〔**Original Communication**〕 Matsumoto Shigaku 40：19～25, 2014
key words：site–directed mutagenesis, superoxide dismutase, *Porphyromanas gingivalis*

Effect of substituting Trp for Leu at position 72 on the structure of *Porphyromonas gingivalis* superoxide dismutase

Miho KOMACHIYA[a,1], Shinya MIZOUE[a,1], Masashi MIHARA[a,2],
Masaki OSAWA[a,2], Yuichiro KIKUCHI[3], Setsuko UEMATSU[4],
Kaname HIRAI[5], Yuichiro OKUBO[6], Akihiro KUROIWA[1],
Kazuhiro YAMADA[2], Fumiyuki YAMAKURA[7] and B. Yukihiro HIRAOKA[6]

[1]*Department of Removable Prosthodontics, School of Dentistry, Matsumoto Dental University*
[2]*Department of Orthodontics, School of Dentistry, Matsumoto Dental University*
[3]*Department of Microbiology, School of Dentistry, Tokyo Dental University*
[4]*Department of Orthodontics and Dentofacial Orthopedics, Osaka University, Dental Hospital, Osaka*
[5]*Department of Oral Microbiology, School of Dentistry, Matsumoto Dental University*
[6]*Division of Hard Tissue Research, Institute for Oral Science, Matsumoto Dental University*
[7]*Department of Chemistry, Juntendo University, School of Health Care and Nursing, Inba, Chiba*

Summary

Porphyromonas gingivalis contains a single constitutive superoxide dismutase (SOD) that is active with either iron or manganese at the active site. The aim of this work was to evaluate the effect of the Leu 72 to Trp mutation on the structure of *P. gingivalis* SOD (*Pg* SOD) using electrophoretic characterization. Leu 72, which is located near the active site metal, is substituted with Trp in aligned amino acid sequences of iron–containing SOD. The results of electrophoretic characterization and the expressed activity of mutant SOD suggest that mutant SOD have the same gross structure as wild–type SOD. We herein conclude that the integrity of Leu 72 is a necessary requisite for the metal–tolerant activity of *Pg* SOD.

Introduction

Superoxide dismutases (SOD; EC 1.15.1.1.) are essential for aerobic life, playing an important protective role against oxidative stress. In some experiments, prokaryotes possess four classes of SODs characterized by their metal ion: nickel, iron, manganese, and Fe/Mn[1)]. The Fe/Mn type SOD is called "cambialistic" SOD, from the Latin cambialis, thus suggesting change and the donation of

（recieved February 28, 2014 ; accepted March 18, 2014）
[a]*These authors contributed equally to this work*

enzymes capable of making a cofactor substitution[2]. Metal replacement studies with Fe–SOD and Mn–SOD produced by several species indicate strict metal cofactor specificity for these enzymes[3]. In contrast, the Fe–SOD, Mn–SOD and cambialistic SOD exhibit a high level of structure homology[4,5]. In each case, the metal ligation sphere is a five–coordinate structure with a trigonal bipyramidal geometry, as shown in Figure 1[5].

The anaerobe *P. gingivalis* synthesizes typical cambialistic SOD[4,6], the amino acid sequence of which appears similar to that of Fe–SOD[7,8]. The SOD of anaerobically grown *P. gingivalis* has iron as a cofactor, although SOD derived from aerobically grown *P. gingivalis* associates with manganese[4]. The mechanism underlying this phenomenon is unclear; therefore, we propose a possible mechanism for the changes in the metal–specific activity based on a comparison of the structure of the wild–type and mutant SOD.

In the preliminary investigations, the constructed Tyr to Phe mutation at amino acid position 77 of *P. gingivalis* SOD (*Pg* SOD) was tested[9]. Tyr (Y) 77 is conserved in aligned amino acid sequences of 50% Fe–SOD proteins (Fig. 2), although it is substituted to Phe (F) in most Mn–SOD proteins (62/63 cases: 98%). The mutant SODs exhibited a protein level of 1/100 the expression observed in the wild–type SODs. There is no simple explanation for this result at the present time; however, it is possible that proteolysis was induced by the expression of the enzyme proteins. In this case, the protein structure of the mutant SOD may exhibit deformation. In contrast, Yamano and Maruyama reported that the substitution of Tyr 77 with Phe in the metal–specific Fe–SOD from hyperthermophilic archaeon, *Sulfolobus solfataricus* dose not change the metal–specific activity of the enzyme[10]. The role of amino acid residues located near the active–site metal, such as Tyr 77, is in any event, unclear.

