Electron Spin Resonance (ESR) Analysis of the Coordination of Nitric Oxide Complex of Iron(II) Myoglobin

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Background and Objective: The ferrous complex of myoglobin containing nitric oxide (NO) is called NO complex of Fe(II) myoglobin (nitrosylmyoglobin). *Staphylococcus xylosus* as starter culture is used for meat products. Although the bacterium contributes to indirect color formation by nitrate reduction or decrease in pH, the direct effect of the bacterium on color formation have not been reported. Our objective was to examine *S. xylosus* for its ability to convert metmyoglobin to a red myoglobin derivative in MRS broth supplemented with metmyoglobin. A model salami without nitrite or nitrate addition was prepared by inoculating *S. xylosus* as starter culture. *S. xylosus* FAX-1 produced nitric oxide (NO) in MRS broth without addition of nitrite and nitrate[1]. The red myoglobin derivative(s) was identified and analyzed by electron spin resonance (ESR) spectroscopy.

Materials and Methods: *S. xylosus* FAX-1 was assessed for its ability to generate a red myoglobin derivative from metmyoglobin in MRS broth[2]. MRS broth supplemented with 2.0 mg/mL metmyoglobin (Sigma) originating from equine heart was designated as MRS-Mb broth. Each sample (0.2 mL culture fluid) was directly transferred to an ESR tube. ESR spectra were recorded on an ESR spectrometer (JES-TE2X, JEOL Co., Ltd.) under the following conditions: microwave power, 4 mW; modulation frequency and width, 100 kHz and 0.5 or 1.0 mT; temperature, 77 K; measurement time, 8 min.

Results and Discussion: *S. xylosus* FAX-1 was capable of converting metmyoglobin to a red myoglobin derivative in MRS broth and the red myoglobin derivative was NO complex of Fe(II) myoglobin. NO complex of Fe(II) myoglobin has two states, namely pentacoordinate and hexacoordinate[3]. Perutz et al.[4] reasonably interpreted that the shift of the Soret band to longer wavelengths that accompanied the axial coordination of a nitrogenous base was the result of a decrease in interaction of the porphyrin a_{2u} orbital with the iron dz^2 orbital, induced by a decrease in Fe-center distance (Fig. 1). From this, the red myoglobin derivative in MRS-Mb culture of FAX-1 was identified as hexacoordinate NO complex of Fe(II) myoglobin. The ESR spectrum (width: 1.0 mT) of pentacoordinate NO complex of Fe(II) myoglobin was considered as a model system [phosphate buffer (pH 4.5) containing metmyoglobin (2.0 mg/mL) , sodium nitrite (0.01%) and sodium ascorbate (0.04%)] whose g factor was around 2 (Fig. 2 c). The ESR spectrum of pentacoordinate NO complex of Fe(II) myoglobin of a different width (0.5 mT) showed three g factors with a rhombic symmetry of g = 2 (Fig. 2 c)[5, 6].

The spectrum of metmyoglobin in MRS broth was determined (Fig. 2 a). A peak can be observed at g ~ 3.5 for metmyoglobin [Fe(III)]. The spectrum of the red myoglobin derivative in MRS-Mb culture of FAX-1 is shown (Fig. 2 b). The conspicuous ^{14}nitrogen hyperfine features at g ~ 2 in the ESR spectrum of Fe (octaethyl-porphyrin dianion) NO indicate a pentacoordinate structure, and a hexacoordinate structure for Fe (octaethyl-porphyrin dianion) $(NO)_2ClO_4$[7]. The ESR spectrum of the red myoglobin derivative in MRS-Mb culture of FAX-1 was similar to that of Fe (octaethyl-porphyrin dianion) NO. Several spectral properties have been used to investigate the coordination state of iron of various hemoprotein complexes with NO at different pH's. When the pH of nitrosylmyoglobin changed from 7.0 to 4.0, it affected the conversion of myoglobin from hexacoordinate to pentacoordinate[8]. Furthermore, Duprat et al.[8] demonstrated that lowered pH produced pentacoordinated NO complex of Fe(II) myoglobin. When FAX-1 was cultured in MRS broth (pH 6.3) for 16 h at 37°C, the pH decreased to 5.8. The MRS-Mb culture was adjusted to pH 4.0 using lactic acid. In a similar way, the pH of the MRS-Mb culture was increased to 7.0 using sodium hydroxide. As shown (Fig. 3 a), the ESR spectrum of MRS-Mb culture at pH 7.0 indicates hexacoordination because of the absence of hyperfine structure around g = 2. When the pH of the MRS-Mb culture was decreased to pH 4.0 with lactic acid, the immediate conversion of hexacoordinate NO complex of Fe(II) myoglobin to pentacoordinate caused the hyperfine structure (Fig. 3 b). The MRS-Mb culture (pH 4.0) was increased once again to pH 7.0 by sodium hydroxide. The ESR spectra recorded immediately prior to and after incubation for 1 h are shown (Fig. 3 c and d), respectively (note the difference in

instrument gain). The intensity of 3-line hyperfine structure of hemoglobin is indicated by ESR spectrum. The ESR spectra indicate complex ESR signals of hexacoordinated and pentacoordinated NO-hemoglobin. The ESR spectra (Fig. 3 c and d) indicated that hexacoordinate and pentacoordinate NO complexes of Fe(II) myoglobin are both present. Recorded after 3 h, the ESR spectrum of the same sample showed a rhombic symmetry of g ~ 2 (Fig. 3 e), and a typical hexacoordinate NO complex of Fe(II) myoglobin identical to that shown (Fig. 2 b). The reversible change of pentacoordinate and hexacoordinate NO complexes of Fe(II) myoglobin indicates that nitrosylprotoporphyrin probably remains in the globin pocket and that it is connected with globin moiety by van der Waals force. In all NO derivatives of myoglobin, hemoglobin, cytochrome *c* peroxidase and cytochrome *c*, the axial ligand (heme fifth ligand) *trans* to the NO group is an imidazole group of histidine residue. Fe(II) in nitrosylheme of hexacoordinate state is located in the porphyrin plane, while the displacements of Fe(II) from the porphyrin plane toward the NO group (Fe-center distance) of pentacoordinate state are 0.2~0.3A (Fig. 1).

