A change of the metal-specific activity of a cambialistic superoxide dismutase from Porphyromonas gingivalis by a double mutation of Gln-70 to Gly and Ala-142 to Gln

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Gln-70, which is located near the active-site metal, is conserved in aligned amino acid sequences of iron-containing superoxide dimutases (Fe-SODs) and cambialistic SOD from Porphyromonas gingivalis, but is complementarily substituted with Gln-142 in manganese-containing superoxide dismutase (Mn-SOD). In order to clarify the contribution of this exchange of Gln to the metal-specific activity of P. gingivalis SOD, we have prepared a mutant of the enzyme with conversions of Gln-70 to Gly and Ala-142 to Gln. The ratios of the specific activities of Mn- and Fe-reconstituted P. gingivalis SOD increased from 1.4 in the wild-type to 3.5 in the mutant SODs. Furthermore, the visible absorption spectra of the Mn- and Fe-reconstituted mutant SODs more closely resembled that of Mn-specific SOD than that of the wild-type SOD. We conclude that a difference in configuration of the Gln residues of P. gingivalis SOD partially accounts for the metal-specific activity of the enzyme.

Key words: glutamine, iron, manganese, metal-recognition, site-specific mutagenesis.

INTRODUCTION

The origin of the metal specificity for the enzymic activity of manganese-containing superoxide dismutase (Mn-SOD; EC 1.15.1.1) and iron-containing superoxide dismutase (Fe-SOD) has been one of the outstanding questions in the field of bioinorganic chemistry. The metal specificity has been divided into two types. The first is a metal-specific type of superoxide dismutase (SOD) that requires the original metals for the activity; that is, manganese-substituted Fe-SODs [1,2] and iron-substituted Mn-SODs [3,4] retain little or no enzymic activity. The other type of SOD uses both metals to exhibit the enzymic activity. These are called cambialistic SODs [5–8]. In spite of these differences in metal specificity, the structures of these SODs are very similar; that is, Mn-SODs [9–12] and Fe-SODs [12–13] and cambialistic SODs ([14] and S. Sugio, B. Y. Hiraoka and F. Yamakura, unpublished work) have a large degree of sequence homology and X-ray structural similarity. The iron and manganese atoms are commonly ligated by three histidine residues, an aspartic acid residue and a solvent molecule. In addition, these metals are surrounded by a similar environment consisting of a group of aromatic amino acid residues. Therefore, significant differences in the active-site environment were not found in Fe-, Mn- or cambialistic SODs. However, three minor differences have been observed in the metal environments (within 8 Å) of the Fe-SODs and Mn-SODs. One difference is that Gln-70 in Fe-SODs is complementarily substituted by Gln-142 in Mn-SODs (note: amino acid numbering is based on the positions in Porphyromonas gingivalis SOD). However, the side-chain amide groups of the glutamine residues are oriented to the same positions in the three-dimensional structures of the Fe- and Mn-SODs. These amide groups are part of a hydrogen-bond network that includes conserved Tyr-35 and the metal-ligand solvent and may connect this tyrosine to catalysis at the metal in the Fe- and Mn-SODs [10–13]. The second difference is that a histidine residue occupies position 142 instead of glutamine in some Fe-SODs [15,16]. In these Fe-SODs, this histidine makes another type of hydrogen-bond network that includes both Tyr-35 and Trp-159 or the metal-ligand solvent, depending on the orientation of the histidine [15,16]. The third difference is that Tyr-77 in Fe-SODs is changed to phenylalanine in Mn-SODs [9–13]. Although a few exceptions have been reported [17,18], these three differences could be the primary candidates to account for the metal specificity of the Fe- and Mn-SOD activity [19], improving on the suggestions by Parker and Blake [20] and Yamakura et al. [21]. Recently, Yamano and Maruyama [22] reported that the substitution of Tyr-77 by Phe in the metal-specific Fe-SOD from Sulfolobus solfataricus did not change the metal-specific activity of the enzyme. Therefore, the third difference may not contribute to the metal-specific activity of Fe- and Mn-SODs.

