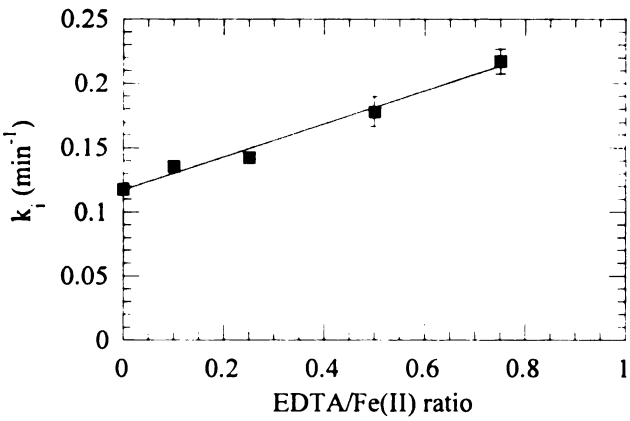


Figure 6. Effect of the EDTA to Fe(II) ratio on the TfdA inactivation rate.

TfdA (20 μg/ml) was reacted with 10 mM TPAA in assay buffer containing 400 μM ascorbic acid and various concentrations of EDTA and Fe(II). The reactions were monitored continuously at 324 nm, and the enzyme inactivation rates were calculated using Equation 2. At EDTA/Fe(II) ratios greater than 1, the enzyme was inactive. The data represent studies using Fe(II) concentrations ranging from 10 to 300 μM.

Kinetic constants for TfdA acting on thiophenoxyacetic acids and phenoxyacetic acids.

Using optimal ascorbic acid in the reaction assays (400 μM for the two sulfur-containing compounds; 50 μM for the others) and examining only the initial rates of product formation, kinetic constants were assessed for TPAA, 4-chlorothiophenoxyacetic acid, 2,4-D, 4-chlorophenoxyacetic acid, and phenoxyacetic acid (Table 2; 2,4-dichlorothiophenoxyacetic acid is not commercially available and was not tested). The K_M values for the thioether compounds were 3- and 9-fold greater than those of the corresponding oxy-ether forms. In both sets of compounds, TfdA exhibited greater affinity for the more highly chlorinated species. Substitution of the ether oxygen in the phenoxyacetates by a sulfur atom reduced the k_{cat} by 13 and 22-fold relative to the

corresponding oxy-ether forms. Thus, the catalytic efficiency of TfdA toward the thiophenoxyacetic acids was greatly reduced (700- to 2,400-fold) compared to the benchmark substrate, 2,4-D. Both thiophenoxyacetic acids tested exhibited the rapid inactivation reaction as detailed for TPAA, while the three phenoxyacetic acids resulted in linear kinetics over the time course examined.

Table 2. Kinetics values for TfdA substrates

Substrate[a]	K_m (mM)	k_{cat} (min^{-1})
TPAA	2.4 ± 0.2	29 ± 1
4-chlorothiophenoxyacetic acid	1.1 ± 0.3	45 ± 2
phenoxyacetic acid	0.74 ± 0.10	647 ± 42
4-chlorophenoxyacetic acid[b]	0.117 ± 0.006	595 ± 15
2,4-D	0.030 ± 0.003	587 ± 23

[a] Ascorbate concentrations were 400 μM for thioether substrates and 50 μM for ether substrates. Values for the thioether substrates were based on initial rates that were calculated using Equation 2.

[b] Values were previously reported (Fukumori and Hausinger, 1993b).

Discussion

TPAA-based assay for TfdA.

TPAA is a substrate for TfdA, and is converted to thiophenol as the sole aromatic product. The release of this thiol allows for use of TPAA in a qualitative, short-term continuous assay for the enzyme. I have chosen to utilize DTDP to monitor released thiol in this assay because the released thiopyridine chromophore exhibits a pH-independent spectrum near pH 7 (Brocklehurst and Little 1973); however, dithiodinitrobenzoate (Ellman's reagent) or other thiol-specific reagents can also be used. It is important to include high concentrations of ascorbic acid in the assay, and the assay is not suitable for quantitative measurements because of the observed rapid inactivation of the enzyme.

Model to explain ascorbic acid dependency and enzyme inactivation during TPAA utilization.

Results from studies to characterize the dependence of ascorbic acid on TPAA degradation and to elucidate the features associated with TfdA inactivation have been integrated into the model illustrated in Figure 7. Significantly, this model is distinct from that involving an uncoupling of α-KG decomposition from substrate hydroxylation, which has been used to explain the requirement for ascorbic acid in several other α-KG-dependent dioxygenases (e.g., (Holme and Lindstedt 1982; Myllylä et al. 1984)). In TfdA, oxidative decarboxylation of α-KG is tightly coupled to hydroxylation of substrate. My model suggests that selected states of TfdA can undergo two distinct oxidative inactivation processes, one of which is reversed by ascorbic acid.

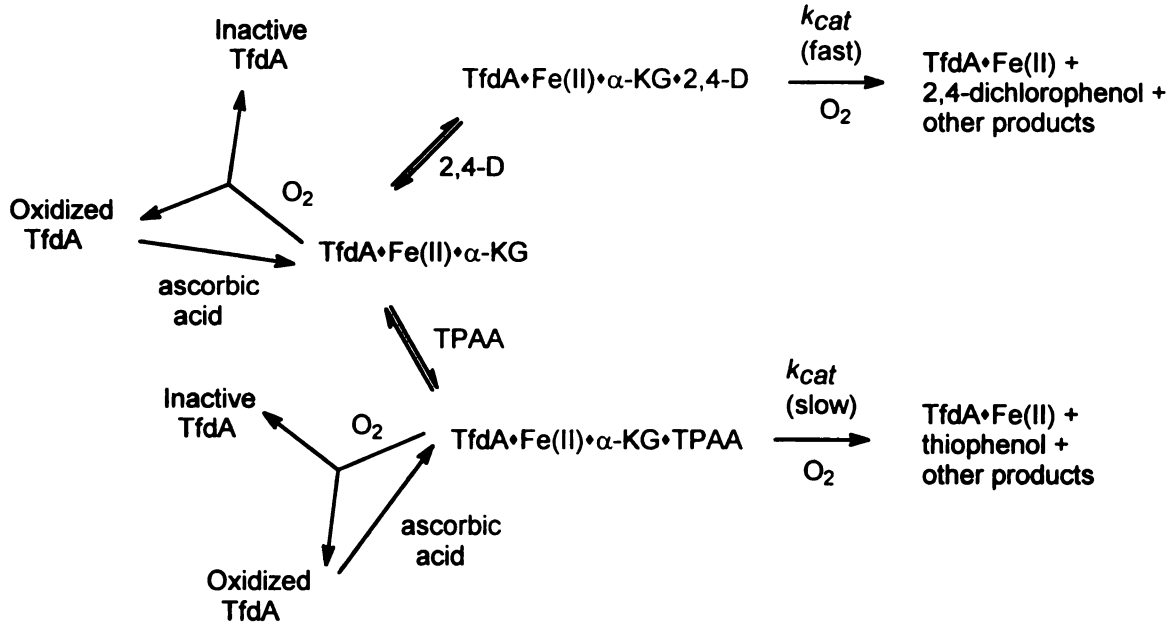


Figure 7. Model of TfdA reactivity with 2,4-D and TPAA.

The active form of the enzyme is shown as the species with bound Fe(II) and α-KG. Spectroscopic studies have provided evidence consistent with the existence of this species (Fukumori and Hausinger, 1993b). This species is suggested to be inactivated in the presence of oxygen via a mechanism that forms an irreversibly inactive species and an ascorbic acid-reversible form of the protein. TfdA binds 2,4-D with high affinity and rapidly catalyzes the decomposition of the substrate. The TfdA·Fe(II)·α-KG·2,4-D species is proposed to be protected from the oxidative inactivation processes. TfdA binds TPAA with lower affinity and catalyzes its decomposition at a reduced rate compared to 2,4-D. The TfdA·Fe(II)·α-KG·TPAA state of the enzyme is capable of undergoing both types of oxidative inactivation reactions

In the absence of any aromatic substrate, the TfdA·Fe(II)·α-KG complex is proposed to react with oxygen resulting in one inactive species that is reversible by ascorbic acid and another species that is irreversibly inactivated. In the presence of 1 mM 2,4-D (a substrate possessing a very low K_M), the enzyme is primarily in the form of the TfdA·Fe(II)·α-KG·2,4-D complex which is stable against the oxidative inactivation reactions, and is rapidly and efficiently converted to products. Ascorbic acid

consumption is greatly reduced in the presence of 2,4-D, compared to the enzyme lacking substrate. By contrast, the rates of ascorbic acid consumption in the presence of TPAA, a poor substrate with a high K_m and low k_{cat} relative to 2,4-D, are twice that of the substrate-free enzyme, suggesting that the TfdA·Fe(II)·α-KG·TPAA complex may be especially susceptible to oxidation. As for substrate-free enzymes, the oxidative reaction partitions between an irreversible state and an inactive state that can be reversed by ascorbate. In the absence of ascorbic acid and the presence of TPAA, the enzyme should be competent to catalyze very limited substrate hydroxylation, but it rapidly undergoes both inactivation processes. The net effect is that TfdA catalyzes very few turnovers before it is completely inactivated. Given the low concentration of enzyme in the assay mixtures, the level of product generated before losing all activity is too low to be detected. A portion of the inactive TfdA can be restored to activity by subsequent addition of ascorbate.

Several aspects of this model are consistent with known iron chemistry. For example, the ascorbate-reversible inactive state(s) of the enzyme may represent Fe(III) species arising from one-electron transfer to oxygen; Fe(III) is readily reduced to Fe(II) by ascorbic acid (Taqui-Khan and Martell 1968). The irreversibly inactivated enzyme species may arise from reaction of the protein with hydroxyl radicals (Berlett and Stadtman 1997) generated via the Fenton reaction:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO^\bullet \qquad \text{(Equation 3)}$$

Hydrogen peroxide may be formed in the TfdA assay mix by the autooxidation of ascorbic acid (Weissberger et al. 1943) or by the dismutation of superoxide formed by

reduction of molecular oxygen by Fe(II) . I note, however, that catalase, superoxide dismutase or hydroxyl radical scavengers failed to protect the enzyme from inactivation, indicating the absence of free H_2O_2, $O_2^{\bullet-}$, and $HO^{\bullet}$. Rather, the inactivating species is likely to be protein associated.

The presence of EDTA is known to enhance the production of hydroxyl radicals or species with similar chemical and kinetic properties by Fenton-type reactions (reviewed in (Goldstein et al. 1993)). Consistent with this, increasing the EDTA/Fe(II) ratio present in the assay (up to a 1:1 ratio) roughly doubled the rate of enzyme inactivation (Figure 6) possibly due to formation of a TfdA·Fe(II)·EDTA complex. In a typical TPAA assay, the amount of EDTA present from enzyme storage corresponded to a ratio of 0.2 EDTA/Fe, so by comparison with Figure 6, less than 20% of the inactivation observed in the typical assays is an artifact due to the presence of EDTA. The postulated protein damage arising from hydroxyl radicals does not appear to give rise to significant protein fragmentation.

What accounts for the differences in TfdA reactivity with 2,4-D and TPAA?

Substitution of the ether oxygen of phenoxyacetates with a thioether atom found in the thiophenoxyacetates results in significant changes in TfdA reactivity toward these compounds (Table 2). The enzyme exhibits 5-fold higher affinity for phenoxyacetic acid over TPAA and more than 10-fold greater affinity for 4-chlorophenoxyacetic acid over 4-chlorothiophenoxyacetic acid, demonstrating that the heteroatom identity influences binding of the substrate. Additionally, the presence of a thioether adjacent to the methylene C-H bond undergoing oxidation reduces the catalytic rate in the range of 13 to

22-fold. The decreased rate is unlikely to arise from changes in the C-H bond strength. For example, the C-H bond strengths in the model compounds methanol and thiomethane are within a few percent of each other (401.9 ± 0.6 and 393 ± 8 kJ mol-1 respectively; (Berkowitz et al. 1994)). Rather, the rate effects are likely to arise from subtle changes due to sulfur being larger and more polarizable than oxygen. For example, the precise docking of substrate to enzyme will be affected by the heteroatom identity (especially if this heteroatom interacts with the enzyme metallocenter as speculated earlier; (Fukumori and Hausinger 1993b)) thus affecting the position and geometry of the C-H bond undergoing oxidative chemistry relative to the metal-bound oxygen atom that is inserted. A positional shift such as this may also explain the high reactivity of the TfdA·Fe(II)·α-KG·TPAA complex toward the oxidative inactivation reactions described above. Alternative explanations for the reduced reactivity of TfdA for thiophenoxyacetic acids versus phenoxyacetic acids could include stabilization of an intermediate carbon-centered radical by the adjacent sulfur atom or influences on oxidative addition by carbon-heteroatom bond polarization.

The studies reported here may provide useful insights for catalytic mechanisms and inactivation reactions of other α-ketoglutarate-dependent dioxygenases. Ascorbic acid requirement for turnover, especially turnover of poor substrates, should not automatically be attributed to uncoupling of the reaction. It is ironic that the very things that assist in TfdA turnover- iron, oxygen, ascorbic acid- can also contribute to the generation of reactive oxygen species that lead to enzyme inactivation.

Acknowledgments

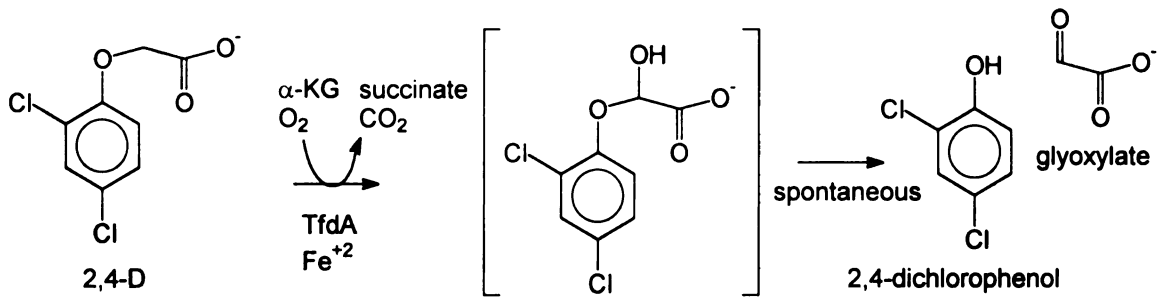
I thank the members of the Hausinger lab for critiquing the manuscript version of this chapter for publication and Michael Washburn for the dehydroascorbic acid preparation.

CHAPTER 3

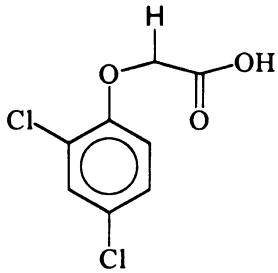
STEREOSPECIFICITY OF PHENOXY HERBICIDE DEGRADATION

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) and structurally related phenoxy herbicides (Figure 8) are widely used for the control of broad-leaved weeds and woody plants. Many studies of the environmental fate of these compounds show that they are degraded rapidly in aerobic soil by biologically-mediated processes (e.g., reviewed in (Loos 1969; Sandmann et al. 1988)). One of the best-characterized phenoxy herbicide-degrading organisms is *Ralstonia eutropha* JMP134 (Don and Pemberton 1981), capable of using 2,4-D as its sole carbon and energy source. The first step in 2,4-D metabolism is its conversion to 2,4-dichlorophenol, catalyzed by the enzyme 2,4-D/α-ketoglutarate (α-KG) dioxygenase or TfdA (from the plasmid pJP4-derived *tfdA* gene designation; (Streber et al. 1987)). This ferrous ion-dependent enzyme couples the oxidative decarboxylation of α-KG to hydroxylation of the substrate at the C-2 carbon to yield a postulated hemiacetal intermediate which spontaneously decomposes to form 2,4-dichlorophenol and glyoxylate (Fukumori and Hausinger 1993a; Fukumori and Hausinger 1993b) (Scheme 13).

