Localization of the Substrate and Oxalacetate Binding Site of Succinate Dehydrogenase*

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Succinate dehydrogenase is composed of two subunits, one of molecular weight 70,000, containing FAD in covalent linkage to a histidyl residue of the polypeptide chain, the other subunit of molecular weight 30,000. The fact that substrate, substrate analogs, and oxalacetate prevent inactivation of the enzyme by thiol-specific agents indicates that a thiol group must be present in close proximity to the flavin. Comparison of the incorporation of radioactivity into each subunit in the presence and absence of succinate or malonate shows that both substrate and competitive inhibitors protect a sulfhydryl group of the 70,000-molecular weight subunit. This indicates that a thiol group of the flavoprotein subunit is part of the active site. Similar investigations using oxalacetate as a protecting agent indicate that the tight binding of oxalacetate to the deactivated enzyme also occurs in the flavoprotein subunit, and may involve the same thiol group which is protected by succinate from alkylation by N-ethylmaleimide. It is clear, therefore, that not only the flavin site but also an essential thiol residue are located in the 70,000-molecular weight subunit.

A second thiol group, located in the 30,000-molecular weight subunit, also binds N-ethylmaleimide covalently under similar conditions, without being part of the active site. Succinate, malonate, and oxalacetate do not influence the binding of this inhibitor to the thiol group of the lower molecular weight subunit.

Using maleimide derivatives of nitroxide-type spin labels, it has been possible to demonstrate the presence of two types of thiol groups in the enzyme which form covalent derivatives with the spin probe. When the enzyme is treated with an equimolar quantity of the spin probe, a largely isotropic electron spin resonance spectrum is obtained, indicating a high probe mobility. When this site is first blocked by treating the enzyme with an equimolar quantity of D-ethylmaleimide, followed by an equimolar amount of spin label, the label is strongly immobilized with a splitting of 64 gauss. It is suggested that the sulfhydryl group which is involved in the immobilized species is at the active site.

Succinate dehydrogenase is composed of two subunits. One, with a molecular weight of 70,000, contains FAD covalently linked to the protein as 8a-[(N)3-histidyl]-FAD (2-4), whereas the other, 30,000 dalton component, does not. In addition, at least one sulfhydryl group is important for catalytic activity (5-10). The presence of a sulfhydryl group at the active site was recently questioned (11, 12), based on the findings that inhibition by MalNEt and bromopyruvate was incomplete, and only partial protection from inactivation was obtained in the presence of substrate or malonate, a competitive inhibitor of the enzyme. It has been shown (9), however, that these findings were due in part to incompletely activated preparations and in part to the anion composition of the reaction mixture (11, 12) which prevented complete reaction with MalNEt of the thiol group at the active site. The ability to obtain succinate dehydrogenase in the fully activated state by incubation at mildly acid pH in the presence of certain anions (13), free of tightly bound oxalacetate (14), has made it possible to establish that a thiol group is indeed essential for the catalytic activity of succinate dehydrogenase and probably occurs at the substrate site. In accord with this, substrates, competitive inhibitors, and anions which activate the enzyme also protect it from inhibition by MalNEt and prevent binding of this inhibitor to the essential thiol group (9, 10).

These results suggested, therefore, that a thiol group must be present in close proximity to the FAD moiety in the active site.

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The abbreviations used are: MalNEt, N-ethylmaleimide; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; esr, electron spin resonance.
center of succinate dehydrogenase (9, 10). Neither the pentapeptide of the covalently bound flavin nor the much larger Felberg and Hollocher (12) have reported that a crucial thiol group involved in the inhibition. By differential labeling with [14C]MalNEt in the presence or absence of malonate, the cysteinyl residue is located on the flavoprotein subunit of succinate dehydrogenase. They conclude, however, that this thiol group is not at the catalytically active site. The location of the thiol group may also be approached theoretically by looking for the oxalacetate binding site, as has been attempted by Winter and King (16). However, although oxalacetate binding is presumably covalent (17) and stable in the native enzyme, being a thiohemiacetal, it dissociates readily on denaturation. In accord with this, methods which dissociate the enzyme and substrates or competitive inhibitors, irreversibly alkylated with MalNEt was used to ascertain the location of the active site cysteinyl residue.

