The quaternary structure of wheat-germ aspartate transcarbamoylase

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1. The molecular mass of aspartate transcarbamoylase purified from wheat germ was found to be 101kDa by sucrose-density-gradient centrifugation, 103kDa by gel-filtration chromatography and 108kDa by polyacrylamide-gel electrophoresis. A mean value of 104 ± 11 kDa was obtained by pooling several replicate results from each method. 2. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate indicated a single size of polypeptide chain of mean molecular mass 37 ± 4 kDa. The ratio of the mean molecular masses of the active and denatured enzymes is 2.8. 3. When the active enzyme was covalently cross-linked at a low protein concentration by dimethyl suberimide, and then examined electrophoretically under denaturing conditions, three size species were observed to predominate, of apparent molecular masses 36, 77 and 106kDa respectively. 4. These results indicate that the intact, fully regulatory enzyme is a simple trimer, slightly larger than the trimeric 'catalytic subunit' of the aspartate transcarbamoylase from *Escherichia coli* [Weber (1968) *Nature (London)* **218**, 1116–1118]. The prevalence of trimeric structures amongst carbamoyl-transferase enzymes is discussed.

It is now nearly two decades since the regulatory properties of aspartate transcarbamoylase (carbamoyltransferase) (EC 2.1.3.2) from *Escherichia coli* were first described (Gerhart & Pardee, 1962). In that period, not only has the *E. coli* enzyme remained one of the most intensively-studied regulatory enzymes, but the enzyme has also been studied in a wide variety of other organisms. A striking feature of these studies is the multiplicity of quaternary structures that have been found. Amongst prokaryotes, these structures range in molecular mass from about 100 to about 500kDa (Bethell & Jones, 1969), and include dimeric (Adair & Jones, 1972), trimeric (Brabson & Switzer, 1975), tetrameric (Chang & Jones, 1974) and complex hexameric forms of the enzyme. Complex hexamers have the structure $(C_3)_2 \cdot (R_2)_3$ where C and R are distinct catalytic and regulatory polypeptide chains; the 'catalytic subunit' C_3 is separable as a catalytically active entity, but lacks the regulatory properties of the native structure. The complex hexamer is exemplified by the enzyme from *E. coli* (Gerhart & Schachman, 1965; Weber, 1968), and there is strong evidence that the enzymes from *Salmonella typhimurium* (O'Donovan & Neuhaard, 1970) and twelve other species of the Enterobacteriaceae (Wild

et al., 1976, 1980) have the same structure, although their polypeptide-chain compositions remain to be elucidated. We may note here that a trimeric structure of about 100kDa makes up the native enzyme from *Bacillus subtilis* (Brabson & Switzer, 1975), as well as the 'catalytic subunits' of the enzymes from *E. coli* and (very probably) other members of the Enterobacteriaceae, none of which exhibit feedback inhibition or other regulatory behaviour.

Prokaryotic aspartate transcarbamoylases do not appear to be included in stable multienzyme complexes, in contrast with the enzyme from several eukaryotes. In animal extracts the enzyme occurs as a component of a large (>600kDa) complex incorporating one or more other enzymes of the pyrimidine-biosynthetic pathway (Hoogenraad *et al.*, 1971; Mori & Tatibana, 1973; Shoaf & Jones, 1973; Kent *et al.*, 1975; Brothers *et al.*, 1978); in a few cases the unit of this complex has been shown to be a large multifunctional polypeptide chain (Coleman *et al.*, 1977). Amongst fungi, large multienzyme complexes incorporating aspartate transcarbamoylase have been demonstrated in *Saccharomyces cerevisiae* (Lue & Kaplan, 1969) and *Neurospora crassa* (Williams *et al.*, 1970).

Somewhat surprisingly, therefore, the enzyme from another group of eukaryotes, the higher plants, appears to be quite small (comparable with the smallest bacterial forms of the enzyme) and there is as yet no evidence that the enzyme forms a stable complex with other enzymes of the pyrimidine pathway. Structural data on two higher-plant aspartate transcarbamoylases have been published. The enzyme from mung bean (*Phaseolus aureus*) was studied by Ong & Jackson (1972), who reported a molecular mass between 83 and 93 kDa. However, Achar *et al.* (1974) reported a molecular mass of 128 kDa for the same enzyme; these workers also suggested a tetrametric structure based on kinetic co-operativity (Savithri *et al.*, 1978) and have reported non-identical subunits of masses 34 and 17 kDa (Rao *et al.*, 1979). It remains to be shown what multiples of these chains make up the native enzyme. The wheat-germ enzyme was studied by one of us (Yon, 1970, 1972), who reported a molecular mass of 100 kDa and suggested a trimeric structure based on kinetic co-operativity. This model was later rejected in favour of a dimeric structure when preliminary measurements of the subunit mass of the purified enzyme suggested a value close to 50 kDa (Grayson & Yon, 1978). In the present paper we report further work on the subunit structure of the wheat-germ enzyme which supports the original trimeric structure proposed by Yon (1972).

