The Nature of the Multiple Forms of Cytoplasmic Aspartate Aminotransferase from Pig and Sheep Heart

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Starch-gel electrophoresis of sheep heart aspartate aminotransferase was carried out over the range pH 7.0–8.5. The enzyme separates into three subforms in the same way as the pig heart enzyme. As the pH was increased the distance migrated by each subform increased by the same amount, so that they remained the same distance apart. Titration of the enzyme over the appropriate pH range was used to calculate the difference in charge between the subforms and it was concluded that they differ by one charged group per dimer from their nearest neighbour on the electrophoretogram over the whole pH range studied. It was also shown that the pig-heart α and β subforms differ by almost one charged group per dimer in the range pH 5.5–5.7 and that the spacing between the subforms on starch-gel electrophoresis at pH 8.0 is the same as that for the sheep enzyme. Since the charge difference between the subforms is maintained over such a wide range of pH, it is concluded that they probably differ from each other in covalent structure, because of the improbability that conformational differences can give rise to such behaviour. The relationship between the subforms and inactive binding of the coenzyme is also examined.

Cytoplasmic aspartate aminotransferase (aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) from pig heart muscle has been shown to exist as a number of electrophoretically distinct forms, designated α , β , γ etc. in order of their increasing mobility towards the anode (Dekker & Rau, 1963; Martinez-Carrion *et al.*, 1967). Martinez-Carrion *et al.* (1967) also found that the subforms with greatest electrophoretic mobility contained larger amounts of an inactively bound form of the coenzyme pyridoxal 5'-phosphate and that this form absorbed light maximally at 340 nm rather than at 360 nm or 430 nm where the actively bound coenzyme absorbs. This form of the coenzyme could not be separated from the enzyme by the mild resolution methods normally used. However, the electrophoretic differences between the subforms remained even after resolution of the actively bound coenzyme. Staining with a specific enzyme stain (Dekker & Rau, 1963) showed that all three of the major subform bands had some enzymic activity. It is well established that the pig heart enzyme is dimeric, consisting of two very similar chains of mol.wt. 46000 (Fasella & Turano, 1970; Ovchinnikov *et al.*, 1973).

The occurrence of subforms of very similar electrophoretic behaviour has been demonstrated for the aspartate aminotransferases of a large number of species including man, rat, dog (Dekker & Rau, 1963) and chicken (Bertland & Kaplan, 1968). This wide distribution, together with the observation that the subforms appear to exist in the living animal and

are not simply artifacts of the preparation (Martinez-Carrion *et al.*, 1967), may be taken to suggest that they have some physiological significance. The subforms have been investigated by a number of workers, but no positive conclusion has been reached as to the nature of the differences between them.

On the basis of extensive physicochemical and chemical investigation Martinez-Carrion *et al.* (1967) concluded that, if differences in primary structure existed between one subform and another, they must be very small. They also found, however, that there were no differences in the conformation of the subforms detectable by optical rotatory dispersion. The nature of the subforms has also been investigated by Banks *et al.* (1968). These workers showed that β and γ forms could be generated from the α form and that prolonged treatment with urea converted all the subforms into the same, fast-moving, electrophoretic form. They suggested that the differences in net charge which the subforms possess at pH 8 are due to differences in the pK of a histidine residue caused by environmental differences. This hypothesis requires that the subforms differ in conformation and not in primary structure.

The present paper shows that sheep heart soluble aspartate aminotransferase also contains subforms which appear to be very similar electrophoretically to those of the pig enzyme. The nature of these subforms is investigated with the object of determining whether the subforms differ in conformation or in primary structure. In the light of some of the findings

the pig heart enzyme subforms are reinvestigated. Throughout the present work the subforms are referred to as α , β and γ solely on the basis of electrophoretic mobility. This designation does not imply any particular spectral or coenzyme-binding property for any of the subforms.

Experimental

Materials

Malate dehydrogenase (EC 1.1.1.37) and NADH were bought from Boehringer, London W.5, U.K. Hydrolysed starch was from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada, and CM-52 cellulose from Whatman Biochemicals, Maidstone, Kent, U.K. L-Serine *O*-sulphate was prepared by the method of Tudball (1962). L-Cysteinesulphinic acid was bought from Sigma (London) Chemical Co., London S.W.6, U.K. Other chemicals were bought from British Drug Houses, Poole, Dorset, U.K.

