Mechanism of Yeast Cytochrome b_2 Action. I. Thermodynamics and Relaxation Kinetics of the Interaction between Cytochrome b_2 and Oxalate†

Darwin Thusius,* Bernadette Blazy, and Alain Baudras

ABSTRACT: Oxalate is the strongest known inhibitor of yeast cytochrome b_2 activity. We have used spectrophotometric titration, temperature-jump relaxation, and calorimetry in an investigation of the interaction between enzyme and inhibitor. The titration data are consistent with noncooperative binding to one site per subunit. This conclusion is corroborated by temperature-jump results which reveal a single relaxation phenomenon which obeys second-order kinetics. Further evidence for a simple binding reaction comes from the observation that the reaction enthalpy estimated from relaxation amplitudes is in good agreement with the value obtained directly with batch calorimetry. The forward and reverse rate constants evaluated from the temperature-jump experiments are, respectively, $1 \times 10^4\ M^{-1}\ \text{sec}^{-1}$ and $15\ \text{sec}^{-1}$. Although considerably smaller than a diffusion-controlled value, the forward rate constant is characterized by an unusually small activation energy of ~3 kcal/mol. This, together with a large unfavorable association activation entropy of −30 eu, suggests that oxalate diffuses freely to the active site, but only a small fraction of the collisions are productive due to severe steric requirements.

Yeast cytochrome b_2 catalyzes the oxidation of L-lactate and other α-hydroxy acids by various one- and two-electron acceptors, including ferricyanide, cytochrome *c*, thionine, and molecular oxygen. The in vivo reaction is believed to involve the transfer of electrons from L-lactate to cytochrome *c* (Pajot and Claisse, 1974). Cytochrome b_2 is a structurally unique protein in that it possesses an equal number of heme and flavine mononucleotide prosthetic groups which can both participate in the catalytic electron transfer mechanism (Morton et al., 1961; Hasegawa and Ogura, 1961; Baudras, 1965). It is therefore believed that cytochrome b_2 will exhibit special mechanistic features found in no other enzymatic system (Morton and Sturtevant, 1964).

Investigations devoted to elucidating the mechanism of cytochrome b_2 mediated electron transfer have included steady-state kinetics of the catalytic reaction at different substrate, product, and acceptor concentrations (Hinkson and Mahler, 1963; Hasegawa and Ogura, 1961), stopped-flow kinetics of enzyme reduction by lactate in the absence of acceptors (Morton and Sturtevant, 1964; Iwatsubo et al., 1968; Capeillère-Blandin et al., 1975), and stopped-flow kinetics of enzyme reoxidation by acceptor molecules (Morton and Sturtevant, 1964; Suzuki and Ogura, 1970). Although it is now generally acknowledged that the flow of electrons in the absence of acceptors follows the path L-lactate → flavine mononucleotide → heme (Morton and Sturtevant, 1964; Suzuki and Ogura, 1970; Capeillère, 1974), differences of opinion exist concerning the mechanism of reoxidation of the reduced enzyme (Morton et al., 1961; Hasegawa and Ogura, 1961; Hinkson and Mahler, 1963; Nicholls, 1966; Suzuki and Ogura, 1970; Forestier and Baudras, 1971). Other discrepancies concern the values of the elementary rate and equilibrium parameters. Thus, two independent kinetic studies are consistent with the rather small second-order rate constant of $1 \times 10^5\ M^{-1}\ \text{sec}^{-1}$ for formation of the Michaelis complex between L-lactate and oxidized cytochrome b_2 (Hinkson and Mahler, 1963; Iwatsubo and Capeillère, 1967), whereas a third investigation indicates a lower limit of $10^6\ M^{-1}\ \text{sec}^{-1}$ (Morton and Sturtevant, 1964). In addition, the conclusion that the elementary equilibrium constant for dissociation of the Michaelis complex is much smaller than the steady-state Michaelis constant K_m (Iwatsubo and Capeillère, 1967) is at variance with results indicating that these two parameters are nearly equal (Hinkson and Mahler, 1963).

The essentially irreversible nature of lactate oxidation at neutral pH limits rapid kinetic measurements to flow methods, which cannot detect processes with half-lives shorter than several milliseconds. In the present study we investigate the interaction between oxidized cytochrome b_2 and oxalate—the strongest known inhibitor of cytochrome b_2 activity. Oxalate binding is completely reversible, allowing the extension of rate measurements to the microsecond region with the temperature-jump relaxation technique. The relaxation experiments are complemented with spectrophotometric and calorimetric titrations. These results together with steady-state rate measurements reported in the companion paper (Blazy et al., 1976) lead to the characterization of some of the elementary kinetic steps involved in cytochrome b_2 catalysis.

Experimental Procedure

Materials. Cytochrome b_2 was prepared by the method of Appleby and Morton (1954, 1959), modified by Spyridakis et al. (1971). The DNA associated with the enzyme was eliminated by the method of Symons (1965). The reduced cytochrome b_2 so obtained can be stored as a precipitate in a 65% anaerobic ammonium sulfate solution at pH 7 for several months without any detectable change in activity.

† From the Laboratoire d'Enzymologie Physicochimique et Moléculaire, Université de Paris-Sud, 91405 Orsay, France (D.T.), and the Centre de Recherche de Biochimie et de Génétique Cellulaires C.N.R.S., 31077 Toulouse-Cedex, France. *Received July 8, 1975.* This work received support from the C.N.R.S. (Laboratory of Professor J. Yon; Grant No. 13) and the D.G.R.S.T. (Laboratory of Professor A. Baudras; Grant No. 72-7-0385).

