

The two enantiospecific dichlorprop/ α -ketoglutarate-dioxygenases from *Delftia acidovorans* MC1 – protein and sequence data of RdpA and SdpA

Anne Westendorf*, Dirk Benndorf, Roland H. Müller, Wolfgang Babel

UFZ Centre for Environmental Research, Department of Environmental Microbiology, Permoserstr. 15, D-04318 Leipzig, Germany

Accepted: August 26, 2002

Abstract

Two α -ketoglutarate-dependent dioxygenases carrying enantiospecific activity for the etherolytic cleavage of racemic phenoxypropionate herbicides [(*RS*)-2-(2,4-dichlorophenoxy)propionate and (*RS*)-2-(4-chloro-2-methylphenoxy)propionate] from *Delftia acidovorans* MC1 were characterized with respect to protein and sequence data. The (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA) appeared as a monomeric enzyme with a molecular weight of 32 kDa in the presence of SDS. N-terminal sequences revealed relationship to α -ketoglutarate-dependent taurine dioxygenase (TauD) and to 2,4-dichlorophenoxyacetate/ α -ketoglutarate-dioxygenase (TfdA). The (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) referred to 36 kDa in the presence of SDS and to 108 kDa under native conditions. Internal sequences of fragments obtained after digestion made evident relationship to TfdA and TauD. Two-dimensional electrophoretic separation resulted in the resolution of up to 3 individual spots with almost identical molecular weights but different isoelectric points with both RdpA and SdpA. The structural differences of these isoenzyme forms are not yet clear.

Key words: chiral herbicides – (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA) – (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) – partial amino acid sequences – *Delftia acidovorans* MC1

Introduction

A few strains are known which are capable of completely degrading the chiral herbicides (*RS*)-2-(2,4-dichlorophenoxy)propionate (2,4-DP; dichlorprop) and (*RS*)-2-(4-chloro-2-methylphenoxy)propionate (MCP; mecoprop) in addition to phenoxyacetate derivatives (2,4-dichlorophenoxyacetate; 2,4-D) (Zipper *et al.* 1996; Ehrig *et al.* 1997; Müller *et al.* 1999). This contrasts with the experience gained with most of the phenoxy herbicide-degrading strains, which have been shown, based on the activity of 2,4-D/ α -ketoglutarate-dioxygenase (TfdA) (Fukumori and Hausinger 1993 a, b; but cf. Saari *et al.* 1999), to be highly specific for the productive degradation of phenoxyacetates (Hägglom 1992; Hoffmann *et al.* 1996). The enzymes of one of the above strains, *Delftia acidovorans* MC1, have been purified and characterized (Müller and Babel 1999; Westendorf *et al.* 2003). Both enzymes were found to be α -ketoglutarate-dependent dioxygenases (Müller *et al.* 2001). This matches the reaction mechanism described for the cleavage of 2,4-D by the respective enzyme, i.e. TfdA, from the canonical strain *Ralstonia eutropha* JMP134 (Fukumori and Hausinger 1993 a, b). Kinetic investigations revealed that one of the enzymes of strain MC1 is highly specific for the cleavage of the R-configuration of 2,4-DP and MCP, i.e. (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase, consecutively designed RdpA. The other enzyme, i.e. (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase and designed SdpA, prefers the S-configuration but also accepts phenoxyacetate derivatives as substrate (Westendorf *et al.* 2003). Sequence data of *tfdA* are known

Corresponding author: Anne Westendorf
e-mail: annewest@umb.ufz.de

(Streber *et al.* 1987; Suwa *et al.* 1996; Mäe *et al.* 1993; Ka *et al.* 1994; Vallaey *et al.* 1996; Hoffmann *et al.* 2001) revealing high similarity in the gene of the various strains of β - and γ -*Proteobacteria* (but form a separate cluster, *tfdA* α , with α -*Proteobacteria*; Itoh *et al.* 2002). The application of primers derived from conserved regions of *tfdA* (Vallaey *et al.* 1996), however, failed to result in amplification products in PCR with genomic DNA from strain MC1 (Müller *et al.* 2001). No data are available on a molecular level with respect to the phenoxypionate-degrading strains. The present investigation is designed to elucidate the protein characteristics of the two different dioxygenases of strain MC1. Special attention is directed to sequence data whose elucidation should enable the assignment of the two enzymes to known proteins by databank comparison. Moreover, these data should favor targeting the genetic basis.

