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# A COMPARISON OF THE THERMAL STABILITY AND SUBSTRATE BINDING CONSTANTS OF PROLYL-*t*RNA SYNTHETASE FROM *PHASEOLUS AUREUS* AND *DELONIX REGIA*

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**Key Word Index**—*Phaseolus aureus*; Leguminosae; *Delonix regia*; thermal stabilization; Pro-*t*RNA synthetase, imino acid specificity; substrate binding.

**Abstract**—Pro-*t*RNA synthetase from *P. aureus* and *D. regia* was protected against thermal denaturation by various substrates; the kinetics of this protection was investigated. The affinity of substrates for each synthetase was studied by a thermal inactivation technique. In the presence of ATP, Pro and several Pro-analogues were bound to each enzyme more efficiently than when ATP was absent. The efficiency of imino acid analogue binding, relative to that of Pro, was greater when ATP was absent. Pyrrolidine and 3-pyrroline were able to bind to the enzyme only in the presence of ATP. The ratio of the ATP/Pro binding constants for the *Delonix* enzyme was greater than that for the *Phaseolus* enzyme. Values for several thermodynamic parameters involved in substrate binding were determined for each synthetase. The results are discussed in relation to the order of substrate binding and the known differences in substrate specificity between the enzymes from *P. aureus* and *D. regia*.

## INTRODUCTION

THE SUBSTRATE specificity of a number of aminoacyl-*t*RNA synthetases from higher plants has recently been investigated in the authors' laboratories.[1–4] Many plants synthesize non-protein amino acids which are able to act as analogue molecules.[5] Such plants appear to have evolved a mechanism at the level of the aminoacyl-*t*RNA synthetases to exclude these potentially toxic compounds from proteins.[1,6] The Pro-*t*RNA synthetase from plants which contain azetidine-2-carboxylic acid (A2C), e.g. *D. regia*, differs in several ways from the enzyme found in plants lacking A2C, e.g. *P. aureus*.[2] The negligible A2C-dependent ATP-PPi exchange catalysed by the *Delonix* enzyme contrasts with the situation with *Phaseolus* enzyme, where A2C is activated with a $V_m$ of 55% of that of Pro.[2] The mechanism by which the *Delonix* enzyme discriminates against A2C is unknown, and so in this investigation we explore more fully the differences in substrate binding between this enzyme and that from *P. aureus*.

Rouget and Chapeville[7,8] and Papas and Mehler[9] have concluded that the binding of

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[1] LEA, P. J. and NORRIS, R. D. (1972) *Phytochemistry* **11**, 2897.
[2] NORRIS, R. D. and FOWDEN, L. (1972) *Phytochemistry* **11**, 2921.
[3] LEA, P. J. and FOWDEN, L. (1973) *Phytochemistry* **12**, 1903.
[4] ANDERSON, J. and FOWDEN, L. (1970) *Biochem. J.* **119**, 677.
[5] FOWDEN, L. (1970) in *Progress in Phytochemistry* (REINHOLD, L. and LIWSCHITZ, Y., eds.), Vol. 2, p. 203, Wiley, New York.
[6] PETERSON, P. J. and FOWDEN, L. (1965) *Biochem. J.* **97**, 112.
[7] ROUGET, P. and CHAPEVILLE, F. (1968) *European J. Biochem.* **4**, 305.
[8] ROUGET, P. and CHAPEVILLE, F. (1968) *European J. Biochem.* **4**, 310.
[9] PAPAS, T. S. and MEHLER, A. H. (1971) *J. Biol. Chem.* **246**, 5924.

substrates to Leu- and Pro-*t*RNA synthetases from *Escherichia coli* occurs in sequential reactions, in which binding of ATP precedes that of the amino acid substrate. We have shown that for most aminoacyl-*t*RNA synthetases from higher plants, ATP is superior to the specific amino acid substrate in protecting individual enzymes against thermal denaturation.[10] Kinetic analysis of thermal inactivation may be used to estimate protection constants ($\pi$) for enzyme-substrate complexes[11,12] in the absence of an enzyme reaction. The protection constant has been equated with the dissociation constant of the enzyme-protector complex.[13] In view of the known instability of many synthetases,[14] especially the Pro-enzyme from A2C-containing plants,[2] it has been difficult to obtain homogeneous enzyme preparations for binding studies. However, since the thermal inactivation technique does not require a highly purified enzyme preparation, we have used this method to estimate the dissociation constants of complexes involving the Pro-*t*RNA synthetase from *Phaseolus* and *Delonix* and its protecting compounds, in order to provide further information regarding the differences in the substrate specificity of these two enzymes.

## RESULTS

### *Enzyme Preparation and Thermal Denaturation Procedure*

The Pro-*t*RNA synthetase from *D. regia* was unstable on DEAE-cellulose whilst the enzyme from *P. aureus* gave variable results after fractionation on hydroxyapatite. Therefore Sephadex G200 or DEAE-cellulose fractions of the *Phaseolus* enzyme and desalted $(NH_4)_2SO_4$ fractions of the *Delonix* enzyme were generally used for experiments.

Substrate protection of Pro-*t*RNA synthetase due to amino acids liberated by proteolysis during the thermal denaturation period was minimized by the use of enzyme preparations having endogenous ATP-$^{32}$PPi exchange rates of not more than 3% that of the Pro-stimulated exchange. $(NH_4)_2SO_4$ fractions of both the *Phaseolus* and *Delonix* enzyme contained negligible ATPase activity: the maximum amount of ATP hydrolysed in these preparations after preincubation at 60° for 20 min was 0·08 $\mu$mol (2% of that present in the assay medium). However, since the binding constant of ATP for both synthetases was very low, it was essential in some experiments to use DEAE-cellulose fractions in which less than 0·02 $\mu$mol of ATP were hydrolysed under the above conditions. $(NH_4)_2SO_4$ fractions containing Pro-*t*RNA synthetase, exhibited high pyrophosphatase activity; more than 4 $\mu$mol of Pi were liberated from PPi after heat treatment at 70° for 15 min, i.e. approximately 75% of the amount of PPi originally present. Therefore DEAE-cellulose or Sephadex G200 fractions of Pro-*t*RNA synthetase, which contained little pyrophosphatase activity, were used in these experiments where PPi was present as a protecting substrate during the thermal treatment. Under the normal assay conditions (see Experimental), less than 7% of Pi was liberated from PPi using $(NH_4)_2SO_4$ fractions.

