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Preference for threonine over serine near the active site metal of superoxide dismutase in *Porphyromonas gingivalis*: Effect of Gly 155 to Ser mutation

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Summary

In this study, we analyzed the cambialistic superoxide dismutases (SODs) of *Porphyromonas gingivalis* (*Pg*–SODs) with a mutation directed at glycine position 155 to introduce serine. Glycine 155 is a highly conserved outer sphere in manganese–containing SODs (Mn–SODs), even though threonine is substituted at this position in most iron–containing SODs (Fe–SODs). Conversion of glycine 155 may affect the metal–specific activity of SODs, including that of cambialistic *Pg*–SODs. Previously, we reported that a *Pg*–SOD Gly155Thr mutant exhibited a substantially changed metal–specific activity from that of a cambialistic type to an Fe–specific type. Although serine and threonine equally contribute to protein function, serine has never been observed at position 155 in SODs. In order to elucidate this phenomenon, we created a *Pg*–SOD mutant Gly155Ser. The specific Fe–dependent activity of this mutant was almost identical to the wild–type SOD, whereas the Mn–dependent activity exhibited a 60% reduction. The ultraviolet–visible absorption of Fe– and Mn–reconstituted mutant SODs did not exhibit characteristic absorption spectra. Similar to the wild–type SODs, the mutant SODs exhibited a single band with identical mobilities after separation by polyacrylamide gel electrophoresis. However, their behavior after anion–exchange chromatography differed from that of the wild–type SODs. Thus, Gly155 is considered to be an essential residue for maintaining the hydrogen–bond network for Mn–specific and Fe/Mn–tolerant activity. Gly155 was possibly retained instead of Ser during the evolution of SODs owing to its increased efficiency in

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maintaining a dimeric structure.

Introduction

Superoxide dismutases (SODs, EC 1.15.1.1) play a protective role against oxidative stress and are essential for the maintenance of aerobic life. SODs in prokaryotes are divided into five classes according to the presence of metal ions, such as Ni, Cu, and either Fe or Mn[1-3]. Fe/Mn–type SODs are also called cambialistic SODs, which suggests that they can use both metals to exert their enzymic activity[4]. Metal replacement studies with Fe– and Mn–SODs produced by several species have indicated a strict metal cofactor specificity for enzyme function[5]. However, Fe–, Mn–, and Fe/Mn–SODs exhibit high structural homology[6,7]. In all cases, the metal ligation sphere exhibits a five–coordinate structure with a trigonal bipyramidal geometry[7].

Porphyromonas gingivalis is an anaerobic bacterium that synthesizes a typical Fe/Mn–SOD[6,8] with similar amino acid sequences to those of Fe–SOD[9,10]. Several minor differences have been observed in the metal environments of Fe– and Mn–SODs. Although Gly155 is conserved in most Mn–SODs, threonine is present at this position in most Fe–SODs[11]. Asp157, which is located two residues away from the conserved 155 position, is a ligand–binding residue (Fig. 1). Previously, we showed that a Gly155Thr (G/T) mutation in *P. gingivalis* SOD (*Pg*–SOD) resulted in an 85% reduction in the enzyme activity upon reconstitution with Mn compared with enhanced activity upon reconstitution with Fe, which indicates that this point mutation converts the cambialistic protein to an iron–specific protein[12]. This is the first report regarding the Fe– and Mn–SOD family of proteins with respect to the direct changes in metal–specific activity and changes induced by site–directed mutagenesis of

