site of azurin is rather rigidly defined by the peptide moiety.

Further structural information has been obtained from NMR studies. Due to the rapid electron spin relaxation associated with tetrahedral nickel(II), we have been able to observe isotropically shifted resonances in the ^{1}H NMR spectrum which can be attributed to protons of the ligand moieties [2]. These resonances shift discontinuously with pH in parallel with the C2 hydrogen of histidine-35, providing direct evidence that this protonation is coupled to a conformational change. ^{113}Cd NMR studies indicate there is little difference between the binding sites of stellacyanin and azurin [3] even though stellacyanin lacks methionine which is one of the copper ligands of azurin. The NMR results are consistent with the suggestion that a closely related donor type, probably cystine sulfur, replaces methionine sulfur in the case of stellacyanin [4].

Finally, metal replacement studies have also proved useful in studies of tree laccase. By substituting mercury(II) for copper(II) in the type 1 site we have for the first time been able to isolate the EPR spectrum of type 2 copper, allowing observation of ligand hyperfine splitting in the perpendicular region [5].

Acknowledgments. This research was supported by NIH grant GM22764. The author would also like to acknowledge the coworkers listed in the references as well as Elizabeth J. Pulliam.

1 H. R. Engeseth, D. R. McMillin and E. L. Ulrich, *Inorg. Chim. Acta, 67,* 145 (1982).
2 J. A. Blaszak, E. L. Ulrich, J. L. Markley and D. R. McMillin, *Biochemistry, 24,* 6253 (1982).
3 H. R. Engeseth, J. D. Otvos and D. R. McMillin, to be submitted.
4 D. L. Tennent and D. R. McMillin, *J. Am. Chem. Soc.,101,* 2307 (1979).
5 M. C. Morris, M. M. Morie-Bebel and D. R. McMillin, to be submitted.

E3

Cryo-Vibrational Spectroscopy of Blue Copper Proteins

WILLIAM H. WOODRUFF* and KENNETH NORTON

Department of Chemistry, University of Texas at Austin, Austin, Tex. 78712, U.S.A.

BASIL I. SWANSON* and HERBERT A. FRY

Los Alamos National Laboratory, Los Alamos, N.M. 87545, U.S.A.

BO G. MALMSTRÖM*

Department of Biochemistry and Biophysics, Chalmers University of Technology, Gothenburg, Sweden

ISRAEL PECHT*

Department of Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel

DAVID F. BLAIR, WILSON CHO, GARY W. CAMPBELL, VANESSA LUM, VINCENT M. MISKOWSKI, SUNNEY I. CHAN* and HARRY B. GRAY*

Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena, Calif. 91125, U.S.A.

Resonance Raman (RR) and Fourier transform infrared (FTIR) spectra at 12 K have been obtained for *Pseudomonas aeruginosa* azurin, spinach plastocyanin, stellacyanin, and tree laccase. The temperature dependence of the azurin, plastocyanin, and stellacyanin spectra have been recorded as have the RR excitation profiles at 12 K. Room temperature RR spectra have been obtained for azurins from *Alcaligenes fecalis, Alcaligenes sp., Bortadella pretussis,* and *Bortadella bronchiseptica*; bean plastocyanin; fungal laccase, human ceruloplasmin; and zucchini squash ascorbate oxidase. Isotope studies employing ^{63}Cu/^{65}Cu and H/D substitution have been performed on the azurins from *Ps. aeruginosa, Alc. fecalis,* and *Alc. sp.* Principal conclusions include the following: The intense RR modes near 400 cm^{-1} include internal ligand deformations and the Cu–S(cys) stretch, rather than the Cu–S(cys) stretch and Cu–N(his-Im) stretches as previously supposed. The Cu–N(his-Im) stretches are assignable to the ubiquitous feature near 265 cm^{-1}, consistent with the frequencies of similar motions in other proteins and in model complexes. Spinach plastocyanin exhibits a frequency shift of 14 cm^{-1} in its cysteine C–S stretching frequency (*ca.* 750 cm^{-1}) upon freezing of the protein solution, suggesting that extra-protein forces (*e.g.*, solvent structure, crystallization, or substrate binding) can influence the conformation of the active site. Above the freezing point of the solvent the plastocyanin RR modes are unusually broad, suggesting either extremely facile dephasing processes, or large inhomogeneous effects due to thermally accessible conformations in the high-temperature form of the active site. No selective enhancement of either the strong or weak RR modes is observed in the S(cys) → Cu charge transfer excitation profiles of azurin, plastocyanin, or stellacyanin at 12 K. The azurin species other than *Ps. aeruginosa* exhibit an 'extra' strong RR peak near 400 cm^{-1} which is, however, seen to be related to an unresolved shoulder in the 12 K *Ps. aeruginosa* spectrum. It is therefore unnecessary to invoke higher coordination numbers than four for copper to explain the RR spectra of the azurins other than *Ps. aeruginosa.* RR peaks appear in the azurin spectrum below 200 K which may be due to methionine C–S stretching modes and Cu–S(met) stretch or methionine C–S–C angle bend. If these low-temperature features are indeed due to such motions, methionine must be

