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# Reduction Kinetics of Bacterial Cytochromes $c_2$

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In a continuing effort to understand the mechanism of electron transfer by *c*-type cytochromes we have extended our investigations of the oxidation and reduction of *Rhodospirillum rubrum* cytochrome $c_2$. We have utilized the oxidant, oxidized azurin, and the reductants $SO_2^-$, $S_2O_4^{2-}$, sodium ascorbate, and reduced azurin. The results of these studies demonstrate that, as found previously with the iron hexacyanides, electron transfer apparently takes place at the exposed heme edge. Furthermore, we report studies on the reduction of ferricytochrome $c_2$ from *Rhodopseudomonas sphaeroides, Rhodopseudomonas capsulata, Rhodomicrobium vannielii,* and *Rhodopseudomonas palustris* by potassium ferrocyanide. Based on the amino acid sequence homology between the various cytochromes $c_2$ and presumed structural homology, the observed rates of electron transport are analyzed in terms of the structure in the region of the exposed heme edge.

In an effort to elucidate the mechanism of electron transfer by *c*-type cytochromes considerable kinetic information has been reported for the oxidation and reduction of horse heart cytochrome *c* (1-6). These data coupled with available chemical and structural information have led to the view that both oxidation and reduction of cytochrome *c* take place at or near the heme edge with considerable participation by the protein moiety (see Ref. 7 for a review). Nevertheless, a number of questions remain, such as the identification of the amino acid side chains at the site of electron transfer, the extent of participation of the protein moiety in regions away from the site of electron transfer, and the relation of studies with nonphysiological reactants (primarily those used to date) to the physiological situation.

Recently, a detailed study of the reaction of *Rhodospirillum rubrum* cytochrome $c_2$ with iron hexacyanides was reported (8) which demonstrated substantial similarity to analogous studies with mammalian cytochrome *c*. This was not surprising in that cytochrome $c_2$ and cytochrome *c* have been shown to have substantial structural homology (9). However, a number of significant differences were noted, including measurably different rates of reduction and oxidation as compared to cytochrome *c* (8). These findings are not interpretable in a quantitative fashion in the context of our present understanding of biological electron transport. In addition, both the oxidation-reduction potential (10) and rates of oxidation and reduction of cytochrome $c_2$ (8) were found to be dependent on pH (pH 5-8), which is not the case with cytochrome *c* (11, 12). This latter observation suggests the possibility of identifying the specific amino acid side chains involved in electron transport by cytochrome $c_2$ through their appropriate p*K* values. Although it appears that cytochrome $c_2$ transfers electrons via the exposed heme edge (8), as proposed for cytochrome *c* (3, 6, 13), this interpretation is limited by the fact that the oxidation and reduction of cytochrome $c_2$ have only been studied in detail with the iron hexacyanides as reactants.

In an effort to further our understanding

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of biological electron transport, we are reporting here studies of the effect of pH, ionic strength, and temperature on the reduction of *R. rubrum* cytochrome $c_2$ by ascorbate, $SO_2^-$, and $S_2O_4^{2-}$. Furthermore, the interaction of cytochrome $c_2$ with the copper protein azurin is reported. Taking advantage of natural chemical modifications of cytochrome $c_2$, we are also reporting the comparative studies of the reduction of cytochrome $c_2$ from five different organisms by potassium ferrocyanide.

## MATERIALS AND METHODS

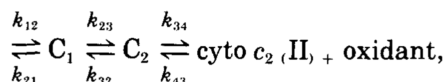
*Rhodospirillum rubrum* cytochrome $c_2$ (R-cyto $c_2$)[4] was purified as described by Bartsch *et al.* (14). *Rhodopseudomonas capsulata* strain Saint Louis cytochrome $c_2$ (C-cyto $c_2$), *Rhodopseudomonas sphaeroides* strain 2.4.1 cytochrome $c_2$ (S-cyto $c_2$), and *Rhodopseudomonas palustris* strain 2.1.37 cytochrome $c_2$ (P-cyto $c_2$) were purified by standard methods (15). *Rhodomicrobrium vannielii* strain 3.1.1 cytochrome $c_2$ (V-cyto $c_2$) was kindly provided by Dr. T. E. Meyer, Department of Chemistry, University of California at San Diego. Azurin was isolated from *Pseudomonas denitrificans* as previously described (16). Studies over a range of pH values were performed in Tris–sodium acetate–glycine–potassium phosphate buffer adjusted to the appropriate pH. Potassium ferrocyanide (Mallinckrodt), sodium ascorbate (American Drug and Chemical Co.), and sodium dithionite (Hardman and Holden) were the best grades available.

Kinetic studies were conducted in a Durrum-Gibson stopped-flow spectrophotometer with a mixing time of 3.5 ms. The temperature was maintained at 20°C unless otherwise noted. Reduction and oxidation of cytochrome $c_2$ were monitored at 418 nm with the heme concentration at 2–3 $\mu$M. All solutions used in the stopped-flow experiments were deoxygenated by bubbling with water-saturated argon gas. Absorption spectra were recorded on a Cary Model 118 spectrophotometer.

## RESULTS

*General.* The reaction of R-cyto $c_2$ with the iron hexacyanides was previously found to be consistent with the mechanism given by Eq. [1] (8).

