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 SODs (Mn-SODs). 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 ratio of the specific activities of Mn- to 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that is, Mn-SODs [9–12], Fe-SODs [12–13] and cambialistic SODs ([14] and S. Sugio, B. Y. Hiraoka and F. Yamakura, unpublished work) have a large degree of sequence homology and X-ray structural similarity.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

Abbreviations used: SOD, superoxide dismutase; Fe-SOD, iron-containing SOD; Mn-SOD, manganese-containing SOD; MBP, maltose-binding protein.

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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 activity. This is the first report of successful changing of the metal-specific activities in Fe-SODs, Mn-SODs or cambialisatic SODs by replacement of the amino acids near the metals by site-directed mutagenesis.

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 *Xmn*I site of the pMAL vector. The downstream primer corresponded to nucleotide 550 to the stop codon of the *sod* sequence and a *Hin*dIII 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 amylose 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 amylose column buffer and applied to a 20-ml amylose 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. Trypsin 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 *Eco*RI/*Hin*dIII 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 Gln (CAA) to Gly (GGA) was introduced at amino acid position 70 and an Ala (GCC) to Gln (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, Jusco, Tokyo, Japan) equipped with a hydroxyapatite column (7.5 × 100 mm, Tonen, Tokyo, Japan) rather than a conventional hydroxyapatite 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}; 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 66700 $M^{-1} \cdot cm^{-1}$, 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