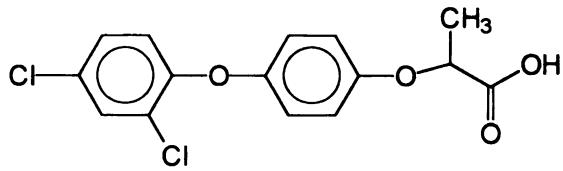


Scheme 13. Reaction catalyzed by TfdA



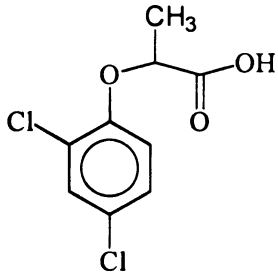
2,4-dichlorophenoxyacetic acid

2,4-D



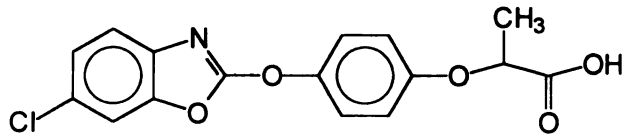
2-[4-(2,4-dichlorophenoxy)phenoxy]-propionic acid

Diclofop



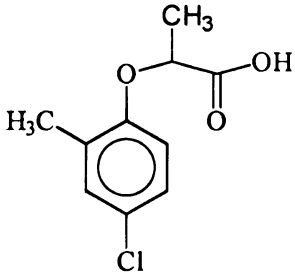
2-(2,4-dichlorophenoxy)propionic acid

Dichlorprop



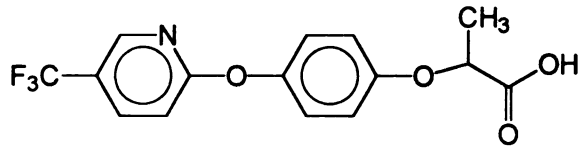
2-[4-[(6-chloro-2-benzoxazolyl)oxy]-phenoxy)propionic acid

Fenoxaprop



2-(2-methyl-4-chlorophenoxy)propionic acid

Mecoprop



X=H 2-[4-[(5-trifluoromethyl)-2-pyridinyl]oxy]-phenoxy]propionic acid

Fluazifop

Figure 8: Phenoxyacetic and phenoxypropionic herbicides

This chapter of my thesis focuses on characterizing the stereochemistry of the reaction catalyzed by TfdA and related herbicide-degrading enzymes. The C-2 position of 2,4-D is prochiral, but the enzymatic hydroxylation reaction may exhibit a stereochemical preference for abstracting either the *pro-R* or *pro-S* hydrogen. Because the immediate product of this reaction is an unstable hemiacetal that yields final products lacking stereochemistry, the stereospecificity of 2,4-D hydroxylation by TfdA cannot be directly determined. Here, I use a 2,4-D analogue containing a methyl group attached to the C-2 carbon (i.e., the herbicide dichlorprop; 2-(2,4-dichlorophenoxy)propionic acid) to block access to one or the other side of C-2 carbon in order to determine the stereochemistry of hydroxylation. Dichlorprop (in the racemic form) was previously shown to be a substrate of TfdA (Fukumori and Hausinger 1993b), but the stereospecificity of the reaction was not determined. Of environmental significance, only the R-isomer of phenoxypropionates are effective herbicides (Dicks 1985; Gerwick et al. 1988; Matell 1953; Nestler and Bieringer 1980). I characterize the reaction with dichlorprop to demonstrate that pJP4-encoded TfdA hydroxylates at the *pro-R* position of 2,4-D (i.e., degrading (S)-dichlorprop). In addition, I demonstrate that cell extracts of *Burkholderia cepacia* RASC (a 2,4-D degrader containing a TfdA with 80% sequence identity to that from pJP4 (Suwa et al. 1996)) has the same stereopreference as JMP134. By contrast, I show that cell extracts of an *Alcaligenes denitrificans* isolate, known to degrade the R-enantiomer of mecoprop (see structure in Figure 8) (Tett et al. 1994) degrades the R-enantiomer of dichlorprop. Although the RASC- and *A. denitrificans*-derived enzymes exhibit opposite stereochemical preferences for dichlorprop utilization, I

show them to be highly related on the basis of cofactor requirements and DNA hybridization studies.

Methods

Chemicals

(+)-(R)-dichlorprop was a kind gift from BASF. Racemic and (+)-(R)-mecoprop were kind gifts from A.H. Marks. Racemic dichlorprop and 2,4-D were purchased from Aldrich.

Bacterial strains

R. eutropha JMP134 (Don and Pemberton 1981) and *B. cepacia* RASC (Suwa et al. 1996) were obtained from the Research on Microbial Ecology lab at Michigan State University. A strain of *A. denitrificans* isolated for its ability to degrade mecoprop (Tett et al. 1994) was obtained from Hilary Lappin-Scott (University of Exeter).

Activity assay with colorimetric detection of phenol derivatives

The standard assay mix contained 1 mM α-KG, 50 μM each of Fe(II) and ascorbate, 1 mM 2,4-D in 10 mM imidazole buffer (pH 6.75), and enzyme. In studies of cell extracts, the concentrations of selected reagents were varied in some experiments as noted in the text. The iron and ascorbate were either supplied together as iron(II) ascorbate salt or separately as $(NH_4)_2Fe(SO_4)_2$ and ascorbic acid. Reactions were carried out at 30 °C, and terminated by addition of EDTA to a final concentration of 5 mM. Phenol production was determined at 510 nm following addition of 4-aminoantipyrene, pH 10 borate buffer and potassium ferricyanide, as described (King et al. 1991). Measured extinction coefficients for the 4-aminoantipyrene adducts were 15,700 M^{-1} cm^{-1}

for 2,4-dichlorophenol, 11,300 M^{-1} cm^{-1} for 2-methyl-4-chlorophenol, and 11,600 for 4-chlorophenol. Values for 2,4-dibromophenol and 2,4,5-trichlorophenol were approximated by using the value for 2,4-dichlorophenol.

Preparation of cell extracts and purified enzyme

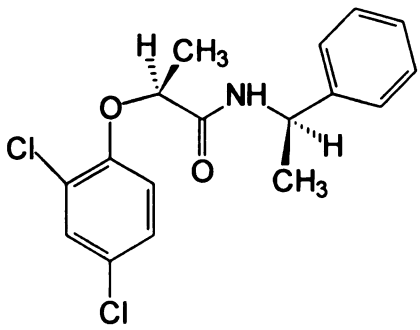
TfdA was purified from *Escherichia coli* DH5α cells carrying the cloned *R. eutropha* JMP134 *tfdA* gene on plasmid pUS311 as described (Fukumori and Hausinger 1993a) by sequential chromatography on DEAE Sepharose and MonoQ (Pharmacia) columns.

2,4-D and mecoprop degraders were grown aerobically at 30°C in MMO medium (mineral salts medium, (Stanier et al. 1966)) with 50 ppm yeast extract and the relevant herbicide at concentrations between 1 and 5 mM, until 60-90% of the herbicide was consumed as measured by disappearance of the chromophore at 230 nm. Alternatively, they were grown on 1/10th strength (3g/l) Trypticase Soy Broth (TrpSoy, Becton Dickinson) to stationary phase as measured by cell optical density at 600 nm. Cell extracts were prepared by resuspending the bacteria in Tris buffer (pH 7.7) with 1 mM EDTA followed by disruption in a French pressure cell and ultracentrifugation (100,000 *x g* , 30 min) or centrifugation in a microcentrifuge (16,000 *x g*, 5 min). Protein concentrations were determined using the BioRad protein assay with bovine serum albumin as the standard.

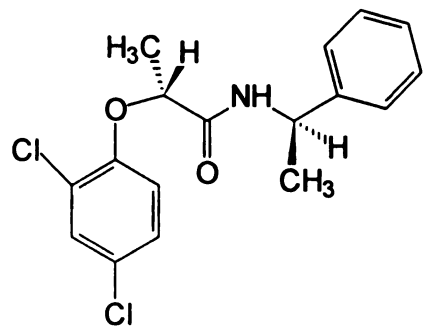
Formation of diastereomers and Gas Chromatography (GC)

The levels of (R)- and (S)-dichlorprop in samples were assessed by using a modification of a published GC method (Blessington et al. 1989). Aqueous reaction

mixtures (1 ml) were acidified with 25 µl of HCl (conc.) and extracted with 1 mL methylene chloride. The organic phase (0.5 mL) was dried under a gentle stream of air, 1 drop of thionyl chloride was added, and the sample was incubated 10 min over a steam bath. Following removal of the thionyl chloride under vacuum, the sample was dissolved in 0.1 ml chloroform with 1 drop of (R)-phenylethylamine added, allowed to react 25 min at room temperature (forming diastereomers as shown in Scheme 14), then dried and transferred to 0.5 ml of ethyl acetate. An aliquot (1 µl) of the sample was injected onto a DB-5 column (polymethylsiloxane with 5% phenyl groups; J & W Scientific), with a temperature program set to increase linearly from 200 to 270°C in 7 min, and the analytes were monitored by electron capture detection (rather than the less sensitive flame ionization detection originally used (Blessington et al. 1989)). Peaks were identified by comparison with the retention times of the diastereomeric amides of authentic (R)-dichlorprop and (RS)-dichlorprop.



(S)-dichlorprop (R)-phenylethylamine



(R)-dichlorprop (R)-phenylethylamine

Scheme 14. Diastereomeric derivatives of dichlorprop for GC/ECD

High Pressure Liquid Chromatography (HPLC)

Dichlorprop was reacted with cell extracts or purified enzyme as described for the activity assay, and the reactions were stopped with 5 mM EDTA. Samples (1 mL) were filtered (0.2 μm pore size) and 20 μl aliquots were injected onto a Nucleodex α-PM column (4.0 mm i.d. x 200 mm length; Machery-Nagel, Germany). The eluent was 30% phosphate buffer (50 mM, pH 3) and 70% methanol at a flow rate of 0.7 ml/min. Species eluting from the column were monitored with UV detection at 230 nm.

DNA hybridization

Genomic DNA of *A. denitrificans* was prepared by phenol/chloroform extraction as described (Sambrook et al. 1989) and digested with *Bam*H1. Following separation on an 8 % polyacrylamide gel, the DNA was transferred (Sambrook et al. 1989) to a Hybond N membrane (Amersham). Probes for $tfdA_{RASC}$ (a 1-kb fragment prepared by *Pst*I digestion of pYB232 (Suwa et al. 1996)) and for $tfdA_{JMP134}$ (an 801-bp *Sty*I fragment of pJP4 (Holben et al. 1992)) were labeled with digoxigenin according to the manufacturer's instructions (Boehringer-Mannheim Biochemicals). The membrane was blotted with the probes at high stringency (68°C, 50% formamide) or low stringency (68 °C, no formamide) using the protocol from the Boehringer-Mannheim Biochemical's manual.

Data analysis

Data were fit to straight lines, exponential decays, or square hyperbola using the program KaleidaGraph (Synergy software). As well, progress curves of

2,4-dichlorophenol production versus time were fitted to Equation 1 (Saari and Hausinger 1998) by non-linear regression using the program KaleidaGraph (Synergy Software).

$$A_t = A_f(1-e^{-k_i t}) \qquad \text{(Equation 1)}$$

In this equation, A_t is the absorbance at time t, A_f is the final absorbance, and k_i is the rate of inactivation.

Results

TfdA from* R. eutropha *JMP134 is specific for (S)-dichlorprop

The stereospecificity of TfdA activity towards dichlorprop was investigated by two methods. The first method involved use of GC to monitor the individual enantiomers remaining during the reaction. As shown in Figure 9 (2 panels), the R- and S-isomers of dichlorprop are readily separated by GC after conversion to the diastereomeric species by reaction with (R)-phenylethylamine. During the reaction of racemic dichlorprop with TfdA, the percentage of S- relative to R-enantiomer decreased over time, suggesting that TfdA acts on the S-enantiomer of dichlorprop (Figure 10). The second method made use of a colorimetric assay for phenol released from either racemic dichlorprop or (R)-dichlorprop (Figure 11), and gave results in agreement with those from the GC method. The R-isomer was not a substrate while the racemic mixture (containing the S-isomer) yielded product 2,4-dichlorophenol.

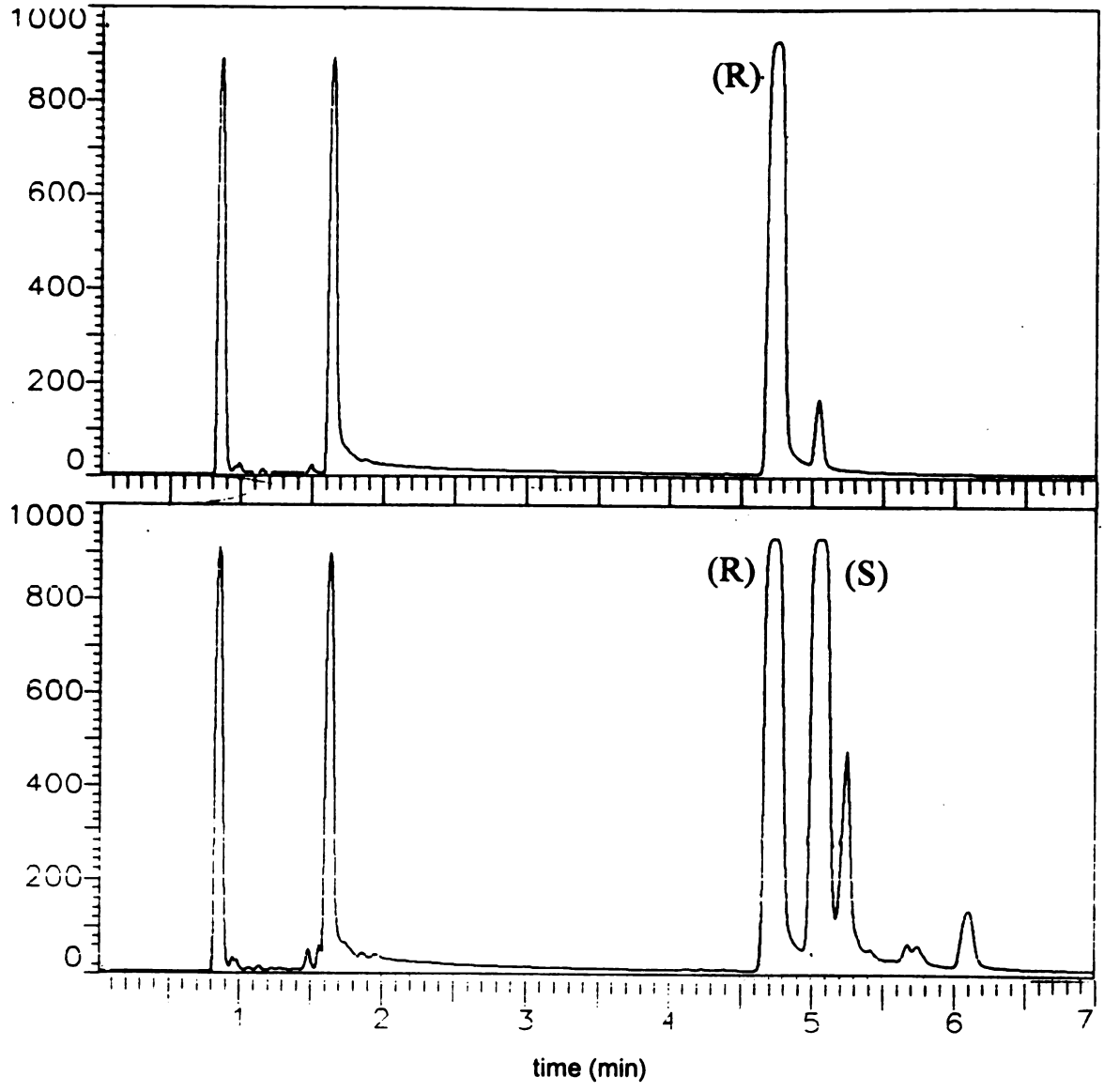


Figure 9. Separation of the diastereomeric derivatives of (R)- and (S)-dichlorprop by GC with ECD detection.

Sample derivatization and chromatographic conditions are as described in the methods.