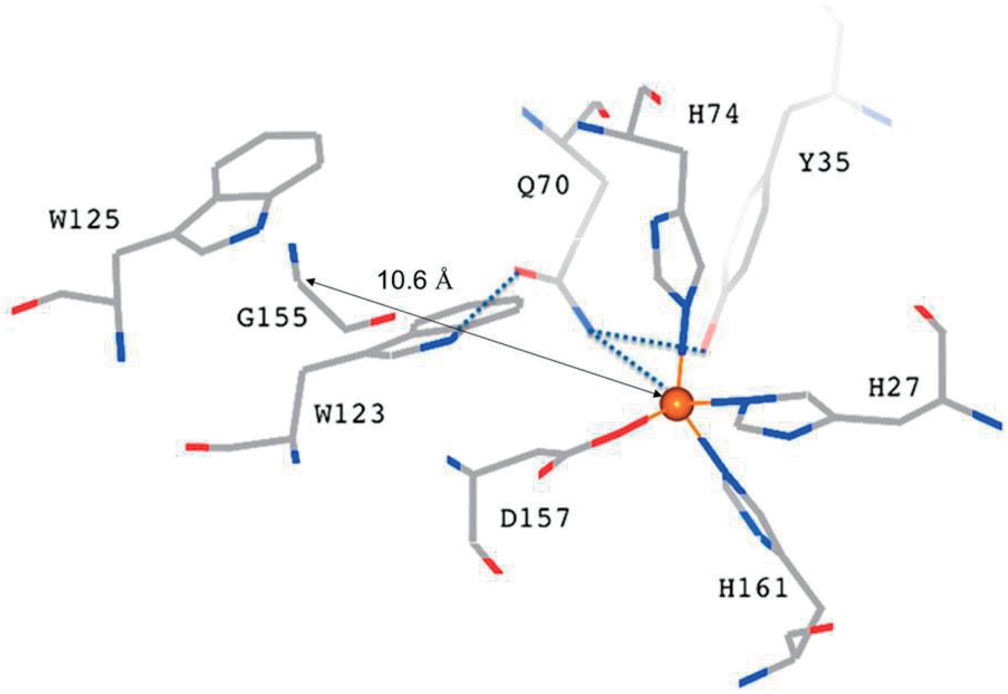

Fig. 1 : The metal ion is depicted as an orange sphere coordinated by side chains of three His (H27, H74, H161) residues, one Asp (D157), and a solvent molecule. Gly155 is located 10.6 Å away from the metal ion. The figure constructed by using Protein Adviser, Fujitsu Kyushu System Eng. Ltd., Fukuoka, Japan based on protein data bank coordinates, PDB: 1D5N.

amino acids other than the active site or second sphere.

In order to elucidate the function of amino acid residues at position 155 in Mn– and Fe–SODs, we analyzed a total of 237 Fe– and Mn–SOD (100 Fe–SODs and 137 Mn–SODs) sequences from the UniProtKB/Swiss–Prot database without redundancy in the species of origin. Surprisingly, no SODs have serine at position 155 in any of the analyzed proteins.

In order to understand the underlying reason for the abovementioned observation, we constructed a SOD point mutant Gly155Ser (G/S mutant) followed by its reconstitution with Fe and Mn and the determination of its various properties. We concluded that serine cannot be replaced with threonine at the 155 position without a considerable functional tradeoff because the hydroxy group of serine cannot replace the contribution of the methyl group at the C–gamma position.

Materials and Methods

Cytochrome *c* and trypsin (reduced chymotrypsin activity, T1426) were obtained from Sigma–Aldrich, MO, USA. Xanthine oxidase (cow milk) was purchased from Roche Diagnostics, Mannheim, Germany. The vector pMAL–c2, amylose resin, and *Escherichia coli* strain TB–1 were obtained from New England Biolabs, MA, USA.

Analytical methods

SOD activity was measured using the standard assay proposed by McCord and Fridovich[13] with a reduction in the final volume of the assay system from 3 to 1.0 ml[11]. One unit of SOD activity was defined as the amount that resulted in a 50% decrease in the rate of reduction of xanthine/xanthine oxidase–induced cytochrome *c* in 50 mM phosphate buffer, pH 7.8. UV–visible spectra of the enzyme were measured using a Hitachi U–3000 spectrophotometer equipped with a micro–cuvette holder. Nondenaturing polyacrylamide gel electrophoresis (PAGE) in slab gels was carried according to the method of Davis[14]. Sodium dodecyl sulfate (SDS)/PAGE was according to the method of Laemmli[15]. The protein concentrations of Fe– and Mn–reconstituted wild–type *Pg*–SODs were estimated using molar absorption coefficients of 9.19×10^4 $M^{-1} \cdot cm^{-1}$ and 8.56×10^4 $M^{-1} \cdot cm^{-1}$ at 280 nm, respectively, measured by the method of Hartree[16], as described by Beyer et al.[17]. These values were also used to estimate the protein concentrations of the Fe– and Mn–reconstituted mutant SODs. DNA sequence was carried out by the dideoxy method using a Gene Rapid sequencer (type Seq 4X4, Amersham Pharmacia Biotech UK Ltd., NJ, USA). The molecular weights of the mutant SODs were determined using a SSQ 7000 electrospray ionization mass spectrometer (Thermo–Quest Finnigan Mat Co., Ltd., CA, USA), as described previously [12]. Metal contents were determined by atomic emission spectrometry using Inductively Coupled Plasma Atomic Emission Spectrometry (Seiko Instruments Inc. SPS–3000).

