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PRO-*t*RNA SYNTHETASE FROM *PHASEOLUS AUREUS* AND *DELONIX REGIA*

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Key Word Index—*Phaseolus aureus: Delonix regia:* Leguminosae; Pro-*t*RNA synthetase; substrate specificity; photoinactivation; imino acid analogues.

Abstract—Pro-*t*RNA synthetase from *Phaseolus aureus* was photoinactivated in the presence of methylene blue or rose bengal. Pro and several imino acid analogues protected the enzyme against dye-mediated photoinactivation but ATP was ineffective. Together with kinetic data, this evidence suggested that a His-residue near the Pro-binding site was involved in the enzyme reaction. In the absence of methylene blue, *Phaseolus* enzyme was stable to light whilst that from *Delonix* was rapidly and reversibly photoinactivated. ATP as well as Pro, protected the *Delonix* enzyme against dye-independent photoinactivation. In the presence of methylene blue, the *Delonix* enzyme was more rapidly photoinactivated than in the absence of the dye. *p*-Chloromercuribenzoate (*p*CMB)-inhibited enzyme from both *Phaseolus* and *Delonix* was reactivated by sulphydryl reducing reagents. Reactivation of *Delonix* enzyme was markedly temperature-dependent whilst *Phaseolus* enzyme was reactivated equally efficiently at all temperatures tested. ATP, *t*RNA, Pro and several analogues of Pro, protected both the *Phaseolus* and *Delonix* enzymes against *p*CMB inhibition. The possible roles of the His-residue and SH group are discussed in relation to the known differences in substrate specificity between the *Phaseolus* and *Delonix* enzymes.

INTRODUCTION

PLANTS which synthesize non-protein amino acids structurally similar to one or more of the amino acids found in proteins have evolved mechanisms at the level of the aminoacyl-*t*RNA synthetases to exclude these analogues from protein molecules.[1–4]

Pro-*t*RNA synthetase from plants containing azetidine-2-carboxylic acid (A2C), e.g. *Delonix regia*, is able to catalyze ATP-^{32}PPi exchange in the presence of imino acid analogues smaller than Pro less efficiently than the enzyme from plants lacking A2C, e.g. *Phaseolus aureus*.[3] The reverse situation is observed for analogues larger than Pro, e.g. methanoproline.[3]

Some analogues of Pro, which fail to stimulate ATP-^{32}PPi exchange catalyzed by particular Pro-*t*RNA synthetases, nevertheless are able to bind to the enzyme.[5] The binding of most imino acid analogues to enzyme from both *Delonix* and *Phaseolus* is more efficient, relative to Pro, in the presence of ATP than in its absence.[5] Thermodynamic and kinetic data have suggested that the binding of substrates elicits a conformational change within

[1] LEA, P. J. and NORRIS, R. D. (1972) *Phytochemistry* **11**, 2897.
[2] PETERSON, P. J. and FOWDEN, L. (1965) *Biochem. J.* **97**, 112.
[3] NORRIS, R. D. and FOWDEN, L. (1972) *Phytochemistry*, **11**, 2921.
[4] LEA, P. J. and FOWDEN, L. (1972) *Phytochemistry* **11**, 2129.
[5] NORRIS, R. D. and FOWDEN, L. (1973) *Phytochemistry* **12**, 2109.

the Pro-*t*RNA synthetase molecule and that the binding of ATP prior to the imino acid substrate modifies the Pro-binding site, causing it to become more rigid.[5]

A sulphydryl group has been implicated at or near the active site of several aminoacyl-*t*RNA synthetases,[6–9] but little is known of its function. An unconfirmed report of Boyko and Fraser suggests that Gly-*t*RNA synthetase from rat liver contains a His residue at the active site,[10] but little additional evidence is available concerning the nature of other functional amino acid residues at the active sites of aminoacyl-*t*RNA synthetases.