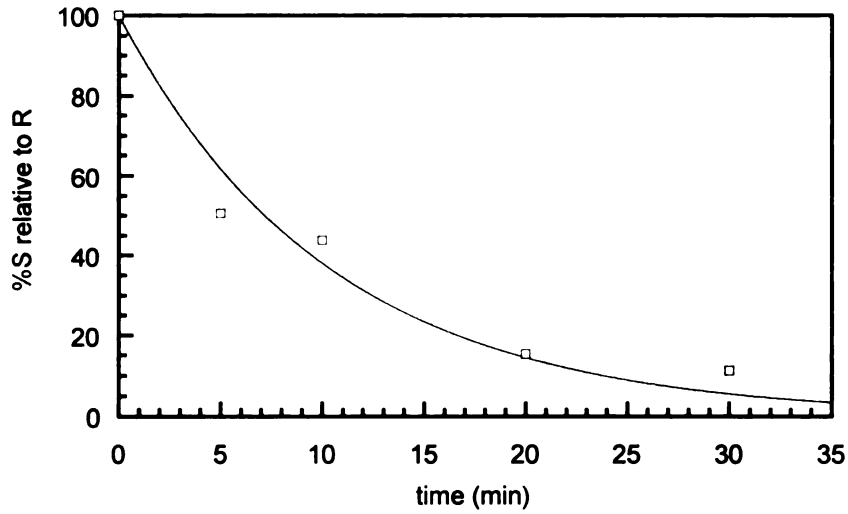


Figure 10. Loss of (S)-dichlorprop (relative to the R-enantiomer) catalyzed by TfdA.

TfdA (50 μg) was incubated with dichlorprop (100 μM) and ascorbic acid, ferrous ion and α-KG (standard concentrations). Following extraction, dichlorprop was converted to the (R)-phenylethylamide diastereomer and analyzed by GC/ECD as described in the methods. Data were fit to an exponential decay with a decay rate of 0.096 ± 0.011 min^{-1}.

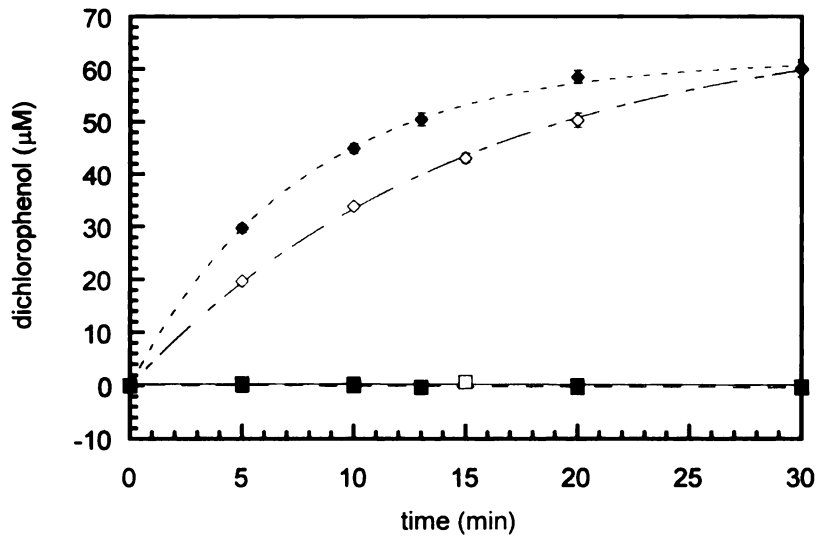


Figure 11. Phenol production by TfdA acting on dichlorprop.

TfdA (10 μg/ml) was incubated with racemic mixtures of dichlorprop at 0.2 mM (◊) and 2 mM (◆) respectively, or with (R)-dichlorprop at the same concentrations (□ and ■, respectively). Phenol was measured colorimetrically as described in the methods. Data for the racemic dichlorprop was fit to equation 1 (k_i = 0.065 ±0.002 and 0.131 ± 0.004 min^{-1} for 0.2 and 2 mM, respectively), while data for the (R) dichlorprop was fit by linear regression (slopes of -0.01 and -0.02 μM min^{-1} for 0.2 and 2 mM, respectively).

Stereospecificity of various phenoxy herbicide degraders towards dichlorprop

In order to compare the stereospecificities of hydroxylation by various phenoxy herbicide-degrading organisms to that observed for the purified *R. eutropha* JMP134 enzyme, additional strains were examined along with JMP134. Similar to the benchmark *R. eutropha* species, *B. cepacia* RASC was originally isolated for its ability to degrade

2,4-D. Moreover, RASC has been shown to possess a TfdA with 80% amino acid identity to that from JMP134 (Suwa et al. 1996). Another isolate was a mecoprop-degrading strain of *Alcaligenes denitrificans*, which had already been shown to degrade the R-enantiomer of this herbicide (Tett et al. 1994; Tett et al. 1997), in contrast with the specificity of $TfdA_{JMP134}$. Neither of the 2,4-D degrading strains grew on minimal medium with dichlorprop (1 mM) as the carbon source, as shown by lack of turbidity of the cultures and unchanged dichlorprop concentrations in the media (data not shown). Thus, studies were carried out using cell extracts of JMP134 and RASC grown on 2,4-D and *A. denitrificans* grown in (R)-mecoprop.

Cell extracts were tested for activity towards 2,4-D, dichlorprop, and mecoprop in the presence of α-KG, ferrous ion and ascorbate while monitoring phenol production. Cell extracts of JMP134 and RASC showed higher activity towards 2,4-D than dichlorprop (Figure 12A), whereas *A. denitrificans* showed activity towards mecoprop and dichlorprop but minimal activity towards 2,4-D (Figure 12B). The activity of *A. denitrificans* extracts towards dichlorprop was completely dependent on the presence of ferrous ion and α-KG, and apparently was lost rapidly (in < 5 min) in the absence of ascorbic acid (Figure 13). The maximal velocity for *A. denitrificans* cell extract activity for (R)-dichlorprop was slightly higher than for (R)-mecoprop (Figure 14), while the K_m values for both substrates were on the order of 0.1 mM. The stereospecificity of dichlorprop hydroxylation by cell extracts of JMP134, RASC, and *A. denitrificans* were determined by using an HPLC column with a chiral stationary phase, which can separate the enantiomers of mecoprop and dichlorprop without the need for extraction or

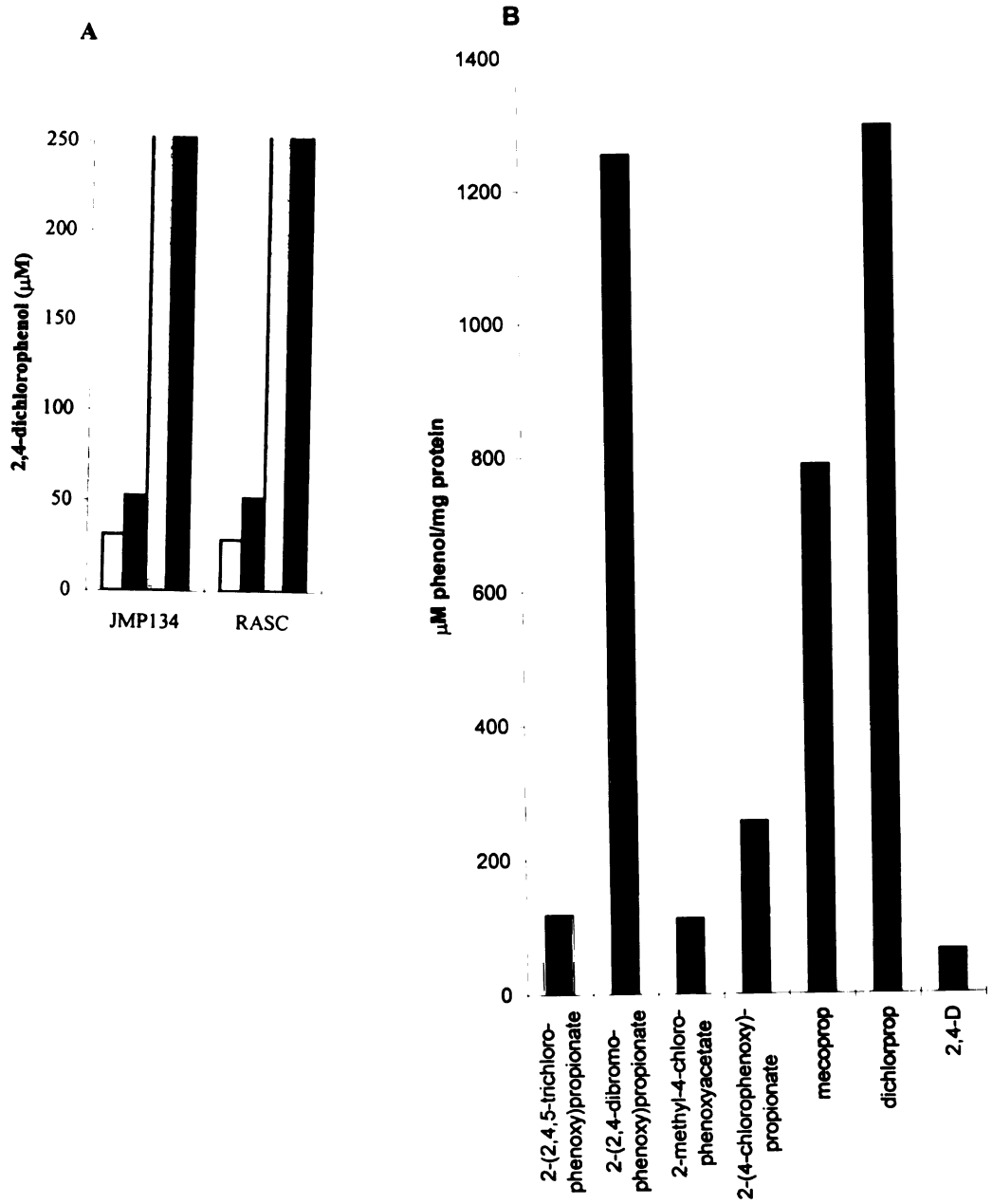


Figure 12. Activity of cell extracts towards phenoxy herbicides.

A. Phenol production by JMP134 and RASC cell extracts (50 μl) incubated with ascorbate, ferrous ion, and α-KG (standard concentrations) and racemic dichlorprop for 5 (open bars) and 20 (black bars) min and towards 2,4-D for 5 (light gray) and 20 (dark gray) min. The high activities (>250 μM) reflect off-scale absorbances. B. Phenol production by MMO-grown *A. denitrificans* cell extract incubated with ascorbate, ferrous ion, and α-KG (standard concentrations) and phenoxy herbicides (1 mM) as indicated.

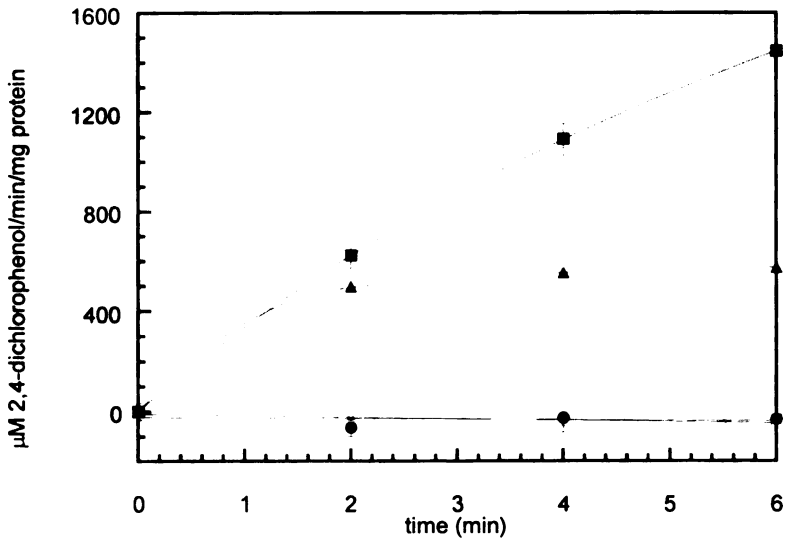


Figure 13. Requirement for α-KG, iron and ascorbate to support activity of *A. denitrificans* cell extract.

Cell extract prepared from trp-soy grown cells as described in methods. Phenol production by *A. denitrificans* cell extract (10 µl) activity incubated with the standard reaction mix containing (R)-dichlorprop (1 mM) (■), and mix from which ascorbate (▲), iron (●) or α-KG (x) had been omitted. Error bars are standard deviation of three cuvettes.

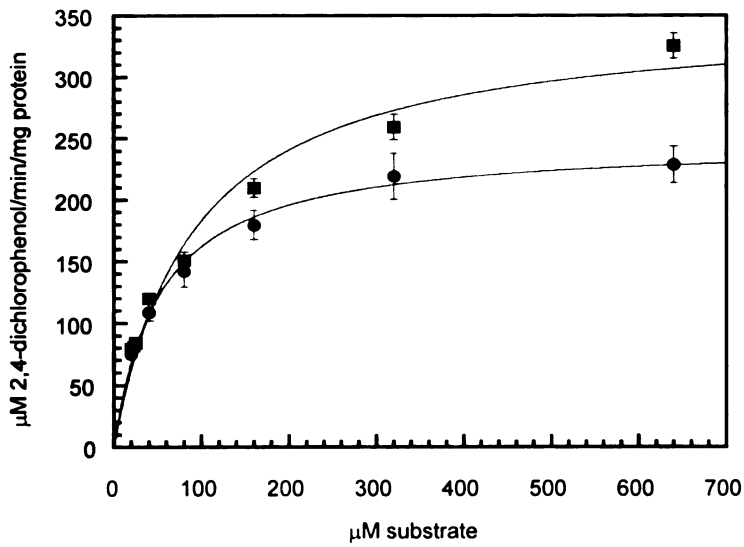


Figure 14. Concentration dependence of *A. denitrificans* cell extract activities towards (R)-mecoprop and (R)-dichlorprop, measured by phenol production.

Cell extract prepared from MMO-grown cells as described in methods. Velocity data (based on 6 minute time points, triplicate samples) were fit to a square hyperbola (i.e. the Michaelis-Menten equation) for (R)-dichlorprop (K_m values of 91 ± 16 µM; V_{max} 351 ± 21 µM/min/mg protein) and for (R)-mecoprop (K_m values of 52 ± 4 µM; V_{max} 247 ± 6 µM/min/mg protein). Mecoprop velocity data corrected for ~1% contamination with a phenolic compound presumed to be 4-chloro-2-methylphenol.

derivatization of the aqueous samples (Machery Nagel product literature; see also Figure 15). In agreement with the GC and phenol assay results carried out with purified TfdA, cell extracts of JMP134 degraded the S-enantiomer of dichlorprop (Figure 16). Similarly, cell extracts of RASC also degraded the S-enantiomer. In contrast, *A. denitrificans* cell extracts degraded the R-enantiomer of dichlorprop (Figure 16B) which concurs with the stereochemistry of intact cells towards mecoprop (Tett et al. 1994).

A. denitrificans *contains a DNA sequence related to tfdA*

The presence of α-KG-dependent herbicide-degrading activity in *A. denitrificans* raises the question of whether the phenol-releasing enzyme in this organism is related in sequence to that found in JMP134 or RASC, despite its use of the opposite enantiomer. To test for possible sequence relatedness, fragments of the *tfdA* genes from these 2,4-D degraders were hybridized to genomic DNA of *A. denitrificans*. The $tfdA_{\text{JMP134}}$ fragment hybridized at low stringency (~60% DNA sequence identity) while the $tfdA_{\text{RASC}}$ fragment hybridized at high stringency (> 90% DNA sequence identity) (Figure 17). At low stringency, a fragment from *R. eutropha* JMP134 containing $tfdA_{JMP134}$ hybridizes to $tfdA_{RASC}$ as well as another smaller band containing an unknown sequence.

