

Preparation and Spectroscopic Studies of Cobalt(II) Derivatives of Blue Copper Proteins

(metal replacement/electronic spectroscopy/charge transfer transitions)

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Contributed by Harry B. Gray, September 11, 1974

ABSTRACT Preparation of cobalt(II) derivatives of type 1 copper proteins has been extended to include bean plastocyanin and azurin from *Pseudomonas aeruginosa*. Fluorescence quenching data suggest that Co(II) binds to the type 1 site of both apoplastocyanin and apoazurin. Electronic absorption spectral measurements have been made for cobalt(II)-stellacyanin, cobalt(II)-plastocyanin, and cobalt(II)-azurin. In each case two visible bands with moderate intensities and two rather strong ultraviolet absorption peaks are observed. The intensity pattern and the separation of the two ultraviolet bands in the Co(II) derivatives correspond closely to the two intense, low-lying absorption peaks in the analogous, type 1 copper proteins. The evidence suggests that the intense, two-band systems represent ligand-to-metal charge transfer transitions in both Cu(II) and Co(II) derivatives. It is proposed that the transitions in each case originate in the $3p\pi$ and $3p\sigma$ orbitals of a cysteine sulfur ligand at the type 1 site. The visible absorption bands in the Co(II) derivatives are assigned to $d-d$ transitions. The large 4P splitting observed indicates that the type 1 Co(II) site is of low symmetry. The $d-d$ band pattern suggests that the high-spin Co(II) center is either distorted tetrahedral or five-coordinate. The observed band intensities are in much closer accord with the values for Co(II) complexes known to have distorted tetrahedral structures.

Recently we reported the successful preparation of a Co(II) derivative (1) of a blue (type 1) (2) copper protein. The electronic absorption spectrum of this derivative, cobalt(II)-stellacyanin [Co(II)St], reveals that the excited states derived from $d-d$ and charge transfer transitions are well separated (1), quite in contrast to the probable situation in the native protein [Cu(II)St].

Clearly, an opportunity now exists for a systematic study of the Co(II) derivatives of type 1 copper proteins that have different reduction potentials. Analysis of the $d-d$ and charge transfer bands of such Co(II) proteins conceivably could generate a reasonable structural hypothesis for the wide variation in potential of type 1 copper. Further, close comparisons of the electronic spectra of analogous Co(II) and Cu(II) proteins should allow firm assignments to be made for the intense, visible absorption bands in the latter derivatives (2).

This paper is concerned with the preparation and spectroscopic study of two new Co(II) derivatives. The proteins are plastocyanin (Pl) from *Phaseolus vulgaris* and azurin (Az) from *Pseudomonas aeruginosa*. Assignments are proposed for

both the visible and ultraviolet absorption bands in Co(II)St, Co(II)Pl, and Co(II)Az, and a discussion of the probable nature of the low-lying electronic transitions in the native type 1 proteins is presented.

MATERIALS AND METHODS

Plastocyanin was extracted from the leaves of 4- to 6-week-old bean plants and purified by the procedure of Wells (3) to an absorbance ratio A_{278}/A_{597} between 1.1 and 1.2. Stellacyanin was extracted and purified as described earlier (1). Azurin was extracted and purified from *Pseudomonas aeruginosa* (strain no. 10145, American Type Culture Collection) by a standard method (4) to a ratio A_{625}/A_{280} of 0.42.

Sigma Tris·HCl buffer solutions (0.025 M pH 8.0) were used in the preparation of cobalt(II) derivatives and for the spectroscopic measurements. The buffer solutions were eluted through Chelex-100 resin to remove adventitious metal ions. Small volumes of 1000 ppm Varian AA standard solution were used as the source of cobalt(II). All other chemicals used were reagent grade.