Materials and methods

Enzyme purification. The (*R*)-phenoxypionate/ α -ketoglutarate dioxygenase (RdpA) and the (*S*)-phenoxypionate/ α -ketoglutarate dioxygenase (SdpA) were purified as described by Westendorf *et al.* (2003). RdpA and SdpA were enriched by this procedure by a factor of 20 and 30, respectively, resulting in 71% and >98% purity.

Electrophoretic and chromatographic separations. For one-dimensional electrophoresis, a PowerEase 500 system was used (Novex, San Diego, USA). SDS PAGE of the enzyme preparation was carried out on 12% Tris glycine pre cast acrylamide gels (Novex). The samples contained 1–2 μ g of protein. The running buffer was composed of 2.9 g/l Tris base, 14.4 g/l glycine and 1.0 g/l SDS. The sampling buffer contained 2 ml glycerol, 4 ml 10% SDS (w/v), 0.5 ml 1% bromophenol blue and 2.5 ml 0.5 M Tris/HCl, pH 6.8 in 10 ml distilled water. The gels were treated at 125 V. Mark 12TM Wide Range Standard (Novex) was applied as a reference.

Peptides obtained from the digested enzymes were separated by peptide electrophoresis with Tris/Tricin buffer as described by Schägger and von Jagow (1987) in Mini Protean II Cell (Bio-Rad, Hercules, CA, USA). The following concentrations of acrylamide were used: separation gel (16% T and 3% C), spacer gel (10% T and 3% C) and stacking gel (4% T and 3% C). Electrophoresis was run with a constant current of 10 mA/gel for 0.5 h and a constant current of 20 mA/gel for 4 h. Ultra low range proteins (Sigma) served as a standard.

Two dimensional electrophoresis was performed as described by Benndorf and Babel (2002).

Gels were stained by following a slightly modified procedure described by Blum *et al.* (1987), or by using Coomassie brilliant blue.

Gel exclusion chromatography was performed by HPLC at 23°C using a Bio-Sil SEC-250 column (300 mm $\times$ 7.8 mm, Biorad) and a mobile phase containing 5 mM Na₂HPO₄, 5 mM NaH₂PO₄, 150 mM NaCl (pH 6.8). The flow rate was 1 ml/min. The molecular weight was determined by using a gel filtration standard (Biorad).

Protein digestion. Before enzymatic and chemical in-gel digestion, bands containing protein separated by PAGE were cut out and the gel slices washed three times with 50% methanol and 5% acetic acid in water for at least 3 h. The gel slices were reduced and alkylated by adding and removing the following solutions: 200 μ l acetonitrile (5 min), 30 μ l 1,4-dithioerythrole in 100 mM ammonium hydrogencarbonate (30 min), 30 μ l 100 mM iodoacetamide in 100 mM ammonium hydrogencarbonate (30 min), 200 μ l acetonitrile (5 min), 200 μ l 100 mM ammonium hydrogencarbonate (10 min) and 200 μ l acetonitrile (5 min). After drying the gel slices by vacuum centrifugation, enzymatic digestion was performed by adding 30 μ l LysC buffer (25 mM Tris/HCl pH 8.5) containing 1.5 μ l LysC (Lys-C, enzyme preparation from *Lysobacter enzymogenes*, Merck, Germany), 1 mM EDTA and 0.05% SDS, and incubating the sample overnight. Chemical digestion was performed by adding 30 μ l BNPS-scatole solution (500 μ g BNPS in 500 μ l 75% acetic acid; Crimmins *et al.*, 1990) and incubating the sample for 1 h at 47°C by shaking. To recover the peptides, the following solution were added and the respective supernatants collected: twice 30 μ l of 50 mM ammonium hydrogencarbonate for 10 min and twice 30 μ l extraction buffer (10 ml acetonitrile, 5 ml dist. water, 1.176 ml 85% formic acid) for 10 min. The unified supernatants were centrifuged under vacuum until a dry pellet was obtained. The latter was solubilized in sample buffer for electrophoretic separation.

