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PARTITIONING OF ELECTROSTATIC AND CONFORMATIONAL CONTRIBUTIONS IN THE REDOX REACTIONS OF MODIFIED CYTOCHROMES *c*

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Summary

The reduction of acetylated, fully succinylated and dicarboxymethyl horse cytochromes *c* by the radicals $CH_3\dot{C}H(OH)$, CO_2^-, O_2^-, and e_{aq}^-, and the oxidation of the reduced cytochrome *c* derivatives by $Fe(CN)_6^{3-}$ were studied using the pulse radiolysis technique. Many of the reactions were also examined as a function of ionic strength. By obtaining rate constants for the reactions of differently charged small molecules redox agents with the differently charged cytochrome *c* derivatives at both zero ionic strength and infinite ionic strength, electrostatic and conformational contributions to the electron transfer mechanism were effectively partitioned from each other in some cases. In regard to cytochrome *c* electron transfer mechanism, the results, especially those for which conformational influences predominate, are supportive of the electron being transferred in the heme edge region.

Introduction

An important approach in the study of structure-function relationship of cytochrome *c* is the use of chemical derivatives of the protein [1]. In this approach the effect of chemical modification of specific amino acid residues upon the conformation, net charge, and reactivity of the protein are important to consider. In some derivatives of cytochrome *c* the modification does not

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Abbreviations: Ac-, acetylated; Suc-, succinylated; Cxm-, carboxymethyl methionine-65,80.

change the overall conformation of the protein, as can be judged from the intact physicochemical properties of the molecules [2], whereas in other cases the native conformation is significantly changed [1].

In the present study, three chemical derivatives of cytochrome *c* were studied: (1) Acetylated cytochrome *c*, in which the ϵ-amino groups of the lysines were transferred into neutral groups. (2) Succinylated cytochrome *c* in which the ϵ-amino groups of the lysines were transferred into negatively charged groups. (3) Carboxymethyl methionine-65,80 cytochrome *c*. The overall charge of the first two derivatives is negative, whereas in the third the charge is positive, similar to that of the unmodified protein. Conformation of the heme region of the first derivative is similar to the native protein, while in the two other derivatives the heme region conformation is very much disrupted. The use of the pulse radiolytic method in studies of biological redox systems is well documented [3—10]. The effects of charge and conformation were studied, and to a large degree partitioned from each other using this method.

Experimental

Materials. Sigma type III horse-heart ferricytochrome *c* was used without further purification. Ac-cytochrome *c* was prepared by reacting cytochrome *c* with acetic anhydride, in the presence of sodium acetate. Suc-cytochrome *c* was prepared by reacting cytochrome *c* with succinic anhydride. Both procedures have been described by Wada and Okunuki [15]. Cxm-cytochrome *c* was prepared by the reaction of cytochrome *c* with bromoacetate in the presence of cyanide, by the method of Schejter and Aviram [16].

The concentration of native Ac- and Suc-cytochromes *c* was determined by the 528 nm, absorbance peak, using $\epsilon = 11.2\ mM^{-1} \cdot cm^{-1}$ [17]. The absorption spectra of Ac- and Suc-cytochromes were found to be identical to that of the native protein, excepting the loss of the 695 nm band in Suc-cytochrome *c* (see Table I). The concentration of Cxm-cytochrome *c* was determined by the 530 nm absorbance peak of the oxidized molecule using $\epsilon = 9.4\ mM^{-1} \cdot cm^{-1}$ [14].

All reagents were of analytical grade, and were used without further purification.

Apparatus and procedures. The Varian linear accelerator of the Hebrew University, the optical and electronic systems and routine procedures have been described elsewhere [3,18]. The pulse radiolytic data were analyzed on-line using a Nova 1200 minicomputer and appropriate interphases developed in the accelerator laboratory.

Analysis of ionic strength effects. The kinetic-ionic strength effects were analysed with either the full Bronsted-Debye-Huckel equation (Eqn. 1) or the Debye-Marcus theory based equation (Eqn. 2) more recently derived by Wherland and Gray [19].

$$\ln k_I = \ln k_0 - \frac{A(Z_A + Z_B)^2 I^{1/2}}{1 + \kappa R^{\neq}_{AB}} + \frac{AZ_A^2 I^{1/2}}{1 + \kappa R_A} + \frac{AZ_B^2 I^{1/2}}{1 + \kappa R_B} \tag{1}$$

$$\ln k_I = \ln k_\infty - 3.576 \left(\frac{e^{-\kappa R A}}{1 + \kappa R_B} + \frac{e^{-\kappa R B}}{1 + \kappa R_A} \right) \left(\frac{Z_A Z_B}{R_A + R_B} \right) \tag{2}$$

TABLE I

PROPERTIES OF NATIVE AND CHEMICALLY MODIFIED CYTOCHROME *c*

Cytochrome *c* derivative	Redox potential (mV)	Z_{Seq} *	Absorption at 695 nm	Spectral changes in the range 400—600 nm	Reaction with CO	Change in the reactivity with oxidase and reductase
Native	+260 [11]	9/8	+	N.A. **	— [11]	N.A.
Acetylated	+155 [12]	—10/—11	~74% that of the native protein	— [14]	— [14]	+ [14]
Succinylated	<—120 [12]	—29/—30	—	Small changes in the Soret band	?	+ [14]
Carboxymethylated	—120 [13]	9/8	—	No changes in positions of the peaks. Lower values at 500—600 nm	+ [14]	+ [14]

* See Procedures for definition of Z_{Seq}.

