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Evidence for the Existence of Two Classes of Sulfhydryl Groups Essential for Membrane-Bound Succinate Dehydrogenase Activity†

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ABSTRACT: Kinetics of the inhibition of activated membrane-bound succinate dehydrogenase by N-substituted maleimides were studied. Three maleimide derivatives having a different hydrophobic character (*N*-ethyl-, *N*-butyl-, and *N*-benzylmaleimide) were tested. The method developed by Ray & Koshland (Ray, W. J., Jr., & Koshland, D. E., Jr. (1961) *J. Biol. Chem. 236*, 1973–1979) was used for analyzing experimental data. The results showed that two classes of sulfhydryl groups, with quite different reactivities, were essential for catalytic activity. The most reactive sulfhydryl groups were located in the substrate site as revealed by the fact that they were protected against alkylation in the presence of succinate or a competitive inhibitor, malonate. However, ligands of the catalytic site did not completely prevent inactivation of succinate dehydrogenase. Analysis of the kinetics of the inhibition observed in the presence of substrate indicated that the slow-reacting sulfhydryl groups did not belong to the active site. Rate constant values of the reaction of each set of sulfhydryl groups with the three maleimide derivatives showed that the most reactive thiols were probably located in a hydrophobic microenvironment since alkylation of this set of sulfhydryl groups was sensitive to the hydrophobic character of the thiol reagent. The reactivity of the other class of sulfhydryl groups was not influenced by the nature of the substituent. When the enzyme was deactivated by oxaloacetate, the two classes of sulfhydryl groups became unreactive with the alkylating agents. Masking of these groups may reflect a conformational change of the enzyme.

Succinate dehydrogenase (EC 1.3.99.1) is a dimeric enzyme composed of one subunit of molecular weight 70 000, containing the flavin residue and one subunit of molecular weight 30 000 (Kenney et al., 1976). The protein is firmly linked to and partially buried in the mitochondrial inner membrane (Merli et al., 1979).

In mitochondria, succinate dehydrogenase is subjected to a complicated regulatory mechanism [see Gutman (1978) for a review]. The enzyme can exist under two forms: an inactive state stabilized by binding of oxaloacetate (Wojtczak et al., 1969) and an active state which is stable in the presence of ligands of the catalytic site, such as succinate and malonate (Kearney, 1957; Gutman, 1977), or other compounds, such as $CoQH_2$[1] (Gutman et al., 1971a,b), ATP (Gutman et al., 1971c), and some inorganic anions (Kearney et al., 1974; Gutman, 1976).

Sensitivity of succinate dehydrogenase toward sulfhydryl reagents has been recognized for a long time (Barron & Singer, 1945; Singer et al., 1956). Although the enzyme contains a large number of sulfhydryl or disulfide groups (Vinogradov et al., 1972; Pagani et al., 1974), only a few of them are reactive with *N*-ethylmaleimide (Kenney, 1975; Kenney et al., 1976).

Succinate dehydrogenase has been described to possess one sulfhydryl group essential for catalytic activity, located in the substrate site (Kenney, 1975), in the immediate vicinity of the flavin moiety (Kenney et al., 1976). In the active form, soluble succinate dehydrogenase was inhibited by *N*-ethylmaleimide (Kenney, 1975). According to Kenney, the kinetics of inhibition was complex but malonate completely protected the essential sulfhydryl group against alkylation. However, Sanborn et al. (1971) and Felberg & Hollocher (1972) observed an incomplete protection of the catalytic activity when the soluble enzyme was allowed to react with *N*-ethylmaleimide in the presence of malonate. They concluded that the crucial cysteine was not at the catalytically active site.

In previous work (Le-Quoc et al., 1979), we showed that the inhibition by thiol reagents of some sulfhydryl-dependent mitochondria functions, including succinate oxidation, was modulated by the energetic state of mitochondria. This fact suggested that energization produces interactions between membrane components which modify the properties of individual enzymes. As shown by Merli et al. (1979), succinate dehydrogenase is partially embedded in the inner mitochondrial membrane. In this environment, the enzyme has some prop-

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[1] Abbreviations used: SDH, succinate dehydrogenase; TTFA, thenoyltrifluoroacetone; Tris, tris(hydroxymethyl)aminomethane; $CoQH_2$, reduced ubiquinone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NAD, β-nicotinamide adenine dinucleotide.

