Aspartate Transcarbamoylase from *Phaseolus aureus*

PARTIAL PURIFICATION AND PROPERTIES

By B. L. ONG and J. F. JACKSON

Department of Agricultural Biochemistry, Waite Agricultural Research Institute,
University of Adelaide, Glen Osmond, S. Austral. 5064, Australia

(Received 4 April 1972)

1. Aspartate transcarbamoylase from 4-day-old radicles of *Phaseolus aureus* was purified 190-fold by $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-cellulose and DEAE-Sephadex chromatography and Sephadex-gel filtration. The partially purified enzyme, which required P_i for maximum stability, had an apparent molecular weight of 83000 ± 5000 . 2. Uridine nucleotides were found to inhibit the activity; UMP was the most potent inhibitor, followed by UDP and UTP. No other nucleotide was found to affect the enzyme, nor could UMP inhibition be overcome by adding another nucleotide. Aspartate gives a hyperbolic substrate-saturation curve, both with and without UMP. The nucleotide inhibitor is non-competitive with respect to this substrate. Carbamoyl phosphate also yields a hyperbolic substrate-saturation curve in the absence of feedback inhibitor, but when UMP is added a sigmoidal pattern results, and the inhibition is competitive with carbamoyl phosphate. 3. The degree of inhibition by UMP is not affected by *p*-chloromercuribenzoate, urea, mild heat pretreatment or change in pH over the range 8.5–10.5, but is affected by temperature. 4. The aspartate analogue, succinate, both activates and inhibits the reaction, depending on the concentrations of aspartate and succinate used. 5. Kinetic studies with the partially purified enzyme showed that the K_m for carbamoyl phosphate (0.091 mM) is much lower than that for aspartate (1.7 mM). A sequential reaction mechanism was inferred from product-inhibition kinetics, with carbamoyl phosphate binding to the enzyme before aspartate, and the product, carbamoylaspartate, being released ahead of P_i . Initial-velocity studies gave a set of parallel reciprocal plots, compatible with an essentially irreversible step occurring before the binding of aspartate.

One of the early steps in the biosynthetic pathway leading to UMP, and eventually to CTP, is the reaction catalysed by aspartate transcarbamoylase (EC 2.1.3.2), where carbamoylaspartate and phosphate are produced from carbamoyl phosphate and aspartate. The enzyme from *Escherichia coli* has been thoroughly examined and found to consist of two types of subunits: a catalytic one, which can carry out the basic reaction given above, and a regulatory subunit, which can bind CTP and which, when attached to the catalytic subunit as in the native enzyme, decreases the activity considerably in the presence of CTP (Gerhart & Pardee, 1962; Gerhart & Schachman, 1965). Aspartate transcarbamoylase from other sources differs from the *E. coli* enzyme, particularly in regulatory and kinetic properties. Thus Bethel & Jones (1969) showed that this enzyme in *Pseudomonas fluorescens* is subject to regulation by UTP, whereas the enzymes from *Bacillus subtilis* and *Streptococcus faecalis* (Prescott & Jones, 1970) are not affected by any pyrimidine nucleotide. These enzymes give hyperbolic substrate-saturation curves in the absence

of effector, in contrast with the sigmoidal relationships found with the native *E. coli* enzyme. Although the partially purified mammalian transcarbamoylases do not appear to be inhibited by pyrimidine nucleotides (Bresnick & Mosse, 1966; Hoogenraad *et al.*, 1971) the plant activity has been reported to be regulated by UMP (Neumann & Jones, 1964; Yon, 1971*a,b*).

The aim of the present investigation is to compare the aspartate transcarbamoylase from *Phaseolus aureus* with the enzyme from other sources, and to establish how its regulatory and other properties fit in with the other early steps in pyrimidine biosynthesis in plant tissue. Preliminary accounts of the work have been given by Ong & Jackson (1970, 1971).

Experimental

Materials

Phaseolus aureus seeds were purchased from the local (Adelaide) seed store. The DEAE-cellulose used was Whatman DE.11 (capacity 1 mequiv./g), and

Pharmacia Fine Chemicals (Uppsala, Sweden) supplied DEAE-Sephadex A-50 (particle size 40–120 μm ; capacity 3.5 ± 0.5 mequiv./g) and Sephadex G-200. Horse cytochrome *c* (grade IV) and bovine serum albumin (grade IV) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), γ -globulin and human ovalbumin were from Mann Research Laboratories (New York, N.Y., U.S.A.) and lactate dehydrogenase was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). [^{14}C]Aspartate and $\text{Na}_2^{14}\text{CO}_3$ were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), carbamoyl[^{14}C]aspartate was from New England Nuclear Corp. (Boston, Mass., U.S.A.), and all other amino acids, carbamoyl phosphate, carbamoylaspartate and nucleic acids and their derivatives were from Sigma Chemical Co. All buffers and inorganic reagents were A.R. grade (British Drug Houses Ltd., Poole, Dorset, U.K.), except tris buffer, which was supplied by Sigma Chemical Co. Glass beads (size 16) were from Sigma Chemical Co.

