The Anomalous Kinetics of Coupled Aspartate Aminotransferase and Malate Dehydrogenase

EVIDENCE FOR COMPARTMENTATION OF OXALOACETATE

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Cytoplasmic aspartate aminotransferase and malate dehydrogenase were purified from pig heart. Kinetic parameters were determined for the separate reaction catalysed by each enzyme and used to predict the course of the coupled reaction:

\[
\text{aspartate} \quad \xrightarrow{\text{EC 2.6.1.1}} \quad \text{oxaloacetate} \quad \xrightarrow{\text{EC 1.1.1.37}} \quad \text{malate}
\]

\[
\text{oxoglutarate} \quad \text{glutamate} \quad \text{NADH} \quad \text{NAD}^+
\]

Although a lag phase should have been easily seen, none was detected. The same coupled reaction was also carried out by using radioactive aspartate in the presence of unlabelled oxaloacetate. The reaction was quenched with HClO₄ after 70 ms and the specific radioactivity of the malate produced in this system was found to be essentially the same as that of the original aspartate. These results show that oxaloacetate produced by the aspartate aminotransferase is converted into malate by malate dehydrogenase before it equilibrates with the pool of unlabelled oxaloacetate and are consistent with a proposal that the enzymes are associated in a complex. However, no physical evidence of the existence of a complex could be found. An alternative means of compartmentation of the intermediate as an unstable isomer is considered.

The enzymes aspartate aminotransferase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) can be used to catalyse the following sequence of reactions:

\[
\text{aspartate} \quad \xrightarrow{\text{EC 2.6.1.1}} \quad \text{oxaloacetate} \quad \xrightarrow{\text{EC 1.1.1.37}} \quad \text{malate}
\]

\[
\text{2-oxoglutarate} \quad \text{glutamate} \quad \text{NADH} \quad \text{NAD}^+
\]

Each enzyme occurs in mitochondria as a cationic isoenzyme and in the cytosol as a distinct anionic isoenzyme (Wieland & Pfeiderer, 1959; Delbruck et al. 1959; Thorne et al., 1963; Boyd, 1966). Both enzymes are particularly abundant in heart muscle and there is now considerable evidence (Safer et al., 1971; La Noue et al., 1973; Williamson et al., 1973) that in this tissue the paired enzymes on either side of the mitochondrial membrane operate a shuttle, the effect of which is to transport reducing equivalents from the cytosol to the mitochondrial interior (Borst, 1963).

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The present paper describes a study of the kinetics of the consecutive reactions catalysed by the enzymes, purified together from pig heart, with particular emphasis on the transit of oxaloacetate between them. Some physical properties are also examined in an attempt to detect complex-formation between the enzymes.

Experimental

Materials

L-Aspartic acid was from BDH, Poole, Dorset, U.K., 2-oxoglutaric acid from Koch–Light, Colnbrook, Bucks., U.K., and L-malic acid and oxaloacetic acid were from Sigma (London) Chemical Co., London S.W.6, U.K. The aspartic acid was found, by using a Technicon amino acid analyser, to be 98.8% pure; malic acid, determined as its hydrazone by the method of Hohorst (1965), was found to be 69.2% pure, and oxaloacetate, determined by reduction with NADH by using malate dehydrogenase, was 80% pure. These values for purity were calculated by
Protein concentration is shown by $E_{280}$ (---). ●, Aspartate aminotransferase activity; ○, malate dehydrogenase activity. Experimental details are given in the text.

Assuming the compounds to be anhydrous. A nuclear-magnetic-resonance spectrum of the oxaloacetate showed no impurities, so the discrepancy was presumed to be due to water.

Rabbit muscle lactate dehydrogenase (530 units/mg) was from Boehringer, London W.5, U.K., and pig heart alanine aminotransferase (93 units/mg) from Sigma. Dowex ion exchangers were bought from Micro-Bio Laboratories Ltd., London W.11, U.K. All other reagents were of analytical grade.

**Preparation of enzymes**

Pig heart ventricles (3 kg) were homogenized in 10 litres of a solution containing 0.15 M-succinic acid, 1 mM-2-oxoglutaric acid, 1 mM-EDTA and approx. 10 μM-pyridoxal 5′-phosphate, adjusted to pH 6.2 with NaOH. The homogenate was centrifuged at 2500 g for 30 min (r av. 10 cm) and 250 g of solid (NH₄)₂SO₄/litre added. After 30 min at 4°C the suspension was centrifuged as before and 250 g of solid (NH₄)₂SO₄/litre added to the supernatant. The suspension was centrifuged as before and the precipitate dissolved and dialysed against 0.01 M-sodium acetate, adjusted to pH 5.4 with acetic acid. The solution (120 ml) was applied to a column of CM-cellulose (35 cm × 4.4 cm) and eluted with a linear gradient beginning with this buffer (1 litre) and ending with 0.1 M-sodium acetate, pH 5.4.