Before use, lactate and ammonium sulfate were removed by passing stock enzyme solution through a Sephadex G-25 column equilibrated with phosphate buffer.

The enzyme concentration (moles of heme/liter) was determined spectrophotometrically by assuming molar extinction coefficients ϵ_{413} = 129 000 for the oxidized form and ϵ_{423} = 183 000 for the reduced form (Pajot and Groudinsky, 1970). The activity (moles of ferricyanide reduced per second per mole of heme) determined under standard conditions (0.2 *M* phosphate, 1.5 m*M* ferricyanide, 66 m*M* DL-lactate, 25°C) was 175 sec^{-1}, and varied by no more than ±10 sec^{-1} for different preparations.

L-Sodium lactate (Sigma grade L-1) concentration was determined enzymatically with cytochrome b_2 in the presence of an excess of ferricyanide. An extinction coefficient of ϵ_{420} = 1040 M^{-1} cm^{-1} was assumed for ferricyanide.

Oxalate solutions were prepared from the sodium salt (Merck A.R.) and buffer from disodium and monopotassium phosphate (Prolabo PR).

Kinetics. Temperature-jump experiments were carried out with a Messanlagen single beam temperature-jump spectrometer equipped with a 75-W tungsten lamp.

The volume and optical path length of the sample cell were 1.5 ml and 0.7 cm. The factor $\delta T/\delta A$ (where T = temperature, A = absorbance) for a solution of phenol red in 0.1 *M* Tris-HCl buffer (pH 7.9) was determined with a Cary 14 spectrophotometer. The same dye solution was then used to estimate the temperature change corresponding to a given discharge voltage of the temperature-jump machine.

Relaxation times for the binding of oxalate to cytochrome b_2 were determined by adding small volumes of a concentrated sodium oxalate solution to a buffered solution of enzyme in the temperature-jump cell; at each oxalate concentration photographs were taken of three acceptable oscillograms. Ten temperature jumps on a 2×10^{-5} *M* oxalate-free enzyme solution at 10°C in the absence of light had no effect on the catalytic activity. Exposure of the same solution to light (480 nm) for 20 min resulted in a 10% loss in activity. To avoid photolysis, reaction solutions were exposed to light only during the ~15-sec periods necessary to balance the instrument signal output before the thermal perturbation. The loss in enzyme activity after a completed series of temperature-jump experiments in the presence of oxalate was typically less than 10–15%. Relaxation times and amplitudes were evaluated graphically from the slopes and intercepts of first-order plots:

$$\ln \delta I_t = -(1/\tau)t + \ln \delta I^0$$

Here δI_t is the instantaneous deviation of the signal (in millivolts) from its final equilibrium value, I^0 is the static signal at t = 0, and δI^0 is the overall signal change. The above relation is only valid for small signal changes ($\delta I \ll I^0$), where $\delta A^0 = \delta I^0/2.3I^0$.

Equilibrium Measurements. Titrations of cytochrome b_2 by oxalate were achieved with a Cary Model 14 spectrophotometer equipped with a slidewire for the 0–0.1 absorbance range. In order to obtain accurate difference spectra the sample and reference cell were not removed from their holders during the titration. Small volumes of stock oxalate solution were added to the sample cell and an equivalent volume of buffer was added to the reference cell containing an identical solution of enzyme. After stirring with platinum wires (which remained in the cells) the difference spectrum was scanned. To correct for small drifts between scans, the baseline was adjusted at 600 nm, where the change in optical density is negligible (Figure 1). Due to the large absorbance of the enzyme solutions and the narrow peaks of the difference spectrum, care was taken to scan slowly enough to allow equilibration of the spectrophotometer slit mechanism. We routinely verified that no significant change in enzyme activity occurred during the titrations.

