Studies on Oxalacetate Carboxylase of Pig Heart Muscle[†]

Part II. Distinction of Oxalacetate Carboxylase from Other Oxalacetate Decarboxylating Enzymes and Some Considerations on the Physiological Significance of the Enzyme

By Takio MORINAGA and Masaharu SHIRAKAWA

*Laboratory of Biochemistry, Faculty of Domestic Science,
Fukuoka Woman's University*

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The existence of oxalacetate carboxylase (OAC-lyase), distinct from other oxalacetate decarboxylating enzymes, was concluded by the following facts: (1) the enzyme action was not effected by ATP, ADP, acetyl CoA and was not stimulated by LTP, different from pyruvate carboxylase and phosphoenol pyruvate carboxykinase; (2) although an appreciable amount of malic enzyme activity was found in the crude extract of pig heart muscle, the purified preparation of OAC-lyase was completely free from this activity; (3) OAC-lyase activity was unaffected by NADP⁺ and L-cysteine, different from the decarboxylating activity of malic enzyme.

The reversibility of OAC-lyase reaction was studied by a tracer technique, and it was found that the reaction was practically irreversible.

As regards the physiological significance of OAC-lyase, a preliminary experiment on the transcarboxylation between oxalacetate and acetyl CoA by OAC-lyase were unsuccessful.

In the preceding paper the purification and enzymic properties of OAC-lyase (EC 4.1.1.3) from pig heart muscle have been described. The enzyme was so unstable that it was difficult to purify above 50-fold over the crude extract.

The enzyme was activated by some bivalent metal ions, among which Mn²⁺ was the most effective and was independent of redox and ergonic nucleotides and avidin. Results ob-

tained were in accord with those of the cod fish enzyme as reported by Schmitt *et al.*¹⁾ and Kosicki.²⁾

As pointed out previously, there remained some obscurities caused by the fact that other OA-decarboxylating enzymes, especially malic enzyme (EC 1.1.1.40), contributed to OAC-lyase activity. Although it is possible to distinguish OAC-lyase from pyruvate carboxylase (EC 6.4.1.1) and PEP-carboxykinase (EC 4.1.1.32) under the suitable conditions of activity measurements, malic enzyme might be confused with OAC-lyase because of its ability to decarboxylate OA under the similar con-

[†] Part I of the series: T. Morinaga and M. Shirakawa, *Agr. Biol. Chem.*, **35**, 1166 (1971).

Abbreviations. OAC-lyase: oxalacetate carboxylase, OA: oxalacetate, PEP: phosphoenol pyruvate, MDH: malate dehydrogenase, LDH: lactate dehydrogenase, BSA: bovine serum albumin, CMC: CM-cellulose, DEAE: DEAE-cellulose.

1) A. Schmitt, I. Bottke and G. Siebert, *Z. physiol. Chem.*, **347**, 18 (1966).

2) G. W. Kosicki, *Biochemistry*, **7**, 4299 (1968).

ditions, even in the absence of NADP^{+} .^{3,4)}

The existence of OAC-lyase in mammalian tissues, whose physiological function is hard to understand in the light of present knowledge of general metabolism, is of considerable interest. In this regard, a preliminary study was carried out on the transcarboxylation between OA and acetyl CoA by this enzyme, like the case of OA-methylmalonyl CoA transcarboxylase (EC 2.1.3.1) in *Propionibacterium* by Swick and Wood.⁵⁾

In the present paper we will describe the distinction of OAC-lyase in pig heart muscle from other OA-decarboxylating enzymes, reversibility of the enzyme action and possibility of the transcarboxylation by OAC-lyase.

METHODS

Enzyme solutions. Preparation of pig heart OAC-lyase was described in the preceding paper. When rat heart and liver were employed, crude extracts were prepared as follows: slices of rat heart and liver were suspended in an equal volume of 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM mercaptoethanol and 0.25 M sucrose, and homogenized in a glass homogenizer. Homogenates were centrifuged (35,000 rpm, 40 min) and the supernatant was collected.

Enzyme activities. OAC-lyase activity was measured by the methods reported previously. Malic enzyme activity was assayed spectrophotometrically according to the method of Hsu and Lardy.⁶⁾ The reaction mixture consisted of 100 μ moles of Tris-HCl buffer (pH 7.4), 0.5 μ mole of L-malate, 1 μ mole of NADP^{+} , 25 μ moles of MgSO_4 , 1 mg of BSA, 0.1 ml of enzyme solution and water to make a final volume of 1.0 ml. L-Malate solution was added to start the reaction and the rate of formation of NADPH was measured spectrophotometrically at 340 m μ using a

Gilford spectrophotometer (Model 200). One unit of enzyme activity was defined as the amount of enzyme which catalyzes the formation of 1 μ mole of NADPH per min.

