

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

PREVIEW

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

By

Ruth Elizabeth Saari

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Center for Microbial Ecology

1998

UMI Number: 9909399

**Copyright 1998 by
Saari, Ruth Elizabeth**

All rights reserved.

**UMI Microform 9909399
Copyright 1998, by UMI Company. All rights reserved.**

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

PREVIEW

Copyright by
RUTH ELIZABETH SAARI
1998

ABSTRACT

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

By

Ruth Elizabeth Saari

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.

PREVIEW

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 Slepkin, 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.

My members of my advisory committee, Drs. Craig Criddle, Shelagh Ferguson-Miller, Rawle Hollingsworth, and Jack Preiss also were helpful in guiding me through my dissertation research. I thank them for the time they took to critique my annual reports and this dissertation, and for helping me to be more hypothesis-driven in my research.

I am grateful to my husband Eric Saari for emotional support during the writing of this dissertation and practical assistance with the details of formatting and printing the document.

TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES.....	xii
LIST OF SCHEMES.....	xiv
LIST OF ABBREVIATIONS	xv
CHAPTER 1: INTRODUCTION	1
<i>2,4-D catabolism by R. eutropha JMP134</i>	2
Ether bond cleavage as the first step of 2,4-D degradation by <i>R. eutropha</i> JMP134 ..	4
Remaining steps in 2,4-D degradation by <i>R. eutropha</i> JMP134.....	6
<i>Environmental/Evolutionary Significance of TfdA</i>	7
<i>Relationship of TfdA to other α-KG-dependent dioxygenases</i>	16
Order of substrate binding and product release.....	22
Uncoupled reactions and the role of ascorbic acid.....	24
Crystallography, chemical modification and mutagenesis studies to identify key residues in catalysis.....	26
Metallocenter spectroscopy of α -KG-dependent dioxygenases and related enzymes	30
Nature of the reactive iron and oxygen-containing intermediate(s).....	32
Hydroperoxy mechanism	32
Oxyferryl mechanism.....	35
Other mechanisms.....	36
<i>Outline of thesis</i>	39
CHAPTER 2: ASCORBIC ACID-DEPENDENT TURNOVER AND REACTIVATION OF 2,4-DICHLOROPHENOXYACETIC ACID/ α -KETOGLUTARATE DIOXYGENASE USING THIOPHENOXYACETIC ACID	41
<i>Introduction</i>	42
<i>Experimental procedures</i>	45
Chemicals.....	45

Purification of TfdA.....	45
Assays of TfdA activity.	46
Synthesis of dehydroascorbic acid.....	48
Synthesis of phenylsulfinylacetic acid and phenylsulfonylacetic acid.....	48
Analysis for proteolysis.....	48
Numerical computations	49
<i>Results</i>	49
Demonstration that TPAA is a substrate for TfdA.	49
Thiophenol production from TPAA requires and consumes ascorbic acid.	51
α -KG decomposition is not uncoupled from substrate hydroxylation.....	58
Oxidative inactivation of TfdA.....	59
Kinetic constants for TfdA acting on thiophenoxyacetic acids and phenoxyacetic acids.	62
<i>Discussion</i>	64
TPAA-based assay for TfdA.....	64
Model to explain ascorbic acid dependency and enzyme inactivation during TPAA utilization.	64
What accounts for the differences in TfdA reactivity with 2,4-D and TPAA?.....	67
<i>Acknowledgments</i>	69
CHAPTER 3: STEREOSPECIFICITY OF PHENOXY HERBICIDE DEGRADATION	70
<i>Introduction</i>	71
<i>Methods</i>	74
Chemicals.....	74
Bacterial strains.....	74
Activity assay with colorimetric detection of phenol derivatives.....	74
Preparation of cell extracts and purified enzyme.....	75
Formation of diastereomers and Gas Chromatography (GC)	75
High Pressure Liquid Chromatography (HPLC).....	77
DNA hybridization.....	77
<i>Results</i>	78
TfdA from <i>R. eutropha</i> JMP134 is specific for (S)-dichlorprop	78
Stereospecificity of various phenoxy herbicide degraders towards dichlorprop	81
<i>A. denitrificans</i> contains a DNA sequence related to tfdA	86
<i>Discussion</i>	90
Stereochemical differences amongst α -KG dependent dioxygenases acting on dichlorprop	90
Environmental and biotechnology significance of bacterial enantiospecificity towards phenoxypropionate herbicides	92
<i>Acknowledgments</i>	95