In this paper, we have tried to determine whether the differences in substrate specificity observed between *Delonix* and *Phaseolus* enzymes reflect differences in the type of functional amino acid residue at their active sites. Evidence is presented for the presence of an imidazole (His) and a SH (Cys) group within the active site of each enzyme, and substrates and inhibitors of Pro-*t*RNA synthetase were used in attempts to determine the relative positions of these two groups within the active sites of the enzymes. Although we have gained considerable insight about conformational features of the active sites,[5] our experiments have not provided a convincing explanation of the specificity differences encountered between the two enzymes.

RESULTS

Enzyme Preparations

It was essential to exclude *t*RNA from enzyme preparations since this molecule protected Pro-*t*RNA synthetase from both *Phaseolus* and *Delonix* against *p*CMB inhibition; the absence of substrate protection against *p*CMB inhibition observed with an enzyme preparation obtained after $(NH_4)_2SO_4$ fractionation was attributable to contaminating *t*RNA. Hence enzyme fractions from Sephadex G75 columns were always used for experiments. The absence of *t*RNA in these latter preparations was confirmed by the negligible aminoacylation of Pro in the absence of added *t*RNA.

The enzyme preparations from *Delonix*, if used for photoinactivation studies, were clarified by centrifugation to remove insoluble protein which was found to decrease the efficiency of photoinactivation and to interfere with substrate protection.

Effect of pH on the Kinetic Parameters of ATP-^{32}PPi exchange

The pH-activity profiles of Pro-*t*RNA synthetase from *Phaseolus* and *Delonix* exhibited two components below pH 8 (Fig. 1). The ability of both the *Phaseolus* and *Delonix* enzyme to catalyze Pro-dependent ATP-^{32}PPi exchange was lost rapidly below pH 6·7 whilst a less steep component of the pH-activity curve was evident between pH 6·7 and 8. ATP-^{32}PPi exchange catalyzed by the *Phaseolus* enzyme was lost less rapidly between pH 6·7 and 8 compared with the *Delonix* enzyme.

The K_m Pro for the ATP-^{32}PPi exchange reaction catalyzed by the *Phaseolus* enzyme exhibited a marked pH dependence (Fig. 2); the slope of the pK_m vs pH graph below pH 6 was calculated as 1·15. The K_m Pro for the *Delonix* enzyme could not be accurately calculated below pH 6·4 because of the low V_m values for Pro.

[6] LOFTFIELD, R. B. (1972) in *Protein Synthesis* (MCCONKEY, E., ed.), Vol. 1, p. 1, Marcel Dekker, New York.
[7] STULBERG, M. P. and NOVELLI, G. D. (1962) *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBACK, K., eds.), Vol. 6, p. 401, Academic Press, New York.
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The K_m ATP for the ATP-^{32}PPi exchange reaction catalyzed by either the *Phaseolus* or *Delonix* enzymes[3] was unchanged between pH 5 and 13, although the low ATP-^{32}PPi exchange activity observed below pH 6·4 precluded accurate measurements of K_m for the latter enzyme.

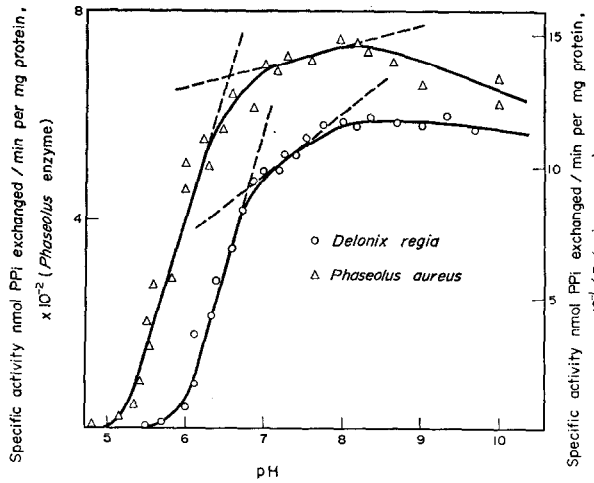

FIG. 1. ATP-^{32}PPi EXCHANGE CATALYSED BY PRO-*t*RNA SYNTHETASE FROM *Delonix regia* AND *Phaseolus aureus* AT DIFFERENT pHs.
Buffers of constant ionic strength (0·1 M) were used throughout the pH range tested; pH 5–6·8, maleic acid-KOH; pH 6–10, Tris-maleate KOH. Small corrections were made for specific buffer effects

