The Reaction of N-Ethylmaleimide at the Active Site of Succinate Dehydrogenase

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SUMMARY

Since 1938 mammalian succinate dehydrogenase has been thought to contain thiol groups at the active site. This hypothesis was questioned recently, because irreversible inhibition by bromopyruvate and N-ethylmaleimide appeared not to satisfy the requisite criteria for reaction at the active site. These recent observations of incomplete inactivation of succinate dehydrogenase by N-ethylmaleimide and incomplete protection by substrates can, however, be explained adequately by the presence of oxalacetate and other strong competitors of the inactivation process in the enzyme used in these studies. Substrates, competitive inhibitors, and anions which activate succinate dehydrogenase protect the enzyme from inhibition by N-ethylmaleimide.

Inhibition of succinate dehydrogenase by N-ethylmaleimide involves at least two second order reactions which are pH dependent, with \( pK_a \) values of 8.0 to 8.2. This pH dependence, the known reactivity of N-ethylmaleimide toward thiols, and the protection by substrate and competitive inhibitors indicate that sulfhydryl residues are required for catalytic activity and perform an essential, not secondary, role in the catalysis. Just as the presence of tightly bound oxalacetate prevents inhibition by N-ethylmaleimide, alkylation of the sulfhydryl residue(s) at the active site prevents the binding of \([^{14}C] \text{oxalacetate} \). Thus, these thiol groups at the active site also may be the site of tight binding of oxalacetate during the activation-deactivation cycle.

In 1938 Hopkins and coworkers (1, 2) reported that heart muscle succinate dehydrogenase is reversibly inhibited by reagents capable of oxidizing thiol groups and that this inhibition is prevented by succinate and malonate, a competitive inhibitor of the enzyme. They suggested that sulfhydryl residues are essential for the activity of the enzyme. These observations were extended to a variety of alkylating and mercaptide-forming thiol reagents (3) and were later confirmed with highly purified preparations of the enzyme (4).

Questions arose more recently concerning the location of the thiol groups involved in this inhibition. Thus, neither the pentapeptide of the covalently bound FAD (5) nor the much larger tryptic FAD peptide (6) contained a cysteinyl residue. This observation may be rationalized by assuming that the substrate binding site is juxtaposed to the flavin in the tertiary structure of the enzyme to form the catalytic site (7).

More recent studies using bromopyruvate (8) or MalNEt (9) as irreversible inhibitors of succinate dehydrogenase suggested that the thiol group(s) reacting was not at the catalytically active site but at a secondary site and that the resulting loss of activity was an indirect effect. This interpretation was based on the observation (8, 9) that inhibition by MalNEt and bromopyruvate was incomplete, and substrate or malonate only partially protected the enzyme from inactivation.

The studies of Sanborn et al. (8) were performed with enzyme preparations that represented mixtures of the activated and deactivated forms of the enzyme and were performed in the presence of relatively high phosphate concentration (8, 9). These circumstances appeared pertinent to the results obtained, because in the deactivated form the enzyme is known to contain tightly bound oxalacetate (10, 11), which may have prevented reaction with the alkylating agents. In addition, phosphate which has long been known to be an activator of the enzyme (12) also may have interfered with reaction of the enzyme with bromopyruvate and MalNEt. The results cited (8, 9) could, therefore, have been due to the experimental conditions.

Succinate dehydrogenase is known to undergo activation-deactivation under a variety of conditions (13) and, as usually isolated, exists as a mixture of activated and deactivated forms. The recent discovery that succinate dehydrogenase can be obtained in the fully activated state by incubation with certain anions at mildly acid pH and that the tightly bound oxalacetate and activating anions subsequently may be removed without significant deactivation (14) provides a way to circumvent the problems inherent in the investigation of the inhibition of the enzyme.

