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Some Kinetic and Other Properties of the Isoenzymes of Aspartate Aminotransferase Isolated from Sheep Liver

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A method for the purification of the mitochondrial isoenzyme of sheep liver aspartate aminotransferase (EC 2.6.1.1) is described. The final preparation is homogeneous by ultracentrifuge analyses and polyacrylamide-gel electrophoresis and has a high specific activity (182units/mg). The molecular weight determined by sedimentation equilibrium is 87100 ± 680. The amino acid composition is presented; it is similar to that of other mitochondrial isoenzymes, but with a higher content of tyrosine and threonine. Subforms have been detected. On isoelectric focusing a broad band was obtained, with pI9.14. The properties of the mitochondrial aspartate aminotransferase are compared with those of the cytoplasmic isoenzyme. The K_m for L-aspartate and 2-oxoglutarate for the cytoplasmic enzyme were 2.96 ± 0.20mM and 0.093 ± 0.010mM respectively; the corresponding values for the mitochondrial form were 0.40 ± 0.12mM and 0.98 ± 0.14mM. Cytoplasmic aspartate aminotransferase showed substrate inhibition by concentrations of 2-oxoglutarate above 0.25mM in the presence of aspartate up to 2mM. The mitochondrial isoenzyme was not inhibited in this way. P_i at pH7.4 inhibited cytoplasmic holoenzyme activity by up to about 60% and mitochondrial holoenzyme activity up to 40%. The apparent dissociation constants for pyridoxal 5'-phosphate were 0.23 μM (cytoplasmic) and 0.062 μM (mitochondrial) and for pyridoxamine 5'-phosphate they were 70 μM (cytoplasmic) and 40 μM (mitochondrial). P_i competitively inhibited coenzyme binding to the apoenzymes; the inhibition constants at 37°C were 32 μM for the cytoplasmic isoenzyme and 19.5 μM for the mitochondrial form.

It has been shown for a variety of tissues that there are two isoenzymes of aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1), one located in the mitochondria and the other in the cytoplasm. The mitochondrial isoenzyme has been purified from a number of these sources (Hook & Vestling, 1962; Wada & Morino, 1964; Nisselbaum & Bodansky, 1964, 1966; Boyd, 1966; Martinez-Carrion & Tiemeier, 1967; Magee & Phillips, 1971; Aoki *et al.*, 1972; Shrawder & Martinez-Carrion, 1973), but the properties reported for the preparations have varied somewhat. Moreover, the amino acid composition of mitochondrial aspartate aminotransferase from mammalian liver has not been established. Expressed as units of activity per mg of tissue, liver is the third richest source of aspartate aminotransferase (Bergmeyer & Bernt, 1963). In this tissue the mitochondrial isoenzyme has important roles in the transfer of reducing equivalents between mitochondria and cytoplasm and in the operation of the urea cycle (Katunuma *et al.*, 1967; Chappell & Robinson, 1968; LaNoue & Williamson, 1971). We have obtained highly purified preparations of the mitochondrial form from sheep liver and we here report details of the isolation procedure and some properties of the purified protein.

As with aspartate aminotransferases from other sources (Braunstein, 1973; Morino & Watanabe, 1969), although the mitochondrial and cytoplasmic isoenzymes catalyse the same reaction, there are differences between their active sites (Campos-Cavieres & Milstein, 1975), and we wished therefore to see if the kinetic properties of the two isoenzymes showed the differences expected from the results obtained for the enzyme from other sources (Fleisher *et al.*, 1960; Boyd, 1961; Nisselbaum & Bodansky, 1964; Michuda & Martinez-Carrion, 1969).

We have found that, unlike the more extensively studied pig heart aspartate aminotransferase, the

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sheep liver apoenzyme has a relatively low affinity for the coenzyme, and this has enabled us to determine the dissociation constants for both isoenzymes.

A brief account of some of this work has been presented (Orlacchio *et al.*, 1976).

Materials and Methods

Materials

Pig heart cytoplasmic aspartate aminotransferase was obtained from Boehringer (London) Corp., London W5 2TZ, U.K., and from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, U.K. Malate dehydrogenase, NADH and 2-oxoglutarate were purchased from Boehringer (London) Corp.; pyridoxal phosphate was from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. L-Aspartate was from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Ampholine was from LKB Instruments, London S.W.20, U.K., and starch hydrolysed was from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada. Acrylamide and *NNN'N'*-tetramethylethylenediamine were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Bisacrylamide and 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride were obtained from Eastman Kodak Co., Rochester, NY, U.S.A. Naphthalene Black, Coomassie Brilliant Blue and Fast Blue B were purchased from Gurr, London N.W. 9, U.K. Ionagar 2 was from Difco Laboratories, Detroit, MI, U.S.A., and polyvinylpyrrolidone was from May and Baker, Dagenham, Essex, U.K. Bovine plasma albumin was obtained from Armour Pharmaceutical Co., Hampden Park, Eastbourne, Sussex, U.K.

Buffers. The 0.15M-NaCl/17.6mM-sodium/potassium phosphate buffer, pH7.6, corresponds to a solution 16.2mM in Na_2HPO_4, 1.4mM in KH_2PO_4 and 0.15M-NaCl (all final concentrations). Sodium succinate buffer (50mM), pH6, 10mM-potassium/sodium phosphate buffer, pH7.4, and 5mM-Tris/HCl buffer, pH8.5, were prepared as described by Dawson *et al.* (1969). Sodium diethylbarbiturate buffers (25mM and 50mM), pH8.6, were prepared as described by Feinstein (1968).

Methods

Enzyme assay. Aspartate aminotransferase activity (at enzyme concentrations in the range 5–15μg/3ml) was measured by the decrease in A_{340} corresponding to the oxidation of NADH in a coupled indicator reaction (Karmen, 1955) with reagent concentrations as used by Boyd (1966). Malate dehydrogenase was dialysed against 0.01M-sodium/phosphate buffer, pH7.4, to remove $(NH_4)_2SO_4$ before use. One unit of aspartate aminotransferase activity is defined as that amount of enzyme that catalyses the formation of 1μmol of oxaloacetate/min at 25°C in 0.20M-sodium/potassium phosphate buffer, pH7.4.