Cobalt(II)-stellacyanin was prepared as described previously (1). The cobalt(II) derivative of plastocyanin was prepared anaerobically at 4°C. In a typical preparation, 4 ml of a 0.1 mM solution of Cu(II) Pl was reduced with ascorbate and then dialyzed under nitrogen versus 0.01 M CN^- in 0.025 M pH 8 Tris buffer (pH measured before CN^- addition) in a Minibeaker Fiber dialysis unit obtained from Bio-Rad. The dialysis versus CN^- was continued for about 2 hr, after which the solution was dialyzed 5 hr versus 0.025 M pH 8 Tris. Approximately 50 μ l of 1000 ppm cobalt(II) standard solution was then added. The uptake of cobalt(II) was found to be complete in about 3 hr. Excess cobalt(II) was removed by dialysis for 2-3 hr versus Tris buffer.

Copper was removed from Cu(I) Az [prepared by dithionite reduction of Cu(II)Az] by overnight dialysis versus 0.05 M CN^- in 0.025 M pH 8 Tris. The apoprotein-cyanide solution was then charged into the Minibeaker. Cyanide removal and cobalt(II) incorporation were carried out as described above for Co(II)Pl.

Cobalt concentrations were measured with a Varian Techtron model AH-5 atomic absorption spectrometer. Protein concentrations were estimated from absorbances at 280 nm. Absorption spectra were measured with a Cary 17 spectrophotometer. Fluorescence spectral measurements were made on a Perkin-Elmer MPF-3 instrument. All pH measurements were made on a Brinkman pH 101 meter at 25°C.

Abbreviations: St, stellacyanin; Az, azurin; Pl, plastocyanin; LMCT, ligand-to-metal charge transfer; kK, 10^3 cm $^{-1}$.

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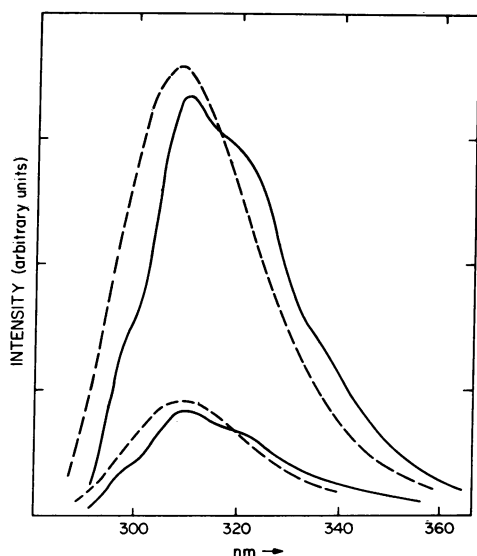


FIG. 1. The fluorescence spectra of azurin (solid line) and plastocyanin (broken line) derivatives: upper curves, apoproteins; lower curves, Co(II) derivatives. Excitation wavelengths: azurin, 270 nm; plastocyanin, 274 nm.

RESULTS AND DISCUSSION

Apoazurin and apoplastocyanin both react readily with aqueous Co(II). The kinetics of formation of these cobalt(II) derivatives have not been studied quantitatively, but we have noted that the rate of reaction in the case of apoplastocyanin increases as the concentration of aqueous Co(II) is raised. It should also be pointed out that, under the conditions employed for Co(II) incorporation, neither Ni(II) nor Fe(II) binds in any specific way to the two apoproteins. Thus, it would appear that type 1 binding sites are somewhat more selective in metal ion replacement reactions than are the Zn(II) ligands in either carbonic anhydrase or carboxypeptidase A (5).

It has been shown previously that the apoderivatives of parsley plastocyanin (6), stellacyanin (7), and azurin (8) fluoresce more strongly than the corresponding native proteins. Partial fluorescence quenching also accompanies Co(II) binding to the three apoproteins under study here (Fig. 1) (1). For apoplastocyanin, we have found that both fluorescence quenching and 330-nm absorption increase linearly with [Co(II)] up to a mole ratio of Co(II) to apoprotein of about 0.85. Both of these spectroscopic measures reach constant levels upon further increases in [Co(II)], corresponding to the binding of one metal ion to each protein molecule.

