Wheat-Germ Aspartate Transcarbamoylase

PURIFICATION AND COLD-LABILITY

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1. Aspartate transcarbamoylase was purified approx. 3000-fold from wheat (*Triticum vulgare*) germ in 15–20% yield. The product has a specific activity of 14 $\mu\text{mol}/\text{min}$ per mg of protein and is approx. 90% pure. The purification scheme includes the use of bio-specific 'imphilyte' chromatography as described by Yon [*Biochem. J.* (1977) **161**, 233–237]. The enzyme was passed successively through columns of CPAD [*N*-(3-carboxypropionyl)aminodecyl]-Sephacrose in the absence and presence respectively of the ligands UMP and L-aspartate. In the second passage the enzyme was specifically displaced away from impurities with which it co-migrated in the first passage. These two steps contributed a factor of 80 to the overall purification. 2. The enzyme is slowly inactivated on dilution at 0°C and pH 7.0, the inactivation being partially reversible. A detailed investigation of the temperature- and pH-dependence of the cold-inactivation suggested that it was initiated by the perturbation of the pK_a values of groups with a moderately high and positive heat of ionization, which were tentatively identified as histidine residues. These findings support a new concept of cold-lability proposed by Bock, Gilbert & Frieden [*Biochem. Biophys. Res. Commun.* (1975) **66**, 564–569].

Ever since the involvement of aspartate transcarbamoylase (EC 2.1.3.2) in the regulation of pyrimidine biosynthesis in *Escherichia coli* was reported as one of the first cases of feedback regulation (Yates & Pardee, 1956), the enzyme has maintained its importance, and studies have been reported on the enzyme from a wide variety of organisms. A recent listing quoted studies from seventeen bacterial, four fungal, one algal, thirteen animal (including nine mammalian) and four higher-plant sources (Grayson, 1978). The higher-plant enzymes that have received attention are from lettuce (*Lactuca sativa*) (Neumann & Jones, 1964), wheat (*Triticum vulgare*)-germ (Yon, 1972, 1973, 1974; Grayson & Yon, 1978), mung bean (*Phaseolus aureus*) (Ong & Jackson, 1972; Achar *et al.*, 1974; Savithri *et al.*, 1978a,b) and cowpea (*Vigna unguiculata*) (Johnson *et al.*, 1973), of which only the wheat-germ and mung-bean enzymes have been purified.

The purification scheme for the wheat-germ enzyme (Yon, 1974) utilized successive chromatography steps on two mixed-function matrices that

contained equal numbers of hydrophobic and positively charged groups but different numbers of negatively charged (carboxy) groups. The yield was low (7%), and repeated use of the method showed that one of the adsorbents was labile and needed to be freshly prepared to give reproducible results, unlike the other adsorbent, CPAD-Sephacrose, which was stable and could be repeatedly washed and re-used. Further, excessive losses of activity in the later stages of purification were traced to cold-lability, to which dilute solutions of the enzyme were particularly prone. These disadvantages necessitated a change in purification methods. It has been shown that, for several enzymes, interaction with an immobilized amphiphilic ampholyte ('imphilyte') such as CPAD-Sephacrose is affected by the presence of free ligands that can bind to the enzyme, and it was suggested that this could be the basis of a purification scheme for such an enzyme (Yon, 1977). We report here the application of such an effect in an improved purification of wheat-germ aspartate transcarbamoylase. In the development of this purification scheme, a detailed study of the cold-inactivation of the enzyme was carried out, which appears to support novel ideas on cold-inactivation advanced by Bock *et al.* (1975). A report of this study is included.

Abbreviation used: CPAD-Sephacrose, *N*-(3-carboxypropionyl)aminodecyl-Sephacrose.

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Experimental

Materials

Unless otherwise stated, the sources of all materials are as stated in Yon (1972) or Yon & Simmonds (1975). Wheat-germ was purchased from a local pharmacy as Bemax (Beecham, Brentford, Middx., U.K.) and was stored at 4°C until required. Hydroxyapatite was made by the method of Dawson *et al.* (1969) and stored at 4°C for several months before use. DEAE-cellulose (DE52) was from Whatman, Maidstone, Kent, U.K.