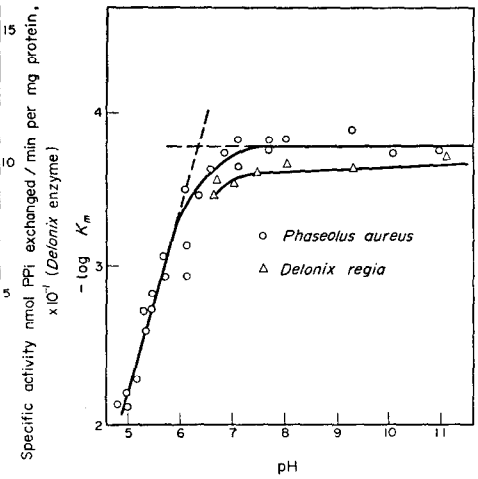

FIG. 2. EFFECT OF pH ON THE K_m FOR PRO IN THE ATP-^{32}PPi EXCHANGE REACTION CATALYZED BY THE PRO-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*.

Effect of Deuterium Oxide (D_2O) on ATP-^{32}PPi exchange

When the ATP-^{32}PPi exchange reaction was performed in an assay mixture containing 90% D_2O, the rate of exchange (at an enzyme-saturating Pro concentration of 10 mM) catalyzed by both the *Delonix* and *Phaseolus* enzyme was decreased by 10–20% compared with the rate observed in H_2O. However the K_m Pro values calculated for either enzyme were not significantly different when assays were performed in D_2O.

Photoinactivation in the Absence of Dye

When Pro-*t*RNA synthetase from *Phaseolus* was irradiated with 'white' light, approximately 15% of the enzyme activity was lost after 1 hr. This photoinactivation was independent of temperature between 10 and 40°. Irradiation with UV light induced a more rapid photoinactivation (~20% enzyme inactivated in 40 min) than did IR light (<5% enzyme inactivated in 1 hr).

In the absence of sulphydryl reducing reagents such as mercaptoethanol, a rapid photoinactivation of the *Delonix* enzyme was observed (Table 1). A dark control indicated that this loss of activity was not due solely to thermal instability of the enzyme at the temperature concerned (Table 1). In contrast with *Phaseolus* enzyme, the Pro-*t*RNA synthetase from *Delonix* was rapidly inactivated by IR light, over 60% of the enzyme activity being lost within 20 min at 20°. Photoinactivation of the *Delonix* enzyme was partially reversed by

subsequent incubation with 40 mM mercaptoethanol (Table 1); when photoinactivation was performed in the presence of mercaptoethanol a marked temperature dependence was observed (Fig. 3). The addition of 15% ethylene glycol together with mercaptoethanol

TABLE 1. THE ABILITY OF VARIOUS REAGENTS TO PROTECT THE Pro-*t*RNA SYNTHETASE FROM *Delonix regia* AGAINST PHOTOINACTIVATION IN THE ABSENCE OF METHYLENE BLUE*

Photoinactivation/preincubation treatment	ATP-^{32}PPi exchange activity remaining cpm
Not photoinactivated	1098
Photoinactivated, 23°	560
Photoinactivated, 23°, +ATP (2 mM)	999
Photoinactivated, 23°, +Pro (4 mM)	1000
Photoinactivated, 23°, +mercaptoethanol (20 mM)	800
Photoinactivated, 23°, +50% ethylene glycol + mercaptoethanol (20 mM)	963
Photoinactivated, 23°, then preincubated with mercaptoethanol (40 mM), 10 min	702
Dark control, 23°	907
Dark control, 0° + 20 mM mercaptoethanol	308
Dark control, 37° + 20 mM mercaptoethanol	1200

* Photoinactivation was carried out in Tris-HCl buffer, pH 8·5 for 20 min at 23° unless otherwise stated.

decreased the rate of enzyme photoinactivation to a greater extent than did mercaptoethanol alone, but it was not possible to prevent photoinactivation completely even in the presence of 50% ethylene glycol and 20 mM mercaptoethanol (Table 1). ATP and Pro also protected the *Delonix* enzyme against photoinactivation (Table 1). First order inactivation kinetics were observed both in the presence and absence of substrates.