The elution pattern is shown in Fig. 1. The early protein peak contains aspartate aminotransferase and malate dehydrogenase, and starch-gel electrophoresis showed both to be anionic (cytosol). The cationic, mitochondrial enzymes were eluted together much later, at a salt concentration of about 0.04 M. The anionic enzymes were pooled, dialysed against 2 mM-sodium phosphate, pH 7.5, and applied to a column of DEAE-cellulose (35 cm × 4.4 cm) equilibrated with the same buffer. This column was eluted with a gradient of sodium phosphate, pH 7.5, from 2 mM (1 litre) to 0.1 M (5 litres). Although there was some overlap, good separation of the two enzymes was achieved by pooling of appropriate fractions, and those of highest specific activity were used in this study. Both enzymes showed their characteristic subforms (Decker & Rau, 1963; Thorne et al., 1963), but were not visibly contaminated by other proteins.

**Enzyme assays**

Aspartate aminotransferase activity was measured by using the NADH-linked assay method of Karmen (1955) with cationic malate dehydrogenase (Boehringer). As shown by Banks et al. (1963) this method gives the same rate as when oxaloacetate (enol form) is measured directly under conditions where the keto-enol tautomerization is not significantly rate-limiting. Malate dehydrogenase was measured by following the steady-state oxidation of NADH at 340 nm and 25°C by using 3 mM-oxaloacetate, 120 μM-NADH and 0.1 M-Tris/HCl, pH 7.6. Throughout this paper a unit of enzyme activity is that amount associated with the oxidation of 1 μmol of NADH per litre per minute at 25°C.
followed reactions Vol. 153 under these syringes. through instrument ensuring cylinder gas 0.073 s. to but actively labelled aspartate measurements separate already determined to cylinder. two giving 1,umol of 280nm of the vessel quenching using (containing 0.5M-NaOH were syringes same reaction, moment was used, the reaction was quenched by vigorously stirred solution. The reaction was determined, which led directly to a rapidly stirred quenching solution. The quench time of this system was determined by using the lactate dehydrogenase-catalysed reduction of pyruvate by NADH. The progress of this reaction was first followed with the instrument in the stopped-flow mode, and then, with the instrument in the quenched-flow mode and by using the same reaction, the whole contents of the drive syringes were driven into the quenching vessel (containing 0.5M-NaOH vigorously stirred at 0°C) by using 0.34kPa (3.3kg/cm²) pressure from a N₂ cylinder. From a knowledge of the progress of the reaction and the final NADH concentration in the quenching vessel the quench time was calculated. It is important to take into account the water that was already in that part of the system occurring after the drive syringes, since this dilutes the NADH in the stop vessel and at the end of the determination this volume is filled with reactants. This volume was determined to be 0.837±0.002ml on the basis of 20 separate measurements of the dilution of radioactively labelled aspartate solution from the drive syringes and also independently by the dilution of Phenol Red. The quench time was determined to be 0.073s.

The lactate dehydrogenase reaction was also used to demonstrate that the constant pressure from the gas cylinder drove the syringes at a uniform rate, thus ensuring that the quench time was constant. For this the instrument was used in the stopped-flow mode, but the contents of the drive syringes were driven out through the drain valve directly and not into the stop syringe. The transmission through the cuvette, which under these conditions contains flowing solution, was followed and found to be constant throughout the operation, showing that the flow rate was constant.

Stopped-flow and quenched-flow experiments with the coupled aspartate aminotransferase and malate dehydrogenase reactions were carried out with both enzymes and NADH in one syringe and aspartate and 2-oxoglutarate in the other. All were dissolved in 0.1M-Tris, adjusted to pH7.6 with HCl. For the quenched-flow experiments the reactants (total volume 4ml) were driven into 10ml of 0.47M-HClO₄ rapidly stirred at 0°C. This process had to be repeated several times in order to obtain enough product for analysis, and after each 'shot' the flow system was washed with water to prevent contamination by unquenched solution in the dead volume.

