Activation of Succinate Dehydrogenase by Anions and pH*

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SUMMARY

Succinate dehydrogenase in the deactivated form becomes activated on incubation at mildly acid pH to an extent dependent on the pH, type of anion present, and the concentration of the anion. The equilibrium level established under one set of conditions can be perturbed by changing either the pH or the anion concentration and the resulting level of activation reflects the new set of conditions. The rate of activation is influenced by the temperature, the anion concentration, and the pH when simple anions (e.g. Br\textsuperscript{-}) are used, but the energy of activation is not affected by either pH or anion concentration. The value found for the activation energy \( E_A \) is 25 Cal per mole. In contrast, when activation of the enzyme is mediated by succinate, malonate, or reduced coenzyme \( Q_10 \), the \( E_A \) value is 31 to 33 Cal per mole. Although this difference in energies of activation suggests that activation by mild acid and anions involves a different mechanism, no difference in the catalytic activity of preparations activated by these different methods is detected.

In previous papers of this series we reported that succinate and substrate competitors (2), reduced CoQ\textsubscript{10} (CoQ\textsubscript{10}H\textsubscript{2})\textsuperscript{1} IDP, ITP (3), and, in intact mitochondria, ATP and all substrates which reduce endogenous CoQ\textsubscript{10} (4) activate mammalian succinate dehydrogenase. The present paper deals with another, but high levels of activation are reproducibly achieved by these different methods.

* This is paper XXIII in the series: Studies on Succinate Dehydrogenase. The previous paper in the series is given in Reference 1. This investigation was supported by grants from the National Institutes of Health (HL 10027), the American Cancer Society (BC 46B), and the National Science Foundation (GB 36570X).

1 The abbreviations used are: CoQ\textsubscript{10} and CoQ\textsubscript{10}H\textsubscript{2}, coenzyme Q\textsubscript{10} and its reduced form; ETP and ETP\textsubscript{a} are nonphosphorylating and phosphorylating submitochondrial particle preparations, respectively, from heart mitochondria; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; MES, 2-(N-morpholinio)ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperazine-N\textsuperscript{-2}-ethanesulfonic acid.

In the same preliminary note data were presented to suggest that succinyl-coenzyme A also activates the enzyme. The samples of succinyl-coenzyme A used in these experiments were found to contain up to 10\% succinate by analysis. This fact and breakdown of succinyl-coenzyme A to succinate to an unknown extent during the activation experiment renders these data invalid.

enzyme undergo transition from the deactivated to the activated state at mildly acid pH values, particularly in the presence of certain anions, and on neutralization the enzyme returns to the deactivated state. At sufficiently high concentrations of these anions activation proceeds even at neutral or mildly alkaline pH. This novel type of activation has a high energy of activation, suggesting a conformational change in the protein, although the value is not as high as for activation by substrates or CoQ\textsubscript{10}H\textsubscript{2}.

These observations have provided an explanation (1) for the observation (6) that succinate dehydrogenase extracted from Complex II with sodium perchlorate is fully activated and have laid the groundwork for the demonstration that activation of the enzyme by several agents involves the dissociation of oxalacetate from a very tight binding site (7).

MATERIALS AND METHODS

Soluble succinate dehydrogenase was purified from an acetone powder of beef heart ETP by a modification (8) of the procedure of Bernath and Singer (9). Ammonium sulfate was removed from the precipitated enzyme by gel exclusion in Sephadex G-60, equilibrated with 50 mM Hepes, pH 7.4 to 7.8.

Conditions for activation and treatment of the enzyme are specified in the text. Succinate dehydrogenase activity was measured at 15° by the PMS-DCIP assay (10, 11), but at fixed (0.1 mM) PMS concentration, and specific activity is defined as micromoles of succinate oxidized per min per mg of protein under these conditions.

Levels of activation are expressed as per cent of the maximal activity, i.e. that obtained under identical assay conditions after preincubation of the enzyme for 6 min with 20 mM succinate at 38°. Protein was determined by the biuret procedure (12).