Acetyl CoA carboxylase activity was measured by the method of Yugari *et al.*⁷⁾ The reaction mixture (0.17 ml) contained 30 μ moles of imidazole hydrochloride buffer (pH 6.5), 1 μ mole of mercaptoethanol, 0.3 μ mole of potassium citrate, 0.1 ml of enzyme solution and an adequate volume of water. After incubation of the mixture at 30°C for 60 min, 0.14 μ mole of acetyl CoA, 1 μ mole of MnCl_2 , 2 μ moles of ATP, 15 μ moles of NaHCO_3 , 1 μ Ci of $\text{NaH}^{14}\text{CO}_3$ (4.9×10^5 cpm) and an adequate volume of water to make a final volume of 0.3 ml were added. After further 10 min incubation at 30°C, the reaction was stopped by the addition of 7 to 8 mg of Dowex 50 -H⁺ until the pH of the mixture fell to 2.0. Excess CO_2 generated was trapped in 20% KOH by aeration and then insoluble matter was centrifuged off. A 0.1 ml aliquot of the supernatant solution was added to the tracer solvent* and was counted for the radioactivity.

OA-acetyl CoA transcarboxylase activity was tested through the identification of malonyl- ^{14}C CoA formed by the enzymic transcarboxylation between acetyl CoA and OA-U- ^{14}C , which had been prepared by enzymic dehydrogenation of L-malate-U- ^{14}C . A 0.2 ml aliquot of the reaction mixture containing 0.05 ml of Medium A**, 0.5 μ mole of pyruvate, 3.6 units of LDH, 3.5 units of MDH, 0.1 ml of enzyme solution and water was incubated at 30°C for 30 min. To the reaction mixture containing OA-U- ^{14}C formed were added 0.1 ml of Medium B*** and 0.1 μ mole acetyl CoA, and the mixture was incubated again for 30 min at 30°C. The reaction was stopped by adding 0.5 ml of 2N HCl and the reaction mixture was centrifuged. 2,4-Dinitrophenyl hydrazine (0.01 ml, 20 mM) was added to the supernatant solution in order to trap ketoacids and the hydrazone formed

7) Y. Yugari, T. Matsuda, T. Mise and M. Suda, *Proc. Symp. Chem. Physiol. Pathol.*, **3**, 43 (1963).

* The tracer solvent: toluene-tritonX100(2:1).

** Medium A contained 2 μ moles of Tris-HCl buffer (pH 8.4), 0.1 μ mole of L-malate, 0.2 μ Ci of L-malate-U- ^{14}C and 0.1 μ mole of NAD^{+} in a final volume of 0.05 ml.

*** Medium B (0.1 ml) contained 40 μ moles of imidazole-HCl buffer (pH 6.5), 1 μ mole of mercaptoethanol, 2 μ moles of ATP and 1 μ mole of MnCl_2 .

3) S. Ochoa, A. H. Mehler and A. Kornberg, *J. Biol. Chem.*, **174**, 979 (1948).

4) W. J. Ruther and H. A. Lardy, *ibid.*, **233**, 374 (1958).

5) R. W. Swick and H. G. Wood, *Proc. Natl. Acad. Sci. U.S.*, **46**, 28 (1960).

6) R. Y. Hsu and H. A. Lardy, *J. Biol. Chem.*, **242**, 520 (1967).

was removed by extraction with ethyl acetate. A small amount of charcoal was then added to the solution and the mixture was centrifuged. The precipitate was washed three times with 1 ml of 1 N HCl and then treated with 1 ml of 4 N ammonium hydroxide. The eluate obtained by centrifugation was evaporated to dryness. The residue was dissolved in 5 ml of the tracer solvent and its radioactivity was measured. The formation of OA-U- ^{14}C was determined preliminarily by measuring radioactivities of 2,4-dinitrophenyl hydrazone formed in the reaction mixture. It was found that 21% of the initial radioactivity was fixed in OA under the above conditions.