The abbreviations used are: MalNEt, N-ethylmaleimide; DCIP, 2,6-dichlorophenolindophenol; Mes, 2-(N-morpholino)ethanesulfonic acid; Hepes, N-2-hydroxyethylpipерази新生-2-ethanesulfonic acid.
Soluble succinate dehydrogenase was obtained from an acetone powder of beef heart mitochondria according to the procedure of Bernath and Singer (15). NaBr was added to the enzyme eluted at the calcium phosphate gel step to a final concentration of 1 M, the pH adjusted to 6.5 with 0.5 M H3SO4, and the enzyme was incubated at 25° until full activation was obtained (15 to 25 min). The solution was taken to 0° and saturated ammonium sulfate was added to a final concentration of 30%. After centrifugation of the resulting suspension at 37,000 × g for 10 min, the supernatant solution was brought to 50% saturation in ammonium sulfate and recentrifuged as above. The precipitated enzyme was dissolved in a minimal volume of buffer and desalted on a column of Sephadex G-50 (Pharmacia) equilibrated with the buffer used in the inhibition studies.

Succinate dehydrogenase activity was measured at 15° by the phenazine methosulfate-DCIP assay (16) at fixed (0.1 mM) phenazine methosulfate concentration. Preincubation of the enzyme with 20 mM succinate for 6 min at 38° results in activation of the deactivated form of the enzyme. The active form of the enzyme is given by the activity without preincubation and the sum of the active and deactivated forms and by the activity after preincubation with succinate, i.e., the maximal activity. Levels of activation are expressed in terms of activity observed without preincubation as a percentage of the maximal activity. Protein was determined by the biuret method (17) and covalently bound flavin was determined as previously described (18) and was used to estimate the succinate dehydrogenase concentration, assuming a molecular weight of 100,000 (19).

For the enzymatic generation of [3H]oxalacetate, 0.35 mM [3H]aspartate (uniformly labeled, the Radiochemical Centre) and 7.0 mM oxalacetate were incubated in 50 mM Hepes, pH 7.0, at 25° for 30 min, in the presence of 0.02 mg/ml of glutamate-oxalacetate transaminase (Boehringer). Under these conditions 70% of the aspartate is converted to oxalacetate. This solution is then taken to 15° and succinate dehydrogenase is added (0.3 nmol of bound flavin per nmol of aspartate originally used). After incubation for 15 min, the enzyme solution is passed through Sephadex G-50 at 4° to remove excess reagents. The enzyme fraction is assayed for radioactivity and bound flavin to determine the extent of the incorporation of oxalacetate. The MalNEt-treated enzyme was prepared by incubation of the enzyme (20 µM) with 0.25 mM MalNEt for 30 min at 15° prior to the oxalacetate incubation step.

N-Ethyl (2,3-14C)maleimide was obtained from the Radiochemical Centre. Concentration of MalNEt was determined spectrophotometrically assuming ε295 = 620 M−1 cm−1 (20). All chemicals used in this research were of reagent grade quality.

In the evaluation of kinetic data on the inhibition by excess MalNEt, it was assumed that two reactive forms of the enzyme were present and thus two rate constants are necessary to describe the composite residual activity. The loss of activity was expressed as the sum of the loss of activity of the individual forms:

\[ A_0 - A = f_1 e^{-k_1 t} + f_2 e^{-k_2 t} \]  

where \( A_0 \) is the fraction of total activity remaining after time \( t \); \( f_1 \) and \( f_2 \) are the fractions of total activity in the two reactive species and are the values obtained from the intercept at zero time. Thus, the pseudo-first order rate obtained after >90% of the enzyme had reacted was extrapolated back to zero time. The values obtained from this line then were subtracted from the observed residual activity at a given time to obtain the pseudo-first order rate for the faster reaction. The slope of this latter line was used to determine \( k_1 \), the pseudo-first order rate constant, and \( k_2 \), the fast second order rate constant for MalNEt inhibition, was evaluated from the relationship \( k_2 = k_1 k_{MalNEt} \). Likewise, the slope for the slower reaction was used to determine \( k_2 \), the pseudo-first order, and \( k_2 \), the slow second order rate constant. (See for example, Fig. 2.)

RESULTS

Inhibition of Succinate Dehydrogenase by MalNEt—One of the first tests for reaction at the active site of an enzyme by an irreversible inhibitor is that complete loss in activity will occur when the inhibitor is in sufficient excess. Recent findings indicate that reaction of succinate dehydrogenase with MalNEt or bromopyruvate (8) did not fulfill this criterion, in contrast to earlier investigations (1-4) on the inhibition of succinate dehydrogenase with thiol-specific reagents. Fig. 1 demonstrates, however, that the inactivation of succinate dehydrogenase essentially goes to completion following pseudo first order kinetics to greater than 90% inactivation in the presence of excess MalNEt.

