Regulation of Mitochondrial Succinate Dehydrogenase by Substrate Type Activators†

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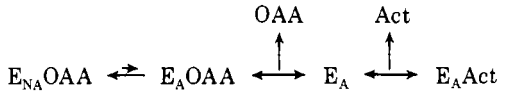
ABSTRACT: The activation of succinate dehydrogenase in submitochondrial particles was measured in the presence of oxaloacetate (a negative modulator) and substrate type activators (succinate, malonate, and fumarate). Quantitative analysis of equilibrium and kinetic experiments led to the following model for activation

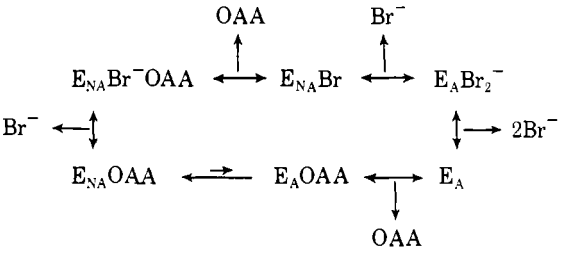


The only stable forms of the enzyme are the nonactive complex with oxaloacetate ($E_{NA}OAA$) and the active complex with the activator (E_AAct). The rate-limiting step in activation is the spontaneous conformation change from the nonactive to the active complex with oxaloacetate (E_AOAA). This reaction is shown to precede both the dissociation of oxaloacetate and the binding of activator. The only stable form of active enzyme is the active 1:1 complex with the activator (E_AAct). The free active enzyme (E_A) is unstable in the sense that both activators and oxaloacetate rapidly react with it forming tight complexes. The reaction between oxaloacetate and the free active enzyme is extremely fast ($k = 1.24 \times 10^6$ M^{-1} s^- 1 at 30 °C and pH 7.4). The role of activator is to stabilize E_A in a complex which cannot react directly with oxaloacetate. Because of the low concentration of E_A, the rate-limiting step in deactivation is the bimolecular reaction between E_A and OAA. The kinetic and thermodynamic parameters were combined in equations which predict for any combination of negative (oxaloacetate) and positive (substrate type activators) modulators the equilibrium level of active enzyme and the rate at which this state is approached. The dissociation constants of the activators from the regulatory site are markedly different from the respective K_s or K_i values. This indicates that the substrate binding site and the regulatory site are not the same. The thiohemiacetal model for deactivation of oxaloacetate is discussed.

Oxaloacetate has been known for many years to be a powerful inhibitor of succinate dehydrogenase (EC 1.3.99.1) (Das, 1937). Wojtczak et al. (1969) were the first to consider this inhibition as deactivation. In the recent years, the role of oxaloacetate as a deactivator has been studied extensively. Oxaloacetate and the enzyme form a very tight complex, which can be dissociated either by activation (Ackrell et al., 1974; Gutman, 1976) or by denaturation. It was suggested that oxaloacetate is covalently linked as a thiohemiacetal (Vinogradov et al., 1972) with a SH group at the active site of the flavoprotein subunit of the enzyme (Kenney et al., 1976).

The reaction of this SH group with MalNEt[1] is blocked by either activators or oxaloacetate (Kenney et al., 1976; Vinogradov et al., 1976). The dissociation constants of succinate and malonate, as calculated from their effect on rate of alkylation, are similar to the respective K_s or K_i values (Vinogradov et al., 1976). Yet, these studies measured the effect of only one modulator, activator or oxaloacetate, on the reactivity of this SH group, but not their simultaneous interaction with the proposed common active site. In order to understand the mechanism of this step, we looked for a quantitative correlation between the active fraction of the enzyme and the modulators' concentrations.

Our studies on activation of succinate dehydrogenase by Br^- (Gutman, 1976) indicated that activation proceeds by two parallel pathways:



The upper pathway is, kinetically, the dominant path for activation. It is characterized by a nonactive loose complex $E_{NA}OAA{\cdot}Br^-$, from which oxaloacetate dissociates while the enzyme retains its nonactive conformation. In a later step, another Br^- is attached to stabilize the active species where the enzyme is protected by two Br^- from reacting with oxaloacetate. The lower pathway, which has a minor role in activation, is the dominating route of deactivation. The rate-limiting step is a bimolecular reaction between free active enzyme and oxaloacetate. The product of this rapid reaction is the extremely unstable complex E_AOAA which transforms to the stable nonactive complex.

