Biochimica et Biophysica Acta 892 (1987) 1–9
Elsevier

BBA 42529

The kinetics of the aerobic oxidation of ferrocytochrome *c* by cytochrome *c* oxidase in solvents of increased viscosity are partially diffusion controlled

Brian B. Hasinoff and John P. Davey

Department of Chemistry and Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X7 (Canada)

(Received 28 May 1986)
(Revised manuscript received 23 December 1986)

Key words: Cytochrome *c* oxidase; Diffusion-controlled kinetics; Viscosity; Kinetics; Ferrocytochrome *c*; Aerobic oxidation

The steady-state spectrophotometrically determined initial velocity kinetics of the aerobic oxidation of ferrocytochrome *c* by cytochrome *c* oxidase were examined for effects of diffusion control in solvents of increased viscosity. Both glycerol/water and sucrose/water proved unsatisfactory as viscosogens due to weak competitive inhibition (K_i values of 2.6 M and 1.6 M, respectively). However, polyethylene glycol (PEG) was satisfactory as a viscosogen. The measured diffusion coefficient of ferrocytochrome *c* in PEG/water was shown to follow closely the Stokes–Einstein equation. In PEG/water mixtures at high ionic strength the minimum association rate constant ($k_{min} = V_{max}/(K_m[E_0])$ is partly diffusion controlled with contributions from diffusion control and chemical activation control being about equal at 5 mPa · s, a viscosity that may be physiologically relevant. This finding can be interpreted to mean that cytochrome *c* oxidase is an enzyme that has evolved to approach its maximum efficiency. The steady-state kinetics were also examined at low ionic strength where multiphasic kinetics are exhibited. The effect of increased viscosity was exhibited over the whole experimentally accessible region indicating that there are effects due to diffusion control on both the high-affinity and low-affinity binding of ferrocytochrome *c*. Several models for diffusion control were examined and a comparison is made with other diffusion-controlled reactions of proteins.

Introduction

Many association reactions involving enzymes and substrates are fast and the possibility that they are proceeding at rates that are partly limited by the diffusion of the reactants towards one another must be considered. This topic has been the subject of a recent review [1]. Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase; EC 1.9.3.1) is the terminal oxidase in the respiratory chain of all eukaryotic organisms, and is an integral constituent of the inner membrane of mitochondria. The essential nature of this enzyme may be exemplified by the fact that it is probably responsible for more than 90% of the oxygen consumption by living organisms on earth. It catalyzes the following reaction

$$\text{ferrocytochrome } c + \tfrac{1}{4}O_2 + H^+ \underset{\text{cytochrome } c \text{ oxidase}}{\rightleftharpoons} \text{ferricytochrome } c + \tfrac{1}{2}H_2O \quad (1)$$

Abbreviation: PEG, polyethylene glycol.

Correspondence: B.B. Hasinoff, Department of Chemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X7, Canada.

The association reaction of cytochrome *c* with cytochrome *c* oxidase has been directly measured to be about $5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at $I = 0.1$ M [2–5]. At lower ionic strengths this rate exceeds 10^8 $M^{-1} \cdot s^{-1}$. This high value, especially for a protein–protein reaction, is suggestive of a process that may be partly influenced by diffusion. The role of diffusion control and cytochrome *c* mobility in the eukaryotic electron-transport chain has been speculated upon [2–4,6].

To test for the influence of diffusion control on a reaction, the viscosity of the solvent is increased by adding non-interacting viscosogens to the solvent, thereby decreasing the diffusion coefficients of the reactants and slowing the rate of collision and hence of the reaction. In looking for the effects of diffusion control in a steady-state polarographic study on cytochrome *c* oxidase at high ionic strength (where the cytochrome *c* readily dissociates from the cytochrome *c* oxidase) the K_m values for both the low-affinity and high-affinity phase of the reaction increased significantly with increases in solvent viscosity [6]. Identical results were found using either sucrose or glycerol as a viscosogen and the V_{max} values were unaffected by the viscosity. Thus it was concluded that cytochrome *c* diffuses between cytochrome *c* reductase and cytochrome *c* oxidase during electron transport. However, in another study [2,3] it was concluded from the magnitude of the activation enthalpy of the reaction of ferrocytochrome *c* with cytochrome *c* oxidase and the lack of any effect on the kinetics in a single solution of increased viscosity that the reaction is not diffusion-limited. However, in the latter study it was not considered that a reaction may be influenced simultaneously by both diffusion and chemical activation resulting in apparent activation energies different from that of the viscosity dependence of water [7–11].

