Role of Cysteine Residues in 4-Oxalomesaconate Hydratase from *Pseudomonas ochraceae* NGJ1

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4-Oxalomesaconate hydratase from *Pseudomonas ochraceae* NGJ1 is unstable in the absence of reducing reagents such as dithiothreitol, and strongly inhibited by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). To study the role of cysteine residues in enzyme catalysis, the eight individual cysteine residues of the enzyme were replaced with serine residues by site-directed mutagenesis. The catalytic properties and chemical modification of wild- and mutant type-enzymes by DTNB showed that (i) none of eight cysteine residues was essential for enzyme catalysis; (ii) the inhibition by DTNB was mostly due to modification of Cys-186; (iii) Cys-96 might be another residue reacting with DTNB, and its modification caused an increase in the *K*ₐₘ-value for 4-oxalomesaconate; (iv) the other six cysteine residues were inaccessible to DTNB, but susceptible to HgCl₂; and (v) only replacement of Cys-186 remarkably improved the stability of the enzyme in the absence of reducing reagent.

**Key words:** 5,5′-dithiobis(2-nitrobenzoic acid); Ellman’s reagent; 4-oxalomesaconate hydratase; *Pseudomonas ochraceae*

Protocatechuic acid is one of the central intermediates in the microbial metabolism of aromatic compounds.1) 4-Oxalomesaconate hydratase (2-hydroxy-4-oxobutyrate-1,2,4-tricarboxylate 2,3-hydro-lyase, EC 4.2.1.83) (OMH) is found on the bacterial catabolic pathways responsible for the *meta*-degradation of protocatechuic acid.2,3) The enzyme catalyzes the reversible hydration of 4-oxalomesaconate to 1,4-carboxy-4-hydroxy-2-oxo-oacidate, which is degraded to oxaloacetate and p-ruvurate by Mg²⁺-dependent aldolase (Fig. 1).4,5) OMH purified from *Pseudomonas ochraceae* (PocOMH) is a dimer with a subunit molecular mass of about 37 kDa.2) PocOMH requires no addition of metal ions for activity, and shows no absorption peak around 400 nm. Thus, PocOMH is distinguishable from both metal-activated hydratases such as *Escherichia coli* 2-hydroxypentadione acid hydratase (EC 4.2.1.80) and hydratases containing the Fe–S cluster, such as *E. coli* dihydroxyacid dehydratase (EC 4.2.1.9).6,7) The catalytic amino acid residues in PocOMH have not been identified. The substrate 4-oxalomesaconate is unstable. It easily lactonizes to 2-pyrone-4,6-dicarboxylic acid under acidic pH, and converts to the enol-form under alkaline pH.8–10) Therefore, the convenient spectrophotometric assay is impractical for examination of the activity versus pH relationship, which is useful in searching for catalytic amino acid residues. Cysteine residues are of particular interest, since PocOMH is activated by various thiol compounds, including dithiothreitol (DTT), cysteine, GSH, and 2-mercaptoethanol, and is strongly inhibited by HgCl₂ and p-chloromercuribenzoate.2,10) Thiol compounds are also required for storage of the enzyme, especially in its highly purified state. However, it is unclear whether the cysteine residues are essential for the catalytic function or structural integrity of the enzyme.

Hara et al. cloned the gene *ligJ* from lignin- assimilating bacteria *Sphingomonas paucimobilis* SYK-6, and identified its gene product LigJ as OMH (SpaOMH).11) SpaOMH purified from recombinant *E. coli* shows a much higher *K*ₐₘ-value for 4-oxalomesaconate than does PocOMH. SpaOMH also shows no apparent requirement of thiol compounds for stability, although it is somewhat activated by cysteine, and strongly inhibited by HgCl₂. Several genes showing a high degree of homology to *ligJ* have been found in other bacterial strains, but their gene products were not

