TIGHTLY BOUND OXALACETATE AND THE ACTIVATION OF SUCCINATE DEHYDROGENASE*

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SUMMARY

Soluble succinate dehydrogenase prepared from acetone powders of submitochondrial particles is almost entirely in the deactivated state and contains 0.5 mole of oxalacetate (OAA) per mole of histidyl flavin. OAA is dissociated by succinate, malonate, IDP, ITP, and high concentrations of anions at elevated temperatures, but not significantly in the cold, with concurrent activation of the enzyme; the high energy of activation observed for OAA release and for activation suggests that a conformation change in the protein is involved. On removal of OAA, a reversible activation-deactivation cycle dependent on the pH is demonstrable. Submitochondrial particles behave similarly but appear to contain 1 mole of tightly bound OAA per histidyl flavin in the deactivated state.

We have recently reported (1) that, in addition to previously known activators of succinate dehydrogenase (SD) (substrates and competitive inhibitors (2), reduced CoQ (3), ATP (4), ITP and IDP (4)), the enzyme is also converted from inactive to active form at low pH (~ pH 6) and reverts to the deactivated state on readjustment to higher pH values. The extent of activation depends on pH and is increased by the presence of certain anions (Cl^-, Br^-, NO_3^-, $SO_4^=$, etc.) in submitochondrial particles; in soluble preparations anions are required for major pH activation (1).

When the pH and anion activated enzyme, soluble or membrane-bound, is incubated with very low concentrations of succinate, fumarate, L- or D- malate activity gradually declines (5). It seemed likely that this loss of activity could be due to the formation of oxalacetate (OAA) from malate, added as such or generated by SD and traces of fumarase present, since the direct oxidation of both L- and D- malate to OAA by SD has been reported (6). In our experiments, however, extensive deactivation occurred with malate added in trace amounts (5-50μM), whereas the dissociation constants of the enzyme for D- and L-malate have been reported to be 1.4 mM and 4.6 mM, respectively (6).

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It is shown in this report that the loss of activity on incubation with these acids is nevertheless due to OAA formation, and further, that SD, as isolated, contains very tightly bound oxalacetate. The relationship between this tightly bound oxalacetate and activation of the enzyme is explored.

MATERIALS AND METHODS

SD was purified by the method of Bernath and Singer (7), except that the starting material was an acetone powder of beef heart ETP (8). For activation, the enzyme was incubated in MES buffer, (2-(N-morpholino)ethanesulfonic acid), neutralized with NaOH, with other additions and conditions as specified, and periodic samples were assayed at 15° by the PMS-DCIP method (9), at fixed PMS concentration (3). Levels of activation are expressed as per cent of the maximal activity, which is that obtained under the same assay conditions after incubation with 25 mM succinate for 5 min at 38°. OAA was measured fluorometrically with malate dehydrogenase + NADH (10).

RESULTS AND DISCUSSION

Effect of Malate on pH-Anion Activated Enzyme - As seen in Fig. 1, when SD in an ETP preparation is activated by incubation at pH 6 the addition of certain dicarboxylic acids induces a loss of activity, according to the nature and concentration of the acid. D-malate is effective in the lowest amounts (5-10μM); succinate has maximal effect at 20-25 μM concentration, while in higher concentration (100μM) it causes activation of the enzyme to a higher level than obtained originally at pH 6.

To test whether the loss of activity might be due to OAA formation by the action of SD, this type of experiment was repeated with concurrent measurement of OAA. In the experiments of Fig. 2, the soluble enzyme was used and the effect of D-malate was studied. Evidently OAA formation occurred directly by the action of SD, and even under anaerobic conditions. The anaerobic experiment is explainable on the following basis: the enzyme at the start contained 2.92 nmoles of histidyl flavin (equated with one active center of SD) and 1.4 nmoles of OAA/mg; by the end of the experiment an additional 1.6 nmoles of OAA/mg was found, which corresponds to the concentration of active SD at the point when malate was added. Hence a single turnover of the flavin is sufficient to account for the OAA produced. The somewhat higher amount of OAA found in the aerobic experiment may be due to slight autooxidizability of the soluble enzyme. The loss of activity is not due to competitive inhibition of SD by OAA, since the amount of OAA carried over into the assay was only about 7 nM, whereas the K_I of SD for OAA has been reported to be almost 3 orders of magnitude higher (1.5 to 6.3 μM and the K_D from absorbance data 4 μM (11, 12). Loss of activity with time is, however, related to OAA production, and the relationship is demonstrable as well after addition of L-malate or low concentrations of succinate.

