LOCALIZATION OF THE OXALOACETATE-BINDING SITE IN THE IRON-FLAVOPROTEIN SUBUNIT OF THE INHIBITOR-SUCCINATE DEHYDROGENASE COMPLEX

Daryl B. Winter and Tsoo E. King
Department of Chemistry
State University of New York
Albany, New York 12222

Received November 26,1973

SUMMARY--The oxaloacetate in the inhibitor-succinate dehydrogenase complex could be displaced by the substrate or inhibitors. The oxaloacetate-binding site is found in the iron-flavoprotein subunit of the enzyme by two methods of dissociation of succinate dehydrogenase into subunits.

It was suggested some time ago that succinate dehydrogenase (SDH)* contains at least two distinct iron-protein moieties (1). Recently these two moieties have been demonstrated by low temperature electron paramagnetic resonance spectroscopic titration (2) and by the chemical separation of an iron protein and an iron-flavoprotein (3). A sulfhydryl group, not labile sulfide, of SDH has been shown as the site for binding with oxaloacetate (OAA) (4). This note reports the direct and related evidence that the OAA binding site is in the iron-flavoprotein subunit of succinate dehydrogenase.

EXPERIMENTAL--The methods of preparations and determinations were described as previously (4) except ^{14}C-labeled OAA was prepared by transamination of the amino group of ^{14}C-labeled asparate to α-ketoglutarate in the presence of transaminase. The gel electrophoresis in sodium dodecylsulfate (SDS) and β-mercaptoethanol was performed according to Weber and Osborn (5).

RESULTS AND DISCUSSION--In confirming our previous results (4), OAA was bound to soluble SDH and the enzyme-inhibitor complex thus formed could be subjected to purification by Sephadex chromatography. However, the maximal binding was effected by incubation of OAA and the dehydrogenase for a few

*Abbreviations: OAA, oxaloacetate; SDS, sodium dodecylsulfate; SDH, succinate dehydrogenase

minutes at 38°. The ratio of OAA to the acid nonextractable flavin in the enzyme was again found to be about 0.7. This ratio is significantly higher than that recently reported by Kearney et al. for the succinate dehydrogenase extracted from acetone powder of beef heart (6).

We previously reported that the bound OAA is in a thiosemiacetal form through a two stage reaction between the enzyme and oxaloacetate. Our conclusion is supported by the recent reports of Wojtczak (7) and Nicholls (8) and their coworkers. However, even the OAA incorporated in a thiosemiacetal could be displaced by substrate and inhibitors as shown in Fig. 1.

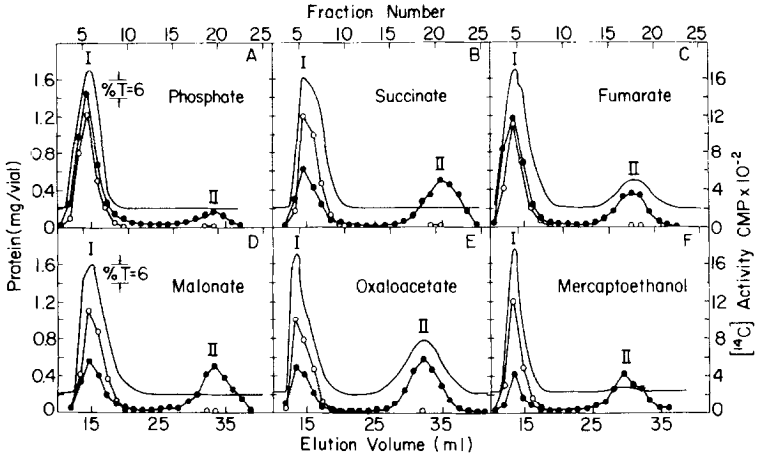

Fig. 1. The purified ^{14}C-labeled OAA-succinate dehydrogenase complex, 10 mg/ml, was incubated with phosphate, 50 mM; succinate 20 mM; fumarate 20 mM; malonate, 20 mM; oxaloacetate, 20 mM; or β-mercaptoethanol, 15 mM; for 10 min at 38°. The mixture was then rapidly cooled to 4° and chromatographed on a Sephadex G-50 column with 50 mM phosphate buffer, pH 7.4, anaerobically. The continuous line is from the automatic recording of transmittance (%T) at 280 nm for protein, the open circles are the protein content per fraction by the biuret method, and the solid circles are the radioactivity (CPM) per vial by a liquid scintillation method. Roman numerals I and II are the peak numbers. Recoveries for radioactivity and protein were greater than 92%.

