Cations in Component Reactions of 'Malic' Enzyme Catalysis

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The 'malic' enzyme (EC 1.1.1.40) has been purified (300-fold) from wheat germ and its abilities to catalyse the decarboxylation and the hydrogenation of oxaloacetic acid and oxaloacetate esters was studied. The free 1-carboxyl group is essential for the interaction of oxaloacetates and substituted oxaloacetates with the enzyme via cations. The free 4-carboxyl group is required for the decarboxylation but is not indispensable for the hydrogenation. At high concentrations, cations inhibit the enzymic hydrogenation of oxaloacetic acid but not that of 4-ethyl oxaloacetate. A plausible inhibitory mechanism is proposed.

Possible similarity between the chemical and the enzymic decarboxylations of oxaloacetic acid have prompted studies on the cation-catalysed decarboxylation (Westheimer, 1963; Rund & Plane, 1964; Claus & Rund, 1969) in the hope that it may serve as a model system for oxaloacetate decarboxylase (Kosicki & Westheimer, 1968) and the 'malic' enzyme (Hsu, Lardy & Cleland, 1967).

The 'malic' enzyme (L-malate-NADP⁺ oxidoreductase, decarboxylating; EC 1.1.1.40) which catalyses the oxidative decarboxylation of malate to pyruvate and CO₂ (Ochoa, Mahler & Kornberg, 1948; Hsu & Lardy, 1967) can be considered as a bifunctional enzyme catalysing two component reactions which require bivalent cations, especially Mn²⁺, as activators:

The 'malic' enzyme is inhibited by high concentrations of cations (Speck, 1949; Rutter & Lardy, 1958; Stickland, 1959). To provide information concerning the nature of cation inhibition and gain some insight into the bifunctionality of the 'malic' enzyme, this work was undertaken to investigate the effect of cations and the structural requirement of substrates for the component reactions of the 'malic' enzyme catalysis. Oxaloacetic acid (compound I) and its mono- and di-ethyl esters (compounds II, III and IV) are of particular interest, because their abilities to form complexes with bivalent cations and to decarboxylate in chemical systems have been established (Steinberger & Westheimer, 1951; C. S. Tsai, T. Y. Lin & E. E. Sharkawi, unpublished work).

MATERIALS AND METHODS

Chemicals. Oxaloacetic acid (compound I) and 4-ethyl oxaloacetate (compound III) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Compound I was recrystallized twice from hexane as white crystals, m.p. 165–166°C. Compound III was crystallized once each from chloroform and benzene to give white crystals, m.p. 102–103°C. Diethyl oxaloacetate (compound IV) was purchased from K & K Laboratories Inc., Plainview, N.J., U.S.A., and purified by vacuum distillation. To synthesize 1-ethyl oxaloacetate (compound II), *tert*.-butyl acetate was prepared from *tert*.-butyl alcohol and acetyl chloride in the presence of dimethyl-aniline (Hauser, Hudson, Abramovich & Shivers, 1955). The condensation of ethyl oxalate with *tert*.-butyl acetate by means of isopropylmagnesium chloride yielded 1-ethyl-4-*tert*.-butyl oxaloacetate (Dubois, Hennequin & Durand, 1963). The hydrolysis of 1-ethyl-4-*tert*.-butyl oxaloacetate with HBr in acetic acid gave compound (II) (Steinberger & Westheimer, 1951), which was crystallized twice from hexane to give white needles, m.p. 53–54°C. The n.m.r. spectrum (CDCl₃) showed chemical shifts (p.p.m.) at 1.40 (triplet, CH₃), 4.42 (quartet, CH₂), 3.95 (singlet, ketonic CH₂) and 6.13 (singlet, enolic CH).

1,4-Dioxan was refluxed over sodium metal before distillation. NADPH was the product of Sigma Chemical Co., St. Louis, Mo., U.S.A.

Purification of enzyme. The 'malic' enzyme from wheat germ has been partially purified (Harary, Korey & Ochoa, 1953); however, the instability of this enzyme necessitated the development of a simplified procedure as described below. A purification of 300-fold was obtained in 1–2 days. All the steps were performed at 0–4°C and the centrifugation was carried out in a refrigerated centrifuge at 7000g for 15 min unless otherwise stated. Acetone-dried wheat germ (100g) (Ochoa, 1955) was stirred for 30 min at room temperature with 1 litre of 0.01 M-sodium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol and centrifuged. The yellow extract was adjusted to pH 5.1

with cold 2M-acetic acid and the precipitate was removed by centrifugation. The supernatant was fractionated with cold (-10°C) 95% ethanol, and the fraction, precipitated between 11.7% and 30.4% ethanol, was dissolved in 40 ml of 0.01M-sodium phosphate buffer, pH 7.4, containing 5mM-2-mercaptoethanol. It was clarified by centrifugation and the clear solution was fractionated with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The fraction, precipitated between 50% and 65% saturation was dissolved in a minimum volume of 0.01M-sodium phosphate buffer, pH 7.4, containing 5mM-2-mercaptoethanol. This solution was delivered on to a Sephadex G-200 column (Sephadex column K25/90) fitted with an upward flow adaptor. Proteins were eluted from the column in an ascending manner with the same buffer. The 5ml-fraction which had the highest specific activity was used for kinetic studies.

