The Effect of Opposing Effectors on Activation Level of Succinate Dehydrogenase: Equilibrium and Kinetic Studies†

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ABSTRACT: The activation of mitochondrial succinate dehydrogenase by various activators is a result of dissociation of oxaloacetate tightly bound to the nonactive enzyme. But, quantitative correlation between the effector concentrations and the active fraction of the enzyme was not at hand. In this study we measured the level of active succinate dehydrogenase equilibrated with a wide range of opposing effectors: oxaloacetate (1–500 μM) and activator (0.02–1.5 M NaBr). The results are compatible with a model assuming two stable forms of the enzyme: a nonactive enzyme-oxaloacetate complex and an active enzyme free of oxaloacetate. The active form is stabilized by binding two Br^- and one H^+. The rate of activation (k_a) and exchange between enzyme bound and free oxaloacetate k(ex) were measured. Both k_a and k_{ex} are hyperbolically dependent on Br^- concentration but differ in magnitude and pH dependence. k_{ex} at infinite Br^- concentration is pH dependent but k_a is not. The two reactions, activation and exchange, also differ in their activation energy being 32 and 21.5 kcal/mol, respectively. It is concluded that, in the course of activation, Br^- interacts at two distinct steps. First to produce a ternary, nonactive [enzyme-oxaloacetate-Br^-] complex. From this complex, oxaloacetate dissociates and the oxaloacetate-free enzyme assumes its active form. Finally, the active enzyme is stabilized by binding another Br^-. The rate-limiting step in deactivation is binding of oxaloacetate to active enzyme. The complex formed undergoes a very rapid transformation to the stable nonactive form. This pathway, under certain conditions, can reverse its direction and contribute to the overall rate of activation. It is suggested that the equilibrium between the two stable forms of the enzyme can be reached by two parallel pathways, each contributing independently to the observed rate of activation, while the final equilibrium is determined by the free energy between the products and the reactants.

The activation of succinate dehydrogenase by various activators has been known for many years (Kearney, 1957; Gutman et al., 1971a,b; Kearney et al., 1974). Only recently, when Ackrell et al. (1974) identified the binding of oxaloacetate to succinate dehydrogenase as the mechanism of deactivation, could a common mechanism be ascribed to the effect of various activators. All activators, substrates, $CoQH_2$[1], anions, IDP (Ackrell et al., 1974), or reductive activation (Gutman and Silman, 1975a,b) remove the oxaloacetate from the enzyme to yield the free active form of it.

In our previous studies (Gutman and Silman, 1975a,b), the reductive activation of succinate dehydrogenase was analyzed and shown to be a simple equilibrium between four stable forms of the enzyme: oxidized and reduced oxaloacetate complexes of the enzyme both nonactive, where the reduced complex is less stable than the oxidized one. The two other forms are the oxidized and reduced forms of the ligand-free active enzyme. Consequently the equilibrium concentrations of the active form of the enzyme are a function of both the poised redox potential and the concentration of free oxaloacetate.

In the present communication we employed a similar approach, combining equilibrium and kinetic studies, in investigating the mechanism of activation of succinate dehydrogenase by anions. It is not likely that, under physiological conditions, anions play a key role in regulation of succinate dehydrogenase; yet because of the following reasons, anions can serve as a good model for studying the mechanism of activation. Unlike other activators (as succinate or $CoQH_2$), Br^- does not reduce the enzyme, nor does it react with high affinity with the substrate binding site as does malonate. When using membrane-bound enzyme, even high Br^- concentrations do not inactivate nor solubilize the succinate dehydrogenase. Finally, due to the relatively low affinity, the anion can be removed very rapidly by either dilution or centrifugation.

The mechanism for activation proposed by Ackrell et al. (1974) was:

$$E_{NA} + OAA \underset{-\text{activator}}{\overset{+\text{activator}}{\rightleftharpoons}} E_A \cdot OAA \rightarrow E_A + OAA$$

This model, as written, cannot account quantitatively for the effect of activator or oxaloacetate concentrations either on the rate of the partial reactions or for the equilibrium level of active enzyme. As will be shown in this study, the activation of succinate dehydrogenase by anions consists of an equilibrium between two stable forms of the enzyme: a nonactive enzyme-oxaloacetate complex (E_{NA}·OAA)[1] and active enzyme stablized as a Br^- complex. The fraction of the active enzyme and the rate of its appearance are both a function of the Br^- and oxaloacetate concentrations.

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[1] Abbreviations: ETP and ETP_H, for nonphosphorylating and phosphorylating submitochondrial particles, respectively; OAA, oxaloacetate; E_A and E_{NA} for active and nonactive forms of succinate dehydrogenase; k'_a and k_a, apparent and maximal rate of activation, respectively; k'_{ex} and k_{ex} apparent and maximal rate of exchange, respectively; k'_d and k_d, apparent and maximal rate of deactivation, respectively; PMS, phenazine methosulfate; DCPIP, dichlorophenoleindophenol; $CoQH_2$, reduced ubiquinone.

Materials and Methods

Beef heart mitochondria and ETP were made according to Ringler et al. (1963); ETP_H was made by the method of Hansen and Smith (1964). Succinate dehydrogenase activity was measured spectrophotometrically using the DCPIP–PMS method (Kimura et al., 1967) as modified by Gutman et al. (1971a). In order to avoid activation in situ, the assay was carried at 13–15 °C.

Prior to use, ETP_H were washed with 0.25 M sucrose, 50 mM Tris-acetate, pH 7.4, and 5 mM $MgSO_4$ and resuspended in 0.18 M sucrose, 50 mM Tris-acetate, pH 7.4, and 5 mM $MgSO_4$ to 20 mg/ml, activated if called for by the experimental procedure (1 mM malonate, 30 min at 30 °C), spun down, and resuspended in the same buffer.