Preparation of aspartate aminotransferase

The pig heart enzyme was prepared as described by Martinez-Carrion *et al.* (1967), method B.

The preparation of sheep heart enzyme was very similar in outline but differed in detail. The hearts (10 kg) were roughly trimmed to remove aorta, auricles and fat, and the ventricle muscle was minced and then homogenized in a solution containing 0.15 M-sodium succinate, 20 mM-2-oxoglutarate, 1 mM-EDTA and 0.1 μ M-pyridoxal phosphate to give a 50% (v/v) homogenate. This was heated to 76°C for 15 min and the supernatant was separated from insoluble material by spinning the contents through a cotton pillow-case inside the spin-dryer of a domestic twin-tub washing machine. The clear supernatant emerging from the spin-dryer 'waste pipe' was cooled to 4°C and 300 g of the $(\text{NH}_4)_2\text{SO}_4$ dissolved in it per litre. After centrifugation at 2500 g for 30 min (r_{av} , 11.7 cm), a further 100 g of $(\text{NH}_4)_2\text{SO}_4$ was dissolved per litre of supernatant. After standing overnight at 4°C the suspension was centrifuged at 4500 g for 1 h (r_{av} , 7.8 cm) and the resulting precipitate taken up in 0.02 M-sodium acetate adjusted to pH 5.4 by adding acetic acid. The resulting suspension (about 100 ml) was dialysed for 24 h against 3 $\times$ 10 litres of cold water and then against 2 litres of the above-mentioned sodium acetate buffer. After centrifugation to remove undissolved material, this solution was applied to a column (10 cm $\times$ 5 cm) of CM-52 cellulose equilibrated with the same sodium acetate buffer, pH 5.4. Elution with this buffer was continued until no more protein emerged, and then 0.06 M-sodium acetate buffer adjusted to pH 5.4 with acetic acid was applied. The yellow solution eluted

by this buffer contained the enzyme, which was precipitated by adding 450 g of $(\text{NH}_4)_2\text{SO}_4$ /litre. The precipitate was taken up in the buffer used to prepare the original homogenate and dialysed against 2 litres of water for 1 h and then for 1 h against 2 litres of 0.02 M-sodium acetate buffer adjusted to pH 5.4 with acetic acid. This solution was chromatographed on CM-cellulose as described previously. After this short dialysis the enzyme is eluted immediately by 0.02 M buffer, leaving impurities behind. The precise reason for differences in chromatographic behaviour dependent on length of dialysis is not known, but the second column fractionation is sufficient to give a preparation which is homogeneous on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate and on ultracentrifugation. Although three bands are visible on starch-gel electrophoresis, all three of these have aminotransferase activity, as shown by a specific enzyme stain (Dekker & Rau, 1963).

Separation of subforms on CM-cellulose

The enzyme (500 mg) was fractionated on a column (45 cm $\times$ 2.5 cm) of CM-52 cellulose with a gradient of increasing pH produced by mixing the contents of two identical bottles connected in series. Each bottle contained 0.02 M-sodium acetate, the nearest to the column had been adjusted to pH 5.4 with acetic acid and the other to pH 5.8. Although separation of the peaks was not good, appropriate pooling of the fractions gave samples which ran homogeneously as α and β respectively on starch-gel electrophoresis. The only fraction containing γ subform also contained β subform in approximately equal amounts. The α fraction had a specific activity of 208 units/mg, β 90 units/mg and the mixture of β and γ rather surprisingly had 140 units/mg.

Resolution of coenzyme and enzyme assay

This was done by the method of Furbish *et al.* (1969). After resolution, the residual activities were always below 0.5% of the holoenzyme and recovery of activity was complete within 20 s of mixing. Activity was measured by the method of Karmen (1955) under conditions described previously (John & Fasella, 1969).

Starch-gel electrophoresis

This was carried out by the method of Barrett *et al.* (1962). The pH of the gel and electrode buffers was adjusted to the values required by adjusting the Tris and LiOH concentrations respectively while keeping the citrate and borate concentrations constant. The voltage was kept constant at 30 V/cm and electrophoresis was continued for 6 h.