Plant material

Batches (250g) of *P. aureus* seeds were surface-sterilized with ethanol for 60s, washed with sterile water, and then soaked in 0.2% (w/v) bleaching powder (May and Baker, Dagenham, Essex, U.K.) for 2–3h. After two rinses with water, the seeds were allowed to imbibe water overnight. The seeds were then germinated on thick, moist blotting paper spread on plastic trays and covered with black plastic sheets. All equipment was sterilized in an autoclave or by ethanol treatment before use. The radicles were harvested after growing for 3 days in the dark at 22–25°C.

Aspartate transcarbamoylase assay

Two assay methods were employed. (a) The colorimetric method of Prescott & Jones (1969) was used during the latter stages of enzyme purification, and for the studies on the inhibition by UMP and denaturing agents. (b) The radiochemical assay of Ong & Jackson (1971) was used for the initial-velocity and product-inhibition kinetics.

Initial rates were linear up to 20% of the limiting substrate consumed with both assay methods, and care was taken to work within this range. Details of incubation mixtures are as described by Ong & Jackson (1971), unless otherwise stated, and a unit of activity is 1 μmol of carbamoylaspartate formed/h at 30°C and at pH 8.5. The Sephadex G-200 fraction was used for kinetic studies, unless otherwise stated. Protein was determined by the method of Lowry *et al.* (1951), after precipitation with 5% (w/v) trichloroacetic acid, with bovine serum albumin as standard.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out as described by Reid & Bielecki (1968).

Calculation of K_m , V_{max} , and the Hill coefficient

Normal Michaelis–Menten substrate-saturation curves were fitted to a computer program based on the statistical treatment for K_m and V_{max} determination, together with associated standard errors, as set out by Wilkinson (1961) and used by Atkinson *et al.* (1961). Results from sigmoidal substrate-saturation relationships were fitted to the empirical Hill equation (Hill, 1913). A computer program for this process, and for the calculation of parameters from initial-velocity data, was kindly supplied by Dr. R. G. Nicholls of this Department.

Results

Purification of the enzyme

Crude extract. A total of 3kg of 3-day-old *P. aureus* radicles was homogenized with 1kg of glass beads and 1.5 litres of 0.2M-tris-HCl buffer (pH 9.0), containing 0.02M-2-mercaptoethanol and 0.002M-EDTA, in a Waring Blendor at 4°C. The homogenate was filtered through a double layer of muslin and centrifuged at 20000g for 40min. The supernatant fraction, which contained 80–95% of the activity, turned slightly brown on standing, but no loss of activity was observed during this period.

$(\text{NH}_4)_2\text{SO}_4$ fractionation at 4°C. Although 15–20% of the activity was recovered in the 0–40%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction, this was discarded. The 40–70%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction, containing the rest of the enzyme activity, was recovered by centrifugation at 10000g for 15min and the pellet was dispersed in 0.02M-potassium phosphate buffer (pH 8), containing 0.002M-2-mercaptoethanol and 0.001M-EDTA (sodium salt), so that the final protein concentration was 20.0mg/ml. After dialysis against two changes of the above buffer (8h each), the turbid solution was centrifuged; all the activity was in the supernatant fraction.

DEAE-cellulose chromatography. The supernatant was loaded on to a column (7cm $\times$ 39cm long) of DEAE-cellulose, which had previously been equilibrated with the above phosphate buffer at 4°C. Approx. 25mg of protein was loaded/ml of bed volume, at a rate of 50ml/h. The column was then developed with a linear KCl gradient (0–0.4M) in the phosphate buffer used above. Aspartate transcarbamoylase activity emerged at approx. 0.1M-KCl; the 8ml fractions collected at this point contained about 1.5mg of protein/ml. Fractions with a specific activity greater than 1.4 μmol of carbamoylaspartate formed/h per mg of protein were pooled, and the

activity was concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$. The 35–75% satn. $(\text{NH}_4)_2\text{SO}_4$ fraction was dissolved in a small volume of the above phosphate buffer, pH 8.0, containing 10% (v/v) glycerol and was dialysed against the same buffer (two changes, 8 h each).

