BBA 63417

Binding of substrates by native and chemically modified isocitrate dehydrogenase

Alkylation of a single methionyl residue of isocitrate dehydrogenase (*threo*-D_S-isocitrate:TPN oxidoreductase (decarboxylating), EC 1.1.1.42) with iodoacetate to produce a carboxymethyl enzyme results in a greater disruption of the ability of the enzyme to catalyze dehydrogenation than decarboxylation[1,2]. Alteration of the kinetic parameters of the enzyme is not responsible for the observed loss of activity, since partially active preparations exhibit Michaelis constants and pH–rate profiles which are essentially identical with those of native enzyme. When modification of enzymic groups results in inactivation, it is not possible to distinguish by kinetic measurements between loss of activity caused by inability to bind substrates and inactivation caused by disruption of a step subsequent to binding. In this study, the direct binding of radioactive isocitrate and α-ketoglutarate by native and carboxymethyl enzyme have been compared in order to clarify the function of the essential methionyl residue.

The TPN-dependent pig-heart isocitrate dehydrogenase (Boehringer, Mannheim) was purified 10-fold to homogeneity[2]. Carboxymethyl enzyme, prepared by reaction of native isocitrate dehydrogenase with 28 mM iodoacetate at pH 5.8 and 30°, exhibits only 5% of its original dehydrogenase activity. New England Nuclear Corp. supplied the DL-[5,6-$^{14}C_2$]isocitrate, 50% of which was found to participate in the enzymatic transfer of hydrogen to TPN^+. The α-[1-^{14}C]ketoglutaric acid was purchased from Calbiochem. Isocitrate dehydrogenase activity was determined spectrophotometrically[2].

Procedure for binding experiments. Substrate binding experiments were conducted at 23° in 0.03 M triethanolamine chloride buffer (pH 7.4). These conditions are similar to those used in determining the Michaelis constants for the substrates, except for the higher protein concentration and the absence of coenzyme in the binding experiments. The reaction mixtures contained substrate, $MnSO_4$ (2 mM) where indicated, EDTA (0.2 mM) and enzyme (6.6 μM). Aliquots of these original solutions were diluted 6-fold with distilled water and counted on a low-background Nuclear-Chicago gas-flow counter to assess the total substrate concentration. Appropriate corrections were made for self absorption. Free substrate was separated from enzyme-bound substrate using a 1-ml syringe ultrafilter (Amicon Corp.) equipped with a Diaflo UM-1 membrane which retains solutes of molecular weight greater than 10 000. The initial 0.2 ml of the filtrate was discarded. The second 0.2–0.4 ml of filtrate was diluted and counted as in the case of the original solution in order to measure the free substrate concentration. The enzyme-bound substrate concentration was calculated from the difference between total and free substrate concentration. In the absence of enzyme, radioactive isocitrate and α-ketoglutarate solutions pass unchanged in concentration through these membranes. The possibility of any leakage of enzyme through the membrane was checked in each run by assaying the undiluted filtrate for isocitrate dehydrogenase activity, a measurement sufficiently sensitive to detect 0.5% of the original protein concentration. A given binding experiment could be completed in an hour, during which interval enzymatic activity remains constant under these conditions. The ultrafiltration technique for measuring direct binding is

similar in principle to the method of ultracentrifugal separation[3,4]; however, it is better suited for proteins of relatively low molecular weight.

Binding of isocitrate. The binding data were analyzed graphically in accordance with the equation[5]:

$$\frac{r}{[\text{substrate}]_{\text{free}}} = \frac{n}{K} - \frac{r}{K} \qquad (1)$$

where r = moles substrate bound per mole protein, n = number of substrate binding sites and K = dissociation constant for the enzyme–substrate complex. This equation, which is valid for the binding of a small molecule by a protein containing independent sites, yields a linear plot of $r/[\text{substrate}]_{\text{free}}$ as a function of r for both isocitrate and α-ketoglutarate (Figs. 1 and 2). The enzyme binds approx. 1 mole of isocitrate per mole of protein with a dissociation constant of 2.25 μM in the presence of a saturating concentration of $MnSO_4$ (Fig. 1; Table I). Under similar conditions of pH and temperature, the Michaelis constant for *threo*-D_S-isocitrate was of the same order of magnitude: 4.1 μM as determined from the effect of this substrate on the initial velocity of oxidative decarboxylation, and 3.9 μM as measured by its ability to protect the enzyme against inactivation by 5,5'-dithiobis-(2-nitrobenzoic acid)[6]. These results suggest that the binding of isocitrate is not significantly affected by TPN^+ in the assay mixture. In the absence of $MnSO_4$, there is a 5-fold increase in the dissociation constant for the enzyme–isocitrate complex as measured by direct binding (Fig. 1; Table I). Although metal ion is not essential for the combination of enzyme and substrate, it apparently contributes to the strength of binding.

