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Catechol 2,3-dioxygenase from the thermophilic, phenol-degrading *Bacillus thermoleovorans* strain A2 has unexpected low thermal stability

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Abstract Catechol 2,3-dioxygenase from the thermophilic *Bacillus thermoleovorans* A2 was purified and characterized. The catechol 2,3-dioxygenase has a molecular mass of 135000 Da and consists of four identical subunits of 34700 Da. One iron per enzyme subunit was detected using atom absorption spectroscopy. Enzyme activity was not inhibited by EDTA, suggesting that the iron is tightly bound. Addition of hydrogen peroxide to the enzyme completely destroyed activity, indicating that the iron was in the divalent state. The isoelectric point of the enzyme was 4.8. The enzyme displayed optimal activity at pH 7.2 and 70°C. The half-life of the catechol 2,3-dioxygenase at the optimum temperature was 1.5 min under aerobic conditions and 10 min in a nitrogen atmosphere. This stability of the enzyme is comparable to the stability of the enzyme from the mesophilic *Pseudomonas putida* mt-2. The stability of the cloned enzyme in *E. coli* extracts was identical to the stability in wild-type extracts, suggesting that no stabilizing factors were present in *Bacillus thermoleovorans* A2 In whole cells the half-life of the enzyme at 70°C was approximately 26 min, when protein synthesis was disrupted by chloramphenicol; however, the activity remained constant when protein synthesis was not inhibited. From these results we concluded that catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2 is not particularly thermostable, but that the organism retains the ability to degrade phenol at high temperatures because of continuous production of this enzyme.

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Introduction

Over the last decade, a number of bacteria have been isolated that can grow at high temperatures. These microorganisms contain enzymes that function at elevated temperatures. Some of these enzymes are stable at temperatures up to 140°C for more than an hour (Müller et al. 1998). Such enzymes are of great interest for industrial applications (Ladenstein and Antranikian 1998; Pennisi 1997).

The search for thermophilic microorganisms has recently been extended to include microorganisms that degrade environmental pollutants (Müller et al. 1998). We have isolated a number of strains that are able to degrade alkanes, phenol, and cresols at temperatures up to 70°C (Mutzel et al. 1996; Duffner et al. 1997). We have cloned and sequenced the genes for the enzymes catalyzing the degradation of phenol and cresols from one of these strains, *Bacillus thermoleovorans* A2 (Duffner and Müller 1998). Although the phenol hydroxylase represents a novel class of two component hydroxylases, the gene of the catechol 2,3-dioxygenase shared 43% homology with *tdnC* coding for the 3-methylcatechol 2,3-dioxygenase from *Pseudomonas putida* UCC2 and 40% with *xylE*, the catechol 2,3-dioxygenase encoded in the TOL plasmid (Duffner and Müller 1998). We describe here the purification, biochemical properties, and thermal stability of the second enzyme in the phenol degradation pathway, the catechol 2,3-dioxygenase.

Materials and methods

Growth conditions

Bacillus thermoleovorans A2 was cultivated at 65°C on mineral salts medium pH 6.5 containing 1 mM phenol, 0.01%

yeast extract, and 0.01% tryptone as described previously (Mutzel et al. 1996). The *Escherichia coli* clone containing the plasmid pA2FD1 (Duffner and Müller 1998) was cultivated in Luria Bertani (LB) medium (Sambrook et al. 1989) with 100 μg/ml ampicillin at 37°C. Cultivations were carried out in 2-l Erlenmeyer flasks containing 500 ml of the sterilized medium and incubated at 37°C for 24 h with vigorous shaking.

Preparation of cell-free extract

Cells were harvested by centrifugation for 20 min at 13000 × *g* and washed twice with 0.05 M phosphate buffer, pH 7.0, at 4°C, and the cell pellet was resuspended in the same buffer in an approximate 1:1 mass ratio of cell weight to buffer; 2 g of wet cells was used in the purification from the wild-type strain and 7 g wet cells were used in the purification from the *E. coli* clone. The suspension of the wild-type cells was sonicated for 15 min (200 W; duty cycle, 30%) with a Branson Sonifier W-450 (Heimann, Schwaebisch Gmünd) at 0°C in an ice bath. The cell suspension from *E. coli* was sonicated for 40 min. Unbroken cells and debris were removed by centrifugation at 4°C and 13000 × *g* for 30 min. The supernatant was stored at −70°C with no significant loss of activity over 2 months.