In this study we examine the effect of three viscosogens on the kinetics of the steady state aerobic oxidation of ferrocytochrome *c* catalyzed by cytochrome *c* oxidase to determine if there is any influence of diffusion. The use of low molecular weight viscosogens was complicated by binding at high molar concentrations of viscosogen. An initial velocity spectrophotometric method was chosen over the polarographic method as the substrate concentration is directly measured. Using the steady-state method more nearly duplicates the situation in the cell, and the effect of viscosity on any rate-limiting step in the overall reaction would show up in the kinetics. In polyethylene glycol/water the effect of viscosity on the association reaction is consistent with the effects of simultaneous diffusion and chemical activation control.

The theoretical diffusion-controlled rate constant for noninteracting uniformly reactive molecules of cytochrome *c* oxidase and cytochrome *c* would be $1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ [1,9] but as the protein reactive sites will only be a small fraction of the total surface area, the diffusion-controlled rate may be considerably less than this value [8,16]. The classical Smoluchowski equation [13] predicts for a diffusion-controlled rate constant k_D (in $M^{-1} \cdot s^{-1}$)

$$k_D = \frac{4\pi N_A D R}{1000} \tag{2}$$

where D (in $cm^2 \cdot s^{-1}$) is the sum of the translational diffusion coefficients of the two reacting species, R (in cm) is the encounter distance, often taken as the sum of the radii of the two reacting molecules, and N_A is Avogadro's number. The Stokes–Einstein expression

$$D = \frac{k_B T}{6\pi r \eta} \tag{3}$$

substituted in Eqn. 2 has often been used to predict the influence of solvent viscosity on k_D, where k_B is the Boltzmann constant, r is the molecular radius, and η is the solvent viscosity. A more accurate empirical form of Eqn. 3 is

$$D = \frac{AT}{\eta^p} \tag{4}$$

where A is an empirical constant and p is a constant depending upon solute size ($p < 1$) [14,15]. With $p = 1$ in Eqn. 4 and the inclusion of a factor f in Eqn. 2, which may include steric, interaction, and probability factors,

$$k_D = \frac{f \cdot 4\pi R A T N_A}{1000 \eta} \tag{5}$$

when $D_S \gg D_E$. More generally, any reaction is

expected to be simultaneously influenced by both diffusion control and chemical-activation control through the Noyes equation [9]

$$k^{-1} = k_D^{-1} + k_C^{-1} \tag{6}$$

where k is the observed association rate constant and k_C is the chemical activation rate constant. The chemical activation rate constant is the rate constant normally observed in the absence of any contribution from diffusion. Substitution of Eqn. 5 in Eqn. 6 predicts that k^{-1} is a linear function of η, and provides an experimental determination of f. The experimental value of f, which is a measure of the departure from Eqn. 2, may then be used with various diffusion models to compare theory and experiment. Association reactions involving myoglobin [10], the separated chains of hemoglobin [11], and carbonic anhydrase [16] have shown the effects of both diffusion and chemical activation control on the kinetics.

It should be noted that Eqn. 6 predicts that an Arrhenius plot of log k vs. T^{-1} should be curved if k_D and k_C have different activation energies. This effect of partial diffusion control on the Arrhenius plots has been observed previously for microperoxidase [7], myoglobin [10] and the separated chains of hemoglobin [11]. Since k is a composite quantity, k_C and k_D contribute at different temperatures proportionately different amounts to k. In this regard distinctly curved Arrhenius plots were observed for both the pre-steady state [2] and steady-state [2,17] binding of cytochrome *c* to cytochrome *c* oxidase. However, these results were interpreted as being due to changes in two-dimensional diffusion of cytochrome *c* associated with phospholipid within the membrane. The break in the Arrhenius plot was said to be due to the transition temperature of this phospholipid.

Experimental procedures

Materials. Bovine heart cytochrome *c* oxidase (Sigma) was dissolved in 0.01 M phosphate buffer (pH 7.4) containing 0.4% Triton X-100 or 0.1% Tween 80, and its concentration was determined spectrophotometrically from the absorbance difference between reduced and oxidised forms of the enzyme at 605 nm on the basis of a millimolar absorption coefficient for heme *a* of 11.0 $mM^{-1} \cdot cm^{-1}$ [18].

Lyophilized cytochrome *c* (Sigma, type VI from horse heart) was used without further purification. Ferrocytochrome *c* (more than 98% reduced) was prepared under spectrophotometric control by an anaerobic gel filtration of dithionite-reduced cytochrome *c* as described elsewhere [5]. Sodium phosphate buffers, pH 7.0, $I = 0.1$ M and pH 7.8, $I = 0.028$ M, were used in the present study. Solutions of the viscosogens, polyethylene glycol 6000 (Baker; molecular weight, 6000–7500), glycerol (Baker; 99.9% anhydrous) and sucrose (Anachemia; reagent grade) were prepared by weight.