The spectroscopic titration data for the reaction of apoplastocyanin with Co(II) together with earlier experiments on Co(II)St (1) strongly suggest that binding occurs at the type 1 site. The mechanism of the fluorescence quenching associated with metal ion binding is unknown. It is probably a local effect, however, as sequence studies have shown that aromatic groups are conspicuously present near a cysteine in both azurin and an algal plastocyanin (9). Nonradiative decay of the fluorescent excited states of the protein aromatic groups through the ligand-to-metal charge transfer (LMCT) states (*vide infra*) does not appear to be the dominant mechanism, however, as the quenching efficiency is comparable in both Co(II) and Cu(II) derivatives. The energy match of the LMCT excited states is much closer in the Co(II) case, and as

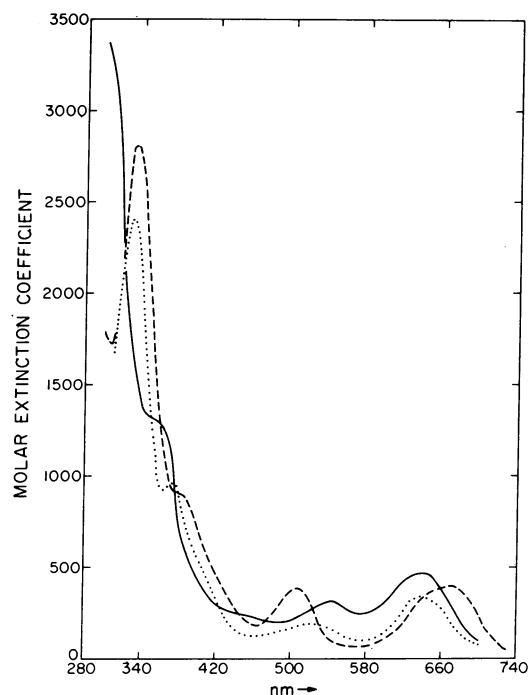


FIG. 2. The electronic absorption spectra of the cobalt(II) derivatives of plastocyanin, stellacyanin, and azurin. The protein concentrations were estimated from A_{280} . Molar extinction coefficients are based on total [Co(II)]. For Co(II)Pl (broken line), [Co(II)] = 0.21 mM and [Pl] = 0.27 mM; for Co(II)St (solid line), [Co(II)] = 0.10 mM and [St] = 0.15 mM; for Co(II)Az (dotted line), [Co(II)] = 0.094 mM, [Az] = 0.12 mM.

a consequence a greatly enhanced probability for nonradiative decay would be expected.

The Co(II) derivatives exhibit two distinct absorption bands in the ultraviolet region (Fig. 2). In each case the more intense band occurs at higher energy. Assignment of these bands to allowed charge transfer transitions may be made with confidence, as no $d-d$ excitations are expected to give rise to such high-energy, intense features. The remarkable correspondence of the separations ($\Delta\bar{\nu}$) of the two bands in the Co(II) derivatives with those between the two most prominent low-energy features in the electronic spectra of type 1 copper proteins strongly suggests that analogous transitions are involved (Table 1). Furthermore, it is highly likely that all the bands under discussion are of the LMCT type, as such transitions would be expected to be significantly red-shifted in the Cu(II) proteins. Generally, a linear correlation (slope about 1) is found between reduction potential and LMCT band position in analogous series of metal complexes (10).

TABLE 1. Comparison of LMCT band positions for type 1 copper proteins and cobalt(II) derivatives

Protein	$\bar{\nu}_{\max}$, kK	$\Delta\bar{\nu}$, kK
Co(II)Pl	26.0, 30.0	4.0
Cu(II)Pl*	12.6, 16.8	4.2
Co(II)Az	26.7, 30.3	3.6
Cu(II)Az*	12.2, 16.0	3.8
Co(II)St	27.4, 32.3	4.9
Cu(II)St*	11.8, 16.6	4.8

* Data from Malkin and Malmström (2).

