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SUBSTRATE DISCRIMINATION BY PROLYL-*t*RNA SYNTHETASE FROM VARIOUS HIGHER PLANTS

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Abstract—Prolyl-*t*RNA synthetase from plants (e.g. *Delonix regia*) containing azetidine-2-carboxylic acid (A2C), activated imino acid analogues larger than proline (Pro) more efficiently than did the enzyme from plants lacking A2C. The reverse situation was observed for analogues, including A2C itself, that are smaller than Pro. The enzyme from A2C-producing species was quite labile and salt-sensitive, with a high pH optima for the ATP-^{32}PPi exchange reaction, whereas the enzyme from non-producer species was stable and insensitive to salts, with a lower pH optimum. Certain analogues of Pro, which failed to stimulate ATP-^{32}PPi in the presence of a particular type of Pro-*t*RNA synthetase, nevertheless could bind to the enzyme and inhibit the esterification of *t*RNA by Pro. In the absence of *t*RNA, no significant ATP-^{32}PPi exchange was catalyzed by the *Delonix* enzyme on addition of A2C; the addition of *t*RNA resulted in a low but real level of activation of the analogue relative to Pro. These findings are discussed in relation to the ability of the enzyme from A2C-producing plants to discriminate against the analogue.

INTRODUCTION

AZETIDINE-2-CARBOXYLIC ACID, the lower homologue of Pro, inhibits the growth of seedlings which do not themselves contain the imino acid.[1] The growth of cultures of *E. coli*[2] and carrot tissue[3] is also inhibited by this analogue. The extent of growth inhibition is correlated with the degree of replacement of the Pro residues in cellular proteins by those of A2C.[2] The incorporation of A2C into protein molecules rests upon an ability of Pro-*t*RNA synthetase to accept A2C as a substrate and transfer it to *t*RNA. For instance, the enzyme from *Phaseolus aureus* (Leguminosae) and *Asparagus officinalis* (Liliaceae), plants in which A2C could not be detected, activated the analogue at a rate of 38% of that determined for Pro.[4]

Plants which synthesize non-protein amino acids structurally akin to one or more of the amino acids found in proteins have evolved mechanisms to exclude these analogues from protein molecules.[5] Discrimination against such analogues seems to depend upon species differences in the substrate specificity of the aminoacyl-*t*RNA synthetases.[4–9] Thus *Convallaria majalis* and *Polygonatum multiflorum* (Liliaceae), plants which contain A2C, have evolved a Pro-*t*RNA synthetase which fails to activate the analogue, thereby protecting these species against their own harmful constituents.[4,8]

[1] L. FOWDEN, *J. Exptl Bot.* **14,** 387 (1963).
[2] L. FOWDEN and M. H. RICHMOND, *Biochim. Biophys. Acta* **71,** 459 (1963).
[3] F. C. STEWARD, J. K. POLLARD, A. A. PATCHETT and B. WITKOP, *Biochim. Biophys. Acta* **28,** 308 (1958).
[4] P. J. PETERSON and L. FOWDEN, *Biochem. J.* **97,** 112 (1965).
[5] L. FOWDEN, D. LEWIS and H. TRISTRAM, *Advances in Enzymology* (edited by F. F. NORD), Vol. 29, p. 89, Interscience, New York (1967).
[6] P. J. LEA and R. D. NORRIS, *Phytochem.* **11,** 2897 (1972).
[7] I. K. SMITH and L. FOWDEN, *Phytochem.* **7,** 1065 (1968).
[8] P. J. PETERSON and L. FOWDEN, *Nature, Lond.* **200,** 148 (1963).
[9] P. J. LEA and L. FOWDEN, *Phytochem.* **11,** 2129 (1972).

A2C was considered originally to form a highly characteristic constituent of some members of the Liliaceae.[10] However, the imino acid has been isolated recently from seedlings of the legume *Delonix regia*,[11] and it is known to form a major constituent of four other legume seedlings, including *Parkinsonia aculeata*.[12] A2C also occurs in small quantities in the nitrogenous fraction of sugar beet.[13] A2C is not detectable by routine chromatographic methods in the dry seeds of these legumes, nor in an unfractionated sugar beet extract, where it occurs at a concentration of about one-fiftieth that of Pro.

The identification of A2C in plants unrelated to the Liliaceae, raises the question whether the properties of the Pro-*t*RNA synthetases are similarly modified in all A2C-containing plants, and, more specifically, whether similar mechanisms for discriminating against the analogue have been evolved. Since A2C is synthesized in these legume species only during the growth of the seedlings,[11] two distinct Pro-*t*RNA synthetases may exist: an ancestral *Phaseolus*-type enzyme in the dry seed, where the imino acid is absent, and a modified enzyme, characteristic of vegetative tissues and synthesized concommitantly with the accumulation of A2C in the growing plant. This paper reports the presence of a single Pro-*t*RNA synthetase in A2C-producing plants, together with a comparative study of the properties and substrate specificities of the Pro-*t*RNA synthetase extracted from; (i) *Phaseolus aureus*, *Hemerocallis fulva* and *Ranunculus bulbosa*, plants lacking A2C; (ii) *Delonix regia*, *Parkinsonia aculeata* and *Convallaria majalis*, plants which synthesize considerable quantities of A2C; and (iii) *Beta vulgaris*, a plant containing only trace amounts of the imino acid. Kinetic data are presented for the enzymes from *Delonix* and *Phaseolus* in a preliminary attempt to understand the mechanism underlying discrimination against A2C.

RESULTS

Enzyme Fractionation

The fractionation scheme employed for purification of the Pro-*t*RNA synthetase was essentially similar for each plant species studied (Tables 1 and 2). PVP at an optimum concentration of 0·3% was essential for efficient extraction of the enzyme from *Delonix*, *Parkinsonia*, *Convallaria* and *Beta*, although it did not influence the extraction of enzyme from other species. The continuous presence of glycerol and dithiothreitol (or mercaptoethanol) throughout the purification procedure was essential to maintain the stability of the Pro-*t*RNA synthetases discriminating against A2C (*Delonix*-type enzyme), although such additives were unnecessary during the early purification steps of enzyme from non-A2C-producer species (*Phaseolus*-type enzyme).

The presence of 15% (v/v) glycerol during the acetone and $(NH_4)_2SO_4$ fractionation steps resulted in an increased yield of Pro-*t*RNA synthetase, due to the enhanced stability of the enzyme when compared with the stability observed during previous preparations from some of the same species.[4,8] The acetone precipitation step produced the most variable results in terms of enzyme activity and different batches of *Delonix*, *Parkinsonia* or *Beta* required slight modification in the amount of acetone required to precipitate the enzyme in a discrete fraction.

[10] L. FOWDEN and F. C. STEWARD, *Ann. Bot. N.S.* **21**, 53 (1957).
[11] M-L. SUNG and L. FOWDEN, *Phytochem.* **8**, 2095 (1969).
[12] L. FOWDEN, unpublished results.
[13] L. FOWDEN, *Phytochem.* **11**, 2271 (1972).