Apparatus and Methods. The cytochrome *c* oxidase catalyzed aerobic oxidation of ferrocytochrome *c* was followed on a Shimadzu UV-260 recording spectrophotometer, by measuring the decrease in absorbance of ferrocytochrome *c* at 550 nm. Concentrations were determined using the following extinction coefficients [19]: ϵ_{550}(red) 27.7 $mM^{-1} \cdot cm^{-1}$ and $\Delta\epsilon_{550}$(red–ox) 18.5 $mM^{-1} \cdot cm^{-1}$. The reaction was initiated by injecting 5 μl of the aqueous stock enzyme solution into the motor-stirred 1 cm reaction cell containing 3.6 ml of solution at 25 °C. Initial velocity measurements were computed from first-derivative time-course records extrapolated back to zero time. At the higher ionic strength, usually 8–10 initial velocity measurements were made and analyzed in Lineweaver–Burk plots by weighted (assuming 2% error in v) linear least-squares analyses (Fig. 1). At the lower ionic strength, 14 initial velocity and up to 7 first-order [3] ([cytochrome] < 0.4 μM) measurements were made. A 5 cm cell was used at the lower substrate concentrations.

In the classical Michaelis–Menten scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$k_{min} = \frac{V_{max}}{[E_0]K_m} = k_1\left[\frac{k_2}{k_2 + k_{-1}}\right]$$

$$k_1 > \frac{V_{max}}{([E_0]K_m)} \tag{7}$$

k_{min} is a minimum value of k_1 even for a more complex reaction scheme [16]. Even if k_{min} is not

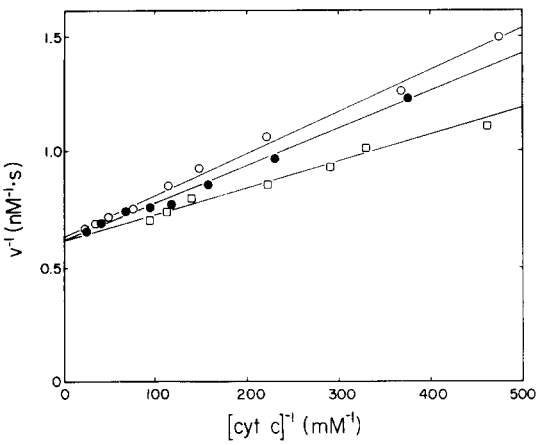

Fig. 1. Lineweaver–Burk plots for the cytochrome-*c*-oxidase-catalyzed oxidation of ferrocytochrome *c* in pH 7.0, 0.1 M ionic strength phosphate buffer in PEG/water mixtures (□, $\eta = 0.93$ mPa·s; ●, $\eta = 3.1$ mPa·s; ○, $\eta = 3.8$ mPa·s). V_{max} remained constant in different PEG/water mixtures.

equal to k_1 it is a quantity that is directly proportional to k_1 (Eqn. 7). At high ionic strengths the cytochrome *c* oxidase reaction is monophasic and is described by

$$v = \frac{V_{max}[\text{cytochrome}]}{K_m + [\text{cytochrome}]} \quad (8)$$

However, at lower ionic strengths nonlinear Lineweaver–Burk plots are obtained. These results have often been analyzed by an equation of the form

$$v = \frac{V_{max1}[\text{cytochrome}]}{K_{m1} + [\text{cytochrome}]} + \frac{V_{max2}[\text{cytochrome}]}{K_{m2} + [\text{cytochrome}]} \quad (9)$$

where the subscripts refer to the low-affinity and high-affinity sites for binding cytochrome *c*. The interpretation of these V_{max} values and K_m values in Eqn. 9, of course, depends upon the enzyme mechanism.

The solution viscosities were measured at 25 °C on a calibrated Wells–Brookfield cone-plate microviscometer to about ±1% and ranged from 0.9 to 6 mPa · s (1 mPa · s = 1 cP = 1 centipoise). Diffusion coefficients of ferrocytochrome *c* in PEG/water mixtures were determined using the Taylor dispersion technique [14,15]. In this method, a small sample (5 μl) of the solute was injected into the solvent as it flowed down a thermostatted 30.5 m long stainless-steel capillary tube (internal diameter, 0.76 mm). Provided that the flow is laminar, the combination of flow and diffusion results in a Gaussian distribution of solute along the tube. The broadened eluted peak was detected by the spectrophotometer as it passed through a 1 cm flow-through cell. The diffusion coefficient was calculated from $D = 0.2310\ r_c^2 t_r/(W_{1/2})^2$, where r_c (in cm) is the capillary radius, t_r (in s) the residence time of ferrocytochrome *c* in the tube and $W_{1/2}$ (in s) is the width at half height of the eluted peak [14,15]. The solvent flow through the capillary was controlled by a peristaltic pump with a flow rate of 0.2 ml/min.