Protein determination. Protein concentration was determined by a modification (Miller, 1959) of the method of Lowry *et al.* (1951), with bovine plasma albumin as a standard.

Agar-gel electrophoresis. This was carried out in agar gel [1.5% (w/v) Ionagar 2 in 25mM-sodium diethylbarbiturate, pH8.6, containing 0.1% NaN_3] with 50mM-sodium diethylbarbiturate, pH8.6, in the reservoir (Feinstein, 1968). The agar gel was melted on a boiling-water bath, and 10ml were layered on a levelled plate (8.2cm × 8.2cm). The gel was allowed to set for 20min; circular wells of 1–2mm diameter were then cut and filled with the sample solutions. The plates were run for 3h at 4.7V/cm and at 4°C. After the run, the plates were first fixed by immersion for 30min in ethanol/acetic acid/water (5:1:5, by vol.) and then covered with filter paper and allowed to dry out at 37°C. They were then stained with a saturated solution of Naphthalene Black in the fixer for 5–10min, and finally destained in the fixer mixture.

Horizontal starch-gel electrophoresis. The technique described by Smithies (1955) as modified by Kristjansson (1963) was followed for the preparation of the gels. Three Tris/borate buffer systems were tried. On completion of electrophoresis (300V for 4h), the gel was sliced in half lengthwise; one half was stained for protein with 1% Naphthalene Black solution in methanol/water/acetic acid (5:5:1, by vol.) and the other for enzyme activity as described by Decker & Rau (1963) with 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride as the staining reagent.

Polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis was carried out at pH8.9, essentially as described by Davis (1964). However, only one gel concentration was used throughout, corresponding to the 'running gel' [7.5% (w/v) acrylamide]. The samples were applied by layering 15–20μl (5–40μg of protein) on top of the gel, after addition of some glycerol or sucrose solution at a concentration of 33% (w/v) (Smith, 1968). The runs were carried out towards the cathode at 1mA/tube for 15min, then at 2mA/tube for another 15min and finally at 3mA/tube for 75min. By the end of the run, the voltage rose to 350V. Each gel was sliced in two lengthwise and one half was tested for protein and the other for enzymic activity as described by Campos-Cavieres & Munn (1973).

Isoelectric focusing. Isoelectric focusing on polyacrylamide plates (21.6cm × 16.5cm × 0.1cm) in the pH range 8.5–10.5 was carried out as described by Campos-Cavieres & Munn (1973) with the following modifications: (i) Ampholine (2.1ml) of pH range 9–11 was used; (ii) ammonium persulphate (0.6ml of a 1%, w/v, solution) was added as final reagent in the mixture; (iii) the electrode solutions were Ampholine

(0.1%, v/v) in the pH range 7–9 and 2M-NaOH respectively; (iv) sheep liver mitochondrial aspartate aminotransferase was dialysed against 5mM-Tris/HCl, pH8.5, for 24h before each run.

Ultracentrifugation studies. Analytical ultracentrifugation was carried out in a Beckman–Spinco model E ultracentrifuge equipped with R.I.T.C. temperature control. Before centrifugation, samples were dialysed for 24h against 0.15M-NaCl/17.6mM-sodium/potassium phosphate buffer, pH7.6. A value of 0.735ml/mg was used for the partial specific volume of mitochondrial aspartate aminotransferase (Polyanovsky & Telegdi, 1965; Magee & Phillips, 1971). Sedimentation-velocity experiments were performed by using Schlieren optics in 4°-sector cells, with 12mm aluminium centre pieces, at 23°C and at a rotor speed of 60000rev./min, giving 260000g ($r_{av.}$ 6.5cm). Meniscus-depletion sedimentation-equilibrium experiments were performed by using the short-column technique of Yphantis (1964). In these runs, 4mm solution columns in a double-sector cell equipped with sapphire windows were used.

Amino acid analyses. Portions of the enzyme were dialysed against distilled water for 24h and then freeze-dried. They were hydrolysed with constant-boiling glass-distilled HCl for 24, 48 and 72h *in vacuo* at 105°C. The hydrolysates were evaporated to dryness over NaOH in a vacuum desiccator, then taken up in 0.5ml of 67mM-sodium citrate buffer, pH2.0. Samples were analysed on a Technicon micro-analyser (Technicon Instruments Co., Chertsey, Surrey, U.K.) by the procedure of Beale & Kent (1968). Cysteine was determined as cysteic acid in 48h hydrolysates after performic acid oxidation (Hirs, 1967).

Purification of isoenzymes. Cytoplasmic aspartate aminotransferase was purified from sheep liver as described by Campos-Cavieres & Munn (1973). The mitochondrial isoenzyme was purified as described below by modifications of the procedure of Boyd (1966).

The centrifugations were performed in an MSE 18 refrigerated centrifuge. All steps were carried out at 0–4°C, unless otherwise specified. Exposure of the preparation to light was restricted as far as possible.

Stage 1. Livers, rapidly removed from sheep immediately after they had been killed and bled out, were diced and rinsed in ice-cold unbuffered 0.25M-sucrose. Portions of about 50g in an equal volume of 0.25M-sucrose were homogenized, first in an all-Perspex homogenizer with a clearance of 1.0mm, and then smaller portions of this homogenate, further diluted with an approximately equal volume of 0.25M-sucrose, were homogenized by nine down-and-up strokes in a Teflon-in-glass homogenizer, clearance 0.12mm. The homogenate prepared in 0.25M-sucrose was centrifuged at 650g for 10min. The supernatant was centrifuged at 12000g for 10min, and the mitochondrial pellet obtained was resuspended in 0.25M-sucrose and washed twice with this solution by alternate centrifugation (12000g, 10min) and resuspension.

