Correlation of the Kinetics of Electron Transfer Activity of Various Eukaryotic Cytochromes c with Binding to Mitochondrial Cytochrome c Oxidase*

(Received for publication, August 18, 1975)

SHELagh FERGUSON-MILLE,† DAVID L. BRAUTIGAN,§ and E. MARGOLIASH

From the Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201

1. A detailed study of cytochrome c oxidase activity with Keilin-Hartree particles and purified beef heart enzyme, at low ionic strength and low cytochrome c concentrations, showed biphasic kinetics with apparent $K_m = 5 \times 10^{-8} \text{M}$, and apparent $K_m = 0.35$ to $1.0 \times 10^{-6} \text{M}$. Direct binding studies with purified oxidase, phospholipid-containing as well as phospholipid-depleted, demonstrated two sites of interaction of cytochrome c with the enzyme, with $K_d \leq 10^{-7} \text{M}$, and $K_d = 10^{-8} \text{M}$.

2. The maximal velocities at low ionic strength increased with pH and were highest above pH 7.5.

3. The presence and properties of the low apparent $K_m$ phase of the kinetics were strongly dependent on the nature and concentration of the anions in the medium. The multivalent anions, phosphate, ADP, and ATP, greatly decreased the proportion of this phase and similarly decreased the amount of high affinity cytochrome c-cytochrome oxidase complex formed. The order of effectiveness was ATP $> ADP > P_i$, and since phosphate binds to cytochrome c more strongly than the nucleotides, it is concluded that the inhibition resulted from anion interaction with the oxidase.

4. At low concentrations bakers' yeast iso-1, bakers' yeast iso-2, horse, and Euglena cytochromes c exhibited very different activities and kinetic patterns in their reactions with cytochrome c oxidase, while at high concentrations all attained the same maximal velocity. The different proportions of low apparent $K_m$ phase in the kinetic patterns of these cytochromes c correlated with the amounts of high affinity complex formed with purified cytochrome c oxidase.

5. The apparent $K_m$ for cytochrome c activity in the succinate-cytochrome c reductase system of Keilin-Hartree particles was identical with that obtained with the oxidase ($5 \times 10^{-8} \text{M}$), suggesting the same site serves both reactions.

6. It is concluded that the observed kinetics result from two catalytically active sites on the cytochrome c oxidase protein of different affinities for cytochrome c. The high affinity binding of cytochrome c to the mitochondrial membrane is provided by the oxidase and at this site cytochrome c can be reduced by cytochrome c1. Physiological concentrations of ATP decrease the affinity of this binding to the point that interaction of cytochrome c with numerous mitochondrial phospholipid sites can competitively remove cytochrome c from the oxidase. It is suggested that this effect of ATP represents a possible mechanism for the control of electron flow to the oxidase.

Cytochrome c is a ubiquitous component of the mitochondrial electron transport chain that is easily prepared and has been studied extensively (1-3). The amino acid sequences have been determined for the protein of over 70 different eukaryotes and, despite residue variations of up to 70% (3-5), no reproducible differences have been observed in the maximal activities with mammalian mitochondrial enzymes (6, 7). This apparent conservation of function is presumably related to the identity of spatial folding of the polypeptide chain, observed for c-type cytochromes even less closely related than those of eukaryotes (8-12). This situation and the apparently constant rate at which amino acid substitutions occur in cytochrome c (13-16) were consistent with the neutral mutation hypothesis for protein evolutionary change (17-20). However, when an examination of the much more extensive data now available demonstrated that the rate of residue variation in cytochrome c was not constant, either in a single line of descent at various evolutionary intervals or in different lines of descent during the same time, the neutral mutation hypothesis was no longer tenable for this protein (21-24). Since maximal activities and spatial structure are the same for the different cytochromes c, the essential mechanism of electron transfer has probably
remained unaltered in the course of eukaryotic evolution. However, if the evolutionary changes are not neutral, some aspect of function must be selected for and is likely to relate to the surface charge topography of cytochrome c, important for optimal interaction with mitochondrial enzyme systems. Changes in binding that result from such evolutionary variations should be most readily observed at nonsaturating concentrations of cytochrome c and under ionic strength conditions that maximize the electrostatic protein interactions known to operate in this case (1-3, 25-30).

Low ionic strength media were therefore chosen to investigate the steady state kinetics of cytochrome c reaction with beef heart cytochrome c oxidase and reductase in order to examine both naturally occurring variants and chemically induced modifications of surface residues. Under such conditions very low concentrations of cytochrome c were effective, revealing a high affinity reaction of the protein with mitochondrial oxidation-reduction enzymes. Different kinetic patterns were found for the cytochromes c of horse, baker's yeast, and Euglena. The apparent $K_m$ values for the biphase kinetics of the reaction of cytochrome c with cytochrome c oxidase were of the same order of magnitude as the dissociation constants for the two binding sites found on the enzyme. The high affinity reaction was very sensitive to anions, the binding being

Low ionic strength media were therefore chosen to investigate the steady state kinetics of cytochrome c reaction with beef heart cytochrome c oxidase and reductase in order to examine both naturally occurring variants and chemically induced modifications of surface residues. Under such conditions very low concentrations of cytochrome c were effective, revealing a high affinity reaction of the protein with mitochondrial oxidation-reduction enzymes. Different kinetic patterns were found for the cytochromes c of horse, bakers' yeast, and Euglena. The apparent $K_m$ values for the biphase kinetics of the reaction of cytochrome c with cytochrome c oxidase were of the same order of magnitude as the dissociation constants for the two binding sites found on the enzyme. The high affinity reaction was very sensitive to anions, the binding being inhibited by phosphate, ADP, and ATP. The ATP was effective in its physiological concentration range, providing a possible control mechanism for the oxidation of ferricytochrome c.

Preliminary reports of part of these observations have appeared elsewhere (31, 32).