When the inhibition is examined in greater detail, it is observed that the inhibition is biphasic and the slower rate follows first order kinetics (Fig. 2). Subtraction of the degree of inactivation corresponding to this slower phase from the experimental values observed during the faster, initial phase indicates that the initial loss in activity also follows first order kinetics. Thus, the inhibition of succinate dehydrogenase by excess MalNEt may be described as the sum of two first order reactions.

In the presence of substrate and competitive inhibitors, little, if any, inactivation by MalNEt occurs (Table I). At low concentrations of oxalacetate and L-malate, succinate dehydrogenase is deactivated (10) and consequently, the degree of activity remaining must be determined after activating the enzyme with succinate.

These results may be contrasted with the results of Sanborn...
**Fig. 2.** Semilog plot of inhibition of succinate dehydrogenase by MalN\(\text{Et}\). Succinate dehydrogenase (1.5 \(\mu\)M) was incubated at 15\(^\circ\) with 0.2 \(\mu\)M MalN\(\text{Et}\) in 50 mM Hepes, pH 7.0. Observed activity remaining at indicated time (---); extrapolation of slower rate to zero time (-----); line obtained after subtraction of slower rate from observed activity (- - -).

**Table I**

*Protection of succinate dehydrogenase by substrate and competitive inhibitors from inactivation by MalN\(\text{Et}\)*

Succinate dehydrogenase (5 to 25 \(\mu\)M) was incubated with the reagents listed in 50 mM Hepes, pH 7.0, at 15\(^\circ\), unless otherwise noted. Then MalN\(\text{Et}\) was added, incubation was continued, and activity was measured at the time indicated.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration(\mu)M</th>
<th>MalN(\text{Et})</th>
<th>Time of incubation min</th>
<th>Activity remaining %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.10</td>
<td>30</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Succinate(a)</td>
<td>16.0</td>
<td>0.10</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Fumarate(a)</td>
<td>4.5</td>
<td>0.09</td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>0.074</td>
<td>0.36</td>
<td>10</td>
<td>100(b)</td>
</tr>
<tr>
<td>L-Malate</td>
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<td>0.20</td>
<td>10</td>
<td>97(a)</td>
</tr>
<tr>
<td>L-Malate</td>
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<td>0.19</td>
<td>10</td>
<td>80(b)</td>
</tr>
<tr>
<td>Malonate</td>
<td>1.0</td>
<td>0.24</td>
<td>30</td>
<td>91</td>
</tr>
<tr>
<td>Malonate</td>
<td>5.0</td>
<td>1.09</td>
<td>40</td>
<td>91</td>
</tr>
</tbody>
</table>

* In incubation at 0\(^\circ\) in 50 mM Tris-HCl, pH 7.4.

PROTECTION BY MALONATE AGAINST INHIBITION BY MALN\(\text{Et}\)---

et al. (8) and Felberg and Hollocher (9) who noted incomplete protection by substrates and competitive inhibitors when succinate dehydrogenase was treated with bromopyruvate and MalN\(\text{Et}\). A likely reason for this was the use of high phosphate concentrations in their experiments. Inorganic phosphate is a known activator of the enzyme (12) and furthermore it is expected to act like nitrate and bromide which, as shown below, protect the active site of the enzyme from inactivation by irreversible inhibitors. The present results strongly indicate that reaction of succinate dehydrogenase is at or near the substrate binding site.

**Fig. 3.** Effect of malonate on inhibition of succinate dehydrogenase by MalN\(\text{Et}\). The enzyme (1.5 \(\mu\)M) was incubated at 25\(^\circ\) for 15 min with 0(A), 1.8(B), 0.5(C), and 48 \(\mu\)M(D) malonate in 50 mM Mes, pH 6.3. The enzyme solution was taken to 15\(^\circ\) and MalN\(\text{Et}\) was added to a final concentration of 0.10 \(\mu\)M. The loss in activity with time was followed using the phenazine methosulfate-DCIP assay at 0.1 \(\mu\M phenazine methosulfate.

