Inorganica Chimica Acta, 56 (1981) L33–L34

Is Phenolate Oxygen of a Tyrosine Residue the Bridging Ligand at the Binuclear Copper Site in Oxyhemocyanin?

JUN KINO, SHINNICHIRO SUZUKI, WASUKE MORI and AKITSUGU NAKAHARA

Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan

Received April 24, 1981

Recent studies on the coordination geometry and the ligating groups around the two copper ions at the binuclear copper site in hemocyanin have, to some extent, disclosed the structural aspects of the active site of this protein [1, 2]. Thus, two or three imidazole nitrogens of histidine residues are believed to be coordinated around each copper ion, whereas no evidence for ligation of cysteinyl or methionyl sulfur has ever been reported. A pair of five-coordinated copper(II)s bridged by an endogenous ligand has also been proposed for the binuclear copper(II) site in oxyhemocyanin by Solomon *et al.* [3–5], Spiro *et al.* [6, 7], and the present authors [8–10]. As for the bridging ligand, a phenolate oxygen of tyrosine residue has been believed to be most acceptable, although there are some contradictions [11, 12]. In the course of a study of methemocyanin prepared by treating *S. lessoniana* oxyhemocyanin with an excess of thiourea, we found recently a fact which contradicts an implication of the phenolate oxygen-bridged copper(II) cluster. In this communication we describe a concerned spectroscopic study of *Sepioteuthis lessoniana* methemocyanin.

The hemocyanin used in this study was isolated and purified from the hemolymph of squid, *S. lessoniana* by ultracentrifugation and extensive dialysis in 50 m*M* Tris–HCl buffer (pH 8.0) at 4 °C, according to the method described previously [13, 14]. The methemocyanin was prepared by incubation of oxyhemocyanin at 4 °C with 290-fold excess of thiourea for 6 hr in the same buffer [15, 16]. The absorption and CD spectra were recorded at room temperature with a Hitachi 323 spectrophotometer and a JASCO MOE-1 magnetic circular dichroism spectrometer, respectively, and represented in Fig. 1. The ESR spectrum was obtained at 77 K by using a JES-FEIX ESR spectrometer, and shown in Fig. 2.

As is clear from Fig. 2, the methemocyanin obtained on addition of 290-fold excess of thiourea exhibited an extremely weak ESR signal, from which the amount of ESR-active copper(II) was estimated to be *ca.* 4% of the total copper, namely, *ca.* 2% of the total active site in the native hemocyanin. The

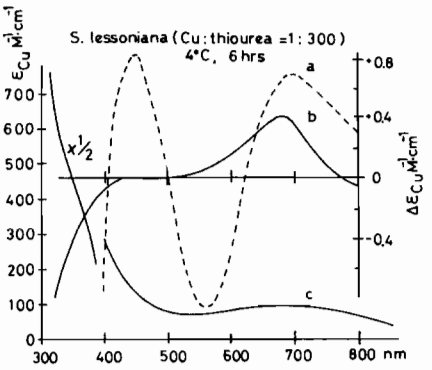

Fig. 1. CD spectrum of squid oxyhemocyanin (a), CD (b) and electronic absorption (c) spectra of oxyhemocyanin treated with thiourea. Condition: hemocyanin, 0.75 m*M*; thiourea, 0.22 M; 50 m*M* Tris-HCl buffer (pH 8.0); room temperature. ϵ_{Cu} and $\Delta\epsilon_{Cu}$ are referred to the mol of Cu(II) ions.

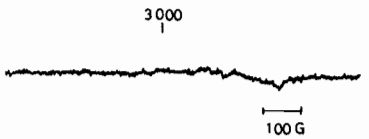

Fig. 2. ESR spectrum of oxyhemocyanin treated with thiourea. Condition: hemocyanin, 0.75 m*M*; 50 m*M* Tris-HCl buffer (pH 8.0); 77 K. Microwave power 5 mW; frequency 9.29 GHz; modulation amplitude 8 gauss; amplitude 1600.

methemocyanin was dialyzed against 2 liters of Tris–HCl buffer solution (pH 8.0) at 4 °C, and resulted in regeneration of *ca.* 35% of oxyhemocyanin. This was evaluated on the basis of CD intensity at 440 nm where oxyhemocyanin exhibited a maximum intensity while methemocyanin gave actually no CD absorption as illustrated in Fig. 1, suggesting the existence of 35% of deoxyhemocyanin in the methemocyanin obtained by the above proceduce. Since the residual species other than the ESR-detectable met-form and the deoxy-form is considered to be ESR-silent met-form, the above described so-called methemocyanin is interpreted as a mixture containing *ca.* 63% of ESR-silent met-form, 35% of deoxy-form and 2% of ESR-detectable met-form. The absorption and CD spectra as illustrated in Fig. 1 actually reflect those of the ESR-silent methemocyanin because the amount of ESR-detectable methemocyanin is negligibly small. The absorption band at 650–700 nm suggests that the coordination geometry around copper(II) is tetragonal, and the weak shoulder at 350 nm is considered to be due to S → Cu(II) charge transfer transition, implying the coordination of thiourea around

