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Interaction of cytochrome *c* with cytochrome *c* oxidase: An understanding of the high- to low-affinity transition

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The steady-state kinetics of high- and low-affinity electron transfer reactions between various cytochromes *c* and cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) preparations were studied spectrophotometrically and polarographically. The dissociation constants for the binding of the first and second molecules of horse cytochrome *c* ($I = 15$ mM) are $5 \cdot 10^{-8}$ M and $1 \cdot 10^{-5}$ M, respectively, close to the spectrophotometric K_m values and consistent with the controlled binding model for the interaction between cytochrome *c* and cytochrome oxidase (Speck, S.H., Dye, D. and Margoliash, E. (1984) Proc. Natl. Acad. Sci. USA 81, 346–351) which postulates that the binding of a second molecule of cytochrome *c* weakens that of the first, resulting in low-affinity kinetics. While the K_m of the polarographically assayed high-affinity reaction is comparable to that observed spectrophotometrically, the low-affinity K_m is over an order of magnitude smaller and cannot be attributed to the binding of a second molecule of cytochrome *c*. Increasing the viscosity has no effect on the V_{max} of the low-affinity reaction assayed polarographically, but increases the K_m. Thus, the transition from high- to low-affinity kinetics is dependent on the frequency of productive collisions, as expected for a hysteresis model ascribing the transition to the trapping of the oxidase in a primed state for turnover. At ionic strengths above 150 mM, the rate of cytochrome *c* oxidation decreases without any correlation to the calculated net charge of the cytochrome *c*, indicating rate-limiting rearrangement of the two proteins in proximity to each other.

Introduction

The steady-state kinetics of cytochrome *c*–cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) interaction in vitro is commonly assayed by monitoring either the oxidation of cytochrome *c* spectrophotometrically or the consumption of oxygen manometrically or polarographically. In both assays, at ionic strengths less than 100 mM, changing the concentration of cytochrome *c* results in biphasic kinetics [1–4], with a high-affinity K_m value equal to the K_D for the binding of the first molecule of cytochrome *c* to cytochrome oxidase [4–7]. However, the polarographically assayed high-affinity V_{max} is 10-fold greater than that observed spectrophotometrically [5,8,9]. The K_m of the low-affinity reaction in the spectrophotometric assay is $1.5 \cdot 10^{-5}$ M [9–11]. In contrast, the polarographic assay displays at least two low-affinity phases. The initial phase has an apparent K_m of $3 \cdot 10^{-7}$ M [4,9] and the second a larger K_m and V_{max}, which are comparable to those observed in the low-affinity spectrophotometrically assayed reaction [8,11] and may indicate similar reactions.

Three main classes of models exist to explain the spectrophotometric kinetics and the ability of two molecules of cytochrome *c* to bind cytochrome oxidase. The first and simplest explanation proposes the existence of two sites of electron transfer with different binding affinities for cytochrome *c* and rates of turnover [3,4]. The inability to detect a second site of electron transfer or observe different pathways of electron flow into the oxidase is inconsistent with this model [12–14], but does not rule it out.

The second class of models proposes one site of electron transfer with the biphasic kinetics resulting from effects associated with the binding of a second molecule of cytochrome *c* on the oxidase at a site not

Abbreviations: TMPD, *N',N',N,N*-tetramethyl-*p*-phenylenediamine; PEG, poly(ethyleneglycol).

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capable of electron transfer [15,16]. An example is the controlled binding hypothesis of Speck et al. [15], in which the binding of the second cytochrome *c* molecule, through electrostatic repulsion increases the rate of ferricytochrome *c* dissociation from the electron transfer site, the proposed rate-limiting step. Other researchers have proposed an allosteric mechanism for achieving the same effect [16].

The third class of models incorporates the fact that associated with the catalysis of electron transfer from cytochrome *c* to oxygen, cytochrome oxidase also translocates protons [17–22]. This coupling of two different processes inherently means that, upon titrating a parameter or species associated with either electron transfer or proton translocation, one should observe non-linear steady-state kinetics, provided that the parameter enters into the two rate expressions differently. Numerous models have been proposed relating electron transfer to proton translocation [21–28]; how they apply to these in vitro assays in which the enzyme is in an uncoupled environment is unknown.

The polarographic assay of oxygen consumption involves the use of an artificial reducing system so that interpretation of the kinetics is complicated, inasmuch as an understanding of the kinetic significance of the artificial reducing system is incomplete. It has been proposed that the high-affinity V_{max} represents the reduction of oxidized cytochrome *c* bound to the oxidase at or near the active site, thereby circumventing product dissociation and resulting in the increased activity relative to the spectrophotometric assay [29]. Examination of several primate cytochromes *c* demonstrated a direct correspondence between the V_{max} of the high-affinity phase and the rate at which *N',N',N,N*-tetramethyl-*p*-phenylenediamine (TMPD) reduced ferricytochrome *c* bound to cytochrome oxidase [7]. However, the basis for the low-affinity phases has not been elucidated.