The preparation of CPAD-Sepharose was essentially as described by Yon & Simmonds (1975), incorporating the buffering method of Porath *et al.* (1973). The charge ratio (Yon & Simmonds, 1979) was 1.0–1.1 and the substitution density was in the range 15–20 µmol/ml of settled gel.

Assay methods

Aspartate transcarbamoylase was assayed as described by Yon (1972). Protein in column effluents was usually monitored as A_{280} , and in other cases was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

The active enzyme was electrophoresed according to the methods recommended by the manufacturers of the apparatus (Shandon Southern, London EC2 5DL, U.K.) in 7.5% (w/v) gel at pH 9.5. Protein bands were stained with Coomassie Brilliant Blue G250 (Weber & Osborn, 1969). Enzyme activity bands were made visible by incubating the gels in 0.1M-glycine adjusted to pH 10, containing 1.0mM-L-aspartate, 0.2mM-carbamoyl phosphate and 2mM- CaCl_2 . Activity was shown as an opalescent precipitate of calcium phosphate after approx. 20 min. To permit densitometric scanning of activity gels, calcium phosphate bands were stained in 0.001% Alizarin Red-S and 0.0005% AlCl_3 in 0.1M- K_3PO_4 , pH 11, for 12–24h.

Results and Discussion

Purification scheme

Extraction and heat treatment. Wheat-germ (500g) was stirred vigorously in 3 litres of distilled water for 30 min at room temperature with an overhead-paddle stirrer. The mixture was centrifuged at 3000 rev./min (r 25 cm) for 30 min at 20°C. The turbid supernatant was strained through a pad of glass wool in a funnel to remove lumps of lipid material. CaCl_2 (1M) was added with stirring to bring the final CaCl_2 concentration to 20mM, and the pH was immediately adjusted to pH 7.0 by adding 1M-NaOH. The extract was heated rapidly to 60°C with efficient stirring to prevent

local overheating. The heating was discontinued and the extract left for 5 min and then rapidly cooled by placing the container in an ice bath. The extract was centrifuged as described above for 15 min and the precipitate discarded.

Acetone fractionation. The supernatant was cooled to 0°C. Acetone (15% of the volume of the supernatant) precooled to -20°C was added slowly with moderate stirring. The extract was left in the cold (0°C) for 1 h. The precipitate was removed by centrifugation at 0°C and discarded. A further quantity of acetone at -20°C (45% of the volume of the heat-treated supernatant) was added with stirring and the mixture again left in the cold for 1 h. The

Fig. 1. Biospecific shift of the elution volume of wheat-germ aspartate transcarbamoylase during chromatography on CPAD-Sepharose

(a) Chromatography in the absence of ligands. The acetone fraction was loaded (A–B) after which the column was washed at pH 8.0 (B–C) and then developed with a gradient from pH 8.0 to 11.0 (C to end of run). (b) Rechromatography of the pooled active fractions from (a) in the presence of UMP and L-aspartate. After loading (A–B) the column was washed at pH 8.5 (B–C) and then developed with a gradient from pH 8.5 to 11.0 (C to end of run). Profiles show total protein (---) and enzyme activity (●). Fractions included in the hatched areas were pooled and used in the following step.

mixture was centrifuged as before. The substantial pellet was resuspended in 250ml of cold (4°C) 0.01M-Tris adjusted to pH7.0 with glycine, and stirred vigorously to disperse lumps. The slurry was centrifuged at 12000 rev./min (*r* 7.5cm) in an MSE High-Speed 18 centrifuge. The pH of the clear light-brown supernatant was checked and re-adjusted to pH7.0 if necessary. This preparation was stored at 4°C until required. It was stable for several weeks. All subsequent operations were performed at room temperature.

CPAD-Sepharose chromatography in the absence of enzyme ligands. A column (200mm×25mm) was packed with CPAD-Sepharose equilibrated with 0.01M-Tris/glycine buffer, pH7.5. The column was loaded with the acetone fraction at room temperature and at a flow rate of approx. 5ml/min maintained by a peristaltic pump. The flow rate was increased to 10ml/min, the loaded column was first washed with 300ml of 0.01M-Tris/glycine, pH8.0, and then developed with a 1500ml gradient from pH8.0 to 11.0 (750ml of 0.01M-Tris/glycine, pH8.0, and 750ml of 0.01M-glycine/NaOH, pH11.0, in the two compartments of the gradient-maker). The enzyme usually began to emerge at pH9.0–9.2. Active fractions were pooled, the trailing edge of the activity peak (see Fig. 1a) being omitted. The pH was adjusted to 7.5 with acetic acid. This step normally takes 3–4h, and it was desirable to proceed to the second chromatography step on the same day.