Reversibility of OAC-lyase reaction

a) *Identification of malic acid by paper chromatography.* OA formed from pyruvate by OAC-lyase was reduced to malic acid in the presence of MDH and NADH, and malic acid was detected chromatographically. The reaction mixture containing 1.5 mmoles of Tris-HCl buffer (pH 7.4), 150 μ moles of pyruvate, 37.5 μ moles of ATP, 12 μ moles of NADH, 12 μ moles of MnCl_2 , 300 μ moles of NaHCO_3 , 1.8 μ moles of acetyl CoA, 10 units of MDH and OAC-lyase in a final volume of 15 ml was incubated at 30°C for 30 min. The reaction was stopped by the addition of an equal volume of ethanol and the mixture was heated for 5 min in a boiling water bath. After the solution had been cooled, coagulated protein was centrifuged off. The supernatant solution was evaporated to dryness. The residue was extracted three times with 80% methanol and the extracts were combined and dried up. The residue was dissolved in a minimal amount of water and applied to paper chromatography using a solvent system, *n*-propanol-ammonia (30%)-water (60:30:10, v/v). Organic acids were detected by spraying bromphenol blue solution.

b) $^{14}\text{CO}_2$ -fixation by OAC-lyase. The reaction mixture (see Table III) was incubated at 30°C for 30 min and 1 ml of 1 N HCl was added to stop the reaction. After excess CO_2 had been removed by aeration, 1 ml of the solution was added to 5 ml of the tracer solvent and the radioactivity was measured with a liquid scintillation counter (Beckman LS 250).

Protein contents. Protein was estimated by Folin-Ciocalteu's method.⁸⁾ BSA was used as the standard.

RESULTS

It was found that OAC-lyase activity was practically indifferent to ADP, ATP, NAD^+ , NADH, NADP^+ and acetyl CoA (Table I). ITP which is required for PEP-carboxykinase activity^{9,10)} exhibited a weak inhibitory effect.

The reversibility of enzymic decarboxylation of OA was investigated. OA which might be formed from pyruvate and NaHCO_3 was attempted to be identified by the following three methods. (a) Changes of absorbancy at 255 $\text{m}\mu$ ¹¹⁾ which is attributable to OA were measured in the reverse enzyme reaction. Results are illustrated in Table II. Enzymic synthesis of OA from pyruvate was not recognized. (b) It was attempted to identify malic acid which might be derived from OA in the presence of MDH and NADH. As shown in Fig. 1, malic acid was not detected. (c) A tracer technique was employed finally. Pyruvate, $\text{NaH}^{14}\text{CO}_3$ and some cofactors were incubated with the enzyme and after removal of remaining $\text{NaH}^{14}\text{CO}_3$, ^{14}C -activity attributable to OA- ^{14}C in the solution was measured. As illustrated in Table III and IV, net incorporation was not detected. It was concluded that the decarboxylation of

TABLE I. EFFECT OF SOME NUCLEOTIDES
Activities were assayed manometrically.

Addition	Final concentration mM	Relative activity
None		100
ATP	0.17	117
ADP	0.10	107
ITP	0.30	61
NAD^+	0.15	99
NADH	0.15	109
NADP^+	1.00	103
Acetyl Co A	0.12	103

9) M. F. Utter, K. Kurahashi and I. A. Rose, *J. Biol. Chem.*, **207**, 803 (1954).

10) H. C. Chang and M. D. Lane, *ibid.*, **241**, 2413 (1966).

8) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 629 (1927).

TABLE II. REVERSIBILITY OF OAC-LYASE
REACTION (A)

Complete system contained, in 3.0 ml, 150 μ moles Tris-HCl buffer (pH 7.4), 0.25 μ mole MnCl_2 , 7.5 μ moles pyruvate, 0.047 μ mole ATP, 15 μ moles NaHCO_3 , 0.08 μ mole acetyl Co A and 0.1 ml OAC-lyase.

Reaction system	O.D. (255 $\text{m}\mu$)	
	Initial	After 10 min
Complete system	1.000	1.000
Acetyl Co A omitted	0.590	0.590
Acetyl Co A and OAC-lyase omitted	0.550	0.550
OAC-lyase only	0.035	0.035
Acetyl Co A only	0.485	0.485
OAC-lyase + Acetyl Co A	0.520	0.520

OA by OAC-lyase was practically irreversible.