In this paper, the interaction of cytochrome *c* with cytochrome oxidase, in an uncoupled environment, is presented for both the spectrophotometric and polarographic assays. The demonstration via ultracentrifugation that the dissociation constant for the binding of a second molecule of cytochrome *c* with cytochrome oxidase was the same as the K_m of the low-affinity phase supports the controlled binding hypothesis [15], as describing the spectrophotometric assay at low ionic strengths. The effects on the rate of oxygen consumption by cytochrome oxidase of viscosity, employment of phylogenetically different cytochromes *c* and the role of TMPD in the artificial reducing system were all consistent with a hysteresis model as describing the mechanistic basis for the high- to low-affinity transition in the polarographic assay. At ionic strengths exceeding 150 mM, demonstrated by Sinjorgo et al. [30] to result in monophasic kinetics, no correlation between changes in the ionic strength and the net charge of the cytochrome *c* was observed, indicating that the rate-limiting step was no longer product dissociation, but instead some form of configurational rearrangement of the two proteins in proximity to each other.

Materials and Methods

Chemicals. Sucrose, Tris and Tween 20 (Sigma), *n*-dodecyl β-maltoside (Boehringer Mannheim), TMPD (Eastman), poly(ethylene glycol) 8000 (Kodak) and ascorbic acid (Aldrich gold-label) were obtained from the indicated sources. TMPD was recrystallized from ethanol.

Cytochrome c. Ustilago sphaerogena (ATCC No. 12421) was grown to stationary-phase on rich medium containing 4 ppm thiamine hydrochloride and 1 ppm $ZnCl_2$ [31], and lysed by treatment with ethyl acetate for 5 h at room temperature as described [32], with 2 mM phenylmethylsulfonyl fluoride added to inhibit proteinase activity. The cytochrome *c* was absorbed on Amberlite IRC-50 following 15-fold dilution of the lysate with water and prepared as previously described [33]. The cytochromes *c* of horse, tuna, *Euglena gracilis*, dusky shark, and *Rhodospirillum rubrum* (c_2) were purified as previously described [5,33–35]. *Paracoccus denitrificans* ($c = 550$) cytochrome *c* was the kind gift of Russel Timkovich (University of Alabama). *Candida krusei* cytochrome *c* (Sigma) and the eukaryotic cytochromes *c* were chromatographed on carboxymethylcellulose.

Cytochrome c oxidase. Purified beef-heart cytochrome *c* oxidase preparations were generous gifts from A.O. Muijsers and B.F. Van Gelder (University of Amsterdam) and Y.-C. Ching (A.T.&T. Bell Laboratories). The kinetic properties of both preparations were found to be similar. The concentration of the enzyme was determined according to Williams [36].

Submitochondrial particle preparation. Beef-heart and horse-heart particle preparations were prepared according to Method 1 of King [37], as modified by Ferguson-Miller et al. [4]. *Ustilago* submitochondrial particles were prepared by disruption of cells, grown to late log phase, by two 15 min cycles of vigorous shaking of cells/100 mM phosphate (pH 7.2)/0.5 mm glass beads (1 : 1 : 1, v/v). Centrifugation at $7000 \times g$ and $10\,000 \times g$ removed the glass beads and cellular debris. Subsequent ultracentrifugation at $45\,000 \times g$ for 90 min sedimented the submitochondrial particles, which were stored as described for the beef and horse preparations [4].

Kinetic studies. The polarographic steady-state, spectrophotometric steady-state and presteady-state assays of cytochrome *c* oxidase activity were according to the procedures of Brautigan et al. [33], Speck et al. [38] and Veerman et al. [39], respectively, employing a Gilson 5/6 oxygraph, Hitachi 557 spectrophotometer and a Durrum Rapid Kinetics System Series D-100 with the

OLIS model 4000 Data System. Because of possible secondary salt effects, ionic strengths were measured with a conductivity meter standardized to sodium chloride.