In a previous paper, we constructed Gly 155 Thr mutant SOD and determined various properties of the produced enzymes[11]. In particular, we found that this mutation changes the metal–specific activity remarkably from a cambialistic type to a type close to that of Fe–specific forms. This is

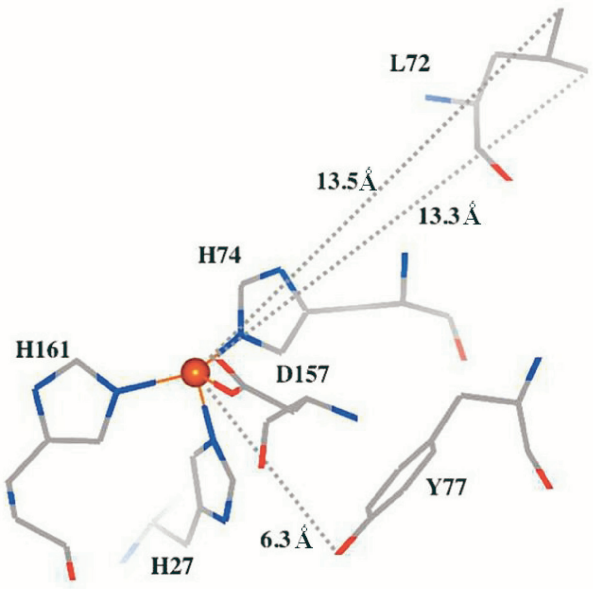

Fig. 1 : Metal binding site of *Pg* SOD. The metal ion as an active site is bound by His 74, His 161 and Asp 157 in the equatorial plane and by His 27 and a solvent molecule in the axial plane. The α –carbon of Leu 72 is located 10.7 Å away from the metal ion, while the δ –carbon in the side chain is located 13.3 to 13.5 Å away from the metal ion. In addition, the hydroxyl group of Trp 77 is located 6.3 Å away from the metal ion. This figure was drawn based on Waals 2013, Altif Laboratories Inc., Tokyo, Japan.

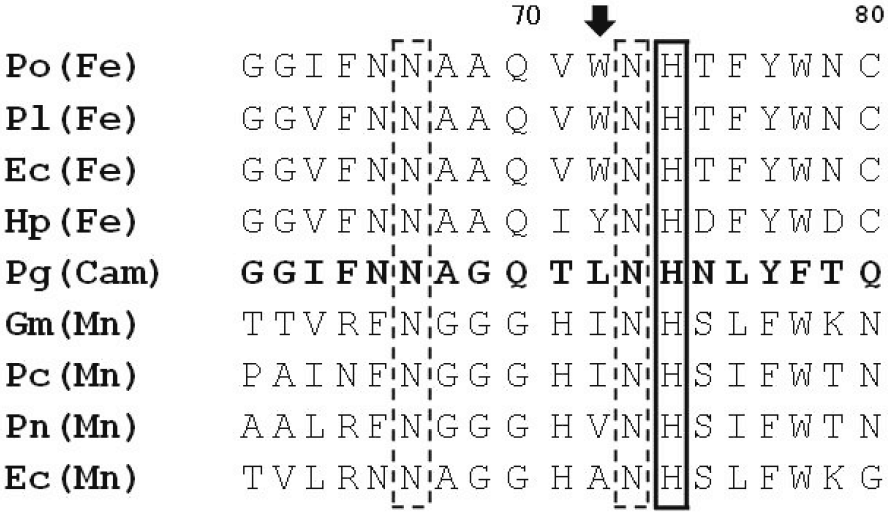

Fig. 2：Comparison of the amino acid sequences, near the target amino acid Leu 72, of the SODs of the following organisms: Po: *Pseudomonas ovalis*, Pl: *Photobacterium leiognathi*, Ec: *Escherichia coli*, Hp: *Helicobacter pylori*, Pg: *P. gingivalis*, Gm: *Ganoderma microsporum*, Pc: *Pneumocystis carinii*, Pn: *Phytophthora nicotianae*.
Data obtained from the UniProKB/SwissProt database. Positions are numbered to correspond to the sequence of *Pg* SOD. The solid and dashed line boxes indicate the positions at which residues are identical and positions regarded to be metal ligands, respectively. The arrow indicates the 72 position. Abbreviations: Fe: Fe–SOD, Cam: cambialistic SOD, Mn: Mn–SOD.