**EXPERIMENTAL PROCEDURE**

**Chemicals**—Sodium ascorbate (Nutritional Biochemicals), TMPD* (Eastman), succinic acid (Sigma grade I) recrystallized from water, cacodylic acid (dimethylsulfoxycetic acid) (J. T. Baker), sucrose (Sigma grade I), Tris base (ultrapure, Schwarz/Mann), ADP and ATP (sodium salts, Sigma, P-L Biochemicals, Miles), Easalol 1130 (Kao Soap Co.), and Tween 20 (Sigma) were obtained from the indicated sources. All other chemicals were of the best quality available.

Cytochrome c—Horse cytochrome c was prepared according to Margoliash and Walske (33); bakers' yeast iso-1 and iso-2 cytochromes c, according to Sherman et al. (34). Euglena cytochrome c (cytochrome c-558) was obtained by an adaptation of the aluminum sulfate extraction procedure (33, 35), blending the partially thawed polymeric material resulting from lyophilization. Keilin-Hartree Particle Preparation—The procedure is that of Keilin and Hartree (6, 36), and again chromatographed on Sephadex G-75 in the ferrous form in 0.1 M ammonium sulfate containing 1 mM EDTA, and 0.7 mM TMPD at 25°. A base-line rate was subtracted from the rate of oxygen consumption in the presence of various concentrations of cytochrome c (0.02 to 8 $\mu $M) to give the steady state velocity. These rates were equivalent to the initial steady state velocity since it was determined spectrophotometrically that the cytochrome c was maintained better than 98% reduced under the conditions of this assay. Under all conditions studied there was no detectable rate of autooxidation of any of the cytochromes c used.

**Succinate-Cytochrome c Reductase Activity**—This activity was measured polarographically with a Gilson model KM oxygen graph with a Yellow Springs Instruments oxygen electrode. An extra thin membrane was used on the electrode to obtain maximal sensitivity. Assays were routinely run in 25 mM cacodylate (Tris), pH 7.8, 250 mM sucrose, 7 mM sodium ascorbate (from a stock solution of 0.5 M sodium ascorbate containing 1 mM EDTA), and 0.7 mM TMPD at 25°. Rates of oxygen consumption were calculated in an O$_2$ ml of buffer (42). Very low base-line rates were measured after the addition of Keilin-Hartree particles (0.04 to 0.46 mg of protein/ml) or purified oxidase (0.037 to 0.15 $\mu $M cytochrome aa$_3$), indicating an insignificant rate of reduction of the enzyme by ascorbate or TMPD. This base-line rate (consisting mainly of the low autooxidation rate of cytochrome c and ascorbate) was subtracted from the rate of oxygen consumption in the presence of various concentrations of cytochrome c (0.02 to 8 $\mu $M) to give the steady state velocity. These rates were equivalent to the initial steady state velocity since it was determined spectrophotometrically that the cytochrome c was maintained better than 98% reduced under the conditions of this assay.

Under all conditions studied there was no detectable rate of autooxidation of any of the cytochromes c used.

**Succinate-cytochrome c oxidase activity** was measured spectrophotometrically using 5-cm light path cells in a Zeiss DMR-21 double-beam spectrophotometer. Cytochrome c samples were oxidized with ferricyanide and desalted on a column of Sephadex G-15 equilibrated in the appropriate assay buffer immediately before use. The Keilin-Hartree particles were preincubated in the presence of succinate (20 mM) and sodium cyanide (0.25 mM) for 3 min in the same buffer as for the polarographic assay to obtain complete inhibition of the oxidase activity. The initial rate of reduction was measured after addition of cytochrome c (0.3 to 7 $\mu $M) using a reduced minus oxidized millimolar extinction coefficient of 18.7.

**Binding of Ferricytochrome c to Cytochrome c Oxidase**—This binding was measured using gel filtration, as described for this system by C. R. Hartzell* and Hartzell and Shaw (43). Samples of cytochrome c oxidase (5 to 20 nmol), both phospholipid-containing and phospholipid-depleted, were mixed with 50 nmol of horse cytochrome c in a total volume of 50 to 100 $\mu $l and chromatographed on columns of Sephadex G-75 (0.7 x 40 cm at 22° and 0.7 x 25 cm at 4°) equilibrated in solutions containing cytochrome c (0 to 20 mM) (Table I and Fig. 10). In the case of Euglena and baker's yeast iso-2 cytochromes c, solutions of cytochrome c oxidase (6 to 10 nmol) in 50 to 100 $\mu $l were used. These were equilibrated to 20 to 40 nmol of the lyophilized proteins and were chromatographed after 10 min. In all cases, the cytochrome oxidase, with any bound cytochrome c, eluted first from the column, completely separated from

*The abbreviations used are: TMPD, N,N',N'-tetramethylphenylenediamine dichloride.
the band of excess cytochrome c. Samples of 0.8 to 1.5 ml of the cytochrome c oxidase-containing band and the amounts of cytochrome c and cytochrome aa₃ were estimated from the oxidized and dithionite-reduced spectra. Correction at 605 nm and 550 nm for the absorbance of the cytochrome c and cytochrome oxidase, respectively, was done as described by Van Buuren et al. (44). The concentration of each component was calculated using a reduced minus oxidized millimolar extinction coefficient of 24 at 605 nm for the cytochrome aa₃, of 20 at 550 nm for the horse and yeast cytochromes c and of 14.0 at 558 nm for Euglena cytochrome c (35, 44). The molar concentrations of cytochrome oxidase were calculated on the basis of 2 mol of heme α/ mol. These concentrations, corrected for the cytochrome c oxidase activity of the Keilin-Hartree particle preparations tested, but the apparent Kₘ for the initial phase of 5 x 10⁻⁸ M over the entire pH range examined. This value was expressed in nanomoles of O₂ per min and s is expressed in micromolar concentrations of cytochrome c.