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**Abbreviations:** DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; IPTG, isopropyl β-thiogalactopyranoside; KE, 20 mm potassium phosphate, pH 7.0, containing 10% (v/v) ethylene glycol; KED, KE containing 1 mM DTT; OMH, 4-oxalomesaconate hydratase; PAGE, polyacrylamide gel electrophoresis; PAR, 4-(2-pyridylazoloresorcinol; PocOMH, *Pseudomonas ochraceae* OMH; RpaOMH, *Rhodopseudomonas palustris* OMH; SpaOMH, *Sphingomonas paucimobilis* OMH
characterized by biochemical methods. Previously, we cloned and sequenced the gene cluster responsible for the meta-degradation of protocatechuate from *P. ochraceae* NGJ1 (formerly *P. ochraceae*) (GenBank accession nos. AB050935 and AB127969). In this study, the gene encoding PocOMH was subcloned into pKF18k and expressed in *E. coli* MV1184. To answer the above question on the role of cysteine residues, the eight individual cysteine residues in PocOMH were replaced with serine residues by site-directed mutagenesis. The wild- and mutant-type enzymes are purified to investigate their kinetic properties, stability, and reaction toward Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid), DTNB]. One of the mutant-type enzymes was found to be more stable than the wild-type enzyme in the absence of reducing reagent. The results obtained in this study are discussed based on the X-ray structure of *Rhodopseudomonas palustris* CGA009 OMH (RpaOMH), which reveals that RpaOMH is a homodimer and that it tightly binds one Zn ion per subunit (Brokhaven Protein Data Bank ID, 2GWG, Forouhar, F., Abashidze, M., Jayaraman, S., Cunningham, K., Ciao, M., Ma, L., Xiao, R., Acton, T. B., Montelione, G. T., Hunt, J. F., and Tong, L., Northeast Structural Genomics Consortium (NESG), Crystal structure of 4-oxalomesaconate hydratase, LigJ, from *Rhodopseudomonas palustris*, Northeast structural genomics target RPR66, released May 23, 2006).

Materials and Methods

Materials. DTNB and 4-(2-pyridylazo)resorcinol (PAR) were obtained from Nacalai Tesque (Kyoto, Japan) and TCI (Tokyo) respectively. 2-Pyrone-4,6-dicarboxylic acid was chemically synthesized as described previously. All other chemicals were commercial products of analytical grade.

DNA manipulations. DNA was extracted from agarose gel with a GeneClean (Qbiogene, Carlsbad, CA). Plasmid DNA was prepared from competent *E. coli* cells with a MiniPrep (Qiagen, Hilden, Germany). Synthetic oligonucleotides, restriction enzymes, a ligation kit, and competent cells of *E. coli* BMH71-18 mutS and MV1184 were obtained from Takara (Kusatsu, Japan). DNA digestion, ligation, and transformation were carried out as described by Sambrook *et al.* DNA sequencing was performed on denatured double-stranded DNA templates with a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Warrington, UK), followed by electrophoresis with an ABI 377 DNA sequencer (Perkin-Elmer, Norwalk, CT). The sequence data were analyzed using the DNASIS program (Hitachi Software Engineering, Yokohama, Japan).

Amino acid sequence. After SDS-polyacrylamide gel electrophoresis (SDS–PAGE), the protein was electroblotted onto a polyvinylidene difluoride membrane (ATTO, Tokyo), and the amino acid sequence was determined with a Procise protein sequencer (Perkin-Elmer). Protein sequence similarities were searched using the FASTA program and the BLAST program. CLUSTAL W was used for sequence alignment.

Cloning of the PocOMH gene. Plasmid pBEE7.5, which was prepared by cloning the *EcoRI* 7.5-kb DNA fragment at *EcoRI* site of pBluescript SK–, harbors the *PocOMH* gene. The gene is 1,029 bp long, corresponding to a protein of 342 amino acid residues with a calculated molecular mass of 38,260 Da, and it has an appropriate ribosome-binding sequence GGAG upstream of the initial ATG codon. A *HincII*-digest of pBEE7.5 gave five major DNA fragments of 3.0, 2.2, 2.0, 1.5, and 0.9 kb on agarose gel electrophoresis. Among these, the 2.2-kb DNA fragment carrying the entire *PocOMH* gene was extracted from the gel, introduced in the *HincII* site of pKF18k to obtain plasmid pKHH2.2, and transformed into *E. coli* BMH71-18 mutS for site directed mutagenesis, or into MV1184 for preparation of wild-type enzyme. An appropriate transformant, in which the *PocOMH* gene was in the same direction as the *lac* promoter, was selected by the restriction analysis of plasmid DNA.