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. In order to clarify the contribution of the amino acid residue to the effects of SOD, we have prepared a mutant from Leu to Trp at the 72 position. Leu 72, which is conserved as Trp in the most Fe–specific SOD, a target amino acid residue for mutation in this study, is located two residues apart from His 74, a ligand binding residue (Fig. 2). Upon non–denaturing polyacrylamide gel electrophoresis (PAGE), the wild–type *Pg* SOD and Leu 72 Trp mutant showed only one band with a SOD activity and the same level of electrophoritic mobility. In addition, the expressed activity of the mutant SOD was approximately 80% of that of wild–type SOD. These result suggest that Leu 72 is a necessary for the metal–tolerant activity with respect to maintaining the functional properties of *Pg* SOD.

Materials and Methods

Cytochrome *c* was obtained from Sigma–Aldrich, MO, USA. The vector M13 mp18 and pUC18 were obtained from TOYOBO, Tokyo. Xanthine oxidase (from cow's milk) was purchased from Roche Diagnostics, Mannheim, Germany. All other reagents were of the highest purity commercially available.

Site–directed mutagenesis of SOD

The *in vitro* mutagenesis of SOD was performed according to previously described methods[6], based on the method described by Kunkel[12]. A mutation of Leu (code: CTC) to Trp (code: TGG) was introduced at amino acid position 72. Mutant cDNA was screened and sequenced to ensure the absence of spurious mutations. Wild–type and mutant SOD were expressed in *Escherichia coli* QC774,

with deletion of the *sod* s gene[13].

Analytical methods

Crude extracts were separated electrophoretically in gels containing 7.5% acrylamide according to the Davis method[14]. The visualized SOD activity was detected in the gel using the photochemical nitro blue tetrazolium stain, as descrived by Beauchamp[15].

The SOD activity was measured by inhibiting the xanthine/xanthine oxidase–induced reduction of cytochrome *c* at a pH 7.8, according to a previous report with a slight modification[6,16]. The protein concentration was estimated according to the method of Hartree[17] using crystalline bovine serum albumin as the standard.

The *Pg* SOD protein amounts were measured using the enzyme–linked immunosorbent assay as described in a previous paper[9] using purified recombinant *P. gingivalis* SOD as the antigen.

Results and Discussion

In this study, the contribution of Leu residues in 72 position was evaluate based on changes to the Trp residues in the structure of *Pg* SOD on electrophoretic characterization. Crude cell extracts were separated via non–denaturing PAGE and stained for the SOD activity (Fig. 3). Electropherogram of *E.coli* DH5 *α* strain, which contains both Mn–SOD and Fe–SOD, showed each SOD and their hybrid forms due to their assembly into a dimer structure. Each of the wild–type (lane 1) and mutant *Pg* SODs (lane 2) displayed a single major band with the same level of mobility for each sample. These results suggest that the Leu 72 Trp mutant has the same gross structure as wild–type SODs. The *Pg* SOD, a cambialistic SOD, demonstrated the same level of mobility for the hy-