RESULTS

Kinetics of Reaction of Horse Cytochrome c with Beef Cytochrome c Oxidase

pH Dependence—The ascorbate-TMPD-cytochrome c oxidase activity of the Keilin-Hartree particles was studied as a function of pH in 25 mM cacodylate buffer, varying the concentration of Tris to change the pH from 5.8 to 8.4. The results (Fig. 1) are represented in Eadie-Hofstee single reciprocal plots in which the negative reciprocal slope gives the apparent Michaelis constant of the reaction and the intercept with the abscissa gives the maximal velocity. Biphase kinetics were observed, with an apparent Kₘ for the initial phase of 5 x 10⁻⁸ M over the entire pH range examined. This value was consistently reproduced with four separate Keilin-Hartree particle preparations. The extrapolated Vₘₐₓ of this initial phase (Fig. 1, inset) was maximal above pH 7.5.

The second phase of the kinetic plots occurred with all the Keilin-Hartree particle preparations tested, but the apparent Kₘ was not as constant as it was for the first phase, varying from 5 to 20 times that of the initial phase. In contrast to the experiments in which the cation concentration was changed, when the anion concentration was varied to change the pH in a Tris-acetate buffer, there was an increase in the apparent Kₘ for both phases of the reaction as the anion concentration was decreased from 54 to 7 mM (pH 5.9 to 8.6) (Fig. 2). At the lowest acetate concentration the kinetics became monophasic. The same results were obtained when the anion concentration was varied in a Tris-cacodylate buffer. The extrapolated Vₘₐₓ of the initial phase (Fig. 2, inset) increased even more dramatically than that when the cation concentration was changed (Fig. 1), with an apparent inflection point at pH 7.2.

These observations suggested that anion concentration had an important influence on the kinetic behavior of the system, whereas it was less affected by the cation concentration in the ranges studied. A further examination of the effects of various anions is described below.

Temperature Dependence—The apparent Kₘ of the initial phase of the kinetics was unaffected by temperature over the range of 11 to 35°. The maximal velocity of this phase doubled for each 10° increase in temperature over the same range.

Enzyme Concentration Dependence—When the concentration of the Keilin-Hartree particles was increased over a 10-fold range there was a 3-fold increase in the apparent Kₘ for the initial phase at pH 7.8 in 25 mM Tris-cacodylate buffer. The extrapolated Vₘₐₓ for this apparent Kₘ, extrapolated to zero enzyme concentration, was 2.8 x 10⁻⁴ M (Fig. 3, inset). This value is remarkably close to that obtained for the dissociation constant for the binding of cytochrome c to cytochrome c-depleted mitochondria (approximately 3 x 10⁻⁴ M) (45, 46). As expected, the Vₘₐₓ (Fig. 3, inset) was linearly dependent on the enzyme concentration over the range examined and extrapolated to zero at zero enzyme concentration. It should be noted that in Fig. 3 higher concentrations of cytochrome c that reveal the second kinetic phase were employed with only two of the five Keilin-Hartree particle concentrations studied.

**Fig. 1.** Effect of varying pH and cation concentration on cytochrome c oxidase activity of Keilin-Hartree particles. Rates of oxygen consumption were measured polarographically as described under "Experimental Procedure." Final concentrations: Keilin-Hartree particle protein, 0.16 mg/ml; horse cytochrome c, 0.09 to 4.9 μM. The concentration of Tris was varied to achieve the indicated pH values: △—△, 6.8; O—O, 6.4; ●—●, 6.9; □—□, 7.5; ■—■, 8.4. v is expressed in nanomoles of O₂ per min and s is expressed in micromolar concentrations of cytochrome c.

**Fig. 2.** Effect of varying pH and anion concentration on cytochrome c oxidase activity of Keilin-Hartree particles. Conditions are as described under "Experimental Procedure" except that the buffer consisted of 50 mM Tris and varying concentrations of acetate to achieve the indicated pH values: △—△, 5.9; O—O, 7.0; ●—●, 7.5; □—□, 7.9; ■—■, 8.6. The Keilin-Hartree particle protein concentration was 0.12 mg/ml.
Eukaryotic Cytochromes c React with Mitochondrial Enzymes

FIG. 3. Effect of enzyme concentration on cytochrome c oxidase activity of Keilin-Hartree particles. Conditions as described under “Experimental Procedure.” Keilin-Hartree particle protein concentrations in milligrams per ml were: A-A, 0.046; O-O, 0.07; O-O, 0.115; O-O, 0.23; D-m, 0.46. The inset shows $K_m$ values (O-O) in micromolar and $V_{max}$ values (O-O) in nanomoles of $O_2$ min$^{-1}$.

One possible explanation of the increase in apparent $K_m$ with enzyme concentration is that at the higher levels of Keilin-Hartree particles a significant proportion of the cytochrome c is bound, so that the total amount of cytochrome c added no longer correctly represents the concentration of free substrate. It is not possible in this case to determine whether such depletion kinetics (47, 48) actually operate since the amount of cytochrome oxidase available for reaction with externally added cytochrome c cannot be estimated with certainty for Keilin-Hartree particles. However, since disruption of the vesicles by treatment with deoxycholate (1 mg/mg of protein) according to Smith and Camerino (49) resulted in an approximately 8-fold increase in the $V_{max}$ of the low apparent $K_m$ phase of the reaction, as little as 12% of the enzyme may be reactive in the intact Keilin-Hartree particles, suggesting that depletion of substrate should not be kinetically important except at the highest enzyme concentrations. However, other estimates of the amount of available oxidase are possible and are considered under “Discussion.”