CPAD-Sepharose chromatography in the presence of UMP and L-aspartate. A column (150mm×25mm) was packed with CPAD-Sepharose equilibrated with 0.01M-Tris containing 200μM-UMP and adjusted to pH8.5 with L-aspartic acid. The pooled fractions from the previous step were adjusted to 200μM in UMP and then loaded on to the column at a flow rate of 10ml/min. The column was washed with 300ml of 0.01M-Tris/UMP/aspartate buffer, pH8.5, and then developed with a 1500ml gradient from pH8.5 to

11.0 (750ml of 0.01M-Tris/UMP/aspartate, pH8.5, in one compartment of the gradient-maker and 750ml of the same mixture adjusted to pH11.0 with 1M-NaOH in the other). Active fractions were pooled, the leading edge (about 25%) of the activity peak being omitted (see Fig. 1b). The pH was decreased to 7.5 with acetic acid. The enzyme was now at high dilution with a low protein concentration; under these conditions its stability is limited (half-life of about 12h). If it was not possible to complete the purification on the same day, the active fractions were adsorbed on to the hydroxyapatite column (next stage) and left there until the following day.

Fig. 2. *Hydroxyapatite chromatography*
The pooled active fractions from the second CPAD-Sepharose step (Fig. 1b) were loaded (A–B) on to a column of hydroxyapatite/cellulose. The column was then washed at pH7.5 (B–C) and developed with a gradient (0–50mm) of sodium phosphate at pH7.5 (C to end of run). Fractions between D and E were pooled. Profiles show protein (---) and enzyme activity (●).

Table 1. *Purification of aspartate transcarbamoylase from wheat-germ*
A unit of enzyme activity catalyses the formation of 1 μmol of carbamoyl aspartate/min under the assay conditions described by Yon (1972).

Stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Aqueous extract	48 500	206	0.0042	1	100
2. CaCl ₂ and heat treatment	25 500	190	0.0075	1.78	92
3. Acetone fractionation	1240	177	0.079	18.8	86
4. CPAD-Sepharose chromatography (ligands absent)	121	127	1.05	250	61.6
5. CPAD-Sepharose chromatography (UMP and L-aspartate present)	11.9	78	6.55	1560	37.8
6. Hydroxyapatite chromatography	4.9	55	11.2	2670	26.7
7. DEAE-cellulose chromatography	2.6	38	13.8	3285	17.4

Hydroxyapatite chromatography. A column (100mm $\times$ 25mm) was packed with a uniform mixture of hydroxyapatite and cellulose (3:1, by packed volume) equilibrated with 0.01M-Tris/UMP/aspartate buffer, pH 7.5. The column was loaded with the pooled active fractions from the previous stage (approx. 175ml) and washed with 250ml of the equilibrating buffer. It was then developed with a concave gradient from 0 to 50mM- Na_2HPO_4 in the equilibrating buffer (200ml of buffer only in the first compartment, and 300ml of 50mM- Na_2HPO_4 in buffer re-adjusted to pH 7.5 in the second). The flow rate was approx. 5 ml/min. The active fractions were pooled. A typical run is shown in Fig. 2.

Concentration on DEAE-cellulose or CPAD-Sephadex. A small column (50mm $\times$ 5mm) was packed with either DEAE-cellulose or CPAD-Sephadex, equilibrated with 0.01M-Tris/UMP/aspartate, pH 7.5. The pooled fractions from the hydroxyapatite step (approx. 100ml) were loaded on to this column at a flow rate of approx. 5 ml/min. After a brief wash with the equilibrating buffer, the enzyme was frontally desorbed by applying a wash of

0.4M-sodium acetate in the equilibrating buffer (adjusted to pH 7.5) in the case of DEAE-cellulose, or 0.1M-glycine/NaOH, pH 10, in the case of CPAD-Sephadex. Fractions (1 ml each) were collected into tubes containing 0.1 ml of 2M-Tris/acetate, pH 7.5. The enzyme activity normally emerged in one or two fractions. These were pooled and sterilized by passage through a Millipore filter (0.2 μm pore size). Glycerol was added to 50% (w/v) concentration. The enzyme was stored in this state at room temperature, when it has a half-life of approx. 2 weeks.