DEAE-Sephadex A-50 chromatography. After adsorption of the above non-diffusible material on to a column ($4.2\text{cm}^2 \times 30\text{cm}$ long) of DEAE-Sephadex, which had been equilibrated with the above phosphate-glycerol buffer at pH 7.5, the column was subjected to a linear gradient of KCl (0.05–0.7 M) in the above phosphate buffer, with 10% (v/v) glycerol being added to aid in the stability of the enzyme. The activity was eluted in a sharp peak at 0.3–0.45 M-KCl, the most active fraction having a specific activity of 20 units/mg of protein.

Sephadex G-200 filtration. The most active fractions were concentrated by precipitation with 80% (w/v) saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was dissolved in a small volume of the above phosphate buffer, pH 8.0, containing 10% (v/v) glycerol. The concentrated solution was passed through a column ($4.2\text{cm}^2 \times 90\text{cm}$ long) of Sephadex G-200, which had previously been equilibrated with the same buffer. Active fractions with a specific activity of 20 units/mg of protein or better were pooled, divided into 2 ml portions and these were snap-frozen in liquid N_2 and stored at -15°C . Enzyme activity preserved in this way was stable for at least 3 months. A summary of the purification is given in Table 1. When a fraction with a specific activity of 30 units/mg of protein was analysed by polyacrylamide-gel electrophoresis at pH 8.5 in tris-glycine buffer, eight to ten protein bands were observed, only one of which was shown to have aspartate transcarbamoylase activity. This activity was demonstrated with samples of the dispersed gel, by using the radiochemical assay (Ong & Jackson, 1971); with this technique only about 1% of the enzyme activity applied to the gel was recovered.

Physical properties

Molecular weight. This was determined in two ways. A column ($0.8\text{cm}^2 \times 51\text{cm}$) of Sephadex G-200, calibrated with human γ -globulin (mol.wt. 160000), lactate dehydrogenase (mol.wt. 140000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000) and cytochrome *c* (mol.wt. 12700), gave an apparent molecular weight of 83000 ± 5000 for both the Sephadex G-200 and the DEAE-cellulose fractions. The glycerol-gradient method of Martin & Ames (1961), with a 5–35% (v/v) glycerol gradient and with catalase as the internal standard, gave 93000 as the apparent molecular weight.

Stability. The presence of 0.02 M-potassium phosphate as well as 10% (v/v) glycerol was required for maintenance of aspartate transcarbamoylase activity in the purified fraction. Thus omission of glycerol, or a decrease in phosphate concentration (to 1 or 0.5 mM), or replacement of phosphate by 0.02 M-tris-HCl buffer (pH 8), all resulted in substantial loss of activity after 24 h at 0°C . The stabilizing effect of phosphate is illustrated in Fig. 1, where the effect of mild heat treatment on the partially purified enzyme is recorded under various ionic conditions. Another bivalent ion, sulphate, added in the form of its ammonium salt, also affords some protection against heat inactivation, whereas 1 mM-aspartate, 1 mg of bovine serum albumin/ml and 30 mM-NaCl gave only slight protection (Fig. 1).

Kinetics

pH optimum. As with the lettuce-seedling enzyme (Neumann & Jones, 1962) and the wheat-germ activity (Yon, 1971*a,b*), the partially purified aspartate transcarbamoylase from *P. aureus* had a relatively high pH optimum, in this case around 10.5. This was observed by using a composite buffer, consisting of potassium phosphate, tris and ethanolamine, each at 0.03 M, covering the pH range 7.5–12.5. The K_m for both aspartate and carbamoyl phosphate did not

Table 1. *Partial purification of aspartate transcarbamoylase*

A total of 3 kg of *P. aureus* radicles was used as starting material. During the first three stages, the radiochemical assay was used to determine enzyme activity, and the colorimetric method was used for steps 4 to 6.

Step no.	Fraction	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg of protein)	Yield (%)
1	Crude homogenate	3500	5.02	0.56	0.11	100
2	20000g supernatant	3400	3.1	0.6	0.19	100
3	40–70% satn. $(\text{NH}_4)_2\text{SO}_4$	156	19.2	10.1	0.53	80
4	DEAE-cellulose eluate	39	14.1	28.4	2.0	56
5	DEAE-Sephadex eluate	20	4.1	37.0	9.1	37
6	Sephadex G-200 eluate	40	0.7	14.8	21.2	30

change significantly between pH 10.5 and 8.5; however, the $V_{\max}$ did vary over this range, being $7.3 \pm 0.7 \mu\text{mol/h}$ per mg of protein at pH 8.5 and $24.9 \pm 2.5 \mu\text{mol/h}$ per mg at pH 10.5.