CHAPTER 4: SPECTROSCOPY OF TfdA	96
<i>Introduction</i>	97
Metal dependence and metallocenter characterization of α -KG-dependent dioxygenases	98
Spectroscopic techniques used with TfdA	101
<i>Procedures</i>	102
Isolation of TfdA and protein assay.	102
TfdA activity.	103
Preparation and analysis of XAS samples	103
Preparation and analysis of EPR and ESEEM samples	103
Preparation and analysis of UV-visible samples.....	104
<i>Results</i>	104
Inhibition of TfdA by selected divalent cations	104
EPR and ESEEM spectroscopies of TfdA	105
UV-visible spectroscopy of TfdA	111
EXAFS of TfdA	111
<i>Discussion</i>	115
Effect of various metals on α -KG-dependent dioxygenases.....	115
Ligands of resting TfdA include histidine, water, and likely aspartate.....	116
α -KG binds iron of TfdA in a bidentate manner	117
2,4-D binding displaces a ligand.....	117
<i>Acknowledgments</i>	119
CONCLUSIONS AND FUTURE DIRECTIONS	121
<i>Model of TfdA reactivity</i>	122
<i>Diversity of bacterial enzymes involved in breaking the ether bond of phenoxy herbicides</i>	126
LIST OF REFERENCES	130

LIST OF TABLES

Table 1. Consumption of ascorbic acid by TfdA.....	55
Table 2. Kinetics values for TfdA substrates.....	63

PREVIEW

LIST OF FIGURES

Figure 1: Ribbon structure of Mn-IPNS	27
Figure 2. TfdA-catalyzed production of thiophenol from TPAA.	50
Figure 3. Effect of ascorbic acid concentration on the production of thiophenol from TPAA by TfdA.....	52
Figure 4. Stoichiometry of thiophenol production and ascorbate consumption during TPAA turnover.....	54
Figure 5. Effect of supplemental ascorbic acid on progress curves for the TfdA-catalyzed decomposition of TPAA.	57
Figure 6. Effect of the EDTA to Fe(II) ratio on the TfdA inactivation rate.....	62
Figure 7. Model of TfdA reactivity with 2,4-D and TPAA.	65
Figure 8: Phenoxyacetic and phenoxypropionic herbicides.....	72
Figure 9. Separation of the diastereomeric derivatives of (R)- and (S)-dichlorprop by GC with ECD detection.....	79
Figure 10. Loss of (S)-dichlorprop (relative to the R-enantiomer) catalyzed by TfdA.	80
Figure 11. Phenol production by TfdA acting on dichlorprop.....	81
Figure 12. Activity of cell extracts towards phenoxy herbicides.	83
Figure 13. Requirement for α -KG, iron and ascorbate to support activity of <i>A. denitrificans</i> cell extract.	84

Figure 14. Concentration dependence of <i>A. denitrificans</i> cell extract activities towards (R)-mecoprop and (R)-dichlorprop, measured by phenol production.....	85
Figure 15. Separation of (R)- and (S)-dichlorprop by HPLC using Nucleodex α -PM column.....	87
Figure 16. Stereospecificity of activity of cell extracts and purified protein towards racemic dichlorprop, measured by chiral HPLC.....	88
Figure 17. Southern blot of <i>A. denitrificans</i> genomic DNA hybridized with <i>A. tfdA</i> _{JMP134} or <i>B. tfdA</i> _{RASC}	89
Figure 18. Stereospecificity of hydroxylations catalyzed by α -KG-dependent dioxygenases.	91
Figure 19. EPR spectra of resting and substrate-bound Cu-TfdA.	108
Figure 20. Two-pulse ESEEM spectra of resting and substrate-bound Cu-TfdA in deuterated versus protonated solvent.	109
Figure 21. Three-pulse ESEEM spectra of resting and substrate-bound Cu-TfdA in deuterated versus protonated solvent.	110
Figure 22. UV-visible spectra of substrate-bound Fe-TfdA.	112
Figure 23. EXAFS spectra of resting and substrate-bound Fe-TfdA.....	113
Figure 24. Proposed ligand identity and geometry for resting and substrate-bound TfdA.....	120
Figure 25 Model of TfdA reactivity.....	126

LIST OF SCHEMES

Scheme 1. Pathway for 2,4-D degradation in <i>R. eutropha</i> JMP134.	3
Scheme 2. Reaction catalyzed by TfdA.	5
Scheme 3. Phenoxyacetic acid and phenoxypropionic acid herbicides.	10
Scheme 4. Reaction catalyzed by ACC oxidase.	18
Scheme 5. Reactions catalyzed by IPNS, DAOCS and DACS.	19
Scheme 6. Reaction catalyzed by HPPD.	20
Scheme 7. Reaction catalyzed by TauD.	21
Scheme 8. Hydroperoxy mechanism for α -KG-dependent dioxygenases.	34
Scheme 9. Oxyferryl mechanism for α -KG-dependent dioxygenases.	37
Scheme 10. Oxyferryl mechanism for IPNS.	38
Scheme 11. Reaction catalyzed by TfdA.	43
Scheme 12. Assay of TfdA using TPAA as a substrate.	44
Scheme 13. Reaction catalyzed by TfdA.	71
Scheme 14. Diastereomeric derivatives of dichlorprop for GC/ECD.	76
Scheme 15. General structure of halogenated alkylphenoxyacetic acids.	124

