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Absorption Spectra of d^{10} Metal Ion Derivatives of Plastocyanin

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Received August 8, 1985

Near-UV absorption studies have been carried out for various metal-replaced derivatives of spinach plastocyanin. Above 250 nm plastocyanin derivatives containing Zn(II), Cd(II), or Cu(II), as well as apoplastocyanin, exhibit very similar absorptions due to phenylalanine and tyrosine residues. In contrast, forms containing Hg(II), Cu(I), or Ag(I) show enhanced absorbance in the UV region. In difference spectra with apoplastocyanin as a reference, the Hg(II) derivative exhibits a maximum around 247 nm ($\Delta\epsilon \simeq 9800 \pm 100$ M^{-1} cm^{-1}) and a shoulder at 280 nm ($\Delta\epsilon \simeq 2100$ M^{-1} cm^{-1}) while the Cu(I) analogue has a strong absorbance at 275 nm ($\Delta\epsilon \simeq 3320 \pm 20$ M^{-1} cm^{-1}) and a weaker shoulder at 310 nm. In Ag(I) plastocyanin a band is apparent at 240 nm ($\Delta\epsilon \simeq 5100 \pm 100$ M^{-1} cm^{-1}). The metal dependence of the absorption spectrum suggests that the metal centers are directly involved in the transitions, and literature data support this view. For the Hg(II) derivative the bands can be assigned as S(Cys) → Hg(II) charge-transfer absorptions analogous to transitions found in mercury thioneins. For the Cu(I) and Ag(I) derivatives the bands are plausibly assigned to metal-centered or charge-transfer transitions involving the radial extension of electron density from the *n*d shell. A similar assignment is advanced for bands, previously assigned as ligand-to-metal charge-transfer transitions, in the spectra of copper thioneins. These results show that UV spectra can sometimes be useful in characterizing protein derivatives containing metal ions that, on the basis of visible spectra, have been regarded as spectroscopically "silent".

Introduction

Plastocyanin is a "blue" copper protein that functions as an electron transferase between cytochrome *f* and the reaction center P700 in the chloroplasts of higher plants.[1] Isolated plastocyanin contains a single Cu(II) ion in a distorted tetrahedral binding site formed by donor atoms from the side chains of a cysteine, a methionine, and two histidine residues.[2] Oxidized plastocyanin shows a strong, broad absorption that maximizes at 597 nm ($\epsilon \simeq 4000$–5000 M^{-1} cm^{-1}) and exhibits shoulders around 460 nm and around 730 nm. The intense absorption at 597 nm and the shoulder at 730 nm have been assigned as the σ and π components, respectively, of S(Cys) → Cu(II) charge-transfer excitation.[3-5] The visible and near-infrared spectra of blue copper proteins have been studied in considerable detail, and analogous transitions can be identified from other blue copper proteins, e.g., azurin and stellacyanin.[3-6] The UV spectrum of these same proteins has been less studied, because it is dominated by absorption from aromatic side chains[7] and is less useful in the characterization of the coordination geometry and the donor environment of copper.

On the other hand, the UV spectrum of plastocyanin should not be difficult to analyze because the protein contains no tryptophan and only two or three tyrosine residues. Recently, Draheim et al. studied the UV spectra of the Cu(II) and Cu(I) forms of plastocyanin (hereafter denoted as $Cu^{II}Pl$ and $Cu^{I}Pl$, respectively) as well as the spectrum of apoplastocyanin (apoPl).[8] They found enhanced UV absorbance from $Cu^{I}Pl$ and attributed the absorbance increase to environmental perturbations of the aromatic side chains. There are, however, several difficulties with this interpretation. One is that the crystallographic work has not revealed any significant conformational differences among these forms.[9-11] To overcome this objection, Draheim et al. suggested that the conformational change might be too small to be resolved in the X-ray work or that the absorbance might be associated with dynamic distortions from the average structure observed by crystallographic methods.