** N.A., not applicable.

Where at 25°C, the constant $A = 1.174$, k_I is the observed rate constant at a given ionic strength, I; Z_A and Z_B are the net charge on species A and B respectively, R_A and R_B are their radii, $R^{\neq}_{AB}$ is the radius of the transition state complex which in these experiments can be taken to be the sum of the radii of the reactants, $\kappa = 0.329\sqrt{I}$, k_∞ is the rate constant at infinite ionic strength and k_0 is the rate constant at zero ionic strength. The kinetic-ionic strength data were curve fitted to Eqns. 1 and 2 by a nonlinear least squares regression analysis done by computer and computer plotted.

The simplified Bronsted-Debye-Huckel equation, Eqn. 3,

$$\ln k_I = \ln k_0 + AZ_AZ_B\sqrt{I} \tag{3}$$

was not used since it has been shown by Ryan and Feinberg [20,21] to be theoretically and thus experimentally valid only if both A and B have very small radii. Eqns. 1 and 2 provide a good estimate of the net protein charge from the kinetic-ionic strength data as compared to the charge of the protein obtained from the primary sequence and prosthetic group A_{Seq} [20,21]. In these calculations, the experimental protein charges Z_{Eq2} and Z_{Eq3} are obtained from the kinetic-ionic strength data when the radius of the protein and small molecule reductant are known along with the charge of the small molecule reductant. A value of −3 was used for the charge of ferricyanide, although it should be noted that under certain solution conditions, ion pairing between potassium ion and $Fe(CN)_6^{3-}$ has been observed [22].

Results

The reducing radicals and the reduced derivatives were produced in situ according to the reactions that are presented in Table II using appropriate concentrations of reagents. In discussing the various free radical reaction. Table II should be referred to.

Reduction by $CH_3\dot{C}H(OH)$. Solutions contained 100 mM ethanol, 10—25 μM of cytochrome *c* derivative, and 2 mM phosphate buffer (pH = 7.0—7.3). Solutions were saturated with N_2O (~25 mM). The ionic strength was ~0.005. The hydrated electrons formed by the pulse are transformed into OH radicals by N_2O (Reaction 1 of Table II). Under our conditions more than 99% of the $OH^{\cdot}$ radicals reacted with ethanol according to Reaction 9. 99% of the $H^{\cdot}$ atoms reacted according to Reaction 6.

The reductions by $CH_3\dot{C}H(OH)$ were first order processes as shown by the proportionality of the rates to the concentrations of the cytochrome *c* derivatives. Second order rate constants obtained from measurements at 550 and 450 nm are shown in Table III. The value for native cytochrome *c* agrees with a previous determination [4].

Reduction by $CO_2^{\cdot-}$. Solutions contained HCOONa (5—100 mM) and cytochrome *c* derivative and were saturated with N_2O (~25 mM). The pH was set by adding phosphate buffer (2 mM, pH = 7.0—7.2). The hydrated electrons, $OH^{\cdot}$ and $H^{\cdot}$ form $CO_2^{\cdot-}$ via Reactions1, 5 and 7.

TABLE II

REACTIONS OCCURRING IN A PULSE IRRADIATED SOLUTION, IN THE PRESENCE OF VARIOUS SOLUTES

Reaction		k ($M^{-1} \cdot s^{-1}$)	Reference
1	$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + OH^- + OH^\cdot$	$5.6 \cdot 10^9$	23
2	$e^-_{aq} + O_2 \rightarrow O_2^-$	$2 \cdot 10^{10}$	23
3	$e^-_{aq} + Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$	$\leqslant 3 \cdot 10^9$	23
4	$H^\cdot + O_2 \rightarrow HO_2^\cdot$	$2 \cdot 10^{10}$	24
5	$H^\cdot + HCOO^- \rightarrow H_2 + CO_2^-$	$4.5 \cdot 10^8$	24
6	$H^\cdot + CH_3CH_2(OH) \rightarrow H_2 + CH_3\dot{C}H(OH)$	$2.8 \cdot 10^9$	24
7	$OH^\cdot + HCOO^- \rightarrow H_2O + CO_2^-$	$5.2 \cdot 10^8$	25
8	$OH^\cdot + (CH_3)_3COH \rightarrow H_2O + \dot{C}H_2C(CH_3)_2OH$	$1.8 \cdot 10^9$	25
9	$OH^\cdot + CH_3CH_2(OH) \rightarrow H_2O + CH_3\dot{C}H(OH)$	$1.4 \cdot 10^{10}$	25
10	$OH^\cdot$ + NAT (red) $\rightarrow$ products	$4.2 \cdot 10^9$	26
11	$CO_2^- + O_2 \rightarrow CO_2 + O_2^-$	$2.3 \cdot 10^9$	27

The reductions followed first order kinetics, rates being proportional to the concentration of the derivative. Second order rate constants at an ionic strength of 0.1 are shown in Table III. The reduction was followed at 550 nm.

