TIGHT BINDING OF OXALOACETATE TO SUCCINATE DEHYDROGENASE

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SUMMARY: [^{14}C]Oxaloacetate forms a stable complex with succinate dehydrogenase which withstands repeated Sephadex filtration. Oxidized glutathione, 2-thenoyltrifluoroacetone, KCN and ageing at +4° at neutral pH do not prevent the enzyme to bind oxaloacetate. The binding is prevented by succinate or malonate but the complex, once formed, can not be split by these compounds, although the enzyme activity can be restored; the reconstitutive property of succinate dehydrogenase is, however, irreversibly lost. Bound oxaloacetate does not exchange with added oxaloacetate, but can be released by perchloric acid. Sonic particles of beef heart mitochondria can also bind oxaloacetate. However, this complex can be split by succinate or malonate.

It has been shown (1 - 3) that oxaloacetate (OA) differs from other competitive inhibitors of succinate dehydrogenase (SD) by the fact that the inhibition develops slowly upon incubation of the enzyme with the inhibitor. The reversal of the inhibition by high concentrations of substrate or by removal of OA was also slow (2). Two alternative explanations of this peculiarity have been proposed (2): 1. SD forms a stable complex with OA; 2. OA induces a slow but reversible conformational inactivation of SD. To check the first possibility we studied the reaction between [^{14}C]OA and SD and found the formation of a relatively stable complex which could not be split by gel filtration. These results were already published in a preliminary form (4). Recently, two reports pertinent to this subject have appeared. Vinogradov et al. (5) showed the formation of a stable complex between SD and OA, resistant to Sephadex filtration, similar to that described by ourselves (4); and Kearney et al. (6) reported on the presence of tightly bound OA in deactivated preparations of SD. In the present paper we describe in more detail our

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study on complex formation between SD and OA and present further data on the properties of this complex. We also provide an evidence that a site on the enzyme molecule involved in the binding of OA is also responsible for the reconstitution of the succinate oxidase system (7, 8).

MATERIALS AND METHODS

Crude soluble, reconstitutively active, SD was obtained from beef heart mitochondria by the method of King (9) with slight modifications (2) and passed through a column of Sephadex G 25, equilibrated with 10 mM Tris-HCl + 1 mM EDTA (pH 7.4), to remove succinate. Partially purified SD was prepared according to Dervartanian and Veeger (10), occasionally omitting the last (2nd) ammonium sulfate precipitation. Enzyme activity was determined spectrophotometrically with phenazine methosulfate and 2,6-dichlorophenolindophenol (11), as described before (2). [^{14}C]OA was obtained from uniformly labelled [^{14}C]aspartate (specific activity 79 mCi/mmol) by incubating with an excess of 2-oxoglutarate and glutamate -oxaloacetate transaminase (EC 2.6.1.10). The efficiency of [^{14}C]OA formation exceeded 75% and the remaining [^{14}C]aspartate was not removed. [^{14}C]OA thus formed was incubated with SD during 30 min at 20°, the mixture was then cooled to 0° and passed through a column (2.2 x 20 cm) of Sephadex G 25 equilibrated with 10 mM Tris-HCl + 1 mM EDTA (pH 7.4). SD eluted in the void volume of the column was collected and the enzymic activity and radioactivity were measured. Peptide-bound flavin was determined according to Cerletti et al. (12).

RESULTS

When SD was preincubated with [^{14}C]OA and the mixture was fractionated on Sephadex two peaks of radioactivity were found, the first one coinciding with the protein and SD activity and the second corresponding to low molecular weight constituents (Fig. 1). The enzyme activity could easily be measured in the effluent, in spite of pretreatment with OA, since the activity was restored by high concentration of succinate in