RESULTS

Activation by pH and Anions—As documented in our preliminary report (5), on lowering the pH of an ETP preparation from pH 7.4 to various pH values in the range of 5.8 to 7.0 and incubating the suspension at 30°, succinate dehydrogenase is gradually activated and the final level of activation reached depends on the pH. The extent of activation of the enzyme at a given pH is quite variable from one particle preparation to another, but high levels of activation are reproducibly achieved in the presence of added anions. In contrast, activation of the soluble enzyme at acid pH never occurs to a significant degree without added anions, but as with particulate preparations, is always observed when suitable anions, such as Cl\textsuperscript{-}, Br\textsuperscript{-}, etc., are also present in the incubation mixture (Fig. 1A). At each pH an apparent equilibrium is reached between activated and deactivated enzyme. On plotting the equilibrium levels versus pH, a sigmoidal curve is obtained (Fig. 1B). The position of
the lower salt concentration deactivation occurred to a level approximating that reached in the control experiment for the same set of conditions (crosses). Maintaining the same pH (crossed circles), or so as to maintain was diluted 1:1 to reduce the Br- concentration to 35 mM while activation level was close to equilibrium, the reaction mixture no activation occurs in the absence of added salts. After the activated by incubation with 70 mM NaBr at pH 7.5, where almost pH. In Fig. 3B, the enzyme in an ETP preparation was activate dehydrogenase by NaCl. Soluble enzyme, protein = 1 mg per ml, was incubated at 20° in 100 mM NaCl in either 100 mM Heps or 100 mM MES at the following pH values: 5.5 (X); 6.07 ( ); 7.0 (A); 7.5 ( ); 8.0 (C). B (center), activation of the soluble enzyme as a function of pH. The points on the curve represent the final levels of activation attained in the experiments of A.

**Effects of Temperature and Anion Concentration on Rate of Activation**—Activation of soluble succinate dehydrogenase at acid pH values in the presence of anions obeys first order kinetics (Fig. 4). The rate is a function of the anion concentration. The variation of the rate constant with Br- concentration (Fig. 4, inset) could be accurately determined in the range 0.5 to 2 M; above this concentration significant inactivation of the enzyme occurred (20 to 30%), which may render the data uncertain. Pronounced increases in rates of activation were observed, however (dotted line in Fig. 4, inset) which may indicate that a different process is taking place. At Br- concentrations below 0.5 M, activation did not reach completion. The probable reason for this is that lower salt concentrations are insufficient to prevent rebinding of the oxalacetate which is released from the enzyme in the course of activation, as discussed in the next paper (13). Consistent with this is the finding that semicarbazide displaced the equilibrium in favor of the activated form (Fig. 5). The initial linear portion of the rate curves was prolonged as the semicarbazide concentration was increased and maximal effect was obtained with 0.1 mM semicarbazide (Fig. 5, Curve D). Semicarbazide did not affect the rate of activation.

**Energy of Activation**—From Arrhenius plots of the effect of temperature on the rate of activation by Br- at various pH values the energy of activation was calculated. Although the observed rate of activation of succinate dehydrogenase varied with pH and Br- concentration (Table I) and also with the nature of the anion (Tables II and III), the Ea value was quite constant (Table I). This value (~25 Cal per mole) is significantly lower than the Ea for activation by succinate, malonate or CoQH2 (33 ± 2 Cal per mole) (Table I and Reference 3). As further shown in Table I, while the rate of activation by anions is distinctly pH dependent, activation by succinate is not, suggesting that different mechanisms are involved in initiating the activation in the two cases.

**Comparison of Different Anions as Activators**—Table II presents representative data for the relative effectiveness of different anions as activators of membrane-bound succinate dehydrogenase. The data reflect the degree of activation reached at apparent equilibrium at two pH values. The adjustment of pH in these, as in other experiments in this paper, was made by the addition of MES or Heps buffers. Suitable control experiments in which the pH was lowered to 6.3 with an ion
The activity of a suspension of ETP was monitored during incubation at 30°C in 200 mM sucrose-50 mM Hepes, pH 7.35, was monitored (X). B, by dilution of anion concentrations. In the control experiment the activation of ETP (1 mg of protein per ml) in 100 mM MES, pH 6.0, was monitored in the absence of anions. The activity of a suspension of ETP (2 mg of protein per ml) in 100 mM glycylglycine, pH 8.0, to yield a final pH of 7.35 (O). In 100 mM glycylglycine, pH 8.0, containing 70 mM NaBr (0) and a third sample with an equal volume of the buffer, without NaBr (O). The incubations were continued at 30°C. In a control experiment, ETP was incubated under the final conditions of Sample 3, in 100 mM sucrose-50 mM Hepes, pH 7.5, containing 70 mM NaBr (0). After 25 min a second sample was diluted with an equal volume of the same buffer, containing 70 mM NaBr (0) and a third sample with an equal volume of the buffer, without NaBr (O). The incubations were continued at 30°C. In the control experiment, ETP was incubated under the final conditions of Sample 3, in 100 mM sucrose-50 mM Hepes, pH 7.5, 35 mM NaBr, protein = 2 mg per ml (X).