Site-directed mutagenesis. Site-directed mutagenesis of the *PocOMH* gene was performed by the oligonucleotide-directed dual amber method using a Mutanexpress Km (Takara). To replace the TGC/T codon of cysteine with the AGC or TCT codon of serine at positions 92, 96, 124, 131, 183, 186, 205, and 260, the following synthetic oligonucleotides were used as mutagenic primers (the amino acid changes and their positions in the amino acid sequences are shown, and the codon changes are underlined): C92S, 5′-pCACAGCTCGTGTGCTATGCGCAGCC-3′; C96S, 5′-pCTGACGCCGAAGCTCAGCTCGTGTGC-3′; C124S, 5′-pAGC-TCGGAATGCTGTTGCAAGGT-3′; C131S, 5′-pT-

Fig. 1. Degradation of 4-Oxalomesaconate in the Protocatechuate Meta-Degradation Pathway. Reactions 1 and 2 are catalyzed by OMH and 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17) respectively.
ACTGCTCCACAGCTTGACCAGT-3’; C183S, 5’-pAAGCATGAAATTGCCTGGTGGAAGA-3’; C186S, 5’-pGTGGTGGAAGATGCGATTTGACG-3’; C205S, 5’-pTGCAAGCTACCCGCCATGCAAAG-3’; and C260S, 5’-pGCTGGTGACAGCTGTGCGTGCAAT-3’. E. coli MV1184 was used as the host for recombinant plasmids. The base changes were confirmed by DNA sequencing with appropriate sequencing primers.

Preparation of wild- and mutant-type enzymes. The wild- and mutant-type enzymes were produced in E. coli MV1184 cells. Recombinant E. coli was grown at 37°C in 800 ml of Luria-Bertani medium containing kanamycin (50 μg/ml). When A_{600} reached about 0.10, 5 mM isopropyl β-thiogalactopyranoside (IPTG) was added, and the culture was continued until late log phase. The bacterial cells were collected by centrifugation, washed 10% ethylene glycol (KE buffer), and disrupted with a Sonifier (Branson, Danbury, CT). After centrifugation to remove cell debris, the supernatant (crude extract) was passed through a Bio-Gel A-1.5 m (Bio-Rad, Hercules, CA) equilibrated with KE buffer. The active fractions were pooled and put on a column (2 x 10 cm) of DEAE-cellulose (Serva, Heidelberg, Germany) previously equilibrated with KE buffer. After washing with 200 ml of KE buffer, the enzyme was eluted with a linear gradient prepared with 100 ml of KE buffer and 100 ml of KED buffer containing 0.5 M KCl. The active fractions were pooled, and the enzyme was precipitated by the addition of solid ammonium sulfate (75% saturation). The precipitate was collected by centrifugation, washed once with 50 mM potassium phosphate, pH 7.0, and stored at -25°C until needed.

All manipulations for enzyme purification were performed at 0-4°C. The bacterial cells were suspended in 10 ml of 20 mM potassium phosphate, pH 7.0, containing 10% (v/v) ethylene glycol and 1 mM DTT (KED buffer) and disrupted with a Sonifier. The disrupted supernatant (crude extract) was passed through a column (3 x 30 cm) of Bio-Gel A-1.5 m (Bio-Rad, Hercules, CA) equilibrated with KED buffer. The active fractions were pooled and put on a column (2 x 10 cm) of DEAE-cellulose (Serva, Heidelberg, Germany) previously equilibrated with KED buffer. After washing with 200 ml of KED buffer, the enzyme was eluted with a linear gradient prepared with 100 ml of KED buffer and 100 ml of KED buffer containing 0.5 M KCl. The active fractions were pooled, and the enzyme was precipitated by the addition of solid ammonium sulfate (75% saturation). The precipitate was collected by centrifugation and dissolved in about 3 ml of 20 mM potassium phosphate, pH 7.0, containing 1 mM DTT. The solution was dialyzed against 200 ml of the same buffer, and then against 200 ml of 1 mM DTT. It was put on isoelectric focusing with a 110-ml glass column, as described by Vesterberg. Sucrose was used to maintain the pH gradient obtained with a 2.5% Pharmalite (pH 4-6.5) (GE Healthcare, Piscataway, NJ), and 1 mM DTT was added to prevent enzyme inactivation. Electrophoresis was done at 900 V for 48 h. The active fractions were pooled and then dialyzed against 200 ml of KED buffer. The solution was passed through a Bio-Gel A-1.5 m column (3 x 20 cm) equilibrated with KED buffer to remove Pharmalite. The purified enzyme preparation was concentrated in a collodion bag apparatus (Sartorius, Göttingen, Germany), and stored at -25°C.