Determination of Equilibrium Level of Activation. Activated ETP_H were suspended in 0.18 M sucrose, 100 mM Hepes, or Mes at the desired pH and NaBr concentration. A freshly prepared solution of oxaloacetate was added and the activity of the enzyme was followed by removing samples for assay at 15 °C. The sampling continued until no further change in activity was observed; this was attained after ca. 10 min (at 30 °C); routinely the activity was measured after 15–30 min of incubation.

To account for possible inactivation, control samples were activated in the cuvette (containing 20 mM succinate, 60 mM KPO_4 buffer, pH 7.6, and 2 mM KCN) for 15 min at 30 °C and cooled to the assay temperature, (15 °C), and the reaction was started by addition of DCPIP and PMS (Kimura et al., 1967). This procedure allows estimation of the extent of competitive inhibition by oxaloacetate which has been carried over. It was verified that, even at high Br^- concentrations, no solubilization of the enzyme took place.

Exchange between Enzyme-Bound and Free Oxaloacetate. ETP_H were washed as before and activated by 0.5 M NaBr, pH 6.2. The mixture was allowed to set until at least 85% of the enzyme was active (8–12 min). The ETP_H were spun down (100 000*g* for 30 min) and resuspended in 0.18 M sucrose, 50 mM K-Pi buffer, and 1 mM NAD to 5–10 mg/ml. [U-^{14}C]-L-Malate (82 mCi/mmol) was added in a tenfold molar excess to the enzyme content. The enzyme was fully deactivated in 2–5 min at 30 °C. The ETP_H were spun down and washed by the same buffer and resuspended to 15 mg/ml in 0.18 M sucrose, 10 mM Tris-acetate, pH 7.2. Exchange was initiated by diluting the ETP_H to 1 mg/ml with 0.18 M sucrose and 100 mM buffer (Hepes or Tris-acetate), at the desired pH and NaBr concentration, plus 1 mM unlabeled oxaloacetate, all at a known constant temperature. At designated times, samples were withdrawn and diluted (1:10) in 0.18 M sucrose and 100 mM Tris-acetate, pH 8, containing 10 mM semicarbazide, precooled to 0 °C. Under such conditions there is neither exchange nor activation by the Br^-. The samples were spun down and resuspended in 0.2 ml of 1% sodium dodecyl sulfate; radioactivity was measured by scintillation counting using Packard Instagel scintillation fluid. Protein content was determined for each sample by the biuret method. To evaluate the efficiency of the washing procedure, a sample at $t = 0$ was denatured by 6% Cl_3CCOOH and then washed by the same procedure. It was verified that the indicated concentration of the detergent did not quench the scintillation. Samples were removed from the exchange reaction mixture and assayed for succinate dehydrogenase activity, with or without prior activation in the cuvette.

[U-^{14}C]Oxaloacetate was made by controlled catalytic oxidation of [U-^{14}C]-L-malate. [U-^{14}C]-L-Malate (35 mCi/mmol); 150 μM in 50 mM glycine buffer, pH 10, was catalytically oxidized by ETP (0.15 mg/ml) at 30 °C in the presence of NAD (1 mM) and $K_3Fe(CN)_6$ (1 mM). ETP, but not ETP_H, contains enough malate dehydrogenase to catalyze the oxidation of malate. The reaction was monitored at 420 nm until all malate was oxidized. The protein was precipitated by $HClO_4$ (0.5 M) which was later neutralized by $KHCO_3$. Excess of $K_3Fe(CN)_6$ was reduced by ascorbate.

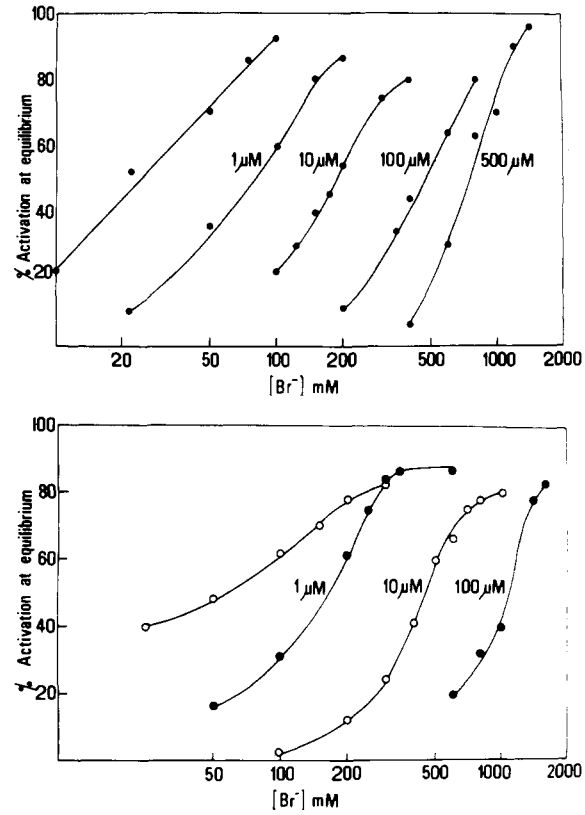


FIGURE 1: The effect of bromide and oxaloacetate concentrations on the activity of succinate dehydrogenase: (top) equilibrated, pH 6.2; (bottom) equilibrated, pH 7.2. ETP_H, washed and activated as described under Materials and Methods, were equilibrated at 30 °C, 0.18 M in sucrose, 100 mM Hepes, at pH 6.2 or 7.2, with the indicated concentrations of oxaloacetate and NaBr. Each point represents the activation level after 30 min of equilibration measured with respect to full activation (100% = 0.34 μmol/min/mg at 13 °C).

