Generation of Aspartate Aminotransferase Multiple Forms by Deamidation

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The development of aspartate aminotransferase subforms *in vitro* was followed by densitometry after thin-film isoelectric focusing. At the same time ammonia production was measured. Each reaction can be expressed in terms of a first-order process in which 2 mol of glutamine or asparagine/mol of dimer are deamidated with a half time of 22 days. The more negatively charged subforms developed *in vitro* were almost fully active. Another process occurred leading to inactivation by coenzyme modification, and this was independent of deamidation. Although the enzyme formed absorbed maximally at 340 nm, it was different from the naturally occurring inactive enzyme that absorbs at this wavelength.

Both mitochondrial and cytoplasmic aspartate aminotransferase (EC 2.6.1.1) from many species exist as a number of electrophoretically distinct subforms (Dekker & Rau, 1963; Bertland & Kaplan, 1968; Michuda & Martinez-Carrion, 1969). These subforms, which are present in fresh tissue (Martinez-Carrion *et al.*, 1967), appear as a series of evenly spaced bands after starch-gel electrophoresis at about pH 8 and are referred to as α , β , γ etc. according to their increasing mobility towards the anode. In an extensive study of the subforms of the pig heart cytoplasmic enzyme Martinez-Carrion *et al.* (1967) separated the subforms by chromatography on CM-Sephadex at pH 5.4 and found that the more negatively charged subforms contained more of the coenzyme bound inactively in a mode which absorbs light maximally at 340 nm rather than at the higher wavelengths characteristic of actively bound pyridoxal phosphate. Although there was a relationship between the subforms separated by the two different methods, the electrophoretic subforms and chromatographic subforms were not exactly correlated. Those obtained by the CM-Sephadex chromatography were referred to as fractions I, II, III and IV by Martinez-Carrion *et al.* (1967). These workers determined the amino acid composition of the subforms and failed to detect any significant differences. However, they recognized that, particularly for those amino acids whose frequency in the molecule is high, a difference of one or two residues per enzyme molecule would not have been revealed. Bossa *et al.* (1968) showed that all of the enzyme is not accounted for in this way, since about 10% of it can only be eluted from the CM-Sephadex column by increasing the ionic strength beyond that used by Martinez-Carrion *et al.* (1967). This fraction itself consists of subforms which are electrophoretically the same as subforms α , β and γ . Bossa *et al.* (1968) showed that this fraction was rich

in carbohydrate, an observation which was confirmed by Denisova & Polyanovsky (1973), who also identified and quantified the sugars. Banks *et al.* (1968) showed that the β - and γ -forms could be generated from the α -form and suggested that the differences in electrophoretic mobility were caused by differences in conformation owing to movement of a histidine residue to a different environment. John & Jones (1974) demonstrated that the β -subform has one more negative charge per dimer than the α -, and that the γ -subform has two more negative charges than the α -. Because this unitary difference in negative charge between the subforms was maintained over a wide pH range John & Jones (1974) concluded that the mechanism generating it was more likely to be a covalent modification than a conformation change, and suggested deamidation of glutamine or asparagine as the most probable reaction.

The fact that the more negative subforms also contain more inactively bound coenzyme has prompted the suggestion that the β -subform contains one inactive monomer and the γ -subform two (Evans & Holbrook, 1974). This suggestion does not account for the fact that, although enzyme containing 340 nm absorbing coenzyme is inactive, both γ - and δ -subforms have significant activity (Martinez-Carrion *et al.*, 1967; Denisova & Polyanovsky, 1973).

Denisova & Polyanovsky (1973) reported that the subforms contained sialic acid and suggested that the amounts present provided the basis for the difference in charge between the subforms.

In the present paper we report results of experiments *in vitro* in which the course of subform generation and coenzyme modification was followed by using quantities of the pure α -subform sufficiently high that if deamidation was occurring the amount of ammonia formed would be measurable. In addition, we have carried out experiments on the relationship between

subform generation and enzyme inactivation caused by modification of coenzyme.

It is clear that the heterogeneity of aspartate aminotransferase is extensive and there are difficulties of nomenclature. Throughout this paper Greek letters will be used to indicate mobility at pH 8 on starch-gel electrophoresis and Roman numerals to indicate peaks eluted after CM-Sephadex chromatography.