closely coordinated to copper in azurin at low temperature. The RR spectra are consistent with a monotonic relationship between the force constant of the Cu–S(cys) bond and the energy of the ligand-field transitions of the various proteins. The isotope studies lead to tentative identification of the RR modes which contain significant contributions from M–L stretches and internal histidine motions. The results provide an initial basis for reliable structural interpretation of the RR spectra of the blue copper proteins.

E4

Electron Transfer Loci on Blue Copper Proteins

I. PECHT*, A. LICHT, O. FARVER and P. FRANK

Dept. of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

The chemical properties of the Cr(II)/(III) couple have been successfully employed to affinity label electron transfer loci on redox proteins. Being strong reductants and able to exchange their ligands sphere fast, the Cr(II) ions can coordinate one or more of the surface residues on the protein while reducing it. Since the Cr(III) produced is effectively substitution inert, any protein residues in the coordination sphere of the Cr(II) during the electron transfer will remain bound to the Cr(III) product. Identification of the Cr(III) binding loci has been achieved primarily through proteolytic cleavage of the different labeled proteins. Spectroscopic methods have been useful in corroborating these assignments.

Several single blue copper proteins have been examined by the above approach. These include the bacterial electron carriers azurin derived from *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. Plastocyanin (from French bean and poplar tree) which serves as an electron mediator in the photosynthetic apparatus and stellacyanin obtained from the lacquer sap of *Rhus vernicifera* have all been studied by this method. More recently it has been shown that even the multicentered blue copper oxidase–laccase can be reductively labeled by this procedure. In this latter case, while 3.3 equivalents of Cr(II)aq were required for full reduction of the protein, only 0.7 Cr(III) ions remained bound to laccase after extensive dialysis. This may indicate a single reduction locus for Cr(II) in this protein.

The cuprous ions in the Cr(III) labeled plastocyanin, azurin and stellacyanin could be fully reoxidized by inorganic or enzymatic agents. While the original, single Cr(III) ion coordinated to azurin and stellacyanin remains bound through several Cr(II) reduction and reoxidation cycles, one can label plastocyanin with at least two Cr(III) ions in two redox cycles.

In the structures of both plastocyanin and *Ps.* azurin the 'northern' end imidazol of His-87 or 117 respectively was considered to be the potential electron transfer site. The analysis of the Cr(III) labeled sites on these two proteins clearly showed that electrons can also be introduced via different loci. These were proposed to proceed via the His 35 region in azurin (Az) and the negative patch on plastocyanin (Pc).

To examine whether the latter electron transfer sites are also involved in the biochemical function of these proteins, their reactivities, in the native and Cr(III) labeled forms were compared. For Pc, photoreduction and oxidation by chloroplasts and by photosystem I reaction centers respectively were studied. For azurin the reactions with cytochrome c_{551} and *Ps.* cytochrome oxidase were investigated. It became apparent that the Cr(III) label attenuated the reactivity of both azurin and plastocyanin with only one of their respective partners. This led to the conclusions that on both proteins: (a) There are probably two distinct and physiologically operative electron transfer sites. (b) One of these sites is centered around the respective Cr(III) labeled region. (c) By elimination, the second is at the exposed, homologous imidazol of His-87 or 117 in Pc and Az respectively.

E5

Inhibition of Amine Oxidases by Cu(II) Complexes and Anions: Mechanistic Implications

DAVID M. DOOLEY*, C. E. COTE and K. C. GOLNIK

Department of Chemistry, Amherst College, Amherst, Mass. 01002, U.S.A.

Copper-containing amine oxidases catalyze the oxidative deamination of primary amines by the following general mechanism [1]:

$$E + RCH_2NH_2 \rightleftharpoons E \cdot RCH_2NH_2 \xrightarrow{H_2O} E_{red} + RCHO\,(+ NH_3) \quad (1)$$

$$E_{red} + O_2 \rightarrow E + H_2O_2\,(+ NH_3) \quad (2)$$

Our interests center on the role(s) of copper in reactions (1) and (2) and on the activation and utilization of O_2 by these enzymes. An important mechanistic question in this regard is whether the oxidation of E_{red} proceeds via sequential one-electron steps or via a single, two-electron step. One-electron oxidation would generate O_2^- as an intermediate, whereas a two-electron oxidation [2] would not. Accordingly we investigated several Cu(II) complexes, previously shown to be superoxide dismutase active, as potential