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erties which are lost when succinate dehydrogenase is removed from the membrane (Ohnishi et al., 1976; Beinert et al., 1977; Ackrell et al., 1980). Our purpose was to study the sensitivity of succinate dehydrogenase toward sulfhydryl reagents when the enzyme is bound to the membrane.

In this report, we studied the kinetics of inhibition of membrane-bound succinate dehydrogenase by *N*-ethylmaleimide and two more hydrophobic analogues. Kinetic results showed that two classes of sulfhydryl groups, having quite different reactivities, are essential for the catalytic activity. In contrast to the data obtained by Kenney (1975) with the soluble enzyme, succinate or malonate only partially protected succinate dehydrogenase toward inactivation by N-substituted maleimide derivatives. The most reactive sulfhydryl groups were protected from alkylation by ligands of the substrate site, indicating that these groups were located in the catalytic site. The other class of cysteinyl residues is not located in the active site, since it is not protected by succinate or malonate. However, the reaction of the two kinds of sulfhydryl groups with alkylating reagent is completely suppressed when succinate dehydrogenase is deactivated by incubation with oxaloacetate. Thus, an interaction of oxaloacetate with the enzyme restricted to the formation of a thiohemiacetal linkage with a cysteinyl residue in the active site (Kenney et al., 1976) is not sufficient to explain the protective effect of this anion.

Experimental Procedures

Maleimide derivatives were purchased from U.S.B., Cleveland, OH.

Rat liver mitochondria were isolated according to Weinbach (1961). Submitochondrial vesicles were obtained by sonication. Mitochondria (10 mg of protein mL^{-1}) were subjected to a hypotonic shock in distilled water, followed by an ultrasonic treatment of six 15-s periods at 4 °C at full setting in a Braun-sonic sonicator. Unbroken mitochondria were eliminated by centrifugation at 9000*g* for 10 min, and sonic particles were collected by sedimentation at 100000*g* for 60 min. The pellet of submitochondrial particles, representing ~25% of the initial supsension, was resuspended in 250 mM sucrose adjusted to pH 7.4 with Tris.

Succinate dehydrogenase was activated by incubating the sonic particles for 15 min at 37 °C with 50 mM β-hydroxybutyrate and 0.1 mM NAD or 50 mM succinate (or malonate) in 50 mM Hepes or 50 mM sodium phosphate, pH 7.6 TTFA (3 mM) and rotenone (10 μg) were also added together with succinate, in order to prevent electron transfer via the respiratory chain.

Succinate dehydrogenase activity was measured spectrophotometrically at 623 nm by using phenazine methosulfate coupled to 2,6-dichloroindophenol as described by Veerger et al. (1969). KCN (1.5 mM), antimycin (1.25 μg), and TTFA (1 mM) were included in the assay medium. TTFA was added to inhibit succinate–ubiquinone reductase activity (Hatefi & Stiggall, 1978). The assay was performed at 25 °C.

Inhibition (expressed as percent of the control) of membrane-bound succinate dehydrogenase activity by N-substituted maleimide derivatives was not influenced by the presence or the absence of TTFA (not shown).

Kinetics analysis of membrane-bound succinate dehydrogenase inactivation by sulfhydryl reagents required the estimation of free sulfhydryl reagent concentrations, since part of the added compound reacted with a large number of sulfhydryl groups present in the membrane. Thus, unreacted N-substituted maleimide derivative was estimated in the supernatant after the particles had been spun down. An excess of cysteine was added, and the remaining cysteine was estimated by a colorimetric assay at 405 nm by using 5,5′-dithiobis(nitrobenzoic acid).

Partition of maleimide derivatives in the 1-octanol–water system was measured at 25 °C after extensive mixing of 0.5 mM thiol reagent with 5 mL of octanol and 5 mL of water. Maleimide derivative concentration in the organic phase was estimated at 300 nm by using molar extinction coefficient values of 600 for *N*-ethylmaleimide and *N*-benzylmaleimide and 625 for *N*-butylmaleimide. Partition coefficient was expressed as the concentration of the compound in the organic phase over its concentration in the aqueous phase.

Protein content was determined according to Jacobs et al. (1956).