Stage 2. Butan-1-ol extraction in the presence of 50mM-sodium succinate buffer (pH6.0) was carried out with the above suspension. The aqueous phase was dialysed against 10mM-sodium/potassium phosphate buffer, pH7.4, to remove dissolved butanol (48h dialysis, several changes of buffer solution).

Stage 3. Sodium succinate buffer, pH6.0, 2-oxoglutarate and pyridoxal phosphate were added to final concentrations of 0.15M, 1mM and 0.01mM respectively, and the solution was then heated in portions of about 100ml on a water bath at 70°C with continuous stirring. It took 2–4min for the preparation to reach 70°C; it was then heated for a further 15min. The suspension was quickly cooled down to 4°C in an ice/water mixture and centrifuged at 6000g for 10min. The mitochondrial isoenzyme was almost completely inactivated if the heat step was carried out in the absence of 2-oxoglutarate.

Stage 4. The proteins precipitated with $(NH_4)_2SO_4$ between 50 and 90% saturation were redissolved in 5mM-Tris/HCl buffer, pH8.5, and dialysed against the same buffer.

Stage 5. The mitochondrial isoenzyme was further purified by DEAE-cellulose chromatography. The protein was applied to a column (2.5cm × 26cm) equilibrated at 4°C with 5mM-Tris/HCl buffer (pH8.5 at 4°C). The buffer flow rate was 40ml/h; fractions of volume 3–4ml were collected. Fractions with the highest specific activity were pooled and concentrated by ultrafiltration.

Stage 6. The protein sample in 0.15M-NaCl/17.6mM-sodium/potassium phosphate buffer (pH7.6) was finally purified by gel filtration on a column (1.0cm × 90cm) of Sephadex G-100 (equilibrated with the same buffer). This yielded two peaks of protein; enzyme activity was associated with one of these.

Kinetic investigations. These employed the same principle as for the standard assay. The concentrations of malate dehydrogenase (5μg in a final volume of 3.0ml) and NADH (0.1mM) were kept constant throughout, but the concentrations of the substrates were varied: 0.04mM-pyridoxal 5′-phosphate and 0.05M-triethanolamine/HCl buffer, pH7.4, were used unless otherwise indicated and the reaction mixture was maintained at 37°C in a thermostatically controlled cuvette holder in a Cecil Instruments CE272 spectrophotometer. The reaction was initiated after 5min incubation by rapidly stirring in the required amount of 2-oxoglutarate. The progress of the reaction was recorded as the decrease in A_{340} corresponding to the oxidation of NADH. The slope of the straight line was linearly related to the concentration of active holoenzyme.

Like aspartate aminotransferase from wheat germ (Orlacchio *et al.*, 1975), sheep liver aspartate aminotransferase has a relatively low affinity for the coenzyme. For the purpose of the comparison of the kinetic properties of the two isoenzymes from sheep liver we have therefore adopted the reasoning of Orlacchio *et al.* (1975) in using the equations derived by Litwack & Cleland (1968) for tyrosine aminotransferase, which also has a low affinity for the coenzyme. It follows that the assumptions made by Litwack & Cleland (1968) have to be accepted here. Under the conditions tested, and for the purpose stated, this seems a reasonable approach, but we cannot claim that it is applicable to all situations.

K_{ic} and K_{id} (the dissociation constants of the pyridoxal and pyridoxamine forms of the holoenzyme) can be determined by plotting $1/v$ versus $1/[A]$ (or $1/[B]$), at two concentrations, c' and c'', of pyridoxal 5'-phosphate (or pyridoxamine 5'-phosphate) by using the following equation

$$K_{ic} \text{ (or } K_{id}) = \frac{c'c''(r-1)}{c''-rc'}$$

where r is the ratio of the slopes of the two straight lines obtained respectively with c' and c'', A is aspartate and B is 2-oxoglutarate.

The inhibition constant K_i for phosphate towards the coenzyme was determined from the ratio of the slopes of the two lines obtained by plotting $1/v$ against $1/C_t$ (where C_t = total coenzyme concentration) in the presence and absence of the inhibitor.

Results

Purification of the mitochondrial isoenzyme

The purification procedure was carried to completion for five separate preparations; details of two of these, (*a*) with the lowest purification factor, but yielding the preparation with the highest specific activity, (*b*) with the best purification factor but lowest specific activity, are presented in Table 1.

Electrophoretic behaviour

Sheep liver mitochondrial aspartate aminotransferase prepared as described above migrated towards the cathode as a single zone on agar-gel electrophoresis at pH 8.6. The electrophoretic pattern is shown, together with that of the cytoplasmic isoenzyme, in Fig. 1. On starch-gel electrophoresis, the mitochondrial isoenzyme migrated towards the cathode as two partially resolved zones with enzymic activity in the Tris/EDTA/borate buffer (pH 8.6) of Hopkinson *et al.* (1970). In other buffer systems tried, only a single broad zone staining for protein and with associated enzymic activity was obtained. Similarly on electrophoresis on polyacrylamide gel, the enzyme yielded a very broad band of protein corresponding to the zone of activity on suitably stained gels when run towards the cathode at pH 8.9.

Isoelectric focusing

Purified samples of the liver mitochondrial isoenzyme (in 5 mM-Tris/HCl, pH 8.5) yielded a broad diffuse zone with enzyme activity and a pI value of 9.14 ± 0.04 (mean $\pm$S.E.M., $n = 4$).

Sedimentation-velocity experiments

The preparation of the mitochondrial isoenzyme obtained after gel filtration showed a single component in the ultracentrifuge (Fig. 2). Sedimentation coefficients ($S_{20,w}$) of 5.45, 5.55, 5.51 and 5.48 S were determined at protein concentrations of 6, 4.3, 3.5 and 3.1 mg/ml respectively.

Table 1. *Purification of sheep liver mitochondrial aspartate aminotransferase*

Data for two representative preparations are given (*a*) with the lowest purification factor, (*b*) with the best purification factor.