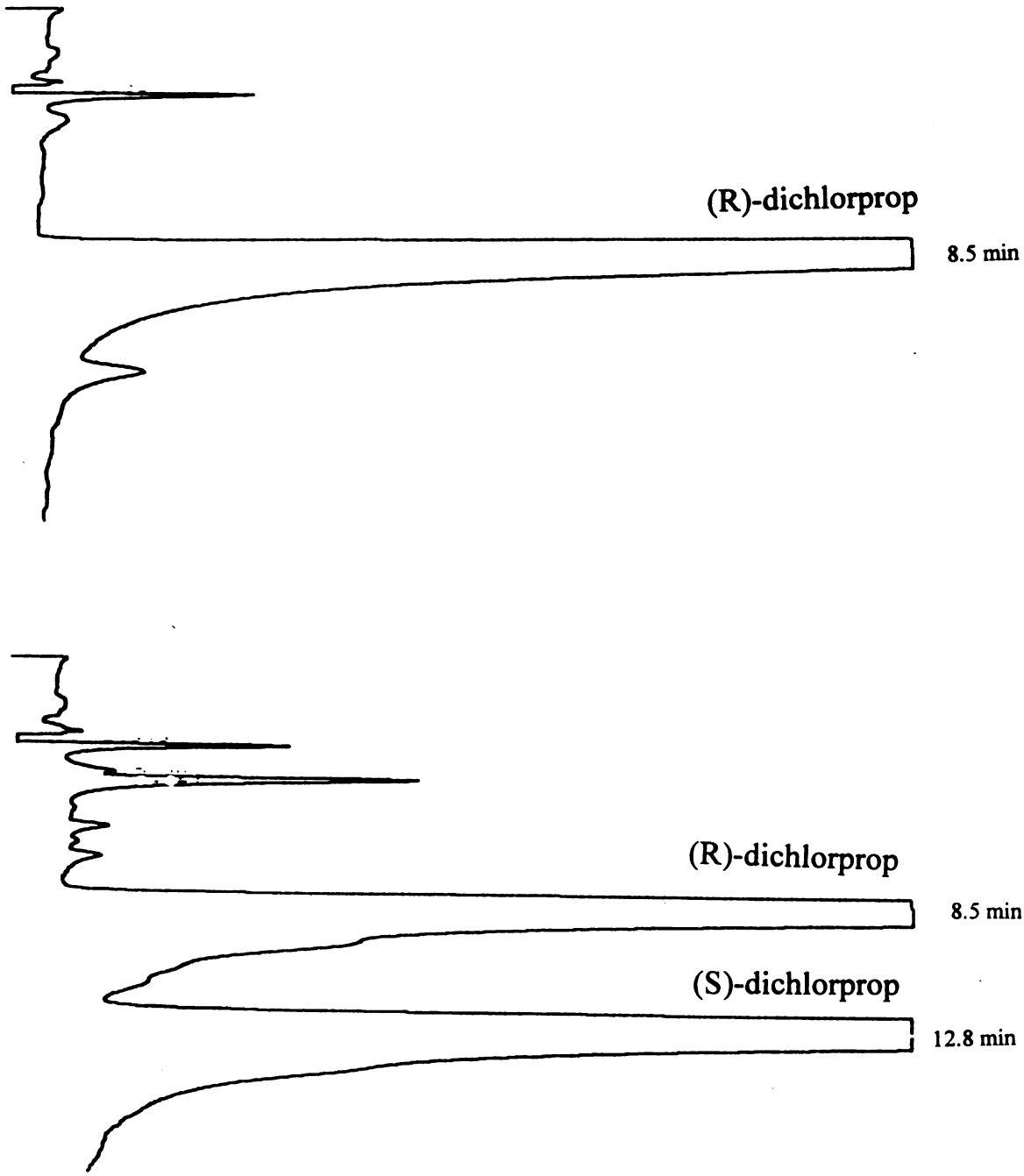


Figure 15. Separation of (R)- and (S)-dichlorprop by HPLC using Nucleodex α-PM column.

Sample preparation and chromatography conditions as described in the methods. Detection at 230 nm.

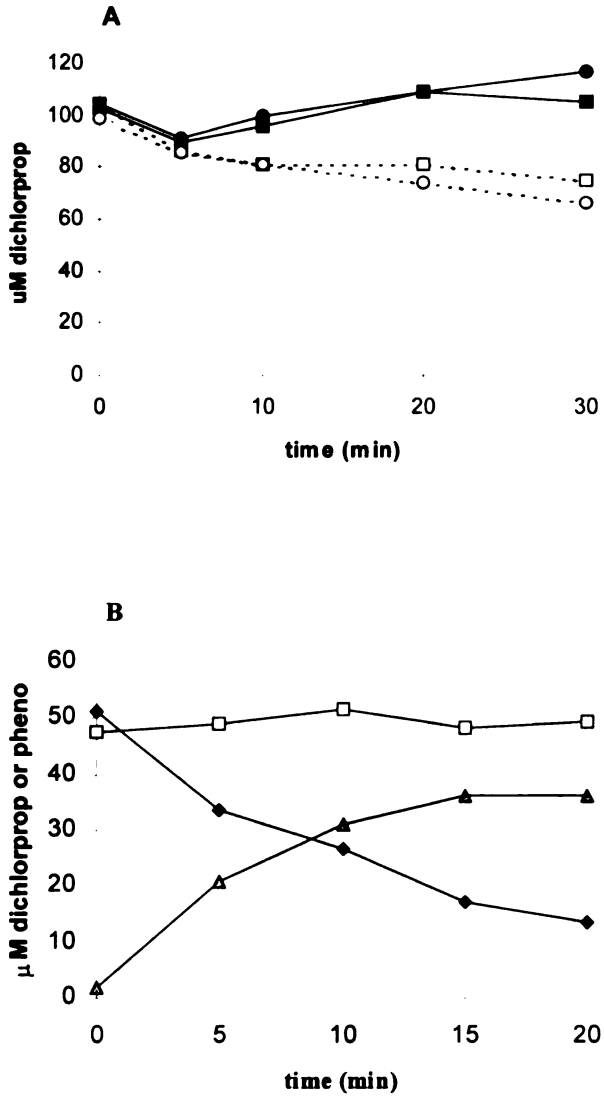


Figure 16. Stereospecificity of activity of cell extracts and purified protein towards racemic dichlorprop, measured by chiral HPLC.

A. Concentrations of (R)- and (S)-dichlorprop (filled and open symbols respectively) remaining after incubation of cell extracts (100 µl) of JMP134 (squares) and RASC (circles) with racemic dichlorprop (0.2 mM) and the standard reaction components B. Concentrations of (R)- and (S)-dichlorprop and 2,4-dichlorophenol (filled, open and gray symbols respectively) remaining after incubation of cell extracts (50 µl) of *A. denitrificans* with racemic dichlorprop (0.1 mM) and the standard reaction components. Dichlorprop was quantitated by using chiral HPLC and phenol was quantitated colorimetrically, as described in the methods.

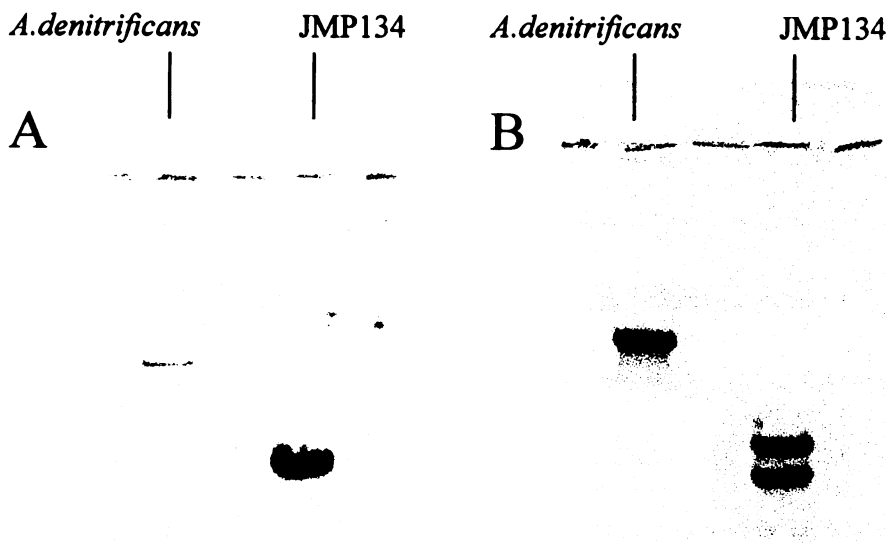


Figure 17. Southern blot of *A. denitrificans* genomic DNA hybridized with A. $tfdA_{JMP134}$ or B. $tfdA_{RASC}$.

Southern blot of *Bam*HI-digested genomic DNA from *A. denitrificans* and *R. eutropha* JMP134 hybridized at low stringency (as described in the methods) with A. $tfdA_{JMP134}$ or B. $tfdA_{RASC}$.

Discussion

Stereochemical differences amongst α-KG dependent dioxygenases acting on dichlorprop

Each of the herbicide-degrading activities examined here appear to exhibit sterospecificity in their α-KG-dependent hydroxylation reactions as shown by their use of a single enantiomer of the phenoxypropionate derivatives. Stereospecificity is a common phenomenon among α-KG-dependent dioxygenases. In hydroxylations which yield a chiral product catalyzed by this class of enzymes, only one of the two possible hydroxylated enantiomers is formed (Figure 18). Proton removal is also stereospecific, and the overall reaction proceeds with retention of configuration. Assuming that these three herbicide degraders also hydroxylate with retention of configuration, and taking into account the data reported here on the stereospecificity, it suggests that when acting on 2,4-D, JMP134 and RASC remove the *pro-R* proton of C-2 and then form the R-enantiomer of the hydroxylated intermediate, whereas *A. denitrificans* has the opposite stereospecificity.

It is somewhat surprising that TfdA of JMP134 (or RASC) acts only on the S-enantiomer of dichlorprop, while the activity of *A. denitrificans* is specific for the R-enantiomer. The stereospecificity of *R. eutropha* JMP134 extracts for the S-isomer of mecoprop and dichlorprop has been confirmed by others (Nickel et al. 1997). Furthermore, the R-specificity of *A. denitrificans* for mecoprop has been reported previously (Tett et al. 1994; Tett et al. 1997). As illustrated above, I have now obtained evidence that the enzyme responsible for herbicide degradation in the latter

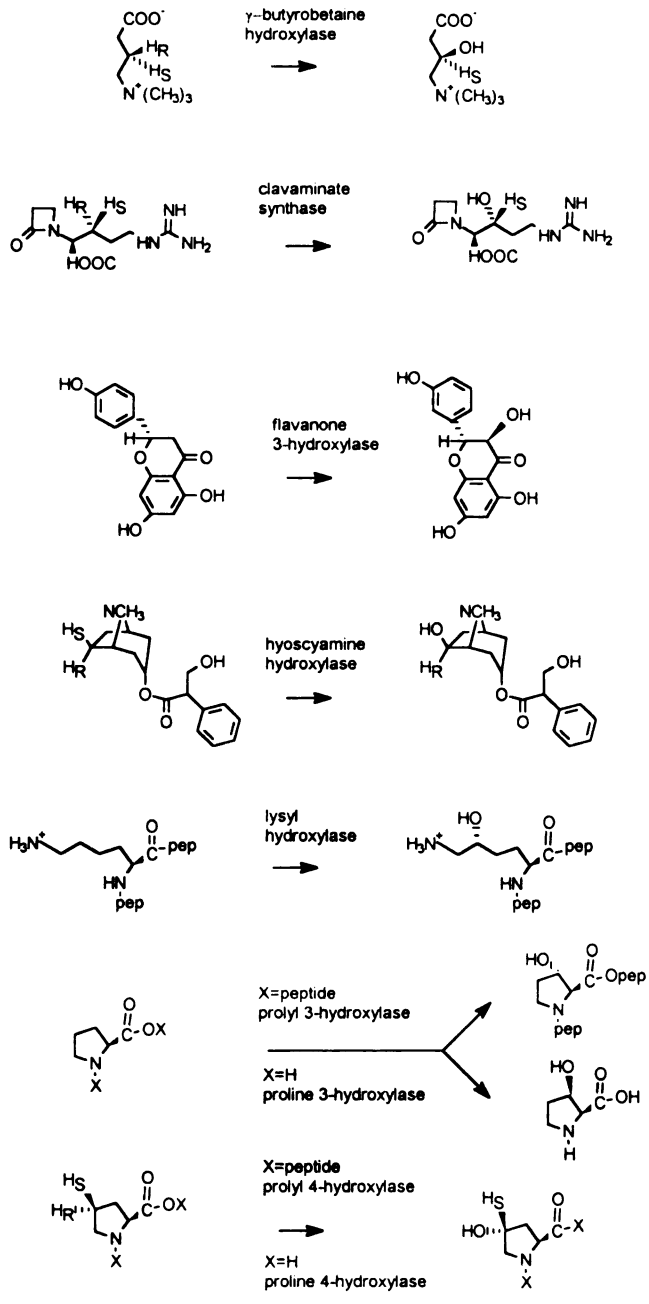


Figure 18. Stereospecificity of hydroxylations catalyzed by α-KG-dependent dioxygenases.

Where known, the stereochemistry of removal of prochiral proton is indicated with R and S subscripts on the protons. References: γ-butyrobetaine hydroxylase (Englard et al. 1985); clavaminate synthase (Baldwin et al. 1993b); flavanone 3-hydroxylase (Britsch and Grisebach 1986); hyoscyamine hydroxylase (Leete and Lucast 1976); lysyl hydroxylase (references from (Jefford and Cadby 1981b)) prolyl 3-hydroxylase (Majamaa et al. 1985), proline 3-hydroylase (Mori et al. 1997), proline 4-hydroxylase (Baldwin et al. 1993a) and prolyl-4 hydroxylase (Fujita et al. 1964). pep = peptide

microorganism is also an α-KG-dependent dioxygenase, and I have shown that *A. denitrificans* possesses a gene highly similar to $tfdA_{RASC}$ and somewhat related to $tfdA_{JMP134}$. Related to these findings, a recent publication (Nickel et al. 1997) reported that α-KG-dependent dioxygenases acting on phenoxypropionates can have opposite stereochemistry; *Sphingomonas herbicidovorans* has two α-KG-dependent dioxygenases for degradation of (S)- and (R)-dichlorprop, as shown by different activities in cell extracts corresponding to differential expression of two different size proteins when grown on one or the other enantiomer. The sequence relationship between the two *S. herbicidovorans* proteins is unknown, but my results comparing the *R. eutropha* JMP134 and *A. denitrificans* systems suggests that the *S. herbicidovorans* enzymes of opposite stereochemistry may be derived from the same ancestral sequence.

Environmental and biotechnology significance of bacterial enantiospecifity towards phenoxypropionate herbicides

The stereochemistry of phenoxypropionate degradation has environmental significance. In particular, only the R-enantiomers of dichlorprop and the other phenoxy herbicides are active towards plants (Dicks 1985; Gerwick et al. 1988; Matell 1953; Nestler and Bieringer 1980). Modern trends in agriculture include an emphasis on application of only the active enantiomer of a chiral product to reduce the environmental load of organopesticides. In the European Union, about 75% of the mecoprop and dichlorprop sold is in the enantiomeric form, with the expectation that within five years 100% will be sold as the R-form (Rodney Akers, BASF, pers. comm.). Currently, BASF uses a proprietary chemical process and A.H. Marks uses a proprietary fermentative

process for preparing the enantiomeric forms (Rodney Akers, BASF, pers. comm.). One potential synthetic application of JMP134 or purified TfdA would be to act on racemic dichlorprop to eliminate the unwanted S-enantiomer, with recycling of the released 2,4-dichlorophenol. A potential application for *A. denitrificans* or the *tfdA*-like gene it contains would be protection of crops from mecoprop and dichlorprop. Cotton shows enhanced tolerance of 2,4-D in the presence of the pJP4-derived *tfdA* transformed either into the plant (Lyon et al. 1993) or into root-associated microbes (Feng and Kennedy 1997). For protection against the chiral phenoxypropionate herbicides, the pJP4-derived gene product would be ineffective (because it would not degrade the herbicidal R-enantiomer), but the gene from *A. denitrificans* could be used since its gene product degrades the R-enantiomer.

Even in cases where only the R-enantiomer of a phenoxypropionate is applied to soils, it is important to note that biologically-mediated interconversion between the enantiomers has been observed. Thus, it is useful to have a technique, such as the derivatization/GC or the chiral HPLC methods used in this research, that can separate the enantiomers within a sample. For example, either (R)- or (S)-dichlorprop in soil was racemized in soil by ~30 days, by a microbially-mediated process (Buser and Müller 1997). The experiments involved adding D_2O to the soil and following the formation of deuterated dichlorprop over time. The deuterium was only found in the C-2 carbon, not on the C-3 methyl, indicating that racemization chemistry only involves the C-2. Diclofop and fenoxaprop in soil also showed interconversion of enantiomers, although the S-enantiomers had a faster rate of inversion than the R form, leading to an equilibrium of 70% R- and 30% S-enantiomer within 3 to 9 days (Wink and Luley 1988). By

contrast, fluazifop in soil was only inverted in one direction; (S)-fluazifop was converted to R-form with a half life of 1-2 days, while the reverse reaction (R to S) did not occur to any appreciable extent (Bewick 1986). The relative rates of degradation of the enantiomers of phenoxypropionate herbicides differ in various reports, presumably reflecting differences in the metabolic capabilities of the microbial communities or isolates. One study showed that the rates of degradation of mecoprop and dichlorprop in the soil were between 7 and 22 d, with the S-enantiomers degrading faster than the R-enantiomers (Müller and Buser 1997). Another study of dichlorprop in soil showed that the S-enantiomer degrades more rapidly ($t_{1/2}$ = 4.4 d) than the R-enantiomer ($t_{1/2}$ = 8.7 d). (Garrison et al. 1996). A similar stereopreference was exhibited by a microbial mixed culture (isolated from a landfill) towards fluazifop: the S-enantiomer was degraded within eight days and while the R-enantiomer was not degraded until after an additional 20 day lag period (Nègre et al. 1993). The opposite stereopreference, for exclusively the R-enantiomer, was observed for two bacterial communities isolated from a marine environment acting on diclorfop (Ludwig et al. 1992) and from wheat rhizospheres acting on mecoprop (Tett et al. 1994). The methods described here are applicable for monitoring stereochemical preferences in environmental samples, cell extracts, and with purified herbicide-degrading enzymes.