Reduction by O_2^-. Solutions contained 100 mM HCOONa and 20—30 μM of the cytochrome *c* derivative, and were reacted with O_2^-. The pH was set to 7.0 with 2 mM phosphate buffer. O_2^- is produced by the reactions 2 and 4 (Table II) or by the combined reactions 5, 7 and 11 (Table II). Immediately after the pulse, only O_2^- and $HO_2^\cdot$ radicals are present in the solution. These two radicals are in equilibrium with a pK = 4.75 [28], and therefore, at pH = 7.0 practically only O_2^- radicals are present.

Of the three derivatives, only Ac-cytochrome *c* is reduced by O_2^-. The two other derivatives have redox potentials similar to that of O_2^- (E^{0*} = —160 mV, 1 M O_2), and this is probably the reason why these molecules do not react

TABLE III

SECOND ORDER RATE CONSTANTS FOR THE REDUCTION AND OXIDATION OF CYTOCHROME *c* AND ITS DERIVATIVES ($M^{-1} \cdot s^{-1}$)

pH $\simeq$ 7.0.

	I	Cytochrome *c* derivatives			
		Native	Ac-	Cxm-	Suc-
Reductant					
e^-_{aq}	0.1	$3.0 \cdot 10^{10}$	$2.0 \cdot 10^{10}$	$3.3 \cdot 10^{10}$	$4.0 \cdot 10^{10}$
CO_2^-	0.1	$1.0 \cdot 10^8$	$1.5 \cdot 10^9$	$1.3 \cdot 10^8$	$4.0 \cdot 10^9$
O_2^-	0.1	$8.0 \cdot 10^5$	$3.5 \cdot 10^5$	No reduction	No reduction
$CH_3\dot{C}H(OH)$	0.005	$1.8 \cdot 10^8$	$2.5 \cdot 10^8$	$3.5 \cdot 10^8$	$1.8 \cdot 10^9$
Oxidant					
$Fe(CN)_6^{3-}$	0.1	$6.9 \cdot 10^6$	$2.1 \cdot 10^5$	$2.7 \cdot 10^8$	$3.8 \cdot 10^7$

fast enough to compete with the self decay of $O_2^{\cdot-}$ at neutral pH. Under the experimental conditions, the self decay of $O_2^{\cdot-}$ by dismutation is negligible as compared to its reaction with Ac-cytochrome *c*, because of the high ratio of the concentrations of the protein to $O_2^{\cdot-}$. The concentration of $O_2^{\cdot-}$ was 0.5—1 μM.

The second order rate constant of the reduction of Ac-cytochrome *c* by $O_2^{\cdot-}$ at $\mu = 0.1$ was $3.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ (Table III). The reduction was followed at 550 nm. The ratio between the rate constant of the reduction of cytochrome [5—8] and of Ac-cytochrome *c* by $O_2^{\cdot-}$ is in agreement with the results of Azzi et al. [29].