Results

Diffusion coefficient of ferrocytochrome c

Values of log D_{cyt} vs. log η in aqueous and PEG/water buffers are plotted in Fig. 2. The failure of the Stokes–Einstein equation (Eqn. 3) to predict *D* for small solute molecules is well known [10,11,14,15,20]. This failure can be particularly acute in polymer/water mixtures where, for example, a $p \approx 0.16$ was observed for Na^+ in PEG/water [20]. Any non-Stokesian behaviour in PEG/water solutions would be a serious limitation to the routine use of PEG as a viscosogen in experiments of this kind. However, a linear least-

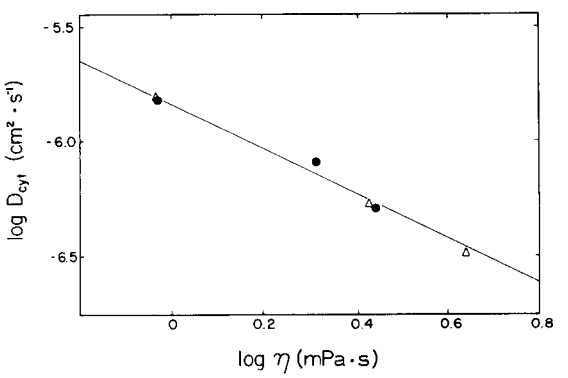

Fig. 2. Log $D_{cytochrome}$ vs. log η, for ferrocytochrome *c* in PEG/water mixtures at 25 °C in pH 7.0, 0.1 M ionic strength phosphate buffer. The straight lines are weighted linear least-squares calculated (●, determined at the experimental Triton X-100 concentration; △ no Triton X-100 present). The least-squares calculated viscosity exponent *p* from the slope is 0.97 ± 0.03.

squares fit to the logarithmic form of Eqn. 4 gives, for ferrocytochrome c, $p = 0.97 \pm 0.03$ (Fig. 2). The lack of any change in the diffusion coefficient in the presence of Triton X-100 indicates that there is no detectable detergent binding to cytochrome c.

Effect of viscosogens on the kinetics

The effect of increasing the solvent viscosity with PEG, sucrose and glycerol on the cytochrome c oxidase kinetics (at an ionic strength where the kinetics are monophasic [5,6]) is shown in Fig. 3. It should be noted that for all three viscosogens the V_{max} values were invariant. This result was reported previously for glycerol and sucrose [6]. It indicates that the turnover to product is not affected by the change in solvent composition as might be expected for an intramolecular process.

The effect of the three viscosogens on the kinetics is clearly different. These results are consistent with both glycerol and sucrose weakly inhibiting the reaction. The high concentrations of glycerol (up to 5 M) and sucrose (up to 1.2 M) used to increase the solvent viscosity competitively inhibit the enzyme. It had previously been reported that there was no difference between sucrose and glycerol on the cytochrome c oxidase steady-state kinetics [6]. However, these results were obtained using a polarographic assay that is known to give

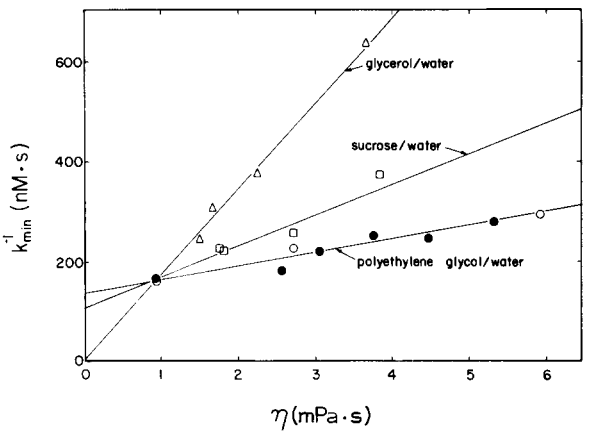

Fig. 3. Plot of the reciprocal minimum association rate constant k_{min}^{-1} for the reaction of ferrocytochrome c with cytochrome c oxidase as a function of solvent viscosity at 25 °C in pH 7.0 phosphate buffer, ionic strength 0.1 M at $[E_0] = 1.2$ nM in the presence of several viscosogens. k_{min} was calculated from $V_{max}/(K_m[E_0])$. Both glycerol and sucrose, as well as other alcohols, are weak competitive inhibitors of the enzyme. The steric factor f is calculated from the slope of the plot with Eqn. 5 in Eqn. 6. (●, enzyme dispersed in Triton X-100 buffer; ○, enzyme dispersed in Tween 80). The highest [PEG] used of 11.9% (w/v) corresponds to a molar concentration of polymer of approx. 20 mM and gives $\eta = 5.9$ mPa·s.

Michaelis parameters much different from those obtained by the spectrophotometric assay [21]. As a test of this hypothesis several smaller alcohols

TABLE I

MICHAELIS CONSTANTS AND DIFFUSION-CONTROL STERIC FACTORS FOR THE REACTION OF FERROCYTOCHROME c WITH CYTOCHROME c OXIDASE

The values below are determined in PEG/water at 25 °C in phosphate buffer.