Acknowledgments

I thank Peter Chapman for suggesting that dichlorprop could function as a probe for the stereochemistry of TfdA-catalyzed hydroxylation; Deborah Hogan for performing the DNA hybridization; and Paul Loconto for allowing me to use the chromatographic equipment at the Engineering Research Labs of MSU, assisting in setting up the GC method and suggesting the use of electron capture detection.

CHAPTER 4

SPECTROSCOPY OF TfdA

The EPR and some of the ESEEM results described in chapter 4 have been published as: Whiting, A. K., Que Jr., L., Saari, R. E., Hausinger, R. P., Fredrick, M. A., and McCracken, J. (1997). "Metal coordination environment of a Cu(II)-substituted α-keto acid-dependent dioxygenase that degrades the herbicide 2,4-D." *J. Am. Chem. Soc.*, 119(14), 3413-3414. The XAS results described in this chapter will be submitted to *J. Biol. Inorg. Chem.* with the title "X-ray absorption spectroscopic analysis of Fe(II) and Cu(II) forms of an herbicide-degrading α-ketoglutarate dioxygenase".

Introduction

The enzyme catalyzing the first step in metabolism of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in the soil bacterium *Ralstonia eutropha* JMP134 (Don and Pemberton 1981) is TfdA, an α-ketoglutarate (α-KG)-dependent dioxygenase (Fukumori and Hausinger 1993a). This enzyme requires ferrous iron for catalysis, with half-maximal activity at ~10 μM Fe(II); no other metals tested (Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II) or Zn(II)) could replace iron for activity (Fukumori and Hausinger 1993b). Some insight into the active site residues has been obtained using amino-acid reactive reagents. The enzyme was inactivated by the histidyl-reactive reagent diethylpyrocarbonate (DEPC), but inactivation could be prevented by inclusion of 2,4-D together with α-KG. The role of histidines in TfdA activity was hypothesized to arise at least in part from these residues serving as metal ligands. In contrast, cysteines are unlikely to function as iron ligands, based on the lack of enzyme inactivation with cysteinyl-reactive reagents (N-ethylmaleimide and iodoacetamide) suggesting that they are not involved in this fashion. The studies in this chapter are focused on spectroscopic characterization of the TfdA metallocenter and comparison of its properties to those of related enzymes.

Metal dependence and metallocenter characterization of α-KG-dependent dioxygenases

Common features shared by TfdA with the other α-KG-dependent dioxygenases include a requirement for ferrous iron, oxygen and α-KG. This class of enzymes carries out the decarboxylation of α-KG to form succinate and CO_2, typically coupled to hydroxylations of target molecules (reviewed in (De Carolis and De Luca 1994; Prescott 1993; Prescott and John 1996)). In some cases (including TfdA) the hydroxylated compound is unstable and decomposes to form the final products. Generally the iron levels required are on the order of micromolar to tens of micromolar. Half-maximal activity of aspartyl β-hydroxylase is obtained with 3 μM iron (Gronke et al. 1990), 3-6 μM for prolyl hydroxylase (Annunnen et al. 1997; Myllylä et al. 1977) similar to the value of 2-4 μM for lysyl hydroxylase (Puistola et al. 1980a), compared to much higher value of ~0.45 mM for thymidine 2'-hydroxylase (Bankel et al. 1972). Optimal activity for taurine dioxygenase (TauD) is seen between 5 and 150 μM Fe(II) (Eichhorn et al. 1997), compared to 30 μM for proline hydroxylase (Lawrence et al. 1996). While no crystal structures have reported for α-KG-dependent dioxygenases, the structure is known for isopenicillin N synthase (IPNS). Although this iron- and oxygen-dependent enzyme does not use α-KG, it is related in sequence to two α-KG dependent enzymes in the isopenicillin biosynthetic pathway (Kovacevic and Miller 1991; Kovacevic et al. 1989; Perry et al. 1988). The ligands to the metal in the resting enzyme (in the manganese form) are His_{214}, Asp_{216}, His_{270}, Gln_{330}, and two water molecules, in an octahedral arrangement (Roach et al. 1995). Upon binding of the substrate L-α-amino-δ-adipoyl-L-

cysteinyl-D-valine (ACV) to Fe-IPNS (anaerobic), the glutamine residue is displaced by a sulfur residue of ACV, and a water is lost (without replacement), leaving the metal five-coordinate with square-pyramidal geometry (Roach et al. 1997). The presence of the histidines, a carboxyl group and a water molecule in the resting enzyme, as well as water displacement upon binding of ACV though its thiol group were predicted by earlier X-ray absorption spectroscopy (XAS) studies, but the fourth protein-derived ligand was incorrectly modeled as a His rather than a Gln, and the Asp was thought to bind through both oxygens of the carboxyl group (Orville et al. 1992; Randall et al. 1993; Scott et al. 1992). Studies using electron spin envelope echo modulation (ESEEM) of Cu(II)-IPNS indicated two equatorially coordinated histidines in the resting enzyme (Jiang et al. 1991). The loss of a water ligand upon substrate binding was also predicted by ESEEM studies, by comparison of spectra in D_2O and H_2O (ibid.) Crystallographic evidence indicated that the oxygen analogue nitrous oxide (NO) can bind the open site on the metal when added to Fe-IPNS·ACV, forming an octahedral complex, with a O-N-Fe bond angle of 120° (Roach et al. 1997).

The two histidyl and one aspartyl ligands to the metallocenter of IPNS are likely to be a feature of α-KG-dependent dioxygenases as well. A motif consisting of His-X-Asp-X_{53-57}-His is observed in many members of this class of enzymes (including TfdA) (Borovok et al. 1996; Jia et al. 1994). Mutation of the His and Asp residues in these motifs reduces the activity to less than 1% of the wild type in flavanone 3β-hydroxylase, proline 4- and prolyl hydroxylase, and lysyl hydroxylase (Lamberg et al. 1995; Lukacin and Britsch 1997; Myllyharju and Kivirikko 1997; Pirskanen et al. 1996). In some cases,

replacement by other residues capable of metal binding allowed partial maintenance of activity (McGinnis et al. 1996; Myllyharju and Kivirikko 1997). The fourth protein ligand to the metallocenter of IPNS, Gln, is not conserved in the α-KG-dependent dioxygenases nor is it essential for IPNS activity (Landman et al. 1997; Sami et al. 1997). In agreement with the genetic evidence consistent with the necessity of histidyl residues for activity, studies of a variety of α-KG-dependent dioxygenases indicate DEPC treatment inactivates the enzymes (Fukumori and Hausinger 1993b; Lawrence et al. 1996; Lukacin and Britsch 1997; Myllylä et al. 1992). In some cases, cysteinyl-reactive reagents (mercurials, iodoacetamide) also interfere with the activity (Halme et al. 1970; Jia et al. 1994; Lindstedt and Lindstedt 1970; Miller and Varner 1979; Popenoe et al. 1969), but this was not observed for TfdA (Fukumori and Hausinger 1993b) and only weakly in 4-hydroxyphenylpyruvate dioxygenase (HPPD; (Taniguchi et al. 1964)). HPPD carries out a reaction analogous to those of the α-KG-dependent dioxygenases, except that the α-ketoacid it uses is internal to the substrate rather than part of a separate molecule (i.e., α-KG).

The binding of substrates in the active site of α-KG-dependent dioxygenases is of necessity different than that of IPNS because the substrates for the former enzymes generally do not contain sulfur and the latter does not use the cosubstrate α-KG. Evidence for the binding mode of α-KG comes from a variety of techniques including circular dichroism and ultraviolet (UV)-visible spectroscopies applied to the α-KG-dependent dioxygenase clavaminate synthase (Pavel et al. 1998). The spectra suggest a bidentate binding mode for α-KG to the iron (i.e., through one of the C-1 carboxyl

oxygen atoms and the C-2 keto group), leading to displacement of two of the ligands from the six- coordinate resting state.

Spectroscopic techniques used with TfdA

To better define the properties of the TfdA metallocenter, I have used a variety of spectroscopic techniques, including electron paramagnetic resonance (EPR), and ESEEM spectroscopies, XAS, and UV-visible spectroscopy. The EPR method employs radiation in the microwave frequency to cause electron spin transitions in an unpaired electron (e.g. from an organic radical or paramagnetic transition metal) exposed to a magnetic field. Typically this technique is performed using a fixed frequency and scanning over varying magnetic field strength. The position of the absorbance maxima (measured by the g parameters) and the modulations of the shoulders (measured by the A parameters) are controlled by the metal ligands. An extension of this technique is ESEEM spectroscopy, which uses multiple discrete pulses of microwave radiation (rather than a continuous wave) to irradiate the sample and monitors the emitted echo. In the two-pulse method, the echo intensity is measured as a function of the time τ between the pulses. The data is typically Fourier transformed into the frequency domain. This technique allows better characterization of the number of certain ligands which electronically couple to the metallocenter. Another technique used, XAS, is based on irradiation of the sample with monochromatic X-radiation, which ejects photoelectrons from the 1s or 2s or 2p shells. Relaxation to the ground state is accompanied by fluorescence emission, which is monitored for varied incident radiation frequencies. On a plot of intensity versus energy, the shape and position of the "edge" at which the energy is high enough to eject the

photoelectrons gives information about the identity, oxidation state, electronic structure, and geometric arrangement of the metal. At energies higher than the edge, there is a region of oscillatory behavior, described as extended X-ray absorption fine structure (EXAFS). Data from this region of the spectrum can be used to determine precise distances (± 0.01 Å) between a metal center and its ligands, and gives some information about the nature of the ligands (reviewed by (Scott 1985)). This behavior arises because a portion of the photoelectrons are back-scattered from atoms of nearby ligands, creating positive and negative interference depending on the atomic distance and the photoelectron wavelength (the wavelength varies with the X-ray energy). Lastly, spectroscopy in the UV-visible region of the electromagnetic spectrum can detect d-to-d transitions or metal-to-ligand- and ligand-to-metal-charge transfer (MLCT and LMCT) transitions in metalloproteins. The absorbance is caused by electronic transitions such as those between d orbitals on a metal or by electrons moving from a d orbital of the metal to an unfilled orbital of the ligand (or the reverse direction). For all these spectroscopic techniques, much information can be obtained by comparison with small model compounds (whose structures can be readily determined by X-ray crystallography) and by looking for differences between the resting enzyme and substrate-bound states.

Procedures

Isolation of TfdA and protein assay.

TfdA was prepared from *Escherichia coli* DH5α cells carrying the *tfdA* gene on plasmid pUS311, as described (Fukumori and Hausinger 1993b). Protein was

concentrated using centricon and centriprep ultrafiltration (Amicon) and the buffer was changed, if necessary, by repeated dilution and re-concentration. Protein concentrations were determined by using the BioRad assay with bovine serum albumin as a standard.

TfdA activity.

TfdA activity towards 2,4-D was measured in reaction mixtures containing α-KG (1 mM), ascorbic acid (50 μM), $(NH_4)Fe(SO_4)_2$ (50 μM or as specified), imidazole buffer (10 mM, pH 6.75) and 2,4-D (1 mM or as specificied). Inhibition by other divalent metals was tested by including the chloride salts (various concentrations) of these metals in the TfdA reaction mix.

Preparation and analysis of XAS samples

Samples were prepared by adding glycerol (final concentration 33-40%) to TfdA in MOPS buffer (pH 7, 10 mM) and then adding the metals and organic cofactors, and freezing in the XAS cuvette on dry ice. Samples were shipped frozen to the lab of Professor Robert Scott at the University of Georgia for XAS data collection, using the Stanford Synchrotron Radiation Lab (SSRL) and analyzed using published methods (Scott et al. 1992).

Preparation and analysis of EPR and ESEEM samples

Cu-TfdA samples for EPR and ESEEM spectroscopies were prepared and analyzed as described (Whiting et al. 1997). In addition, Cu-TfdA samples in the presence of D_2O and H_2O were prepared. The protein was exchanged into buffer containing MOPS (50 mM, pH or pD 7) in either H_2O or D_2O by two cycles of concentration and dilution (5-fold). Cu was added slowly dropwise to the concentrated

protein and then α-KG, 2,4-D or both was added to aliquots (the metals and substrates were prepared in the respective deuterated or protonated MOPS buffer). Samples were frozen by slow immersion into an ethanol-dry ice bath. Two-pulse and three-pulse ESEEM sequences were run. ESEEM data were fitted under the guidance of Professor John McCracken in the Chemistry department at Michigan State University. Fe-TfdA·NO samples were prepared anaerobically and EPR spectra were collected by Eric Hegg in Professor Lawrence Que's lab at the University of Minnesota using TfdA I provided.

Preparation and analysis of UV-visible samples

α-KG (0.75 mM), 2,4-D (0.75 mM) and/or NO were added anaerobically to TfdA (0.75 mM) that was reconstituted with stoichiometric amounts of ferrous ion, in MOPS buffer (25 mM, pH 6.9). Absorbance spectra were recorded over the UV and visible range by Eric Hegg.

Results

Inhibition of TfdA by selected divalent cations

TfdA requires added ferrous ion for activity; no other divalent metals tested were able to replace Fe(II) in supporting turnover by TfdA (Fukumori and Hausinger 1993b). Nevertheless, some other metals are capable of interacting with TfdA, since they showed inhibition of the Fe(II)-dependent activity (relative to 50 μM Fe(II)) as measured colorimetrically by phenol release. Cu(II) showed the strongest inhibition, completely abolishing TfdA activity at the lowest concentration tested, 10 μM, while Zn(II) and

Mn(II) inhibited by 75% and 50% respectively at this same concentration. Co(II) showed moderate inhibition; i.e., reducing activity by 40% at 50 μM. Ni(II) was weakly inhibitory (80% active at 500 μM) while Mg(II) did not affect the activity at concentrations up to 500 μM. Adam Whiting extended these studies to show that Cu(II) is a competitive inhibitor with respective to Fe(II), with a K_i of 1-3 μM (Whiting et al. 1997), which indicates that this cation is likely binding to the same site as is Fe(II). This finding is an important for interpreting the spectroscopic studies of Cu-substituted TfdA, since it suggests that copper binds the same site on the protein as does iron.

EPR and ESEEM spectroscopies of TfdA

To study a ferrous ion-dependent metalloprotein by EPR spectroscopy, it is necessary either to use a substitute divalent metal that is paramagnetic or to add the radical NO to the Fe(II) form of the enzyme, since Fe(II) alone is EPR silent. We carried out both alternatives in these studies, using Cu-substituted TfdA and NO-bound Fe(II)-TfdA. EPR spectra of Cu(II)-TfdA indicates that the metallocenter ligands are tetragonally arranged, with a mix of N/O ligands in the equatorial plane (Figure 19), (Whiting et al. 1997)). The spectrum is unchanged if 2,4-D is added, but does show changes if α-KG is added. The same changes can be produced by glutarate, indicating that the observed features of the EPR spectrum depend only on the carboxyl group, not the keto group. The ternary complex Cu-TfdA·α-KG·2,4-D has an EPR signal characteristic of a more rhombic (non-symmetrical) environment. The EPR spectrum of the ternary complex resembles those of substrate-bound Cu-substituted enolase and ribulose-1,4-bisphosphate carboxylase/oxygenase (Dickinson et al. 1980; Styring and

Branden 1985) which have 6 oxygen ligands. The slightly broader signals for the TfdA complex compared to these two enzymes suggests the presence of a nitrogen ligand. The presence of nitrogen ligands was confirmed by ESEEM analysis: Cu-TfdA, with or without α-KG, showed peaks indicating at least two imidazoles serving as metal ligands. The signal for one of these imidazoles is lost in the ternary Cu-TfdA·α-KG·2,4-D complex. This disappearance indicates either displacement of one of the imidazoles upon 2,4-D binding to the Cu-TfdA·α-KG complex or rearrangement of the principle axes so one of the imidazoles is axial rather than equatorial and thus contributes minimally to the ESEEM spectrum.

