Comparative Kinetic Studies of Cytochromes *c* in Reactions with Mitochondrial Cytochrome *c* Oxidase and Reductase†

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ABSTRACT: Kinetic studies of the reactions of selected eukaryotic and prokaryotic cytochromes *c* with mitochondrial cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1) using a standardized complex IV preparation from beef heart are reported. Data on reactions with NADH-linked cytochrome *c* reductase (complexes I and III) are included. The concentration ranges employed provide a basis for quantitative demonstration of a general rate law applicable to oxidase reactions of cytochrome *c* of greatly differing reactivities. Results are interpreted on the basis of a modified Minnaert mechanism (Minnaert, K. (1961) *Biochim. Biophys. Acta 50,* 23), assuming productive complex formation between cytochrome *c* and free oxidase in addition to further complex binding of a second cytochrome *c* molecule to the initially formed oxidase complex. Kinetic constants so obtained are consistent with the assumption that binding is the dominant parameter in reactivity, and can be rationalized most simply on this basis.

Comparative biochemical approaches to elucidate structural bases for cytochrome *c* function by kinetic studies of reactions of cytochrome *c* from both prokaryotic and eukaryotic sources with cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) have been proposed (Kamen, 1972). Generally, data available—largely from comparison of reaction rates at single concentrations—show that as electron donors to the mitochrondrial oxidase most bacterial cytochromes *c*, as well as algal cytochromes *f*, are inactive. However, such information is of limited significance. Relative reactivities of two cytochromes *c* can differ depending upon the single concentration chosen for comparison and further large kinetic differences between cytochromes can be observed at concentrations of cytochrome *c* less than 1 μM which otherwise would be unnoticed at the higher concentrations generally employed in these surveys (Ferguson-Miller et al., 1976).

In this paper we describe kinetic analyses of the reaction of selected cytochromes *c* with a standard, reproducible beef heart cytochrome *c* oxidase preparation and include some observations as well on the mitochondrial cytochrome *c* reductase catalyzed reactions. Results obtained establish the generality of the rate law previously deduced for the reaction of horse cytochrome *c* with oxidase (Errede et al., 1976). The kinetic constants obtained from this rate law provide a quantitative measure of cytochrome *c* function with oxidase and therefore provide a sounder basis for deductions made from such comparisons. A number of mechanisms are consistent with this rate law, but we have chosen one most plausible at present for detailed discussion in accordance with the objective of these studies—clarification by comparative biochemical approach of the structural parameters which govern reactivity of cytochrome *c* with mitochondrial cytochrome oxidase.

Materials and Methods

Cytochromes. References for the methods of preparation and α band extinction coefficients are given in Table I for the cytochromes used. Horse cytochrome *c*, *Rhodospirillum rubrum* cytochrome c_2, *Paracoccus denitrificans* cytochrome c_{550} and *Rhodomicrobium vanielii* cytochrome c_2 were treated to remove adventitiously bound ions and possible small fractions of polymeric cytochrome according to the methods previously described (Errede et al., 1976).

Prior to use, 2 mL of a solution of approximately 2 mM cytochrome *c* was reduced by addition of excess sodium ascorbate. The excess reductant was removed by gel filtration chromatography on Sephadex G-25 fine (1.0 × 25 cm) equilibrated with the buffer to be used thereafter. The concentrations of the sodium dithionite-reduced cytochromes were determined based on the extinction coefficients of Table I.

For purposes of the survey of oxidase and reductase activities, the procedure for removal of bound ions was modified to accommodate the small quantities of cytochrome *c* required for these experiments. In place of the Tris base dialysis, 0.2 mL of a 0.5 mM solution of cytochrome *c* was subjected to gel filtration chromatography on Sephadex G-25 fine (1 × 15 cm) equilibrated in 20 mM Tris base and 10 μM EDTA. The middle portion of the cytochrome band eluted from the column was collected, neutralized with cacodylic acid, and oxidized by addition of Tris-Co(III)-*o*-phenanthroline or reduced by addition of excess sodium ascorbate. The cytochrome *c* solution was then treated essentially as previously described. This procedure could not be used for cytochromes *c* with p*I* values >9 as they were found to precipitate on the Sephadex column.

Preparation of Mitochondrial Complexes. Cytochrome oxidase (complex IV) was prepared according to the method of Fowler et al. (1962) and NADH cytochrome *c* reductase (complexes I-III) according to the method of Hatefi et al. (1961). Protein concentrations were determined by both the modified Biuret (Gornal et al., 1949) and modified Lowry (Miller, 1959) procedures. The preparations—divided into small aliquots to avoid repeated freeze thawing—were stored

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TABLE I: Comparison of Cytochrome *c* Properties and Rates of Reaction with Mitochondrial Oxidase and Reductase.

Cytochrome *c*[a]	Properties: Absorption maxima (nm)	Properties: Extinction coefficient (mM^{-1} cm^{-1})	Properties: $E_{m,7}$ (mV)	Properties: pI	Rel rates of reaction: Oxidase[b,c] (%)	Rel rates of reaction: Reductase[g] Zero order (%)	Rel rates of reaction: Reductase[g] First order (%)
Mitochondrial *c* type cytochromes							
Mammalian							
Horse[n]	550	27.6[o]	261[p]	10.04[q]	100[d]	100	100
Protozoa							
Euglena gracilis (ATCC 12716)[r]	558	26.3[r]	244[s]	Basic[t]	283[e]	102	102
Cr. oncopelti[r]	557	24.7[r]	254[s]	Basic[t]	220[e]	98	61
Cr. fasciculata[u]	555	29.7[v]	280[v]	9.9[v]	154[e]	101	85
T. pyriformis[w]	553	27.6[w]	245[w]	Acidic[w]	0.8	1[h]	
Bacterial c_2 type cytochromes							
Photosynthetic							
R. rubrum S1 (ATCC 11170)[x]	550	26.9[y]	320[z]	6.2[x]	0.9	69	48
R. photometricum (SP113)[aa]	550.5	*bb*	341[z]		0.2	51	14
R. molischianum iso 1 (ATCC 14031)[cc]	549	29.2[cc]	388[z]	9.8[cc]	<0.05	81	13 (6)
R. molischianum iso 2 (ATCC 14031)[cc]	549	29.8[cc]	305[z]	9.4[cc]	0		25
R. fulvum iso 1[aa]	549.5	*dd*	371[z]		<0.05	73	9 (6)
Rhodopseudomonas sphaeroides 2.4.1 (ATCC 17023)[aa]	550	30.8[ee]	353[ff]	6.3[gg]	1.0	93	102
Rhodopseudomonas capsulata SL (ATCC 23782)[aa]	550	*bb*	368[ff]	7.1[gg]	0.6	66	129
Rhodopseudomonas palustris 37 (ATCC 17007)[aa]	551.5	26.7[y]	367[z]	9.7[gg]	0.6	66	55
Rhodopseudomonas palustris x[aa]	551.5	*hh*	367[z]		0.9	65	68
Rhodopseudomonas palustris 6 (ATCC 17001)[aa]	551.5	*hh*	348[z]		1.3	69	99
Rhodopseudomonas viridis[aa]	550.5	*hh*	297[z]		0.4	28	6
Rhodomicrobium vannielii (ATCC 17100)[aa]	550	*hh*	357[ff]	7.9[gg]	0.3	70	56
Nonphotosynthetic							
P. denitrificans (ATCC 13543)[ii,jj]	550	26.8[jj]	250[kk]	4.5[jj]	4.4	62	28
Bacterial c_{551} type cytochromes							
Photosynthetic							
R. tenue[aa]	552.5	*ll*	405[z]		0	12[i]	1
R. gelatinosa[aa]	553	*ll*		9.6[gg]		0	
Nonphotosynthetic							
Ps. aeruginosa (ATCC 10145)[mm]	551	28.3[mm]	286[mm]	4.7[mm]	<0.05	2[j]	
Bacterial c_{555} type cytochromes							
Photosynthetic							
C. limicola (NCIB 8346)[nn,oo]	555	26.2[oo]	145[oo]	10.5[pp]	27[f]		6
Pr. aestuarii[aa,qq]	555	20.4[rr]	103[rr]	4.7[rr]	0	0	
Soluble algal and related *c* type cytochromes							
Algae							
Porphyra tenera[ss]	553	21.7[ss]	335[ss]	3.5[ss]	<0.05	1[k]	
Spirulina maxima[tt]	553	*uu*			<0.05	3[l]	
Protozoan							
Euglena gracilis (ATCC 12716)[t]	552	29.3[t]	371[vv]	5.5[gg]	<0.05	2[m]	

[a] References for methods of preparation are given in the footnotes in this column for each cytochrome *c*. [b] Assay conditions: 0.1 M Mes, pH 6.0; 5 μM cytochrome *c*; 121.5 nM oxidase; 25 °C. [c] Relative rates of reaction were calculated on the basis of values of k' for each cytochrome. [d] Conditions were as specified in *b* except that 8.8 nM oxidase was used. [e] Conditions were as specified in *b* except that 3.5 nM oxidase was used. [f] Conditions were as specified in *b* except that 24 nM oxidase was used. [g] Assay conditions: 50 mM Hepes, pH 7.5; 250 μM KCN; 100 μM NADH; 5 μM cytochrome *c*; 1.8 ng of complexes I–III; 25 °C. Unless specified, initial rates of reduction were ≥98% inhibited by the addition of 4.4 ng of antimycin. [h] 20% inhibition by antimycin. [i] 84% inhibition by antimycin. [j] 10% inhibition by antimycin. [k] 35% inhibition by antimycin. [l] 23% inhibition by antimycin. [m] 14% inhibition by antimycin. [n] Type IV purchased from Sigma Chemical Co. [o] Schejter et al., 1963. [p] Margalit and Schejter, 1973. [q] Barlow and Margoliash, 1966. [r] Pettigrew et al., 1975b. [s] Pettigrew et al., 1975c. [t] Pettigrew, 1975. [u] The method for purification of *Cr. oncopelti c* was used (Pettigrew et al., 1975b). [v] Kusel et al., 1969. [w] Yamanaka et al., 1968. [x] Bartsch et al., 1971. [y] Bartsch, 1963. [z] Pettigrew, unpublished results. [aa] General method for bacterial cytochrome *c* purification, as outlined by Bartsch (1977). [bb] The value of the extinction coefficient for *R. rubrum* c_2 was used. [cc] Flatmark et al., 1970. [dd] The value of the extinction coefficient for *R. molischianum* iso-1-c_2 was used. [ee] Meyer, unpublished results. [ff] Pettigrew et al., 1975b,c. [gg] Kamen et al., 1972. [hh] The value of the extinction coefficient for *Rps. palustris* 37 c_2 was used. [ii] Formerly, *Micrococcus denitrificans*. [jj] Scholes et al., 1971. [kk] Kamen & Vernon, 1955. [ll] The value of the extinction coefficient for *Ps. aeruginosa* c_{551} was used. [mm] Horio et al., 1960. [nn] Formerly, *Chlorobium thiosulfatophilum*. [oo] Meyer et al., 1968. [pp] Gibson, 1961. [qq] *Prosthecochloris aestuarii* (formerly, *Chlorobium limicola*) is part of the *Chloropseudomonas ethylica* strain 2K consortium from which the cytochrome c_{555} was isolated. [rr] Shioi et al., 1972. [ss] Katoh, 1960. [tt] Ambler & Bartsch, 1975. [uu] The value of the extinction coefficient for *Porphyra tenera* c_{553} was used. [vv] Perini et al., 1964.

at a concentration of 25 mg of protein per mL of TSH[1] (50 mM Tris, 0.67 M sucrose, 1 mM histidine adjusted to pH 8.0 with HCl) at −70 °C. The molar concentration of oxidase was determined spectrophotometrically using an extinction coefficient (Δ605–630 nm) of 16.5 mM^{-1} cm^{-1} for the sodium dithionite reduced enzyme (Griffiths & Wharton, 1961). Prior to use for assay the stock solutions of oxidase or reductase were diluted to 0.1–0.4 mg per mL of ice cold water. These solutions, kept at 4 °C, gave reproducible activities for at least 3 h.

