Purification, biochemical properties and substrate specificity of a catechol 1,2-dioxygenase from a phenol degrading *Acinetobacter radioresistens*

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Abstract A catechol 1,2-dioxygenase (C1,2O) has been purified to homogeneity from *Acinetobacter radioresistens* grown on phenol as the sole carbon and energy source. The C1,2O appears to be a homodimer, with a molecular mass of 78 000 Da. At relatively high ionic strengths (0.5 M Na_2SO_4) subunit dissociation occurs and the monomeric unit (38 700 Da) is shown to be active. This phenomenon has never been observed before in dioxygenases. The purified C1,2O contains 0.96 iron(III) ions per unit and spectroscopic measurements suggest the presence of one high-spin iron(III) ion in an environment characteristic of intradiol cleaving enzymes. The NH_2-terminal amino acid sequence has been determined and compared to the primary structures of intradiol rings cleaving dioxygenases from other *Acinetobacter* strains revealing 45% homology with the benzoate-grown *A. calcoaceticus* ADP-1 and an identity of only one of the 20 amino acids sequenced for the phenol-grown *A. calcoaceticus* NCIB 8250.

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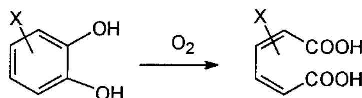
Key words: Catechol 1,2-dioxygenase; Intradiol dioxygenase; Phenol degradation; *Acinetobacter radioresistens*; Subunit dissociation

1. Introduction

The aerobic catabolism of aromatic compounds has been extensively investigated for a variety of microorganisms and for different natural and xenobiotic compounds [1–4]. In particular many microorganisms use a catabolic sequence for the degradation of aromatic compounds called the β-ketoadipate pathway in which ring cleaving dioxygenases play a key role [5,6].

Among these enzymes [7–9] catechol 1,2-dioxygenases catalyze the intradiol cleavage of catechols to *cis,cis*-muconic acids with the incorporation of molecular oxygen (see Scheme 1), the first step of this pathway.



Scheme 1.

Intradiol dioxygenases have been purified from a variety of organisms comprising *Pseudomonas, Alcaligenes, Nocardia, Rhodococcus* and also *Acinetobacter calcoaceticus*, always showing a quaternary structure composed of at least two subunits with one to two iron(III) ions per dimer and a widely diversified substrate specificity [10–16].

The bacterium *Acinetobacter radioresistens* [17], subject of the present investigation, has been isolated from soil adjacent to an activated sludge pilot plant at the Politecnico di Torino (Turin, Italy) (unpublished observations). This strain has been selected for its fast phenol catabolism, when used as the sole energy and carbon source (unpublished observations). Usually the phenol degradation occurs by converting them into the corresponding catechols which are subsequently transformed by ring cleaving dioxygenases to muconic acids [2,4].

In the present study we report the isolation of a catechol 1,2-dioxygenase from a novel strain of *A. radioresistens* grown on phenol as sole carbon and energy source. The purified dioxygenase is shown to be a mononuclear Fe(III) homodimeric enzyme which dissociates into active subunits upon increasing the ionic strength. The implications of these findings are discussed.

2. Materials and methods

2.1. Chemicals and biochemicals

The chemical and biochemical compounds used in the present study were of the highest purity available (Acros Organics, Belgium; Pharmacia Biotech, Sweden; Sigma Chemical Co., USA).

Chromatographic media used were DE52 diethylaminoethyl cellulose (Whatman International Ltd., UK), Q Sepharose High Performance, Superdex 75, and Sephacryl S-100 (Pharmacia Biotech, Sweden). Protein standards were purchased from Sigma Chemical Co., USA, Pharmacia Biotech, Sweden, and Bio-Rad Laboratories, USA. Catechol, protocatechuic acid and methyl catechols were purchased from Aldrich Chemical Co., USA.

