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

MASTER

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Work carried out during the period:
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SUMMARY

The reduction of acetylated, fully succinylated and dicarboxymethyl horse cytochromes *c* by the radicals $\text{CH}_3\dot{\text{C}}\text{H OH}$, CO_2^- , O_2^- , and e^-_{aq} , and the oxidation of the reduced cytochrome *c* derivatives by $\text{Fe}(\text{CN})_6^{3-}$ 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

In order to understand the mechanism of electron transfer of the cytochromes *c*, the relationship between their structure and function have to be thoroughly studied. One important approach to this problem 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 change the overall conformation of the protein, as can be judged from the intact physico-chemical properties of these molecules (2), whereas in other cases the native conformation is significantly changed (1).

In the present study, cytochrome *c* derivatives in which the ϵ -amino groups of the lysines were transformed into neutral groups by acetylation, or into negatively charged groups by succinylation, were studied (see Table 1). In the case of acetylated cytochrome *c* (ACC), the conformation of the protein is only slightly perturbed (1), as indicated by the minor changes in the physico-chemical properties of this derivative. In the case of succinylated cytochrome *c* (SUCC), the conformation of the protein is much more disrupted and open than that of the native protein and the 695 nm band is lost. A third and important derivative studied was the dicarboxymethyl methionine-65, 80-cytochrome *c* (DCMC). Like SUCC, it has disrupted heme coordination and a more open conformation than native protein, but doesn't have any significant change in the charge of the protein.

The effects of charge and conformation were studied, and to a large degree, partitioned from each other using the pulse radiolytic technique (3-6). The application of this technique to other biological systems, including cytochrome *c* has been reviewed (6).

The application of the pulse radiolytic method in this kinetic redox study is particularly appropriate, since by generating powerful reductants, the large free energy driving force for the reaction becomes essentially a constant and permits the observation of electrostatic and conformational influences on the kinetics.

EXPERIMENTAL

Materials. Sigma type III horse-heart ferricytochrome *c* was used without further purification. ACC was prepared by reacting cytochrome *c* with acetic anhydride, in the presence of sodium acetate. SUCC was prepared by reacting cytochrome *c* with succinic anhydride. Both procedures have been described Wada and Okunuki (11). DCMC was prepared by the reaction of cytochrome *c* with bromoacetate in the presence of cyanide, by method of Schejter and Aviram (12).

The concentration of native cytochrome *c* (NAT), ACC, and SUCC were determined by the 528 nm absorption peak, using $\epsilon = 11.2 \text{ mM}^{-1} \text{ cm}^{-1}$ (13). The absorption spectra of ACC and of SUCC were found to be identical to that of the native protein, excepting the loss of the 695 nm band in SUCC (see Table 1). The concentration of DCMC was determined by the 530 nm absorption peak of the oxidized molecule using $\epsilon = 9.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (10).

Water used was triply distilled. Argon and N_2O were supplied

by Matheson. T-Butanol (Merck) and ethanol (Fluka) contained no impurities detectable by ultraviolet absorption spectrometry.

$K_3Fe(CN)_6$ and $HCOONa$ were supplied by B.D.H., $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ by Mallinckrodt. All reagents were of analytical grade, and were used without further purification.

Apparatus. The Varian linear accelerator of the Hebrew University and the optical and electronic systems have been described elsewhere (14). The irradiation cell was a 1 x 1 x 2 cm rectangular flow-through cell fabricated of spectrosil. The optical path was 3 cm, by passing the analytical light three times through the cell. Spectra of solutions were taken on a Cary 14 spectrophotometer and pH measurements were made with a radiometer pHM 52 digital pH meter.

Procedures. Solutions were prepared in glass bottles less than 15 hours before use. Deaeration of solutions and saturation with N_2O were accomplished by sweeping with argon or N_2O for about 15 minutes, in large glass syringes equipped with capillary standard taper joints. Irradiation was carried out no more than 0.5 hours after sweeping with the gas.

The absorbed dose per pulse was determined routinely using the spectrum of hydrated electrons produced by pulsing 10 mM aqueous ethanol at pH = 9.5-10, taking $\epsilon_{578} = 1.06 \times 10^4 M^{-1} cm^{-1}$, and $G(e_{aq}^-) = 2.75$ molecules/100 ev (15). Doses used ranged between 200 and 1000 rads per pulse (equivalent to 0.5-3 μM of e_{aq}^-). The temperature of the solutions was $20 \pm 2^\circ C$. The pulse radiolytic data were analyzed on-line using a Nova 1200 minicomputer and appropriate interphases developed in the accelerator laboratory.