Preparation and Calculation of Ionic Strength of Buffers Used for Kinetic Assays. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffers were prepared by the addition of sodium hydroxide to a solution of either 0.2 or 0.02 M Mes and 0.1 M Hepes (acid forms). The amount of sodium hydroxide added was equivalent to the concentration of conjugate base of the buffer required for the desired pH. The solutions were then diluted to give a final concentration of 0.1 or 0.01 M Mes and 0.04 M Hepes. The ratio of base to acid for a given pH was calculated, using the Henderson–Hasselbach equation and pK_a (25 °C) values of 6.1 and 7.5 for Mes and Hepes, respectively (Good et al., 1966). The ionic strength, I, of the buffers was determined by the usual relation:

$$I = (1/2)(1/\rho)\sum_i c_i(z_i)^2 \qquad (1)$$

with density, ρ, assumed to be unity for the concentrations of buffers used, taking into account that both Mes and Hepes as zwitterions in the protonated form behave as dipoles in solution.

Assay for Cytochrome Oxidase Activity. The kinetics of the oxidase catalyzed reaction were monitored by disappearance of ferrocytochrome *c* measured spectrophotometrically. The decrease in absorbance of the α peak was determined for concentrations of cytochromes *c* between 1 and 60 μM, that of the β peak for concentrations greater than 60 μM, and that for the γ peak for concentrations less than 1 μM. Experiments were performed in the presence of 0.1 M Mes, pH 6.0, or 0.01 M Mes, pH 6.0, and 10 μM EDTA at 25 °C. A pH below neutrality was chosen for all assays to avoid deviations from pseudo-first-order kinetics apparent above pH 7.0 (Yonetani & Ray, 1965) attributable to the increasing equilibrium concentration of an ascorbate-unreactive monomer form of cytochrome *c* under conditions of increasing pH (Greenwood & Palmer, 1965; Wilson & Greenwood, 1971). Buffer, EDTA, and an appropriate amount of ferrocytochrome *c* were added to 1- or 3-mL cuvettes so that the final volumes after addition of oxidase were 1 or 3 mL, respectively (3-mL cuvettes were utilized for concentrations of cytochrome *c* less than 1 μM). Prior to assay, the mixtures in the cuvettes were placed in a metal block that was immersed in a temperature-regulated water bath. After temperature equilibration, a cuvette was placed in the temperature-regulated sample holder of the spectrophotometer. The reaction was initiated by addition of an appropriate amount of oxidase. An Aminco-Chance Model DW-2 dual wavelength recording spectrophotometer, operated in the split beam mode, was used for all measurements.

Calculation of Rates of Ferrocytochrome c Oxidation Catalyzed by Cytochrome Oxidase. Rates of ferrocytochrome *c* were calculated as pseudo-first-order rate constants, k_{obsd}. The values for $-k_{obsd}$ were obtained from the slope of plots of $\ln[A_t - A_\infty]$ vs. time where A_t was the absorbance at time t and A_∞ the absorbance at completion of reaction. A computer program was used to calculate $-k_{obsd}$ by a least-squares method.[2] When possible the reaction was allowed to go to completion, and the corresponding value for A_∞ was verified by addition of a trace of potassium ferricyanide. When reactions were very slow, as for the reactions of *R. rubrum* c_2 and *Rm. vannielii* c_2 with oxidase, the first-order time course would be verified only for several cytochrome *c* concentrations and then A_∞ determined by addition of potassium ferricyanide after 3 half-lives of oxidation. The rate designated k' was defined by eq 2

$$k' = k_{obsd}/[\text{oxidase}] \qquad (2)$$

where [oxidase] was the molar concentration of oxidase used.

Calculation of Kinetic Constants for the Reaction of Cytochrome c with Oxidase. It has been demonstrated (Smith & Conrad, 1956; Minnaert, 1961; McGuinness & Wainio, 1962; Yonetani & Ray, 1965; Errede et al., 1976) that the reaction of ferrocytochrome *c* with cytochrome oxidase obeys the following general rate law:

$$\text{velocity} = k'[\text{oxidase}][\text{ferrocytochrome } c] \qquad (3)$$

where k'[oxidase] is the pseudo-first-order rate constant, k_{obsd}, for this reaction; k' is a function of total cytochrome *c* concentration (Smith & Conrad, 1956; Errede et al., 1976). The data obtained for a wide range of cytochrome *c* concentrations were plotted as k' vs. k'[C] (analogous to V/S vs. V plots) and as $1/k'$ vs. [C] (analogous to V/S vs. S plots). Because of the large concentration ranges employed, it was advantageous to examine data using both methods of analysis. The former allowed best visualization of the data in the lower concentration range while the latter was best for the higher concentration range.

Method 1. Under conditions in which the data for the above plots were nonlinear and two distinct slopes were apparent, data were analyzed using eq 3 with k' defined by eq 4.

$$k' = \frac{\alpha_1 + \alpha_2[\text{C}]}{1 + \beta_1[\text{C}] + \beta_2[\text{C}]^2} \qquad (4)$$

The four constants in eq 4 can be approximated by satisfying two limiting cases, as in cases I and II of Table II. At these extremes, plots of k' vs. k'[C] and $1/k'$ vs. [C] become linear so that associated constants can be calculated from the slopes and intercepts of either plot (Table II). First-order approximations for the constants α_1 and β_1 are derived by calculating the slope and intercept for the linear region of plots of k' vs. [C]. First-order approximations for the constants α_2 and β_2 are made by using the linear region of plots of $1/k'$ vs. [C] together with the estimate for β_1 (see Table II). The estimates of these constants for each cytochrome can be refined using a computer program[3] to optimize the coefficients in eq 4 so that the best fit to *all* the data throughout the concentration range studied is obtained. Values for the constants obtained in this manner are valid if data that satisfy the requirements specified by the limiting cases of Table II are included in the optimization calculation.

Method 2. For cases in which two distinct slopes are not

[1] Abbreviations used: TSH, 50 mM Tris, 0.67 M sucrose, 1 mM histidine adjusted to pH 8.0 with HCl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; *C., Chlorobium; Cr., Crithidia; P., Paracoccus; Ps., Pseudomonas; Pr., Prosthecochloris; R., Rhodospirillum; Rm., Rhodomicrobium; T., Tetrahymena.*

[2] This program was provided by P. Lert and J. McGinness, University of California, San Diego.

[3] The program used is a modified version of a curve-fitting program kindly provided by Dr. M. A. Cusanovich, University of Arizona.

apparent in the plots of k' vs. $k'[C]$ and $1/k'$ vs. [C], first-order approximations for the coefficients of eq 4 are found by solving several sets of simultaneous equations of the form:

$$k_i' = \alpha_1 + \alpha_2 k_i'[C_i] + \beta_1 k_i'[C_i] + \beta_2 k_i'[C_i]^2 \qquad (5)$$

where k_i' is the experimentally observed rate constant for the C_i concentration of cytochrome c, $i = 1$–4.

The estimates so obtained are then used in the computer program that optimizes each of the constants so that the best fit to eq 3 with k' as defined by eq 4 is obtained for the experimental data throughout the concentration range used. Again, reliable estimates for the constants obtained by the curve fitting method require that the concentration range of cytochrome c studied include concentrations that meet the criteria for establishment of both limiting cases (Table II).

Method 3. For cases in which the plots of $1/k'$ vs. [C] and k' vs. $k'[C]$ appeared linear throughout the concentration range studied, the data were analyzed using eq 6 to define the relationship between k' and total cytochrome c concentration

$$k' = \frac{\gamma}{1 + \delta[C]} \qquad (6)$$

with γ and δ defined as $k_1k_3/(k_2 + k_3)$ and k_1/k_2, respectively, by Minnaert's mechanism IV (Minnaert, 1961). γ was obtained from the value of the ordinate intercept ([C] = 0) and δ from the negative of the slope for plots of k' vs. $k'[C]$. Values for the slope and intercept were obtained using a linear regression analysis for the data pairs, k' and $k'[C]$.

NADH-Cytochrome c Reductase Reaction. The reduction of ferricytochrome c catalyzed by complexes I–III was monitored by following the increase in absorbance of the α band for cytochrome c spectrophotometrically. Experiments were performed at 25 °C in the presence of 50 mM Hepes (pH 7.5), 20 μM EDTA, 250 μM KCN, and 100 μM NADH. 5 μM cytochrome c and 1.8 ng of complexes I–III were used in all assays. The specificity of the reduction for each cytochrome c was verified by addition of 4.4 ng of antimycin to the assay mixture. (Inhibition of cytochrome c reduction by antimycin indicates that the reaction monitored is specifically reduction of cytochrome c by complex III.) Zero-order rates were calculated from the initial linear region of the reaction and first-order rates were calculated from the linear portion of plots of $\ln[A_t - A_\infty]$ vs. time according to the method of Smith et al. (1974).

Results

Comparison of Cytochrome c Reactivity with Mitochondrial Cytochrome c Oxidase and Reductase. Representative cytochromes c from several structural categories (see Ambler, 1973) were assayed for their ability to serve as electron donors to cytochrome oxidase and electron acceptors for NADH-cytochrome c reductase. The results of this survey are given in Table I. Oxidase and reductase rates are reported as percent of the respective values obtained for horse c. The values for two physical-chemical properties of these cytochromes, thermodynamic electrochemical potential and isoelectric point, have also been included in this table for comparison with kinetic results.

The kinetics of oxidase catalyzed oxidation of cytochrome c were shown to follow a first-order time course (see Materials and Methods) for all cytochromes except where rates were indicated to be <0.05% of horse c. In those instances a slow, cyanide sensitive oxidation could be observed if long reaction times were allowed. The time required for oxidation of one-half the amount of cytochrome c present was compared with that for horse c. Using this criterion it was estimated that first-order rates of oxidation for these cytochromes could be no greater than the 0.05% reported.