Inhibition by nucleotides. The most effective nucleotide inhibitor was found to be UMP, which, under the assay conditions used, resulted in an almost complete inhibition at 1 mM. As shown in Fig. 2, UDP and UTP are less effective, the concentration giving 50% inhibition being 1.3 and 2.7 mM respectively, compared with 0.3 mM for UMP. Inhibition by 0.3 mM-UMP was not relieved by up to 5 mM concentrations of GMP, GTP, IMP, AMP, ornithine and ATP, and, in the absence of UMP, the rate of reaction was not affected by TMP, TTP, uridine, thymidine, CMP, CDP, CTP, ATP, ornithine, dihydro-orotate or orotate, all at 3 mM, in the presence of 1 mM-carbamoyl phosphate.

Effect of substrate concentration on UMP inhibition. Unlike the *E. coli* enzyme (Gerhart & Pardee, 1963), the plant aspartate transcarbamoylase gives hyperbolic substrate-saturation curves in the absence of UMP. In the presence of the nucleotide inhibitor, hyperbolic curves are still observed when aspartate concentration is varied (Fig. 3a); however, a sigmoidal relationship is evident when the variable substrate is carbamoyl phosphate (Fig. 3b). The effect of UMP addition in altering the carbamoyl phosphate-saturation curve to a sigmoidal shape is to raise the apparent K_m for carbamoyl phosphate. Increasing the UMP concentration raises the K_m for carbamoyl phosphate still further, whereas the K_m for aspartate and the $V_{\max}$ for the enzymic reaction are little changed (Table 2). It is evident then that inhibition by UMP is competitive with respect to carbamoyl phosphate but not with respect to aspartate. Application

Fig. 1. Protection of the enzyme against heat inactivation by various reagents

The partially purified enzyme was dialysed for 8 h against 0.025 M-tris-HCl-0.002 M-2-mercaptoethanol-0.001 M-EDTA-10% (v/v) glycerol buffer, pH 7.5, (resulting in some loss of activity). The various reagents were added to portions of the non-diffusible material, which were then incubated at 45°C, portions being removed at intervals for determination of aspartate transcarbamoylase activity by the radiochemical method at 30°C. Additions were: ○, 25 mM-potassium phosphate; □, 30 mM-(NH₄)₂SO₄; ●, 30 mM-NaCl; △, 1 mM-aspartate; ▲, 1 mg of bovine serum albumin/ml; ▲, no addition.

Fig. 2. Inhibition of aspartate transcarbamoylase by uridine nucleotides

Nucleotides found to give inhibition were: ○, UMP; □, UDP; ●, UTP. The colorimetric assay was used. Each reaction mixture contained 0.02 mg of protein.

Fig. 3. Substrate-saturation curves in the presence and absence of UMP

(a) Aspartate concentration varied with: ●, no UMP added; Δ , with 0.26 mM-UMP. (b) Carbamoyl phosphate concentration varied: ●, in the absence of UMP; Δ , with 0.028 mM-UMP; ○, with 0.083 mM-UMP. The colorimetric assay was used in all cases.

of the empirical Hill equation (Changeaux, 1963) to the results suggests that there are at least three binding sites for UMP per enzyme molecule (Table 2). The concentration of UMP required to give 50% inhibition did not alter significantly between pH 8.5 and 10.5.

Inhibition by denaturing reagents. Because the *E. coli* aspartate transcarbamoylase is activated by *p*-chloromercuribenzoate and because both *p*-chloromercuribenzoate and urea lead to a splitting of the enzyme into subunits without loss of catalytic activity and with loss of control by CTP (Gerhart & Pardee, 1962), the effect of these compounds on the plant enzyme was investigated. In the absence of UMP, no activation was observed with these compounds; in fact, a steady decrease in activity was observed as the concentration of reagent was raised in the assay mixture. Inhibition was almost complete at 6 mM-*p*-chloromercuribenzoate, 2.5 M-urea and 0.8 M-guanidine-HCl buffer. An excess of GSH completely reversed the inhibition by *p*-chloromercuribenzoate. In the presence of *p*-chloromercuribenzoate, the plant enzyme was still inhibited by UMP (Fig. 4), in contrast with the results obtained for the *E. coli* enzyme and its inhibitor, CTP (Gerhart & Pardee, 1962).

Studies on changes in temperature. Although V_{max} and K_m for aspartate were shown to increase with increasing temperature, the K_m for carbamoyl phosphate did not change significantly over the temper-

ature range 12.5–37°C (Table 3). This could indicate a lower activation energy for the binding of carbamoyl phosphate with the enzyme, compared with that for aspartate (cf. Dixon & Webb, 1958). Aspartate transcarbamoylase from *P. aureus* displayed hyperbolic substrate-saturation curves over the temperature range. This is in contrast with the *E. coli* enzyme, which we found to give a sigmoidal relationship above 20°C, changing to hyperbolic substrate-saturation curves at temperatures less than 15°C. O'Donovan & Neuhaud (1970) report a similar transition to hyperbolic curves at low temperatures, again with the enzyme from *E. coli*.