When necessary, *t*RNA was removed from enzyme preparations by Sephadex G75 in order to exclude any effects due to the binding of this polynucleotide to the enzyme and to enable the protection constants to be compared more accurately with the $K_m$ values determined by the ATP-$^{32}$PPi exchange reaction.

Most preparations of Pro-*t*RNA synthetase from *Phaseolus* gave reproducible results

[10] NORRIS, R. D. and FOWDEN, L. (1973) *Biochim. Biophys. Acta* in press.
[11] BURTON, K. (1951) *Biochem. J.* **48,** 458.
[12] CHUANG, H. Y. K., ATHERLY, A. G. and BELL, F. E. (1967) *Biochem. Biophys. Res. Commun.* **28,** 1013.
[13] CHUANG, H. Y. K. and BELL, F. E. (1972) *Arch. Biochem. Biophys.* **152,** 502.
[14] LOFTFIELD, R. B. (1971) in *Proteins Syntheis* (MCCONKEY, E., ed.), Vol. 1, p. 1, Marcel Dekker, New York.

when 200–500 $\mu$g protein was present per ml preincubation medium, although purer preparations required the addition of bovine serum albumin (100–300 $\mu$g/ml) in order to obtain linear denaturation kinetics. The concentration of enzyme protein in the preincubation medium was kept below 50 $\mu$g/ml in order to minimize the action of proteases and pyrophosphatase.[15,16]

Since the Pro-enzyme from *Delonix* was cold-labile, enzyme preparations from this plant were warmed at 30° for 10 min in the presence of 40 mM mercaptoethanol, prior to thermal denaturation, to convert all the Pro-*t*RNA synthetase molecules into a catalytically active form (Norris and Fowden, in preparation). This procedure was not necessary with the *Phaseolus* enzyme if fresh preparations were used. However, the Pro-*t*RNA synthetase from some batches of *Phaseolus* appeared to be somewhat cold labile and the enzyme was then pretreated in the same manner as the *Delonix* enzyme.

No reversal of the heat inactivation was observed after the partially heat-denatured enzyme from *Phaseolus* had been stored at room temperature for up to 20 hr after the heating procedure.

The ATP-$^{32}$PPi exchange reaction was generally used to measure residual Pro-*t*RNA synthetase activity because this method was extremely sensitive when compared with the alternative aminoacylation assay procedure. The heat denaturation profiles as measured by the ATP-$^{32}$PPi exchange or the *t*RNA esterification reactions were not significantly different. However, the poor aminoacylation of Pro and difficulties in obtaining suitable *t*RNA preparations from *Delonix* made the latter method unreliable.

*General Kinetics and Heat Stability*

Thermal denaturation of the Pro-*t*RNA synthetase from *Phaseolus* obeyed first order kinetics over a temperature range of 45–70° in the absence of substrates or sulphydryl reagents. However, some preparations yielded non-linear inactivation curves over this range, presumably because freshly denatured enzyme molecules could stabilize the remaining active enzyme. A first order curve was usually obtained in these cases by the addition of bovine serum albumin to the preincubation medium. The Arrhenius plot of the logarithm of the rate constants of thermal inactivation against $1/T$°K was linear over the range 50–70° for the *Phaseolus* enzyme and the energy of activation ($E_a$) for the inactivation reaction was calculated from the slope of the graph ($=E_a/2{\cdot}3R$) as 60 kcals/mol.

In the absence of sulphydryl reducing reagents, the Pro-enzyme from *Delonix* was extremely heat labile, exhibiting an anomalous and poorly-reproducible heat denaturation curve. In the presence of 40 mM mercaptoethanol or dithiothreitol the enzyme was stabilised considerably (Fig. 1) and exhibited the normal type of denaturation profile. The apparent thermal inactivation of the *Delonix* enzyme occurring between 30 and 40° in the absence of sulphydryl reducing reagents was partially reversible, because some ATP-$^{32}$PPi exchange activity was regained by warming the inactivated enzyme for 10 min at 30° with 40 mM mercaptoethanol. The rate constants of thermal inactivation in the presence of mercaptoethanol were approximately first order over a temperature range of 40–52° although they were non-linear in the absence of this compound.

Bovine serum albumin and glycerol partially protected both the *Delonix* and *Phaseolus* enzymes against thermal denaturation at 45° and 50° respectively, but at temperatures above 60° little protection was afforded to the *Phaseolus* enzyme by these reagents (Table 1).

[15] Moustafa, E. and Proctor, M. H. (1962) *Biochim. Biophys. Acta* **63**, 93.
[16] Anderson, J. W. and Fowden, L. (1969) *Plant Physiol.* **44**, 60.

The relationship between temperature and $V_m$ of the ATP-$^{32}$PPi exchange catalysed by the Pro-*t*RNA synthetase from both *Phaseolus* and *Delonix* exhibited two marked breaks (see Fig. 2). The almost constant initial rates of exchange at temperatures above 40° were not caused by insufficient saturation of the enzyme with its substrates.[17] The $K_m$ values for both the *Delonix* and *Phaseolus* enzymes did not change significantly between 25 and 56°.

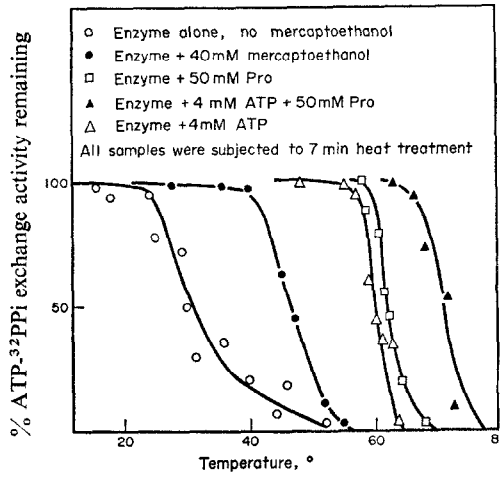


FIG. 1. THERMAL DENATURATION PROFILE OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.