Results

Inhibition of Membrane-Bound Succinate Dehydrogenase by N-Substituted Maleimide after Activation of the Enzyme. When sonic particles were incubated in the presence of 50 mM β-hydroxybutyrate in 50 mM Hepes, pH 7.5, for 15 min, succinate dehydrogenase was activated through $CoQH_2$ production via the respiratory chain. The catalytic activity was 1.5 times greater than the activity of a sample incubated in the absence of a respiratory substrate. In the same conditions, incubation of membrane fragments with 50 mM succinate (plus 3 mM TTFA and 10 μg of rotenone) induced a 2.5-times activation of the enzymatic activity. If NADH was used as an activator of the enzyme, in experimental conditions similar to those described by Ackrell et al. (1974), the maximal enzymatic activity was quite comparable to that obtained after incubation of sonic particles with β-hydroxybutyrate.

Maleimide derivatives *N*-ethylmaleimide (Figure 1A, trace a) or the more hydrophobic compound *N*-benzylmaleimide (Figure 1B, trace a) strongly inhibited succinate dehydrogenase activity, when the enzyme was activated by the means of β-hydroxybutyrate oxidation.

As can be seen in Figure 1, trace b, succinate only partially protected the enzyme against inhibition. Malonate gave quite similar results (not shown).

The hydrophobic *N*-benzylmaleimide appeared to be a more potent inhibitor than *N*-ethylmaleimide (parts A and B of Figure 1).

In the experiments described in Figure 1, the thiol reagents were incubated for 4 min at 38 °C in a 50 mM phosphate buffer. Similar results were obtained after incubation for a longer time at 25 °C.

According to Kenney (1975), the phosphate medium used by Felberg & Hollocher (1972) should be responsible for the discrepancies observed in the two laboratories about the protective effect of succinate against *N*-ethylmaleimide inactivation. Using membrane-bound enzyme, we got partial protection, whether the experiments were performed in Hepes or phosphate buffer.

Graphical Analysis of Reaction Kinetics. In addition to *N*-ethyl- and *N*-benzylmaleimides, a third derivative, *N*-butylmaleimide, was tested. The longer alkyl chain of this compound increased its hydrophobic character compared to that of *N*-ethylmaleimide [as revealed by the distribution of these compounds in the two phases of the 1-octanol–water system (Table I)].

When succinate dehydrogenase was activated through β-hydroxybutyrate oxidation, the plot on semilog paper of the remaining activity, measured in the presence of an excess of the three N-substituted maleimides as a function of time, was not linear (Figure 2, traces a). This suggested that two classes of sulfhydryl groups, with different reactivities were involved in the inhibitory process.

