Characterization of an intradiol dioxygenase involved in the biodegradation of the chlorophenoxy herbicides 2,4-D and 2,4,5-T

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Received 30 January 1997; revised version received 25 February 1997

Abstract Hydroxyquinol 1,2-dioxygenase, an intradiol dioxygenase, which catalyzes the cleaving of the aromatic ring of hydroxyquinol, a key intermediate of 2,4-D and 2,4,5-T degradation, was purified from *Nocardioides simplex* 3E cells grown on 2,4-D as the sole carbon source. This enzyme exhibits a highly restricted substrate specificity and is able to cleave hydroxyquinol (K_m for hydroxyquinol as a substrate was 1.2 μM, V_{max} 55 U/mg, K_{cat} 57 s^{-1} and K_{cat}/K_m 47.5 μM s^{-1}), 6-chloro- and 5-chlorohydroxyquinol. Different substituted catechols and hydroquinones are not substrates for this enzyme. This enzyme appears to be a dimer with two identical 37-kDa subunits. Protein and iron analyses indicate an iron stoichiometry of 1 iron/65 kDa homodimer, α_2 Fe. Both the electronic absorption spectrum which shows a broad absorption band with a maximum at 450 nm and the electron paramagnetic resonance spectra are consistent with a high-spin iron(III) ion in a rhombic environment typical of the active site of intradiol cleaving enzymes.

Key words: Hydroxyquinol 1,2-dioxygenase; Intradiol dioxygenase; Chlorophenoxyalkanoic herbicide; 2,4-D; 2,4,5-T; *Nocardioides simplex*

1. Introduction

2,4-Dichlorophenoxyacetate (2,4-D) is at present the most commonly used herbicide since another phenoxy-herbicide, 2,4,5-T, has been banned in many countries due to its high toxicity. Furthermore, their biodegradation intermediates 2,4-dichloro- and 2,4,5-trichloro-phenols (2,4-DCP and 2,4,5-TCP) are still used as biocides and constitute a major group of environmental pollutants [1].

Nocardioides simplex 3E, isolated by enrichment culture techniques under selective pressure of 2,4,5-T, completely mineralizes both 2,4-D and 2,4,5-T and the corresponding chlorophenols [2].

Based on identification of 2,4-D and 2,4,5-T degradation pathways and activity of key enzymes it was suggested that degradation of both herbicides by *N. simplex* 3E proceeds through the formation of trihydroxylated benzene [3].

We report here the identification, purification and preliminary characterization of an enzyme from this strain which catalyzes the splitting of the aromatic ring of hydroxyquinol with high specificity.

2. Material and methods

2.1. Enzyme production and purification

Nocardioides simplex 3E isolated by enrichment culture techniques from soils treated with chloroaromatic pesticides was grown in a 10-l fermentor with 7 liters of a mineral medium containing 2,4-D as the sole source of carbon. Conditions of cultivation and preparation of cell free extracts were as previously described [4].

Cell free extract was brought to 37% saturation with powdered $(NH_4)_2SO_4$ and stirred for 1 h at 4°C before centrifugation. The pellet was discharged and the supernatant solution was brought to 67% $(NH_4)_2SO_4$ saturation. The precipitate was collected by centrifugation, dissolved in 40 ml of 50 mM Tris-HCl buffer pH 7.8 (buffer A) and dialyzed against 5 liters of the same buffer.

