Extremophiles (1998) 2:439–446

ORIGINAL PAPER

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Gentisate 1,2-dioxygenase from *Haloferax* sp. D1227

Received: November 19, 1997 / Accepted: May 12, 1998

Abstract Gentisate 1,2-dioxygenase from the extreme halophile *Haloferax* sp. D1227 (*Hf*. D1227) was purified using a three-step procedure. The enzyme was found to be a homotetramer of 42000 ± 1000 Da subunits, with a native molecular weight of 174000 ± 6000 Da. The optimal salt concentration, temperature, and pH for enzyme activity were 2 M KCl or NaCl, 45°C, and pH 7.2, respectively. The gene encoding *Hf*. D1227 gentisate 1,2-dioxygenase was cloned, sequenced, and expressed in *Haloferax volcanii*. The deduced amino acid sequence exhibited a 9.2% excess acidic over basic amino acids typical of halophilic enzymes. Four novel histidine clusters and a possible extradiol dioxygenase fingerprint region were identified.

Key words Gentisate 1,2-dioxygenase · Extreme halophile · Archaea · Halophilic enzymes · Ring-fission dioxygenases

Introduction

Under aerobic conditions, aromatic compounds are transformed by monooxygenases and dioxygenases into dihydroxylated central intermediates including gentisate, catechol, and protochatechuate, which are subsequently cleaved by ring-fission dioxygenases (Gibson and Subramanian 1984). Intradiol dioxygenases such as catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase cleave between the two hydroxyl groups (*ortho* cleavage) and typically contain nonheme Fe^{3+}, whereas extradiol dioxygenases such as catechol 2,3-dioxygenase and protocatechuate 4,5-dioxygenase cleave at a bond proximal to one of the two hydroxyl groups (*meta* cleavage) and contain Fe^{2+}.

Phylogenetic analyses have indicated that while enzymes within each class are evolutionarily related, intradiol and extradiol dioxygenases have arisen from different ancestors (Eltis and Bolin 1996; Harayama et al. 1992; Neidle et al. 1988). Gentisate 1,2-dioxygenase cannot be classified as either intradiol or extradiol because the cleavage of gentisate occurs between the carboxyl substituent and the proximal hydroxyl group (Fig. 1). Gentisate 1,2-dioxygenases from *Moraxella osloensis*, *Pseudomonas testosteroni*, *Pseudomonas acidovorans*, *Bacillus stearothermophilus* PK1, and *Rhodococcus erythropolis* S-1 have been purified and characterized (Crawford et al. 1975; Harpel and Lipscomb 1990; Kiemer et al. 1996; Suemori et al. 1993). The enzymes from *P. testosteroni* and *P. acidovorans* have been demonstrated to contain active-site Fe^{2+} with coordination sites for both the substrate and O_2, suggesting that gentisate 1,2-dioxygenases in these organisms are mechanistically more similar to extradiol dioxygenases.

Haloferax sp. D1227 (*Hf*. D1227), an extreme halophile isolated from soil contaminated with highly saline oil brine near Grand Rapids, Michigan, is the only reported aerobic archaeon utilizing aromatic compounds (i.e., benzoic acid, cinnamic acid, and 3-phenylpropionic acid) as sole carbon and energy sources for growth (Emerson et al. 1994). During our study of the 3-phenylpropionic acid metabolism by *Hf*. D1227 (Fu and Oriel, submitted), gentisate 1,2-dioxygenase activity was demonstrated in cell extracts grown on 3-phenylpropionic acid, cinnamic acid, or benzoic acid. Examination of the gentisate 1,2-dioxygenase from this extreme halophile was therefore undertaken. In this article, we report the purification and characterization of gentisate 1,2-dioxygenase from *Hf.* D1227, as well as the cloning, sequencing, and expression of the encoding gene.

Communicated by W.D. Grant

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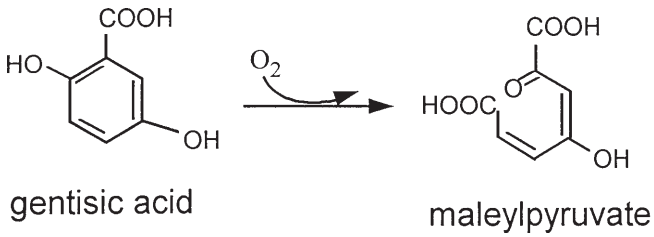