In the present study we measured the interaction between substrate type activator and oxaloacetate with the enzyme. The level of the active enzyme is ascribed to equilibria between two complexes and their respective free ligands: nonactive cc[illegible] with oxaloacetate, active complex with activator, and the free acids. It is of interest to point out that the ratios of dissociation constants of the opposing effectors differ markedly from the

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[1] Abbreviations used: Act, activator; OAA, oxaloacetate; E_A and E_{NA}, active and nonactive forms of the enzyme, respectively; SDH, succinate dehydrogenase; ETP_H, phosphorylating submitochondrial particles; PMS, phenazine methosulfate; DCPIP, dichlorophenol indophenol; MalNEt, *N*-ethylmaleimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

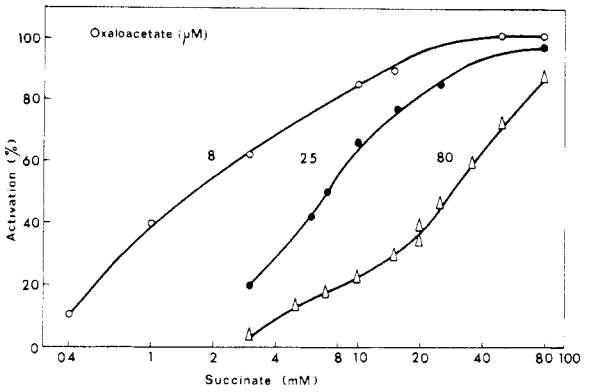

FIGURE 1: Equilibrium level of active succinate dehydrogenase in presence of succinate and oxaloacetate. ETP_H (1 mg/mL) was equilibrated with the indicated concentration of oxaloacetate and succinate at 30 °C for 30 min (0.25 M sucrose–25 mM Mes–1 mM KCN (pH 7.4)). Samples were removed and assayed at 15 °C (100% = 0.32 μmol min^{-1} mg^{-1}).

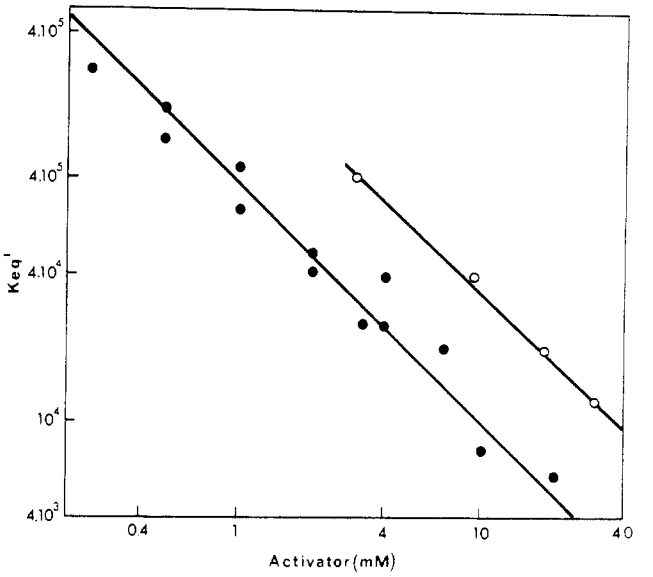

FIGURE 2: Dependence of observed equilibrium constant on activator concentration. Each point represents the reciprocal of oxaloacetate concentration giving 50% deactivation in presence of the indicated activator concentration. Data collected from Hill plots of experiments as in Figure 1. (○) Succinate; (●) malonate.

values reported for their interaction at the active (substrate-binding) site.

The mechanism of activation can be described by a simple model which accounts both for equilibrium level as well as for rates of activation.

Materials and Methods

ETP_H were prepared from beef heart mitochondria (Ringler et al., 1963) according to Hansen and Smith (1964). Succinate dehydrogenase activity was measured by the PMS-DCPIP[1] method as described before (Gutman, 1976). Samples from the activation reaction (containing 1 mg/mL ETP_H, 0.25 M sucrose, 25 mM Mes (pH 7.4), and the indicated concentrations of oxaloacetate and activators) were removed and assayed immediately at 15 °C. The rate of DCPIP reduction was measured from the initial slopes. Rate constants were calculated by least-square analysis. [U-^{14}C]Oxaloacetate, exchange of enzyme bound oxaloacetate, and determination of equilibrium level of enzyme-bound [^{14}C]oxaloacetate were all measured as described before (Gutman, 1976).