The kinetic-ionic strength effects were analysed with either the full Bronsted-Debye-Huckel equation (Equation 1) or the Debye-Marcus theory based equation (Equation 2) more recently derived by Wherland and Gray (16).

$$\ln k_I = \ln k_0 - \frac{\underline{A}(Z_A + Z_B)^2 I^{\frac{1}{2}}}{1 + \kappa R_{AB}^{\ddagger}} + \frac{\underline{A} Z_A^2 I^{\frac{1}{2}}}{1 + \kappa R_A} + \frac{\underline{A} Z_B^2 I^{\frac{1}{2}}}{1 + \kappa R_B} \quad (1)$$

$$\ln k_I = \ln k_{\infty} - 3.576 \left[\frac{e^{-\kappa R_A}}{1 + \kappa R_B} + \frac{e^{-\kappa R_B}}{1 + \kappa R_A} \right] \left[\frac{Z_A Z_B}{R_A + R_B} \right] \quad (2)$$

Where at 25°C, the constant $\underline{A} = 1.174$, 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, $R_{AB}^{\ddagger}$ 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 where all charges are shielded from each other and electrostatic interactions at minimized. k_0 is the rate constant at zero ionic strength, where electrostatic interaction are at their maximum. The kinetic-ionic strength data was curve fitted to Equations 1 and 2 by a nonlinear least squares regression analysis done by computer and computer plotted.

The simplified Bronsted-Debye-Huckel equation, Equation 3,

$$\ln k_I = \ln k_0 + \underline{A} Z_A Z_B \sqrt{I} \quad (3)$$

was not used since it has been shown by Ryan (17) and Feinberg (18) to

be theoretically and thus experimentally incorrect to use Equation 3 for reactions involving redox proteins. Equation 3 is valid only if both A and B have very small radii; this is clearly not the case in reactions with cytochrome *c* and Equations 1 and 2 are used instead. Large errors are generated when either protein-protein or small molecule-protein kinetic-ionic strength reaction are evaluated by Equation 3. Feinberg and Ryan (17,18) have also shown that both Equations 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 Z_{Seq} . In these calculations, which are used in this work to partition electrostatic and conformational contributions to the observed rates, the experimental protein charges $Z_{Eq 2}$ and $Z_{Eq 3}$ 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 (19).

RESULTS

The reducing radicals and the reduced derivatives were produced *in situ* according to the reactions that are presented in Table 2 using appropriate concentrations of reagents. In discussing the various free radical reaction, Table 2 should be referred to.

Reduction by $CH_3\dot{C}HOH$

Solutions contained 100 mM ethanol, 10-25 μ M 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

Reaction 1 of Table 2. OH radicals react with the ethanol present in the solution according to Reaction 9, and form $\text{CH}_3\dot{\text{C}}\text{HOH}$ radicals. Under the experimental conditions employed, over 99% of the $\text{OH}^\bullet$ radicals reacted with the ethanol. The $\text{H}^\bullet$ atoms formed by the pulse react with the ethanol via Reaction 6 and produce also $\text{CH}_3\dot{\text{C}}\text{HOH}$ radicals. Under the experimental conditions, over 99% of the H atoms reacted with the ethanol.

The reduction of the cytochrome *c* derivatives by $\text{CH}_3\dot{\text{C}}\text{HOH}$ was a pseudo-first order process, under our experimental conditions, as demonstrated by the rates being proportional to the concentrations of the cytochrome *c* derivative. Second order rate constants at $\mu = 0.1$ are shown in Table 3. The reduction process was followed at 550 nm and at 450 nm. In order to make a significant comparison, the rate of reduction of native cytochrome *c* was also determined under similar experimental conditions. The value of the rate constant was found to be equal to the formerly published value (4), as shown in Table 3.

Reduction by CO_2^- ~~~~~

Solutions contained HCOONa (5-100 mM, depending on the desired ionic strength), and 10-30 μM of 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 formed by the pulse are transformed into $\text{OH}^\bullet$ radicals by Reaction 1. $\text{OH}^\bullet$ and $\text{H}^\bullet$ radicals react with formate according to Reactions 5 and 7, and thus form CO_2^- radicals.