Fig. 1. The reaction catalyzed by gentisate 1,2-dioxygenase

Materials and methods

Materials

All chemicals used in this study were reagent grade. Benzoic acid and cetyltrimethylammonium bromide (CTAB) were purchased from Aldrich (Milwaukee, WI, USA). Catechol, protocatechuic acid, gentisic acid, ferrous ammonium sulfate, 2,2′-dipyridyl, ascorbic acid, sodium dodecyl sulfate (SDS), and polyvinylidene difluoride (PVDF) membrane were obtained from Sigma (St. Louis, MO, USA). Phenyl Sepharose CL-4B, Bio-Gel hydroxyapatite, and Superose^{TM}12 prepacked columns were from Pharmacia (Uppsala, Sweden). *N*,*N*,*N*′,*N*′,-tetramethylethylenediamine (TEMED), ammonium persulfate, acrylamide, bisacrylamide, restriction enzymes, and T4 DNA ligase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). X-Gal, isopropylthio-β-D-galactoside (IPTG), and *Taq* DNA polymerase were obtained from Life Technologies (Grand Island, NY, USA). GENECLEAN II Kit was obtained from BIO 101 Inc. (La Jolla, CA, USA).

Microorganisms, plasmid vectors, media, and growth conditions

Haloferax sp. D1227 has been described (Emerson et al. 1994). *Haloferax volcanii* WFD11 was obtained from Dr. W.F. Doolittle (Dalhousie University, Halifax, Canada). pBluescript SK(+) was purchased from Strategene (La Jolla, CA, USA). Plasmid vector pMDS30 was a gift from Dr. M.L. Dyall-Smith and has been described (Kamekura et al. 1996).

For growth of *Hf.* D1227 on benzoic acid, BS3 mineral salts medium (Emerson et al. 1994) of the following composition (in g/l) was used: $(NH_4)_2SO_4$, 0.33; KCl, 6.0; $MgCl_2{\cdot}6H_2O$, 12.1; $MgSO_4{\cdot}7H_2O$, 14.8; KH_2PO_4, 0.34; $CaCl_2{\cdot}H_2O$, 0.36; and NaCl, 100. Before sterilization, 1 ml/l of a trace element solution (Widdel and Bak 1992) was also added and the pH was adjusted to 6.9 with KOH. Filter-sterilized benzoic acid was added after sterilization. For rich medium (BSYT), BS3 mineral salts medium was supplemented with 3 g/l yeast extract and 3 g/l tryptone. *E. coli DH5α* was grown on LB medium (Maniatis et al. 1982). For solid media, 15 g/l Bacto-agar was added before autoclaving.

Cells were cultured aerobically in erlenmeyer flasks containing 1:4 volume of medium. Flasks were incubated at 37°C on a rotary shaker at 200 rpm. Growth was monitored by measuring $OD_{600\,nm}$ on a Gilford DU spectrophotometer.

Enzyme assay and protein determination

Unless stated otherwise, gentisate 1,2-dioxygenase was assayed at room temperature by measuring the formation of maleylpyruvate at 334 nm for 30 s with a Perkin Elmer (Norwalk, CT, USA) double-beam 124 spectrophotometer (Lack 1959). The reaction mixture (2 ml) contained 0.25 mM gentisic acid, 50 µl enzyme, and 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl. The reference cuvette contained the same reaction mixture with the exception of gentisic acid. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol maleylpyruvate/min. The molar absorption coefficient for maleylpyruvate ($\varepsilon = 10800$) (Crawford et al. 1975) was used to calculate enzyme activity.

Protein concentrations of the enzyme preparations were determined by the method of Bradford (1976) with bovine serum albumin dissolved in 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl as the standard.

Preparation of cell-free extracts

Mid-log-phase cells grown on 3 mM benzoic acid were harvested by centrifugation at 10600 × *g* for 30 min. After washing twice with 1:5 culture volume of 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, cells were resuspended in 10 ml of the same buffer. Then, 2.5-ml aliquots of the suspension were sonicated four times (30 s at 50 W followed by 2 min of cooling) in an ice bath using a 4710 Series ultrasonic homogenizer (Cole-Parmer, Chicago, IL, USA). After removing unbroken cells and cell debris by centrifugation at 47800 × *g* for 30 min (4°C), 5 mM ascorbic acid was added to the supernatant to stabilize the enzyme.

Enzyme purification

Cell-free extract (10 ml) was loaded onto a phenyl Sepharose CL-4B column (6 cm × 1.7 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl and 5 mM ascorbic acid. The sample was eluted with the same buffer at a flow rate of 14 ml/h. Fractions (0.55 ml) with high activity were pooled, diluted with an equal volume of 10 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl and 5 mM ascorbic acid, and loaded onto a hydroxyapatite column (3 cm × 1.7 cm) equilibrated with the same buffer. The sample was eluted with 60 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl and 5 mM ascorbic acid at a flow rate of 7 ml/h. Fractions (0.55 ml) with high activity were pooled and concentrated to 200 µl using a Centricon-10 concentrator (MW cutoff, 10000) (Amicon, Beverly, MA, USA). The concentrated sample was then loaded onto a Superose^{TM}12 prepacked

column (30 cm × 1 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl and 5 mM ascorbic acid and eluted with the same buffer at a flow rate of 6 ml/h. Fractions (0.55 ml) with the highest activity were pooled and stored at 4°C. All chromatographic steps were performed at 4°C except gel filtration, which was carried out at room temperature on a Pharmacia FPLC (fast performance liquid chromatography) system.

