probable conformation in solution to the neutralization degree, to the nature of the counterions, and to the ionic strength.

The four limiting spectra corresponding to the $\alpha$ helix, $\beta$, unordered, and extended structures have been obtained and used to propose an interpretation of each of the experimental CD spectra. The transitions are considered by introduction of only two contributions depending on the neutralization degree and on the DP: $\alpha$-unordered, unordered-extended, or $\beta$-unordered forms. This treatment is original and gives very good concordance between experimental and calculated spectra.

The limiting forms can be used when discussing the structure of other peptides and proteins. This work proves that there is characteristic DP for the existence of each ordered form and implies that over DP = 10, there is practically no variation in the CD characteristics of limiting structures. These results can be used to get a better resolution of CD spectra on proteins by taking into account the influence of the length of each sequence.

Finally, the stability of each oligomer is discussed and the DP is shown to have a large influence on the most stable conformation for a given solvent condition.

## References and Notes


(1) M. Rinaudo and A. Domard, *Biopolymers*, 14, 2035 (1975).
(2) M. Goodman, A. S. Verdini, C. Toniolo, W. D. Phillips, and F. A. Bovey, *Proc. Natl. Acad. Sci. U.S.A.*, **64**, 444 (1969).
(3) M. Goodman, F. Naider, and C. Toniolo, *Biopolymers*, **10**, 1719 (1971).
(4) A. Yaron, E. Katchalski, A. Berger, G. D. Fasman, and H. A. Sober, *Biopolymers*, **10**, 1107 (1971).
(5) G. M. Bonora and C. Toniolo, *Biopolymers*, **13**, 2179 (1974).
(6) (a) M. L. Tiffany and S. Krimm, *Biopolymers*, **6**, 1379 (1968); (b) *ibid.*, **6**, 1767 (1968); (c) *ibid.*, **8**, 347 (1969); (d) S. Krimm, J. E. Mark, and M. L. Tiffany, *ibid.*, **8**, 695 (1969); (e) M. L. Tiffany and S. Krimm, *ibid.*, **11**, 2309 (1972).
(7) A. J. Adler, N. J. Greenfield, and G. D. Fasman, *Methods Enzymol.*, **27**, 675 (1973).
(8) M. Rinaudo and A. Domard, *C. R. Hebd. Seances Acad. Sci., Ser. C*, **277**, 339 (1973).
(9) I. Tinoco, Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.*, **38**, 1317 (1963).
(10) Y. P. Myer, *Macromolecules*, **2**, 624 (1969).
(11) N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969).
(12) B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, **31**, 476 (1959).
(13) S. Lifson and A. Roig, *J. Chem. Phys.*, **34**, 1963 (1961).


# Ionic Strength and pH Effects on the Rates of Reduction of Blue Copper Proteins by $Fe(EDTA)^{2-}$. Comparison of the Reactivities of *Pseudomonas aeruginosa* Azurin and Bean Plastocyanin with Various Redox Agents


**R. C. Rosenberg, Scot Wherland, R. A. Holwerda, and Harry B. Gray***

*Contribution No. 5246 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125.*


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**Abstract:** The rates of the anaerobic reduction of *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* laccase and stellacyanin by $Fe(EDTA)^{2-}$ have been measured in the pH range 5.6–7.8. The results in each case have been analyzed in terms of electron transfer to protonated and deprotonated protein species in rapid equilibrium. Rate constants for the protonated ($k_a$) and deprotonated ($k_b$) forms and p$K$ values are as follows: azurin, $k_a = 2.4\ (10^3)$, $k_b = 1.0\ (10^3)\ M^{-1}\ s^{-1}$, p$K$ = 6.4; plastocyanin, $k_a = 5.5\ (10^4)$, $k_b = 3.1\ (10^4)\ M^{-1}\ s^{-1}$, p$K$ = 6.1; laccase, $k_a = 4.6\ (10^2)$, $k_b = 1.6\ (10^2)\ M^{-1}\ s^{-1}$, p$K$ = 6.8; stellacyanin, $k_a = 5.7\ (10^5)$, $k_b = 5.1\ (10^5)\ M^{-1}\ s^{-1}$, p$K$ = 6.4. The ionic strength dependences for the reduction of azurin and plastocyanin have also been measured. Marcus theory has been employed to analyze the ionic strength data, and protein self-exchange rate constants ($k_{11}$) that include correction for electrostatic effects have been calculated. The electrostatics-corrected $k_{11}$ values ($M^{-1}\ s^{-1}$) for azurin are as follows: 7 ($10^{-3}$) [$Fe(EDTA)^{2-}$], 2 ($10^4$) (cytochrome *c*), and 8 ($10^7$) (cytochrome $c_{551}$). For plastocyanin, $k_{11}$ values are 10 [$Fe(EDTA)^{2-}$], 2 ($10^6$) (cytochrome *c*), and approximately 4 ($10^{10}$) (cytochrome *f*); all rate constants except the one based on cytochrome *f* are corrected for electrostatic effects. It is proposed that $Fe(EDTA)^{2-}$ cannot penetrate the hydrophobic region surrounding the blue copper center in azurin and is forced to transfer an electron over a relatively long distance (>3 Å). The redox centers in cytochromes apparently can approach the plastocyanin and azurin blue copper centers, resulting in efficient electron transfer. For both blue copper proteins, electron-transfer reactivity is greatest when a physiological partner is involved.


We have previously reported[1] second-order rate constants and activation parameters for the reduction of the blue (or type 1)[2,3] copper in *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* laccase and stellacyanin by $Fe(EDTA)^{2-}$. In order to compensate for the different potentials of the type 1 copper in the four proteins, relative Cu(II)/Cu(I) self-exchange rates were calculated from Marcus theory and found to vary over ten orders of magnitude, according to 2 ($10^{10}$) (stellacyanin) > 1 ($10^5$) (plastocyanin) > 7 ($10^2$) (azurin) > 1 (laccase).[1] As our previous studies did not give careful consideration to medium effects on the rate constants, we report in this paper the nature of the pH and ionic strength dependences for the $Fe(EDTA)^{2-}$ reductions. Employing Marcus theory with inclusion of electrostatic effects, we have analyzed in detail the ionic strength dependences of azurin and plastocyanin electron-transfer reactions. This analysis has allowed us to calculate electrostatics-corrected, blue-protein self-exchange rate constants ($k_{11}$) from cross reaction kinetic data. The $k_{11}$ values for azurin and plastocyanin based on cross reactions with $Fe(EDTA)^{2-}$ and horse heart cytochrome *c* are compared with those estimated for probable physiological substrates, and the results are discussed in terms of the accessibility of the copper site to each redox agent.