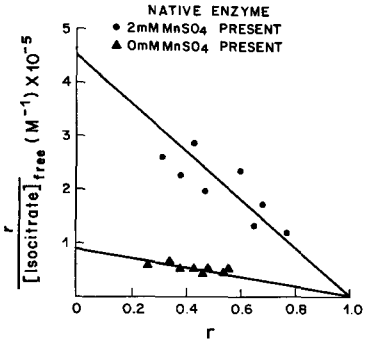
The inactive carboxymethyl enzyme is still capable of binding isocitrate, as evidenced by a value of "n" not significantly different from that of the native enzyme (Table I). Its characteristic dissociation constant (5.50 μM), as measured in the presence of Mn^{2+} is only twice as high as that of the unmodified enzyme, suggesting that impairment of the binding of isocitrate cannot be the major cause of the inactivation.

Binding of α-ketoglutarate. Native isocitrate dehydrogenase binds approx. 1 mole of α-ketoglutarate per mole of enzyme with a dissociation constant of 4.62 μM in the presence of $MnSO_4$ (Fig. 2; Table I). The metal ion strengthens the binding of this substrate; K for α-ketoglutarate is 10.3 μM in the absence of $MnSO_4$.

TABLE I

BINDING OF [5,6-$^{14}C_2$]ISOCITRATE AND α-[1-^{14}C]KETOGLUTARATE BY NATIVE AND CARBOXYMETHYL ENZYME

Substrate	*Enzyme preparation*	*$MnSO_4$ (2 mM)*	*K_{diss} (μM)*	*n*
(I) Isocitrate	Native	+	2.25	1.05
	Native	−	11.5	1.37
	CM-enzyme	+	5.50	1.08
(II) α-Ketoglutarate	Native	+	4.62	1.10
	Native	−	10.3	1.32
	CM-enzyme	+	2.30	1.24
	CM-enzyme	−	11.4	1.32



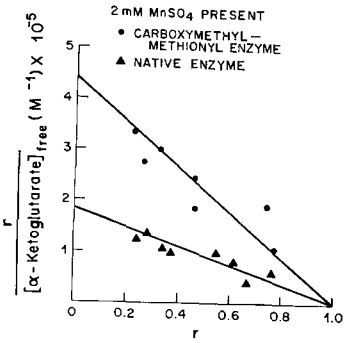


Fig. 1. Binding of [5,6-$^{14}C_2$]isocitrate by native enzyme in the presence and absence of $MnSO_4$. In the presence of $MnSO_4$, $K = 2.25\ \mu M$ with $n = 1.05$; whereas in the absence of $MnSO_4$, $K = 11.5\ \mu M$ and $n = 1.37$. The lines here shown have been normalized to $n = 1.0$ to facilitate comparison of the dissociation constants.

Fig. 2. Binding of α-[1-^{14}C]ketoglutarate by native and carboxymethyl enzyme. For the native enzyme, $K = 4.62\ \mu M$, with $n = 1.10$; while for carboxymethyl enzyme $K = 2.30\ \mu M$, with $n = 1.24$. The lines here shown have been normalized to $n = 1.0$ to facilitate comparison of the dissociation constants.

Binding of α-ketoglutarate is not hindered in the inactive carboxymethyl enzyme; in fact, the dissociation constant is somewhat lower in the modified as compared to the native enzyme, when measured in the presence of Mn^{2+} (Table I; Fig. 2). An indirect indication of the competence of the carboxymethyl enzyme to bind metal ion is given by the 5-fold decrease in the dissociation constant when $MnSO_4$ is added to the reaction mixture.