TABLE II: Limiting Cases for Determination of Characteristic Constants of Equation 4.

If	
$\alpha_1 > 10\alpha_2[C]$	$\alpha_2[C] > 10\alpha_1$
$1 + \beta_1[C] > 10\beta_2[C]^2$	$\beta_1[C] + \beta_2[C]^2 > 10$
Then	
$k' = \frac{\alpha_1}{1 + \beta_1[C]}$	$k' = \frac{\alpha_2/\beta_1}{1 + \{\beta_2/\beta_1\}[C]}$
Plot: $1/k'$ vs. [C]	
$1/k' = 1/\alpha_1 + \{\beta_1/\alpha_1\}[C]$	$1/k' = \beta_1/\alpha_2 + \{\beta_2/\alpha_2\}[C]$
β_1 = slope/intercept	β_2/β_1 = slope/intercept
α_1 = 1/intercept	α_2/β_1 = 1/intercept
Plot: k' vs. $k'[C]$	
$k' = \alpha_1 - \beta_1 k'[C]$	$k' = \alpha_2/\beta_1 - \{\beta_2/\beta_1\}k'[C]$
β_1 = −slope	β_2/β_1 = −slope
α_1 = intercept	α_2/β_1 = intercept

The kinetics of NADH-cytochrome c reductase catalyzed reduction of cytochrome c were biphasic, characterized by an initial linear phase zero order in cytochrome c and a terminal phase first order in cytochrome c (Smith et al., 1974). Although Smith et al. (1976) have reported that *R. rubrum* c_2 and *P. denitrificans* c_{550} show only first-order kinetics for reduction, under the conditions employed here we have found zero-order kinetics persist throughout the initial 50% of the reaction. However, the zero-order phase was apparently absent or minimal for reaction of *R. molischianum* iso-2 c_2 and *C. limicola* c_{555} with complexes I–III. *R. molischianum* and *R. fulvum* iso-1-cytochromes c_2 also showed anomalous kinetics at pH 7.5. At this pH the first 15–20% of the reaction was apparently zero order. However, the remaining portion of the reaction showed two distinct first-order rates, indicative of the presence of two species of cytochrome c in solution. The contribution of both rates to the total reaction was approximately equal. The value for the slower first-order rate is given in parentheses in Table I. The kinetics of reaction for these two cytochromes at pH 6.0 showed no evidence of zero-order kinetics and only one first-order rate which was 6% of that for horse c at the same pH.

When initial rates of reaction were ≤3% of horse c, lengthy reaction times prevented determination of any possible first-order rate. Furthermore, in these instances the reduction of cytochrome c was <35% inhibited by the addition of antimycin, indicating that the reduction rates measured might not represent interaction of the cytochrome c with complexes I–III.

Kinetic characterization of the oxidase reaction was undertaken for selected cytochromes shown to be of particular interest from each structure category. Such characterization was not possible for cytochromes with rates of reaction <0.05% of horse c and for *T. pyriformis* c because of the very limited amount of this particular cytochrome available. Attempts to develop detailed kinetic analyses of mitochondrial pyridine-linked cytochrome c reductase system (complexes I–III) are not indicated at present because of the difficulties in interpretation which arise from the existence of biphasic kinetics.

General Kinetic Properties of Cytochrome c Reaction with Oxidase. The kinetics of oxidation for horse c, *Cr. fasciculata* c, *C. limicola* c_{555}, *P. denitrificans* c_{550}, *R. rubrum* c_2, and *Rm. vannielii* c_2 were found to follow a first-order time course for the disappearance of ferrocytochrome c. Plots of $\ln[A_t - A_\infty]$

TABLE III: Values for the Coefficients of Equation 4.[a]

Cytochrome	I (mM)	α_1 ($M^{-1}\,s^{-1}$)	α_2 ($M^{-2}\,s^{-1}$)	β_1 (M^{-1})	β_2 (M^{-2})	γ ($M^{-1}\,s^{-1}$)	δ (M^{-1})
Horse *c*	44	2.50×10^8	2.49×10^{13}	6.21×10^6	2.39×10^{11}		
	4	1.90×10^8	5.04×10^{13}	1.20×10^7	1.10×10^{12}		
Cr. fasciculata c	44	7.94×10^7	2.30×10^{13}	1.40×10^6	2.07×10^{11}		
C. limicola c_{555}	44	3.65×10^6	1.14×10^{11}	9.03×10^4	7.83×10^8		
P. denitrificans c_{550}	44	6.05×10^5	1.98×10^{10}	1.29×10^5	5.61×10^8		
R. rubrum c_2	44	9.70×10^4	$(1.51 \times 10^9)^b$	2.58×10^4	$(2.12 \times 10^8)^b$	9.75×10^4	1.17×10^4
	4	8.63×10^5	1.03×10^{11}	3.56×10^5	2.27×10^{10}	7.91×10^5	1.68×10^5
Rm. vannielii c_2	44	2.55×10^4	$(1.66 \times 10^8)^b$	6.98×10^3	$(3.27 \times 10^7)^b$	2.62×10^4	2.39×10^3

[a] Conditions for assay are given in the legend to Figure 1. [b] Concentrations of cytochrome c_2 were not adequate for valid estimation of the constants.

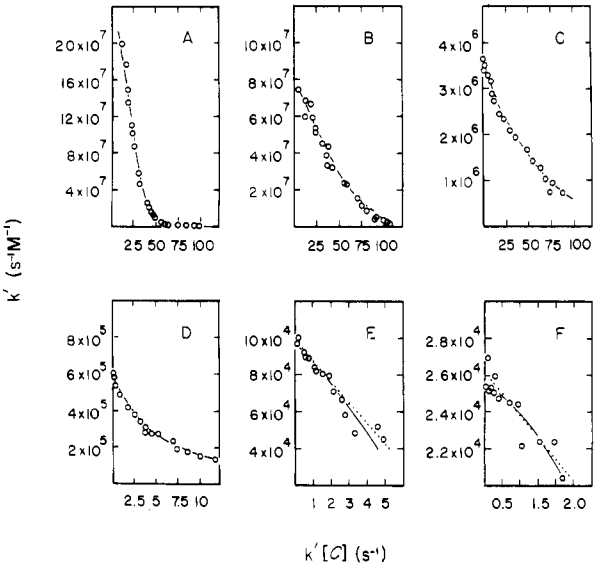

FIGURE 1: Comparison of rate dependence on total cytochrome *c* concentration as shown by plots of k' vs. k'[C]. All assays were performed at 25 °C in 0.1 M Mes, pH 6.0 (I = 44 mM) and 10 μM EDTA. (A) Horse cytochrome *c*: 0.06–0.70 μM cytochrome *c* with 1.3 nM oxidase; 0.50–10 μM cytochrome *c* with 4.2 nM oxidase; 2–5 μM cytochrome *c* with 8.8 nM oxidase; and 7.5–160 μM cytochrome *c* with 37 nM oxidase. (B) *Cr. fasciculata c*: 0.098–1.33 μM cytochrome *c* with 1.6 nM oxidase; 1.20–15.1 μM cytochrome *c* with 3.3 nM oxidase; 15.1–92.8 μM cytochrome *c* with 8.1 nM oxidase. (C) *C. limicola* c_{555}: 0.096–4.82 μM cytochrome c_{555} with 4.8 nM oxidase; 4.82–121 μM cytochrome c_{555} with 24 nM oxidase. (D) *P. denitrificans* c_{550}: 0.70–2.40 μM cytochrome c_{550} with 8.1 nM oxidase; 2.4–110 μM cytochrome c_{550} with 121.5 nM oxidase. (E) *R. rubrum* c_2: 1.23–110 μM cytochrome c_2 with 61 nM oxidase. (F) *Rm. vannielii* c_2: 1.22–110 μM cytochrome c_2 with 121.5 nM oxidase.

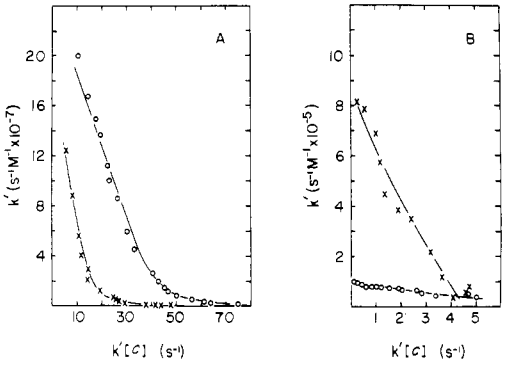

FIGURE 2: Comparison of rate dependence on total cytochrome *c* concentration at ionic strengths 4 mM and 44 mM for horse *c* (A) and *R. rubrum* c_2 (B). Assay conditions at I = 44 mM (○) are as defined for Figure 1. Assay conditions at I = 4 mM (X): 25 °C, 0.01 M Mes, pH 6.0, 10 μM EDTA. (A) Horse *c*: 0.049–0.197 μM cytochrome *c* with 1.2 nM oxidase; 0.296–1.48 μM cytochrome *c* with 2.3 nM oxidase; 1.48–29.6 μM cytochrome *c* with 17.5 nM oxidase. (B) *R. rubrum* c_2: 0.30–1.46 μM cytochrome c_2 with 24 nM oxidase; 0.73–29.9 μM cytochrome c_2 with 48 nM oxidase; 29.9–103 μM cytochrome c_2 with 95 nM oxidase.

vs. time were linear for at least 4 to 5 half-lives (95%) of oxidation without any apparent deviations. The first-order rate constant, k_{obsd}, was directly proportional to oxidase concentration between 1 and 40 nM heme *a* for horse cytochrome *c* and between 50 nM and 400 nM heme *a* for *R. rubrum* c_2. Therefore, using concentrations of oxidase within the limits of 1 to 400 nM to obtain reasonable reaction rates with the various cytochromes, it could be assumed that the direct proportionality between k_{obsd} and oxidase concentration held. The rates, however, were found to decrease with increasing total cytochrome *c* concentration in all cases.

These observations established eq 3 as applicable to the reaction of oxidase with cytochromes *c* of greatly differing reactivities (Table I). However, the dependence of k' on total cytochrome *c*, [C], was different for each of these cytochromes *c*. Increasing the concentration of horse cytochrome *c* from 1 to 100 μM resulted in a decrease greater than 25-fold in k'. For approximately the same increase in total cytochrome *c* concentration there was only a 25% decrease in k' for *Rm. vannielii* cytochrome c_2. Decrease in k' with increasing cytochrome *c* concentration fell between these extremes for the other cytochromes *c* studied. Hence, it was concluded that the differences in activities observed could be compared quantitatively by defining the relationship between k' and total cytochrome *c* concentration for each.