Description/model	Parameter	for $I = 0.10$ M [a]	for $I = 0.028$ M [b]	
			high affinity	low affinity
Steric factor (experimental)	f (%)	0.48 ± 0.03	0.006 – 0.014	0.09 – 0.2
Chemical activation [c]	k_C ($\mu M^{-1} \cdot s^{-1}$)	8.9 ± 0.3	33 – 110	1.4 – 1.9
Michaelis constant ($\eta = 0$ mPa·s)	K_m (μM)	1.5 ± 0.1	0.08 – 0.18	32 – 39
Michaelis constant ($\eta = 0.93$ mPa·s)	K_m (μM)	1.8 ± 0.1	0.4 – 0.9	36 – 44
Maximum velocity [d]	V_{max} (nM·s^{-1})	6.0 ± 0.3	2.7 – 3.7	24 – 27
Half-cone solid angle of entry	θ (°)	21	5.0 – 6.6	12.3 – 16.0

[a] Errors are linear fitting errors (Ref. 23) from 6-parameter non-linear least-squares analyses.

[b] Upper and lower bounds (Ref. 23) from non-linear least-squares analyses are shown here as some parameters are not well determined due to the nature of the model and limitations due to the experimentally accessible range of substrate concentration that could be used.

[c] In the encounter complex mechanism k_C is a composite quantity (Eqn. 11).

[d] Refers to $[E_0] = 0.45$ nM.

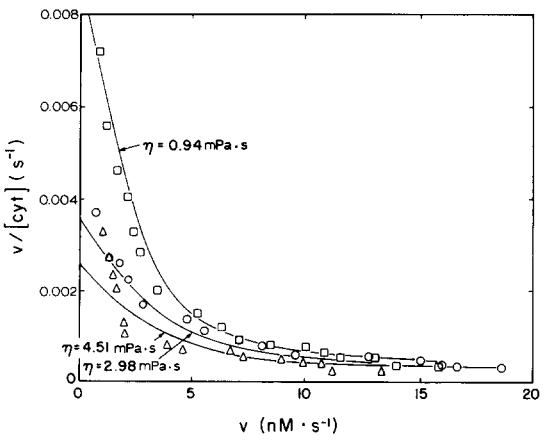

Fig. 4. Eadie–Hofstee plots of the aerobic oxidation of ferrocytochrome *c* by cytochrome *c* oxidase in PEG/water solutions of varying viscosity (□, $\eta = 0.94$; ○, $\eta = 2.98$ and △, $\eta = 4.51$ mPa·s) in pH 7.8, 0.028 M ionic strength phosphate buffer. The lines were calculated using the best-fit parameters described in Table I. For the $\eta = 0.94$ mPa·s data, four points at low v are used in the analysis plot-off scale.

that do not cause any significant change in solvent viscosity were also found to inhibit competitively cytochrome *c* oxidase with K_i values of 860 ± 70 mM for isopropanol, 480 ± 50 mM, for methanol and 350 ± 30 mM for *n*-propanol. The cause of this inhibition is not known; however, it has been reported that 830 mM isopropanol causes a decrease in the hydrodynamic radius of the Triton X-100 micelle from 44 Å to 38 Å due either to the solubilization of alcohol in the micelle layer or to the hydrophobic core [22]. Studies on the nature of this weak inhibition will be further reported on. Thus, subtracting the viscosity component, as determined in PEG/water, from both the glycerol/water and sucrose/water results of Fig. 3, competitive inhibition constants, K_i, of 1600 ± 200 mM for sucrose and of 2600 ± 300 mM for glycerol, are obtained.

The large ($M_r = 6000$) neutral PEG molecule with ether linkages and terminal $-$OH groups would not be expected to associate to any significant degree to cytochrome *c* oxidase. Also, the binding of ferrocytochrome has been demonstrated to be largely electrostatic in nature. Thus in the absence of any association the effect PEG exerts on the kinetics is due to the decrease in diffusion coefficients of the reactants. In accord with Eqns. 5 and 6, k_{min}^{-1} for the oxidation of ferrocytochrome *c* in PEG/water depends linearly on the solvent viscosity (Fig. 3), which is good evidence for the effect of diffusion control on the kinetics. The non-zero intercept indicates a significant chemical activation component to the reaction, with the k_D^{-1} term of Eqn. 6 being 20% of the k_C^{-1} term at 1 mPa · s.

With Eqn. 5 substituted in Eqn. 6, and from the slope and from the intercept of Fig. 3, the minimum value of the steric factor f and the chemical activation k_C, respectively, were calculated (Table I). The value of A in Eqns. 4 and 5 was estimated from $D = 1.30 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1} at 20 °C in aqueous solution for ferrocytochrome *c* [24]. R was calculated from $R = r_E + r_{cytochrome}$ with $r_E = 72$ Å, as determined by hydrodynamic studies [25], and $r_{cytochrome} = 16.6$ Å [26]. Thus the k_D calculated from Eqn. 2 is $1 \cdot 10^{10}$ M$^{-1} \cdot$ s^{-1} at $\eta = 0.93$ mPa · s and 25 °C.