An alternative possibility is that the UV spectrum of $Cu^{I}Pl$ contains additional bands, e.g., metal-centered or charge-transfer transitions involving the Cu(I) center. Previously, we have shown that the charge-transfer spectrum of a metalloprotein can be effectively analyzed through systematic spectral studies of metal-substituted derivatives of the protein.[12] Here we present an analysis of the UV spectra of various plastocyanin derivatives containing d^{10} metal ions.

Experimental Section

Materials. Plastocyanin was extracted from spinach leaves and purified by following the procedure of Markley et al.[13] until the absorbance ratio A_{278}/A_{597} was between 1.1 and 1.2. Tris·HCl and phosphate buffer solutions were passed through Chelex 100 resin to remove any adventitious metal ions. For the reconstitution steps, metal ions were added as aliquots of atomic absorption standard solutions. All other chemicals were analytical reagent grade.

Apoplastocyanin and its metal-containing derivatives, e.g., $Zn^{II}Pl$, were prepared anaerobically at 4 °C by following the procedure described by McMillin et al.[3] with minor modifications. In brief, $Cu^{II}Pl$ was reduced by the addition of sodium ascorbate under a nitrogen atmosphere and then dialyzed in a hollow-fiber device for 2 h against 0.025 M Tris buffer (pH 8.05 at 5 °C) containing 0.01 M KCN. The solution was then dialyzed against the Tris buffer for 3 h to remove the excess CN^-. To the apoPl obtained was added 1.0–1.25 equiv of the metal ion, i.e., Zn(II), and the solution was stirred at 4 °C for 2 h to allow for metal uptake. Finally, the excess metals were removed by dialyzing against 0.025 M pH 8.05 Tris·HCl or 0.1 M pH 7.0 phosphate buffer.

Protein concentrations were estimated spectrophotometrically by the biuret method.[14] The concentration of free thiol group was estimated by spectrophotometric methods using 5,5′-dithiobis(2-nitrobenzoic acid).[15] Copper concentrations were determined with biquinoline in a glacial acetic acid medium.[16] Mercury was also estimated spectrophotometrically with dithizone,[17] while Cd, Zn, and Ag were determined by atomic absorption. Within experimental error (ca. 5%), the metal to protein stoichiometry was 1:1. In the case of zinc the agreement was ±10%.

The atomic absorption measurements were obtained with a Perkin-Elmer 2380 spectrophotometer. The electronic absorption spectra were

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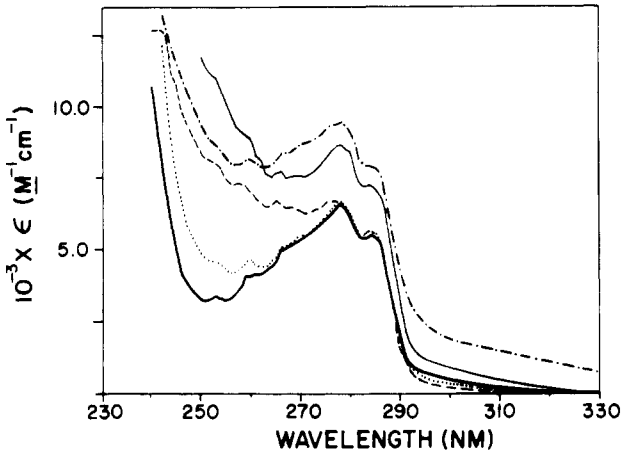

Figure 1. UV absorption spectra of plastocyanin derivatives in 0.025 M pH 7.8 Tris buffer at 20 °C: apoPl (—, thick trace); $Cu^{II}Pl$ (···); $Ag^{I}Pl$ (- - -); $Hg^{II}Pl$ (—, thin trace); $Cu^{I}Pl$ (-·-).

recorded with a Cary 17D spectrophotometer. The difference absorption spectra of $Cu^{II}Pl$, $Cu^{I}Pl$, $Hg^{II}Pl$, and $Ag^{I}Pl$ were obtained vs. an equal concentration of the reference, apoPl. The CD spectra were run in a Cary 60 spectrophotometer in a 0.025 M pH 7.8 Tris-HCl buffer at 20 °C.