When removal of DTT was necessary, the enzyme solution was passed through a column (1.7 x 25 cm) of Sephadex G-50 (GE Healthcare), previously equilibrated with 20 mM potassium phosphate, pH 7.0, containing 10% ethylene glycol (KE buffer).

Determination of enzyme activity. 4-Oxalomesaconate was prepared by alkaline hydrolysis of 2-pyrene-4,6-dicarboxylic acid. 2-Pyrene-4,6-dicarboxylic acid (20 mg) was incubated at 15°C for about 1 h in 6 ml of 83 mM NaOH. Then the solution was neutralized with HCl. To determine the concentration of 4-oxalomesaconate, a sample (20 μl) of the solution was incubated at 25°C in 3 ml of 0.1 M Tris-acetate, pH 8.0, containing 5 mM potassium phosphate, pH 8.0, 2 mM MgCl₂, 3 μg of the purified PocOMH, 0.14 mM NADH, 15 units of malate dehydrogenase (Sigma, St. Louis, MO), 15 units of lactic dehydrogenase (Sigma), and 3 μg of purified 4-hydroxy-4-methyl-2-oxoglutarate aldolase. The last enzyme was prepared from recombinant E. coli as described previously. The amount of 4-oxalomesaconate was estimated from the decrease in A₃₆₅, which indicates the total amount of oxaloacetate and pyruvate (Fig. 1).

The activity of OMH was determined at 25°C in the forward reaction. The standard assay mixture (3 ml) contained 50 mM Tris-acetate, pH 8.0, 0.08 mM 4-oxalomesaconate, and the enzyme. The decrease in A₃₆₅ was measured. The activity was calculated with an E₅₀₀ of 6 mm⁻¹ cm⁻¹. One unit of enzyme activity was defined as μmol of substrate reacted per min under the assay conditions, and the specific activity was defined as unit per mg of the enzyme. Protein was determined by the method of Lowry et al., as modified by Bennett. Bovine serum albumin was used as a standard. A subunit molecular mass of 38 kDa was used for calculation of the kcat value.

Chemical modification with DTNB. The enzyme solution (2.7 ml) containing 0.42-0.88 mg of enzyme in KE buffer was mixed with 0.3 ml of 1 M Tris-Cl, pH 8.0, and 0.1 ml of 10 mM DTNB dissolved in 50 mM potassium phosphate, pH 7.0. The mixture was incubated at 25°C, and the increase in A₂₅₂ due to the formation of 2-nitro-5-thiobenzoate diion was monitored. The number of SH groups was calculated as described by Riddle et al. During incubation, a sample (2-50 μl) was withdrawn to measure residual enzyme activity under the standard assay conditions. To determine the total number of SH groups in the enzyme, 1% (w/v) SDS was added to the mixture.

Determination of enzyme-bound Zn ion. The purified enzyme (0.92 mg) in 3 ml of 20 mM Tris-Cl, pH 7.5, containing 1 mM mercaptoethanol was mixed with 0.15 ml of 1.25 mM PAR (50% ethanolic solution). After incubation at 25°C for 10 min, 0.35 ml of 10% (w/v) SDS was added. Alternatively, the enzyme in 3 ml of 20 mM Tris-Cl, pH 7.5, was mixed with 0.35 ml of 10% (w/v) SDS and boiled for 10 min prior to the addition of PAR. The increase in A₅₉₀ was measured with the blank mixture without enzyme. The amount of Zn ions was calculated with an E₅₀₀ of 66 mm⁻¹ cm⁻¹.

Other analytical methods. Disc PAGE was done with
7.5% gel at 4 °C by the method of Davis. SDS–PAGE was done with 12% gel by the method of Laemmli. Proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass of the native enzyme was determined by gel filtration on Sephadex G-200 (Aldrich, Milwaukee, WI), previously equilibrated with KED buffer, as described previously. UV and visible absorption spectra were measured with a UV-300 spectrophotometer (Shimadzu, Kyoto, Japan). The CD spectrum in the UV region was measured with a cuvette (light path, 1 mm) using a J-820 CD polarimeter (Jasco International, Tokyo). The enzyme samples (0.55–0.74 mg/ml) in KED buffer were scanned 10 times. Each spectrum was corrected by subtracting the appropriate blank.