Experimental

Materials

Aspartate aminotransferase was prepared by method (B) of Martinez-Carrion *et al.* (1967). Malate dehydrogenase (EC 1.1.1.37) and NADH were from Boehringer, London W.5, U.K. Filters and other apparatus for sterilizing solutions were from Millipore (U.K.), London N.W.10, U.K. Ion-exchange celluloses (microgranular grades CM-52 and DE-52) were supplied by Whatman Biochemicals, Maidstone, Kent, U.K., and CM-50 Sephadex was from Pharmacia (G.B.), London W.5, U.K. Ampholine was from LKB Instruments, South Croydon, Surrey, U.K. Pyridoxal 5'-phosphate and 2-oxoglutaric acid were supplied by Koch-Light Laboratories, Colnbrook, Bucks., U.K., and L-cysteinesulphinic acid was from Sigma (London) Chemical Co., London S.W.6, U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

CM-Sephadex chromatography

Fractionation of the subforms was carried out on CM-Sephadex using the method described by Martinez-Carrion *et al.* (1967). The concentrations of the eluting buffer solutions used were as described by Martinez-Carrion *et al.* (1967) and they were made by diluting a stock solution (sodium acetate adjusted to pH 5.40 by adding acetic acid, final $[\text{Na}^+] = 1.0\text{M}$). Dilution of this gave pH values very near to those described by Martinez-Carrion *et al.* (1967). This procedure gave fractions up to IV. When more tightly bound material was being investigated the elution procedure was extended with another linear gradient running from 0.11M-sodium acetate (500ml) to 0.55M-sodium acetate (500ml). Each of these solutions was made by diluting the stock sodium acetate solution (1.0M, pH 5.4) without adjustment of pH.

Enzyme incubation

After separation on CM-Sephadex the α -subform was dialysed into sodium phosphate buffer, pH 7.4 ($[\text{Na}^+] = 0.01\text{M}$). Any ammonia in the solution was removed by standing overnight in an evacuated desiccator containing concentrated H_3PO_4 . An equal

volume of the phosphate buffer was treated in the same way and used throughout the experiments as a control. This treatment was effective in decreasing dissolved NH_3 to insignificant amounts. The solutions were sterilized by filtration through Millipore filters (0.2 μm pore size) into sterile tubes, which were equipped with ground-glass Thunberg stoppers. The bulb of the stopper contained 3 ml of octan-2-ol to prevent bacterial contamination. The contents were maintained at 25°C and precautions were taken to prevent contamination during sampling. Samples of the solution were examined microscopically during the incubation and found not to be contaminated with micro-organisms.

Ammonia determinations

Ammonia was measured by the method of Conway (1962). Samples of the enzyme (volume between 0.4 ml and 1 ml, containing 10–20 mg of enzyme) were placed in the outer chamber of the diffusion vessel and 1 ml of 1M-HCl was added to the inner chamber. The enzyme sample was made alkaline by adding 1 ml of saturated K_2CO_3 and the vessel was covered and left for 2 h for ammonia to diffuse into the HCl. The remaining HCl was titrated by using a microburette containing $\text{Ba}(\text{OH})_2$ (0.1M approx.) standardized against HCl immediately before use.

Quantification of subform generation

The amount of each subform present in the sample was measured by separation by isoelectric focusing on thin films of polyacrylamide gel [5% (w/v) acrylamide, 0.05% (w/v) N,N' -methylenebisacrylamide, 2% (w/v) Ampholine covering pH range 4–7 and 0.06% (w/v) potassium persulphate]. The thin films were prepared by pouring the solution on to glass plates (8 cm $\times$ 18 cm) bound with four layers of masking tape to determine the gel thickness. A Perspex plate was carefully lowered to cover the solution and exclude all air. The gel was left to set for 2 h. The enzyme sample (approx. 12 μg) was applied on 5 mm squares of Whatman 3MM paper near the anode. Electrofocusing was performed, by using paper strips soaked in 40mM- H_2SO_4 and 65mM-ethanolamine at anode and cathode respectively, at 3 W for 6 h on a cooled plate covered to prevent evaporation. The focused subforms were stained and destained by the method of Vesterberg (1972). After destaining the gels were soaked for 2 h in 10% (v/v) glycerol, cut out and fixed on microscope slides by allowing to dry. The slides were scanned using a Joyce-Loebl Chromoscan microdensitometer.

The area under each peak was measured by photocopying the trace. The whole length of the scan was cut out and the weight of the paper was taken to represent the total protein. The paper was then cut at

points midway between the peaks and the separate peaks weighed. The extent of negative charge development was measured by allowing one mol of charge per dimer for protein in the β -position and two mol of charge per dimer for protein in the γ -position.