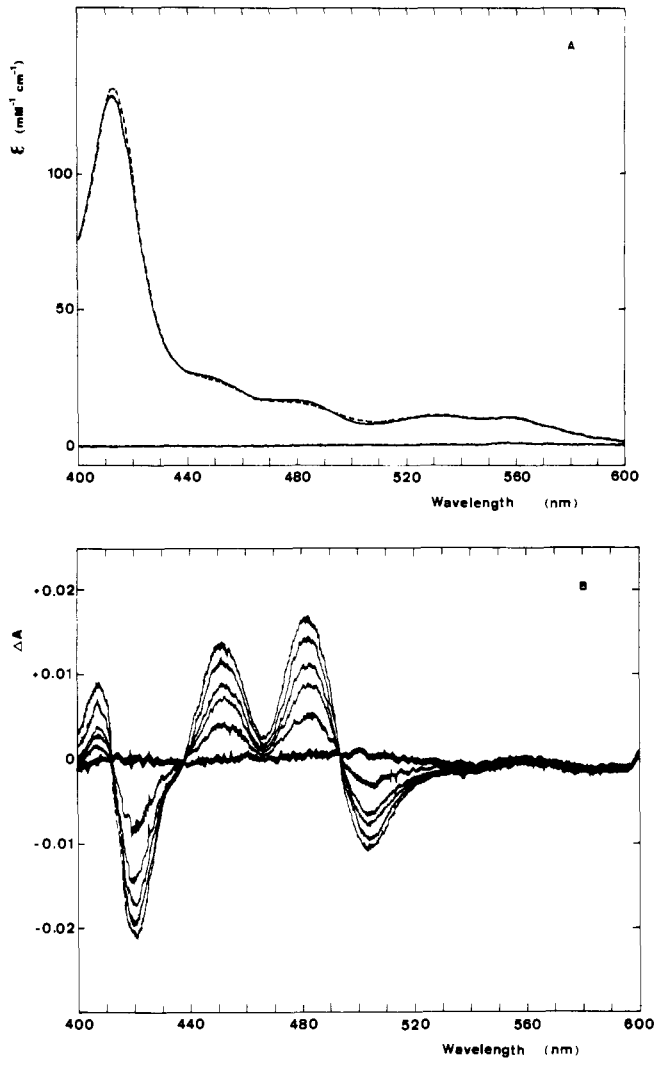

FIGURE 1: (A) Perturbation of cytochrome b_2 absorption spectrum by oxalate. The 482-nm region corresponds to the absorption of FMN; at 452 nm both heme and flavine absorb and at 408 nm heme only absorbs. The oxidized form of the enzyme is dissolved in buffer alone (0.2 *M* phosphate, 1 m*M* EDTA, pH 7.2, 20°C; dashed line) or in the presence of 50 m*M* oxalate (same buffer; solid line). (B) Difference spectrum between free oxidized enzyme and its complex with oxalate. Same conditions as in A. Progressive additions of oxalate to the sample cell (l = 1 cm) and of buffer to the reference cell; both contain 14.7 μM ferricytochrome b_2.

Calorimetric Measurements. Reaction heats for oxalate binding to cytochrome b_2 were determined with an LKB 10700-2 batch calorimeter in the differential mode. We verified that the thermopiles of the sample and reference cells had identical responses by demonstrating no signal change when mixing 4 ml of buffer with 2 ml of oxalate in each cell.

The enthalpy change was measured at 20 m*M* oxalate. Small corrections were necessary to account for the enzyme heat of dilution and the fact that the binding sites were not entirely saturated.

Table I: Spectrophotometric Titrations of Different Cytochrome b_2 Preparations by Oxalate.

Enzyme Preparation[a]	nC_E° (μM)	C_L° (mM)	K^{-1}_{482nm} (mM)	K^{-1}_{452nm} (mM)	K^{-1}_{420nm} (mM)	K^{-1}_{350nm} (mM)	$\Delta\epsilon_{482nm}$ (M^{-1} cm^{-1})	$\Delta\epsilon_{452nm}$ (M^{-1} cm^{-1})	$\Delta\epsilon_{420nm}$ (M^{-1} cm^{-1})	$\Delta\epsilon_{350nm}$ (M^{-1} cm^{-1})
A	14.7	0.12–32.0	1.13 ±0.09	1.20 ±0.10	0.86 ±0.08		1460 ±30	1200 ±30	1570 ±40	
B	18.8	id.	1.33 ±0.03	1.29 ±0.07	1.08 ±0.08		1430 ±90	1160 ±20	1840 ±30	
A*	26.1	id.	1.63 ±0.05	1.48 ±0.05	1.59 ±0.09	1.58 ±0.07	1500 ±10	1200 ±10	1780 ±30	990 ±10
B*	24.0	id.	1.29 ±0.03	1.19 ±0.03	1.20 ±0.10	1.28 ±0.04	1510 ±90	1270 ±70	1080 ±30	800 ±10
C*[b]	21.9	0.49–29.0	4.30 ±0.20				1970 ±40			

[a] A and B, freshly prepared enzyme; A* and B*, the same preparations after 1 month storage as a precipitate in ammonium sulfate; C*, a third ammonium sulfate stored enzyme preparation; conditions, 0.2 M phosphate, 1 mM EDTA, pH 7.20, 20°C. [b] All conditions identical except pH is 7.93.

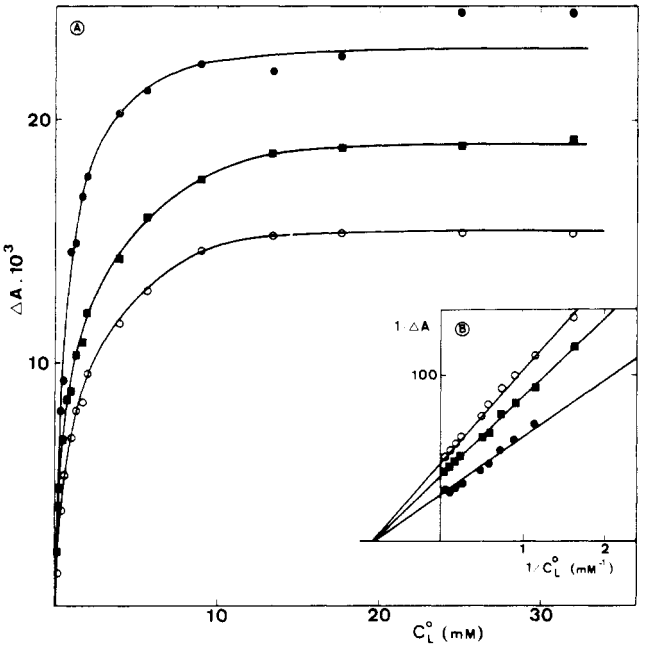

FIGURE 2: Titration of cytochrome b_2 by oxalate. The changes of absorbance at three selected wavelengths [λ 482 nm (■); λ 452 nm (○); λ 420 nm (●)] are plotted as a function of oxalate concentration (A) and in the form of a double-reciprocal plot (B).