Titration of enzyme

The enzyme sample was dialysed extensively against water (4×2 litres for a total of 36h). A known volume (10ml) containing 15–20mg of protein was adjusted to pH9 by adding NaOH and then titrated by adding 5mM-HCl, 10 μ l at a time, with a Lang-Levy micro-pipette. The pH was measured with a Pye model 920 pH-meter, the expanded scale of which gives a full-scale deflexion for a change in pH of 1.4 units. The pH-meter was standardized at pH7 for the whole of the titration and checked at pH9.2 and 4.01 with other standard buffers when the readings obtained were 9.1 and 4.15 respectively. A sample of the water used for the initial dialysis was also titrated over this range and the readings were subtracted from the enzyme titrations. The concentration of the pig heart enzyme monomer was determined by using $\epsilon_{280} = 7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Birchmeier *et al.*, 1973). The same extinction coefficient was also used for the sheep heart enzyme, since this was the value calculated by measuring the extinction at 388nm of an enzyme sample treated with 0.1M-NaOH. This treatment liberates all the pyridoxal phosphate for which $\epsilon_{388} = 6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Peterson & Sober, 1954).

Reaction with serine sulphate

The enzyme (6mg in 3ml of 0.02M-acetic acid adjusted to pH7.5 with solid Tris) was treated with serine sulphate at a final concentration of 0.04M. The pH was kept at 7.5 for 1h by adding solid Tris and then the sample was dialysed for 2h each against three successive 2-litre volumes of water. The residual activity was less than 0.1% of the original. Incubation with 0.01M-serine sulphate yielded 50%-inactive enzyme.

Results and Discussion

Variation of electrophoretic mobility with pH

Banks *et al.* (1968) compared the electrophoretic migration of the mixture of pig heart aspartate aminotransferase subforms at pH8.6, 8.0 and 7.5 and altered conditions so that at each pH the least rapidly migrating subform had moved the same distance from the origin. They observed that at pH7.5 the subforms were better separated than at pH8.0, and at pH8.6 they did not separate at all. From this they concluded that the subforms have different net negative charges at pH7.5 but that they become substantially the same as the pH is raised. The sheep heart enzyme also separates into subforms on starch-gel electrophoresis and we have carried out a similar experiment with the enzyme from this species. However, instead of choosing conditions so that the slowest-running form always ran the

same distance, the times and voltages for each separation were kept constant and only the pH was varied. The resulting stained gels are shown in Plate 1. Although the absolute distance migrated increases with increasing pH, the distance between the subforms is the same at each pH. The simplest explanation for this appears to be that, although the absolute net charge of each subform increases by the same amount with increasing pH, the difference in net charge between the subforms is constant over this pH range.

Heterogeneity and the binding of coenzyme

One possible explanation for the existence of subforms is that they result directly from differences in binding of the coenzyme in that both monomers of subform α have the coenzyme bound in the active 360nm-absorbing mode, both monomers of subform γ have the coenzyme bound in the inactive 340nm-absorbing mode and that subform β is a hybrid of these two extremes. This explanation cannot be true for the sheep heart subforms, since fractionation of this enzyme on CM-cellulose gives a fraction containing approximately equal amounts of subforms β and γ with no subform α , and yet has 75% of the specific activity of the α subform. Further, it shows no significant absorption peak at 340nm before resolution and can be separated from 90% of its coenzyme, leaving only about 10% of the inactive 340nm-absorbing form.

The γ form of the pig heart enzyme also has some activity, as shown by staining the starch-gel-electrophoresis blocks with a specific enzyme stain (Dekker & Rau, 1963; Martinez-Carrion *et al.*, 1967), thus proving that in this subform the coenzyme is not all bound in the inactive mode.

The bands also become less distinct at higher pH values, suggesting that some hybridization may be occurring while electrophoresis is in progress.