Thus, the magnitude of the shift could be calculated if the reduction potentials of the Co(II) derivatives were known. With such potentials lacking, all that can be said is that the 13–16 kK (13,000–16,000 cm^{-1}) shift is reasonable for an LMCT assignment. For comparison, the lowest LMCT band in CuBr_4^{2-} falls 18 kK below the corresponding absorption system in CoBr_4^{2-} (11).

Definitive assignments for the two LMCT bands cannot be made until the ligand environment of type 1 copper is known. We tentatively suggest, however, that the transitions in question originate in the occupied $3p\pi$ and $3p\sigma$ orbitals of cysteine sulfur (cys-S^-). There is a substantial amount of evidence that suggests one of the type 1 ligands is cys-S^- (1, 6, 12), and the observed band intensity pattern is readily understood in terms of lower energy π and higher energy σ LMCT components (13–15). In the Cu(II) derivatives, $d-d$ transitions are expected to fall in the same region as the lowest LMCT bands (1). However, the available evidence now indicates that such $d-d$ bands are masked by the more intense LMCT absorptions.

The cobalt(II) proteins reveal two well-separated, somewhat unsymmetrical absorption bands in the visible region (Fig. 2). The weak feature at 410 nm in the spectrum of Co(II)Az is attributable to a trace amount of a cytochrome impurity. It should also be noted that the spectrum of Co(II)St shown in Fig. 2 is of better quality than the one we previously reported at higher pH (1), owing mainly to improved preparative procedures. The molar extinction coefficient of 450 at 640 nm is somewhat larger than our earlier value (1).

Electron spin resonance spectral measurements at 4.2°K confirm that each of the three Co(II) proteins possesses a high-spin ground state (D. R. McMillin and H. B. Gray, unpublished results). Thus, assignment of the visible absorption bands to two or more $d-d$ transitions in a high-spin cobalt(II) center may be made with confidence. Energetic considerations further suggest that all the transitions in question are to levels derived from the 4P (free ion) excited state of Co^{2+} (16).

The observed energies and intensities of the $d-d$ bands rule out octahedral coordination for Co(II) (16). The high degree of splitting of 4P strongly suggests that the Co(II) occupies a binding site of very low symmetry, probably involving a coordination number of four or five. Electronic spectroscopic differences between distorted tetrahedral and five-coordinate high-spin Co(II) complexes are hard to find. Examination of a large selection of high-spin model complexes has revealed that the molar extinction coefficients of the principal $d-d$ bands in five-coordinate cases fall somewhat below typical values for distorted tetrahedral examples (17). Generally, the ϵ values for the $d-d$ bands of five-coordinate, high-spin Co(II) complexes

are smaller than 250 (17), even in cases where thioether and tertiary phosphine ligands are present (18, 19). As all three Co(II) proteins exhibit ϵ values considerably greater than 250, a distorted tetrahedral coordination geometry appears the more attractive of the two models under consideration. In fact, it may be noted that the $d-d$ band patterns of the type 1 Co(II) proteins are similar to those observed for the anion derivatives of Co(II) carbonic anhydrase (20), and it is highly likely that the latter derivatives possess distorted tetrahedral Co(II) centers (17, 20). Particularly striking are the close $d-d$ spectral similarities between the HS^- derivative of Co(II) carbonic anhydrase and Co(II)St, although the latter exhibits somewhat smaller ϵ values. We must emphasize, however, that the observed $d-d$ band patterns of the type 1 Co(II) proteins only require a low binding-site symmetry, and a determination of coordination number cannot be made with confidence until a much larger body of physical data is available for these systems.

We thank the National Science Foundation for support of this research. D.R.M. acknowledges National Institutes of Health Research Fellowship no. 1F02 GM 57661-01. This is Contribution no. 4960 from the Arthur Amos Noyes Laboratory.

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