Comments on purification of the enzyme

The progress of a typical purification is given in Table 1; the purity of the final product was tested by polyacrylamide-gel electrophoresis (Fig. 3). In previous tests in this laboratory, several enzymes showed differences in chromatography behaviour in the absence and presence of their biospecific ligands when chromatographed on 'imphilytes' (immobilized amphiphilic ampholytes) such as CPAD-Sephadex (Yon, 1977). For wheat-germ aspartate transcarbamoylase, the effect of the ligand UMP (a potent end-product inhibitor) was to increase the affinity of the adsorbent for the enzyme, an effect that was slightly enhanced by L-aspartate. When the adsorbed enzyme was displaced by an increasing pH gradient,

Fig. 3. Polyacrylamide-gel electrophoresis of the purified enzyme

Duplicate 7.5% acrylamide gels were each loaded with approx. 50 μg of protein. After electrophoresis one of the gels was stained with Coomassie Brilliant Blue (a) and the other was treated to show enzyme activity (b). The gels were scanned in a Joyce-Loebl densitometer; the vertical (absorbance) scale is logarithmic. 'O' denotes the origin and 'TD' the position of the tracking dye, marked by a 'spike' of Indian ink injected into the gel. The band at the origin of the lower gel is an artefact that also occurred in a control gel from which the enzyme was omitted.

Fig. 4. Reversible cold-inactivation of wheat-germ aspartate transcarbamoylase

The enzyme, purified to the acetone-fractionation stage, was diluted 10-fold into 0.1M-sodium citrate buffer, pH 7.0. The diluted enzyme was divided into three portions, which were rapidly cooled to 0°C at zero time. At 10, 30 and 60 min thereafter, one of the portions was rapidly warmed to 25°C and was assayed immediately (●) and at intervals over the next 25 min (○), by using an incubation time of 3 min in the assay.

Fig. 5. Inactivation of the enzyme at various values of pH and temperature

The enzyme, purified to the acetone-fractionation stage, was diluted 10-fold into portions of 0.1M-sodium citrate adjusted to various pH values between 5.0 and 8.0 and at temperatures of 0(Δ), 6(▲), 17(○) and 25°C (●). Samples were withdrawn at intervals and assayed for residual activity, which is expressed as a fraction of the activity of a sample at pH 8.0 and 25°C assayed immediately after dilution. Results are shown for the following times after dilution: (a) 1 min, (b) 20 min and (c) 3 h. The continuous curves are theoretical, for the co-operative uptake of 3H⁺/enzyme molecule; each has been fitted by eye to a set of experimental points. The broken lines are fitted to sets of experimental data that do not fit well to theoretical lines.

there was a distinct shift of the eluted enzyme peak towards higher pH values in the presence of UMP (Yon, 1977). The present purification scheme includes the maximization of this shift in two chromatographic

passages. In the second passage (Fig. 1b) the enzyme was specifically displaced away from impurities with which it co-migrated in the first passage. There was still, however, a considerable overlap between the main zones of protein and of the enzyme. To minimize the contaminating proteins in the zone-overlap region, a portion of the enzyme activity was sacrificed from the trailing edge of the enzyme peak in the first passage, and from its leading edge in the second passage (unhatched areas in Fig. 1).

Cold-lability

In the present purification scheme, all chromatographic steps were performed at room temperature. In previous schemes, serious losses occurred in all chromatographic steps at 4°C and increased as the enzyme approached purity. These losses were traced to cold-lability of the enzyme. Experiments on cold-lability were performed in 0.1M-citric acid adjusted to various pH values by adding 10M-NaOH. The pK_a values of the carboxy groups of citric acids were negligibly perturbed by the temperature changes described below.