On the other hand, the glutamine residue of a cambialistic SOD from Porphyromonas (Bacteroides) gingivalis is located at position 70, the same as for the first type of Fe-SODs [6], but another cambialistic SOD from Propionibacterium shermanii has a histidine residue at position 142 [14], the same as that of the second type of Fe-SODs. Although the positions of the glutamine and histidine residues of these cambialistic SODs are the same as those of Fe-SODs, these SODs still show enzymic activity with manganese. Therefore, the metal-specific activity of the cambialistic SODs may be controlled not only by the factors described above but also by other unknown factor(s). Since no unique differences in the structures of cambialistic SODs have been observed compared with Fe- and Mn-SODs, as described above, the metal-specific activities of the Fe- and Mn-SODs may also be controlled by a mechanism similar to that of the cambialistic SODs. Therefore, it is extremely important to clarify the role of...
the positional difference of the glutamine residue in the metal-specific activity of *P. gingivalis* SOD by conversion of amino acids at positions 70 and 142 by using site-directed mutagenesis.

In this study, we prepared a mutant *P. gingivalis* SOD that possessed a double mutation of Gln-70 → Gly and Ala-142 → Gln and examined these replacements on the metal-specificity of the enzymic activity of the mutant enzyme. We conclude that the complementary exchanging of the glutamine residues in Mn-Fe-SODs reveals part of the structural basis of the metal-specific activities in Fe-SODs, Mn-SODs or cambialisatic activity. This is the first report of successful changing of the SODs reveals part of the structural basis of the metal-specific activity. We conclude that the positional difference of the glutamine residue in the metal-specific activities in Fe-SODs, Mn-SODs or cambialisatic activity. This is the first report of successful changing of the SODs reveals part of the structural basis of the metal-specific activity.

**EXPERIMENTAL**

**Materials**

The vector pMAL-c2, amylose resin and *Escherichia coli* strain TB-1 were obtained from New England Biolabs. Cytochrome *c* and xanthine oxidase were obtained from Sigma and Roche Diagnostics, respectively.

**Construction of maltose-binding protein (MBP)/SOD protein expression vector**

The *sod* coding sequence was amplified from pKD210, which contains the 573-bp *sod* gene of *P. gingivalis* ATCC 33277 [23], by PCR and was ligated into pMAL-c2. The upstream primer corresponded to nucleotides 1–20 of the *sod* sequence that gave a 5' blunt end for cloning into the XmnI site of the pMAL vector. The downstream primer corresponded to nucleotide 550 to the stop codon of the *sod* sequence and a HindIII site was added. The coding *sod* gene was inserted downstream of the *malE* gene of *E. coli*, which encodes MBP, resulting in the expression of a MBP–SOD fusion protein.

**Induction, overexpression and purification of MBP–SOD fusion protein**

After the transformed *E. coli* cells were grown in rich broth medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 2 g/l glucose and 100 mg/l ampicillin) to early log phase at 37 °C, 0.3 mM isopropyl-β-D-thiogalactoside was added and incubation was continued for 2.5 h. Collected cells were suspended in 50 ml of amylase column buffer (20 mM Tris/ HCl pH 7.4, 200 mM NaCl and 1 mM EDTA) and disrupted by ultrasonic treatment. The supernatant was obtained by centrifugation and diluted 1:5 with amylase column buffer and applied to a 20-ml amylase resin column at a flow rate of 38 ml/h. The fusion protein was eluted with the same buffer containing 10 mM maltose. The eluted protein (1 mg/ml) was digested with trypsin (1 % w/w, Sigma, sequencing grade) at 42 °C for 4 h. Because a recognition site specific for factor Xa in the pMAL-c2 plasmid was cleaved only by about 50 % or less at high temperature (42 °C) and after long incubation (48 h) by factor Xa, we used trypsin for the cleavage of the fusion protein. Tryptsin digests exhibited two major protein bands showing molecular masses that corresponded to MBP (42700 Da) and wild-type SOD (21500 Da) in SDS/PAGE (results not shown). The digests were applied to a Q-Sepharose column (10 ml) equilibrated with 20 mM Tris/HCl, pH 8.0 (Q buffer). The protein was eluted with a linear gradient of 6 column vols. of 0–75 mM NaCl in Q buffer and was found to be a single band by SDS/PAGE (results not shown). A yield of 15–20 mg of purified wild-type enzyme was obtained from 1 litre of induced bacterial culture.