2.2. Microorganism and culture conditions

The *Acinetobacter* strain used in the present study was isolated from the soil surrounding an activated sludge pilot plant used for the study of biodegradation of a variety of pollutants at the Politecnico di Torino (Turin, Italy) (unpublished observations). The species was determined to be *A. radioresistens* by fatty acid gas chromatography and AFLP (amplified fragment length polymorphism) at the LMG (Laboratorium voor Microbiologie, Universiteit Gent, Belgium) Culture Collection Centre. An API 20 NE kit (Biomérieux, France) was used for purity check. The strain is conserved at −80°C in 40% glycerol.

The bacterium was cultured in a modified minimal medium [18]: solution A contains $(NH_4)_2SO_4$ 12.6 g/l, KH_2PO_4 5.0 g/l; solution B contains NaCl 0.75 g/l, $CaCl_2{\cdot}2H_2O$ 0.66 g/l, $MgCl_2{\cdot}6H_2O$ 1.60 g/l; and solution C is composed of $FeCl_3{\cdot}2H_2O$ 0.58 g/l, EDTA 0.78 g/l. The final medium was prepared by mixing 2 parts of solution A, 2 parts of solution B, 1 part of solution C and 22.5 parts of water. The carbon source (phenol) was added to a final concentration of 400 mg/l. The final pH after sterilization was 7.8. The bacterium was grown at 30°C with 1% inoculum.

The growth was monitored by measuring the optical density at 600

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Abbreviations: C1,2O, catechol 1,2-dioxygenase; C2,3O, catechol 2,3-dioxygenase; EPR, electron paramagnetic resonance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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nm and the phenol consumption analyzed through HPLC on a C18 analytical column (250×4.6 mm) with 50% acetonitrile/H_2O isocratic at 1 ml/min and monitoring the absorbance at 254 and 270 nm. Culture broth (20 μl) was injected after centrifugation and filtration and the phenol peak area was estimated.

2.3. Preparation of enzyme extracts

The purification procedure was carried out in 50 mM Tris-SO_4 buffer, pH 8.0, at 4°C unless otherwise stated.

Wet cells (27 g) were suspended in 80 ml of 50 mM Tris-SO_4, pH 8.0. Cells were broken by sonication with a Sonics and Materials (USA) Vibra-Cell model VC-375 ultrasonic processor at the maximum power (5 min total time), taking care to maintain the temperature below 10°C with a freezing bath. The suspension was then centrifuged for 60 min at 50 000 rpm, 4°C.

The clear supernatant obtained after centrifugation was directly applied to a DE52 cellulose column (2.6×20 cm) previously equilibrated with 50 mM Tris-SO_4, pH 8.0. The column was washed with at least 400 ml of the same buffer and then the bound proteins were eluted with a linear gradient of 0–250 mM Na_2SO_4 in the same buffer at 2 ml/min (total buffer volume: 1.6 l).

The fractions containing the highest C1,2O activities were collected, concentrated by ultrafiltration, washed to reduce the ionic strength and then concentrated again to less than 5 ml.

The concentrated DE52 fractions were injected into a HPLC System Gold (Beckman, USA) composed of a 125 Solvent Module and a 168 diode array UV-visible detector interfaced to a personal computer and a Q Sepharose high performance column (Pharmacia, Sweden) (1.6×16 cm) previously equilibrated with 20 mM Tris-SO_4, pH 8.0 (buffer A). After protein injection the binary buffer gradient reported in Fig. 1 was applied (buffer B: 20 mM Tris-SO_4, pH 8.0 and 500 mM Na_2SO_4).

2.4. Enzyme assay

The catechol 1,2-dioxygenase (intradiol) activity was monitored spectrophotometrically following the formation of *cis,cis*-muconic acid at 260 nm and 25°C ($\varepsilon_{260} = 16\,000\ M^{-1}\ cm^{-1}$). The assay mixture generally contained in 1 ml total: 20 μl catechol 10 mM, 975 μl Tris-SO_4 50 mM pH 8.0, 1 mM EDTA and 5 μl enzyme solution. The catechol 2,3-dioxygenase (extradiol) activity was determined by measuring the formation of 2-hydroxymuconic semialdehyde at 390 nm under the same conditions reported for intradiol activity [19].

One unit of enzymatic activity is defined as the amount of enzyme producing 1 μmol of *cis,cis*-muconate per minute at 25°C. The kinetic parameters were determined by fitting the data with a non-linear least-squares fitting program to a typical Michaelis-Menten equation.