**Table I.** Rate Constants

A. pH Dependences

| Protein | pH | $k$, $M^{-1}$ $s^{-1}$ | $\sigma^a$ |
|---|---|---|---|
| Azurin[b] | 5.80 | 2.25 ($10^3$) | 0.00708 |
| | 6.40 | 1.87 ($10^3$) | 0.00113 |
| | 6.80 | 1.36 ($10^3$) | 0.00155 |
| | 7.00 | 1.31 ($10^3$) | 0.00102 |
| | 7.40 | 1.19 ($10^3$) | 0.000767 |
| | 7.80 | 1.08 ($10^3$) | 0.000515 |
| Stellacyanin[c] | 5.00 | 2.72 ($10^2$) | 9.65 |
| | 5.50 | 2.79 ($10^2$) | 3.39 |
| | 6.00 | 2.70 ($10^2$) | 5.08 |
| | 6.40 | 2.47 ($10^2$) | 6.40 |
| | 6.90 | 2.04 ($10^2$) | 1.45 |
| | 7.40 | 2.14 ($10^2$) | 0.22 |
| | 7.80 | 2.09 ($10^2$) | 2.81 |
| Plastocyanin[d] | 5.73 | 48.1 | 0.9 |
| | 5.93 | 45.9 | 1.3 |
| | 6.11 | 41.1 | 1.0 |
| | 6.31 | 41.3 | 1.0 |
| | 6.53 | 35.3 | 0.7 |
| | 6.74 | 36.4 | 0.6 |
| | 6.93 | 36.0 | 0.8 |
| | 7.15 | 34.5 | 0.8 |
| | 7.34 | 33.2 | 0.7 |
| | 7.72 | 30.3 | 0.8 |
| | 7.87 | 30.9 | 0.5 |
| Laccase[e] | 4.92 | 0.354 | 0.001 |
| | 5.40 | 0.421 | 0.009 |
| | 5.92 | 0.421 | 0.002 |
| | 6.32 | 0.335 | 0.006 |
| | 6.72 | 0.300 | 0.003 |
| | 7.33 | 0.215 | 0.001 |
| | 7.70 | 0.166 | 0.001 |
| | 4.94 | 1.54 | 0.02 |
| | 5.39 | 1.74 | 0.03 |
| Laccase[f] | 5.92 | 1.78 | 0.0034 |
| | 6.33 | 1.62 | 0.05 |
| | 6.79 | 1.33 | 0.01 |
| | 7.36 | 0.967 | 0.009 |
| | 7.77 | 0.853 | 0.004 |

B. Ionic Strength Dependences

| $[Fe(EDTA)^{2-}]$ | $\mu$, M | $k_{obsd}$, $s^{-1}$ | $\sigma$ |
|---|---|---|---|
| Azurin (pH 6.8, 25.5 ± 0.1 °C, 5mM phosphate) | | | |
| 0.3 mM | 0.01 | 0.349 | 0.007 |
| | 0.035 | 0.394 | 0.009 |
| | 0.075 | 0.416 | 0.018 |
| | 0.130 | 0.444 | 0.012 |
| | 0.200 | 0.453 | 0.014 |
| | 0.350 | 0.510 | 0.015 |
| 0.4 mM | 0.010 | 0.452 | 0.014 |
| | 0.035 | 0.532 | 0.013 |
| | 0.075 | 0.558 | 0.014 |
| | 0.130 | 0.583 | 0.009 |
| | 0.200 | 0.612 | 0.017 |
| | 0.350 | 0.676 | 0.022 |
| Plastocyanin (pH 6.9, 25.1 ± 0.2 °C, 3 mM phosphate) | | | |
| 1.0 mM | 0.05 | 31.1 | 1.0 |
| | 0.10 | 49.5 | 1.7 |
| | 0.20 | 75.9 | 1.2 |
| | 0.30 | 89.1 | 4.0 |
| | 0.50 | 113 | 4.0 |

[a] For the azurin data, the standard deviations of the second-order rate constants are those of the slopes from the $1/\sigma^2$ weighted least-squares fits of the concentration dependences at each pH. For the other proteins, the standard deviations are those from the mean of the multiple determinations performed. [b] 25.0 °C, $\mu$ = 0.2 M, 0.05 M from buffer. [c] 25.2 °C, $\mu$ = 0.5 M, 0.26 M from buffer, 0.5 mM $Fe(EDTA)^{2-}$. [d] 25.0 °C, $\mu$ = 0.2 M, 0.18 M from buffer, 1.0 mM $Fe(EDTA)^{2-}$. [e] 25.6 °C, $\mu$ = 0.5 M, 0.3 M from buffer, 1.0 mM $Fe(EDTA)^{2-}$. [f] 25.6 °C, $\mu$ = 0.5 M, 0.3 M from buffer, 5.0 mM $Fe(EDTA)^{2-}$.

**Table II.** Best Fit Parameters

| Protein | $pK^a$ | $k_a$, $M^{-1}$ $s^{-1}$ [b] | $k_b$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|
| Azurin | 6.42 ± 0.01 | 2.4 ± 0.2 ($10^3$) | 1.0 ± 0.1 ($10^3$) |
| Stellacyanin | 6.4 ± 0.3 | 5.7 ± 0.95 ($10^5$) | 5.1 ± 0.88 ($10^5$) |
| Plastocyanin | 6.1 ± 0.2 | 5.5 ± 0.4 ($10^4$) | 3.1 ± 0.1 ($10^4$) |
| Laccase[c] | 6.78 ± 0.01 | 4.64 ± 0.02 ($10^2$) | 1.55 ± 0.01 ($10^2$) |
| Laccase[d] | 6.78 ± 0.01 | 3.9 ± 0.6 ($10^2$) | 1.46 ± 0.01 ($10^2$) |

[a] Standard errors of the nonlinear least-squares treatment are those quoted. [b] For all proteins except azurin, second-order rate constants were calculated assuming a first-order dependence on $Fe(EDTA)^{2-}$. [c] 1 mM $Fe(EDTA)^{2-}$. [d] 5 mM $Fe(EDTA)^{2-}$.

