FORMATION OF ENZYME BOUND CARBON DIOXIDE IN THE REDUCTIVE CARBOXYLATION OF ALPHA-KETOGLUTARATE BY ISOCITRATE DEHYDROGENASE

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L_s -Isocitrate NADP oxidoreductase (E.C. 1.1.1.42) catalyses the reversible oxidative decarboxylation of isocitrate to alpha-ketoglutarate and carbon dioxide with the specific participation of NADP. No free intermediate has been isolated in the overall reaction nor have attempts to demonstrate the existence of oxalosuccinic acid as a free intermediate or in the enzyme bound form been successful (Siebert et al., 1957; Plaut, 1963; Carsiotis, 1958). With large quantities of fairly purified enzyme and labeled carbon dioxide, we attempted to demonstrate enzyme bound oxalosuccinate in the overall reverse reaction by use of the 'Chase' technique successfully employed to demonstrate substrate binding for several enzymes (Krishnaswamy et al., 1962; Nishimura et al., 1963; Anderson and Meister, 1966). Enzyme bound carbon dioxide was demonstrated both in the presence and absence of NADP or of alpha-ketoglutarate; the extent of binding was significantly greater in the presence of NADP or of alpha-ketoglutarate. Enzyme bound oxalosuccinate was not found under these conditions. Prior incubation of carbon dioxide with the enzyme exerted a specific protective effect against inhibition by PCMB of the oxidation of isocitrate.

MATERIALS AND METHODS

Isocitric dehydrogenase was prepared from pig heart according to the procedure of Grafflin and Ochoa (1950) and assayed as described by

Siebert et al. (1957). One unit is the amount of enzyme which causes a change in absorbancy of 0.01 O.D. unit per minute at 340 mμ at room temperature under standard conditions of incubation; specific activity is the number of units per mg of protein. DL-isocitric acid was purchased from Fluka A.G, Buchs. SG. NADP and NADPH were products of Sigma Chemicals. Radioactive bicarbonate was obtained from Atomic Energy Establishment Trombay, Bombay. Alpha-ketoglutarate was purchased from Light Chemicals. Other chemicals used were of reagent grade. Isocitrate was chromatographically identified using n-butanol: acetic acid: water solvent system (4:1:1). (Rf = 0.52). For details of experiments reported in the communication, see under footnotes to Table 1 and Fig.1.

RESULTS AND DISCUSSION

Experiments described in Table 1 illustrate binding of carbon dioxide to the enzyme. The formation of isocitrate-C^{14} by the overall reverse reaction catalyzed by the enzyme was measured in experiment A. The dilution of carbon dioxide-C^{14} by addition of excess carbon dioxide-C^{12} during the first incubation was considerably greater than when added during the second incubation along with the other reactants. Thus, there was more radioactivity in isocitrate formed in incubates B, D and F compared to the corresponding controls. This would strongly indicate binding of carbon dioxide-C^{14} to the enzyme in a manner that it is available for formation of isocitrate-C^{14}. Such binding could take place independently to a significant extent (B). However, it was considerably enhanced in the presence of NADPH or alpha-ketoglutarate (D and F). Approximately 2500 counts per minute would be expected in the isocitrate formed in incubates C, E and G if equilibration between labeled and unlabeled carbon dioxide had reached completion and all of the NADPH was utilized for reaction. Much less radioactivity than this was found in C, E and G; thus appreciable catalytic formation of isocitrate did not take place in the 15 second interval

TABLE 1. FORMATION OF ENZYME BOUND CARBON DIOXIDE IN THE REDUCTIVE CARBOXYLATION OF ALPHA-KETOGLUTARATE BY ISOCITRATE DEHYDROGENASE[a]

Additions during First incubation, 30", 25°C	Additions during Second incubation, 15", 25°C	Isocitrate-C^{14} formed total c.p.m.[b]	
		Expt. I[c]	Expt. II[c]
A. ENZ+$C^{14}O_2$+α-KG	NADPH	36,900	38,000
B. ENZ+$C^{14}O_2$	α-KG+NADPH+$C^{12}O_2$	6,200	4,700
C. ENZ+$C^{14}O_2$+$C^{12}O_2$	α-KG+NADPH	100	300
D. ENZ+$C^{14}O_2$+NADPH	α-KG+$C^{12}O_2$	12,900	11,100
E. ENZ+$C^{14}O_2$+$C^{12}O_2$+NADPH	α-KG	400	390
F. ENZ+$C^{14}O_2$+ α-KG	NADPH+$C^{12}O_2$	17,050	13,000
G. ENZ+$C^{14}O_2$+ α-KG+$C^{12}O_2$	NADPH	620	543