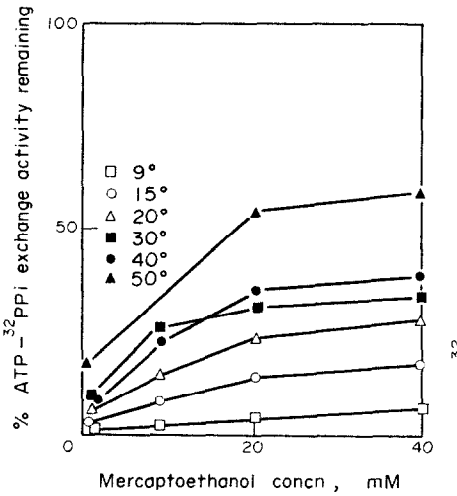

FIG. 3. EFFECT OF MERCAPTOETHANOL CONCENTRATION AND TEMPERATURE ON PHOTOINACTIVATION OF Pro-*t*RNA SYNTHETASE FROM *Delonix regia*.
The photoinactivation time was 17 min, in the presence of 15% ethylene glycol.

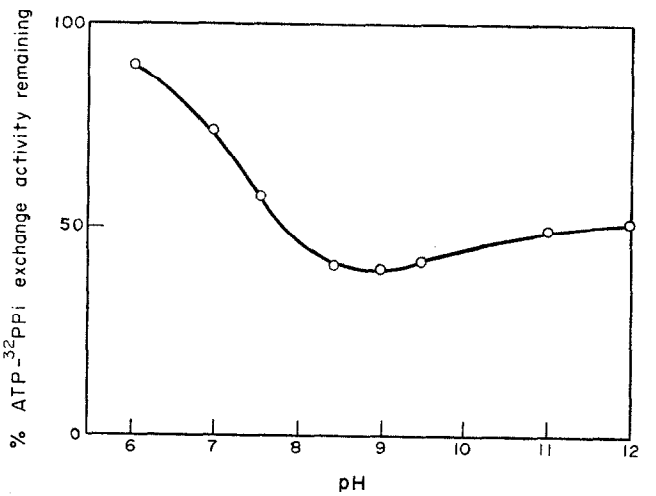

FIG. 4. pH DEPENDENCE OF METHYLENE BLUE PHOTOINACTIVATION OF Pro-*t*RNA SYNTHETASE FROM *Phaseolus aureus*.
Photoinactivation time 15 min. Buffers: Tris-maleate-KOH or NaOH-KCl (0·05 M).

Photoinactivation in the Presence of Methylene Blue or Rose Bengal

The effectiveness of photoinactivation of the *Phaseolus* enzyme in the presence of methylene blue varied with pH (Fig. 4). Small corrections of the rate constants of photoinactivation were made to allow for a slight dye-independent photoinactivation. Dark controls run at the same temperature as the photoinactivation system showed no reduction in enzyme activity.

TABLE 2. ABILITY OF SUBSTRATES OF THE PRO-*t*RNA SYNTHETASE FROM *Phaseolus aureus* TO PROTECT THE ENZYME AGAINST PHOTOINACTIVATION IN THE PRESENCE OF METHYLENE BLUE*

Substrate added to photoinactivation medium	ATP-^{32}PPi exchange activity remaining (% original activity)†
None	41
+L-Proline (4 mM)	85
+ATP (2 mM)	42
+L-Proline (2 mM) + ATP (2 mM)	88
+L-Azetidine-2-carboxylic acid (4 mM)	65
+L-Thiazolidine-4-carboxylic acid (12 mM)	65
+L-Thiazolidine-4-carboxylic acid (4 mM)	49
+*N*-Methylglycine (20 mM)	40
+D-Proline (40 mM)	49
+AMP (4 mM)	42
+PPi (4 mM)	43
+Pyrrolidine (40 mM)	45
+3-Pyrroline (40 mM)	41
+L-Proline amide (8 mM)	60
+ATP (2 mM) + pyrrolidine (40 mM)	42
+*t*RNA (*Phaseolus*, 1 mg)	60

* Photoinactivation (or preincubation) was conducted in the presence of 0·01% methylene blue at 10° for 20 min in Tris-HCl buffer, pH 8·5.