Amino acid sequencing. Peptides separated by PAGE were blotted onto PVDF membranes (Jin and Cerletti 1992). Gels were washed for 5 min in distilled water and for 15 min in 10 mM CAPS buffer pH 11. Blotting was performed for 2 h at 3 mA/cm² with 10 mM CAPS buffer pH 11 (Trans Blot, Semi-Dry Transfer Cell, Biorad, USA). After staining with Coomassie brilliant blue, the bands were cut and applied to N-terminal sequencing (PROCISE® 491 cLC Protein Sequencer or PROCISE® 473A Protein Sequencer, Applied Biosystems; Forster City, CA, USA).

Results

One-dimensional SDS PAGE of the enzyme preparation resulted in distinct protein bands corresponding to molecular weights of about 32 kDa for SdpA and to 36 kDa for RdpA. Gel exclusion chromatography of the preparations of RdpA under native conditions resulted in a protein fraction corresponding to about 108 kDa which showed cleavage activity for (*R*)-2,4-DP. In the presence of SDS, the 108 kDa band disappeared in favor of a band with an apparent molecular weight of 36 kDa. This pattern indicates a trimeric structure of the R-specific enzyme. By contrast, with SdpA no fraction could be detected which corresponded to a protein larger than the monomeric form in gel exclusion chromatography. The protein appeared at a retention time corresponding to a molecular weight of about 23.7 kDa under native conditions. The enzyme separated under these conditions showed specific activity corresponding to that found before. Applying this fraction to SDS PAGE or performing gel exclusion chromatography in the presence of SDS resulted in separation patterns which correlated this protein to a mass of about 32 kDa.

Table 1. Isoelectric points (pI) and molecular weight (MW) of the isoforms of RdpA (R) and SdpA (S)

Spot	pI	MW [kDa]
R1	5.9	36.3
R2	5.86	35.9
R3	5.8	35.7
S1	6.33	32.8
S2	6.16	32.5

Data were obtained from electropherograms by software analysis (Phoretix 2D, 5.01, NonLinear Dynamics Ltd., G.B.)

Table 2. N-terminal sequences of the isoforms of RdpA and SdpA and internal sequences from selected peptide fragments of the two proteins

Position		Sequence	Accession No
N-terminal	R-Spot1	MHAALSPLSQRFERI	P 83310
	R-Spot2	MHAALSPLSQRFERI	
	R-Spot3	MHAALSPLSQRFEETAVQPLTGGV	
	S-Spot1	MQTTLQITPTGATLG	P 83309
	S-Spot2	MQTTLQITPTGATLGATVTGVHLAT	
Internal	R-Frac1E	SIEGYPEVQMIRREANESGRVIGDDXHTXII	P 83310
	R-Frac5C	ETLSPTMQATIEGLN	
	R-Frac8C	NEILDAFHTYQVIYFPGQAITNEQHIAFSR	
	S-Frac1E	LGHVQQAGSAYI	P 83309
	S-Frac2E	VIVGNTAG	

R, S refers to RdpA and SdpA; Spot designates the respective proteins in the 2D gels (Fig. 1 for SdpA; RdpA not shown); Frac refers to the protein fractions after enzymatic (E) and chemical (C) digestion

The purified enzymes were treated by 2D-electrophoresis which resulted in the resolution of the respective enzymes into 2–3 individual protein spots. This is convincingly shown for SdpA in Fig. 1. Apparently, the isoforms were characterized by an almost identical molecular weight but differed in their charges (Table 1). A similar pattern was observed for RdpA (Table 1). Although the latter preparation contained significant impurities which could not be eliminated by the purification protocol applied, three spots were clearly identified to correspond to RdpA by admixture of crude extracts of pyruvate grown cells (not shown). The individual protein spots with respect to RdpA and SdpA preparations were used to determine N-terminal amino acid sequences. The results are shown in Table 2. The sequences were clearly identical with respect to the isoforms of RdpA and SdpA except position 14 in spot R3 which may be attributed to analytical uncertainties in this late cycle of sequencing.