Inhibition of the *P. aureus* enzyme by UMP was not affected by mild-heat pretreatment, which entailed heating the enzyme at 60°C for 10 min and then cooling quickly in ice. However, when the temperature of the reaction mixtures was varied, the degree of inhibition by UMP was observed to be dependent on temperature. As shown in Fig. 5, at any given UMP concentration the degree of inhibition is greater at lower temperatures, and increasing the UMP concentration appears to cause an increased activation energy for the overall enzyme reaction. When the Hill equation was applied to these data (Fig. 6), the results suggested that the changes in inhibition patterns at the various temperatures were not due to a change in the number of UMP-binding sites per enzyme molecule, as the parallel lines (Fig. 6) indicate

Table 2. *Effect of UMP on some kinetic parameters*

Activity was determined in all cases by the colorimetric procedure. The statistical treatment of Wilkinson (1961) was used for determination of $V_{\max}$, and the kinetic constant, K , where aspartate was the variable substrate, and the empirical Hill (1913) equation was used for the parameters with carbamoyl phosphate. For aspartate, therefore, $K_m = K$ and for carbamoyl phosphate $K_m = \sqrt[n]{K}$, K_m being defined as the substrate concentration giving an initial velocity of $V_{\max}/2$. The Hill coefficient, n , was calculated from the slope of a plot of $\log[v/(V_{\max} - v)]$ versus $\log[\text{carbamoyl phosphate}]$, where aspartate was the variable substrate, and from the fitted empirical Hill relationship where carbamoyl phosphate was varied.

Fixed substrate	Variable substrate	UMP concentration (mM)	K (mM)	$V_{\max}$ ($\mu\text{mol/h per mg of protein}$)	Hill coefficient (n)
Carbamoyl phosphate (3 mM)	Aspartate	—	0.48 ± 0.06	2.70 ± 0.11	1.10
Carbamoyl phosphate (3 mM)	Aspartate	0.26	0.47 ± 0.05	2.11 ± 0.07	1.08
Aspartate (3.3 mM)	Carbamoyl phosphate	—	0.013 ± 0.003	3.03 ± 0.05	1.40 ± 0.06
Aspartate (3.3 mM)	Carbamoyl phosphate	0.03	0.09 ± 0.02	3.71 ± 0.12	2.43 ± 0.13
Aspartate (3.3 mM)	Carbamoyl phosphate	0.08	1.10 ± 0.05	3.53 ± 0.06	2.47 ± 0.08

Fig. 4. *Effect of p-chloromercuribenzoate on UMP inhibition*

Transcarbamoylase activity was determined by the colorimetric method. ●, No *p*-chloromercuribenzoate; ○, 1.1 mM *p*-chloromercuribenzoate; □, 2.2 mM *p*-chloromercuribenzoate; ▲, 4.4 mM *p*-chloromercuribenzoate.

no change in interaction coefficient between 5°C and 45°C. The slope of an Arrhenius plot for the reaction catalysed by the partially purified *E. coli* aspartate transcarbamoylase over the temperature range 4.5–25°C was unchanged by the addition of 0.1 mM-CTP to the reaction mixture. An inhibition of approx. 20% was observed at 37°C.

Activation and inhibition by succinate. The aspartate analogues succinate and maleate have been shown both to activate and to inhibit the *E. coli* aspartate transcarbamoylase under certain conditions (Gerhart & Pardee, 1963). We have found a similar result with the *P. aureus* enzyme. Thus succinate behaves as an activator at low concentrations of both succinate and aspartate, and as an inhibitor at high succinate concentrations and at high aspartate concentrations (Fig. 7).

Initial-velocity studies. Lineweaver–Burk plots obtained by varying the concentration of aspartate at a constant carbamoyl phosphate concentration, and vice versa, were linear. When these experiments were carried out at several concentrations of the fixed substrate, a system of parallel straight lines was obtained in each case (Fig. 8). According to Cleland (1963*a,b*), this indicates that there is an essentially irreversible step in the reaction mechanism somewhere between the binding of the two substrates. A re-plot of intercepts on the perpendicular axis versus the reciprocal of the fixed substrate concentration gave a straight line in each case, with a similar $V_{\max}$ value and suggested K_m values of 0.091 mM and 1.7 mM for carbamoyl phosphate and aspartate respectively.

Product inhibition. The inhibitory effects of products in changing slopes and intercepts of reciprocal

Table 3. Effect of temperature on some kinetic parameters

The $V_{\max}$ values listed were obtained from the aspartate substrate-saturation experiment. Similar values showing a consistent increase in $V_{\max}$ with increasing temperature were also obtained from the experiments where carbamoyl phosphate concentration was varied. The enzyme used in this experiment was the DEAE-Sephadex fraction (Table 1).

