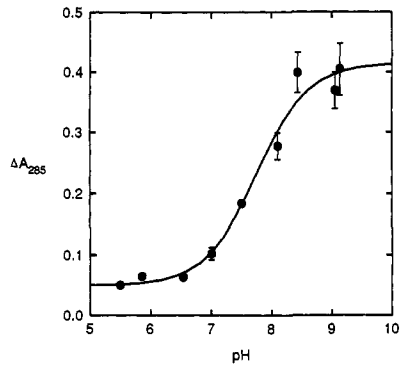

Figure 2. Dependence of ac_4rfH_2-sensitized DMUD splitting efficiency on pH, as measured by the increase in absorbance at 285 nm after a 3-min irradiation at 436 nm in phosphate- or borate-buffered (0.03 M), argon-purged (Oxiclear filter for trace O_2 removal) aqueous solution. The concentration of ac_4rfH_2 was 12 μM. Points are averages of at least three determinations ±SD. The curve was generated by fitting a titration expression $[\Delta A_{min}/(1 + 10^{pH-pK_a}) + \Delta A_{max}/(1 + 10^{pK_a-pH})]$ to the data. The values of ΔA_{min} and ΔA_{max} were thereby found to be 0.049 ± 0.015 and 0.41 ± 0.02, respectively.

$DMUD^{\bullet-}$ to produce DMU and $DMU^{\bullet-}$. Chain propagation occurs by electron transfer from $DMU^{\bullet-}$ to a neutral DMUD. The newly formed $DMUD^{\bullet-}$ can subsequently split. Termination steps that compete with propagation are loss of an electron by either the monomer radical anion or dimer radical anion (presumably to the flavin radical $ac_4rfH^{\bullet}$). This mechanism parallels one we recently proposed for a possible chain reaction in dimer radical cation splitting initiated by a protonated, oxidized flavin.[3k] It differs, however, from a radical cation chain reaction[3l] in which the propagating species was the sensitizer radical cation and sensitization plots were linear in $[dimer]^{-1}$.

The high splitting efficiency of DMUD[11] made possible the determination of a pH profile for the reaction. When photolysis of 1.2×10^{-5} M flavin and 3.4 mM DMUD in solutions ranging from pH 5.5 to 9.1 was carried out for 3 min, a very clear result emerged (Figure 2). Splitting efficiency followed a titration curve centered at pH 7.7. Under these conditions, flavin anion sensitized splitting was approximately 8-fold greater than neutral flavin sensitized splitting (i.e., $\Delta A_{max} = 0.41 \pm 0.02$ and $\Delta A_{min} = 0.049 \pm 0.015$; Figure 2). Possible explanations for this are inefficient electron transfer from ac_4rfH_2 ($pK_a \sim 7$)[12] and/or faster back electron transfer to the protonated radical in the geminate pair $\{dimer^{\bullet-}\ ac_4rfH_2^{\bullet+}\}$. It is also possible that one or more steps subsequent to initiation of the chain reaction are sensitive to pH and this contributes to the observed pH profile (e.g., less efficient chain termination by $ac_4rf^{\bullet-}$; $pK_a \sim 8$[12b] for $ac_4rfH^{\bullet}$).[10]

Previously, photosensitized dimer splitting by oxidized[4,13] and reduced flavins[4] has been carried out in alkaline solution. In the case of oxidized flavins, this was a consequence of the need to form the deprotonated dimer.[14] In the present study, deprotonation of the reduced flavin is apparently necessary. It is implausible that the reduced flavin monoanion would be more efficient at abstracting an electron from the dimer than would the neutral reduced flavin. Thus, the findings reported here virtually rule out electron abstraction from dimers by reduced flavins in aqueous solutions. The active sites of photolyases are hydrophobic,[15] however, and there can be significant solvent polarity effects on dimer radical anion splitting efficiency.[3a] Therefore, whether photolyases employ $FADH_2$ in its deprotonated form ($FADH^-$)[5b] requires further studies of the natural system.

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Redesign of a Type 2 into a Type 1 Copper Protein: Construction and Characterization of Yeast Copper-Zinc Superoxide Dismutase Mutants

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The essential features of type 1 copper proteins appear to be two histidine imidazoles and a cysteine thiolate coordinated to Cu^{2+} in a trigonal planar geometry with one or two additional weak axial ligands.[1] The type 2 copper protein copper-zinc superoxide disumtase, CuZnSOD, contains a copper binding site (four histidines) and a zinc binding site (three histidines and an aspartate) in each of its two identical subunits, and Cu^{2+} can be bound to either or both sites.[2,3] We reasoned that substitution of histidine residues by cysteine in either site would give us new types of metal-binding sites containing histidines and cysteines and that the properties of Cu^{2+} bound to these sites would make an interesting comparison with the properties of the natural type 1 copper proteins.[4-7]