In the experiments for the determination of the pH optimum the following buffers were used: 50 mM sodium acetate (pH 4.0–5.7), 50 mM phosphate buffer (pH 6.0–7.7), 50 mM Tris-sulfate (pH 7.5–8.5), and 50 mM NaOH-glycine (pH 8.5–10.0).

2.5. Determination of concentration

The concentration and the extinction coefficients at 280 and 440 nm of the pure proteins were determined using the spectrophotometric UV absorption method of Scopes [20]. The Bradford and the Warburg and Christian methods [21,22] were used for the estimation of protein concentrations in extracts and partially purified protein solutions.

2.6. Spectroscopic and analytical methods

UV-visible absorbance spectra were recorded utilizing a Cary 3E spectrophotometer (Varian Ltd., Australia) interfaced to a personal computer.

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

The iron content was determined using the iron colorimetric titration procedure with *ortho*-phenanthroline [23].

The molecular mass of the purified enzyme was determined using SDS-PAGE (12–15% polyacrylamide), native gradient PAGE (5–25% polyacrylamide), and gel filtration on Sephacryl S-100 and Superdex 75 (Pharmacia Biotech, Sweden). Molecular mass markers ranging from 14 000 to 140 000 Da were used for all of these procedures (Bio-Rad Laboratories, USA, and Pharmacia Biotech, Sweden).

SDS-PAGE was performed according to a modification of the Laemmli method [24]. The protein content in samples was 2–5 μg. The proteins were stained with Coomassie R-350.

The isoelectric point was determined by analytical IEF Phast System electrophoresis (Pharmacia Biotech, Sweden). The marker was the p*I* calibration kit from Pharmacia Biotech (Sweden).

The primary sequence was determined for the first 20 amino acids using an Applied Biosystems 470A gas-phase sequencer (USA), equipped with an on-line model 120A phenylthiohydantoin (PHT) derivatives analyzer [25]. The electrophoretic band corresponding to C1,2O was blotted into an Immobilon P membrane (Millipore, USA) and then cut for use in the analyzer.

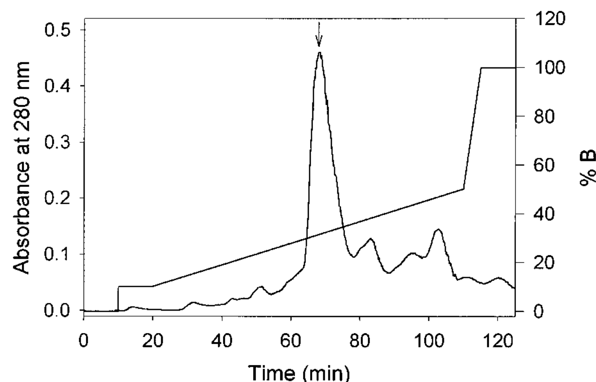

Fig. 1. Q Sepharose HP chromatography of catechol 1,2-dioxygenase from *A. radioresistens*. The arrow indicates the peak containing the C1,2O activity. The Na_2SO_4 gradient is also shown (100% B: 0.5 M Na_2SO_4, Tris-SO_4 20 mM pH 8.0).

3. Results and discussion

3.1. Purity of the enzyme

The results of the purification are summarized in Table 1. As shown in Fig. 1, a single major band appeared at half gradient on Q Sepharose HP corresponding to C1,2O as determined by activity measurements.

Two purification steps led to electrophoretically homogeneous preparations of catechol 1,2-dioxygenase. The pure C1,2O with specific activity of 24.5 U/mg is obtained after a 15.0-fold enrichment, and a yield of 54%.

As shown in Fig. 2, the purified enzyme preparation migrates as a single protein band on SDS-PAGE (15%). It is also eluted as a single symmetrical peak from Sephacryl S-100 or Q Sepharose HP columns.