Rates of activation were determined with a soluble enzyme preparation and E_0 values were calculated from Arrhenius plots. Malonate activated samples were incubated for 1 min in the assay cuvette in the presence of succinate to allow full dissociation of the malonate-enzyme complex prior to starting the assay. Activation energies for the activation of soluble succinate dehydrogenase by bromide. Soluble enzyme, protein = 1 mg per ml, was incubated at 10°C in 50 mM MES, pH 6.3, and NaBr at 0.5 mM (X) and 1.0 mM (O) concentrations. Ordinates, the concentration of the deactivated form of the enzyme, expressed as the difference between the maximal activity attained by incubating an aliquot of the enzyme with 250 mM chloride at 38°C for 6 min and the activity at time = t induced by incubation with bromide. Inset, the dependence of the rate constant on bromide concentration. For details see text.

**Fig. 4 (center).** First order rate plots for the activation of soluble succinate dehydrogenase by bromide. Soluble enzyme, protein = 1 mg per ml, was incubated at 10°C in 50 mM MES, pH 6.3, and NaBr at 0.5 mM (X) and 1.0 mM (O) concentrations. Ordinates, the concentration of the deactivated form of the enzyme, expressed as the difference between the maximal activity attained by incubating an aliquot of the enzyme with 200 mM succinate at 38°C for 6 min and the activity at time = t induced by incubation with bromide. Inset, the dependence of the rate constant on bromide concentration. For details see text.

**Fig. 5 (right).** Effect of semicarbazide on activation with suboptimal anion concentration. Soluble succinate dehydrogenase was incubated at 25°C in 50 mM MES, pH 6.3, protein = 5 mg per ml, with NaCl and semicarbazide-HCl to give a final chloride concentration of 250 mM. Semicarbazide-HCl concentrations were as follows. Curve A, none; B, 20 mM; C, 50 mM; D, 100 mM. Ordinates, concentration of deactivated enzyme expressed as in Fig. 4.

**Table I**

<table>
<thead>
<tr>
<th>Activator</th>
<th>Molarity</th>
<th>pH</th>
<th>Protein</th>
<th>20° k_app</th>
<th>15° k_app</th>
<th>Activation energy (kcal/mole)</th>
</tr>
</thead>
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<tr>
<td>Succinate</td>
<td>0.02</td>
<td>6.2</td>
<td>5.0</td>
<td>0.06</td>
<td>30.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>7.4</td>
<td>5.0</td>
<td>0.06</td>
<td>30.7</td>
<td></td>
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<tr>
<td>Malonate</td>
<td>0.004</td>
<td>6.3</td>
<td>1.0</td>
<td>0.02</td>
<td>31.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>7.5</td>
<td>1.0</td>
<td>0.01</td>
<td>33.4</td>
<td></td>
</tr>
<tr>
<td>Bromide</td>
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<td>6.3</td>
<td>5.0</td>
<td>0.15</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>6.3</td>
<td>5.0</td>
<td>0.13</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.5</td>
<td>5.0</td>
<td>0.25</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>7.5</td>
<td>5.0</td>
<td>0.13</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
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<td>6.4</td>
<td>1.0</td>
<td>0.06</td>
<td>26.4</td>
<td></td>
</tr>
</tbody>
</table>

Table III presents similar data for activation of the soluble, purified enzyme at pH 6.0. It may be seen that cations do not influence the degree of activation attained at a given pH. The order of effectiveness of Cl^-, S0_4^2-, and acetate was not materially different from that observed with membranal preparations.