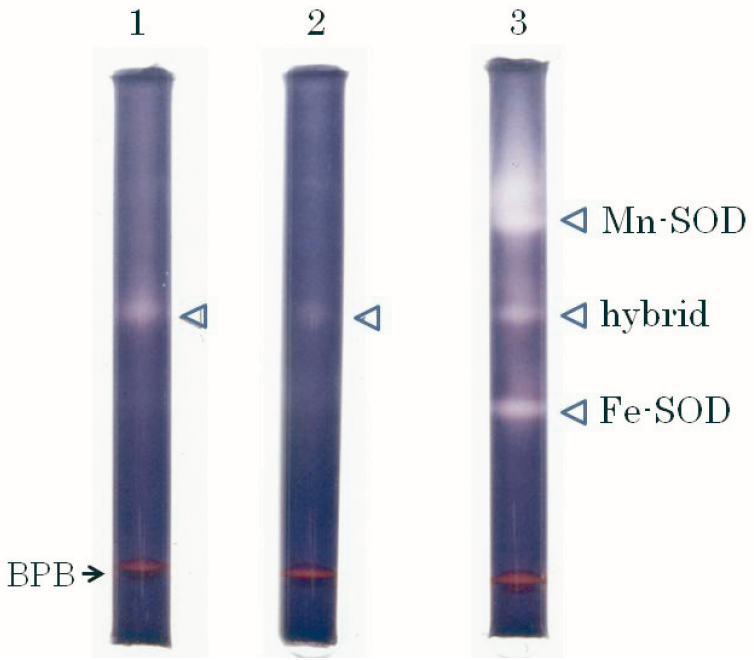

Fig. 3 : Activity–stained non–denaturing PAGE of the crude extracts of the wild–type and mutant SODs. Lanes: 1, *E. coli* DH5 *α* ; 2, wild–type *Pg* SOD; 3, Leu 72 Trp mutant SOD. Approximately 100 μg of protein was applied to each gel for activity staining. Nitro blue tetrazolium was reduced by the superoxide anion, which is generated from riboflavin with illumination. The gels became uniformly dark purple except at positions containing SOD (achromatic zones; triangle mark). BPB, marker dye, bromophenol blue.

brid form of Mn–SOD and Fe–SOD in *E.coli* (lane 3). Similar results have been commonly observed in other studies[4,18].

In order to elucidate the functions of Leu and Trp at position 72 in Mn–SODs and Fe–SODs, respectively, we used 97 Mn– and Fe–SOD sequences obtained from the UniProtKB/Swiss–Prot database. Among 34 Fe–SODs, 18 (53%) had Trp (W) at position 72. The second most frequent amino acid was Ile (I), Lys and Tyr (Y; 3/34 cases each). NoFe–SOD was found to have any Leu residues in this position.

Among the 63 Mn–SODs, 35 (56%) had Ile (I) at this position. The second most frequent amino acid was Val (V; 10/63 cases), followed by Ala (A; 6/63 cases). Leu (L) was present in only four cases involving Mn–SOD. Therefore, it is likely that the integrity of Leu 72 is a necessary requisite for the metal–tolerant activity of *Pg* SOD.

The wild–type and mutant *Pg* SODs exhibited an SOD activity of 2.45 ± 0.72 (mean ± the standard deviation; n=3) and 1.96 ± 0.22 units/mg protein, respectively. The expression enzyme–protein levels were evaluated using an enzyme–linked immunoabsorbent assay[16]. The mutant SOD exhibited a lower expression than the wild–type–SOD, and the SOD–protein levels were almost 80% (1.58 ± 0.31 μg/mg of total protein) of that of the wild–type SOD (1.92 ± 0.54 μg/mg of total protein). Therefore, we conclude that the Leu 72 Trp mutation mismatches part of the structure basis of cambialistic SODs, such as *Pg* SOD, namely, that Trp in the 72 position is well–suited for the structure basis of the iron–specific activity. In order to confirm this possibility, we are preparing double or more mutations, including Leu 72 Trp mutation, in our next study aimed at understanding the role of amino acid residues located near the active site of *Pg* SOD.

References

1) Abreu IA and Cabelli DE (2010) Superoxide dismutases–a review of the metal–associated mechanistic variations. Biochim Biophys Acta**1804**: 263–74.
2) Martin ME, Byers BR, Olson MO, Salin ML, Arceneaux JE and Tolbert C (1986) A *Streptococcus mutans* superoxide dismutase that is active with either manganese or iron as a cofactor. J Biol Chem **261**: 9361–7.
3) Kirby T, Blum J, Kahane I and Fridovich I (1980) Distinguishing between Mn–containing and Fe–containing superoxide dismutases in crude extracts of cells. Arch Biochem Biophys **201**: 551–5.
4) Amano A, Shizukuishi S, Tamagawa H, Iwakura K, Tsunasawa S and Tsunemitsu A (1990) Characterization of superoxide dismutases purified from either anaerobically maintained or aerated *Bacteroides gingivalis* . J Bacteriol **172**: 1457–63.
5) Sugio S , Hiraoka BY and Yamakura F (2000) Crystal structure of cambialistic superoxide dismutase from *Porphyromonas gingivalis* . Eur J Biochem **253**: 49–56.
6) Hiraoka BY, Yamakura F, Sugio S and Nakayama K (2000) 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. Biochem J **345**: 345–50.
7) Amano A, Shizukuishi S, Tsunemitsu A, Maekawa K and Tsunasawa S (1990) The primary structure of superoxide dismutase purified from anaerobically maintained *Bacteroides gingivalis* . FEBS Lett **272**: 217–20.
8) Nakayama K (1990) The superoxide dismutase–encoding gene of the obligately anaerobic bacterium *Bacteroides gingivalis* . Gene **96**: 149–50.
9) Osawa M, Mihara M, Kikuchi Y, Okubo Y, Yamada K, Hirai K and Hiraoka BY (2010) Immunoassay based on a polyclonal antibody for the quantification of *Porphyromanas gingivalis* SOD: a preliminary report. Matsumoto Shigaku **36**: 1–6.
10) Yamano S and Maruyama T (1999) An azide–insensitive superoxide dismutase from a hyperthermo-