Determination of Kinetic Constants from k' Dependence on Total Cytochrome c Concentration. Replots of the data for k' dependence on cytochrome *c* concentration (only plots of k' vs. k'[C]) are shown in Figure 1. Those in Figures 1A-D are clearly nonlinear. Therefore, the data were analyzed using eq 4 to define the dependence of k' on cytochrome *c* concentration and constants were obtained according to Method 1. The optimized values of the constants α_1, α_2, β_1, and β_2 for horse *c*, *Cr. fasciculata c*, *C. limicola* c_{555}, and *P. denitrificans* c_{550} are given in Table III. Using the appropriate form of eq 4 and these values, the corresponding curves in Figures 2A-D were calculated. The excellent fit to the data established eq 4 as a basis for adequate definition of k' dependence on cytochrome *c* concentration for the reaction of these cytochromes with oxidase. The concentration ranges of cytochrome *c* employed for the determination of the kinetic constants for horse *c* and *Cr. fasciculata c* included concentrations of cytochrome *c* that rigorously satisfied all of the limiting conditions specified in Table II. The concentrations of *C. limicola* c_{555} and *P. denitrificans* c_{550} included in the estimates of the constants for

these cytochromes met the specifications of the limiting conditions of Table II except that $\alpha_2 \simeq 4\alpha_1$ for the highest concentration of each.

The data for *R. rubrum* c_2 and *Rm. vanielii* c_2 (Figures 1E and 1F) show more scatter than for the other cytochromes *c* studied. It should be recalled that the total decrease in k' for a 100-fold increase in cytochrome *c* concentration for each is small. However, the degree of accuracy that can be obtained in this assay system for the values of k' is generally no better than ±5%. Thus, much of the scatter is within the limits of accuracy of the experiment.

Assuming linear plots shown in Figures 1E and 1F, the constants γ and δ of eq 6 were calculated according to Method 3. The resulting values for each cytochrome are given in Table III. The dotted curves in Figures 1E and 1F were calculated using the corresponding values for γ and δ along with the appropriate form of eq 6. The data were also analyzed using eq 4 and Method 2 for obtaining values for the constants α_1, α_2, β_1, and β_2. The solid curves in Figures 1E and 1F were calculated using the values of these constants given in Table III and the appropriate form of eq 4. Neither of the two limiting conditions for case II of Table I was satisifed for the concentrations of *R. rubrum* c_2 and *Rm. vannielii* c_2 employed. Concentrations of *R. rubrum* $c_2 > 0.25$ mM and *Rm. vannielii* $c_2 > 0.65$ mM would be required to meet the conditions that would make visible the effects of the case II constants, α_2 and β_2. (Concentrations of *R. rubrum* $c_2 > 0.6$ mM and *Rm. vannielii* $c_2 > 1.5$ mM would be necessary to satisfy the conditions specified by Table II.) Therefore, under conditions in which it was practical to measure rates of reaction with oxidase for these two cytochromes, k' dependence on total cytochrome *c* concentration was largely defined by the special conditions of limiting case I, or equivalently eq 6.

Effect of Ionic Strength. The influence of ionic strength on the reaction of horse cytochrome *c* with cytochrome oxidase has been well documented (Wainio et al., 1960; Davies et al., 1964; van Gelder et al., 1975). A comparison of the rate dependence on total cytochrome *c* concentration was made for horse *c* (Figure 2A) and *R. rubrum* c_2 (Figure 2B) using assay buffers at an ionic strength of 44 mM (0.1 M Mes, pH 6.0) and 4 mM (0.01 M Mes, pH 6.0). The tenfold change in ionic strength did not alter the form of the rate law given by eq 3 with k' defined by eq 4. The values of the constants of eq 4 were calculated according to Method 1 for horse *c* and Method 2 for *R. rubrum* c_2 data obtained at $I = 4$ mM. (The data at $I = 44$ mM were treated as described previously.) The values so obtained for α_1, α_2, β_1, and β_2 are given in Table III and were used to calculate the corresponding curves in Figures 2A and 2B using the appropriate form of eq 4. All limiting conditions of Table I were satisfied for the estimation of constants for both horse *c* and *R. rubrum* c_2 for the data obtained under assay conditions of 4 mM ionic strength. Nonetheless, the data for *R. rubrum* c_2 do not appear curved (points marked "X" in Figure 2B). Therefore, constants γ and δ of eq 6 were also calculated according to Method 3 and the resulting values are given in Table III.

Mechanisms for the Interpretation of Kinetic Results

The functional competence of variant cytochromes *c* as well as chemically modified cytochromes can be quantitated on the basis of values for the constants of eq 4 obtained by application of kinetic analyses as described. However, any number of mechanisms which define these constants can result in rate expressions of the form deduced from experiment. Therefore, no unique basis for interpretation exists. It is necessary to consider the kinetic results obtained as defined by several plausible mechanisms and only within this framework can an evaluation of the structure–function relationships be made.

Although a number of proposals have been made to account for the general features of the oxidation of cytochrome *c* catalyzed by cytochrome oxidase as defined by eq 3 (Slater, 1949; Smith & Conrad, 1956; Minnaert, 1961; McGuinness & Wainio, 1962; Hollocher, 1962, 1964; Cope, 1963; Nicholls, 1964; Yonetani & Ray, 1965), none account for the exact dependence of k' on total cytochrome *c* concentration. Equation 4 includes additional terms in total cytochrome *c* concentration in both denominator and numerator which are not present in the rate laws previously proposed. The denominator terms of the rate law represent the distribution of oxidase in its various forms at steady state. The quadratic term in total cytochrome *c* concentration indicates that under certain conditions two molecules of cytochrome *c* are bound to the oxidase at the moment of electron transfer. Such higher order complexes of cytochrome *c* and oxidase were considered by Hollocher (1962) and predicted to be observable at concentrations of cytochrome $c > 0.1$ mM (by inference from the predicted binding constant of $<10^4$ M^{-1}). Experiments that measure stoichiometry for cytochrome *c* binding to *c* depleted mitochondria or that directly measure cytochrome *c* binding to oxidase support the kinetic evidence for (cytochrome)$_2$–oxidase complexes (Nicholls, 1965; Erecinska, 1975; Ferguson-Miller et al., 1976).

It is important to note that, although ultimately the net reaction being studied is the oxidation of ferrocytochrome *c* and the reduction of oxygen to water, terms in oxygen concentration do not appear in the rate law (eq 3 and 4). Stopped-flow measurements by Gibson et al. (1965) indicate that reduction of O_2 by oxidase is very rapid relative to other electron transfer processes. Furthermore, polarographic measurements of oxygen uptake catalyzed by this system directly demonstrate that rates of reaction are independent of oxygen concentration until the oxygen tension is reduced to less than 10% of that for an air-saturated solution. For the highest concentration of cytochrome *c* employed (160 μM), approximately 20% of the oxygen present in an air-saturated solution is consumed and the oxygen tension is never rate limiting. Therefore, for deduction of mechanisms, the oxidation of cytochrome *c* catalyzed by cytochrome oxidase can be considered equivalent to a one-substrate, enzyme-catalyzed reaction.

Any number of mechanisms consistent with the general rate law given by eq 3 and 4 can be devised. We have described two (Errede et al., 1976) based on modification of the generally accepted "Mechanism IV" of Minnaert in which we have taken into account complexes involving two molecules of cytochrome *c* per oxidase. These are termed "dependent site" and "dead end" mechanisms. They involve diametrically opposed assumptions in that the former postulates productive complexes, the latter nonproductive. As examples of others conceivable, we may mention a variation of the productive complex scheme in which—contrary to the "dependent site" mechanism—two independent sites of binding exist ("independent site" mechanism), as well as a fourth scheme in which free cytochrome *c* can participate in electron transfer with a molecule of bound cytochrome *c*—termed the "exchange" mechanism. The detailed schemes, together with functions relating rate law parameters to reaction constraints are given in Table IV. The assumptions involved in predicting first-order kinetics at all substrate concentrations as demanded by the general rate law are that "on" and "off" constants (or in the case of the "dead end" mechanism their ratio) are equal for ferrocytochrome *c* and ferricytochrome *c*. Arguments can be presented which support choice of one rather than another, but it is not possible

TABLE IV: Comparison of Oxidase Mechanisms.[a]

A. "Dependent Site" Mechanism

$$S + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \overset{k_3}{\rightarrow} V \underset{k_6}{\overset{k_5}{\rightleftarrows}} P + E$$

$$S + U \underset{k_8}{\overset{k_7}{\rightleftarrows}} W \overset{k_9}{\rightarrow} X \underset{k_{12}}{\overset{k_{11}}{\rightleftarrows}} P + U$$

$$S + V \underset{k_{14}}{\overset{k_{13}}{\rightleftarrows}} Y \overset{k_{15}}{\rightarrow} Z \underset{k_{18}}{\overset{k_{17}}{\rightleftarrows}} P + V$$

Rate Equation:

$$\text{velocity} = \frac{k_1^\circ + K_1k_2^\circ[C]}{1 + K_1[C] + K_1K_2[C]^2}[E^\circ][S]$$

Definition of Mechanistic Constants:

	Limiting rate of reaction	Binding
site 1:	$k_1^\circ = \frac{k_1k_3}{k_2 + k_3}$	$K_1 = \frac{k_1}{k_2}$
site 2:	$k_2^\circ = \frac{k_7k_9}{k_8 + k_9}$	$K_2 = \frac{k_7}{k_8}$

B. "Independent Site" Mechanism

$$S + E_1 \underset{k_2}{\overset{k_1}{\rightleftarrows}} U_1 \overset{k_3}{\rightarrow} V_1 \underset{k}{\overset{k_5}{\rightleftarrows}} P + E_1$$

$$S + E_2 \underset{k_8}{\overset{k_7}{\rightleftarrows}} U_2 \overset{k_9}{\rightarrow} V_2 \underset{k_{12}}{\overset{k_{11}}{\rightleftarrows}} P + E_2$$

Rate Equation:

$$\text{velocity} = \frac{(k_1^\circ + k_2^\circ) + (K_1k_2^\circ + K_2k_1^\circ)[C]}{1 + (K_1 + K_2)[C] + K_1K_2[C]^2}[E^\circ][S]$$

Definition of Mechanistic Constants:

	Limiting rate of reaction	Binding
site 1:	$k_1^\circ = \frac{k_1k_3}{k_2 + k_3}$	$K_1 = \frac{k_1}{k_2}$
site 2:	$k_2^\circ = \frac{k_7k_9}{k_8 + k_9}$	$K_2 = \frac{k_7}{k_8}$

C. "Dead End Complex" Mechanism

$$U \underset{k_2}{\overset{k_1}{\rightleftarrows}} S + E \overset{k_3}{\rightarrow} P + E \underset{k_6}{\overset{k_5}{\rightleftarrows}} V$$

$$W \underset{k_8}{\overset{k_7}{\rightleftarrows}} S + U \overset{k_9}{\rightarrow} P + U \underset{k_{12}}{\overset{k_{11}}{\rightleftarrows}} X$$

$$Y \underset{k_{14}}{\overset{k_{13}}{\rightleftarrows}} S + V \overset{k_{15}}{\rightarrow} P + V \underset{k_{18}}{\overset{k_{17}}{\rightleftarrows}} Z$$