Results

The Effect of Oxaloacetate and Activator Concentration on the Fraction of Active Enzyme. If the role of activator is to stabilize the active form of the enzyme, then the fraction of the active enzyme should depend on concentrations of both negative and positive modulators. This is documented in Figure 1 where the fraction of active enzyme, equilibrated (30 min, 30 °C) with oxaloacetate and succinate, is drawn with respect to succinate concentration. It is evident that, while the same fraction of active enzyme can be achieved by different concentrations of oxaloacetate and succinate, it is uniquely determined by their ratio. Hill plots of such titrations measured either with succinate (plus KCN) or malonate yield parallel straight lines with a slope of $n = 1$.

The apparent equilibrium constant for deactivation [$K'_{eq} = ((SDH_{nonactive})/(SDH_{active}))\ (1/OAA)$] can be calculated from the oxaloacetate concentration which, in the presence of a given activator concentration, deactivates 50% of the enzyme (Gutman and Silman, 1976) (for correlation of K'_{eq} with defined parameters, see Discussion). The titrations shown in Figure 1 were repeated at various oxaloacetate and activator concentrations and the corresponding K'_{eq} values were calculated. The dependence of K'_{eq} on the activator concentration is given in Figure 2. It is evident that the higher the activator concentration is, the higher the oxaloacetate concentration needed to deactivate the enzyme. It should be pointed out that, unlike activation by Br^-, where the slope of the log-log plot is 2, the slope measured for substrate type activators is 1. This implies that the active complexes formed with Br^- or malonate have different stoichiometries.

Determination of Dissociation Constants Ratio of Ligands at the Regulatory Site. As seen in Figure 2, compared with succinate, malonate is a better stabilizer of the active enzyme. This difference can be accurately determined. We equate the nonactive fraction with the oxaloacetate–enzyme complex (Ackrell et al., 1974; Gutman and Silman, 1975; Gutman, 1976):

$$(SDH_{nonactive}) = (E_{N_A}OAA)$$

and the active fraction with the free plus activator stabilized enzyme

$$(SDH_{active}) = (E_A) + (E_A Act)$$

In the presence of high ligand concentrations, where the free enzyme concentration is negligible (see Discussion), we approximate

$$(E_A \cdot Act) + (E_{N_A}OAA) = 1$$

Under such conditions, the following equations:

$$K_{OAA} = \frac{(E)(OAA)}{(E \cdot OAA)} \quad (1)$$

$$K_{Act} = \frac{(E)(Act)}{(E \cdot Act)} \quad (2)$$

can be combined in eq 3:

$$\frac{1}{(SDH_{active})} = \frac{K_{Act}}{K_{OAA}} \frac{(OAA)}{(Act)} + 1 \quad (3)$$

This equation relates the reciprocal of the active fraction of the enzyme with either (OAA), or with $(Act)^{-1}$, with an intercept of 1. This equation justifies the empirical correlation of active

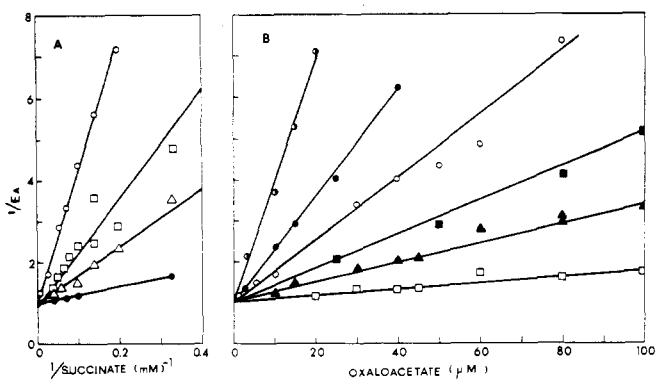

FIGURE 3: The dependence of active fraction of succinate dehydrogenase on oxaloacetate and activator concentration. ETP_H was equilibrated 30 min at 30 °C with indicated ligand concentration and assayed as in Figure 1. The lines are the best fit by least-square analysis. (A) The reciprocal of active fraction is drawn vs. 1/(succinate). The intercepts with the abscissa at the respective oxaloacetate concentrations are (○) 30 mM succinate for 80 μM oxaloacetate; (□) 17 mM succinate for 50 μM oxaloacetate; (△) 9.5 mM succinate for 25 μM oxaloacetate; (●) 3 mM succinate for 8 μM oxaloacetate. (B) The reciprocal of the active fraction is drawn with respect to oxaloacetate. The concentration of malonate and respective intercepts with abscissa are: (◑) 0.25 mM malonate and 3 μM oxaloacetate; (●) 0.5 mM and 7 μM; (○) 1 mM and 125 μM; (■) 2 mM and 24 μM; (▲) 4 mM and 42 μM; (□) 10 mM and 130 μM.