Experimental

Materials

Sephadex G-200 was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. Proteins used as molecular-weight standards were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Chemicals for polyacrylamide-gel electrophoresis and all buffer components were from BDH, Poole, Dorset, U.K. Dimethyl suberimide was obtained from Pierce and Warriner (U.K.) Ltd., Chester, U.K. Substrates and other materials used in assays have been listed previously (Grayson *et al.*, 1979).

Enzyme preparation

For sucrose-density-gradient centrifugation the enzyme was purified as described by Yon (1974). For gel-filtration and electrophoretic experiments the enzyme was purified by the method of Grayson *et al.* (1979) or Yon (1981). The latter preparation was also used in cross-linking experiments.

Sucrose-density-gradient centrifugation

The method used was that of Martin & Ames (1961). Sucrose gradients (5–20%, w/v) were formed in 5 ml centrifuge tubes with 0.05 M-Tris/HCl,

pH 7.5, as buffer. Linearity of the gradients was confirmed by refractometry. Triplicate tubes were loaded with 0.2 mg of aspartate transcarbamoylase, 1.0 mg of catalase and 1.0 mg of ovalbumin. Centrifugation was on an MSE Superspeed 50 instrument for 16 h at 4°C and 30 000 rev./min (r_{av} , 7.1 cm). Fractions (approx. 0.2 ml) were collected and assayed for aspartate transcarbamoylase, catalase (Beers & Sizer, 1952) and ovalbumin (A_{280}). The molecular weight of aspartate transcarbamoylase was calculated independently relative to catalase and ovalbumin as standards, as described by Martin & Ames (1961).

Gel-filtration chromatography

The enzyme was filtered through a column (80 cm × 2.5 cm) of Sephadex G-200 equilibrated with 0.05 M-Tris/HCl, pH 7.5, at room temperature. Blue Dextran or DNA was used as void-volume marker and L-tryptophan as internal-volume marker. The column was calibrated both internally and externally in several runs with combinations of the following proteins (assay methods in parentheses): catalase (Beers & Sizer, 1952), liver alcohol dehydrogenase (Bergmeyer *et al.*, 1974), lactate dehydrogenase (Bergmeyer & Bernt, 1974), bovine serum albumin (A_{280}), haemoglobin, myoglobin and cytochrome *c* (all A_{410}).

Polyacrylamide-gel electrophoresis

Electrophoresis of the native enzyme was performed in the discontinuous buffer system of Davis (1964), with 7.5% (w/v) polyacrylamide gels and with ammonium persulphate as initiator. When the acrylamide concentration was varied for the construction of Ferguson (1964) plots, this was done by diluting the stock acrylamide solution [30% (w/v) acrylamide/0.8% (w/v) bisacrylamide] with distilled water before addition of the buffer and initiator solutions. For the calibration of Ferguson plots, the sets of polymers of bovine serum albumin and of haemoglobin produced by cross-linking with glutaraldehyde (Payne, 1973) were used.

Electrophoresis of the enzyme in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969), with 7.5 or 10% polyacrylamide gels. Gels were calibrated by glutaraldehyde-cross-linked myoglobin or haemoglobin (Payne, 1973), as well as by mixtures of well-known proteins.

Cross-linking by reaction with dimethyl suberimide

The enzyme was cross-linked by the method of Davies & Stark (1970) after dialysis into 0.2 M-triethanolamine/HCl, pH 8.5. The cross-linked enzyme was left overnight in 1% sodium dodecyl

sulphate/1% 2-mercaptoethanol and then examined by electrophoresis (Weber & Osborn, 1969).

Molecular weights of calibration proteins

The molecular weights of standard proteins and of their denatured subunits were taken from Weber & Osborn (1969) or Darnall & Klotz (1972).

Results

Molecular mass of the native enzyme

The molecular mass of the native enzyme was determined by several methods.

Sucrose-density-gradient centrifugation by the method of Martin & Ames (1961) gave a value of 101 ± 9 kDa (mean $\pm$ s.d. of ten determinations, i.e. five replicates in each of which the molecular weight was calculated independently by reference to catalase and ovalbumin). Gel-filtration chromatography on Sephadex G-200 gave a value of 103 ± 12 kDa (mean $\pm$ s.d. for five runs). Hence, gel filtration of the purified enzyme gives the same result as given by gel filtration of an unpurified extract of wheat germ (Yon, 1970), indicating no size changes during the purification of the enzyme.

The molecular mass was also determined electrophoretically by the method of Hedrick & Smith (1968). The electrophoretic mobility of the enzyme was measured in gels of various acrylamide contents, and primary Ferguson plots (mobility against percentage of acrylamide) were constructed (Ferguson, 1964; Hedrick & Smith, 1968). The slope of this plot is related to molecular size; thus a comparison with the slopes of Ferguson plots of standard proteins enables a molecular mass to be estimated, on the assumption that the proteins compared have similar shapes and partial specific volumes. A typical result is given in Fig. 1. Repeated determinations with different batches of the enzyme over a period of several years gave a value of 108 ± 11 kDa (mean $\pm$ s.d. of 13 determinations).

When the individual results of all three methods were pooled (28 determinations) a value of 104 ± 11 kDa was obtained.