Binding studies. Gel-filtration measurements of the binding of ferricytochrome *c* to purified beef cytochrome oxidase were as described [40,5,7]. The binding of ferricytochrome *c* to purified beef cytochrome oxidase was also measured employing a Beckman model E analytical ultracentrifuge with a model AND rotor, run at 52100 rpm, monitoring the absorbance across the cell at 410 nm and 420 nm [41]. The employment of a sufficiently large centrifugal force sediments the free cytochrome oxidase and its complexes with cytochrome *c* as a tight front, while free cytochrome *c* remains evenly distributed throughout the cell during the course of the experiment. Employing a calibration curve made for both cytochrome *c* and cytochrome oxidase at 410 nm and 420 nm to correct for the large slit-width and distortions in the spectrophotometer of the ultracentrifuge, the total concentration of cytochrome oxidase being sedimented (free and in various complexes with cytochrome *c*), the total concentration of cytochrome *c* in the presence of the sedimenting oxidase (free and bound) and the concentration of free cytochrome *c* (that behind the front) can be obtained. Assuming that binding at the first site precedes that at the second, the concentrations of cytochrome oxidase with two cytochromes *c* and with only one bound, could be calculated from the concentration of bound cytochrome *c*, measured as the difference between the amount sedimenting with the enzyme and that not. Since the ratio for the dissociation constants for the binding of the first and second molecules of cytochrome *c* is 10^3, it was appropriate to assume that complete occupancy of the high-affinity site preceded binding at the second site. Dissociation constants were then calculated for a minimum of four different free cytochrome *c* concentrations. The occupancy of the second site varied up to a maximum of 35%, above which titrations were not possible due to the high absorbance of the proteins. Titration of the high-affinity binding was not possible using this ultracentrifugation procedure due to the inability of the spectrophotometer, associated with the ultracentrifuge, to quantify such low concentrations of cytochrome *c*. In conducting these experiments, *n*-dodecyl β-maltoside was used in place of Tween 20 to reduce the background absorbance; this change in detergent has been shown to increase the specific activity without significantly altering the K_m [42], an observation we have confirmed.

Calculation of kinetic parameters. Determination of the K_m of the high-affinity phase involves simple linear regression analysis of the data, omitting those data points displaying significant overlap with the low-affinity reaction. To calculate the K_m of the low-affinity reaction, the following expression was employed which corrects for the overlap between the high- and low-affinity phases:

$$K_m = c_f \left[\frac{TN^L_{max} - TN^H_{max}}{TN_{obs} - TN^H_{max}} - 1 \right]$$

In this expression c_f is the concentration of free cytochrome *c* resulting in a specific low-affinity activity TN_{obs}, with TN^H_{max} and TN^L_{max} being the turnover numbers of the high- and low-affinity reactions extrapolated to infinite substrate concentration. This expression assumes that:

(1) A molecule of oxidase can only catalyze either high- or low-affinity activity but not both simultaneously; namely, there is only one site of electron transfer.

(2) The transition from high-affinity activity to low-affinity activity is dependent on the concentration, to the first power, of cytochrome *c* in solution. This requires that initial steady-state conditions apply, so that all the ferrocytochrome *c* in solution is available for reaction, that no significant amount of oxidized cytochrome *c* is present and that substrate depletion does not occur. Furthermore, the transition from high- to low-affinity reactivity is first order with respect to cytochrome *c* concentration and can be approximated by $[H] \times [c]/[L]$ in which $[c]$, [H] and [L] are the concentrations of the free cytochrome *c*, cytochrome oxidase catalyzing high-affinity turnover and cytochrome oxidase catalyzing low-affinity turnover, respectively. While this is obviously correct for the spectrophotometric assay in which the transition from high- to low-affinity activity is dependent on the binding of an additional molecule of cytochrome *c*, it is only an approximation for the polarographic assay in which the rate equation of the low-affinity phase is not known.

(3) The K_m of the high-affinity reaction is at least 1.5 orders of magnitude smaller than that of the low-affinity reaction, such that under low-affinity conditions the concentration of uncomplexed enzyme is negligible.

Results

Dissociation constants of cytochrome c-cytochrome oxidase complexes

The K_D for the binding of the first molecule of horse cytochrome *c* to purified beef cytochrome oxidase, measured by gel filtration in 25 mM acetate (adjusted to pH 7.9 with Tris)/0.25% Tween 20, was found to be $5 \cdot 10^{-8}$ M, in agreement with earlier measurements [4–7]. Increasing the ionic strength with KCl to 37 mM and 52 mM resulted in K_D values of $1.1 \cdot 10^{-7}$ M, and $3.0 \cdot 10^{-7}$ M, indistinguishable from the K_m values reported by Sinjorgo et al. [30] for the high-affinity reaction spectrophotometrically assayed under comparable conditions.

TABLE I

Comparison of dissociation constants and Michaelis-Menten constants of the polarographic assay

Cytochrome *c*	K_{D1} [a] (M)	K_{D2} [b] (M)	High affinity	Low affinity	
			K_m (M)	K_{m1} [c] (M)	K_{m2} [d] (M)
Horse	$5 \cdot 10^{-8}$	$1.1 \cdot 10^{-5}$	$3 \cdot 10^{-8}$	$8.4 \cdot 10^{-7}$	$1.7 \cdot 10^{-5}$
Fungal [e]	n.d. [f]	$1.2 \cdot 10^{-5}$ [g]	$6 \cdot 10^{-9}$	$7.7 \cdot 10^{-7}$	n.d.
Spider monkey [h]	$1 \cdot 10^{-8}$	n.d.	$1. \cdot 10^{-8}$	$1.1 \cdot 10^{-6}$	n.d.