When the EPR spectroscopy was carried out in the presence of imidazole, two equatorial imidazoles were observed in addition to the two histidines of Cu-TfdA. This indicates that there are at least two open binding sites for the resting enzyme. The spectrum in the presence of α-KG is less readily interpreted, but suggests that free imidazole binds this complex as well. Free imidazole does not bind the ternary complex with 2,4-D and α-KG.

TfdA containing the native metal, complexed with NO, was also studied by EPR. The results show (surprisingly) little difference between the nitrosylated resting enzyme and the substrate-bound Fe-TfdA·NO·α-KG and Fe-TfdA·NO·α-KG·2,4-D complexes. Thus the shape of the EPR signal appears to be dominated by the Fe-N-O coupling with little electronic interaction arising from other ligands.

The presence of water ligand(s) in various states of the Cu-bound enzyme was studied by using ESEEM spectroscopy and comparison of the spectra in D_2O and H_2O. In

general, deuterium gives a much more intense modulation of the envelope for Cu centers than do protons. The shape of the envelope for a metal center with multiple ligands can be described, to a first approximation, as the product of the contributions from the individual ligands. Thus, by dividing the ESEEM spectrum in the presence of D_2O by that in the presence of H_2O (keeping the other conditions the same), it is possible to isolate the contribution of D_2O to the spectrum (McCracken et al. 1987; Mims et al. 1984). The modulations show up as broad peaks in the plots of echo intensity versus τ with a distance between the peaks of ~500 nsec, corresponding to a peak at 2 MHz in the Fourier transform (e.g Figure 20A). Strong deuterium modulation of the ESEEM was observed for the resting enzyme, which was not much changed by the addition of α-KG (e.g. Figure 20B and Figure 21B). Subsequent addition of 2,4-D (with α-KG present) caused a loss of deuterium modulation, as shown by the peak at 2 MHz for the comparison of Cu-TfdA·α-KG to Cu-TfdA·α-KG·2,4-D (Figure 20C). The disappearance of the deuterium modulation upon addition of 2,4-D suggests that the herbicide binds by displacing a water, or possibly by shifting the water to a long axial position. The effect was not as clear with 2,4-D addition to the resting enzyme (i.e., compare Cu-TfdA with Cu-TfdA·2,4-D, Figure 21C) because of the deep deuterium modulation in the Cu-TfdA spectrum which attenuates the spectrum, but possibly 2,4-D also displaces a water from the resting enzyme.

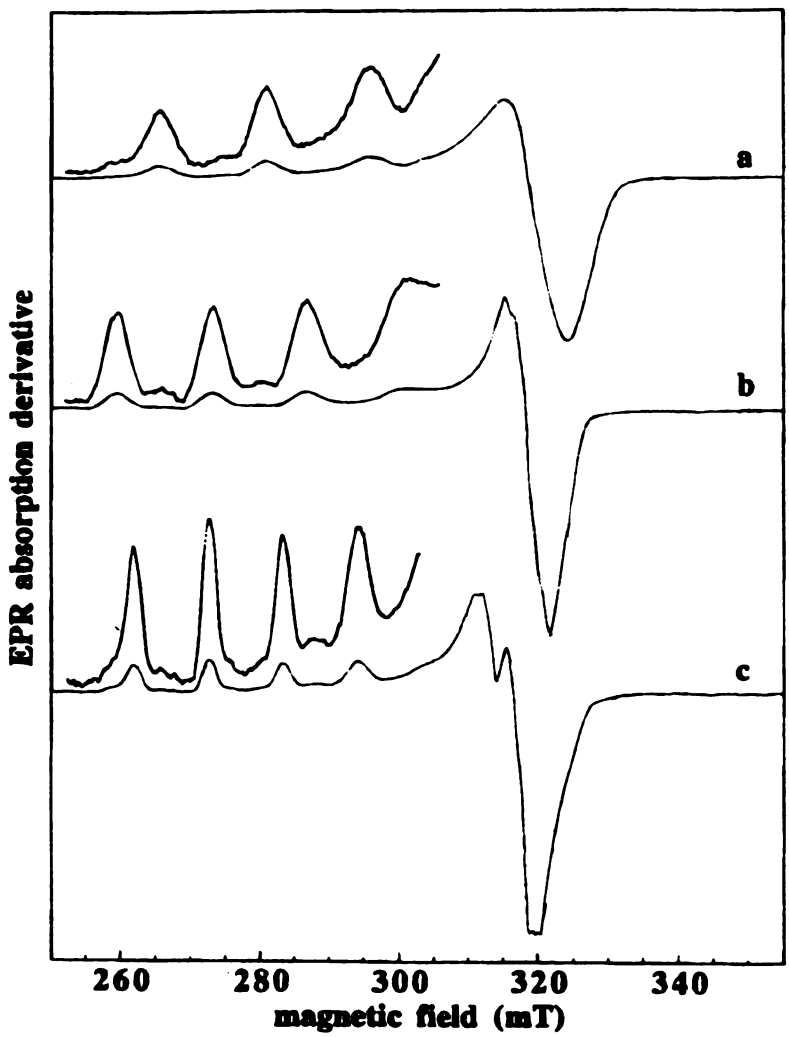


Figure 19. EPR spectra of resting and substrate-bound Cu-TfdA.

(A) Cu-TfdA ($g_{\perp}$=2.07, $g_{\parallel}$=2.29, $A_{\parallel}$=16.1 mK, 4.2 mT fwhm at 273 mT) (B) Cu-TfdA + 5 mM α-KG ($g_{\perp}$=2.07, $g_{\parallel}$=2.36, $A_{\parallel}$=14.9 mK, 4.2 mT fwhm at 273 mT) (C) Cu-TfdA + 5 mM α-KG + 5 mM 2,4-D ($g_{\perp}$=2.08, $g_{\parallel}$=2.37, $A_{\parallel}$=12.0 mK, 2.2 mT fwhm at 273 mT). Insets show $g_{\parallel}$ with intensity magnified 5 times. Spin quantitation indicates that all of the added Cu is bound in all three cases. EPR spectra were obtained at 20 K as single 4 min scans from 220 to 380 mT using 0.20 mW power at 9.23 GHz, 1.0 mT modulation amplitude.

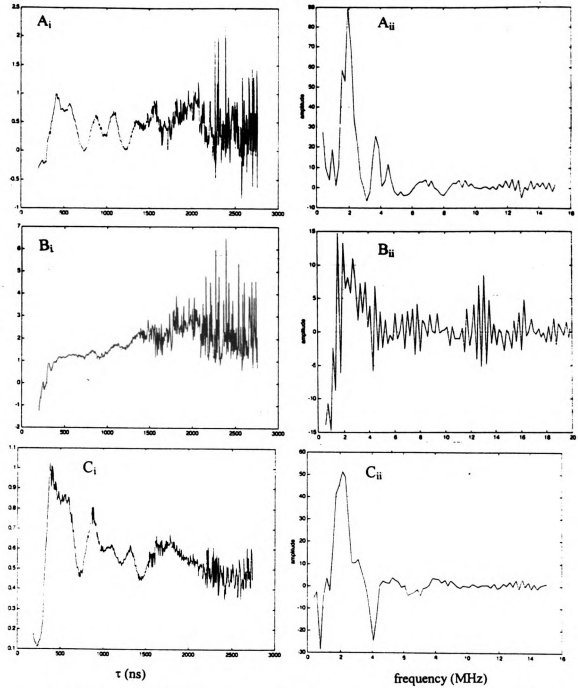


Figure 20. Two-pulse ESEEM spectra of resting and substrate-bound Cu-TfdA in deuterated versus protonated solvent.

Spectrum in D_2O/spectrum in H_2O for (A) Cu-TfdA (B) Cu-TfdA divided by Cu-TfdA·α-KG (C) Cu-TfdA·α-KG divided by Cu-TfdA·α-KG·2,4-D (i) echo intensity versus τ (ii) Fourier transform- normalized intensity versus frequency. Two pulse ESEEM spectra were obtained at 4.2 K as the average of four scans with τ from 0.12 to 3 μsec,. field of 3050 G, 8.8-8.9 GHz.

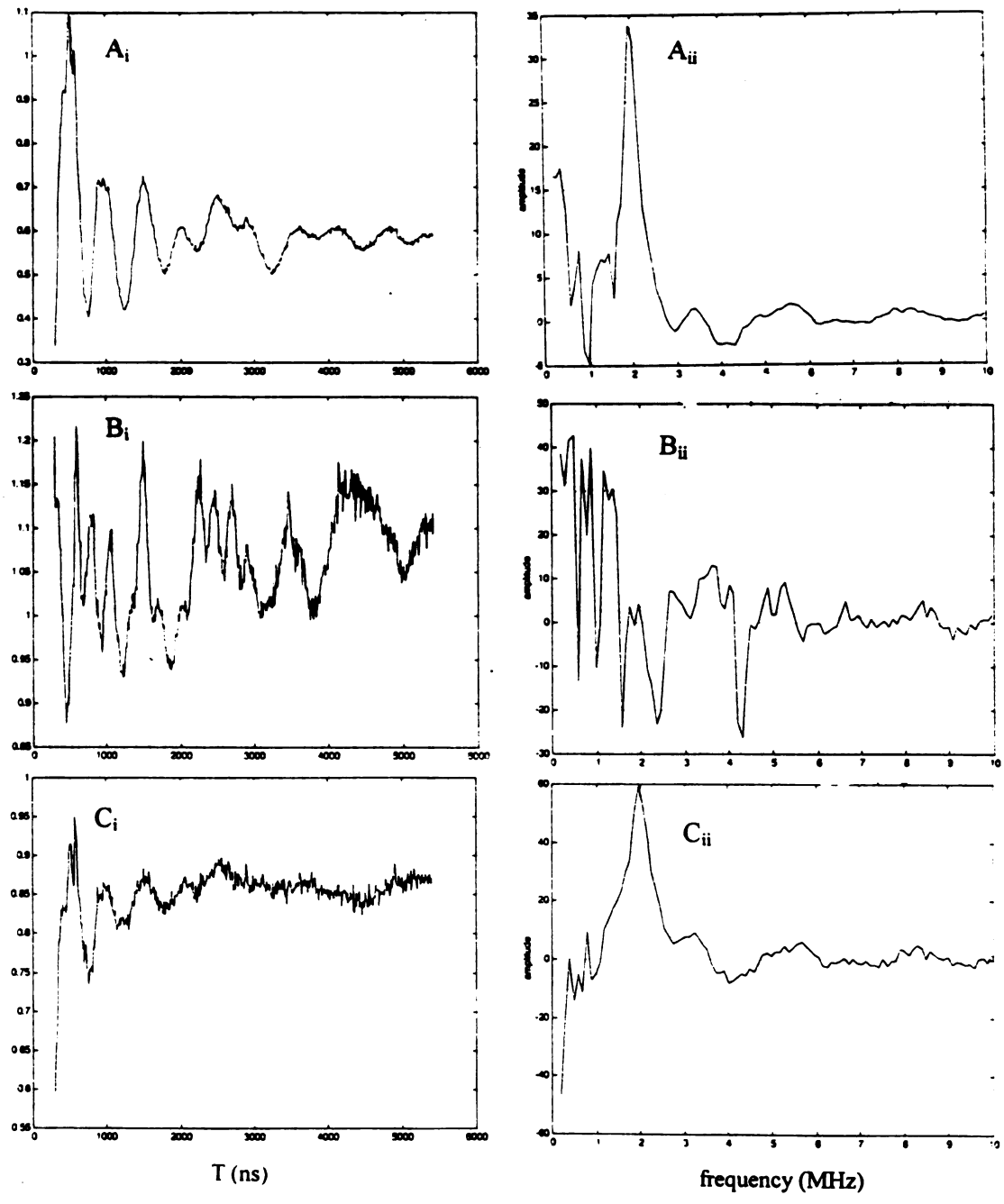


Figure 21. Three-pulse ESEEM spectra of resting and substrate-bound Cu-TfdA in deuterated versus protonated solvent.

Spectrum in D_2O/spectrum in H_2O for (A) Cu-TfdA (B) Cu-TfdA divided by Cu-TfdA·α-KG (C) Cu-TfdA divided by Cu-TfdA·2,4-D. Two pulse ESEEM spectra were obtained at 4.2 K as the average of four scans with τ=0.250 μsec, T from 0.12 to 3 μsec, constant field of 3050 G, 8.8-8.9 GHz,

UV-visible spectroscopy of TfdA

The UV-visible spectrum of Fe-TfdA under anaerobic conditions develops a MLCT transition centered at approximately 524 nm with an absorbance coefficient (ε) of 190 $M^{-1}cm^{-1}$ (Figure 22). By comparison with Fe(II) model compounds incubated with pyruvate, this spectrum suggests bidentate binding of iron via one oxygen atom of the carboxylated group and the keto group of the α-KG. Upon binding of 2,4-D as well, the absorbance is somewhat blue-shifted and becomes sharper so that the shoulders become more resolved. The fact that absorbance is still observed in this region upon addition of 2,4-D indicates that α-KG remains chelated in the ternary complex. Upon addition of NO to the Fe-TfdA·α-KG·2,4-D complex, an intense (ε=1300 $M^{-1}cm^{-1}$) absorbance at 445 nm is generated, characteristic of nitrosylated Fe(II), indicating that there is a free site capable of binding NO or that this gas displaces a ligand. It is not possible to determine whether α-KG remains bound in a bidentate fashion upon addition of NO because of the high background around 500 nm.

EXAFS of TfdA

Both copper- and iron-substituted TfdA samples were studied by using XAS methods. Representative plots of edge and EXAFS data collected with TfdA are shown in Figure 23. The portion of the curve from which the EXAFS was obtained (higher energy than the edge, Figure 23A) can be considered as the sum of the behavior that would be observed for the metal in the absence of ligands (i.e., as a gas) plus the oscillatory behavior due to the ligands (the EXAFS, χ); the latter can be isolated using curve-fitting techniques. These EXAFS data were transformed to **k** space, where **k** is the

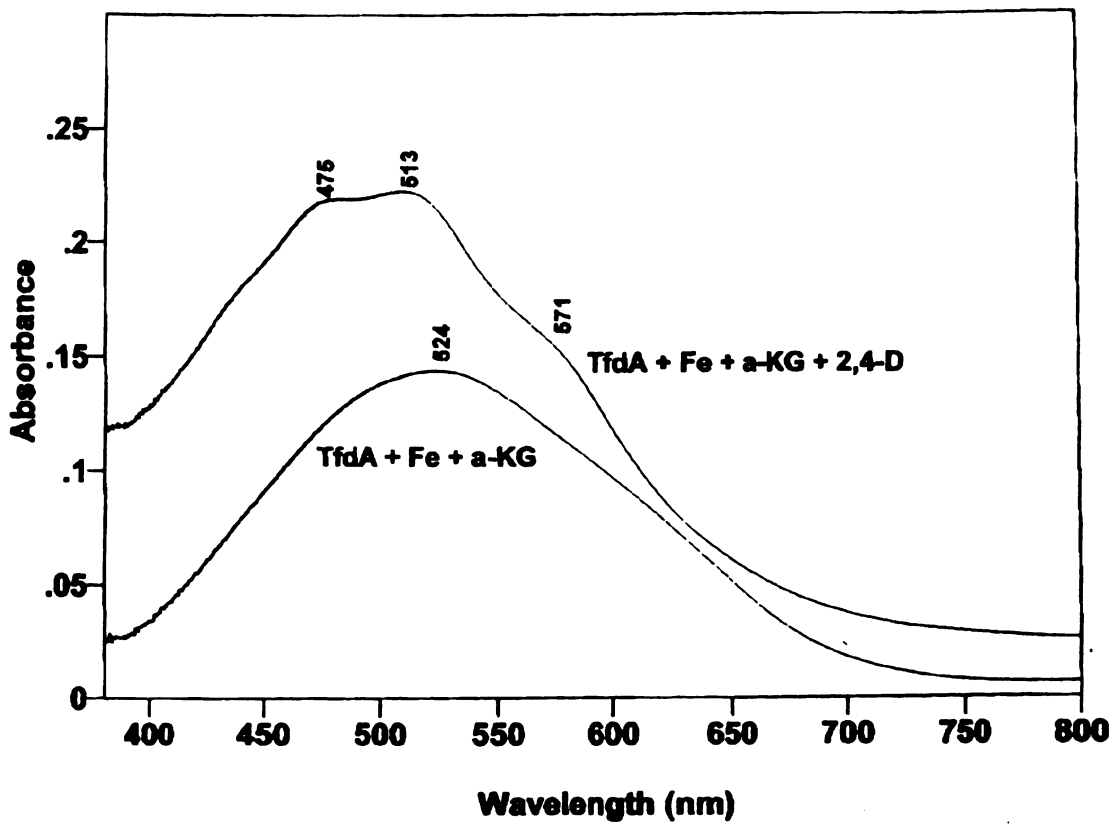


Figure 22. UV-visible spectra of substrate-bound Fe-TfdA.