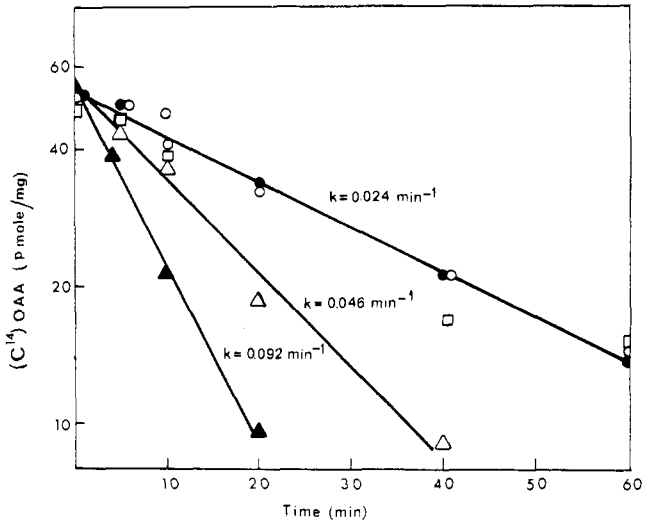

FIGURE 4: Effect of Br^- and malonate on exchange between enzyme bound and free oxaloacetate. ETP_H, labeled with [^{14}C]oxaloacetate as described under Materials and Methods, were suspended in 0.18 M sucrose–50 mM Mes containing 1 mM unlabeled OAA (30 °C). At times, samples were withdrawn, diluted 1:10 in 0.18 M sucrose–100 mM Tris-acetate (pH 8.0) containing 10 mM semicarbazide, and spun down. Protein and radioactivity of pellet were determined. The exchange was measured in the presence of: (○) 2 mM malonate (pH 7.4); (△) 0.15 M NaBr (pH 7.2); (▲) 0.5 M NaBr (pH 7.2); (□) control, no activator added (pH 7.2); (●) control, no activator added (pH 7.4). The corresponding K'_{eq} (see text) are: (●) 5×10^4 M^{-1}; (▲) 6×10^4 M^{-1}; (△) 1×10^6 M^{-1}.

enzyme and activator concentrations used by Kearney (1957).

Analysis of results measured with known concentrations of effectors is given in Figure 3. The intersection of the lines with the abscissa and the concentration of the second effector allows us to calculate K_{Act}/K_{OAA}. Figure 3A relates $1/(SDH_{active})$ vs. 1/(succinate). The ratio $K_{succinate}/K_{OAA}$ measured with 80, 50, 25, and 8 μM oxaloacetate is 375, 340, 380, and 375, respectively. Figure 3B relates $1/(SDH_{active})$ vs. (oxaloacetate). The value of $K_{malonate}/K_{OAA}$ ratio is 77, 97, 83, 76, 66, and 71 as measured with 10, 4, 2, 1, 0.5, and 0.25 mM malonate, respectively. For fumarate this ratio is 3000.

In a previous study (Gutman, 1976), we found with ETP_H, equilibrated with a constant amount of [^{14}C]oxaloacetate and increasing Br^- concentrations, a linear dependence of nonactive enzyme vs. bound oxaloacetate. It corresponded to 1 mol of bound oxaloacetate per mol of nonactive enzyme. In a similar experiment using malonate instead of Br^-, the same linear dependence was observed. In order to correlate the amount of bound oxaloacetate to the malonate concentration, we employ eq 4, analogous to eq 3.

$$\frac{1}{(E_{NA}[^{14}C]OAA)} = \frac{K_{OAA}}{K_{Act}} \frac{(Act)}{(OAA)} + 1 \qquad (4)$$

This treatment of the experimental results yields a straight line, indicating that 1 mol of oxaloacetate is bound in the nonactive complex and 1 mol of activator suffices to stabilize the active complex. The ratio $K_{malonate}/K_{OAA}$ measured is 84, in agreement with the results obtained from Figure 3B.