[a] The dissociation constant for the binding of the first molecule of cytochrome *c*, as measured by gel filtration.
[b] The dissociation constant for the binding of the second molecule of cytochrome *c*, as measured by ultracentrifugation.
[c] The cytochrome *c* concentration range referred to as low affinity by Ferguson-Miller et al. [4], and Smith et al. [9] (approx. 0.5 to 3 μM).
[d] The cytochrome *c* concentration range studied by Slater [8] (10–60 μM).
[e] Kinetically, the cytochromes *c* of *C. krusei*, yeast iso-1, and *U. sphaerogena* were indistinguishable.
[f] Not determined.
[g] Measured using *C. krusei* cytochrome *c*.
[h] Data taken from Osheroff et al. [7].

Determination of the K_D for the binding of a second molecule of cytochrome *c* by gel filtration may be inaccurate due to interactions with the column matrix that will skew large dissociation constants. To address this concern and the uncertainty in the literature concerning the value of the dissociation constant for the binding of a second molecule of cytochrome *c* to cytochrome oxidase [4,7], an ultracentrifugation procedure, described under Materials and Methods, was employed. The K_D for the binding of a second molecule of horse cytochrome *c* was found to be $1.1 \cdot 10^{-5}$ M, while that for a second molecule of *C. krusei* cytochrome *c* was $1.2 \cdot 10^{-5}$ M. In both measurements the standard deviations were $3 \cdot 10^{-6}$ M.

The inability to detect the binding of a second molecule of cytochrome *c* to cytochrome oxidase by observing perturbations in the absorption spectra of the two proteins [14] may indicate only that the binding of the second molecule of cytochrome *c* does not cause any alterations in the electronic transitions of the hemes. This is consistent with the second molecule of cytochrome *c* binding at a site not capable of electron transfer and with a large dissociation constant.

Steady-state kinetic studies

A comparison of the kinetic and binding properties of different cytochromes *c* is presented in Table I. All displayed biphasic kinetics, with the high-affinity reactions of the three fungal proteins having a V_{max} approximately one-tenth that for the horse protein. Because of limitations on the accuracy and sensitivity of the polarogrpahic assay, the fungal proteins could be assigned only an upper limit of $6 \cdot 10^{-9}$ M for the K_m of the high-affinity phase, and any slight differences between their high-affinity reactions could not be observed. This combination of very small K_m and V_{max}