	Total activity (units)		Total protein (mg)		Specific activity (units/mg of protein)		Yield (%)		Purification factor	
Stage	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
1. Dialysed butanol extract	11000	9515	3235	4680	3.4	2.0	100	100	1	1
2. Heated butanol extract	10100	6075	1174	1160	8.6	5.2	92	64	2.5	2.6
3. $(NH_4)_2SO_4$ precipitate	5400	4970	300	450	18.0	11.0	49	52	5.3	5.5
4. Pooled DEAE-cellulose fractions	2200	1670	18.6	21.6	117.4	77	20	18	34.5	39
5. Pooled Sephadex fractions	1020	1200	5.6	9.9	182	121	9.3	13	53.5	61

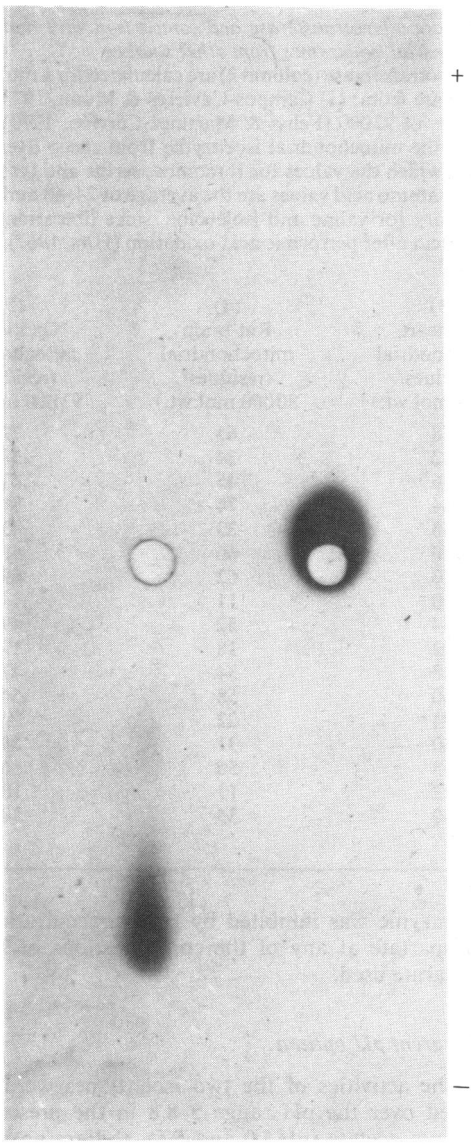

Fig. 1. *Agar-gel electrophoresis of purified sheep liver aspartate aminotransferase isoenzymes*
Electrophoresis was carried out at pH 8.9 as described under 'Methods'. Samples (20 μl) were loaded in the centre well. Naphthalene Black was used as stain. (*a*) Mitochondrial, (*b*) cytoplasmic aspartate aminotransferase.

Sedimentation equilibrium

Protein samples were dissolved in 0.15M-NaCl/17.6mM-sodium/potassium phosphate buffer (pH 7.6, $I = 0.2$) at concentrations of 0.022–0.071% (w/v).

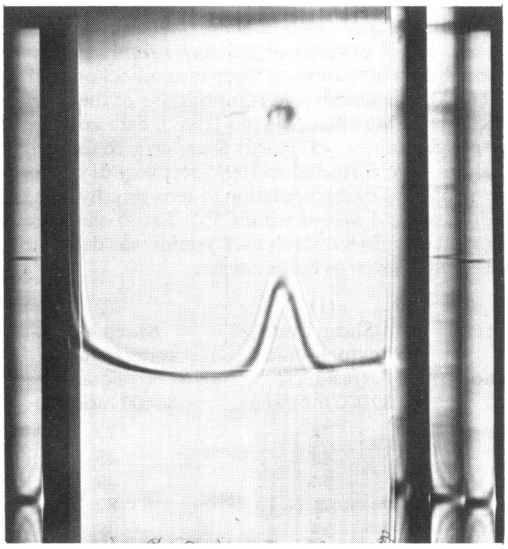

Fig. 2. *Sedimentation-velocity pattern for purified mitochondrial aspartate aminotransferase*
Enzyme concentration was 6mg/ml in 0.15M-NaCl/17.6mM-sodium/potassium phosphate buffer, pH 7.6; bar angle = 50°; rotor speed 56000rev./min. Photograph was taken 54min after reaching maximum speed. Sedimentation was from left to right.

They were centrifuged at 16000rev./min (18500*g*) for 25h and then the speed was increased to 18000rev./min (23500*g*) and maintained for 17h (at 22°C). Plots of the Rayleigh fringe displacement versus the square of the distance from the centre of rotation were linear for all runs; thus the preparations were homogeneous within the experimental error (Bowen, 1970). A mol.wt. of 87100 ± 680 (mean ± S.E.M., $n = 10$) was obtained.

Amino acid composition

The amino acid composition of sheep liver mitochondrial aspartate aminotransferase together with that of the cytoplasmic isoenzyme (Campos-Cavieres & Munn, 1973) and those of mitochondrial aspartate aminotransferases from other sources (given for comparison) are presented in Table 2.

Sheep liver mitochondrial aspartate aminotransferase contains 772 amino acid residues (tryptophan not included), for an apoenzyme mol.wt. of 86600.

Michaelis constants

The L-aspartate concentration was varied between 2.00 and 9.00mM for the cytoplasmic isoenzyme (10 μg) and between 0.233 and 1.5mM for the mitochondrial isoenzyme (8 μg). The 2-oxoglutarate con-

Table 2. *Amino acid composition of sheep liver mitochondrial aspartate aminotransferase and comparison with that of the cytoplasmic isoenzyme from sheep liver and mitochondrial isoenzymes from other sources*

The numbers of residues of sheep liver mitochondrial aspartate aminotransferase (column 2) are calculated for a mol. wt. of 86600. The amino acid compositions of the other preparations are from: (1) Campos-Cavieres & Munn, 1973; (3) Michuda & Martinez-Carrion (1969), data corrected for a mol.wt. of 92000 (Feliss & Martinez-Carrion, 1970); (4) Magee & Phillips (1971); (5) Scandurra & Cannella (1972). For the mitochondrial isoenzyme from sheep liver the analyses were corrected to 100% recovery of DL-norleucine, after which the values for threonine, serine and tyrosine were obtained by extrapolation to zero-time hydrolysis. Most other amino acid values are the average of 24, 48 and 72h hydrolyses and agreed within 5%. Extrapolation was not necessary for valine and isoleucine, since liberation was constant after 24h hydrolysis. Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967), and the result is given as half-cysteine.