Tightly-Bound OAA in the Enzyme - As noted above, the enzyme used in the experi-

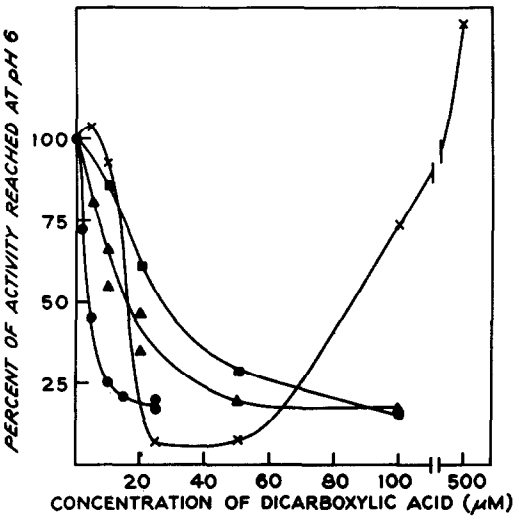

Fig. 1.

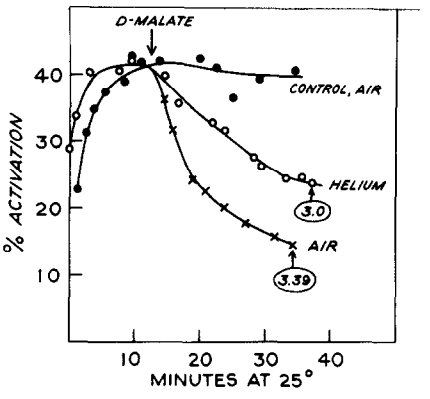

Fig. 2.

Fig. 1. Effect of dicarboxylic acids on activation of SD. ETP, pre-treated with Antimycin A (1 nmole/mg protein) was incubated in 100 mM MES buffer, pH 6.0, protein = 2 mg/ml, for 15 min at 30°. Dicarboxylic acids were then added to give the concentrations indicated on the abscissa. The activities determined after 15 min further incubation are plotted on the ordinate as the percentage of the control, incubated without added dicarboxylic acid. Control activity during 15-30 min incubation was 66-70% of the maximal activity. X, succinate; ■, L(-)malate; ▲, fumarate; ●, D(+)malate.

Fig. 2. Formation of OAA from D(+)malate by SD. Soluble enzyme was activated by incubation at 25° in 100 mM MES - 50 mM NaCl, pH 6.1; protein = 1 mg/ml. After 13 min, D(+)malate was added to 100 μM final concentration. At the end of the experiment samples were deproteinized with perchloric acid (3%, v/v), and the neutralized extracts analyzed for OAA. The numbers encircled on the graph are the total amounts of OAA found in nmoles/mg protein. The anaerobic experiment was conducted under He and samples were withdrawn anaerobically with a syringe.

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ments of Fig. 2, as isolated, contained 0.48 nmoles of OAA per mole of histidyl flavin. Each of several enzyme preparations examined contained OAA in 1:2 ratio to histidyl flavin and all were 85 to 95% deactivated. The OAA present in the enzyme is exceedingly tightly bound, so that neither Sephadex passage, dialysis, nor treatment with NADH + malate dehydrogenase releases it. It is dissociated on precipitation of the protein with perchloric acid and has been identified as OAA both by the malate dehydrogenase reaction and by gas-liquid chromatography* (13). The bound OAA is also released on incubation with agents which activate the enzyme (e.g., succinate, malonate, IDP, certain anions at

*We are grateful to Dr. J. I. Salach for the gas-liquid chromatographic analysis.

acid pH) but only at elevated temperatures; both the release of OAA and activation by these agents are extremely slow in the cold.