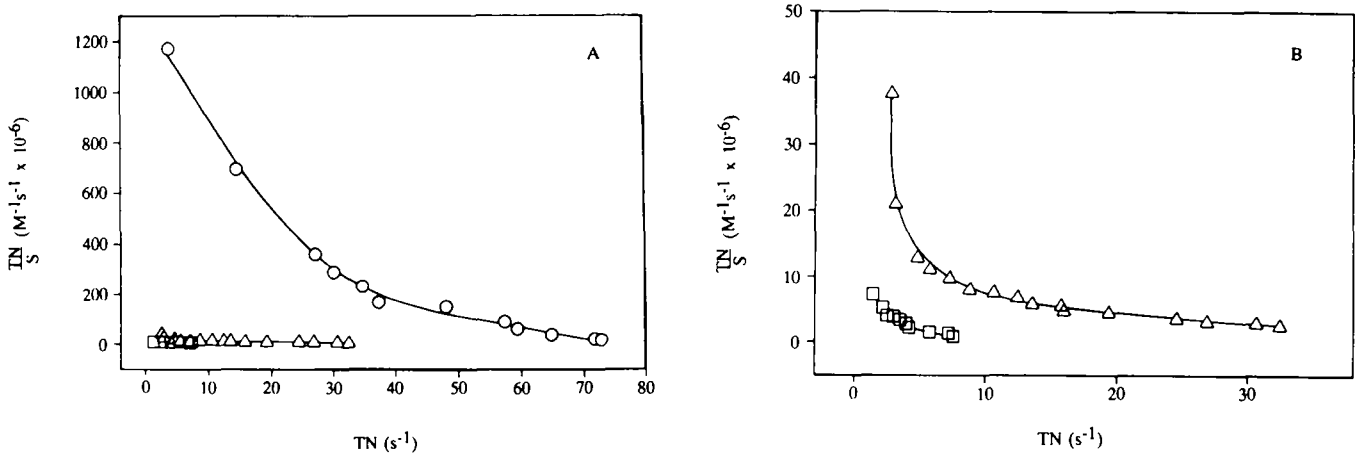

Fig. 1. Comparison of steady-state assays for the interaction of horse cytochrome *c* with beef-heart submitochondrial particles. Polarographic measurement of steady-state oxygen consumption by 2.5 nM cytochrome oxidase in 25 mM acetate-Tris (pH 7.9), 0.7 mM TMPD, 5 mM ascorbate (○); the same conditions except that TMPD was absent (△); spectrophotometric analysis of the initial steady-state rates of the oxidation of horse ferrocytochrome *c* by 0.2 nM cytochrome oxidase in 25 mM acetate-Tris (pH 7.9) (□). (A) and (B) show the same data plotted on two different scales, displaying the biphasic nature of the kinetics for all three assay systems, and the large influence of TMPD.

values made it difficult to detect the high-affinity reaction for the fungal proteins, explaining why earlier researchers observed only the low-affinity reaction [5]. Correction for overlap with the high-affinity reaction, employing Eqn. 1, yielded K_m values of $7.7 \cdot 10^{-7}$ M and $8.4 \cdot 10^{-7}$ M for the fungal and horse cytochromes *c*, respectively. These values are similar to that of the primate spider-monkey cytochrome *c* [7] and over an order of magnitude smaller than the K_D for the binding of a second molecule of cytochrome *c* to the enzyme.

Role of TMPD in the polarographic assay

A comparison of the spectrophotometric and polarographic kinetics in the presence of both TMPD and ascorbate or with ascorbate alone (Fig. 1), demonstrated that in the absence of TMPD, the kinetic parameters derived from both assays were very similar. The slight difference, less than 40%, in the V_{max} of the high-affinity reaction, may be attributed to the accumulation of product, ferricytochrome *c* in the spectrophotometric assay. This does not occur in the polarographic measurement inasmuch as the ascorbate maintains the cytochrome *c* fully reduced under these conditions [43] avoiding such a problem typically associated with initial steady-state spectrophotometric determinations.

The presteady-state rate of the TMPD reduction of *C. krusei* cytochrome *c* complexes to purified beef cytochrome oxidase, in 25 mM acetate-Tris (pH 7.9) was found to be 4 to 5 s^{-1}, similar to the polarographically derived steady-state V_{max} for the high-affinity phase of the reaction.

Effects of viscosity on the polarographic assay

Increasing the viscosity with PEG had no discernible effect on the V_{max} of the initial low-affinity phase of the polarographic assay, but did cause an increase in the apparent K_m (Fig. 2). At the higher concentrations of cytochrome *c*, associated with the titration of the binding of a second molecule to cytochrome oxidase, the effects of viscosity changed to a decrease in the V_{max} with no alteration in the apparent K_m, reinforcing the proposal that the two phases employ different mechanisms. Even though the differences between these two low-affinity phases are relatively small, they are significant considering the high precision of low-affinity polarographic measurements and the relatively low background activity they are measured against. These effects of viscosity were also observed employing glycerol or sucrose (data not shown).

Ionic strength effects on the spectrophotometric assay

Increasing the ionic strength from 15 mM to 750 mM elicited two types of inhibition of the spectrophotometrically determined rate of eukaryotic cytochrome *c* oxidation by horse-heart, beef-heart, and *U. sphaerogena* submitochondrial particle preparations. The later preparation displays an approx. 20-fold greater specific activity than the other preparations and as such avoids long reaction times and/or high enzyme concentrations, even at ionic strengths resulting in a 90% inhibition, so that possible artifacts due to substrate depletion or slow solvent effects on the integrity of the submitochondrial particles were minimized. As Table II summarizes, between 15 mM and 100 mM ionic strength, the inhibition decreased with increasing cytochrome *c* concentration, such that at the higher concentrations of cytochrome *c*, characteristic of the low-affinity reaction, the rate of reaction was ionic strength independent. Thus, the inhibition observed at concentrations of cytochrome *c*, which at ionic strengths below 100 mM exhibit a mixture of high- and low-affinity activities, must represent inhibition of the residual high-affinity activity.