The cytochrome *c* oxidase kinetics were also examined in PEG/water mixtures in low ionic strength ($I = 0.028$ M; pH 7.8) phosphate buffer and resulted in sharply concave Eadie–Hofstee plots (Fig. 4) as has been well documented before [6]. Clearly the data show that the viscosity also has an effect under conditions of low ionic strength. These plots have been interpreted in terms of a variety of different mechanisms [27–31]. More recently, a single catalytic site model involving two-dimensional diffusion between a catalytic and membrane site [29,31] has been proposed. Obviously any interpretation of the low-ionic strength viscosity data depends upon the mechanism chosen to interpret the data. The results of Table I are based on a fit of the low-ionic strength data of Fig. 4 to Eqn. 9, into which Eqns. 5–7 are substituted. The parameters of Table I and the calculated lines of Fig. 4 were obtained from a 6-parameter non-linear least squares [23] fit. The parameters for the low ionic strength data are not well defined due to the sharp curvature and greater experimental error at low [cytochrome] and small v, and consequently only the non-linear bounds [23] are reported in Table I. For dependent-site and independent-site mechanisms the k_{min} has an interpretation similar to that of Eqn. 7 and in the dead-end complex mechanism the k_{min} values are direct measures of association rate constants [27].

The low-ionic-strength Michaelis and diffusion parameters of Table I are based on two binding sites on the enzyme. However, it is acknowledged that the limited conclusions reached regarding the low-ionic strength data are dependent upon the actual mechanism. The single catalytic site mechanism [29,31] yields a much more complex expression than Eqn. 9. If this mechanism were operative then a simple interpretation of the viscosity dependence at low-ionic strength data is not possible. Other mechanisms could involve different interpretations of these parameters.

Discussion

Diffusion models with steric constraints

The diffusion of a protein to a sterically hindered site on a larger enzyme molecule may result in a significant reduction in the diffusion-controlled rate constant [1,8,12]. The simple surface reactivity model [10,11] assumes that only a fraction of the effective surface area of the enzyme, σ_E is reactive. A value of σ_E may be roughly estimated from the ratio of the cross-sectional area of the substrate to the surface area of the enzyme, $\sigma_E = \pi r^2_{cytochrome}/4\pi r^2_E$. Further factoring f into $f = \sigma_E \sigma_{cytochrome}$ and assuming $\sigma_{cytochrome} \approx \frac{1}{2}$, a calculated value of f may be obtained. The value of $\sigma_{cytochrome} \approx \frac{1}{2}$ may be a good estimate as pulse radiolyses studies on the reaction of hydrated electrons with ferricytochrome c give $f = 0.40$ after correction for electrostatic effects [32]. The value of f thus calculated is 0.7% and is in good agreement with the experimental f at high ionic strength but not with f at low ionic strength. It has been noted that this model is oversimplified [1]; however, it does have the advantage that it contains no adjustable parameters. The calculated f should also contain an electrostatic factor, f_{elec}, which for oppositely charged reactants is greater than 1. However, f_{elec} cannot be rigorously estimated. The effect of an f_{elec} greater than 1 would be to increase the calculated f. Some progress has been made in this regard [33] in a model that includes both monopole and dipole interactions.

The more sophisticated Schurr–Schmitz solid-angle diffusion model [8], which considered both translational and rotational diffusion, gives a theoretical $f = (1 - \cos\theta)\theta r_E/(r_E + r_{cytochrome})$, where θ is the minimum half-cone solid angle (in rad) necessary for reaction at a hemispherical site. While no estimate of θ is available, lacking the structure of cytochrome c oxidase, a value of θ can be estimated from the experimental f values of Table I, and they range from 5 to 21°. These are minimum values of θ as k_{min} is the minimum value of the association rate constant (Eqn. 7). Several other diffusion models have also been employed [1,11,16].

A comparison of the experimental f at low ionic strength indicates that in aqueous buffer ($\eta = 0.93$ mPa · s) the faster-reacting high-affinity site is largely (approx. 80%) diffusion controlled, while the low affinity site is largely chemical-activation controlled. For the low affinity site at a viscosity of about 7 mPa · s the contributions from diffusion and chemical activation control are about equal. It should be noted that these conclusions regarding the low affinity site depend upon the actual mechanism. At high ionic strength the contributions are equal at about 5 mPa · s. The viscosity in the intermembrane space is unknown. However, estimates of the viscosity of the cytoplasm of the cell range from 2 to 70 mPa · s depending upon the cell studied and the experimental method used [34]. Thus under physiological conditions these reactions may be significantly influenced by diffusion of cytochrome c through the intermembrane space. Since the diffusion-controlled rate of reaction is an upper limit, the degree to which it has been achieved is some measure of the evolution of the enzyme [16]. Thus under high viscosity physiological conditions any increase through evolution in the chemical activation rate constant would not result in a proportionate increase in the overall k_{min}.