Results

Thermodynamics. Each subunit of tetrameric cytochrome b_2 possesses a binding site for oxalate (Capeillère, 1974). In the simplest mode of ligand binding to a multisite macromolecule the binding loci are identical and independent. The equilibrium properties of such a system are characterized by a unique equilibrium constant defined as (Van Holde, 1971):

$$K = \bar{C}_B/\bar{C}_F\bar{C}_L \quad (1)$$

where $\bar{C}_F$ is the equilibrium concentration of free sites, $\bar{C}_B$ is the equilibrium concentration of occupied sites, and $\bar{C}_L$ is the equilibrium concentration of free ligand. Scheme I gives the relationships between the n elementary equilibrium constants and the intrinsic association constant for an arbitrary number of sites.

The individual constants are defined as $K_i = \bar{C}_{EL_i}/\bar{C}_{EL_{i-1}}\bar{C}_L$. Assuming the above model and the condition $\epsilon_L = 0$, the following hyperbolic concentration dependence is

Scheme I

$$\begin{aligned} E + L &\rightleftharpoons EL & K_1 &= (n)K \\ EL + L &\rightleftharpoons EL_2 & K_2 &= [(n-1)/2]K \\ EL_i + L &\rightleftharpoons EL_{i+1} & K_i &= [(n-i+1)/i]K \\ EL_{n-1} + L &\rightleftharpoons EL_n & K_n &= (1/n)K \end{aligned}$$

predicted for the spectrophotometric titration of macromolecule by ligand:

$$\Delta A/C_E^0 = n\Delta\epsilon/(1 + K/\bar{C}_L)$$

$$\Delta\epsilon = \epsilon_B - \epsilon_F \quad (2)$$

where ϵ_F and ϵ_B are the intrinsic molar extinction coefficients of free and occupied sites, respectively. The superscript 0 denotes total molar concentration.

The visible absorption spectrum of ferricytochrome b_2 and the spectrum observed at a saturating concentration of oxalate are given in Figure 1A. The binding of ligand results in small (≤3%) perturbations of both the heme and flavine absorption bands. Difference spectra obtained in a titration of enzyme by oxalate are presented in Figure 1B. The clean isosbestics in a wide range of ligand concentration strongly suggest identical binding sites. Nonidentical sites would imply more than two intrinsic extinction coefficients, resulting in deviations from the behavior of Figure 1B.

Changes in absorbance as a function of total ligand concentration at three different wavelengths are presented in Figure 2. The hyperbolic behavior is consistent with independent binding sites. This conclusion was tested quantitatively by fitting the titration data to eq 2 with an iterative procedure which found the "best" values of K and $n\Delta\epsilon$ satisfying the usual least-squares criterion. The input to the program was the raw data $\Delta A/C_E^0$ vs. C_L^0 and an initial K value obtained from total ligand concentration at 50% saturation. In each iteration free ligand concentration $\bar{C}_L$ was calculated from the total concentration C_L^0 using the equilibrium constant of the previous iteration according to eq 3,

$$\bar{C}_L = C_L^0 - \bar{C}_B = C_L^0 - S[1 - (1 - 4P/S^2)^{1/2}]/2 \quad (3)$$

where $S = K^{-1} + nC_E^0 + C_L^0$ and $P = nC_E^0C_L^0$. It was assumed that $n = 4$, as concluded by Capeillère (1974) working at a lower ionic strength than used here. Since the absolute error in ΔA was reasonably constant, weighting the data was unnecessary. Table I presents the results of the curve-fitting procedure for two different enzyme prepara-

Table II: Calorimetric Reaction Heats.

Temp (°C)	$\Delta H°$ (kcal/mol)	No. of Runs
12.0	−5.3 ± 0.3	2
16.2	−5.2 ± 0.1	1
20.5	−5.2 ± 0.5	2
23.5	−6.5 ± 0.4	2

tions. The small standard errors associated with K and $n\Delta\epsilon$ indicate that the titration data are in very good agreement with binding to identical and noninteracting sites.

Although differences between equilibrium constants determined at different wavelengths are evident, as well as differences between parameters for independent enzyme preparations, the largest discrepancies are not much larger than estimated 90% confidence intervals (i.e., twice the standard errors given in Table I). Only freshly prepared enzyme was used in the calorimetric and kinetic experiments described below.

It will be noted that increasing the pH from 7.2 to 8.0 increases the dissociation equilibrium constant by a factor of three. From the $\Delta\epsilon_{482}$ value at the higher pH we may conclude that the structural change responsible for the decreased affinity modifies the difference spectrum at this wavelength by about 25% compared to pH 7.2.

In the above titrations $C_L{}^0$ was at least five times larger than $nC_E{}^0$. Therefore, to a good approximation $C_L{}^0 \simeq \bar{C}_L$. We may then graphically examine the fit of the data to eq 2 with the familiar linear representation of $1/\Delta A$ vs. $1/C_L{}^0$

$$1/\Delta A = 1/n\Delta\epsilon C_E{}^0 + (K/n\Delta\epsilon C_E{}^0)/C_L{}^0 \qquad (4)$$

(eq 4). The straight lines of Figure 2B were calculated with the parameters obtained in the iterative numerical analysis. Only those points are included which satisfy the condition $C_L{}^0 > 10(nC_E{}^0)$. Although providing a convenient visual confirmation of noncooperative binding, we recall that fitting titration data to the nonlinear expression of eq 2 is preferable to a least-squares analysis of the linearized form due to the problem of correctly weighting data for double reciprocal plots (Cleland, 1967).