Measurement of charge differences between subforms by titration

The determination of how the subforms differ from each other will be greatly assisted by measuring the actual difference in net charge between them. Plate 1 shows that each of the subforms moves 12mm further at pH8 than at pH7. In order to determine the increase in net charge responsible for the increase in distance migrated, a known amount of the enzyme was titrated with standard acid. The number of equivalents of acid required to adjust the solution from pH8 to 7 is a direct measure of the net change in charge, since it represents the number of H⁺ ions bound per dimer. A titration of the sheep heart enzyme, shown in Fig. 1, was used to calculate this value which was 3.1 H⁺ ions bound per dimer. Thus

Fig. 1. Titration of sheep heart enzyme

The sheep heart enzyme (10 ml, containing $0.18 \mu\text{mol}$ of dimer) was titrated with 5 mM-HCl.

each subform migrates 12 mm further at pH 8 than at pH 7 because it has increased its net negative charge by three per dimer. A change in net negative charge of one per dimer would therefore represent a distance of 4 mm. (This value is also given by calculations from the gels run at other pH values.) Since the distance between each of the subforms is 4 mm, subform β must have one more negative charge per dimer than subform α , and subform γ two more negative charges per dimer than subform α .

Electrophoretic mobility of the covalently modified enzyme

The glutamate analogue, serine sulphate, reacts stoichiometrically with aspartate aminotransferase, inactivating it, and at the same time introducing a carboxyl group at the active site (John & Fasella, 1969; John *et al.*, 1973). This has been seen to produce increasing electrophoretic mobility of the pig heart enzyme subforms (A. Giartosio, personal communication). One would expect this group to be completely unprotonated in the region of pH 8. Plate 2 shows a starch-gel electrophoretogram of the α subform, α subform totally inactivated with serine sulphate and α subform which was 50% inactivated with serine sulphate. The positions of the respective bands are identical with those of native subforms α , β and γ , confirming that the native subforms each differ from their nearest neighbour by one negative charge per dimer.

Charge differences of pig heart enzyme subforms at acid pH

The subforms of the pig heart enzyme are clearly separable at pH 8 (Martinez-Carrion *et al.*, 1967; Banks *et al.*, 1968). The appearance of these starch-gel electrophoretograms at this pH is very much like that of the sheep heart enzyme in that several evenly spaced bands are visible with the slowest band staining most intensely. It is also known that the pig heart enzyme subforms separate by isoelectric focusing and that subforms α and β have isoelectric points of 5.69 ± 0.02 and 5.53 ± 0.02 respectively (Marino *et al.*, 1969). Thus subform β is more negatively charged than subform α even at this low pH, and again a titration of the enzyme over the appropriate pH range can be used to measure the difference in negative charge. If subform β has one negative charge per dimer more than subform α then it has to bind one equivalent of protons more than subform α in order to become isoelectric, and in order to do this on isoelectric focusing it migrates to a zone 0.16 pH unit lower and obtains the necessary protons from the buffer. In the titration the number of equivalents of acid required to bring about the same pH change is a direct measure of the number of extra H^+ ions that are bound. The titration of the pig heart enzyme is very similar to that of the sheep heart enzyme. Between pH 5 and 5.5 it is essentially linear and over this range 0.85 equivalent of acid is required to bring about a pH change of 0.16 units.

It can be concluded therefore that the unit negative charge difference between subforms α and β is almost unchanged from pH 5.6 to 8.0 in the pig heart enzyme and at least from pH 7.0 to 8.5 in the sheep heart enzyme.

Theoretical treatment

If the only difference between the subforms was that the environment of an ionizable group differed from one subform to another, thus altering its pK , then the differences would be greatest midway between the two pK values in question and would diminish at higher and lower pH values.

Just how far apart the pK values would have to be to give the effects observed may be determined from the equation:

$$W = \frac{1}{\frac{[\text{H}^+]}{K_1} + 1} - \frac{1}{\frac{[\text{H}^+]}{K_2} + 1}$$

in which W is the fractional difference in charge between the subforms and K_1 and K_2 are the dissociation constants for the group in question in its two different environments. This equation predicts that the charge difference between subforms α and β

EXPLANATION OF PLATE I

Electrophoresis of the subforms of sheep heart enzyme at different pH values

The starch-gel electrophoresis was carried out as described in the text. The pH of both gel and electrode buffers were as indicated in the photograph.