Site–directed mutagenesis of SOD

Construction of the expression vector, induction of protein overexpression, and purification of the maltose–binding protein (MBP)/SOD proteins, was carried out according to previously described methods[8]. Briefly, the sod coding gene was inserted downstream of the *malE* gene of *E. coli*, which encodes MBP. This resulted in the expression of a fusion MBP–SOD protein. Transformed *E. coli* TB–1 were cultured in rich broth medium. After isolation of the fusion proteins from cell supernatants using an amylose column, the eluted protein was digested with trypsin. The SOD proteins were

further purified from the tryptic digests using a Q–Sepharose column (Amersham Biosciences NJ, USA) until a single band was obtained via SDS–PAGE.

The *Eco*R I/*Hind* III fragment of SOD/pMal–c2, corresponding to nucleotides 76–573 of sod, was ligated into M13mp19 for mutagenesis. In vitro mutagenesis of SOD was performed using the Mutan–K system (Takara Biomedicals, Tokyo, Japan), based on the method described by Kunkel[18] according to manufacturer's recommendation. A substitution of Gly (GGA) to Ser (AGC) was introduced at amino acid position 155. The mutant cDNA was screened and sequenced to ensure the absence of spurious mutations. The mutant SOD was expressed and purified by similar 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[8]. In order to remove minor components from the reconstituted proteins, we used a HPLC system (Shimadzu LC–10A, Japan) equipped with a CHT–10 hydroxyapatite column (12 × 88 mm, Bio–Rad, CA, USA).

Results

Characterization of the mutant SODs

Purified wild–type and SOD mutant proteins produced single bands upon resolution by SDS–PAGE (data not shown). 1 L of bacterial culture yielded 25–30 mg of purified wild–type enzyme. However, the yield of G/S mutant protein was decreased to one–sixth that of wild–type SOD. The mutant enzyme was eluted with a slight delay compared to wild–type SOD after application onto a Q–Sepharose column for the final purification stage (Fig. 2). While the elution peaks for both the wild–type and the mutant proteins were symmetrical, the mutant protein peak was slightly broader.

The SOD G/S mutants were sequenced for exclusion of spurious mutations and also analyzed with mass spectrometer to accurately determine the molecular mass. MS revealed molecular masses of 21,531 Da for the G/S mutant SOD, which was identical to the theoretical calculated value. The

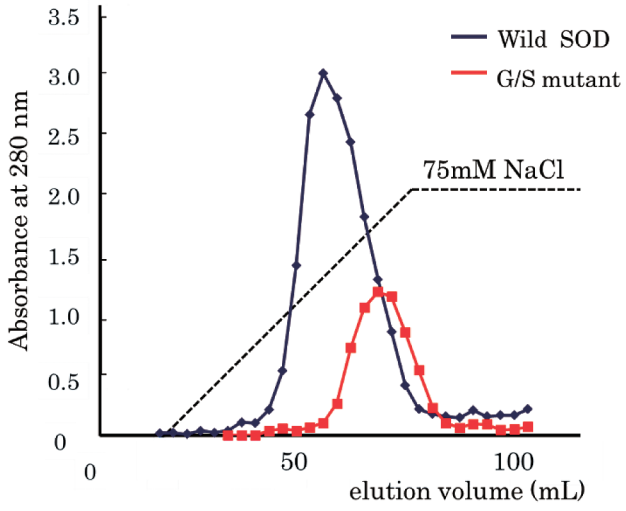

Fig. 2 : Q–Sepharose chromatography of the final purification stage for wild–type and G/S mutant SOD, using a linear NaCl gradient from 0 to 75 mM in the 50 mM Tris–Cl buffer (pH 8.0).