Estimation of native molecular mass and subunit molecular weight

The molecular weight of the native gentisate 1,2-dioxygenase was determined by gel filtration chromatography as described in the purification procedure. The column was calibrated with bovine thyroglobulin (M_r, 669000), horse spleen apoferritin (M_r, 443000), sweet potato β-amylase (M_r, 200000), yeast alcohol dehydrogenase (M_r, 150000), bovine serum albumin (M_r, 66000), and bovine erythrocyte carbonic anhydrase (M_r, 29000) in the MW-GF-1000 kit (Sigma).

The subunit molecular weight of gentisate 1,2-dioxygenase was obtained by CTAB-PAGE performed as described by Eley et al. (1979). A 4% concentrating gel and a 12% separating gel were used. The samples were desalted by diafiltration with 100 mM potassium phosphate buffer (pH 7.0) using Centricon-10 before electrophoresis. Proteins were stained with Coomassie brilliant blue R-250 (Garfin 1990). Molecular weight standards SDS-6H from Sigma were used. CTAB-PAGE was done at 30 mA and room temperature with cold-water cooling of the buffer chamber.

N-terminal amino acid sequencing and peptide sequencing

The purified enzyme was run on SDS-PAGE and electroblotted onto PVDF membrane by the method of Matsudaira (1987). The protein band stained with Coomassie brilliant blue R-250 was excised and submitted for N-terminal amino acid sequencing. For peptide sequencing, enzyme on the membrane was digested with trypsin at 37°C for 20 h and the peptides were separated by HPLC using a C_{18} column (0.8 mm × 250 mm) with a gradient of 5% acetonitrile to 50% acetonitrile. Both N-terminal amino acid sequencing and peptide sequencing were performed on a 494 Procise protein sequencer (Applied Biosystems, Weiterstadt, Germany) using automated Edman degradation at the Michigan State University (MSU) Macromolecular Structure Facility.

Preparation of *Haloferax* sp. D1227 genomic DNA

Mid-log-phase cells of *Hf.* D1227 grown in 100 ml BSYT medium were harvested by centrifugation at 10600 × *g* for 30 min. After washing twice with 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, the pellet was resuspended in 50 ml distilled water for cell lysis. After removal of cell debris by centrifugation at 47800 × *g* for 30 min, the supernatant was extracted once with an equal volume of phenol and twice with an equal volume of phenol/chloroform. DNA in the aqueous phase was precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at −16°C overnight. After centrifugation, the pellet was rinsed with 1 ml of 70% ethanol, air-dried, and resuspended in 1 ml of sterile redistilled H_2O. This preparation was then treated with RNase at a final concentration of 25 μg/ml (Maniatis et al. 1982).

Cloning and sequencing of the *Haloferax* sp. D1227 gentisate 1,2-dioxygenase gene

A part of the open reading frame for the *Hf.* D1227 gentisate 1,2-dioxygenase gene was first obtained by polymerase chain reaction (PCR) amplification of the *Hf.* D1227 genomic DNA using two degenerate primers derived from the purified *Hf.* D1227 gentisate 1,2-dioxygenase. The entire nucleotide sequence of the coding and flanking regions of the gene was then obtained by chromosomal walking using PCR techniques. The overall strategy for cloning the *Hf.* D1227 gentisate 1,2-dioxygenase gene is depicted in Fig. 2. For this, a 226-bp fragment (segment a) of the *Hf.* D1227 gentisate 1,2-dioxygenase gene was first obtained by PCR amplification of *Hf.* D1227 genomic DNA using forward primer PO45 [5′-GCIGA(A/G)CA(A/G)GA(A/G)CCIAA(A/G)GA-3′] and reverse primer PO44 [5′-TAICCIGT(A/G)TTIACIGG(A/T/C/G)AC-3′], corresponding to N-terminal sequences AEQEPKE and YGTNVPV from the intact enzyme and a tryptic peptide of the enzyme, respectively. Segment a was sequenced using PO44 and PO45 as sequencing primers.

