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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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-

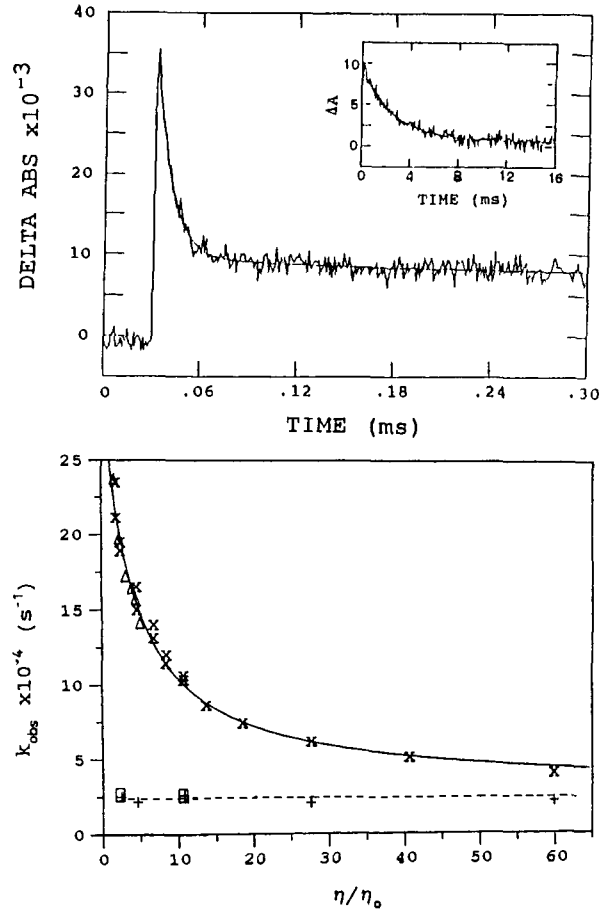

Figure 1. (Upper) Decay of ^{3}Zncyt, monitored at 460 nm, in a solution containing 10.0 μM Zncyt and 20.0 μM pc(II) in ca. 2.5 mM phosphate buffer at pH 7.0 that contains 60% by weight glycerol, at 25 °C. The solid line is a biexponential fit. Inset: The same, over longer time, to show recovery of the 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 (△) 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.

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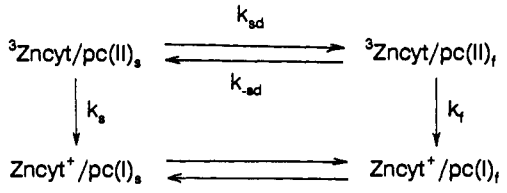
Scheme I



forced by covalent amide cross-links, which impede protein rearrangement.[16-18] Replacement of iron(II) by zinc(II) does not perturb conformation of cytochrome *c* and its association with other proteins.[19,20] The triplet state ^{3}Zncyt is a strong reductant ($E°$ = −0.88 V).[21]

The solvent was a phosphate buffer at pH 7.0 whose relative viscosity (η/η_0 = 1.00) was adjusted by adding buffered glycerol up to 80% by weight or by adding buffered or pure ethylene glycol up to 60% by weight. The ionic strength of the final solvent was always low, nominally 2.5 mM. The positions and widths of the Zncyt and pc(II) absorption bands were unaffected by incubation in even the most viscous buffers. The rate constants for the natural decay of ^{3}Zncyt are 120 ± 10, 120 ± 10, 110 ± 10, 90 ± 5, and 83 ± 6 s^{-1} in buffers with relative viscosities of 1.00, 4.40, 10.7, 27.6, and 59.8, respectively. These rate constants were unaffected by incubation, and their variation barely exceeds the error bounds. The viscous solvents evidently do not perturb the proteins. The noncovalent complex Zncyt/pc(II) was formed in the aqueous buffer; its concentration and solution viscosity were then adjusted as described above. The covalent complex Zncyt/pc(II) was prepared, purified, and characterized by published methods.[22] Flash photolysis experiments were done as before.[22,23]

Cupriplastocyanin quenches ^{3}Zncyt by electron transfer.[23] This quenching is biexponential (Figure 1) at all viscosities because Zncyt exists both free and bound to pc(II). The two exponential processes are well separated in time.[23-25] The rate constant of the slower process increases, and its fraction of the total quenching decreases, as pc(II) concentration increases; this slower process is the bimolecular reaction in eq 1. The rate constant of the faster

$$^3\text{Zncyt} + \text{pc(II)} \rightarrow \text{Zncyt}^+ + \text{pc(I)} \quad (1)$$

process is independent of pc(II) concentration and of ioinic strength (in the range 2.5–10 mM).[23] Its fraction of the total quenching increases as pc(II) concentration, and with it the bound fraction of Zncyt, increases; this faster process is the unimolecular reaction in eq 2, the subject of this study. The decay behavior of ^{3}Zncyt in viscous buffers (Table I in the supplementary material) is identical to its behavior in aqueous buffers.[23]

$$^3\text{Zncyt/pc(II)} \xrightarrow{k_{obsd}} \text{Zncyt}^+\text{/pc(I)} \quad (2)$$