We prepared five single histidine-to-cysteine mutants using oligonucleotide-directed mutagenesis on the cloned CuZnSOD gene from *Saccharomyces cerevisiae*.[8] The mutant genes were expressed in *Escherichia coli* in the T7 RNA polymerase expression system,[9] and the mutant proteins were purified to homogeneity.[10] We describe here our characterization of the most stable copper site mutant, H46C (His 46 to Cys 46), and the most stable zinc site mutant, H80C (His 80 to Cys 80).[11] Apoprotein was prepared using procedures reported previously for the bovine protein,[12] except that 0.25 mM dithiothreitol was added to the

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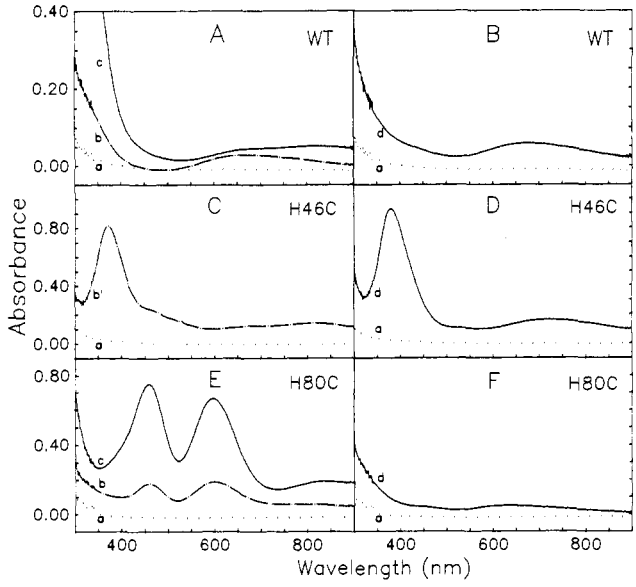

Figure 1. Electronic absorption spectra of Cu_2Cu_2 and Cu_2Zn_2 derivatives of recombinant SOD wild type (WT) from *S. cerevisiae* and single His to Cys mutants (copper site, H46C, and zinc site, H80C) at room temperature, in 100 mM sodium acetate buffer, pH 5.5, referenced against the same buffer. Subunit concentrations of the protein samples were WT, 0.40 mM; H46C, 0.48 mM; H80C, 0.42 mM. a: Apoprotein b: Apoprotein plus 1.0 equiv of Cu^{2+} per subunit b′: Apoprotein plus 0.8 equiv of Cu^{2+} per subunit c: Apoprotein plus 2.0 equiv of Cu^{2+} per subunit d: Apoprotein plus 1.0 equiv of Zn^{2+} and 1.0 equiv Cu^{2+} per subunit. (When >0.8 equiv of Cu^{2+} is added to apo-H46C under these conditions, the intensity of the 371-nm band is observed to diminish.[11] When 1.0 equiv of Zn^{2+} is added first, the full 1.0 equiv of Cu^{2+} may then be added without loss of intensity of the 371-nm band.)

dialysis buffer as an antioxidant and removed by dialysis in the final step. Residual copper was <1% on the basis of atomic absorption.

The visible spectral changes associated with addition of Cu^{2+} alone or Zn^{2+} followed by Cu^{2+} to the recombinant wild type yeast apo-CuZnSOD are very similar to those observed previously for bovine CuZnSOD.[13] In Figure 1 Ab, Cu^{2+} was bound to the copper site only, giving a broad absorption due to a d–d transition around 650 nm. In Figure 1Ac, both sites contained Cu^{2+}, and the spectrum corresponds to the superposition of the transition at 650 nm, due to Cu^{2+} in the distorted tetragonal copper site, and a new d–d transition around 820 nm, due to Cu^{2+} in the distorted tetrahedral zinc site. When Zn^{2+} was added prior to Cu^{2+} (Figure 1Bd), the zinc site was blocked and Cu^{2+} was bound only to the copper site. Thus only the bands due to Cu^{2+} in the copper site (650 nm) and the imidazolate-to-copper charge-transfer transitions (shoulder at ~450 nm) are seen.[13]

Titrations of the copper site mutant apo-H46C with Cu^{2+} alone or Zn^{2+} followed by Cu^{2+} resulted in spectroscopic changes in the d–d region (Figures 1Cb′ and 1Dd) that were similar to those seen for the wild type apoprotein. The resemblance of the d–d regions of the visible absorption spectra of wild type and the H46C mutant for both the Cu_2Zn_2 and copper-substituted derivatives suggests that the geometries of the copper and zinc sites are not greatly changed by the substitution of cysteine for histidine 46 in the copper site.