We have measured the enthalpy change for oxalate binding to cytochrome b_2 directly with a microcalorimeter. Table II gives the results of these experiments at four different temperatures. Taking 1 mM for the intrinsic dissociation constant at 20°C, we calculate −5 ± 1.5 eu for the intrinsic reaction entropy at this temperature. In relating the calorimetric results to a multistep model, it must be borne in mind that the enthalpies are identical for all steps, but the reaction entropies depend on the elementary equilibrium constants, K_i. For $n = 4$, we calculate $\Delta S°_1 = -2 \pm 2$ eu, $\Delta S°_2 = -4 \pm 2$ eu, $\Delta S°_3 = -5 \pm 2$ eu, and $\Delta S°_4 = -8 \pm 2$ eu. A nonnegligible heat capacity is suggested at the higher temperatures, but the effect was not further investigated.

Kinetics. The temperature perturbation of solutions of ferricytochrome b_2 results in an absorbance change whose half-life is at least as short as the resolution time of our temperature-jump instrument (~5 μsec). Although this signal change could arise from a structural transition of the enzyme, it may simply represent a direct effect of temperature on the flavine and heme extinction coefficients.

An unresolvable fast phase persists in the presence of oxalate, but in addition a decay is observed in the millisecond region. First-order plots were consistent with a single relaxation time. It proved convenient to study the slow relaxation

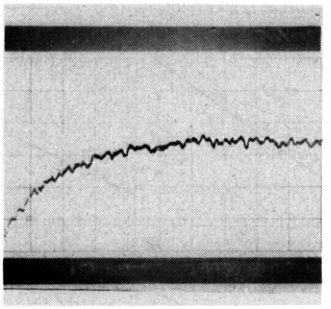

FIGURE 3: Temperature-jump oscillogram: vertical axis, 1 mV per division; horizontal axis, 20 msec per division; initial signal, 1.16 V; machine time constant, 1 msec; ligand concentration, 2.07 mM. In all experiments the temperature perturbation was 10.0(±0.5)°C.

time at 452 nm, where the amplitude of the fast effect is practically negligible and the oxalate difference spectrum exhibits a maximum (Figure 1B). It is noteworthy that even though the extinction coefficients of free enzyme and complex differ by only ~3%, the precision of our temperature-jump technique was such that reasonably accurate decay curves (Figure 3) could be obtained over a wide range of ligand concentration.

The simplest microscopic mechanism for multiple ligand binding is the stoichiometry of Scheme I. In the limit of identical and noninteracting sites, the kinetics can be defined in terms of an intrinsic association rate constant k_a and an intrinsic dissociation rate constant k_d. The specific rate constants of the elementary steps are related to the intrinsic parameters by simple statistical factors determined by the number of unoccupied binding sites per macromolecule. For $n = 4$, we have the reactions shown in Scheme II.

Near equilibrium a four-step reaction will in general give rise to four kinetic phases, or for absorbance measurements:

$$\delta A = \sum_{i=1}^{4} \delta A_i{}^0 e^{-t/\tau_i} \qquad (5)$$

where the τ_i and $\delta A_i{}^0$ are relaxation times and amplitudes, respectively. Assuming the above restrictions on the rate constants, it can be shown that the inverse relaxation times differ by integers (Eigen, 1967):

$$1/\tau_i = i[k_a(\bar{C}_E + \bar{C}_L) + k_d] \qquad (6)$$

where i runs from 1 to 4. The relaxation amplitudes may be expressed as shown in eq 7 (Thusius, 1972, 1975;. Jovin, 1976), where $\Delta\epsilon^*$ and ΔH^* are normal parameters which are linear combinations of the actual extinction coefficients and enthalpy changes. The normal gamma factor Γ_i^* gives

$$\delta A_i{}^0 = \Delta\epsilon_i^*(\Delta H_i^* \delta T/RT^2)\Gamma_i^* \qquad (7)$$

the concentration dependence of the ith amplitude. It has been shown (Eigen, 1967) that noncooperative binding results in all normal reaction heats being identically equal to zero except ΔH_1^*. Therefore only one relaxation phase is experimentally detectable. With ligand in large excess, it follows from eq 6 that the observed inverse relaxation time is as shown in eq 8. The ratio between the experimental forward and reverse rate constants $K = k_a/k_d$ is the intrinsic equilibrium constant of eq 1. Figure 4 demonstrates that over a tenfold change in oxalate concentration and at four different temperatures the reciprocal relaxation times are in satisfactory agreement with eq 8. Values of the intrinsic association and dissociation rate constants are listed in Table III together with the kinetic equilibrium constants.