Temperature (°C)	K_m (carbamoyl phosphate) (mM)	K_m (aspartate) (mM)	$V_{\max}$ ($\mu\text{mol/h}$ per mg of protein)
12.5	0.063 ± 0.013		
	0.078 ± 0.006		
15		0.511 ± 0.076	0.041 ± 0.002 0.0406 ± 0.0018
20	0.045 ± 0.008 0.051 ± 0.007		
25		0.793 ± 0.261	0.086 ± 0.014 0.0856 ± 0.0144
27.5	0.059 ± 0.006 0.049 ± 0.004		
37	0.072 ± 0.011 0.055 ± 0.014		
37.5		1.650 ± 0.514	0.194 ± 0.041

Fig. 5. Arrhenius plot at various UMP concentrations

Initial rates (v) were determined with the colorimetric method. $\circ$, No UMP; $\square$, 0.1 mM-UMP; $\bullet$, 0.2 mM-UMP; $\triangle$, 0.3 mM-UMP; $\blacksquare$, 0.6 mM-UMP; $\blacktriangle$, 0.8 mM-UMP.

plots can be used to diagnose the steady-state mechanism (Cleland, 1963*a,b*). Accordingly, when reciprocal plots for carbamoyl phosphate and aspartate were obtained from reactions carried out in the presence of

Fig. 6. Hill plot for UMP inhibition at various temperatures

The colorimetric assay was used after incubation at the following temperatures: $\circ$, 5°C; $\blacktriangle$, 15°C; $\square$, 25°C; $\bullet$, 35°C; $\triangle$, 45°C.

phosphate and carbamoylaspartate in turn, the inhibition patterns shown in Fig. 9 were obtained. Clearly the reciprocal plots for aspartate in the presence of either product show non-competitive inhibition. An analysis of the standard error of the intercept on the vertical axis for the case where carbamoylaspartate is used as inhibitor for the

Fig. 7. Effect of succinate on aspartate transcarbamoylase activity

Activity was determined by the colorimetric procedure. $\circ$, 0.5 mM-aspartate; $\bullet$, 3.5 mM-aspartate.

Fig. 8. Initial-velocity studies

Rates were determined by the radiochemical procedure and are recorded here as $\mu\text{mol/h}$ per mg of protein. (a) Aspartate is the variable substrate, at the following carbamoyl phosphate concentrations: $\bullet$, 0.04 mM; $\circ$, 0.1 mM; $\triangle$, 2.0 mM. (b) Carbamoyl phosphate is the variable substrate, at the following aspartate concentrations: $\triangle$, 0.3 mM; $\bullet$, 0.4 mM; $\square$, 0.6 mM; $\blacktriangle$, 1.0 mM; $\circ$, 2.0 mM. The lines shown are those fitted by using the statistical treatment of Wilkinson (1961).

Fig. 9. Product-inhibition studies

The radiochemical procedure was used in determining initial velocities (v), recorded as $\mu\text{mol/h}$ per mg of protein. The lines shown are those calculated from the statistical treatment of Wilkinson (1961). (a) Aspartate is the variable substrate and phosphate is the inhibitor. $\circ$, No phosphate; $\square$, 100 mM-phosphate; $\bullet$, 300 mM-phosphate. (b) Aspartate is the variable substrate and carbamoylaspartate is the inhibitor. $\circ$, No carbamoylaspartate; $\square$, 150 mM-carbamoylaspartate; $\bullet$, 300 mM-carbamoylaspartate. (c) Carbamoyl phosphate is the variable substrate and phosphate is the inhibitor. $\circ$, No phosphate; $\square$, 25 mM-phosphate; $\bullet$, 50 mM-phosphate. (d) Carbamoyl phosphate is the variable substrate and carbamoylaspartate is the inhibitor. $\circ$, No carbamoylaspartate; $\square$, 50 mM-carbamoylaspartate; $\bullet$, 100 mM-carbamoylaspartate.

carbamoyl phosphate reciprocal plot (Table 4) suggests that the intercepts from both the 0.1 M- and 0.05 M-carbamoylaspartate experiments are significantly different from that for the inhibitor-free case. Thus this must also represent non-competitive inhibition. The same type of analysis for phosphate inhibition when carbamoyl phosphate was the variable substrate indicates that the point of intersection of each of the three lines with the vertical axis is not significantly different (Table 4), and so this must be a case of competitive inhibition.

These results are consistent with an ordered Bi Bi

mechanism, where carbamoyl phosphate and then aspartate bind to the enzyme, followed by release of carbamoylaspartate and phosphate, in that order.