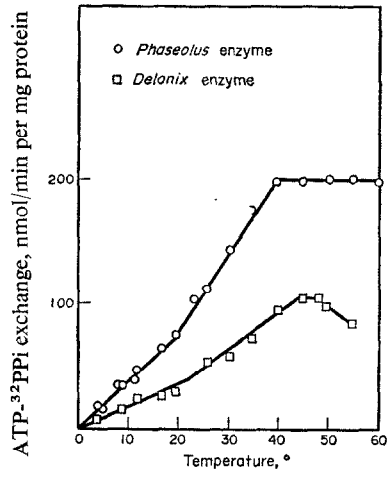


FIG. 2. VARIATION OF INITIAL RATE OF PRO-DEPENDENT ATP-$^{32}$PPi EXCHANGE WITH TEMPERATURE.

*Stabilization by Substrates*

The presence of Pro or ATP during the thermal inactivation step markedly stabilized the Pro-*t*RNA synthetase from *Delonix*; at concentrations providing maximum protection, Pro was slightly more effective than ATP (Fig. 1). This contrasted with the protection conferred by these substrates upon the Pro-enzyme from *Phaseolus* where ATP was superior to the imino acid substrate; for a 7 min heating period, the respective $t^{1/2}$ values (the temperature at which 50% of the ATP-$^{32}$PPi exchange activity remained after heating for a fixed time interval) determined, (a) in the absence of substrates, (b) in the presence of Pro (50 mM), or (c) ATP (4 mM) were 66°, 73° and 77°. When ATP and Pro were both present during the thermal inactivation step the enzyme was stabilized more effectively than when either substrate was present alone; the $t^{1/2}$ values obtained for the *Delonix* and *Phaseolus* enzymes (7 min heating period) were 70° and 84° respectively.

First order kinetics for inactivation were observed over a wide range of Pro and ATP concentrations and the Arrhenius plots for the Pro-enzyme and ATP-enzyme complexes were linear over the range of temperatures tested, i.e. 50–60° for the *Delonix* and 60–78° for the *Phaseolus* enzymes.

At pH 8, in the absence of $Mg^{2+}$, ATP did not protect either the *Delonix* or *Phaseolus* enzyme against thermal denaturation (Fig. 3); but $Mg^{2+}$ was not an essential requirement for the binding of Pro (Fig. 4) nor did it confer added stability on the enzyme at pH 8 in the absence of substrates. However, at pH 6·5, $Mg^{2+}$ (10 $\mu$mol) appeared to stabilize the

[17] DIXON, M. and WEBB, E. C. (1964) *Enzymes*, Chap. IV, p. 145, Longmans, London.

*Delonix* enzyme, the rate constant of thermal inactivation being reduced by more than 50% in the presence of $Mg^{2+}$.

The *Phaseolus* enzyme was not protected against thermal inactivation by a homologous (*Phaseolus*) or heterologous (*Delonix*) *t*RNA preparation (0·3 mg) at temperatures above 55°, but when the enzyme from *Phaseolus* was heated at 45° with a homologous *t*RNA preparation, the rate constant of thermal inactivation was reduced by 8%. In the absence of mercaptoethanol, the *Delonix* enzyme appeared to be stabilized at 43° by a homologous *t*RNA preparation, the rate constant of inactivation being decreased 10–20% by *t*RNA. In the absence of mercaptoethanol this protection was not so marked.

TABLE 1. THE ABILITY OF PROTEIN AND GLYCEROL TO PROTECT THE Pro-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia* AGAINST THERMAL DENATURATION

| Heat treatment | Additions | % ATP-$^{32}$PPi activity remaining |
|---|---|---|
| *Phaseolus* enzyme | | |
| 50°, 18 hr | none* | 10 |
| | 15% glycerol* | 35 |
| | 40 mM mercaptoethanol* | 7 |
| | 1·5 mg bovine serum albumin* | 20 |
| | 3 mg *Phaseolus* protein* | 30 |
| 65°, 30 min | none* | 12 |
| | 15% glycerol* | 15 |
| | 0·5 mg bovine serum albumin* | 15 |
| *Delonix* enzyme (40 mM mercaptoethanol present) | | |
| 45°, 16 min | none* | 20 |
| | 15% glycerol* | 50 |
| | 1·5 mg bovine serum albumin* | 30 |
| | 3 mg *Delonix* protein† | 37 |

* 500 μg protein/reaction tube of an $(NH_4)_2SO_4$ fraction after passage through Sephadex G75.

† Diluted after thermal treatment to protein concentration of 500 μg/tube.

*Effect of pH on Substrate Protection*

The degree of protection against heat denaturation conferred by substrates of the *Delonix* or *Phaseolus* Pro-*t*RNA synthetases varied markedly with pH (Figs. 3–5). In the absence of $Mg^{2+}$, the *Phaseolus* enzyme was apparently protected by ATP at pH values between 5·5 and 6·5 (Fig. 3), although the enhanced stability of the enzyme itself in this pH region (in the presence or absence of $Mg^{2+}$) may partly account for this (Fig. 5).

*Specificity of Stabilization*

Several analogues of Pro which did not stimulate ATP-$^{32}$PPi exchange catalysed by Pro-*t*RNA synthetase from either *Delonix* or *Phaseolus* were able to protect both enzymes against thermal inactivation. Increasing concentrations of protecting substrate increasingly protected each enzyme against thermal denaturation and in most cases first order inactivation kinetics were obtained over a wide temperature range. The rate constants of inactivation calculated for different concentrations of protecting substrates were plotted by a

method similar to that of Lineweaver and Burk. The value of the protection constant at 45° ($\pi^{45°}$) was calculated by extrapolation of a Van't Hoff plot ($-\log \pi$ vs. $1/T°K$) obtained by determining $\pi$ at several different elevated temperatures (see Ref. 13). The Van't Hoff plots were linear for each protecting compound over the range of temperature used to calculate the $\pi$ values. Enzyme preparations from *Delonix* always contained 40 mM mercaptoethanol to ensure approximately first order decay kinetics.