Other methods

Starch-gel electrophoresis was carried out by the method described by Banks *et al.* (1968). Enzyme activity was measured using the method devised by Karmen (1955), but by using 0.5 mM-2-oxoglutarate and 2.5 mM-aspartate in Tris/HCl, pH 7.5 ($[Cl^-] = 0.1$ M) and is expressed in units of $\mu\text{mol/min}$. Absorption spectra were determined by using a Beckman model 25 recording spectrophotometer. Concentration of aspartate aminotransferase was determined by using $\epsilon_{280} = 7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Birchmeier *et al.*, 1973).

Results

Subform generation and ammonia production

The subforms of this enzyme are very readily observed by using starch-gel electrophoresis at pH 8, but the bands are never sharp, and measuring the protein present in each position is difficult because the spaces between the bands are small. Nevertheless the method can be used to give a good qualitative assessment of the course of subform generation. The results of an experiment in which the pure α -subform was maintained at 25°C for 25 days and sampled at intervals are shown in Fig. 1. In similar experiments, improved resolution was achieved by isoelectric focusing. Densitometric traces of gels obtained are shown in Fig. 2. It is clear from these experiments that there are two types of subform developing. The most rapid change is from α to β , but there is a slower reaction that gives rise to minor bands which will be called α' , β' . Although the existence of some of the minor bands is not clear from the densitometer traces, the original gels showed them clearly. An example of a gel from a sample after 13 days is shown in Fig. 3.

Fig. 4 illustrates an experiment in which both subform generation and ammonia production were followed. The results are expressed as mol of NH_3 per mol of enzyme dimer and subform generation as mol of negative charge developed per mol of dimer. It should be remembered that the quantification of this latter value is a slight underestimate, because the value derived does not allow for negative charge arising in the development of the minor subforms. The evidence of Fig. 4 shows quite clearly that as NH_3 is produced negative charge is developed and that within the fairly large experimental errors these two processes coincide stoichiometrically. This provides strong support for the proposal that the subforms

Fig. 1. Development of subforms demonstrated by starch-gel electrophoresis

After elution from CM-Sephadex the α -subform was dialysed into phosphate buffer and NH_3 was removed as described in the text. This procedure was carried out at 4°C and took 2 days. Thereafter the enzyme was incubated at 25°C. The first sample, indicated as 0, was taken at the beginning of the 25°C incubation and further samples were taken at the times indicated.

formed *in vitro* from the native α -subform arise by deamidation of either asparagine or glutamine. The minor subforms can be explained by proposing the slower deamidation of another asparagine or glutamine residue giving a carboxy group in a different environment. We estimate, using the method described previously (John & Jones, 1974), that at the isoelectric pH of each of the new subforms the carboxy groups formed in the fast and slow reactions are respectively 85 and 20% ionized. The pK values corresponding to these ionizations are 4.5 and 5.1. The half-time of the faster process is 22 days and that of the slower about 200 days. Ionization of both types of carboxy group would be complete at higher pH, so that starch-gel electrophoresis at pH 8 would not distinguish between β - and α' -forms. This would explain why fractions II and III separated at 5.4 on CM-Sephadex, but both ran as β -form at pH 8.

Changes in enzyme activity and coenzyme absorption spectrum

Fig. 5 shows changes in absorption spectrum of the enzyme that occur during the experiment. There is a progressive decrease in the band ascribed to actively bound coenzyme and a simultaneous increase at lower wavelengths. These spectral changes are accompanied by loss of enzyme activity. The form giving the new absorption band is not the pyridoxamine form of the enzyme, since addition of 2-oxoglutarate does not affect it. The absorbance maximum appears at 330 nm rather than at 340 nm seen for the naturally occurring inactive form. The rate of the reaction giving these changes varied from one experiment to another despite apparently constant pH,

Fig. 2. Course of subform development demonstrated by thin-layer isoelectric focusing. Densitometer scans of isoelectrofocusing gels obtained as described in the text. (a) Freshly prepared α -subform; (b) preparation at beginning of 25°C incubation; (c), (d), (e) and (f) were from samples taken at 3, 13, 17 and 23 days respectively.

temperature and buffer composition. Attempts to find out if the uncontrolled parameter was dissolved oxygen failed because of bacterial contamination of samples left open to the air. We have not pursued this aspect further because evidence, presented later in this paper, shows that the modified coenzyme formed *in vitro* is different from that formed *in vivo*. However, the fact that the rate of deamidation is constant from one experiment to another, whereas coenzyme modification occurs at different rates suggests that the two processes are independent.