When assaying an individual synthetase by the ATP-^{32}PPi exchange procedure, it is important to exclude any exchange due to contaminating synthetases. In this respect, the most important step in the fractionation scheme is that of heat denaturation. The Pro-*t*RNA synthetase, isolated from plants not containing A2C (*Phaseolus*-type enzyme), was more stable towards heat-denaturation than any of the other aminoacyl-*t*RNA synthetases.[14]

TABLE 1. THE STEPWISE PURIFICATION OF THE Pro-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*

Phaseolus aureus

Fraction	Total protein (mg)	Specific activity (units)*	Relative purification	Yield (%)	Contaminating synthetases†
Homogenate‡	1400	4·6	1	100	1330
Protamine sulphate				96	1250
Acetone§	350	15·0	3·2	80	504
$(NH_4)_2SO_4$§	17·2	270	58	71	39
Heat denaturation	14·6	320	69	71	0
DEAE-cellulose	3	1215	290	55	0
Hydroxylapatite	0·8	1655	350	20	0

Delonix regia

Fraction	Total protein (mg)	Specific activity (units)*	Relative purification	Yield (%)	Contaminating synthetases†
Homogenate‡	590	1·36	1	100	2312
Protamine sulphate				93	2027
Acetone§	132	4·15	3·1	69	970
$(NH_4)_2SO_4$§	12	40	30	60	135
Heat denaturation	11·5	42	31	59	17
DEAE-cellulose	2	101	75	27	4

* Specific activities are expressed as nmol PPi exchanged/min/mg protein.
† Expressed as % of pro-*t*RNA synthetase in each fraction.
‡ After removal of endogenous amino acids by Sephadex G25.
§ Cuts as in Experimental.

Thus, even in crude homogenates, it was possible to eliminate all ATP-^{32}PPi exchange due to contaminating enzymes. Although the Pro-*t*RNA synthetase from A2C-producer plants (*Delonix*-type enzyme) was quite thermolabile, it could be stabilized markedly by Pro, and so heat denaturation again had considerable value in reducing ATP-^{32}PPi exchange attributable to other synthetases.

The *Phaseolus*-type enzyme was stable when fractionated on a DEAE-cellulose column, but the *Delonix*-type enzyme became inactivated. However, by using a short column and an elution buffer supplemented with ATP and 40% glycerol, 10% of the activity was recovered. This activity could be further increased by preheating the enzyme fractions at 37° for 10 min in the presence of 80 mM dithiothreitol before assay. The final fractionation

[14] R. D. NORRIS and L. FOWDEN, unpublished results.

of the *Phaseolus aureus* enzyme on hydroxylapatite resulted in low yields and an unstable enzyme preparation, so that DEAE-cellulose fractions were normally used for experiments.

TABLE 2. THE STEPWISE PURIFICATION OF Pro-*t*RNA SYNTHETASE FROM SEVERAL HIGHER PLANT SPECIES

Species	Fraction	Total protein in homogenate (mg)	Specific activity (units)*	Contaminating synthetases† (%)
Parkinsonia aculeata	Homogenate‡	495	1·21	1811
	Acetone§		3·7	750
	$(NH_4)_2SO_4$§		41·0	124
	DEAE-cellulose		49	18
Convallaria majalis	Homogenate‡	416	0·91	1414
	Acetone§		2·3	709
	$(NH_4)_2SO_4$§		31·5	101
Beta vulgaris (var. *media*)	Homogenate‡	100	0·5	2060
	Acetone§		1·9	768
	$(NH_4)_2SO_4$§		23·0	120
Hemerocallis fulva	Homogenate‡	169	3·1	1950
	Acetone§		8·6	609
	$(NH_4)_2SO_4$§		79·0	98
	DEAE-cellulose		190·0	30
Ranunculus bulbosa	Homogenate‡	129	3·7	1657
	Acetone§		14·0	599
	$(NH_4)_2SO_4$§		173·0	98

* Specific activities are expressed as nmol PPi exchanged/min/mg protein.
† Expressed as % of Pro-*t*RNA synthetase in each fraction.
‡ After removal of endogenous amino acids by Sephadex G25.
§ Cuts as in Experimental.

Contaminating nucleic acid present in the various DEAE-cellulose preparations was usually less than 2% for *Phaseolus*-type enzymes. However, up to 8% nucleotide material was often present in *Delonix*-type enzyme obtained at this stage. The purification achieved for these synthetases compares favourably with those for other plant synthetases,[7,9,15–18] and the Pro-enzyme from *Phaseolus aureus* exhibited a single protein band on polyacrylamide gel electrophoresis.

pH Optima

The *Phaseolus*-type Pro-*t*RNA synthetases exhibited fairly sharp pH optima around 7·5–8 for the ATP-^{32}PPi exchange reaction (Table 3); activity fell more rapidly on the acid than on the alkaline side of the optimum pH. In contrast the pH curves for the *Delonix*-type enzymes showed flatter pH optima, in the region of pH 8·4–9·4, and little ATP-^{32}PPi exchange was observed below pH 6·3.

Various buffers were tested at similar molarities; generally Tris-HCl was the most effective in promoting Pro-dependent ATP-^{32}PPi exchange, but Tris-maleate-KOH was equally effective at high pH values. Imidazole, glycine and borate buffers were 60–85% as effective

[15] M. M. ATTWOOD and E. C. COCKING, *Biochem. J.* **96,** 616 (1965).
[16] J. W. ANDERSON and L. FOWDEN, *Biochem. J.* **119,** 677 (1970).
[17] E. MOUSTAFA, *Biochim. Biophys. Acta* **76,** 280 (1963).
[18] A. B. LEGOCKI and J. PAWELKIEWICZ, *Acta Biochim. Polon.* **14,** 313 (1967).

as Tris-HCl, but phosphate buffer was only 25–40% as effective. All buffers tested gave approximately the same pH optimum for a given enzyme.

TABLE 3. pH OPTIMA FOR PRO-*t*RNA SYNTHETASES FROM VARIOUS PLANT SPECIES USING THE ATP-^{32}PPi EXCHANGE

Species	pH optimum	Species	pH optimum
Phaseolus aureus	7·5–7·8	*Delonix regia*	9·0–9·4
Hemerocallis fulva	7·6–7·9	*Parkinsonia aculeata*	8·8–9·2
Ranunculus bulbosa	7·5–7·8	*Convallaria majalis*	8·4–8·7
Beta vulgaris	7·9–8·3	*Polygonatum multiflorum*	8·4–8·8

All pH optima were calculated for 0·1 M tris-maleate-KOH buffer.

The optimum pH for Pro esterification of *t*RNA using Pro-*t*RNA synthetase from either *Phaseolus* or *Delonix* was in the region of 7·2–7·5. In both cases, a sharp pH optimum was observed, with little Pro esterification occurring above pH 8·3. Alkaline hydrolysis of the ester link between Pro and *t*RNA did not totally account for the lower aminoacylation of Pro-*t*RNA at pH values higher than 7·8.

General Stability

The enzyme from *Phaseolus* was fully stable for several weeks at 2° as a DEAE-cellulose fraction and at −20° as an $(NH_4)_2SO_4$ precipitate although it was less stable if stored as a frozen solution. Pro-*t*RNA synthetase from *Ranunculus* and *Beta* was less stable under similar conditions but still retained some activity after several weeks.