## Experimental Section

Azurin was extracted and purified from *P. aeruginosa* (ATCC 10145) by a slight modification of procedures given by Ambler.[4] In the present case, the pH of the acetone powder extract was lowered only to 4.0 before the diluted extract was applied to a CM-cellulose column (30 × 4.5 cm Whatman CM 23 equilibrated with 0.05 M $NH_4OAc$, pH 4.0). Subsequent stepwise elution and further purification were performed as described elsewhere.[4] The purified oxidized azurin had an $A_{625}/A_{280}$ ratio of 0.44 and showed one band on starch gel electrophoresis.[5] A slight hint of a band at 415 nm was observed in the most concentrated azurin solutions, indicating only a trace of cytochrome impurity was present. The yield of purified azurin was 280 mg from 180 g of the acetone powder. Purified azurin was dialyzed into 0.05 M $NH_4OAc$ (pH 4.0) buffer, millipore filtered, and stored at 4 °C. Before each series of kinetic experiments, the azurin was dialyzed against the requisite buffer solution.

Bean plastocyanin, *Rhus* laccase, and stellacyanin were extracted and purified by standard methods.[1] Reagent grade chemicals were used without further purification and deionized distilled water was used to prepare all solutions. Solutions of $Fe(EDTA)^{2-}$ were prepared as previously described.[1] Nitrogen gas was passed through two chromous scrubbing towers to remove all oxidizing impurities.

All kinetic experiments and data analysis were performed as previously described[1] except that a weighting factor of $1/\sigma^2$ was used in the nonlinear least-squares fit of the pH dependence data.

## Results

The pH dependences of the rate constants ($k$) for reduction of azurin, stellacyanin, plastocyanin, and laccase by $Fe(EDTA)^{2-}$ are given in Table I. Detailed concentration dependences establish that the azurin reaction is first order in both reducing agent and total oxidized protein over the entire pH range accessible in phosphate-buffered media. Five to eight different $Fe(EDTA)^{2-}$ concentrations were examined between 0.4 and 4.5 mM at each pH in the interval 5.8–7.8.

As $Fe(EDTA)^{2-}$ does not have a $pK_a$ in the pH 6–7 region,[6] it is reasonable to interpret the observed variation in $k$ with pH for each of the proteins containing only type 1 copper in terms of a rapid equilibrium between protonated and deprotonated protein species. With $K_a$ the acid ionization constant for this equilibrium, we have the following relationships.

$$-d[Cu(II)]_{tot}/dt = k_a[Cu(II)H^+][Fe(EDTA)^{2-}] + k_b[Cu(II)][Fe(EDTA)^{2-}] = k[Cu(II)]_{tot}[Fe(EDTA)^{2-}] \quad (1)$$

$$k = (k_a[H^+] + k_bK_a)/(K_a + [H^+]) \quad (2)$$

Values for $k_a$, $k_b$, and $K_a$ for azurin, plastocyanin, stellacyanin, and laccase were obtained from nonlinear least-squares fits of the data in Table I to eq 2 and are given in Table II. Figure 1 shows the experimental data and calculated curve for each protein. As the results in Table II indicate, the rates of reduction of azurin, plastocyanin, and stellacyanin by $Fe(EDTA)^{2-}$ are dependent on the degree of protonation of a group with a $pK_a$ of ~6.3. In each case the rate is greatest at low pH. For *Rhus* laccase the situation is more complicated,

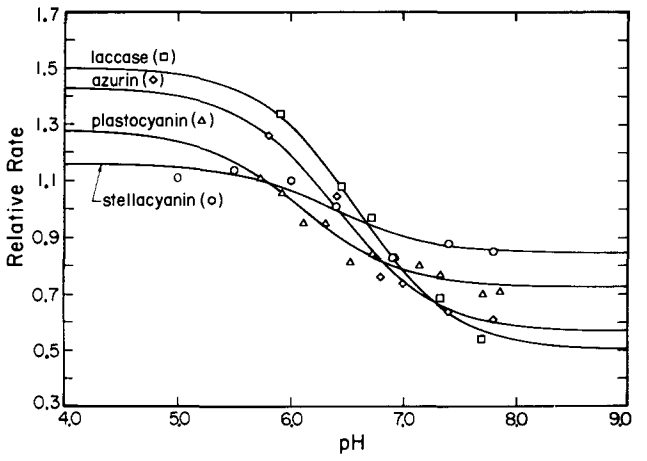


**Figure 1.** Dependence of the rate constants on pH. The rates for each protein are normalized to the mean of $k_a$ and $k_b$ as given in Table II. The best-fit curves are shown for each protein. Data symbols are laccase, □; azurin, ◇; plastocyanin, △; and stellacyanin, ○.

as the reduction rate reaches a maximum at pH 5.9, indicating that more than one ionizable group influences the electron-transfer process. Lack of complete data in the lower pH region precludes a detailed analysis of the laccase-$Fe(EDTA)^{2-}$ reaction. However, it appears that this reaction is also influenced by the state of ionization of a group with a $pK_a$ between 6 and 7, and the fitting was done for data from pH 5.9 to 7.8.