In the noncovalent complex ^{3}Zncyt/pc(II) the rate constant k_{obsd} decreases with increasing viscosity (Figure 1). Since the values determined in glycerol and ethylene glycol solutions of equal viscosities are equal, and since all 23 values fall on the same smooth curve, the decrease in k_{obsd} is not caused by specific protein–solvent interactions, but by the increase in viscosity. It must be intrinsic to the noncovalent complex. In the covalent complex ^{3}Zncyt/pc(II), however, k_{obsd} = (2.4 ± 0.2) × 10^4 s^{-1} regardless of viscosity.[26]

This dependence can be explained by the mechanism in Scheme I. It shows two precursor complexes or reactants that interconvert by surface diffusion (sd).[27] So do the corresponding successor complexes or products, but their possible structural difference is unimportant. The two precursor complexes are designated s (for slow or less reactive) and f (for fast or more reactive); these designations of the corresponding successor complexes are mere labels. Of the four microscopic rate constants in Scheme I and in eq 3 derived from it, only k_{sd} and k_{-sd} are expected to depend on viscosity; they should be inversely proportional to it. At very

$$k_{obsd} = k_s + \frac{k_{sd}k_f}{k_{-sd} + k_f} \quad (3)$$

high viscosity, $k_{sd} \ll k_s$ and $k_{obsd} \approx k_s$. Indeed, the k_{obsd} values for the noncovalent complex converge to those for the covalent complex as relative viscosity increases (Figure 1); the two extrapolated lines merge at $\eta/\eta_0 \approx 150$. In the viscosity range covered by the experiments, when $k_{-sd} \ll k_f$, electron transfer is controlled by surface diffusion, and eqs 4 and 5 obtain. Fitting

$$k_{obsd} \approx k_s + k_{sd} \quad (4)$$

$$1/k_{sd} = A\eta/\eta_0 + B \quad (5)$$

of k_{obsd} with eqs 4 and 5 (the solid curve in Figure 1) yields k_s = 2.7 × 10^4 s^{-1} and $1/k_{sd} = (0.94\eta/\eta_0 + 3.56)$ μs. In aqueous solution, in which η/η_0 = 1.00, k_{sd} = 2.2 × 10^5 s^{-1}. Previously determined rate constants for the reaction in eq 2 were (2.5 ± 0.4) × 10^5 s^{-1} in the preformed complex (at low ionic strength) and (2.8 ± 0.6) × 10^5 s^{-1} in the encounter complex (at high ionic strength).[23] In this previous study we considered the possibility of structural rearrangement of both complexes. The present study confirms that both electron-transfer reactions are controlled by rearrangement. Since $k_{off} < 1 \times 10^4$ s^{-1},[23] complex dissociation does not interfere with surface diffusion.

The agreement between the directly determined k_{obsd} = (2.4 ± 0.2) × 10^4 s^{-1} and the fitted k_s = 2.7 × 10^4 s^{-1} shows that the covalent complex has the same orientation as the less reactive form of the noncovalent complex, ^{3}Zncyt/pc(II)$_s$. In this orientation the strong donor ^{3}Zncyt can reduce pc(II) from the initial binding site even though the electron-transfer paths from this site to the copper site are not optimal.[28] But reduction from a different location on the pc(II) surface, in the orientation ^{3}Zncyt/pc(II)$_f$, is faster. We intend to pinpoint this optimal location in future studies with pc mutants.

The photoinduced reaction in ^{3}Zncyt/pc(II), with a driving force of 1.2 eV, is gated. Since Zncyt and cyt have the same topography and electrostatic properties, they probably diffuse at similar rates on the pc surface. Then the thermal reaction within cyt(II)/pc(II), with a driving force of 0.10 eV, probably is not gated because the electron-transfer step (1300 ± 200 s^{-1})[16,17] is slower than rearrangement. The possibility that similar electron-transfer reactions may be gated or not gated depending on the driving force should be kept in mind when independence of the observed rate constant on driving force is taken as evidence for gating.

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Supplementary Material Available: Characteristics of the faster quenching process (1 page). Ordering information is given on any current masthead page.

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