Phosphate exhibited little ability to release any bound OAA, while on the other hand, oxaloacetate (not labeled), malonate, fumarate and succinate displaced about 70, 60, 25, and 60%, respectively, of the ^{14}C-labeled OAA from the inhibitor-enzyme complex. The degree of displacement was, in general, inversely related to the dissociation constant of the substrate-enzyme complex or the inhibitor-enzyme complex. It is known that the magnitude of K_i is in the order of K_i^{OAA} (1.5×10^{-6} M (9)) < $K_i^{malonate}$ (4.5×10^{-5} M (10)) < $K_i^{fumarate}$ (2×10^{-3} M (11)). Although $K_m^{succinate}$ (1.2×10^{-3} M (10)) is much larger than K_i^{OAA} the capability for succinate to release ^{14}C-OAA was only slightly inferior to that for OAA. This fact may be due to K_i being a true equilibrium constant whereas K_m is a complex kinetic constant; no true dissociation constant of the succinate-SDH complex has been reported and cannot be determined by simple methods.

The ability of mercaptoethanol to displace OAA in the enzyme-inhibitor complex was also examined in order to test the competitive effect of a sulfhydryl with the thiosemiacetal and for the practical application of separation of the subunits in dissociation medium by SDS gel electrophoresis. As shown in Fig. 1F, mercaptoethanol displaced about 70% of the bound OAA. This thiol, however, is not a competitive inhibitor. It does not compete with succinate for the enzyme but rather with the enzyme sulfhydryl for OAA. These facts are in line with our conclusion (4) that a thiosemiacetal is formed between OAA and the enzyme.

Although the behavior of β-mercaptoethanol complicated the design of our experiments for the localization of the OAA binding site in the subunit of succinate dehydrogenase, the SDS gel electrophoresis in a dissociating medium was still a useful tool for our probe. Consequently we subjected the ^{14}C-labeled OAA-enzyme complex to the conventional gel electrophoresis in SDS and β-mercaptoethanol (5). The results of the distribution pattern of radioactivity and protein is shown in Fig. 2. The first major protein

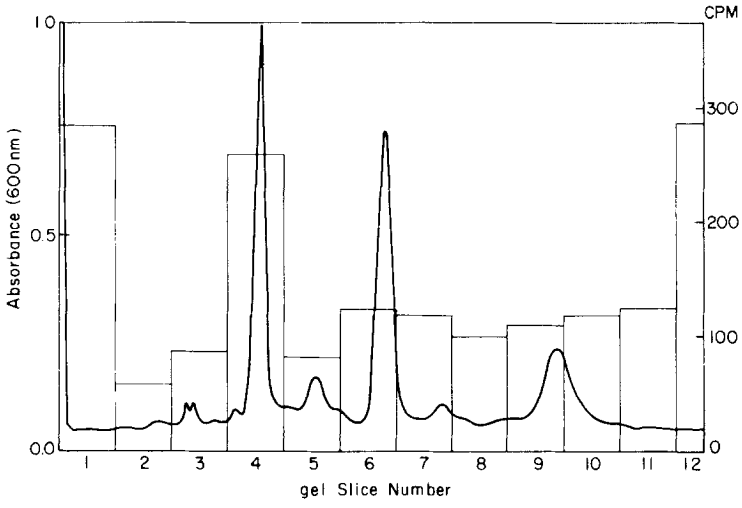

Fig. 2. The distribution of protein and radioactivity in the subunit fractions of the inhibitor-enzyme complex. Approximately 3 mg of a purified ^{14}C-labeled OAA-SDH complex was incubated 5 min in a mixture containing 0.01% SDS, 0.005% β-mercaptoethanol and 50 mM phosphate buffer, pH 7.4. The mixture was separated by large scale gel electrophoresis adapted from the method of Weber and Osborn at 40 milli-amphere. The gel was uniformly sliced at approximately 5 mm thickness. The gel slice was solubilized in an "NCS Solubilizer" (purchased from Amersham/Searle) and counted for radioactivity in a liquid scintillation counter. The radioactivity is presented in bars using the left ordinate. The gel was also stained by Coomassie Blue and the stained gel was scanned at a wavelength of 600 nm as represented by the continuous solid curve. The first protein peak (in slice 4) was the iron-flavoprotein subunit (Fp) of 70,000, the second protein peak (in slice 6) was an impurity which was substantiated from evidence of other experiments (to be published); and the last protein peak (in slices 9-10) was the iron protein subunit (Ip) of 27,000. The dye front is not shown. The initial blue stain and radioactivity was due to the denatured polymerized aggregate impermeable to the gel, probably resulting from the extremely low mercaptoethanol concentrations employed.

peak equivalent to a molecular weight of 70,000* was the iron-flavoprotein subunit and the last protein peak of 27,000* was the iron protein subunit of the dehydrogenase. It is clear only the first peak possessed significant, above the background, radioactivity bound to the protein. The radioactivity in slice number 1 was due to some denatured polymerized aggregate which

*These figures were obtained from a "standard" curve which consisted of proteins of known molecular weights.

did not penetrate the gel whereas that in number 12 was from the tail end of the released free OAA.