Rate Equation:

$$\text{velocity} = \frac{k_1^\circ + K_1k_2^\circ[C]}{1 + K_1[C] + K_1K_2[C]^2}[E^\circ][S]$$

Definition of Mechanistic Constants:

	Limiting rate of reaction	Binding
site 1:	$k_1^\circ = k_3$	$K_1 = \frac{k_1}{k_2}$
site 2:	$k_2^\circ = k_9$	$K_2 = \frac{k_7}{k_8}$

D. "Exchange" Mechanism

$$S + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \overset{k_3}{\rightarrow} V \underset{k_6}{\overset{k_5}{\rightleftarrows}} P + E$$

$$S + V \underset{k_8}{\overset{k_7}{\rightleftarrows}} U + P$$

Rate Equation:

$$\text{velocity} = \frac{k_1^\circ + k_1^\circ(k_7/k_2)[C]([E^\circ][S])}{1 + [K_1 + k_7/(k_2 + k_3)][C] + K_1[k_7/(k_2 + k_3)][C]^2}$$

Definition of Mechanistic Constants:

Limiting rate of reaction:	$k_1^\circ = k_1k_3/(k_2 + k_3)$
Binding:	$K_1 = k_1/k_2$
Exchange reaction:	k_7/k_2
	$k_7/(k_2 + k_3)$

[a] Symbols: S = ferrocytochrome *c*; P = ferricytochrome *c*; E = free oxidase; U = ferrocytochrome *c*-oxidase complex; V = ferricytochrome *c*-oxidase complex; W = 2 ferrocytochrome *c*-oxidase complex; X=Y = ferrocytochrome *c* + ferricytochrome *c*-oxidase complex; Z = 2 ferricytochrome *c*-oxidase complex; E° = total oxidase; C = total cytochrome *c*, i.e., S + P. The subscripts used for E, U, and V in mechanism B refer to oxidase site 1 and site 2. Reverse rate constants (i.e., k_4, k_{10}, k_{16}) are omitted, as they are taken as equal to zero (see text).

at present to reject definitively any of these, except possibly the "exchange" mechanism.[4] At present the "dependent site" formulation is the simplest on which to base an interpretation of the kinetic constants determined. This mechanism as presented in Table IVA is a direct extension of Minnaert's "Mechanism IV" in that an electron transfer occurs between cytochrome *c* and an oxidase molecule that is already complexed with one molecule of cytochrome *c*. No assumptions concerning the nature of interaction of the oxidase with two molecules of cytochrome *c* need be made. It is important to note that the site 2 electron transfer occurs only when a sufficient concentration of cytochrome *c*-oxidase complex has been formed and thus refers only to reaction of the second bound cytochrome *c*. The assumptions required to obtain the expected form of the rate equation are: (1) reverse reaction (reduction of cytochrome *c*) is negligible; (2) reaction of cytochrome *c* with ferricytochrome *c*-oxidase complex is equivalent to reaction with ferrocytochrome *c*-oxidase complex ($k_7 = k_{13}$, $k_8 = k_{14}$, $k_9 = k_{15}$, $k_{11} = k_{17}$, and $k_{12} = k_{18}$); (3) the "on" and "off" constants for complex formation are independent of the oxidation state of cytochrome *c* ($k_1 = k_6$, $k_2 = k_5$, $k_7 = k_{12}$, and $k_8 = k_{11}$).

Justification of these assumptions can be provided by the following considerations. Under aerobic assay conditions ferrocytochrome *c* is quantitatively oxidized to ferricytochrome *c* in the presence of oxidase. Addition of an oxidant, potassium ferricyanide, to the reaction mixture at the apparent completion of reaction directly establishes that complete oxidation of substrate has occurred. Therefore, the equilibrium for this reaction strongly favors the forward reaction (oxidation). The observation that k_{obsd} is independent of the initial ratio of ferricytochrome *c* to ferrocytochrome *c* (Smith & Conrad, 1956; Errede et al., 1976) suggests that the equilibrium binding constant for reactants is indistinguishable from that for products. Yonetani & Ray (1965), using the method of initial rate analysis, measured K_m values for ferrocytochrome *c* re-

[4] An extended discussion is given elsewhere (Errede, 1976). This paper is based on data in the Doctoral thesis by B. J. Errede (1976), University of California at San Diego, La Jolla, California 92093.

TABLE V: Values for the Kinetic Constants Defined by the "Dependent Site" (A), "Dead End Complex" (A), and "Independent Site" (B) Mechanisms.[a]

Cytochrome	I (mM)		$k_1°$ (M^{-1} s^{-1})	K_1 (M^{-1})	$k_2°$ (M^{-1} s^{-1})	K_2 (M^{-1})
Horse *c*	44	A	2.50×10^8	6.21×10^6	4.01×10^6	3.84×10^4
		B	2.47×10^8	6.17×10^6	2.48×10^6	3.87×10^4
	4	A	1.90×10^8	1.20×10^7	4.20×10^6	9.20×10^4
		B	1.87×10^8	1.19×10^7	2.78×10^6	9.27×10^4
Cr. fasciculata c	44	A	7.94×10^7	1.40×10^6	1.65×10^7	1.48×10^5
		B	7.04×10^7	1.23×10^6	9.05×10^6	1.69×10^5
C. limicola c_{555}	44	A	3.65×10^6	9.03×10^4	1.26×10^6	8.67×10^3
		B	2.54×10^6	8.06×10^4	1.11×10^6	9.72×10^3
P. denitrificans c_{550}	44	A	6.05×10^5	1.29×10^5	1.54×10^5	4.36×10^3
		B	4.62×10^5	1.24×10^5	1.43×10^5	4.51×10^3
R. rubrum c_2	44	A	9.70×10^4	2.56×10^4	*b*	*b*
		B	*b*	*b*	*b*	*b*
	4	A	8.65×10^5	3.56×10^5	2.90×10^5	6.37×10^4
		B	7.02×10^5	2.72×10^5	1.64×10^5	8.32×10^4
Rm. vannielii c_2	44	A	2.55×10^4	6.98×10^3	*b*	*b*
		B	*b*	*b*	*b*	*b*

[a] The values for the constants were calculated using the values for the coefficients of eq 4 given in Table III and the appropriate functions defined by the rate laws derived for each mechanism in Table IV. [b] The limiting conditions for estimation of constants α_2 and β_2 were not satisfied. Therefore, values for these constants cannot be determined.

action with oxidase and K_i values for inhibition of the reaction by ferricytochrome *c*. They found the values for K_i were, within experimental error, identical with the values for K_m under conditions where the kinetics were pseudo-first-order. These results directly demonstrate that the *ratio* of the "on" constant to the "off" constant for ferrocytochrome *c* binding to the oxidase was kinetically indistinguishable from the *ratio* of the "on" and "off" constants for ferricytochrome *c* binding to the oxidase.

The equivalence of the individual constants can be rationalized further on the basis of the nature of the product and the reactant involved. The primary difference between product and reactant is the valence state of the heme-iron. The surface of the protein presumably is not significantly changed by oxidation and reduction. Because the binding constants for product and reactant are equivalent, it is plausible that the rate of complex formation and complex dissociation could be the same for ferricytochrome *c* and ferrocytochrome *c*.

The rate equation derived from the mechanism presented in Table IVA defines the constants α_1, α_2, β_1, and β_2 of eq 4 in terms of ratios of rate constants for the proposed reaction sequence. We introduce and define notation to represent these ratios for the rate equation in Table IVA. The constants $k_1°$ and $k_2°$ represent the limiting values of k' obtained at zero cytochrome *c* concentration for the site 1 and site 2 reactions, respectively. K_1 represents the high affinity binding constant and K_2 the low affinity binding constant. A similar system of notation is used for all mechanisms shown in Table IV.

In Table V values for the kinetic constants corresponding to the four mechanisms as bases for calculations[5] are shown. The major finding, referring to the "A" series in the table, is that large relative decreases in values for K_1 and K_2 ($\geq$ one order of magnitude) are paralleled by decreases in $k_1°$ and $k_2°$, which is consistent with the assumption that binding is a dominant parameter.

[5] The method for derivation of rate equations from each mechanism presented as well as details of calculations of kinetic constants are given elsewhere (Errede, 1976).

Discussion

Relative Oxidase and Reductase Activities of Cytochromes c. The results from the comparison (Table I) of oxidase activities for *c*-type cytochromes from the structural categories represented here are in agreement with results obtained from similar surveys that have been published previously (Yamanaka, 1972; Davis et al., 1972; Smith et al., 1973b, 1976). The inclusion of relative rates of reaction with NADH-cytochrome *c* reductase for these cytochromes greatly extends the heretofore limited comparisons involving this electron transfer function of cytochrome *c*. A striking observation is the dramatic differences in relative rates of reaction that are possible for a given cytochrome *c* with these two mitochondrial redox systems. In particular, the activities measured for the various cytochromes c_2 bear out the previous observations of Elsden et al. (1953) and Davis et al. (1972) that this group of cytochromes generally exhibits good reactivities with mitochondrial reductase but poor reactivities with mitochondrial oxidase. This difference in electron donor and acceptor functions of the c_2 cytochromes with oxidase and reductase may be indicative of differences in structural requirements for reaction with these two mitochondrial redox components. The absence of any correlation between thermodynamic electrochemical potential and isoelectric point with observed activities of either oxidase or reductase indicates that neither parameter is a sufficient determinant for mitochondrial cytochrome *c* function.

It should be noticed that the relative rates of NADH-cytochrome *c* reductase reaction are significantly different when measured as initial rates than when measured from the first-order portion of the reaction. This difference is compatible with

the interpretation made by Smith et al. (1974) concerning the significance of the biphasic kinetics observed for the reductase reaction. They concluded that the initial zero-order rate is a consequence of a rate-limiting electron transfer prior to reduction of cytochrome *c* by cytochrome c_1 of complex III. Also, this observation reinforces the necessity for reporting both initial and first-order rates for any comparisons of reductase activities, until such time that the kinetics for this system are better defined.

The kinetics of reduction for *R. molischianum* and *R. fulvum* iso-1-cytochromes c_2 indicate the presence of two species in solution at pH 7.5 and one species at pH 6.0. The p*K* for pH titrations of the 695-nm absorbance band of these cytochromes is anomalously low; for *R. molischianum* it is 7.3 and for *R. fulvum* 6.9 (M. Cusanovitch, unpublished). Therefore, at pH 7.5 both the species that absorbs at 695 nm and the species that absorbs less at this wavelength should be present, while at pH 6.0 only the former should be present.