Amino acid	(1) Sheep liver cytoplasmic (residues/ 89000 mol.wt.)	(2) Sheep liver mitochondrial (residues/ 86600 mol.wt.)	(3) Pig heart mitochondrial (residues/ 92000 mol.wt.)	(4) Rat brain mitochondrial (residues/ 80000 mol.wt.)	(5) Ox kidney mitochondrial (residues/ 93000 mol.wt.)
Asx	78	77	78	65	72
Thr	44	45	32	34	38
Ser	54	49	46	45	47
Glx	71	82	84	74	81
Pro	54	38	38	33	43
Gly	63	66	70	66	58
Ala	58	66	70	62	69
Cys	11	9	10	11	14
Val	49	50	54	52	49
Met	20	18	22	18	19
Ile	35	44	48	34	47
Leu	72	55	60	58	54
Tyr	24	30	24	22	27
Phe	38	32	40	31	35
Lys	40	54	58	50	56
His	22	19	22	17	18
Arg	37	38	40	35	38

centration was varied between 0.05 and 0.2mM for the cytoplasmic isoenzyme and between 0.185 and 2mM for the mitochondrial isoenzyme. The values of K_m (L-aspartate) were 2.96 ± 0.20mM and 0.40 ± 0.12mM for the cytoplasmic and mitochondrial isoenzymes respectively. The corresponding values for K_m (2-oxoglutarate) were 0.093 ± 0.010mM and 0.98 ± 0.14mM. The values in each case are the means $\pm$S.D. from two or three separate experiments and were obtained by replotting the apparent maximum velocities by the procedure of Velick & Vavra (1962) (Fig. 3).

Substrate inhibition

As previously noted for pig heart cytoplasmic aspartate aminotransferase (Jenkins *et al.*, 1959; Turano *et al.*, 1960), the cytoplasmic isoenzyme was inhibited at concentrations of 2-oxoglutarate greater than 0.25mM in the presence of concentrations of L-aspartate less than 2mM. This inhibition was not demonstrable for the mitochondrial isoenzyme with 2-oxoglutarate at concentrations up to 2mM and at L-aspartate concentrations as low as 0.25mM. Neither isoenzyme was inhibited by high concentrations of L-aspartate at any of the concentrations of 2-oxoglutarate used.

Apparent pH optima

The activities of the two isoenzymes were compared over the pH range 5–8.8 in the presence of sodium acetate (pH5.0 and 5.5), sodium/potassium phosphate (pH5.9 and 6.2), sodium cacodylate (pH5.9–7.4), triethanolamine/HCl (pH7.0–7.8) and Tris/HCl (pH7.8–8.8). There was no significant difference between the two enzymes, both having broad maxima of activity between pH6.5 and 8.5. As Cheng *et al.* (1971) have pointed out, this kind of apparent dependence of activity on pH may result in part from changes in species of the buffer ions. The activities of the sheep liver isoenzymes were the same for the pairs Tris/HCl and triethanolamine/HCl and for triethanolamine/HCl and sodium cacodylate, but in phosphate the enzymic activity was lower than in cacodylate. The effect of phosphate on the activities of the isoenzymes at pH7.4 was examined in more detail.

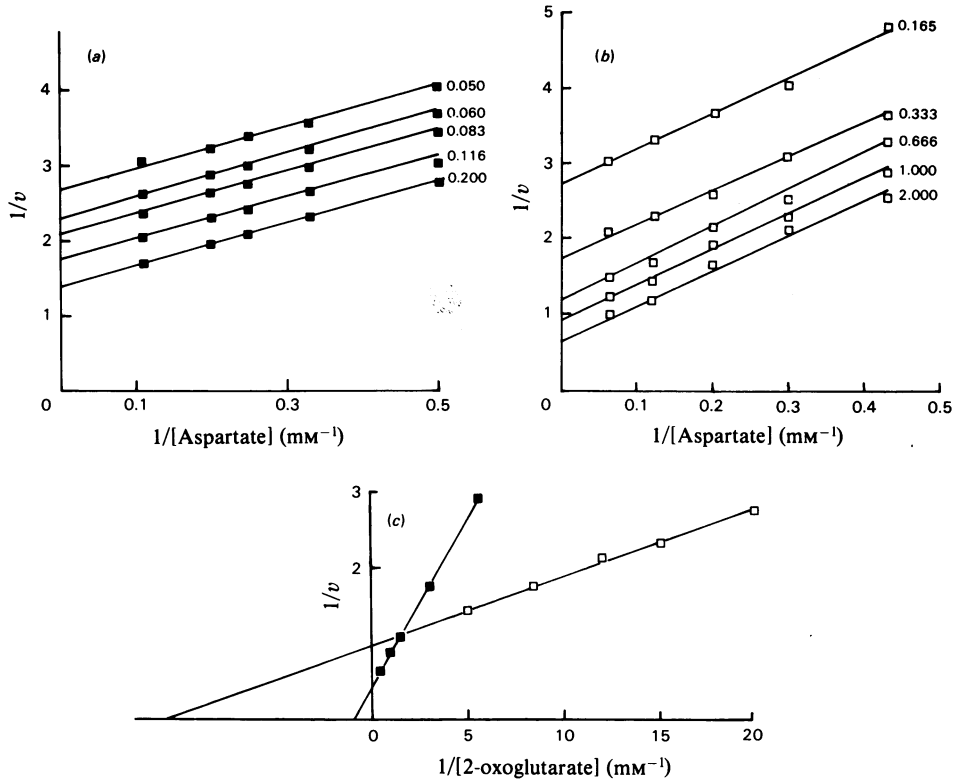

Fig. 3. *Transamination of* 2-*oxoglutarate and* L-*aspartate by* (*a*) *cytoplasmic and* (*b*) *mitochondrial aspartate aminotransferase* (*a*,*b*) Lineweaver–Burk plots of initial velocities (v = decrease in A_{340}/5 min) against aspartate concentrations at a series of fixed 2-oxoglutarate concentrations. (*c*) Secondary plots of the intercepts of $1/v$, (■) from (*a*) and (□) from (*b*), as a function of the co-substrate concentration.