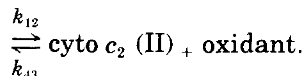
cyto $c_2$ (III) + reductant [1]



$$\underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C_2 \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} \text{cyto } c_2 \text{ (II)} + \text{oxidant},$$

where $C_1$ is a reductant–cyto $c_2$ (III) complex and $C_2$ is an oxidant–cyto $c_2$ (II) complex. The second-order rate constants for complex formation ($k_{12}$ and $k_{43}$) have been reported as well as the limiting first-order rates in both directions ($k_{23}$, $k_{32}$) (8). For the reactions of R-cyto $c_2$ to be reported here (reduction by azurin, ascorbate, $SO_2^-$, and $S_2O_4^{2-}$ and oxidation by azurin), no evidence for complex formation was found and Eq. [2] was applied. The notation $k_{12}$ and $k_{43}$ has been retained to relate the work presented here to that reported for the iron hexacyanides.

cyto $c_2$ (III) + reductant [2]



$$\underset{k_{43}}{\overset{k_{12}}{\rightleftharpoons}} \text{cyto } c_2 \text{ (II)} + \text{oxidant}.$$

The reaction of ferri-R-cyto $c_2$ with sodium ascorbate was found to obey pseudo first-order kinetics at pH values of 7.0 or less; however, at alkaline pH the reaction was biphasic with a slow kinetic species detectable. The contribution of the slow kinetic species to the total absorbance change increased with increasing pH. Plots of the pseudo first-order rate constants vs [ascorbate] were linear in all cases ([ascorbate] = 1 to 50 mM).

The reduction of ferricytochrome $c_2$ with sodium dithionite obeyed pseudo first-order kinetics ($[S_2O_4^{2-}]$ = 10 to 500 $\mu$M) for all experimental conditions used here as long as oxygen was omitted ($[O_2] < 1$ $\mu$M). Second-order plots ($[S_2O_4^{2-}]$ vs $k_{obs}$) were nonlinear and the data were analyzed utilizing the rate law given in Eq. [3] which has previously been applied to the reduction of ferri-horse heart cytochrome $c$ by sodium dithionite (1, 6), where $k_1$ is the second-order rate constant for $S_2O_4^{2-}$ reduction and $k_2$ the second-order rate constant for $SO_2^-$ reduction. $K_{eq}$ is the equilibrium constant for the breakdown of $S_2O_4^{2-}$

[4] Abbreviations used: R-cyto $c_2$, *Rhodospirillum rubrum* cytochrome $c_2$; C-cyto $c_2$, *Rhodopseudomonas capsulata* strain Saint Louis cytochrome $c_2$; S-cyto $c_2$, *Rhodopseudomonas sphaeroides* strain 2.4.1 cytochrome $c_2$; P-cyto $c_2$, *Rhodopseudomonas palustris* strain 2.1.37 cytochrome $c_2$; V-cyto $c_2$, *Rhodomicrobrium vannielii* strain 3.1.1 cytochrome $c_2$; HIPIP, high-potential iron-sulfur protein.

to $2SO_2^-$ and was determined by electron paramagnetic resonance as described previously (6). This mechanism requires that both $SO_2^-$ and $S_2O_4^{2-}$ reduce cytochrome $c_2$ and that plots of $k_{obs}$ $[S_2O_4^{2-}]^{-1}$ vs $[S_2O_4^{2-}]^{-1/2}$ be linear; this was found to be the case for all conditions investigated here.

$$k_{obs} = k_1 [S_2O_4^{2-}] + k_2 K_{eq}^{1/2} [S_2O_4^{2-}]^{1/2}. \quad [3]$$

The reaction of R-cyto $c_2$ with azurin was monitored at both 600 and 552 nm yielding identical results. Pseudo first-order kinetics were found at all azurin concentrations (2 to 20 $\mu$M) for both oxidation and reduction of R-cyto $c_2$ (heme concentration, 1 $\mu$M). Second-order plots were linear in all cases.

The reduction of cytochrome $c_2$ from various sources by ferrocyanide obeyed pseudo first-order kinetics ([ferrocyanide] = 0.030 to 1 mM) and as analyzed in terms of Eq. [1].

*Effect of ionic strength.* Table I presents second-order rate constants for the reaction of R-cyto $c_2$ with ascorbate, $S_2O_4^{2-}$, $SO_2^-$, and oxidized and reduced azurin at various ionic strengths. In all cases, the rate of reaction was decreased by increasing ionic strength indicating the interaction of oppositely charged reactants. With ascorbate, $S_2O_4^{2-}$, and $SO_2^-$, a positive charge at the site of electron transfer on R-cyto $c_2$ is indicated. As the charge at the site of electron transfer on azurin is unknown, the interpretation in this case is not unequivocal; however, the results are consistent with a plus charge on cytochrome $c_2$ and a minus charge on azurin (both proteins have a net negative charge at pH 7.0). Table II summarizes the second-order rate constants for the reaction of R-cyto $c_2$ with a variety of oxidants and reductants. The values presented were obtained from plots of ln $k_{12}$ or ln $k_{43}$ vs $\mu^{1/2}$ by extrapolation to infinite dilution as described by the Debye–Hückel relation (17). Although the absolute values of the rate constants and apparent charges at infinite dilution are questionable due to the uncertainties in the application of the Debye–Hückel equation, the relative values are useful for comparative purposes. For comparison, previous results with the iron hexacyanides and the high-potential iron-sulfur protein (HIPIP) from *Chromation vinosum* (18) are included in Table II.