Under the experimental conditions employed, the reduction of cytochrome *c* derivatives were observed to undergo pseudo-first order kinetics and the rates were proportional to the concentration of the derivative.

Second order rate constants at an ionic strength of 0.1 are shown in Table 3. The reduction was followed at 550 nm.

Reduction by O_2^- ~~~~~

Solutions contained 100 mM HCOONa and 20-30 μ M 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 of O_2 with e^-_{aq} (Reaction 2) and with H $\cdot$ atoms (Reaction 4). It is also produced by reaction of CO_2^- formed in Reactions 5 and 7 with O_2 via Reaction 11. 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 $pK = 4.75$ (25), and therefore, at pH = 7.0 practically only O_2^- radicals are present.

Of the three derivatives, only ACC is reduced by O_2^- . SUCC and DCMC have redox potentials similar to that of O_2^- ($E^{\circ*} = -160$ mV, 1M O_2), and this is probably the reason why these molecules do not react fast enough to compete with the self decay of O_2^- at neutral pH. Under the experimental conditions, the self decay of O_2^- by dismutation is negligible as compared to its reaction with ACC, because of the high ratio of the concentrations of ACC and of O_2^- . The concentration of O_2^- was 0.5 - 1 μ M.

The second order rate constant of the reduction of ACC by O_2^- at $\mu = 0.1$ was $3.5 \times 10^5 M^{-1} s^{-1}$ (Table 3). The reduction was followed at 550 nm and was found to be a pseudo-first order process. The ratio between the rate constant of the reduction of NAT (5) and of ACC by O_2^- is in agreement with the results of Azzi et al. (26).

Reduction by e^-_{aq} ~~~~~

Solutions contained 10-30 μM of the cytochrome *c* derivative, t-Butanol in a concentration 10^4 times that of the protein, and phosphate buffer (45 mM, pH=6.9-7.1, $\mu = 0.1$). Solutions were saturated with argon, in order to prevent reactions of the primary radicals with O_2 . Most of the $\text{OH}^\bullet$ radicals formed by the pulse are scavenged by t-Butanol in Reaction 8, Table 2. The decay of the absorption of e_{aq}^- at 58--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 3.

Oxidation by $\text{Fe}(\text{CN})_6^{3-}$ after rapid reduction by e_{aq}^-
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Solutions contained $\text{K}_3\text{Fe}(\text{CN})_6$, cytochrome *c* derivatives (10-20 μM), t-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 $\text{OH}^\bullet$ radicals were scavenged by t-butanol by Reaction 8, assuming a similar rate of reaction with $\text{OH}^\bullet$ radicals for cytochrome *c* (Reaction 10, Table 2), and its derivatives. The t-butanolic radicals do not react with cytochrome *c* (4), and presumably do not react with its derivatives either.

e_{aq}^- react both with cytochrome *c* derivatives and with $\text{Fe}(\text{CN})_6^{3-}$ ions (Reaction 3, Table 2). In most of the experiments, the concentration of $\text{Fe}(\text{CN})_6^{3-}$ was smaller or equal to the concentration of cytochrome *c* derivatives, so that more than 95% of e_{aq}^- react with the protein.

Immediately after the pulse was completed, a fast increase of

absorption at 550 nm or a fast decrease of absorption at 450 nm were observed. This fast process is the reduction of the protein by e_{aq}^- , which was finished within 10 μ sec after the pulse. Then, another absorption change, in the opposite direction was followed. This later process is the reoxidation of the reduced derivative by $Fe(CN)_6^{3-}$. Under our experimental conditions, the oxidation by $Fe(CN)_6^{3-}$ was a pseudo-first order process. The dependence of the observed rate constant on the concentration of $Fe(CN)_6^{3-}$ ions, in the oxidation of ACC, SUCC, and DCMC is shown in Figure 1. Second order rate constants are summarized in Table 3.

Kinetic-ionic strength studies of cytochrome *c* redox reactions.
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 The dependence of the rate constant for the reduction of ACC by CO_2^- on ionic strength was studied and is shown in Figure 2 and is curve fitted to Equation 2. Also the dependence of the rate constant for the ferricyanide oxidation of ACC, SUCC, and DCMC (after rapid reduction by e_{aq}^-) upon ionic strength was determined and the results are shown in Figures 3-5. The effective or net protein charges are predicted from Equation 1 and 2 were determined as were both the k_o and k_∞ rate constants. These results are shown in Table 4. Also shown in the same table are some results from previous work with native cytochrome *c* (NAT).

