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IONIC STRENGTH, pH, AND THE ELECTROSTATIC CORRECTION OF REDOX PROTEIN REACTION RATES

Benjamin A. Feinberg and Warren V. Johnson

Department of Chemistry
The University of Wisconsin-Milwaukee
Milwaukee, Wisconsin 53201

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SUMMARY: A kinetic study was made of the oxidation of reduced Chromatium vinosum high potential iron-sulfur protein (HiPIP) by ferricyanide as a function of both ionic strength and pH. From the ionic strength results at each of several pH's, the calculated net charge of the protein was found to become more negative as the pH increased and compared well with the net charge of the protein based on the primary sequence. Also the rates were ionic strength dependent at each pH. When the rates were corrected for electrostatic interactions between the negatively charged ferricyanide and the protein at each pH, two apparent pK_a's were observed at about pH 7 and 10. It is concluded that since the net redox protein charge does change with pH, that in rate-pH profile and other kinetic studies, especially of nonphysiological redox couples, the second order rate constants need to be electrostatically corrected. Through the electrostatic correction of these rates, it is then possible to partition purely electrostatic effects from mechanistically significant ionizations of specific amino acid residues.

INTRODUCTION

An important difference between electron transfer reactions of redox proteins and classical enzymic reactions is that in electron transfer reactions, there is no bond breaking and/or bond making. This difference results in a redistribution of the relative importance of the various contributions to the transition state free energy (1). More specifically the Gibbs free energy change associated with the electrostatic interactions between redox proteins become important and observable with changing ionic strength. A commonly used method for detecting mechanistically important ionizations in both redox and other reactions is to examine the rate of the reaction as a function of pH. In the case of redox proteins, however, the change of the charge of the

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protein with changing pH can seriously mask mechanistically important ionizations since the electrostatic contribution to the observed kinetics can be significant. For this reason it appears necessary to correct the rate constant at each pH for electrostatic interactions and further, through such corrections, to partition the influence of the overall charge of the protein from the influence of mechanistically important ionizations of specific amino acid residues.

In more recent work, Feinberg, et al. (2) have electrostatically corrected second order rate constants in order to partition electrostatic and conformational contribution to the kinetics of cytochrome c derivatives. In order to explore further the importance and need to electrostatically correct the second order rate of redox proteins reactions (especially for nonphysiological redox couples such as this) a detailed kinetic study of the oxidation of reduced Chromatium vinosum high potential protein (HiPIP) by ferricyanide was done as a function of both ionic strength and pH.

EXPERIMENTAL PROCEDURE

Potassium ferricyanide and sodium dithionite were obtained from Fisher; 2-mercaptoethanol was obtained from Aldrich, and Tris base from Sigma. All other chemicals used were of reagent grade and only double distilled water was used. All pH measurements were made with a Corning Model 12 pH meter with Corning 476050 combination electrode. Spectrophotometric measurements were made with a Varian Cary 17 spectrophotometer. HiPIP was isolated and purified as described by Bartsch (3, 4). For the kinetic experiments extinction coefficients determined by Dus were used (5). Kinetic studies were carried out with a Durrum stopped flow spectrophotometer interfaced with a Northern Scientific NS-560 time averaging computer. Data were treated by a regression analysis of the linear form of the first order rate law and second order rates were determined under pseudo-first order conditions.

All buffers were deaerated 30 minutes with nitrogen that had passed through an oxygen scrubbing tower. For buffers with pH's of 10.15 and 11.3 a drying tube containing sodium hydroxide was attached atop a miriad bottle. Stock solutions of reduced HiPIP were prepared by the addition of a slight excess of dithionite, passage over a small Sephadex G-25 column preequilibrated at the appropriate pH and ionic strength, collected in a ground glass stoppered

vial and diluted to the appropriate concentration with buffer. In all cases the rate of the reaction was measured at three different excesses (10 fold and greater) of ferricyanide to demonstrate second order kinetics.

Electrostatic Corrections. The kinetic-ionic strength data were analyzed by a non-linear least squares regression analysis of Equation 1 derived by Wherland and Gray (6) from the Marcus theory of outer sphere electron transfer reactions and Debye potential theory:

$$\ln k_I = \ln k_\infty - 3.576 \left[\frac{e^{-\kappa R_A}}{1 + \kappa R_B} + \frac{e^{-\kappa R_B}}{1 + \kappa R_A} \right] \left[\frac{Z_A \cdot Z_B}{R_A + R_B} \right] \quad (1)$$

where k_I is the observed rate constant at ionic strength I, Z_A and Z_B are the net charges of the reactants of A and B respectively, R_A and R_B are the radii of A and B respectively, $\kappa = .329\ I^{\frac{1}{2}}\ \text{Å}^{-1}$, and k_∞ is the rate constant at infinite ionic strength (i.e., the rate that is independent of electrostatic interactions since the charges of reactants are fully shielded from each other by the ions of the electrolyte solution). The rate constant at infinite ionic strength is the electrostatically corrected rate constant, k_∞. From the curve fit both the apparent net charge of the protein and k_∞ are obtained. The predicted redox protein charge from Equation 1 was compared to the net charge of the protein as determined from the sequence (Z_{seq}) at a particular pH.