Selection of Cytochromes c for Detailed Kinetic Analyses. To compare oxidase function of various cytochromes *c* by use of defined kinetic constants and to correlate differences found with structure, we were required to examine proteins which were associated with a wide range of function and for which details of structure were well established. Accordingly, we selected those available which best satisfied these conditions. Three-dimensional structures for a number of mitochondrial cytochromes *c* were known, e.g., horse (Dickerson et al., 1971; Dickerson, 1972), tuna (Swanson et al., 1977; Takano et al., 1977), and bonito (Ashida et al., 1971, 1973; Tanaka et al., 1975). Well-resolved structures for prokaryotic forms at hand were those of the cytochrome c_2 from the nonsulfur purple photosynthetic bacterium, *Rhodospirillum rubrum* (Salemme et al., 1973a,b) and the cytochrome c_{550} from the facultative denitrifier, *Paracoccus denitrificans* (Timkovich & Dickerson, 1973, 1976), as well as tentative structures for the cytochrome c_{551} of the facultative aerobe, *Pseudomonas aeruginosa* (Dr. R. E. Dickerson, personal communication), and the green photosynthetic anaerobe, *Chlorobium limicola v. thiosulfatophilum* (Korszun & Salemme, 1977). Primary structures were also available for the following: the atypical mitochondrial cytochrome *c* of the protozoan *Crithidia fasciculata* (Hill & Pettigrew, 1975); the cytochrome *c* from the protozoan *Tetrahymena pyriformis* (Tarr & Fitch, 1976), which was unreactive with the beef heart oxidase (Yamanaka et al., 1968); the cytochrome c_2 from a budding form of photosynthetic prokaryote, *Rhodomicrobium vanniellii* (Ambler et al., 1976), which among cytochromes c_2 most closely resembled the mitochondrial cytochromes *c*; and two cytochromes c_{555} (van Beeuman et al., 1976) from two green photosynthetic bacteria, *Prosthecochloris aestuarii* and *C. limicola v. thiosulfatophilum,* the former of which exhibited no reactivity with oxidase while the latter homologous protein was active. Although tertiary structures for the first three of these cytochromes *c* are not available, it is likely that they exhibit the same general "cytochrome *c* fold" as do those for which a tertiary structure is known (Dickerson et al., 1976). Such an assumption is reasonable in view of the similarity in tertiary structures for cytochrome *c* as diverse in source and function as horse heart cytochrome *c*, *R. rubrum* cytochrome c_2, *P. denitrificans* cytochrome c_{550}, *C. limicola v. thiosulfatophilum* cytochrome c_{555}, and *Ps. aeruginosa* cytochrome c_{551}. It is reasonable to anticipate that kinetic studies of these cytochromes *c* can provide clues for identification of structural features that control reactivity in the oxidase system of mitochondria.

Generality of the Rate Law Deduced for Reaction of Horse c with Oxidase and Requirements for Quantitative Comparison of Cytochrome c Function. We have documented the variations in rates of oxidation catalyzed by the cytochrome oxidase preparation in Table I and demonstrated for each cytochrome *c* tested the validity of the general rate law previously deduced for reaction of horse cytochrome *c* with the oxidase (Errede et al., 1976). Thus one can compare cytochrome *c* function on the basis of the kinetic constants, α_1, α_2, β_1, and β_2, of eq 4, evaluated with regard to the limiting conditions outlined in Table I. Experiments must be designed so that the concentration range of each cytochrome *c* studied is determined by preliminary estimates of these constants. This range needs to be extended, within practical limits, so that adequate weight is given to the final estimation of all four constants of eq 4. Any kinetic constants reported without regard to these precautions cannot be considered meaningful. In the present study concentrations of cytochrome *c* could be used in the determination of constants for horse *c*, *Cr. fasciculata c*, *C. limicola* c_{555}, and *P. denitrificans* c_{550} that provided reliable estimates for both site 1 and site 2 constants. However, as mentioned above, available concentrations of *R. rubrum* c_2 and *Rm. vannielii* c_2 were adequate only for the estimation of the site 1 constants.

The rate expression that has been deduced for the reaction of various cytochromes *c* with beef heart mitochondrial oxidase may be generally applicable to the oxidation of ferrocytochrome *c* catalyzed by other biological redox systems. It has been reported that the reaction of cytochrome *c* with yeast cytochrome *c* peroxidase (ferrocytochrome *c*:hydrogen-peroxide oxidoreductase, EC 1.11.1.5) is first order at all concentrations of cytochrome *c* studied (Beetlestone, 1960). Furthermore, the rate dependence on cytochrome *c* concentration has been found to be biphasic as for the oxidase reaction (Kang et al., 1977). Therefore, it appears likely that the same considerations for experimental design and evaluation of reported kinetic constants necessary for the reaction of cytochrome *c* with oxidase also will be necessary for the definition of the cytochrome *c*–yeast peroxidase system.

Effects of Ionic Strength. A tenfold change in ionic strength did not alter the form of the established rate law. The minor change in the binding constant, K_1, obtained at the two ionic strengths for horse cytochrome *c* was consistent with the observations of van Gelder et al. (1975). However, significant differences in the rates, k', were obtained for each cytochrome *c* concentration at these two ionic strengths (Figure 2A), consistent with the differences in the four constants obtained for the two assay conditions (Table III). On the other hand a tenfold increase in the values of the constants for *R. rubrum* c_2 accompanied the tenfold decrease in ionic strength; this result was consistent with the expectation that a much lower salt concentration would be more effective in influencing binding of a cytochrome more weakly interactive with oxidase. Under conditions of lower ionic strength (where binding increased) the *R. rubrum* c_2 data satisifed all conditions of Table I for the estimation of the constants of eq 4. However, the values for the site 1 constants, $k_1°$ and K_1, were near those for the site 2 constants, $k_2°$ and K_2 (Table V). The similarity in these values would account for the apparent linearity of the plot of k' vs. k'[C] even though the curve calculated according to eq 4 (Figure 2B, X) provided an excellent fit to the data.

It has been demonstrated that the kinetic constants for the reaction of two cytochromes, horse *c* and *R. rubrum* c_2, with oxidase reflect different sensitivities to changes in ionic strength. Therefore, it may be expected that relative reactivities for a group of cytochromes would differ depending upon the ionic strength of the assay medium employed. Hence, kinetic comparison of oxidase reactions with various cytochromes *c*

TABLE VI: Presence (+) or Absence (0) of Cationic Groups Structurally Analogous to Those at the Front of Horse *c*.[a]

Cytochrome	Relative binding		Position of cationic groups							
	K_1	K_2	13	25	27	72	73	79	86	87
Horse *c*	1000	10	+	+	+	+	+	+	+	+
Cr. fasciculata c	200	40	+	0	0	+	+	+	+	+
P. denitrificans c_{550}	20	1	+	0	+	0	0	+	0	+
R. rubrum c_2	4	*b*	+	0	+	0	0	+	0	0
Rm. vannielii c_2	1	*b*	0	+	0	+	0	+	+	0
T. pyriformis c	*b*	*b*	0	0	0	0	+	+	0	+
Ps. aeruginosa c_{551}	*b*	*b*	+	+	0	0	0	0	0	0

[a] See text and Figure 3. [b] Binding constants were not determined.

would require determinations at several ionic strengths.

Correlation of Structural Differences of Cytochromes c with Observed Kinetic Differences for Reaction with Mitochondrial Oxidase. Using the "dependent site" mechanism the relative values of K_1 and K_2 can be identified unambiguously as the kinetic binding constants for the cytochromes *c* compared, but the values obtained for the limiting rates of reaction, $k_1°$ and $k_2°$, represent a complex function of rate constants (Table IV, A). These values reflect both the efficiency of cytochrome *c* interaction with oxidase and the rate constant for electron transfer. Therefore, the constants $k_1°$ and $k_2°$ per se are less useful for establishing functional classes of the cytochromes *c* compared. Nonetheless, restriction of the functional definition of cytochrome *c* to the relative values of the kinetic binding constants does provide clues as to features of the cytochrome *c* surface that effect interaction with the oxidase.

We emphasize that the in vitro assay method inherently involves uncertainties in evaluation of cytochrome *c* function that may not reflect the in vivo case. Davies et al. (1976) have shown that the cytochrome *c* of *P. denitrificans* membranes remains firmly bound and does not appear to dissociate from the membrane during electron transport. The *P. denitrificans* membrane is a respiratory system analogous to that of the mitochondrial membrane (John & Whatley, 1975). Therefore, it may be that the need to get cytochrome *c* "on" and "off" the oxidase site during mitochondrial electron transport does not exist as it does in the in vitro assay system. However, one may reasonably expect that the surface residues required for binding in the in vitro assay will be those residues involved in the interaction of cytochrome *c* with oxidase in the mitochondria.

Although the sequences for the mitochondrial cytochromes *c* [horse *c* (Margoliash et al., 1961), *Cr. fasciculata c* (Hill & Pettigrew, 1975), and *T. pyriformis c* (Tarr & Fitch, 1976)], and the bacterial c_2-type cytochromes [*Rm. vannielii* c_2 (Ambler et al., 1976), *R. rubrum* c_2 (Dus et al., 1968), and *P. denitrificans* c_{550} (Timkovich et al., 1976)] are analogous, very few amino acids are invariant. The sequences for *Ps. aeruginosa* c_{551} (Ambler, 1963), *C. limicola* c_{555}, and *Pr. aestuarii* c_{555} (van Beeumen et al., 1976) resemble each other more than they do the mitochondrial cytochromes *c* or the bacterial cytochromes c_2. Evaluation of the structural perturbations resulting from the numerous amino acid substitutions (relative to horse cytochrome *c*) throughout the molecule is not possible as yet.

The interaction of cytochrome *c* with cytochrome oxidase has been shown to be largely electrostatic in nature (Wainio et al., 1960; Davies et al., 1964; Person & Fine, 1960, 1961; Smith & Conrad, 1956). More specifically evidence indicates that the positive charges provided by the lysyl residues of cytochrome *c* effect appreciable interaction of cytochrome *c* with oxidase (Takemori et al., 1962; Wada & Okunuki, 1968, 1969). Furthermore, investigations involving (1) chemical modification of specific cationic, surface amino acid residues of cytochrome *c* (Wada & Okunuki, 1968, 1969; Margoliash et al., 1973; Brautigan & Ferguson-Miller, 1976; Staudenmayer et al., 1976, 1977), (2) monovalent antibody binding to cytochrome *c* (Smith et al., 1973a), and (3) reactions of inorganic redox reagents with cytochrome *c* (see Cusanovich, 1978, for a recent review of these investigations) provide evidence, in toto, consistent with the following premise. The binding of cytochrome *c* to the oxidase involves the interaction of cationic residues at the "front"[6] of the cytochrome *c* with appropriate groups on the oxidase in order to achieve the proper orientation of the heme edge most accessible to solvent (tetrapyrrole rings II and III) for electron transfer. We may proceed to consider the possible significance of the distribution of charged amino acid groups at the front of the molecule.