*pH effects.* The reaction of $S_2O_4^{2-}$ and $SO_2^-$ with ferricytochrome $c_2$ as a function of pH is presented graphically in Fig. 1A. The rate constant for $SO_2^-$ reduction goes through a maximum at pH 9 while the $S_2O_4^{2-}$ rate constant decreases with increasing pH. This pattern is similar to that observed for the reduction of horse heart cytochrome $c$ (6). Ascorbate reduction is complex in that at pH values above 7 the reaction is biphasic. However, both kinetic species present at alkaline pH follow second-order kinetics. This observation is in sharp contrast to the reduction of ferricytochrome $c_2$ or $c$ by ferrocyanide where two kinetic species are observed at alkaline pH but the slow kinetic species

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE OXIDATION AND REDUCTION OF *R. rubrum* CYTOCHROME $c_2$ AS A FUNCTION OF IONIC STRENGTH[a]

| $\mu$ | Ascorbate, $k$ ($M^{-1}$ $s^{-1}$) | $SO_2^-$, $k \times 10^{-7}$ ($M^{-1}$ $s^{-1}$) | $S_2O_4^{2-}$, $k \times 10^{-4}$ ($M^{-1}$ $s^{-1}$) | Azurin (reduced), $k \times 10^{-4}$ ($M^{-1}$ $s^{-1}$) | Azurin (oxidized), $k \times 10^{-4}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|
| 0.018 | – | – | – | 4.1 | – |
| 0.035 | – | 8.8 | 9.2 | 3.6 | 9.2 |
| 0.045 | 68 | – | – | – | – |
| 0.055 | 52 | 8.7 | 8.3 | 3.3 | – |
| 0.085 | 47 | 8.3 | 5.7 | 2.7 | – |
| 0.135 | 27 | 7.8 | 3.3 | 2.5 | 0.5 |
| 0.535 | 10 | 3.6 | 1.6 | – | – |

[a] The buffer generally was 0.01 M potassium phosphate, pH 7.0, supplemented with various amounts of NaCl to give the indicated ionic strengths. Heme concentration, 2–5 $\mu$M; reaction temperature, 20 ± 0.2°C.

TABLE II

THE EFFECT OF IONIC STRENGTH ON THE REDOX KINETICS OF *R. rubrum* Cytochrome $c_2$[a]

| | $E_{m,7}$ (mV) | $k_i$ ($M^{-1}$ $s^{-1}$) | Apparent charge on cytochrome $c_2$ |
|---|---|---|---|
| Reductant | | | |
| Ferrocyanide[b] | 410 | $5.0 \times 10^6$ | +1.3 |
| HIPIP[c] | 320 | $1.4 \times 10^6$ | Product of charges = −2.3 |
| Azurin | 300 | $4.8 \times 10^6$ | Product of charges = −0.8 |
| $S_2O_4^{2-}$ | 150 | $2.5 \times 10^5$ | +0.7 |
| Ascorbate | 58 | 170 | +2 |
| $SO_2^-$ | −740 | $1.1 \times 10^8$ | +0.7 |
| Oxidant | | | |
| Ferricyanide[b] | 410 | $1.3 \times 10^7$ | +0.7 |
| HIPIP[c] | 320 | $2.2 \times 10^5$ | Independent of ionic strength |
| Azurin | 300 | $1.9 \times 10^6$ | Product of charges = −6.9 |

[a] $k_i$ was obtained from the extrapolation of plots of ln $k_{obs}$ vs $\mu^{1/2}$ to zero ionic strength and hence represents the rate constant at infinite dilution. The values of $k_{obs}$ used in each case are given in Table I. The apparent charge was obtained from the slope of a plot of ln $k_{obs}$ vs $\mu^{1/2}$ and the known charge of the reactant used.

[b] Data from Ref. 8.

[c] Data from Ref. 18.

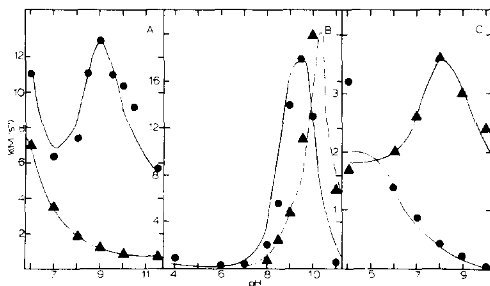


FIG. 1. Effect of pH on the oxidation and reduction of R-cyto $c_2$. Solid lines generated as described in the text. (A) Reduction by $SO_2^-$ (●, ordinate × $10^{-7}$) and $S_2O_4^{2-}$ (▲, ordinate × $10^{-4}$). (B) Reduction by ascorbate: ●, fast kinetic species (acid form, ordinate × $10^{-3}$); ▲, slow kinetic species (alkaline form). (C) Oxidation by potassium ferricyanide (▲, ordinate × $10^{-6}$) and reduction by potassium ferrocyanide (●, ordinate × $10^{-4}$).

has a rate constant that is independent of the reduction concentration (8, 12). Figure 1B represents the second-order rate constants for ascorbate reduction, with both kinetic species having sharp pH optima.