The very dramatic difference between the spectra of the wild type derivatives and those of the H46C mutant, however, is the appearance of a new, strong absorption band at 371 nm ($\epsilon \geq 2100$ M^{-1} cm^{-1}) for the Cu_2Cu_2 derivative (Figure 1Cb′) and at 379 nm ($\epsilon \geq 1900$ M^{-1} cm^{-1}) for the Cu_2Zn_2 derivative (Figure 1 Dd), which was assigned to a sulfur-to-copper charge-transfer transition. The energy of this transition is much higher than that of type 1 copper proteins, which is typically observed at ~600 nm. Its

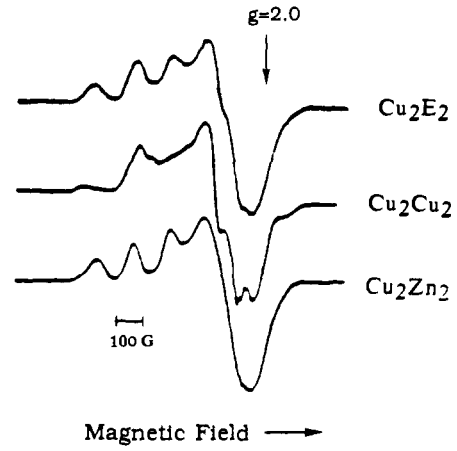

Figure 2. X-band electron paramagnetic resonance (EPR) spectra of Cu_2E_2, Cu_2Cu_2, and Cu_2Zn_2 derivatives of the zinc site mutant H80C, in 100 mM sodium acetate buffer, pH 5.5 at 90 K. Protein sample concentrations in subunit: Cu_2E_2 and Cu_2Cu_2 derivatives, 0.38 mM; Cu_2Zn_2 derivative, 0.49 mM. Instrument settings: microwave frequency, 9.50 GHz; microwave power, 20 mW.

position is consistent with our expectations, since four-coordinate tetragonal and five-coordinate copper(II)–thiolate and –mercaptide model complexes typically display relatively high energy charge-transfer transitions.[14-16] The only known Cu(II)–thiolate model complexes that have low-energy S-to-Cu(II) charge-transfer transitions similar to those of the type 1 proteins are tetrahedral or nearly so.[16,17]

By contrast, addition of Cu^{2+} to the zinc site mutant apo-H80C resulted in the appearance of two strong absorption bands at considerably lower energy, i.e., at 458 nm ($\epsilon \geq 1800$ M^{-1} cm^{-1}) and at 597 nm ($\epsilon \geq 1600$ M^{-1} cm^{-1}) (Figure 1Ec). Addition of Zn^{2+} prior to addition of Cu^{2+} effectively blocked the formation of these new strong bands (Figure 1Fd).

The visible spectra of most type 1 copper proteins are dominated by an intense band at 600–625 nm,[4,5,18] but in certain cases an additional band is observed at ~450 nm. A particularly striking example of the latter case is the type 1 center in nitrite reductase (λ_{max} = 450, 585 nm),[19] whose X-ray crystal structure at 2.3 Å recently became available.[20] Our H80C-Cu_2Cu_2SOD mutant also gives two intense bands in the visible region (Figure 1Ec). Gerwirth and Solomon[6] have recently assigned the intense band at 600 nm of the type 1 copper protein plastocyanin as a cysteine S $p\pi$-to-Cu(II) charge-transfer transition and have suggested that charge-transfer transitions from cysteine S pseudo-σ and σ orbitals to Cu(II) must lie among the weaker bands that are at higher energy than the 600-nm band. Although detailed assignment of the electronic spectrum of our protein must await further experiments, we have observed, using resonance Raman spectroscopy (data not shown), that Cu–S vibrations typical of type 1 copper are enhanced when either the 458- or 597-nm bands are irradiated, indicating that both bands have S-to-Cu(II) charge-transfer character.[21]

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The EPR spectrum of H46C-Cu_2Zn_2SOD ($g_\perp$ = 2.05, $g_\parallel$ = 2.23, $A_\parallel$ = 144 G) resembles that of wild type Cu_2Zn_2SOD ($g_\parallel$ = 2.09, $g_\parallel$ = 2.26, $A_\parallel$ = 132 G), i.e., it is typical of a type 2 copper protein. This result demonstrates that the presence of a thiolate ligand is not sufficient in itself to produce the unusual EPR spectrum with small parallel hyperfine coupling constant characteristic of type 1 copper proteins. The EPR spectra of H80C-Cu_2E_2SOD ($g_\perp$ = 2.08, $g_\parallel$ = 2.26, $A_\parallel$ = 130 G) and H80C-Cu_2Zn_2SOD ($g_\perp$ = 2.07, $g_\parallel$ = 2.27, $A_\parallel$ = 134 G) are virtually identical to those of the corresponding wild type derivatives (wild type Cu_2E_2SOD: $g_\perp$ = 2.06, $g_\parallel$ = 2.26, $A_\parallel$ = 144 G), indicating that the mutation in the zinc site had little effect on the nature of the copper site (see Figure 2). By contrast, the EPR spectrum of H80C-Cu_2Cu_2SOD (Figure 2) appears to be the sum of the spectra of a type 2 Cu(II) site, similar to that of H80C-Cu_2E_2SOD and H80C-Cu_2Zn_2SOD, and a stellacyanin-like type 1 Cu(II) site.[22,23]