To facilitate the structural comparison the presence (+) or absence (0) of cationic residues in the positions structurally analogous to those at the front of horse cytochrome *c* is summarized in Table VI for the cytochromes being compared and the relative values of the binding constants are also given. In addition, schematic diagrams of the chain folding for each cytochrome *c* showing the location of cationic residues at the front of the molecule are illustrated in Figure 3. The figures of the three cytochromes (*Cr. fasciculata c*, *T. pyriformis c*, and *Rm. vannielii* c_2), for which only primary structures are known, were made assuming, as remarked previously, that chain folding was similar to that for the cytochromes with defined three-dimensional structures.

At the front surface of horse cytochrome *c*, lysines are found at positions 13, 25, 27, 72, 73, 86, and 87 (Figure 3). The only negatively charged residues are in peripheral regions (glutamate-21, aspartate-50, and glutamate-69). Thus an essentially continuous ring of positive charge characterizes the front surface. However, the tops of horse *c* and of each of the cytochromes *c* studied have both negative and positive residues; the C-terminal and N-terminal helices on this surface approach each other so that inter- as well as intrahelix ion pairs can be formed. Hence, it is difficult to estimate effective charge and to assess any possible functional contribution of cationic residues of this region.

The cytochrome *c* of *Cr. fasciculata* does not have cationic groups equivalent to horse lysines-25 and -27 (Figure 3); the absence of positive charge at the right front surface can account for the fivefold lower value of K_1 obtained for this cytochrome

[6] The convention that has been adopted defines the "front" of the cytochrome *c* molecule as the surface surrounding the heme edge of tetrapyrrole rings II and III (roughly, residues 12–17, 27–28, 70–73, and 77–87 for horse *c*). Part of the C-terminal helix (residues 88–95 for horse *c*) defines the "top" of the molecule. The "bottom" of the molecule is formed by the peptide chain below the propionic acid side chains of the heme (roughly, residues 38–58 for horse *c*).

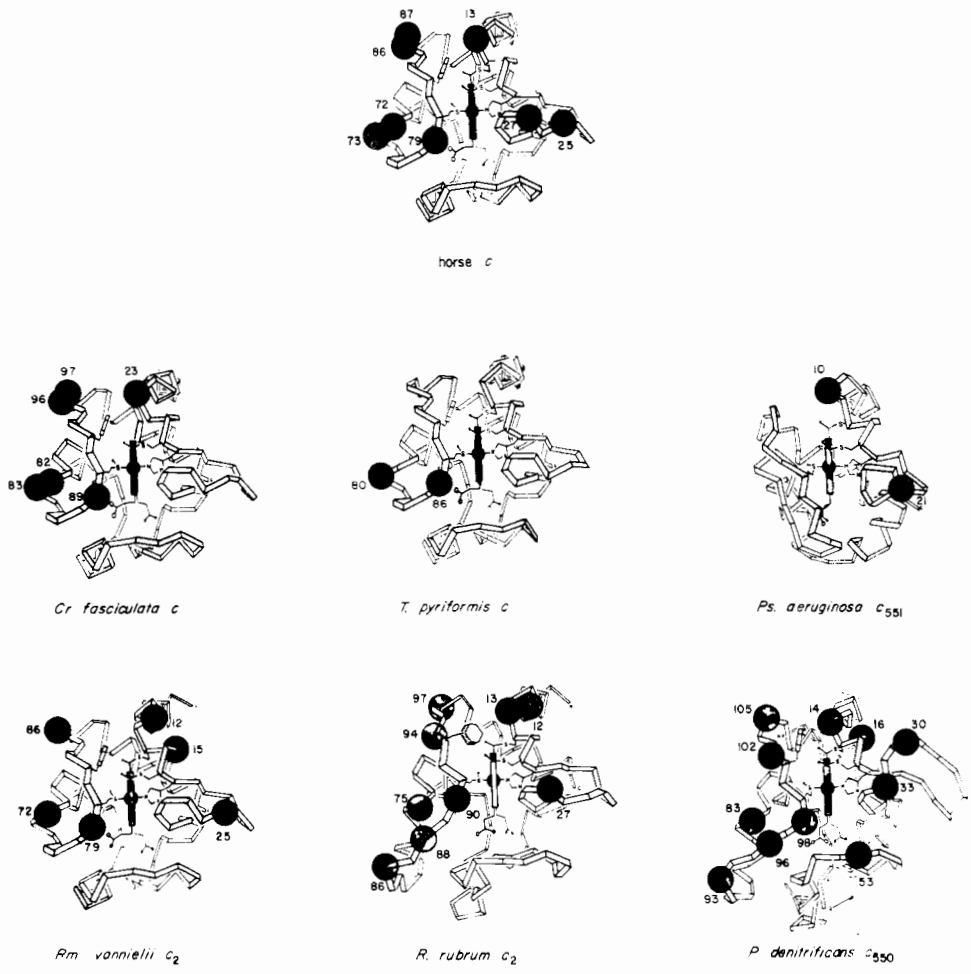

FIGURE 3: Comparison of the location of cationic residues at the front of selected cytochromes *c*. Schematic representation of the front view for the folded polypeptide chain with the heme viewed on edge. The heme–iron extraplanar ligands, histidine nitrogen, and methionine sulfur are shown. The location of cationic residues is indicated by the large shaded circles. (Adapted from figures for tuna *c*, *R. rubrum* c_2, *P. denitrificans* c_{550}, and *Ps. aeruginosa* c_{551} courtesy of Professor R. E. Dickerson.)

c relative to horse *c*. It appears that only the positive charge at position 27 is crucial for binding since identical values for K_1 have been obtained for horse *c* and yeast iso-1-cytochrome *c* which does not have a cationic group at the position equivalent to horse lysine-25 (Ferguson-Miller et al., 1976). The relative value for *Cr. fasciculata c* K_2 is four times greater than that for horse *c*. The mechanistic interpretation of kinetic results is consistent with either two distinct acceptor sites on the oxidase for cytochrome *c* or one acceptor site for two cytochrome *c* molecules. In the former case, the two sites could have different specificities and the absence of charge at the lower right front could be required for optimal site 2 binding. In the latter case, we suggest that the absence of positive charge in the region of lysine-27 slightly changes the orientation of the first bound cytochrome *c* (relative to the orientation for horse *c*) so that one site may better accommodate a second bound cytochrome for electron transfer.

Although detailed kinetic analysis was not undertaken for the reaction of *T. pyriformis c*, the extremely low reactivity observed (Table I) for this protozoan mitochondrial cytochrome *c* can be explained by the absence of positive charges compared with horse *c* (Table VI and Figure 3). The total absence of charge at the right front is particularly striking. Likewise, the observed lack of reactivity (Table I) for *Ps. aeruginosa* c_{551} can be correlated with the more hydrophobic front surface of this molecule (Figure 3) due to the absence of lysines equivalent to those of horse *c* (Table VI).

The comparison of cationic residues at the front surface of the c_2-type cytochromes, *Rm. vannielii* c_2, *R. rubrum* c_2, and *P. denitrificans* c_{550}, is not straightforward. It is apparent for *R. rubrum* c_2 and *P. denitrificans* c_{550} that the distribution of cationic groups in this region is significantly different from horse *c* (Figure 3). In addition, the insertions and deletions in the peptide backbone (relative to horse *c*) have the effect of slightly changing the orientation of several regions of the peptide chain for the bacterial cytochromes so that sequentially analogous residues are not strictly structurally analogous. The 70's loop insertion shared by *R. rubrum* c_2 and *P. denitrificans* c_{550} causes the lysine sequentially analogous to horse lysine-72 to be displaced backward from the front of the molecule; likewise, a single residue deletion after the methionine sixth ligand in the former causes the lysine corresponding to horse lysine-87 to be similarly displaced (Figure 3). In contrast, the three residue deletion in this region for *P. denitrificans* c_{550} results in lysine-105 assuming an orientation comparable to horse lysine-87. Lastly, the single residue deletion at the position equivalent to horse *c* valine 11 changes the orientation of the N-terminal helix residues for all three c_2-type cytochromes. Consequently, it is unlikely that the ϵ-amino group of *Rm. vannielii* c_2 lysine-12 can assume the position above tetrapyrrole ring II as in the orientation for horse cytochrome *c* lysine-13. The summary of conserved cationic residues presented in Table VI takes into account these considerations.

The absence of the cationic groups for *P. denitrificans* c_{550}

specified in Table VI is consistent with the 50-fold lower site 1 binding constant obtained for this cytochrome *c* relative to that for horse *c*. Also, the increased positive charge at the lower front of the c_{550} may be unfavorable for assumption of the correct orientation in binding to the oxidase. These differences in charge distribution have much less effect on the site 2 binding constant for *P. denitrificans* c_{550}, which is only tenfold lower than that for horse *c*. The charge distribution for *R. rubrum* c_2 is similar to that for *P. denitrificans* c_{550} (Figure 3) and, therefore, similar features will be responsible for the decreased binding constants obtained for these two cytochromes relative to horse *c*. The even lower binding constant for *R. rubrum* c_2 could be attributed to the additional loss of a cationic group equivalent to horse lysine-87 and possibly to the slight change in orientation of the C-terminal helix position, which brings the negative charges contributed by residues 98, 99, and 100 closer to the front surface of *R. rubrum* c_2 than in either *P. denitrificans* c_{550} or horse *c*. The extremely low binding constant obtained for *Rm. vannielii* c_2 can be attributed to the absence of a cationic group structurally equivalent to lysine-13 in addition to the absence of residues corresponding to horse lysines-27, -73, and -87.

Availability of the tertiary structure for oxidase-reactive, reductase-unreactive *C. limicola v. thiosulfatophilum* cytochrome c_{555} (Korszun & Salemme, 1977) as well as that of the wholly unreactive homologous *P. aestuarii* cytochrome c_{555} should provide a crucial comparison for insight into structural requirements for cytochrome *c* function.

General Conclusions

Despite many uncertainties currently unavoidable, the present comparison clearly implicates the cationic residues of the peptide chain surrounding the heme edge of tetrapyrrole rings II and III in the interaction of cytochrome *c* with oxidase. As noted, the very low reactivities found for *T. pyriformis c* and *Ps. aeruginosa* c_{551} can be accounted for on the basis of the relatively hydrophobic front surfaces of these proteins. The results for the comparison of *P. denitrificans* cytochrome c_{550} and *R. rubrum* c_2 clearly demonstrate that location rather than the "net" charge of this surface is a determining factor for binding. Cationic residues at both the left and right front appear necessary for interaction with oxidase. Participation of lysines-13, -27, -72, and/or -73, and -86 and/or -87 is implicated in the binding of cytochrome *c* to oxidase; the absence of any one of these cationic residues can effect a change in the observed binding constant for the particular cytochrome *c* studied (Table VI). These results are in agreement with the hypothesis of Salemme (1976) regarding the interaction of cytochrome *c* with another redox protein, cytochrome b_5.