Results

Equilibrium of Succinate Dehydrogenase with Oxaloacetate. Figure 1 relates the equilibrium level of succinate dehydrogenase measured in presence of oxaloacetate and Br^- at a constant pH. Each point represents the equilibrium level of active enzyme in ETP_H, at a given concentration of oxaloacetate and Br^-. It is evident that the level of activation is a function of both concentrations: the higher the oxaloacetate level, the more Br^- is needed to keep the enzyme active. The relationship between the active enzyme fraction and the concentration of effectors is pH dependent. At the higher pH, a lower concentration of oxaloacetate will deactivate the enzyme at any Br^- concentration.

To find the correlation between the level of activation and the amount of oxaloacetate bound to the enzyme, we repeated the equilibration experiment using [^{14}C]oxaloacetate. ETP_H were equilibrated at pH 6.2 and various Br^- concentrations with 1 μM [U-^{14}C]-oxaloacetate. After equilibration the particles were spun down and the radioac-

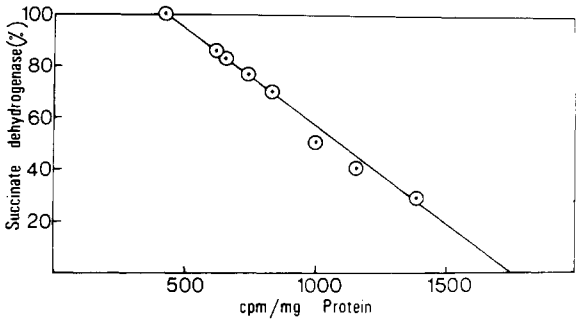


FIGURE 2: Correlation between equilibrium level of active succinate dehydrogenase and its bound oxaloacetate content. ETP_H were washed and resuspended in 0.18 M sucrose, 100 mM Hepes, and 1 μM [^{14}C]oxaloacetate to 0.5 mg/ml, as in Figure 1 (top). NaBr was added to various concentrations (0–0.2 M) and, after 15 min equilibration at 30 °C, the particles were spun down at 30 °C and resuspended in 0.18 M sucrose, 50 mM Tris-acetate, and 5 mM $MgSO_4$. The enzymic activity and radioactivity were measured (100% activity = 0.333 μmol/min/mg at 13 °C; 1400 cpm/mg = 1 mol of oxaloacetate/mol of succinate dehydrogenase).

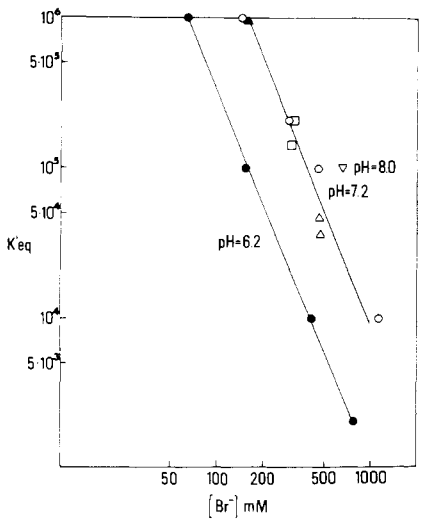


FIGURE 3: The variation of the apparent equilibrium constant for activation with respect to bromide concentration. The values of K'_{eq} were calculated from the data presented in Figure 1 and other experiments. The pH of the experiments is indicated on the figures. The different symbols are for different experiments.

tivity and catalytic activity of the pellet were measured. Figure 2 correlates the equilibrium level of active enzyme measured from the pellet with the radioactivity of the pellet. The linearity between these two variables is evident. The difference in bound oxaloacetate between fully active and completely nonactive enzyme is 1 mol of oxaloacetate per mole of enzyme, in accord with Ackrell et al. (1974). The oxaloacetate content associated with the active enzyme is probably trapped in or between the vesicles, but a washing procedure to remove it is difficult to devise; any changes in oxaloacetate or Br^- concentration of the washing solution will perturb the equilibrium, while a wash with unlabeled oxaloacetate will initiate an exchange between free and enzyme-bound molecule oxaloacetate (see below). The 1:1 correlation between nonactive enzyme and increase in bound oxaloacetate is in accord with the previous suggestion (Gutman and Silman, 1975a,b) that the stable forms of the enzyme which participate in equilibrium with oxaloacetate are nonactive enzyme in a oxaloacetate complex and oxaloacetate-free, active enzyme.

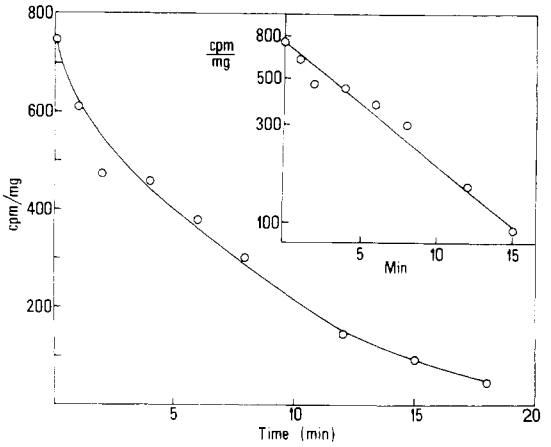


FIGURE 4: The time course for exchange of enzyme-bound oxaloacetate with free ligand. ETP_H were labeled by [^{14}C]OAA as described under Materials and Methods. The exchange was measured at 30 °C against 1 mM unlabeled OAA in 0.18 sucrose, 100 mM Tris-acetate, pH 7.2, and 300 mM NaBr. The reaction was stopped and radioactivity monitored as described under Materials and Methods. (Insert) Semilogarithmic plot of radioactivity vs. time.