The activation energy for OAA release and that for activation by 500 mM Br^- at pH 6.3 have been compared and found to be identical (18.1 Kcal/mole). The E_A for OAA release by substrates and $CoQ_{10}H_2$ has not yet been determined but E_A for activation by these agents is higher (~33 Kcal/mole (2, 3)) than the value for Br^- activation quoted above. The latter is nevertheless high enough to suggest that a conformation change either precedes or is involved in the dissociation of OAA.

Although the identical E_A values and the observed ratio of OAA to histidyl flavin (1:2) suggest the possibility that one OAA might inhibit 2 active centers, thereby accounting for the almost completely deactivated state of the preparation, it is evident (Fig. 3) that the apparent rate constants for OAA release and activation by Br^- are not the same. It appears instead that conversion to the active enzyme is twice as fast as OAA release; this would preclude a simple one-step mechanism for release of OAA from the OAA-enzyme complex. It is tempting to speculate that the enzyme may exist in an inactive dimeric structure, with one OAA shared between 2 monomeric units, and that OAA is released only after conversion of the structure to an activated form. In this connection, however, it should be noted that all types of soluble preparations exist largely as a dimer (14, 15) in

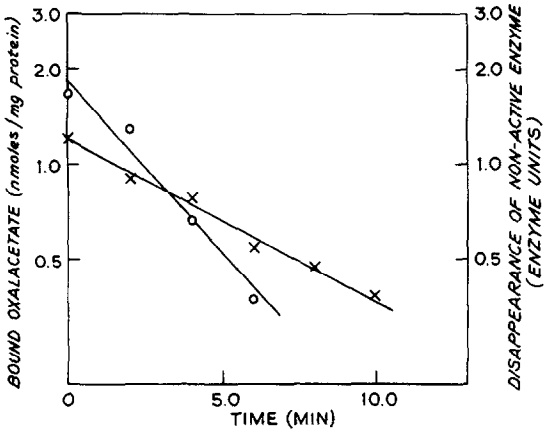

Fig. 3. Rate of activation and removal of OAA. Soluble enzyme was incubated at 20° in 50 mM MES - 500 mM NaBr, pH 6.3; protein = 5 mg/ml. Units of nonactivated enzyme remaining were calculated as maximal activity minus observed activity at the indicated time intervals. For determination of bound OAA aliquots of the incubation mixtures containing 1 mg of protein were first treated at 0° for 5 min with malate dehydrogenase (5 units) and NADH (10 nmoles) to remove free OAA; enzyme-bound OAA was then liberated by perchloric acid, which also served to stop further malate dehydrogenase action, and determined in the neutralized perchlorate extract. X, bound OAA; O, non-activated enzyme.

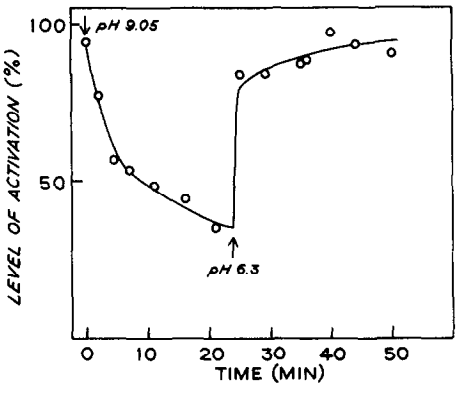

Fig. 4.

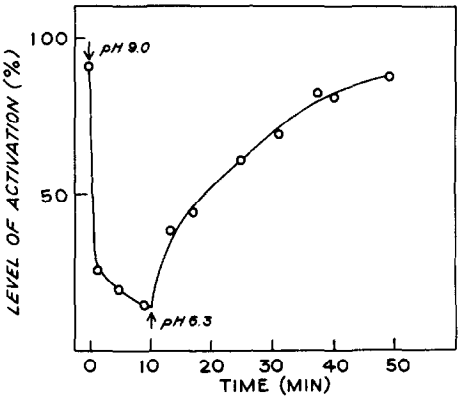

Fig. 5.