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Protection by succinate and competitive inhibitors toward inhibition by MalN\(\text{Et}\) is dependent upon the relative concentrations of enzyme, protecting agent, and MalN\(\text{Et}\). Although at intermediate and high concentrations substrates activate the enzyme, at very low (<0.1 \(\mu\)M) concentrations succinate and fumarate deactivate the enzyme, as do oxalacetate and d- and L-malate (11). Deactivation by fumarate is due to oxidation by oxalacetate by the dehydrogenase (10, 11). In addition, succinate and malate can reduce the enzyme, while fumarate can oxidize it. These multiple reactions may obscure protective effects against alkylating agents. In contrast, malonate does not alter the oxidation-reduction state of the enzyme and, thus, with fully activated preparations its only action is the formation of a reversible enzyme-inhibitor complex. Its effect on the inhibition of succinate dehydrogenase by MalN\(\text{Et}\) can, therefore, be unambiguously interpreted. Fig. 3 presents the results of such an investigation. It is seen that increasing concentrations of malonate decrease the rate of inhibition of succinate dehydrogenase at a fixed concentration of MalN\(\text{Et}\), and that at sufficiently high concentrations of malonate essentially complete protection is obtained (Fig. 3, Table I).

Incorporation of Oxalacetate into MalN\(\text{Et}\)-treated Enzyme---

Inubation of fully activated succinate dehydrogenase with \(^{14}\)C-oxalacetate results in deactivation of the enzyme. Analysis of the enzyme fraction after Sephadex passage to remove excess reagents shows that 1 mol of tightly bound oxalacetate (measured by specific radioactivity) is incorporated per mol of enzyme.* If the enzyme is treated with MalN\(\text{Et}\) prior to incubation with \(^{14}\)C-oxalacetate, less than 0.1 mol of oxalacetate is incorporated per mol of flavin. Thus, it is emphasized that not only does oxalacetate prevent the inhibition of succinate dehydrogenase by MalN\(\text{Et}\) but also the enzyme previously inhibited with MalN\(\text{Et}\) does not bind \(^{14}\)C-oxalacetate.

* Chemical analysis indicates that 0.5 mol is assayable as oxalacetate, the remainder in part as \(\text{CO}_2\) plus pyruvate and an additional as yet unidentified compound. W. C. Kenney, B. A. C. Ackrell, and E. B. Kearney, (1974) unpublished results.
Inhibition by MalNEt of active form of succinate dehydrogenase

Succinate dehydrogenase preparations containing a fraction of the enzyme in active form were obtained by limited activation at mildly acidic pH (14) in lieu of bromide incubation after the calcium phosphate step. Sephadex chromatography was performed in 1 mM Hepes, pH 7.0, and aliquots of the enzyme (2 to 10 µM) then were incubated at 0°C with MalNEt at the concentrations and pH given. The fraction of the enzyme in the active form is determined as described under “Materials and Methods.” The loss in maximal activity (activity at zero time minus the observed activity) was followed with time until a plateau, or constant, level was obtained. Buffers used were: pH 7.0 and 7.8, 50 mM Hepes; pH 6.0, 50 mM Mes.

<table>
<thead>
<tr>
<th>Fraction of succinate dehydrogenase in active form</th>
<th>MalNEt</th>
<th>pH</th>
<th>Loss of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>µM</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>100%</td>
<td>0.10</td>
<td>7.0</td>
<td>95</td>
</tr>
<tr>
<td>73</td>
<td>0.25</td>
<td>7.0</td>
<td>63</td>
</tr>
<tr>
<td>54</td>
<td>0.70</td>
<td>7.0</td>
<td>57</td>
</tr>
<tr>
<td>32%</td>
<td>0.50</td>
<td>7.0</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>7.0</td>
<td>3</td>
</tr>
<tr>
<td>73</td>
<td>0.25</td>
<td>7.8</td>
<td>63</td>
</tr>
<tr>
<td>54</td>
<td>0.70</td>
<td>7.8</td>
<td>60</td>
</tr>
<tr>
<td>18%</td>
<td>0.25</td>
<td>7.8</td>
<td>22</td>
</tr>
<tr>
<td>54</td>
<td>0.70</td>
<td>6.0</td>
<td>55</td>
</tr>
<tr>
<td>60%</td>
<td>0.50</td>
<td>6.0</td>
<td>70</td>
</tr>
</tbody>
</table>

* Bromide-treated enzyme.