Results and Discussion

The Location of Metal Binding. There is good evidence that the various metals each bind at the blue copper site. In the case of Cd(II) the evidence has been previously summarized.[18] In addition, recent NMR experiments have resolved couplings between the ^{113}Cd nucleus and various protons that have been assigned to the four ligands of the blue copper site.[19] Thiol titration experiments are also consistent with metal binding at the blue copper site. Thus, it has already been shown that the binding of Cu(II), Hg(II), and Ag(I) as well as Cd(II) blocks the lone thiol group, which can be titrated in apoPl.[12,18] In this study we have shown that Zn(II) also masks the thiol group.

Competition experiments also strongly suggest that the metal ions bind at the same site in the protein. For some years it has been known that the addition of Ag(I) or Hg(II) causes partial bleaching of $Cu^{II}Pl$ solutions.[20] This suggests that Ag(I) and Hg(II) compete for the copper-binding site, and X-ray studies have confirmed that Cu(II) is replaced by Hg(II).[2] We have carried out further competition studies in 0.02 M pH 7.8 Tris·HCl buffer at 20 °C. The addition of 1–2 equiv of Ag(I) or Hg(II) to $Cu^{II}Pl$ induces bleaching at 600 nm, the reaction being nearly quantitative in the case of Hg(II). Contrariwise, the addition of similar levels of Zn(II) or Cd(II) has no effect on the absorption at 600 nm, and neither Zn(II) nor Cd(II) is taken up. On the other hand, the visible absorption bands of $Cu^{II}Pl$ develop when 1 equiv of Cu(II) is added to a solution of $Zn^{II}Pl$ or $Cd^{II}Pl$, albeit at a much slower rate than when Cu(II) is added to apoPl. Moreover, metal analyses show that loss of Zn(II) or Cd(II) occurs with the uptake of Cu(II). The stoichiometry of metal binding and the displacement studies provide strong support for the hypothesis that the various metal ions favor a common binding site in the protein. It also follows that the binding affinity decreases in the order Hg(II) > Cu(II), Ag(I) > Cd(II), Zn(II).

Silver is unusual in that if excess Ag(I) is added to apoPl or $Cu^{II}Pl$ in Tris-HCl or Tris-HNO_3 buffer, more than 1 equiv of metal is retained in the protein solution even after exhaustive dialysis. This may mean that Ag(I) binds at additional sites, e.g., at surface methionines, or that silver adheres to the protein in some insoluble form. In any case, the blue copper site appears to be the preferred binding site, as the protein's thiol group is completely masked after the addition of 1 equiv of Ag(I) and the UV absorbance is fully developed. This result and a kinetics study of a mercurial reagent reacting with $Cu^{II}Pl$[20] establish that the UV absorbance changes are connected with the binding of specific metal ions in the blue copper site.

Table I. Spectral Data for Plastocyanin Derivatives

protein	ϵ_{278}, M^{-1} cm^{-1}	$\epsilon_{278}/\epsilon_{250}$	protein	ϵ_{278}, M^{-1} cm^{-1}	$\epsilon_{278}/\epsilon_{250}$
apoPl	6500 ± 100	2.1	$Cu^{I}Pl$	9500 ± 150	1.0
$Cu^{II}Pl$	6700 ± 100	1.4	$Hg^{II}Pl$	8700 ± 140	0.6
$Cd^{II}Pl$	6600 ± 100	1.5	$Ag^{I}Pl$	6700 ± 140	0.8
$Zn^{II}Pl$	6600 ± 100	1.8			