Cu(II).* It has been known that coordination of phenolate oxygen around a tetragonal copper(II) gives rise to a charge transfer band at 400–450 nm [17, 18]. In the present study, however, no such band was found in the range of 400–500 nm either in the optical absorption or circular dichroism spectrum as is clear from Fig. 1. This implies that phenolate oxygen of a tyrosine residue is not involved in coordination as the bridging ligand at the binuclear copper site of oxyhemocyanin, contrary to the prospects of many investigators [3–7, 19]. In the light of spectroscopic data on binuclear copper(II) complexes involving alkoxide-bridge (370–440 nm: $p_\pi(O) \rightarrow d(Cu(II))$ charge transfer band) [20], participation of a seryl or threonyl OH as bridging ligand is supposed also unlikely in the same way. Oxide, O^{2-} or hydroxide, OH^- bridge might be the most leading candidate for the bridging ligand at the binuclear copper site of oxyhemocyanin.

Acknowledgement

This work was supported by a Grant-in-Aid for Special Project Research (No. 511311) from the Ministry of Education, Science and Culture of Japan.

*The same kind of charge transfer shoulder ($\epsilon < 1000$) was observed at around 350 nm for Cu(bipyridine)$^{2+}$ upon addition of 10-fold excess thiourea at pH 8.0 in water-methanol (1:1) system.

References

1 H. Beinert, *Coord. Chem. Rev., 33,* 55 (1980).
2 R. Lontie and C. Gielens, in 'Metalloproteins', U. Weser Ed., Georg Thieme Verlag, Stuttgart, New York, (1979) p. 62.
3 N. C. Eickman, R. S. Himmelwright and E. I. Solomon, *Proc. Nat. Acad. Sci. U.S.A., 76,* 2094 (1979).
4 R. S. Himmelwright, N. C. Eichman, C. D. LuBien and E. I. Solomon, *J. Am. Chem. Soc., 102,* 5378 (1980).
5 R. S. Himmelwright, N. C. Eickman, C. D. LuBien, K. Lerch and E. I. Solomon, *J. Am. Chem. Soc., 102,* 7339 (1980).
6 J. M. Brown, L. Powers, B. Kincaid, J. A. Larrabee and T. G. Spiro, *J. Am. Chem. Soc., 102,* 4210 (1980).
7 J. A. Larrabee and T. G. Spiro, *J. Am. Chem. Soc., 102,* 4217 (1980).
8 W. Mori, S. Suzuki, M. Kimura, Y. Sugiura and A. Nakahara, *J. Inorg. Biochem., 13,* 89 (1980).
9 S. Suzuki, W. Mori, J. Kino, Y. Nakao and A. Nakahara, *J. Biochem., 88,* 1207 (1980).
10 A. Nakahara, W. Mori, S. Suzuki and M. Kimura, in 'Biomimetic Chemistry, Advanced in Chemistry Series, No. 191', D. Dolphin, C. Mckenna, Y. Murakami, and I. Tabushi Eds., American Chemical Society, Washington, D. C., (1980) p. 341.
11 E. J. Wood and W. H. Bannister, *Biochim. Biophys. Acta, 154,* 10 (1968).
12 N. Shaklai and E. Daniel, *Biochemistry, 9,* 564 (1970).
13 T. Omura, T. Fujita, F. Yamada and S. Yamamoto, *J. Biochem., 50,* 400 (1961).
14 W. Mori, O. Yamauchi, Y. Nakao and A. Nakahara, *Biochem. Biophys. Res. Commun., 66,* 725 (1975).
15 F. Ghiretti, in 'Physiology & Biochemistry of Haemocyanin', W. A. Rombaus Ed., Academic Press, New York, (1968) p. 75.
16 W. Mori and A. Nakahara, in 'Bioinorganic Chemistry II' (in Japanese) H. Tanaka, A. Nakahara and S. Fukui Eds., Kagaku dozin, Kyoto (1976) p. 107.
17 A. R. Amundsen, J. Whelan, B. Bosnich, *J. Am. Chem. Soc., 99,* 6730 (1977).
18 R. C. Lanoschoot, J. A. M. Vanhest and J. Reedijk, *Inorg. Chim. Acta, 46,* 41 (1980).
19 B. Salvate, P. Zatta, A. Ghiretti-Magaldi and F. Ghiretti, *FEBS Letts., 32,* 35 (1973).
20 Y. Nishida and S. Kida, *J. Inorg. Nucl. Chem., 38,* 451 (1976).