Fig. 4. Effect of pH on the OAA-free enzyme. Soluble enzyme was activated fully by incubation at 25° in 50 mM MES - 500 mM NaBr , pH 6.3; protein = 16 mg/ml. The OAA released was removed by passing the mixture (2.2 ml) through a column (2 x 21 cm) of Sephadex G-50, equilibrated with the same buffer and saturated with N_2. The enzyme in the first 30% of the exclusion volume was concentrated with 0.6 sat. $(NH_4)_2SO_4$, redissolved in 50 mM MES, pH 6.3, and desalted on a second column of Sephadex G-50, equilibrated with 50 mM MES, pH 6.3, under N_2. The enzyme was 90-95% activated and contained no OAA. The enzyme solution was then brought to pH 9.0 by the addition of Tris base (protein = 2.73 mg/ml) and incubated at 25° . At the time indicated by the arrow, the solution was acidified to pH 6.3 with MES (free acid) and the incubation continued (protein = 1.36 mg/ml).

Fig. 5. Effect of pH on OAA-free ETP. ETP was incubated for 23 min at 30° in a mixture of 50 mM MES, pH 6.3, Antimycin A (1 nmole/mg protein), 1 mM KCN, and 1 mM NADH. The mixture was cooled, more NADH was added, equivalent to a final concentration of 0.75 mM, and the mixture was flushed with N_2 and spun at 144,000 x g for 15 min. The particles, resuspended in 50 mM MES, pH 6.3 were 90% activated with respect to SD activity and contained no OAA. An aliquot of the suspension was then adjusted to pH 9 with Tris base (protein = 13.6 mg/ml), incubated at 30°, and the changes in activation level monitored. After 10 min an aliquot was readjusted to pH 6.3 with MES (free acid) (protein = 10.1 mg/ml) and the incubation continued.

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the concentration range used in the present studies, including the Davis-Hatefi preparation (16), which is fully activated and contains no OAA.

Activation-Deactivation in OAA-free Preparations - The presence of firmly bound OAA is not the only reason for the activation-deactivation characteristics of the enzyme. As shown in Fig. 4, preparations freed from OAA by incubation with Br^- at pH 6.3, are deactivated on incubation at pH 9, following which readjustment of the pH to 6.3 causes gradual, full return of the activity. The cycle shown in Fig. 4 requires no added anions but occurs solely as a result of changes in an ionizing group(s) on the enzyme.

Behavior of Submitochondrial Particles - In Complex II SD is largely in the deactivated form but the ratio of deactivated/activated enzyme varies from preparation to preparation. Measurement of bound OAA and histidyl flavin shows for the deactivated fraction of the enzyme the same 1:2 relationship found in soluble preparations extracted from acetone powders of ETP. In two ETP preparations examined, however, a 1:1 ratio was observed. The OAA was not released significantly by incubation of ETP at pH 6 and 30° but was fully dissociated when 100 to 200 mM Br^- was also present, with concurrent activation of SD. OAA was also released and SD activated on incubation with NADH, under the conditions of the $CoQ_{10}H_2$-mediated activation (3). The OAA-free particle obtained after centrifugation contained SD in fully activated form, which was then deactivated at alkaline pH and activated at acid pH (Fig. 5).

Thus it appears that in both the soluble enzyme and its membrane-bound form the presence of firmly bound OAA tends to mask pH activation, which is readily observed with OAA-free enzyme. This may account for variability encountered in activation of SD in different ETP samples by pH alone (i.e., no added inorganic anions), since the OAA content might vary and samples containing a high ratio of OAA to flavin would tend to be activated only on dissociation of the OAA, which requires the addition of anions or other activators. In terms of catalytic activity the deactivated form obtained on incubation of the OAA-free enzyme at alkaline pH is indistinguishable from the inactive OAA-bound enzyme, while the acid-activated form can only be observed after removal of any bound OAA.

At present it is not possible to assess what role these processes play in the rapid regulation of SD in intact mitochondria. It may be recalled that efforts to relate the dramatic activation of the enzyme by ATP in tightly coupled mitochondria to OAA removal have been unsuccessful (4). It is also important to emphasize that the present data do not permit the conclusion that OAA-containing, deactivated enzyme is inactive because of OAA inhibition, but it is conceivable that OAA may be bound at a second, regulatory site and thereby induce the inactive conformation. Release of OAA and return of activity may both be a consequence of return to the active conformation. The occurrence of a conformation change in OAA binding in a pseudo-irreversible manner has, in fact, been suggested (12).

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