The Effect of Malonate on the Exchange between Free and Bound Oxaloacetate. In the presence of Br^-, the exchange between free and bound oxaloacetate is faster than the rate of activation, an indication that the dissociation of oxaloacetate precedes the appearance of the active enzyme (Gutman, 1976). Induction of such exchange by malonate would signify that the same pathway is common for activation by malonate and Br^-. Comparing the effects of two activators on a parameter not essentially associated with activation calls for conditions where their activation potential should be the same. This activation potential is quantitated by the value K'_{eq}; thus, in our experiment we used such concentrations (malonate, 2 mM, and $NaBr^-$, 0.5 M), which stabilize the active enzyme to the same extent. As seen in Figure 4, in the absence of activator there is a slow exchange of the enzyme bound oxaloacetate (k = 0.024 min^{-1}). This rate is slower than activation (see below). NaBr, 0.5 M ($K'_{eq} = 6 \times 10^4$ M^{-1}), enhances the exchange to 0.092 min^{-1}, but 2 mM malonate or 20 mM succinate (not shown) with the corresponding K'_{eq} values of 5×10^4 M^{-1} and 2×10^4 M^{-1} had no effect on rate of exchange. Even 0.15 M Br^-, a concentration at which the active enzyme is stabilized to 1/20 ($K'_{eq} = 10^6$ M^{-1}) compared with the level attained by 2 mM malonate, induces a significant enhancement of the exchange. Thus malonate or succinate activates the enzyme in a mechanism where dissociation of oxaloacetate does not precede the appearance of the active enzyme.

Effects of Malonate and Oxaloacetate on the Kinetics of Activation. Activation of succinate dehydrogenase is a reversible reaction which proceeds in a pseudo-first-order kinetics to a state of equilibrium. Measurement of the rate of this reaction (k_{obsd}) and the equilibrium level of active enzyme allow calculation of the pseudo-first-order rate constant of the partial reactions: activation and deactivation (Gutman, 1976).

$$k_{obsd} = k'_{activation} + k'_{deactivation} \qquad (5)$$

$$\frac{(SDH_A)}{(SDH_{NA})} = \frac{k'_{activation}}{k'_{deactivation}} \qquad (6)$$

Figure 5 depicts the effects of modulators on k_{obsd}. In Figure 5A we increased the concentration of malonate from 1 to 10 mM while oxaloacetate concentration was constant (40 μM). As seen, the higher the concentration of malonate, the higher is the final level of active enzyme (evident by increase of $A_\infty - A_i$) but the apparent rate of activation (k_{obsd}) is slower. When malonate concentration is constant (Figure 5B) (4 mM) and oxaloacetate concentration increases (from 15 to 100 μM),

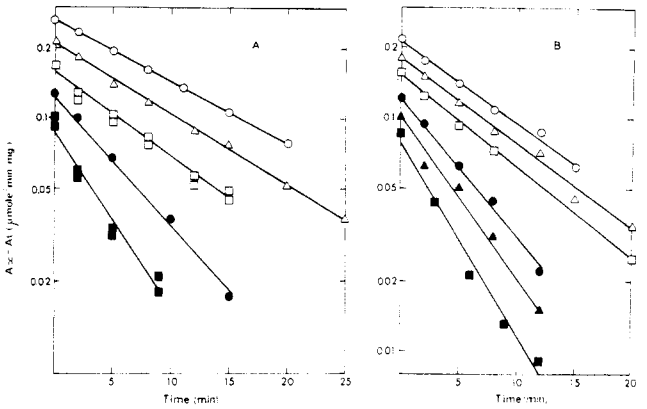

FIGURE 5: Kinetic analysis for approach to equilibrium experiments in presence of oxaloacetate and activator. ETP_H were washed by oxaloacetate (at the concentrations given in the figure) in 0.25 M sucrose–25 mM Mes (pH 7.4), and resuspended in the same buffer and indicated oxaloacetate concentration to 1 mg/mL. The activation (30 °C) was initiated by addition of malonate and samples were removed and assayed at 15 °C. Full activity and equilibrium level of active enzyme were measured in triplicate. The lines were drawn by best fit, least-mean-squares analysis. (A) Oxaloacetate, 40 μM; malonate, variable; (○) 10 mM malonate, k = 0.0612 min^{-1}, r = 0.994; (△) 5 mM malonate, k = 0.0704 min^{-1}, r = 0.998; (□) 2.5 mM malonate, k = 0.083 min^{-1}, r = 0.992; (●) 1.5 mM malonate, k = 0.138 min^{-1}, r = 0.998; (■) 1 mM malonate, k = 0.177 min^{-1}, r = 0.974. (B) Malonate, 4 mM; oxaloacetate, variable; (○) 15 μM oxaloacetate, k = 0.0782 min^{-1}, r = 0.996; (△) 30 μM oxaloacetate, k = 0.0853 min^{-1}, r = 0.994; (□) 45 μM oxaloacetate, k = 0.0864 min^{-1}, r = 0.996; (●) 60 μM oxaloacetate, k = 0.137 min^{-1}, r = 0.997; (▲) 80 μM oxaloacetate, k = 0.175 min^{-1}, r = 0.984; (■) 100 μM oxaloacetate, k = 0.192 min^{-1}, r = 0.993.

we observe a decrease in equilibrium level of active enzyme (decrease in $A_\infty - A_t$) accompanied by acceleration of rate of activation.