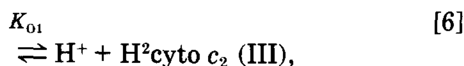
Previous kinetic studies with the iron hexacyanides and the oxidation–reduction potential as a function of pH were interpreted in terms of three p$K$ values in each oxidation state of R-cyto $c_2$ (8). However, a recent study (Dr. G. Pettigrew, personal communication) of the oxidation–reduction potential of cytochrome $c_2$ has been interpreted in terms of two p$K$ values for ferri-R-cyto $c_2$ (6.2, 8.4) and one for ferro-cyto $c_2$ (7.0). Reevaluation of our previous results leads us to believe that the interpretation of Pettigrew is a more accurate description of the situation. Nevertheless, the kinetic data for ascorbate, $SO_2^-$, and $S_2O_4^{2-}$ reduction and ferricyanide oxidation (8) clearly require at least two p$K$ values above 7. To accommodate all available information we propose three p$K$ values for ferri-cyto $c_2$ (~6.2, 8.4, and 9.5) and two for ferro-cyto $c_2$ (~7.0 and 9.5). The p$K$ value at 9.5 in each oxidation state would be undetectable by equilibrium measurements. Equation [4] presents the expression for the observed rate constant for reduction and Eq. [5] presents it for oxidation.

$$k_{obs} = \frac{k_{Ra} + (k_{Rb} K_{O1})/[H^+] + (k_{Rc} K_{O1} K_{O2})/[H^+]^2 + (k_{Rd} K_{O1} K_{O2} K_{O3})/[H^+]^3}{1 + (K_{O1})/[H^+] + (K_{O1} K_{O2})/[H^+]^2 + (K_{O1} K_{O2} K_{O3})/[H^+]^3}. \quad [4]$$

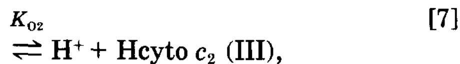
$$k_{obs} = \frac{k_{Oa} + (k_{Ob} K_{R1})/[H^+] + (k_{Oc} K_{R1} K_{R2})/[H^+]^2}{1 + (K_{R1})/[H^+] + (K_{R1} K_{R2})/[H^+]^2}. \quad [5]$$

The expressions for the rate and equilibria processes used to derive Eq. [4] are given by Eqs. [6]–[12],

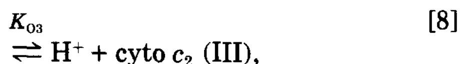
$$\mathrm{H_3cyto}\ c_2\ \mathrm{(III)} \overset{K_{01}}{\rightleftharpoons} \mathrm{H^+ + H^2cyto}\ c_2\ \mathrm{(III)}, \quad [6]$$



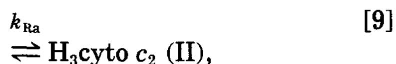
$$\mathrm{H_2cyto}\ c_2\ \mathrm{(III)} \overset{K_{02}}{\rightleftharpoons} \mathrm{H^+ + Hcyto}\ c_2\ \mathrm{(III)}, \quad [7]$$



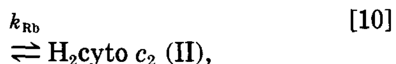
$$\mathrm{Hcyto}\ c_2\ \mathrm{(III)} \overset{K_{03}}{\rightleftharpoons} \mathrm{H^+ + cyto}\ c_2\ \mathrm{(III)}, \quad [8]$$



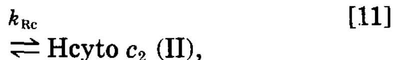
$$\mathrm{H_3cyto}\ c_2\ \mathrm{(III) + R} \overset{k_{Ra}}{\rightleftharpoons} \mathrm{H_3cyto}\ c_2\ \mathrm{(II)}, \quad [9]$$



$$\mathrm{H_2cyto}\ c_2\ \mathrm{(III) + R} \overset{k_{Rb}}{\rightleftharpoons} \mathrm{H_2cyto}\ c_2\ \mathrm{(II)}, \quad [10]$$

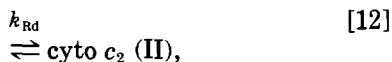


$$\mathrm{Hcyto}\ c_2\ \mathrm{(III) + R} \overset{k_{Rc}}{\rightleftharpoons} \mathrm{Hcyto}\ c_2\ \mathrm{(II)}, \quad [11]$$



and

$$\mathrm{cyto}\ c_2\ \mathrm{(III) + R} \overset{k_{Rd}}{\rightleftharpoons} \mathrm{cyto}\ c_2\ \mathrm{(II)}, \quad [12]$$



where R represents the reductant. Similar expressions can be written for the ferro form of the cytochrome to yield Eq. [5].