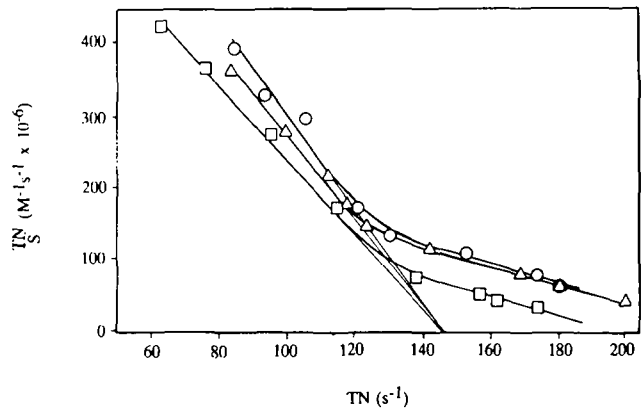

Fig. 2. Effect of viscosity on the polarographically assayed low-affinity reactions of horse cytochrome *c* with beef-heart submitochondrial particles. The rate of oxygen consumption by 5 nM cytochrome oxidase in the absence of polyethylene glycol (○), in the presence of 5% (w/v) poly(ethylene glycol) (△), and 10% (w/v) poly(ethylene glycol) (□), were assayed as described in Fig. 1 for the polarographic assay with TMPD. The data plotted represent the average of a minimum of three complete sets of determinations, with each point measured in duplicate and yielding a standard deviation of less than 10%, typically less than 5%.

Above 150 mM there is a strong dependence of the rate of cytochrome *c* oxidation on the ionic strength. The slopes of the plots of the logarithm of the turnover numbers versus the square roots of the ionic strengths for different cytochrome *c* concentrations, were approximately the same, indicating that the ionic strength derived inhibitions were primarily the result of decreases in the rate constants and not due to changes in the apparent K_m.

Increasing the ionic strengh decreased the rate of oxidation by the ustilago particle preparation of the bacterial cytochromes *c* (*P. denitrificans* and *R. rubrum*), indicating that, though these proteins have a net negative charge, they behave in their interaction with cytochrome oxidase as though they were cationic. However, because of the extremely low activities dis-

TABLE II

Effects of ionic strength on the spectrophotometrically assayed initial steady-state rate of oxidation of various cytochromes c by Ustilago sphaerogena cytochrome oxidase

Samples were prepared and analyzed as described for Fig. 1. The initial buffer was 25 mM acetate, titrated to pH 7.9 with Tris, supplemented with KCl to achieve the various ionic strengths. The concentration of *U. sphaerogena* cytochrome oxidase, prepared as submitochondrial particles, was typically 0.2 nM.

Cytochrome *c*	[*c*] [a] (μM)	TN [b] (s^{-1})	$-m_I$ [c]	$-m_{II}$ [d]	TN_{II} [e] (s^{-1})
Horse	12.5	160	0.33	3.8	$3.8 \cdot 10^3$
	6.3	146	0.52	4.3	$3.7 \cdot 10^3$
	2.5	98	0.69	4.6	$2.7 \cdot 10^3$
	1.3	70	0.91	n.d. [f]	n.d.
Tuna	2.0	175	0.86	4.9	$4.6 \cdot 10^3$
Dusky shark	1.8	121	1.2	4.8	$4.6 \cdot 10^3$
Euglena gracilis	1.6	38	1.6	3.7	$1.1 \cdot 10^2$
	0.8	20	2.2	4.7	$1.7 \cdot 10^2$
Yeast iso-1	4.9	205	0.48	3.9	$1.2 \cdot 10^4$
	2.0	180	1.0	4.1	$6.2 \cdot 10^3$
	1.0	135	3.0	3.9	$2.7 \cdot 10^3$
Candida krusei	4.4	202	0.65	3.8	$1.1 \cdot 10^4$
	1.8	155	0.92	3.9	$5.1 \cdot 10^3$
	0.9	96	1.1	3.7	$2.1 \cdot 10^3$
Ustilago sphaerogena	1.0	76	1.4	4.6	$2.7 \cdot 10^3$
Paracoccus denitrificans [g]	4.2	2.2	1.1		3.0
	2.1	1.7	1.1		2.3
Rhodospirillum rubrum [g]	2.1	1.6	1.8		2.4

[a] The concentration of reduced cytochrome *c* initially in the cuvette prior to the addition of cytochrome oxidase.

[b] The initial specific steady-state activity observed in buffer containing no added KCl.

[c] Slope of the effect of increasing ionic strength below 100 mM on the initial steady-state rate of cytochrome *c* oxidation.

[d] Slope of the effect of increasing ionic strength above 150 mM on the initial steady-state rate of cytochrome *c* oxidation.

[e] Extrapolation of the specific activities observed above 150 mM ionic strength to zero ionic strength.

[f] Not determined.

[g] The bacterial cytochromes *c* displayed only a monophasic decrease in the initial steady-state rate of cytochrome *c* oxidation with increasing ionic strength.

played with eukaryotic cytochrome oxidases, more detailed kinetic analyses were not undertaken.

The effects of ionic strength were independent of whether potassium chloride, lithium chloride or potassium cacodylate were employed as effectors. The only differences observed arose from secondary salt effects on their ionizations. In contrast, employing Tris-acetate as both the buffer and the ionic strength effector resulted in a strong inhibition between 25 mM and 250 mM (I = 15 mM–110 mM), approx. 5-fold greater than with the other salts.