Enzyme assay

Unless otherwise stated, catechol 2,3-dioxygenase activity (catechol: oxygen 2,3-oxidoreductase [decyclizing], EC number 1.13.11.2) was determined at 60°C. The standard assay volume of 1 ml contained 50 mM phosphate buffer, pH 7.0, and 100 μM of substrate. The enzyme reaction was started by adding an appropriate amount of enzyme. The formation of the *meta* ring-cleavage product was followed spectrophotometrically by monitoring the increase at 375 nm for catechol ($\varepsilon = 33000\,M^{-1}$), at 379 nm for 4-methylcatechol ($\varepsilon = 28000\,M^{-1}$), at 378 nm for 3-methylcatechol ($\varepsilon = 13400\,M^{-1}$), at 379 nm for 4-chlorocatechol ($\varepsilon = 36100\,M^{-1}$), and at 343 nm for 3,4-dihydroxybenzoic acid ($\varepsilon = 13200\,M^{-1}$), as substrates (Hirose et al. 1994). The absorbance was read every 3 s after addition of the enzyme, and the initial slope of the curve obtained was used for calculation of the activity. The amount of enzyme catalyzing the conversion of 1 μmol substrate per minute was defined as 1 U.

Protein determination

Protein determination was carried out by the methods of Lowry et al. (1951) and Bradford (1976) with bovine serum albumin as standard.

Purification of the catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2

All purification steps were carried out in oxygen-free buffers (saturated with helium), and the samples were stored under nitrogen atmosphere at −70°C. Purification was achieved by applying the crude cell-free extract to a column containing 5 ml DMAE-Sephacel (Pharmacia, Sweden) equilibrated with 20 mM phosphate buffer, pH 7.0, and eluted with a linear gradient of KCl (0.0–0.2 M). The active fractions were pooled and concentrated by ultrafiltration and applied to a Pharmacia Superdex 200 HR 16/60 column, equilibrated with 200 mM phosphate buffer, pH 7.0. The active fractions were pooled and applied to a Pharmacia Mono Q 10/10 column, equilibrated with 20 mM phosphate buffer, pH 7.0, and eluted with a linear gradient (0–0.2 M KCl).

Purification of the C230 from the *E. coli* clone containing the plasmid pA2FDl

Crude extract from the *E. coli* clone containing the plasmid pA2FDl, which carries the gene for the catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2 cloned in pUC18 (Yanisch-Perron et al. 1985), was prepared as described earlier. The soluble crude extract from *E. coli* was heated to 56°C in a hot water bath under stirring, incubated for 5 min under nitrogen atmosphere, and then cooled rapidly to 4°C in an ice-saline bath. Protein precipitate was removed by centrifugation at 13000 x *g* for 20 min. The conditions for enzyme separation using anionic exchange chromatography (Mono Q) and gel permeation chromatography were as described for the enzyme from the wild-type strain.

Polyacrylamide gel electrophoresis (PAGE)

Proteins were electrophoretically separated by the discontinuous sodium dodecyl sulfate (SDS) system described by Laemmli (1970). The Low Molecular Weight Calibration Kit (Pharmacia, Uppsala, Sweden) was used as a calibration standard in the determination of molecular mass. Native PAGE was carried out as described without the addition of SDS to the gels or gel-loading buffer.

Other analytical methods

Protein molecular weights were estimated by gel permeation chromatography with a TSK 3000sw column (Pharmacia) using ferritin (440000 Da), catalase (232000 Da), aldolase (158000 Da) (Pharmacia), bovine serum albumin (BSA, 67000 Da), and α-chymotrypsin (23000 Da) (Sigma, München) as standards. The isoelectric point of the C230 was determined by isoelectric focusing (IEF) with the Multiphor II-System (Pharmacia) using an Ampholine PAG plate in a PH range 3.5–9.5 (Pharmacia) and IEF standards (Pharmacia). The iron content was measured with the aid of atom emission spectroscopy at 259.9 and 273 nm (ICP-AES; Plasma II, Perkin Elmer, Überlingen, Germany) using 300 μl of the purified enzyme at a concentration of 890 μg protein/ml.

Chemicals

Catechol, 3-methylcatechol, and 4-methylcatechol were obtained from Merck (Darmstadt, Germany); 3,4-dihydroxybenzoic acid was obtained from Sigma (München, Germany); chloramphenicol was from Boehringer (Mannheim, Germany); and 4-chlorocatechol was a kind gift from Dr. S. Fetzner, Oldenburg. All other chemicals were from Merck (Darmstadt, Germany) or Boehringer (Mannheim, Germany) and were of the highest purity available.