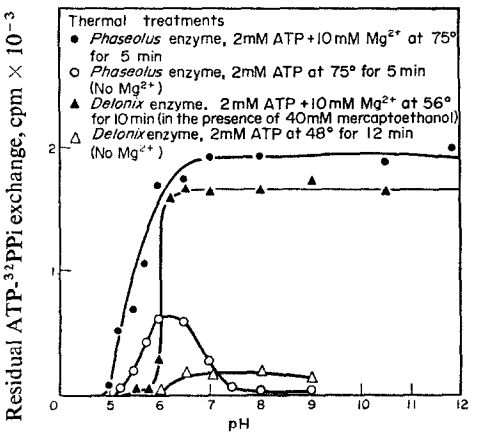


FIG. 3. EFFECT OF pH UPON THE ATP-DEPENDENT PROTECTION OF Pro-*t*RNA SYNTHETASE AGAINST THERMAL INACTIVATION.

A series of buffers of constant ionic strength (0·05 M) was used over the pH range and slight rate corrections made for specific buffer effects; pH 5–6·8, maleic acid–KOH; pH 6–10, Tris–maleate–KOH; pH 11–12 hydroxide-chloride. After the thermal treatment, the pH was adjusted to 8 by the addition of 0·2 M Tris and either HCl or KOH. Cpm in unheated controls: *Phaseolus* enzyme, 2300; *Delonix* enzyme, 2100.

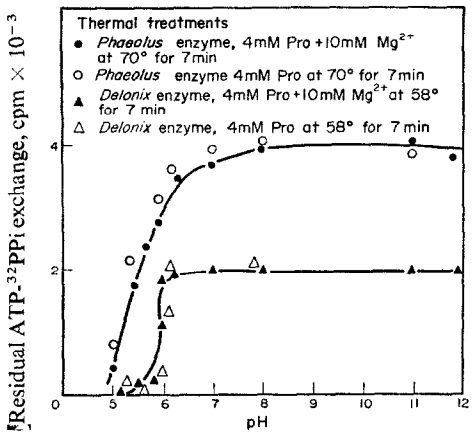


FIG. 4. EFFECT OF pH UPON THE PRO-DEPENDENT PROTECTION OF Pro-*t*RNA SYNTHETASE AGAINST THERMAL INACTIVATION.

(*Delonix* enzyme was preincubated in the presence of 40 mM mercaptoethanol.) For buffer see Fig. 3. Cpm in unheated controls: *Phaseolus* enzyme, 4200; *Delonix* enzyme, 2500.

In the absence of ATP, the $\pi^{45°}$ values for the binding of Pro to the *Phaseolus* or *Delonix* enzyme (Table 2) were considerably lower than the $K_m^{45°}$ values of $1{\cdot}5 \times 10^{-4}$ M and $2{\cdot}6 \times 10^{-4}$ M respectively, calculated for the ATP-$^{32}$PPi exchange reaction. Most analogues of Pro were also efficiently bound in comparison with Pro (Table 2).

In the presence of ATP, Pro was bound to both the *Delonix* and *Phaseolus* enzymes more efficiently than in its absence. However, in the presence of ATP, most Pro analogues were bound less efficiently in relation to the binding of Pro (Tables 2 and 3). In most instances the $\pi$ analogue/$\pi$ proline ratio in the presence of ATP bore more resemblance to the $K_m$ analogue/$K_m$ proline ratio for the ATP-$^{32}$PPi exchange reaction than did the $\pi$ analogue/$\pi$ proline ratio determined in the absence of ATP (Table 3). The $\pi$ ATP/$\pi$ proline (–ATP) ratio for the *Phaseolus* enzyme was 0·01 whilst this ratio for the *Delonix* enzyme was 0·1–0·2. In the presence of 20 mM Pro, $\pi^{45°}$ values for ATP were reduced to $5 \times 10^{-10}$M and $2–3 \times 10^{-8}$ M for the *Phaseolus* and *Delonix* enzymes respectively.

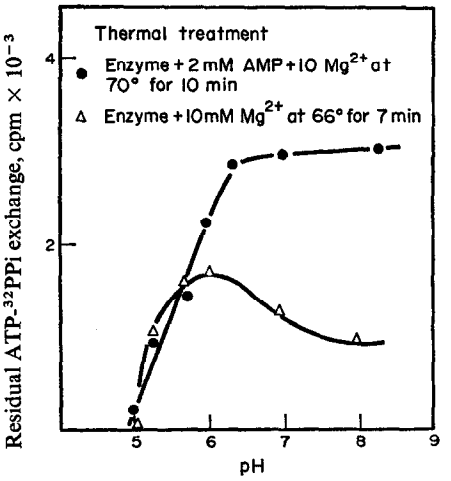


FIG. 5. EFFECT OF pH UPON THE AMP-DEPENDENT PROTECTION OF Pro-*t*RNA SYNTHETASE FROM *Phaseolus* AND UPON THE ENZYME IN THE ABSENCE OF SUBSTRATES.

For buffers see Fig. 3. Cpm in unheated controls: enzyme + $Mg^{2+}$ + AMP, 4000; enzyme + $Mg^{2+}$ (−AMP), 3600.

Cyclopentane carboxylic acid and L-baikiain were not bound to the enzyme in the presence or absence of ATP, but *cis*-3-hydroxy-L-proline protected the *Delonix* enzyme slightly against thermal inactivation in the absence of ATP ($\pi^{45°} \sim 10^{-1}$ M). Tetrahydrofuran and tetrahydrothiophen protected the *Phaseolus* and *Delonix* enzymes only in the presence of ATP; $\pi$ values could not be obtained with any degree of reproducibility ($> 10^{-1}$ M at 45°).