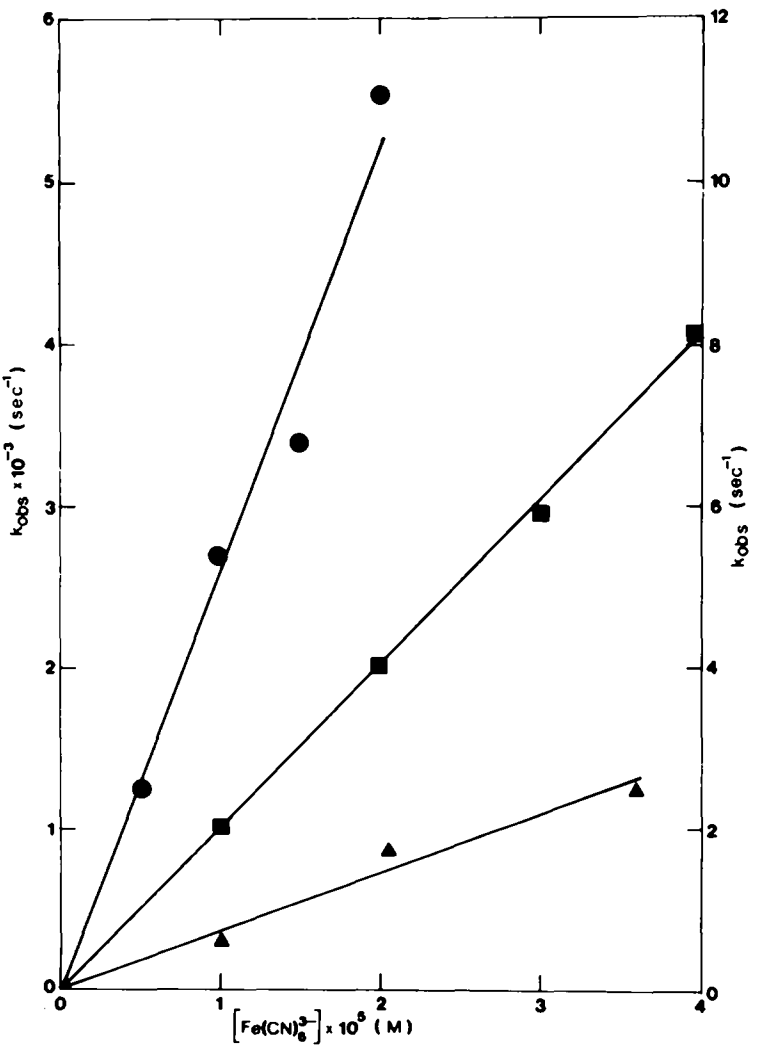


Fig. 1. The dependence of the rate of oxidation of Ac-, Suc-, Cxm-cytochrome *c* by $Fe(CN)_6^{3-}$ on the concentration of $Fe(CN)_6^{3-}$. $I = 0.1$ (phosphate); pH = 7.1. The concentration of the derivatives: $1 \cdot 10^{-5}$ M. ●, Cxm-cytochrome *c* (left scale); ▲, Suc-cytochrome *c* (left scale); ■, Ac-cytochrome *c* (right scale).

Reduction by e^-_{aq}. Solutions containd 10—30 μM of the cytochrome *c* derivative, *tert*-butanol in a concentration 10^4 times that of the protein, and phosphate buffer (45 mM, pH = 6.9—7.1, $I = 0.1$) and were saturated with argon. Most of the OH˙ radicals formed by the pulse are scavenged by *tert*-butanol in Reaction 8, Table II. The decay of the absorption of e^-_{aq} at 580—600 nm was followed. Under our experimental conditions, the decay was pseudo-first order, and its rate was proportional to the concentrations of the derivatives. Second order rate constants are summarized in Table III.

Oxidation by $Fe(CN)_6^{3-}$ *after rapid reduction by* e^-_{aq}. Solutions contained $K_3Fe(CN)_6$, cytochrome *c* derivatives (10—20 μM), *tert*-butanol (100—200 mM), and phosphate buffer in a concentration determined by the desired ionic strength. The pH was 7.0—7.2. Solutions were saturated with argon. Over 99% of the OH˙ radicals were scavenged by *tert*-butanol by Reaction 8, assuming a similar rate of reaction with OH˙ radicals for cytochrome *c* (Reaction 10, Table II), and its derivatives. The *tert*-butanolic radicals do not react with cytochrome *c* [4], and presumably do not react with its derivatives either.