$$E_A + OAA \rightleftharpoons E_{NA} \cdot OAA$$

The apparent equilibrium constants for the above reaction (K'_{eq}) were calculated from the data given in Figure 1, and from other experiments. The variation of K'_{eq} with pH and [Br^-] is presented in Figure 3, where the log K'_{eq} decreases linearly with an increase in log [Br^-]. The slope of the line is $n = 2$, both for pH 6.2 and 7.2. The effect of pH is evaluated from the difference between the two lines: a tenfold increase in [H^+] is associated with a tenfold decrease in K'_{eq}. The value measured at pH 8.0, 800 mM Br^- (1.1×10^{-5} M^{-1}), is also compatible with the exponent of -1 for the effect of proton on the equilibrium (calculated value 1.05×10^{-5} M^{-1}).

Exchange between Enzyme-Bound and Free Oxaloacetate. The binding of oxaloacetate to succinate dehydrogenase is destabilized upon reduction of the enzyme, with concomitant accelerated exchange between free and enzyme-bound oxaloacetate (Gutman and Silman, 1975a,b). An analogous case is the decrease of K'_{eq} by high Br^- concentration. Figure 4 demonstrates that, in presence of 0.3 M NaBr, pH 7.2, enzyme-bound oxaloacetate is exchanged with free oxaloacetate. As shown in the insert, the reaction follows first-order kinetics. As the exchange was measured in the presence of 1 mM oxaloacetate, no trace of active enzyme was detected during the course of the reaction. On the other hand, activation in the cuvette showed that no inactivation took place and the enzyme retained all its enzymic activity.

The experiment of Figure 4 was repeated at the pH and Br^- concentrations as shown in Figure 5, where the observed first-order rate constants for the exchange (k'_{ex}) are related to Br^- on a double reciprocal plot. This presentation will be justified in the Discussion. The saturation curves at the two pH's are identical; it is only the maximal rate (k_{ex}) which is pH dependent. This property was also varified by a Hill plot analysis of the data given in Figure 5. The points of the two curves fit a single straight line with $n = 1$.

The Effect of pH and [Br^-] on Rate of Activation. The rate of activation was measured as a function of pH and Br^- concentration. In these experiments, no oxaloacetate was added and the final level of active enzyme was essen-

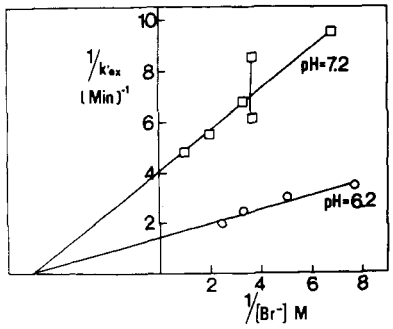


FIGURE 5: The effect of pH and Br^- concentration on the rate of the exchange reaction. The first-order rate constants for the exchange reaction were measured from experiments similar to that given in Figure 4. The curve for pH 6.2 (O–O) was measured with 100 mM Hepes; (□–□) pH 7.2 with 100 mM Tris-acetate (30 °C).

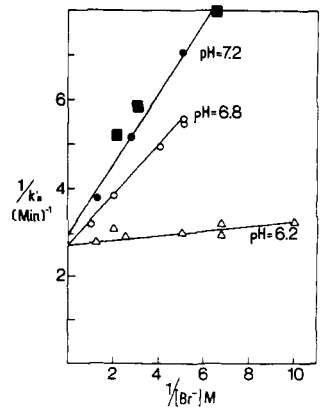


FIGURE 6: The effect of pH and Br^- concentration on the rate of activation of succinate dehydrogenase. The rate of activation was measured, at 30 °C at the pH and Br^- concentrations indicated in the figure, by following with time the appearance of active enzyme. The first-order rate constants of the activation are drawn with respect to Br^- concentration on a double reciprocal scale. For pH 7.2, closed circles (●) represent rate constant measured in activation experiments, and squares (■) are rate constants calculated from approach to equilibrium in deactivation experiments (see Figure 7). (O–O) pH 6.8; (△–△) pH 6.2.

tially 100%. Figure 6 depicts (on a double reciprocal plot) the increase of the first-order rate constant of activation, k'_a, with the $[Br^-]$. The maximal rate k_a is the same at all pH values, but the lower the pH, the less Br^- needed to attain this rate. A Hill plot of the data for pH 6.8 and 7.2 yielded two parallel lines with $n = 1$.

Approach to Equilibrium Kinetics. The kinetics of approach to equilibrium for a reversible first-order (or pseudo-first-order) reaction follows apparent first-order kinetics. The observed rate constant is $k'_{obsd} = k'_a + k'_d$ and the final level of equilibrium is $[E_A]/[E_{NA}] = k'_a/k'_d$, where k'_a and k'_d are the respective apparent first-order rate constants for activation and deactivation. By measuring the kinetics of approach to equilibrium in presence of variable concentration of oxaloacetate and Br^-, the effect of oxaloacetate and Br^- on the rate of deactivation can be measured.

A typical time course of such a reaction is given in Figure 7, demonstrating the effect of oxaloacetate concentration on the rate and the level of equilibrium. The rate constants for activation, calculated from such experiments are essentially the same as those measured from activation kinetics in absence of oxaloacetate. This is demonstrated in Figure 6 where k'_a values calculated from both types of experiments fit the same line.