Table 1
Purification of catechol 1,2-dioxygenase from *A. radioresistens*

Step	#	Volume (ml)	Activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	1	90	979	600	1.63	100	1.00
DE 52 cellulose	2	350	643	102	6.30	65.7	3.86
Q Sepharose HP	3	20	524	21.4	24.5	53.5	15.0

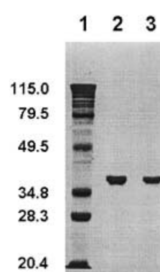

Fig. 2. SDS-PAGE (15%) of the purified catechol 1,2-dioxygenase from *A. radioresistens*. Lane 1: protein standards, molecular masses in kDa are indicated on the left; lanes 2 and 3: catechol 1,2-dioxygenase 5.0 μg and 2.5 μg respectively.

3.2. Molecular mass and subunit composition

The subunit molecular mass was estimated to be 38 700 Da from the relative mobility on a denaturing SDS-PAGE (15%). An estimate of the molecular mass of the native purified enzyme was obtained from the relative mobilities in native 5–25% gradient polyacrylamide slab gels and was calculated to be 39 000 Da suggesting that a single unit composes the active enzyme. This result was not confirmed using gel filtration chromatography on Sephacryl S-100 column (0.7×120 cm) or Superdex 75 (1.6×60 cm) equilibrated with 25 mM Tris-sulfate and 100 mM Na_2SO_4. Under these conditions the molecular mass was 78 000–79 000 Da, consistent with a dimeric quaternary structure of the enzyme. In order to understand the differences in the estimates of the molecular mass observed using different techniques we tried to change the ionic strength of the gel filtration buffer and indeed we observed a large spreading of the protein sample at molecular masses lower than 78 000 Da using 25 mM Tris-sulfate pH 8.0 and 400 mM Na_2SO_4 as the chromatographic buffer. Activity measurements on samples after gel filtration at high salt concentrations revealed the presence of an enzyme with the same activity both when the assays were performed at low or at high ionic strengths (50 mM Tris-sulfate pH 8.0 or 0.4 M Na_2SO_4 in the same buffer). To our knowledge this behavior has not been observed for any other dioxygenase known to date and it suggests that the increase in ionic strength results in a dynamic subunit dissociation which produces a still active 'sticky' monomeric form of the enzyme as also confirmed by native polyacrylamide gradient gels.

3.3. Spectroscopic properties and metal content

Visible and EPR spectra of the purified enzyme were consistent with the presence of high-spin Fe(III) ions (0.96 mol of iron per mol of enzyme) in the active site. The broad band centered at 440 nm in the electronic absorption spectrum (Fig. 3) is indicative of a ligand to Fe(III) charge transfer transition, characteristic of tyrosinate coordination to the iron ion [8,9]. The extinction coefficients at 205, 280 and 440 nm were calculated, using the method by Scopes [20], to be

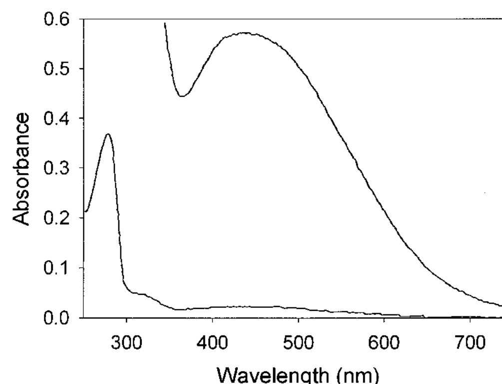

Fig. 3. UV-visible electronic absorption spectrum of the purified catechol 1,2-dioxygenase from *A. radioresistens* in 20 mM Tris-SO_4 pH 8.0. Enzyme concentration: upper spectrum 0.27 mM, lower spectrum 0.027 mM.

$\varepsilon_{205}{}^{1\ mg/ml} = 29.9$, $\varepsilon_{280}{}^{1\ mg/ml} = 0.720$, and $\varepsilon_{440}{}^{1\ mg/ml} = 0.0550$, $A_{280}/A_{440} = 12.6$.

The X-band EPR spectrum of the purified enzyme in frozen buffer at 4.2 K shows an intense signal at g = 4.3 typical of high-spin iron(III) ions in rhombic environments (data not shown).