**Results**

**Multiple sequence alignment**

The deduced amino acid sequences of OMHs from various bacterial strains were compared. PocOMH showed a high degree (80–99%) of homology toward OMHs from *Comamonas testosteroni* BR6020 and *R. palustris* CGA009, and a medium degree (63–68%) of homology toward OMHs from *Arthrobacter keyseri* 12B, *S. paucimobilis* SYK-6, and *Sphingomonas*. sp. LB126.

Fig. 2. Alignment of the Deduced Amino Acid Sequences of OMHs from Various Bacterial Strains.

Sequences: a, *P. ochraceae* NGJI (GenBank accession no. AB050935); b, *R. palustris* CGA009 (TrEMBL accession no. Q6N0R4); c, *A. keyseri* 12B (GenBank accession no. AF331043); d, *S. paucimobilis* SYK-6 (GenBank accession no. AB073227). The amino acid residues of each sequence are numbered on the right. Identical residues are shown by plus signs. Cysteine residues replaced by site-directed mutagenesis are underlined.

**Purification and properties of recombinant PocOMH**

The PocOMH gene was subcloned from pBEE7.5 to pKF18k to obtain plasmid pHKH2.2. *E. coli* MV1184 harboring pHKH2.2 was grown in Luria-Bertani medium containing kanamycin and IPTG. The wild-type enzyme was purified about 14-fold with a yield of 34% from the crude extract of *E. coli* by gel filtration column chromatography, ion exchange column chromatography, and isoelectric focusing (Table 1). The purified enzyme preparation was near homogeneity on SDS–PAGE (Fig. 3). The molecular mass (72 kDa) and the subunit molecular mass (38 kDa) were estimated by gel filtration on Sephadex G-200 and SDS–PAGE respectively. The N-terminal amino acid sequence (MIIDVHGHYTT-A-...) was identified by Edman degradation.
PAALGAWR), determined by Edman degradation, completely coincided with that deduced from the nucleotide sequence. The recombinant enzyme showed properties similar to those of PocOMH purified from *P. ochraceae* in respect of pI, absorption spectrum, optimum pH, and substrate specificity. The \( K_m \) for 4-oxalomesaconate and the \( V_{\text{max}} \) were determined to be 10.7 \( \mu \text{M} \) and 116 \( \mu \text{mol/min/mg} \) respectively by double reciprocal plot. The \( K_m \)-value was significantly lower than that (138 \( \mu \text{M} \)) of SpaOMH.11) Citrate inhibited the reaction competitively, with a \( K_i \)-value of 0.41 \( \mu \text{M} \), determined by the method of Dixon.33) When the enzyme was preincubated with various reagents at 25°C for 10 min in 50 \( \mu \text{M} \) potassium phosphate, pH 7.0, none of the following reagents (each 1 \( \mu \text{M} \)) affected the enzyme activity: EDTA, 1,10-phenanthroline, 8-hydroxyquinoline, 2,2'-dipyridine, tiron, Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{3+}\), Co\(^{3+}\), and Ni\(^{2+}\). Cu\(^{2+}\) strongly inhibited the enzyme, and Zn\(^{2+}\) weakly inhibited it. Although diethylpyrocarbonate (5 \( \mu \text{M} \)) reacted with several histidine residues in the enzyme as judged by the increase in \( A_{235} \), no inhibition was detectable (data not shown). The purified enzyme gradually lost activity in the absence of a reducing reagent. At 4°C, more than 90% of activity was lost for 1 d (Fig. 4). Brief incubation of the inactivated enzyme with DTT restored about 74% of the activity. The inactivated enzyme showed mobility on disc PAGE similar to that of the native enzyme (data not shown), suggesting that the inactivation involved no gross change in the molecular form of the enzyme. When stored at −25°C in KED buffer, no appreciable loss of enzyme activity was observed for at least 2 months.

**Measurement of enzyme-bound Zn ion**

A recent X-ray crystallographic study demonstrated that RpaOMH binds one Zn ion per subunit. This appears to conflict with the observation that the activity

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**Fig. 3.** Analysis of Enzyme Preparations by SDS–PAGE.

