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Preparation and Spectroscopic Studies of Cobalt(II)-Stellacyanin

(blue copper protein/metal replacement/electronic spectroscopy/cysteine ligand)

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ABSTRACT The cobalt(II) derivative of the "blue" copper protein stellacyanin has been prepared, and its visible-ultraviolet spectrum is reported. Tryptophan fluorescence quenching and *p*-mercuribenzoate titration results strongly suggest that Co(II) and Cu(II) compete for the same stellacyanin binding site and that a cysteine sulfur atom is coordinated in both cases. This interpretation is supported by the finding of an intense band at 355 nm in Co(II)-stellacyanin attributable to a charge transfer transition of the $RS^- \rightarrow$ Co(II) type. The visible absorption spectrum of Co(II)-stellacyanin exhibits band maxima at 540, 625, and 655 nm. These bands are attributable to *d–d* transitions originating in a high-spin Co(II) center. It is suggested that a correspondence exists between charge transfer bands observed at 355 and 300 nm in the Co(II) derivative to those found at 604 and 450 nm in the native protein. It is concluded that the intense 604-nm peak in Cu(II)-stellacyanin is attributable to a cys-S $\rightarrow$ Cu(II) charge transfer transition.

The intense blue color of laccase, stellacyanin, and many other metalloproteins is associated with a distinctive cupric site, designated Type 1 or "blue." Although many studies of the electronic structure of the Type 1 copper site have appeared (1, 2), the assignment of the intense absorption band at about 600 nm remains uncertain. One hypothesis is that the "blue" site is strongly distorted, giving rise to a *d–d* transition of enhanced intensity by admixture of *p* orbital character into the ground state of Cu(II) (3, 4). Another possibility is that the color of the "blue" proteins arises from a charge transfer band (5). Falk and Reinhammar (6) have recently examined the circular dichroic spectra of several "blue" proteins, concluding that the number of bands observed cannot be satisfactorily explained considering *d–d* transitions alone. Williams (7) has suggested that the intense "blue" copper band may be assigned as charge transfer from an RS^- group to Cu(II) in an approximately tetrahedral coordination environment.

Derivatives containing environmentally sensitive spectroscopic and magnetic probes such as Co(II) and Ni(II) in place of enzymatic zinc atoms have been valuable in elucidating the strength, coordination number, and symmetry of active-site ligand fields in many metalloenzymes (8–10). The metal replacement technique apparently has not been used previously in studying the structure of the "blue" copper site, however. Substitution of Co(II) at a Type 1 copper site should provide an excellent test of the proposed (7) $RS^- \rightarrow$ Cu(II) charge transfer assignment, as $RS^- \rightarrow$ Co(II) and *d–d* bands would be expected to be well separated and therefore susceptible to a reasonably detailed analysis. Here we report the successful replacement of Cu(II) by Co(II) in stellacyanin, a one-copper "blue" protein from the lacquer tree *Rhus vernicifera*.

MATERIALS AND METHODS

Reagent-grade chemicals were used without further purification. Buffer solutions were extracted with dithizone in CCl_4 to remove trace metal contaminants. Glutathione and the sodium salt of *p*-hydroxymercuribenzoate were obtained from Sigma.

Stellacyanin was extracted and purified essentially by the method of Reinhammar (11). Lacquer acetone powder was obtained from Saito and Co., Ltd., Tokyo. Apostellacyanin, prepared by the method of Morpurgo *et al.* (12), was dialyzed for 24 hr against several changes of 25 mM pH 8.1 Tris buffer (pH 8.5; corrected to 5°) after copper removal. A 2-fold excess of $Co(ClO_4)_2$ was then added to prepare Co(II)-stellacyanin [Co(II)St]. Spectral changes accompanying formation of the cobalt derivative were complete within 24–36 hr. All steps in the preparation of Co(II)St were carried out at 5°.