After removal of $(NH_4)_2SO_4$ the enzyme solution was loaded onto a DEAE-Toyopearl column (25/30) previously equilibrated with buffer A, washed with 200 ml of the same buffer, and a linear gradient from buffer A to buffer A plus 0.5 M NaCl (2 l) with a flow rate of 4 ml/min was applied. Active fractions were pooled and an equal volume of buffer A was added. The enzyme solution was then loaded onto a Q-Sepharose FF column (28/16) previously equilibrated with buffer A plus 0.2 M NaCl, washed with 50 ml of the same buffer, and a linear gradient from 0.2–0.5 M NaCl in the same buffer (800 ml) with a flow rate of 1.5 ml/min was applied. The enzyme eluted with 0.31–0.35 M NaCl. Active fractions were pooled and 3.5 M solution of ammonium sulfate was added to the final concentration of 0.7 M. The enzyme solution was loaded onto a column of phenyl-Superose CL-4B (20/4) previously equilibrated with 0.7 M of ammonium sulfate in buffer A. The column was washed with 10 ml of the same buffer with ammonium sulfate and a linear gradient of 0.7–0 M ammonium sulfate (100 ml) with a flow rate of 1 ml/min was applied. The enzymatic activity eluted at 0.6–0.1 M of ammonium sulfate. Active fractions were pooled and concentrated to 0.5 ml by ultrafiltration with Amicon membrane PM 10 and Centricon 10. The concentrated solution was loaded onto a column of Superdex 200 (16/70) equilibrated with buffer A plus 0.2 M of NaCl and eluted with the same buffer and a flow rate of 0.5 ml/min. Active fractions were combined and loaded onto a Mono-Q 5/5 column in a Pharmacia FPLC system. The column was previously equilibrated with buffer A plus 0.2 M NaCl. The elution was performed by broken gradient of 0.2–0.5 M NaCl in buffer A (35 ml). The enzyme was eluted with 0.32–0.35 M NaCl. Hydroxyquinol was used as a substrate for enzyme assays during the purification.

2.2. Enzyme activity assay

The activity of HQ 1,2-DO was assayed either polarographically with a YSI 5300 device (USA) as described by Sze and Dagley [5] in 50 mM MES buffer (pH 6.5) or spectrophotometrically with a Shimadzu UV 160 spectrophotometer (Japan) according to the modified method of Tiedje et al. [6]. The reaction mixture contained 0.1 M MES buffer (pH 6.5), 0.1 mM hydroquinol and the enzyme. The reaction was started by the addition of substrate. Activity was measured by determining the increase in absorption at 243 nm which correlates with the formation of maleylacetate, molar extinction 4440 M^{-1} cm^{-1} [6].

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetate; 2,4,5-T, 2,4,5-trichlorophenoxyacetate; HQ, hydroxyquinol; CHQ, chlorohydroxyquinol; HQ 1,2-DO, hydroxyquinol 1,2-dioxygenase; CHQ 1,2-DO, chlorohydroxyquinol 1,2-dioxygenase; 2,4,5-TCP, 2,4,5-trichlorophenol; EPR, electron paramagnetic resonance

PII S0014-5793(97)00297-4

One unit of enzyme activity was defined as the amount (mg) of enzyme that catalyzed the formation of 1 μmol of product per min.

Protein concentration, during purification, was determined using the A_{280}/A_{260} method of Warburg and Christian [7], and the concentration of the purified enzyme was determined using the A_{280}/A_{205} method of Scopes [8].

2.3. Analytical methods

The absorption spectrum of the enzyme was recorded using a Shimadzu UV 160 or a Varian Cay 3 spectrophotometer.

The content of metal ions in the purified enzyme was determined using atomic absorption spectroscopy with flame atomization, spectrophotometer model AAS 5100/Zeeman, Perkin Elmer.

X band EPR spectra were recorded at 4.2 K on a Bruker ER200 spectrometer equipped with an Oxford continuous flow ESR 900 cryostat.

Electrophoresis in 12.5% polyacrylamide gel in the presence of SDS was performed according to a modification of the Laemmli method [9]. The protein content in samples was 2–5 μg. The low molecular weight kit MW-SDS-70L from Sigma (USA) was used as standard. The proteins were stained with Coomassie R-250 [10].

For the estimation of molecular mass under non-denaturing conditions protein was applied on a Superdex-200 (16/70) and eluted with buffer A plus 0.2 M NaCl. The molecular weight was calculated from a standard linear regression curve for the reference proteins (M_r 17 800–150 000).