Specific radioactivity of malate

The specific radioactivity of malate produced in the quenched-flow experiments was determined after its separation from the other reactants and products. Solutions from these experiments in 0.1M-HCl were applied to a column (30cm×1cm) of anion-exchange resin (Dowex AG 50W-X8, 200–400 mesh) and eluted with 4mm-HCl. The radioactive eluate was concentrated and applied to a column (30cm×1cm) of cation-exchange resin (Dowex AG 1-X8, 200–400 mesh) and eluted with a linear gradient made from 850ml of water in one vessel and the same volume of 33mm-HCl in the other. This procedure gave good separation of malate from all the other components in the system.

Malate was determined by the method of Hummel (1949). The radioactivity of the sample was determined in a Packard Tri-Carb scintillation counter by using a scintillation solution made by dissolving 14g of 2,5-diphenyloxazole in 1600ml of toluene and adding 400ml of methoxyethanol and 100ml of Hyamine 10X hydroxide solution (10%, w/v, in methanol). The efficiency of counting was determined by using the [14C]aspartic acid used as substrate as an internal standard.

Results

Time-course of coupled enzyme reactions

The formation of product by a system in which two enzymes catalyse consecutive reactions, as shown in eqn. (1):

$$A \rightarrow B \rightarrow P$$

may be predicted by eqn. (2) so long as the first reaction proceeds at constant velocity and the concentration of B is low enough for the second reaction to be first order. The values of v and k, which represent the steady-state velocity of the first enzyme and first-order rate constant for the second enzyme respectively, will of course be proportional to the concentrations of those enzymes.

$$[P] = vt + \frac{v}{k}(e^{-kt} - 1)$$
axis, \( \tau \), is \( 1/k \) and the linear, steady-state slope is \( v \).
The rate of the overall reaction approaches the steady-state rate exponentially with a constant equal to the first-order rate constant for the second reaction. If the steady-state concentration of \( B \) is low (compared with the \( K_m \) for both the second enzyme and \( B \)) then the equation can be used to predict the course of the reaction until the first reaction deviates from zero order or significant back reaction occurs. It has been shown to predict satisfactorily the course of the coupled reaction catalysed by rabbit muscle pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) (Hess & Wurster, 1970). We have found that it also predicts accurately the course of the coupled reaction between alanine aminotransferase (EC 2.6.1.2) from pig heart and lactate dehydrogenase from rabbit muscle (Fig. 2). Even in cases where the steady-state concentration of the intermediate is significant compared with \( K_m \) for the second enzyme, so that the reaction course eventually diverges from that predicted by eqn. (2), the early part of the reaction should still conform to the equation.

The course of the coupled reactions catalysed by aspartate aminotransferase and malate dehydrogenase might also be expected to be predicted by the same equation. Fig. 3(a) shows the reduction of

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**Fig. 2. Kinetics of the coupled alanine aminotransferase-lactate dehydrogenase reaction**

(a) Pyruvate (6\( \mu \)M) in one syringe was mixed with rabbit muscle lactate dehydrogenase (0.2mg/ml, 110 units/ml) and NADH (70\( \mu \)M) in the other syringe. (b) Alanine (0.5\( \mu \)M) and 2-oxoglutarate (50\( \mu \)M) in one syringe were mixed with pig heart alanine aminotransferase (0.02mg/ml, 1.6 units/ml), rabbit muscle lactate dehydrogenase (0.2mg/ml) and NADH (0.22\( \mu \)M) in the other syringe. The reactions were carried out at 18°C in 0.1M-Tris/HCl buffer, pH 7.5, and followed at 365nm.