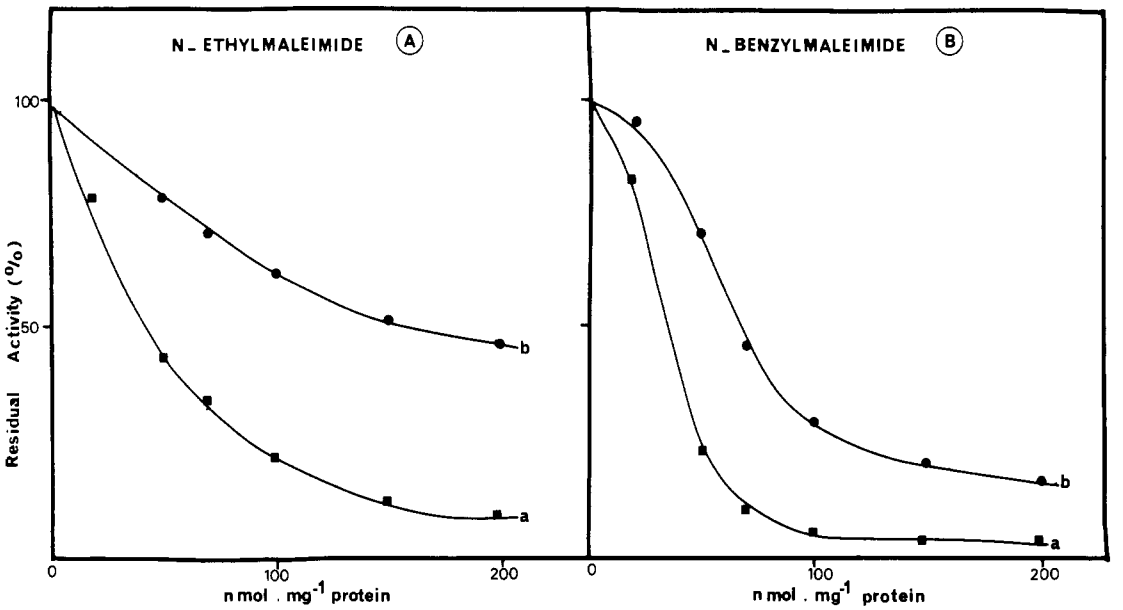


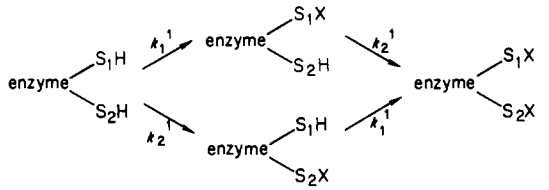
FIGURE 1: Dose–response curve for the inhibition of succinate dehydrogenase activity by *N*-ethylmaleimide (A) and *N*-benzylmaleimide (B) after activation of the enzyme either by succinate or through β-hydroxybutyrate oxidation. Sonic particles (5 mg of protein) were first treated with 50 mM β-hydroxybutyrate (a) or 50 mM succinate (plus 3 mM TTFA and 10 μg of rotenone) (b) for 15 min in 50 mM sodium phosphate, pH 7.6, at 37 °C. Incubation with the sulfhydryl reagent was then carried out for 4 min at 38 °C in the same medium.

Table I: Influence of the Substituent on the Partition Coefficient and on the Rate Constant Values of the Inhibition of Succinate Dehydrogenase by Maleimide Derivatives

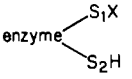
maleimide derivative	partition coefficient in 1-octanol–water system	second-order rate constants (min^{-1} mM^{-1})	
		k_1^2	k_2^2
N-ethylmaleimide	3.0	3.63	0.41
N-butylmaleimide	5.5	4.33	0.51
N-benzylmaleimide	∞[a]	15.40	0.58

[a] The low sensitivity of the spectrophotometric estimation did not allow us to detect any benzylmaleimide in the lower phase (water phase).

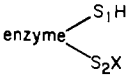
According to theoretical considerations established by Ray & Koshland (1961), the scheme of the reaction between the maleimide derivative and succinate dehydrogenase can be represented by



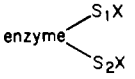
where $-S_1H$ and $-S_2H$ represent the two classes of sulfhydryl residues and X represents the alkylating group (k_1^1 and k_2^1 are the rate constants for pseudo-first-order reactions). The complex



is partially reactive and has only a fraction *F* of the activity of the enzyme, whereas the forms



and



are both inactive.

If k_1^1 is much greater than k_2^1, the equation of the inactivation reaction will be

$$A/A_0 = (1 - F)e^{-k_1^1 t} + Fe^{-k_2^1 t} \quad (1)$$

A/A_0 represents the enzyme activity divided by that at zero time.

Extrapolation of the straight line, corresponding to the slower reaction, back to the ordinate axis can give *F* and the slope gives k_2^1. At a given time, substraction of the values obtained by extrapolation from the observed values of A/A_0 gives a straight line with the slope k_1^1.

The traces c on Figure 2 were determined by this way. k_1^1 and k_2^1 are the pseudo-first-order rate constants for reaction with the two kinds of sulfhydryl groups essential for succinate dehydrogenase activity, in the presence of an excess of sulfhydryl reagent.

In the range of concentrations tested, reaction with *N*-ethylmaleimide with both classes of sulfhydryl groups was shown to follow second-order kinetics (Figure 3). Since the particles used in this study contain a lot of thiols which react with *N*-ethylmaleimide or analogues, the free thiol reagent concentration was different from the initial one. Corrections were made by estimating free maleimide concentrations in the supernatant after the membrane vesicles had been eliminated. These corrected concentrations are used in Figure 3.

As shown in Table I, the rate constants of the reaction with the fast-reacting sulfhydryl groups increase with the hydrophobic character of the reagent, while the second class of sulfhydryl groups is much less sensitive to the inhibitor hydrophobicity.