The rate of deactivation is a linear function of oxaloacetate concentration, indicating that the rate-limiting step in the deactivation is the second-order reaction with oxaloacetate. The second-order rate constants for deactivation measured at 300 and 450 mM NaBr (pH 7.2) are 2×10^4 and 6×10^3 M^{-1} min^{-1}, respectively. The dependence of rate of deactivation on $[Br^-]$ is shown in Figure 8. The rate is a linear function of $1/[Br]^2$. As will be shown in the Discussion, this linearization is not empirical but predicted by the model.

The Effect of Temperature on the Rates of Exchange and Activation. The results just presented indicate that the exchange of oxaloacetate and activation of the enzyme might proceed at independent rates. The fact that these two reactions are not controlled by the same rate-limiting step is further demonstrated by the difference in their activation energy (Figure 9). The activation energy for exchange is 21.5 kcal/mol, while for activation, $E_a = 32$ kcal/mol.

Discussion

We shall first consider the equilibrium and the kinetics experiments separately and later discuss them as a whole.

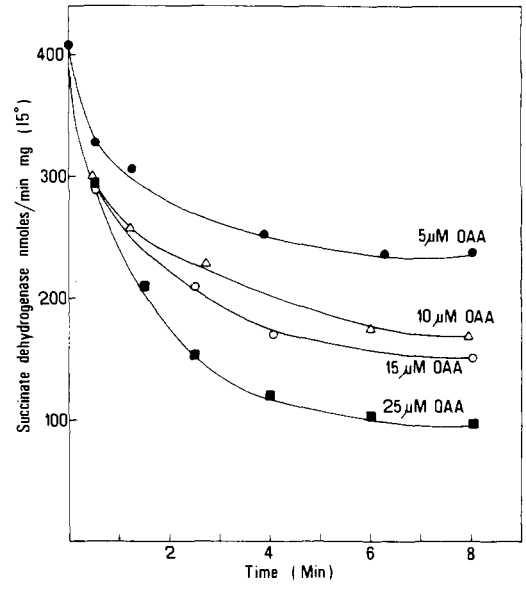


FIGURE 7: The time course for the approach to equilibrium kinetics. The deactivation was initiated by adding the indicated OAA concentration to activated ETP_H suspended in 0.18 M sucrose, 100 mM Hepes, pH 7.2, and 300 mM NaBr. Oxaloacetate concentrations were: (O) 5 μM; (△) 10 μM; (●) 15 μM; (■) 25 μM.

First we have to define the species which are in equilibrium and correlate them with the observed parameters. The most stable form is the nonactive enzyme-oxaloacetate complex; it does not dissociate upon dilution, by passing through a Sephadex column or reduction with malate dehydrogenase (Kearney et al., 1972). There is a strict 1:1 stoichiometric relationship between the membrane-bound enzyme and oxaloacetate (Kearney et al., 1972; Ackrell et al., 1974; Figure 2). Characterized by a dissociation constant (for oxidized enzyme in absence of anions) of $\sim 7 \times 10^6$ M^{-1} (Gutman and Silman, 1975a,b; Zimakova et al., 1970).

The other stable form of the enzyme is oxaloacetate-free active enzyme. This is the final product of any mode of activation (Kearney et al., 1972). Other forms of active en-

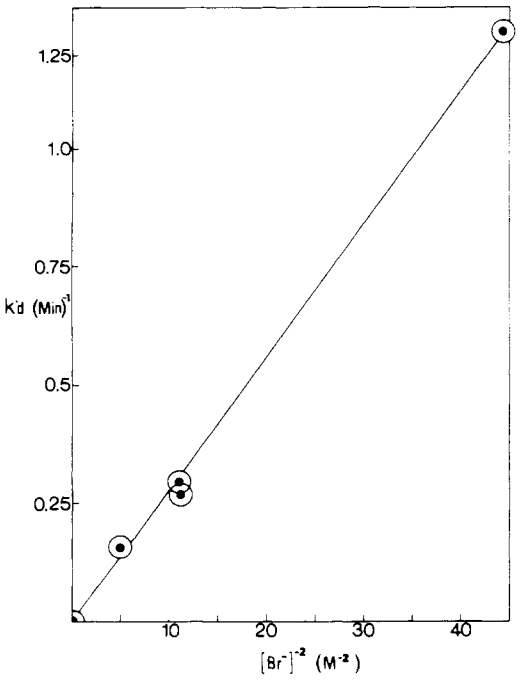


FIGURE 8: The effect of Br^- concentration on the first-order rate constant for deactivation. The experiment was carried out at 30 °C, in 0.18 M sucrose and 100 mM Tris-acetate, pH 7.2. Deactivation was initiated by addition of 25 μM oxaloacetate. Rate constants were calculated from the approach to equilibrium kinetics as in Figure 7.

