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

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WILLIAM C. KENNEY, PATRICK C. MOWERY, RICHARD L. SENG, AND THOMAS P. SINGER

From the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 and the Division of Molecular Biology, Veterans Administration Hospital, San Francisco, California 94121

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 nitrooxide-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 N-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 8α-[N\(^{\text{V}}\)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\(^4\) and bromopyruvate was incomplete.

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4The 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 (23-residue) tryptic FAD peptide contains a cysteinyl residue (15). Questions, therefore, arose concerning the location of the thiol group involved in the inhibition. By differential labeling with $[^{14}C]$MalNEt in the presence or absence of malonate, Felberg and Hollocher (12) have reported that a crucial thiol group 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 (14). 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 into its subunits also cause extensive release of the tightly bound oxalacetate (16). Since it has not been possible to obtain covalent adducts which survive denaturation between the enzyme and substrates or competitive inhibitors, irreversible alkylation 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-$^{14}$C]maleimide (specific radioactivity, 4.8 mCi/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 Hatfield (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 0.25 m sodium perchlorate.

Enzymatic analyses were performed at $15^\circ$ as described previously (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted 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% H$_2$O$_2$ 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 E1 spectrometer outfitted 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 dithiothreitol (for incorporation experiments). Excess radioactive material was removed from the protein by precipitation with 15% (w/v) trichloroacetic acid followed by centrifugation at 12,800 $g$ 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 15% (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 polyacrylamide 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. Covaably bound flavin was determined fluorometrically (23); this was used to estimate the succinate dehydrogenase concentration, assuming a molecular weight of 100,000 (2).
The incorporation of radioactivity is obtained when malonate is present as presented in Fig. 3. As is apparent from Fig. 3A, the rate and level of alkylation of the 30,000- and 70,000-molecular weight subunits of succinate dehydrogenase. These results are 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. 3. As is apparent from Fig. 3A, the rate and level of alkylation of the 30,000 molecular weight subunit are the same whether or not malonate is present during the incubation with MalNEt. Fig. 3B 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 by subjecting the results to analysis by the Guggenheim method, as described in Ref. 25. Treatment of the data of Fig. 3B 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 activity site sulfhydryl, with nonessential sulfhydryl groups on the same subunit (Fig. 3B, 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.
Fig. 3. Time course of incorporation of [14C]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 H2O2 and analyzed for 14C incorporation. A, 30,000-molecular weight subunit. B, 70,000-molecular weight subunit. □, incubation with malonate; ◇, no malonate present; Δ, difference between unprotected and malonate-protected samples.

Fig. 4. Kinetics of inactivation of succinate dehydrogenase with MalNEt and of [14C]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 sulfhydryl 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 sulfhydryl 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 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 modulators. 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 sulfhydryl 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 sulfhydryl 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 10 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 concomitantly 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 \([^{14}C]\)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 cysteiny1 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 \([^{14}C]\)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)-2,2,5,5-tetramethylpyrroldinoxyl was used. Furthermore, 3-(2-maleimidomethylcarbamoyl)-2,2,5,5-tetramethylpyrroldinoxyl is still strongly immobilized, showing a decrease in splitting of the outer hyperfine lines of less than 0.5 gauss. Furthermore, these 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 dicarbonyl 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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