3.4. Amino acid NH_2-terminal sequence

In Table 2 is reported the NH_2-terminal sequence of the purified C1,2O from *A. radioresistens* compared to that of isofunctional enzymes from strains of *A. calcoaceticus* [26,27]. The comparison reveals 45% identity between the C1,2O enzyme from *A. radioresistens* and the benzoate-grown *A. calcoaceticus* ADP-1 whereas the similarities between the enzyme from *A. radioresistens* and that from phenol-grown cells of *A. calcoaceticus* NCIB 8250 are not significant because when compared to previous alignments reported for a series of intradiol dioxygenases only one out of 20 amino acids is identical [28].

3.5. Substrate specificity, effects of pH and temperature, isoelectric point

In Table 3 the substrate specificity of the purified C1,2O is reported. Catechol and methyl substituted catechols are converted by the enzyme. Both intradiol and extradiol cleavages of 3-methylcatechol are observed. No activity with 3-chlorocatechol or protocatechuic acid was detected. The kinetic parameters for catechol cleavage were estimated to be: $K_M = 3.24$ μM, $K_{cat} = 15.8\ s^{-1}$ per subunit, and the activation energy was $E_a = 3570$ cal/mol.

The purified C1,2O shows a plateau of maximal activity from pH 6.0 to 8.5, the activity rapidly decreases outside this range. The optimal temperature range for activity is 37–47°C. The purified enzyme retained 100% activity after 1 month storage at 4°C.

The isoelectric point was estimated to be 5.0.

Table 2
NH_2-terminal sequence alignment of catechol 1,2-dioxygenases from *A. radioresistens* and other known *Acinetobacter* strains [25,26]

C1,2O	1				5					10					15					20
A. radioresistens	**Thr**	**Ala**	**Ala**	**Asn**	**Val**	**Lys**	**Ile**	**Phe**	**Asn**	**Thr**	**Glu**	**Glu**	**Val**	**Gln**	**Asn**	**Phe**	**Ile**	**Asn**	**Leu**	**Leu**
A. calcoaceticus ADP1			Met	Glu	**Val**	**Lys**	**Ile**	**Phe**	**Asn**	**Thr**	Gln	Asp	**Val**	**Gln**	Asp	**Phe**	Leu	Arg	Val	Ala
A. calcoaceticus NCIB8250								Met	**Asn**	Arg	Gln	Gln	Ile	Asp	Ser	Leu	Val	Gln	Gln	Met

Table 3
Substrate specificity of catechol 1,2-dioxygenase from *A. radioresistens*

Substrate	Activity (%)	Extra/Intra (%)
Catechol	100	0
3-Methylcatechol	14.4	3.2
4-Methylcatechol	16.5	0
3-Chlorocatechol	0	0
Protocatechuate	0	0

4. Conclusions

The catechol 1,2-dioxygenase from *A. radioresistens* purified to homogeneity in the present study corresponds to 3% of the total protein of the *A. radioresistens* and contains about 1 mol of iron ions per mol of the 38 700 Da unit.

Both the UV-visible absorption and the EPR spectra reported in the present paper indicate the presence of high-spin iron(III) ions in an environment similar to that observed for other intradiol cleaving dioxygenases [10–16].

Preliminary results for the presently purified dioxygenase show substrate specificities similar to isofunctional enzymes from other sources [10–13].

Comparison of the NH_2-terminal sequence of the purified C1,2O from *A. radioresistens* and that of the benzoate-grown *A. calcoaceticus* ADP-1 reveals 45% identity whereas for the phenol-grown *A. calcoaceticus* NCIB 8250 an unexpectedly much lower similarity was observed.

The quaternary structure of the enzyme isolated here has been observed to change when the ionic strength is varied. At low ionic strengths the enzyme appear to be a dimer $(\alpha Fe)_2$, upon increasing the ionic strength it monomerizes but still retains its catalytic activity. Such behavior has never been observed for any dioxygenase although it has been studied in detail for a series of enzymes like dehydrogenases, kinases, aldolase etc. [29–31].

Further experiments are currently being performed in order to better characterize the present enzyme in terms of catalytic properties and substrate specificities. Furthermore the changes in quaternary structure observed for the first time in dioxygenases will be the subject of additional investigations.

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