30 Da difference between the observed and the calculated molecular masses of the G/S mutant and wild–type SOD (21,501 Da) was similar to the value calculated by the replacement of glycine (57 Da) and serine (87 Da) residues in the protein structure. Thus, the plasmid construction, site–directed mutagenesis of SOD, and purification of the SOD protein were successful.

After the reconstitution of the purified mutant SOD with Fe or Mn, the purified Fe– and Mn–reconstituted mutant SODs were subjected to non–denaturing PAGE along with wild–type SOD. Figure 3 shows that each mutant and its corresponding wild–type SOD resulted in a single major band with an identical sample mobility. The Fe–reconstituted mutant enzyme exhibited a protein tailing effect.

Active site spectra of the mutant SODs

The UV–visible absorption spectra of both Fe–substituted and wild–type *Serratia marcescens* Mn–SOD and *Pseudomonas ovalis* Fe–SOD, which are metal–specific SODs[22–24], are shown in Figs. 4. In the wild–type Mn–reconstituted samples, slight absorption was observed around 480 nm; how-

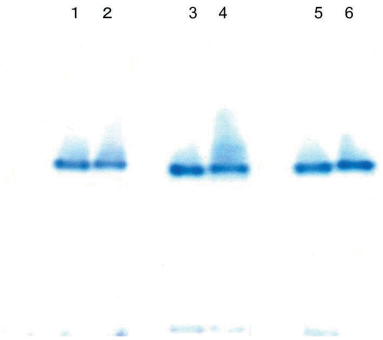

Fig. 3：Non–denaturing PAGE of purified Fe– and Mn–reconstituted wild–type and mutant SODs. Each sample (2 μg) was applied and stained with Coomassie Brilliant Blue G–250 after electrophoresis. Lane 1, purified wild–type; lane 2, mutant SOD; lane 3, Fe–reconstituted wild–type; lane 4 Fe–reconstituted mutant SOD; lane 5, Mn–reconstituted wild–type; lane 6, Mn–reconstituted mutant SOD.

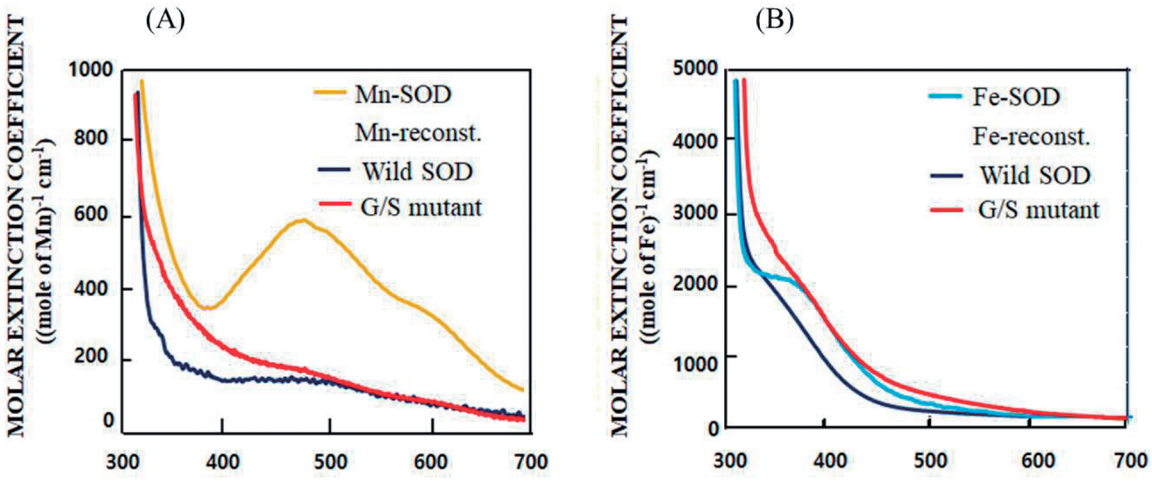

Fig. 4：UV–visible absorption spectra of Fe– and Mn–reconstituted wild–type and G/S mutant *Pg*–SODs. (A) Absorption spectra of *Serratia marcescens* Mn–SOD, Mn–reconstituted wild–type and G/S mutant *Pg*–SODs. (B) Absorption spectra of *Pseudomonas ovalis* (Fe–specific type), Fe–reconstituted wild–type and G/S mutant *Pg*–SODs. The spectra were measured in 10 mM potassium phosphate buffer pH 7.8.

ever, the shoulder at 350 nm was not observed in the Fe–reconstituted samples. Moderate absorption was observed throughout the visible area in the Mn–reconstituted sample of the G/S mutant enzyme, but no characteristic absorption was observed (Fig. 4A). A moderate shoulder, similar to that of the wild–type, was also observed in the Fe–reconstituted preparation of the G/S mutant enzyme (Fig. 4B).