The dependence of the rate constants of the partial reactions on the modulator concentrations is given in Figure 6. As shown in Figure 6A, the rate of deactivation increases linearly with oxaloacetate concentration demonstrating the bimolecular nature of the rate-limiting step. The reactants of this rate-limiting step are oxaloacetate and the free active enzyme E_A. As discussed by Vinogradov et al. (1976), the rate of inactivation of enzyme by irreversible inhibitor reacting with the substrate binding site is

$$-\frac{d(E)}{dt} = k[I]\left(\frac{K_s}{K_s + [S]}\right) E_0$$

This is a differential equation for a bimolecular second-order reaction where the concentration of the free enzyme is lowered by the presence of the substrate with the respective K_s. The same equation is applicable in our case for describing the dependence of the pseudo-first-order rate of deactivation ($k'_{deactivation}$) on activator concentrations. Under conditions where [I] is high enough to ensure pseudo-first-order kinetics, as in our case (Figure 5), the observed rate of deactivation can be related with malonate concentration as in eq 7.

$$\frac{1}{k'_{deactivation}} = \frac{1}{k_b[I]}\left(1 + \frac{[S]}{K_s}\right) \qquad (7)$$

where k_b is the second-order rate constant for binding of oxaloacetate. Redrawing Figure 6B as $1/k'_{deactivation}$ vs. [malonate] yields a straight line (r = 0.9979) with intercept of the abscissa at $K_{malonate}$ = 0.166 mM. The true second-order rate constant for binding of oxaloacetate, calculated from the intercept of the ordinates, is $k_b = 1.24 \times 10^6$ M^{-1} s^{-1}. It is a surprise that the true rate of activation (k_a) is affected neither

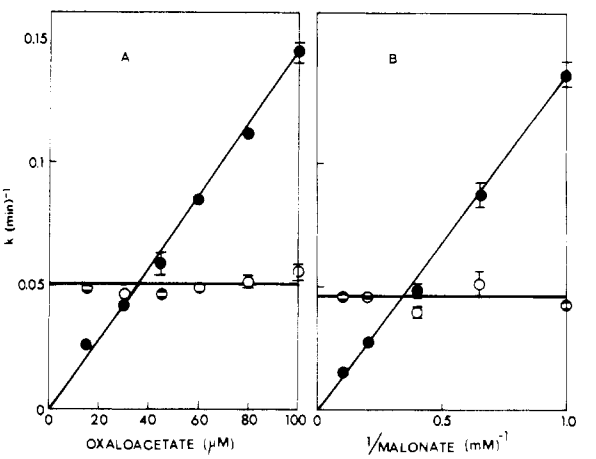

FIGURE 6: The dependence of the first-order rate constant of activation and pseudo-first-order rate constant of deactivation on the effectors concentrations. The rate constants were calculated from the data given in Figure 6A and 6B using eq 5 and 6. (A) Results extracted from Figure 6A. (B) Results extracted from Figure 6B. (●) $k'_{deactivation}$; (○) $k_{activation}$.

by activator nor oxaloacetate concentration (Figure 6A and 6B). It is in contrast with the effect of Br^- on the rate of activation (Gutman, 1976). This is taken as further evidence that the mode of activation by substrate is different from that caused by Br^-. We conclude that, in the presence of substrate type activators, activation is a true first-order conversion of nonactive complex to an active form, prior to the dissociation of oxaloacetate. Finally the accuracy of our quantitative analysis is documented in Table I where the predicted rate constants and the final level of active enzyme are compared with the measured parameters. It should be emphasized that the only parameter needed to calculate the rates of activation by fumarate was $K_{fumarate}/K_{OAA}$.