Table II. CD Data for Plastocyanin Derivatives

protein	λ_{max}, nm	ellipticity, deg M^{-1}	protein	λ_{max}, nm	ellipticity, deg M^{-1}
apoPl	254	+46	$Cu^{I}Pl$	253	+280
	278	−66		285	−59
$Cu^{II}Pl$	254	+86		315	+36
	278	−53	$Hg^{II}Pl$	249	+580
	305	+33		278	−160
			$Ag^{I}Pl$	260	+53
				278	−66

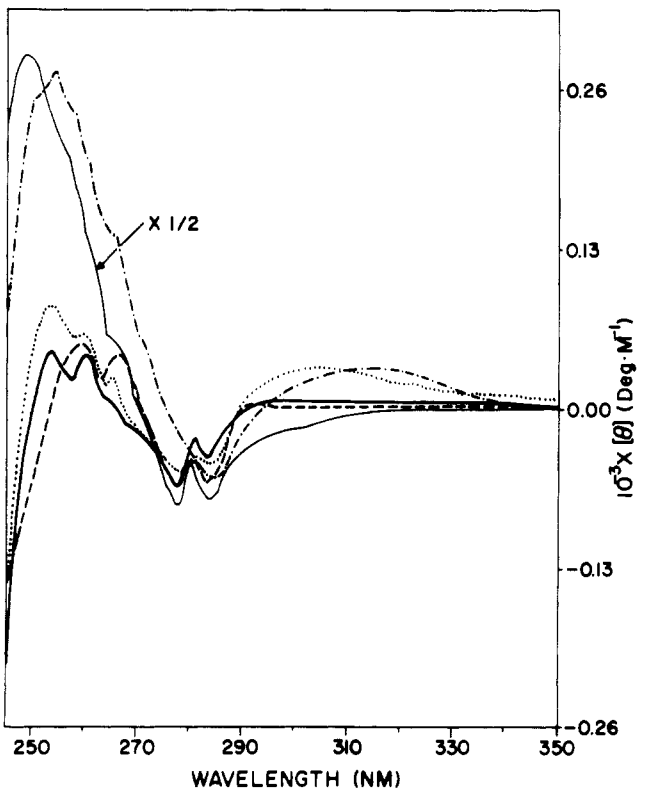

Figure 2. CD spectra in the near-UV region in 0.025 M pH 7.8 Tris buffer at 20 °C: apoPl (—, thick trace); $Cu^{II}Pl$ (···); $Ag^{I}Pl$ (- - -); $Hg^{II}Pl$ (—, thin trace); $Cu^{I}Pl$ (-·-).

Near-UV Absorption Spectra of the Metal Derivatives. The near-UV absorption spectra of $Hg^{II}Pl$ and $Ag^{I}Pl$, as well as those of apoPl, $Cu^{II}Pl$, and $Cu^{I}Pl$, are presented in Figure 1. The spectra of $Zn^{II}Pl$ and $Cd^{II}Pl$ have also been measured but, for the sake of clarity, are not shown. They track almost exactly the spectra of apoPl and $Cu^{II}Pl$, respectively. All of the spectra exhibit a peak around 278 nm and shoulders around 284, 273, 269, 259, and 252 nm.

Compared with the other spectra, the near UV spectra of $Hg^{II}Pl$, $Ag^{I}Pl$, and $Cu^{I}Pl$ exhibit significantly increased absorbance. In particular, significant increases in ϵ_{278}, the molar absorptivity at 278 nm, are observed for $Cu^{I}Pl$ and $Hg^{II}Pl$ (Table I). Even larger increases in absorptivity are observed around 250 nm, and there is a pronounced increase in the ϵ_{250} of $Ag^{I}Pl$ as well (Table I).