**Fig. 3. Kinetics of the coupled aspartate aminotransferase-malate dehydrogenase reaction**

(a) Oxaloacetate (1.9\( \mu \)M) in one syringe was mixed with malate dehydrogenase (2 units/ml) and NADH (30\( \mu \)M) in the other syringe. (b) and (c) Aspartate (5\( \mu \)M) and 2-oxoglutarate (1\( \mu \)M) in one syringe were mixed with malate dehydrogenase (2 units/ml), aspartate aminotransferase (0.4 unit/ml) and NADH (0.15\( \mu \)M) in the other syringe. The reactions were carried out at 18°C in 0.1M-Tris/HCl buffer, pH 7.5, and followed at 365nm.
oxaloacetate by NADH catalysed by malate dehydrogenase and Figs. 3(b) and 3(c) show the course of the coupled reaction in which aspartate and 2-oxoglutarate are converted first into oxaloacetate and then into malate. Data replotted from Fig. 3(a) show the reaction to be first order with a rate constant \( k = 2.5 \text{s}^{-1} \). The rate of the overall reaction calculated from Figs. 3(b) and 3(c) is \( 2.5 \mu \text{M/s} \), which agrees well with that expected on the basis of manual mixing experiments at lower enzyme concentrations. We have determined the \( K_m \) for malate dehydrogenase and oxaloacetate in a separate experiment to be \( 24 \mu \text{M} \) and \( V_{\text{max}} \), at the enzyme concentration used in the stopped-flow experiment, to be \( 60 \mu \text{M/s} \), from which values it was calculated that the steady-state concentration of oxaloacetate required for the malate dehydrogenase to give the observed overall rate in the coupled reaction should be \( 1.1 \mu \text{M} \). This is so far below the \( K_m \) that eqn. (2) would be expected to predict the course of the reaction accurately. Fig. 4 shows the actual course of the reaction together with that predicted by eqn. (1) by using \( k = 2.5 \text{s}^{-1} \) and \( v = 2.5 \mu \text{M} \). No lag is apparent under conditions where one would be expected. The experiment shown in Fig. 2 demonstrates clearly that the apparatus is capable of detecting the lag phase. Further, this particular experiment has been carried out on a second Durrum stopped-flow spectrophotometer and in the Laboratory of Molecular Enzymology, University of Bristol, on a stopped-flow spectrophotometer built in that Department. The same results were obtained in each case. Particular care was taken with this experiment, because of an artifact observed as a result of a small amount of diffusion that may occur if the solutions are left standing in the drive syringes before mixing. As a result of this diffusion small amounts of oxaloacetate are formed in part of the flow system that is supposed to contain only 2-oxoglutarate and a 'burst' is observed as the oxaloacetate is rapidly reduced when it meets the dehydrogenase and NADH from the other half of the flow system. The artifact can be completely eliminated by carrying out each measurement without leaving the solutions standing in the drive syringes.

Although, because of the noise level, it is difficult to put a lower limit on the possible lag that may be present in the progress curves shown in Figs. 3(b) and 3(c), it seems fair to claim that it is no longer than 0.05s, whereas theoretically it should be 0.4s. Thus 50ms after mixing, when the concentration of oxaloacetate produced by the aminotransferase can only be 0.125 \( \mu \text{M} \), the malate dehydrogenase is working at a rate that requires a concentration of \( 1 \mu \text{M} \). Two possible explanations for this apparent paradox were considered. First, it is possible that the oxaloacetate produced by the aminotransferase reaches the malate dehydrogenase before diffusing into the whole solution, a proposal that implies some sort of association between the enzymes. The second possible explanation is that the aminotransferase gives rise to oxaloacetate as an isomer identical with that used as substrate for malate dehydrogenase and that conversion of this isomer into the equilibrium mixture of forms present in normal oxaloacetate solutions is slow relative to transit between the enzymes.

It may be helpful at this stage to point out that the different isomers postulated cannot be the well-known keto and enol forms of oxaloacetate, since, although the aminotransferase does produce the keto form of oxaloacetate (Banks et al., 1963), this is also the predominant form (84\%) in solutions of the compound (Banks, 1961). Compartmentation of enzymically produced oxaloacetate cannot therefore be on this basis.

Specific radioactivity of malate produced by the coupled enzymes from radioactive aspartate

The proposals mentioned above require that oxaloacetate produced by the aminotransferase would not achieve equilibrium with oxaloacetate in free solution before being converted into malate. To see if this requirement was fulfilled \(^{14} \text{C} \text{aspartate} \) was used as the substrate for aspartate aminotransferase and the coupled reaction was carried out in the presence of unlabelled 8.5\( \mu \text{M} \)-oxaloacetate. The reaction was quenched after 0.07s and the specific radioactivity of the malate produced was measured. The specific radioactivity of the malate was predicted for three theoretical cases.
Table 1. Relative specific radioactivities of \([^{14}C]\) malate produced from \([^{14}C]\) aspartate

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<th>Relative specific radioactivities</th>
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<tr>
<td></td>
<td>[Oxaloacetate]</td>
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<tr>
<td>Expt. 1</td>
<td>48</td>
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<tr>
<td>Expt. 2</td>
<td>41</td>
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(1) If the labelled oxaloacetate produced by aspartate aminotransferase equilibrates rapidly with the unlabelled oxaloacetate then the relative specific radioactivity \(r_1\) of the malate produced should be less than that of the original aspartate, in accordance with eqn. (3). This equation was obtained by making the simplifying assumption that the oxaloacetate concentration produced enzymically does not become high enough (about 7-fold lower than \(K_m\)) to decrease binding of external oxaloacetate significantly:

\[
r_1 = \frac{vt + \frac{v}{k}(e^{-kt} - 1)}{vt + \frac{v}{k}(e^{-kt} - 1) + [B](1 - e^{-kt})}
\]

where \(r_1\) is the ratio of the specific radioactivity of malate to that of aspartate; \([B]\) is the concentration of unlabelled oxaloacetate; \(t\) is the quench time and \(v\) and \(k\) are as in eqn. (2).