Catalytic properties

The specific activities and metal contents of the Fe– and Mn–reconstituted wild–type and mutant SODs are given in Table 1. Each metal–reconstituted SOD contained nearly stoichiometric amounts of Fe or Mn and negligible amounts of the other metals, which suggests the specific metal–reconstitution of the enzymes. In order to correct for the influence of different metal contents on the apparent activity of each SOD preparation, we expressed the specific SOD activities as units of (mg protein)$^{-1}$ (mol of Fe and/or Mn)$^{-1}$. The wild–type Fe– and Mn–reconstituted SODs showed intermediate specific activity values compared to previous reports[19–21].

Discussion

Column chromatography using Q–Sepharose showed broader and slightly delayed peak elution for the mutant SOD compared with that of the wild–type SOD (Fig. 2). This phenomenon was not clear because the proteins did not undergo any further posttranslational modifications compared to their wild–type counterparts[28]. However, the replacement of glycine with serine may have led to a conformational change in the enzyme. *Pg*–SOD, a dimeric protein, is adsorbed onto column sorbents by a series of forces, including hydrogen bonding, hydrophobic interactions, and electrostatic forces. These forces are also responsible for the maintenance of the protein quaternary structures[26]. The walls of the substrate (i.e., superoxide) entry channel are formed by the contributions from the amino acid residues of both chains of the fundamental dimeric unit found in all Fe– and Mn–SODs, and the residues lining the channel may stabilize the dimer interface[27]. Edwards et al.[28] created a Gln142 (note that amino acid numbering is based on the position in *Pg*–SOD) mutant of *E. coli* Mn–SOD, in which the position of the glutamine residue was complimentarily substituted by Gln70 in the Fe–specific type (Fig. 1), which as a result disrupted the hydrogen–bond network at the active site and considerably affected the protein structure. Miller and Wang[29] reported that a Gln142Glu mutant of *E. coli* Mn–SOD possessed thermal stability and disfavored Mn ion binding, whereas the opposite was observed for the wild–type enzyme. Thus, it is possible that a glycine or threonine residue at position 155 near the active site is well suited for the stabilization of the substrate entry channel and dimeric structure. However, a serine residue at this position may perturbs this structure.

Glycine, serine, threonine, alanine, cysteine, and proline are typically grouped together owing to their small size; a substitution between small side chain groups is frequently observed in nature and may exhibit favorable effects[30]. However, the reason for the strict conservation of threonine in all proteasomal active sites is unclear, especially because its replacement with a serine residue in archeal proteasomes did not exhibit altered rates of substrate hydrolysis[31,32]. In addition, although threonine is equally represented as serine in certain genomes and is an effective nucleophile similar to serine, it is never observed at the active site of the large family of trypsin–like proteases that utilize the Asp/His/Ser triad[33,34].

Here, we analyzed 237 Fe– and Mn–SOD sequences obtained from the UniProtKB/Swiss–Prot

database to elucidate the function of amino acids at position 155 (Table 2). Among the 100 eubacterial Fe–SODs, none of them had serine residues at position 155. Threonine was the most commonly substituted amino acid among Fe–SODs. Furthermore, among the 137 Mn–SODs, none of them had a serine residue at this position. We believe that the absence of serine at this position may be attributed to the van der Waals interactions between the methyl groups combined with the hydrogen bonding interaction of the hydroxy group near the active center. These interactions may possibly stabilize the G/T mutant SOD conformation but not the G/S mutant SOD conformation. Based on our findings, we believe that the absence of bare–hydroxy groups in threonine allows it to fit at position 155 over serine residues.