Results

Purification of the catechol 2,3-dioxygenase

Catechol 2,3-dioxygenase was purified from both the wild-type strain *Bacillus thermoleovorans* A2 and from the *E. coli* clone containing the plasmid pA2FD1. Both procedures yielded pure enzyme with identical mobilities in native and SDS-PAGE (Fig. 1). The specific activities of the C230 in the crude extracts from the wild-type strain and *E. coli* were comparable (0.4 U/mg protein for *Bacillus thermoleovorans* A2 and 0.37 U/mg for the *E. coli* clone). However, the yield of the purified enzyme from *E. coli* was twofold higher than that from the wild-type strain, possibly because of the use of a heat precipitation step to remove thermolabile *E. coli* proteins (Table 1).

Relative molecular mass and isoelectric point

By gel filtration, a molecular mass of $135\,000 \pm 10\,000$ Da was estimated for the catechol 2,3-dioxygenase. In SDS-PAGE, a unique band at $34\,700 \pm 1000$ Da (Fig. 1) was obtained, consistent with a homotetrameric structure of the enzyme. SDS-PAGE molecular weight determination is in good agreement with the theoretically calculated molecular mass of 35 487 Da from the deduced amino acid sequence (Duffner and Müller 1998). The isoelectric point of the catechol 2,3-dioxygenase was 4.8.

Iron content

The iron content of the purified catechol 2,3-dioxygenase was determined by atom absorption spectroscopy; 0.87 iron atoms per subunit was detected, suggesting that 1 iron atom per subunit was present. This result is in agreement with the iron ratio that has been obtained for extradiol dioxygenases from mesophilic organisms (Nakai et al. 1983; Harayama and Rekik 1989). This result suggests that the iron in the catechol 2,3-dioxygenase is tightly bound and not lost during purification. This conclusion is supported by the fact that the addition of EDTA in a final concentration of 1 mM had no effect on enzyme activity and that the addition of Fe^{2+} did not increase the activity. The addition of 0.6% H_2O_2 to the colorless solution of the purified enzyme completely inactivated the enzyme, which points to a ferrous state iron as prosthetic group.

Substrate specificity and kinetic properties

Catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2 has a high specificity for catechol. Other substrates are only attacked at very low ratios. The relative catalytic activities of the enzyme with other substrates are given in Table 2. The apparent K_m value for catechol was 2 µM. At high

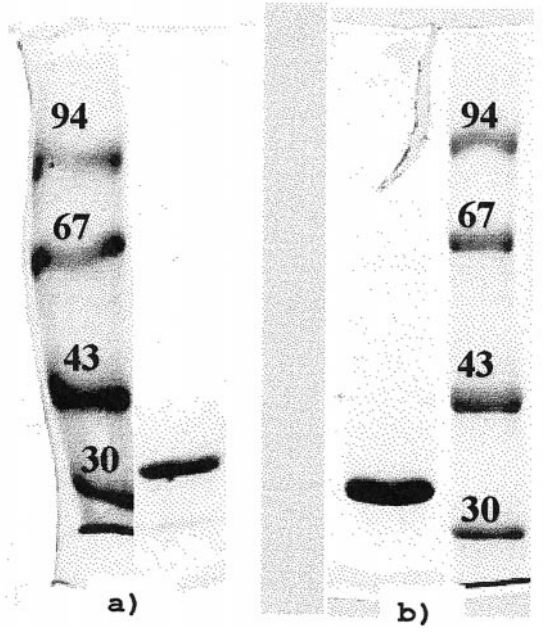

Fig. 1a,b. Polyacrylamide gel electrophoresis of purified catechol-2,3-dioxygenase from the **a** wild-type strain *Bacillus thermoleovorans* A2 and the **b** *E. coli* clone pFDA2 with standard proteins (MW given in kDa)

Table 1. Purification of catechol-2,3-dioxygenase from the *E. coli* clone containing the plasmid pFDA2 started with 7 g cells (wet weight)

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification factor (x-fold)	Yield (%)
Cell-free extract	435	0.37	161	1	100
Heat precipitation	210	0.7	147	1.9	91
Mono-Q anion exchanger, pH 7.0	6.4	1.8	12	4.9	66
Superdex 200 gelfiltration	5.2	2	10.4	5.4	6.5
Mono-Q anion exchanger pH	0.64	9	5.8	24	3.8

Table 2. Substrate specificity of catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2

Substrate	Relative activity (%)
Catechol	100
3-Methylcatechol	17
4-Methylcatechol	8
4-Chlorocatechol	16
3,4-Dihydroxybenzoic acid (protocatechuic acid)	2