Reaction with Purified Cytochrome c Oxidase—To determine whether the reaction pattern described above was unique to membrane-bound cytochrome c oxidase, experiments were carried out with a purified oxidase preparation. Fig. 4 shows that a kinetic behavior very similar to that observed with Keilin-Hartree particles was also obtained with the pure enzyme. At low enzyme concentrations the apparent $K_m$ is $5 \times 10^{-8}$ M, identical with that found with the membrane preparations, while at high enzyme concentrations there is an increase of $K_m$, again suggesting the possibility of substrate depletion (see “Discussion”). Interestingly, the second kinetic phase, with the high apparent $K_m$, was not as prominent with the pure oxidase as with the Keilin-Hartree particles (compare Figs. 1 and 4).

Effect of Anions on Cytochrome c Oxidase Activity

The monovalent anions, cacodylate, acetate, and chloride, had identical inhibitory effects. As shown in Fig. 5A, when the concentration was doubled a 2-fold increase of the apparent $K_m$ of the initial phase of the kinetics was observed. However, phosphate that is present as the divalent anion at the pH of the assay (pH 7.8) had a much larger effect over the same concentration range (Fig. 5B). Furthermore, the inhibition exerted by phosphate appeared to be qualitatively different from that of the monovalent anions, being largely uncompetitive. At a concentration of 25 mM, three-quarters or more of the initial low $K_m$ phase of the kinetics had been eliminated or transformed into a high $K_m$ phase, while at 50 mM no initial phase whatsoever could be detected.

The pattern of inhibition produced by the polyvalent anions, ADP and ATP, was similar to that observed with phosphate (Fig. 6), except that the nucleotides were effective at much lower levels, well within their physiological concentration ranges of up to 5 mM (50). Even at concentrations as low as 3 mM, ATP virtually eliminated the low apparent $K_m$ phase of the kinetics, while ADP merely caused a 33% decrease in the apparent maximal velocity of this phase.

The various effects of anions cannot be explained solely on the basis of ionic strength since the monovalent and the polyvalent anions exhibited qualitatively different types of inhibition.

Comparison of Horse, Bakers’ Yeast, and Euglena Cytochromes c Reactions with Cytochrome c Oxidase

Examination of the reactions of four different cytochromes c with beef heart Keilin-Hartree particle oxidase revealed clear differences in the low apparent $K_m$ phase of the kinetics (Fig. 7). Both yeast cytochromes c had about double the maximal velocity of the horse protein for the low apparent $K_m$ phase. The kinetic plot for the Euglena protein extrapolated to the
same maximal velocity as for the yeast cytochromes, but gave an apparent $K_m$ value 10 times that obtained for either the horse or the yeast proteins. This overall kinetic picture (Fig. 7) may be interpreted to indicate that there are two separate enzymically active sites on the oxidase which horse cytochrome $c$ recognizes with different affinities, as suggested by the two phases of the kinetic plot ($K_m = 4 \times 10^{-8} M$ and $K_m = 1 \times 10^{-9} M$). According to such an interpretation the yeast cytochromes react with both these sites with equal high affinity ($K_m = 4 \times 10^{-8} M$), while the Euglena cytochrome $c$ reacts with both with equal low affinity ($4 \times 10^{-9} M$).

A study of the enzyme concentration dependence of the reaction of the yeast cytochrome $c$ with Keilin-Hartree particle oxidase revealed another difference from the reaction of the horse protein. Comparing Figs. 3 and 8, it is apparent that the kinetics of the yeast cytochromes $c$ showed a more marked dependence on enzyme concentration than those for horse cytochrome $c$. There is strong curvature of the kinetic plot for the iso-2 cytochrome $c$ of yeast at the highest enzyme concentration employed even though at the same and higher enzyme concentrations horse cytochrome $c$ still gave a linear initial kinetic phase. Iso-1 cytochrome $c$ of yeast behaved identically with the iso-2 cytochrome $c$. The curvature suggests that with the yeast proteins there is a larger degree of substrate depletion, supporting the idea that both postulated kinetic sites on the oxidase bind the yeast cytochromes $c$ strongly, while only one of them has high affinity for the horse protein. With the pure cytochrome $c$ oxidase the difference in activity between the horse and yeast proteins at low concentrations was even more striking (Fig. 9).

An alternative explanation for these results would be that only one kinetically active site exists on the oxidase, and that the yeast cytochromes $c$ have twice the turnover of the horse protein at that site. However, the direct binding studies reported below, that show the presence of two cytochrome $c$
FIG. 8 (left). The effect of enzyme concentration on yeast iso-2 cytochrome c activity with Keilin-Hartree particle cytochrome c oxidase. Conditions are as described under "Experimental Procedure." Final concentrations: yeast cytochrome c, 0.04 to 2.4 μM; Keilin-Hartree particle protein (mg/ml), □□□, 0.046; ○○○, 0.07; ●●●, 0.115; □□□, 0.23. v/s is given in nanomoles of O₂ per min per micromolar cytochrome c × 10⁻¹.

FIG. 9 (right). Activity of horse and yeast iso-2 cytochromes c with purified cytochrome c oxidase. The conditions are as described under "Experimental Procedure" and in the legend to Fig. 4. Final concentrations: cytochrome aa₃, 0.038 μM; yeast iso-2 cytochrome c (○○○), 0.05 to 0.5 μM; horse cytochrome c (△△△), 0.05 to 7.7 μM.

Binding of Cytochrome c to Purified Cytochrome c Oxidase

The remarkably low apparent Kₐ values obtained for the initial phase of the kinetics were very near the dissociation constant for cytochrome c with cytochrome c-depleted mitochondria (45, 46). Direct estimates of the binding of cytochrome c to purified cytochrome c oxidase were therefore carried out to examine whether the effects of the anions and of the cytochromes c of various species on the kinetics were related to changes in binding. A tightly bound complex was formed by mixing cytochrome c and cytochrome aa₃, which was not dissociated by gel filtration under the conditions of the experiment. The amount of complex so formed was dependent on the concentration and type of anion present (Table I). The high affinity complex with horse cytochrome c approached a ratio of 1 molecule of cytochrome c to 1 molecule of cytochrome aa₃ in 25 mM Tris cacodylate, while yeast iso-2 cytochrome c yielded a value of about 2, and the Euglena protein gave a ratio much smaller than 1. This is strikingly similar to the kinetic results described above that indicated the presence of two active sites on the oxidase with different affinities for cytochrome c. The same 1:1 ratio was obtained with horse cytochrome c at 22 and 4°C (Table I), indicating a temperature independence of the binding analogous to the temperature independence of the apparent Kₐ of the initial phase of the kinetics. This similarity is further strengthened by the effect of phosphate, ADP, and ATP, all of which decrease the amount of tightly bound complex formed, while lower ionic strength conditions cause an increase. There was no significant difference in the binding of cytochrome c to a phospholipid-depleted as compared to a phospholipid-containing preparation of cytochrome c oxidase.