A 900-bp fragment (segment b) upstream and overlapping segment a was obtained by PCR amplification of the ligation mixture of *Sac*I-digested genomic DNA and pBluescript SK(+) with primers T_3 from pBluescript SK(+) [5′-AATTAACCCTCACTAAAGGG-3′] and PO67 [5′-GCCGAATTGGTTTCCGAAGT-3′], which is 88 bp downstream of the 5′-end of segment a. Partial sequence of segment b was obtained using PO67 as the sequencing primer. Segment c, a 644-bp fragment downstream and overlapping segment a, was obtained by PCR amplification of the ligation mixture of *Sal*I-digested genomic DNA and pBluescript SK(+) with T_7 primer from pBluescript SK(+) [5′-GTAATACGACTCACTATAGGGC-3′] and primer PO66 [5′-TCTGGAAGTGGGAAGACATC-3′], which is 82 bp upstream of the 3′-end of segment a. Segment c was sequenced using T_7 and PO66 as sequencing primers. A 264-bp fragment (segment d) downstream and overlapping segment c was obtained by PCR amplification of the ligation mixture of *Kpn*I-digested genomic DNA and pBluescript SK(+) with primers T_7 and PO71 [5′-TGTTTCCGACG-ATGTCGTTC-3′], which is 54 bp upstream of the 3′-end of segment c. The sequence of segment d was determined using T_7 and PO71 as sequencing primers.

The PCR reaction mixtures in a total volume of 100 μl contained 2 μl of DNA template (∼100 ng), 20 mM Tris-HCl

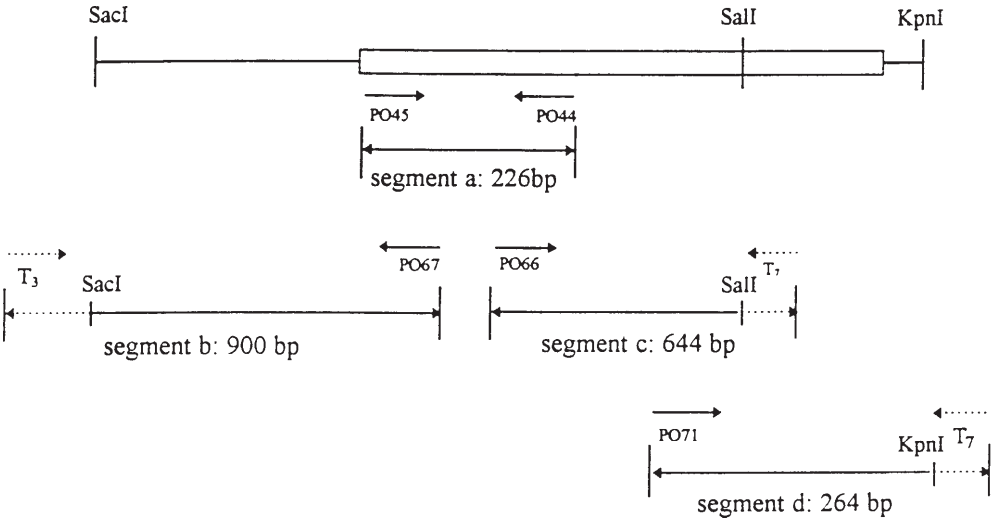


Fig. 2. Strategy for cloning the coding and flanking regions of the *Haloferax* sp. D1227 (*Hf.* D1227) gentisate 1,2-dioxygenase gene. The coding region is enclosed in the *box. Solid lines* represent *Hf.* D1227 sequences and *dashed lines* represent pBluescript SK(+) sequences

(pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2.5 U of *Taq* DNA polymerase, and 30 pmol of each primer. PCR reactions were carried out on a Perkin Elmer GeneAmp PCR system 2400, and thermal cycling conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. A final extension was performed at 72°C for 6 min. PCR products were purified with the GENECLEAN II kit following the protocol recommended by the manufacturer. Nucleotide sequencing was done at the Nucleic Acid Sequencing Facility of Michigan State University with an ABI Prism sequencer (Applied Biosystems).

Expression of Hf. *D1227 gentisate 1,2-dioxygenase gene in* Haloferax volcanii *WFD 11*

A 1353-bp fragment encoding the gentisate 1,2-dioxygenase gene and its flanking regions was amplified from the genomic DNA of *Hf.* D1227 using primers PO92 [5′-GCGGAAAGCTTTGGGAGTAC-3′] and PO93 [5′-TAGGTACCTACCCGGCCTGG-3′]. PO92 corresponds to nucleotides 10 to 29 of the sequence shown later in this report in Fig. 4, except that a G at position 21 was changed to a T to introduce a *Hin*dIII restriction site. PO93 corresponds to nucleotides 1346 to 1363 of the sequence with two random nucleotides T and A added to the 5′-end for effective digestion at the *Kpn*I site. PCR reaction and product purification were carried out as described.

After double digestion with *Hin*dIII and *Kpn*I, this 1353-bp fragment was ligated to plasmid pMDS30, which had been cut with the same two enzymes. Transformation of the resulting plasmid into *E. coli DH5α* and the plasmid isolation from *E. coli* transformants were performed using standard methods (Maniatis et al. 1982). Transformation of the resulting plasmid into *Haloferax volcanii* WFD 11 was carried out using the polyethylene glycol (PEG) method described by Holmes and Dyall-Smith (1990). Transformants were selected on rich BSYT plates supplemented with 15% sucrose and 0.3% novobiocin. Confirmed to contain the 1353-bp insert by PCR amplification with primers PO92 and PO93, one colony was inoculated into 10 ml BS3 medium containing 5 mM pyruvate and 0.3% novobiocin, and gentisate 1,2-dioxygenase activity was measured as described in Enzyme assay.