The differences between the second-order rate constants for the protonated and deprotonated forms of the oxidized blue proteins are quite small, being less than a factor of 3 in all cases. This small difference is consistent with the ionizable group being located near, but not necessarily at, the site of preferred electron transfer. We consider it unlikely that this group is a ligand of the copper, as the visible absorption spectra of these proteins are unchanged over the pH range where ionization occurs.[7]

The type of pH dependence observed for the rate of reduction of blue copper by $Fe(EDTA)^{2-}$ has been observed for other negatively charged reductants. For example, a protonated form of *Rhus* laccase is reduced 23 times faster by $Fe(CN)_6^{4-}$ than the corresponding deprotonated enzyme.[8] Based on the calculated p$K$ of 6.41, it was suggested that the ionizable group on laccase is a histidine residue. The interpretation of the small pH dependence observed with $Fe(EDTA)^{2-}$ is probably more complex, at least in the case of plastocyanin. Both spinach and bean plastocyanins possess only two histidines,[9] and Markley and co-workers have shown[10] by NMR measurements that the residues in the former protein have p$K$ values below 5. Close sequence analogies between spinach and bean plastocyanins[10] suggest that it would be unwise to propose histidine involvement as the origin of the observed pH effects on the reduction rates. Indeed, it should be emphasized that a detailed interpretation of the pH dependences is not possible at this time, as factors such as variable binding of salt and buffer ions and slight protein conformational changes may well be involved.

We have also investigated the effects of ionic strength variations on the reduction rates of azurin and plastocyanin. At pH 6.8 and 6.9, respectively, the rates of reduction of oxidized azurin and plastocyanin increase with increasing ionic strength (Table I). Analysis of the ionic strength dependences will be considered in a subsequent section.

## Discussion

In recent papers[1,11] we have applied the relative Marcus theory[12] to the reactions of inorganic reagents with proteins. The standard Marcus theory equation is

$$k_{12} = \sqrt{k_{11}k_{22}Kf} \tag{3}$$

where $k_{11}$ and $k_{22}$ are the self-exchange rates, $K$ is the equilibrium constant, $f$ is ca. 1 for small driving forces, and $k_{12}$ is the cross reaction rate constant. If the protein always uses the same activation mechanism in cross reactions and if there are no strong associations between the protein and the reagents, the calculated $k_{11}$ should be a true constant; alternatively, if the contribution to the activation free energy attributable to protein-reagent interactions is different for each small molecule oxidant or reductant, the calculated self-exchange rate could vary substantially. Such variation has been observed in the reactions of HiPIP with several inorganic reagents.[11]

Before we can assess the possible importance of specific interactions leading to protein structural changes in the transition state, we must first take simple protein-reagent electrostatic effects into account. We proceed by expressing the free energies that correspond to each parameter in eq 3 in the following way.[13]

$$\Delta G_{12}^\circ = \Delta G_r^\circ + w_{12} - w_{21} \tag{4}$$

$$\Delta G_{11}^* = \Delta G_{11}^{**} + w_{11} \tag{5}$$

$$\Delta G_{22}^* = \Delta G_{22}^{**} + w_{22} \tag{6}$$

$$\Delta G_{12}^* = \Delta G_{12}^{**} + w_{12} \tag{7}$$

The asterisked $\Delta G$ terms are activation free energies with the subscripts as defined above, and $\Delta G_{12}^\circ$ is the observed free-energy change. The double-asterisked $\Delta G$ terms and $\Delta G_r^\circ$ are corresponding quantities that are independent of electrostatic effects. The $w$ terms represent the electrostatic work required to bring the reagents together from infinite separation to the separation and orientation in the transition state; $w_{12}$ is defined as the work needed to bring the protein and reagent together, and $w_{21}$ is the work required to bring the two products together. In terms of free energies, the Marcus formula is

$$\Delta G_{12}^* = (\Delta G_{11}^* + \Delta G_{22}^* + \Delta G_{12}^\circ + w_{12} + w_{21} - w_{11} - w_{22})/2 \tag{8}$$

The problem of evaluating eq 8, then, becomes one of evaluating the work terms. A simple but reasonable approach to this problem is to consider the protein to be a sphere possessing a totally symmetric charge distribution. The dielectric within the sphere is lower than that of the medium, but its value is not required.[14] The equation for the potential energy in this case is[15]

$$V = \tfrac{1}{2}\left[\frac{e^{\kappa R_1}}{1+\kappa R_1} + \frac{e^{\kappa R_2}}{1+\kappa R_2}\right]\left(\frac{Z_1 Z_2 e^2}{\epsilon}\right)\left(\frac{e^{-\kappa r}}{r}\right) \tag{9}$$

where $R_1$ is the protein radius at the distance of closest approach by the average small ion in solution, $R_2$ is the similarly defined radius of the reagent [estimated to be 4 Å for $Fe(EDTA)^{2-}$ from examination of a molecular model], $Z_1$ and $Z_2$ are the respective charges on the protein and its redox partner, $r$ is the distance of closest approach (taken as $R_1 + R_2$ in these calculations), $e$ is the charge on an electron, $\epsilon$ is the dielectric of the medium (taken as 78.3 for water at 25 °C), and $\kappa$ is the ionic strength parameter, equal to $0.329\,(\mu)^{1/2}$ Å$^{-1}$ in water at 25 °C. The protein radius may be estimated from[14]

$$R_1^3 = \frac{3}{4\pi}\frac{M}{N}(\bar{\nu}_2 + \delta_1 \nu_1^\circ) \tag{10}$$

where $M$ is the molecular weight of the protein, $N$ is Avogadro's number, $\bar{\nu}_2$ is the partial specific volume of the protein, $\delta_1$ is the effective solvation, and $\nu_1^\circ$ is the partial specific volume of water. Taking $\bar{\nu}_2 = 0.73$ cm$^3$/g, $\delta_1 = 0.2$, and $\nu_1^\circ = 1$,[14] we have

$$R_1 = 0.717\,M^{1/3} \tag{11}$$

Table III. Parameters Derived from Ionic Strength Dependences

| | Eq 14 | | Eq 13 | | Eq 16 | | Charge from sequence | pI |
|---|---|---|---|---|---|---|---|---|
| | $k$ ($\mu$ = 0) | $Z_1$ | $k$ ($\mu$ = 0) | $Z_1$ | $k$ ($\mu$ = 0) | $Z_1$ | | |
| Azurin | 1.1 ($10^3$)[a] | −0.15 | 4.0 ($10^2$)[a] | −3.53 | 8.0 ($10^2$)[a] | −1.10 | −1[b] | 5.4[c] |
| Plastocyanin | 2.0 (10)[d] | −0.55 | 0.57[d] | −8.84 | 0.77[d] | −7.19 | −8[e] | <6[f] |

[a] In $M^{-1}$ $s^{-1}$. [b] See ref 4a. [c] T. Horio, I. Sekuzu, T. Higashi, and K. Okunuki, *Haematin Enzymes, Symp. Int. Union Biochem., 1959*, **19**, 302 (1961). [d] In $s^{-1}$ [e] See ref 9b. [f] P. R. Milne and J. R. E. Wells, *J. Biol. Chem.*, **245**, 1566 (1970).