Spectrophotometric titrations at 250 nm with *p*-mercuribenzoate (13) were used to determine the number of reactive sulfhydryl groups in apo- and Co(II)-stellacyanin at pH 8.1. Solutions of *p*-mercuribenzoate were standardized against glutathione. Cobalt concentrations were determined with a Varian Techtron model AH-5 atomic absorption spectrophotometer. Cobalt standards were prepared in the same dithizone-extracted buffer used in spectroscopic determinations. Stellacyanin concentrations were obtained with the 604 and 280 nm extinction coefficients reported by Malmström *et al.* (14).

Absorption and fluorescence spectra were recorded on Cary 17 and Perkin-Elmer MPF-3 spectrophotometers, respectively. Fluorescence spectra of apo- and Co(II)-stellacyanin were measured several hours apart, and a solution of the native protein was run concurrently with each for the purpose of making intensity comparisons. Before spectra were recorded, excess cobalt was removed from Co(II)-stellacyanin samples by stirring them in the presence of Chelex 100 resin (50% by volume, resin pre-equilibrated with metal-free buffer), resulting in a change in pH from 8.1 to 9.5. All pH measurements were performed on a Brinkman pH 101 meter.

RESULTS

Titrations of apostellacyanin with *p*-mercuribenzoate showed that 1.1 ± 0.2 mol of the mercurial are taken up per mol of protein, in agreement with the results of Morpurgo *et al.* (12). No titratable –SH groups were revealed in Co(II)-stellacyanin, and it was found that the spectrum characteristic

Abbreviations: St, stellacyanin.
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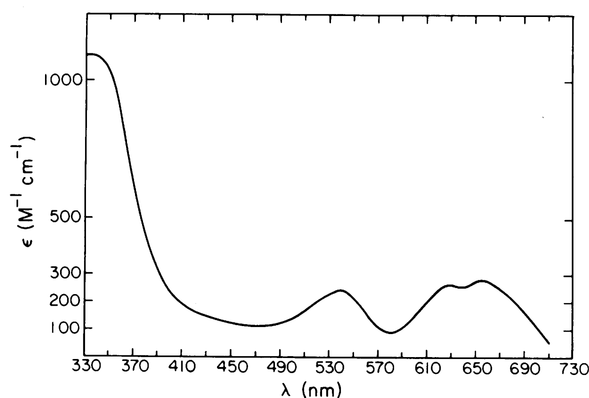

FIG. 1. Electronic absorption spectrum of cobalt(II)-stellacyanin in pH 9.5 Tris buffer at 25°.

of the cobalt derivative does not develop when $Co(ClO_4)_2$ is mixed with apostellacyanin titrated with one equivalent of *p*-mercuribenzoate. Furthermore, the "blue" absorption band does not appear when an excess of $Cu(ClO_4)_2$ is added to Co(II)St. Upon excitation at 280 nm, the fluorescence maxima of apo- and Co(II)- stellacyanin are at 330 and 333 nm, respectively. The relative intensities of the fluorescence maxima were found to be as follows:

$$I_{apo}/I_{native} = 4.7$$

$$I_{native}/I_{Co(II)St} = 1.6$$

Fig. 1 shows the visible absorption spectrum of cobalt(II)-stellacyanin corrected for contributions from residual native protein (about 2%). Resolution of the lowest energy absorption system into peaks at 625 and 655 nm is evident immediately after the cobalt derivative is treated with Chelex 100 resin. A higher energy maximum is observed at 540 nm. Spectra measured several hours after the Chelex 100 procedure show a single low-energy maximum at 640 nm, which has slightly increased intensity in comparison to the better resolved system. A decrease in 350-nm absorbance accompanies the loss of splitting in the 640-nm absorption band. The extinction coefficients given in Fig. 1 should be regarded as accurate only to ± 30%. Uncertainties in correcting for the strong absorption of residual native stellacyanin in the vicinity of 604 nm have prevented a more precise estimation of ε values. The ultraviolet absorption spectrum of the Co(II) derivative has also been examined over the interval 250–400 nm. In addition to the intense protein absorption at 280 nm, there are two other distinct features, a band maximum at 355 nm and a shoulder indicating a peak at about 300 nm.