Experiments will be presented which show that there is one sulphydryl group on each subunit which reacts rapidly with MalNEt. It will also be shown that the fast reacting sulphydryl group on the 70,000-molecular weight subunit can be protected by substrate and inhibitors. Finally, data involving spin label analogs of MalNEt will be used to detail further the environments around the reactive sulphydryl groups.

**MATERIALS AND METHODS**

N-Ethyl[2,3-14C]maleimide (specific radioactivity, 4.8 mCl/mmol) was obtained from the Radiochemical Centre, and the ESR spin label derivatives were obtained from SYVA. All chemicals were reagent grade.

Succinate-Coenzyme Q reductase was isolated from beef heart mitochondria by the method of Ziegler and Doeg (18). Succinate dehydrogenase was prepared by the procedure of Davis and Hatefi (3) for the experiment documented in Fig. 1. In subsequent experiments, the enzyme was isolated in the absence of succinate (19) using a single extraction with ethyl ether and sodium perchlorate.

Enzymatic analyses were performed at 15°C as described previously (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was run on gels (5 x 100 mm) containing 10% acrylamide as described by Weber and Osborn (20). After staining with Coomassie blue, the gels were sliced and dissolved in 0.2 ml of 30% H3PO4 and incubated at 40°C until solution occurred (approximately 48 hours). Radioactive counting was conducted with a Beckman LS-150 liquid scintillation counter, using Aquasol (New England Nuclear) as the scintillation medium. ESR spectra were taken with a Varian E4 spectrometer equipped with an E257 variable temperature accessory. In experiments in which the enzyme was incubated with MalNEt, the reaction was stopped at the indicated time by transferring an aliquot of the reaction mixture to a cuvette containing 20 mM succinate (for activity measurements) or to a centrifuge tube containing a 200-fold molar excess of diethiothreitol (for incorporation experiments). Excess radioactive material was removed from the protein by precipitation of the latter with 5% (w/v) trichloroacetic acid followed by centrifugation at 12,800 x g for 10 min. The precipitate was resuspended in 1 ml of 5% (w/v) trichloroacetic acid and centrifuged as above. The washing was repeated using 1 ml of 5% (w/v) trichloroacetic acid, and the resulting pellet was dissolved in 0.4 ml of 10 mM sodium phosphate, pH 7.0, containing 0.1% (w/v) sodium dodecyl sulfate. Prior to polycrylamide electrophoresis, mercaptoethanol was added to a final concentration of 75 mM, and the solution was incubated for 2 hours at 38°C.

Protein was estimated by the biuret (21) or Lowry (22) methods. Covalently bound flavin was determined fluorimetrically (23); this was used to estimate the succinate dehydrogenase concentration, assuming a molecular weight of 100,000 (2). Spin Labeling of Succinate Dehydrogenase—As will be presented under "Results," studies using nitroxide-type spin labels indicated that two types of sulphydryl groups were present in the enzyme. One type yielded an ESR spectrum which indicated that the probe was only weakly immobilized; the spectrum of the other type indicated that the probe was strongly immobilized. The differential labeling of these two types of thiol groups was performed as follows. All experiments were conducted in 50 mM Hepes buffer, pH 8.0. In order to estimate the quantities of spin label or MalNEt needed to incubate with the enzyme, a bound flavin content of 7 nmol/mg of protein was assumed. To remove traces of diethiothreitol, the enzyme was passed through a Sephadex G-50 (fine) column (0.9 x 20 cm) prior to use. Three labeling methods were used. For preliminary experiments, succinate dehydrogenase (approximately 20 mg/ml) was incubated with spin label (1 mM) for 1 min at 0°C. It was isolated by precipitation in 50% ammonium sulfate at 0°C followed by centrifugation at 20000 x g, 10 min. 2°. The pellet was quickly rinsed with Hepes buffer and then dissolved.

To label the weakly immobile sites predominantly, succinate dehydrogenase (4.4 mg/ml) was allowed to react with spin label (19 μM), in the presence of 2 mM malonate as a protection agent for the active site, for 3 min at 15°C. Unreacted label was removed via passage through a Sephadex G-50 column (0.9 x 20 cm) equilibrated with 2 mM malonate in addition to the Hepes buffer. The enzyme was precipitated by ammonium sulfate fractionation and redissolved as described above.

The strongly immobilized sites were labeled by allowing the enzyme (approximately 4.5 mg/ml) to react with MalNEt (32 μM) for 1 min at 15°C, then with spin label (50 μM) for 3 min at 15°C. Isolation of the enzyme was accomplished as described for the preliminary experiments.