TfdA (0.75 mM) was reconstituted anaerobically and electronic absorbance was recorded from 400 to 800 nm; spectra corrected for background absorbance of Fe-TfdA. Sample contain Fe-TfdA + 0.75 mM α-KG (lower spectrum) or Fe-TfdA + 0.75 mM α-KG + 0.75 mM 2,4-D (upper spectrum)

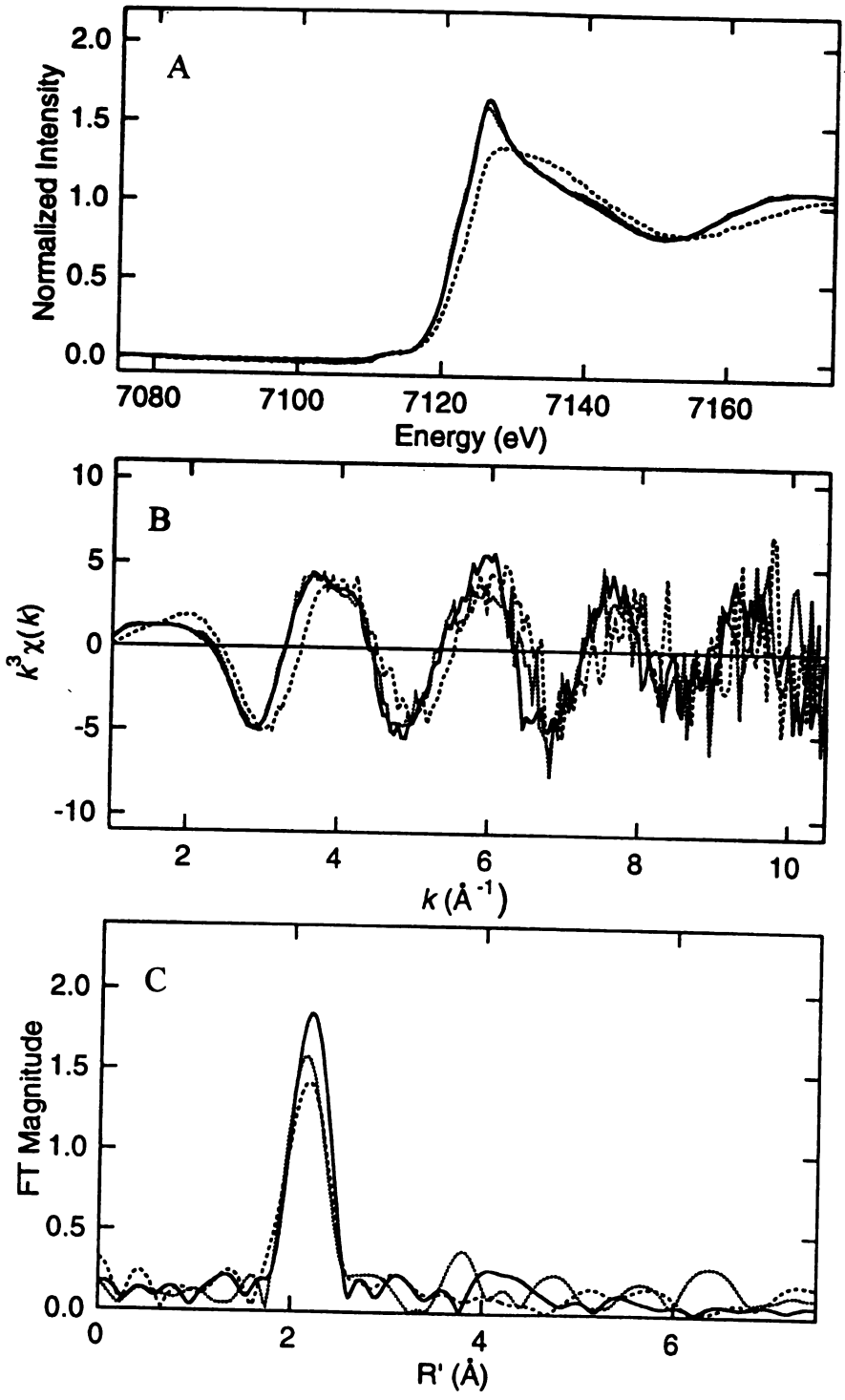


Figure 23. EXAFS spectra of resting and substrate-bound Fe-TfdA.

Solid line: Fe-TfdA long dashed line: Fe-TfdA + α-KG short dashed line: Fe-TfdA + 2,4-D (A) Fluorescence intensity versus X-ray energy (B) **k**-space (C) Fourier transform into R′ space

photoelectron wave vector and is related to its wavelength λ by the equation $\mathbf{k}=2\pi/\lambda$ (Fig 23B). A Fourier transform is typically used to convert this data into density of electron scatterers as a function of distance R′ (Fig. 23C); the actual distance R from the metal is related to R′ by addition of a phase shift (typical values -0.5 to -0.2Å) that depends on the particular scatterer.

The copper-substituted enzyme showed an intense peak at 2 Å and two weaker peaks at 3 and 4 Å in R′ space, characteristic of histidines in the ligand sphere, and agreed with spectra for $[Cu(imid)_4]^{2+}$. The spectrum was best fit assuming 4-coordination, or possibly 5-coordinate if there is an axial ligand with a long bond, and the ligands include ~ 2 histidines. No change was observed upon binding of α-KG, whereas the signature of one of the histidine peaks was lost when 2,4-D was added (with or without α-KG present). For copper, Jahn-Teller distortion can occur, so that the histidine could appear to be displaced but may simply reflect rearrangement of the ligand to an axial position. When the physiological metal was used in the XAS studies, resting TfdA showed ~2 imidazole ligands from a total coordination number of five or six according to EXAFS analysis, which is supported by comparison of the area of the pre-edge region in the XAS spectrum with corresponding areas in the spectra of five- and six-coordinate model compounds. No changes in the EXAFS were observed for Fe-TfdA upon binding of α-KG, but one histidine was lost upon binding of 2,4-D. Because Fe sites are much less affected by Jahn-Teller effects than Cu sites, the loss of the imidazole ring cannot be fit by modeling a rearrangement in which the principle axis rearranges so that the histidyl residue switches from being an equatorial to an axial ligand.

Discussion

Effect of various metals on α-KG-dependent dioxygenases

The strong inhibition of TfdA by Cu(II) and moderate inhibition by Mn(II) and Zn(II) is consistent with results for other α-KG-dependent dioxygenases, although the exact patterns differ. Cu(II) and Zn(II) strongly inhibit both flavanone synthase (an α-KG-dependent desaturase) (Britsch 1990), flavanol hydroxylase (Britsch and Grisebach 1986), and taurine dioxygenase (Eichhorn et al. 1997); the latter enzyme was also somewhat inhibited by Co(II). Zn(II) was a competitive inhibitor with respect to Fe(II) for lysyl hydroxylase (Puistola et al. 1980b). Zn(II) and Pd(II) are competitive inhibitors with respect to Fe(II) in prolyl hydroxylase (Rapaka et al. 1976; Tuderman et al. 1977). For proline 4-hydroxylase, Co(II) and Zn(II) completely inhibited the hydroxylation, while Mn(II), Cu(II), and Hg(II) were somewhat inhibitory (Lawrence et al. 1996). Proline 3-hydroxylase is inhibited by Co(II), Zn(II) and Cu(II) (Mori et al. 1996). Hyoscamine 6β-hydroxylase is strongly inhibited by Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II), somewhat by Ca(II) or Fe(III), and not by Mg(II) (Hashimoto and Yamada 1987) . Thymidine 2'-hydroxylase is inhibited strongly by Co(II), Ni(II), Cu(II) and Zn(II), and somewhat by Mn(II) (Bankel et al. 1972). The internal α-ketoacid dioxygenase HPPD was not inhibited by Li(I), Mg(II),Ca(II), Cr(III), Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Rb(I), Sr(II), Pd(II), Cd(II), Sn(II),Cs(I), Be(II), Pb(II) or MoO_4^{2-} but it was inhibited 30-50% by 1 mM Fe(II), Y(III) and Ln(III) (Lindblad et al. 1977). Surprisingly, Fe(II) was not required for activity of HPPD, which was attributed to the fact that the iron is tightly bound in this enzyme and not lost during the purification

(*ibid.*). Proline 3-hydroxylase exhibits less than 10% activity with Co(II), Cu(II) or Zn(II) at 1 mM (Mori et al. 1997)

Ligands of resting TfdA include histidine, water, and likely aspartate

The metallocenter in resting TfdA is five- or six-coordinate, as indicated by EPR of the Cu form and XAS of the Fe form. The EXAFS data on Cu(II)-TfdA only shows four ligands, but Jahn-Teller distortion can occur with this metal, resulting in longer axial bonds, making the axial ligands difficult to detect by EXAFS. At least two of the ligands are likely to be histidines, based on spectroscopic evidence from both ESEEM and XAS methods. The presence of two histidine ligands is consistent with the involvement of the His_{113}-X-Asp_{115}-X_{51}-His_{167} sequence in metal binding. Similar motifs have been shown to be conserved in other α-KG-dependent dioxygenases and these residues are thought to be metallocenter ligands according to evidence derived from site-directed mutagenesis studies and by comparison with the structure of IPNS (Borovok et al. 1996; Jia et al. 1994; Lamberg et al. 1995; Lukacin and Britsch 1997; McGinnis et al. 1996; Myllyharju and Kivirikko 1997; Pirskanen et al. 1996; Roach et al. 1995; Roach et al. 1997). Thus, the spectroscopically observed histidine ligands in TfdA likely include His_{113} and His_{167}. Additionally, I speculate that Asp_{115} is also a ligand, consistent with the observation of five or six N/O ligands for Fe(II)-TfdA. The deep deuterium modulation of the ESEEM spectra of Cu-TfdA suggest multiple water ligands and a relatively water-accessible active site.

α-KG binds iron of TfdA in a bidentate manner

Both the Cu- and Fe-TfdA species bind α-KG. Addition of α-KG to the copper form of the protein perturbed the EPR signal compared to that in resting enzyme, but α-KG likely does not chelate the metal ion. No differences were noted by XAS. Upon addition of α-KG to Fe-TfdA under anaerobic conditions this cofactor chelates the metal through its C-1 carboxyl oxygen and its keto oxygen, as indicated by the generation of a MLCT band in the UV-visible spectrum characteristic of bidentate binding. The lack of change in the EXAFS upon binding of α-KG is somewhat surprising in view of the close proximity of the α-KG necessary to allow charge transfer from the metal. A plausible explanation is that α-KG oxygens displace water (oxygen) ligands, which are not distinguishable from the α-KG oxygens by EXAFS (e.g. EXAFS data of IPNS predicted bidentate binding of a carboxyl group (Randall et al. 1993), but crystallography indicated it was monodentate, with the second oxygen coming from a water ligand (Roach et al. 1995)). The binding of cosubstrate to Fe-TfdA resembles that reported for the clavaminate synthase (Pavel et al. 1998) in that α-KG is thought to bind in a bidentate fashion. The total number of ligands for Fe-TfdA according to the XAS analysis was five or six; the latter would agree with octahedral coordination reported for clavaminate synthase (*ibid.*) and IPNS (Roach et al. 1995).

2,4-D binding displaces a ligand

The binding of 2,4-D to enzyme was difficult to interpret because it depended on the type of spectroscopy in use. Substrate added to Cu-TfdA caused little change in the EPR spectrum, whereas changes were observed in the EXAFS for both the copper and

iron forms of the enzyme. Analyses of the EXAFS data for both the iron and the copper forms indicate that one of the imidazoles is displaced compared to the resting state, which does not agree with the EPR results on the copper form of the enzyme. It must be kept in mind, however, that the XAS results on the copper form are ambiguous because of possible Jahn-Teller distortion.

The ternary complex Cu-TfdA·α-KG·2,4-D was analyzed by EPR and ESEEM and indicated that one imidazole is either displaced or rearranged to an axial position (compared to Cu-TfdA·α-KG), that a water ligand is lost, and that the center is hexacoordinate. XAS of the copper form of the ternary complex also indicates that there is one less histidine than in Cu-TfdA The ternary complex Fe-TfdA·α-KG·2,4-D also is distinct from the Cu-TfdA·α-KG species, as shown by UV-visible spectroscopic studies. The numbers of histidine ligands in the two species were not compared because of the lack of detail in the Fe-TfdA·NO EPR spectra and the difficulty of maintaining anaerobic conditions while preparing the XAS samples.

As pointed out by Hegg and Que (Hegg and Que Jr. 1997), the motif of two histidines and one carboxyl group forming the ligands on one side of the iron ("2-His-1-carboxylate facial triad") seems to be widespread among non-heme iron(II) enzymes. The main evidence for this motif is from crystal structures of five oxygenases for different classes of enzymes. In tyrosine hydroxylase (TyrH) and 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), the carboxyl group is provided by a glutamyl residue; for IPNS and iron superoxide dismutase, it is provided by an aspartyl residue, and for soybean lipoxygenase, from the carboxyl group of a terminal isoleucine. The data reported here

are compatible with TfdA being included in the "2-His-1-carboyxlate facial triad" family as ilustrated schematically in Figure 24

Acknowledgments

The metal inhibition studies were performed by myself and Adam Whiting. The EPR and UV-visible measurements and analysis were performed in the lab of Dr. Lawrence Que (University of Minnesota) by Adam Whiting and Eric Hegg on enzyme I prepared. The ESEEM data collection and analysis was performed by Dr. John McCracken (Michigan State University), Matthew Fredrick and myself. The XAS measurements and data analyses were performed by members of Dr. Robert Scott's (University of Georgia) laboratory including Chris Colangelo, Jun Dong, Christina Stålhandske and Nate Cosper on frozen samples I prepared.