**Site-directed mutagenesis of SOD**

An *EcoRI/HindIII* fragment of sod/pMal-c2, which corresponds to nucleotides 76–573 of the *sod* sequence, was ligated into M13mp19 for mutagenesis. *In vitro* mutagenesis of SOD was introduced by the Mutan-K system (Takara Biomedicals, Tokyo, Japan), which is based on the method described by Kunkel [24] under conditions recommended by the manufacturer. A mutation of Gin (CAA) to Gly (GGA) was introduced at amino acid position 70 and an Ala (GCC) to Gin (CAG) mutation was introduced at amino acid position 142. Mutant cDNA was screened and sequenced to ensure the absence of spurious mutations. Mutant SOD was expressed and purified by the same methods as the wild-type SOD (described above).

**Preparation of metal-reconstituted proteins**

Fe- and Mn-reconstituted wild-type and mutant SODs were prepared according to the acid-guanidine hydrochloride denaturation method described in a previous paper [7]. In order to remove minor components in the reconstituted proteins, we used an HPLC system (system 800, Jasco, Tokyo, Japan) equipped with a hydroxypapitate column (7.5 × 100 mm, Tonen, Tokyo, Japan) rather than a conventional hydroxyapitate column alone.

**MS**

The molecular masses of the wild-type and the mutant SODs were determined with a TSQ 700 electrospray ionization mass spectrometer (Thermo-Quest Finnigan Mat, San Jose, CA, U.S.A.). The analytical conditions were as follows: spray voltage, 4.5 kV; electron multiplier, 1500 V; manifold vacuum, 7.0 × 10⁻⁶ torr; manifold temperature, 70 °C; capillary temperature, 150 °C; scan range, *m/z* 500–3000; scan time, 5 s. The SODs were dissolved in a mixture of methanol and 0.5 % acetic acid (1:1, v/v) to a final concentration of 10 pmol/µl and infused into the ion source of the TSQ 700 with a pump.

**Analytical methods**

SOD activity was measured by inhibition of the xanthine/xanthine oxidase-induced reduction of cytochrome *c* at pH 7.8 [25], with reduction of the final volume of the assay system from 3 to 0.75 ml [7]. Ultraviolet and visible spectra of the enzyme were measured with a Hitachi U-3000 recording spectrophotometer equipped with a micro-cuvette holder. Metal contents were determined by using atomic absorption spectrometry with a Hitachi Z-9000 atomic absorption spectrophotometer. PAGE in slab gels was carried out according to the method of Davis [26] with a few modifications. SDS/PAGE was performed according to Laemmli [27]. Protein concentrations of Fe- and Mn-reconstituted wild-type *P. gingivalis* SODs were estimated by using molar absorption coefficients of 73100 and 34600–3000, respectively, which were measured by the method of Lowry et al. [28], as described in a previous paper [7]. These values were also used to estimate protein concentrations of the Fe- and Mn-reconstituted mutant SODs.