In Table II experimental values of f and half-cone angles θ are compared for several protein reactions that exhibit evidence of diffusion control. A comparison of the half-cone angles of entry θ calculated [8] from the experimental values of f is more valid than a comparison of the experimental f values as the proteins and substrates vary considerably in size. On this basis the half-cone angle of entry θ for ferrocytochrome c to cytochrome c oxidase is, at high ionic strength, between that found for the carbonic anhydrase reac-

TABLE II

COMPARISON OF DIFFUSION-CONTROL PARAMETERS OF SOME PROTEIN REACTIONS

Reaction	f (exp) (%)	θ [a] (°)	Ref.
Ferricytochrome $c + e_{aq}^-$	40	114	32
Ferrimyoglobin + e_{aq}^-	17	86	32
Yeast alcohol dehydrogenase + NADH	3.3	48	38
Carbonic anhydrase + CO_2	1.4	40	16
Carbonic anhydrase + HCO_3^-	0.5	25	16
Cytochrome c oxidase + ferrocytochrome c [b]	0.48	21	this work
α-Hb chain + CO	0.3	23	11
β-Hb chain + CO	0.2	22	11
Cytochrome c oxidase + ferrocytochrome c [c]	0.09–0.2	12–16	this work
Mb + CO	0.13	18	10
Cytochrome c oxidase + ferrocytochrome c [d]	0.006–0.014	5–7	this work

[a] The half-cone angle of entry θ is calculated [8] from the experimentally determined values of f in the column to the left.
[b] High ionic strength, $I = 0.1$ M.
[c] Low ionic strength, $I = 0.028$ M, low-affinity site.
[d] Low ionic strength, $I = 0.028$ M, high-affinity site.

tions [16] and CO binding to the more open heme structures of the α and β chains of hemoglobin. However, the half-cone angle of entry θ for the high affinity site at low ionic strength is smaller even than that for the restricted entry of CO to Mb [10].

The possibility may also be considered that a weakly bound encounter complex (E · · · S) is formed prior to transformation to the enzyme-substrate complex ES.

$$E + S \underset{k_{-D}}{\overset{k_D}{\rightleftharpoons}} E \cdots S \underset{k_{-tr}}{\overset{k_{tr}}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where k_D and k_{-D} are pure diffusion-controlled association and dissociation rate constants, respectively [7,12,35]. From Eqn. 7 [35] we obtain

$$k_{min}^{-1} = \frac{1}{k_D} + \frac{k_{-D}}{k_D k_{tr}} + \frac{k_{-D} k_{-tr}}{k_D k_{tr} k_2} \tag{10}$$

All of the viscosity dependence will, as in Eqn. 6, be found in the $1/k_D$ term, as the viscosity dependence of k_{-D}/k_D in the remaining terms cancels out [35].

Eqn. 10 is of the same form as Eqn. 6, but with

$$k_C^{-1} = \frac{k_{-D}}{k_D k_{tr}} + \frac{k_{-D} k_{-tr}}{k_D k_{tr} k_2} \tag{11}$$

The viscosity dependence of Eqns. 6 and 10 are identical and thus the experimental f values and half-cone angles θ remain unchanged in Tables I and II. However, the intercept at a viscosity of 0 mPa · s is a composite quantity (Eqn. 11) and can be given no simple interpretation in the absence of the knowledge of the individual rate constants. Though it may be a reasonable postulate it should, however, be noted that there is no experimental evidence for the existence for the existence of an encounter complex between cytochrome c and cytochrome c oxidase.

Diffusion-controlled reactions of large molecules have not been well studied. The reaction rate of disulfide anion radicals produced by pulse radiolysis on the surface of proteins ranged from 2 to 50% of the diffusion-controlled rate for 13 different proteins [36]. It was concluded from this study that rotation of the proteins within an encounter complex brought the reactive groups into contact. The binding of *lac* repressor protein to operator DNA proceeds at about 10% of the diffusion-controlled rate [37] and this has been interpreted in terms of a mechanism involving one-dimensional sliding [1] to the operator binding site.

In summary, the oxidation of ferrocytochrome c by cytochrome c oxidase has been shown to be viscosity dependent at both high and low ionic strengths. It would thus appear that any translational movement of cytochrome c in the inter-

membrane space during mitochondrial electron transport between its membrane-bound redox partners would be subject to diffusion effects.