RESULTS AND DISCUSSION

Electrostatically Corrected Rates. The second order rate constants were obtained for the oxidation of reduced Chromatium vinosum HiPIP by ferricyanide as a function of ionic strength and pH and the results are shown in Table 1. Clearly, at a given pH, the observed second order rates increase since the electrostatic repulsion between the negatively charge ferricyanide (-3) and the relatively negative HiPIP [(pI = 3.67 (5)] is decreased by electrolyte shielding as the ionic strength increases. When the rates are corrected for the electrostatic interactions (pH 5, $k_\infty = 4.1 \times 10^3\ M^{-1}s^{-1}$; pH 7, $k_\infty = 5.1 \times 10^3\ M^{-1}s^{-1}$; pH 8, $k_\infty = 6.2 \times 10^3\ M^{-1}s^{-1}$; pH 9.4, $k_\infty = 6.6 \times 10^3\ M^{-1}s^{-1}$; pH 10.2, $k_\infty = 6 \times 10^3\ M^{-1}s^{-1}$; pH 11.3, $k_\infty 5.5 \times 10^3\ M^{-1}s^{-1}$) they are observed to change as a function of pH as shown in Figure 1. The results contrast strongly with the rate at different pH's at $\mu \simeq .200$ in which all the rates are about 3.50

Table 1. Rate of HiPIP oxidation by ferricyanide as a function of pH and ionic strength, μ.

pH	μ	$k\ M^{-1}s^{-1} \times 10^{-3}$	pH	μ	$k\ M^{-1}s^{-1} \times 10^{-3}$
5.0	.0051	1.05	9.35	.0298	.800
	.0351	1.60		.0598	1.35
	.0651	2.13		.0898	2.06
	.1051	2.80		.1298	2.52
	.2051	3.51		.2298	3.87
7.0	.0088	.546	10.15	.0151	.477
	.0338	1.23		.0451	1.31
	.0588	1.68		.0751	1.89
	.1088	2.41		.1151	2.37
	.1588	3.05		.2151	3.35
	.2088	3.30			
			11.3	.0166	.391
7.95	.0136	.506		.0466	.937
	.0436	1.30		.0766	1.43
	.0736	1.85		.1166	1.91
	.1136	2.54		.2166	3.28
	.2136	3.66			

$\times 10^{3} M^{-1}s^{-1}$. Thus in earlier work on the rate vs pH study of the ferricyanide oxidation of Chromatium HiPIP, no variation of rate was observed with electrostatically uncorrected rates (7). More critically Figure 1 yielded two apparent pK_a's at pH≈7 and pH≈9. Strong evidence has been obtained to show that the first $pK_a \approx 7$ is due to the participation of the sole His 42 in the redox mechanism (8).

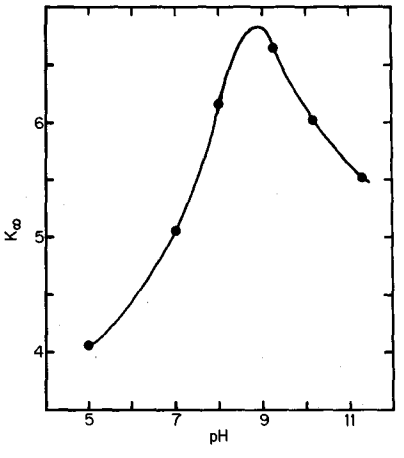

Figure 1. Plot of electrostatically corrected second order rates of oxidation of HiPIP by ferricyanide versus pH.

Table 2

pH	Z Eq 1	Z Seq
5.0	-2.0	-2.2
7.0	-3.6	-3.9
7.95	-4.5	-4.1
9.35	-5.2	-5.3
10.15	-4.7	-7.0
11.3	-5.2	-10.2

Net protein charge and pH. In regard to protein charge, using Z=-3 and R=4.0 Å for ferricyanide, and R=15.1 Å for HiPIP, the predic charge for HiPIP as a function of pH (as calculated from the kinetic ionic strength data and Eq 1) is shown in Table 2 and compared with the net charge of the protein based on the primary sequence (9) using standard pK_a's (10). Detailed titration and protein charge studies similar to those of Gurd (10) have not yet been done for any of the HiPIP's, thus the standard pK_a's are only approximate. As can be seen in Table 2, there is (1) a significant change in the charge of the protein as the pH is increased (HiPIP) becomes more negative as deprotonation occurs), (2) Eq 1 generally provides a good approximation of the net charge of the protein from the kinetic-ionic strength data and (3) electrostatic interactions between the reactants change as a function of pH.

It is concluded that because of the significant changes in the net charge of HiPIP (and redox proteins in general) with pH, it is necessary to obtain electrostatically corrected rate constants for many redox protein reactions. This means, too, that the ionic strength at which kinetic studies are done is also important and again, electrostatically corrected rate constants eliminate many of the problems associated with variations in ionic strength.

Mechanistically significant ionizations of specific amino acid residues can be more clearly revealed from rate-pH profiles with electrostatic correction of rates. Thus far these conclusions apply to many of the non-physiological small molecule-protein and protein-protein redox reactions that are actively being explored to define the electron transfer mechanism of several redox proteins, including the high potential iron-sulfur proteins.

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