The substrate specificity of the enzyme was determined in 50 mM MES buffer pH 6.5 by polarography with a Clark DW 1 oxygen electrode using a YSI 5300 device (USA) and spectrophotometrically with freshly prepared solutions of di- and trihydroxylated compounds. Trihydroxylated substrated solutions were prepared under anoxic (nitrogen) conditions.

2.4. Analysis of kinetic data

K_m and V_{max} values were calculated from 1/S versus 1/V plots. K_{cat} values were determined using a molecular mass of 65 kDa for the enzyme. Inhibition constants were estimated graphically using the Dixon method [11].

Chemicals, biochemicals and reagents were purchased from Sigma (USA) and Serva (Germany); HQ was from Aldrich (USA).

6-Chloro-, 2-methoxy-6-chloro- and 5-chlorohydroxyquinols were synthesized by Yu. Shuruchin according to a modification of the method described in [12]. Q-Sepharose FF and phenyl-Sepharose-CL-6B were from Pharmacia (Sweden), DEAE-Toyopearl-650M was from Toyo Soda (Japan), and reagents for electrophoresis from Bio-Rad (USA).

3. Results and discussion

The purification procedure is summarized in Table 1. Six purification steps led to an electrophoretically homogeneous preparation of HQ 1,2-DO. The pure enzyme with specific activity of 55 U/mg was obtained after a 785-fold enrichment, with a yield of about 60%.

The purified enzyme retained 100% activity after 2 months storage at 4°C in the pH range 7.0–9.5. HQ 1,2-DO was active from pH 6.5 to 10.5 with the optimal activity at pH 7.5. For pH values higher than 8 it was impossible to determine the pH dependence of the enzyme reaction because of the rapid autoxidation of hydroxyquinol and chlorohydroxyquinols under basic conditions.

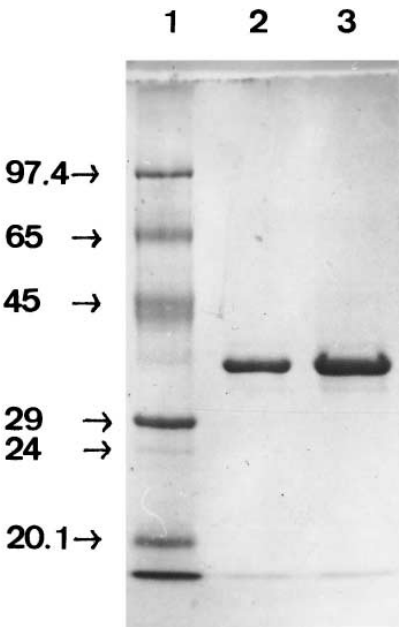

Fig. 1. SDS-PAGE (12% polyacrylamide) of HQ 1,2-DO. Lane 1 contains the low molecular mass protein standards of 20 100, 24 000, 29 000, 45 000, 65 000, and 97 400 Da. Lanes 2 and 3 correspond to HQ 1,2-DO, 3.0 and 6.0 μg.

Kinetic parameters for HQ 1,2-DO from *N. simplex* 3E were estimated in relation to HQ: $K_m = 1.2$ μM, $V_{max} = 55$ U/mg, $K_{cat} = 57$ s^{-1} and $K_{cat}/K_m = 47.5$ μM^{-1} s^{-1}. (K_{cat} was calculated for a molecular weight of 65 kDa).

The enzyme is active at 10–60°C with optimal activity at 50–55°C under test conditions. Heating of the enzyme at 30, 40, and 45°C for 10 min led to 20, 74 and 94% decrease of activity respectively.

The enzyme is remarkably specific. Only HQ, 5CHQ, and 6CHQ are the substrates. The oxidation rates of 6CHQ and 5CHQ by HQ 1,2-DO are about 5 and 2.4% of the oxidation rates of unsubstituted HQ, 2.75 and 1.3 U/mg respectively. The range of halogen-, methyl- and methoxy-substituted catechols, 4-nitrocatechol, chloro- and 2,6-dichloroquinol, 3,4-dihydroxybenzaldehyde tested are not substrates, but were found to be inhibitors of HQ turnover (Table 2). 4,5-Dibromocatechol and chloroquinol were the strongest inhibitors of HQ 1,2-DO. Hydroquinol, 4-fluorocatechol, and 2,6-dichlorohydroquinol were weak inhibitors.