Using the equations given above and the p$K$ values and rate constants given in Table III, the solid lines shown in Fig. 1 were calculated. Figure 1A is for $SO_2^-$ and $S_2O_4^{2-}$ reduction, Fig. 1B ascorbate reduction, and Fig. 1C ferrocyanide reduction and ferricyanide oxidation with the data taken from a previous publication (8).

Excluding the slow kinetic species observed with ascorbate at alkaline pH, excellent agreement is obtained between the kinetic and equilibrium measurements and among the various reductants. For ferrocyanide and ascorbate reduction, the agreement between the calculated and observed rate constants at pH 4 is poor (Fig. 1) suggesting another p$K$ below pH 4 for ferri-cyto $c_2$.

The contribution of the second kinetic phase observed for ascorbate reduction at alkaline pH increases with increasing pH. Thus, a plot of the log of the fraction of the total absorbance represented by the fast kinetic species (acid form) divided by the fraction as the slow kinetic species (alkaline form) is linear (Fig. 2). These data yield a p$K$ of 9.4 with a slope of 2 ($n$, the number of protons, = 0.5); these values are in excellent agreement with the pH titration of the 695-nm absorption band (p$K$ = 9.3) and ferrocyanide reduction (p$K$ = 9.6) (8).

*Temperature.* Table IV summarizes thermodynamic data for the reduction of R-cyto $c_2$ by ascorbate, $SO_2^{2-}$, and $S_2O_4^{2-}$ and compares these results to those reported for the analogous reactions with

TABLE III

Comparison of p$K$ Values and Limiting Rate Constants for the Oxidation and Reduction of R-cyto $c_2$ by a Variety of Reactants

| | p$K_1$ | p$K_2$ | p$K_3$ | $k_a$ ($M^{-1}\ s^{-1}$) | $k_b$ ($M^{-1}\ s^{-1}$) | $k_c$ ($M^{-1}\ s^{-1}$) | $k_d$ ($M^{-1}\ s^{-1}$) |
|---|---|---|---|---|---|---|---|
| Reductant | | | | | | | |
| Ferrocyanide | 6.2 | 8.4 | 9.5 | $2 \times 10^5$ | $5 \times 10^4$ | $10^4$ | $10^3$ |
| $S_2O_4^{2-}$ | 6.2 | 8.4 | 9.5 | $10^5$ | $2.2 \times 10^4$ | $10^4$ | $8 \times 10^3$ |
| Ascorbate (fast) | 6.2 | 8.8 | 9.8 | 30 | 2 | $3 \times 10^3$ | 0.5 |
| Ascorbate (slow) | – | 9.6 | 10.6 | – | 0.4 | 26 | 0.1 |
| $SO_2^-$ | 6.2 | 8.4 | 9.5 | $1.6 \times 10^8$ | $5 \times 10^7$ | $1.7 \times 10^8$ | $6 \times 10^7$ |
| Equilibrium | 6.2 | 8.4 | 9.5 | – | – | – | – |
| Oxidant | | | | | | | |
| Ferricyanide | 7.0 | 9.5 | – | $1.8 \times 10^6$ | $3.6 \times 10^6$ | $1.5 \times 10^6$ | – |
| Equilibrium | 7.0 | 9.5 | – | – | – | – | – |

the iron hexacyanides (8). For all cases, reduction is primarily an enthalpic process with oxidation having a much larger entropic contribution. The similarities in the thermodynamic parameters again suggest a common site of reduction.

*Comparative studies.* The reactions of cytochrome $c_2$ from four sources at different ionic strengths are summarized in Table V. The data presented are in terms of $k_{12}$ and $k_{23}$ since in all cases the data are interpretable in terms of Eq. [1] with second-order plots becoming independent of [ferrocyanide] at high concentrations. To compare the various cytochromes $c_2$, Debye-Hückel plots (17) were made and the value of $k_{12}$ at infinite dilution and the apparent charge at the site of electron transfer were determined. Table VI presents the value of $k_{12}$ at infinite dilution, $k_{23}$, the apparent charge, the isoelectric point, and the oxidation-reduction potentials of the cytochromes $c_2$ studied. Also included in Table VI are the same parameters for R-cyto $c_2$ (8) and horse heart cytochrome $c$ (6) for comparison. Most notable are the wide range of values for $k_{12}$ and the lack of correlation of the oxidation-reduction potential with $k_{12}$ and $k_{34}$. P-cyto $c_2$ represents an anomaly in that $k_{23}$ is ionic strength dependent, a finding not observed with the other cytochromes examined. In all cases a positive charge at the site of electron transfer was observed and, except for V-cyto $c_2$ and horse heart cyto $c$, the apparent charge roughly correlated with the value of $k_{12}$.

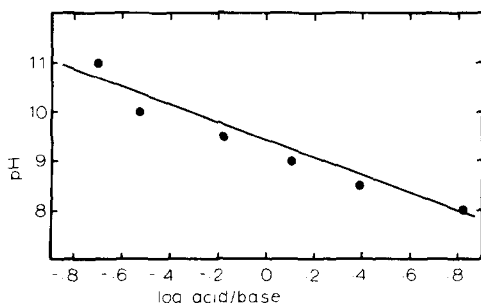



FIG. 2. Relation between pH and the acidic and basic forms of R-cyto $c_2$ for ascorbate reduction. The ratio acid/base was derived from the fraction of the fast and slow kinetic species as described in the text.