† Corrected for photoinactivation in the absence of methylene blue.

Pro, several Pro-analogues and *t*RNA protected the enzyme against dye-mediated photoinactivation (Table 2), first order kinetics of enzyme inactivation being observed both in the presence and absence of substrates. ATP, AMP, PPi, pyrrolidine and 3-pyrroline failed to protect the enzyme from photoinactivation. The presence of ATP in this photoinactivation system did not modify the protective effect of Pro or imino acid analogues, although with some purer preparations a slight decrease in the rate of inactivation (5–10%) was observed. Pyrrolidine and 3-pyrroline failed to protect the enzyme in the presence of ATP.

When photoinactivation was carried out in the presence of rose bengal at pH 8 in the presence or absence of substrates, similar results to those for the methylene blue system were obtained.

The study of methylene blue-mediated photoinactivation with the *Delonix* enzyme was complicated by the presence of a rapid dye-independent inactivation. The enzyme was inactivated more rapidly in the presence of methylene blue than in its absence but it was difficult to evaluate the extent of substrate protection against the dye-mediated inactivation

because these compounds also affected normal photoinactivation (Table 3). However a decreased protection by ATP compared with Pro was observed for methylene blue-mediated photoinactivation.

TABLE 3. PHOTOINACTIVATION OF Pro-*t*RNA SYNTHETASE FROM *Delonix regia* IN THE PRESENCE AND ABSENCE OF METHYLENE BLUE*

	Photoinactivation treatment	ATP-^{32}PPi exchange activity remaining (% original activity)
	No methylene blue	52
	+methylene blue	13
	+methylene blue + ATP (2 mM)	61
	+methylene blue + Pro (4 mM)	67
(a)	No methylene blue + mercaptoethanol (20 mM)	78
	+methylene blue + mercaptoethanol (20 mM)	35
	+methylene blue + mercaptoethanol (20 mM) + Pro (4 mM)	74
	+methylene blue + mercaptoethanol (20 mM) + ATP (4 mM)	62
	No methylene blue + mercaptoethanol (20 mM) + Pro (4 mM)	93
(b)	No methylene blue + mercaptoethanol (20 mM) + ATP (2 mM)	93
	% protection by ATP in absence of methylene blue	68†
	% protection by Pro in absence of methylene blue	68
	% protection by ATP in the presence of methylene blue	42
	% protection by Pro in the presence of methylene blue	60

* Photoinactivation was carried out at 23° for 20 min using 0·01 % methylene blue.

† Cal. as follows: [(100–(a)) − (100–(b))]/[100–(a)] × 100.

p*CMB Inhibition*

Pro-*t*RNA synthetase from *Delonix* and *Phaseolus* (20–30 μg enzyme protein) was completely inhibited by 5×10^{-4} M *p*CMB. *p*CMB-inhibited enzyme from *Phaseolus* was reactivated by the addition of sulphydryl reducing compounds. This reactivation was very rapid at temperatures between 0 and 40°. *p*CMB-inhibited enzyme from *Delonix* was not significantly reactivated by sulphydryl reducing reagents under the conditions illustrated in Table 4. However, partial reactivation was achieved by incubation of the enzyme with mercaptoethanol or dithiothreitol (20 mM) at temperatures between 15 and 40°, this reaction being time and temperature dependent (Fig. 5). In order to prevent reactivation of the enzyme during the assay period, it was essential to dilute the partially reactivated enzyme preparations so that the final concentration of sulphydryl reducing reagents present in the assay medium was less than 1 mM. The ability of the different thiol reagents to effect reactivation of the *Phaseolus* enzyme was in the order mercaptoethanol > thioglycollic acid > reduced glutathione > cysteine. Reactivation of the *Delonix* enzyme was also dependent on the concentration of sulphydryl reducing reagent used, and the maximum

level of reactivation was achieved with 20 mM mercaptoethanol (or dithiothreitol) at a temperature of 30°. In contrast with the 87% reactivation obtained for *p*CMB-inhibited enzyme from *Phaseolus*, *Delonix* enzyme was never reactivated by more than 65%.