TABLE 2. $\pi^{45°}$ VALUES FOR SUBSTRATE PROTECTION OF PROLYL-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*

| Protecting compound | *Phaseolus* | | *Delonix* | |
|---|---|---|---|---|
| | $\pi^{45°}$ | | $\pi^{45°}$ | |
| | No ATP | 4 mM ATP | No ATP | 4 mM ATP |
| | Molarity | | | |
| L-Proline | $2{\cdot}5 \times 10^{-5}$ | $3\text{–}6 \times 10^{-8}$ | $0{\cdot}8\text{–}1 \times 10^{-4}$ | $\sim 2 \times 10^{-7}$ |
| 3,4-Dehydro-DL-proline† | $1 \times 10^{-5}$ | $\sim 10^{-7}$ | $1 \times 10^{-3}$ | $5 \times 10^{-6}$ |
| *cis*(*exo*)-3,4-Methano-L-proline | $2\text{–}5 \times 10^{-3}$ | $10^{-2}$–$10^{-3}$* | $1 \times 10^{-3}$ | $1\text{–}5 \times 10^{-4}$ |
| D-Proline | $0{\cdot}7\text{–}1{\cdot}3 \times 10^{-1}$ | $10^{-2}$* | $\sim 10^{-1}$* | $> 10^{1}$* |
| *N*-Methylglycine | $7\text{–}8 \times 10^{-4}$ | $0{\cdot}8\text{–}1 \times 10^{-4}$ | $> 10^{1}$ | $\sim 10^{-2}$* |
| L-Thiazolidine-4-carboxylic acid | $1\text{–}5 \times 10^{-2}$ | $5 \times 10^{-1}$* | $\sim 0{\cdot}5\text{–}1 \times 10^{-1}$ | $10^{-2}$–$10^{-3}$* |
| L-Azetidine-2-carboxylic acid | $3 \times 10^{-5}$ | $\sim 2 \times 10^{-6}$ | $3\text{–}6 \times 10^{-3}$ | $8 \times 10^{-4}$ |
| Pyrrolidine | $> 10^{1}$ | $4\text{–}6 \times 10^{-7}$ | $\infty$ | $3 \times 10^{-6}$ |
| 3-Pyrroline | $\infty$ | $1\text{–}2 \times 10^{-6}$ | $\infty$ | $7 \times 10^{-5}$ |
| ATP | $2\text{–}3 \times 10^{-7}$ | | $2\text{–}3 \times 10^{-5}$ | |
| AMP | $1{\cdot}2 \times 10^{-6}$ | | $\geqslant 10^{-3}$ | |

* $\pi^{45°}$ values calculated from data obtained with concentrations of analogues giving less than 25% protection.

† $\pi$ values corrected for D form assuming a protection equivalent to that exhibited by D-Pro.

The rate constants of thermal inactivation of the *Phaseolus* and *Delonix* enzymes were reduced by less than 10% when heated for 7 min at 67° and 47° respectively in the presence of 4 mM PPi. Adenine, GTP, UTP, ITP and CTP all failed to bind to the enzyme although ADP exhibited a slight protective effect.

TABLE 3. COMPARISON OF THE RELATIVE EFFICIENCY OF VARIOUS PRO-ANALOGUES TO CATALYSE ATP-$^{32}$PPi EXCHANGE AND PROTECT THE PRO-*t*RNA SYNTHETASE FROM *Delonix regia* AND *Phaseolus aureus* AGAINST THERMAL INACTIVATION

| | *Phaseolus aureus* | | | *Delonix regia* | | |
|---|---|---|---|---|---|---|
| Protecting compound | $K_m$ analogue* / $K_m$ Proline | −ATP $\pi$ analogue / $\pi$ Proline | +ATP $\pi$ analogue / $\pi$ Proline | $K_m$ analogue / $K_m$ Proline | −ATP $\pi$ analogue / $\pi$ Proline | +ATP $\pi$ analogue / $\pi$ Proline |
| 3,4-Dehydro-DL-proline | 2 | 0·4 | ~2 | 4 | 10 | 20 |
| *cis*(*exo*)-3,4-Methano-L-proline | ∞ | ~$10^2$ | ~$10^3$ | 25 | 10 | $10^2$ |
| D-Proline | ∞ | 4 × $10^2$ | ~$10^4$ | ∞ | ~$10^3$ | 2 × $10^4$ |
| *N*-Methylglycine | 3 × $10^2$ | 30 | ~2 × $10^3$ | 1·5 × $10^3$ | $10^4$ | 5 × $10^4$ |
| L-Thiazolidine-4-carboxylic acid | $10^2$ | $10^3$ | $10^7$ | 2 × $10^2$ | ~8 × $10^2$ | ~5 × $10^3$ |
| L-Azetidine-2-carboxylic acid | 10 | 1·2 | ~40 | ∞ | ~40 | 4 × $10^3$ |
| Pyrrolidine | 15 | >$10^4$ | ~10 | 14 | ∞ | 15 |
| 3-Pyrroline | 6 | ∞ | ~20 | 14 | ∞ | ~300 |

* Data obtained from Ref. 2.

### *Thermodynamic Constants Associated with Substrate Protection*

The energy of activation ($E_a$) for the thermal inactivation reaction was estimated for complexes of Pro-*t*RNA synthetase with either Pro or ATP or Pro ATP, the substrates being present during the thermal treatment at saturating protective concentrations (Table 4).

TABLE 4. THERMODYNAMIC CONSTANTS ASSOCIATED WITH THE PROLYL-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*

| | *Phaseolus* | | | | *Delonix* | | | |
|---|---|---|---|---|---|---|---|---|
| Enzyme system* | $E_a$* (kcal/mol) | $-\Delta F$* (kcal/mol) | $-\Delta H$* (kcal/mol) | $-\Delta S°$* (cal/mol/°K) | $E_a$* (kcal/mol) | $-\Delta F$* (kcal/mol) | $-\Delta H$* (kcal/mol) | $-\Delta S°$* (cal/mol/°K) |
| Enzyme alone | 60 | | | | 45 | | | |
| Enzyme-Pro complex | 95 | 6·8 | 71 | 200 | 70 | 6·0 | 65 | 185 |
| Enzyme-ATP complex | 110 | 9·6 | 80 | 220 | 65–70 | 6·8 | 90 | 260 |
| Enzyme-Pro-ATP complex (Pro and ATP at saturating protective concentrations) | 120 | | | | 115 | | | |
| Enzyme-Pro-ATP complex (ATP at saturating concentrations Pro variable) | | 11·0 | 67 | 175 | | 9·7 | 75 | 205 |

* See text for details.

