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Contribution of the amino acid residues located near the active site metal to the metal–specific activity of *Porphyromonas gingivalis* SOD induced by a double mutation of Leu 72 Trp and Leu 76 Phe

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Summary

The role of superoxide dismutase (SOD) as a radical scavenger in *Porphyromonas gingivalis* is well documented. *P. gingivalis* SOD (*Pg* SOD), which is characterized by a metal–tolerant activity, can use either iron or manganese as a cofactor. Leu 72 and Leu 76, located near the active–site metal, are characteristic amino acid sequences of *Pg* SOD proteins, although they are substituted to Trp in the 72 position and Phe in the 76 position in most iron–containing SOD (Fe–SOD) proteins. In the present study, we constructed a mutant of the enzyme with changes from Leu 72 to Trp and Leu 76 to Phe. This mutant SOD was examined with respect to its metal–dependent activity and structural characterization. We herein conclude the integrity of Leu 72 and Leu 76 is a necessary requisite for the metal–tolerant activity of *Pg* SOD.

Introduction

The Gram–negative obligate anaerobe *P. gingivalis* is one of the organisms most strongly asso-

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ciated with chronic adult periodontitis[1–3]. The actions of superoxide dismutases (SOD: EC 1.15.1.1.), which are essential for aerobic life due to their important protective effects against oxidative stress, may contribute to the virulence of this organism. These enzymes, along with catalase and peroxidase, belongs to a specific cellular system that has evolved for cellular protection against oxidative stress. Interestingly, a previous study was unable to detect either a catalase or peroxidase activity in *P. gingivalis*[4].

Eubacteria and archaea typically contain SODs that require manganese or iron as a cofactor, abbreviated as Mn–SOD and Fe–SOD, respectively. Metal replacement studies with Fe–SODs and Mn–SODs, which are produced by several bacterial species, indicate strict metal cofactor specificity for these enzymes[5]. However, some bacterial species such as *Propionibacterium sherimanii*[6] and *P. gingivalis*[7,8], have been found to use the same protein moiety to form either Fe–SOD or Mn–SOD. This feature is termed cambialism, from the Latin "cambialis", suggesting change and the donation of enzymes capable of making cofactor substitution[9]. Despite these differences in metal specificity, Mn–SODs, Fe–SODs and cambialistic SODs are closely related and similar in both amino acid sequence and three–dimensional structure[10–13]. For example, their active metal atoms are commonly ligated by three histidine residues, an aspartic acid residue and a water molecule (Fig. 1). In addition, these metals are surrounded by a similar environment consisting of a group of aromatic amino acid residues. Therefore, there are no significant differences in the active site environment between Fe–, Mn– and cambialistic SODs. However, some minor differences have been observed in the active metal ion environments of Fe–SODs and Mn–SODs. Gly 155 is conserved in most Mn–SODs,

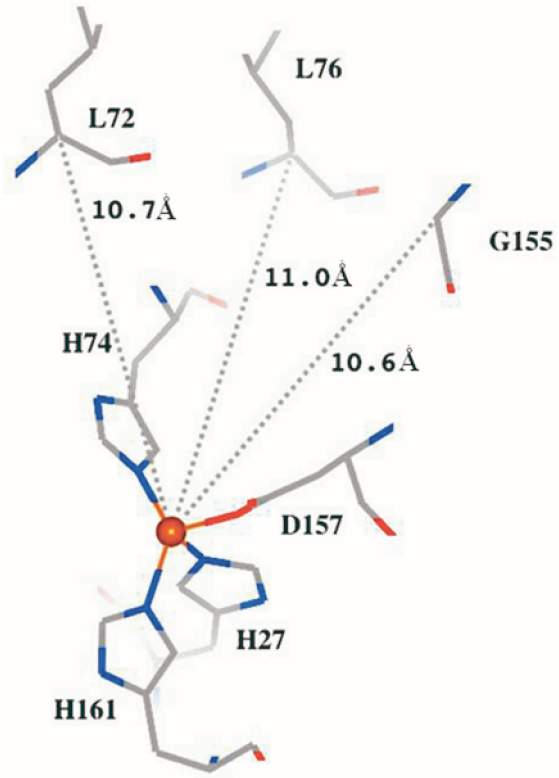

Fig. 1：Metal binding site of *Pg* SOD. The metal ion as an active site is bound by His 74, His 161 and Asp 157 in the equatorial plane and His 27 and a solvent molecule in the axial plane. The α–carbon of Leu 72 and Leu 76 is located 10.7 and 11.0 Å away from the metal ion, respectively, while the δ–carbon in the side chain is located 12.4 to 13.6 Å away from the metal ion.
This figure was drawn based on Waals 2013, Altif Laboratories Inc., Tokyo, Japan