When the enzyme (purified to the acetone-fractionation stage) was diluted 10-fold into citrate buffer at pH 7.0 and 25°C, and the temperature then decreased to 0°C, a progressive loss of activity occurred (Fig. 4, ●). When the temperature was subsequently re-adjusted to 25°C, a lag of some 10 min occurred, after which the enzyme began to recover activity slowly (Fig. 4, ○). Providing the exposure to 0°C did not exceed 1 h, the lag period and the initial rate of recovery appeared to be independent of the time of exposure. For very long periods of inactivation (24 h) at pH 7.0 (or any lower pH) and 0°C, the enzyme was completely and irreversibly inactivated.

Shortly after we first noted cold-lability in this enzyme, Bock *et al.* (1975) suggested that cold-lability in rabbit muscle phosphofructokinase could be attributed to the temperature-dependence of the pK_a values of critical ionizable residues. This prompted us to examine the cold-inactivation of wheat-germ aspartate transcarbamoylase as a function of both temperature and pH. The results (Fig. 5) show the residual enzyme activity after exposure of the 10-fold diluted acetone fraction to pH values in the range 5.2–7.5 for various times, at temperatures of 0, 6, 17 and 25°C. The experimental points in many cases fit quite well to a set of theoretical curves (the continuous lines in Fig. 5) each of which represents the deprotonated fraction of a putative set of ionizable groups on the enzyme. For the curves shown, this deprotonated fraction is a highly-co-operative function of [H⁺] concentration (Hill coefficient, *h*, = 3). The theoretical curves are displaced towards higher pH values as the temperature is lowered, suggesting that the pK_a value of the putative ionizable groups is temperature-

Fig. 6. Time course of cold-inactivation

The pH corresponding to 50% residual activity, in curves such as those of Fig. 5, are plotted against time after dilution, for temperatures of 0 (▲), 6 (△), 17 (●) and 25°C (○). After 30 min the residual activity at 0°C was less than 50% at all pH values tested.

Scheme 1. Mechanism for the cold-inactivation of aspartate transcarbamoylase

ΔH_{ion}^0 , heat of ionization

sensitive. The strong inference of these observations is that, for short times of exposure to moderately low temperatures, the residual activity reflects exactly the deprotonation state of a set of residues on the protein whose heat of ionization is positive and quite large. Application of the van't Hoff isochore to the curves of Fig. 5 suggests a value of approx. 28 kJ/mol for the heat of ionization. The residues most likely to have this heat of ionization and a $\text{p}K_a$ close to 6 at room temperature are those of histidine (Edsall & Wyman, 1958). Our results on cold-inactivation of this enzyme, for moderate inactivation times and decreases in temperature, provide strong support for the cold-inactivation theory of Bock *et al.* (1975), which may, indeed, apply to many more cold-labile enzymes (Bock & Frieden, 1978).

The inactivation becomes more complex at very low temperatures and after long periods of inactivation, as shown by the deteriorating fit of the experimental points to the theoretical curves

(---, Fig. 5). The pH value corresponding to 50% residual activity (a pseudo- $\text{p}K_a$ value) is plotted against time in Fig. 6. At 25 and 17°C this value starts at 5.35 and 5.50 respectively and slowly increases to reach constant values of 6.0 and 6.3 after approx. 30 min. This behaviour suggests the slow approach to an equilibrium state. At 6 and 0°C, however, this value continues to increase throughout the whole period (3 h) of the experiment.

These observations suggest, as a minimal mechanism for the cold-inactivation of the enzyme, the mechanism shown in Scheme 1. The active deprotonated form of the enzyme, represented by E, is rapidly and reversibly inactivated by the co-operative uptake of three protons (suggested by the Hill coefficient of the curves in Fig. 5) when the temperature or the pH is lowered. The inactive protonated enzyme EH_3 slowly changes to an inactive form $\text{E}'\text{H}_3$ over a period of approx. 30 min; this change is reversible and the reverse change is also slow. During this change, the $\text{p}K_a$ of the sensitive groups increases slightly, probably reflecting changes in their microenvironment. At low temperatures (6°C or less), form $\text{E}'\text{H}_3$ slowly changes to an inactive form, $\text{E}''\text{H}_3$; this change is irreversible. The existence of forms E, E' and E'' remains to be demonstrated, and it will be interesting to see whether structural, spectroscopic or other changes can be correlated with temperature and pH in a manner that parallels inactivation, thus providing independent support for Scheme 1.

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