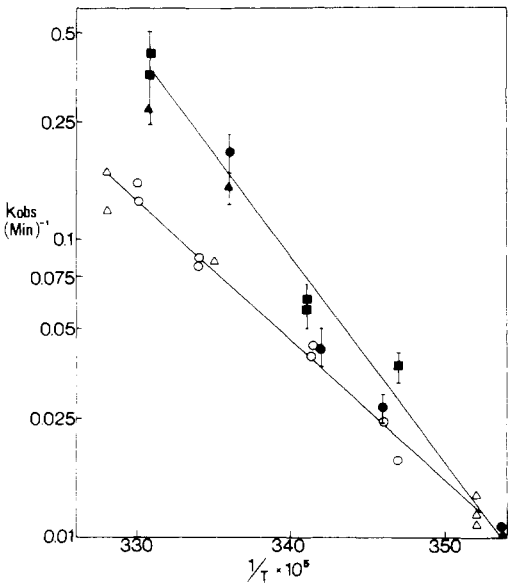


FIGURE 9: Arrhenius plot for activation of succinate dehydrogenase and exchange of enzyme-bound oxaloacetate. The activation (closed symbols, ▲, ●) was measured at different temperatures in 180 mM sucrose, 130 mM NaBr, and 100 mM Hepes, pH 6.2. The exchange reaction (open symbols △, ○) was measured in 180 mM sucrose, 50 mM Tris-acetate, pH 7.2, 500 mM NaBr, and 1 mM OAA. For activation, E_a = 32 kcal/mol. For exchange reaction, E_a = 21.5 kcal/mol.

zyme, such as E_A·OAA (Ackrell et al., 1974), were proposed but, due to its transient nature, it should not be considered in analysis of equilibrium state. As shown in Figure 2, at equilibrium, there is a linear dependence of the nonactive fraction of the enzyme on the content of activator-removable oxaloacetate, with a stoichiometry of 1 mol of oxaloacetate per mole of enzyme; thus in equilibrium analysis we shall equate the free and the complex forms of the enzyme with the respective active and nonactive fraction of succinate dehydrogenase. (The experimental conditions selected were such that the free ligands are very close to their total concentration.)

The relationship between the effector concentrations and the stable forms of the enzyme can be described by assuming that the enzyme participates in two independent equilibria.

$$E_A + OAA \rightleftharpoons (E_{NA} \cdot OAA) \qquad (1)$$

$$K_{OA} = \frac{[E_{NA} \cdot OAA]}{[E_A][OAA]}$$

and

$$E_A + nH^+ + mBr^- \rightleftharpoons (E_AH^+{}_n\, Br^-{}_m) \qquad (2)$$

$$K_{HB} = \frac{[E_AH^+{}_nBr^-m]}{[E_A][H^+]^n[Br^-]^m}$$

Regarding the high affinity of succinate dehydrogenase for oxaloacetate (Zimakova et al., 1970, Gutman and Silman, 1975a,b) and the high concentrations of Br^- used in our experiments, we approximate

$$[E_{total}] = [E_{NA} \cdot OAA] + [E_A \cdot H^+{}_nBr^-{}_m]$$

Under such assumptions we derived the following equation, which related the empirial relationship of Figure 3 (K'_{eq}) with defined equilibrium constants:

$$K'_{eq} = \frac{[E_{NA}]}{[E_A][OAA]} = \frac{K_{OA}}{K_{HB}} \frac{1}{[H^+]^n[Br^-]^m}$$

The log–log plot of this function in Figure 3 yielded $n = 1$ and $m = 2$.

Kinetic studies of deactivation demonstrate that the rate of this reaction is a function of the two ligands. The linear dependence of k'_d on oxaloacetate indicates a second-order reaction with oxaloacetate binding in the rate-limiting step, as in reaction 1.

$$\frac{d[E_{NA}\cdot OAA]}{dt} = k_d[OAA][E_A]$$

The effect of Br^- is more complex. The concentration of E_A which participates in reaction 1 is a function of $[H^+]$ and $[Br^-]$, as given by reaction 2. Thus the kinetic equations will take the following form

$$\frac{d[E_{NA} \cdot OAA]}{dt} = k_d[OAA][E_A] = k_d[OAA] \times \frac{[E_A \cdot H^+{}_n \cdot Br^-{}_m]}{[H^+]^n[Br^-]^m} \frac{1}{K_{HB}}$$

and

$$k_{obsd} = \frac{k_d}{K_{HB}[H^+]^m} \frac{1}{[Br^-]^m} [OAA]$$

The same exponent for $[Br^-]$, $m = 2$, is derived from Figure 8, where k'_d is shown to be linear function of $1/[Br^-]^2$.

The nature of the interaction of Br^- with the succinate dehydrogenase is ambiguous. On one hand, Br^- can activate at concentrations too low to be ascribed to chaothropic effect. (See the 0 and 10 μM oxaloacetate curves in Figure 1.) On the other hand, the activating anions can be arranged in a series very similar to the lyothropic series (Kearney et al., 1974). It is likely that we have a case similar to that described by Aviram (1973a,b). Chaothropic anions were found to alter both conformation and spectrum of

Table I: Comparison between Rates of Exchange and Activation Measured at Various pH and Bromide Concentrations.[a]

	[Br⁻] (mM)								
	∞			500			200		
pH	k_{ex} (min⁻¹)	k_a (min⁻¹)	$\frac{k_{ex}}{k_a}$	k'_{ex} (min⁻¹)	k'_a (min⁻¹)	$\frac{k'_{ex}}{k'_a}$	k'_{ex} (min⁻¹)	k'_a (min⁻¹)	$\frac{k'_{ex}}{k'_a}$
6.2	0.7	0.38	1.85	0.5	0.37	1.3	0.34	0.33	1.03
7.2	0.25	0.33	0.74	0.18	0.21	0.85	0.124	0.141	0.9

[a] Data from Figures 5 and 6.

acid cytochrome *c*. This effect, observed at concentrations too low to have a chaothropic effect, was due to interaction of the anion at a site different from the heme group (Lanir and Aviram, 1975).