**RESULTS**

**Number and Location of Sulphydryl Groups**—We first wanted to define the number and subunit location of sulphydryl groups in succinate dehydrogenase and also ascertain whether both substrate and inhibitors react with the same active site sulphydryl group. The incorporation of [14C]MalNEt was, therefore, studied in the absence of or presence of succinate, malonate, or oxalacetate. Fig. 1 shows the incorporation of radioactivity into each of the subunits of succinate dehydrogenase under conditions in which 70% inhibition had occurred in the sample containing no protective agents. The level of incorporation of radioactivity into the 30,000-molecular weight subunit is the same whether or not malonate, succinate, or oxalacetate are present in the incubation medium. The incorporation into the 70,000-molecular weight subunit is significantly decreased, however, if succinate, malonate, or oxalacetate are present in the incubation medium, and this decrease is substantially the same regardless of the protecting agent employed. The additional incorporation in the larger subunit in the absence of protective agents corresponds to approximately 0.65 mol of MalNEt incorporated per mol of flavin, which would be expected under these conditions in which 70% inhibition of enzymatic activity had occurred. A further test for the essentiality of this sulphydryl group is to ascertain that the kinetics of alkylation correspond to the kinetics of inactivation (24). Since succinate, oxalacetate, and malonate afford equal protection, the latter was used for reasons presented earlier (9) in investigating the active site.

**Kinetics of MalNEt Incorporation into Succinate Dehydrogenase**—Fig. 2 shows the incorporation of [14C]MalNEt into succinate dehydrogenase both in the absence and presence of malonate as a function of time. In this figure, the difference in incorporation is also plotted. It is seen that for the latter, the level of incorporation approaches 1 mol of MalNEt per mol of flavin, representing the titration of the active site thiol group. It is possible to show that the kinetics of incorporation is consistent with the kinetics of inhibition in this experiment.
Location of Substrate Site of Succinate Dehydrogenase

Fig. 1. Incorporation of [14C]MalNEt into subunits of succinate dehydrogenase. The enzyme (1.1 μM) was incubated in 50 mM Hepes, pH 8.0, at 15° for 9 min with [14C]MalNEt (25 μM) in the absence of or presence of malonate (0.32 mM), succinate (16 mM), or oxalacetate (0.09 mM). The reaction was stopped with dithiothreitol and the protein was prepared for and subjected to polyacrylamide gel electrophoresis as described under "Methods," which results in the resolution of the 70,000 (1.5 cm) and 30,000 (4 cm) molecular weight subunits. The gels were then sliced, solubilized in H2O2, and counted for 14C incorporation.

However, as will be shown below, two sulfhydryl groups are reacting with MalNEt at different rates. Because of this, a small difference between two large numbers is obtained, especially in the early time samples. Secondly, this type of experiment does not distinguish between the incorporation into the 70,000- and 30,000-molecular weight subunits.

These problems can be overcome, however, by subjecting an aliquot of the protein to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Methods," and following the incorporation of radioactivity into each of the subunits of succinate dehydrogenase. These results are presented in Fig. 2. As is apparent from Fig. 2A, the rate and level of alkylation of the 70,000 molecular weight subunit are the same whether or not malonate is present during the incubation with MalNEt. Fig. 2B shows the time course of incorporation into the flavoprotein subunit. A substantial decrease in incorporation of radioactivity is obtained when malonate is present as a protective agent.

In order to determine the rate constant for incorporation, it is necessary to know the level obtained at infinite time. This may be ascertained by inspection of the data obtained as in Fig. 2 or Fig. 3E, or by subjecting the results to analysis by the Guggenheim method, as described in Ref. 25. Treatment of the data of Fig. 2B indicates that one site is labeled with MalNEt and that the value for the incorporation at infinite time is 3500 cpm. In the procedure used, 90% of the radioactivity applied can be accounted for in the gel fragments, and 90% of this is associated with the two subunits of succinate dehydrogenase. Thus, 70 to 75% of the theoretical counts per site should be obtained under the experimental conditions employed. The value of 3500 cpm is 74% of theoretical for the amount of protein subjected to polyacrylamide gel electrophoresis, assuming one site to be alkylated. From this it may be concluded that there is a site on the 30,000-molecular weight subunit which is readily alkylated by MalNEt. Secondly, 1 thiol residue is present on the flavoprotein subunit which is protected by malonate from alkylation.