TABLE IV

THERMODYNAMIC PARAMETERS FOR THE REACTION OF *R. rubrum* CYTOCHROME $c_2$ WITH ANIONIC REACTANTS

| | $\Delta H\ddagger$ (kcal/mol) | $T\Delta S\ddagger$ (kcal/mol)[a] | $\Delta G\ddagger$ (kcal/mol)[a] |
|---|---|---|---|
| Reductant | | | |
| Ferrocyanide[b] | 11.4 | 0.5 | 10.9 |
| $S_2O_4^{2-}$ | 16.0 | 2.6 | 13.4 |
| Ascorbate | 13.0 | −2.3 | 15.3 |
| $SO_2^-$ | 10.0 | 1.6 | 8.4 |
| Oxidant | | | |
| Ferricyanide[b] | 5.2 | −3.0 | 8.2 |

[a] 20°C.
[b] Data from Ref. 8.

## DISCUSSION

In terms of the reduction of ferricytochrome $c_2$, the results presented here strongly support the view that all reactants interact at a common site on the cytochrome. This conclusion is derived from the similar apparent charge at the site of electron transfer (Table II), the identical effects of pH in terms of pK

TABLE V

SECOND-ORDER RATE CONSTANTS FOR THE REDUCTION OF VARIOUS CYTOCHROMES $c_2$ AS A FUNCTION OF IONIC STRENGTH

| $\mu$ | *R. palustris* (strain 2.1.37) | | *R. capsulata* | | *R. sphaeroides* | | *R. vannielii* | |
|---|---|---|---|---|---|---|---|---|
| | $k_{12} \times 10^{-5}$ ($M^{-1}$ $s^{-1}$) | $k_{23}$ ($s^{-1}$) | $k_{12} \times 10^{-5}$ ($M^{-1}$ $s^{-1}$) | $k_{23}$ ($s^{-1}$) | $k_{12} \times 10^{-5}$ ($M^{-1}$ $s^{-1}$) | $k_{23}$ ($s^{-1}$) | $k_{12} \times 10^{-4}$ ($M^{-1}$ $s^{-1}$) | $k_{23}$ ($s^{-1}$) |
| 0.038 | – | – | 3.9 | 500 | – | – | – | – |
| 0.042 | 4.4 | 200 | 2.8 | 500 | 17.8 | 303 | 7.0 | 22 |
| 0.068 | 2.4 | 111 | 2.4 | 500 | 8.5 | 294 | 3.1 | 42 |
| 0.118 | 1.6 | 111 | 1.5 | 500 | 3.9 | 238 | 1.9 | 37 |
| 0.168 | 0.9 | 91 | – | – | – | – | – | – |
| 0.218 | 0.6 | 98 | 0.6 | 500 | 1.5 | 244 | 5.9 | 29 |

TABLE VI

THE EFFECT OF IONIC STRENGTH ON THE REDUCTION KINETICS OF CYTOCHROMES $c_2$

| Source | $E_0'$ (mV) | p*I* | $k_{12}$ ($M^{-1}$ $s^{-1}$) | $k_{23}$ ($s^{-1}$) | Apparent charge |
|---|---|---|---|---|---|
| *R. capsulata* | 368 | 7.1 | $1.3 \times 10^6$ | 500 | +0.7 |
| *R. palustris* (strain 2.1.37) | 362 | 9.7 | $1.3 \times 10^6$ | 90-200 | +0.7 |
| *R. vannielii* | 356 | 7.9 | $4.0 \times 10^5$ | 32 | +1.0 |
| *R. sphaeroides* | 351 | 5.5 | $4.2 \times 10^7$ | 270 | +1.6 |
| *R. rubrum* | 324 | 5.9 | $5.0 \times 10^6$ | 250 | +1.3 |
| Horse heart cytochrome *c* | 261 | 10.4 | $1.0 \times 10^6$ | 132 | +1.3 |

values on the reduction process (Table III), and the similar thermodynamic activation parameters (Table IV). We have previously demonstrated that R-cyto $c_2$ interacts with iron hexacyanides via a complex mechanism (Eq. [1], Ref. 8). We have no reason to think that this same mechanism is not operative for the reactants used here, even though no complexes were detected for the interaction of R-cyto $c_2$ with $SO_2^-$, $S_2O_4^{2-}$, ascorbate, and oxidized and reduced azurin (and HIPIP, Ref. 18). The lack of detectable complexes may be attributed to the lifetime of the complex. In terms of ferricytochrome $c_2$ reduction, ferrocyanide is the weakest reductant ($E_0'$ approximately ~400 mV). Yet the rate of electron transfer ($k_{23}$) is near the limit of the stopped flow (250 $s^{-1}$, Ref. 8). If driving force is a significant factor in the reaction then all other reductants studied should have rates of electron transfer ($k_{23}$) much greater than 250 $s^{-1}$ and would not be detectable by available mixing methods. Thus, it is our view that the second-order rate constants reported here and elsewhere (8, 18) monitor complex formation.