Protection by Succinate against Inhibition. Besides the finding that there are two classes of sulfhydryl groups essential for the catalytic activity of the enzyme, the partial protective effect of succinate against inactivation by maleimide derivatives deserved some attention. Figure 2 (traces b) shows that, in the presence of succinate, a linear representation was obtained, indicating the involvement of a single set of sulfhydryl groups in the inactivation reaction. The identity between the slopes obtained in the presence of succinate and the slope corresponding to the reaction of *N*-alkylmaleimide derivatives (*N*-

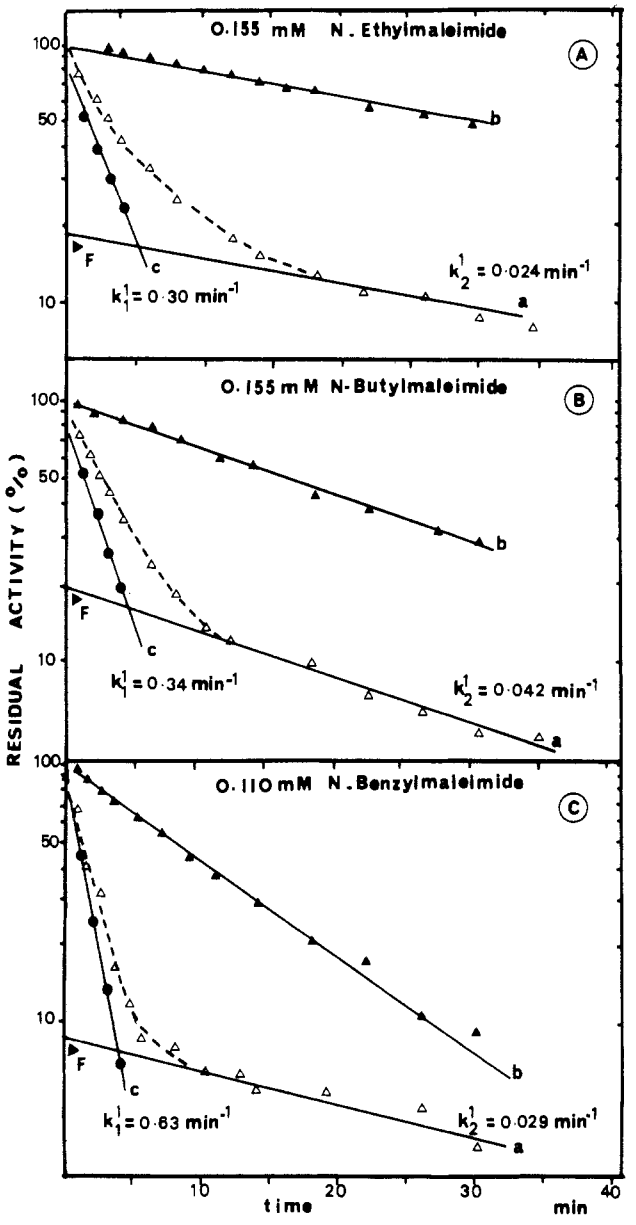


FIGURE 2: Kinetics of the inhibition of membrane-bound succinate dehydrogenase by *N*-maleimide derivatives at 25 °C. Succinate dehydrogenase was activated through 50 mM β-hydroxybutyrate oxidation (a) or by 50 mM succinate (plus 3 mM TTFA and 10 μg of rotenone) (b). At a given time, the values of the residual activity corresponding to the reaction with the slow-reacting sulfhydryl groups were extrapolated and substracted from the observed remaining activity. The slope of the resulting line (c) was used to determine the k_1^1 value.

ethylmaleimide and *N*-butylmaleimide) with the weakly reactive sulfhydryl groups is evident. The same results were obtained with the unsubstituted maleimide and *N*-methylmaleimide (not shown).

This suggested that the partial protection ensured by succinate was due to the masking of sulfhydryl groups located in the substrate site and that the other set of sulfhydryl groups did not belong to the active site. The cysteinyl residues situated in the substrate site were much more reactive than the other class. Similar results were obtained by using a competitive inhibitor, malonate, instead of succinate.