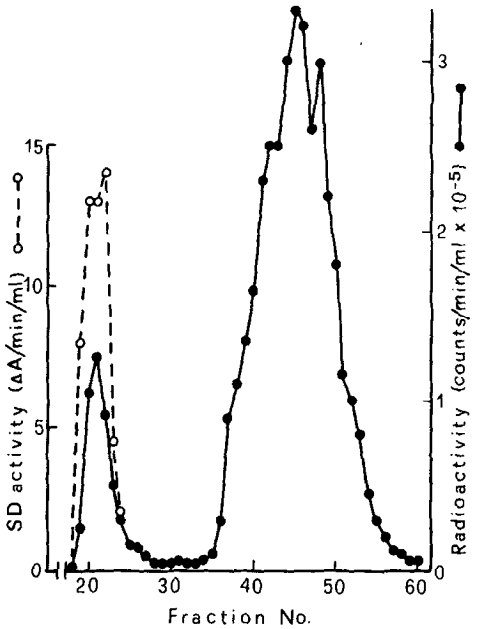

Fig. 1. Formation of a stable complex between oxaloacetate and succinate dehydrogenase. Partially purified SD (last ammonium sulfate precipitation omitted) was incubated in 10 mM Tris-HCl + 1 mM EDTA (pH 7.4) with 40 μM [^{14}C]OA and 2.5 ml of the mixture was subjected to Sephadex filtration. Fractions of 1.0 ml were collected and examined for SD activity (o- - -o) and radioactivity (●——●). SD activity is expressed as change in the absorbance at 600 nm in the assay system per min per ml of the effluent.

the assay medium (2). When [^{14}C]OA was replaced by [^{14}C]aspartate or [^{14}C]malonate Sephadex filtration resulted in a complete separation of the enzyme and the radioactivity (not shown).

The elution of a part of [^{14}C]OA in the void volume of the column was observed with both the crude as well as the purified enzyme preparations, but not with human γ-globuline nor the matrix fraction of beef heart mitochondria. When the effluent containing SD-OA complex was subjected to a second Sephadex filtration, practically all radioactivity was again eluted in the void volume. This procedure could be repeated several times with the same result.

To get more information on the site of binding, the effect of some factors known to interact with SD was studied. Oxidized glutathione is an oxidizing agent for protein SH groups and has been reported to prevent

the reconstitution of the succinate oxidase system from soluble SD (7). Also cyanide may slowly react with sulfhydryl groups and is known to dissociate SD from the respiratory chain (7, 13, 14). Cyanida can also bind to non-heme iron, as does 2-thenoyltrifluoroacetone, a known iron-chelating agent. The latter inhibits particle-bound, but not soluble, SD (14). Pretreatment of SD with either 50 mM oxidized glutathione or 0.2 mM thenoyltrifluoroacetone had no effect on the binding of [^{14}C]OA. The effect of 2 mM KCN was less clear. In some experiments it somewhat diminished the binding of OA whereas in others it was without effect. Ageing of SD at +4° for 19 hours at neutral pH, resulting in the loss of most of the enzyme activity, had little effect on OA binding. However, ageing at 30° and pH 9.5 during 2 to 3 hours strongly diminished or completely abolished the binding.

When high concentration of succinate (Table I) or, even better, malonate (not shown) was present <u>during</u> the incubation of SD with [^{14}C]OA the binding was largely diminished. However, if succinate or malonate was added <u>after</u> preincubation of the enzyme with OA, i.e. when the SD-OA complex was already formed, bound OA was not released even after 10 min incubation at 20° (Table I). This observation is of particular importance since it is known (2) that the inhibition is almost completely released under these conditions.

When the complex of [^{14}C]OA with SD was incubated with unlabelled OA and passed through Sephadex thereafter, more than 80% radioactivity was recovered in the fraction containing the enzyme, indicating that OA, once bound to SD, is not easily exchangeable with external OA. Bound OA could be, however, released when the enzyme was precipitated with perchloric acid. About 90% radioactivity was then released into the supernatant and was tentatively identified by means of thin layer chromatography (15) and extraction (16) as a mixture of oxaloacetate and pyruvate. The latter was formed apparently by non-enzymatic decarboxylation of OA during the analytical procedures.

Table I. Effect of succinate on the binding of oxaloacetate to succinate dehydrogenase. Preparations of partially purified SD (last ammonium sulfate precipitation omitted) were incubated with [^{14}C]OA. Succinate was added either from the beginning of incubation (Expt. 1) or after 20 min and the incubation continued for next 10 min (Expt. 2). The mixture was then fractionated on Sephadex column.

Expt. No.	Additions to the incubation	[^{14}C]OA bound to SD (% of total radioactivity)
1.	None	12.0
	40 mM succinate present from the beginning of incubation	2.4
2.	None	9.7
	40 mM succinate added after 20 min	8.4

A possibility that OA forms a Schiff base with SD was checked. The SD-OA complex was therefore treated with borohydride (17) and precipitated with perchloric acid thereafter. The radioactivity was released into the supernatant, exactly as without borohydride treatment, showing that no Schiff base was formed.

The ratio of OA to peptide-bound flavin was determined in fractions containing SD-OA complex. The amount of OA was calculated from the specific radioactivity of [^{14}C]aspartate. The following values for the OA/flavin ratio were obtained in three experiments: 1.3, 0.7 and 0.7.