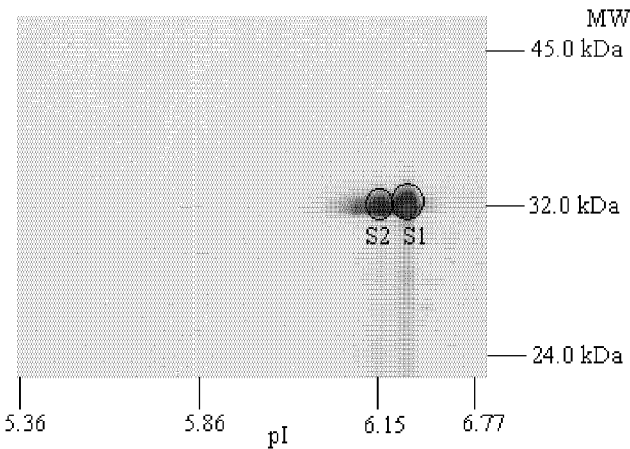


Fig. 1. Part of the 2D-gel of SdpA. Isoelectric focusing from right to left, SDS PAGE from up to down.

D. acidovorans MC1. The molecular weights of the monomers, 32 and 36 kDa respectively, are in the range found for analog enzymes from other sources (Fukumori and Hausinger 1993b; Nickel *et al.* 1997). With TfdA from *R. eutropha* JMP134 a dimeric structure was deduced (Fukumori and Hausinger 1993b). Surprisingly, with the two enzymes of strain MC1, isomeric enzyme forms were detected being separated from each other by only slight differences in their isoelectric points. The structural reasons are not yet apparent. The existence of isoenzymes is a likely explanation of the complex, multiphasic characteristics observed in estimating the kinetic properties with preparations of both RdpA and SdpA (Westendorf *et al.* 2003).

The two dioxygenases from *D. acidovorans* MC1 were shown to be related to other α -ketoglutarate-dependent dioxygenases. According to the N-terminal and two internal sequences, SdpA showed closest homology to taurine dioxygenase (TauD) from *E. coli* (Eichhorn *et al.* 1997) and *Pseudomonas aeruginosa* (36%/43% identical/similar to *E. coli* enzyme) and to TfdA from strains JMP134 and RASC (18%/20% identical/similar to JMP134) based on 44 amino acids aligned. With respect to RdpA, homology with other α -ketoglutarate-dependent dioxygenases was detected from sequence data of various internal fragments, according to which highest homology was attributed to TfdA (32%/37% identical/similar; strain JMP134), followed by TauD (21%/30% identical/similar; *E. coli*) on the basis of 101 amino acids aligned. The present sequence data are considered an appropriate basis to target the genetic background and elucidate the whole structure of these enzymes.

Acknowledgements

This investigation was kindly supported by grant no. 13-8811.61/94 from the Saxon Ministry of the Environment and Agriculture (AW) and by E.C. (HERBICBIOREM, QLK3-CT-1999-00041) (DB).