Effect of potassium phosphate on holoenzyme activity

Samples of the cytoplasmic and mitochondrial isoenzymes were incubated with excess pyridoxal 5′-phosphate to ensure that they were in the holoenzyme form. They were then dialysed overnight against 2mM-triethanolamine/HCl buffer (pH7.4). A sufficient quantity of enzyme was placed into each assay mixture containing all the normal components at pH7.4 (see under 'Methods') with a range of phosphate concentrations from 9 to 150mM. As shown in Fig. 4, increasing concentrations of potassium phosphate are inhibitory toward both holo-isoenzymes. At 100mM-phosphate, cytoplasmic aspartate aminotransferase activity is inhibited by approx. 60%, whereas the mitochondrial isoenzyme activity is inhibited by approx. 40%, compared with their activities in the absence of phosphate.

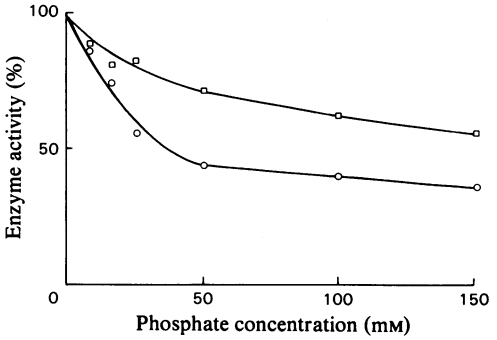

Fig. 4. *Effect of potassium phosphate on the activity of the cytoplasmic* (○) *and mitochondrial* (□) *holo-isoenzymes of sheep liver aspartate aminotransferase*
Samples of the enzyme preparations were incubated under the standard conditions in the presence of phosphate at a range of concentrations. The final concentrations of the substrates were 9mM-L-aspartate and 0.2mM-2-oxoglutarate for the cytoplasmic enzyme and 1.5mM-L-aspartate and 2mM-2-oxoglutarate for the mitochondrial form.

Resolution of the enzyme

The enzyme may be resolved from its bound pyridoxal 5′-phosphate by the method of Scardi *et al.*

(1963). The cytoplasmic and mitochondrial isoenzymes of aspartate aminotransferase from sheep liver were resolved more than 95% and more than 90% respectively from their bound coenzyme by dialysis for 2h at room temperature against an excess of 0.1M-L-glutamic acid in 0.5M-potassium phosphate buffer, pH6. After further extensive dialysis against 0.5M-potassium phosphate buffer and then distilled water, the resolved isoenzymes each had less than 5% of the activity of the corresponding original holo-

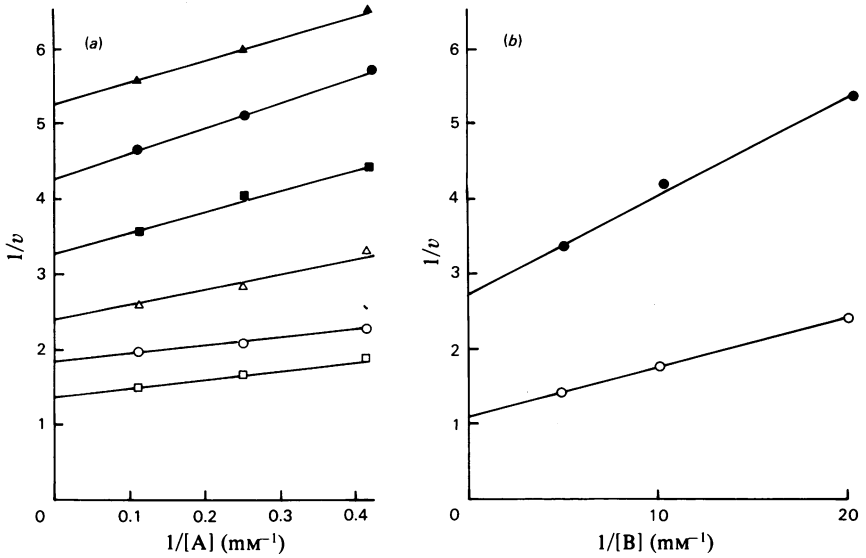

Fig. 5. *Effect of different concentrations of 2-oxoglutarate and pyridoxal 5'-phosphate on the activity of cytoplasmic aspartate aminotransferase as a function of aspartate concentration*

(*a*) Double-reciprocal plots of the initial velocity (decrease in A_{340}/5min) as a function of aspartate (A) concentration at three different concentrations of 2-oxoglutarate (B) at two concentrations of pyridoxal 5'-phosphate for cytoplasmic aspartate aminotransferase (10μg): △, ▲, 0.05mM, ○, ●, 0.1mM, and □, ■, 0.02mM-2-oxoglutarate respectively; ▲, ●, ■, 0.166μM and △, ○, □, 0.83μM-pyridoxal 5'-phosphate respectively. (*b*) Secondary plots of the intercepts of 1/*v* versus 1/[B]. The upper line (●) refers to a coenzyme concentration of 0.166μM and the lower line (○) to 0.83μM.