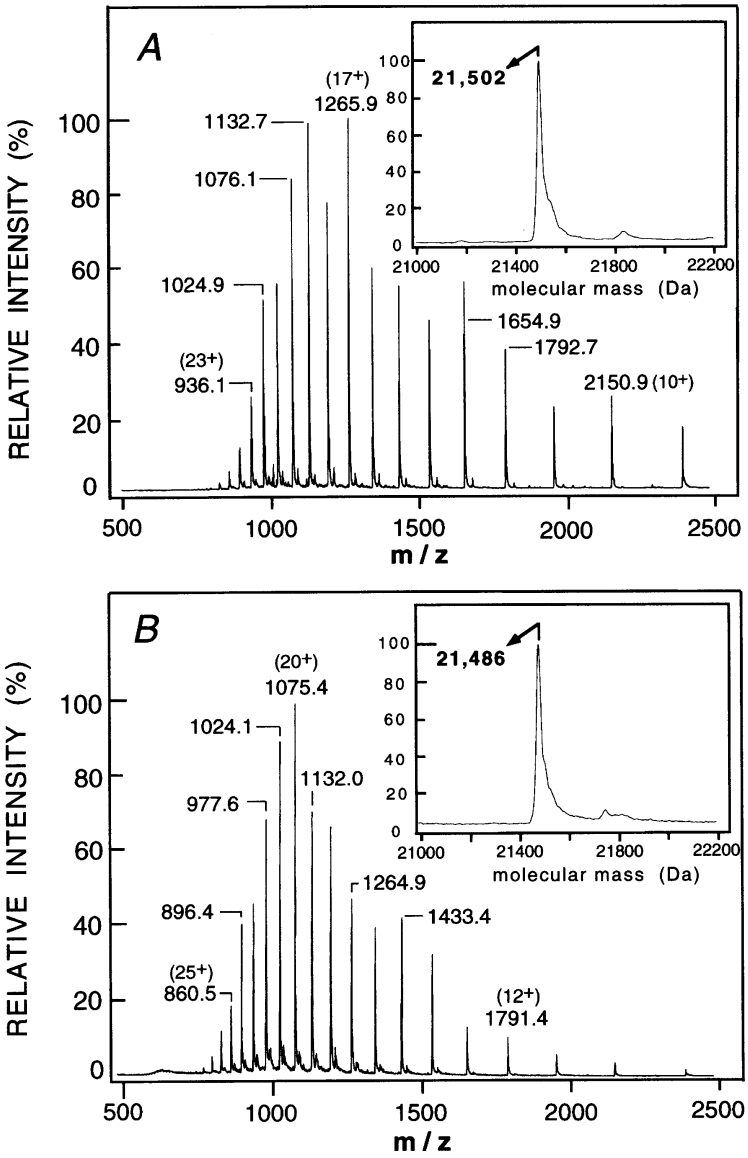

Figure 1 MS of wild-type (A) and mutant (B) SOD

Samples were prepared and analysed as described in the Experimental section. The insets represent the reconstructed molecular-mass profiles obtained from these spectra. Inset *y* axes show relative intensity (%).

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-

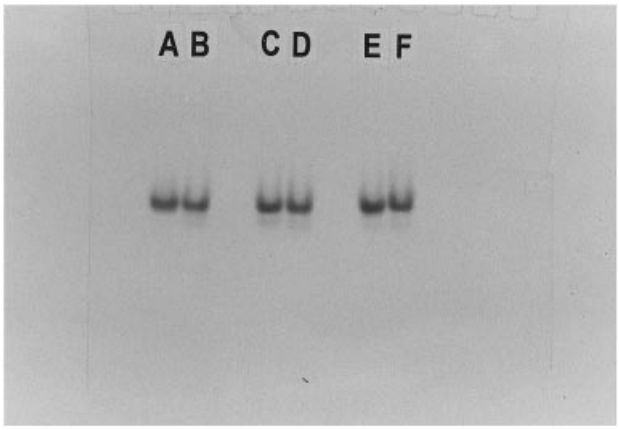

Figure 2 PAGE of purified, and Fe- and Mn-reconstituted wild-type and mutant *P. gingivalis* SODs

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.

Sample	Specific activity	Metal contents (g of atoms/mol of dimer)	
		Fe	Mn
Purified enzymes			
Wild-type SOD	1436 ± 112	1.20 ± 0.05	0.15 ± 0.01
Mutant SOD	1015 ± 58	1.00 ± 0.06	0.16 ± 0.01
Fe-reconstituted enzymes			
Wild-type SOD	1656 ± 94	1.53 ± 0.06	0.004 ± 0.002
Mutant SOD	830 ± 43	1.48 ± 0.06	0.002 ± 0.001
Mn-reconstituted enzymes			
Wild-type SOD	2275 ± 138	0.026 ± 0.038	1.25 ± 0.014
Mutant SOD	2872 ± 152	0.024 ± 0.022	1.39 ± 0.036