The high reactivity of *N*-benzylmaleimide with the sulfhydryl groups of the catalytic site (as indicated by the k_1^2 value; see Table I) can account for the fact that poor protection is observed in the presence of succinate (Figure 2C, trace b). The same result was obtained by using *N*-phenylmaleimide,

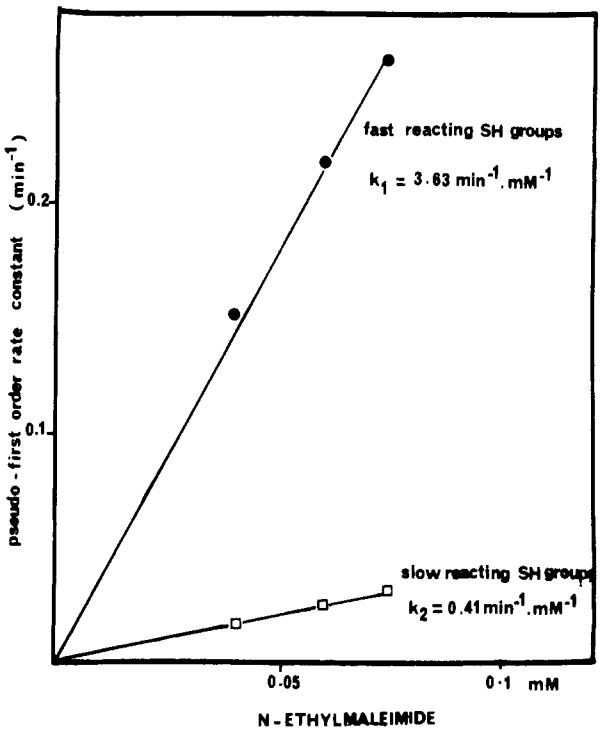


FIGURE 3: Influence of *N*-ethylmaleimide concentrations on the pseudo-first-order constants of succinate dehydrogenase inactivation. First-order rate constants were obtained from graphs similar to that of Figure 2 by using different *N*-ethylmaleimide concentrations. The slope of the lines gave the second-order constant values.

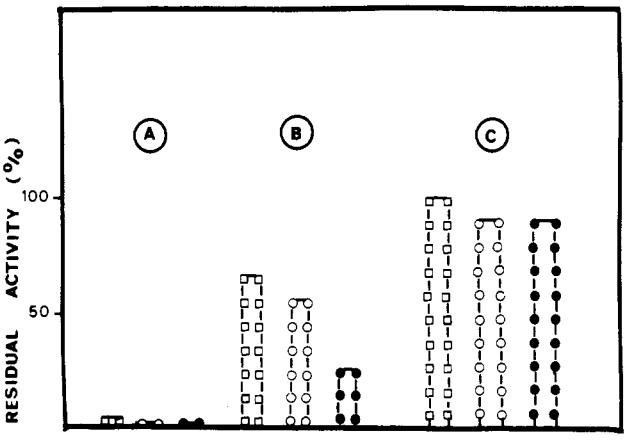


FIGURE 4: Comparative inhibition of membrane-bound succinate dehydrogenase by maleimide derivatives measured with activated or deactivated enzyme. Membrane-bound SDH was activated by treatment with 50 mM β-hydroxybutyrate (A) or 50 mM succinate plus 3 mM TTFA and 10 μg of rotenone (B) or desactivated by 1.2 mM oxaloacetate plus 10 μg of rotenone (C). Membrane-bound SDH was then allowed to react for 20 min at 25 °C with 0.15 mM SH reagent, *N*-ethylmaleimide (□), *N*-butylmaleimide (○), or *N*-benzylmaleimide (●), in 50 mM Hepes, pH 7.5. The oxaloacetate deactivated particles (C) were washed and centrifuged at 100000*g* and then reactivated by incubation with 50 mM succinate plus 1.5 mM TTFA for 15 min at 37 °C. The enzyme activity was measured as described under Experimental Procedures.

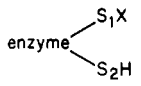
another *N*-aryl-substituted compound, which was equally highly reactive with the sulfhydryl groups of the active site (not shown).

Modification of Enzyme Thiol Groups' Sensitivity Induced by Specific Ligands. The above results show that membrane-bound succinate dehydrogenase, when in the activated state, was very sensitive toward sulfhydryl reagents and that ligands of the active site partially protected the enzyme from inactivation. This is also illustrated on Figure 4A,B.