Fractionation of subforms generated *in vitro*

Fraction I (77 mg) was prepared and maintained at 25°C. At the start this fraction was found to be pure α -subform, but after 2 weeks the preparation consisted almost entirely of faster-running subforms. Re-fractionation on CM-Sephadex gave two peaks that were eluted early, approximately in the position of fraction I, and containing 7 and 14 mg of protein respectively. The first to be eluted was shown by starch-gel electrophoresis to be predominantly δ -

Fig. 3. Isoelectric focusing of α -subform after 13 days incubation

The gel shown is that used to give the densitometer trace of Fig. 2(c).

Fig. 4. Correlation of negative charge development and NH_3 production

Negative charge developed ($\circ$) and NH_3 produced ($\bullet$) were measured as described in the text. The continuous line is theoretical for a first-order process in which 2 mol/mol of dimer react with a half time of 22 days.

Fig. 5. Changes in absorption spectrum

Absorption spectra were determined of samples taken after incubation at 25°C for 4, 8, 18 and 27 days. The spectrum with the highest absorbance at 430 nm was that at the start of the incubation and the times of the remaining spectra are in order of decreasing absorbance at this wavelength. Spectra were determined in 0.2 M-succinate at pH 7 since at this pH no precipitation occurred and in the presence of this dianion the active enzyme absorbs at 430 nm rather than at 360 nm.

form and the second predominantly γ -form. Neither showed an absorption peak at 340 nm and they had specific activities of 135 and 155 units/mg respectively (in our assay the α -subform has a specific activity of 190 units/mg). The properties of these subforms prepared *in vitro* from pure α -subform are therefore very different from those of the γ - and δ -subforms separated in the same way by using freshly prepared

enzyme. Fraction I prepared by Martinez-Carrion *et al.* (1967) consisted of γ - and δ -subforms and had 68 % of its coenzyme absorbing at 340 nm and was only 20 % as active as the α -subform. The remainder of the enzyme was not eluted by the normal gradient, which runs to 0.11 M-sodium acetate, pH 5.4. However, two more peaks, together accounting for 20 mg of protein, were eluted with a gradient running from 0.11 to 0.55 M-sodium acetate, pH 5.4. This material therefore behaves as fraction V. Examination of the absorption spectrum showed that the first peak contained only about half of the coenzyme of the α -subform and the later fraction contained almost no coenzyme. The specific activities of these two fractions were 20 and 6 units/mg respectively. Starch-gel electrophoresis showed that these two were predominantly β - and γ -forms.

We propose therefore that *in vitro*, under the conditions that we have used, the α -subform undergoes two types of reaction which are independent of each other. One of these types of reaction involves de-

amidation of amides, either asparagine or glutamine, to give carboxy groups the negative charges of which are fully expressed at pH 8, but which are distinguishable by techniques such as isoelectric focusing, which operate nearer the pK of these groups. This deamidation has only a small effect on the activity of the enzyme.

The other reaction is modification of the coenzyme to a derivative which no longer binds tightly to the apoenzyme. It leads to loss of activity and increase in absorbance at 330 nm. A reaction that would fulfil these requirements would be oxidation to 5'-phosphopyridoxic acid. If this proposal is right, one would expect apoaminotransferase to bind tightly to CM-Sephadex.

Chromatography of apoaminotransferase on GM-Sephadex

A sample of freshly prepared α -subform was separated from its coenzyme and applied to the column of CM-Sephadex used in the routine preparation of subforms. The column was eluted first with the gradient to 0.11 M-sodium acetate, pH 5.4, and then with the extension to 0.55 M-sodium acetate, pH 5.4. All of the apoenzyme remained firmly bound to the column throughout. The apoaminotransferase clearly has a high affinity for CM-Sephadex, but this affinity is even higher than that for the subforms eluted as fraction V. It seems probable that the extra negative charge developed by deamidation was sufficient to compensate for the high affinity conferred by loss of coenzyme. Fraction V prepared during the routine fraction of freshly prepared enzyme into subforms has been shown to be rich in carbohydrate (Bossa *et al.*, 1968; Denisova & Polyanovsky, 1973). However, we thought it worthwhile to examine this fraction for the possibility that at least some of it bound tightly to CM-Sephadex, because it was apoenzyme and not because it contained carbohydrate.

