COMPARATIVE KINETIC-IONIC STRENGTH STUDY OF TWO DIFFERENTLY CHARGED CYTOCHROMES c: EFFECTS ARE LIMITED TO OVERALL CHARGE

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Received October 3,1977

SUMMARY: The reduction kinetics of two differently charged cytochromes c, horse cytochrome c and Rhodosprillum rubrum cytochrome c_2, by ferrous $EDTA^{2-}$ were studied as a function of ionic strength. Since both proteins have nearly the same heme edge region, but have very different overall surface charge, this comparative study served as a direct test of the utility of small nonbinding non-physiological redox agents in the study of the charge of electron transfer sites of redox proteins. Calculations based on the ionic strength-kinetic data yielded protein charges of +10 and +2.3 for cytochrome c and cytochrome c_2 respectively and compared well with values of +9 and +3 for the overall charge of the proteins based on acidic and basic amino acid residues. It is concluded that ionic strength effects upon the redox kinetics with such nonbinding nonphysiological redox agents reflect the influence of the overall protein charge and not the localized charge of the presumed site of electron transfer.

INTRODUCTION

Oxidation-reduction proteins and enzymes are of particular biological and chemical interest since they catalyze the transformation of a variety of substrates and also play an important role in the transfer and modulation of energy throughout living cells. As an important member of the mitochondrial terminal respriatory chain, cytochrome c has received an enormous amount of attention over the last few decades with regard to both its structure (1,2) and function (2,3). Notwithstanding this

ISSN 0006-291X

extensive body of work, the detailed mechanism of the electron transfer of cytochrome c and its interaction with redox donor/-acceptors has not been fully elucidated. Two approaches to cytochrome c electron transfer mechanism have been (1) the study of the oxidation-reduction of cytochromes with nonphysiological oxidant/reductants and (2) enzymic studies of cytochrome c with its biological redox donor/acceptors. As a result of the non-physiological studies, it appears that the obvious pathway for the electron to and from the cytochrome heme iron is via the exposed heme edge at the front side of the molecule (4-6).

The exposed heme edge of the cytochrome molecule is surrounded by several basic lysine amino acid residues and further, the overall charge distribution on the cytochrome surface is asymmetric, with the front heme edge region being the most positive. Although it has been known for some time that the lysine residues play an important role in the biological oxidation of cytochrome c only recently have Ferugson-Miller et al. demonstrated by chemical derivative studies that the lysines in the upper left area of the heme edge play a crucial role in the binding of cytochrome to the oxidase (7). They were careful to point out that the enzymic kinetic studies demonstrated only the effects of binding and did not give information as to whether or not electron transfer kinetic changes have occurred.

In regard to the oxidation and reduction of cytochromes c by small nonbinding nonphysiological redox agents much work has been done (3). Use has also been made of the kinetics of redox proteins with small redox agents as a function of ionic strength to probe the nature of the charge present at the site or region of electron transfer (8,9). In order to assess more fully the

use of such kinetic-ionic strength experiments, a rigorous experimental test was devised. A comparative study was made of the reduction of two differently charged cytochromes c, horse cytochrome c and Rhodospirillum rubrum cytochrome c_2, by ferrous $EDTA^{2-}$ as a function of ionic strength. Since both proteins have nearly the same heme edge region, but have very different pI values, these experiments serve to evaluate clearly whether it is localized or overall protein charge that effects the observed kinetics.

MATERIALS AND METHODS

Horse heart cytochrome c was purchased from Sigma (Grade VI) and was purified by carboxymethyl cellulose (CMC) chromatography as described by Brautigan et al. (10). Rhodospirillum rubrum was cultured, harvested, and the cytochrome c_2 isolated and purified to homogeneity by CMC gradient chromatography as described by Sponholtz et al. (11). The overall charges of horse cytochrome c and cytochrome c_2 were calculated to be +9 and +3 respectively using the method described by Koppenol et al. (12).