The crude extract (35 \( \mu \text{g} \), lane 1) and the purified enzyme preparations (1–2 \( \mu \text{g} \), lanes 2–10) were put on SDS–PAGE. Lanes 1 and 2, wild-type enzyme; lane 3, C92S; lane 4, C96S; lane 5, C124S; lane 6, C131S; lane 7, C183S; lane 8, C186S; lane 9, C205S; lane 10, C260S; lane M, marker proteins.

**Table 1.** Purification of PocOMH from *E. coli* Harboring pKHH2.2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>168</td>
<td>1324</td>
<td>7.88</td>
<td>100</td>
</tr>
<tr>
<td>Bio-Gel A-1.5 m</td>
<td>82.0</td>
<td>1402</td>
<td>17.1</td>
<td>106</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>10.2</td>
<td>1013</td>
<td>99.3</td>
<td>76.5</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>4.0</td>
<td>452</td>
<td>113</td>
<td>34.1</td>
</tr>
</tbody>
</table>
of PocOMH is hardly affected by various metal chelators. Hence, the Zn content of PocOMH was measured with a metallochromic chelator PAR. When purified wild-type enzyme was incubated at 25°C for 10 min with PAR in the presence of 1 mM mercaptoethanol, no decrease in enzyme activity was found, and a small amount of Zn ions, possibly due to non-specific contaminants, was observed. The molar ratio (r) of Zn ions to enzyme subunit was estimated to be 0.14. Incubation at 25°C in 1% (w/v) SDS prompted the release of Zn ions (r = 0.43). When the enzyme was boiled for 10 min in 1% (w/v) SDS prior to the addition of PAR, the release of Zn ions was further prompted (r = 1.07). This value is close to 1.00 if non-specific contaminants are corrected for. These results suggest that PocOMH possesses one Zn ion per subunit, and that it strongly binds to the enzyme protein and reacts with the metal chelators only under denaturing conditions.

**Inactivation of wild-type PocOMH by DTNB**

When wild-type PocOMH (subunit concn., 6.8 μM) was incubated at 25°C with an excess amount of DTNB (0.32 mM) in the presence of 1% (w/v) SDS, about 7.2 SH groups (molar ratio against enzyme subunit) were modified by DTNB. This is in agreement with the cysteine content deduced from the nucleotide sequence of the PocOMH gene. In the absence of SDS, about one SH group rapidly reacted with DTNB, and then an additional one SH group reacted at a slower rate concomitant with a significant loss of enzyme activity (Fig. 5A). No further reaction of SH groups was observed during prolonged incubation (30 min). Possibly, the other six SH groups are buried in the enzyme molecules and are inaccessible to the bulky DTNB molecules, unless the enzyme is unfolded with denaturing agents. Citrate (10 mM), a weak competitive inhibitor, weakly repressed both the increase in A_{412} and the loss of enzyme activity.

**Properties of the mutant-type enzymes**

The eight individual cysteine residues in PocOMH, Cys-92, 96, 124, 131, 183, 186, 205, and 260, were replaced with serine residues by site-directed mutagenesis. Each mutant-type enzyme was purified to near homogeneity (12–71 fold purification with a yield of 25–34%) (Fig. 3). All the mutant-type enzymes were active, suggesting that none of the eight cysteine residues is essential for enzyme catalysis (Table 2). C92S showed the lowest k_{cat} and K_m values, although the k_{cat}/K_m value was slightly higher than that of the wild-type enzyme. C124S showed the highest k_{cat}/K_m value, whereas C96S and C183S showed somewhat lower values. The kinetic constants of C96S were markedly different from those of the wild-type enzyme. K_m increased about 8-fold, and k_{cat} increased about 2-fold. The increase in the K_m-value suggests that Cys-96 is involved in the binding of 4-oxalomesaconate. The alteration in the kinetic constants was not striking with other mutant-type enzymes.