In the presence and absence of ATP, there was a pronounced temperature sensitivity of the $\pi$ values. The enthalpy ($\Delta H$) for the binding of various substrates was calculated from the slope of the Van't Hoff plots of $\pi$ values [slope $= (\Delta H + RT)/2{\cdot}3R$]. However, the temperature sensitivity of the $K_m$ values for Pro in the exchange reaction showed a much smaller temperature dependence; the $K_m$ values for the *Phaseolus* enzyme ranged from 1·3 × $10^{-4}$ M at 30° to 4–5 × $10^{-4}$ M at 56° whilst the $K_m$ values for the *Delonix* enzyme

ranged from $2 \times 10^{-4}$ M at 30° to 3–5 $\times 10^{-4}$ M at 48°. These values correspond to an enthalpy change for the exchange reaction of not more than −10 kcal/mol for each enzyme. The $\Delta H$ value for A2C-binding to the Pro-*t*RNA synthetase in the presence of ATP was calculated as −50 to −55 kcal/mol for the *Delonix* enzyme and −55 to −60 kcal/mol for the *Phaseolus* enzyme.

The free energy change ($\Delta F$) due to substrate binding was calculated from the $\pi^{45°}$ values [$-\Delta F = 2{\cdot}3RT \log (1/\pi^{45°})$] and from this value, the entropy change ($-\Delta S$) was estimated from the equation $-\Delta S° = (\Delta H - \Delta F)/T$ (Table 4).

## DISCUSSION

The ability of substrates to protect aminocyl-*t*RNA synthetase against thermal inactivation is well documented.[3,10,18–20] The differences in thermal stability between the *Delonix* and *Phaseolus* Pro-enzyme were very marked but in the presence of both Pro and ATP the *Delonix* enzyme was stabilized to a greater extent as measured by the increase in $t^{1/2}$ values. The $t^{1/2}$ and $\pi$ values for substrate protection suggested that Pro was relatively more efficient as a protective substrate for the Pro-*t*RNA synthetase from *Delonix* compared with the *Phaseolus* enzyme. The anomalous heat denaturation curve of the *Delonix* enzyme in the absence of mercaptoethanol may reflect a rapid oxidation of an SH group on the enzyme since the synthetase could be partially reactivated by the addition of a sulphydryl reducing reagent.

The breaks in the temperature/initial velocity profile for ATP-$^{32}$PPi exchange catalysed by both the *Delonix* and *Phaseolus* enzymes may be due to different molecular forms of the Pro-*t*RNA synthetase. The *Delonix* enzyme exists as two molecular forms; an active form which is present mainly at temperatures above 23° and an inactive form which is present below 23°. The transition temperature between these forms (23°) corresponds to the lower break in the temperature/velocity profile. Similar temperature-dependent changes in enzyme activity have been reported for urease,[21] fumarate hydratase[22] and the *E. coli* Pro-*t*RNA synthetase.[23,24] The inflection in the temperature/velocity profile at 40° might be related to the possibility that the overall ATP-$^{32}$PPi exchange reaction involves two steps; (a) binding of ATP and Pro to the enzyme, and (b) pyrophosphorylase of ATP. Since the temperature coefficient of each of these steps may be different, the overall rate of exchange would then be limited by the reaction with the lower temperature coefficient.[25] Since the temperature coefficient of ATP and Pro binding is high (Table 4) and the binding of PPi to the enzyme is relatively inefficient, it is possible that the limiting factors which account for the constant rate of exchange above 40° are the binding of PPi and pyrophosphorolysis of ATP. The activity vs. temperature curves for all other aminoacyl-*t*RNA synthetases so far studied have shown no unusual features.[26,27] However, the Pro-*t*RNA synthetase from *E. coli*

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exhibits a 'flat curve'[28] for the esterification reaction, but unlike the plant enzyme showed a conventional curve for the exchange reaction.

The ability of substrates to protect Pro-*t*RNA from *Delonix* and *Phaseolus* was markedly pH dependent. The sudden drop in protection at pH 6 for the *Delonix* enzyme may reflect a dissociation of the enzyme into inactive subunits such as occurs with aldolase.[29] The enhanced stability of the *Phaseolus* enzyme at pH 6 may reflect the changes of the group ionization constants which occurs on changing the pH,[30] leading to a more stable or less soluble form of the enzyme. The absolute requirement for $Mg^{2+}$ in the ATP-binding reaction is clearly illustrated by the heat denaturation data for both the *Delonix* and *Phaseolus* enzymes. The absence of a $Mg^{2+}$ requirement for Pro binding also suggests that a metal-ion bond is not involved in binding the carboxyl group of the imino acid to the enzyme.

The exclusion of *t*RNA from the enzyme preparations used in the thermal inactivation systems allowed an accurate estimation of the substrate dissociation constants to be made. Some aminoacyl-*t*RNA synthetases have shown a complex dependence on the presence of *t*RNA[31–34] and in several instances protection of an individual synthetase by the specific *t*RNA has been demonstrated.[35,36] Although the *Phaseolus* enzyme appeared not to be protected against thermal inactivation by *t*RNA at temperatures above 55°, this may merely reflect an alteration in the tertiary structure of the polynucleotide at this temperature which prevents it binding to the enzyme. The protection observed at lower temperatures by the addition of *t*RNA to both the *Delonix* and *Phaseolus* enzymes might also be accounted for by non-specific effects or by contaminating polynucleotides in the *t*RNA preparations.[37] The ability of reagents, such as glycerol and protein, to protect aminoacyl-*t*RNA synthetases against thermal denaturation has also been described elsewhere.[13,38]

The $\pi^{45°}$ value for Pro in the absence of ATP for the *Phaseolus* enzyme was much lower (compared with the $K_m$ value for Pro-dependent ATP-$^{32}$PPi exchange) than those observed for Pro-enzyme from rat liver[12] or *E. coli*[39] or for several other synthetases from higher plants[10] and bacteria.[40] Previous measurements of the protective ability of individual analogues made at single temperatures and substrate concentrations[2] cannot be strictly related to the $\pi$ values because of differences in; (a) the slopes of the Van't Hoff plots,(b) the denaturation kinetics in the absence of mercaptoethanol, which accentuates substrate protection, and (c) the maximum protective concentration of substrates required for each enzyme.