In most of the experiments, the concentration of $Fe(CN)_6^{3-}$ was smaller or

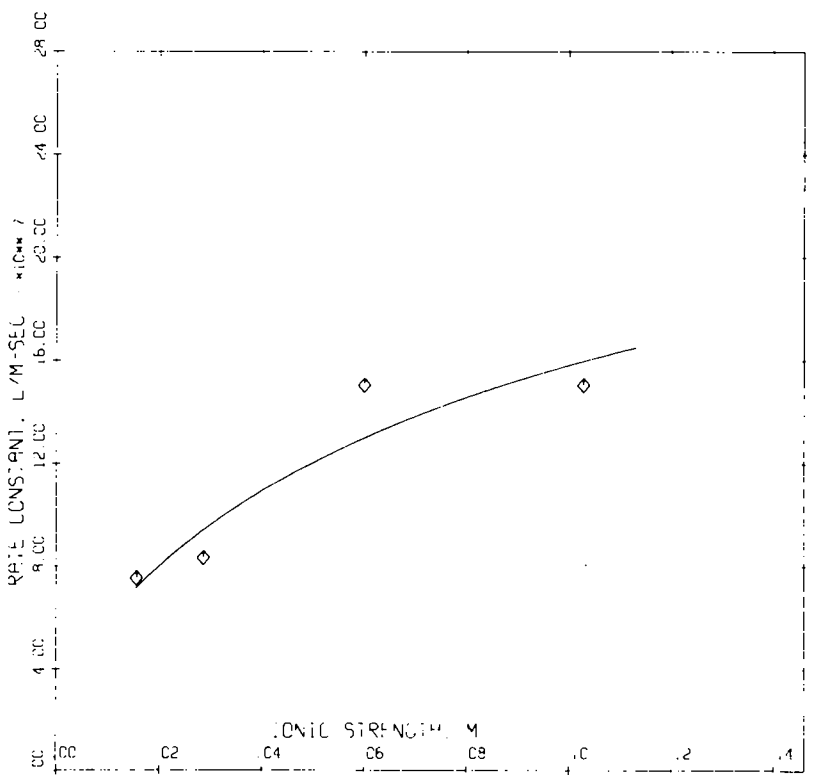
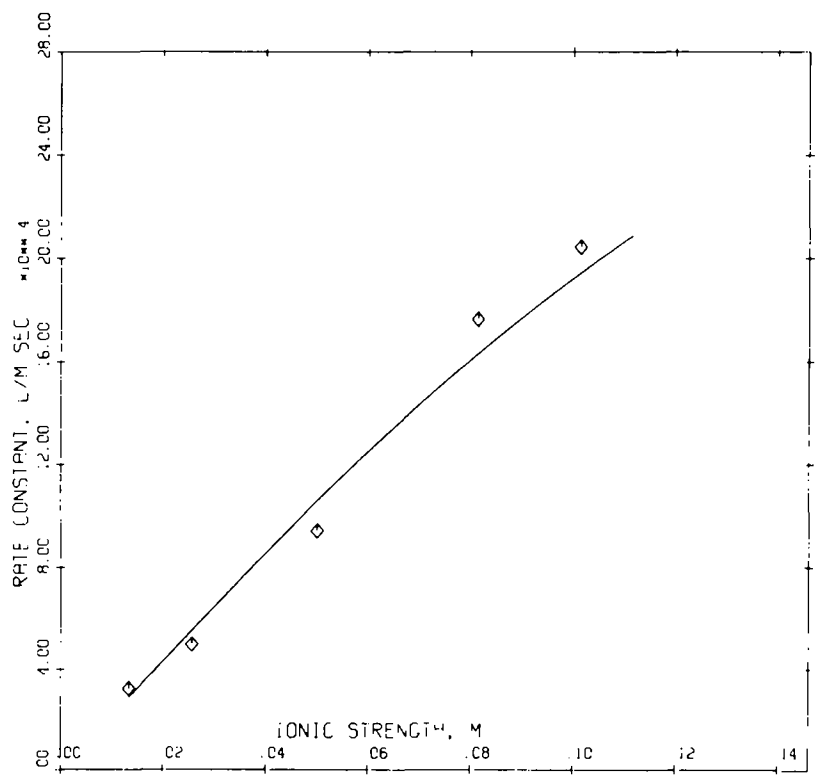


Fig. 2. The variation of the second order rate constant as a function of ionic strength for the reaction Ac-cytochrome *c* (Ox) with CO_2^-. The solid line is the best curve fit to Eqn. 2 and the diamonds are the experimental points.

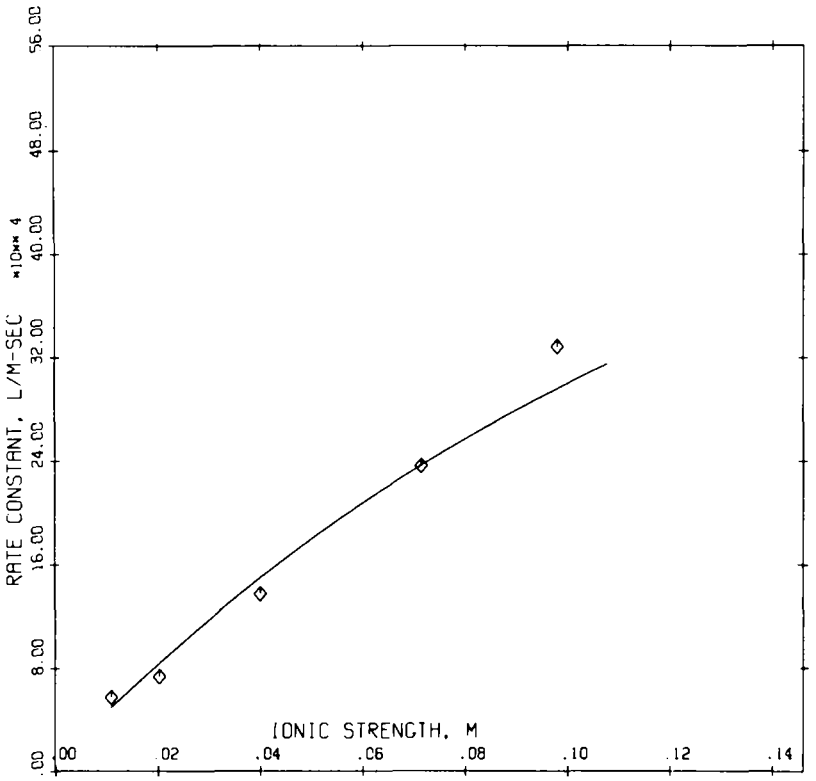
equal to the concentration of cytochrome *c* derivatives, so that more than 95% of e^-_{aq} reaction with the protein (reaction 3, Table II).