The kinetics of the reduction of the cytochromes by ferrous $EDTA^{2-}$ were done using chronoamperometry as a substitute for stopped flow kinetics. The details of this methodology are discussed elsewhere (13). In this method ferrous $EDTA^{2-}$ is generated in situ in a solution of ferric EDTA and the cytochrome. The electrochemical current response reflecting the interaction of the ferrous $EDTA^{2-}$ with the cytochrome was then used to evaluate the second order homogenous rate constants for the reduction process for a given protein at a given ionic strength. All kinetics were done at pH 7.0 and ionic strength was increased by using concentrated pH 7 phosphate buffer. Experiments were also done in which the ionic strength was increased with sodium chloride and the rates obtained were identical to those obtained by Gray for the same reaction for horse cytochrome c (8), thus demonstrating the validity of chronoamperometry as an alternative to stopped flow kinetics.

Treatment of the kinetic-ionic strength data was done using the Marcus theory treatment as derived by Wherland and Gray (14) which takes into consideration the coulombic interaction between the reactants and also differences in their size as shown in Equation (1):

$$\ln k = \ln k_\infty - 3.756\left[\frac{e^{-\kappa R_1}}{1 + \kappa R_2} + \frac{e^{-\kappa R_2}}{1 + \kappa R_1}\right]\left[\frac{Z_1 Z_2}{R_1 + R_2}\right] \qquad (1)$$

where k is the observed rate constant, k_∞ is the rate constant

TABLE 1. Kinetic constants for the reduction of horse cytochrome c and R. rubrum cytochrome c_2 by ferrous $EDTA^{2-}$

Horse cytochrome c		R. rubrum cytochrome c_2	
μ	$k \times 10^{-4}$ $M^{-1}s^{-1}$	μ	$k \times 10^{-6}$ $M^{-1}s^{-1}$
.037	2.60	.016	1.10
.046	1.76	.040	.92
.055	1.40	.090	.72
.076	1.00		
.112	.70		

at infinite ionic strength, R_1 and R_2 are the radii of the reacting species (16.5 Å for cytochrome and 4 Å for ferrous $EDTA^{2-}$) Z_1 and Z_2 are the overall charges on the reacting species (ferrous $EDTA^{2-}$ = -2) $\kappa = .329\sqrt{\mu}$ $Å^{-1}$ where μ is the ionic strength. Their work demonstrated the inadequacies of the classical Debye-Huckel treatment.

RESULTS AND DISCUSSION

The second order kinetic rate constants for the reduction of both horse cytochrome c and Rhodospirillum rubrum cytochrome c_2 by ferrous $EDTA^{2-}$ were determined by chronoamperometry. The results are shown in Table 1 and plotted as a curve fitted function of Equation (1) in Figure 1 for cytochrome c and cytochrome c_2 respectively. In fitting the data to Equation (1), with the charge of ferrous $EDTA^{2-}$ being -2, the Z values for the redox proteins were calculated to be $Z_{\text{cytochrome c}} = +10$ and $Z_{\text{cytochrome } c_2} = +2.3$; these Z values represent the charge of the protein interacting with the ferrous $EDTA^{2-}$ and they are remarkably close to the overall protein charge values of +9 and +3 calculated on the basis of the negative and positive amino acid residues (12).