Discussion

Interpretation of spectrophotometric kinetis

The controlled binding hypothesis of Speck et al. [15] assumes that in both high- and low-affinity phases of the steady-state rection of cytochrome *c* with cytochrome oxidase, followed spectrophotometrically, the rate-limited step is product dissociation. The basis for this assignment has been amply discussed [15,16,44]. So far, the data supportive of the model have focused on the high-affinity reacton, in that (a) the K_m was shown to be identical to the K_D for the binding of the first molecule of cytochrome *c* with cytochrome oxidase; (b) chemical modification of cytochrome *c* equally effects the high-affinity K_m and K_D values [6]; and (c) stopped-flow studies have demonstrated that the formation of free cytochrome *c*, from the 1 : 1 complex with cytochrome oxidase, occurs at a rate comparable to the steady-state high-affinity V_{max} [16]. However, the essential feature of the model is that the binding of a second molecule of cytochrome *c* to the enzyme, at a site not capable of electron transfer, will through electrostatic repulsion increase the rate of product dissociation from the electron transfer site, thus resulting in low-affinity kinetics. The ultracentrifugation procedure employed in this paper has allowed the measurement of the dissociation constant for the binding of the second molecule of cytochrome *c*, $1 \cdot 10^{-5}$ M, which, as the model predicts, is close to the K_m of the low-affinity reaction. Thus, both the high- and low-affinity kinetic phases of the spectrophotometric assay behave as predicted by the controlled binding hypthesis [15].

Models have been proposed explaining the biphasic kinetics in the spectrophotometric assay as representing two different states of the oxidase, which displays different properties towards cytochrome *c* [23,27,28]. Our results do not contradict a conformational change as being involved in the transition from high- to low-affinity kinetics. However, the observation that K_m for the low-affinity phase is similar to the K_D for the binding of a second molecule of cytochrome *c* means that whatever model is proposed must take into account that under low-affinity conditions two molecules of cytochrome *c* are bound to the oxidase. Thus, the low-affinity kinetics may be catalyzed by an altered enzyme, whose transformation is elicited by the binding of a second molecule of cytochrome *c*.

The model recently proposed by Michel and Boshard [27] is contrary to our data, since throughout both high- and low-affinity phases, only one molecule of cytochrome *c* is bound. In addition, by using the sum of two independent Michaelis-Menten equations (see Eqn. 6) to calculate the kinetic parameters for the high- and low-affinity reactions, the mathematics is actually describing two independnet sites of electron transfer. This is contrary to their own single-site model which ascribes

the high- to low-affinity transition to a conformational change in the enzyme, and as such, a molecule of cytochrome oxidase cannot catalyze simultaneously both high- and low-affinity reactions.

Interpretation of polarographic kinetics

This correspondence between the kinetic parameters of the spectrophotometric assay and the binding constants does not hold for the polarographic assay. Even though the K_m of the high-affinity reaction is the same as the dissociation constant for the binding of the first molecule of cytochrome *c* to cytochrome oxidase, the K_m of the subsequent low-affinity phase in the polarographic assay is over one order of magnitude smaller than the K_D for the second molecule. Therefore, the high- to low-affinity transition involves only a 1:1 complex of cytochrome *c* with cytochrome oxidase. Since the transition occurs as a result of increasing the concentration of free cytochrome *c*, the resultant higher turnover rate must represent a change in the mechanism of interaction, presumably involving a change in the conformation of the two proteins separately or in complex [45,46]. That such conformational changes occur have been shown from circular dichroism spectra for the interaction of various cytochromes *c* with either cytochrome oxidase or cytochrome *c* peroxidase (Ref. 47; unpublished data). Thus, it can be proposed that following interaction, the enzyme is primed for turnover, so that if the next molecule of substrate binds prior to the enzyme relaxing back to the less active form, a high turnover rate will result, namely the low-affinity kinetic phase.

Such a hysteresis model has two readily testable aspects. One is that the transition from high- to low-affinity kinetics should display a dependence on the frequency of productive collisions and not on the concentration of substrate, such as may be achieved by changing the diffusion coefficients of the reactants by altering the viscosity of the medium. As shown above, increasing the viscosity with PEG, glycerol or sucrose does not affect the V_{max}, but does result in higher K_m values of the initial low-affinity phase. Earlier work had revealed similar effects, though no interpretation was given [48,49]. The second testable consequence is that the K_m of the initial low-affinity phase of the polarographic assay is representative of the lifetime of the primed state of the enzyme, not that of the cytochrome *c*. Employment of cytochromes *c* isolated from widely divergent sources with different properties should not alter the K_m, assuming that these cytochromes *c* have a similar rate of productive association. Confirming that this is indeed the case, it was found that cytochromes *c* varying by as much as 5-fold in their high-affinity K_m values, vary by less than 50% in their low-affinity K_m values (see Table I). This small variation may represent slight differences in the rates of productive association.