Discussion

The properties described here for the partially purified *P. aureus* enzyme are similar to those so far reported for other plant aspartate transcarbamoylases. Thus a preliminary report by Yon (1971a) shows that the wheat-germ enzyme, purified 20-fold, has a molecular weight of approx. 100000 and so, like

Table 4. Analysis of standard errors for the intercepts from carbamoyl phosphate reciprocal plots with product inhibition

The standard error of $1/V_{\max}$ is given by the relationship (standard error of $V_{\max}/V_{\max}^2$).

	Inhibitor	$V_{\max}$ ($\mu\text{mol/h per}$ mg of protein)	Standard error of $V_{\max}$	$1/V_{\max}$	Standard error of $1/V_{\max}$
Carbamoyl- aspartate	(0)	2.3311	0.0265	0.4290	0.0049
	(50mM)	2.0870	0.0713	0.4792	0.0164
	(100mM)	1.9205	0.1511	0.5207	0.0410
Phosphate	(0)	1.4238	0.0381	0.7023	0.0188
	(25mM)	1.3144	0.1374	0.7608	0.0795
	(50mM)	1.4143	0.1695	0.7071	0.0847

the *P. aureus* enzyme, it is similar in size to the catalytic subunit of the *E. coli* aspartate transcarbamoylase (Gerhart & Schachman, 1965). Both the wheat-germ enzyme and the one from lettuce seedlings (Neumann & Jones, 1964) are subject to inhibition by uridine nucleotides, and as described here for the *P. aureus* aspartate transcarbamoylase, the order of effectiveness is UMP > UDP > UTP. No other nucleotides have been found to inhibit these plant transcarbamoylases.

Unlike the native *E. coli* system, these plant enzymes all give hyperbolic substrate-saturation curves for aspartate and carbamoyl phosphate, and for aspartate in the presence of the inhibitor UMP. However, when the carbamoyl phosphate substrate-saturation relationships are investigated in the presence of the allosteric-type inhibitor, UMP, a sigmoidal curve results with both the *P. aureus* and wheat-germ enzymes. The Hill coefficient for UMP inhibition is given as 3 for the wheat-germ enzyme (Yon, 1971b), a value also obtained for the *P. aureus* transcarbamoylase. The competitive nature of this inhibition with respect to the substrate carbamoyl phosphate for the *P. aureus* and the wheat-germ enzymes may have important implications for the overall control of the pyrimidine biosynthetic pathway in plants (Ong & Jackson, 1972). We did not find any change in the degree of inhibition by UMP when the pH of the reaction mixture was lowered from 10.5 to 8.5. In a preliminary communication, Yon (1971b) reports that UMP inhibition is pH-dependent for the wheat-germ activity, the enzyme being completely insensitive to this inhibitor below pH 7.5, and that above this pH value, UMP inhibition increases to a maximum at around pH 10.

Inhibition of the native *E. coli* enzyme by its allosteric inhibitor, CTP, can be abolished by including urea or *p*-chloromercuribenzoate in the reaction mixture, or by a mild heat pretreatment of the enzyme (Gerhart & Pardee, 1962). They encountered no loss of activity under the conditions

used; in fact, activity was doubled both by the heat treatment and by 50 μM -*p*-chloromercuribenzoate. These results were interpreted in terms of a breakdown of the native enzyme into its composite catalytic and regulatory subunits. The *P. aureus* enzyme was not activated by this type of treatment, and the pattern of inhibition by UMP was unaltered. There is as yet no evidence for distinct catalytic and regulatory subunits in the plant enzyme. The *P. aureus* enzyme does share at least one property with the native *E. coli* enzyme, that of activation and inhibition by the aspartate analogue, succinate. However, this was interpreted for the *E. coli* enzyme as being caused by interactions between adjoining catalytic subunits (Gerhart & Pardee, 1963). The stabilization of the *P. aureus* activity by P_1 has not been reported for any other aspartate transcarbamoylase, although it does appear that 0.1M-phosphate will protect the native *E. coli* enzyme from loss of CTP inhibition caused by heat pretreatment (Gerhart & Pardee, 1962).

The lower degree of UMP inhibition observed with the plant enzyme when the temperature of the reaction mixture was raised is similar to the effect of temperature on the baker's-yeast aspartate transcarbamoylase reaction (Kaplan & Messmer, 1969). With this enzyme, a decrease in inhibition by its feedback inhibitor, UTP, was observed as the temperature was raised. Kaplan & Messmer (1969) interpreted this result in terms of dissociation of the enzyme into subunits lacking regulatory sites, since, like the *E. coli* aspartate transcarbamoylase, baker's-yeast transcarbamoylase has a tendency to lose feedback-type inhibition after heat pretreatment at 60°C (Kaplan *et al.*, 1967). The *E. coli* enzyme is known to be dissociated into subunits by this type of heat pretreatment (Gerhart & Pardee, 1963). However, this may not be the explanation for the plant enzyme, since we found no change in the interaction coefficient over the temperature range used to study UMP inhibition, and there is as yet no evidence for

Scheme 1. Suggested kinetic mechanism for *P. aureus* aspartate transcarbamoylase

the existence of separate regulatory subunits from heat pretreatment or other results.