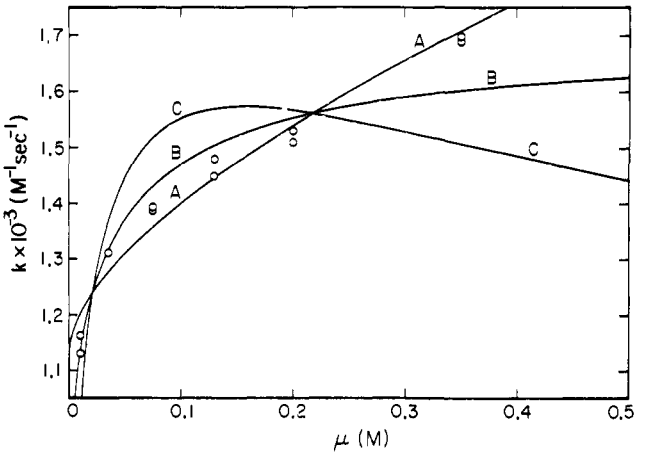


Figure 2. Dependence of $k$ on $\mu$ for the reaction of azurin with $Fe(EDTA)^{2-}$. The curves are the best fits to eq 14 (A), 16 (B), and 13 (C).

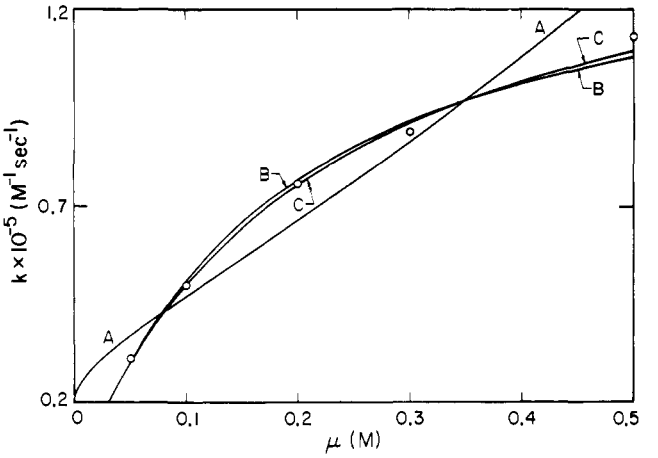


Figure 3. Dependence of $k$ on $\mu$ for the reaction of plastocyanin with $Fe(EDTA)^{2-}$. The curves are the best fits to eq 14 (A), 16 (B), and 13 (C).

Two general methods can be tested to estimate the charge on the protein, given the information available. One is to sum the charge contributions from the individual amino acids, assuming they have the normal p$K$'s of the free residues. The second general method is to fit the known ionic strength dependence to a protein charge, given the reagent charge and all radii. In the latter calculation, there are several approaches that could be chosen. Two of these arise from the standard treatment of ionic strength dependences as activity coefficient effects, and the third is a Marcus theory result. The activity coefficient theory leads to the equation[16]

$$\ln k = \ln k_0 - \frac{Z_1^2 \alpha\sqrt{\mu}}{1 + \beta R_1\sqrt{\mu}} - \frac{Z_2^2\alpha\sqrt{\mu}}{1 + \beta R_2\sqrt{\mu}} + \frac{(Z_1 + Z_2)^2\alpha\sqrt{\mu}}{1 + \beta R_\pm\sqrt{\mu}} \quad (12)$$

which reduces to

$$\ln k = \ln k_0 + \frac{(2Z_1Z_2 + Z_2^2)\alpha\sqrt{\mu}}{1 + \beta R_1\sqrt{\mu}} - \frac{Z_2^2\alpha\sqrt{\mu}}{1 + \beta R_2\sqrt{\mu}} \quad (13)$$

under the assumption that the activated complex and protein have the same radius ($R_1 = R_\pm$) and that the charge on the protein does not vary. Only when the ionic strength is assumed to be quite low, and all of the radii assumed to be approximately equal, does the commonly quoted relationship, eq 14, result.

$$\ln k = \ln k_0 + 2\alpha Z_1 Z_2\sqrt{\mu} \quad (14)$$

The Marcus theory treatment with work terms (eq 4-8) may be written

$$\Delta G_{12}^* = \tfrac{1}{2}(\Delta G_{11}^{**} + \Delta G_{22}^{**} + \Delta G_r^\circ) + w_{12} \quad (15)$$

where $w_{12}$ is the only ionic strength dependent term. Expanding $w_{12}$ (from eq 9), the following expression for the ionic strength dependence of the rate constant is obtained.

$$\ln k = \ln k_0 - 3.576\left(\frac{e^{-\kappa R_1}}{1 + \kappa R_2} + \frac{e^{-\kappa R_2}}{1 + \kappa R_1}\right)\frac{Z_1Z_2}{R_1 + R_2} \quad (16)$$

The $\ln k_0$ term in this case refers to infinite (not zero) ionic strength.

The fits of the ionic strength data for the azurin and plastocyanin reactions are shown in Figures 2 and 3. Parameters are set out in Table III. In all cases, the charge has the same sign as predicted from the isoelectric point and the amino acid composition; however, eq 14 always gives a much smaller value for the charge than either of the other two equations or the amino acid composition. Equally good fits to eq 13 and 16 could be obtained with smaller radii and smaller charges, but the choice of any radius smaller than that of the protein itself would be totally arbitrary and would introduce another variable. The agreement between the total charge calculated for the protein from the sequence and from the treatment of the ionic strength data with eq 16 is encouraging. Thus we have used the parameters derived from the fits to eq 16 in the work term calculations.