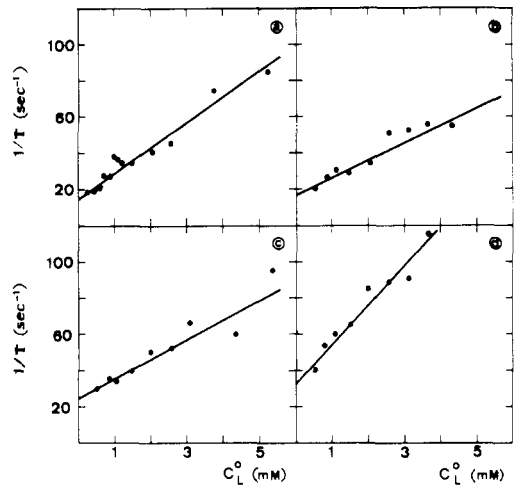

FIGURE 4: Reciprocal relaxation times. The straight lines are best least-squares fits of data to eq 8. Final temperature; (a) 21°C; (b) 25°C; (c) 30°C; (d) 37°C. Cytochrome b_2 concentration: 30 μM.

$$1/\tau = k_a C_L^0 + k_d \tag{8}$$

In an effort to establish whether kinetic phenomena exist beyond the slow time limit of our temperature-jump technique we rapidly mixed oxalate with oxidized cytochrome b_2 in a stopped-flow machine and followed the absorbance at 350 or 482 nm.[1] With [oxalate] = 1×10^{-2} M and [enzyme] = 2×10^{-5} M no change in absorbance could be detected up to 10 sec, indicating that the binding reaction is essentially over within the mixing time of the flow machine.

The temperature dependence of the association rate constant k_d was described by a linear Arrhenius plot; from the slope we calculate 9 ± 0.5 kcal/mol for the intrinsic dissociation activation enthalpy $\Delta H_d^{\ddagger}$. This value together with k_d determined at 21°C gives an intrinsic activation entropy $\Delta S_d^{\ddagger}$ of −23 ± 2 eu. The small temperature dependence of k_a precluded an accurate graphical estimation of the association activation enthalpy. On the other hand, this parameter can be evaluated from the overall enthalpy change obtained in our calorimetric experiments using the relation $\Delta H_a^{\ddagger} = \Delta H_d^{\ddagger} + \Delta H^{\circ}$. A reasonable estimate of ΔH° at 21°C is −6 ± 0.5 kcal/mol (Tables II and III), yielding $\Delta H_a^{\ddagger} \sim 3$ kcal/mol at this temperature. The corresponding activation entropy is $\Delta S_a^{\ddagger} = \Delta S_d^{\ddagger} + \Delta S^{\circ} \sim -28$ eu. The relaxation amplitude results given below suggest that ΔH° may increase by 1–2 kcal/mol in going from 20 to 37°C, which is the temperature range of the kinetic experiments, implying a yet smaller $\Delta H_a^{\ddagger}$ and more negative $\Delta S_a^{\ddagger}$ at higher temperatures.

In a temperature-jump technique employing spectrophotometric detection, relaxation amplitudes are functions of reaction enthalpies and extinction coefficients. If ligand is in large excess the amplitude for the reaction of Scheme I is given by eq 9, where ΔH° is the intrinsic reaction enthalpy,

$$\delta A^0 = nl\Delta\epsilon \left(\frac{\Delta H^{\circ}}{RT^2}\delta T\right)\left[\frac{C_E^0}{(1 + C_L^0 K)(1 + 1/C_L^0 K)}\right] \tag{9}$$

[1] We thank Dr. Iwatsubo for help in carrying out the stopped-flow experiments in his laboratory.

Table III: Oxalate Binding to Cytochrome b_2; Kinetic Results at pH 7.2.

T (°C)	$k_a \times 10^3$ (M^{-1} sec^{-1})	k_d (sec^{-1})	K^{-1} (mM)	$\Delta H^{\circ c}$ (kcal/mol)
21.0	14.3 ± 1.4	14 ± 1.5	1.0	−6.5
25.0	13.0 ± 1.4	18 ± 2	1.4	−8.3
25.0	10.2 ± 1.1[a]	16 ± 2[a]	1.6[a]	−8.1[a]
26.0	3.1 ± 0.2[b]	22 ± 0.7[b]	7.0[b]	−17[b]
30.0	10.8 ± 1.3	24 ± 3	2.2	−8.5
37.0	22 ± 2	32 ± 3	1.5	−11

[a] Different enzyme preparation than employed in other runs. [b] pH 7.98. [c] Calculated from amplitude expression of eq 9 assuming $n = 4$ and $\Delta\epsilon = 511\ M^{-1}\ cm^{-1}$.

δT is the temperature change, and l is the optical path length.

Equation 9 predicts an asymmetric bell-shaped curve for a plot of $\delta A^0/C_E^0$ vs. C_L^0. Figure 5 shows that the experimental amplitudes do in fact conform to this behavior. As pointed out elsewhere (Winkler, 1969; Thusius et al., 1973; Thusius, 1973), the C_L^0 value corresponding to the maximum of such a curve is equal to the dissociation equilibrium constant K^{-1}. In the present work estimates of K^{-1} obtained in this way were used to prime an iterative least-squares program which employs all data points to find the best statistical value of the equilibrium constant and the quantity $n\Delta\epsilon(\Delta H^{\circ}/RT^2)\delta T$ (Thusius, 1973). These parameters were then used to calculate the theoretical curves of Figure 5.

To calculate the association enthalpy from the relaxation amplitudes we need an estimate of $n\Delta\epsilon$ in eq 9. Actually this quantity is only an apparent value which is a function of the bandwidth of the temperature-jump optical system. In order to assure high signal-to-noise ratios we worked with the largest slits of our Bosch and Lomb monochromator, resulting in a bandwidth of 20 nm at half-peak height. This is comparable to the width of the 452-nm band of the enzyme–oxalate difference spectrum.