TABLE 4. THE ABILITY OF SULPHYDRYL REDUCING REAGENT TO REVERSE THE *p*CMB-INHIBITED Pro-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia*

Reducing compound	Reactivation (%) *P. aureus* Inhibition temp.* 0°	15°	*D. regia* Inhibition temp.* 0°	15°
Dithiothreitol	90	88	7	20
Mercaptoethanol	85	89	0	17
Thioglycollic acid	82	80	0	14
Reduced glutathione	72	76	0	5
Cysteine	70	70	0	5

* 400 μg enzyme protein was inhibited with 5×10^{-5} M *p*CMB at either 0° or 15°, then incubated for 10 min at 15° in the presence of various sulphydryl reducing reagents (40 mM). Aliquots of enzyme were then taken and diluted to reduce the concentration of reducing compounds in the assay medium to 0·5 mM.

Substrate Protection Against p*CMB Inhibition*

Substrates of the Pro-*t*RNA synthetase partially protected the *Phaseolus* or *Delonix* enzymes against *p*CMB inhibition (Table 5). This protection was only observed over a narrow range of *p*CMB concentration (Fig. 6). Certain analogues of Pro which did not stimulate ATP-^{32}PPi exchange catalyzed by the enzyme from either *Delonix* or *Phaseolus*,[3] protected the synthetase against *p*CMB inhibition. For example, enzymes from *Delonix*

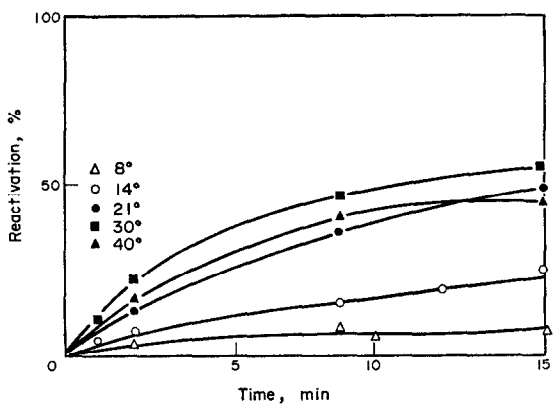

FIG. 5. REACTIVATION OF Pro-*t*RNA SYNTHETASE FROM *Delonix regia* AFTER *p*CMB INHIBITION. Enzyme was inhibited by 5×10^{-4} M *p*CMB then incubated at various temps. in the presence of 20 mM mercaptoethanol. Aliquots were withdrawn periodically and assayed by the ATP-^{32}PPi exchange reaction.

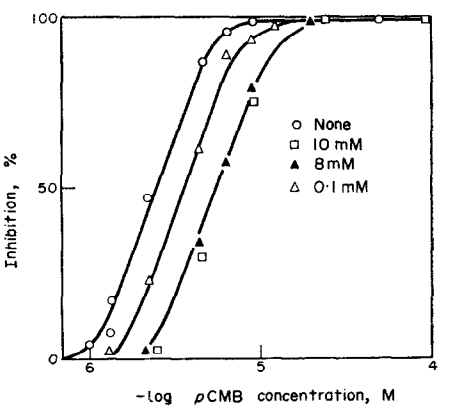

FIG. 6. *p*CMB INHIBITION CURVE FOR Pro-*t*RNA SYNTHETASE FROM *Phaseolus aureus*. Concentration of Pro in preincubation medium is shown in Fig. Concentration of enzyme/tube = 10 μg protein.

and *Phaseolus* were protected by A2C and methanoproline, respectively. The level of protection against *p*CMB inhibition conferred on the *Phaseolus* enzyme by the addition of ATP and Pro together was not significantly greater than that observed when Pro was added alone (Table 5). Similarly, the degree of protection conferred by *t*RNA was not increased by the addition of either ATP or Pro.