Results

Purification of gentisate 1,2-dioxygenase from *Haloferax* sp. D1227

The soluble gentisate 1,2-dioxygenase from *Hf.* D1227 was purified 207 fold from cell-free extracts of benzoic acid-grown cells in three steps with 54% yield to give a preparation with a specific activity of 187 U/mg (Table 1). This preparation was electrophoretically homogeneous, showing one band on an CTAB-PAGE gel (results not shown). All purification steps were performed in the presence of 2 M KCl because *Hf.* D1227 gentisate 1,2-dioxygenase lost its activity irreversibly in low-salt buffers. Ascorbic acid, which proved to be an stabilizer for the enzyme, was added to all the purification buffers.

Molecular weight and subunit structure

The molecular weight of the native purified *Hf.* D1227 gentisate 1,2-dioxygenase was estimated to be 174000 ± 6000 by gel filtration. The subunit molecular weight of the purified enzyme estimated by CTAB-PAGE was 42000 ± 1000. These results suggest that *Hf.* D1227 gentisate 1,2-dioxygenase is a tetramer composed of four equally sized 42-kDa subunits. CTAB-PAGE instead of SDS-PAGE was used in subunit molecular weight estimation in this study because it has been demonstrated (Monstadt and Holldorf 1990) that electrophoresis with this cationic detergent is a more accurate method to determine the subunit molecular weight of halophilic proteins, as halophilic proteins only

Table 1. Purification of gentisate 1,2-dioxygenase from *Haloferax* sp. D1227 (*Hf.* D1227)

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	114	104	0.9	100	1
Phenyl Sepharose Cl-4B	19	100	5.3	96	6
Hydroxyapatite	1.7	84	49.4	80	55
Superose 12	0.3	56	186.7	54	207

bind a small amount of SDS, resulting in reduced mobility and overestimation of the molecular weight. The gel filtration column was calibrated in the presence of 2 M KCl with non-halophilic marker proteins from Sigma, yielding a calibration curve identical to that in measured in the absence of salt.

N-terminal amino acid sequencing and peptide sequencing

Amino acid residues at the N-terminus of *Hf.* D1227 gentisate 1,2-dioxygenase were determined to be A-E-Q-E-P-K-E-L-L-E-M-S-T-D-T-E-R-L-L-E-E-N-D-L-R-P-L-W-E-V-E-K-D-F-G-N-Q-F-G-G-. The N-terminal sequence of one tryptic peptide of this enzyme was also obtained: V-A-V-P-V-N-T-G-Y-R-. This peptide sequence along with the N-terminal sequence was used in gene cloning and sequencing (see following). Among the five purified gentisate 1,2-dioxygenases, only N-terminal sequences of *P. testosteroni* and *P. acidovorans* enzymes have been reported, and these have no sequence similarity with that of *Hf.* D1227 gentisate 1,2-dioxygenase.

Effect of salt, pH, and temperature on the activity and stability of the purified *Haloferax* sp. D1227 gentisate 1,2-dioxygenase

The salt dependence of *Hf.* D1227 gentisate 1,2-dioxygenase activity, shown in Fig. 3, exhibited the highest activity in the presence of 2 M KCl. NaCl could substitute for KCl, yielding the same 2 M optimum. The optimal pH was pH 7.2 and the optimal temperature for the activity was 45°C (data not shown). The salt, pH, and temperature dependence of the enzyme activity were determined by measuring the initial reaction rate over 30 s, during which time no inactivation of the enzyme was observed under all conditions studied.

The purified *Hf.* D1227 gentisate 1,2-dioxygenase lost activity rapidly and irreversibly in salt concentrations less than 2 M KCl, with 40% of activity remaining when the enzyme was kept in 1.5 M KCl for 30 min in ice and assayed. This enzyme was also heat labile; incubation at 55°C for 10 min caused 96% loss of activity. The enzyme was stable over a narrow pH range (pH 6.5–8.0). In 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, the enzyme was stable for as long as 3 days at 4°C when 5 mM ascorbic acid or dithiothreitol (DTT) was added, but retained little activity following storage for 1 week under these conditions.