The assays contained, in 1 ml, 50 mM potassium phosphate buffer, pH 7, and 0.5 U of enzyme. The substrate concentration was 0.1 mM

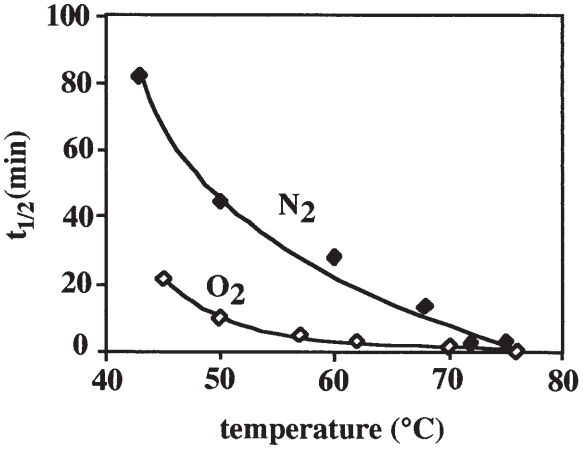

Fig. 2. Temperature dependence of the half-lifes of purified catechol-2,3-dioxygenase from *Bacillus thermoleovorans* A2 under aerobic (O_2) and anaerobic (N_2) conditions

substrate concentrations, catechol inhibited the reaction. The inhibitor constant, determined according to Bisswanger (1994), was 3.9 mM.

Effect of pH and temperature on the activity

The pH optimum of the catechol 2,3-dioxygenase was 7.2. Determinations were carried out in universal buffer (Britton and Robinson 1931), phosphate buffer, and Tris-HCl buffer. Tris buffer showed an inhibitory effect on the catechol 2,3-dioxygenase. In comparison with phosphate buffer, the remaining enzyme activity in Tris-HCl at pH 7.0 was only 15%. The inhibitory effect of Tris-HCl on the activity of catechol 2,3-dioxygenase was also described for the enzyme from *Pseudomonas putida* mt-2 (Nakai et al. 1983).

The observed temperature optimum of 70°C for the enzyme was slightly higher than the growth optimum of the wild-type strain. However, at this temperature the enzyme was not very stable. The half-life of the purified enzyme at 70°C was 1.5 min under aerobic conditions. This instability has been reported previously for mesophilic enzymes and was attributed to the oxidation of iron to the ferric state (Harayama and Kok 1992; Polissi and Harayama 1993). Therefore, we checked enzyme stability in the absence of oxygen, where the enzyme was slightly more stable with a half-life of 10 min (Fig. 2). The half-lives of the enzyme in crude extracts of the wild-type strain and of *E. coli* were similar (Table 3), indicating that no stabilizing factors had been removed during purification.

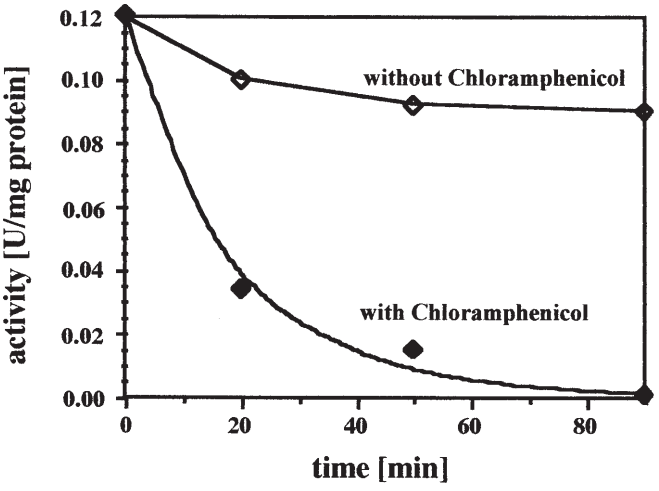

Fig. 3. Activity of catechol 2,3-dioxygenase from *Bacillus thermoleovorans* A2 at 70°C in whole cells in the absence (*open symbols*) and in the presence (closed symbols) of 340 µg/ml chloramphenicol: 600 ml of a grown culture (OD_{600} = 1,2) was resuspended in fresh medium with and without chloramphenicol. At given time intervals, 10-ml samples were removed and cells were harvested by centrifugation. Crude extracts for activity measurements were prepared as described in Methods

To test the stability of the enzyme in whole cells of *Bacillus thermoleovorans* A2, we incubated freshly grown cells at 70°C in medium containing chloramphenicol, which inhibited further protein synthesis. Decrease of catechol 2,3-dioxygenase activity in whole cells was slower than in crude extracts, indicating that in vivo the enzyme has a higher stability (Fig. 3). However, the observed half-life of 26 min is still low. In cells incubated without the antibiotic, the activity remained almost constant (Fig. 3), which implies that strain *Bacillus thermoleovorans* A2 retains its ability to cleave catechol at 70°C by this enzyme through continuous de novo synthesis of the enzyme.