**RESULTS**

Figure 1 shows the results of MS of the purified wild-type and mutant *P. gingivalis* SODs. Deconvolution of the protein mass spectra (Figures 1A and 1B, insets) revealed molecular masses of 21502 Da for wild-type SOD (calculated mass, 21501 Da) and 21486 Da for mutant SOD (calculated mass, 21487 Da), which were almost consistent with the calculated values of each of the
SODs. The difference between the observed molecular masses (16 Da) was close to that between the calculated values (14 Da) of the double replacement of glutamine with glycine (−57 Da) and alanine with glutamine (+43 Da) in the protein structure. These data show that the construction of the plasmid, the mutation of the sod gene and the purification of the SODs were properly processed.

After metals of the purified wild-type and mutant SODs were reconstituted with iron or manganese, the purified, Fe-reconstituted and Mn-reconstituted wild-type and mutant SODs were subjected to non-denaturing PAGE. Each of the wild-type and mutant SODs gave a single major band (>95%) and a faster-moving minor band (<5%), with the same mobilities for each sample (Figure 2). Table 1 shows the specific activities and metal contents of the purified, Fe-reconstituted and Mn-reconstituted wild-type and mutant SODs. Each metal-reconstituted SOD contained nearly stoichiometric amounts of iron or manganese and negligible amounts of the other metals, suggesting that each metal-reconstituted enzyme contained little non-specific binding metal. To correct for the influence of different metal contents on the apparent activities of each of the SOD preparations, we expressed the specific activities of the SODs as units mg of protein−1 mol of Fe and/or Mn−1 mol of subunit−1. The wild-type Fe- and Mn-reconstituted SODs showed similar specific activities as described previously [7]. The ratio of the specific activities of Mn-reconstituted SOD to Fe-reconstituted SOD was about 1.4 in the wild-type SOD and 3.5 in the mutant SOD. Therefore, the metal-specific activity of P. gingivalis SOD changed, by the double mutation of amino acids, from being a little more active with Mn compared with Fe, to Mn being dominant. In order to estimate the differences in the metal site, we compared the visible absorption spectra of the Fe-recon-
Figure 2  PAGE of purified, and Fe- and Mn-reconstituted wild-type and mutant P. gingivalis SODs

Each sample (7 μg) was applied and stained with Coomassie Brilliant Blue G-250 after electrophoresis. The samples were: purified wild-type (lane A) and mutant (lane B) SODs, Fe-reconstituted wild-type (lane C) and mutant (lane D) SODs, and Mn-reconstituted wild-type (lane E) and mutant (lane F) SODs.

Table 1  Activities and metal contents of purified, and Fe- and Mn-reconstituted enzymes of the native and mutant P. gingivalis SODs

Values are given as means ± S.D. Specific activity is given in terms of units/mg of protein per mol of Mn and/or Fe per mol of subunit.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity</th>
<th>Metal contents (g of atoms/mol of dimer)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fe</td>
</tr>
<tr>
<td>Purified enzymes</td>
<td></td>
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</tr>
<tr>
<td>Wild-type SOD</td>
<td>1436 ± 112</td>
<td>1.20 ± 0.05</td>
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<tr>
<td>Mutant SOD</td>
<td>1015 ± 58</td>
<td>1.00 ± 0.06</td>
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<tr>
<td>Fe-reconstituted enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type SOD</td>
<td>1656 ± 94</td>
<td>1.53 ± 0.06</td>
</tr>
<tr>
<td>Mutant SOD</td>
<td>830 ± 43</td>
<td>1.48 ± 0.08</td>
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<tr>
<td>Mn-reconstituted enzymes</td>
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<td></td>
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<tr>
<td>Wild-type SOD</td>
<td>2275 ± 138</td>
<td>0.026 ± 0.038</td>
</tr>
<tr>
<td>Mutant SOD</td>
<td>2872 ± 152</td>
<td>0.024 ± 0.022</td>
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</tbody>
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DISCUSSION