EXPLANATION OF PLATE 2

Starch-gel electrophoresis of the α subform modified with L-serine O-sulphate

The electrophoresis was carried out at pH 8.2. The samples are as follows: (1) native enzyme; (2) native α subform; (3) α subform modified with serine sulphate; (4) α subform modified to 50% of residual activity with serine sulphate.

approaches a maximum of 1 midway between the two pK values as the pK values become further apart. The value of W is directly proportional to the distance between the subforms.

From the equation it can be deduced that in order to give rise to the observed behaviour the shift in pK caused by the proposed conformational difference would have to be at least 5 pH units, i.e. from a pK of about 4.5 to at least 10. Such a change in the ionizing properties of a single group is so far outside the range normally accepted for environmental pK shifts as to be ruled out. It is, however, conceivable that the unit charge difference is made up by contributions from several groups in which all the pK values are altered by conformational change. For this to be the correct explanation it would be necessary for the sum of the contributions coincidentally to be equal to 1 over the pH range under discussion. It is much more probable that the reason for the maintenance of differences between the subforms over such a wide range of pH is that the responsible ionizable group is present in one subform but not in the other and that its pK is either above or below the pH range studied. If the value of 0.85 of a unit charge calculated for the difference between the pig heart enzyme subforms is sufficiently accurate then the pK of the group concerned must be somewhere near the pH at which such a fractional difference in the charge is observed. In order to have a residual charge of 0.85 at pH 5.3 and a whole unit charge at pH 8 a group must have a pK of 4.5. However, we consider that the results are not sufficiently precise to allow this claim to be made with great certainty.

It has been shown that the pig heart α subform is slowly converted into more negatively charged forms (Banks *et al.*, 1968). We have observed the same phenomenon with the sheep heart enzyme. This behaviour could be explained by the generation of a neutral amino acid from a basic one, but it is very difficult to envisage how this could occur. It is, however, possible that an acidic amino acid residue could be derived from a neutral one either by hydrolysis of glutamine or asparagine, or by oxidation of a cysteine residue. All of the available evidence points quite strongly to amide hydrolysis. First, Martinez-Carrion *et al.* (1967) measured 3.8 cysteine residues per monomer for their fraction IV, which is almost pure subform α and 4.2 cysteine residues per monomer for their fraction I, which consists of subforms β and γ only. The pure subform α contains five cysteine residues per monomer (Bossa *et al.*, 1973; Ovchinnikov *et al.*, 1973) and of these only four are available for titration by thiol reagents (Birchmeier *et al.*, 1973). If oxidation of cysteine were to account for the difference in the subforms Martinez-Carrion *et al.* (1967) would have been 25% in error, whereas an error of only 3% in their estimation of amide content is necessary to explain their tentative sugges-

tion that the subforms do not differ in this respect. Secondly, if cysteine residues were responsible for the differences in negative charge then these differences would be expected to diminish with increasing pH, since at high pH cysteine itself carries a negative charge. Thirdly, there is some evidence, discussed above, that the group in question has a pK near 4.5.

The generation of subforms by hydrolysis of amides is not a newly described phenomenon. A very well characterized example is given by rabbit muscle aldolase (Lai *et al.*, 1970; Midelfort & Mehler, 1972). This enzyme is a tetramer in which at one position near the C-terminus each of the chains may contain an asparagine residue or its hydrolysis product, so that five subforms are apparent on electrophoresis. The hydrolysis was found to occur *in vivo*, with a half-time of 8 days. The lens protein α -crystallin also undergoes an aging process resulting in subforms of increasing negative charge and this has been attributed to amide hydrolysis (Palmer & Papaconstantinou, 1969; Delcour & Papaconstantinou, 1972). Further, cytochrome *c* undergoes conversion into several multiple forms as a result of hydrolysis of more than one amide in its structure and the rates of hydrolysis differ (Flatmark, 1966).

It has been proposed (Robinson *et al.*, 1970) that the deamidation of proteins could serve as a means of incorporating 'planned obsolescence' into the molecules. If this is so then the question of whether the formation of inactively bound coenzyme is a consequence of amide hydrolysis takes on some physiological significance. The observations of Marino *et al.* (1972) that formation of the faster-running subforms and formation of inactively bound coenzyme are both accelerated by high concentrations of $(NH_4)_2SO_4$ at pH 9.2, conditions that are just those required for hydrolysis of amides (Flatmark, 1966), support the proposal that the two processes are in some way linked.

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