Co(II)-substituted *Octopus vulgaris* Hemocyanin

SHINNICHIRO SUZUKI*, SADAYOSHI HIROSE, SATOSHI SAWADA and AKITSUGU NAKAHARA
Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan
Received May 24, 1985

Abstract

Co(II)-substituted hemocyanin (Co(II)Hc) of the octopus, *Octopus vulgaris*, has been prepared by dialysis of apohemocyanin against Co(II) ion and subsequent Chelex-treatment. The blue 50%-Co(II)Hc (half-apo Co(II)Hc), in which binuclear coppers are replaced in the hemocyanin by a single Co(II), exhibits two absorption maxima at 560 ($\epsilon_{Co} = 250$) and 594 nm ($\epsilon_{Co} = 320$ M^{-1} cm^{-1}) and a shoulder near 610 nm, all of which are attributed to a d–d transition of high spin Co(II) (S = 3/2) with a tetrahedral geometry. The magnetic circular dichroism (MCD) spectrum in this region also suggests the existence of a tetrahedral Co(II) species in the protein. The visible absorption and MCD spectra of octopus 50%-Co(II)Hc are quite similar to those of squid 50%-Co(II)Hc described in the previous paper (S. Suzuki, J. Kino, M. Kimura, W. Mori and A. Nakahara, *Inorg. Chim. Acta, 66,* 41 (1982)). The formation of half-apo Co(II)Hc demonstrates that the binuclear copper sites in native octopus hemocyanin may differ from each other in coordination geometry, as in other molluscan hemocyanins, squid and snail hemocyanins. The coordination environment of the active-site Co(II) substituted for Cu in the octopus hemocyanin is the same as that of the corresponding active site of the squid hemocyanin.

Introduction

Hemocyanins (Hc) in molluscs and arthropods are dioxygen carriers containing two adjacent coppers at the active sites. Recent X-ray crystal analysis of spiny lobster hemocyanin demonstrated that each active-site copper in the presumably deoxy-form has three histidine imidazoles as ligands [1]. However, the geometries of the coupled copper sites are still ambiguous.

Substitution of Co(II) ion for Cu ion in hemocyanins might provide useful information on the structural environment of the copper sites, since Co(II) complexes exhibit characteristic visible absorption and MCD spectra due to the d–d transitions. Cobalt(II)-substituted hemocyanins (Co(II)Hc) have already been prepared from hemocyanins of squid [2], edible snail [3], octopus [4], horseshoe crab [5], and crab [3, 4]. The copper pair at the active site in molluscan hemocyanins was replaced by only one tetrahedral Co(II) ion [2–4].

We report here the preparation and spectroscopic study of 50%, 75%, and 125% Co(II)-substituted *Octopus vulgaris* hemocyanins, in which the extents of incorporation of Co(II) are 50%, 75%, and 125% (excess Co binding), respectively. The 50%-Co(II) hemocyanin (50%-Co(II)Hc) involves one tetrahedral Co(II) ion per coupled active site, as does 50% Co(II)-substituted squid hemocyanin [2]. The three Co(II)-derivatives might disclose the geometries of the other two adjacent active sites.

Experimental

Materials

Oxyhemocyanin (oxyHc) was isolated from octopus hemolymph (*Octopus vulgaris*) and purified by the previous method [2, 5]. OxyHc shows three absorption maxima at 281 ($\epsilon_{Cu} = 31\,700$), 351 ($\epsilon_{Cu} = 8250$), and 583 nm ($\epsilon_{Cu} = 485$ M^{-1} cm^{-1}) in 60 mM tris-HCl buffer (pH 8.0). The purity of oxyHc was examined on the basis of the ratio of the absorption coefficients at 281 and 351 nm ($\epsilon_{281}/\epsilon_{351} = 3.84$). The CD spectrum displays two negative peaks at 348 ($\Delta\epsilon_{Cu} = -11.7$) and 562 nm ($\Delta\epsilon_{Cu} = -0.98$ M^{-1} cm^{-1}), and two positive peaks at 447 ($\Delta\epsilon_{Cu} = 1.11$) and 695 nm ($\Delta\epsilon_{Cu} = 0.81$ M^{-1} cm^{-1}). The ϵ_{Cu} and $\Delta\epsilon_{Cu}$ are expressed per mol of Cu ion. All reagents used were of the highest grade commercially available.

In order to remove Cu from the hemocyanin, dialysis of oxyHc against a tris-HCl buffer containing 30 mM KCN was carried out twice at 4 °C for 4 days. Residual Cu ion in apoHc was about 10% of total copper, as determined by electronic absorption and atomic absorption spectroscopy. The Co(II)Hc was prepared by dialysis of the apoHc (about 5 ml) against 200 ml of 60 mM tris-HCl buffer (pH 8.0) containing 2.5 mM $CoCl_2 \cdot 6H_2O$ (Co: 99.999% purity,

*Author to whom correspondence should be addressed.