In addition to the tightly bound complexes, additional reversible binding to the oxidase preparations could be measured by the gel filtration technique (Fig. 10). With horse cytochrome c in 25 mM Tris cacodylate, a dissociation constant of approximately 10⁻⁴ M was observed for the 2nd molecule of the protein binding to the oxidase, the first being tightly bound, as stated above. This dissociation constant was of the same magnitude, and may in fact correspond to the apparent

| Ratio of cytochrome c to cytochrome aa₃ |
|---------------------------------------|
|                                       |
| 22°C                                  |
| Horse cytochrome c                     |
| Tris-cacodylate, 25 mM                 | 0.04 |
| Tris-cacodylate, 25 mM + ATP, 3 mM     | 0.45 |
| Tris-cacodylate, 25 mM + ADP, 3 mM     | 0.62 |
| Tris-cacodylate, 5 mM                  | 1.40 |
| Tris-phosphate, 25 mM                  | 0.34 |
| Yeast iso-2 cytochrome c               | 0.36 |
| Tris-cacodylate, 25 mM                 | 1.92 |
| Euglena cytochrome c                   | 0.63 |
| Tris-cacodylate, 25 mM                 |     |

|                                       |
|                                       |
| 4°C                                   |
| Horse cytochrome c                     |
| Tris-cacodylate, 25 mM                 | 0.04 |
| Tris-cacodylate, 25 mM + ATP, 3 mM     | 0.35 |
| Tris-cacodylate, 25 mM + ADP, 3 mM     |     |
| Tris-cacodylate, 5 mM                  | 2.04 |
| Tris-phosphate, 25 mM                  |     |
| Yeast iso-2 cytochrome c               |     |
| Tris-cacodylate, 25 mM                 |     |
| Euglena cytochrome c                   |     |
| Tris-cacodylate, 25 mM                 |     |
not practical to measure a dissociation constant smaller than under the same low ionic strength conditions employed for the Reaction of Horse Cytochrome c with Keilin-Hartree Particle

tions of cytochrome c (0.5 to 20 $\mu$M) by the gel filtration technique employed. $K_m$ values for the low affinity phase of the cytochrome c oxidase reaction (54) that might reflect the same allosteric change in the enzyme resulting in a decrease in the affinity for cytochrome c. However, a more likely explanation for the $K_m$ values obtained in the spectrophotometric reductase assay is that the large excess of cytochrome over enzyme required for this assay precludes the observation of the high affinity 1:1 reaction.

The identity of the $K_m$ values for the Keilin-Hartree particle cytochrome c oxidase and reductase activities obtained polargraphically, as well as that of purified oxidase, suggests that the same binding site, provided by the oxidase, is involved in both the oxidation and reduction reactions.

**DISCUSSION**

**Relation of Kinetics of Cytochrome c Oxidation to Binding with Purified Oxidase**—The discovery of an extremely low apparent $K_m$ region of the reaction of cytochrome c with cytochrome c oxidase in Keilin-Hartree particles and with purified enzyme has made it possible to observe large differences in the reactivities of the cytochromes c of various species and strong inhibitory effects of polyvalent anions on oxidase activity.

The present experiments indicate that this low apparent $K_m$ phase, together with the commonly studied higher $K_m$ phase of the kinetics of cytochrome c oxidation, are related to the two binding sites of different affinity that were found for cytochrome c on purified cytochrome c oxidase. Indeed, a value was obtained for the apparent $K_m$ of the initial kinetic phase (2.8 x
In the experiments presented in this paper. A second site was also found at which cytochrome c binds to the oxidase with a $K_a$ of $1.2 \times 10^{-5}$ M, a value similar to the apparent $K_a$ for the second phase of the oxidation kinetics ($0.35$ to $1.0 \times 10^{-4}$ M). These results are in accord with a number of previous observations. A single high affinity site was indicated by studies of the ascorbate reducibility of oxidase-bound cytochrome c (55) and demonstrated directly by binding measurements. The presence of an additional low affinity binding site ($K_a = 10^{-4}$ M), postulated by Nicholls (96) has been independently confirmed by Hartzell and Hartzell and Shaw (43). In addition, tightly bound complexes containing up to 2 molecules of cytochrome c/molecule of cytochrome aa$_3$ have been observed under a variety of conditions (31-60). Although it is possible that the similarities between the kinetics and the binding behavior are coincidental or the result of measurement artifacts, it seems most likely that the kinetics are in fact a measure of the binding of cytochrome c to cytochrome c oxidase.

This conclusion was further supported by the similarity of the effects of the polyvalent anions, phosphate, ADP, and ATP, on both kinetics and the binding (compare Figs. 5, 6, and 10, and Table I). In addition, parallel variation of the activity and binding behavior was observed for three different cytochromes c examined. Yeast cytochrome c showed twice the activity of the horse protein at low concentrations and formed twice the amount of high affinity complex, while Euglena cytochrome c showed much lower activity at low concentrations and corresponding lower complex formation (Fig. 7 and Table I).