The mechanism of activation proposed by Ackrell et al. (1974) postulated an active intermediate complex, E_A·OAA, whose appearance is a function of the activator concentration.

$$E_{NA} \cdot OAA \underset{-Ac}{\overset{+Ac}{\rightleftharpoons}} E_A \cdot OAA \rightleftharpoons E_A + OAA$$

It was further concluded that rate of activation was faster than the release of enzyme-bound oxaloacetate. We tested this model by shifting the equilibrium from both ends toward the middle. High concentration of activator will favor the appearance of E_A·OAA and E_A, while high concentration of oxaloacetate will shift the equilibrium in the other direction. Thus in presence of both activator and oxaloacetate, E_A·OAA will be a favored species. According to Ackrell's model, this complex should participate in exchange reaction but, as it is an active complex, the exchange reaction must proceed in presence of catalytically active enzyme. As described in the Results, this is not the case. The exchange can proceed in a rate faster than the rate of activation (had oxaloacetate been omitted) without any trace of active enzyme, contrary to the prediction of Ackrell's model. Thus, the nonactive succinate dehydrogenase complex can exchange its oxaloacetate by dissociating without reaching the active conformation of the enzyme.

The exchange represents a reaction taking place at equilibrium; during the course of the reaction there is no net change in the concentration of any of the reactants and the measured reaction is a random transformation between existing equilibrium-stable forms. Thus variation in rate of exchange results from variation in the equilibrium concentration of the exchanging species. The mechanism of the exchange is not a direct displacement by free oxaloacetate. Were this the mechanism, the rate would be a function of oxaloacetate concentration—which is not the case; the maximal rate of exchange against 1 mM oxaloacetate (pH 6.2, saturating Br^-, 13 °C) is $k_{ex} = 0.04$ min⁻¹, while under comparable conditions (pH 6.3, 0.75 M NaBr, 13 °C) the rate of release of oxaloacetate against zero concentration of free oxaloacetate is $k = 0.069$ min⁻¹ (Ackrell et al., 1974).

A mechanism compatible with the experimental results is as follows

$$E_{NA} \cdot OAA + Br^- \rightleftharpoons E_{NA} \cdot OAA \cdot Br^-_n \underset{}{\overset{k_{ex}}{\rightleftharpoons}} E_{NA} \cdot Br^-_n + OAA$$

All components in these reactions are in equilibrium. High oxaloacetate concentrations will lower the concentration of $E_{NA} \cdot Br^-$ to a very low level—as evident by the lack of activation. But this will not slow the rate of dissociation of E_{NA}·OAA·Br^- which is a first-order reaction independent of oxaloacetate.

$$\frac{-d[\text{Enzyme} \cdot [^{14}C]OAA]}{dt} = k_{ex}[E_{NA} \cdot [^{14}C]OAA \cdot Br^-_n]$$

In the presence of 1 mM oxaloacetate we can approximate

$$[E_{total}] = [E_{NA} \cdot OAA] + [E_{NA} \cdot OAA \cdot Br^-_n]$$

At infinite Br^- concentration, all of the enzyme will be as E_{NA}·OAA·Br^-_n and the rate of exchange will be maximal (k_{ex}). At lower Br^- concentrations, the observed rate of exchange k'_{ex} will decrease in proportion to the equilibrium level of the exchanging species. As expressed by the following equation

$$\frac{k'_{ex}}{k_{ex}} = \frac{[E_{NA} \cdot OAA \cdot Br^-_n]}{E_{total}} = \frac{K_{EOBr}[E_{NA} \cdot OAA][Br^-]^n}{E_{total}}$$

where

$$K_{EOBr} = \frac{[E_{NA} \cdot OAA \cdot Br^-_n]}{[E_{NA} \cdot OAA][Br^-]^n}$$

This equation can be rearranged to

$$\frac{1}{k'_{ex}} = \frac{1}{k_{ex}} + \frac{1}{k_{ex}K_{EOBr}} \frac{1}{[Br^-]^n}$$

Which justified the analysis of the results by the double reciprocal plot (Figure 5). The exponent of Br^- in this reaction is $n = 1$, the same as obtained from a Hill plot. K_{EOBr} calculated from Figure 5 is $K_{EOBr} = 5$ M⁻¹. Interestingly this equilibrium constant is pH independent while the kinetic one (k_{ex}) varies with pH. Apparently only the protonated form of E_{NA}·OAA·Br^- can participate in the exchange reaction. The nature of the protonated group controlling this reaction can be studied by a correlation of k_{ex} with pH.

The effect of pH and $[Br^-]$ on rate of activation are completely different from their effect on exchange. The same maximal rate of activation k_a is obtained at all measured pH values (Figure 6); it is the dependence of the rate on Br^- concentration which varies with pH. We conclude that the mechanism leading to the appearance of active succinate dehydrogenase is not identical with that controlling the exchange. A comparison between rate constants for exchange and activation is given in Table I. The rates of these two reactions can vary independent of each other. At pH 6.2 exchange is faster or equal to rate of activation. This is compatible with the linear mechanism[2] described below and the equilibrium equation derived before:

[2] Experimental data do not allow distinction between the two possible pathways, $E_{NA}H^+Br^- + Br^- \rightarrow E_{NA}H^+Br^-_2$ (k_a) $\rightarrow E_AH^+Br^-_2$ and the one written above. Other formulations might be possible too.

$$E_{NA} \cdot OAA \cdot H^+Br^- \overset{k_{ex}}{\rightleftharpoons} E_{NA}H^+ \cdot Br^- + OAA$$

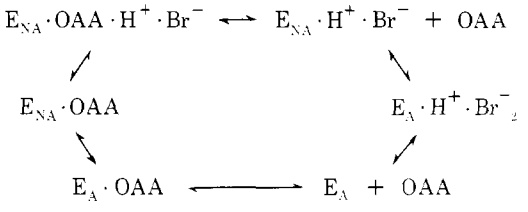
$$E_{NA}H^+ \cdot Br^- \overset{k_a}{\rightleftharpoons} E_AH^+Br^-$$

$$E_AH^+Br^- + Br^- \rightleftharpoons E_AH^+Br^-_2$$

The distinction between activation and exchange is also supported by the activation energies of the two reactions (Figure 9). For activation, the E_a = 32 kcal/mol is similar to that observed by Singer et al. (1973), 33 kcal/mol. But the exchange has a significantly lower activation energy E_a = 21.5 kcal/mol.