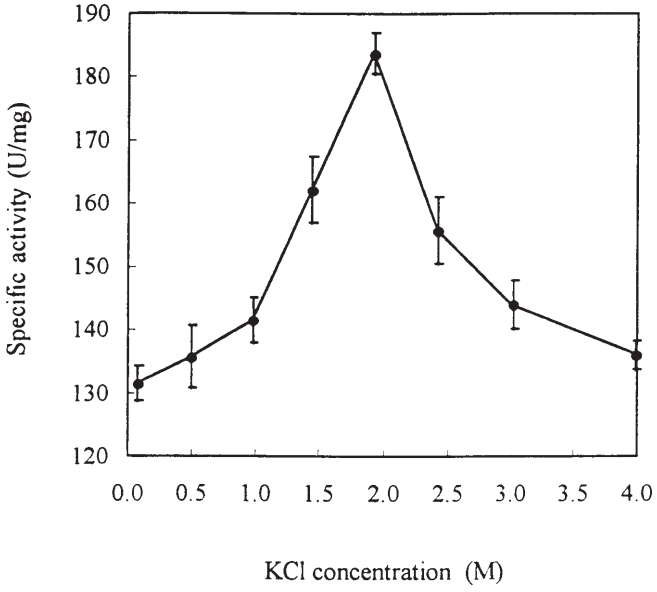


Fig. 3. Effect of salt concentration on *Hf.* D1227 gentisate 1,2-dioxygenase activity

Catalytic properties and product identification

The V_{max} of the gentisate 1,2-dioxygenase reaction was 187 ± 24 U/mg, and the K_m value for gentisic acid was 95 ± 8 μM. Flushing the reaction mixture with nitrogen gas caused 89% loss of enzyme activity, confirming the involvement of O_2 in the reaction. Catechol and protecatechuate were not substrates for this enzyme. The high-affinity Fe^{2+} chelator 2,2′-dipyridyl was an strong inhibitor for the enzyme, with an K_i of 1.2 mM.

As a result of enzymatic gentisate oxidation, a compound with an absorbance maximum at 334 nm was observed that was lost on acidification. These properties are characteristic of the expected maleylpyruvate product (Lack 1959).

Cloning and sequencing of the *Haloferax* sp. D1227 gentisate 1,2-dioxygenase gene

The gene encoding *Hf.* D1227 gentisate 1,2-dioxygenase was cloned and sequenced as described in Materials and methods. The determined nucleotide sequence and the de-

duced amino acid sequence are shown in Fig. 4. The open reading frame of this gene contains 1074 bp, which encodes a protein of 358 amino acids with a molecular weight of 40 547 Da. The deduced protein sequence of *Hf.* D1227 gentisate 1,2-dioxygenase had a 9.2% excess acidic over basic amino acids with an isoelectric point of 4.15. A putative promoter sequence, TTAT, similar to the archaeal box A consensus TTA(T/A) usually located 27 ± 4 bp upstream from the ATG start codon (Dennis 1993), was observed 25 bp upstream from the ATG translation initiation codon. Four histidine clusters, H-X-H, were found in the sequence, whose function is unclear although it has been shown that histidine residues are involved in both iron ligand binding and catalytic function in eubacterial extradiol dioxygenases (Eltis and Bolin 1996). When compared with sequences of ring-cleavage dioxygenases, a segment of *Hf.* D1227 gentisate 1,2-dioxygenase sequence encoding three residues (His, Tyr, and Glu) conserved in the eubacterial extradiol dioxygenase fingerprint region (Eltis and Bolin 1996) was identified (Fig. 4).

