Journal of Bioenergetics and Biomembranes, Vol. 25, No. 6, 1993

Inhibition of Membrane-Bound Succinate Dehydrogenase by Fluorescamine

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Received June 15, 1993; accepted May 24, 1993

Fluorescamine rapidly inactivated membrane-bound succinate dehydrogenase. The inhibition of the enzyme by this reagent was prevented by succinate and malonate, suggesting that the group modified by fluorescamine was located at the active site. The modification of the active site sulfhydryl group by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) did not alter the inhibitory action of fluorescamine. However, the protective effect of malonate against fluorescamine inhibition was abolished in the enzyme modified at the thiol.

KEY WORDS: Fluorescamine; succinate dehydrogenase; heart mitochondria; submitochondrial particles; primary amine.

INTRODUCTION

The sensitivity of succinate dehydrogenase (SDH) (EC 1.3.99.1) to thiol-binding reagents has been recognized for many years (Hopkins and Morgan, 1938; Hopkins *et al.*, 1938). Inhibition of the enzyme by these kinds of reagents resulted from the modification of a sulfhydryl group located at the active site (Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984; Phillips *et al.*, 1987; Jay, 1991; Jay *et al.*, 1991). This thiol, although not essential for substrate binding or catalysis (Hederstedt and Heden, 1989), could influence the binding of dicarboxylates, probably by steric hindrance when a larger group or a charged group were attached to it (Kotlyar and Vinogradov, 1984b; Hederstedt and Heden, 1989; Jay *et al.*, 1991; Schroder *et al.*, 1991). The inhibition of SDH by a histidine-specific reagent has also been reported, and the participation of an imidazole ring in the initial step of succinate oxidation has been suggested (Vik and Hatefi, 1981). In addition, the inactivation of membrane-bound and soluble SDH by phenylglyoxal and 2,3-butanedione has shown the presence of an essential arginine residue(s) which interacts with dicarboxylate to form the primary enzyme–substrate complex (Kotlyar and Vinogradov, 1984b).

The results of the present study describe the effect of fluorescamine (4-phenylspiro[furan-2(3*H*), 1′-phthalan]-3,3′-dione), a reagent for primary amines, on membrane-bound SDH.

MATERIAL AND METHODS

Mitochondria from bovine heart were prepared by the polytron technique as reported before (Jurkovitz *et al.*, 1974). Mitochondria were used for the preparation of submitochondrial particles (EPTH) as indicated by Lee and Ernster (1966). Since membrane-bound SDH is inactivated in this preparation by the presence of tightly bound oxaloacetate (Wojtczak *et al.*, 1969), the enzyme was activated as indicated (Jay, 1991).

Inhibition experiments were carried out at 25°C in a medium containing 200 mM sucrose, 50 mM HEPES (pH 7.8), 1 mM cyanide, and succinate or malonate, at the concentrations indicated in the

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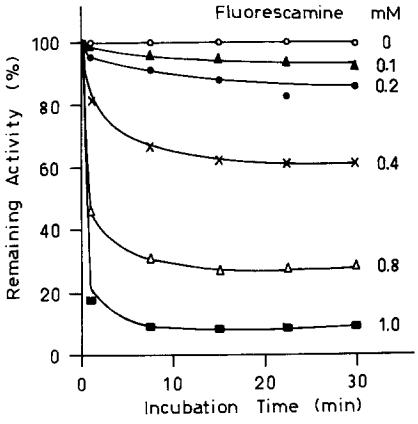

Fig. 1. Inactivation of membrane-bound SDH by fluorescamine. Submitochondrial particles (42 μg protein/ml) were incubated in a medium containing 200 mM sucrose, 50 mM HEPES-NaOH (pH 7.8), and 1 mM NaCN. The reaction was initiated by the addition of the indicated concentrations of fluorescamine. At the times indicated on the abscissa, the appropriate amount of ETPH was withdrawn and their succinate: PMS reductase activity was assayed as described in Materials and Methods. 100% of activity represents 1.4 μmol succinate oxidized per min per mg protein.