Preincubated at given pH for 30 min at 25°C prior to MalNEt treatment.

Inhibition of Active Form of Succinate Dehydrogenase—Succinate dehydrogenase generally is isolated as a mixture of activated and deactivated forms and thus requires prior incubation with substrate or various other agents (13) in order for the full activity to be expressed. When a mixture of activated and deactivated forms is incubated with MalNEt, inhibition follows the kinetic pattern of the curve given in Fig. 2. However, on further incubation of the MalNEt-treated enzyme with succinate at 38°C for 6 min, considerable catalytic activity is obtained in assays at 15°C. Inasmuch as inhibition by MalNEt is irreversible, the return of activity must be due to activation of the deactivated form of the enzyme by succinate. Thus, at any time during MalNEt treatment the activity measured without prior incubation with succinate represents the activated form of the enzyme which has not yet been alkylated, whereas the additional activity found in assays after activation with succinate represents the deactivated (and uninhibited) enzyme fraction. Of the two enzyme forms, then, only the activated form is susceptible to inhibition by MalNEt (Table II), and as a corollary, the deactivated form is not inhibited by this agent. Control experiments have shown that dilution of the enzyme into the assay reaction mixture containing succinate stops the inhibition by MalNEt. Thus, another parameter which must be closely controlled is the means by which the residual enzymatic activity is measured, that is, whether one examines the total activity after succinate activation or the residual activity of the activated form. Use of fully activated enzyme has made it possible in a large part to circumvent this problem and thus to demonstrate that succinate dehydrogenase is inactivated completely by MalNEt.

Effect of MalNEt Concentration, Temperature, and pH on Rate Constants for Inactivation—Table III presents data of the effect of various parameters on the inhibition of succinate dehydrogenase by MalNEt. The second order rate constants, k1 and k2, are obtained by dividing the pseudo-first order rate constants, calculated as described under “Materials and Methods,” by the MalNEt concentration. Over a fairly wide range in MalNEt concentration, k1 is constant at 3.8 min⁻¹ mM⁻¹ at pH 7.0. This, however, is not so for k2 which decreases with increasing MalNEt concentration. These findings are suggestive of the possibility of a MalNEt binding site, i.e., an approach to saturation.

As expected, the rate constants increase with temperature (Table III). From these data an activation energy of 9 to 10 kcal/mol is obtained for the reaction of MalNEt with succinate dehydrogenase.

The rate of inhibition of succinate dehydrogenase by MalNEt increases with increasing pH (Table III). The relationship of the observed rate constant, kobs, with pH can be expressed as:

\[
k_{\text{obs}} = \frac{k_{\text{max}}}{1 + [H^+] / K_a}
\]

which can be rewritten in the form

\[
\frac{1}{k_{\text{obs}}} = \frac{1}{k_{\text{max}}K_a[H^+]} + \frac{1}{k_{\text{max}}}
\]

where \(K_a\) is the dissociation constant of a group reacting and \(k_{\text{max}}\) is the second order rate constant for the conjugate base of this group. Plotting \(1/k_{\text{obs}}\) versus \(H^+\) will yield values for \(k_{\text{max}}\) and \(K_a\). Considering the data of Table III for pH ≥ 7.0, values of \(k_{\text{max}} = 42\ \text{min}^{-1}\ \text{mM}^{-1}\) and \(K_a = 8.0\) are obtained for the fast step. At acid pH values deviation from linearity occurs, indicating that the protonated form, i.e., —SH, may also react, although very slowly. Similar values for the slow step are \(k_{\text{max}} = 10\ \text{min}^{-1}\ \text{mM}^{-1}\) and \(K_a = 8.2\). Considering the known reactivity of MalNEt, the values for \(K_a\) are consistent with the participation of thiol residues in the inactivation step.
Effect of Anions on Inhibition of Succinate Dehydrogenase by MalNEt—Certain anions are known to activate succinate dehydrogenase (14), but the combination of activating anions with the enzyme has not yet been demonstrated. Protection of the enzyme by anions against MalNEt inhibition offered an opportunity to gather evidence for such combination. It was found that with increasing concentration of these anions, the rate of inactivation of the enzyme by MalNEt decreased correspondingly. Inasmuch as the enzyme was fully activated, the effect cannot be due to activation. Thus, anions which activate succinate dehydrogenase also complex with the enzyme, at or near the active site, and in so doing, like malonate, succinate, or oxalacetate, protect the enzyme. Indeed, it may be such an enzyme-anion complex formation which accounts for activation of the enzyme by anions.