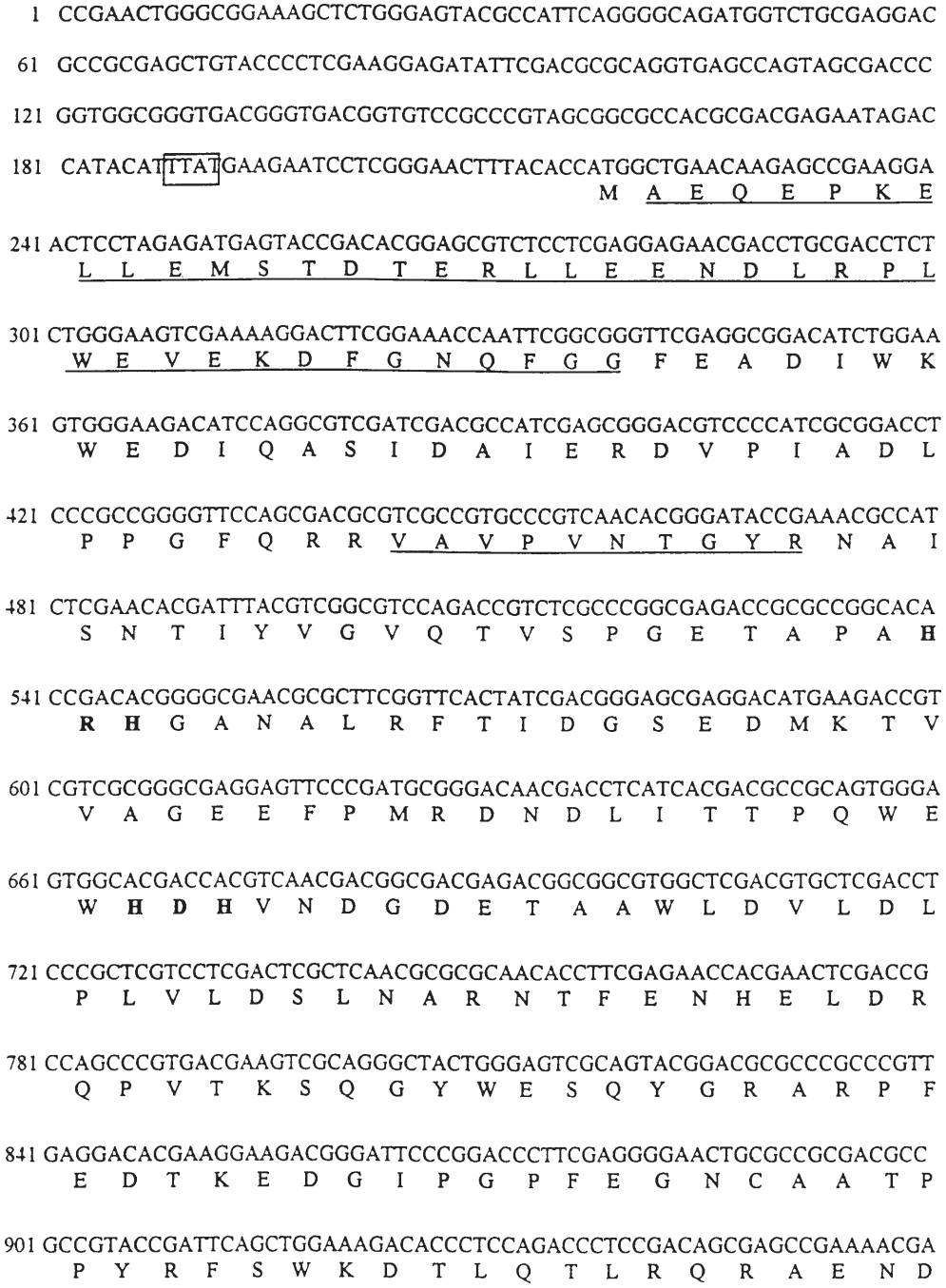
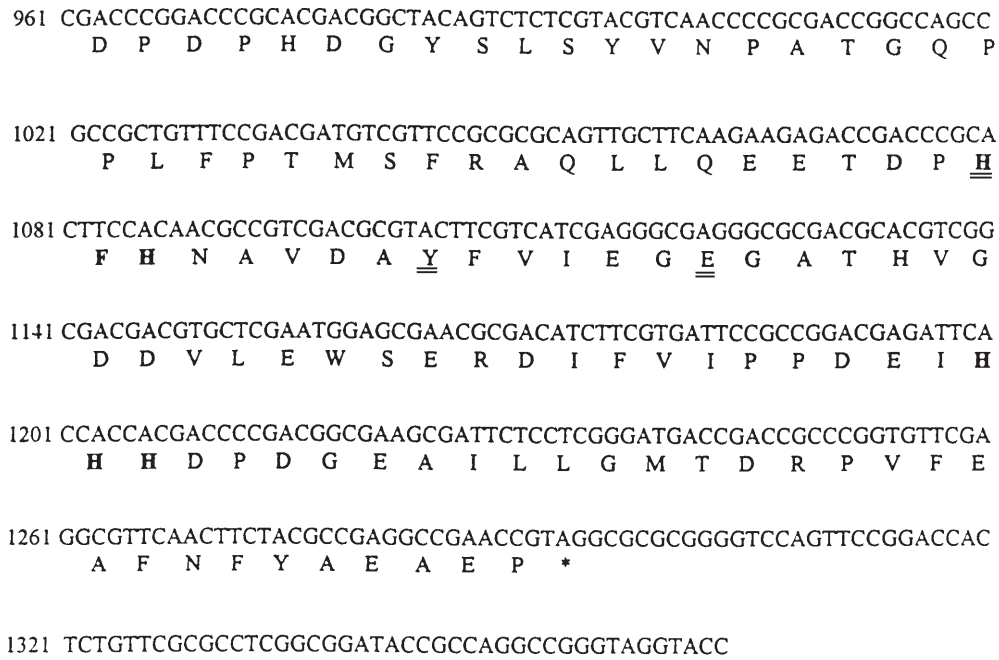


Fig. 4. Nucleotide sequence of the *Hf.* D1227 gentisate 1,2-dioxygenase gene and the deduced amino acid sequence. Amino acid sequences determined by protein sequencing are *underlined*, a putative promoter sequence is *boxed*, the histidine clusters are in *bold letters*, and the putative conserved residues are *double-underlined*

Fig. 4. *Continued*



Expression of the *Haloferax* sp. D1227 gentisate 1,2-dioxygenase gene in *Haloferax volcanii* WFD 11

The gentisate 1,2-dioxygenase activity in the *Hf. volcanii* transformant was 4.8 ± 0.5 U/g protein, which was 0.54% of the activity in *Hf.* D1227. No increase of enzyme activity was detected when 0.5 mM benzoic acid was added as an inducer to the growth medium. This low activity is believed to be real, because it was readily reproducible and because no activity was detected in either the *Hf. volcanii* transformant containing only shuttle vector pMSD30 without insert or in non transformed *Hf. volcanii* grown in benzoic acid.