A complicated situation is observed at pH 7.2 where exchange is slower than activation. It is an inevitable conclusion that at this pH we encounter an activation pathway which does not incorporate the exchanging species as an intermediate. Accordingly we concluded that the same equilibrium can be formed by two parallel reactions consisting of a close circular pathway. A simplified model, which does not include all steps discussed above, is given in Scheme I.

Scheme I



The upper part of the scheme is the mechanism dominating at pH 6.2. The lower one is the alternate pathway contributing to activation at pH 7.2 and high Br^- concentration. The latter one was already proposed to account for the effect of OAA and Br^- on rate of deactivation (vide supra). It also bears resemblance to Ackrell's model, except that the step where the activator interacts with the enzyme is not the same. Ackrell et al. (1974) proposed that the activator reacts with $E_{NA}OAA$, while we place it as ligand stabilization of the active enzyme.

The rate of the reaction $E_AOAA \rightarrow E_{NA}OAA$ is extremely fast. Zimakova et al. (1970) reported a rate constant of 4×10^5 M^{-1} min^{-1} (25 °C). At pH 7.4 and in absence of anions, the deactivation still follows second-order kinetics, with rate constant $\sim 1.5 \times 10^6$ M^{-1} min^{-1} (30 °C) (Gutman and Berger, 1973). Consequently E_AOAA is a very unstable form and can act as an intermediate in the direction of activation only under conditions where the concentrations of both free oxaloacetate and free active enzyme are extremely low so that the equilibrium is shifted to the right. This is achieved at less acid pH, in presence of high [Br^-], and in absence of oxaloacetate.

The last point for discussion is the difference between various preparations of succinate dehydrogenase. With membranal preparations, the E_a for activation is the same (30–33 kcal/mol) for succinate, malonate (Kearney, 1957), $CoQH_2$ (Gutman et al., 1971a), and pH 6 (Singer et al., 1973), or pH 6.2 + Br^- (Figure 9). (A known deviation from this value is in the reductive activation of the enzyme (Gutman and Silman, 1975a,b), where it is the reduced form of the enzyme which undergoes activation.) Soluble preparations are activated by malonate or succinate with an activation energy of 30–33 kcal/mol, by pH 6 or anions, E_a = 20–25 kcal/mol (Kearney et al., 1972), and some soluble enzyme preparations (made by ClO_4^- extraction) activated by succinate are with E_A = 22 kcal/mol (Coles et al., 1974). An inevitable conclusion is that the removal of the enzyme from its natural environment in the membrane results in some major changes in the enzyme which are reflected by the observed variations in the mechanism of the activation. If the physiological importance of the activation is kept in mind, then the membranal preparation should be the favored material for investigation.

References

Ackrell, B. A. C., Kearney, E. B., and Mayr, M. (1974), *J. Biol. Chem. 249,* 2021.

Aviram, I. (1973a), *Eur. J. Biochem. 40,* 631.

Aviram, I. (1973b), *J. Biol. Chem. 248,* 1894.

Coles, C. J., Tisdale, H. D., Kenney, W. C., and Singer, T. P. (1974), *J. Biol. Chem. 249,* 381.

Gutman, M., and Berger, A., unpublished results.

Gutman, M., Kearney, E. B., and Singer, T. P. (1971a), *Biochemistry 10,* 2726.

Gutman, M., Kearney, E. B., and Singer, T. P. (1971b), *Biochemistry 10,* 4763.

Gutman, M., and Silman, N. (1975a), *Mol. Cell. Biochem. 7,* 177.

Gutman, M., and Silman, N. (1975b), in Flavins and Flavoprotein, Singer, T. P., Ed., New York, N.Y., Academic Press (in press).

Hansen, M., and Smith, L. A. (1964), *Biochem. Biophys. Acta 81,* 214.

Kearney, E. B. (1957), *J. Biol. Chem. 229,* 363.

Kearney, E. B., Ackrell, B. A. C., and Mayr, M. (1972b), *Biochem. Biophys. Res Commun. 49,* 115.

Kearney, E. B., Ackrell, B. A. C., Mayr, M., and Singer, T. P. (1974), *J. Biol. Chem. 249,* 2016.

Kearney, E. B., Mayr, M., and Singer, T. P. (1972a), *Biochem. Biophys. Res. Commun. 46,* 531.

Kimura, T., Hauber, J., and Singer, T. P. (1967), *J. Biol. Chem. 242,* 4987.

Lanir, A., and Aviram, I. (1975), *Arch. Biochim. Biophys.* (in press).

Ringler, R. L., Minakami, S., and Singer, T. P. (1963), *J. Biol. Chem. 238,* 801.

Singer, T. P., Kearney, E. B., and Ackrell, B. A. C. (1973), in Mechanisms in Bioenergenetics, Azzone, G. F., Ernster, L., Papa, S., Quaglairiello, E., Siliprandi, N., Ed., New York, N.Y., Academic Press, p 485.

Thorn, M. B. (1962), *J. Biochem. 85,* 116.

Zimakova, N. I., Shvetov, Y. N., and Vinogradov, A. (1970), *Biokhimiya 35,* 973.