Electrostatics clearly play an important role in the chemistry of the interaction of cytochrome $c_2$ and the various reactants studied. A positive charge at the site of electron transfer is found irrespective of the nature of the oxidant or reductant (Table II) or the isoelectric point of the cytochrome (Table VI). This latter point establishes that a specific region on the cytochrome surface which is positively charged mediates electron transfer (8). However, the reactions cannot be completely described in terms of electrostatic effects because the rate of complex formation (Table II), is not proportional to the net charge on the oxidant or reductant. Also, for the reaction of R-cyto $c_2$ and oxidized HIPIP, no ionic strength dependence was noted, yet quite rapid rates were observed. Available evidence suggests that the chromophores of HIPIP and azurin are not solvent accessible (19, 20); hence, direct interaction with the heme of cytochrome $c_2$ is prohibited. Thus, it could be anticipated that steric restrictions might play an important role in controlling the rate of interaction of cytochrome $c_2$ with HIPIP and azurin. However, both HIPIP and azurin interact readily with cytochrome $c_2$ at rates approaching those observed with the small iron hexacyanides; thus, steric restrictions appear to be minimal. In view of the rapid rates of electron transfer between the redox proteins studied and the apparent lack of steric restrictions, a mechanism involving some type of electron tunneling is suggested. Thus, both orientation relative to a specific site of electron transfer and electron tunneling can be proposed as operative components of an electron transfer mechanism between these redox proteins.

The different p*K* values observed for the oxidized and reduced forms of the R-cyto $c_2$ most likely result from local changes in the environment of the participating side chains. This conclusion is derived from the requirement for oxidation and reduction to take place at the same site (Eq. [1], and Ref. 8) and the fact that the oxidized and reduced forms of the molecule have almost identical three-dimensional structures (9). Hence, the p*K* values for ferricytochrome $c_2$ at 6.2, 8.4, and 9.5 could be altered in the ferro form to 7.0, 9.5, and >11, respectively. Alternatively, the p*K* values at 6.2 and 8.4 could be altered

to <4.0 and 7.0, respectively, with the p*K* at 9.5 the same in both oxidation states. Available data cannot discriminate between these two possibilities as measurements below pH 4 or above pH 11 are not possible with the oxidants and reductants used to date. Nevertheless, p*K* changes on the order of 1 to 2 pH units are required which suggest rather substantial modifications of the chemical environment of the participating groups. Analysis of the kinetic data for reduction is difficult because two types of pH effects are noted (Fig. 1). The monovalent anions $SO_2^-$ and ascorbate have maximum rates at approximately pH 9, while $S_2O_4^{2-}$ and ferrocyanide show a continually decreasing rate from low to high pH. Nevertheless, the same p*K* values can be used for all reductants. The origin of these differences cannot be established at this time although the number of charges on a particular reductant may be important.

In terms of the exposed heme edge as the site of electron transfer, the p*K* value at 6.2 in the ferri form can be assigned to the propionic acid side chain of pyrrole ring 3. This group is extensively hydrogen bonded with serine-49 and tyrosine-46 hydroxyl groups within 4 Å (9) and the ε-amino group of lysine-90 within 3.5 Å [distances calculated from available coordinates (21)]. Thus, an altered p*K* for this group is likely and its close proximity to the heme edge and to the heme itself is suggestive of its participation. The alkaline p*K* (9.5 in the ferri form) can readily be assigned to a lysine or tyrosine residue with lysine-75, -88, and -90 and tyrosine-46, -48, -52, and -70 all in close proximity to the heme edge in R-cyto $c_2$. The p*K* at 8.4 in the ferri form is a more difficult assignment as it is lower than is generally observed for ε-amino groups and phenolic hydroxyls. Alternatively, ionizations away from the site of electron transfer could result in the perturbation of the solvent shell of cytochrome $c_2$ with attendant modifications at the site of electron transfer resulting in changes in all apparent rates. This latter possibility is difficult to investigate quantitatively and cannot be excluded at this time.

Ferrocyanide and ascorbate reduction and titration of the 695-nm band at alkaline pH are all consistent with the conversion of cytochrome $c_2$ to a new molecular species at alkaline pH (8). This conversion is complex as nonintegral values of *n* are obtained with a p*K* of approximately 9.4. The alkaline species formed is reducible by ascorbate (second-order reaction) but not reducible by ferrocyanide (8). Furthermore, ascorbate reduction of the alkaline form indicates at least two p*K* values (9.6 and 10.6, Table III). Thus, in the pH range 9–10, two molecular species of ferricytochrome $c_2$ are at equilibrium [rate of inner conversion, $\sim 0.02\ s^{-1}$ (8)] with each species having two ionization states.