Discussion

The apparent equilibrium constant K'_{eq} can be expressed by defined equilibrium parameters. Using the same assumptions needed to derive eq 3 or 4, the following relationship is obtained:

$$K'_{eq} = \frac{(SDH_{nonactive})}{(SDH_{active})}(OAA) = \frac{K_{Act}}{K_{OAA}}\frac{1}{(Act)^n} \qquad (8)$$

where n is the number of activator molecules in the active enzyme complex. For inorganic anions as Br^-, n = 2 (Gutman, 1976). For dicarboxylic activators n = 1 (Figure 2). This indicates that at the regulatory site 1 mol of substrate type activator suffices to stabilize the active form.

The parallel lines drawn for succinate and malonate in Figure 2 reflect the difference in relative stabilities of the corresponding active complexes with respect to the common nonactive complex with oxaloacetate (see Table II). The validity of the assumptions needed to derive eq 3, 4, and 8 is demonstrated by the fact that the same ratio is measured either by enzymic activity or from the amount of bound [^{14}C]oxaloacetate.

Our equilibrium studies measured only the ratio between the association constants, not the absolute values. Still we can compare the relative binding constants of the various ligands to the regulatory site (as measured above), with those measured for the substrate binding site (K_i or K_s). These values appear in Table II. The K_i and K_s were measured with fully activated enzyme; thus these values reflect the relative affinities of oxaloacetate with respect to substrates or competitive in-

TABLE I: Measured and Calculated Rate Constants for Activation of Membrane Bound Succinate Dehydrogenase by 40 mM Fumarate in Presence of 25 μM Oxaloacetate.

	Measured[a]	Predicted
k'_{obsd}	0.116 ± 0.002 min^{-1}	0.119[b] min^{-1}
$k'_{activation}$	0.0436 ± 0.004[b] min^{-1}	0.048 ± 0.0067[c] min^{-1}
$k'_{deactivation}$	0.0726 ± 0.0035[b] min^{-1}	0.071[d] min^{-1}
E_A/E_{NA}	0.59 ± 0.075	0.53

[a] Mean of three experiments. [b] Calculated according to eq 5 and 6. [c] Mean value for k_a taken from Figures 7a and 7b. [d] Calculated from eq. 7, using $k_b = 1.24 \times 10^6$ M^{-1} and $K_{fumarate} = 6.4$ mM.

TABLE II: Dissociation Constants of Modulators of Succinate Dehydrogenase from the Substrate Binding Site and the Regulatory Site.

	Substrate binding site		Regulatory site	
Ligand	K_s (μM)	K_{ligand}/K_{OAA}	K (μM)	K_{Act}/K_{OAA}
Oxaloacetate (K_i)	1.5[a]		2.13	
Malonate (K_i)	18[a]	12	166	78 ± 10
Succinate	110[b]	73	800	370 ± 18
Fumarate	1300[a]	866	6400	3000

[a] Dervartanian and Veeger (1964). [b] Zeijlemaker et al. (1969).

hibitor reacting at substrate binding site. If the same site was functioning both in substrate oxidation and in regulation, then the ratios measured by the two methods should be the same. Since they differ markedly, we should consider the possibility that the site where oxaloacetate regulates is not the one where substrate is oxidized.

These conclusions are further supported by the difference between $K_{malonate}$ (166 μM) as calculated from the data presented in Figure 6B and K_i for malonate (10 μM, Vinogradov et al., 1976; 18 μM, Dervartanian and Veeger, 1964). Table II lists the K_{Act} values for the other ligands when reacting with the regulatory site. In all cases there is a marked difference between K_{Act} and the corresponding values for dissociation from the substrate binding sites.

It is impossible for the reaction of the same ligand with the same site to have two dissociation constants. Consequently the enzyme has two sites, one for substrate oxidation and the other for regulation. The possibility that the two K values reflect negative cooperativity between two identical sites of the dimeric form of the enzyme is ruled out. This is because linear correlations between nonactive enzyme and bound oxaloacetate were measured under conditions where the enzyme was mostly dissociated (Kenney et al., 1976).

The thiohemiacetal linkage of oxaloacetate with the nonactive enzyme first suggested by Vinogradov et al. (1972) was based on the fact that, after binding 25 mol of Ag^+ to SH groups per mol of the succinate dehydrogenase, very little oxaloacetate was bound to the enzyme. Later, Kenney et al. (1976) documented that one SH group, essential for enzymic activity, is barred from reacting with MalNEt either by oxaloacetate or by activators. This does not mean that the two effectors prevent the reaction by the same mechanism. Vinogradov et al. (1976) demonstrated that malonate or succinate protect this SH group by associating with the enzyme at a site with dissociation constants identical with the corresponding K_i or K_s values. Thus there is no doubt that this SH group is at the active site and binding of substrate protects it from reacting with alkylating agents.