To check whether OA can bind to particulate SD, sonic particles from beef heart mitochondria were incubated with [^{14}C]OA and separated by centrifugation. It was found (Table II) that [^{14}C]OA was taken up by the particles whereas [^{14}C]aspartate was not. Particles in which SD had been destroyed by alkali treatment (18) lost most of their ability to bind OA. Contrary to what was observed with soluble SD, OA bound to particulate SD could be released by subsequent addition of succinate or malonate.

DISCUSSION

The presence of minute amounts of OA in some preparations of sol-

Table II. Binding of [^{14}C]oxaloacetate to submitochondrial particles. Sonic particles from beef heart mitochondria (7 mg protein/ml) were incubated 30 min at 20° with 8 μM [^{14}C]OA in the medium containing 120 mM KCl + 10 mM Tris-HCl (pH 7.4), separated by centrifugation at 100,000 x g during 45 min and washed twice. SD-free particles were obtained by incubating sonic particles at pH 9.5 during 2.5 hours under aerobic conditions (18). In samples with [^{14}C]aspartate, glutamate-oxaloacetate transaminase was omitted and 2-oxoglutarate was replaced by glutamate in the system generating [^{14}C]OA.

Additions to the incubation	Radioactivity bound to the particles (counts/min/mg protein)
Sonic particles containing SD	
[^{14}C]OA (control)	2,480
[^{14}C]OA + 40 mM malonate present from the beginning of incubation	200
[^{14}C]OA + 40 mM malonate added after 10 min	230
[^{14}C]Aspartate	30
SD-free particles	
[^{14}C]OA	460
[^{14}C]Aspartate	35

uble SD has already been described (2). Recent observations of Vinogradov et al. (5), Kearney et al. (6) and of the present investigation provide sufficient experimental evidence for the existence of a stable complex between SD and OA. The lower value of 0.7 for the ratio of OA to SD flavin, found in the present study, is close to that observed by Vinogradov et al. and somewhat higher than the value of 0.5 found by Kearney et al. The difference may be due to the fact that Kearney et al. determined OA formed by the enzyme itself whereas Vinogradov et al. and ourselves measured the binding of added OA. The amount of OA measured by the radioactivity of uniformly labelled [^{14}C]OA may be underestimated due to a partial decarboxylation, and it seems likely that SD flavin may be overestimated. Therefore, the true value of OA to flavin ratio in the stable

complex formed from added OA may be even 1:1 or higher. This is also suggested by Vinogradov et al. (5). The SD-OA complex described by Kearney et al. (6) was slowly split by high concentrations of succinate and malonate whereas in Vinogradov's and our experiments OA could not be released from the complex by the substrate or malonate once it was formed. This may be due to a different proportions of OA and SD in the complexes.

The site and mode of binding of OA to SD is not quite clear. Vinogradov et al. (5) postulate the formation of thiosemiacetal with a sulfhydryl group of the enzyme. However, in their hands the binding was prevented only when all 25 SH groups of SD (19) were blocked by Ag^+ titration. After such extensive treatment the enzyme conformation must be seriously altered (cf. 19) and other potential binding sites may also be affected. The fact that the complex can be split by denaturing the protein with perchloric acid should also be taken into consideration when speculating on the binding mechanism. The present experiments with cyanide and thenoyltrifluoroacetone exclude non-heme iron as a possible binding site.

OA bound to soluble SD can not be released by succinate or malonate whereas the catalytic activity could be fully restored under appriopriate conditions (2). This indicates that the tight binding occurs at a site different from the catalytic center of the enzyme. However, the ability of SD to bind to membrane fragments devoid of SD and to reconstitute succinate oxidase is irreversibly lost after OA treatment (2). This suggests that OA binds to a site on the enzyme molecule which is responsible for the reconstitution. This is also supported by the observation that OA is less tightly bound to submitochondrial particles and can be removed therefrom by an excess of succinate or malonate.

It can be therefore concluded that SD posseses two affinity sites for OA. One of them is the catalytic site of the enzyme which "recognizes"

OA as substrate analogue and reacts with it in a competitive way. The second one is most likely a site involved in the reconstitution of succinate oxidase and can form a stable complex with OA.

A tight binding of OA does not exclude, of course, that a conformational change of the enzyme occurs concomitantly, as already suggested by ourselves (2) and recently shown by Kearney et al. (6).

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