The only reason the primed state of cytochrome oxidase can be observed is that the rate of turnover in the polarographic assay is consonant with the rate of relaxation. Had the reaction been slower, as in the spectrophotometric assay, such trapping would not have been observed. Since the rate-limiting step in the spectrophotometric assay is generally considered to be product dissociation, and in the polarographic assay an artificial reducing system is present so that product dissociation is not necessary, the approx. 5-fold greater V_{max} of the high-affinity reaction of the polarographic assay has been proposed to represent TMPD reduction of cytochrome *c* in the vicinity of the active site [7]. In agreement with this is the observation depicted in Fig. 1, above, that omission of TMPD from the polarographic assay results in biphasic kinetics with K_m and V_{max} values very close to those for the spectrophotometric assay. Consistent with such a role for TMPD is that the presteady-state rate of TMPD reduction of ferricytochrome *c* bound to cytochrome oxidase is the same as the V_{max} of the high-affinity phase of the polarographic assay [7], as shown above in the case of *C. krusei* cytochrome *c*.

Though a detailed understanding of the mechanistic basis of the low-affinity reaction in the polarographic assay is not yet available, the V_{max} of the initial phase is ascribed to a combination of rate-limiting reduction of product, ferricytochrome *c*, near the electron transfer site, and reaction of free ferrocytochrome *c* from solution. At even higher concentrations of cytochrome *c*, characteristic of titration of the binding of the second molecule of cytochrome *c*, the K_m is similar to the K_D for this binding event and increasing the viscosity did not alter the K_m but inhibited the rate of reaction. These results are characteristic of the V_{max} being representative of rate-limiting product dissociation as demonstrated by Hasinoff and Davey [50] for the spectrophotometric assay. Such a conclusion is further borne out by the V_{max} of the second low-affinity polarographic phase being comparable to that observed for the low-affinity phase of the spectrophotometric assay.

Influence of ionic strength on spectrophotometric kinetics

At ionic strengths above 150 mM, it has been shown that only monophasic kinetics are observed in the spectrophotometric assay [30]. The kinetically significant charge products ($ZA \cdot ZB$) for this reaction, determined from the relationship observed between the logarithm of the rate of turnover and the square root of the ionic strength [51–53], for *U. sphaerogena; C. krusei,* and baker's yeast iso-1 cytochromes *c* with *U. sphaerogena* submitochondrial particles are 4.6, 3.8 and 3.0, respectively (Table II). Since the cytochrome oxidase used with each cytochrome *c* is the same, the ratio of the charge products equals the ratio of the kinetically significant charges of the cytochromes *c*. The calculated

net charges at pH 7.9, based upon the amino acid sequences, are +1, +3 and +5 for the *U. sphaerogena*, *C. krusei* and baker's yeast iso-1 cytochromes *c*, respectively [54–56]. Therefore, one would expect the ratio of the kinetically significant charge products of the three fungal proteins to be 1 : 3 : 5, and not the observed 1.2 : 1 : 1. The basis for expecting the kinetically significant charge product to reflect the net charges of the two proteins is the assumption that they can be represented as point charges, which are far apart and freely diffusible. However, once the rate-limiting step involves the proteins in proximity to each other, the distribution of charges relative to the interaction domains becomes kinetically significant and the observed charge product is not the same as the overall net charge. Thus, the rate-limiting step of the high ionic strength spectrophotometric kinetics must involve cytochrome *c* and cytochrome oxidase already in proximity to each other.

Questions have been raised concerning the calculation of the charge products for the reactions of macromolecules, and whether corrections for the distance of nearest approach should be included [57–60]. While such calculations change the absolute values of the kinetically significant charge products, no changes in the ratios employed above will result. Hence, the conclusion that the rate-limiting step for the oxidation of cytochrome *c* by cytochrome oxidase, at ionic strengths exceeding 100 mM, involves the two proteins in proximity to each other is independent of such concerns.

Though our data do not yield a mechanistic interpretation of the monophasic reaction observed at high ionic strengths, it is likely that, in accordance with Sinjorgo et al. [30], the monophasic reaction is derived from the high-affinity reaction pathway observed at lower ionic strengths. Increasing ionic strength presumably shields various charges on the proteins, such that the complex formed between them is altered and the rate-limiting step becomes rearrangement, to allow electron transfer. This is in contrast to low ionic strength conditions, in which the rate-limiting step in the spectrophotometric assay is product dissociation. The observation that monophasic kinetics replaces biphasic kinetics at ionic strengths slightly exceeding physiological is intriguing as a potential form of regulation.

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