Immediately after the pulse was completed, a fast increase of absorbance at 550 nm or a fast decrease of absorbance at 450 nm were observed. This fast process is the reduction of the protein by e^-_{aq}, which was finished within 10 μsec after the pulse. Then, another absorbance change, in the opposite direction, was followed. This later process is the reoxidation of the reduced derivative by $Fe(CN)_6^{3-}$. The dependence of the observed first order rate constant on the concentration of $Fe(CN)_6^{3-}$ ions, in the oxidation of the three derivatives, is shown in Fig. 1. Second order rate constants are summarized in Table III.

Kinetic-ionic strength studies of cytochrome c redox reactions. The dependence of the rate constant for the reduction of Ac-cytochrome *c* by CO_2^- on ionic strength was studied and is shown in Fig. 2 where it is curve fitted to Eqn. 2. Also the dependence of the rate constant for the ferricyanide oxidation of all three derivatives (after their rapid reduction by e^-_{aq}) upon ionic strength was determined and the results are shown in Figs. 3—5. The effective or net protein charges are predicted from Eqns. 1 and 2 and were



Figs. 3—5. The variation of the second order rate constant as a function of ionic strength for the oxidation of Ac-cytochrome *c* (Fig. 3), Suc-cytochrome *c* (Fig. 4) and Cxm-cytochrome *c* (Fig. 5) by $Fe(CN)_6^{3-}$. The solid lines are the best curve fits to Eqn. 2 and the diamonds are the experimental points.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56.00
48.00
40.00
32.00
24.00
16.00
8.00
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IONIC STRENGTH, M
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.10
.12
.14

Fig. 4.

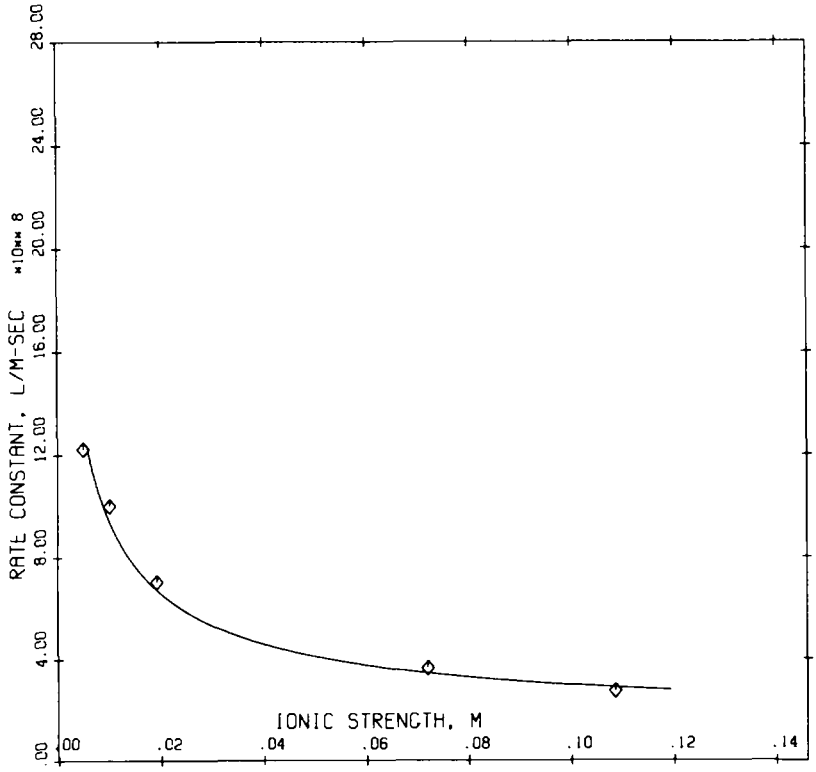
RATE CONSTANT, L/M-SEC
×10** 8
28.00
24.00
20.00
16.00
12.00
8.00
4.00
.00
IONIC STRENGTH, M
.00
.02
.04
.06
.08
.10
.12
.14

Fig. 5.

TABLE IV

KINETIC-IONIC STRENGTH STUDIES OF NATIVE AND MODIFIED CYTOCHROME *c*

Reaction (PH $\simeq$ 7)	$Z_{Eq\ 2}$	$Z_{Eq\ 1}$	Z_{Seq}	k_∞ ($M^{-1} \cdot s^{-1}$)	k_0 ($M^{-1} \cdot s^{-1}$)	Ref.
Cytochrome *c* (red) + $Fe(CN)_6^{3-}$	7.7	4.6	8 *	$3.0 \cdot 10^6$	$1.1 \cdot 10^9$	30
Cytochrome *c* (red) + $Fe(CN)_6^{3-}$	4.4	3.1	8	$4.4 \cdot 10^6$	$3.2 \cdot 10^8$	3
Cxm-cytochrome *c* (red) + $Fe(CN)_6^{3-}$	3.1	1.0	8	$1.7 \cdot 10^8$	$1.9 \cdot 10^9$	This work
Ac-cytochrome *c* (red) + $Fe(CN)_6^{3-}$	−5.8	−9.2	−11	$5.9 \cdot 10^5$	$4.3 \cdot 10^2$	This work
Suc-cytochrome *c* (red) + $Fe(CN)_6^{3-}$	−5.0	−8.2	−30	$7.9 \cdot 10^5$	$1.5 \cdot 10^3$	This work
Cytochrome *c* (Ox) + CO_2^- (pH 6.6)	6.9	6.3	9	$6.4 \cdot 10^8$	$6.5 \cdot 10^9$	5
Ac-cytochrome *c* (Ox) + CO_2^-	−8.0	−11.1	−10	$2.6 \cdot 10^8$	$1.3 \cdot 10^7$	This work