We estimated the effective extinction change of eq 9 in the following way. The mean absorbance change $\Delta\bar{A}$ at wavelength λ_0 measured with a spectrophotometric system having a bandwidth $\Delta\lambda$ is:[2]

$$\Delta\bar{A} = \int_{\lambda_0 - \Delta\lambda}^{\lambda_0+\Delta\lambda} S_M(\lambda)S_L(\lambda) \times \Delta A(\lambda)d\lambda \Big/ \int_{\lambda_0 - \Delta\lambda}^{\lambda_0+\Delta\lambda} S_M(\lambda)S_L(\lambda)d\lambda$$

where $\Delta\bar{A}$ is related to the desired extinction change by $\Delta\bar{A} = e^{-2.3l\Delta\epsilon}$, ΔA is the absorbance measured with an infinitely small bandwidth, $S_M(\lambda)$ is a monochromator band-pass function, and $S_L(\lambda)$ is a spectral function of light source and photodetector. To a good first approximation $S_L(\lambda)$ may be considered wavelength independent in our temperature-jump experiments (tungsten lamp; $\lambda_0 = 452$ nm). If we approximate $S_M(\lambda)$ as a triangular function of λ centered at λ_0 the above expression becomes:

$$\Delta\bar{A} = [1/(\Delta\lambda)^2]\int_{\lambda_0-\Delta\lambda}^{\lambda_0} [\Delta\lambda - (\lambda_0 - \lambda)]e^{-2.3l\Delta\epsilon'}d\lambda + [1/(\Delta\lambda)^2]\int_{\lambda_0}^{\lambda_0+\Delta\lambda} [\Delta\lambda + (\lambda_0 - \lambda)]e^{-2.3l\Delta\epsilon'}d\lambda$$

[2] This formula was suggested by Carl Roland Rabl.

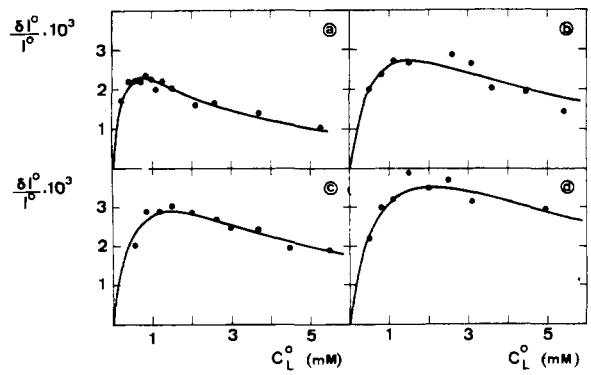

FIGURE 5: Relaxation amplitudes. The curve represents the best nonlinear least-squares fit of the data to eq 9. Conditions are as described in Figure 4.

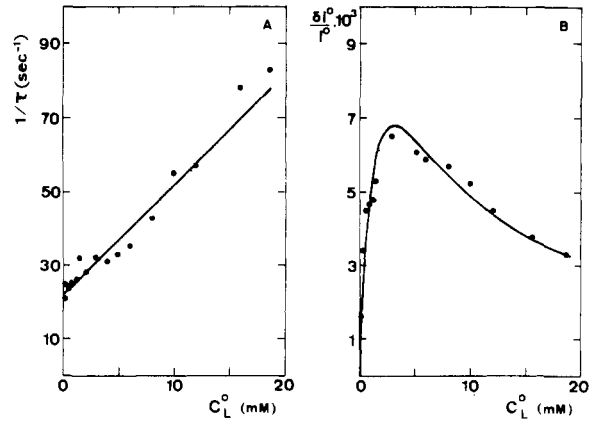

FIGURE 6: Reciprocal relaxation times and relaxation amplitudes at pH 7.93; final temperature, 26°C; enzyme concentration, 30.0 μM.

where $\Delta\epsilon'$ is the true extinction change measured in the titrations at a narrow (0.08 nm) bandwidth. Extinction changes 2 nm apart were taken over a range of 20 nm on either side of 452 nm (Figure 1B) and the above integrals were evaluated numerically with a programmed electronic calculator. From $\Delta\bar{A}$ we estimate 511 M^{-1} cm^{-1} for the apparent extinction change, which is 40% of the true value. Reaction enthalpies obtained from the temperature-jump studies are included in Table III.

In principle the apparent $\Delta\epsilon$ could be determined directly by measuring the difference in absorbance produced on adding a saturating amount of oxalate to cytochrome b_2 in the temperature-jump cell. However, we found an accurate determination unfeasible due to difficulties in thoroughly mixing the reactants and assuring complete diffusion into the "dead space" of the microcell without removing the latter from the cell holder.

The effect of pH on the kinetic and thermodynamic parameters was examined by carrying out a series of temperature-jump experiments at pH 7.9 (Figure 6). Increasing the pH from 7.2 to 7.9 decreases the association rate constant k_a by a factor of three and increases the dissociation rate constant k_d by only ~40%. Thus, the decrease in affinity with increased pH noted in the titration experiments of Table I arises nearly entirely from the pH dependence of k_a. At 25°C the intrinsic thermodynamic parameters derived from the relaxation amplitudes vary from $\Delta H° = -8$ kcal/mol and $\Delta S° = -13$ eu at pH 7.2 to $\Delta H° = -17$ kcal/mol and $\Delta S° = -47$ eu at pH 7.9. In light of this pH dependence the reaction enthalpies and entropies are only overall values containing contributions from one or more protolytic reactions.