Delonix-type enzymes were unstable at all stages of purification, and complete loss of activity occurred within 16 hr at 2° in Tris-HCl buffer. Enzyme stored in 50% (v/v) glycerol was stabilized to some extent, only 50% of the activity being lost in 20 hr: 20% (v/v) ethylene glycol provided less protection against inactivation than 20% glycerol whilst 2 M sucrose had only a slight stabilizing effect. Maximum stabilization was achieved by including dithiothreitol or mercaptoethanol in the medium, together with 50% glycerol. In this manner, the *Delonix*-type enzyme could be stored for 6 days with only 30% loss of activity.

Pro-*t*RNA synthetase from *Delonix* or *Parkinsonia* could be reactivated, after complete loss of activity after storage for 16 hr, by incubation for 10 min at 37° with glycerol together with dithiothreitol or mercaptoethanol. The extent of reactivation depended on the concentration of glycerol used, the nature of the sulphydryl compound and the age of the enzyme. Up to 70% reactivation could be achieved using 40 mM dithiothreitol, whilst mercaptoethanol was less effective. Neither thioglycollate nor glutathione effected any reactivation and incubation with glycerol alone did not have any significant effect. Maximum reactivation was demonstrated 3–4 days after enzyme preparation.

Heat Stability and Urea Denaturation

Pro-*t*RNA synthetases from *Phaseolus aureus*, *Ranunculus bulbosus* and *Hemerocallis fulva* were relatively stable to temperatures which denatured other synthetases.[14] The thermal stability of the enzyme was considerably enhanced by the presence of either ATP or Pro (Table 4). However, the enzyme from *Delonix* or *Parkinsonia* was extremely labile to heat: the addition of either ATP or Pro stabilized the enzyme to a great extent, Pro being more efficient than ATP in this respect. This is in contrast with results obtained for

the *Phaseolus*-type Pro-*t*RNA synthetase, and most other plant synthetases,[14] where ATP is superior to the amino acid substrate in protecting the synthetase against thermal denaturation. The *Phaseolus*-type enzyme was not protected against heat inactivation by glycerol, protein or *t*RNA, although these reagents had a slight protective effect on the *Delonix*-type enzyme.

TABLE 4. THE ABILITY OF SUBSTRATES OF THE Pro-*t*RNA SYNTHETASE FROM VARIOUS PLANTS TO PROTECT THE ENZYME AGAINST THERMAL DENATURATION

Plant species	$t_{\frac{1}{2}}$°* Additions to preincubation medium			
	None	ATP (2 μmol)	Pro (20 μmol)	ATP + Pro (2 μmol) (20 μmol)
Phaseolus aureus	65	73	70	78
Ranunculus bulbosa	51	—	—	—
Parkinsonia aculeata	25	55	58	67
Delonix regia	25	56	59	67

* The temperature at which 50% of ATP-^{32}PPi exchange activity remains after preincubation of $(NH_4)_2SO_4$ fractions of Pro-*t*RNA synthetase for 5 min. $MgCl_2$ (10 μmol) and Tris-HCl, pH 8 (100 μmol) were present throughout the preincubation period.

Thermal denaturation of the enzyme from *Phaseolus aureus* obeyed first order kinetics in the presence or absence of substrates, but the denaturation kinetics observed with the Pro-*t*RNA synthetase from *Delonix* appeared to be more complex. The degree of protection afforded by Pro against the heat inactivation of the Pro-*t*RNA synthetase from *Phaseolus aureus* was calculated in terms of the protection constant (π)[19] which is a measure of the dissociation constant for the enzyme-Pro complex. (The Arrhenius plot of the rate constants of thermal inactivation against $1/T$°K was linear over the range 50–72°.) Increasing concentrations of Pro, increasingly protected the enzyme against thermal denaturation and π was calculated as $2{\cdot}1 \times 10^{-5}$ M at 40° by the method of Burton.[19]

TABLE 5. THE ABILITY OF ANALOGUES OF Pro TO PROTECT THE Pro-*t*RNA SYNTHETASES FROM *Phaseolus aureus* AND *Delonix regia* AGAINST THERMAL DENATURATION

Analogue	% protection*		Analogue	% protection*	
	P. aureus	*D. regia*		*P. aureus*	*D. regia*
L-Proline	100	100	*N*-Methyl-L-alanine	25	13
L-Azetidine-2-carboxylic acid	44	48	*cis*-3-Hydroxy-L-proline	39	
cis(*exo*)-3,4-Methano-L-proline	18	60	*trans*-3-Hydroxy-L-proline	24‡	16‡
3,4-Dehydro-DL-proline	75	80	4-Methylene-DL-proline	5	0
L-Thiazolidine-4-carboxylic acid	52‡	34‡	*cis*-4-Hydroxy-L-proline	5	13
			trans-4-Methyl-L-proline	8	10
N-Methyl-glycine	38‡	13‡	*trans*-4-Hydroxy-L-proline	15	16
N-Ethyl-glycine	3	21	Pyrrolidine	10†	

* Protection was measured by preincubating DEAE-cellulose fractions for 5 min at 40° (*Delonix* enzyme) or 70° (*Phaseolus* enzyme) with the analogues at concentrations of 12 mM. Residual ATP-^{32}PPi exchange activity was then determined. The following analogues gave little protection against thermal denaturation with either synthetase: D-azetidine-2-carboxylic acid; 4-hydroxymethyl-L-Pro; 1-aminocyclopentane-1-carboxylic acid; cyclopentane carboxylic acid; L-baikiain; L-pipecolic acid; *cis*-4-chloro-L-Pro; *trans*-4-chloro-L-Pro; pyrrole-2-carboxylic acid; pyrrole; Δ′-pyrroline-5-carboxylic acid.

† Uncorrected for inhibition during assay.

‡ 40 mM conc. used for these three analogues.

[19] K. BURTON, *Biochem. J.* **48,** 458 (1951).

The protective effect afforded by various Pro analogues against the thermal inactivation of the Pro-enzyme from *Delonix* and *Phaseolus* is illustrated in Table 5. Both enzymes are protected to similar extents by individual analogues: minor discrepancies might be ascribed to the different order of thermal stability of the two enzymes, although an attempt was made to reduce such inconsistency to a minimum by selecting a denaturation temperature falling at an equivalent point on each heat inactivation curve.

Denaturation of the Pro-*t*RNA synthetase from *Phaseolus* or *Beta* with 4 M urea was complete within 5 min; little ATP-^{32}PPi exchange activity remained after 1 min when enzyme from *Parkinsonia* or *Delonix* was preincubated under the same conditions.

Chloromercuribenzoate (p*CMB*) *inhibition*

Similar curves for *p*CMB inhibition were observed for the Pro-*t*RNA synthetase from *P. aureus* and *D. regia*. Both enzymes were completely inhibited by 5×10^{-4} M *p*CMB. The *p*CMB-inhibited enzyme from *P. aureus* could be reactivated by the addition of sulphydryl compounds at 40 mM concentration: 80% reactivation was achieved with dithiothreitol and 70% with mercaptoethanol, whilst thioglycollate and glutathione were slightly less effective. However, sulphydryl compounds failed to reactivate the *p*CMB-inhibited enzyme from *Delonix*.