Since the low apparent $K_a$ kinetics were observed with both Keilin-Hartree particles and purified cytochrome c oxidase, and the high affinity binding occurs with both mitochondria and purified enzyme, it is concluded that the enzymically effective high affinity binding site for cytochrome c in mitochondria is provided by the cytochrome c oxidase protein, not the mitochondrial membrane.

Furthermore, the kinetics of cytochrome c reaction with the succinate-cytochrome c reductase system of Keilin-Hartree particles yielded a single phase with an apparent $K_a$ identical with that for the reaction with the oxidase. This suggests that the high affinity binding site provided by the oxidase also serves the reaction between cytochrome c and cytochrome c oxidase, thus providing a specific association between cytochrome c, and cytochrome oxidase in the mitochondrial membrane. Such a complex has indeed been obtained.

Influence of Anions—Numerous effects have been described (see Refs. 1-3, 46, 61) of both cations and anions on the reaction of cytochrome c with cytochrome c oxidase, in the high apparent $K_a$ range of the kinetics that has so far been examined. In the low apparent $K_a$ phase it was found that the nature and concentration of the anions in the medium had a major effect. Monovalent anions, such as chloride, cacodylate, and acetate, were competitive inhibitors of the reaction, while the polyvalent anions, phosphate, ADP, and ATP, appeared to cause the disappearance of the high affinity reaction site in an uncompetitive manner, or its conversion into a low affinity site. At the same ionic strength the polyvalent anions were much more effective inhibitors than the monovalent anions. Since some anions hardly bind at all to cytochrome c (cacodylate) and some bind only to ferricytochrome c (chloride), while others bind to both ferri- and ferrocytochrome c (for example, phosphate) (36, 62-64), it is possible that the different varieties of inhibition observed are the result of different types or sites of binding to cytochrome c. However, the dissociation constants for ATP and ADP are at least 1 order of magnitude larger than for phosphate (63, 64), while the inhibitory effects on the reaction between cytochrome c and cytochrome c oxidase vary in the opposite order, ATP being more effective than ADP, which is itself more effective than phosphate. It is therefore probable that the polyvalent anion inhibition is the result of binding to the oxidase. Since ATP induces a spectral change in the cytochrome aa$_3$ of coupled mitochondria (54) in concentrations similar to those needed for the inhibition of the binding of cytochrome c to the oxidase, it is possible that both effects reflect a structural change induced in the oxidase by the polyvalent anions.

When the pH of the reaction mixture was varied by changing the monoanion concentration (acetate or cacodylate) at a constant cation concentration (Tris), a monophasic titration of the maximal activity was observed (see Fig. 2). It is interesting that the inflection point is at pH 7.2, close to the pK for EPR-detectable changes between low spin and high spin forms observed with purified cytochrome c oxidase (38, 65). It would thus appear that the form of cytochrome oxidase that reacts with cytochrome c at high affinity may be related to the high pH EPR detectable species.

Reactions of Various Cytochromes c with Cytochrome c Oxidase—Major differences were observed in the reactivity at low concentrations of four cytochromes c with cytochrome c oxidase. These correlated remarkably well with variations in the binding characteristics of these cytochromes with the purified enzyme. Differences occurred in the apparent $K_a$ values, while the maximal velocities of all four cytochromes c were very similar. It thus appears that evolutionary changes in primary structure have resulted in changes in the surface topography of the molecule leading to changes in binding, while maintaining unaltered the essential mechanism of electron transfer.

The differences in activity and binding of these cytochromes c can be explained in terms of changes in primary structure. Of particular relevance are variations in positively charged lysyl and arginyl residues that have been implicated in the interaction between cytochrome c and the oxidase (1-3, 25-30). All the cytochromes tested have lysine or arginine at position 13. In addition, lysine is present at position 11 in the yeast iso-1 and yeast iso-2 proteins, that have high affinity binding at both oxidase sites, while valine is at position 11 in the horse protein which shows high affinity binding at only one site, and glutamic acid is in the homologous position in Euglena cytochrome c which has only low affinity binding (4, 5). Furthermore, horse and yeast cytochromes c, which display high affinity binding, have a lysine at position 27, where Euglena cytochrome c, that has no high affinity binding, carries a serine. In contrast, yeast iso-1 cytochrome c with a lysine at position 22, has the same kinetic behavior as yeast iso-2 cytochrome c with glutamic acid in that position. Histidyl...
residue 26, proposed as the binding site for phosphate in the horse protein from NMR evidence (66) also varies in the species examined. Yeast iso-2 and Euglena cytochromes c have asparagine at position 26 and are still inhibited by phosphate. This is consistent with the present proposal that phosphate inhibition is not a result of binding to the cytochrome c. In summary, it is likely that positive charges at positions 11, 13 and 27 are important for binding, while the residues at 22 and 26 do not significantly affect either the kinetics or binding.

If it is a general evolutionary phenomenon among the cytochromes c of eukaryotic species that changes in amino acid sequence result in changes in binding characteristics, it is clear that any investigation of functional variability has to encompass a wide range of cytochrome c concentrations in order to detect these differences. For example, Euglena cytochrome c has been reported to be twice as active as horse cytochrome c in reacting with beef cytochrome c oxidase (67) at a single concentration. From Fig. 7 it can be seen that depending on the concentration range employed one can obtain results which show that Euglena cytochrome c is more active, as active or less active than horse cytochrome c. All these merely reflect the fact that Euglena cytochrome c has a single kinetic phase in its reaction with beef cytochrome oxidase, the $K_m$ value for which is between the $K_m$ values for the two phases of the reaction of horse cytochrome c.

Furthermore, any detailed examination of the functional concomitants of evolutionary changes in structure will have to consider not only the reactions of a series of cytochromes c with the oxidase of a single species, as done in this paper, but the complete matrix of possible interactions of the cytochromes with the oxidases of all the species considered. In the present comparison, the two varieties of yeast cytochrome c give higher activity with the beef oxidase than does the horse protein, but do not distinguish between the two enzymically active sites. Since horse cytochrome c is phylogenetically and functionally closer to the beef protein than the yeast cytochromes c, it is possible that the ability to distinguish between the two enzyme binding sites is of prime physiological importance.