Discussion

Iwatsubo and Capeillère (1967) have obtained an indirect estimate of the cytochrome b_2–oxalate binding constant by following rates of enzyme denaturation in urea. Their experiments gave $K^{-1} = 1.4$ mM at 25°C and pH 7.6, in reasonable agreement with our direct determination by spectrophotometric titration at 20°C and pH 7.2. The titration data are consistent with noncooperative binding, indicating that the sites for oxalate are identical and isolated from one another on the enzyme surface.

Calorimetry gives $\Delta H° = -5.3$ (±0.3) kcal/mol for the intrinsic reaction heat at 12–20°C. Oxalate binding is therefore enthalpy driven. The intrinsic entropy change is "normal": $\Delta S° = -5$ (±1.5) eu.

Scheme II

$$E + L \underset{k_d}{\overset{4k_a}{\rightleftharpoons}} EL$$

$$EL + L \underset{2k_d}{\overset{3k_a}{\rightleftharpoons}} EL_2$$

$$EL_2 + L \underset{3k_d}{\overset{2k_a}{\rightleftharpoons}} EL_3$$

$$EL_3 + L \underset{4k_d}{\overset{k_a}{\rightleftharpoons}} EL_4$$

Our temperature-jump results confirm the site independence implied in the equilibrium experiments. A single relaxation time is observed whose concentration dependence conforms to that expected for a simple bimolecular binding to equivalent and noninteracting sites. The kinetic behavior argues against ligand-induced or preexistent enzyme structural changes, since a more complex mechanism would in general give rise to more than one relaxation phenomenon, and in many cases would result in nonlinear plots of $1/\tau$ vs. oxalate concentration. A priori one might expect ligand exchange reactions of the type:

$$EL_i + EL_j \rightleftarrows EL_{i-1} + EL_{j+1}$$

to play a role in the microscopic mechanism. These processes are thermodynamically dependent, and therefore cannot be detected by equilibrium studies. Regarding rates near equilibrium, it can be shown that if the direct substitution reactions of the independent equilibria (Scheme II) are much faster than the above ligand exchanges, the kinetics will be described by a single decay with the time constant $1/\tau = k_a(C_E + C_L) + k_d$. It seems likely that this condition is in fact satisfied, since the large excess of oxalate will promote direct substitution rates over exchange rates between enzyme–ligand complexes.

Relaxation amplitudes provide an independent check on the validity of a model used to rationalize relaxation times, since times and amplitudes are functions of different system parameters. We were able to demonstrate that the amplitude concentration dependence for oxalate binding to cytochrome b_2 was in good quantitative agreement with the behavior predicted for Scheme II. In addition, the admittedly approximate reaction heats extracted from the amplitudes correlate well with the enthalpy change determined directly by calorimetry.

In view of the apparent one-step nature of the binding reaction, the small association rate constant of 1×10^4 M^{-1}

sec^{-1} was a surprising result. Theoretical as well as experimental evidence indicates 10^8–10^9 M^{-1} sec^{-1} for typical diffusion-controlled rate constants for binding of a small ligand to a protein (Amdur and Hammes, 1966). Second-order rate constants orders of magnitude smaller than this are usually taken as an indication of a complex mechanism, such as initial ligand binding followed by a conformational change. On the other hand, the association activation enthalpy for the oxalate-cytochrome b_2 system is quite small (~3 kcal/mol) and characteristic of a diffusion controlled process; the "slow" reaction rate relative to other ligand-protein associations arises from a very unfavorable activation entropy, $\Delta S_a^{\ddagger} \simeq -30$ eu. The fact that $\Delta S_a^{\ddagger}$ is far from the overall entropy $\Delta S^{\circ} = -5$ eu implies that the activated complex is significantly different from product structure in regard to steric effects.

The most straightforward interpretation of these observations is that oxalate binding involves little deformation of enzyme structure (one relaxation time; simple second-order kinetics; unusually small $\Delta H_a^{\ddagger}$) but requires stringent steric alignment in forming the activated complex ($\Delta S_a^{\ddagger}$ large and negative). In terms of collision theory, only a small number of encounters (1 in 10^7) between enzyme and oxalate possess the correct orientation for effective binding. On the other hand, the system recovers most of its original randomness in passing from transition state to stable complex ($\Delta S^{\circ} \simeq 0$). Admittedly, the pH dependence of k_a renders this interpretation provisional, since heats and entropies of rapid protolytic reactions may contribute significantly to the observed activation parameters.

We note that while ligand binding to enzymes has been studied in a large number of cases with rapid kinetic techniques, relatively few investigations have included temperature dependences of rate parameters. The present results point to the importance of determining activation enthalpies and entropies before concluding that an abnormally small second-order rate constant reflects the presence of one or more significantly populated intermediates.

Morton and Sturtevant (1964) have reported that kinetic properties of cytochrome b_2 may vary, depending on the yeast used as source and the method of preparation. Variations were even found among samples prepared by the same method. In this regard we add that the purification procedure used here (Spyridakis et al., 1971) is an improved version of the earlier method. Furthermore, all but one series of kinetic experiments were carried out with enzyme from the same preparation. Finally, differences in thermodynamic and kinetic parameters for independent enzyme preparations (Tables I and II) can be largely attributed to experimental error, and in any case do not invalidate our interpretation of the molecular events involved in formation of the enzyme–ligand complex.

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