* Titration data gives a charge of 6.3 and 5.3 for horse ferri and ferrocytochrome *c* respectively, (red), reduced; (Ox), oxidized.

determined as were both the k_0 and k_∞ rate constants. These results are shown in Table IV. Also shown in the same table are some results from previous work with native cytochrome *c*.

Discussion

Conformational effects. Two main classes of cytochrome *c* derivatives were studied: (a) acetylated cytochrome *c* in which positively charged lysines are converted to neutral groups. This molecule is negatively charged but has only minor conformational changes in the heme region compared to cytochrome *c*, as is indicated by maintenance of the 695 nm absorption band and by its redox potential (Table I). (b) Succinylated and carboxymethylated cytochrome *c*, where significant disruption of the heme region occurs, as is indicated by the loss of the 695 nm absorption band, the the lowering of the redox potential (Table I). Suc-cytochrome *c* is negatively charged whereas in the Cxm-cytochrome *c* the overall charge is unaltered. In both the heme region is more exposed to the external environment compared to either the native or the acetylated protein.

Kassner [32,33] and Dickerson [34] have suggested a correlation between the redox potential and the hydrophobicity of the heme environment: a less hydrophobic environment (a more open conformation or exposed heme edge region) favors the Fe(III) state, and lowers the redox potential of the protein. Thus the redox potential of the derivative is an indicator of an open conformation in the region of the heme crevice or edge. Since the $CH_3\dot{C}H(OH)$ radical is an uncharged reducing agent, it was used to resolve the effect of the opening of the conformation (and altered redox potential) upon the reactivity of the cytochrome *c* without electrostatic contributions to reactivity. The observed order of reactivity of $CH_3CH(OH)$ with the cytochromes *c* derivatives was: Suc- >> Cxm- > Ac- $\simeq$ native cytochrome *c*. These results are particularly interesting since the slower rates are observed where the redox potential differences between the protein and reducing agent are becoming larger in going from Suc-cytochrome *c* to the native molecule. This is the opposite direction

expected on the basis of redox potential, and thus demonstrates the overriding influence of progressively greater heme edge exposure upon reactivity.

According to the Marcus [35] theory an increase in the reaction rate is expected as the difference in redox potential between the reactants increases. The fact that our results contradict this expectation further indicates the role of conformation upon the observed kinetics; however, the Marcus theory is most valid when the $\Delta G^{0'}$ ($\Delta E^{0'}$) of the reaction is relatively small, a condition which is not fulfilled in this and many other similar studies.

In further considering conformational effects, two observations are relevant: (1) the rate of reduction of Cxm-cytochrome *c* by CO_2^- is somewhat higher than the CO_2^- reduction of native cytochrome *c*. Since both protein molecules have the same charge, the increase in rate of Cxm-cytochrome *c* is attributed to its more open conformation. (2) Ferricyanide oxidized both Suc-cytochrome *c* (in spite of its quite negative charge) and Cxm-cytochrome *c* faster than native or acetylated cytochrome *c*. As noted earlier in Ac-cytochrome *c* the charge is more negative but the heme edge region is left intact much like the native molecule and the ferricyanide oxidation of Ac-cytochrome *c* is slower than native cytochrome *c* (see Table III). A similar effect has been previously observed for the oxidation of non-relaxed cytochrome *c* which has a more open conformation than that of relaxed cytochrome *c* [3,36].

Partitioning of electrostatic and conformational contributions to redox kinetics. The positive charge of cytochrome *c* [11] plays a major role in its interaction with biological redox donor/acceptors as well as with nonbiological redox agents. More critically it has been shown by chemical modification of specific lysine residue of horse cytochrome *c*, that certain positively charged lysines are necessary for the binding of cytochrome *c* to cytochrome *c* oxidase and are thus required for biological activity [37,38]. One way electron transfer protein reactions differ from classical enzyme reactions is that in the former there is no covalent bond making or breaking and this results in a redistribution of the various contributions to the transition state free energy. The result of this redistribution is that electrostatic interactions between redox proteins and their reductants/oxidants become important and observable in kinetic-ionic strength experiments.

In order to obtain more quantitative information regarding both electrostatic and conformational effects upon mechanisms (and a partitioning of these effects) the experimental results are discussed in terms of k_0 and k_∞ rate constants which are obtaind from the kinetic ionic strength studies as noted in Experimental; k_0 is the rate constant at zero ionic strength where electrostatic interactions are at their maximum and k_∞ is the rate constant at infinite ionic strength were electrostatic interactions are nil. In the latter case, the influence of redox potential and conformation are predominant.