Acknowledgement

The financial support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

References

1 Berg, O.G. and Von Hippel, P.H. (1985) Annu. Rev. Biophys. Chem. 14, 131–160
2 Veerman, E.C.I., Wilms, J., Dekker, H.L. Muijsers, A.O., Van Buuren, K.J.H., Van Gelder, B.F., Osheroff, N., Speck, S.H. and Margoliash, E. (1983) J. Biol. Chem. 258, 5739–5745
3 Osheroff, N., Speck, S.H., Margoliash, E., Veerman, E.C.I., Wilms, J., König, B.W. and Muijsers, A.O. (1983) J. Biol. Chem. 258, 5731–5738
4 Antalis, T.M. and Palmer, G. (1982) J. Biol. Chem. 257, 6194–6206
5 Yonetani, T. and Ray, G.S. (1965) J. Biol. Chem. 240, 3392–3398
6 Swanson, M., Speck, S.H., Koppenol, W.H. and Margoliash, E. (1982) in Electron Transport and Oxygen Utilization (Chien Ho, ed.), pp. 51–56, Elsevier/North-Holland, Amsterdam
7 Hasinoff, B.B. (1981) Arch. Biochem. Biophys. 211, 396–402
8 Schurr, J.M. and Schmitz, K.S. (1976) J. Phys. Chem. 80, 1934–1936
9 Noyes, R.M. (1961) Prog. React. Kinet. 1, 129–160
10 Hasinoff, B.B. and Chrishti, S.B. (1982) Biochemistry 21, 4275–4278
11 Hasinoff, B.B. and Chrishti, S.B. (1983) Biochemistry 22, 58–61
12 Eigen, M. and Hammes, G.G. (1963) Adv. Enzymol. 25, 1–38
13 Von Smoluchowski, M. (1917) Z. Phys. Chem. Stoechiom. Verwandlschaftsl. 92, 129–168
14 Evans, D.F., Tominaga, T. and Chan, C. (1979) J. Soln. Chem. 8, 461–478
15 Evans, D.F., Tominaga, T. and Davis, H.T. (1981) J. Chem. Phys. 74, 1298–1305
16 Hasinoff, B.B. (1984) Arch. Biochem. Biophys. 233, 676–687
17 Wilms, J., Dekker, H.L., Boelens, R. and Van Gelder, B.F. (1981) Biochim. Biophys. Acta 637, 168–176
18 Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688
19 Margoliash, E. (1954) Biochem. J. 56, 535–543
20 Phillips, H.O., Marcinkowsky, A.E., Sachs, S.B. and Kraus, K.A. (1977) J. Phys. Chem. 81, 679–682
21 Smith, L., Davies, H.C. and Nava, M.E. (1979) Biochemistry 18, 3140–3146
22 Teh, H.C., Ong, G.H., Ng, S.C. and Gan, L.M. (1985) J. Disp. Sci. Technol. 6, 255–262
23 IBM SHARE Library (1964) Program no. SDA 3094
24 Margoliash, E. and Listgarten, J. (1962) J. Biol. Chem. 237, 3397–3405
25 Saraste, M., Penttilä, T. and Wikström, M. (1981) Eur. J. Biochem. 115, 261–268
26 Wherland, S. and Gray, H.B. (1977) in Biological Aspects of Inorganic Chemistry (Addison, A.W., Cullen, W.R., Dolphin, D. and James, B.R., eds.), pp. 289–368, John Wiley, New York
27 Errede, B. and Kamen, M.D. (1978) Biochemistry 17, 1015–1027
28 Ferguson-Miller, S., Brautigan, D.L., Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115
29 Speck, S.H., Dye, D. and Margoliash, E. (1984) Proc. Natl. Acad. Sci., USA 81, 347–351
30 Sinjorgo, K.M.C., Meijling, J.H. and Muijsers, A.O. (1984) Biochim. Biophys. Acta 767, 48–56
31 Margoliash, E. and Bosshard, H.R. (1983) TIBS 8, 316–320
32 Hasinoff, B.B., Licht, A. and Pecht, I. (1984) Biochim. Biophys. Acta 767, 627–634
33 Van Leeuwen, J.W. (1983) Biochim. Biophys. Acta 743, 408–421
34 Wojcieszyn, J.W., Schlegel, R.A., Wu, F.-S. and Jacobson, K.A. (1981) Proc. Natl. Acad. Sci. USA 78, 4407–4410
35 Hammes, G.G. (1978) in Principles of Chemical Kinetics, p. 222, Academic Press, New York
36 Sommer, J., Jonah, C., Fukuda, R. and Bersohn, R. (1982) J. Mol. Biol. 159, 721–744
37 Riggs, A.D., Bourgeois, S. and Cohn, M. (1970) J. Mol. Biol. 53, 401–417
38 Hasinoff, B.B., Dreher, R. and Davey, J.P. (1987) Biochim. Biophys. Acta 911, 53–58