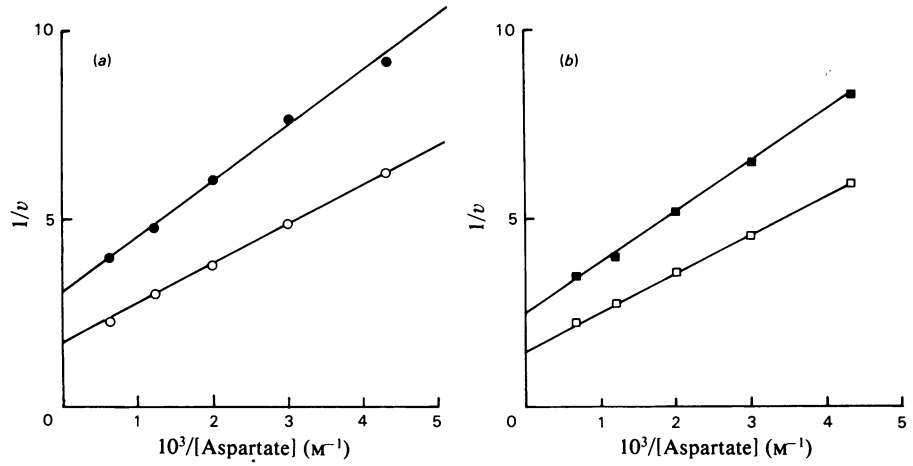

Fig. 6. *Double-reciprocal plots of the initial velocity (decrease in A_{340}/5min) as function of aspartate concentration at 2mM-2-oxoglutarate for mitochondrial aspartate aminotransferase* (8μg)

(*a*) At two concentrations of pyridoxal 5'-phosphate: ●, 0.066μM; ○, 0.2μM; (*b*) at two concentrations of pyridoxamine 5'-phosphate: ■, 0.066mM; □, 0.2mM.

enzyme. In the presence of excess pyridoxal 5'-phosphate both isoenzymes recovered more than 90% of their original activity.

Steady-state kinetics

Double-reciprocal plots, i.e. $1/v$ versus 1/substrate concentration, with a saturating concentration of coenzyme, at different concentrations of the second substrate, give a series of parallel straight lines (Fig. 3). This is compatible with the formation of two binary substrate–holoenzyme complexes [Ping Pong mechanism (Cleland, 1963) similar to that described in, e.g., pig heart (Jenkins *et al.*, 1959; Turano *et al.*, 1960) and wheat germ (Orlacchio *et al.*, 1975) aspartate aminotransferase]. When the coenzyme concentration was not saturating, the slopes of the lines obtained were dependent on coenzyme concentration (Figs. 5 and 6). At saturating concentrations of 2-oxoglutarate a plot of $1/v$ versus 1/[aspartate] should give straight lines which do not converge at the intercept on the $1/v$ axis for different concentrations of coenzyme. With the sheep liver cytoplasmic enzyme this condition cannot be easily verified because high concentrations of 2-oxoglutarate are inhibitory. Therefore the data shown in Fig. 5(*a*) obtained with three concentrations of 2-oxoglutarate up to a maximum of 0.2mM were replotted (Fig. 5*b*). The two lines obtained for different concentrations of coenzyme do not converge on the ordinate, which shows that the intercepts of these lines are dependent on coenzyme concentration, as for aspartate aminotransferase from wheat germ (Orlacchio *et al.*, 1975).

The apparent dissociation constants K_{ic} and K_{id} for the coenzymes were determined as described under 'Methods'. K_{ic} for the cytoplasmic isoenzyme (data in Fig. 5) was 0.23μM; K_{id} was calculated to be 70μM. K_{ic} and K_{id} for the mitochondrial isoenzyme (data in Fig. 6) were 0.062μM and 40μM respectively.

By assuming that K_{ic} and K_{id} are equilibrium constants, and by means of the relationship $\Delta G^0 = RT \ln k$, it was possible to calculate that the ΔG^0 of binding of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate respectively was −39.3 and −24.7kJ/mol at 37°C for the cytoplasmic isoenzyme and −42.6 and −26.1kJ/mol at 37°C for the mitochondrial isoenzyme. Therefore the Δ (ΔG^0) of binding between the two forms of coenzyme is −14.6kJ/mol for cytoplasmic aspartate aminotransferase and −16.5kJ/mol for the mitochondrial form.

K_{ic} and K_{id} are true equilibrium constants only if the rate constants for coenzyme binding and dissociation from the protein are large compared with those for other steps of the enzymic reaction (Litwack & Cleland, 1968). The following checks have been made. (*a*) The reactions between apoenzyme and pyridoxal 5'-phosphate (and/or the Schiff base between aspartate and pyridoxal 5'-phosphate) appears to be fast enough to allow steady-state-kinetics measurements to be made, as shown by the fact that when the transamination reaction was started by adding pyridoxal 5'-phosphate to the reaction mixture and measuring the transamination rate every minute for 10min, a constant rate was observed with no lag phase. (*b*) Preincubation of apoenzyme with pyridoxal 5'-phosphate for different times from 1 to 10min gave identical reaction rates, showing that an equilibrium is established between the apoenzyme plus the coenzyme and the holoenzyme. However, the identity of K_{ic} and K_{id} with the respective equilibrium constants is not proved.

Inhibition of the apo-isoenzymes by phosphate ions

Phosphate behaved as a competitive inhibitor of the coenzyme for both cytoplasmic and mitochondrial

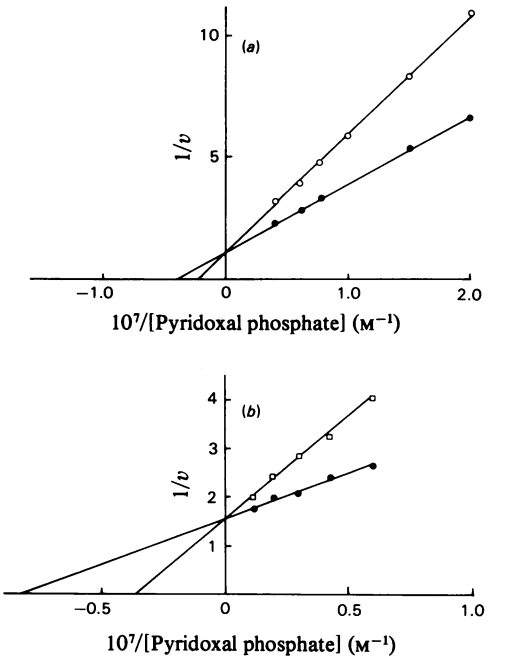

Fig. 7. *Inhibition of* (*a*) *mitochondrial and* (*b*) *cytoplasmic isoenzymes of apo-(aspartate aminotransferase) by phosphate ions*