Conclusion: With *S. xylosus* FAX-1, metmyoglobin in MRS broth (pH 5.8) was found to undergo conversion to hexacoordinate NO complex of Fe(II) myoglobin. When the pH of the MRS-Mb culture changed from 5.8 to 4.0, it affected the conversion from hexacoordinate to pentacoordinate NO complex of Fe(II) myoglobin (nitrosylmyoglobin formed in cured meat) . This conversion process was reversible.

Pertinent literatures: 1) Morita, H., Sakata, R. and Nagata, Y. 1998. J. Food Sci. in press. 2) Morita, H., Yoshikawa, H. Sakata, R., Nagata, Y. and Tanaka, H. 1997. J. Bacteriol., 179: 7812-7815. 3) Hori, H., Ikeda-Saito, M. and Yonetani, T. 1981. J. Biol. Chem. 256: 7849-7855. 4) Perutz, M.F., Kilmartin, J.V., Nagai, K., Szabo, A. and Simmon, S.R. 1976. Biochemistry 15: 378-387. 5) Kon, H. 1968. J. Biol. Chem. 243: 4350-4357. 6) Pegg, R.B., Shahidi, F., Gogan, N.J. and DeSilva, S.I. 1996. J. Agric. Food Chem. 44: 416-421. 7) Addison, A.W. and Stephanos, J.J. 1986. Biochemistry 25: 4104-4113. 8) Duprat, A.F., Traylor, T.G., Wu, G.-Z., Coletta, M., Sharma, V.S., Walda, K.N. and Magde, D. 1995. Biochemistry 34: 2634-2644.

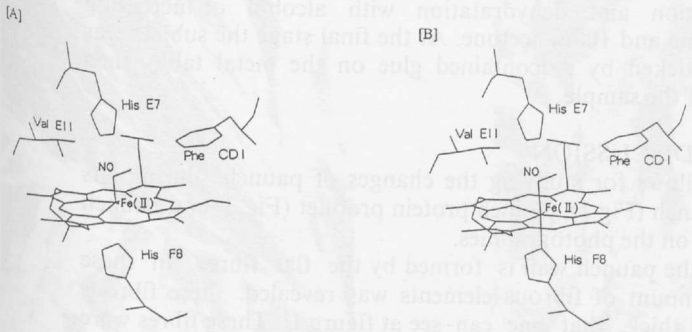

Fig. 1 - Illustrations of pentacoordinate [a] and hexacoordinate [b] NO complex of Fe(II) myoglobin. Nitrosylmyoglobin formed in cured meatis pentacoordinate NO complex of Fe(II) myoglobin.

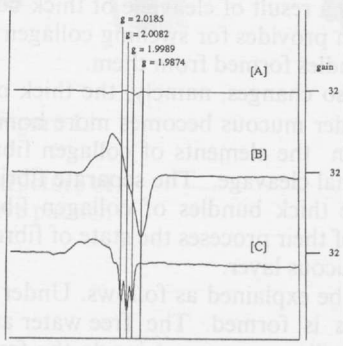

Fig. 2 - Characteristic ESR spectra at 77 K, 1.0 mT and 8 min: [a] metmyoglobin in MRS broth; [b] NO complex of Fe(II) myoglobin converted by *S. xylosus* FAX-1; [c] NO complex of Fe(II) myoglobin formed by reaction of myoglobin with NO from nitrite.

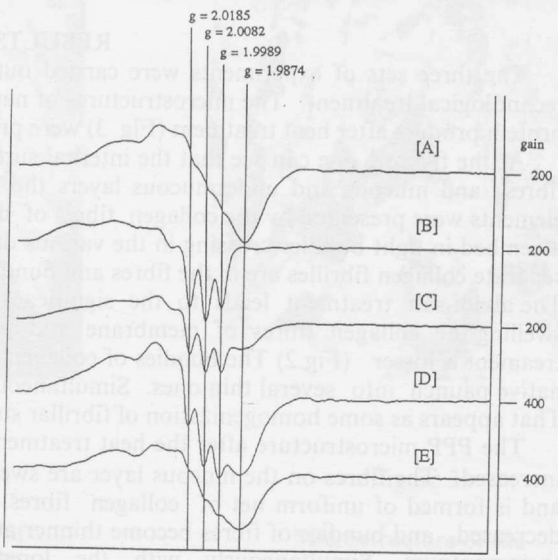

Fig. 3 - Characteristic ESR spectra at 77 K, 1.0 mT and 8 min: [a] pH of MRS-Mb culture increased to 7.0; [b] pH of MRS-Mb culture determined immediately decreased to 4.0; [c] pH of MRS-Mb culture determined after 1 h increased to 7.0 from 4.0; [d] pH of MRS-Mb culture determined after 2 h increased to 7.0 from 4.0; [e] pH of MRS-Mb culture determined after 3 h increased to 7.0 from 4.0.