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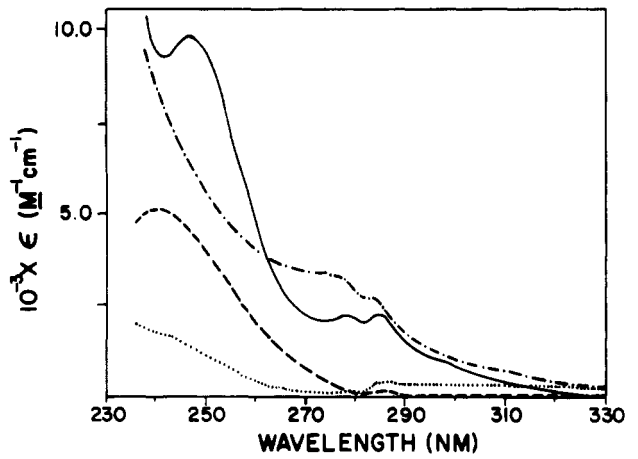

Figure 3. Absorption difference spectra with apoPl as a reference at 20 °C in 0.025 M pH 7.8 Tris buffer: $Ag^{I}Pl$ (--); $Hg^{II}Pl$ (—); $Cu^{I}Pl$ (-·-); $Cu^{II}Pl$ (···).

CD Spectra in the Near-UV Region. Corresponding CD spectra are presented in Figure 2, and selected molar ellipticities are presented in Table II. The spectra are conveniently separated into distinct regions. Above 290 nm $Cu^{II}Pl$ and $Cu^{I}Pl$ each exhibit a broad positive feature while $Hg^{II}Pl$ exhibits a negative shoulder in this region. Between 270 and 290 nm all derivatives exhibit negative ellipticities, and in comparison with apoPl, $\Delta\epsilon_{284}$ is enhanced (more negative) relative to $\Delta\epsilon_{278}$ in the metalated forms. Between 250 and 270 nm all forms show positive ellipticities, and especially pronounced maxima are observed for $Hg^{II}Pl$ and $Cu^{I}Pl$. Finally, below 250 nm the ellipticity of all derivatives decreases toward negative values.

Band Assignments. The absorption bands in the near-UV spectrum of apoPl can be attributed to the aromatic side chains. Spinach plastocyanin contains six phenylalanines and three tyrosines, and by analogy with the UV spectra of the free amino acids,[12] the peak at 278 nm and the shoulder around 284 nm may be assigned to the tyrosine residues while the shoulders below 278 nm may be assigned to the phenylalanines. Below 250 nm, the onset of the $n \rightarrow \pi^*$ transitions of the amide groups tends to cause a sharp increase in the molar absorptivity.[21] As noted earlier, Draheim et al. discussed the enhanced UV absorptivity of $Cu^{I}Pl$ and proposed that a conformational transition attends the reduction of $Cu^{II}Pl$ with the result that aromatic chromophores are left in an altered environment.[8] However, it is most unlikely that such an effect could explain the increased absorptivity in the UV spectrum of $Cu^{I}Pl$. In the first place, the sheer magnitude of the absorbance increase (46% at 278 nm) can hardly be reconciled with an environmental perturbation; complete deprotonation of a tyrosine OH function gives rise to a much smaller effect.[22] Secondly, analogous transitions are observed from the reduced states of other blue copper proteins including azurin and stellacyanin, whose UV spectra are dominated by tryptophan residues.[23] It is also significant that the change in UV absorbance is very sensitive to the nature of the d^{10} metal ion that is present. One could argue that this reflects differences in protein conformation; however, the pattern of UV absorbance changes shows no correlation with the ionic radii (Zn(II) < Cu(I) < Cd(II) < Hg(II) < Ag(I)) or with the apparent binding constants.[24] Moreover, as judged by changes in the tyrosine absorbance around 284 nm, the various metal ions appear to affect the protein conformation in a similar way; vide infra. Finally, as discussed below, additional transitions *are expected* for precisely those d^{10} derivatives that show enhanced UV absorbance.

