PN 1217

Enzymic activation and cleavage of D- and L-malate

The recent note by TUBOI AND KIKUCHI[1] on the enzymic cleavage of malate to glyoxylate and acetyl-CoA in *Rhodospirillum spheroides* extracts prompts us to report parallel experiments which also demonstrate the occurrence of this reaction but lead to different conclusions on its nature.

Rhodospirillum rubrum cells were grown on glutamate–DL-malate medium[2] or D-malate–glutamate (or ammonia)–phosphate medium[3]. *Escherichia coli* K12 was grown on glucose–tryptophan[4]. Extracts of cells were prepared by sonication and centrifugation at 100000 × *g* for 60 min.

TABLE I

ENZYMIC ACTIVATION OF D- AND L-MALATE

The test system contained (in μmoles): Tris–HCl buffer, pH 7.5 (100), $MgCl_2$ (8), CoA (0.2), crystalline ATP (10), GSH (10), D-malate, L-malate or succinate (100), neutralized NH_2OH–HCl (625) and enzyme. Volume, 1.0 ml. Incubation, 30 min at 38°. Values are μmoles of hydroxamic acid formed and have been corrected for smaller amounts found when substrate was omitted. Sheep liver was a dialyzed 0.3 to 0.6 satd. $(NH_4)_2SO_4$ fraction of crude extract. *R. rubrum*, 5.4 (1) and 3.5 (2) mg protein; *E. coli*, 2.5 mg protein; sheep liver, 20 mg protein.

Substrate	*R. rubrum* (1)	*R. rubrum* (2)	*E. coli*	*Sheep liver*
L-Malate	0.50	0.53	0.52	0.23
D-Malate	0.33	0.53	0.52	0.12
Succinate	4.70	4.05	4.30	0.56

The system of KAUFMAN[5] was used to assay malate activation. As seen in Table I, both L-malate and D-malate were converted to the corresponding malyl-CoA compounds in extracts of *R. rubrum.*, *E. coli*, and sheep liver. As anticipated this reaction required ATP, CoA, and Mg^{2+} and presumably proceeded according to Reaction 1.

$$\text{L- or D-malate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{L- or D-malyl-CoA} + \text{ADP} + P_i \qquad (1)$$

Since these extracts were even more active in catalyzing the conversion of succinate to succinyl-CoA, the question arose as to whether Reaction 1 was an unspecific activity of succinyl-CoA synthetase[5] (EC 6.2.1.4). This seems unlikely since purified succinyl-CoA synthetase from pig heart[5] and from spinach[6] does not act on L-malate. The malohydroxamic acid formed in the test system was extracted under mild conditions and placed on Whatman No. 1 paper. In two solvent systems, water–saturated butanol and isoamyl alcohol–formic acid, a "delayed" iron-positive spot was obtained of R_F 0.36 and 0.19, respectively. However, both succinomonohydroxamic and malomonohydroxamic acids had the identical R_F value in each solvent. Qualitatively it was noted that after spraying with $FeCl_3$ the purplish color appeared almost at once with succinomonohydroxamic acid but only after some delay with malomonohydroxamic acid. Moreover the D-malic acid (Mann Laboratories) and L-malic acid (California Corporation) were essentially pure by elementary analysis and specific rotation data and could not contain significant amounts of succinate which has a low affinity (K_m = 0.015 M) for the enzyme. No acethydroxamic acid (*i.e.* acetyl-CoA formation) was detected in these experiments.

TABLE II

ENZYMIC ACYLATION OF CoASH BY D- AND L-MALATE

The assay system was that of Table I except GSH and NH_2OH were omitted and 1.50 μmoles CoASH were used. Incubation, 30 min at 38°. Values in parentheses obtained after heating at end of incubation for 1.5 min at 100° before sulfhydryl determination.

Substrate	−SH (μmoles)			
	R. rubrum (5.4 mg)		*E. coli* (2.5 mg)	
	1	2	1	2
None	0.25	0.57 (0.56)	0	0.08
L-Malate	0.61	0.82 (0.56)	0.37	0.37
D-Malate	1.13	1.18 (0.48)	0.63	0.59* (0)
Succinate	1.50	1.37	—	1.25* (0)

* No succinyl-CoA accumulated with D-malate, 0.89 μmole accumulated with succinate as substrate.