Since the conformation of Ac-cytochrome *c* is only slightly different from that of the native molecule, the influence of charge can be examined without the interference of large conformational influences. Thus while the k_∞ for the CO_2^- reduction of cytochrome *c* and its acetylated derivative are nearly the same ($6.4 \cdot 10^8\ M^{-1} \cdot s^{-1}$ vs. $2.6 \cdot 10^8\ M^{-1} \cdot s^{-1}$ respectively), the influence of electrostatic repulsion is seen in the more than two orders of magnitude dif-

ference in the k_0 rates of $6.5 \cdot 10^9\ M^{-1} \cdot s^{-1}$ vs. $1.3 \cdot 10^7\ M^{-1} \cdot s^{-1}$ respectively. The same pattern is observed even more dramatically in the oxidation of these two protein molecules by $Fe(CN)_6^{3-}$. The k_0 rate of Ac-cytochrome *c* is about six orders of magnitude slower than that for cytochrome *c* ($4.3 \cdot 10^2\ M^{-1} \cdot s^{-1}$ vs. $3.2 \cdot 10^8\ M^{-1} \cdot s^{-1}$ respectively). In contrast the electrostatically corrected rates, k_∞, are similar and differ only by an order of magnitude ($5.9 \cdot 10^5\ M^{-1} \cdot s^{-1}$ vs. $4.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$) (Table IV).

It is interesting to note, in light of the earlier discussion of conformational effects, that the k_∞ value of ferricyanide oxidation of Cxm-cytochrome *c* is about two orders of magnitude greater than the similarly charged native cytochrome *c* ($1.7 \cdot 10^8\ M^{-1} \cdot s^{-1}$ vs. $4.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$); this difference in rate is understandable in terms of both the greater exposure of the heme edge region of Cxm-cytochrome *c* and its lower redox potential. In examining the electrostatically informative k_0 values for their ferricyanide oxidation of Cxm-cytochrome *c*, it is consistent that their k_0 values differ by less than an order of magnitude which is expected since their charge is the same. The rate constants at $I = 0.1$ shown in Table III corroborate the above interpretation.

In Table III, the rates of e^-_{aq} reduction are shown for cytochrome *c* and its derivatives. They are all nearly identical in the range of $2\text{—}4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. The hydrated electron is among the most powerful of reductants and is thus one of the least discriminating with regard to either electrostatic or conformational effects. It is also possible, but not discernible from this study, that e^-_{aq} reduces cytochrome *c* and its derivatives by a mechanism different from $CH_3CH(OH)$ or CO_2^- as has been previously suggested [4].

Net protein charge and kinetics. As noted in Experimental the kinetic-ionic strength experiments also provide the net protein charge, and these results are shown in Table IV. The predicted net protein charge (e.g. $Z_{Eq\,2}$) for the native and modified cytochromes *c* compare well with the net protein charge obtained from the sequence and prosthetic group, Z_{Seq}. The notable exception is the charge predicted for Cxm-cytochrome *c*. Although it would be interesting to postulate for the ferricyanide oxidation reactions that the decrease of charge on native and Cxm-cytochrome *c* is due to ferricyanide binding, this is not the case since in this work the concentration of ferricyanide used is low and the amount of ferricyanide-cytochrome *c* complex formed is very small [3,40]. The close correlation between the predicted charges and Z_{Seq} for both the positively and negatively charged cytochromes *c* demonstrate the role of net protein charge on the observed kinetics of electron transfer.

In showing consistency between the predicted net protein charges and k_∞, it is important to note that the k_∞ rates of ferricyanide oxidation of cytochrome *c* as compared to Ac- and Suc-cytochromes (approx. $3.5 \cdot 10^6\ M^{-1} \cdot s^{-1}$ vs. 5.9 and $7.9 \cdot 10^5\ M^{-1} \cdot s^{-1}$ respectively) are nearly identical to the results of LeBon and Cassatt [39] on ferricyanide oxidation of native cytochrome ($k = 8.0 \cdot 10^6\ M^{-1} \cdot s^{-1}$) and trifluoroacetylated cytochrome *c* ($k = 3.0 \cdot 10^5\ M^{-1} \cdot s^{-1}$) both at $I = 0.1$. Since the values for k_∞ are already electrostatically corrected, the difference between native and Ac-cytochrome, and native and trifluoroacetylated cytochrome cannot be adequately explained by differences in electrostatic interactions as once thought.

The results presented and discussed in this work are consistent with and support the conclusion that cytochrome *c* electron transfer takes place in the heme edge region. Through the combination of different cytochrome *c* derivatives with differently charged redox agents and the influence of ionic strength, the contributions of conformation and net protein charge (electrostatics) to its function (kinetics) have been significantly partitioned from each other and a clearer view of their role in the electron transfer mechanism has been obtained.

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