The parameters employed in making the work term calculations and the resulting calculated protein self-exchange rate constants are set out in Table IV for azurin and plastocyanin reactions with $Fe(EDTA)^{2-}$ and horse heart cytochrome $c$. The electrostatic corrections to the predicted $k_{11}$ values are modest both for the large charge on plastocyanin and the small one on azurin, as the protein self-exchange and the cross reaction work terms compensate. The calculated self-exchange rate of 7 ($10^{-3}$) $M^{-1}$ $s^{-1}$ for azurin[17] based on $Fe(EDTA)^{2-}$ is notably lower than the value of 2 ($10^4$) $M^{-1}$ $s^{-1}$ obtained from the cross reaction with horse heart cytochrome $c$. Further, it is of interest that the azurin self-exchange rate based on the cross reaction with a possible physiological partner, cytochrome $c_{551}$, is calculated (Table IV) to be extremely high [8 ($10^7$) $M^{-1}$ $s^{-1}$]. Thus, our analysis of azurin reactivity indicates that a large variation in protein-reagent interaction oc-

**Table IV.** Calculation of Protein Self-Exchange Rates[a]

| Protein | Reagent | $k_{12}$ | $\Delta G_{12}^*$ | $\Delta G_{22}^*$ | $\Delta G_{12}^\circ$ | $w_{12}$ | $w_{21}$ | $w_{11}$ | $w_{22}$ | $\Delta G_{11}'$ | $k_{11}'$ | $\Delta G_{11}$ | $k_{11}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azurin[b] | $Fe(EDTA)^{2-}$ [c] | 1.2 ($10^2$)[d] | 13.224 | 11.333[c] | −4.843 | 0.078 | 0.039 | 0.009 | 0.493 | 19.958 | 1 ($10^{-2}$) | 20.343 | 7 ($10^{-3}$) |
| | Cyt *c* (III) | 1.1 ($10^3$)[e] | 13.290 | 13.97[f] | 1.614 | −0.118 | −0.051 | 0.015 | 0.406 | 10.97 | 5.6 ($10^4$) | 11.56 | 2 ($10^4$) |
| | Cyt $c_{551}$ (II)[g] | 6.1 ($10^6$)[h] | 8.186 | 11.348[i] | −1.614 | 0.022 | 0.033 | 0.009 | 0.079 | 6.638 | 8 ($10^7$) | 6.671 | 8 ($10^7$) |
| Plastocyanin[j] | $Fe(EDTA)^{2-}$ | 5.3 ($10^4$)[d] | 10.996 | 11.333 | −5.304 | 0.594 | 0.297 | 0.506 | 0.493 | 15.963 | (10) | 16.071 | 1 (10) |
| | Cyt *c* (II) | 1 ($10^6$)[k] | 9.257 | 14.060 | −2.306 | −0.497 | −0.563 | 0.506 | 0.530 | 6.760 | 7 ($10^7$) | 8.856 | 2 ($10^6$) |

[a] $\mu$ = 0.1 M, 25 °C; rate constants are given in $M^{-1}$ $s^{-1}$; free energies and work terms are given in kcal/mol; all primed quantities are uncorrected for electrostatic interactions. [b] $R_1$ = 17.2 Å ($M$ = 13 900; see ref 4a); $E$ = 330 mV (*Ps. aer.*) (T. Yamanaka in "The Biochemistry of Copper", J. Peisach and W. E. Blumberg, Ed., Academic Press, New York, N.Y., 1966, p 275); $E$ = 290 mV (*Ps. fl.*) [P. M. Wood, *Biochim. Biophys. Acta*, **357**, 370 (1974)]; $Z_1 = -1.10$. [c] $k_{22}$ = 3 ($10^4$) $M^{-1}$ $s^{-1}$ (see ref 11); $Z_2 = -2$; $Z_2' = -1$; $R_2$ = 4 Å. [d] Values corrected to $\mu$ = 0.1 M (see ref 1). [e] C. Greenwood, A. Finazzi-Agro, P. Guerrieri, L. Avigliano, B. Mondovi, and E. Antonini, *Eur. J. Biochem.*, **23**, 321 (1971). [f] Horse heart cytochrome *c* parameters are $R_2$ = 16.6 Å, $Z_2$ = 7.5 (oxidized), $Z_2'$ = 6.5 (reduced); for sequence, histidine p*K* values, and $M$, see R. E. Dickerson and R. Timkovich, "The Enzymes", Vol. 11, 3d ed, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1975, pp 397–547; $E$ = 260 mV [R. Margalit and A. Shejter, *Eur. J. Biochem.*, **32**, 492 (1973)]. [g] Cytochrome $c_{551}$ (*Ps. aer.*) parameters are $R_2$ = 14.4 Å, $Z_2 = -2$ (oxidized), $Z_2' = -3$ (reduced); for sequence and $M$, see Dickerson and Timkovich cited in footnote *f*; $E$ = 260 mV (R. A. Holwerda, S. Wherland, and H. B. Gray in ref 24). [h] See ref 21. [i] $k_{22}$ is calculated to be 3 ($10^4$) $M^{-1}$ $s^{-1}$ from $k_{12}$ = 5.3 ($10^4$) $M^{-1}$ $s^{-1}$ for the cross reaction of cyt $c_{551}$ (II) and $Co(phen)_3^{3+}$ (cross reaction data from J. V. McArdle, Ph.D. Thesis, California Institute of Technology, 1976); $R$ [$Co(phen)_3^{3+}$] = 7 Å. [j] $R_1$ = 15.8 Å ($M$ = 10 700; see ref 9b); $Z_1 = -7.19$; $E$ = 350 mV (bean) (N. Sailasuta, F. C. Anson, and H. B. Gray, to be submitted for publication); $E$ = 360 mV (parsley) (P. M. Wood, cited in footnote *b*). [k] For parsley plastocyanin (P. M. Wood, cited in footnote *b*).

curs in the transition state, with kinetic access to the blue copper center decreasing according to cytochrome $c_{551} \gg$ cytochrome $c \gg Fe(EDTA)^{2-}$. As various physical studies have shown that the blue copper is sequestered in a hydrophobic region away from solvent,[18] such a wide variation in electron-transfer reactivity is not surprising.[19] It is probable that $Fe(EDTA)^{2-}$, which is least reactive, cannot penetrate the hydrophobic region and is forced to transfer an electron to copper over a distance that could be as great as 3 or 4 Å.[11] With the cytochromes, especially cytochrome $c_{551}$, electron transfer is greatly facilitated, presumably by a pathway in which the heme edge and the copper center are brought into close proximity. The fact that cytochrome $c_{551}$ is the best electron-transfer agent yet found for azurin supports the proposal[20] but these two proteins are physiological partners.