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References

Ambler, R. P. (1963) *Biochem. J. 89,* 349.

Ambler, R. P. (1973) *Syst. Zoology 22,* 554.

Ambler, R. P. (1976) in *Handbook of Biochemistry and Molecular Biology, Proteins* (Fasman, G. D., Ed.) Vol. III, p 292, CRC Press, Cleveland, Ohio.

Ambler, R. P., & Bartsch, R. G. (1975) *Nature (London) 253,* 285.

Ambler, R. P., Meyer, T. E., & Kamen, M. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 472.

Ashida, T., Ueki, T., Tsukihara, T., Sugihara, A., Takano, T., & Kakudo, M. (1971) *J. Biochem. (Tokyo) 70,* 913.

Ashida, T., Tanaka, N., Yamane, T., Tsukihara, T., & Kakudo, M. (1973) *J. Biochem. (Tokyo) 73,* 463.

Barlow, G. H., & Margoliash, E. (1966) *J. Biol. Chem. 24,* 1473.

Bartsch, R. G. (1963) in *Bacterial Photosynthesis* (Gest, H., San Pietro, A., & Vernon, L. P., Eds.) p 475, Antioch Press, Yellow Springs, Ohio.

Bartsch, R. G. (1977) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) Plenum Pub. Corp., New York, N.Y., (in press).

Bartsch, R. G., Kakuno, T., Horio, T., & Kamen, M. D. (1971) *J. Biol. Chem. 246,* 4489.

Beetlestone, J. (1960) *Arch. Biochem. Biophys. 89,* 35.

Borden, D., & Margoliash, E. (1976) in *Handbook of Biochemistry and Molecular Biology, Proteins* (Fasman, G. D., Ed.) p 268, Vol. III, CRC Press, Cleveland, Ohio.

Brautigan, D. L., & Ferguson-Miller, S. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1598.

Cope, F. W. (1963) *Arch. Biochem. Biophys. 103,* 352.

Cusanovich, M. (1978) *Bioorg. Chem. 4,* 117.

Davies, H. C., Smith, L., & Wasserman, A. R. (1964) *Biochim. Biophys. Acta 85,* 238.

Davies, H. C., Pinder, P. B., Nava, M., & Smith, L. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1598.

Davis, K. A., Hatefi, Y., Salemme, F. R., & Kamen, M. D. (1972) *Biochem. Biophys. Res. Commun. 49,* 1329.

Dickerson, R. E. (1972) *Sci. Am. 226,* 58.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O., Samson, L., Cooper, A., & Margoliash, E. (1971) *J. Biol. Chem. 246,* 1511.

Dus, K., Sletten, K., & Kamen, M. D. (1968) *J. Biol. Chem. 243,* 5507.

Elsden, S. R., Kamen, M. D., & Vernon, L. P. (1953) *J. Am. Chem. Soc. 75,* 6347.

Erecinska, M. (1975) *Arch. Biochem. Biophys. 103,* 352.

Errede, B. (1976) Doctoral Thesis, University of California at San Diego.

Errede, B., Haight, G. P., & Kamen, M. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 113.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251,* 1104.

Flatmark, T., Dus, K., de Klerk, H., & Kamen, M. D. (1970) *Biochemistry 9,* 1991.

Fowler, L. R., Richardson, S. H., & Hatefi, Y. (1962) *Biochim. Biophys. Acta 64,* 170.

Gibson, J. (1961) *Biochem. J. 79,* 151.

Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240,* 888.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 5,* 467.

Gornall, A. G., Bardwill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177,* 751.

Greenwood, C., & Palmer, G. (1965) *J. Biol. Chem. 240,* 3660.

Griffiths, D. E., & Wharton, D. C. (1961) *J. Biol. Chem. 236,* 1850.

Hatefi, Y., Haavik, A. G., & Jurshuk, P. (1961) *Biochim. Biophys. Acta 64,* 106.

Hill, G. C., & Pettigrew, G. W. (1975) *Eur. J. Biochem. 57,* 265.

Hollocher, T. C. (1962) *Biochem. J. 79,* 151.

Hollocher, T. C. (1964) *Nature (London) 202,* 1006.
Horio, T., Higashi, T., Sasagawa, M., Jusai, K., Nakai, M., & Okunuki, K. (1960) *Biochem. J. 77,* 194.
John, P., & Whatley, F. R. (1975) *Nature (London) 254,* 495.
Kamen, M. D. (1972) *Proteins, Nucleic Acids, Enzymes 18,* 753.
Kamen, M. D., & Vernon, L. P. (1955) *Biochim. Biophys. Acta 17,* 100.
Kamen, M. D., Dus, K. M., Flatmark, T., & de Klerk, H. (1972) in *Electron and Coupled Energy Transfers in Biological Systems* (King, T. E., & Klingenberg, M., Eds.) Vol. 1, Part A, p 243, Marcel Dekker, New York, N.Y.
Kang, C. H., Ferguson-Miller, S., & Margoliash, E. (1977) *J. Biol. Chem. 252,* 919.
Katoh, S. (1960), *Plant Cell Physiol. 1,* 91.
Korszun, Z. R., & Salemme, F. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 5244.
Kusel, J. P., Suriano, J. R., & Weber, M. M. (1969) *Arch. Biochem. Biophys. 133,* 293.
Margalit, R., & Schejter, A. (1973) *Eur. J. Biochem. 32,* 492.
Margoliash, E., Smith, E. L., Keil, G., & Tuppy, H. (1961) *Nature (London) 192,* 1121.
Margoliash, E., Miller, S. F., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L., & Morrison, M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3245.
McGuinnes, E. T., & Wainio, W. W. (1962) *J. Biol. Chem. 237,* 3273.
Meyer, T. E., Bartsch, R. G., Cusanovich, M. A., & Mathewson, J. H. (1968) *Biochim. Biophys. Acta 153,* 854.
Miller, G. L. (1959) *Anal. Chem. 31,* 964.
Minnaert, K. (1961) *Biochim. Biophys. Acta 50,* 23.
Nicholls, P. (1964) *Arch. Biochem. Biophys. 106,* 25.
Nicholls, P. (1965), in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) Vol. II, p 764, University Park Press, Baltimore, Md.
Perini, F., Kamen, M. D., & Schiff, J. A. (1964) *Biochim. Biophys. Acta 88,* 74.
Person, P., & Fine, A. (1960) *Science 132,* 43.
Person, P., & Fine, A. (1961) *Arch. Biochem. Biophys. 94,* 392.
Pettigrew, G. W. (1975) Doctoral Thesis, University of Edinburgh.
Pettigrew, G. W., Aviram, I., & Schejter, A. (1975a) *Biochem. J. 149,* 155.
Pettigrew, G. W., Leaver, J. L., Meyer, T. E., & Ryle, A. P. (1975b) *Biochem. J. 147,* 291.
Pettigrew, G. W., Meyer, T. E., Bartsch, R. G., & Kamen, M. D. (1975c) *Biochim. Biophys. Acta 430,* 197.
Salemme, F. R. (1976) *J. Mol. Biol. 102,* 563.
Salemme, F. R., Freer, S. T., Xuong, Ng. H., Alden, R. A., & Kraut, J. (1973a) *J. Biol. Chem. 248,* 3910.
Salemme, F. R., Kraut, J., & Kamen, M. D. (1973b) *J. Biol. Chem. 248,* 7701.
Schejter, A., George, P., Glauser, S. C., & Margoliash, E. (1963) *Biochim. Biophys. Acta 73,* 641.
Scholes, P. B., McLain, G., & Smith, L. (1971) *Biochemistry 10,* 2072.
Shioi, Y., Takamiya, K., & Nishimura, M. (1972) *J. Biochem. (Tokyo) 71,* 285.
Slater, E. C. (1949) *Biochem. J. 44,* 305.
Smith, L., & Conrad, H. (1956) *Arch. Biochem. Biophys. 63,* 403.
Smith, L., Davies, H. C., Reichlin, M., & Margoliash, E. (1973a) *J. Biol. Chem. 248,* 237.
Smith, L., Nava, M. E., & Margoliash, E. (1973b) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) Vol. II, p 629, University Park Press, Baltimore, Md.
Smith, L., Davies, H. C., & Nava, M. E. (1974) *J. Biol. Chem. 249,* 2904.
Smith, L., Davies, H. C., & Nava, M. E. (1976) *Biochemistry 15,* 5827.
Staudenmayer, N., Smith, M. B., Smith, H. T., Spies, Jr., F. K., & Millett, F. (1976) *Biochemistry 15,* 3198.
Staudenmayer, N., Ng, S., Smith, M. B., & Millett, F. (1977) *Biochemistry 16,* 600.
Stellwagen, E., & Shulman, R. G. (1973) *J. Mol. Biol. 80,* 559.
Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., & Dickerson, R. E. (1977) *J. Biol. Chem. 252,* 759.
Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R., & Dickerson, R. E. (1977) *J. Biol. Chem. 252,* 776.
Takemori, S., Wada, K., Ando, K., Hosokawa, M., Sekuzu, I., & Okunuki, K. (1962) *Biochem. J. 52,* 28.
Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T., & Kakudo, M. (1975) *J. Biochem. (Tokyo) 77,* 147.
Tarr, G. E., & Fitch, W. M. (1976) *Biochem. J. 159,* 193.
Timkovich, R., & Dickerson, R. E. (1973) *J. Mol. Biol. 79,* 39.
Timkovich, R., & Dickerson, R. E. (1976) *J. Biol. Chem. 251,* 4033.
Timkovich, R., Dickerson, R. E., & Margoliash, E. (1976) *J. Biol. Chem. 251,* 2197.
van Beeumen, J., Ambler, R. P., Meyer, T. E., Kamen, M. D., Olson, J. M., & Shaw, E. K. (1976) *Biochem. J. 159,* 757.
van Gelder, B. F., van Buuren, K. J. H., Wilms, J., & Verboom, C. N. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., & Siliprandi, N., Eds.) p 63, North-Holland Publishing Co., Amsterdam, Netherlands.
Wada, K., & Okunuki, K. (1968) *J. Biochem. (Tokyo) 64,* 667.
Wada, K., & Okunuki, K. (1969) *J. Biochem. (Tokyo) 66,* 249.
Wainio, W. W., Eichel, B., & Gould, A. (1960) *J. Biol. Chem. 235,* 1521.
Wilson, M. T., & Greenwood, C. (1971) *Eur. J. Biochem. 22,* 11.
Wood, F. E., & Cusanovich, M. A. (1975) *Bioorg. Chem. 4,* 337.
Yamanaka, T. (1972) *Adv. Biophys. 3,* 227.
Yamanaka, T., Nagata, Y., & Okunuki, K. (1968) *J. Biochem. (Tokyo) 63,* 753.
Yonetani, T., & Ray, G. (1965) *J. Biol. Chem. 240,* 3392.