The molecular mass of the native enzyme was estimated to be 65 kDa by gel filtration as reported in Section 2. The

Table 1
Purification of *N. simplex* 3E hydroxyquinol 1,2-dioxygenase

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	150	4500	16.7	0.004		
Ammonium sulfate fractionation	140	2100	315.0	0.15	2.14	100
DEAE-Toyopearl TSK-Gel	140	610	262.3	0.43	6.13	83.3
Q-Sepharose FF	100	98	234.2	2.39	34.07	74.3
Phenyl-Sepharose	36	41	219.4	5.35	76.26	69.7
Superdex-200	4.5	7.0	204.1	29.16	416	64.8
Mono-Q 5/5	0.82	3.46	190.3	55	784.6	60.4

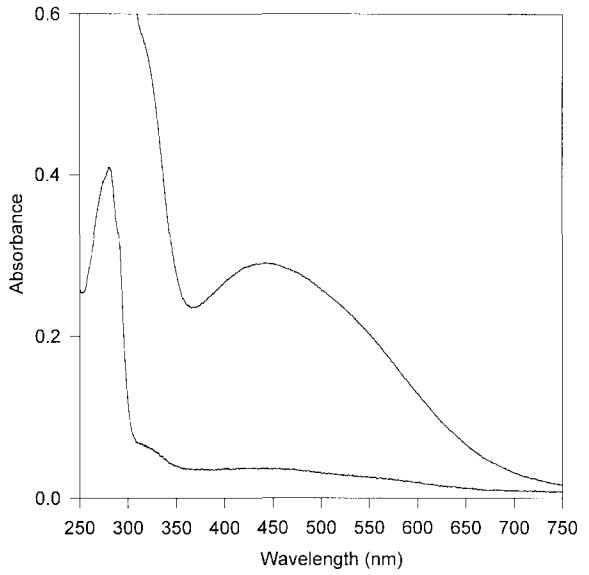

Fig. 2. Electronic absorption spectrum of the purified HQ 1,2-DO from *Nocardioides simplex* 3E 0.08 mM in 50 mM Tris-HCl buffer pH 7.8. The lower spectrum is taken from the same sample diluted 8 times.

molecular mass of the denatured protein as determined by SDS-PAGE was found to be 37 kDa (Fig. 1). On the basis of these results HQ 1,2-DO appears to be a homodimer.

The AAS of the HQ 1,2-DO showed the presence of 1 mol iron/mol enzyme (420 mg of protein contained 0.35 mg of Fe).

The enzyme solution is reddish-brown and in the visible spectrum the enzyme shows a broad, low intensity band centered at 450 nm ($\varepsilon_{450} = 3646\ M^{-1}\ cm^{-1}$, $\varepsilon_{450} = 0.0588$ mg $ml^{-1}\ cm^{-1}$) (Fig. 2).

The extinction coefficients at 280 nm and 205 nm were determined to be 0.65 and 31.05 mg $ml^{-1}\ cm^{-1}$, respectively, using the method of Scopes [8].

The EPR spectrum of the purified enzyme in frozen buffer at 4.2 K is shown in Fig. 3. The signal at $g = 4.3$ is characteristic of high spin iron(III) ions in rhombic environments similar to those observed for other intradiol cleaving dioxygenases.

Several HQ 1,2-DOs have been purified to date from different organisms like *Trichosporon cutaneum* [13], *Phanerochaete chrysosporium* [14], *Azotobacter* sp. [15], *Streptomyces rochei* 303 [16]. Recently HQ 1,2-DO was purified from *Burkholderia cepacia* AC 1100, degrading the herbicide 2,4,5-T, but this enzyme was specific for HQ and was not able to use CHQ [17].