TABLE 6. THE EFFECT OF VARIOUS SALTS UPON PRO-STIMULATED ATP-^{32}PPi EXCHANGE BY PRO-*t*RNA SYNTHETASE FROM VARIOUS PLANT SPECIES*

Plant species	Concentration of salt (M) required to inhibit ATP-^{32}PPi exchange by 50%				
	LiCl	NaCl	KCl	CsCl	NH_4Cl†
Delonix regia	0·25	0·3	0·37	0·5	0·35
Parkinsonia aculeata	0·23	0·27	0·4	0·51	0·38
Phaseolus aureus	1·2	1·43	2·1	1·7	1·1
Beta vulgaris	1·2	1·5	>2	>2	1·0

* $(NH_4)_2SO_4$ fractions of Pro-*t*RNA synthetase were used for these experiments after passage through Sephadex G25.
† Adjusted to pH 8.

Inhibition by Salts

Salts had little inhibitory effect on the ATP-^{32}PPi exchange reaction catalyzed by *Phaseolus*-type enzyme, but *Delonix*-type enzymes were much more sensitive (Table 6). Monovalent group I cations inhibited ATP-^{32}PPi exchange according to their charge/radius ratio, the smallest ions being the most inhibitory. Anions were inhibitory in the order I > Br > Cl and divalent anions were generally about twice as effective as monovalent anions in the inhibition of ATP-^{32}PPi exchange catalyzed by both types of enzyme. These results are consistent with general non-specific ion effects. NH_4^+ and K^+ were equally effective as inhibitors of the *Delonix* enzyme but NH_4^+ was the stronger inhibitor of enzyme from *Phaseolus* (Table 6).

ATP-Mg *requirements*

A concentration of 15 mM Mg^{2+} was most efficient in promoting ATP-^{32}PPi exchange with either the *Delonix* or *Phaseolus* enzyme. Mn^{2+} and Co^{2+} were 25 and 15% as effective

as Mg^{2+} in promoting ATP-^{32}PPi exchange when compared at concentrations of 10 mM, but Zn^{2+}, Ni^{2+} and Cd^{2+} were utilized only slightly. Mn^{2+} (5 mM) inhibited Mg^{2+} (10 mM)-dependent Pro-stimulated ATP-^{32}PPi exchange of both enzymes by 60–70%. Although there may be several equilibrium forms of ATP-Mg-PPi complexes, we have assumed that the enzyme only utilized the monomagnesium complex.[20] The K_m values calculated for the ATP-Mg complex under optimum reaction conditions for enzymes from *Delonix* and *Phaseolus* were $1{\cdot}8 \times 10^{-4}$ M and 8×10^{-5} M respectively.

The optimum ATP/Mg ratio for the *t*RNA esterification reaction using Pro-*t*RNA synthetase from *Phaseolus* was determined as 0·7, and Mg^{2+} concentrations above 15 mM were progressively inhibitory. Spermidine and spermine at concentrations of 60 mM were only 10–20% as effective as 10 mM Mg^{2+} in the esterification reaction and these polyamines could not replace Mg^{2+} as a requirement for ATP-^{32}PPi exchange.

Amino Acid Substrate Specificity

ATP-^{32}PPi *exchange reaction.* The ability of the Pro-*t*RNA synthetase from various plants to utilize analogues of Pro as substrates for the ATP-^{32}PPi exchange is illustrated in Table 7. With enzyme from a given species, the K_m values for most analogues, relative to the value for Pro, reflected the efficiency of analogue activation when expressed as comparative V_{max} values. Lineweaver–Burk plots for Pro activation with *Phaseolus*-type enzymes were linear over all concentrations of amino acid substrate tested. However, plots obtained for Pro activation with enzyme from *Delonix, Convallaria* and *Parkinsonia* were linear up to substrate concentrations of 3 mM, but at higher concentration the reaction rates were higher than those predicted by the Lineweaver–Burk treatment. This phenomenon could only be demonstrated in purified preparations and crude homogenates exhibited classical Michaelis–Menten kinetics. K_m values for Pro determined with purified preparations of the *Delonix*-type Pro-*t*RNA synthetase were generally calculated by extrapolation of the linear part of the Lineweaver–Burk plot. The anomalous kinetics observed at high concentrations of Pro did not significantly affect the K_m or V_{max} values of analogues relative to Pro because most analogues also behaved anomalously at high concentrations. No inhibition of Pro-dependent ATP-^{32}PPi exchange was observed when A2C, thiazolidine-4-carboxylic acid or methanoproline were added to the assay medium in 20-fold excess of Pro, using the purified enzyme from either *Delonix* or *Phaseolus*. A2C at a concentration of 15 mM did not increase the rate of exchange observed with saturating concentrations of Pro.

Aminoacyl transfer to t*RNA.* Transfer of analogue aminoacyl residues to *t*RNA was followed indirectly by measuring the degree at which aminoacylation of *t*RNA by ^{14}C- or ^{3}H-Pro was inhibited by unlabelled analogues (Table 8). *t*RNA was always the limiting component in the reaction mixtures. The aminoacylation of *t*RNA by A2C was also followed directly by determining the incorporation of labelled analogue into *t*RNA.

The enzyme from *P. aureus* utilized ^{14}C-A2C for esterification of a homologous *t*RNA preparation at a rate approx. 50–70% of that determined for ^{14}C-Pro. In one experiment, 0·2 nmol of Pro were incorporated per mg *t*RNA after incubation for 8 min compared with 0·12 nmol of A2C over the same period. In contrast, the enzyme from *Delonix* transferred the analogue at only about 5% of the rate observed with Pro.

[20] F. X. COLE and P. R. SCHIMMEL, *Biochemistry* **9**, 3143 (1970).

TABLE 7. KINETIC PARAMETERS OF THE ATP-^{32}PPi EXCHANGE STIMULATED BY VARIOUS ANALOGUES, USING PRO-*t*RNA SYNTHETASES EXTRACTED FROM A RANGE OF PLANTS

Plant species		L-Proline ($\times 10^4$)	3,4-Dehydro-DL-proline	L-Azetidine-2-carboxylic acid	*cis*(*exo*)-3,4-Methano-L-Pro
Parkinsonia aculeata	K_m	4·35	$2·2 \times 10^{-3}$		$7·1 \times 10^{-3}$
	V_m		49	0–5	42
Delonix regia	K_m	1·82	$7·8 \times 10^{-4}$		$4·6 \times 10^{-3}$
	V_m		49	0–5	$v = 22$ (12 mM)
Convallaria majalis	K_m	4·5	$1·4 \times 10^{-3}$		$2–3 \times 10^{-3}$
	V_m		44	0–5	36
Beta vulgaris	K_m	4·5	$5·0 \times 10^{-4}$	$2·2 \times 10^{-3}$	
	V_m		89	73	< 3
Hemerocallis fulva	K_m	6·25	$7·4 \times 10^{-4}$	$5·3 \times 10^{-3}$	
	V_m		87	75	< 3
Phaseolus aureus	K_m	1·37	$2·8 \times 10^{-4}$	$1·43 \times 10^{-3}$	
	V_m		93	55	< 2
Ranunculus bulbosa	K_m	2·9	$3·64 \times 10^{-4}$	$2·0 \times 10^{-3}$	∞
	V_m		73	66	0