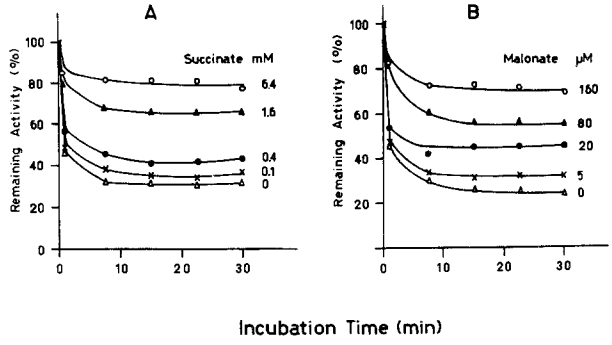

Fig. 2. Effect of succinate and malonate on the inactivation of membrane-bound SDH by fluorescamine. ETPH were modified by fluorescamine (800 μM) as described in Fig. 1 but in the presence of the indicated concentrations of succinate (A) or malonate (B). In the absence of fluorescamine, the enzyme was perfectly stable in the presence of succinate or malonate.

figures. The reaction was started by the addition of ETPH (42 μg protein/ml) followed by the addition of fluorescamine (from a 100 mM solution, dissolved in acetone), the concentrations of which are indicated in the legends of the figures; the addition of acetone to control experiments indicated that this solvent did not have any effect on the dehydrogenase activity. Aliquots from these media were withdrawn at the indicated times, and the dehydrogenase activity was measured by adding 10 mM succinate (or the amount needed to complete this concentration in the case of the media already having succinate), 0.064 mM dichloroindophenol (DCIP), and 1.4 mM phenazine methosulfate (PMS) (final volume 3.0 ml); see Mowery *et al.*, 1977.

Protein concentration was determined by the method of Lowry *et al.* (1951).

RESULTS

Fluorescamine is a reagent which reacts directly with primary amines to yield fluorescent products (Weigele *et al.*, 1972a,b). For assay, peptides or proteins are buffered to an appropriate pH (≥ 7) (Udenfriend *et al.*, 1972) and then fluorescamine, dissolved in a nonhydroxylic solvent, is added. The reaction with the primary amino groups is completed very fast ($t_{1/2} \sim 100$–500 msec) and the excess reagent is destroyed by hydrolysis in a reaction with a halftime of 5–10 sec. All these properties have made fluorescamine a very useful reagent in the study of primary amines in biochemistry (Udenfriend *et al.*, 1972; Vandekerckhove and Montagu, 1974).

In agreement with this scheme of reaction, Fig. 1 shows that the addition of fluorescamine to membrane-bound SDH in a sucrose medium at pH 7.8 induced a rapid loss of the succinate PMS reductase activity. The degree of inactivation reached a different stable level, depending on the concentration of fluorescamine added (0.1–1.0 mM), indicating that no further reaction took place after the destruction of the reagent.

In order to obtain more information on the site of action of fluorescamine, the membrane preparations were incubated in media containing succinate or malonate. If an irreversible inhibitor acts by combining with a group at the active site, it might be expected that the presence of the substrate or competitive inhibitor will protect against inhibition. As shown in Fig. 2 increasing concentration of succinate (0.1–6.4 mM, Fig. 2A) or malonate (5–160 μM, Fig. 2B) progressively diminished the level of inactivation induced by 800 μM fluorescamine.

Since succinate dehydrogenase contains a sulfhydryl group at the active site, it was of interest to find out whether the modification of the thiol could alter somehow the reactivity of fluorescamine toward the catalytically active site. As shown in Table I (line 8) DTNB-treated enzyme had the same reactivity toward fluorescamine as that exhibited by the control particles under these conditions (line 5). However, the

Table I. Effect of DTNB on the Reactivity of Membrane-Bound Succinate Dehydrogenase toward Fluorescamine[a]

Conditions	Remaining activity (%)	Inhibition by fluorescamine (%)	Protection by malonate (%)
1. Control	100	—	—
2. Control → DTT	100	—	—
3. DTNB	7	—	—
4. DTNB → DTT	62	—	—
5. Fluo	17	83	—
6. Fluo → DTT	17	83	—
7. DTNB → Fluo	0	—	—
8. DTNB → Fluo → DTT	10	84	—
		(with respect to line 4)	
9. Mal	100	—	—
10. Mal → Fluo	79	21	75
11. DTNB → Mal → DTT	63	—	—
12. DTNB → Mal → Fluo → DTT	11	83	0
		(with respect to line 11)	

