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

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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

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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

inhibitors of bovine plasma and pig kidney amine oxidase [3, 4].

Cu(II) complexes of 1,10-phenanthroline (phen) and 2,2'-bipyridine (bipy) strongly inhibited both amine oxidases. Lysine, tyrosine, and salicylate complexes, as well as Cu^{2+}(aq.), inhibited the plasma but not the kidney amine oxidase. No inhibition by the free ligands, by $Cu(EDTA)^{2-}$, or bovine erythrocyte SOD was observed. $Cu(phen)_2(NO_3)_2$, Cu^{2+}(aq.), and $Cu(salicylate)_2$ displayed linear noncompetitive inhibition against amine substrates at saturating O_2 concentrations with either pig kidney or beef plasma amine oxidase. Since neither the free ligands nor Cu^{2+}(aq.) inhibit the kidney enzyme at concentrations where $Cu(phen)_2(NO_3)_2$ and $Cu(bipy)_2Cl_2$ are effective, the observed inhibitory action must be attributed to the complexes. Two plausible inhibition mechanisms are (1) dismutation of O_2^-, which is an intermediate in the reoxidation reaction; or (2) direct oxidation of E_{red} by the Cu(II) complexes. Steric requirements for the second mechanism should be less restrictive than for mechanism (1) as outer-sphere electron transfer is possible over substantial distances, but O_2^- dismutation is an inner-sphere process that requires the Cu(II) complex to penetrate to the O_2 reduction site. Thus if O_2^- is tightly bound within the dioxygen-reduction site, its accessibility to Cu(II) complexes of varying size and charge would be mechanistically important. On this basis the reactivity pattern we have observed can be rationalized: only those complexes with hydrophobic ligands and a net positive charge inhibited pig kidney amine oxidase, but the active site in the plasma enzyme may be much more accessible to external reagents.

N_3^- and SCN^- are Cu(II)-specific inhibitors of several amine oxidases [1, 5–7]. Ligand-to-metal charge-transfer bands are evident at ~365 nm ($\epsilon \sim$ 3,000 M^{-1} cm^{-1}) and ~400 nm ($\epsilon \sim 6{,}000$ M^{-1} cm^{-1}) when SCN^- and N_3^-, respectively, are added to beef plasma or pig kidney amine oxidase. The intensities and energies establish that the anions are equitorially coordinated. Changes in the Cu(II) ligand field, as judged by absorption, circular dichroism and EPR spectroscopy, are consistent with a simple ligand substitution reaction. Preliminary NMR evidence indicates that H_2O is the leaving group, as previously demonstrated for the pig plasma enzyme [6]. Depending on the amine concentration both anions are linear noncompetitive or linear uncompetitive inhibitors of beef plasma amine oxidase with O_2 saturating; under the same conditions these anions were strictly linear uncompetitive inhibitors of the pig kidney enzyme. By determining in detail the inhibition mechanism of anions and the Cu(II) complexes, we should be able to learn much about the role(s) of copper in the amine oxidases.

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E6

Activation of Dioxygen by Electron-transfer Reduction in the Presence of Protons or Transition Metal Complexes

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Electrochemical studies of molecular oxygen in aprotic and aqueous media confirm that the primary electron-transfer step for the reduction of O_2 is a one-electron process, which is followed by chemical disproportionation reactions to yield H_2O_2 under acidic conditions (an overall two-electron process) and OH^- under basic conditions (an overall four-electron process) [1]. Spectroscopic, electrochemical, and kinetic measurements have been used to characterize the reactions of O_2^-, $HO_2^{\bullet}$, H_2O_2, and HO_2^- in aprotic solvents. Superoxide ion (O_2^-) is a strong Bronsted base (*e.g.*, deprotonates butanol and water), an effective nucleophile (*e.g.*, reacts rapidly with alkyl halides and esters by an S_N2 mechanism), and a moderate one-electron reducing agent [2]. It rapidly degrades polyhalogenated hydrocarbons (CCl_4, $HCCl_3$, and DDT) to oxygenated products [3]. For substrates with oxidizable hydrogen atoms (hydrazines, hydroxylamines, and reduced flavins), O_2^- is an effective one-electron oxidizing agent [4]. Superoxide also readily oxygenates benzyl and dehydroascorbic acid [5], and couples with reduced paraquat to yield an oxygenating reagent [6]. Hence, a primary route for the biochemical activation of $^3O_2$ to an effective electron acceptor, oxidase, monooxygenase, and dioxygenase is an initial reduction to superoxide ion.

Reduction of O_2 in the presence of protons and transition metal ions yields highly reactive perhydroxyl and peroxide intermediates [7, 8].

$$O_2 + H^+ + e^- \xrightarrow[DMF]{+0.12\ V\ vs\ NHE} HO_2^{\bullet} \rightarrow 1/2\ H_2O_2 + 1/2\ O_2 \quad (1)$$