0020-1693/85/$3.30

Aldrich Chemical Company, Inc.) under N_2 for 20 h. Dialysis of the resulting Co(II)Hc was continued for 3 days against nine changes (each 200 ml) of tris-HCl buffer. The concentration of Co(II) in the product was determined by atomic absorption spectroscopy. The Co(II) ion incorporated in apoHc was 125% of the total sites for Cu in native Hc, namely 2.5 g atoms of Co(II) per mol of protein (the smallest subunit having a copper pair capable of O_2-binding). This sample is termed 125%-Co(II)Hc. Excess Co(II) in 125%-Co(II)Hc was treated with 100–200 mesh-Chelex 100 resin (about 0.5 g, pre-equilibrated with tris-HCl buffer) at 4 °C for 30 min. The Co(II)Hc containing 1.5 g atoms of Co(II) per mol of protein, 75% Co(II)-substituted hemocyanin (75%-Co(II)Hc), was obtained by the first Chelex-treatment. When the second Chelex-treatment (about 1 g) was carried out with 75%-Co(II)Hc for 30 min, 50%-Co(II)Hc (half-apo Co(II)Hc) containing 1 g atom of Co(II) per mol of protein formed.

Measurements

The absorption and MCD spectra were measured at room temperature with a Union SM 401 spectrometer and a JASCO J-500 A spectropolarimeter attached with an electromagnet (1.2 T), respectively. The MCD spectra were corrected for the base line with a JASCO DP-501 data processor. The concentration of Cu or Co was determined with a Nippon Jarrel Ash AA-1 atomic absorption spectrophotometer.

Results and Discussion

The electronic absorption spectrum of blue 50%-Co(II)Hc is shown in Fig. 1. The visible absorption band (d–d band) of Co(II), with two maxima at 560 (ϵ_{Co} = 250) and 594 nm (ϵ_{Co} = 320 M^{-1} cm^{-1}) and a shoulder near 610 nm, is quite similar to that of 50%-Co(II)Hc derived from squid hemocyanin (*Sepioteuthis lessoniana*) [2]. However, the molar absorbance (ϵ_{Co}) at 594 nm is considerably lower than that of the corresponding band of octopus Co(II)Hc reported by Salvato *et al.* [4]. As the d–d

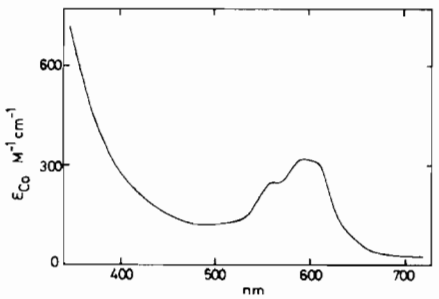

Fig. 1. Visible absorption spectrum of octopus 50%-Co(II)Hc (half-apo Co(II)Hc) at room temperature. The ϵ_{Co} is expressed per mol of Co(II) ion.

bands of tetrahedral Co(II) complexes usually reveal high absorptivities ($\epsilon_{Co} > 300$) [6], the molar absorbance of the d–d band of octopus 50%-Co(II)-Hc strongly suggests that there is a high-spin tetracoordinate (tetrahedral) Co(II) complex in the protein. Figure 2 shows the MCD spectra of 125%-,

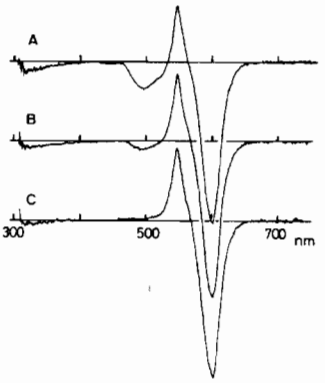

Fig. 2. MCD spectra of (A) 125%-, (B) 75%-, and (C) 50%-Co(II) hemocyanins at room temperature. The intensities ($\Delta\epsilon_m$) expressed per mol of total Co(II) at 547 and 602 nm are (A) 0.16 and −0.46 M^{-1} cm^{-1} T^{-1}, (B) 0.31 and −0.72 M^{-1} cm^{-1} T^{-1}, and (C) 0.52 and −1.1 M^{-1} cm^{-1} T^{-1}, respectively. The $\Delta\epsilon_m$ values at 500 nm in signals A and B are −0.080 and −0.036 M^{-1} cm^{-1} T^{-1}, respectively.