The non–denaturing PAGE and Q–Sepharose column elution pattern were contradictory. However, the results of PAGE suggested that, despite the differences in metal binding, the mutant SODs possessed the same gross electric character and a similar dimeric structure compared to those of wild–type SODs, except for the subtle structural changes, which were not detectable in PAGE.

The G/S mutation in *Pg*–SOD did not alter the Fe–dependent activity, and the Fe–reconstituted G/S mutants exhibited almost identical Fe–dependent activity values compared with those of the wild–type enzyme (Table 1). However, the Mn–dependent activity exhibited a 60% reduction. Thus, the *Pg*–SOD G/S mutation changed the metal–specific activity of the enzyme from a metal–tolerant type (1 : 1.5 Fe/Mn ratio, Table 1) to being slightly more efficient with Fe (1 : 0.5 Fe/Mn ratio) because a threefold increase in the Fe–dependent activity (from 0.5 to 1.5) was obtained. Earlier, we reported similar results in which an *E. coli* Mn–SOD Gly155Thr mutant[35] exhibited a 25% higher specific activity in the Mn–containing mutant compared with that of the wild–type Mn–SOD. However, Schwartz et al.[36] reported that the iron–supported activity in a Gly70Gln/Gln142Ala *E. coli* Mn–SOD double mutant was 6.5% that of *E. coli* Fe–SOD, which was in contrast to the 0% activity obtained with Fe–substituted Mn–SOD. Thus, these mutations induced a metal–specific activity from a strictly iron–inactive form to a somewhat iron–active form. Hunter et al. confirmed that the Gly70Gln/

Table 1：Activity levels and metal content of Fe– and Mn–reconstituted enzymes of the wild–type and G/S mutant of *Pg*–SOD. Values are presented as the mean ± standard deviation.

Sample	Specific activity (units/mg of protein/mol of Mn and/or Fe/mol of subunit)	Metal content (mol/mol of dimer) Fe	Mn
Fe–reconst. enzymes			
Wild–type SOD	1,667 ± 84 (n = 5)	1.38 ± 0.01	0.029 ± 0.000
G/S mutant SOD	1,893 ± 183 (n = 4)	1.43 ± 0.01	0.016 ± 0.001
Mn–reconst. enzymes			
Wild–type SOD	2,515 ± 159 (n = 5)	0.029 ± 0.000	1.46 ± 0.01
G/S mutant SOD	984 ± 56 (n = 5)	0.016 ± 0.001	1.67 ± 0.01

The Fe extinction activities of the wild–type and mutant enzymes were similar, and the Mn–dependent activity exhibited a considerable decrease. The ratio of the specific activities of Fe– to Mn–reconstituted enzymes was approximately 0.7 in the wild–type enzymes and 1.9 in the G/S mutant enzymes.

Table 2：Amino acid residues at position 155 in Mn– and Fe– SODs.

100 eubacterial Fe–SODs	Thr 63 (63%)	Val 15 (15%)	Gly 11 (11%)	Ala 4 (4%)	Cys 4 (4%)	Leu 2 (2%)	Asn 1 (1%)
50 eukaryotic Mn–SODs	Gly 46	Ala 2	Cys 1	Gln 1			
87 eubacterial Mn–SODs	Gly 54	Gln 11	Val 9	Ala 6	Leu 5	Met 1	Thr 1
Total 137 Mn–SODs	Gly 100 (73%)	Gln 12 (8.6%)	Val 9 (6.6%)	Ala 6 (5.8%)	Leu 5 (3.6%)	other (0.7% ea)	

Gln142Ala *E. coli* Mn–SOD double mutant exhibited a higher specific activity in the *E. coli* Mn–containing mutant compared with that of the wild–type Mn–SOD[37]. When combined with the results of this study, this evidence suggests that the metal–specific activity of Mn–SOD is regulated by many factors, including residues other than Gly155 and Gln142. Nakamura et al.[38] crystallographically analyzed the cambialistic SOD of the obligate aerobic hyperthermophilic archaebacterium *Aeropyrum pernix*. The metal ion in the Mn–coordinating enzyme is in a trigonal bipyramidal coordination with five ligands. However, when the enzyme was coordinated with Fe, an additional water molecule was observed, and the metal was observed to be in an octahedral coordination with six ligands. This additional water molecule occupied the proposed superoxide binding site. A detailed analysis of the reaction mechanism of *A. pernix* SOD may provide new insights into the relationship between metals and their coordinating environments.