Plant species		*N*-Methyl glycine	*N*-Methyl-L-alanine	L-Thiazolidine-4-carboxylic acid
Parkinsonia aculeata	K_m	$4·5 \times 10^{-2}$		
	V_m	22	5*	70*
Delonix regia	K_m	$\sim 3 \times 10^{-1}$		$2–5 \times 10^{-2}$
	V_m	14	3*	66*
Convallaria majalis	K_m			
	V_m	19	< 3*	
Beta vulgaris	K_m			
	V_m	50	10*	22*
Hemerocallis fulva	K_m	$1·0 \times 10^{-1}$		
	V_m	74	11*	
Phaseolus aureus	K_m	$6·7 \times 10^{-2}$	$6·8 \times 10^{-2}$	$\sim 2 \times 10^{-2}$
	V_m	80	17*	35*
Ranunculus bulbosa	K_m	$1·4 \times 10^{-1}$		
	V_m	80	9	41*

All analogues were tested at a range of concentrations up to saturation values except where indicated. Results are expressed with respect to the L-form of the amino acid. K_m values are expressed as molar concentrations with respect to L-form. V_m or v was expressed as a % of the V_m for Pro. The following compounds failed to stimulate pyrophosphate exchange with any enzyme preparations at concentrations of 20 mM in the reaction mixture: pyrroline-2-carboxylic acid: pyrrole-2-carboxylic acid; cyclopentane carboxylic acid; L-baikiain. Low ATP-^{32}PPi exchange (< 5%) was observed with *cis*-3-hydroxyproline, 4-methylene-DL-proline and *N*-ethyl-glycine with both the *Delonix* and *Phaseolus* enzymes.

* Values extrapolated from Lineweaver–Burk plots due to insolubility of the analogue at high concentrations.

Specific Inhibitors

Various simple heterocyclic compounds or Pro-analogues with a modified carboxyl group were tested as specific competitive inhibitors of Pro-dependent ATP-^{32}PPi exchange using the Pro-*t*RNA synthetase from *Phaseolus* and *Delonix* (Table 9). Dixon plots[21] were linear in most cases. However, slight deviations from linearity were observed at high inhibitor concentrations when the enzyme from *Delonix* was used and K_is were then calculated by extrapolation of the linear part of the graph.

[21] M. DIXON, *Biochem. J.* **55**, 170 (1953).

TABLE 8. THE EFFECT OF PRO ANALOGUES ON THE TRANSFER OF ^{14}C- OR ^{3}H-PRO TO *t*RNA USING ENZYME FROM *Phaseolus* OR *Delonix*

Substrate	% inhibition of ^{14}C- or ^{3}H-Pro incorporation into *t*RNA* *Phaseolus aureus*	*Delonix regia*	Substrate	% inhibition of ^{14}C- or ^{3}H-Pro incorporation into *t*RNA* *Phaseolus aureus*	*Delonix regia*
L-Azetidine-2-carboxylic acid	55	19	4-Methylene-DL-proline	0	10
cis(*exo*)-3,4-Methano-L-proline	36	27	*N*-Methyl-glycine	25	10
3,4-Dehydro-DL-proline	76	45	*N*-Ethyl-glycine	0	10
L-Thiazolidine-4-carboxylic acid	50	22	*N*-Methyl-L-alanine	12	
cis-3-Hydroxy-L-proline	10	20			

The following analogues (at concentrations 500-fold in excess of the Pro concentration) inhibited ^{14}C- or ^{3}H-Pro incorporation into *t*RNA by 5–15% (using either enzyme): *trans*-3-hydroxy-L-proline; *cis*-4-hydroxy-L-proline; *trans*-4-methyl-L-proline. The following analogues failed to inhibit ^{3}H-Pro incorporation into *t*RNA: *cis*-4-chloroproline; *trans*-4-chloroproline; 4-hydroxymethyl-L-proline; *trans*-4-hydroxy-L-proline; L-baikiain; L-pipecolic acid.

* Analogues were present in the incubation mixture at a concentration 120-fold in excess of that of Pro. For assay conditions see Experimental.

Effect of t*RNA*

Certain $(NH_4)_2SO_4$ fractions of the Pro-*t*RNA synthetase from *Delonix*, which were contaminated with nucleic acids, occasionally catalyzed a slight activation of A2C (5–8% of that of Pro). However, purer $(NH_4)_2SO_4$ or DEAE-cellulose fractions failed to activate A2C significantly. Addition of yeast RNA (8 mg) to this purer enzyme lowered the V_{max} determined for Pro by 20% and increased the A2C-stimulated ATP-^{32}PPi exchange to a level equal to 15–20% of that for Pro, although yeast RNA produced little effect when added to less pure preparations. *t*RNA from *Delonix* (2 mg) also promoted the activation of A2C to about 20–25% of that observed for Pro. In the presence of yeast *t*RNA, A2C slightly inhibited Pro-dependent ATP-^{32}PPi exchange. However, no detailed kinetic studies were performed.

TABLE 9. KINETIC PARAMETERS FOR SPECIFIC INHIBITORS OF THE PRO-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*

Inhibitor	*Phaseolus aureus* K_i*	K_i (inhibitor) / K_m (proline)	*Delonix regia* K_i*	K_i (inhibitor) / K_m (proline)
Pyrrolidine	2×10^{-3}	14·5	$2{\cdot}5 \times 10^{-3}$	13·7
Tetrahydrofuran	3×10^{-1}	2200	9×10^{-2}	490
Tetrahydrothiophen	3×10^{-1}	2200	1×10^{-1}	550
3-Pyrroline	$0{\cdot}8 \times 10^{-3}$	5·8	$2{\cdot}5 \times 10^{-3}$	13·6
Pyrrole	$4{\cdot}5 \times 10^{-2}$	330	4×10^{-2}	220
2-Pyrrolidinone	1×10^{-1}			
DL-Proline amide	$4{\cdot}7 \times 10^{-4}$			

L-Azaproline ethyl ester and DL-proline ethyl ester did not inhibit ATP-^{32}PPi exchange when added to the incubation medium at a concentration 100 × that of Pro. L-Proline methyl ester was only slightly (5%) inhibitory at concentrations 20 × that of proline.

* K_is (expressed as molarities) were calculated according to the method of Dixon[21] by measuring ATP-^{32}PPi exchange at several different concentrations of Pro and inhibitor.

DISCUSSION

The procedures used for the purification of Pro-*t*RNA synthetase allowed unambiguous interpretation of the results, as in most cases the levels of contaminating synthetases were reduced to acceptable limits (Tables 1 and 2).