75%-, and 50%-Co(II) hemocyanins. The MCD spectrum of 125%-Co(II)Hc in Fig. 2A presents two negative bands at 500 and 602 nm and a positive band at 547 nm, bearing a strong resemblance to that of squid 130%-Co(II)Hc (excess-Co(II)Hc [2]. The decreasing Co(II)-concentration in the protein (Fig. 2A to Fig. 2C) leads to a significant reduction in the negative band at 500 nm. The 500 nm-band completely disappears in the MCD spectrum of 50%-Co(II)Hc, the visible absorption spectrum of which is shown in Fig. 1. Both the shape and the amplitude of the MCD band in Fig. 2C are analogous to those of the MCD band of squid half-apo Co(II)Hc [2], being indicative of a tetrahedral Co(II) center. Both the absorption spectrum and the MCD spectrum of octopus 50%-Co(II)Hc reveal that the Co(II), possibly located in either of the coupled active sites, is coordinated in a tetrahedral geometry. The site other than the tetrahedral Co(II) site is probably vacant, though the protein contains a small amount of the residual Cu. Consequently, octopus 50%-Co(II)Hc is termed octopus half-apo Co(II)Hc, like squid half-apo Co(II)Hc [2]. In Figs. 2A and B, two strong MCD bands at 547 and 602 nm are also attributable to a tetrahedral Co(II) complex, whereas a weak band at 500 nm is assigned to an octahedral Co(II) chromophore [2, 7]. The octahedral Co(II) species in 125%- and 75%-Co(II) hemocyanins are estimated to be 1.5 g atoms and 0.5 g atom per mol of protein, respectively, because these samples, like half-apo Co(II)Hc, probably involve 1 g atom of a tetrahedral Co(II)

which is apparently difficult to remove by the Chelex-treatment. In order to confirm the above estimation, the intensity of the strongest negative band at 602 nm in Figs. 2A and B was corrected for the estimate of the tetrahedral Co(II)-concentration. The corrected value of $\Delta\epsilon_m$ of 125%- and 75%-Co(II)Hc is -1.1 M^{-1} cm^{-1} T^{-1}, the same as the $\Delta\epsilon_m$ value of half-apo Co(II)Hc in Fig. 2C. Therefore, 125%- and 75%-Co(II)Hc probably involve 1 g atom of the tetrahedral Co(II) ion in the coupled active sites. Nevertheless, we couldn't conclude whether or not the octahedral Co(II) species (0.5 g atom) in 75%-Co(II)Hc exists in the active site coupled with the tetrahedral Co(II) site, since the octahedral Co(II) exhibits a negative 500 nm band quite similar to that of 125%-Co(II)Hc, in which an octahedral Co(II) ion (at least 0.5 g atom) nonspecifically bound to the protein is obviously involved.

In conclusion, Co(II) ion as a probe of the active site in octopus hemocyanin unequivocally demonstrates that the coupled active sites are not geometrically equivalent as in squid [2] and snail [3] hemocyanins. Moreover, the coordination environment of the active site replaced Cu by Co(II) is the same as that of the corresponding site of the squid hemocyanin.

Acknowledgements

We should like to thank Dr. Masayuki Takagi of this College for supply of octopus hemolymph. This work was supported by a Grant-in-Aid for Scientific Research B (No. 58470034) from the Ministry of Education, Science and Culture of Japan.

References

1 W. P. J. Gaykema, W. G. J. Hol, J. M. Vereijken, N. M. Soeter, H. J. Bak and J. J. Beintema, *Nature, 309,* 23 (1984).
2 S. Suzuki, J. Kino, M. Kimura, W. Mori and A. Nakahara, *Inorg. Chim. Acta, 66,* 41 (1982).
3 R. Witters and R. Lontie, *Life Chem. Rep., Suppl. Ser., 1,* 285 (1983).
4 B. Salvato, E. Tealdo, M. Beltramini and J. Peisach, *Life Chem. Rep., Suppl. Ser., 1,* 291 (1983).
5 S. Suzuki, J. Kino and A. Nakahara, *Bull. Chem. Soc. Jpn, 55,* 212 (1982).
6 I. Bertini, in I. Bertini, R. S. Drago and C. Luchinat (eds.), 'The Coordination Chemistry of Metalloenzymes: The Role of Metals in Reactions Involving Water Dioxygen and Related Species', Reidel, Dordrecht, 1983, p. 1.
7 B. L. Vallee and B. Holmquist, in D. W. Darnall and R. G. Wilkins (eds.), 'Methods for Determining Metal Ion Environment in Proteins: Structure and Function of Metalloproteins', Elsevier/North-Holland, New York, 1980, p. 27.