When sonic particles were incubated in the presence of oxaloacetate, succinate dehydrogenase was stabilized under the inactivated state and the specific activity of the enzyme was very low. N-Substituted maleimide derivatives were added to oxaloacetate-treated membranes; after a 20-min reaction at 25 °C, the sonic particles were spun down in order to eliminate the excess of reagents. Succinate dehydrogenase was reactivated by 50 mM succinate before enzymatic measurements. It can be seen in Figure 4C that the catalytic activity of the reactivated enzyme was not inhibited by maleimide derivatives. Even *N*-benzylmaleimide, which was the more potent inhibitor when the enzyme was in the activated state, had no effect.

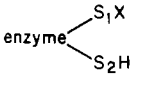
Discussion

The inhibition of membrane-bound succinate dehydrogenase by N-substituted maleimide derivatives follows biphasic kinetics. This would suggest that two classes of sulfhydryl groups are required for catalytic activity. Rate constants for the two classes of sulfhydryl groups are quite different. The most reactive set reacts 8–30 times faster (depending on the sulfhydryl reagent) than the second class. The alkylation rate for the most reactive class increased with the partition coefficient of the inhibitor in 1-octanol–water system (Table I). This result would mean that these sulfhydryl groups are located in a hydrophobic environment and is in accordance with electron spin resonance studies on soluble succinate dehydrogenase, using nitroxide derivatives of maleimide, reported by Kenney et al. (1976). When the general scheme proposed by Ray & Koshland (1961) is applied to the special case of succinate dehydrogenase, it can be concluded that alkylation of the most reactive cysteinyl residues gave a modified enzyme



which is not fully inactive. It should be noticed that the activity of this form, given by the *F* factor value (see eq 1 and Figure 2), is dependent on the nature of the inhibitor. When the sulfhydryl reagent is an *N*-alkylmaleimide [methyl (not shown), ethyl, or butyl radical], the *F* value is ~20% of the total activity, whereas the *F* value obtained in the presence of an *N*-arylmaleimide [*N*-phenylmaleimide (not shown) or *N*-benzylmaleimide] is only ~9%. The steric hindrance of the benzene ring or an electronic effect of the substituent can explain this lower value.

The results derived from protection studies by succinate or malonate indicate that the highly reactive class of sulfhydryl groups belongs to the substrate site. But these groups seem to play only an indirect role in the chemical process of succinate oxidation, since the alkylated form



still possesses a residual activity. The less-reactive sulfhydryl groups are not protected by the substrate, and the rate constant values are almost insensitive to the nature of the maleimide substituent (Table I). However, our results show that, although weakly reactive and not located in the active site, these cysteinyl residues are essential for optimal catalytic activity of succinate dehydrogenase.

On the other hand, the experiments of this report show that when the enzyme is deactivated by oxaloacetate, both classes of cysteinyl residues, crucial for enzymatic activity, are no more reactive with N-substituted maleimides, even with the most hydrophobic derivative. Three hypothesis can be put forward to explain this result. (1) Oxaloacetate reacts with the two kinds of cysteinyl residues via a thiohemiacetal formation (Vinogradov et al., 1972); however, this proposal is not consistent with the fact that, according to Ackrell et al. (1974) and Gutman (1976), the ratio mole of oxaloacetate/mole of histidyl-bound flavin is 1. (2) Oxaloacetate binding, possibly at a regulatory site, induces a conformational change of the enzyme in such a way that sulfhydryl groups are masked, as postulated by Gutman (1978). (3) Binding of oxaloacetate and formation of a thiohemiacetal with the cysteinyl residue of the active site (Kenney et al., 1976; Kenney, 1975; Coles et al., 1979) lead to a modification of the enzyme conformation so that the other sulfhydryl groups become unreactive.

The partial protective effect of succinate or malonate against inactivation that we observed represents the main difference between the results of Kenney (1975), who studied the soluble form of the enzyme, and our results. This difference is important since it supports—together with kinetic analysis of the inhibition process—the conclusion that two sets of sulfhydryl groups are essential for the catalytic activity. The reaction of the inhibitor with the most reactive groups causes a rapid loss of the enzyme activity. Modification of the other set of sulfhydryl groups, although being a slow process, induces an important decrease of the catalytic activity. As can be seen in Figure 2 (traces b), alkylation of cysteinyl residues in the presence of succinate is responsible for 40–60% inhibition of the enzymatic activity after 20-min reaction with *N*-ethylmaleimide or *N*-butylmaleimide. This absence of total protection by ligands of the active site that we report with the particulate enzyme may be due to its location in the membrane.