Since, under the experimental conditions used, loss in activity seems to be restricted to alkylation of a single cysteinyl residue at the active site, the rate of alkylation of this residue may be compared with the rate of loss of catalytic activity. Fig. 4 shows that the kinetics of alkylation is essentially identical with the kinetics of inactivation of the enzyme. Since the two rates are the same, in spite of the large number of thiol groups present in the enzyme, it is possible to demonstrate the requirement of a cysteinyl residue, located on the flavoprotein subunit of succinate dehydrogenase, which is essential for the catalytic activity of the enzyme.

Electron Spin Resonance Studies Using Nitroxide Derivatives of Maleimide—The above experiments demonstrated that, under appropriate conditions, MalNEt would preferentially react with one sulfhydryl group on each enzyme subunit. The group on the smaller subunit is the most reactive, followed by the active site sulfhydryl, with nonessential sulfhydryl groups on the same subunit (Fig. 2B, squares) reacting slowly. We, therefore, believed that succinate dehydrogenase may be amenable to studies with nitroxide spin-labeled maleimide derivatives. We first tried to distinguish between the 30,000-molecular weight and substrate site sulfhydryl groups, and then investigated whether substrates, activators, or inhibitors induced environmental changes. Finally, we investigated the effect of distance between the nitroxide label and maleimide in order to probe the environment of the active group further.
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FIG. 3. Time course of incorporation of $^{14}$C MalNEt into succinate dehydrogenase subunits. Aliquots of the protein, prepared as described in Fig. 2, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The portions of the gel containing the 30,000- and 70,000-molecular weight subunits were solubilized in H$_2$O$_2$ and analyzed for $^{14}$C incorporation. A, 30,000-molecular weight subunit. B, 70,000-molecular weight subunit. \(\Delta\), incubation with malonate; \(\bullet\), no malonate present; \(\Delta\), difference between unprotected and malonate-protected samples.

FIG. 4. Kinetics of inactivation of succinate dehydrogenase with MalNEt and of $[^{14}$C]MalNEt alkylation of an essential cysteinyl residue. The enzyme was incubated with MalNEt as described in Fig. 2. The per cent of untreated enzyme remaining was determined from the best fit line of Fig. 3B (triangles) by the Guggenheim method (25).

To gain insight into the ESR spectrum of spin-labeled succinate dehydrogenase, the enzyme was labeled at 0° with a 10-fold excess of 3-(maleimidomethyl)-2,2,5,5-tetramethylpiperidinoxyl, as described under “Methods.” The spectrum (Fig. 5A) appears to be a superposition of at least two ensembles of labels, one of which is strongly immobilized, with a splitting between the outer hyperfine lines of 64 gauss. The weakly immobilized population (33 gauss) could be isolated by treating the enzyme with half-molar amounts of spin label while protecting the active site with malonate. The resulting spectrum (Fig. 5B), therefore, represents the cysteine on the 30,000-molecular weight subunit. The strongly immobilized spectra could also be isolated by treating the enzyme with an equimolar amount of MalNEt prior to labeling with spin label (Fig. 5C). This represents the active site sulphydryl group on the 70,000 subunit. These contentions are further supported by phenazine methosulfate assays. The enzyme used in Fig. 5B lost less than 10% of its original activity during incubation, whereas the latter lost 90%.

Since we are able to selectively label the reactive sulphydryl groups on the enzyme, we tried to ascertain its conformational lability. One might expect that the enzyme may be conformationally inert when labeled at the active site, and, indeed, such is the case. Adding a good activator, 0.5 M in NaBr, or a deactivator, oxalacetate, or substrate or oxidant (succinate or phenazine methosulfate) had no effect. Phenazine methosulfate and succinate together reduce the spin label, probably because the latter acts as an electron acceptor from phenazine methosulfate, which is reduced by residual active enzyme. Variation of the temperature between 15-35° also had no effect.

Labeled enzyme which had its active site protected by malonate was similarly unaffected by substrates and modifiers. In each experiment, the enzyme was fully activated, and its activity declined less than 10% during the labeling experiments. When oxalacetate was added, the enzyme was only 30% activated. Yet, no change was detectable in its ESR spectrum. One difference was that succinate now reduced the weakly immobilized signals, leaving residual strongly immobilized signals largely unchanged.