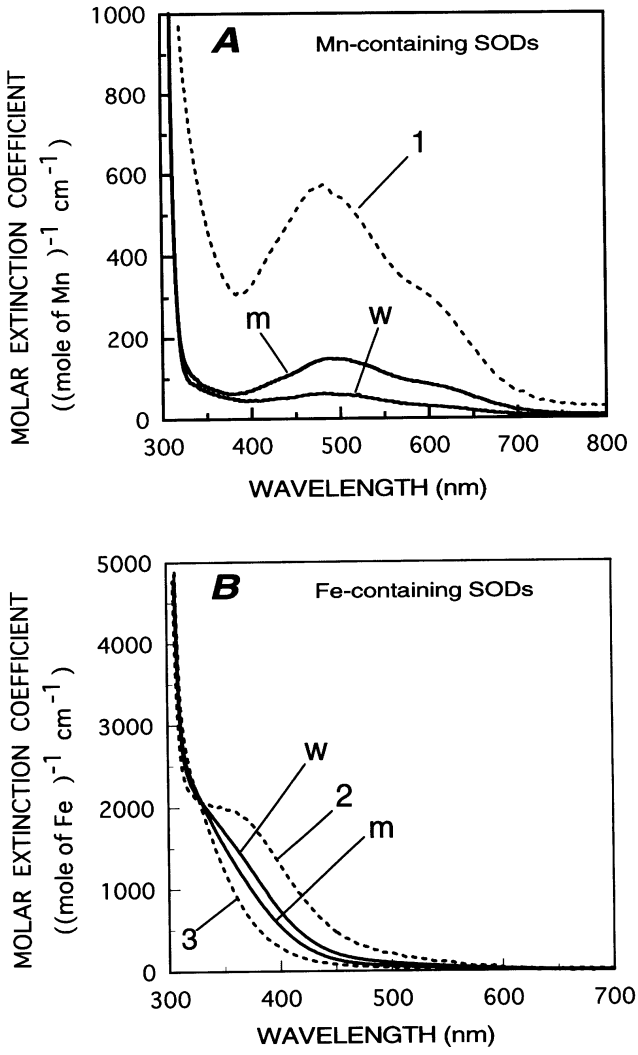

Figure 3 Comparison of the visible absorption profiles of Fe- and Mn-reconstituted wild-type and mutant *P. gingivalis* SODs

(**A**) Absorption spectra 1, w and m represent a Mn-specific type of Mn-SOD from *Serratia marcescens*, Mn-reconstituted wild-type *P. gingivalis* SOD and mutant SOD, respectively. (**B**) Absorption spectra 2, 3, w and m represent an Fe-specific type of Fe-SOD from *Pseudomonas ovalis*, Fe-substituted *S. marcescens* Mn-SOD, Fe-reconstituted wild-type *P. gingivalis* SOD and mutant SOD, respectively. The spectra were measured in 10 mM potassium phosphate buffer, pH 7.8.

stituted and Mn-reconstituted wild-type and mutant SODs (Figure 3). The visible absorption spectra of Fe-SOD from *Pseudomonas ovalis* and both Fe-substituted and native *Serratia marcescens* Mn-SOD, which are metal-specific SODs [2,29], are also shown in Figure 3 as a comparison. Although the extinction coefficient at 489 nm of the Mn-reconstituted wild-type SOD (62 $M^{-1}\cdot cm^{-1}$) was only about one-ninth of that of the Mn-specific Mn-SOD (570 $M^{-1}\cdot cm^{-1}$), the extinction coefficient of the Mn-reconstituted mutant SOD (145 $M^{-1}\cdot cm^{-1}$) increased more than 2-fold over that of the Mn-reconstituted wild-type enzyme and more closely resembled that of Mn-SOD. As shown in Figure 3(B), a small difference was observed between the absorption spectra of the wild-type and mutant Fe-reconstituted SODs. The wild-type SOD spectra more closely resembled those of Fe-specific Fe-SOD from *P. ovalis* and the mutant SOD spectra more closely resembled those of the Fe-substituted form of Mn-specific SOD from *S. marcescens*.

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^{3+} 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