Comparison of the rates of reduction of the various cytochromes $c_2$ studied (Table VI) indicates a wide range in values of both $k_{12}$ (100-fold) and $k_{23}$ (20-fold). Ideally, in a comparative analysis the difference observed can be assigned to specific parameters. In this regard no relation between oxidation–reduction potential or isoelectric point and the values of $k_{12}$ and $k_{23}$ can be detected (Table VI). This is not surprising and points to the more likely explanation that differences in structure in the vicinity of the site of electron transfer are responsible for the wide range of reaction rates. In the discussion that follows, structural differences in the vicinity of the exposed heme edge will be analyzed. Table VII presents the amino acid composition in the region of the exposed heme edge for the cytochromes $c_2$ used here. The side chains given in Table VII were obtained from inspection of the three-dimensional structure of R-cyto $c_2$ (9). The assignments for cytochrome $c_2$ from other sources were made on the basis of amino acid sequence homology. The basic assumption utilized is that the various cytochromes $c_2$ are structurally homologous. Hence, replacement of amino acid residues at a particular position in the amino acid sequence will not influence strongly the overall structural features of the molecule. This assumption is justified in our view by the striking structural homology between mammalian cytochrome *c* and bacterial cytochrome $c_2$ (9). Thus, the closely

TABLE VII

AMINO ACID RESIDUES IN THE VICINITY OF THE HEME EDGE

| Source | Amino acid[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 27[b] | 28 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 88 | 89 | 90 |
| *R. rubrum* | K | V | Y | A | Y | S | D | S | Y | K | S | K |
| *R. capsulata* | a | T | F | K | | K | | | I | | T | G |
| *R. sphaeroides* | | T | F | K | | G | E | G | M | | G | |
| *R. palustris* | – | | F | T | | | P | L | N | S | T | |
| *R. vannielii* | G | | F | N | | | | A | M | – | T | |
| Horse heart | | T | F | T | | T | | A | N | – | T | |

[a] A blank space indicates the same residue as in *R. rubrum;* a dash indicates sequence deletion at the indicated position.

[b] Position.

related bacterial cytochromes $c_2$ can be expected to show little or no differences in their backbone conformations.

Figure 3 represents an attempt to visualize the situation an approaching reactant would encounter in the region of the heme edge. Presented in this figure are only the charged or potentially hydrogen bonding residues taken from Table VII. The figure is roughly to scale (in two dimensions) with solid lines representing distances of less than 3 Å, dashed lines less than 4 Å, and dotted lines less than 5 Å. The distances were determined from the coordinates of ferricytochrome $c_2$ from *Rhodospirillum rubrum* (21) and assigned to other cytochromes $c_2$ from sequence homology. Net charges cannot be calculated because of the extensive hydrogen bond networks likely present (9); nevertheless, a reasonable explanation based on charge can be proposed for the wide range in rates of complex formation ($k_{12}$). S-cyto $c_2$ has four $\epsilon$-amino and two carboxyl groups available and has a much larger rate constant than R-cyto $c_2$, which has three $\epsilon$-amino and two carboxyl groups. V-cyto $c_2$ has one $\epsilon$-amino and two carboxyl groups, consistent with its relatively slow rate of complex formation. C-cyto $c_2$ has four $\epsilon$-amino and two carboxyl groups but is missing lysine-90 (replaced by glycine), the side chain most proximal to the heme edge. Thus, it can be suggested that in *R. capsulata* the loss of lysine-90 is partially compensated but not sufficiently to bring the rates up to the level of *R. rubrum* and *R. sphaeroides* cytochromes $c_2$. *R. palustris* cytochrome $c_2$ has only one $\epsilon$-amino group in the immediate vicinity of the exposed heme edge; however, the carboxyl group present at position 50 in the other cytochromes $c_2$ has been replaced by a proline. Thus, a rate of complex formation comparable to that of *R. capsulata* is observed. The foregoing discussion is speculative; nevertheless, it does suggest a rational explanation for the observed rates and serves as a basis for the design of further experiments.

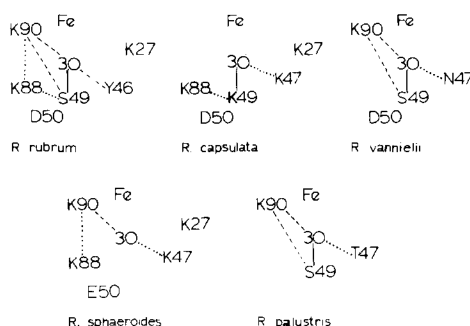



FIG. 3. Structure of cytochrome $c_2$ in the vicinity of the exposed heme edge. Solid lines represent distances of less than 3 Å, dashed lines less than 4 Å, and dotted lines less than 5 Å. From the single-letter code for amino acids: K, lysine; Y, tyrosine; S, serine; D, aspartic acid; N, asparagine; E, glutamic acid; T, threonine. In addition, Fe represents the heme iron and 30 represents a carboxyl oxygen of the propionic acid side chain of pyrrole ring 3.

To summarize we have shown that a wide variety of nonphysiological reactants interact with cytochrome $c_2$ from *R. rubrum* with kinetic parameters consistent with a common mechanism. Moreover, cytochrome $c_2$ from a variety of sources

reacts with potassium ferrocyanide, consistent with an identical mechanism. Kinetic results with physiological reactants (HIPIP and azurin discussed here) are generally consistent in that charged interactions are as observed with the nonphysiological reactants. However, the physiological reactants do not appear to be severely sterically restricted suggesting that electron transfer can take place over a relatively large distance (>5 Å). Analysis of available kinetic data in terms of the primary and tertiary structure in the region of the exposed heme edge yields a consistent interpretation of the observed kinetic differences and similarities and implicates the participation of specific amino acid side chains.

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