The inefficient binding of pyrrolidine and 3-pyrroline to either enzyme in the absence of ATP suggests that the carboxyl group of Pro may be required in order to stabilize the enzyme against thermal inactivation. The role of the carboxyl group, however, must be secondary, since cyclopentane carboxylic acid did not act as a protective substrate. The $\pi^{45°}$ values for substrate binding in the absence of ATP indicated that analogues smaller

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than Pro, e.g. A2C, 3,4-dehydroproline and *N*-methylglycine, protected the *Delonix* enzyme less efficiently, relatively to Pro, than they did the *Phaseolus* enzyme. This seems to confirm the hypothesis that the *Delonix* enzyme possessed a larger active site.[2] Possibily the postioning of smaller analogues at this larger site on the *Delonix* enzyme leads to an incorrect alignment of the carboxyl group with some functional group within the enzyme, thus reducing the magnitude of the conformational change in the enzyme molecule that constitutes a necessary part of an efficient stabilization mechanism. The ability of methanoproline to protect the *Phaseolus* enzyme and of A2C to protect the *Delonix* enzyme against thermal denaturation, together with the ability of D-Pro to bind to both enzymes, suggests that the orientation of the carboxyl group of the substrate required for the binding reaction is less exacting than that required to promote the exchange reaction.

In the presence of ATP, Pro was more efficiently bound to both the *Delonix* and *Phaseolus* Pro-enzyme than when ATP was absent. $\pi^{45°}$ values for ATP were lower than the corresponding values for Pro with both the *Delonix* and *Phaseolus* enzymes and so it is probable that the nucleoside triphosphate binds to the synthetase before the imino acid substrate thereby causing a modification of the Pro binding site which restricts the binding of certain larger Pro analogues. In this manner, catalysis of ATP-$^{32}$PPi exchange would not be promoted by bulkier analogues or by compounds in which the carboxyl group became incorrectly aligned with the ATP molecule, e.g. D-proline, thiazolidine-4-carboxylic acid, *cis*-3-hydroxyproline. The binding of pyrrolidine and 3-pyrroline only in the presence of ATP suggests that the nucleoside triphosphate causes a conformation change in the enzyme that modifies the Pro-binding site. According to current theories of aminoacyl-*t*RNA synthetase mechanism, the carboxyl group of the amino acid substrate is closely associated with the $\alpha$-$PO_4^{3-}$ group of ATP at the active site of the enzyme.[14,41,42] Hence ATP would stabilize the protein in the region where the carboxyl group of the amino acid would fit thus allowing pyrrolidine and 3-pyrroline to elicit a conformational change in the enzyme which would be impossible in the absence of ATP. The increase in the $\pi$ analogue/$\pi$ proline ratios observed for most analogues on addition of ATP, presumably reflects a reduced flexibility of the Pro binding site after binding of the nucleoside triphosphate. However, this increased rigidity about the binding site reflects itself differentially in respect of different analogues for the *Delonix* and *Phaseolus* enzymes. Thus with the *Phaseolus* enzyme, *N*-methylglycine was bound 60 times and A2C 30 times more efficiently (relative to Pro) in the presence of ATP, than in its absence; corresponding figures for the *Delonix* enzyme were 5 and 100 respectively. However, the $\pi$A2C/$\pi$Pro ratio for the *Delonix* enzyme was considerably higher than the corresponding value for *Phaseolus* enzymes either in the presence or absence of ATP. Therefore the ability of the *Delonix* enzyme to discriminate against A2C may be envisaged in terms of the inefficiency of this analogue to elicit a suitable conformational change in the larger Pro-binding site of this enzyme.

The thermodynamic data lend further support to the conclusions reached above. The activation energy for the inactivation reaction in the presence of ATP and Pro was approximately the same for both enzymes. For many proteins the energy of inactivation for the thermal denaturation reaction is considerably higher than the energy of activation under non-denaturing conditions.[43] The high $E_a$ values reported here for the higher plant

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Pro-*t*RNA synthetases are similar to those recorded for the denaturation reaction of many enzymes.[25] The substrate binding abilities of Pro and ATP as measured by $\pi$ values was also reflected by the differences in the $E_a$ values. The change in enthalpy associated with the formation of the enzyme-protector complexes was quite large compared with the negligible overall enthalpy change for the ATP-$^{32}$PPi exchange reaction. This discrepancy is probably due to the rate limiting temperature coefficient of pyrophosphorolysis as described above. The large enthalpy change on binding presumably indicates that a high degree of ordering of the protein intramolecular structure occurs on the binding of either ATP or Pro in agreement with the above postulate that Pro-analogues elicit a conformational change in the enzyme. The $\Delta H$ value of the Pro-enzyme complex for the *Delonix* enzyme was considerably smaller, in comparison with the $\Delta H$ value of the ATP-enzyme complex, than that evaluated for the *Phaseolus* enzyme. The large enthalpy change observed for the ATP-Pro-enzyme complex with both enzymes suggest that even though ATP may cause a conformational change in the enzyme, a further change in molecular ordering occurs when Pro is subsequently bound.

A comparison of the enthalpy changes for Pro and A2C in the presence of ATP indicates that the analogue molecule causes comparatively less intramolecular ordering of the *Delonix* enzyme than of the *Phaseolus* enzyme, but whether this is responsible for the discriminatory ability of the Pro-*t*RNA synthetase from *Delonix* remains to be conclusively proved.

## EXPERIMENTAL

*Plant materials.* Seed of *P. aureus* was obtained commercially. *D. regia* seeds were the gift of Professor L. B. Thrower (Hong Kong).

*Amino acids and analogues.* All samples were tested for purity by PC. D-Proline was obtained from Calbiochem. All other analogues were obtained from sources previously described,[2] except 3-pyrroline which was obtained from Koch-Light Ltd. and pyrrolidine from British Drug Houses.

*Radioisotopic chemicals.* L-[U-$^{14}$C]Pro (265 mCi/mmol) and sodium [$^{32}$P]orthophosphate were obtained from the Radiochemical Centre, Amersham. [$^{32}$P]Pyrophosphate was prepared by the method of Bell[44] to give a product of specific activity 0·2–0·4 $\mu$Ci/$\mu$mol.

*Determination of protein and nucleic acids.* Protein was determined by the Lowry method[45] with bovine serum albumin as a standard. The amount of RNA in the synthetase preparation was measured by the method of Key and Shannon[46] or by the orcinol method;[47] yeast RNA was used as a standard.