Malic enzyme activity was measured at each stage in the purification of OAC-lyase (Table V). Although the crude extract contained considerable amounts of malic enzyme,

TABLE III. REVERSIBILITY OF OAC-LYASE
REACTION (B)

Reaction system contained, in 0.1 ml, 10 μ moles Tris HCl buffer (pH 7.4), 1 μ mole pyruvate, 1 μ mole MgCl_2 , 3 μ moles NaHCO_3 , 0.06 μCi $\text{NaH}^{14}\text{CO}_3$, 0.25 μ mole ATP, 0.46 μ mole acetyl Co A and 0.05 ml enzyme solution.

Enzyme concentration mg/ml	$^{14}\text{CO}_2$ fixed counts/min
0.1	7.6
1.0	9.5
2.0	4.0
5.0	2.5
10.0	7.5

FIG. 1. Paperchromatogram for the Detection of Malate.

Complete reaction system was described in "Methods."

Solvent system: *n*-propanol-ammonia (30%)–water (60:30:10 v/v). A: Complete system. A': Complete system + D,L-malate. A'': Complete system + D,L-lactate. B: Pyruvate, ATP and acetyl CoA were omitted from complete system. C: Pyruvate (only). D: Malate (only). E: Lactate (only).

TABLE IV. REVERSIBILITY OF OAC-LYASE
REACTION (C)

Reaction systems are the same as described in Table III.

Reaction system	$^{14}\text{CO}_2$ fixed counts/min
Complete	13.0
Acetyl Co A omitted	13.5
ATP omitted	21.5

TABLE V. MALIC ENZYME ACTIVITY OF DIFFERENT
OAC-LYASE PREPARATIONS

Purification stage ^{a)}	Activity		Activity ratio Malic/ OAC
	OAC- lyase units/ml	Malic enzyme units/ml	
Crude extract	0.36	0.04	0.11
After salting-out	1.14	0.18	0.16
After CMC-treatment	0.30	0.01	0.03
After DEAE-treatment	0.16	0	0
After Sephadex-treatment	0.22	0	0

^{a)} As regards the purification stage, see the preceding report.

the activity ratio of malic enzyme to OAC-lyase at each stage decreased markedly along with the progress of purification, especially at the stage of CMC-column chromatography. Malic enzyme adsorbed on CMC-column was not eluted at the same conditions as the case of OAC-lyase, but was eluted at pH 9.0 with a good yield (Table VI). Typical elution patterns of malic enzyme and OAC-lyase from Sephadex G-200 column are shown in Fig. 2. OAC-lyase and malic enzyme were eluted in nearly the same fractions, suggesting that both enzymes had approximately the same order of molecular size.

Acetyl CoA carboxylase activity in OAC-

TABLE VI. ELUTION OF MALIC ENZYME FROM CMC-COLUMN

One g of wet CMC, which had been pretreated with 5 mM phosphate buffer (pH 6.0), was added to 5 ml of buffered enzyme solution (4 mM phosphate, pH 6.0) and the mixture was stirred for 10 min. After centrifugation, the precipitate was washed by 5 ml of 5 mM phosphate buffer (pH 6.0), and then malic enzyme adsorbed was eluted by 5 ml of 1 M tris-HCl buffer (pH 6.0, 7.4 or 9.0). Malic enzyme activity of each eluate was measured spectrophotometrically.

pH of elution buffer	Relative activity ^{a)}
6.0	54
7.4	67
9.0	98

^{a)} The activity of the initial enzyme solution was taken as standard (100).

TABLE VII. ACETYL Co A CARBOXYLASE ACTIVITY OF THE PURIFIED PREPARATION OF OAC-LYASE

Enzyme preparation	¹⁴ CO ₂ fixed counts/min
OAC-lyase preparation of pig heart muscle	21
Rat heart extract	304
Rat liver extract	14,449

lyase preparations were measured. Experiments were carried out in order to avoid any confusion by the enzyme contaminated for the detection of enzymic transcarboxylation between OA and acetyl CoA. As shown in Table VII, the purified OAC-lyase preparation of pig heart muscle contained little amount of acetyl CoA carboxylase, but a little more was found in rat heart extract and much more in rat liver extract.

OA-acetyl CoA transcarboxylase activity of pig heart OAC-lyase was studied. Transcarboxylase activity was measured by the determination of malonyl-¹⁴C CoA formed by the enzymic transcarboxylation between OA-U-¹⁴C and acetyl CoA. OA-U-¹⁴C was prepared by the enzymic dehydrogenation of L-malate-U-¹⁴C in the presence of MDH and NAD⁺ with about 20% yield. As described above, OAC-lyase preparations contained little amount of acetyl CoA carboxylase and its contribution to the measurements was negligible. To avoid further contributions of

FIG. 2. Gel Filtration on Sephadex G-200.