Further differences, not inconsistent with the above accessibility argument, are associated with the activation parameters for the reduction of azurin by $Fe(EDTA)^{2-}$ and ferrocytochrome $c_{551}$. With $Fe(EDTA)^{2-}$ as reductant, $\Delta H^{\ddagger}$ is very small (2.0 kcal/mol) and the reaction is essentially controlled by a large, negative activation entropy (−37 eu).[1] In the case of ferrocytochrome $c_{551}$, however, $\Delta H^{\ddagger}$ is much larger (10.6 kcal/mol) and $\Delta S^{\ddagger}$ is positive (8.6 eu).[21,22] The reactions of HiPIP also show distinctly different activation parameters along with different predicted self-exchange rates.[11] In both HiPIP and azurin, it is generally found that the cross reaction that gives the higher predicted self-exchange rate is characterized by a relatively favorable activation entropy, whereas the more forbidden reactions involve minimal $\Delta H^{\ddagger}$ but highly unfavorable $\Delta S^{\ddagger}$ values. The efficient reaction modes appear, then, to be those for which the interaction between the protein and its oxidant or reductant, although perhaps requiring as much as 15 kcal/mol in activation energy, permits close approach to the buried redox site. We assume that the inefficient reactions are blocked from such penetration (e.g., by $\Delta H^{\ddagger} \geq$ 30 kcal/mol) and thus find a lower energy pathway by transferring an electron from a large distance, with very unfavorable entropic but minimal enthalpic activation requirements.

The electrostatics-corrected self-exchange rate for plastocyanin based on $Fe(EDTA)^{2-}$ is 10 $M^{-1}$ $s^{-1}$.[23] The reactivity order, azurin ≪ plastocyanin, is the same with or without corrections for electrostatic interaction. Thus our earlier conclusion[1] that the copper center in plastocyanin is inherently more reactive than that in azurin appears to be secure. The electrostatics-corrected self-exchange rate for plastocyanin based on the cytochrome *c* cross reaction, 2 ($10^6$) $M^{-1}$ $s^{-1}$, is substantially smaller than the uncorrected value [1 ($10^8$) $M^{-1}$ $s^{-1}$]. Taking either value, however, it is apparent that cytochrome *c* is a better electron-transfer agent for plastocyanin than is $Fe(EDTA)^{2-}$. As with azurin, the largest $k_{11}$ for plastocyanin, 4 ($10^{10}$) $M^{-1}$ $s^{-1}$, is obtained from the cross reaction with a physiological partner, cytochrome *f*.[24] Although the estimate of 4 ($10^{10}$) $M^{-1}$ $s^{-1}$ [22] has not been corrected for electrostatic effects, as the requisite data for cytochrome *f* are not available, it is apparent that the reactivity of the blue copper center in plastocyanin depends critically on the nature of its redox partner.

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## References and Notes

(1) S. Wherland, R. A. Holwerda, R. C. Rosenberg, and H. B. Gray, *J. Am. Chem. Soc.*, **97**, 5260 (1975).
(2) (a) R. Malkin and B. G. Malmström, *Adv. Enzymol.*, **33**, 177 (1970); (b) J. A. Fee, *Struct. Bonding (Berlin)*, **23**, 1 (1975).

(3) (a) D. R. McMillin, R. A. Holwerda, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1339 (1974); (b) D. R. McMillin, R. C. Rosenberg, and H. B. Gray, *ibid.*, **71**, 4760 (1974), and references cited therein.
(4) (a) R. P. Ambler and L. H. Brown, *Biochem. J.*, **104**, 784 (1967); (b). R. P. Ambler and M. Wynn, *ibid.*, **131**, 485 (1973).
(5) O. Smithies, *Adv. Protein Chem.*, **14**, 65 (1959).
(6) G. Schwarzenbach and J. Heller, *Helv. Chim. Acta*, **34**, 576 (1951).
(7) S. P. W. Tang and J. E. Coleman, *J. Biol. Chem.*, **243**, 4286 (1968).
(8) R. A. Holwerda and H. B. Gray, *J. Am. Chem. Soc.*, **97**, 6036 (1975).
(9) (a) M. D. Scawen, J. A. M. Ramshaw, and D. Boulter, *Biochem. J.*, **147**, 343 (1975); (b) P. R. Milne, J. R. E. Wells, and R. P. Ambler, *ibid.*, **143**, 691 (1974).
(10) J. L. Markley, E. L. Ulrich, S. P. Berg, and D. W. Krogmann, *Biochemistry*, **14**, 4428 (1975).
(11) J. Rawlings, S. Wherland, and H. B. Gray, *J. Am. Chem. Soc.*, **98**, 2177 (1976).
(12) R. A. Marcus, *J. Phys. Chem.*, **67**, 853 (1963).
(13) A. Haim and N. Sutin, *Inorg. Chem.*, **15**, 476 (1976).
(14) C. Tanford, "Physical Chemistry of Macromolecules", Wiley, New York, N.Y., 1961, pp 466–467.
(15) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).
(16) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism", 2d ed, Wiley, New York, N.Y., 1961, p 150.
(17) The actual self-exchange rate of azurin has not been determined, but it is known to be slow on the NMR time scale (H. A. O. Hill, private communication). Thus the experimental $k_{11}$ for azurin may fall between the values predicted from the $Fe(EDTA)^{2-}$ and cytochrome *c* cross reactions.
(18) (a) N. Boden, M. C. Holmes, and P. F. Knowles, *Biochem. Biophys. Res. Commun.*, **57**, 845 (1974); (b) L. Avigliano, A. Finazzi-Agro, and B. Mondovi, *FEBS Lett.*, **38**, 205 (1974); (c) A. Finazzi-Agro, G. Rotilio, L. Avigliano, P. Guerrieri, V. Boffi, and B. Mondovi, *Biochemistry*, **9**, 2009 (1970).
(19) A similarly large variation in calculated $k_{11}$ values for HiPIP was taken as evidence that different pathways of electron transfer to the relatively buried redox center (the $Fe_4S_4S_4^*$ cluster) are employed by different reagents.[11]
(20) E. Antonini, A. Finazzi-Agro, L. Avigliano, P. Guerrieri, G. Rotilio, and B. Mondovi, *J. Biol. Chem.*, **245**, 4847 (1970).
(21) P. Rosen and I. Pecht, *Biochemistry*, **15**, 775 (1976).
(22) A similar difference may be noted in activation parameters for the reduction of bean plastocyanin by $Fe(EDTA)^{2-}$ [$\Delta H^{\ddagger} = 2.1$ kcal/mol, $\Delta S^{\ddagger} = -29$ eu (pH 7.0, $\mu = 0.2$ M)][1] and the reduction of parsley plastocyanin by ferrocytochrome *f* [$\Delta H^{\ddagger} = 10.5$ kcal/mol, $\Delta S^{\ddagger} = 11$ eu (pH 7.0, $\mu = 0.1$ M)] [P. M. Wood, *Biochim. Biophys. Acta*, **357**, 370 (1974)].
(23) The experimental $k_{11}$ for plastocyanin is not known, but NMR measurements at 50 °C have established that the value is smaller than 4 ($10^3$) $M^{-1}$ $s^{-1}$ [J. K. Beattie, D. J. Fensom, H. C. Freeman, E. Woodcock, H. A. O. Hill, and A. M. Stokes, *Biochim. Biophys. Acta*, **405**, 109 (1975)].
(24) This estimate is based on a $k_{22}$ for cytochrome *f* taken from a cross reaction with $Fe(CN)_6^{3-}$ [R. A. Holwerda, S. Wherland, and H. B. Gray, *Annu. Rev. Biophys. Bioeng.*, **5**, 363 (1976)].