Subunit molecular mass and quaternary structure

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969) gave a single band (see Fig. 2a), indicating subunits of a single size. After an initial discrepant value of 52 kDa (Grayson & Yon, 1978), a careful re-examination of the molecular mass of this band gave a lower value of 37 ± 4 kDa (mean $\pm$ s.d. for seven determinations). The reason for the earlier discrepancy is not known. The lower value is obtained consistently with the improved purification method described by one of us (Yon, 1981).

The ratio of the mean molecular weights of the

Fig. 1. Determination of the molecular mass of wheat-germ aspartate transcarbamoylase by polyacrylamide-gel electrophoresis

(a) Primary Ferguson plots of the enzyme (O) and calibrating proteins (●). The calibrating proteins were the monomer of bovine serum albumin (1) and its glutaraldehyde-cross-linked dimer (2), trimer (3) and tetramer (4). (b) Replots of slopes versus molecular masses. The arrow indicates the slope of the enzyme's primary plot. Mobilities (R_m) were measured relative to the tracking dye, Bromophenol Blue.

native enzyme and its subunit is 2.8. Hence a simple trimer seems the most likely quaternary structure. However, since the uncertainty in both values is of the order of 10%, a tetrameric or dimeric structure cannot be ruled out on these results.

Fig. 2. Polyacrylamide-gel electrophoresis of wheat-germ aspartate transcarbamoylase cross-linked by dimethyl suberimidate and denatured by sodium dodecyl sulphate

The enzyme (0.1 mg/ml) was unmodified in (a) and modified by dimethyl suberimidate at 0.05, 0.15, 0.50 and 1.50 mg/ml in (b), (c) (d) and (e) respectively. The electrophoretic origin is at the top of each gel and the position reached by the tracking dye is indicated by the spike of Indian ink near the bottom. The apparent molecular masses of the three prominent bands in (d) are indicated.

Cross-linking by dimethyl suberimidate

The enzyme was cross-linked intramolecularly by the bifunctional reagent dimethyl suberimidate (Davies & Stark, 1970). The protein concentration was kept quite low (0.1 mg/ml) to minimize intermolecular cross-linking, and the concentration of dimethyl suberimidate was varied. Electrophoretograms of the denatured products (Weber & Osborn, 1969) are shown in Fig. 2. Three prominent protein bands were observed in the electrophoretogram of the cross-linked enzyme, of apparent molecular masses 36, 77 and 106 kDa, i.e. very nearly in the ratio 1:2:3; increasing the concentration of dimethyl suberimidate concomitantly increased the higher-molecular-mass species and decreased the low-molecular-mass species. These results are consistent with the cross-linking of a trimeric enzyme of similar-sized subunits. At the higher concentrations of dimethyl suberimidate minor additional protein bands of molecular mass greater than 106 kDa could be seen, suggesting a small amount of intermolecular cross-linking. This may be due in part to aggregation of the enzyme (Grayson & Yon, 1978).

Discussion

On the basis of the results presented above, the most likely quaternary structure of wheat-germ aspartate transcarbamoylase is a simple trimer of molecular mass 104 kDa. This structure would be consistent with two other observations. Firstly, kinetic studies showed that the end-product inhibitor UMP interacted heterotropically with the substrate, carbamoyl phosphate, each ligand having a sigmoidal saturation curve in the presence of the other. For either ligand, the maximum Hill coefficient of co-operativity was just under 3, suggesting three or more interacting sites for each ligand; moreover, the kinetic data were shown to fit quite well to a trimeric concerted-transition mechanism (Yon, 1972). Secondly, it has been shown that the cold-lability of the enzyme is strongly pH-dependent; curves of inactivation against pH suggested that the inactivation is initiated by the co-operative uptake of three protons, possibly by histidine residues (Grayson *et al.*, 1979). We therefore postulate a trimeric structure for the enzyme to supersede our previous suggestion that the enzyme is an aggregating dimer (Grayson & Yon, 1978).

At the gross quaternary level of structure, the wheat-germ enzyme therefore resembles the enzyme from *Bacillus subtilis* (Brabson & Switzer, 1975) and the catalytic subunit of the enzyme from *E. coli* (Weber, 1968) and possibly other members of the Enterobacteriaceae; however, unlike these bacterial enzymes, it expresses a highly co-operative regulatory mechanism in this relatively small structure. The similarity in quaternary structure is surprising when viewed against the multiplicity of different structural types that have been reported for prokaryotes and eukaryotes (see the introduction). Possibly the 100 kDa trimer is a primitive feature that has survived in some widely divergent contemporary species. The concept of an archaic trimeric transcarbamoylase is supported by the frequency with which trimers are found among enzymes associated with carbamoyl phosphate metabolism, especially ornithine transcarbamoylase; Vickers (1981) recently listed 20 such enzymes, to which could be added the pyr 1-3 multienzyme complex (Jones, 1981) from mammalian cells, which is a trimer of 200 kDa chains (Coleman *et al.*, 1977). To clarify the possibility of evolutionary relatedness, it will be of interest to compare aspects of primary structure in the wheat germ and bacterial enzymes.

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