DISCUSSION

Although the kinetics of incorporation of MalNEt into succinate dehydrogenase is complex, by examination of the incorporation into each of the two subunits of the enzyme, the general picture is clear. The most rapidly reacting sulphydryl group, located on the 30,000-molecular weight subunit, is labeled approximately four times faster than the fast reacting cysteinyl residue located on the flavoprotein subunit and required for catalytic function of the enzyme. In addition to the above two groups, a second sulphydryl group on the flavoprotein subunit is alkylated (Fig. 3B, open squares). The rate...
of alkylation of this group is too slow for it to be essential for activity. The concentration of malonate employed is approximately 100 times its $K$, and no loss in activity is observed when succinate dehydrogenase is incubated with MalNEt in the presence of high concentrations of malonate (9). This low level of incorporation of MalNEt into the 70,000-molecular weight subunit, therefore, cannot represent incomplete saturation of the substrate site thiol group. Furthermore, this does not appear to be a site protected by malonate, for if it were, incorporation of MalNEt would be expected to approach 2 mol per mol of flavin in the absence of malonate in the experiment documented in Fig. 3B (i.e. ca. 7000 cpm).

It should be mentioned that, upon prolonged incubation of MalNEt with succinate dehydrogenase, additional net incorporation is observed in the flavoprotein subunit in the absence or presence of malonate. This secondary reaction appears to follow the incorporation of MalNEt into the essential sulfhydryl group of the flavoprotein, rather than occurring concurrently with it. Whether this is a result of exposure of another sulfhydryl group after alkylation of the active site or a more general unfolding of the enzyme is not clearly understood. Detailed kinetic analysis, however, would predict that more than one thiol group is required for catalytic activity, since inhibition of succinate dehydrogenase by MalNEt follows biphasic kinetics (9). Indeed, the kinetics of incorporation of [3H]MalNEt into this subsequent site is comparable to the kinetics of inhibition of the slow phase for enzymatic inactivation; however, the data are not sufficiently exact for definitive conclusions to be made.

The experiments in this report, which deal with events that occur during the initial, rapid loss in activity when succinate dehydrogenase is treated with MalNEt, lead to the conclusion that the cysteinyl residue, essential for catalytic activity and protected by substrates or competitive inhibitors from inhibition by sulfhydryl reagents, is located on the 70,000-molecular weight subunit of succinate dehydrogenase which also contains the covalently bound FAD moiety. The proximity of this residue to the flavin must be brought about by the tertiary structure of the enzyme.

Oxalacetate appears to bind in the tightly bound (thiohemiacetal) form with the same sulfhydryl group as the one whose alkylation is prevented by succinate or malonate. The evidence for this is: (a) as documented in Fig. 1, oxalacetate prevents alkylation of this sulfhydryl group; and (b) prior alkylation of succinate dehydrogenase by MalNEt prevents the binding of [14C]oxalacetate (9).

The reactive cysteine in the 30,000-molecular weight subunit is in an aqueous environment, as is witnessed by the high degree of mobility of spin labels attached to it (Fig. 5D). Furthermore, this spin label serves as a facile electron acceptor for succinate oxidation. Due to its colligative properties, the soluble enzyme exists primarily as a dimer at the concentrations needed for ESR studies. It seems possible, therefore, that an enzyme containing bound succinate can transfer electrons to a spin label on the surface of its neighboring molecule.

The active site sulfhydryl appears to inhabit a hydrophobic environment. Attached spin labels are strongly immobilized, despite spacer groups between the nitroxide ring and the maleimide. Thus, the weakly immobilized signals were still visible when 3-(maleimido)-2,3,5,5-tetramethylpyrrolidinooxyl was used. Furthermore, 3-(2-maleimidoethylcarbamoyl)-2,3,5,5-tetramethylpyrrolidinooxyl is still strongly immobilized, showing a decrease in splitting of the outer hyperfine lines of less than 0.5 gauss. Furthermore, those labels are not readily accessible as electron acceptors for succinate unless some mediator such as phenazine methosulfate is present. The general competition between anions for binding at this site (9, 26) further attests to its hydrophobicity. Its structure and composition make it particularly suitable for binding certain dicarboxyl compounds, including oxalacetate. Whether the formation of an enzyme-anion complex, or whether a conformational change (27), or thiohemiacetal formation in the case of oxalacetate binding (17), are also needed to explain the enzymology awaits further chemical and biophysical exploration.

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