Most Mn–SODs exhibit two characteristic absorption bands: (1) at approximately 450–480 nm with a molar extinction coefficient of 240–900 $M^{-1} \cdot cm^{-1}$ per Mn and (2) at approximately 600 nm[1,30]. Although slight absorption was observed throughout the visible range, the characteristic absorption bands were not observed in the Mn–reconstituted sample of the G/S mutant enzyme (Fig. 4A). In the Fe–reconstituted samples, a moderate shoulder similar to that of the wild type was observed in the G/S mutant enzyme (Fig. 4B). These results suggested that the metal environment of the mutant SODs was very similar to that of the cambialistic SODs. The mutant enzymes exhibited increased activity dependence on Fe, while retaining their cambialistic properties. Thus, it was concluded that Ser substitution decreases the Mn–dependent activity.

In conclusion, our results probably support the proposal that Gly155, which is located away from the active site, is an essential residue for maintaining the hydrogen–bonding network for Mn–specific and Fe/Mn–tolerant SOD activity, as well as also for maintaining the dimeric structure of SODs. Thr155 is an essential residue for the Fe–specific activity for similar reasons. However, the G/S mutant lost the Mn–specific activity and according to the decrease of Mn–specific activity, the Fe/Mn–tolerance was lost. Ser155 may perturbs the substrate entry channel and, therefore, the dimeric structure of *Pg*–SOD. Furthermore, the Gly155Val mutant, which did not change its metal–tolerant type, changed from an efficient Mn user compared to the wild–type (1 : 1.5 Fe/Mn ratio, Table 1) to a more efficient Fe user (1.4 : 1 Fe/Mn ratio; unpublished results). Further analysis of mutant SODs is currently underway.

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Conflicts of Interest The authors have declared that no competing interests exist.

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抄録：*Porphyromonas gingivalis* SOD の活性中心金属の近位には，トレオニンがセリンよりも優先的に選択される：155位 Gly を Ser に変異させた影響

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歯周病原菌の一種 *Porphyromonas gingivalis* にとって，酸化ストレス防御機構としてスーパーオキシドジスムターゼ（SOD）は生育に必須の酵素である．原核生物には鉄を含む酵素（Fe–SOD）とマンガンを含む酵素（Mn–SOD）の２種が存在し，活性中心の構造が近似しているにも拘わらず各々の活性は金属に対して厳格な選択性を示すのが一般的であるが，*P. gingivalis* SOD（*Pg*–SOD) は Fe と Mn の何れでも活性を示す特徴がある．私達は，この性質を支持する構造的な特徴を明らかにすべく，活性中心近傍に局在するアミノ酸残基の役割を検討している．以前の研究で，N 末端から155番目のアミノ酸残基は，Mn–SOD ではグリシン（Gly）が，Fe–SOD ではトレオニン（Thr）が特徴的に存在することに着目し，*Pg*–SOD の Gly155を Thr に変異させて完全な Fe 依存性 SOD に変化したと報告した．そこで，*Pg*–SOD の Gly 155をセリン（Ser）に置換した変異酵素を作製し，Gly 残基の生理的意義を検討した．

変異体の Fe 依存活性は野生型 SOD とほとんど同一であったが，Mn 依存活性は60％と顕著に減少した．Fe および Mn で再構成された変異体の紫外―可視部吸収スペクトルは，Fe に依存する環境に近い事を示していた．変異体はポリアクリルアミドゲル電気泳動的に野生型 SOD と同様の移動度を持つ単一のバンドを示した．一方，陰イオン交換クロマトグラフィーの挙動は野生型 SOD の挙動とは異なり，変異体は野生型とは異なる酵素表面上の電荷が生じており，総体の構造が変化している事が示唆された．

Thr と Ser は同じヒドロキシ・アミノ酸としてタンパク質機能に等しく寄与している筈であるが，Ser は調べた237種の Fe–および Mn–SOD の155位で存在しなかった．従って，Ser の剥き出しのヒドロキシ基により155位では酵素の構造が維持できない可能性が考えられた．一方，155位の Gly は，Mn–SOD および金属寛容性 SOD の活性発現を維持する必須の残基であると考えられた．