Discussion

The enzyme purified in this study is the first catechol 2,3-dioxygenase that has been purified from an thermophilic organism. Although several enzymes have been detected in extracts from thermophilic microorganisms (Buswell 1974; Adams and Ribbons 1988; Dong et al. 1992; Natarajan et al. 1994), none of them has been purified to homogeneity. Several *meta*-cleaving enzymes have been purified and characterized from mesophilic microorganisms (Nozaki et al. 1963; Müller et al. 1982; Nakai et al. 1983; Hirose et al. 1994; Schmid et al. 1997; Kita et al. 1999). The enzyme purified here is very similar to its mesophilic counterparts. Like most catechol 2,3-dioxygenases, it consists of four identical subunits of about 35 kDa and contains divalent iron in the catalytic center. This finding is in agreement with

Table 3. Comparison of the stabilities of the catechol 2,3-dioxygenases from the thermophilic *B. thermoleovorans* A2 and the mesophilic *P. putida* mt-2

B. thermoleovorans A2				*P. putida* mt-2	
Under N_2		Under O_2		Under O_2	
Temp. (°C)	$\tau_{1/2}$ (min)	Temp. (°C)	$\tau_{1/2}$ (min)	Temp. (°C)	$\tau_{1/2}$ (min)
50	45	50	10.0	55	73.8
60	28	57	4.8	63	21.1
68	14	62	3.3	65	18.0
72	7	70	1.5	68	14.7
75	2.3	76	0.3	71	6.1

From Adam and Ribbons (1988)

the observation that in the sequence of the enzyme from *B. thermoleovorans* A2 all amino acid residues that are thought to be involved in iron and substrate binding in mesophilic enzymes are conserved (Duffner and Müller 1998). The K_m value of 2 μM for catechol is very similar to the 3 μM observed for the enzyme from *Pseudomonas putida* mt-2 (Nakai et al. 1983). Another similarity that these enzymes share is the well-known sensitivity of extradiol ring-cleavage enzymes toward oxygen (Buswell 1974; Müller et al. 1982; Pollissi and Harayama 1993; Kaschabek et al. 1998). A remarkable contrast between the two enzymes is the high specificity of the catechol 2,3-dioxygenase of *Bacillus thermoleovorans* A2 for catechol compared to the enzyme from *Pseudomonas putida* mt-2 (Hirose et al. 1994).

Surprisingly, the temperature stability of the enzyme from *Bacillus thermoleovorans* A2 is not higher than that of the mesophilic enzyme from *Pseudomonas putida* mt-2 (see Table 3). The half-life of 1.5 min of the purified enzyme in the presence of oxygen is certainly not enough to support growth of the organism on phenol as sole substrate under these conditions. Incubation of the enzyme in the absence of oxygen increased the stability, indicating that oxidation of iron is an important inactivating factor. However, the enzyme requires oxygen for activity. In whole cells, this protection of the enzyme against oxidation is more efficient as the half-life in vivo was 26 min. This time seems to be long enough to allow the organism to maintain a constant level of activity by continuous de novo synthesis of the enzyme.

The higher stability in whole cells may also be the result of a reactivation of inactivated enzyme by the action of a ferredoxin as is described for the catechol 2,3-dioxygenase from the TOL plasmid (Polissi and Harayama 1993; Hugo et al. 1998). However, such a reactivation cannot be the reason for the observed high activity in cells without inhibition of protein synthesis because this reactivation does not require de novo protein synthesis. One possibility we cannot exclude from our data is that a ferredoxin, if it is present in *Bacillus thermoleovorans* A2, is also thermolabile and has to be resynthesized to maintain the activity as we cannot distinguish between oxidation and thermal inactivation in whole cells.

Nevertheless, it is clear that the catechol 2,3-dioxygenase is not thermostable and loses its activity even when no oxygen is present, and therefore this enzyme has to be synthesized continuously to maintain a level of activity high enough to enable growth at high temperature. Although this seems to be a rather ineffective way to cope with a high temperature, it gives the organism the ability to maintain growth on phenol at temperatures up to 70°C.

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