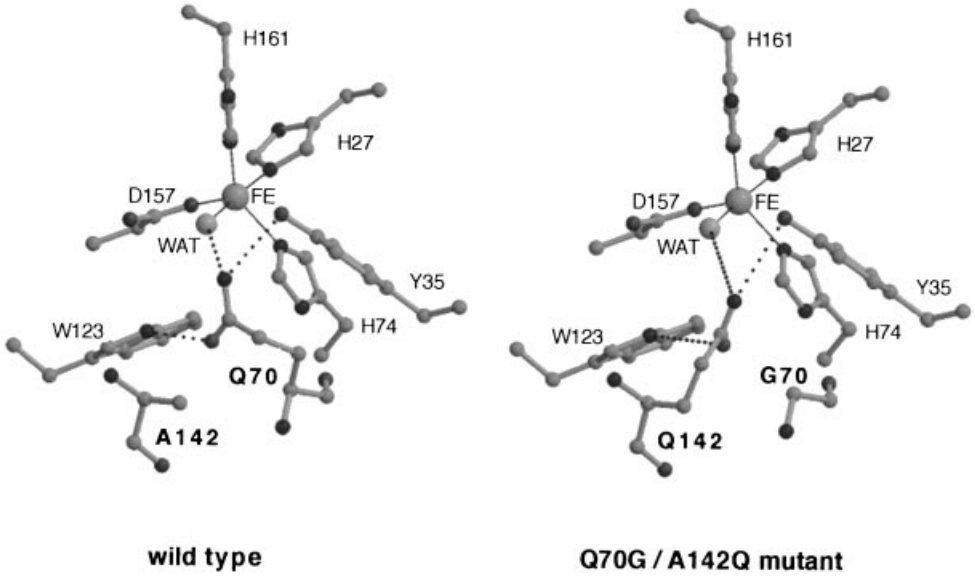

Figure 4 Active site of the wild-type *P. gingivalis* SOD and predicted position of the glutamine 142 residue in the mutant SOD

(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.

wild-type SOD may favour the Mn^{2+} state and that of the mutant SOD may shift a little in favour of Mn^{3+}, 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 the metal and the metal–environment interaction of the cambialistic SOD more closely resembling those of Mn-specific Mn-SOD.

Although X-ray crystallographic studies are required to establish the complementary exchange of the glutamine residue in the mutant *P. gingivalis* SOD, we believe that the amide group of Gln-142 in the mutant SOD is located in a position similar to that occupied by Gln-70 in the wild-type SOD and is a part of the hydrogen-bond network including Tyr-35 and metal-coordinated solvent for the reasons described below. (i) The mutant Mn-reconstituted and Fe-reconstituted SODs showed spectra similar to those of the Mn-specific type of Mn-SOD [29] and of Fe-substituted Mn-SOD [29], respectively, in which Gln-142 is a part of the hydrogen-bond network. (ii) The mutant Fe- and Mn-reconstituted SODs exhibited comparable amounts of enzymic activity to that of the wild-type SODs. A recent study of a Gln-143 → Asn mutant of human Mn-SOD (corresponding to residue 142 in *P. gingivalis* SOD) showed that addition of a new water molecule forms three new hydrogen bonds that link the ligand solvent, Asn-143 and Tyr-34 (Tyr-35 in *P. gingivalis* SOD) [31]. This mutant SOD shows 2–3 orders of magnitude less overall catalytic activity, suggesting that a large difference in the hydrogen-bond network may result in a large loss of the enzymic activity. These results support the idea that Gln-142 in the mutant *P. gingivalis* SOD may be located to maintain the same hydrogen-bond network as the wild-type SOD. (iii) A molecular model of the double mutant *P. gingivalis* SOD can be constructed from the crystal structure of the wild-type enzyme at 1.8 Å resolution (S. Sugio, B. Y. Hiraoka, and F. Yamakura, unpublished work) without any steric hindrance from other amino acid residues (Figure 4). Although the predicted position of the side-chain amide of Gln-142 in the mutant SOD is slightly different ($\approx$ 1.7 Å) from that of Gln-70 in the wild-type SOD (Figure 4), this difference might be small enough to be adjusted by fine tuning of the active-site structure of the mutant SOD to form the hydrogen-bonding network. However, we predicted that this difference in the orientation of the amide nitrogen could cause differences in the state of the active-site metal by possible effects such as difference in the direction of the dipole moment of the amide group and/or difference in the strength of the hydrogen-bond network to the ligand solvent. In order to determine the actual position of the amide group of Gln-142 in the mutant 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 enzymic 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. gigivalis* 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

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.

We thank Dr. Joseph A. Gardner for his critical manuscript review. We thank Professor T. Matsumoto (Showa Women's University) for the measurement of the SOD metal contents. We also thank Dr. K. Murayama and Ms. H. Taka for the MS measurements of the SODs. Part of this study was supported by a Scientific Research Special Grant from Matsumoto Dental University to B.Y.H.

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Received 5 October 1999; accepted 8 November 1999