Table III. Difference Spectra of Plastocyanin Derivatives

protein	λ_{max}, nm	$\Delta\epsilon$, M^{-1} cm^{-1}
$Hg^{II}Pl$	247	9800 ± 100
	280	2130 ± 20
$Cu^{I}Pl$	275	3320 ± 20
	310	710 ± 20
$Ag^{I}Pl$	240	5100 ± 100

On the assumption that the component chromophores are not strongly coupled, the additional transitions are resolved in difference spectra with apoPl as a common reference (Figure 3). Before we discuss the rather broad transitions that are indicated, it can be noted that *in each* difference spectrum, including those obtained with $Zn^{II}Pl$ and $Cd^{II}Pl$, we find a relative minimum around 282 nm and small relative maxima, or small shoulders, around 278 and 285 nm. Without question, these effects are attributable to perturbations of the tyrosine absorbance. Since the modulations are absent when difference spectra are calculated with $Cu^{II}Pl$ as a reference, it follows that the alteration in the environment of one or more of the tyrosines occurs in the apoprotein. Because this effect is a minor one and because any of three different tyrosines could be involved, we will not attempt an interpretation. However, it should be emphasized that no significant difference in the tyrosine environments can be discerned among the various metalated forms.

The difference spectra are most easily analyzed when the metal ions are grouped according to families in the periodic table, and the group IIB (12)[36] metal ions are discussed first. Apart from the onset of a steeply rising absorbance below 250 nm in the case of $Cd^{II}Pl$, the spectra of the Cd(II) and Zn(II) derivatives of plastocyanin are little changed from that of apoPl. In contrast, the $Hg^{II}Pl$ difference spectrum exhibits clearly resolved maxima around 247 and 280 nm (Table III) and tails down to around 320 nm. Corresponding features are observed in the CD spectrum, which reveals a negative tail in the neighborhood of 300 nm, a strongly negative $\Delta\epsilon$ in the region of 280 nm, and a strong positive maximum at 248 nm.

From their study of mercury(II) thionein Vašák et al. have shown that thiolate → Hg(II) charge-transfer bands occur in this spectral region.[25] In the thioneins the metal is believed to be bound by four sulfur donors disposed in a pseudotetrahedral fashion about the metal. Since the blue copper site also contains a thiolate donor and has a pseudotetrahedral geometry, the bands in difference spectrum of $Hg^{II}Pl$ can be assigned in a like manner. More specifically, the transitions might be assigned as the S(Cys) $\sigma \rightarrow$ Hg(II) and S(Cys) $\pi \rightarrow$ Hg(II) components originating from distinct "lone pairs" of the cysteine sulfur in line with bands observed in the spectrum of $Cu^{II}Pl$.[4] On the other hand, the 247-nm transition could correspond to the S π transition, in which case the 280-nm band might be attributed to a transition to a triplet CT state that assumes oscillator strength via a spin/orbit coupling mechanism.[26] The transition(s) associated with the increase in absorbance below 240 nm in the difference spectrum of $Hg^{II}Pl$ is (are) too poorly resolved for assignment.

CT transitions analogous to those of $Hg^{II}Pl$ are not resolved for $Cd^{II}Pl$ and $Zn^{II}Pl$, probably because the bands occur at higher energies. Day and Seal examined the CT spectra of group IIB, halides, and they found that the transition energies increased as the metal was varied in the order Hg(II) << Cd(II) < Zn(II).[26] A similar trend has been observed in the metallothioneins.[25] Due to relativistic effects, the 6s orbital of mercury is very stable,[27]

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and this may explain why its CT transitions occur at relatively low excitation energies.