Pro-*t*RNA synthetase in various stages of purification from either *Delonix* or *Parkinsonia* failed to activate A2C significantly, irrespective of whether the enzyme originated from seedlings or dry seeds. Thus it appears that the ancestral-type enzyme which activates A2C has been eliminated (or severely reduced in amount) by evolutionary processes, leaving a single enzyme throughout the plant cytoplasm which discriminates against the imino acid.

The kinetic data for the Pro-*t*RNA synthetase from seedlings of *Beta vulgaris* (Table 7) indicates that the enzyme from this species can activate A2C with a V_{max} of 73% of that of Pro. This fact, together with the data for other Pro analogues, suggests that the *Beta* enzyme resembles the Pro-*t*RNA synthetases present in species lacking A2C (Table 7). Therefore, it appears that this plant has had no need to evolve a Pro-enzyme capable of discriminating against A2C, because the analogue never reaches a concentration in the plant cell which would lead to effective competition with Pro at the active site of the synthetase.

The Pro-*t*RNA synthetases from the various plants tested exhibited K_m values for Pro (ATP-^{32}PPi exchange assay) in the range $1{\cdot}32$–$6{\cdot}25 \times 10^{-4}$ M, figures similar to those determined for enzyme from *E. coli*[22,23] and rat liver.[24] Whereas the bacterial and animal enzymes activated analogues, with V_{max} values similar to the V_{max} for Pro, the plant enzymes usually activated the same compounds at rates 20–80% of that of Pro.

The Pro-*t*RNA synthetase from the 3 plants which synthesize A2C in high concentrations activated 3,4-dehydroPro (used at enzyme-saturating concentrations) at rates slightly less than 50% of the corresponding V_{max} values determined for Pro (Table 7). Plants possessing a Pro-*t*RNA synthetase capable of utilizing A2C as a substrate activated 3,4-dehydroPro more effectively and V_{max} and K_m values were very similar to those determined for Pro (Table 7).

N-Methylglycine, *N*-ethylglycine and *N*-methylalanine act as substrates for the *E. coli* synthetase by virtue of their possession of an imino N with suitable attached C atoms that give the molecules configurations resembling that of Pro.[23] The K_m values for these analogues, using Pro-*t*RNA synthetases from various higher plants were of the same order as those determined for the *E. coli* enzyme,[23] although V_{max} values never reached 100% of that of Pro.

The data (Table 7) indicated that *N*-substituted derivatives that are less bulky than Pro were activated more efficiently relative to Pro by the Pro-enzyme from species failing to discriminate against A2C (*Phaseolus*-type enzyme), than by the enzyme from plants selecting against A2C (*Delonix*-type enzyme).

Table 7 also gives ATP-^{32}PPi exchange data for analogues larger than Pro. Thiazolidine-4-carboxylic acid, in which the C-4 atom of Pro is replaced by the more bulky S atom is activated more efficiently by the *Delonix*-type enzyme than by the *Phaseolus*-type enzyme. *cis*-(*exo*)-3,4-Methanoproline, in which an exocyclic methylene group bridges the 3 and 4 C atoms of Pro, was activated by the enzyme from A2C-producer plants (*Delonix*-type) relatively effectively with a V_{max} of about 25–40% of that observed for Pro. The enzyme from plants which activated A2C (*Phaseolus*-type) however did not activate methanoproline,

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although it catalyzed a significant activation of thiazolidine-4-carboxylic acid. This activation of thiazolidine-4-carboxylic acid in comparison with the negligible activation of methanoproline no doubt reflects the different spatial arrangement of substituent groups within these two analogues both of which are larger than proline.

Analogues slightly smaller or slightly larger than Pro protected both enzymes against thermal inactivation relatively efficiently and even compounds such as *trans*-3-hydroxy-L-Pro, with fairly bulky side chains, bound to the enzyme. However, most Pro analogues with bulky substituent groups were either inefficient or failed completely to protect either type of enzyme against heat inactivation (Table 5). Several compounds of a similar size to Pro, such as cyclopentane carboxylic acid and pyrrole-2-carboxylic acid were also ineffective in this respect.

Certain analogues which were not activated by the Pro-enzyme from either *Delonix* or *Phaseolus*, protected the synthetase against heat denaturation (Table 5). Thus the enzymes from *Delonix* and *Phaseolus* were protected against thermal inactivation by A2C and methanoproline, respectively, indicating that these compounds can bind to the enzyme.

These results suggest that the geometry of the active site of the Pro-*t*RNA synthetase from plants which discriminate against A2C is such that the activation of molecules larger than Pro is possible. By displaying this flexibility, the fit of smaller analogues is presumably too loose to enforce the correct configurational alignment of the carboxyl group of the analogue with the α-PO_4^{3-} group of ATP. Conversely, the active sites of the Pro-*t*RNA synthetase from species activating A2C are presumed to be smaller, thus facilitating the formation of ligand bonds to analogues less bulky than Pro whilst additionally imposing a constraint on the molecules leading to the correct positioning of their COO^- group. This view is strengthened by the results obtained with specific competitive inhibitors (Table 9). Pyrrolidine is equally inhibitory to the ATP-^{32}PPi exchange catalyzed by enzyme from either *Phaseolus* or *Delonix*, as reflected by the similar K_i/K_m ratios. Various other inhibitors of similar size to Pro were equally efficient in inhibiting ATP-^{32}PPi exchange catalyzed by both types of enzyme. Tetrahydrofuran and tetrahydrothiophen seemed to inhibit the enzyme from *Delonix* more strongly than from *Phaseolus*. Although their exact stereochemistry is not known, these latter two heterocyclics may have an internal bond angle at the C-2 atom that is larger than that at the equivalent position in pyrrolidine. The distance between the heterocyclic atom and the C-2/C-3 axis is also greater in tetrahydrothiophen (and possibly in tetrahydrofuran) and, for this reason, these two molecules may fit more effectively into the wider active site of the *Delonix* enzyme than into the smaller site of the *Phaseolus enzyme*. Similarly, the smaller size of 3-pyrroline compared with the pyrrolidine ring of Pro, allows this molecule to inhibit the *Phaseolus* enzyme more efficiently than the *Delonix* enzyme.

Unshared electrons pairs may be required feature of a molecule before it can bind as a substrate to the synthetase. This view is supported by the observation that: (i) compounds such as tetrahydrofuran and tetrahydrothiophen, with unshared electron pairs, are able to act as inhibitors of the Pro-*t*RNA synthetase; and (ii) compounds similar in size to Pro, but with a reduced charge on the heterocyclic N atom due to resonance or with N replaced by C, failed to stimulate ATP-^{32}PPi exchange or to protect either type of Pro-enzyme against thermal inactivation (Tables 5 and 7). However, the inability of aromatic analogues to support ATP-^{32}PPi exchange may be partly due to the planar position of their carboxyl groups with respect to the ring.