![Fig. 5](https://example.com/fig5.png)

**Table 2. Kinetic Constants of the Wild- and Mutant-Type Enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>k_{cat} (s^{-1})</th>
<th>K_m (μM)</th>
<th>k_{cat}/K_m (s^{-1}μM^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>75.4</td>
<td>10.7</td>
<td>7.05</td>
</tr>
<tr>
<td>C92S</td>
<td>24.1</td>
<td>2.90</td>
<td>8.31</td>
</tr>
<tr>
<td>C96S</td>
<td>159</td>
<td>87.0</td>
<td>1.83</td>
</tr>
<tr>
<td>C124S</td>
<td>104</td>
<td>7.94</td>
<td>13.1</td>
</tr>
<tr>
<td>C131S</td>
<td>74.1</td>
<td>8.38</td>
<td>8.84</td>
</tr>
<tr>
<td>C183S</td>
<td>33.9</td>
<td>8.51</td>
<td>3.98</td>
</tr>
<tr>
<td>C186S</td>
<td>81.3</td>
<td>9.94</td>
<td>8.18</td>
</tr>
<tr>
<td>C205S</td>
<td>56.0</td>
<td>6.18</td>
<td>9.06</td>
</tr>
<tr>
<td>C260S</td>
<td>60.8</td>
<td>7.33</td>
<td>8.29</td>
</tr>
<tr>
<td>DTNB-C186S (+DTT)</td>
<td>151</td>
<td>41.9</td>
<td>3.60</td>
</tr>
<tr>
<td>DTNB-C186S (+DTT)</td>
<td>80.3</td>
<td>8.04</td>
<td>9.99</td>
</tr>
</tbody>
</table>

*C186S was treated with DTNB, as described in Fig. 5. After removal of DTNB by gel filtration on Sephadex G-50, the enzyme was stored in KE buffer (+DTT) or KED buffer (+DTT).*

In the absence of DTT, only C186S fully retained activity during storage at 4°C for 1 d, or −25°C for 2 months (Fig. 4). Other mutant-type enzymes were less stable and significantly inactivated, like the wild-type enzyme. DTT had no effect on the activity of C186S, while other mutant-type enzymes were reactivated to various extents. C186S and the wild-type enzyme showed almost identical heat stability. When heated at various temperatures for 10 min in KE buffer, both enzymes lost half of their activity at about 60°C, and were completely inactivated at 65°C (data not shown).

All mutant-type enzymes except for C186S were strongly inhibited by DTNB, whereas C186S was
slightly activated, possibly due to the change in kinetic properties of the enzyme (Fig. 6). The time course of reaction revealed that DTNB rapidly modified about one SH group of C186S without a decline in enzyme activity (Fig. 5B). These results suggest that inhibition by DTNB was primarily due to modification of Cys-186. The DTNB-treated C186S remained fully active even in the absence of DTT, and showed different kinetic properties from those of C186S (Table 2). Both the $k_{cat}$ and the $K_m$ of C186S markedly increased after the reaction with DTNB. DTNB-treated C186S was reversed to C186S by treatment with 1 mM DTT. All the mutant-type enzymes completely lost activity with incubation (25°C, 10 min) with 1 mM HgCl$_2$.

The CD spectra in the UV region (200–260 nm) of C96S, C183S, and C186S were almost identical to that of the wild-type enzyme, with two negative peaks, at 209 and 218 nm, indicating no gross change in the secondary structure on amino acid replacement (Fig. 7).

**Discussion**

Purified recombinant PocOMH showed physical and catalytic properties similar to those of OMH purified from *P. ochraceae*, and also required reducing reagents such as DTT for stability. Since SpaOMH has seven cysteine residues per subunit and is stable without reducing reagents, there may be particular cysteine residues responsible for the instability of PocOMH. Site-directed mutagenesis showed that none of the eight cysteine residues in PocOMH are directly involved in enzyme catalysis. The cysteine residues in fumarase (EC 4.2.1.2) also lie on the outside of the active site, and their modification leads to enzyme inactivation. DTNB inactivates wild-type PocOMH by modifying two SH groups per subunit. The first SH group was identified as Cys-186. This is based on the observation that C186S is not inhibited by DTNB (Fig. 6), and shows no increase in $A_{412}$ correlated with the decrease in enzyme activity (Fig. 5B). Cys-186 might be located near the active site, since citrate, a weak competitive inhibitor, weakly restrains modification by DTNB (Fig. 5A). Although the second SH group reacting at a very high rate with DTNB could not be specified by measuring $A_{412}$, owing to the interference of Cys-186, its modification appeared not to be inhibitory to the enzyme activity (Fig. 5B). Modification of the second SH group causes a significant increase in $K_m$ and $k_{cat}$ (Table 2), suggesting the importance of this SH group in substrate binding. Among the eight mutant-type enzymes, only C96S showed a similar increase in the kinetic constants. Hence, it is inferred that the second SH group is Cys-96. Probably, Cys-96 and Cys-186 are exposed to the solvent so as to react rapidly with DTNB. The other six cysteine residues might be buried in the enzyme molecule, and might react with HgCl$_2$ but not with DTNB.