whereas threonine occupied this position in most Fe–SODs[8]. The site is located two residues from Asp 157, a ligand to the metal ion and 11 Å from the metal ion. We have previously shown that the Gly 155 Thr mutation in *P. gingivalis* SOD (*Pg* SOD) results in an 85% reduction in the enzyme activity when the protein is reconstituted with manganese, compared to an enhanced activity when reconstituted with iron, indicating that this single mutation converts the cambialistic protein to an iron–type protein[14]. This is the first successful report regarding the Fe– and Mn–SOD family with respect to changes in the metal–specific activity, not only direct changes, but also those induced by the site–directed mutagenesis of an amino acid other than an active site or second sphere. Based on the result obtained using site–directed mutagenesis, we focused on Leu 72 and Leu 76, which are located near the active–site metal, although they are substituted to Trp in the 72 position and Phe in the 76 position in most Fe–SOD proteins. In our preceding paper, we constructed a Leu 72 Trp mutant enzyme and discussed its electrophoretic characterization[15]. We found that the Leu 72 is necessary for the metal–tolerant activity in order to maintain the functional properties of *Pg* SOD. In the present study, we constructed a mutant of the enzyme with changes in Leu 72 to Trp and Leu 76 to Phe, reconstituted with Fe and Mn, and determined various properties of this mutant. We herein conclude that the integrity of Leu 72 and Leu 76 is a necessary requisite for the metal–tolerant activity of *Pg* SOD.

Materials and Methods

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

Analytical methods

The SOD activity measurements were carried out using the standard assay of McCord and Fridovich[16] with a reduction in the final volume of the assay system from 3 to 1.0 ml[8]. One unit of SOD activity was defined as the amount resulting in a 50% decrease in the rate of reduction of xanthine/xanthine oxidase–induced cytochrome *c* in 50 mM of phosphate buffer, at a pH 7.8. The metal content was determined via atomic absorption spectrometry using a Hitachi Z–9000 atomic absorption spectrophotometer.

Polyacrylamide gel electrophoresis (PAGE) in slab gels was carried out in accordance with the method of Davis[17], with a few modifications. The protein concentrations of Fe– and Mn–reconstituted wild–type *Pg* SODs were estimated using molar absorption coefficients of $6.98 \times 10^4\ M^{-1} \cdot cm^{-1}$ and $7.01 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 280 nm, respectively, which were measured according to the method of Hartree[18], as described by Beyer *et al.*[19] using bovine serum albumin as a standard, as reported in a previous paper[20]. These values were also used to estimate the protein concentrations of the Fe– and Mn–reconstituted mutant SODs. 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 previously described[20].

Site–directed mutagenesis of SOD

The construction of the expression vector, as well as induction of the overexpression and purification of maltose–binding protein (MBP)/SOD proteins, was carried out according to previously described methods[20], based on the technique reported by Kunkel[21]. Briefly, the coding *sod* gene was inserted downstream of the *malE* gene of *E. coli*, which encodes MBP, resulting in the expression of a fusion protein including SOD proteins. The transformed *E. coli* TB–1 was grown in rich broth medium. After the fusion protein was isolated from the supernatant of the cells using an amylose column, the eluted protein was digested with trypsin. SOD was further purified from the digests by using a Q–Sepharose column to a single band via SDS–PAGE. A total of 15 to 20 mg of purified enzymes was obtained from 1 liter of induced bacterial culture.

The *in vitro* mutagenesis of SOD was introduced using the Mutan–K system (Takara Biomedicals, Tokyo) under conditions recommended by the manufacturer. The mutation of Leu (code: CTC) to Trp (code: TGG) was introduced at amino acid position 72, while that of Leu (code: CTC) to Phe (code: TTC) was introduced at amino acid position 76. The mutant cDNA was screened and sequenced in order to ensure the absence of spurious mutations. The mutant SOD was expressed and purified using the same methods as those employed for 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[20]. In order to remove minor components in the reconstituted proteins, we used the HPLC system (Shimadzu LC–10A, Japan) equipped with a CHT–10 hydroxyapatite column (12×88 mm, Bio–Rad, CA, USA).

Results

Characterizatiion of the mutant SODs

Leu 72 Trp and Leu 76 Phe double mutant SODs were screened and sequenced to ensure the absence of spurious mutations and additionally subjected to mass spectroscopy in order to accurately measure the molecular mass. The protein mass spectrum revealed a molecular masses of 21,609 Da, which is almost identical to the calculated value of the SOD (21,608 Da). The difference between the observed and calculated molecular masses of the wild–type SOD (21,501 Da), which is 107 Da, was close to that noted between the calculated values of the replacement of leucine to tryptophan (73 Da) and leucine to phenylalanine (34 Da) in the protein structure. These data show that the construction of the plasmid, mutation of the *sod* gene and the purification of the SOD were properly conducted.