Isocitrate dehydrogenase is capable of combining with both α-ketoglutarate and isocitrate in the absence of coenzyme, as demonstrated by the direct binding experiments. In the case of isocitrate, the dissociation constants observed were similar to those measured kinetically, suggesting that the presence of TPN^+ does not appreciably influence the strength of binding of substrate. It has previously been shown that the dissociation constant of TPNH is the same in the presence and absence of α-ketoglutarate and bicarbonate[6]. For α-ketoglutarate, however, the binding constants here reported are approximately two orders of magnitude lower than the Michaelis constant for this substrate (0.5 mM) calculated from its effect on the rate of reductive carboxylation[6]. ROSE[7] has invoked coenzyme-induced conformational change to account for the requirement for TPNH for the exchange of ^3H in the solvent with one of the hydrogens on the β-carbon atom of α-ketoglutarate and reported a dissociation constant of 0.13 mM for α-ketoglutarate, a value similar to the Michaelis constant. The product α-ketoglutarate competes with isocitrate in the isocitrate dehydrogenase reaction, exhibiting an inhibition constant of $20\ \mu M$, as measured in the presence of TPN^+. This value is distinct from both the direct binding constant observed in the absence of other substrates and the dissociation constant obtained in the presence of TPNH. The implication is that, in contrast to the situation with isocitrate, the strength of binding of α-ketoglutarate is exquisitely sensitive to either form of the coenzyme and that at least in the direction of reductive carboxylation, the order of addition of substrates to the enzyme is not strictly random.

The experiments reported in this paper demonstrate that the carboxymethyl

enzyme is still capable of binding isocitrate, α-ketoglutarate and Mn^{2+}. Since TPN^+ and TPNH, in contrast to isocitrate, do not protect against inactivation by iodoacetate, there is no indication that the coenzyme binding site is affected by this reagent. All the available information thus points to an involvement of methionine in catalysis at a step subsequent to formation of the enzyme–substrate complex.

This work was supported by U.S. Public Health Service Grant 5-R01-AM12379 and by U.S. Public Health Service Career Development Award 7-K4-GM-9576.

Biochemical Research Laboratory,
Massachusetts General Hospital and
Department of Biological Chemistry,
Harvard Medical School,
Boston, Mass. (U.S.A.)

ROBERTA F. COLMAN

1 R. F. COLMAN, *Biochem. Biophys. Res. Commun.*, 28 (1967) 222.
2 R. F. COLMAN, *J. Biol. Chem.*, 243 (1968) 2454.
3 S. F. VELICK, J. E. HAYES, JR. AND J. HARTING, *J. Biol. Chem.*, 203 (1953) 527.
4 R. F. COLMAN AND C. FRIEDEN, *Biochem. Biophys. Res. Commun.*, 22 (1966) 100.
5 I. M. KLOTZ, in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. IB, Academic Press, New York, 1953, p. 771.
6 R. F. COLMAN, *Biochemistry*, 8 (1969) 888.
7 Z. B. ROSE, *J. Biol. Chem.*, 235 (1960) 928.

Received August 11th, 1969

Biochim. Biophys. Acta, 191 (1969) 469–472

BBA 63418

Partial purification and some properties of bovine heart arylamidase

Arylamidases are found widely distributed in nature and are capable of hydrolyzing aminoacyl-β-naphthylamides[1–4]. Enzymes isolated from different sources appear to have differing requirements for catalysis. This communication reports on the partial purification of bovine heart arylamidase, its specificity and the effect of selected thiol reagents and alkylating agents on enzymic activity.

Bovine heart arylamidase is a soluble enzyme since essentially all the enzyme activity found in heart homogenates is recoverable in 14 600 × *g* supernatant fractions (Table I). The enzyme has been purified from homogenates to the extent of 1261-fold by $(NH_4)_2SO_4$ fractionation, column hydroxylapatite adsorption and DEAE-Sephadex column chromatography (Fig. 1; Table I). The DEAE-Sephadex pooled peaks of enzyme show one enzyme band by the zymogram technique[3] and eight bands of protein by disc electrophoresis[5] (Fig. 2). These data suggest that either several enzyme peaks are inactivated during electrophoresis or that different aggregates of enzymes or isozymes may exist and be separable by DEAE-Sephadex but are indistinguishable by disc gel electrophoresis. Impurities may also be present.

Abbreviation: PMSF, phenylmethylsulfonyl fluoride.