DISCUSSION

Several results strongly suggest that cobalt(II) and copper(II) compete for the same binding site in stellacyanin: the titratable -SH group in apostellacyanin is no longer accessible in either Cu(II)St or Co(II)St; blocking the sulfhydryl group with *p*-mercuribenzoate prevents Cu(II) or Co(II) uptake; the intense 604-nm absorption band characteristic of native stellacyanin does not appear when Cu(II) is added to Co(II)St, but does appear if no cobalt is present (12); and the tryptophan fluorescence of Cu(II)St and Co(II)St are both partially quenched relative to that of the apoprotein. The mechanism of tryptophan fluorescence quenching in Co(II)- and Cu(II)-stellacyanin is probably related to metal ion binding to a cysteine residue, as Hg^{2+} and Ag^{+} give rise to a similar effect (12). It should be noted, however, that the *p*-mercuribenzoate titration and tryptophan fluorescence quenching results do not require that a sulfhydryl ligand be involved in both cases. A protein conformational change induced by metal binding could cause the titratable sulfhydryl group to become inaccessible to *p*-mercuribenzoate even if it were not a ligand.

The ultraviolet absorption spectrum of Co(II)St provides the strongest evidence for a cys-S to Co(II) linkage. The intense band at 355 nm is attributable to a charge transfer transition of the $RS^- \rightarrow Co(II)$ type, as its position is in excellent accord with similar features at about 350 nm in Co(II)-thiol model complexes (7, 15). The band in Co(II)St at 300 nm most likely represents another charge transfer excitation involving a ligand at the binding site. The visible absorption peaks in Co(II)St are undoubtedly due to *d–d* transitions. The positions and intensities of these bands are suggestive of a distorted tetrahedral, high-spin Co(II) center. The visible spectrum of Co(II)St, for example, is quite similar to that of Co(II)-carbonic anhydrase. Both the spectral and magnetic properties of the latter Co(II) enzyme have been interpreted successfully in terms of distorted tetrahedral coordination (16). The visible spectral differences between distorted tetrahedral and five-coordinate Co(II) centers are very subtle (9), however, and additional experimental information will be required before a firm assignment of the metal ion coordination in Co(II)St can be made.

The visible spectrum of native stellacyanin (14) may now be assigned with reasonable confidence. A roughly linear correlation between charge transfer band position and standard reduction potential is expected for a series of metal ions possessing a given ligand environment (17). Thus, the two charge transfer excitations observed at 355 and 300 nm in Co(II)St should be substantially red-shifted in Cu(II)St. The "blue" band at 604 nm (ε 4080), therefore, most likely represents cys-S → Cu(II) charge transfer, as proposed by Williams (7). The band at 450 nm (ε 960) would then be attributed to the second charge transfer excitation, analogous to the 300-nm band in Co(II)St. The two ultraviolet bands in Co(II)St are separated by 5100 cm^{-1}, which accords well with the 5700 cm^{-1} splitting between the 604- and 450-nm peaks in Cu(II)-St.

The other prominent absorption feature in native stellacyanin is the near-infrared peak at 850 nm (ε 790). An extensive study of Cu(II) electronic spectra has revealed that both distorted tetrahedral and five-coordinate complexes exhibit a *d–d* band with appreciable intensity in the 700 to 900-nm region (18). The distorted tetrahedral cases include Cu(II)-carboxypeptidase A, which absorbs at 795 nm (ε 124) (18). Based on available spectral data, then, assignment of the 850-nm peak in native stellacyanin to a *d–d* transition would appear to be reasonable. The presence of an RS^- ligand could explain the fact that the intensity of the band is somewhat higher than that of, for example, Cu(II)-carboxypeptidase A. Intensification of *d–d* absorption bands in complexes containing heavy donor ligand atoms is commonly observed (19).

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