[a] Submitochondrial particles (1 mg protein/ml) were incubated at 25°C in a mixture containing 200 mM sucrose, 50 mM HEPES-NaOH (pH 7.8), and the following reagents added in the order indicated: 0.1 mM DNTB (during 20 min), 6.0 mM malonate (Mal) (during 5 min), 1.5 mM fluorescamine (Fluo) (during 10 min), and 2.0 mM dithiothreithol (DTT) (during 5 min). After incubation, all the samples were cooled on ice, centrifuged, and their remaining activities measured as indicated in Material and Methods.

protective effect of malonate (Table I, line 10) against the inactivation by fluorescamine was lost when the active-site sulfhydryl was modified by DTNB (Table I, line 12).

DISCUSSION

Fluorescamine is a reagent that was specifically synthesized (Weigele *et al.*, 1972a,b) to react with substances containing primary amino groups. In this regard, an examination of the characteristics of the interaction between fluorescamine and membrane-bound SDH, illustrated in Fig. 1, clearly suggests that the inhibition of the enzyme activity could be the consequence of the modificatin of an amino group. In addition, the protection pattern exerted by succinate and malonate (Fig. 2) suggests that the group modified by fluorescamine is located in the region of the active site.

With respect to the composition of amino acids forming the active site of SDH, this has not been completely defined. Chemical modification studies have indicated the presence of a highly reactive cysteine residue located near the binding site of substrate (Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinograodov, 1984a,b; Phillips *et al.*, 1987; Jay, 1991; Jay *et al.*, 1991). A role for a specific histidine residue in the catalytic mechanism, as a proton donor/acceptor, and for an arginine group(s) in the binding of substrate has also been proposed on the basis of chemical inhibition and site-directed mutagenesis studies (Vik and Hatefi, 1981; Kotlyar and Vinogradov, 1984b; Schroder *et al.*, 1991).

With respect to the role of the active-site cysteine, Hederstedt and Heden (1989) demonstrated that the thiol is not required for substrate binding or catalysis. The *B. subtilis* succinate dehydrogenase binds succinate and oxalacetate normally; yet it has alanine replacing cysteine. The cysteine near the active site could curtail enzyme activity, when it was chemically modified, most likely by interfering with the binding of substrate to the arginine residue. Indeed, succinate dehydrogenase could not be equally inhibited by the arginine-specific reagent phenylglyoxal in the *p*-chloromercuribenzoate-treated enzyme (Kotlyar and Vinogradov, 1984b). This effect of the thiol modification has been ascribed to the fact that cysteine is located adjacent, in the amino acid sequence, to the conserved arginine residue thought to interact with dicarboxylates (Schroder *et al.*, 1991). In this respect Table I (line 12) shows that the protective action of malonate against fluorescamine inhibition was lost when the SH group was modified by DTNB, confirming the fact that malonate could no longer interact with its binding site when the thiol was blocked

(Kotlyar and Vinogradov, 1984b). On the other hand, it was interesting to observe that the reactivity of fluorescamine toward succinate dehydrogenase was not altered when the active-site sulfhydryl was modified (Table I, line 8), suggesting that the group sensitive to fluorescamine is located in the active site in a relatively distant position from both the cysteine and arginine residues.

Since fluorescamine is a specific reagent for primary amines and it does not react with the guanidine group of arginine *in vitro* (Udenfriend *et al.*, 1972) or the active-site arginine residue in SDH (present results), at this point we do not have evidence concerning the identity of the group(s) modified when the enzyme is inactivated by the dye. In a sequence of $\sim$ 26 amino acids, thought to contribute to the active site of fumarate reductase and succinate dehydrogenase, a residue of glutamine in a conserved position appears as the only possible target for fluorescamine (Schroder *et al.*, 1991). However, any amino group brought close to the active site by the folding of the protein could equally react with the reagent. Further investigation should be done in order to determine whether an amino group(s) could play a role in the catalytic mechanism of succinate dehydrogenase.

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