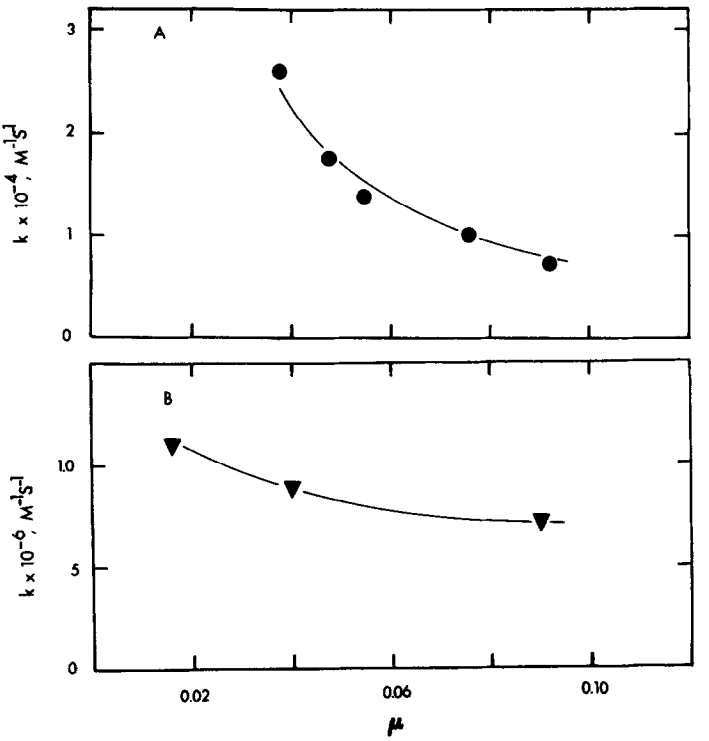

FIGURE 1. Rate of reduction of (A) horse cytochrome c and (B) R. rubrum cytochrome c_2 with ferrous $EDTA^{2-}$ as a function of ionic strength. Solid line is best fit of Eq. (1) to the data. In both (A) and (B), cytochrome = 10^{-4} M, ferrous $EDTA^{2-}$: (A), 2×10^{-5} and (B), 1×10^{-5} M.

The kinetic rates for cytochrome c_2 at a given ionic strength are larger than those for horse cytochrome c almost certainly because of the greater redox potential of cytochrome c_2 of +320 mV compared to +262 mV for horse cytochrome.

In a comparative interpretation of the X-ray and primary structure of horse and R. rubrum cytochromes, Salemme et al. have demonstrated clearly that the greatest similarity between the two cytochromes is the front heme edge region (15). Even though the calculated overall charges and pI's are quite different (pI = +10 for horse cytochrome c, pI = +6.2 for cytochrome c_2) virtually all the lysines present in the biological

binding domain (7) of horse heart cytochrome c are conserved in cytochrome c_2; thus, in spite of the great similarity of the heme edge region, the protein charges calculated from the ionic strength-kinetic data reflect only the overall surface charge of the proteins, and not the localized charge of the heme edge region. These results are expected in light of work by Koppenol et al. (12) which has shown that the cytochrome molecule behaves strictly as a monopole except at distances very close to its surface. More specifically if the heme edge region were to have determined the coulombic interaction between the cytochrome and ferrous $EDTA^{2-}$, then the kinetic-ionic strength data would have yielded the same $Z_{protein}$, since the heme edge regions are nearly the same.

In light of the remarkable agreement between the experimentally determined charges of the proteins compared with the calculated overall charge based on primary structure, it is concluded that the ionic strength dependence of the observed kinetics reflects the overall charge of the reactants and more importantly the overall charge of the protein. Lastly, it is apparent that the use of such kinetic-ionic strength studies as a means for determining the charge of electron transfer sites of redox proteins deserves reevaluation and reinterpretation.

ACKNOWLEDGEMENTS: Both B.A.F. and M.D.R. acknowledge the Donors of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. B.A.F. also gratefully acknowledges two awards from The University of Wisconsin-Milwaukee Graduate School Research Committee in support of this research. One author, B.A.F., would also like to thank Professor Paul Loach of Northwestern University, Evanston, Illinois, for the gift of lyophilized Rhodospirillum rubrum cells used in starting our cultures. The excellent technical assistance of J. Lang, R. Friedrich, L. Neeck, R. Simondsen, and G. Aprahamian in the culturing, isolation, and preparation of cytochrome c_2 is gratefully acknowledged.

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