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Cobalt-substituted derivatives of *Carcinus* hemocyanin

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Summary. Four derivatives of *Carcinus maenas* hemocyanin containing Co(II) in the active site have been prepared under different experimental conditions. Two of them contain one Co(II) ion/active site and most probably represent isomeric forms containing Co(II) either in the 'fast-reacting' or in the 'slow-reacting' position within the active site. A third derivative contains two Co(II) ions active site, which reproduces the metal/protein stoichiometry of native hemocyanin. The fourth derivative is a metal hybrid form containing one Cu(I) ion and one Co(II) ion/active site. The derivatives have been characterized by absorption, circular dichroic and fluorescence spectroscopies. The results indicate that in all derivatives the metal is bound with a low coordination number, in agreement with the presence of three histidine residues/copper ion in the native protein. The two alternative metal-binding positions have different structures as shown by the different spectroscopic properties of the bound Co(II) ions. A marked hyperchromic effect on the optical absorption of Co(II) is observed as a result of the presence of a metal ion in the neighbouring metal-binding position in the active site.

Key words: Hemocyanin - Cobalt - Substitution - Active site

Introduction

The isomorphous substitution of the native metal ions in metalloproteins with Co(II) is a useful technique for studying the structure of the metal-binding site, especially in the case of metalloproteins containing more than one metal-binding site. Information on the coordination sphere of the metal site can be obtained from the spectroscopic and magnetic properties of Co(II) derivatives (Bertini 1983). Also, the influence of the protein matrix on the reactivity toward substitution can be probed by comparing the mechanism of metal ion binding. With superoxide dismutase, for instance, the binding of Co(II) to either the Cu(II) or Zn(II) site is strongly pH-dependent, in agreement with charge effects related to the ionization of specific amino acid residues (Calabrese et al. 1979; Salvato et al. 1989). The influence of the protein matrix is particularly marked for those proteins, like hemocyanin (Hc), in which the metal site is not accessible to solvent. In Hcs, the active site consists of a copper pair (type-3 site). Imidazole residues (three/copper ion) serve as metal ligands (Volbeda and Hol 1989). We have recently developed a method for Co(II) substitution employing excess SCN^- in the incubation medium. The anion acts both as a ligand for Co(II) and possibly as a conformational modulator of the protein structure. In this paper we report the preparation and a preliminary spectroscopic characterization of different Co(II) derivatives of *Carcinus maenas* hemocyanin.

Materials and methods

Native and apo *Carcinus* Hc were prepared as described elsewhere (Salvato et al. 1974; Beltramini et al. 1984). Co(II) binding was achieved by incubating apo-Hc in 0.1 M Tris/HCl pH 7.0 containing 10 mM $MgSO_4$ and 0.1% Tween 80. The concentration of Co(II) was 0.5 mM or 4.0 mM and that of SCN^- 50 mM or 250 mM for the mononuclear or binuclear derivative, respectively. The Co(II) derivatives were purified by exhaustive dialysis against 20 mM phosphate pH 7.0 containing 2.0 mM EDTA and then against pure buffer. Protein concentration was determined by the absorbance at 278 nm ($A = 1.24\ \mathrm{ml\ mg^{-1}\ cm^{-1}}$) and on the basis of a molecular mass of 75 kDa for the species containing one active site. Metal concentration was determined by atomic absorption using the standard addition method to correct for protein matrix effects.

Results and discussion

In the presence of 0.5 mM $CoCl_2$ and 50 mM SCN^-, negligible exchange of protein-bound copper with Co(II) occurs. Under the same conditions, apoHc effi-

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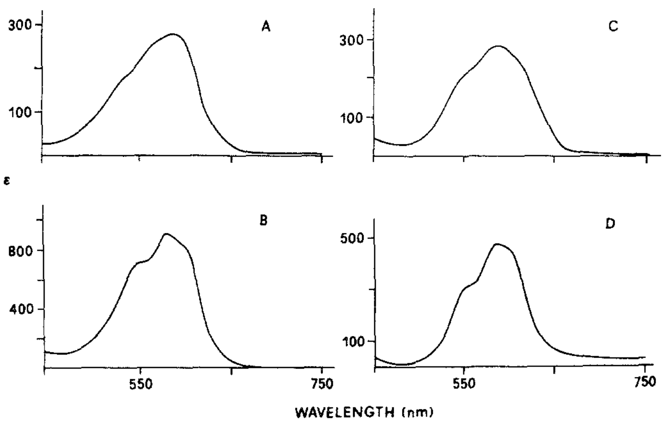