Enzyme from *N. simplex* 3E, degrading both phenoxyalkanoic herbicides 2,4,-D and 2,4,5-T, catalyzed the *ortho* cleavage of both HQ and CHQ.

So, at the first time HQ 1,2-DO, splitting both substituted and non-substituted HQ, was isolated from the culture degrading phenoxyalkanoic herbicides. Like the corresponding enzymes HQ 1,2-DO from *Azotobacter* sp. [15] and 6-CHQ 1,2-DO from *Streptomyces rochei* 303 [16], the enzyme from *N. simplex* 3E was found to be a homodimer with 37 kDa subunits, the pH and T° optima for these enzymes were also very similar.

Preliminary spectroscopic studies (visible spectra and EPR) of the purified enzyme indicate the involvement of high spin Fe(III) ions (1 mol of iron per homodimer) in the catalytic process. The broad band centered at 450 nm in the electronic absorption spectrum is indicative of a ligand to Fe(III) charge transfer transition, typical of tyrosinate coordination to the metal, whereas the EPR spectrum suggests the presence of high spin iron(III) in a rhombic environment. Therefore both spectroscopic techniques indicate an active metal site very similar to those observed for other intradiol dioxygenases like catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase [18–21].

Acknowledgements: This work was supported by a NATO Linkage Grant HT 951032, the Russian Program 'New Methods of Bioengineering (Environmental Biotechnology)'. We appreciate the contribution of the CNR Gruppo Nazionale per la Difesa dia Rischi.

Table 2
Inhibition constants (K_i) of different aromatic compounds for hydroxyhydroquinol 1,2-dioxygenase from *N. simplex* 3E

Inhibitor	Inhibition constant (mM)
Hydroquinone	150.0
Chlorohydroquinone	2.35
2,6-Dichlorohydroquinone	20.0
Methoxyhydroquinone[a]	
Catechol	10.0
3-Chlorocatechol	7.0
4-Chlorocatechol	10.0
3,5-Dichlorocatechol	2.0
4,5-Dichlorocatechol	7.8
4-Nitrocatechol	10.0
4,5-Dibromocatechol	4.8
4-Fluorocatechol	100.0
3,4-Dihydroxybenzaldehyde	9.9

[a]Does not inhibit.

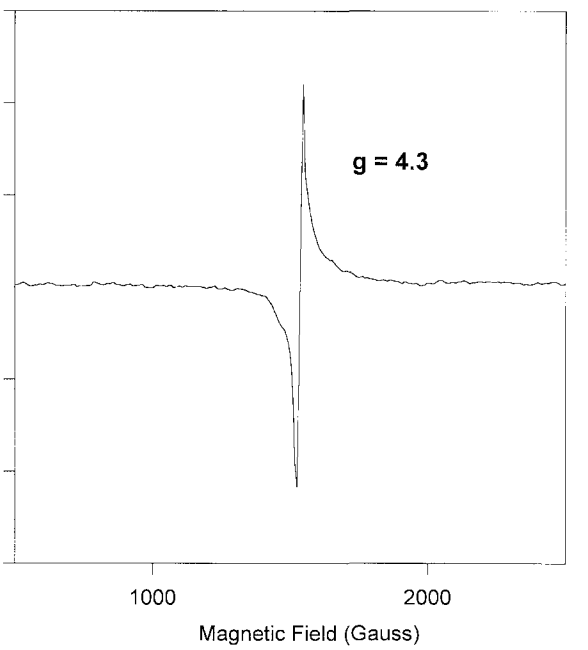

Fig. 3. EPR spectrum of the purified HQ 1,2-DO from *Nocardioides simplex* 3E 0.8 mM in 50 mM Tris-HCl buffer pH 7.8. Instrumental conditions: scan rate 1000 G/min, modulation amplitude 2 Gauss, modulation frequency 100 kHz, microwave frequency 9.24 GHz, microwave power 2 mW, receiver gain 800, temperature 4.2 K.

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