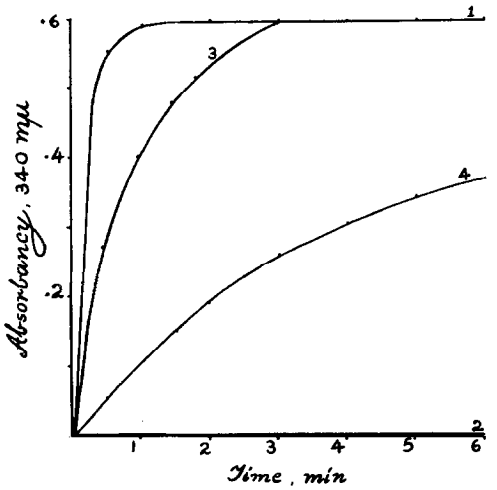
a The reaction mixtures (final volume, 3.1 ml) contained Tris-HCl buffer pH 7.4, 100μ moles; magnesium chloride, 5μ moles; sodium bicarbonate-C^{14} 1.34μ moles (Sp.act. 14.45μ c/μ mole); alpha-ketoglutarate (α-KG), 12μ moles; NADPH, 0.4 μ mole and potassium bicarbonate, 3500μ moles and 8.5 mg of enzyme (4200 units). The first incubation was followed by addition of a mixture of reactants indicated in the second incubation. Addition of 1 ml of 6 N HCl after the second incubation to stop the reaction completely removed carbon dioxide-C^{14}; enzymatically formed labelled isocitrate was determined in aliquots of the protein free supernatants using a windowless gas flow counter.

b Identity of isocitric acid thus formed was established by use of the material isolated in the forward reaction catalyzed by the enzyme. It was also chromatographically characterised.

c When enzyme heated at 100°C for 10 minutes was used in incubations A, B, D and F, radioactive isocitric acid was not formed.

before the reaction was arrested. When the second incubation was prolonged, there was a rapid and progressive decrease in radioactivity in isocitrate in B, D and F indicating a rapid exchange between enzyme bound carbon dioxide-C^{14} and free carbon dioxide-C^{12}. In incubate F, it was not possible to trap any enzyme bound oxalosuccinate as its 2,4-dinitrophenylhydrazone derivative. Presumably, under acid conditions used, a rapid decarboxylation of the beta keto acid formed in stoichiometry to the enzyme might have occurred.

Fig. 1. PROTECTION BY ENZYME BOUND CARBON DIOXIDE AGAINST INHIBITION BY PARACHLOROMERCURIBENZOATE (PCMB).



The reaction mixtures contained in a total volume of 3 ml., Tris-HCl buffer, pH 7.4, 100 μmoles; sodium bicarbonate, 50 μmoles; DL-isocitric acid, 9 μmoles; manganese chloride, 2 μmoles; NADP, 0.4 μmole and enzyme, 0.613 mg (125 units). Incubations were carried out at room temperature (25°C). PCMB was used at a concentration of 0.1 μmole. Conditions of incubation were as follows:

1 - (Enz+CO_2)1' + (H_2O)1' + (Isocitric acid+Mn^{++} +NADP)6'
2 - (Enz+PCMB)1' + (H_2O or CO_2)1' + (Isocitric acid+Mn^{++} +NADP)6'
3 - (Enz+CO_2)1' + (PCMB)1' + (Isocitric acid+Mn^{++} +NADP)6'
4 - Same as 3; concentration of enzyme used was 0.123 mg (25 units).

Susceptibility of isocitrate dehydrogenase to sulfhydryl inhibitors like parachloromercuribenzoate (PCMB) is well recognised (Lotspeich and Peters, 1951; Siebert et al., 1957). Protection against inhibition by PCMB has been observed by prior incubation of the enzyme with isocitrate or NADP in the presence of manganese; it was also noticed that alpha-ketoglutarate and oxalosuccinate were protective in a similar manner while carbon dioxide was not effective(Siebert et al., 1957; Carsiotis, 1958). However, under our experimental conditions, it has been possible to demonstrate clearly such a protective effect of carbon dioxide by prior incubation and binding with the

enzyme (Fig.1). Requirement for a divalent metal ion for such binding was not observed. After a brief incubation of the enzyme with PCMB, addition of carbon dioxide did not result in reversal of inhibition. Degree of inhibition and reversal depended on the enzyme concentration. A study of such protective effect by carbon dioxide of the reductive carboxylation of alpha-ketoglutarate presented difficulties. Much higher concentrations of PCMB are required for 50 percent inhibition of the activity; this catalytic activity is at least 10-15 times lower than the oxidative decarboxylation catalyzed by a unit of enzyme.

As a result of these experiments, it is reasonable to assume that formation of enzyme bound carbon dioxide involves a sulfhydryl group of the enzyme as a point of combination, just as in the case of isocitrate or NADP reported earlier (Ochoa and Weisz-Tabori, 1948; Carsiotis, 1958; Siebert et al., 1957). Study of binding of carbon dioxide to the enzyme offers scope for investigations to understand this phenomenon in relation to the mechanism of the overall reaction and the precise chemical nature of such binding to a site on the enzyme.

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