LIST OF ABBREVIATIONS

AA	L- δ -(α -aminoadipoyl)
ACC	l-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

PREVIEW

CHAPTER 1

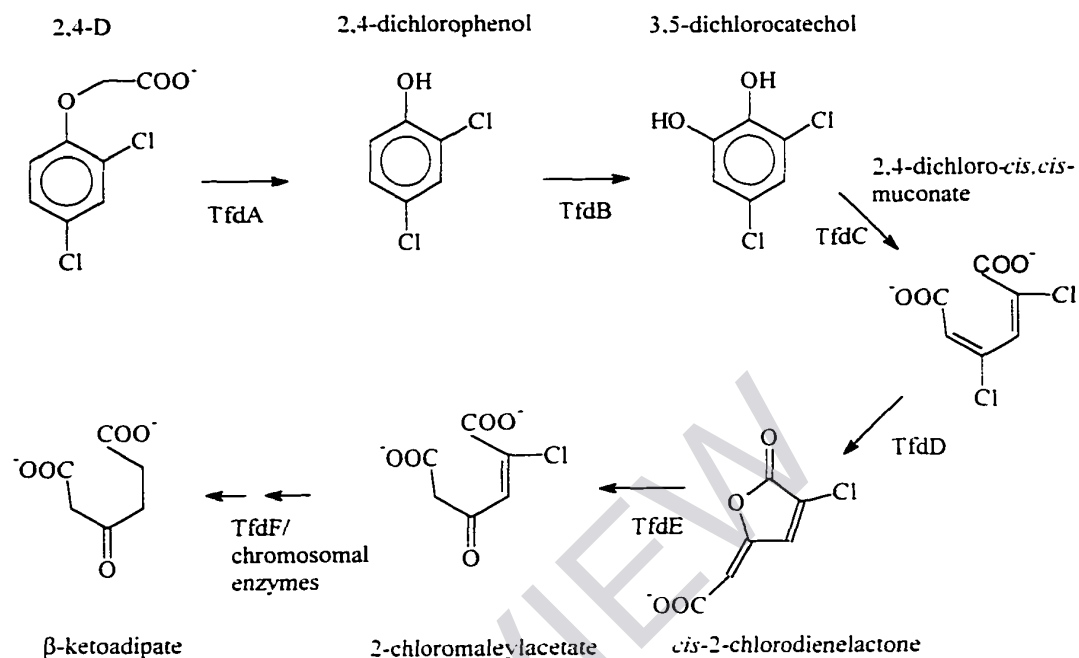
INTRODUCTION

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.

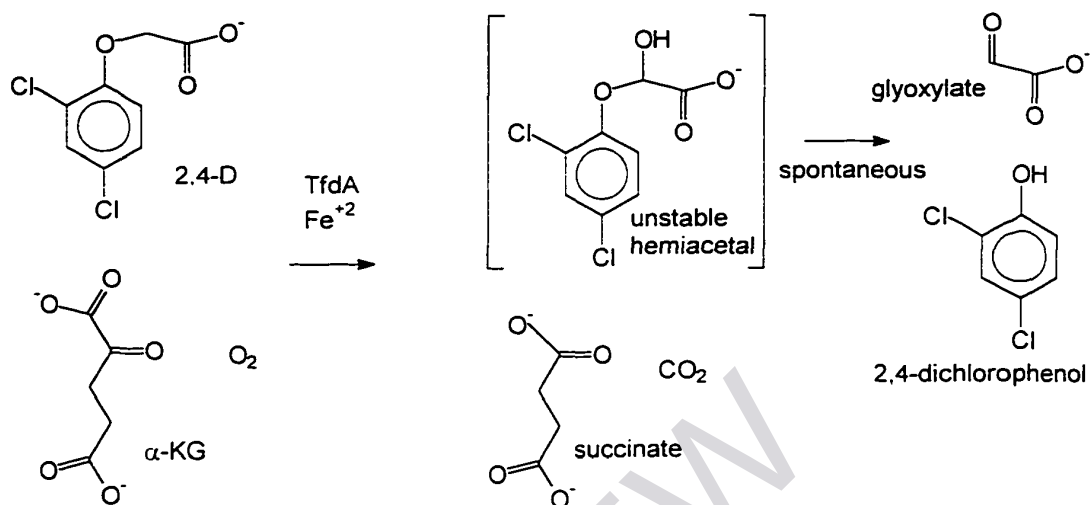


Scheme 1. Pathway for 2,4-D degradation in *R. eutropha* JMP134.

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 (Stenson 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