Measurement of enzymic activities. The decarboxylase activity of the 'malic' enzyme was determined by following the initial rate of CO_2 evolution at $30 \pm 0.5^{\circ}\text{C}$ in a Gilson differential respirometer. The reaction mixture (2.5 ml) contained 200 μmol of sodium acetate buffer, pH 5.1, 2.5 μmol of MnCl_2 , 0.5–5 μmol of oxaloacetic acid or its esters and an appropriate amount (2–5 μg of protein) of enzyme. The dehydrogenase activity of the 'malic' enzyme was measured by following the initial rate of oxidation of NADPH in a Beckman DB spectrophotometer equipped with a recorder, a scale expander and a thermostat circulator maintained at $30 \pm 0.5^{\circ}\text{C}$. The reaction mixture (3.0 ml) contained 250 μmol of glycylglycine buffer, pH 7.4, 2.5 μmol of MnCl_2 , 0.5 μmol of NADPH, 0.5–5 μmol of oxaloacetic acid or its esters and an appropriate amount (2–5 μg of protein) of enzyme. To study the effect of cation, varied concentrations (0.025–2.5 μmol) of MnCl_2 were used.

Rates of chemical decarboxylation. Rates of chemical decarboxylation were determined manometrically in a Gilson differential respirometer. For the spontaneous decarboxylation, the reaction mixture (2.5 ml) contained 200 μmol of sodium acetate buffer, pH 3.2, and 15 μmol of oxaloacetate. For the Mn^{2+} -catalysed decarboxylation, the reaction mixture (2.5 ml) contained 200 μmol of sodium acetate buffer, pH 5.1, 15 μmol of oxaloacetate and 2.5 μmol –2.5 mmol of MnCl_2 .

Other analytical methods. Melting points were obtained by means of a Fisher-Jones hot-stage apparatus and pH measurements were made with a Radiometer TTTle. N.m.r. spectra were taken with a JEOLCO n.m.r. spectrophotometer, model JNM-C-60, with tetramethylsilane as the internal standard. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The 'malic' enzyme from wheat germ has been partially purified (Harary *et al.* 1953). However, the instability of this enzyme necessitates the development of a simplified procedure which is presented in Table 1. A purification of 300-fold was achieved in 1–2 days.

When tested for the decarboxylation, oxaloacetic acid (compound I) and its 1-ethyl ester (compound II) decarboxylate spontaneously; however, only

compound (I) is active in the cation- and the 'malic' enzyme-catalysed reactions. 4-Ethyl oxaloacetate (compound III), though inactive in the decarboxylation chemically or enzymically, is a competitive inhibitor ($K_i = 5.0 \pm 0.6 \times 10^{-4} \text{M}$) for the decarboxylation of compound (I) (Fig. 1). Both compound (I) and compound (III) are reduced by NADPH in the presence of Mn^{2+} and the 'malic' enzyme. Compound (II) and compound (IV) are

Table 1. Purification of 'malic' enzyme from wheat germ

Step	Specific activity	Yield (%)
1 Crude extract	0.035	100
2 pH precipitation	0.08	50
3 Ethanol fractionation	0.38	18
4 Ammonium sulphate fractionation	1.3	16
5 Sephadex G-200 chromatography	11.5	7.2

Fig. 1. 'Malic' enzyme-catalysed decarboxylation of oxaloacetic acid (●) in the presence of 0.5 mM (○) and 1 mM (△) 4-ethyl oxaloacetate.

Table 2. Kinetic parameters for enzymic hydrogenation of oxaloacetates

Oxaloacetates	K_m (mM)	V_{max} ($\mu\text{M} \cdot \text{min}^{-1}$)
Oxaloacetic acid (compound I)	1.1	25.6
1-Ethyl oxaloacetate (compound II)	0	0
4-Ethyl oxaloacetate (compound III)	0.24	1.9
Diethyl oxaloacetate (compound IV)	0	0

inactive both as substrates and inhibitors. Table 2 shows that the K_m value for the hydrogenation of compound (I) is in good agreement with the value found for the enzymic decarboxylation (2.8×10^{-3} M).