DISCUSSION

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Conformational effects. There are two main classes of cytochrome *c* derivatives that were studied. Firstly there are those in which the lysine residues have been modified. In ACC several of the positively

charged lysines are converted to a negatively charged functional group, leaving a modified protein that is now negatively charged but having only minor changes in its conformation in the heme edge region as indicated by the maintenance of the 695 nm absorption band and redox potential change (See Table 1).

The second class of derivatives examined were those in which there was significant disruption of the heme region as indicated by the loss of the 695 nm absorption band and lowering of the redox potential (See Table 1). All the lysines (and possibly a tyrosine) are modified by succinylation, thus producing a negatively charged and conformationally disrupted and open cytochrome *c*. In contrast, the dicarboxymethylation of methionines 65 and 80 of horse cytochrome *c* provided a derivative which has its charge unaffected but which is, like SUCC, conformationally open and disrupted as shown in Table 1.

In light of the physicochemical properties of these derivatives, it can be summarized that the heme edge region of both SUCC and DCMC is relatively more exposed to the external environment than either NAT or ACC.

In regard to the redox potentials of the native and modified cytochrome *c*, Kassner (29,30) and Dickerson (31) 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 $\text{CH}_3\dot{\text{C}}\text{HOH}$ 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 $\text{CH}_3\dot{\text{C}}\text{HOH}$ with the cytochromes *c* was: $\text{SUCC} \gg \text{DCMC} > \text{ACC} \approx \text{NAT}$ (See Table 3). These results are particularly interesting when it is noted that the slower rates are observed in spite of the fact that the redox potential differences between the protein and reducing agent are becoming larger in going from SUCC to NAT. This is the opposite direction expected on the basis of redox potential, and thus demonstrates the overriding influence of progressively greater heme edge exposure (NAT to SUCC) upon reactivity.

In the Marcus theory of outer sphere electron transfer processes (32), many reactions of cytochromes *c* with metal complexes have been examined. According to this 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 ΔG° (ΔE°) of the reaction is relatively small, a condition which is not fulfilled in this and many other similar studies. In further considering conformational effects, the rate of reduction of DCMC by CO_2^- is somewhat higher than the CO_2^- reduction of NAT. Since both NAT and DCMC have the same charge, the increase in rate of

DCMC is again attributed to its more open conformation.

Another manifestation of increased reactivity due to increased heme edge exposure is found in the observation that ferricyanide oxidized both SUCC (in spite of its quite negative charge) and DCMC faster than NAT or ACC. As noted earlier in ACC the charge is more negative but the heme edge region is left intact much like NAT and the ferricyanide oxidation of ACC is slower than NAT (See Table 3). A similar effect has been previously observed for the oxidative of non-relaxed NAT which has a more open conformation than that relaxed (3,33).

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 Partitioning of electrostatic and conformational contributions
 to redox kinetics.
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Native horse cytochrome *c* is a positively charged molecule (7). The charge of the protein 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 (34,35). In this light a close examination of the electrostatic interactions of NAT and its derivatives with charged redox agents is especially interesting and informative. More generally for this work, 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) it is quite informative to discuss the experimental results in terms of k_0 and k_∞ rate constants. Both k_0 and k_∞ are obtained from the kinetic ionic strength studies as noted in the Experimental Methods; 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 where electrostatic interactions are nil. In the latter case, only the influence of redox potential and conformation are predominant.

Since the conformation of ACC is only slightly different from NAT, the influence of charge can be examined without the interference of large conformational influences. Thus while the k_∞ for the CO_2^- reduction of NAT and ACC are nearly the same ($6.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ vs. $2.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ respectively), the influence of electrostatic repulsion is seen in the more than two orders of magnitude difference in the k_0 rates of $6.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ vs. $1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ respectively. The same pattern is observed even more dramatically in the oxidation of ACC and NAT by $\text{Fe}(\text{CN})_6^{3-}$ (after fast reduction by e_{aq}^-). The k_0 rate of ACC is slightly more than six orders of magnitude slower than that for NAT ($4.3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ vs. $3.24 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ respectively). In contrast the electrostatically corrected rates, k_∞ , are similar and they differ only by an order of magnitude ($5.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ vs. $4.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) as shown in Table 4.