In conclusion, we have shown that substitution of a cysteine in place of a histidine ligand in either the copper or zinc sites of CuZnSOD can be used to prepare new, relatively stable copper–cysteinate proteins. Future studies of these and related mutant proteins will focus on their structural, spectroscopic, and electrochemical characteristics as well as their reactivities in electron-transfer reactions.

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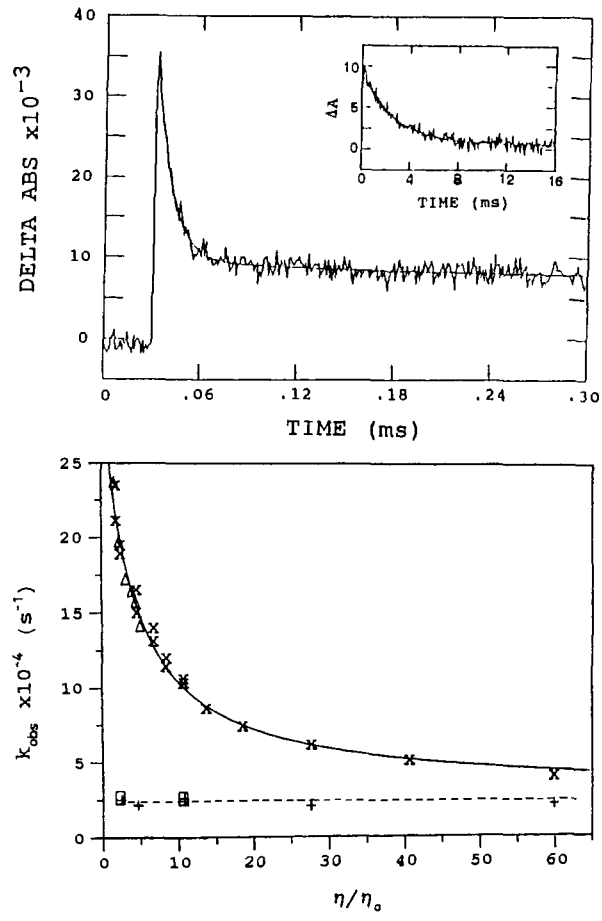

Figure 1. (Upper) Decay of ^{3}Zncyt, monitored at 460 nm, in a solution containing 10.0 μM Zncyt and 20.0 μM pc(II) in ca. 2.5 mM phosphate buffer at pH 7.0 that contains 60% by weight glycerol, at 25 °C. The solid line is a biexponential fit. Inset: The same, over longer time, to show recovery of the ground state. (Lower) Unimolecular quenching of the triplet state within the noncovalent (curved points) and covalent (horizontal points) ^{3}Zncyt/pc(II) complexes, at 25 °C, in 2.5 μM phosphate buffer at pH 7.0 whose relative viscosity was adjusted with glycerol (×) or with ethylene glycol (Δ) for the noncovalent complex and with glycerol for the covalent complex. Concentrations: 10.0 μM Zncyt and 20.0 μM pc(II) for the noncovalent complex; 10.0 μM *N*-acylurea derivatives, chromatographic fractions 1 (□) and 5 (+),[22] for the covalent complex. The solid line is the best fit to eqs 4 and 5, and the dashed line is the best linear fit.

Photoinduced Electron Transfer from Zinc Cytochrome *c* to Plastocyanin Is Gated by Surface Diffusion within the Metalloprotein Complex

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Electron-transfer reactions between metalloproteins have been vigorously studied.[1-4] Their rates and specificity depend on the transfer paths and on protein–protein orientations. Both paths and orientations are modulated by dynamical processes. A pair of metalloproteins can form multiple complexes, and an orientation that is optimal for recognition and binding need not be optimal for reaction. Experimental[5-8] and theoretical[9,10] studies of gating, electron transfer controlled by structural change, have begun. We report here that electron transfer in the noncovalent complex ^{3}Zncyt/pc(II)[11] is gated and determine the rate constant for the controlling process, surface diffusion, by which the associated proteins rearrange from the orientation optimal for binding to the one optimal for reaction.

Cytochrome *c*[12] ($E°$ = 0.26 V vs NHE) binds to plastocyanin[13] ($E°$ = 0.36 V) so that the positive patch around the exposed heme edge abuts an area within the broad negative patch remote from the copper atom.[14,15] This configuration is noninvasively rein-

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