The freshly prepared enzyme (300 mg) was applied to the CM-Sephadex column and eluted by using the usual method up to 0.11 M-sodium acetate, pH 5.4. After elution of fractions I-IV (about 180 mg, of which 77 mg was in fraction IV itself) the column was treated with 20 ml of 0.1 M-pyridoxal phosphate dissolved in 0.11 M-sodium acetate, pH 5.4, and then elution with 0.11 M-sodium acetate, pH 5.4, was continued. A total of 30 mg of protein was eluted in this way in a broad peak of varying specific activity. The lowest-specific-activity protein (46 units/mg) was found in the earliest fraction and the highest (176 units/mg) was eluted later. However, there was still some enzyme bound to the column, which was detectable because it was yellow. In other preparations fraction V accounted for about 20% of the protein applied to the column.

Discussion

Before embarking on these experiments it seemed likely that the material absorbing in the region of 330 nm formed *in vitro* was due to the same inactive form of the coenzyme that is found in large amounts in the faster-running subforms obtained from freshly prepared enzyme. It is clear now that this is not so, because Martinez-Carrion *et al.* (1967) showed, and we have confirmed, that this naturally occurring inactive coenzyme cannot be separated by the treatment usually used to resolve the active coenzyme. The inactive coenzyme formed *in vitro*, although absorbing at similar wavelength, is very readily separated from the apoenzyme. If the reaction *in vitro* described in the present paper does occur *in vivo* it would probably not cause inactivation, because the deficient coenzyme would be replaced by circulating pyridoxal phosphate. Our observation that none of the tightly bound inactive coenzyme is formed *in vitro* seems to indicate that some essential component was absent from our experimental system.

We believe that the present work provides good evidence that the more negatively charged subforms arise *in vitro* by deamidation. The origin of the subforms *in vivo* is, however, less certain, since any process which creates an extra negative charge without altering the size or shape of the molecule would give rise to the electrophoretic subforms. In this context the observations of Denisova & Polyanovsky (1973) about sialic acid content should be considered. These workers found that their preparations of the subforms, which they showed electrophoretically to be predominantly α , β and γ , contained respectively 0.34, 0.5 and 5.2 mol of sialic acid/mol of dimer. The difference in charge between α - and β - and between β - and γ -forms based on these values would be 0.16 and 4.7. To explain the electrophoretic properties of the subforms these values should be 1 and 2, and the experimental and theoretical values seem to us too far apart for sialic acid content to provide a satisfactory explanation. It is, however, possible that material electrophoresing in the β -position is heterogeneous, with the extra negative charge being added either by deamidation or by addition of sialic acid.

The way in which the more negatively charged subforms arise *in vivo* may well provide the first steps in the normal intracellular degradation of this enzyme. Dice & Goldberg (1975, 1976) have clearly shown that proteins with the most rapid rates of breakdown *in vivo* are those with the lowest isoelectric points. Although we have not shown that deamidation occurs *in vivo* it seems unlikely that this process, which occurs with a half-time of 20 days *in vitro* at pH 7.5 and 25°C, should be entirely absent *in vivo*. One might expect at 37°C that it would be faster, perhaps bringing it closer to the observed

half-time for intracellular breakdown of aspartate transaminase.

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References

- Banks, B. E. C., Doonan, S., Lawrence, A. J. & Vernon, C. A. (1968) *Eur. J. Biochem.* **5**, 528–529
- Bertland, L. H. & Kaplan, N. O. (1968) *Biochemistry* **7**, 134–142
- Birchmeier, W., Wilson, K. J. & Christen, P. (1973) *J. Biol. Chem.* **248**, 1751–1759
- Bossa, F., John, R. A., Barra, D. & Fasella, P. (1968) *FEBS Lett.* **2**, 115–117
- Conway, E. J. (1962) *Microdiffusion Analysis and Volumetric Error*, pp. 94–97, Crosby Lockwood and Son, London
- Dekker, L. E. & Rau, E. M. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 144–149
- Denisova, G. F. & Polyanovsky, O. L. (1973) *FEBS Lett.* **35**, 129–132
- Dice, J. F. & Goldberg, A. L. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3893–3897
- Dice, J. F. & Goldberg, A. L. (1976) *Nature (London)* **262**, 514–516
- Evans, R. W. & Holbrook, J. J. (1974) *Biochem. J.* **143**, 643–649
- John, R. A. & Jones, R. E. (1974) *Biochem. J.* **141**, 401–406
- Karmen, A. (1955) *J. Clin. Invest.* **341**, 131–138
- Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F. & Fasella, P. (1967) *J. Biol. Chem.* **242**, 2397–2409
- Michuda, C. M. & Martinez-Carrion, M. (1969) *J. Biol. Chem.* **244**, 5920–5927
- Vesterberg, O. (1972) *Biochim. Biophys. Acta* **257**, 11–19