It is interesting to note, in light of the earlier discussion of conformational effects, that the k_{∞} value of ferricyanide oxidation of DCMC is about two orders of magnitude greater than the similarly charged NAT ($1.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ vs. $4.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$); this difference in rate is understandable in terms of both the greater exposure of the heme edge region of DCMC and its lower redox potential. In examining the electrostatically informative k_o 's for the ferricyanide oxidation of DCMC, it is consistent that their k_o values differ by less than an order of magnitude which is expected since their charge is the same. The rate constants at $\mu = 0.1$ shown in Table 3 corroborate the above interpretation.

Also in Table 3, the rates of e_{aq}^- reduction are shown for NAT and the derivatives. They are all nearly identical in the range of $2-4 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$. 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 NAT and its derivatives by a mechanism different from CH_3CHOH or CO_2^- as has been previously suggested (4).

Net protein charge and kinetics

As noted in Experimental Methods the kinetic-ionic strength experiments also provide the net protein charge, and these results are shown in Table 4. The predicted net protein charge (e.g. $Z_{\text{Eq } 2}$) for the native and modified cytochromes compare well with the net protein charge obtained from the sequence and prosthetic group, Z_{Seq} . The notable exception is the charge predicted for DCMC.

Although it would be interesting to postulate for the ferricyanide oxidation reactions that the decrease of charge on NAT and DCMC is due to ferricyanide binding, this is not the case since in this work the concentration of ferricyanide used is not high enough to insure stoichiometric binding of Fe(CN)_6^{3-} to NAT or DCMC and the amount of ferricyanide-cytochrome *c* complex formed is very small^[3]. The close correlation between the predicted charges and Z_{Seq} for both the positively and negatively charged cytochromes *c* further demonstrate the role of *net* protein charge on the observed kinetics of electron transfer.

As noted earlier both ACC and SUCC have large negative charges as obtained from the kinetic-ionic strength data (see Table 4). Arising from the same analysis are of course the k_{∞} rate constants. In showing consistency between the predicted net proteins charges and k_{∞} , it is important to note that the k_{∞} rates of ferricyanide oxidation of NAT) as compared to ACC and SUCC (ca. $3.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ vs 5.9 and $7.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ respectively) are nearly identical to the results of LeBon and Cassatt (36) on ferricyanide oxidation of NAT and trifluoroacetylated cytochrome *c* (TFA) in which at $\mu=0.1$, $k=8.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for NAT and $3.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for TFA. Since the values for k_{∞} are already electrostatically corrected, the difference between NAT and ACC, and NAT and TFA can not 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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References

1. Ferguson-Miller, S., Brautigan, D. L. and Margoliash, E. (1977) in: "The Porphyrines" (Dolphin, D., -ed.) Vol. 5, Academic Press, New York.
2. Brautigan, D. L., Ferguson-Miller, S., Tarr, G. E. and Margoliash, E. (1978) J. Biol. Chem. 253, 140-148.
3. Ilan, Y., Shafferman, A. and Stein, G. (1976) J. Biol. Chem. 251, 4336-4345.
4. Shafferman, A. and Stein, G. (1974) Science 183, 428-430.
5. Seki, H., Ilan, Y., Ilan, Y. A. and Stein, G. (1976) Biochim. Biophys. Acta 440, 573-586.
6. Shafferman, A. and Stein, G. (1975) Biochim. Biophys. Acta 416, 287-317.
7. Dickerson, R. E. and Timkovich, R. (1975) in: "The Enzymes" (Boyer, P. D. - ed.) Vol. 11, pp. 397-547, Academic Press, New York.
8. Ilan, Y. (1978) Ph.D. Thesis, The Hebrew University, Jerusalem.
9. Cooper, S. C., Lambek, J. and Erecinska, M. (1975) FEBS Lett. 59, 241-244.
10. Stellwagen, E. (1968) Biochemistry 7, 2496-2501.
11. Wada, K., and Okunuki, K. (1968) Biochemistry 64, 667-681.
12. Schejter, A., and Aviram, I. (1970) J. Biol. Chem. 245, 1552-1557.
13. Margoliash, E. and Frohwit, N. (1959) Biochem. J. 71, 570-572.
14. Lichtin, N. N., Shafferman, A. and Stein, G. (1973) Biochim. Biophys. Acta 314, 117-135.
15. Hart, E. J. and Fielden, E. M. (1970) in: "Manual of Radiation Dosimetry" (Holm, N. W. and Berry, R. J. - eds.) pp. 331-335, Marcel Dekker Inc., New York.
16. Wherland, S. and Gray, H. B. (1976) Proc. Natl. Acad. Sci., USA. 73, 2950-2954.
17. Ryan, M. D. and Feinberg, B. A. (1978) Bioelectrochem. Bioenerg. 5, 480-485.
18. Feinberg, B. A. and Ryan, M. D. (1978) in: "Topics in Bioelectrochemistry and Bioenergetics" (G. Milazzo, ed.) John Wiley and Sons, New York, in press.