The results of the PAGE, in which the three mutant SODs showed a single major band ( > 95%), and a faster-moving minor band (< 5%), with the same mobilities as the wild-type SODs (Figure 2), suggests that all the mutant SODs had the same gross structure as wild-type SODs. The double mutation of P. gingivalis SOD changed the efficiency of the metals for the activity of the enzyme from a little efficient with Mn (1:1.4 for Fe/Mn) to Mn being dominant (1:3.5 for Fe/Mn; Table 1). Because the spectral profiles of the wild-type and mutant SODs are similar to that of the Mn-specific Mn-SOD, whose active-site manganese is predominantly in the Mn$^{2+}$ state, and reduction of Mn-SOD to the Mn$^{2+}$ state leads to loss of the visible spectrum [30], the equilibrium between Mn$^{2+}$ and Mn$^{3+}$ in the Mn-reconstituted
SOD may shift a little in favour of Mn
$closely resembles the Fe-substituted Mn-specific SOD from
absorption spectrum of the Fe-reconstituted mutant SOD more
the Mn-reconstituted mutant SOD (Figure 3A). Furthermore, the
increase in the molar extinction coefficient of the spectrum of
wild-type SOD may favour the Mn$sup+ state and that of the mutant
SOD may shift a little in favour of Mn$sup2+, which results in an
increase in the molar extinction coefficient of the spectrum of
the Mn-reconstituted mutant SOD (Figure 3A). Furthermore, the
absorption spectrum of the Fe-reconstituted mutant SOD more
closely resembles the Fe-substituted Mn-specific SOD from S.
marcescens than that of the wild-type SOD (Figure 3B). This
evidence, together with the results of the metal-specific activity of
the mutant SOD, suggests that the double mutation of Gln-70
Gly and Ala-142→Gln results in the catalytic environment of
hydrogen-bond network to the ligand solvent. In order to
determine the actual position of the amide group of Gln-142 in the wild-type SOD, X-ray crystallographic studies on Fe- and Mn-
reconstituted mutant SODs are now underway in our laboratory.
We conclude that the complementary exchanging of the glutamine residue participates in the metal specificity of the en-
yzmic activity of cambialistic SODs and may also be important
for that of the metal-specific types of Fe- and Mn-SODs.
However, since the change of the metal-specific activity into a
Mn-specific type was not complete in the mutant P. gingivalis
SOD (Table 1), other differences in amino acid residues may also
contribute to the Mn-specific activity of the cambialistic SOD.
From the results of this study, we propose that the metal-specific activity of Fe-, Mn- and cambialistic SODs may be controlled
cumulatively by differences in several unknown amino acids,
located apart from the active-site metals, in addition to the
exchange of the glutamine residue. Although a few amino acid
residues besides the primary candidate have been found to be
conservatively different in Fe-SOD and Mn-SOD [19,20], it is
very difficult to predict the effect of these differences in amino

Figure 4 Active site of the wild-type P. gingivalis SOD and predicted position of the glutamine 142 residue in the mutant SOD

(Left-hand panel) Active site of the wild-type SOD. Iron (Fe) is co-ordinated by histidine 74, aspartic acid 157 and histidine 161 as planar ligands and histidine 27 and a solvent molecule (WAT) as axial ligands. The dotted lines represent the hydrogen-bond network among tyrosine 35, glutamine 70 and the co-ordinated solvent. (Right-hand panel) The predicted active site of the mutant SOD. Position of the glutamine 142 was predicted by using a Biopolymer in Insight-II from Molecular Simulation.
acids on the state of active metals by X-ray structural data [9–16]. A recent study on the directed molecular evolution of aspartate aminotransferase, which consisted of a combination of cycles of random mutations and selection steps on the enzyme, showed that a mutant enzyme with 17 amino acid substitutions, most of which were not located close enough to interact directly with the substrate, was successfully obtained for the conversion of substrate specificity [32]. This method may be useful to search for unknown amino acids that contribute to the metal-specific activities of Fe-, Mn- and cambialistic SODs.

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REFERENCES


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