Effect of Adenine and Hypoxanthine Nucleotides—We have reported that IDP and ITIP (5 to 10 mM) activate succinate dehydrogenase in soluble and inner membrane preparations, whereas mononucleotides, including cyclic AMP and IMP, and GTP, ATP, and ADP at comparable concentrations do not (4, 16, 17). ATP, however, activated succinate dehydrogenase in tightly coupled mammalian mitochondria when added in low concentration (19 to 190 μM), but the effect was not seen when the mitochondria were damaged by swelling or other means (4). In plant mitochondria and submitochondrial particles, ATP and ADP (0.5 to 0.8 mM) activate more effectively than ITIP or IDP and the effect is not dependent on mitochondrial integrity (18).

In view of the demonstration that a number of anions are capable of activating succinate dehydrogenase, it seemed possible...
that activation by IDP and ITP might simply be a case of anion activation. An experiment was conducted, therefore, in order to compare ADP and ATP with the corresponding inosine phosphates at an acid pH (pH 6.2) at which anion effects were most likely to be detected. At pH 6.2 ADP and ATP indeed activate soluble succinate dehydrogenase to a moderate extent but far less than equivalent concentrations of IDP or ITP, and, as in studies conducted at pH 7.4 (4), IDP was again more effective than ITP. It would appear, therefore, that activation by IDP and ITP is not merely due to their anionic nature, since other purine nucleotides with comparable pK values for the phosphate group are much less effective and since, at comparable concentrations, inorganic phosphate itself is a relatively poor activator of the soluble enzyme (2).

**DISCUSSION**

Despite extensive studies of the activation-deactivation cycle of succinate dehydrogenase by substrate and competitive inhibitors in several laboratories, the reversible activation of the enzyme by pH and anions was not noted. An exception is the activating effect of phosphate (2, 19), which was ascribed at the time to its ability to combine with the substrate site (15, 20), since substrates and competitive inhibitors activated in a similar manner. Since the substrate and competitive inhibitors are also anions, it might be expected that activation by simple anions would proceed by the same mechanism, being bound at the substrate site but more weakly, and thus differences in concentration requirements merely reflect differences in binding affinities for the enzyme. The observation that the $E_a$ value for anion activation is some 8 Cal per mole lower than that for activation by other agents, however, suggests that high concentrations of simple anions induce activation by a somewhat different mechanism than substrates, competitive inhibitors or CoQ$_{10}$H$_2$. Although precise data for the $E_a$ value for activation have only been obtained for Br$^-$ and phosphate, in studies comparing activation by other anions at different temperatures very similar temperature dependence was noted. Thus, the value of 25 Cal per mole may be provisionally accepted as characterizing activation of the enzyme by simple anions. Although this value is considerably lower than the $E_a$ for activation by succinate, malonate, and CoQ$_{10}$H$_2$, it is still high enough to be compatible with a conformational change in the enzyme. The conformational alteration involving activation by Br$^-$ may be different, however, from that occurring under the influence of substrates or CoQ$_{10}$H$_2$.

In addition to the lower $E_a$ value for activation, the effects of Br$^-$ are characterized by greater apparent rate constants. Thus, activation by Br$^-$ can be readily measured at 15°C (Table I), whereas activation by substrates and CoQ$_{10}$H$_2$ is barely perceptible at this temperature. Similarly, 0.8 M NaClO$_4$ used by Davis and Hatefi (21) for the extraction of the enzyme from Complex II fully activates succinate dehydrogenase (1) during extraction at 0°C.

The finding that acid pH and anions can fully activate succinate dehydrogenase is potentially useful in studies on the mechanism of action of the enzyme. Thus, until now, studies of the mechanism of intramolecular electron transport in succinate dehydrogenase have been hampered by the fact that the preparations used were partly in the activated, partly in the deactivated state (22). When succinate was used as an activator, it could not be rapidly removed without deactivating the enzyme and in its presence reduction by succinate could not be measured. With malonate-activated preparations, on the other hand, displacement of the competitive inhibitor by succinate in rapid-freezing ESR experiments, involving millisecond intervals, could not be assumed to be much more rapid than reduction of the enzyme by the substrate (17). The discovery that simple anions can fully activate the enzyme overcomes this problem, since the presence of these anions probably does not interfere with the catalytic cycle and, in any event, the activating anion may be readily removed without causing deactivation (19).

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