Fig. 1. Cobalt derivatives of *Carcinus* Hc in 50 mM phosphate pH 7.5. Absorption spectra of (**A**) mononuclear derivative prepared by Co(II) addition to apo-Hc; (**B**) binuclear derivative; (**C**) mononuclear Co(II)-Hc prepared by removing one Co(II) ion from the binuclear derivative; (**D**) hybrid copper-Co(II) prepared by metal exchange from native Hc. The molar absorption coefficient is calculated on the basis of a molecular mass of 75 kDa for the species containing one active site

ciently binds Co(II). The kinetics of the process can be described by a single-exponential function with an apparent rate constant of 0.13 h^{-1}. The increase of the Co(II)/Hc ratio as a function of time parallels the increase of absorbance in the spectral region (maximum wavelength 565 mm) corresponding to the d-d absorption of Co(II). The limiting Co(II)/protein stoichiometry, equal to one EDTA-resistant Co(II) ion/active site, is reached after approximately 30 h.

The position of the absorption maximum and the value of the absorption coefficient (Fig. 1A) are indicative of imidazole coordination to Co(II) with a low coordination number (Bertini 1983), in agreement with the presence of three histidine residues/metal site (Volbeda and Hol 1989). A strong ellipticity is exhibited in the visible region by the Co(II)-Hc complex (Fig. 2A). The optical properties of the derivative, together with the resistance of native Hc to the direct substitution, indicate that the metal binding occurs at the active site.

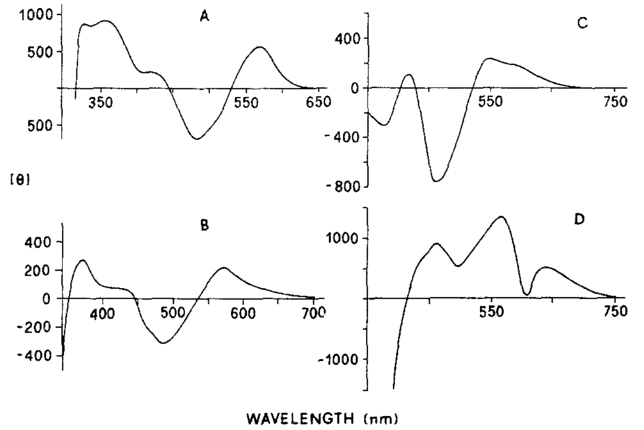

Fig. 2. Circular dichroic spectra of Co(II) derivatives of *Carcinus* Hc. Conditions as in Fig. 1

After long incubation times we observed an increase in the Co(II)/Hc stoichiometry to values above 1, suggesting that the process continues with the binding of additional Co(II) ions. This second process is quite slow and requires 90 h to increase the metal/protein ratio up to 1.2. This additional Co(II) is also resistant to EDTA treatment.

By increasing the SCN^- concentration up to 250 mM and that of Co(II) to 4 mM, apo-Hc yields a derivative containing, after purification, two EDTA-resistant Co(II) ions/active site. The derivative exhibits an absorption spectrum (Fig. 1B) similar to that of the mononuclear derivative. However, small differences in the position of maxima are evident. The molar absorption coefficient, in the order of 900 $M^{-1} cm^{-1}$, indicates that each metal ion is bound in a trigonal- or tetrahedral-like complex. This value is higher than that expected for two Co(II) ions loaded in independent sites, suggesting the existence of an interaction between the two metal ions in the active site. The circular dichroic spectrum of the binuclear derivative (Fig. 2B) is similar to that of mononuclear Co(II)-Hc but the ellipticity is 50% smaller.