Further evidence for Reaction 1 was obtained by measuring acylation of substrate amounts of CoASH[7] in presence of malate and ATP with extracts of *R. rubrum* and *E. coli*. While some disappearance of CoASH occurred with ATP alone, the addition of L-malate, D-malate, or succinate resulted in a large further disappearance of CoASH. The thioester accumulating with D- or L-malate, ATP and CoASH, like succinyl-CoA, was hydrolyzed by heating for 1.5 min at 100° and pH 7.5 (see below). Acetyl-CoA is stable to this treatment. Succinyl-CoA formation was assayed optically by means of the coupled succinyl-CoA:3-oxo-acid CoA transferase (EC 2.8.3.5) and β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) reactions[8]. Only when succinate was the substrate did succinyl-CoA accumulate. This and the chromatographic data above demonstrated unequivocally that the thioester formed from D- or L-malate, ATP and CoASH was malyl-CoA and not succinyl-CoA.

The enzymic cleavage of malate, in presence of ATP and CoA, to acetyl-CoA (and presumably glyoxylate) was demonstrated by addition of oxaloacetate to trap any acetyl-CoA formed as citrate through the action of citrate synthase ("condensing

TABLE III

ENZYMIC CLEAVAGE OF D- AND L-MALATE TO ACETYL CoA

The reaction mixture contained (in μmoles): Tris–HCl buffer, pH 7.5 (100), $MgCl_2$ (8), GSH (10), oxaloacetate (10), enzyme, and where indicated ATP (10), CoA (1), L- or D-malate or acetate (100). Volume 1.0 ml. Incubation, 30 min at 38°. *R. rubrum*, 3 mg; *E. coli*, 5 mg; sheep liver, 20 mg. Values are μmoles of citrate formed.

Additions	*R. rubrum*			*E. coli**	*Sheep liver**
	1	2	3		
ATP, CoA	0.30	0.30	0.30	0.08	0.12
ATP, CoA, L-malate	0.44	0.40	0.40	0.11	0.20
ATP, CoA, D-malate	0.37	0.36	0.34	0.17	0.20
ATP, CoA, acetate	5.93	6.16	—	—	—
CoA	—	—	0.06	—	—
CoA, L-malate	—	—	0.09	—	—
CoA, D-malate	—	—	0.07	—	—

* Incubation, 60 min; 50 units of citrate synthase also added.

enzyme", EC 4.1.3.7). As shown in Table III, extracts of *R. rubrum* formed significant amounts of citrate under these conditions. Extracts of *E. coli* and sheep liver also showed some activity. In *R. rubrum* extract, D-malate was about one-half as active as L-malate in the cleavage reaction although it is converted to malyl-CoA at least as rapidly as L-malate; and ATP as well as CoA was required for the overall conversion to citrate, *i.e.*, acetyl-CoA.

To determine whether the cleavage reaction proceeded via free malyl-CoA according to Reaction 2 or by a malate cleavage Reaction 3, analogous to the citrate cleavage reaction[9], L-malyl-CoA and D-malyl-CoA were prepared from the corresponding acids via their mixed anhydrides[10]. It is not known which carboxyl of malate

$$\text{Malyl-CoA} \rightleftharpoons \text{acetyl-CoA} + \text{glyoxylate} \qquad (2)$$

$$\text{Malate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{glyoxylate} + \text{ADP} + P_i \qquad (3)$$

was esterified but it is reasonable to assume that a mixture of α-thioester and the required β-thioester resulted. The L- and D-malyl-CoA compounds were unstable at neutral pH and were completely hydrolyzed at 100° after 1.5 min, as measured by CoASH release. When either L- or D-malyl-CoA in substrate amounts (2.2 μmoles) was incubated with *R. rubrum* extract, Mg^{2+}, GSH and oxaloacetate, the amount of citrate formed (0.05 μmole) was not significantly greater than in the control without thioester (0.02 μmole). Although some spontaneous and enzymic deacylation of malyl-CoA occurred under these conditions, 55 % of added thioester was recovered. Enzymic CoASH release from L-malyl-CoA, in presence of *E. coli* extract, was increased by ADP and P_i demonstrating the reactivity of L-malyl-CoA in the reverse Reaction 11 and presumably ATP synthesis. Sheep liver catalyzed a rapid hydrolysis of L-malyl-CoA.

These experiments demonstrate the occurrence of two separate enzymes catalyzing Reactions 1 and 3 and indicate that, contrary to the conclusion of TUBOI AND KIKUCHI[1], free malyl-CoA is not the substrate of the malate-cleavage reaction which presumably proceeds via enzyme-bound intermediates as does the citrate-cleavage enzyme[9]. D-Malate appears to react as such, since no conversion of D-malate to L-malate could be demonstrated in these and other enzyme preparations.

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