References

- Benndorf, D., Babel, W. (2002): Assimilatory detoxification of herbicides by *Delftia acidovorans* MC1: induction of two chlorocatechol 1,2-dioxygenases as a response to chemostress. *Microbiology* **148**, 2883–2888.
- Blum, H., Beier, H., Gross, H. J. (1987): Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.
- Crimmins, D. L., McCourt, D. W., Thoma, R. S., Scott, M. G., Macke, K., Schwartz, B. D. (1990): In situ chemical cleavage of proteins immobilized to glass-fiber and polyvinylidenedifluoride membranes: cleavage at tryptophan residues with 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine to obtain internal amino acid sequence. *Anal. Biochem.* **187**, 27–38.
- Ehrig, A., Müller, R. H., Babel, W. (1997): Isolation of phenoxy herbicide-degrading *Rhodospirillum rubrum* species from contaminated building material. *Acta Biotechnol.* **17**, 351–356.
- Eichhorn, E., Ploeg, J. R., Kertesz, M. A., Leisinger, T. (1997): Characterisation of α -ketoglutarate-dependent taurine dioxygenase from *Escherichia coli*. *J. Biochem.* **37**, 23031–23036.
- Fukumori, F., Hausinger, R. P. (1993a): *Alcaligenes eutrophus* JMP 134 “2,4-dichlorophenoxyacetate monooxygenase” is an α -ketoglutarate-dependent dioxygenase. *J. Bacteriol.* **175**, 2083–2086.
- Fukumori, F., Hausinger, R. P. (1993b): Purification and characterization of 2,4-dichlorophenoxyacetate/ α -ketoglutarate dioxygenase. *J. Biol. Chem.* **268**, 24311–24317.
- Hägglblom, M. M. (1992): Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol. Rev.* **103**, 29–72.
- Hoffmann, D., Müller, R. H., Kiesel, B., Babel, W. (1996): Isolation and characterization of an alkaliphilic bacterium capable of growing on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid. *Acta Biotechnol.* **16**, 121–131.
- Hoffmann, D., Kleinstaub, S., Müller, R. H., Babel, W. (2001): Development and application of PCR primers for the detection of the *tfd* genes in *Delftia acidovorans* P4a involved in the degradation of 2,4-D. *Acta Biotechnol.* **21**, 321–331.
- Jin, Y., Cerletti, N. (1992): Western blotting of transforming growth factor β -2. Optimization of the electrophoretic transfer. *Appl. Theor. Electrophoresis* **3**, 85–90.
- Ka, J. O., Holben, W. E., Tiedje, J. M. (1994): Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. *Appl. Environ. Microbiol.* **60**, 1106–1115.
- Itoh, K., Kanda, R., Sumita, Y., Kim, H., Kamagata, Y., Suyama, K., Yamamoto, H., Hausinger, R. P., Tiedje, J. M. (2002): *tfdA*-like genes in 2,4-dichlorophenoxyacetic acid-degrading bacteria belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Azospira* cluster in α -Proteobacteria. *Appl. Environ. Microbiol.* **68**, 3449–3454.
- Mäe, A. A., Marits, R. O., Ausmess, N. R., Koiv, V. M., Heinäru, A. L. (1993): Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes. *J. Gen. Microbiol.* **139**, 3165–3170.
- Müller, R. H., Jorke, S., Kleinstaub, S., Babel, W. (1999): *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichlorprop and mecoprop and the herbicides 2,4-D and MCPA. *Microbiol. Res.* **154**, 241–246.
- Müller, R. H., Babel, W. (1999): Separation of two dichlorprop/ α -ketoglutarate dioxygenases with enantiospecific properties from *Comamonas acidovorans* MC1. *Acta Biotechnol.* **19**, 349–355.
- Müller, R. H., Kleinstaub, S., Babel, W. (2001): Physiological and genetic characteristics of two bacterial strains utilizing phenoxypionate and phenoxyacetate herbicides. *Microbiol. Res.* **156**, 121–131.

- Nickel, K., Suter, M. J. F., Kohler, H. P. E. (1997): Involvement of two α -ketoglutarate-dependent dioxygenases in the enantioselective degradation of (R)- and (S)-mecoprop by *Sphingomonas herbicidovorans* MH. J. Bacteriol. **179**, 6674–6679.
- Saari, R. E., Hogan, D. A., Hausinger, R. P. (1999): Stereospecific degradation of the phenoxypropionate herbicide dichlorprop. J. Mol. Catal. B: Enzymatic **6**, 421–428.
- Schägger, H., von Jagow, G. (1987): Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**, 368–379.
- Streber, W. R., Timmis, K. N., Zenk, M. H. (1987): Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. J. Bacteriol. **169**, 2950–2955.
- Suwa, Y., Wright, A. D., Fukumori, F., Nummy, K. A., Hausinger, R. P. (1996): Characterization of a chromosomally encoded 2,4-dichlorophenoxyacetic acid/ α -ketoglutarate-dioxygenase from *Burkholderia* sp. strain RASC. Appl. Environ. Microbiol. **62**, 2464–2469.
- Vallaes, T., Fulthorpe, R. R., Wright, A. M., Soulas, G. (1996): The metabolic pathway of 2,4-dichlorophenoxyacetic acid degradation involves different families of *tfdA* and *tfdB* genes according to PCR-RFLP analysis. FEMS Microbiol. Ecol. **20**, 163–172.
- Westendorf, A., Müller, R. H., Babel, W. (2003): Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* MC1 initiating the degradation of phenoxypropionate and phenoxyacetate herbicides. Acta Biotechnol. **23**, 11–24.
- Zipper, C., Nickel, K., Angst, W., Kohler, H. P. E. (1996): Complete microbial degradation of both enantiomers of the chiral herbicide mecoprop [(R,S)-2-(4-chloro-2-methylphenoxy)propionic acid] in an enantioselective manner by *Sphingomonas herbicidovorans* sp. nov. Appl. Environ. Microbiol. **62**, 4318–4322.