THE BINDING SITE FOR OXALOACETATE ON SUCCINATE DEHYDROGENASE

by

Andrei D. Vinogradov*, Daryl Winter and Tsoo E. King
Department of Chemistry
State University of New York at Albany
Albany, New York 12222

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SUMMARY--Oxaloacetate, a competitive inhibitor of succinate dehydrogenase, bound with a sulfhydryl group of the enzyme to abolish the enzymic activity. Subsequently a thiosemiacetal was apparently formed to render the inhibition practically irreversible. The dehydrogenase, after taking up 25 silver equivalents per flavin, bound little oxaloacetate.

The experiments by Hopkins and coworkers (1) have decisively established succinate dehydrogenase as an enzyme requiring sulfhydryl groups for its action. This discovery has been widely confirmed and further extended (for example, 2, 3, and references cited therein). It has been found that oxaloacetate is one of, if not the, most powerful competitive inhibitors of succinate dehydrogenase (4). However, in spite of diligent studies in many laboratories for nearly 40 years, the locus of the inhibitory action has been only conjectural (cf. 3-7). From recent experiments, we have evidence to show that the abolition of enzymic activity occurs when oxaloacetate binds with the dehydrogenase through a sulfhydryl group. The potency of oxaloacetate is evidently due to the rapid formation of a thiosemiacetal between the carbonyl group of the inhibitor and a sulfhydryl of the enzyme.

EXPERIMENTAL--The reconstitutively active** succinate dehydrogenase was prepared by our method (8) and contained 8 nonheme iron and 8 acid-labile

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*Permanent address: Department of Animal Biochemistry, School of Biology, Moscow State University, Moscow, U. S. S. R.

**See Ref. 3 for description of reconstitutive activity of succinate dehydrogenase. Briefly, the reconstitutively active preparation can be reincorporated into a particle which contains all respiratory components except succinate dehydrogenase, to re-form succinate oxidase. The reconstituted succinate oxidase shows all the properties of the intact succinate oxidase.

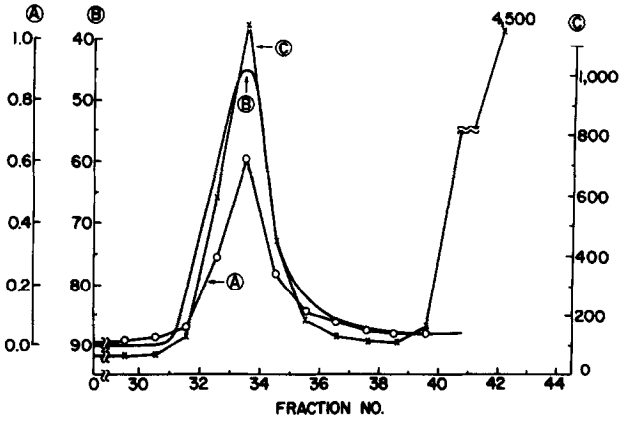

Fig. 1. The incorporation of ^{14}C-oxaloacetate to succinate dehydrogenase. The reaction mixture contained 5 mg succinate dehydrogenase equivalent to 20 nmoles of acid non-extractable flavin, 13 mM ^{14}C-uniformly labelled oxaloacetate and 0.1 M phosphate buffer, pH 7.8 in a total volume of 1 ml. After incubation at room temperature for 10 min., it was layered on a column of Sephadex G-50 (Grade Fine) which had been equilibrated with 0.1 M phosphate buffer. The elution was effected by 0.1 M phosphate, pH 7.8. The fractions were collected at 6 drops per minute and each fraction contained 20 drops or approximately 1 ml depending upon the surface tension of the fractions. The protein content of each fraction was determined by the Lowry method in mg per 0.5 ml eluate as shown in ordinate A and by automatic scanning at 280 nm by a LKB UV Monitor in % of T as shown in Ordinate B. The radioactivity of each fraction was determined in a liquid scintillation counter and expressed in net counts per minute per 0.5 ml eluate (i.e., less the background counts of about 40 cpm) as shown in Ordinate C.

The succinate dehydrogenase was prepared as previously described up to the first ammonium sulfate precipitate form (8). The ^{14}C-labelled oxaloacetate was prepared from ^{14}C-uniformly labelled malate (Amersham-Searle) in the presence of recrystallized malate dehydrogenase and DPN. The DPNH formed was regenerated by DPNH oxidase. Unreacted malate was not removed in the experiment but its presence does not affect the purpose and conclusion of the experiment. The details of these aspects will be reported later.

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sulfide per flavin. The details of the methods and conditions of the operations are presented in the legends to the table and the figure.

RESULTS AND DISCUSSION--This dehydrogenase could readily bind oxaloacetate as demonstrated by the experiment with ^{14}C-labelled inhibitor. As shown in Fig. 1, the oxaloacetate-enzyme complex was stable enough to withstand passage through a Sephadex column. Indeed, the binding with

TABLE I. The binding of oxaloacetate to succinate dehydrogenase*

	nmoles of oxaloacetate bound per mg of dehydrogenase	Oxaloacetate bound per flavin
1. Succinate dehydrogenase fresh, untreated	2.6	0.65
2. Succinate dehydrogenase, after uptake of 25 equivalents of Ag^+	0.48	0.12

*All binding experiments with ^{14}C-labelled oxaloacetate were conducted under the same conditions described in the legend of Fig. 1. The titration of succinate dehydrogenase by Ag^+ was performed amperometrically (9) until 25 silver equivalents were consumed per mole of flavin.

oxaloacetate was irreversible, just as that with N-ethylmaleimide (cf. 6, 7). The initial rate of the inhibition depended upon the ratio of the substrate to the inhibitor in the system; however, once the oxaloacetate was bound, it could not be easily replaced by succinate. The oxaloacetate bound to the enzyme was equivalent to approximately two-thirds of the flavin (cf. Table I). This observation may be due to the fact that not all molecules of the dehydrogenase in the preparation have viable active sites and thus some are incapable of binding with oxaloacetate. When the dehydrogenase was initially titrated with Ag^+, approximately 25 equivalents of silver per flavin (Table I) were required to abolish both enzymic activity and the binding of oxaloacetate.

The possibility that labile sulfide was the group responsible for the binding of these inhibitors to the enzyme was eliminated by analyzing the acid labile sulfide content after binding was completed. Experiments of this type showed no difference between the labile sulfide present in the enzyme as isolated or in the presence of succinate, oxaloacetate or N-ethylmaleimide.

These observations are in agreement with, and strongly reinforce the results of, a model study previously reported (6, 7). The model study

shows that oxaloacetate reacts with cysteine and that the rate and behavior of this reaction exhibits the same characteristics as the reaction of the enzyme with the inhibitor. From the results reported in this communication and those from the model study (7), we think that oxaloacetate binds one sulfhydryl group of the Hopkins type eventually to form a covalent linkage. Through the reaction of the α,β-activated carbonyl group of oxaloacetate with a sulfhydryl at an active site of the dehydrogenase, a thiosemiacetal is formed. It is apparent, then, that the reaction between the enzyme and the inhibitor consists of two steps. The non-covalent binding with a sulfhydryl takes place first and thus inhibits the enzymic activity competitively with succinate. This is followed by the formation of a covalent bond which "stabilizes" the enzyme-inhibitor complex in an essentially irreversible state, and thus differs from the mechanism for malonate inhibition. It is most likely that the very same sulfhydryl, but not acid labile sulfide, binds with succinate to form the active enzyme-substrate complex.

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