# Modification of the Reactive Sulfhydryl Group in Phosphofructokinase

**Kenneth J. Schwartz, Y. Nakagawa, and E. T. Kaiser***

*Contribution from the Departments of Chemistry and Biochemistry, University of Chicago, Chicago, Illinois 60637.* *Received January 2, 1976*

**Abstract:** Modification of rabbit muscle phosphofructokinase (PFK) (ATP:D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) with α-bromo-4-hydroxy-3-nitroacetophenone (BHNA) led to inactivation of the enzyme. The minimum molar ratio of BHNA to PFK (based on a molecular weight of 380 000) sufficient to abolish most (>93%) of the catalytic activity at pH 8 was 5:1. Under these conditions, one sulfhydryl group per protomer molecular weight of 93 000 was modified, and the reaction of the most rapidly reacting thiol group per protomer with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was abolished. Carbamidomethylation of six thiol groups per protomer resulted in a 61% loss of catalytic activity. Incubation of the partially carbamidomethylated enzyme with a fourfold molar excess of BHNA led to the modification of an additional thiol group per protomer and to an overall loss of 82% of the activity exhibited in a control experiment. Protection against inactivation of PFK by a fourfold molar excess of BHNA at pH 7.00 was afforded in the order: magnesium adenosine 5′-triphosphate (MgATP) > magnesium inosine 5′-triphosphate (MgITP) > ATP > ITP. At pH 8.00, the order was MgATP > ATP > MgITP. Fructose 6-phosphate (F6P), fructose 1,6-diphosphate (FDP), cyclic 3′,5′-adenosine monophosphate (cAMP), adenosine 5′-monophosphate (AMP), and citrate offered no protection. Modification with a fourfold molar excess of BHNA did not denature the enzyme or alter its aggregation state significantly. Incorporation of 1 mol of BHNA per mole of protomer resulted in a 40% quenching of the native enzyme fluorescence. The CD spectrum of this BHNA-modified enzyme showed that the bound chromophore was immobilized. The binding of cAMP to PFK was unaffected by modification with a fourfold molar excess of BHNA, but the binding of ATP at its single detectable site (apparent $K_d = 3.2$ μM) was abolished. Spin-labeling of the most reactive thiol group per protomer with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide abolished its reaction with DTNB and resulted in a 40% loss of catalytic activity. No quenching of the native protein fluorescence accompanied this modification. Because modification of the single very reactive thiol group per protomer of rabbit muscle PFK does not result in complete abolition of the enzyme's catalytic activity, it cannot be an essential active site functional group. While there appears to be an intimate connection between at least one MgATP binding site and this thiol group, the observation that BHNA-modified PFK still exhibits a low level of catalytic activity suggests that the thiol group does not directly bind MgATP. The reactive thiol plays no role in the binding of cAMP. Finally, depending on the modifying reagent, a conformational change may accompany sulfhydryl modification and influence catalytic activity and binding at one or more MgATP sites.

Although phosphofructokinase (PFK) is an important enzyme in the regulation of glycolysis, relatively little is known about the constitution of its active site(s) and about its mechanism of action. In 1967, Paetkau and Lardy[1] showed that sulfhydryl groups were necessary for the catalytic activity of the muscle enzyme. Froede et al.[2] modified sheep heart PFK with the sulfhydryl-specific reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate (PCMB), and concluded that the sulfhydryl groups were not at the catalytic site but linked to it by a conformational change. The evidence for the absence of sulfhydryl groups from the active site was twofold: (1) the degree of inactivation was dependent on the sulfhydryl reagent employed, and (2) neither substrate, fructose 6-phosphate (F6P) or adenosine 5′-triphosphate (ATP), protected the enzyme from inactivation by these reagents. No evidence supporting the hypothetical conformational change was presened. On the other hand, Younathan et al.[3] suggested the proximity of thiol groups to the catalytic site of rabbit muscle PFK, since magnesium adenosine 5′-triphosphate (MgATP) and F6P protected the enzyme against inactivation by iodoacetamide, and F6P prevented the modification of two thiol groups per protomer by DTNB.