As documented in this study the substrate binding site is not the regulatory site. How then can oxaloacetate protect the substrate binding site while reacting at a different site? This ambiguity can be explained by assuming that oxaloacetate protects the essential SH group not by forming thiohemiacetal but by altering the conformation of the enzyme around its active site. This model accounts both for the observations of Kenney et al. (1976) and ours. Oxaloacetate alters the configuration of the enzyme and in its nonactive form the substrate binding site is closed (as evident from lack of reactivity of MalNEt with the essential SH group). Activators which displace oxaloacetate from the regulatory site activate the enzyme (Ackrell et al., 1974) but as they also react as substrates and $K_s < K_{Act}$ they occupy the active site thus blocking the SH from reacting with MalNEt.

As demonstrated in Figure 2, 1 mol of succinate or malonate, compared with $2Br^-$, suffices to stabilize the active enzyme. If the negative charges of the activator play a role in activation, then due to the proximity of the two carboxyls, it is unlikely to find with double-charged ligand a complex analogous to the single charged species E_{NA}·OAA·Br^- (Gutman, 1976). This complex is the exchanging species from which oxaloacetate dissociates prior to the formation of the active enzyme. We measured the rate of exchange with 2 mM malonate and 0.5 M Br^-, effector concentrations which stabilize the active enzyme to the same level. The Br^- enhanced exchange while malonate did not (Figure 4). The inability of malonate to induce exchange is further emphasized by the fact that 0.15 M Br^-, a concentration which hardly stabilizes the active enzyme in comparison with 2 mM malonate, still accelerates the exchange. Thus the mechanism of activation by malonate is not identical with that carried out by Br^-, the pathway where exchange precedes activation is absent. As activation by malonate proceeds through a single pathway, the kinetic analysis of activation is much simpler.

Our kinetic studies were carried out under conditions where all components—activator, free oxaloacetate, active and nonactive enzyme—approached their equilibrium concentration. Because of the high concentrations of the ligands with respect to the enzyme, both activation and deactivation followed pseudo-first-order reaction. For such systems the observed rate constant of the approach to final equilibrium is the sum of the forward and backward rate constants and the final level equal to their ratio.

As documented in Figure 6, the rate of deactivation is inversely dependent on malonate concentration and increases linearly with oxaloacetate concentration, indicating that the rate-limiting step in deactivation is the bimolecular reaction between oxaloacetate and the free form of active enzyme (E_A + OAA → E_{NA}OAA) (Gutman, 1976). A surprising observation is that the true rate constant of activation is independent of malonate concentration (Figure 6B), indicating that in the course of activation the active enzyme precedes the formation of the enzyme-malonate complex. Furthermore, the rate of activation is independent of oxaloacetate concentration (Figure 6A), suggesting that activation takes place before the dissociation of oxaloacetate from the complex (E_{NA}·OAA → E_AOAA) (where dissociation preceding activation then rate of activation would depend on $(\text{oxaloacetate})^{-1}$ the same way as deactivation varies with $(\text{malonate})^{-1}$). These conclusions are combined in the following model which accounts both for

kinetic and equilibrium behavior of the enzyme.

$$E_{N_A}OAA \underset{}{\overset{k_a}{\rightleftharpoons}} E_AOAA \underset{k_b}{\rightleftharpoons} E_A + OAA$$

$$E_A + \text{activator} \leftrightarrow E_A \text{ activator}$$

This model accounts for the following observations: (1) neither activator nor oxaloacetate concentration affect the true rate of activation (Figure 6) but both affect its equilibrium level; (2) both activator and oxaloacetate concentration affect the rate of deactivation (Figure 6); (3) substrate type activators activate the enzyme in a mechanism where activation precedes dissociation (Figure 4).

This model is in accord with the two assumptions made before: (A) in the presence of oxaloacetate, the concentration of free enzyme E_A is negligible (this is because of the high affinity of E_A for oxaloacetate (Table II) and the rapid reaction between them); (B) the concentration of E_AOAA is very small. This is evident from the fact that, in the absence of activator, enzyme equilibrated with oxaloacetate is virtually devoid of catalytic activity; as the assay is very sensitive the fraction of E_AOAA cannot exceed 0.1% (see also Gutman, 1976).

Finally, as demonstrated in Table I, this model can combine kinetic equations and thermodynamic parameters to predict the rate of activation and its final level for any combination of oxaloacetate and substrate type activators.

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