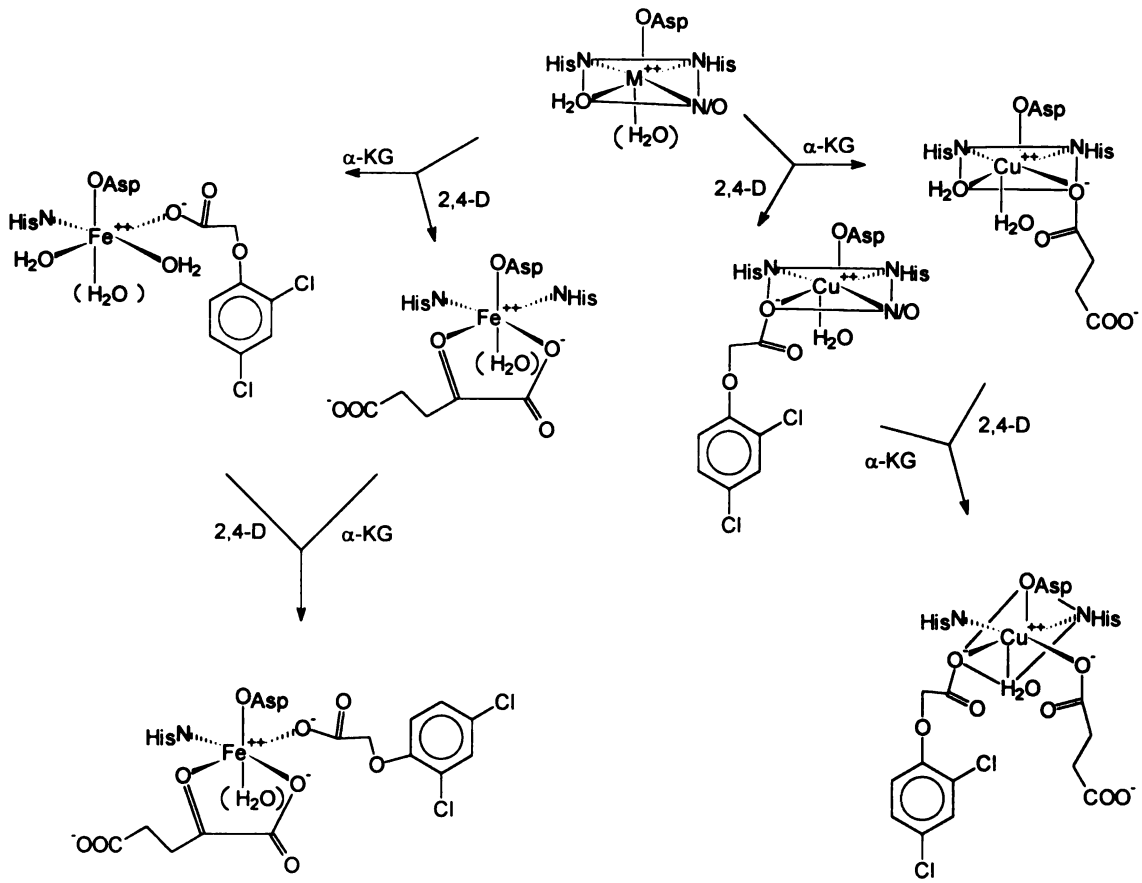


Figure 24. Proposed ligand identity and geometry for resting and substrate-bound TfdA.

M indicates either Fe(II) or Cu(II). The presence of the histidines in the resting enzyme is supported by both EXAFS, EPR and ESEEM. The presence of an aspartyl ligand is consistent with the EXAFS results and predicted by sequence comparisons of TfdA with other α-KG-dependent enzymes and with IPNS. The binding mode for α-KG for the iron-substituted enzyme was demonstrated by UV-visible spectroscopy. Some difference exist between the EXAFS and EPR/ESEEM spectroscopy results with respect to the effect of substrate binding to Cu-TfdA; the figure shows an option consistent with the latter. Displacement of a histidine upon binding of 2,4-D to Fe-TfdA was predicted by EXAFS. The presence of multiple water ligands was shown for Cu-TfdA by ESEEM spectroscopy in deuterated solvent, and is consistent with EXAFS and EPR results.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

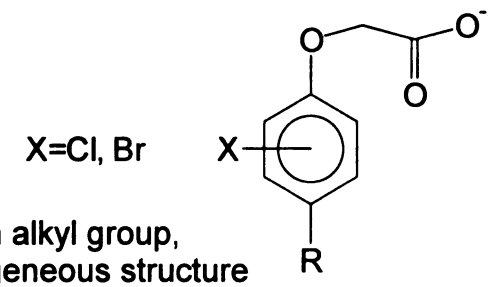
Model of TfdA reactivity

My kinetics and spectroscopy studies reported in this thesis give insight into the mechanism and metallocenter properties of TfdA. My results may help other scientists to better understand alternative α-KG-dependent enzymes, particularly the subclass that show ~30% sequence identity to TfdA (TauD (Eichhorn et al. 1997) and two putative α-KG-dependent dioxygenases identified by their related open reading frames from *Sacharomyces cerevisiae* and *Mycobacterium tuberculosis*). Work is ongoing in the Hausinger lab to spectroscopically characterize TauD and to express the gene encoded by the open reading frame from *S. cerevisiae*. Ascorbic acid is necessary or stimulatory to the activity of many α-KG-dependent dioxygenases and related enzymes, including prolyl hydroxylase (Nietfeld and Kemp 1981; Tuderman et al. 1977), TfdA (Fukumori and Hausinger 1993b), proline 3-hydroxylase (Mori et al. 1996) and hyoscyamine 6β-hydroxylase (Hashimoto and Yamada 1987). For prolyl hydroxylase, this requirement for reductant was shown to be due to uncoupled reaction cycles which left the metallocenter in an oxidized (e.g., Fe(III)) state, which could be reduced by addition of ascorbic acid (De Jong and Kemp 1984; Myllylä et al. 1984). For TfdA, I showed that when TPAA is the substrate, ascorbic acid is required for activity, but the enzyme was not uncoupled. Rather, TfdA was inactivated in an oxygen-dependent process prior to turnover, in a reaction that was partly ascorbate-reversible. This alternate role for ascorbic acid is important to keep in mind when interpreting results with other α-KG dependent dioxygenases, as opposed automatically attribute an ascorbate requirement to

uncoupling. I think the reversible type of inactivation I observed with TPAA as a substrate is a result of oxidation of the metallocenter; however, addition of Fe(II) did not re-activate the enzyme, likely indicating that the oxidized metal was not freely exchangeable. The ability of ascorbic acid to reverse this type of inactivation indicates that it has access to the active site, consistent with studies of prolyl hydroxylase showing competitive inhibition of hydroxybenzenes and benzoic acids with respect to ascorbate (Majamaa et al. 1986).

My examination of substrate analogs has provided insight into the regio- and stereospecificity of the reaction carried out by TfdA from *Ralstonia eutropha* JMP134. By use of an analog in which the ether oxygen was replaced by sulfur (i.e., TPAA), I showed that the reaction was regioselective for the C-2 methylene, since no oxidation occurred on the sulfur. An analog with a methyl group on the C-2 carbon (i.e., dichlorprop) was used to show that the reaction was stereospecific for catalyzing hydroxylation at the *pro(S)* position, since only the (R)-enantiomer (which has a methyl group blocking hydroxylation from the R-position) was a substrate. The studies that I have carried out with substrate analogues demonstrate the utility of this approach for characterizing TfdA. Additional studies with alternative compounds are in progress by others in the Hausinger lab. Such studies can better define the activity of TfdA-like enzymes in the environment, as well as allow further characterization of the TfdA active site structure and mechanism. As well, characterization of the substrate range may lead to bioremediation applications for structurally related recalcitrant compounds such as the breakdown products of alkylphenol polyethoxylates. The parent compounds are widely

used industrial surfactants, and chlorination of wastewaters can produce recalcitrant brominated and chlorinated carboxylated degradation products (Ball and Reinhard 1985; Ball et al. 1989; Reinhard et al. 1982) that include halogenated alkylphenoxyacetic acids (Scheme 15), which are structurally similar to the phenoxy herbicides.



Scheme 15 General structure of halogenated alkylphenoxyacetic acids

My spectroscopic studies have revealed the identity of some of the ligands for the iron of TfdA and the changes that occur upon binding of substrates. The presence of two histidines out of five (or possibly six) N/O ligands in the resting enzyme was confirmed by electron paramagnetic resonance (EPR), electron spin echo envelope modulation (ESEEM), and extended X-ray absorbance fine structure (EXAFS) spectroscopies. The two histidines are likely residues 113 and 167, and one of the unspecified N/O ligands is likely Asp115, based on their forming a His-X-Asp-X_{53-57}-His metal-binding motif as has been seen for other α-KG-dependent dioxygenases (Borovok et al. 1996; Jia et al. 1994) and would be consistent with a 2-His-1-carboxylate facial triad as has been seen for other non-heme dioxygenases (Hegg and Que Jr. 1997). Site-directed mutagenesis studies in progress in the Hausinger lab are also consistent with these residues being important to activity (Deborah Hogan, pers. comm.). The binding of α-KG to the metallocenter was

demonstrated to be bidentate, as shown by generation of a metal-to-ligand charge transfer band in the anaerobic enzyme. This result provides direct evidence for the binding mode suggested previously on the basis of prolyl hydroxylase inhibitor studies; i.e., compounds that were shown to be capable of chelating the iron (e.g., dicarboxypyridines) were potent competitive inhibitors with respect to α-KG (Majamaa et al. 1984). At least two solvent-exchangable sites remain when α-KG is bound, as indicated by the ability of two free imidazoles to bind the metallocenter (observed by EPR spectroscopy), probably at sites normally bound by water molecules. The presence of bound water also is indicated by ESEEM spectroscopy in the presence of deuterated solvent. The UV-visible spectral results extends previous studies with clavaminate synthase (Pavel et al. 1998) which also indicated formation of this α-KG-dependent charge-transfer band by showing that the bidentate binding is maintained in the presence of hydroxylatable substrate, and provided evidence that an additional site exists to which the oxygen analog NO can bind. Some differences were seen between the EPR/ESEEM and EXAFS results upon substrate binding to TfdA. XAS results indicated loss of one of the His ligands upon binding of 2,4-D to the ferrous (and possibly the cupric) form of the enzyme, whereas no changes were observed by EPR methods upon binding of this substrate to the cupric form, but changes are observed with α-KG binding. These studies have specified some of the metallocenter ligands in the resting and substrate-bound states of the enzyme, but there is still much to learn about the geometry of the active site and what happens at the metallocenter during turnover. In particular, a crystal structure of TfdA or another α-KG-dioxygenase would be helpful, and collaborative efforts are underway between the

labs of Robert Hausinger and Peter Roach (University of Oxford, UK) to prepare suitable crystals of TfdA and TauD. The insights gained from the use of substrate analogs and from spectroscopy of TfdA are combined into a model of its reactivity in Figure 25.

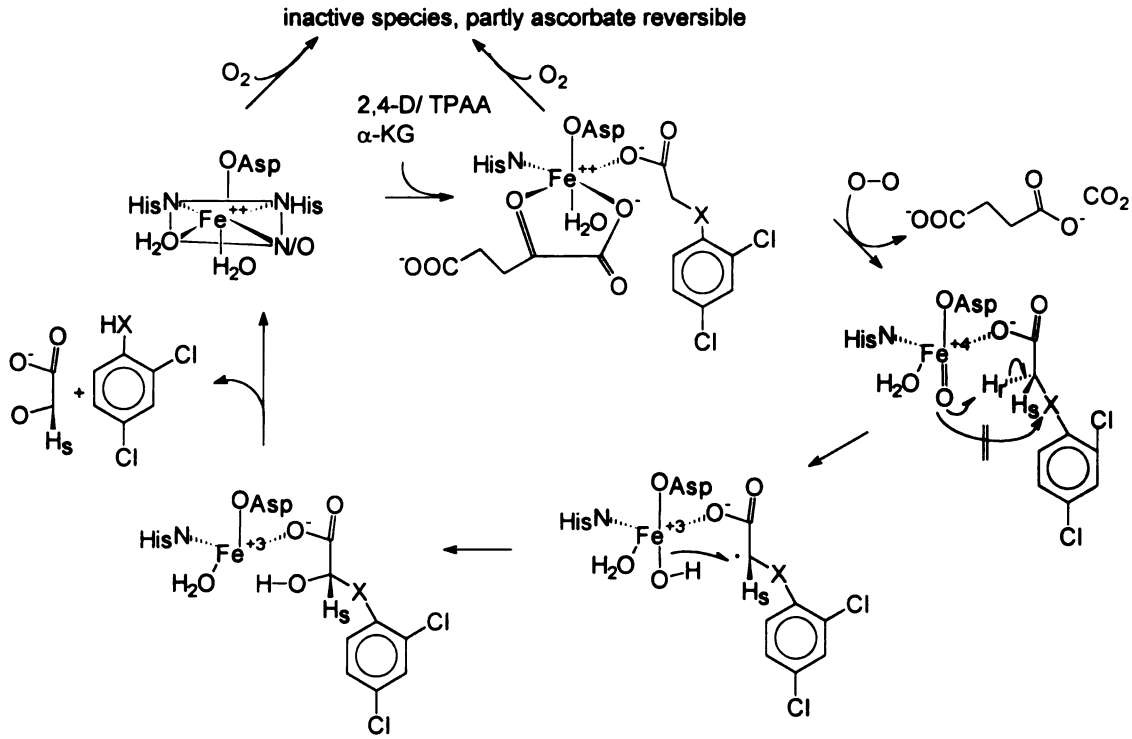


Figure 25 Model of TfdA reactivity

The oxidative inactivation of TfdA is based on results described in chapter 2, the stereochemistry of oxidation is based on results from chapter 3, and the metallocenter ligands are based on data and discusion in chapter 4. The nature of the oxidizing intermediate is based on studies of other α-KG dependent dioxygenases as described in the introduction (chapter 1).

Diversity of bacterial enzymes involved in breaking the ether bond of phenoxy herbicides

The diversity within selected 2,4-D/α-KG dioxygenases was studied at the level of substrate specificity and sequence. Two strains isolated as 2,4-D degraders,

Burkholderia cepacia RASC and *R. eutropha* JMP134 showed preference for this compound rather than its chiral analog dichlorprop, and only degraded the S-enantiomer of dichlorprop. By contrast, *Alcaligenes denitrificans,* a strain isolated for its ability to degrade the chiral herbicide mecoprop, only degraded the R-enantiomer of dichlorprop, and had little activity towards 2,4-D. Opposite stereopreferences have also been observed in a dichlorprop-degrading isolate *Sphingomonas herbicidovorans* which has α-KG-dependent dioxygenases, one of which degrades (R)-dichlorprop and the other which degrades (S)-dichlorprop (Nickel et al. 1997). As I reported in Chapter 4, genomic DNA of the *A. denitrificans* hybridized at high stringency to the *tfdA* gene of RASC, but not JMP134. The techniques reported here for determining stereospecificity of dichlorprop degradation could be applied to cell extracts or enzymes from other strains.

To extend the environmental significance of studies of TfdA to identify wider degrees of difference in the ether-cleaving enzymes of phenoxy herbicide degraders (i.e., members of other classes of enzymes), other techniques than studying the stereopreference are necessary. There is obviously more genetic diversity of 2,4-D ether-cleavage activities than currently can be characterized by *tfdA*-like genes, as shown by the large proportion of 2,4-D degraders among of soil isolates (Dunbar et al. 1997; Ka et al. 1994a; Ka et al. 1994b; Vallaeys et al. 1997) which failed to hybridize or be amplified by *tfdA*-specific probes or primers. The *tftA* and *tftB* genes of *B. cepacia* AC1100 encode the subunits of a non-heme NADH-dependent monooxygenase (Danganan et al. 1995; Xun and Wagnon 1995) that catalyzes cleavage of the ether bond in phenoxy herbicides; hence, *tftA* is one candidate for screening 2,4-D degraders to detect additional diversity.

For strains which do not seem to contain either *tfdA* or *tftA/tftB*, the enzyme class might be identified biochemically by observing cell extract activity with various cofactors, or genetically by complementation using either a plasmid which carries genes for the 2,4-D degradative pathway downstream from *tfdA* (Top et al. 1996) or a 2,4-D$^-$ mutant. Since hybridization would only detect ≥ 60% similarity, it would be advisable to check first whether the activity is due to a very distantly related α-KG dioxygenase by including this cofactor among those being tested. A good candidate class of enzymes that might carry out this reaction are cytochrome P450 enzymes. P450's capable of releasing phenols from phenoxy herbicides have been detected in plants (Topal et al. 1993) and mammals (Mehmood et al. 1996), and overlaps in the types of reactions carried out are seen in the giberellin biosynthetic pathway, in which some steps can be carried out both by a-KG-dependent dioxygenases and by P450s (reviewed by (Hedden 1997)). Diversity might also be observed in the pathways of 2,4-D degradation in anaerobes, perhaps involving hydrolases. Although the relative rates of degradation of phenoxy herbicides are generally much slower under anaerobic conditions (Harrison et al. 1998; Martens 1978; McBain et al. 1997; Sattar and Paasivirta 1980; Vink and van der Zee 1997; Yoshida and Castro 1975), in some cases phenoxy herbicide disappearance with concomitant phenol production (i.e., indicating cleavage of the ether bond) has been observed in reducing environments (Bryant 1992; Chang et al. 1998; Gibson and Suflita 1990; Gibson and Suflita 1993; Mikesell and Boyd 1985).

The studies described here give insight into the mechanism of TfdA with respect to the regio- and stereo-specificity, the active site, and the role of reductant. Additional

spectroscopic, genetic and kinetic experiments would fill in additional details about the mechanism and possible origin of this enzyme; some of these studies, such as site-directed mutagenesis of putative ligands, crystallization, and testing of substrate analogs, are currently in progress.

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