The second order rate constant for the reaction between MalNEt and the enzyme-modifier (anion) complex, $k_m$, and the dissociation constant, $K_D$, for this complex can be calculated following the treatment of O'Sullivan and Cohn (21). The relationship of interest is:

$$k^* = \frac{k_m}{k_1} - \frac{K_D}{k_1} \left( \frac{k^*}{k_1 - 1} \right)$$

$k^*$ is the observed rate constant at a given concentration of modifier and $k_1$ is the constant obtained in the absence of modifier. A plot of $k^*/k_1$ versus $-(k^*/k_1 - 1)/[modifier]$ will have a slope of $K_D$ and an intercept on the ordinate of $k_m/k_1$ from which $k_m$ can be obtained. Table IV lists the dissociation constants and second order rate constants of several anions and modifiers obtained in this way.

Perturbations due to ionic strength differences do not explain these results, because the inhibition at 150 mM Hepes, pH 7.0, was comparable to that at 50 mM Mes, pH 7.0. The ionic strength of the former buffer is comparable to the conditions for the highest anion concentration used for the determination of the results of Table IV.

**DISCUSSION**

Fully activated succinate dehydrogenase is completely inhibited by MalNEt. The reaction follows biphasic kinetics (Fig. 2) which may be described as the sum of two pseudo first order reactions, a fast and a slow one, the latter being 10 to 17% of the former. Substrate and competitive inhibitors protect the enzyme from inactivation (Table I), indicating that MalNEt may be reacting at the active site of the enzyme. All this confirms the long held concept that —SH groups are essential for catalytic activity and are located at the substrate site. Further evidence for this is the finding of a pK, of 8.0, compatible with the ionization of an —SH group, for the rate constant for inhibition.

Although there are a large number of cysteinyl residues in the enzyme (22, 23), most of these do not react under the conditions used in this study. Unpublished results of the author have shown that when inhibition by MalNEt reached 90%, at most 2 mol of MalNEt were incorporated per mol of the enzyme inactivated with MalNEt. Incorporation of these residues was not acid labile; thus, acid labile sulfide of the iron-sulfur centers of the enzyme is not involved. Furthermore, acid hydrolysis of the enzyme inactivated with MalNEt has shown directly that the radioactivity is associated with cysteinyl residues (9).

Vinogradov et al. (24) have postulated that the tightly bound oxalacetate in the deactivated form of the enzyme forms a thiohemiacetal with a cysteinyl residue in the enzyme, and several laboratories have isolated a stable enzyme-oxalacetate complex (10, 25, 26). Inasmuch as the deactivated form containing tightly bound oxalacetate is not inhibited by MalNEt, whereas the active form (Table II), it may well be that the sulfhydryl group involved in thiohemiacetal formation is essential for enzymatic activity and on activation and removal of oxalacetate this residue becomes susceptible to attack by MalNEt, with subsequent inhibition.

It has been reported (8) that the apparent second order rate constant for the initial inhibition of succinate dehydrogenase by bromopyruvate (and MalNEt) is 1 to 2 min$^{-1}$ mm$^{-1}$ at 22$^\circ$ and pH 7.0. The value obtained in the present study is 7.0 min$^{-1}$ mm$^{-1}$ at 25$^\circ$ (Table III) and by interpolation of the temperature dependence of the inhibition (Table III) it is 5.6 min$^{-1}$ mm$^{-1}$ at 22$^\circ$. This disparity is not due to differences in evaluating the data, for the $k_1$ value for the fast reaction calculated in the present study closely parallels the rate constant for the initial loss in activity. The lower rate constant obtained in the earlier studies (8) may be in part due to the use of phosphate buffer. This anion is known to activate succinate dehydrogenase (12), and, like other activating anions (Table I), may decrease the effectiveness of MalNEt and bromopyruvate as alkylating agents.