**Cytochrome c Oxidase Available for Reaction and Turnover Numbers**—An accurate estimate of the amount of oxidase participating in the Keilin-Hartree particle assay system is necessary to calculate turnover numbers for the enzyme, and on that basis to draw conclusions about the physiological and mechanistic significance of the high affinity binding site for cytochrome c on cytochrome c oxidase. Since Keilin-Hartree particles are a heterogeneous population of membrane vesicles such an estimate is difficult to obtain. As given under "Results," disruption of these vesicles with deoxycholate caused an 8-fold increase in the maximal activity of the low $K_m$ phase of the kinetics, suggesting that as little as 12% of the total oxidase may be available in the intact preparation. The maximal turnover numbers for the high affinity phase calculated on this assumption are in the range of 150 to 200 s$^{-1}$.

Since the purified oxidase shows a similar high affinity kinetic region, one would expect to be able to obtain a more precise estimate of the turnover number. In fact, the values obtained with the purified enzyme, 30 to 50 s$^{-1}$, are much lower than those estimated for the Keilin-Hartree particles. If this latter estimate is correct and applicable to the membrane-bound enzyme, at least 50% of the enzyme in the Keilin-Hartree particles must be available to externally added cytochrome c to yield similar turnover numbers. However, the turnover numbers calculated for the purified oxidase may not be accurate since at the higher enzyme levels necessary for polarographic studies and in the absence of solubilizing detergent in the assay medium, the phospholipid-containing enzyme preparation may exist in a micellar form that does not allow free access of cytochrome c to all of the enzyme.

Another estimate of the available enzyme in Keilin-Hartree particles can be obtained indirectly by correcting the kinetic plots for depletion of the free cytochrome c due to binding. Assuming various concentrations of available oxidase and a $K_D$ of $3 \times 10^{-8}$ M, on the basis of results discussed above, the data represented in Fig. 3 at the two highest enzyme levels were corrected for substrate depletion (Fig. 12). By this method, assuming 50% of the enzyme to be available, the apparent $K_m$ values (1.4 and 1.7 x $10^{-9}$ M) obtained from the limiting initial slopes were considerably lower than the assumed $K_D$ value. On the other hand, the apparent $K_m$ values (both $3.0 \times 10^{-9}$ M) obtained by assuming 25% enzyme to be available, were identical with the $K_D$. These results suggest that 25% is a good estimate for the amount of cytochrome c oxidase available for reaction with cytochrome c in this Keilin-Hartree preparation. Furthermore, the turnover numbers for the high affinity reaction, calculated on the assumption of 25% available enzyme, 80 to 100 s$^{-1}$, are in excellent agreement with maximal values calculated for mitochondria (100 s$^{-1}$ (61)).

It should be noted that there is considerable variation in the amount of cytochrome c oxidase available to externally added cytochrome c among preparations of Keilin-Hartree particles. Thus, calculations such as those just presented apply only to the individual preparation employed.

**Physiological Significance**—If the tightly bound form of cytochrome c is physiologically important, several questions arise concerning the mechanics and control of electron transfer...
demonstrated the existence of a cross-over point between the early observations of Chance and Williams (81, 82) that mitochondria. This type of kinetic control would account for chrome c and cytochrome oxidase observed here, and there is no obvious reason why the inhibition could not occur in any single reaction within the chain, but must occur at either respiratory control cannot be the result of a change of rate of reduction equilibrium with the phosphate potential, so that colleagues (78-80). They find that under numerous conditions the concept of respiratory control developed by Wilson and his flow between cytochrome c1 and cytochrome aa3. This kinetic control mechanism is not in accord with the polyvalent anions which is inaccessible for reduction by cytochrome c1. However, in the absence of high affinity binding, there will be competition for the cytochrome c between the low affinity oxidase site (K0 = 3 x 10^-6 M) under physiological conditions. Since the amount of cytochrome c is very near that of cytochrome aa3 in most mitochondria (74), the former will essentially be totally the oxidase. As suggested above, this process may function as the oxidase would be accomplished by lowering the affinity of the tight binding site to a value equivalent to that of the mitochondrial phospholipid. Since, of the anions studied, ATP is most effective in converting the oxidase to a low affinity form, and since it is the predominant adenine nucleotide on the outside of the inner mitochondrial membrane (76, 77) variations in its concentration, that reflect the overall phosphate potential, might well influence the proportion of cytochrome c bound to the oxidase and thereby control the rate of electron flow between cytochrome c1 and cytochrome aa3.

This kinetic control mechanism is not in accord with the concept of respiratory control developed by Wilson and his colleagues (78-80). They find that under numerous conditions the components of the respiratory chain remain in oxidation-reduction equilibrium with the phosphate potential, so that respiratory control cannot be the result of a change of rate of any single reaction within the chain, but must occur at either end of it. It is, however, difficult to ignore the dramatic effect of ATP through this portion of the respiratory chain. One issue is whether the dissociation of cytochrome c from the oxidase is the rate-limiting step in the reaction. Using a dissociation constant of 3 x 10^-4 M (45, 46) and an "on" constant of 5 x 10^7 M^-1 s^-1 (44, 52, 68-70) for the reaction of cytochrome c with cytochrome oxidase, an "off" constant of 1.5 s^-1 is obtained. This last value precludes the possibility that cytochrome c dissociates from the oxidase during each oxidation-reduction cycle, even at the lowest estimated turnover rate. However, the reported rate constant of 5 x 10^7 M^-1 s^-1 was measured under conditions of high ionic strength, typically 100 mm phosphate buffer. It is possible that the value for low ionic strength conditions may be considerably larger, even as great as 3 x 10^8 M^-1 s^-1, as reported for the reaction of ferrocytochrome c with cytochrome c peroxidase (71). If the rate constant does indeed approach a value of about 10^8 M^-1 s^-1, an "off" constant of 30 s^-1 is obtained, suggesting the possibility that the tightly bound cytochrome c dissociates from the oxidase after two or four electrons have been transferred, in response to oxidation-reduction dependent structural changes in the oxidase (52, 72; see also Ref. 73). Whether cytochrome c dissociates from the high affinity binding site only after several oxidation-reduction cycles, or not at all, cytochrome c1 must be capable of reducing cytochrome c while it is bound to the oxidase. This was already suggested by the kinetics of the succinate-cytochrome c reductase system, as discussed above.