philic archaeon, *Sulfolobus solfataricus* . J Biochem **125**: 186–93.
11) Yamakura F, Sugio S, Hiraoka BY, Ohmori D and Yokota Y (2003) Pronounced conversion of the metal–specific activity of superoxide dismutase from *Porphyromonas gingivalis* by the mutation of a single amino acid (Gly155Thr) located apart from the active site. Biochemistry **42**: 10790–9.
12) Kunkel TA (1985) Rapid and efficient site–specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA **82**: 488–92.
13) Carlioz A and Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli* : is superoxide dismutase necessary for aerobic life? EMBO J **5**: 623–30.
14) Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann N Acad Sci **121**: 404–27.
15) Beauchamp C and Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44**: 276–87.
16) McCord JM and Fridovich I (1968) The reduction of cytochrome c by milk xanthine oxidase. J Biol Chem **243**: 5753–60.
17) Hartree EF (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem **48**: 422–7.
18) Dougherty HW, Sadowski SJ, Baker EE (1978) A new iron–containing superoxide dismutase from *Escherichia coli* . J Biol Chem. 1978 Jul 25; **253**(14): 5220–3.

抄録：*Porphyromonas gingivalis*スーパーオキシドジスムターゼの構造における72位LeuをTrpに置換した影響

小町谷美帆[1*]，溝上真也[1*]，三原正志[2*]，大澤雅樹[2*]，菊池有一郎[3]，上松節子[4]，
平井　要[5]，大久保裕一郎[6]，黒岩昭弘[1]，山田一尋[2]，山倉文幸[7]，平岡行博[6]

[1](松本歯大・歯科補綴学講座)
[2](松本歯大・歯科矯正学講座)
[3](東京歯科大・微生物学講座)
[4](大阪大・歯学部付属病院)
[5](松本歯大・口腔細菌学講座)
[6](松本歯大・総合歯科医学研究所　硬組織疾患制御再建学講座)
[7](順天堂大・医療看護学部　化学教室)

＊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.

歯周病原菌*Porphyromonas gingivalis*にとって，スーパーオキシドジスムターゼ（SOD）は酸化ストレスから菌体を保護するために不可欠の酵素である．原核生物にはマンガンを含む酵素（Mn–SOD）と鉄を含む酵素（Fe–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は何れの金属でも活性を持ち，含有する金属によってそれに応じた化学的性質を示す特徴がある．私達は，このような活性の金属依存性を寛容にしている構造的な特徴を明らかにすべく，活性中心近傍の個々のアミノ酸残基の役割を検討してきた．今回，N末端から72番目のLeu（Leu72）に注目した．Fe–SODにおいて同位置はTrpに置き変わっているため，LeuをTrpに変異させることによってFe–SODに近似した構造になれば，野生型酵素よりも高い活性になる事が期待された．そこで，Leu72をTrpに置換した変異酵素を作製し，性質を検討した.

Kunkelの方法により，*Pg* SODのLeu72（code: CTC）をTrp（code: TGG）にする部位特異的変異を導入した．変異酵素は電気泳動的に野生型酵素と同一の挙動を示し，総体の構造が野生型酵素と同等であると考えられた．一方，変異酵素の活性には殆ど変化がなかった．これらの結果から，Leu72 Trp変異は*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の一つであろうと結論付けた.