Because of the inherent complication in separating the two subunits without dissociating the enzyme-inhibitor complex by the SDS-mercaptoethanol gel electrophoresis as described, we sought another method. The subunits of SDH can be separated, although incompletely, by the action of trichloroacetic acid (3). A sample of ^{14}C-OAA-enzyme complex, 5.0 ml containing 58 mg protein, was mixed with 1.0 ml 4 M trichloroacetic acid at pH 7.8. The mixture was frozen in liquid nitrogen and then thawed; this cycle was repeated 3 times. It was then centrifuged to separate the supernatant and the pellet fractions. After appropriate washing the pellet contained 1320 cpm per mg protein whereas the supernatant showed only 475 cpm/mg. This experiment

TABLE I. Distribution of the Radioactivity and of the Iron Protein (Ip) and the Iron-Flavoprotein (Fo) Subunits in SDH and in Subunit Fractions*

	CPM/mg found	CPM/mg calculated	Fp:Ip
Succinate dehydrogenase untreated	-	-	1:1
Supernatant fraction	475	475	1:5
Pellet fraction	1320	1400	1:0.65

*The SDH as well as the supernatant and the pellet fractions was subjected to the gel electrophoresis in a medium containing SDS and β-mercaptoethanol. The Coomassie Blue-stained gel was scanned and the areas under the iron-flavoprotein (Fp) (mol. wt. 70,000) and the iron protein (Ip) (27,000) bands were integrated and the ratio of Fp:Ip is presented in the table. The ^{14}C-labeled OAA-SDH complex was treated with TCA as described in the text. The supernatant and the pellet fractions were retained for ^{14}C determination. The calculated value as shown in the last column was obtained by taking the figure 475 cpm/mg protein in the supernatant fraction as entirely due to Fp.

verifies the conclusion previously obtained by direct gel electrophoresis, that the OAA binding site is in the iron-flavoprotein subunit. The radioactivity in the supernatant fraction, which ideally contains only the iron protein subunit from the trichloroacetic acid treatment, might be due to contamination with the iron flavoprotein subunit. Likewise, the pellet fraction (iron flavoprotein) in all probability might also contain the iron protein subunit. Consequently, experiments employing SDS-gel electrophoresis of the pellet and the supernatant fractions were conducted to determine the extent of contamination. As shown in Table 1, cross contamination did occur heavily in both fractions. However, with allowance made for the observed contamination, it is evident that the oxaloacetate-binding site is indeed in the iron-flavoprotein subunit of the inhibitor-enzyme complex.

ACKNOWLEDGMENTS--This work was supported by grants from the United States Public Health Service (GM-16767 and HL-12576).

References

1. King, T. E., Advances in Enzymology, 28, 155 (1966).
2. Ohnishi, T., Winter, D. B., Lim, J., and King, T. E., Biochem. Biophys. Res. Commun., 53, 231 (1973).
3. Davis, K. A., and Hatefi, Y., Biochemistry 10, 2509 (1971).
4. Vinogradov, A. D., Winter, D. B., and King, T. E., Biochem. Biophys. Res. Commun., 49, 441 (1972).
5. Weber, K., and Osborn, M., J. Biol. Chem., 244, 4409 (1969).
6. Kearney, E. B., Ackrell, B. A. C., and Mayr, M., Biochem. Biophys. Res. Commun., 49, 1115 (1972).
7. Priegnitz, A., Brzhevskaya, O. N., Wojtczak, L., Biochem. Biophys. Res. Commun., 51, 1034 (1973).
8. Kappen, L. S., Brodie, J. D., and Nicholls, P., Biochem. Soc. Trans. 1, 877 (1973).
9. DerVartanian, D., Dissertation, University of Amsterdam, 1964.
10. Keilin, D., and King, T. E., Proc. Roy. Soc., Ser. B152, 163 (1960).
11. Ryan, C. A., and King, T. E., Biochim. Biophys. Acta, 62, 269 (1962).