After reconstituting the purified mutant SODs with iron or manganese, the purified, Fe–reconstituted and Mn–reconstituted mutant SODs were subjected to non–denaturing PAGE, along with those of the wild–type SODs. All of the mutant and wild–type SODs displayed a single major band with the same motility for each sample (Fig. 2), with the exception of the metal–reconstituted enzyme, which exhibited tailing of proteins brought about by the ease of denaturation.

Catalytic properties

Table 1 shows the specific activity levels and metal content of the Fe–reconstituted and Mn–reconstituted wild–type and mutant SODs. Each metal–reconstituted SOD contained nearly stoichio-

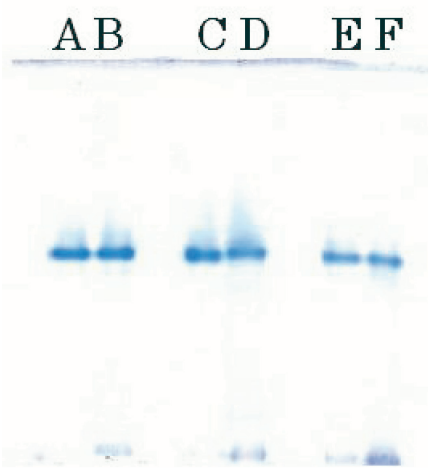

Fig. 2 : Non–denaturing PAGE of purified and Fe– and Mn–reconstituted wild–type and mutant *Pg* SODs. Each sample (2 μg) was stained with Coomassie Brilliant Blue G–250 after electrophoresis. The samples were as follows: purified wild–type (Lane A) and mutant (Lane B) SODs, Fe–reconstituted wild–type (Lane C) and mutant (Lane D) SOD and Mn–reconstituted wild–type (Lane E) and mutant (Lane F) SODs.

Table 1 : Activity levels and metal content of Fe– and Mn–reconstituted enzymes of the wild–type, Leu 72 Trp and Leu 76 Phe mutant of *Pg* SOD. The values for the wild–type are cited from a previous paper: reference no 14. The values are presented as mean ± the standard deviation (n=3).

Sample	Specific activity (units/mg of protein/mol of Mn and/or Fe/mol of subunit)	Metal contents (mol/mol of dimer)	
		Fe	Mn
Fe–reconst. enzymes			
Wild–type SOD	1,598 ± 118	1.41 ± 0.004	0.004 ± 0.002
Mutant SOD	1,295 ± 125	1.33 ± 0.12	0.033 ± 0.000
Mn–reconst. enzymes			
Wild–type SOD	2,653 ± 159	0.034 ± 0.046	1.49 ± 0.018
Mutant SOD	1,690 ± 120	0.012 ± 0.003	1.63 ± 0.112

metric amounts of iron or manganese with a negligible amount of the other metal, thus suggesting that each metal–reconstituted enzyme contained negligible amounts of non–specific metals. In order to correct for the influence of different metal contents on the apparent activity of each SOD preparation, we expressed the specific activity levels of the SODs as units per mg protein divided by the moles of Fe and/or Mnper mol of subunit. The ratio of the specific activity of Fe–SOD to Mn–SOD was 0.77 in the mutant SOD and 0.60 in the wild–type SOD. Therefore, the metal–specific activity of *Pg* SOD was not changed from that of a metal–tolerant type to an iron–specific type by the double mutation in the 72 and 76 position.

Discussion

In order to elucidate the functions of leucine and tryptophan at position 72 and those of leucine and phenylalanine at position 76 in the *Pg* SODs, we used 97 Mn– and Fe–SOD sequences obtained from the UniProtKB/Swiss–Prot database, without redundancy of the species of origin. The amino acids at positions 72 and 76 in the *Pg* SOD, which is located two amino acids before and after the first ligand His 74, were analyzed. Among 63 Mn–SODs, including 16 *Eubacteriae* and 47 eukary-

Table 2：Amino acid residues at positions 72 and 76 in 97 Fe– and Mn–SODs. The values were obtained from the UniProtKB/SwisProt database.