Discussion

Gentisate 1,2-dioxygenase from *Hf.* D1227 is similar in molecular weight and subunit structure to that from *Pseudomonas testosteroni*, *P. acidovorans*, and *Moraxella osloensis* (Crawford et al. 1975; Harpel and Lipscomb 1990), all being tetramers composed of four equal-sized subunits of molecular weight about 40 kDa. The gentisate 1,2-dioxygenase from *Bacillus stearothermophilus* is a homohexamer with a subunit molecular weight of 40 kDa, and that of *Rhodococcus erythropolis* is a homooctamer with a subunit molecular weight of 43 kDa (Kiemer et al. 1996; Suemori et al. 1993). Gentisate 1,2-dioxygenase from *Hf.* D1227 demonstrates a K_m value for gentisate similar to that of the two *Pseudomonas* enzymes, and has a pH optimum (around pH 7.0) similar to four of the purified eubacteria enzymes. The exception is the *Rhodococcus* enzyme, exhibiting maximal activity at pH 8.5. *Hf.* D1227 gentisate 1,2-dioxygenase differs from the eubacterial counterparts in the requirement of 2 M KCl or NaCl for enzyme activity and stability. By possessing additional acidic residues (glutamic acid and aspartic acid), halophilic proteins are highly adapted to function in a high-salt milieu (Dennis and Shimmin 1997; Dym et al. 1995; Frolow et al. 1996). Compared to the chemical neutrality of bulk protein for nonhalophiles (Reistad 1970), *Hf.* D1227 gentisate 1,2-dioxygenase contains a 9.2% excess acidic over basic amino acids, which is typical of halophilic proteins (Prub et al. 1993). The acidic amino acid content of the nonhalophilic gentisate 1,2-dioxygenases has not been determined.

Four of the purified eubacterial gentisate 1,2-dioxygenases required exogenously added Fe^{2+} for enzyme activity. Although the gentisate 1,2-dioxygenase from *Hf.* D1227 did not require added Fe^{2+} for activity, $Fe(NH_4)_2(SO_4)_2$ could partially restore the lost activity of older *Hf.* D1227 enzyme preparations and 2,2′-dipyridyl was an inhibitor for this enzyme, suggesting the presence of Fe^{2+} in the enzyme.

Sequence comparison by BLAST showed no GenBank sequence similar to the *Hf.* D1227 gentisate 1,2-dioxygenase gene. This is not surprising given this enzyme's archaeal origin and the absence of eubacterial gentisate 1,2-dioxygenase sequences in the database. Although the expression of gentisate 1,2-dioxygenase in *Hf. volcanii* verified the function of the cloned gene, the expression level was quite low. Several factors might contribute to this low activity. First, the pMDS30 vector used does not have a promoter adjacent to the *Hf.* D1227 gentisate 1,2-dioxygenase gene insert, and the control elements associated with the gentisate pathway were apparently not included with the

cloned gene because induction of expression by benzoate was not observed. Second, the stability of the gentisate 1,2-dioxygenase and its mRNA in *Hf. volcanii* transformants is unknown.

In summary, gentisate 1,2-dioxygenase from the extreme halophile *Haloferax* sp. D1227 is similar to its eubacterial counterparts in terms of subunit size and metal participation. The enzyme also possesses the properties characteristic of halophilic enzymes, including the requirement of 2 M KCl or NaCl for activity and stability and an excess of acidic over basic amino acids. It will be of interest to determine whether the four histidine clusters in the *Hf.* D1227 gentisate 1,2-dioxygenase gene are features shared by eubacterial gentisate 1,2-dioxygenases. Although a segment of the *Hf.* D1227 gentisate 1,2-dioxygenase contains the histidine, tyrosine, and glutamic acid residues conserved in the eubacterial extradiol dioxygenase fingerprint region, further genetic and structural studies are required to confirm that these residues are involved in metal binding and active site function of the dioxygenase.

Acknowledgments We thank Michael L. Dyall-Smith for plasmid vectors and assistance. We are extremely grateful to Robert Hausinger for help with purification. We also thank Olga Maltseva and Tae-Kyou Cheong for many helpful discussions. This work was partially supported by the MSU Biotechnology Research Center.

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