19. Eaton, W. A., George, P. and Hanania, G. I. H. (1967) J. Phys. Chem. 71, 2016-2021.
20. Bambenek, M. and Ross, A. B. (1973) Selected Specific Rates of Reactions. NBS report No. NSRDS-NBS 43.
21. Anbar, M., Ross, F. and Ross, A. B. (1975) Selected Specific Rates of Reactions. NBS report No. NSRDS-NBS 51.
22. Dorfman, L. M. and Adams, G. E. (1973) Reactivity of the Hydroxyl Radical in Aqueous Solutions. NBS report No. NSRDS-NBS 46.
23. Nilsson, L. (1972) Israel J. Chem. 10, 1011-1019.
24. Ilan, Y. A. Rabani, J. (1976) Int. J. Radiat. Phys. Chem. 8, 609-611.
25. Bielski, B. H. J. and Allen, A. O. (1977) J. Phys. Chem. 81, 1048-1050.
26. Azzi, A., Montencucco, C. and Richter, C. (1975) Biochem. Biophys. Res. Comm. 898-904.
27. Peterman, B. F. and Morton, R. A. (1977) Can. J. Biochem. 55, 796-803.
28. Matthew, J. B., Friend, S. H., Bothelo, L. H., Lehman, L. D., Hanania, G. I. H., and Gurd, F. R. N. (1978) Biochem. Biophys. Res. Commun. 81, 416-421.
29. Kassner, R. J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2263-2267.
30. Kassner, R. J. (1973) J. Amer. Chem. Soc. 95, 2674-2677.
31. Dickerson, R. E., Takano, R. and Kallai, O. B. (1973) in: "Conformation of Biological Molecules and Polymers" (Bergman, E. D. and Pullman, B. - eds.) Vol. 5, pp. 695-707, Jerusalem Academic Press.
32. Sutin, N. (1977) Adv. in Chem. 162, 156-172.
33. Ilan, Y. and Shafferman, A. (1978) Biochim. Biophys. Acta 501, 127-135.
34. Smith, H. T., N. Studenmayer, and Millet, F. (1977) Biochemistry 16, 4971-4974.
35. Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1978) J. Biol. Chem. 253, 149-159.
36. LeBon, T. R., and Cassatt, J. C. (1977) Biochem. Biophys. Res. Commun. 76, 746-750.

Table 1

Properties of native and chemically modified cytochrome *c*

Derivative	Redox potential (mV)	Z_{Seq}^a	Absorption at 695 nm	Spectral changes in the range 400-600 nm	Reaction with CO	Change in the reactivity with oxidase and reductase
Cytochrome <i>c</i> (native)	+260 ^b	9/8	yes	-	no ^b	-
ACC	+155 ^c	-10/-11	~74%
that of the /
native protein	no ^e	no ^e	yes ^e
SUCC	<-120 ^c	-29/-30	no	small changes
in the Soret band	?	yes ^e
DCMC	-120 ^d	9/8	no	no changes in
positions of
the peaks.
lower ϵ values
at 500-600 nm	yes ^e	yes ^e

^aSee Procedures for definition of Z_{Seq} . ^breference (7). ^creference (8). ^dreference (9).

^ereference (10).

Table 2

Reactions occurring in a pulse irradiated solution,
in the presence of various solutes