*Enzyme fractionation.* P. aureus. (a) *Homogenate.* All fractionation procedures were carried out at 5°. Seed meal (16 g) were ground for 2–4 min in a mortar with 190 ml 0·1 M Tris–HCl buffer pH 8, containing 15% (v/v) glycerol, 0·3% PVP (soluble, approx. MW 44 000), 10 mM $MgCl_2$ and 40 mM mercaptoethanol. The homogenate was then centrifuged at 24 000 *g* for 20 min. (b) *Acetone fractionation.* Acetone (31 ml) at −20° was added to 160 ml supernatant from (a) and precipitation allowed to proceed for 15 min at −20°. Sedimented protein (15 000 *g*, 10 min) was discarded and a further 16 ml acetone added to the supernatant. The protein ppt. at this stage was redissolved in 48 ml 0·1 M Tris–HCl buffer pH 8, containing 15% glycerol and 40 mM mercaptoethanol. (c) $(NH_4)_2SO_4$ *fractionation.* 16·5 g $(NH_4)_2SO_4$ was added to 48 ml of the redissolved acetone fraction and protein ppt. allowed to proceed for 30 min. After centrifugation (25 000 *g*, 15 min), a further 6 g of $(NH_4)_2SO_4$ were added to the supernatant and the ppt. redissolved in 10 ml 0·05 M Tris–HCl buffer, containing 40 mM mercaptoethanol, 15% glycerol and 10 mM $MgCl_2$. (d) *Sephadex G75 and G200 chromatography.* The enzyme from step (c) was applied to a column of either Sephadex G75 or Sephadex G200 equilibrated with 0·05 M Tris–HCl buffer pH 8, containing 15% glycerol and 40 mM mercaptoethanol. (e) *DEAE-cellulose chromatography.* Step (c) enzyme was desalted on a column of Sephadex G25 and fractionated on DEAE-cellulose column as previously described.[2] (f) *Hydroxyapatite chromatography.* Step (c) enzyme was desalted on a column of Sephadex G25 and fractionated as previously described.[2]

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*Fractionation of* Delonix *enzyme.* The procedure followed that for the *Phaseolus* enzyme to step (d) except that the $(NH_4)_2SO_4$ fractionation and Sephadex G75 chromatography was carried out at 23°. Different batches of *Delonix* seed required slight modifications of the acetone fractionation; if the homogenate was a golden-brown colour, Pro-*t*RNA synthetase was usually fractionated between 40 and 52 ml acetone/160 ml crude supernatant. However if the homogenate was a dark-brown colour, the ppt. fractionated between 31–43 ml acetone/160 ml homogenate. This difference was probably due to the higher levels of phenolic oxidation products and galactomannans in the darker extracts which led to an earlier ppt. of the protein fractions containing Pro-*t*RNA synthetase activity.

For most experiments either DEAE-cellulose or Sephadex G75 (or G200) fractions were used. Hydroxyapatite fractions were unstable and exhibited non-linear thermal denaturation kinetics of poor reproducibility and hence were not used for thermal denaturation experiments.

*tRNA isolation.* The modification of the general phenol method[48] as described by Vanderhoef *et al.*[49] was used as previously described.[2] Carbohydrates were removed from *Phaseolus* *t*RNA preparations by DEAE-cellulose chromatography.

*Thermal denaturation and assay procedures.* Enzyme preparations were heated to 30° in the presence of mercaptoethanol for 10–15 min prior to thermal inactivation at elevated temps. Thermal inactivation was carried out in thin walled test tubes in thermostatically controlled water baths. Generally each tube contained in a vol. of 1 ml:100 mM Tris–HCl buffer, pH 8 (adjusted for each inactivation temp.); 0·1–0·3 mg bovine serum albumin, 10 mM $MgCl_2$ and 50–300 $\mu$g enzyme preparation; the concentration of Pro, analogues of Pro, ATP and other substrates tested for protection was varied according to the experiment to be performed. After the tubes had been heated at the selected temp. for the appropriate time intervals, they were plunged into ice for 20–30 sec and then left at room temp. Negligible amounts of denaturation occurred during heating to and cooling from the inactivation temp. Within 10 min of the cooling period the reagents necessary for the assay of residual ATP-$^{32}$PPi exchange were added to the tubes in a vol. of 0·9 ml so that the final concentrations of ATP and Pro were 2 mM and 50 mM respectively. In those instances where analogues were employed during the thermal denaturation reaction, Pro was added to such a concentration that any inhibitory effect of the analogue on Pro-dependent ATP-$^{32}$PPi exchange would be minimized. In practice it was found that all the analogues tested did not significantly inhibit Pro-dependent exchange even when present at concentrations 20-fold in excess of the saturating Pro concentration (10 mM). When pyrrolidine and 3-pyrroline were used as protecting substrates the Pro concentration was adjusted so that the inhibitory effects of these compounds on the Pro-dependent ATP-$^{32}$PPi exchange was negligible; in practice a concentration of 200 mM Pro in the incubation medium was sufficient to prevent such inhibition.

*Assay procedures.* (a) ATP-$^{32}$PPi *exchange.* Incubation mixtures generally contained: Tris–HCl buffer pH 8 (200 $\mu$mol), $MgCl_2$ (400 $\mu$mol), ATP (4 $\mu$mol), $^{32}$PPi (4 $\mu$mol) 8, imino acid (100 $\mu$mol) and enzyme preparation; total vol. 2 ml. Reactions were allowed to proceed at 40° for 16 min then terminated by the addition of 5% (w/v) TCA. Labelled ATP was separated from unchanged $^{32}$PPi by absorbing the nucleotide on to charcoal.[50] (b) *Assay of Pro-tRNA formation.* This followed the method previously described;[2] the enzyme preparation, after the thermal denaturation procedure, was diluted so as to maintian a constant concentration of Pro in the incubation mixture (30 nmol). (c) *Assay of ATPase.* Enzyme preparations at various stages of purification were heated in the presence of 4 mM ATP over the same range of temp. used for the thermal inactivation of the Pro-*t*RNA synthetase, and aliquots taken at various time intervals for orthophosphate analysis by the method of Lowry and Lopez.[51] (d) *Assay of pyrophosphatase.* This followed that described for the assay of ATPase except that the enzyme preparations were heated in the presence of 4 mM PPi. The presence of PPase was more rapidly monitored by heating the enzyme preparation in the presence of radioactive PPi and measuring the residual ATP-$^{32}$PPi exchange catalysed by the addition of a known concentration of Pro.

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