Although a thiolate → Cu(I) CT assignment has been invoked for bands in the near UV spectra of copper(I) thioneins,[28,30] such transitions must occur at much higher energies for the monovalent ions of group IB (11).[36] The reason is that ligand-to-metal CT energies depend upon the oxidizing character of the metal center, and the electron affinity of Cu^+ is 10 eV less positive than even that of Zn^{2+}. In keeping with the expectation that qualitatively different types of transitions are involved, the $\Delta\epsilon$ values in the CD spectra of $Ag^{I}Pl$ and $Cu^{I}Pl$ are significantly smaller than those in the spectra of $Hg^{II}Pl$.

The bands in the difference spectrum of $Cu^{I}Pl$ could more plausibly be assigned as 3d → 4s (Rydberg) transitions. McClure and others have studied 3d → 4s transitions of Cu(I) centers in sodium halide matrices in great detail, and they find that the transitions occur in the 250–300-nm region of the spectrum.[31,32] Although the absorptivities of these systems are about an order of magnitude smaller than that of the 270-nm transition of $Cu^{I}Pl$, Cu(I) is bound in an octahedral coordination site in the halide lattices. In plastocyanin the site symmetry is much lower, and the LaPorte selection rule will be relaxed. According to this model the bands at 275 and 310 nm in $Cu^{I}Pl$ would be assigned as analogues of the $^1A_1 \rightarrow {}^1E$ and $^1A_1 \rightarrow {}^1T_2$ transitions, respectively, which are associated with the 3d → 4s configurational change in tetrahedral symmetry. Alternatively, the bands might be assigned as charge-transfer-to-solvent (CTTS) transitions. Stevenson and co-workers have proposed that CTTS transitions occur in the near-UV spectra of a variety of Cu(I) complexes.[33] Still another possibility would be to assign the bands as metal-to-ligand charge-transfer transitions terminating in the π^* orbitals of the ligating imidazole groups. Analogous transitions have been assigned in the UV spectra of related pyrazole-containing systems.[34,37]

The latter three possibilities are not necessarily easy to distinguish. Indeed, a Rydberg state may be an intermediate in a CTTS process.[35] Moreover, all three types of transitions involve a radial displacement of electron density away from the Cu(I) center. The fact Ag^+ has a higher ionization energy than Cu^+ may explain why the analogous transitions of $Ag^{I}Pl$ are shifted to higher energies.

A final comment is in order regarding the positive band that occurs in the CD spectrum of $Cu^{II}Pl$ around 305 nm. This is obviously a metal-dependent transition because it is not in the spectrum of apoPl or $Ag^{I}Pl$. In view of the ease of reduction of Cu(II), this transition is quite possibly a charge-transfer transition that originates from a deep-lying molecular orbital mainly located on one or more of the ligand moieties.

Conclusions

This work speaks to the utility of UV studies in characterizing metalloproteins. The identification of S(Cys) → Hg(II) CT charge-transfer transitions in $Hg^{II}Pl$ implies that similar transitions occur in the spectra of the Hg(II) derivatives of other blue copper proteins. When multiple metal-binding sites exist, these bands could be useful in establishing the location of mercury binding. In a like manner the transitions that occur in the UV spectrum of $Cu^{I}Pl$ should be observable from the reduced states of the other blue copper proteins. Pecht and co-workers have, in fact, observed analogous bands in the spectra of reduced azurin and reduced stellacyanin, although specific band assignments were not discussed.[23] Possible assignments are Rydberg transitions (3d → 4s), metal-to-ligand charge-transfer transitions involving the imidazole ligands, and charge-transfer-to-solvent transitions, all of which involve the radial displacement of a 3d electron. Since none of these types of transitions depends upon the presence of a cysteine sulfur and since histidine residues commonly serve as ligands in copper-containing proteins, analogous transitions may be observed in other Cu(I) proteins. Finally, it should be noted that these results do not rule out the possibility that the aromatic residues experience different environments in $Cu^{II}Pl$ and $Cu^{I}Pl$. However, such effects are not responsible for the principal changes in the near-UV absorbance that occur on reduction.

Acknowledgment. This work was supported by Grant No. GM 22764 from the National Institutes of Health.

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