The present product-inhibition studies suggest an ordered Bi Bi mechanism for the *P. aureus* enzyme, as shown in Scheme 1. This mechanism has also been put forward for the catalytic subunit of *E. coli* aspartate transcarbamoylase (Porter *et al.*, 1969) and for the mouse spleen enzyme (Hoogenraad *et al.*, 1971). Although the initial-velocity work (Fig. 8) gives the appearance of a series of parallel lines for the reciprocal plots, characteristic of a Ping Pong mechanism, a result like this is not at all positive, since the lines may intersect far to the left of the ordinate. The mammalian hexokinases, which are known to have a sequential mechanism, behave in this way (Ning *et al.*, 1969), as does 3-hydroxyanthranilate oxidase (Ogasawara *et al.*, 1966). In the present case, K_{ia} , K_a , K_b and V_{max} , to use Cleland's (1970) notation, were calculated from the initial-velocity results, by using a computer program based on the rate equation for the ordered Bi Bi mechanism of Cleland (1970). This showed that, for the *P. aureus* enzyme, K_{ia} is less than K_a , and is not significantly different from zero. Since K_{ia} is the dissociation constant for the carbamoyl phosphate-enzyme complex, it is likely that the binding of carbamoyl phosphate to this plant enzyme is an essentially irreversible process.

References

- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1961) *Biochem. J.* **80**, 318–323
- Bethel, M. R. & Jones, M. E. (1969) *Arch. Biochem. Biophys.* **134**, 352–365
- Bresnick, E. & Mosse, H. (1966) *Biochem. J.* **101**, 63–69
- Changeaux, J. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 497–504
- Cleland, W. W. (1963a) *Biochim. Biophys. Acta* **67**, 104–137
- Cleland, W. W. (1963b) *Biochim. Biophys. Acta* **67**, 188–196
- Cleland, W. W. (1970) *Enzymes*, 3rd. edn., 2, 1–65
- Dixon, M. & Webb, E. C. (1958) *Enzymes*, p. 160, Longmans Green, London
- Gerhart, J. C. & Pardee, A. B. (1962) *J. Biol. Chem.* **237**, 891–896
- Gerhart, J. C. & Pardee, A. B. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 497–504
- Gerhart, J. C. & Schachman, H. K. (1965) *Biochemistry* **4**, 1054–1062
- Hill, A. J. (1913) *Biochem. J.* **7**, 471–480.
- Hoogenraad, N. J., Levine, R. L. & Kretchmer, N. (1971) *Biochem. Biophys. Res. Commun.* **44**, 981–988
- Kaplan, J. G. & Messmer, I. (1969) *Can. J. Biochem.* **47**, 477–479
- Kaplan, J. G., Duphil, M. & Lacroute, F. (1967) *Arch. Biochem. Biophys.* **119**, 541–551
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Neumann, J. & Jones, M. E. (1962) *Nature (London)* **195**, 709–710
- Neumann, J. & Jones, M. E. (1964) *Arch. Biochem. Biophys.* **104**, 438–447
- Ning, J., Purich, D. L. & Fromm, H. J. (1969) *J. Biol. Chem.* **244**, 3840–3846
- O'Donovan, G. A. & Neuhaud, J. (1970) *Bacteriol. Rev.* **34**, 278–343
- Ogasawara, N., Gander, J. E. & Henderson, L. M. (1966) *J. Biol. Chem.* **241**, 613–619
- Ong, B. L. & Jackson, J. F. (1970) *Proc. Aust. Biochem. Soc.* **3**, 17
- Ong, B. L. & Jackson, J. F. (1971) *Anal. Biochem.* **42**, 289–293
- Ong, B. L. & Jackson, J. F. (1972) *Biochem. J.* **129**, 583–593
- Porter, R. W., Modebe, M. O. & Stark, G. R. (1969) *J. Biol. Chem.* **244**, 1846–1859
- Prescott, L. M. & Jones, M. E. (1969) *Anal. Biochem.* **32**, 408–419
- Prescott, L. M. & Jones, M. E. (1970) *Biochemistry* **9**, 3783–3793
- Reid, M. S. & Bielecki, R. L. (1968) *Anal. Biochem.* **22**, 374–381
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Yon, R. J. (1971a) *Biochem. J.* **121**, 18p
- Yon, R. J. (1971b) *Biochem. J.* **124**, 10p