Column size: 1.5 × 52 cm. Enzyme solution applied: 5 ml. Elution buffer: 50 mM phosphate buffer (pH 6.0). Fraction: 5 ml/tube. Flow rate: 20 ml/hr.

—: Protein, ■: Malic enzyme, ▲: OAC-lyase.

TABLE VIII. TRANSCARBOXYLATION BETWEEN OA AND ACETYL Co A

Radioactivity of L-malate-U- ^{14}C used was 4.41×10^5 counts/min in each test.

Enzyme preparation	$^{14}\text{CO}_2$ fixed	
	In the presence of avidin counts/min	In the absence of avidin counts/min
OAC-lyase preparation from pig heart muscle	292	25
Rat heart extract	361	—
Rat liver extract	493	0

biotin dependent enzymes,^{11~16)} experiments were carried out in the presence or absence of avidin. As shown in Table VIII, the transcarboxylation by OAC-lyase was practically not observed in the presence of avidin.

DISCUSSION

The correlation of four kinds of OA-decarboxylating enzymes are schematically illustrated in Fig. 3, together with their distribution in cell fractions. Reactions (A), (B), (C) and (D) are catalyzed by pyruvate carboxylase, OAC-lyase, malic enzyme and PEP-carboxykinase respectively.

OAC-lyase of pig heart muscle was activated by Mn^{2+} but its activity was not affected by ADP, ATP, acetyl CoA or avidin, different from OA-decarboxylating action of pyruvate

FIG. 3. Pyruvate Cycle and OA-Decarboxylating Enzymes.

A: Pyruvate carboxylase, B: OAC-lyase, C: Malic enzyme, D: PEP-carboxykinase.

The framed compounds are members of the pyruvate cycle.

carboxylase.^{17,18)} OAC-lyase was not activated by ITP, which is required for OA-decarboxylating action of PEP-carboxykinase.^{9,10)} The same had been observed in the case of OAC-lyase in cod fish¹¹⁾ and rat liver.¹⁹⁾

It was known that malic enzyme was bi-functional, namely it decarboxylates not only L-malate oxidatively but also OA non-oxidatively.³⁾ The possibility that OAC-lyase activity might be due to malic enzyme was excluded by following facts: (1) the optimal pH of OAC-lyase was 7.0, which was apparently higher than that of OA-decarboxylating activity of malic enzyme (4.5);²⁰⁾ (2) the activity of OAC-lyase was independent of NADP^+ and L-cysteine which stimulate malic enzyme activity,⁴⁾ and also independent of L-malate which is an inhibitor of malic enzyme;⁶⁾ (3) the activity ratio of malic

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enzyme to OAC-lyase decreased markedly through the purification processes, especially in the step of CMC-column chromatography.

It was assumed from the results of gel filtration by Sephadex G-200 that the molecular size of OAC-lyase was approximately the same with that of malic enzyme (Fig. 2) and hence the possibility that OAC-lyase might be generated by dissociation of malic enzyme to its subunits was practically excluded.

Reversibility of the reaction catalyzed by OAC-lyase was investigated by measurements of ^{14}C -incorporation into OA from ^{14}C -labelled bicarbonate. As the results, net incorporation was not observed (Table II, III and IV) and it was concluded that the decarboxylating reaction of OA by OAC-lyase was practically irreversible.

Although pyruvate carboxylase plays an important metabolic role in the synthetic reaction of OA^{17,21)} (see Fig. 3), the physiological function of OAC-lyase should be found rather in the decarboxylating reaction of OA, but little has been known yet in this regard. We tentatively assumed the existence of a

transcarboxylation reaction between OA and acetyl CoA by OAC-lyase, referring to OA-methylmalonyl CoA transcarboxylase (EC 2.1.3.1) which had been found in *Propionibacterium* by Swick and Wood.⁵⁾ ^{14}C -Exchange between OA- ^{14}C and malonyl CoA was studied without success.

The metabolic function of OAC-lyase remains still in vague. According to the pyruvate cycle proposed by Katz *et al.*,²²⁾ OA is reduced to malate with the consumption of NADH and malate formed is decarboxylated to pyruvate with the formation of NADPH, which is required for lipogenesis.^{23,24)} The pathway from OA to pyruvate by OAC-lyase will be of no use for organisms only except the case in which NADPH is supplied in excess by the pentose cycle and elsewhere.

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