The Hc-bound Co(II) is labile to CN^- treatment which first causes a conversion of Co(II) high-spin to low-spin and then removes the metals from the active site (Salvato et al. 1986). This ligand was, therefore, used to remove one Co(II) ion from binuclear Co(II)-Hc under the following conditions: 10 mM CN^- in 20 mM phosphate pH 8.0 containing 50 mM SCN^-. The resulting mononuclear derivative is characterized by the absorption and circular dichroic spectra shown in Figs. 1C and 2C, respectively. The absorption coefficient, as well as the ellipticity, are again typical for a mononuclear Co(II)-Hc derivative, although the position of spectral features is distinctly different from that of the mononuclear derivative prepared from the apoprotein.

The fluorescence quantum yield of the derivatives was also measured since this parameter is correlated with the number of metal ions in the active site. The quantum yield of apo-Hc is quenched upon Co(II) binding. In both the mononuclear and the binuclear derivatives, the quantum yield ratio F/F_0 (F is the quantum yield of the derivative and F_0 that of apo-Hc) is 0.6. This quenching effect decreases by removing one Co(II) ion from the binuclear derivative by CN^-. Taking into account the spectroscopic properties of the derivatives and the sequential character of the preparative reactions, we suggest that the nature of the different substituted derivatives can be interpreted in the framework of the model previously proposed for the Cu-containing Hc species (Beltramini et al. 1984; Salvato and Beltramini 1987; Beltramini et al. 1986). Thus, the mononuclear derivative prepared from apo-Hc binds Co(II) in the 'fast-reacting' or more accessible position within the metal-deprived active site [f-Co(II)-Hc] while in the binuclear derivative the metal occupies both the 'fast-reacting' and the 'slow-reacting' position. By CN^- treatment, the Co(II) ion present in the 'fast-reacting' position is removed so that the remaining me-

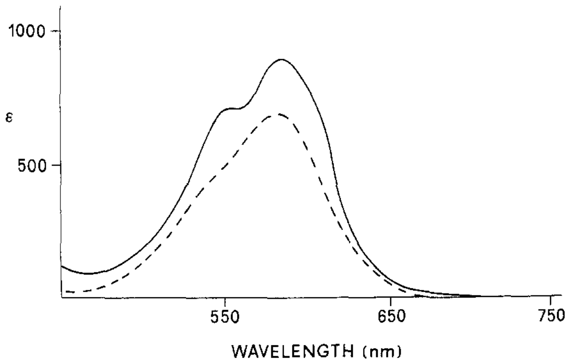

Fig. 3. Comparison of the absorption spectrum of *Carcinus* binuclear Co(II)-Hc (—) with the spectrum resulting by the addition of the spectra of the two mononuclear derivatives in Fig. 1A and 1C (---)

tal ion is present in the 'slow-reacting', less accessible, binding position [s-Co(II)-Hc]. This attribution is in agreement with the observed fluorescence properties of the derivatives since only the metal ion present in the 'fast-reaching' position exerts effects on the emission quantum yield of Hc (Ricchelli et al. 1983; Beltramini et al. 1986). Furthermore, s-Co(II)-Hc is found to be inert toward exogenous ligands which, in contrast, are coordinated by f-Co(II)-Hc and by the binuclear derivative. The two binding positions are not equivalent, not only on the basis of their interaction kinetics with the metal but also from the spectroscopic point of view: the combination of the spectra of the mononuclear derivatives does not simulate spectrum of the binuclear Co(II)-Hc (Fig. 3).

It has been reported that native Hc is capable of exchanging the copper bound in the active site with Cu(I) present in the bulk medium, provided that a suitable mobilizing agent is added to the reaction medium (Cox and Elliott 1974). We have, therefore, used a similar approach to exchange copper with Co(II). In the same incubation medium used for the preparation of the binuclear Co(II) derivative from apo-Hc, the native protein is found to exchange one copper ion with one Co(II) ion/active site. The absorption and circular dichroic spectra of this metal hybrid are shown in Figs. 1D and 2D respectively. The maximum absorption coefficient, in the order of 450 M^{-1} cm^{-1}, is higher than that exhibited by mononuclear Co(II)-Hc. This observation further supports the idea that the spectroscopic properties of bound Co(II) depend on the occupancy of the neighbouring metal-binding position within the binuclear site.

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