In A2C-producing species, Pro is more efficient in stabilizing the Pro-enzyme against thermal denaturation than is ATP, but for the *Phaseolus*-type enzyme the opposite situation

is encountered (Table 4). The thermolability of the *Delonix*-type enzyme, together with its sensitivity to urea and salts, contrasts sharply with the stability of the *Phaseolus*-type enzyme, suggesting that the former enzyme may dissociate into subunits more readily. The stabilizing action of polyols also supports this view because dissociation of bacterial Pro-*t*RNA synthetase into subunits can be prevented by high concentrations of glycerol.[25] The reactivation phenomenon is also explicable in terms of a subunit rearrangement reproducing a catalytically active form of the enzyme. Similar ageing or reactivation phenomena have been reported for a number of bacterial synthetases.[25,26]

The results relating to the incorporation of analogues into *t*RNA using the indirect method (Table 8) indicate that some compounds, which fail to stimulate ATP-^{32}PPi exchange with a particular enzyme, nevertheless may be transferred to *t*RNA. When the direct method of incorporation was applied using labelled A2C, with enzyme and *t*RNA from *Delonix*, little transfer of the analogue into *t*RNA was observed, although the indirect method implicated considerable transfer. These results may merely reflect the greater uncertainties surrounding the use of the indirect method, and now fuller, more exacting quantitative information is obviously required. However, it does seem that, in the presence of *t*RNA, Pro-adenylate formation catalyzed by *Delonix* may be competitively inhibited by A2C, and that this inhibition causes a reduction in the amount of Pro transferred to *t*RNA.

The requirement of *t*RNA for the inhibition of Pro-dependent ATP-^{32}PPi exchange by A2C may be explained by assuming that the binding of *t*RNA produces a conformational change in the enzyme. This is supported by the fact that the pH optima for the ATP-^{32}PPi exchange and *t*RNA esterification differ and that a low A2C-dependent exchange was observed when *Delonix t*RNA was added to reaction mixtures containing the purified *Delonix* enzyme.

The absence of A2C-dependent ATP-^{32}PPi exchange previously observed with the Pro-*t*RNA synthetase from *Convallaria majalis* and *Polygonatum multiflorum*[4,8] perhaps may be ascribed to the absence of *t*RNA from the preparations. Since unfractionated *t*RNA preparations were generally used in our experiments, modifying effects due to the binding of inactive *t*RNAs,[27] organelle-specific *t*RNAs, or inhibitors[28] cannot be ruled out.

Perhaps the slight activation of A2C by the *Delonix* enzyme in the presence of *t*RNA can be rationalized with an *in vivo* need to discriminate against the analogue by one of the following explanations: (i) in physiological situations, the imino acid substrate may bind to the enzyme prior to *t*RNA; in such circumstances A2C presumably would not compete with Pro at the active site; or (ii) any A2C-adenylate formed is subject to a *t*RNA-induced hydrolysis—a similar situation is known where Ile*t*RNA from *E. coli* hydrolyzes the Val-adenylate complex bound to the bacterial Ile-*t*RNA synthetase.[29,30]

A more detailed study of the role played by *t*RNA in the discrimination against A2C will form the subject of a separate investigation.

EXPERIMENTAL

Materials. Plant materials. Seed of *Phaseolus aureus*, *Beta vulgaris* and *Convallaria majalis* were obtained commercially. *Hemerocallis fulva* was collected from Nuffield Gardens, Regent's Park, London NW1,

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and *Ranunculus bulbosa* leaves were obtained from a woodland site in Essex. *Delonix* and *Parkinsonia* seeds were the gifts of Professor L. B. Thrower (Hong Kong) and Professor E. A. Bell (Austin, Texas) respectively.

Amino acids and analogues. All samples were tested for purity by PC. The L-isomers of the normal protein amino acids were high grade commercial samples. Other amino acids were obtained as follows: L-azetidine-2-carboxylic acid, natural isolate from beet (H. Knobloch, Paris);[13] 4-methylene-DL-Pro (natural isolate from *Eriobotrya japonica*),[31,32] *trans*-4-hydroxy-L-Pro (Roche Products Ltd.); 3,4-dehydro-DL-proline (Dr. B. Witkop, Bethesda, Md.); *trans*-4-methyl-L-proline (Dr. R. C. Sheppard, Cambridge); L-baikiain (natural isolate from *Caesalpinia tinctoria*, R. Watson); *cis*-4-hydroxy-L-Pro (natural isolate from *Santalum album*, Dr. A. N. Radhakrishnan, Vellore, India);[33] *cis*-3-hydroxy-L-Pro, and *trans*-3-hydroxy-L-Pro (Dr. F. Irreverre, Bethesda, Md.); L-thiazolidine-4-carboxylic acid (Mann Research Laboratories); *cis*(*exo*)-3,4-methano-L-proline (isolate from *Aesculus parviflora*);[34] *N*-methylglycine, *N*-ethylglycine and *N*-methyl-L-alanine (Professor A. H. Mehler, Milwaukee, Wisconsin); D-A2C (Calbiochem); 4-hydroxymethyl-L-Pro (Dr. A. C. Hulme, Norwich); cyclopentane carboxylic acid (Koch Light Ltd.); pyrrole-2-carboxylic acid (Emmanuel Ltd.); *cis*-4-chloro-L-Pro and *trans*-4-chloro-L-Pro (Dr. J. Kollonitsch, Rahway, N.J.); Δ′-pyrroline-5-carboxylic acid (Mr. H. Tristram, London); L-azaproline ethyl ester (Dr. J. S. Morley, I.C.I. Pharmaceuticals Division, Macclesfield). Other compounds used in the analogue studies were from various commercial sources. DL-Proline amide was synthesized by the method of Price *et al.*[35]

Radioisotopic chemicals. L-[U-^{14}C]Pro (265 mCi/mmol) and L-[G-^{3}H]Pro (670 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. DL-[4-^{14}C]Azetidine-2-carboxylic acid (10 mCi/mmol) was obtained from Calbiochem. [^{32}P]Pyrophosphate was prepared from sodium [^{32}P]orthophosphate by pyrolysis to give a product of specific activity 0·2–0·4 μCi/μmol.

Other chemicals. Hydroxylapatite was prepared by the method of Tiselius as described by Levin.[36] Yeast nucleic acid was obtained from Koch Light Laboratories.

Methods. Determination of protein and nucleic acids. The Folin method as modified by Lowry *et al.*[37] was generally used, with bovine serum albumin as a standard. Protein in eluates from DEAE-cellulose and hydroxylapatite columns was calculated from extinction values measured at 260 and 280 nm.[38] The amount of RNA in the synthetase preparation was measured by the method of Key and Shannon.[39] RNA was precipitated with $HClO_4$ and after washing the pellet with H_2O, hydrolysed in 0·3 N KOH at 37° for 20 hr. The hydrolysate was adjusted to pH 1·5, centrifuged, and the nucleotide content in the supernatant determined spectrophotometrically.