(2) If the oxaloacetate from the aminotransferase binds to the dehydrogenase before it equilibrates with the unlabelled oxaloacetate but the binding of this unlabelled oxaloacetate is not significantly decreased, then the relative specific radioactivity of the malate should be given by

\[
r_2 = \frac{vt}{vt + [B](1 - e^{-kt})}
\]

(3) If the labelled oxaloacetate reaches the malate before equilibration with the unlabelled oxaloacetate and binding of the unlabelled oxaloacetate is also effectively prevented, then the relative specific radioactivity of the malate \(r_2\) will clearly be 1.

Table 1 shows the results of two such quenching experiments. The value of \(v\) was calculated from a separate determination of aminotransferase activity of the sample and \(k\) from a separate experiment with malate dehydrogenase and low concentrations of oxaloacetate of the type shown in Fig. 3(a). The molar ratio of aminotransferase to dehydrogenase was higher (2:1) than in the experiment of Fig. 3 (0.4:1) and was chosen for two reasons. First, sufficient reaction had to occur within the quench time of the instrument to allow measurement of the product malate, and, secondly, the theoretical lag time for the reaction had to be longer than the quench time in order to distinguish satisfactorily between the cases described above.

The results shown in Table 1 show that the oxaloacetate produced by the aminotransferase is not diluted by the external unlabelled oxaloacetate before being converted into malate by the dehydrogenase. The procedure used to purify the malate gave a yield that was 75% of that expected if case 3 was operating with no handling losses. The results are clearly inconsistent with any scheme involving rapid equilibration with the pool of unlabelled oxaloacetate before conversion into malate.

Several types of experiment were carried out in attempts to detect complex-formation between the enzymes. No aggregation to a unit of higher molecular weight was observed when the enzymes were chromatographed on Sephadex G-200 even under circumstances designed to favour such aggregation. For example, in an experiment in which a Sephadex G-200 column (100cm x 1.6cm) was equilibrated with aspartate aminotransferase (1mg/ml in 0.1M Tris/HCl, pH 7.5), and then malate dehydrogenase (1ml, 30mg/ml) chromatographed on the column, the elution position of the dehydrogenase was identical with that observed when it was chromatographed on the same column in the absence of the aminotransferase. This was repeated in the presence of substrates at the concentrations used in the stopped-flow experiments and found once more to give the same elution pattern. Further, the anomalous kinetic observations of the type shown in Fig. 3 are also observed when using commercial malate dehydrogenase (Boehringer), which is cationic and therefore presumably mitochondrial. When this enzyme and the cytoplasmic aminotransferase were treated together with glutaraldehyde, cross-linking of each enzyme occurred readily, but no aggregate of the two enzymes could be detected (R. A. John, unpublished work). It is, of course, difficult to prove the absence of complex-formation, but the available evidence is against it.

An alternative explanation is that the intermediate oxaloacetate is produced by the aminotransferase in the same conformation as that used by the dehydrogenase, that this is not the conformation which
predominates at equilibrium in solution and that rotation from the less to the more stable conformer is slow relative to transit between the enzymes. This proposal has some support from binding studies using fixed stereoisomers which show that both enzymes bind dicarboxylic acids with the two carboxylic groups in the cis position (Jenkins et al., 1959; Angielski & Rogulska, 1962; Yoshida, 1965; Khomutov et al., 1968; Braunstein, 1970). It also seems probable that the predominant form of oxaloacetate in solution would be trans, since this would maximize charge separation. An estimate of how slow this process must be in order to explain these results can be obtained by calculation of the transit time between the enzymes. The experiments were carried out at concentrations between 1 and 0.1 \( \mu M \) so that the average distance between the enzymes would be between 0.1 \( \mu M \) and 0.3 \( \mu M \) (taking the average distance to be that which would separate them if they were arranged in a regular cuboid lattice). From the values given by Webb (1963), the transit time would be a few microseconds. It is not clear why rotation should be so slow for oxaloacetate, and indeed compartmentation may be on some other basis, e.g. different hydrated forms of oxaloacetate. However, this hypothesis could provide the basis for a functional compartmentation within the cellular environment and thereby be significant to the role of oxaloacetate as a key metabolite.

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References