Fig. 2. Effect of Mn^{2+} concentrations on the 'malic' enzyme-catalysed hydrogenation of 0.1 mM- (●), 0.5 mM- (○) and 1 mM- (Δ) oxaloacetic acid.

Fig. 3. Effect of Mn^{2+} concentrations on the cation catalysed decarboxylation of 6mM-oxaloacetic acid (●) in the presence of 20% (○) and 40% (Δ) 1,4-dioxan (by volume).

The K_m value for the hydrogenation of compound (III) is close to the K_d value for the decarboxylation of compound (I). This is consistent with the two component reactions taking place at the same site of the enzyme molecule.

Fig. 2 shows that the 'malic' enzyme-catalysed hydrogenation of oxaloacetic acid is activated by appropriate concentrations of cations but is inhibited at high cation concentrations. By lowering the polarity of solvents, an identical inhibitory effect was observed for the cation-catalysed decarboxylation of oxaloacetic acid at high cation concentrations (Fig. 3). At a saturating substrate concentration (5×10^{-3} M), the cation inhibition gives a linear Hill plot with a coefficient of 1.7 (Fig. 4). Since oxaloacetic acid gives the Hill coefficient of 1.0 at non-inhibitory cation concentration (1×10^{-3} M), the inhibition presumably involves the formation of an oxaloacetate-metal (1:2) complex as proposed by Gelles & Salama, 1958:

The formation of complex (V) may lead to the retardation of decarboxylation at high cation concentrations. Compound (III), which is esterified

Fig. 4. Hill plot for Mn^{2+} inhibition of the 'malic' enzyme-catalysed hydrogenation of oxaloacetic acid (5 mM).

at the 4-carboxyl group and does not form the complex (V), is not inhibited by high cation concentrations in the 'malic' enzyme-catalysed hydrogenation (Fig. 5).

DISCUSSION

Chemical studies of oxaloacetic acid (compound I) and its ethyl esters (compounds II, III and IV) can be briefly summarized as follows for $R-O-CO-CO-CH_2-CO-O-R'$:

- (I) oxaloacetic acid, $R = H$, $R' = H$;
 (II) 1-ethyl oxaloacetate, $R = C_2H_5$, $R' = H$;
 (III) 4-ethyl oxaloacetate, $R = H$, $R' = C_2H_5$;
 (IV) diethyl oxaloacetate, $R = C_2H_5$, $R' = C_2H_5$.

Only compound (I) and compound (II), which possess the ionizable 4-carboxyl groups, undergo decarboxylation in an aqueous solution at an appropriate pH range. The monoanion is the major active species which decarboxylates spontaneously by the proposed mechanism (Tsai, 1967):

The decarboxylation of compound (I), which chelates with cations to form an α -oxocarboxylate complex (VI), is facilitated by cations according to the following mechanism (Steinberger & Westheimer, 1951; Gelles & Hay, 1958):

The decarboxylation of compound (II) which forms a β -oxocarboxylate complex (VII) is not promoted by cations. 4-Ethyl oxaloacetate (compound III) forms the complex (VI) with cations but does not decarboxylate because its 4-carboxyl group is esterified. Diethyl oxaloacetate (compound IV)

where R = H or C₂H₅

does not have free carboxyl groups and is inactive both in the complex-formation and the decarboxylation.

If oxaloacetates form an active complex analogous to the complex (VI) with the 'malic' enzyme via cations (Hsu, Lardy & Cleland, 1967), the structure-activity relationship of the chemical systems described above should be applicable to the enzymic system. Indeed, this is what was observed. Of four compounds [(I)-(IV)] tested, compound (I) and compound (III) interact with the 'malic' enzyme and are substrates for the dehydrogenation reaction. Although only compound (I) is the active substrate for the decarboxylation reactions, compound (III) acts as its competitive inhibitor. Since compound (II) and compound (IV) are esterified at the 1-carboxyl group, which prevents these compounds from forming the complex (VI), they are neither substrates nor inhibitors for the two-component reactions of the 'malic' enzyme catalysis. The free 1-carboxyl group is essential for the interaction

Fig. 5. Effect of Mn^{2+} concentrations on the 'malic' enzyme-catalysed hydrogenation of 1 mM-4-ethyl oxaloacetate.

is retarded by a lowering polarity of solvents at high cation concentration. It is interesting to speculate that there may exist a hydrophobic region at or near the active site of the 'malic' enzyme. Since the decarboxylation of oxaloacetic acid proceeds via a charge dispersion as the reaction passes into the transition state (Tsai, 1967), its rate should be enhanced by a lowering polarity of solvent (Ingold, 1953). The hydrophobic facilitation of the rate (Rund & Plane, 1964) may be, in part, responsible for the catalytic efficiency of the 'malic' enzyme.

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