In the absence of DTT, only C186S maintained catalytic activity in storage at 4°C, while the other mutant-type enzymes were markedly inactivated (Fig. 4). Therefore, Cys-186 was largely responsible for the instability of PocOMH in the absence of a reducing reagent. The heat stability and CD spectrum in the UV region of C186S were almost indistinguishable from those of the wild-type enzyme. The wild-type enzyme inactivated in the absence of DTT was fully reactivated by the subsequent incubation with DTT (Fig. 4). The dimeric structure of the enzyme remained unaltered after inactivation, as tested by Disc PAGE. These results suggest that the
instability of PocOMH was due to the formation of an S–S bond between Cys-186 and another intramolecular SH group. This SH group could not be specified by examining the inactivation and reactivation of the mutant-type enzymes (Fig. 4). Cys-186 is located on a flexible loop, as described below. Assuming that Cys-186 forms an S–S bond with either of the two SH groups near Cys-186, all mutant-type enzymes except for C186S are expected to be inactive in the absence of DTT, as shown in Fig. 4.

The present study also appears to explain the different properties of SpaOMH and PocOMH. SpaOMH lacks both Cys-96 and Cys-186 (Fig. 2), and thus shows a higher $K_m$-value and higher stability in the absence of a reducing reagent than PocOMH.

Forouhar et al. elucidated the X-ray structure of RpaOMH. The amino acid sequence of RpaOMH, including the positions of the eight cysteine residues, is highly homologous to that of PocOMH (Fig. 2). RpaOMH has one Zn ion per subunit. Three conserved histidine residues, His-6, 8, and 178, are located at the bottom of the $\beta$ barrel inside the subunit, and coordinate to the Zn ion. It is appropriate to assume that the active site is around the Zn ion, since the Zn ion coordinated by these six histidine groups can promote the heterolysis of its bound H$_2$O, like human carbonic anhydrase (EC 4.2.1.1). Although some biological function is expected of the HGGG motif (residue nos. 223–226), the GGG tripeptide moiety is deep in the molecule, and appears to be independent of enzyme catalysis. The imidazole group of His-223 is located near the Zn ion, and may be involved in enzyme catalysis. Unfortunately, the region between Thr-181 and Thr-190, in which Cys-183 and Cys-186 are included, is missing in the reported structure. Computer-aided prediction of protein secondary structure using the DNASIS program suggests that this missing region forms a loop structure. Probably, the loop is disordered and invisible in the electron density map. Since various metal chelators hardly react with the Zn ion in native PocOMH, this loop may lie just above the Zn ion so as to protect it from chelators.

In RpaOMH, Cys-260 is located on a turn segment and a further five cysteine residues, Cys-92, 96, 124, 131, and 205, on $\alpha$-helices. The SH groups of these six residues are at a short distance (0.7–1.9 nm) from the Zn ion. The SH groups of Cys-92 and 96, which are at a distance of about 0.7 nm, face the cleft in the enzyme molecule, and probably affect the substrate binding. Cys-124, 131, 205, and 260 lie deep in the molecule. They are also sterically hindered by the surrounding amino acid residues, and may be ineffective in enzyme catalysis. These structural features appear to be mostly compatible with the reactivity of Cys-96 and the inertness of Cys-124, 131, 205, and 260 in the reaction of PocOMH toward DTNB.

In this study, we investigated the role of cysteine residues in PocOMH, and found that none of the eight cysteine residues participates directly in enzyme catalysis. We also found that Cys-186 is mainly responsible for the inactivation of PocOMH. The SH group of Cys-186 on the flexible loop easily reacts with DTNB and other intramolecular cysteine residues in the absence of a reducing reagent, and consequently may interfere with the access of 4-oxalomesaconate to the active-site Zn ion. It is unclear whether the reactivity of Cys-186 is involved in the cellular physiology. The present study perhaps gives a clue as to the structure and function of OMH.

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