Enzyme fractionation. Gel electrophoresis was performed by the method of Ornstein and Davis (see Davis[40]). All operations were carried out in a cold room at 2–4°. (a) *Homogenate. Phaseolus*, *Convallaria*, *Parkinsonia* or *Delonix* seed meal (16 g) was ground for 2–4 min in a mortar with 190 ml 0·1 M tris-HCl buffer pH 8·0, containing 15% (v/v) glycerol, 0·3% PVP (soluble, approx. MW 44 000), 10 mM $MgCl_2$, and 25 mM mercaptoethanol. Leaves of *Ranunculus* and *Hemerocallis* and seedlings of *Beta* were homogenized in an M.S.E. Atomix blender for 3 × 2 min with 3 × their weight of extraction medium. The macerate was pressed through muslin and centrifuged for 20 min at 24 000 *g*. An aliquot of the supernatant was passed through a Sephadex G25 column to remove endogenous free amino acids before the total synthetase activity was measured. (b) *Protamine sulphate.* 1 vol. of 0·5% protamine sulphate was added to 5 vol. of supernatant obtained in (a). After standing 10 min, the precipitated nucleic acids and protein were discarded. (c) *Acetone fractionation. Delonix:* acetone (31 ml) at −20° was added to 160 ml of protamine sulphate supernatant and precipitation allowed to proceed for 15 min at 20°. The sedimented protein (15 000 *g*, 10 min) was discarded and a further 12 ml acetone added to the supernatant. The protein precipitated at this stage was redissolved in 48 ml of 0·1 M tris-HCl buffer, pH 8 containing 15% glycerol and 25 mM mercaptoethanol. When acetone fractionation was applied in a similar manner to protamine sulphate supernatants containing enzymes from *Convallaria* and *Parkinsonia*, the Pro-*t*RNA synthetase appeared in the 25–43 ml acetone fraction: correspondingly, enzyme from *Phaseolus* appeared in the 28–42 ml fraction and from *Beta*, *Ranunculus* and *Hemerocallis* in the 23–42 ml fraction. (d) $(NH_4)_2SO_4$ *fractionation.* $(NH_4)_2SO_4$ (17 g) was added to 48 ml of the redissolved acetone fraction and protein precipitation was allowed to proceed for 15 min. After centrifugation (25 000 *g*, 15 min), a further 4 g of $(NH_4)_2SO_4$ were added to the supernatant

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and the precipitate redissolved in 10 ml of 0·05 M tris-HCl buffer, containing 25 mM mercaptoethanol, 5% glycerol and 10 mM $MgCl_2$. (e) *Heat denaturation.* Pro was added to the redissolved $(NH_4)_2SO_4$ fraction to give a final concentration of 20 mM and the solution heated for 15 min at 52·5° (*Delonix* and *Parkinsonia* enzyme) and 64° (*Phaseolus* enzyme). This step was omitted for the fractions from *Beta*, *Ranunculus*, *Convallaria* and *Hemerocallis*. The solutions of *Phaseolus*-type enzymes were then dialyzed directly against 0·02 M tris-HCl buffer, pH 8, containing 25 mM mercaptoethanol, 15% glycerol and 10 mM $MgCl_2$ for 8 hr (3 changes of buffer). Fractions from *Delonix*, *Parkinsonia* and *Convallaria* were desalted by passage through a small column of Sephadex G25 equilibrated with the above buffer. Precipitated protein was removed by centrifugation. (f) *DEAE-cellulose fractionation.* (i) *Phaseolus* and *Hemerocallis*. A column was packed with pre-swollen Whatman DE52 cellulose equilibrated with 0·02 M tris-HCl buffer, pH 7·4, containing 15% glycerol. The dialyzed fraction was applied and the column then eluted with a linear salt gradient obtained by mixing 0·02 M tris-HCl buffer, pH 7·4 (180 ml), with 0·02 M tris-HCl buffer, pH 8, containing 0·2 M KCl. All buffers also contained 15% glycerol and 25 mM mercaptoethanol. (ii) *Delonix* and *Parkinsonia*. A smaller DEAE-cellulose column was used with equilibration and elution buffers containing 40% glycerol, 40 mM ATP and 80 mM dithiothreitol. An elution gradient of 0·1–0·2 M KCl was employed with 70 ml of buffer in each reservoir. Fractions were preincubated at 37° for 10 min prior to assay. (g) *Hydroxylapatite chromatography. Phaseolus* enzyme. Fractions of eluate from the DEAE-cellulose column containing the enzyme were desalted on a column of Sephadex G25 and applied to a small column of hydroxylapatite equilibrated with 0·005 M phosphate buffer, pH 7·2, containing 25% glycerol, and 25 mM mercaptoethanol. The enzyme was eluted with a linear gradient obtained by mixing 0·005 M phosphate buffer, pH 7·2 (80 ml), and 0·15 M phosphate buffer, pH 8 (80 ml). Both buffers contained 25% glycerol and 25 mM mercaptoethanol.

t*RNA isolation.* The modification of the general phenol method[41] described by Vanderhoef *et al.* was used,[42] with the further modification that double the suggested volume of buffer was employed for the initial extraction of *t*RNA from mung bean and *Delonix* seed meal. Carbohydrates were removed by the methoxyethanol method.[43] The *t*RNA was reprecipitated with cetyltrimethylammonium bromide,[44] dissolved in 0·05 M tris-HCl buffer, pH 8·9, and incubated for 30 min at 37° to deacylate *t*RNA; it was then dialyzed against 0·02 M tris-HCl buffer pH 7·6 for 6 hr. The 260:280 ratio for *Phaseolus t*RNA (1·75) compared favourably with those obtained by Vanderhoef *et al.*,[42] but the 260:220 ratio was lower (0·95). *Delonix t*RNA exhibited a lower 260:280 ratio (1·1–1·4).

Assay procedures. (a) ATP-^{32}PPi *exchange.* Incubation mixtures generally contained: tris-HCl buffer (100 μmol), $MgCl_2$ (140 μmol), ATP (2 μmol), ^{32}PPi (2 μmol), amino acid (10 μmol) and enzyme preparation; total vol. 1 ml. The pH of the incubation mixture with *Phaseolus*, *Hemerocallis*, *Beta* and *Ranunculus* enzymes was 8·0 whilst the *Convallaria*, *Delonix* and *Parkinsonia* enzymes were assayed at pH 8·9. Concentrations of the various reactants were varied according to the nature of the experiments involved. Reactions were allowed to proceed for 16 min at 40° (*Delonix*, *Parkinsonia* and *Convallaria*) or for 20 min at 42° (*Phaseolus*, *Beta*, *Ranunculus* and *Hemerocallis*) and 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.[45] The specific activity of enzyme preparations was expressed as μmol PPi exchanged/min/mg protein (see Davie *et al*).[46] (b) *Assay of Pro-*t*RNA formation.* Incubation mixtures contained $MgCl_2$ (3 μmol), ATP (2 μmol), tris-HCl buffer, pH 7·3 (50 μmol), *t*RNA preparation (0·05–0·125 mg), ^{3}H-Pro (3 μmol, 670 mCi/mmol) or ^{14}C-Pro (15 nmol, 265 mCi/mmol) or ^{14}C-A2C (15 nmol, 10 mCi/mmol) and enzyme preparation (50–200 μg protein); total vol. 1 ml. Reactions were allowed to proceed for 8 min at 37° and were terminated by addition of 5% (w/v) ice-cold TCA (0·5 ml). The precipitates were collected on glass-fibre discs (Whatman GF/A) and washed with TCA and Hokin's reagent as described by Vanderhoef *et al.*;[42] radioactivity was determined by scintillation counting.

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