	Reaction	$k (M^{-1}s^{-1})$	Reference
1	$e_{aq}^- + N_2O \xrightarrow{H_2O} N_2 + OH^- + OH^\cdot$	5.6×10^9	20
2	$e_{aq}^- + O_2 \rightarrow O_2^{\cdot -}$	2×10^{10}	20
3	$e_{aq}^- + Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$	$\leq 3 \times 10^9$	20
4	$H^\cdot + O_2 \rightarrow HO_2^\cdot$	2×10^{10}	21
5	$H^\cdot + HCOO^- \rightarrow H_2 + CO_2^{\cdot -}$	4.5×10^8	21
6	$H^\cdot + CH_3CH_2OH \rightarrow H_2 + CH_3\dot{C}HOH$	2.8×10^9	22
7	$OH^\cdot + HCOO^- \rightarrow H_2O + CO_2^{\cdot -}$	5.2×10^8	22
8	$OH^\cdot + (CH_3)_3COH \rightarrow H_2O + \dot{C}H_2C(CH_3)_2OH$	1.8×10^9	22
9	$OH^\cdot + CH_3CH_2OH \rightarrow H_2O + CH_3\dot{C}HOH$	1.4×10^{10}	23
10	$OH^\cdot + NAT(red) \rightarrow \text{products}$	4.2×10^9	24
11	$CO_2^{\cdot -} + O_2 \rightarrow CO_2 + O_2^{\cdot -}$	2.3×10^9	21

Table 3

Second order rate constants for the reduction and oxidation of cytochrome *c* and its derivatives ($M^{-1}s^{-1}$); pH=7.0.

Reductant	μ	NAT	ACC	DCMC	SUCC
e^-_{aq}	0.1	3.0×10^{10}	2.0×10^{10}	3.3×10^{10}	4.0×10^{10}
CO_2^-	0.1	1.0×10^9	1.5×10^8	1.3×10^9	4.0×10^8
O_2^-	0.1	8.0×10^5	3.5×10^5	no reduction	no reduction
$CH_3\dot{C}HOH$	0.0005	1.8×10^8	2.5×10^8	3.5×10^8	1.8×10^9
<u>Oxidant</u>					
$Fe(CN)_6^{3-}$	0.1	6.9×10^6	2.1×10^5	2.7×10^8	3.8×10^7

Table 4. Kinetic-ion strength studies of native and modified cytochromes *c*.

Reaction (pH=7)	$Z_{\text{Eq } 2}$	$Z_{\text{Eq } 1}$	Z_{Seq}	$k_{\infty} (\text{M}^{-1} \text{s}^{-1})$	$k_o (\text{M}^{-1} \text{s}^{-1})$	Ref.
NAT(red) + $\text{Fe}(\text{CN})_6^{3-}$
(Not preequilibrated
with $\text{Fe}(\text{CN})_6^{3-}$)	7.7	4.6	8 ^a	3.0×10^6	1.1×10^9	27
NAT(red) + $\text{Fe}(\text{CN})_6^{3-}$
(preequilibrated with
$\text{Fe}(\text{CN})_6^{3-}$)	4.4	3.1	8	4.4×10^6	3.24×10^8	3
DCMC(red) + $\text{Fe}(\text{CN})_6^{3-}$
(preequilibrated)	3.1	1.0	8	1.7×10^8	1.9×10^9	This
work
ACC(red) + $\text{Fe}(\text{CN})_6^{3-}$
(preequilibrated)	-5.8	-9.2	-11	5.9×10^5	4.3×10^2	This
work
SUCC(red) + $\text{Fe}(\text{CN})_6^{3-}$
(preequilibrated)	-5.0	-8.2	-30	7.9×10^5	1.5×10^3	This
work
NAT(ox) + CO_2^- (pH 6.6)	6.9	6.3	9	6.4×10^8	6.5×10^9	5
ACC(ox) + CO_2^-	-8.0	-11.1	-10	2.6×10^8	1.3×10^7	This
work

^aTitration data gives a charge of 6.3 and 5.3 for horse ferri and ferrocytochrome *c* respectively (28).

Figure Legends

Figure 1. The dependence of the rate of oxidation of ACC, SUCC and DCMC by $\text{Fe}(\text{CN})_6^{3-}$ on the concentration of $\text{Fe}(\text{CN})_6^{3-}$. $\mu = 0.1$ (phosphate); $\text{pH} = 7.1$. The concentration of the derivatives: $1 \times 10^{-5} \text{ M}$.

○ - DCMC, ▲ - SUCC (left scale), ■ - ACC (right scale).

Figure 2. The variation of the second order rate constant as a function of ionic strength for the reaction ACC (ox) with CO_2^- . The solid line is the best curve fit to Equation 2 and the diamonds are the experimental points.

Figure 3-5. The variation of the second order rate constant as a function of ionic strength for the oxidation of ACC (Fig 3), SUCC (Fig 4) and DCMC (Fig 5) by $\text{Fe}(\text{CN})_6^{3-}$. The solid lines are the best curve fits to Equation 2 and the diamonds are the experimental points.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5