Another question is whether the large changes in affinity of cytochrome c for the oxidase caused by the polyvalent anions is likely to control electron transfer between cytochrome c1 and cytochrome aa3 under physiological conditions. Since the amount of cytochrome c is very near that of cytochrome aa3 in most mitochondria (74), the former will essentially be totally bound to the high affinity site (K0 = 3 x 10^-6 M) if it is available. However, in the absence of high affinity binding, there will be competition for the cytochrome c between the low affinity oxidase site (K0 = 10^-4 M) and the much larger number of phospholipid sites of equal affinity for cytochrome c (K0 = 10^-4 M) (75). Thus, an effective removal of cytochrome c from the oxidase would be accomplished by lowering the affinity of the tight binding site to a value equivalent to that of the mitochondrial phospholipid. Since, of the anions studied, ATP is the most effective in converting the oxidase to a low affinity form, and since it is the predominant adenine nucleotide on the outside of the inner mitochondrial membrane (76, 77) variations in its concentration, that reflect the overall phosphate potential, might well influence the proportion of cytochrome c bound to the oxidase and thereby control the rate of electron flow between cytochrome c1 and cytochrome aa3.

This kinetic control mechanism is not in accord with the concept of respiratory control developed by Wilson and his colleagues (78-80). They find that under numerous conditions the components of the respiratory chain remain in oxidation-reduction equilibrium with the phosphate potential, so that respiratory control cannot be the result of a change of rate of any single reaction within the chain, but must occur at either end of it. It is, however, difficult to ignore the dramatic effect of ATP at physiological concentrations on the reaction between cytochrome c and cytochrome oxidase observed here, and there is no obvious reason why the inhibition could not occur in mitochondria. This type of kinetic control would account for the early observations of Chance and Williams (81, 82) that demonstrated the existence of a cross-over point between cytochrome c and cytochrome a, and represents an additional possibility besides the several mechanisms suggested for such control (83).

Furthermore, the requirements for the suggested mode of control of electron flow through the terminal segment of the respiratory chain are uniquely met by the physicochemical properties of eukaryotic cytochromes c and the structure of mitochondria. Of all the members of the respiratory chain, cytochrome c is the only water-soluble component which is capable of easily moving on and off the inner membrane (84), is located at the outer surface of the inner membrane (85) where its binding can be influenced by the cytoplasmic environment, and is strongly cationic so that it can react with the oxidase or bind inactively to the mitochondrial phospholipid (see Refs. 1 and 2). The system also requires the existence of the outer membrane of mitochondria to maintain cytochrome c in the vicinity of its binding sites.

Implications for Spatial Organization—The present correlations between the kinetics of reaction of cytochrome c with its binding to cytochrome c oxidase indicate that there are two kinetically active binding sites of different affinities on the enzyme, and that cytochrome c1 must be so located that it can reduce the molecule of cytochrome c bound at the high affinity site. When the polyvalent anions affect the structure of cytochrome oxidase, the high affinity binding site becomes one of low affinity and the cytochrome c attached to it can no longer be reduced directly by cytochrome c1. This model requires a particular structural orientation of cytochrome c1 and cytochrome aa3 in the membrane that can only be maintained by specific protein-protein interactions. This implies that a modification of the usual fluid mosaic model of mitochondrial membrane structure (86, 87) is required to allow for stable functional protein complexes, rather than the independent diffusion of each protein in the membrane. There is a second site available for reaction with cytochrome c on the enzyme which is inaccessible for reduction by cytochrome c1. The additional low affinity binding to the membrane phospholipid is enzymically inactive and provides a means for competitive removal of the cytochrome c from low affinity binding to the oxidase. As suggested above, this process may function as control of electron transport through the terminal segment of the respiratory chain.

It must be emphasized that the model just described does not of itself specify the detailed mechanism by which electrons are transferred from one cytochrome to another. A spatial arrangement of oxidase, cytochrome c1, and cytochrome c should be possible that would allow for electron transfer between these components, even by such a structurally restrictive mechanism as heare edge interactions.

Acknowledgments—We wish to acknowledge the generous gifts of Teflon membrane from Dr. W. B. Elliot, Euglena cells from Dr. W. Fitch, cytochrome oxidase from Dr. T. E. King and Dr. C. R. Hartzell and Emasol 1130 from Kao Soap Co. We are also indebted to Dr. C. R. Hartzell for his method of determining binding of cytochrome c to cytochrome c oxidase and for discussing his results before publication.

REFERENCES
1. Margoliash, E., and Schejter, A. (1966) Adv. Protein Chem. 21, 115-286
2. Lemberg, R., and Barrett, J. (1973) Cytochromes, Academic Press, London
3. Dickerson, R. E., and Timkovich, R. (1975) in The Enzymes...
Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes c with binding to mitochondrial cytochrome c oxidase.

S Ferguson-Miller, D L Brautigan and E Margoliash

*J. Biol. Chem.* 1976, 251:1104-1115.

Access the most updated version of this article at [http://www.jbc.org/content/251/4/1104](http://www.jbc.org/content/251/4/1104)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/251/4/1104.full.html#ref-list-1](http://www.jbc.org/content/251/4/1104.full.html#ref-list-1)