The enzyme preparations (15μg of cytoplasmic isoenzyme and 10μg of mitochondrial isoenzyme) were incubated for 5min at 37°C in 0.05M-triethanolamine/HCl, pH7.4, in the absence (●) or in the presence of phosphate (○, final concn. 16.6μM; □, final concn. 33.3μM), pyridoxal 5'-phosphate in the concentration ranges indicated and 9mM-L-aspartate for cytoplasmic apo-(aspartate aminotransferase) or 1.5mM-L-aspartate for mitochondrial apo-(aspartate aminotransferase). The reaction was started by the addition of 2-oxoglutarate, 0.2mM for the cytoplasmic isoenzyme and 2mM for the mitochondrial form. The initial velocity is expressed as decrease in A_{340}/5min.

isoenzymes. The K_i calculated from the ratio of the slopes of the two lines obtained by plotting $1/v$ against $1/C_t$ in the absence and presence of phosphate at 37°C (Fig. 7) were 32μM for cytoplasmic aspartate aminotransferase and 19.5μM for the mitochondrial isoenzyme.

From these values of K_i, the ΔG^0 of binding of phosphate to the apoenzyme was calculated to be −26.5kJ/mol for the cytoplasmic enzyme and −28.2kJ/mol for the mitochondrial form.

Discussion

The method developed here for the purification of the sheep liver mitochondrial aspartate aminotransferase allows the preparation of this enzyme to a high degree of purity. The specific activity of sheep liver mitochondrial aspartate aminotransferase (120–180units/mg) approaches that of the cytoplasmic isoenzyme (217units/mg; Campos-Cavieres & Munn, 1973) and is high compared with other purified preparations of mitochondrial aspartate aminotransferase from liver (Wada & Morino, 1964; Boyd, 1966; Shrawder & Martinez-Carrion, 1973).

Both sheep liver aspartate aminotransferase isoenzymes contain a similar number of amino acid residues, but there are some differences in their amino acid compositions (Table 2). The high lysine content, which is also characteristic of mitochondrial aspartate aminotransferase from other sources (see Table 2), might account for its behaviour on electrophoresis and isoelectric focusing.

The sheep liver isoenzymes have similar coenzyme-binding peptides (Campos-Cavieres & Milstein, 1975), so it seems likely that they have similar sequences in all those portions of their chains that constitute the active site. This contention holds for pig heart aspartate aminotransferase isoenzymes (Doonan *et al.*, 1974). Despite this similarity some difference must exist between the micro-environment of the active site of cytoplasmic and mitochondrial aspartate aminotransferase from sheep liver to account for the differences in their kinetic properties.

The isoenzymes differ in their substrate affinities and in the dissociation constants of the coenzymes. The holoenzymes are also inhibited by P_i to different extents. This latter effect is probably due to competition at one of the substrate sites (Cheng *et al.*, 1971), but we cannot at the present time exclude the possibility that there is competition with coenzyme binding. Phosphate at concentrations of 18.6μM and 33.3μM (Fig. 7) behaves as a competitive inhibitor of the binding of the coenzymes to the apo-isoenzymes, and even in the experiments with holoenzyme (Fig. 4) some apoenzyme may be generated by displacement of coenzyme during the incubation.

Pyridoxamine 5′-phosphate is more readily dissociated from the protein and also recombines more slowly than pyridoxal 5′-phosphate (Meister *et al.*, 1954; Torchinsky, 1963). The greater stability of the pyridoxal phosphate–holoenzyme compared with that of the pyridoxamine phosphate–holoenzyme can be explained by the Schiff-base formation between pyridoxal phosphate and the ε-amino group of a lysine at the active site (Torchinsky, 1963). The overall lower affinity for the coenzyme by the sheep liver isoenzymes as compared with other preparations from mammals has made it possible to determine the dissociation constants. In this way, the energetic contribution of the aldimine bond between the aldehyde group of pyridoxal 5′-phosphate and an ε-amino group of a lysine residue at the active site was calculated for both isoenzymes. These values, −14.6kJ/mol for the cytoplasmic isoenzyme and −16.5kJ/mol for the mitochondrial protein, are close to those reported for wheat-germ aspartate aminotransferase (−9.6kJ/mol; Orlacchio *et al.*, 1975) and for rat tyrosine aminotransferase (−11.7kJ/mol; Borri Voltattorni *et al.*, 1975).

The ratios of the K_m values for the cytoplasmic and mitochondrial isoenzymes for each substrate are similar to those reported for the enzyme from other sources (Braunstein, 1973), although the absolute values differ according to assay conditions (ionic strength, anion and cation effects; Boyde, 1968).

At 2mM-L-aspartate, the cytoplasmic isoenzyme underwent substrate inhibition by concentrations of 2-oxoglutarate higher than 0.25mM. This is likely to be due to the lower K_m for 2-oxoglutarate of the cytoplasmic isoenzyme, since the mitochondrial isoenzyme did not show this inhibition. However, neither isoenzyme was inhibited at higher concentrations of L-aspartate.

We consider that the differences in the kinetic parameters between the two isoenzymes of aspartate aminotransferase are related to differences at their active sites, such as in the coenzyme-binding peptide (Campos-Cavieres & Milstein, 1975) and in the associated micro-environment, and that the latter is determined to some extent by the surface properties of the proteins. The differences in surface properties (revealed by their behaviour on electrophoresis, isoelectric focusing and antigenicity) may be the features that determine the final distribution of the isoenzymes in the cell, but this implies the existence of an appropriate recognition mechanism for the mitochondrial form in one or other of the mitochondrial membranes.

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