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Dihydrofolate Reductase Hysteresis and Its Effect on Inhibitor Binding Analyses†

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ABSTRACT: *Escherichia coli* dihydrofolate reductase was shown to follow slow transient kinetics (hysteresis). Nonlinear reaction velocities were detected during the enzyme assay and required 10–15 min to reach a steady-state rate. The degree of hysteresis was influenced by the enzyme concentration and the order of substrate addition. Incubation of the enzyme with NADPH before addition of dihydrofolate resulted in slow initial velocities that increased up to 2-fold during the course of the assay. Increasing the enzyme concentration from 0.2 to 1 nM resulted in diminished hysteresis. NADPH-initiated reactions were linear at all enzyme concentrations tested. Certain drugs had profound effects on hysteresis. Pyrimethamine practically eliminated the hysteresis of dihydrofolate-started reactions, whereas trimethoprim augmented the nonlinearities in the sense that hysteresis was detected in both enzyme- and NADPH-started reactions. The shape of these reaction tracings makes trimethoprim appear to be a slow binding inhibitor. However, trimethoprim is not a slow-binding inhibitor when assayed under conditions that eliminate hysteresis. Contrary to this, sulfamethoxazole did not affect hysteresis or augment inhibition of the enzyme by trimethoprim. Sulfamethoxazole alone (at 6 mM) did not inhibit the enzyme. A simple procedure has been developed to circumvent hysteresis and allow reliable determinations of K_i values of both weak and tight binding inhibitors. For example, K_i values for pyrimethamine, trimethoprim, and methotrexate were found to be 214 nM, 1.3 nM, and 0.021 nM, respectively.

Dihydrofolate reductase (EC 1.5.1.3) is the site of action of several important drugs including methotrexate, trimethoprim, and pyrimethamine (Hitchings & Burchall, 1965). Presently, there is much interest in understanding the mechanisms by which these inhibitors bind to their target enzyme with such high affinity. The X-ray crystal structures of the *Escherichia coli* enzyme–methotrexate complex (Matthews et al., 1977) and the *Lactobacillus casei* enzyme–methotrexate–NADPH complex (Matthews et al., 1978) revealed many residues that intereact with methotrexate, but there is no satisfactory explanation that completely accounts for its greatly enhanced binding compared to dihydrofolate (Hood & Roberts, 1978). In fact, there is mounting evidence that the binding of ligands to dihydrofolate reductase is a complex process, and a variety of protein conformations with different binding affinities have been detected by NMR, fluorescence, and kinetic techniques (Feeney et al., 1977; Dunn et al., 1978; London et al., 1979; Williams et al., 1979). However, kinetic analyses can be difficult to perform and interpret. Both substrates are fairly unstable and can degrade to a number of products (Blakley, 1969; Lowry et al., 1961). Background (nonenzymic) changes in 340-nm absorbance are common (Baccanari, 1978), and most inhibitors of interest have low K_i values (1 nM or less). Several years ago, Jackson et al. (1977) studied methotrexate inhibition of the enzyme from several mammalian sources and measured its slow off-rate by a variety of means. More recently the general theory of tight-binding inhibitors has been expanded by Cha (1975, 1976) to include analysis of slow-binding and slow, tight-binding inhibitors (Williams & Morrison, 1979). This type of inhibition involves a two-step inhibitor binding process which includes the slow isomerization of one enzyme conformation to another and is characterized by nonlinear reaction velocities. Williams et al. (1980), studying the enzyme from *Streptococcus faecium*, noted that inhibition by several compounds, including trimethoprim and *p*-aminobenzoyl glutamate, was time dependent and interpreted these data to indicate the compounds were slow-binding inhibitors. In this study, we report conditions under which hysteresis (a slow transient velocity) is observed with uninhibited *E. coli* dihydrofolate reductase and examine its relationship to inhibitor binding. We also present a method of circumventing hysteresis and determining tight-binding K_i values.

Materials and Methods

Trimethoprim and pyrimethamine were obtained from Burroughs Wellcome Co., Greenville, NC. Sulfamethoxazole (a product of Roche) was also obtained from Wellcome. The various NADPH preparations were from Sigma Chemical Co. and represented many different "lots" manufactured over a 2-year period. We also tested NADPH from P-L Laboratories and Boehringer Mannheim. Folic acid was purchased from Calbiochem and NADP was from Boehringer Mannheim. All

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