The striking protection by malonate documented in this paper (Fig. 3, Table IV) is in contrast to a previous report (9) that concentrations of malonate $K_D$ are required for effective protection against inactivation by MalNEt. A value of 3.4 $\mu$m has been obtained for the dissociation constant, $K_D$, of the succinate dehydrogenase-malonate complex (Table IV). This is no greater than the $K_A$ values for competitive inhibition by malonate reported in the literature (from 25 to 41 $\mu$m at 22–25$^\circ$ and 38$^\circ$; respectively (12)).

The lack of effectiveness of malonate in the earlier studies (8, 9) again may be due to the presence of phosphate. Thus, not only a malonate-enzyme complex but also a phosphate-enzyme complex must be considered and the association-dissociation of these various complexes would influence the amount of free enzyme available for alklylation by MalNEt.

According to the treatment of O'Sullivan and Cohn (21), protection by malonate against MalNEt inhibition could be the

<table>
<thead>
<tr>
<th>Modifier</th>
<th>$K_D$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malonate</td>
<td>0.0084</td>
<td>0.23</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Bromide</td>
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<td></td>
</tr>
<tr>
<td>Formate</td>
<td>50.0</td>
<td>0.23</td>
</tr>
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</table>

* 50 mM Mes, pH 7.0.

Dissociation constants and second-order rate constants for interaction of various anions and modifiers with succinate dehydrogenase

Constants were determined as described in the text. Unless otherwise noted, inactivation with 0.10 mM MalNEt was at 15$^\circ$ in 50 mM Mes, pH 7.0.
result of either no reaction or of a slow reaction of the enzyme-malonate complex with MalNEt. In the former case the intercept in plots of equation 4, \( k_{\text{app}}/k_1 \), is zero, in the latter case, a positive number. The data in Table IV shows that the intercept is in fact zero.

Felberg and Hollocher (9) have found by differential labeling with \(^{14}C\)-MalNEt in the presence and absence of malonate that a crucial cysteine is located on the flavoprotein subunit (70,000 molecular weight). Although they concluded that this residue is not at the active site, in the light of the foregoing discussion it is highly probable that the cysteiny1 residue characterized by Felberg and Hollocher (9) and the one implicated in the present studies are the same. Inasmuch as in the present paper this sulphydryl group is implicated to be at the substrate site, it would follow that the substrate site is on the flavin subunit. This, in turn, offers the possibility of identifying the substrate site by using \(^{14}C\)-MalNEt.

An important implication of the present experiments concerns the tight binding of oxalacetate. This compound has been reported to inhibit succinate dehydrogenase in two ways: the first an immediate competitive inhibition and the second a "pseudo-irreversible" inhibition which develops slowly, yields a lower \( K_I \) value, but still shows competitive characteristics (27). It has been suggested that the secondary inhibition may involve a conformational change in the enzyme from an activated to a de-activated form (10, 28). Recent results have established the importance of oxalacetate in the regulation of succinate dehydrogenase (10). Although as yet there is no conclusive evidence that the succinate binding site and oxalacetate binding site are the same, the following evidence indicates that this is the case. The MalNEt-treated enzyme, inhibited at the substrate site, does not incorporate oxalacetate in tight binding, whereas the active form does. Conversely, tightly bound oxalacetate, like malonate, prevents the inhibition by MalNEt (Table I).

Anions, particularly those known to activate succinate dehydrogenase (19), e.g. nitrate and bromide, markedly influence the kinetics of inhibition by MalNEt not only in decreasing the apparent second order rate constant (Table IV), but also in eliminating the biphasic characteristics observed in the absence of anions (not shown). The enzyme-anion complex reacts with MalNEt, but at a much slower rate than the free enzyme (Table IV). A site appears to exist on the enzyme which binds anions, but a second site is still free to react with MalNEt and result in inactivation (Table IV).

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REFERENCES
The reaction of N-ethylmaleimide at the active site of succinate dehydrogenase.

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