	Fe–SOD (34 cases)			Mn–SOD (63 cases)		
position		cases			cases	
72	Trp	18	53%	Ile	35	56%
	Ile	3	9	Val	10	16
	Lys	3	9	Ala	6	10
	Tyr	3	9	Leu	4	6
76	Phe	21	62%	Ile	34	54%
	Leu	6	18	Leu	22	35
	Ile	5	15	Phe	4	6

otes, 35 had isoleucine (56%) at position 72 (Table 2). The second most frequent amino acid was valine. Among 34 Fe–SODs, including seven *Archaea*, 12 *Eubacteriae* and 15 eukaryotes, 18 (53%) had tryptophan at this position. The second amino acid was isoleucine, lysine and tyrosine. No Fe–SOD was found to have any Leu residues in this position. Meanwhile, at position 76, the most frequent amino acid was isoleucine (54%), followed by leucine as the second most frequent amino acid. Among 34 Fe–SODs, 21 (62%) had phenylalanine at this position. Leucine was obtained in six cases. However, the overall amino acid sequence of *Pg* SOD resembles that of Fe–SODs[8,13,24]. Therefore, both Leu 72 and Leu 76 are candidates for the element governing the cambialistic nature of *Pg* SOD.

The results of non–denaturing PAGE suggested that all the mutant SODs had the same gross structure as the wild–type SODs, despite the difference in metal. The double mutation of *Pg* SOD resulted in a decrease in both the iron– and manganese–specific activity, using an xanthine/xanthine oxidase system to measure the activity; namely, these values were approximately 75% of that of wild–type SOD (Table 1). The ratio of the specific activity levels of Fe–reconstituted SOD to Mn–reconstituted SOD suggested that the double mutation of Leu 72 Trp and Leu 76 Phe results in a catalytic environment for metal in addition to metal–environment interactions of *Pg* SOD, without changes in the original characteristics of the enzymes. Therefore, we conclude this mutation mismatches part of the structure basis of *Pg* SOD, namely, that Trp in the 72 position and Phe in the 76 position are well–suited for the structure basis of an iron–specific–activity. Two Leu residues in the 72 and 76 positions are also well–suited for the structure basis of a metal–tolerant activity.

Based on the results of this study, we propose that the metal–specific activity of Fe–, Mn– and cambialistic SODs may be cumulatively controlled based on differences in several unknown amino acids located apart from the active–site metal. Although a few amino acid residues besides the primary candidate have been found to be conservatively different in Fe– and Mn–SODs[22], it is very difficult to predict the effects of these differences in the amino acids of active metals based on structural data[10–12,23].

We herein conclude that the integrity of Leu 72 and Leu 76 is a necessary requisite for the metal–tolerant activity of *Pg* SOD. We are currently preparing a triple mutation including Leu 72 Trp and Leu 76 Phe mutations in our next study aimed at understanding the roles of amino acid residues located near the active site of *Pg* SOD.

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抄録：*Porphyromonas gingivalis SOD*における活性中心近傍に局在するアミノ酸残基の金属特異的活性における関与：Leu72 TrpおよびLeu76 Pheの２残基変異による検討

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＊の著者は，この論文における貢献度が同等である事を示す.

スーパーオキシドジスムターゼ（SOD）は酸化ストレス防御機構であり，歯周病原菌*Porphyromonas gingivalis*のような偏性嫌気性菌でさえも生育に必須の酵素である．*P. gingivalis* SOD（*Pg* SOD）の特徴は，金属酵素としては稀な事に活性の金属依存性に寛容で，鉄でもマンガンでも活性を示す．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．今回，N末端から72番目のLeu（Leu72）と76番目のLeu（Leu76）に注目した．鉄イオンに依存して活性を示すSOD（Fe–SOD）においては，この位置はTrpとPheに置き換わっている．そこで，*Pg* SODのLeu72をTrpに，Leu76をPheに置換した変異酵素を作製した．金属依存性が鉄イオンで高くなった場合，Trp72とPhe76はFe–SODの金属特異性を支持する役割を持つと結論づけられる．一方，金属依存性が変異酵素で変化していない場合，Leu72とLeu76は*Pg* SODの金属寛容性を支持する役割を持つと結論づけられる．以上の仮説を実証するため，金属依存活性と構造的な性質を検討した．

Kunkelの方法により，*P.g.* SODのLeu72（code: CTC）をTrp（code: TGG）に，Leu76（code: CTC）をPhe（code: TTC）へ部位特異的変異を導入した．変異酵素は，FeおよびMn再構成酵素を調製して性質を検討した．

ポリアクリルアミド電気泳動の結果，変異酵素は野生型酵素と同一の挙動を示し，分子の構造が野生型酵素と同等であると考えられた．また，野生型酵素の比活性の比率（Fe/Mn)は0.60であり，変異酵素のそれ（0.77）と大きな差がなく，酵素活性値が野生型の75％に低下していた．これらの結果から，Leu72とLeu76が*Pg* SODの金属寛容性を支持する役割を持つアミノ酸残基の候補になり得ると結論付けた.