TABLE 5. THE ABILITY OF SUBSTRATES AND INHIBITORS OF THE PRO-*t*RNA SYNTHETASE FROM *Delonix regia* AND *Phaseolus aureus* TO PROTECT THE ENZYME AGAINST *p*CMB INHIBITION*

Protecting compound	Protection (%) *Phaseolus*	*Delonix*
L-Proline (4 mM)	46	29
L-Azetidine-2-carboxylic acid (4 mM)	38	17
(40 mM)	46	
L-Thiazolidine-4-carboxylic acid (4 mM)	20	
(40 mM)	40	23
N-Methylglycine (40 mM)	15	
D-Proline (40 mM)	13	10
cis(exo)-3,4-Methano-L-Proline (4 mM)	17	18
3,4-Dehydro-DL-Proline (4 mM)	45	24
L-Proline amide (4 mM)	38	
L-Proline methyl ester (4 mM)	40	
Pyrrolidine (40 mM)	0	0
3-Pyrroline (40 mM)	0	0
ATP (2 mM)	38	27
AMP (2 mM)	17	0
PPi (8 mM)	3	2
*t*RNA (homologous-0·05 mg)	47	4
ATP (2 mM) + Pro (4 mM)	46	40
ATP (2 mM) + pyrrolidine (40 mM)	38	27
ATP (2 mM) + 3-pyrroline (40 mM)	38	28

* *Phaseolus* enzyme: protein concentration, 7 μg/ml; *p*CMB, 5×10^{-6} M. *Delonix* enzyme: protein concentration, 40 μg/ml; *p*CMB, $2{\cdot}5 \times 10^{-4}$ M.

The efficiency of protection against *p*CMB inhibition conferred by Pro and Pro-analogues can be defined in terms of $P^{\frac{1}{2}}$, i.e. the concentration of substrate that protects the enzyme from *p*CMB inhibition to an extent of 50% of the maximum protection possible at a fixed *p*CMB concentration. The $P^{\frac{1}{2}}$ values for Pro at several different points on the *p*CMB inhibition curve were determined by plots analogous to those of Lineweaver and Burk[11] [(concentration of protecting substrate)$^{-1}$ vs (% protection relative to that observed at saturating protective concentrations of substrate)$^{-1}$]. No significant difference in the $P^{\frac{1}{2}}$ values were noted at *p*CMB concentrations which caused between 40 and 100% inactivation of the enzyme. $P^{\frac{1}{2}}$ values were most accurately determined when the concentration of *p*CMB used still produced some inhibition irrespective of the level of protective substrate. The $P^{\frac{1}{2}}$ values determined for Pro and a number of Pro analogues are illustrated in Table 6 for the *Phaseolus* enzyme.

[11] LINEWEAVER, H. and BURK, D. (1934) *J. Am. Chem. Soc.* **56**, 658.

Insensitivity of Pro-tRNA Synthetase to Sodium Azide

Sodium azide (10^{-3} M) failed to inhibit Pro-*t*RNA synthetase from *Phaseolus* or *Delonix* in the absence of substrates. However, a slight inhibition of ATP-^{32}PPi exchange (5–8%) catalyzed by the *Delonix* enzyme was observed when sodium azide was present in the assay medium at a concentration of 10^{-1} M. This was probably due to a salt effect (see Ref. 3).

TABLE 6. THE $P^{\ddagger}$ VALUES* FOR SUBSTRATE PROTECTION OF PRO-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AGAINST *p*CMB INHIBITION AND A COMPARISON WITH OTHER KINETIC PARAMETERS

Substrate	$P^{\ddagger}$ (M)	π† (M)	K_m‡ (M)
L-Proline	$1{\cdot}25 \times 10^{-4}$	$2{\cdot}5 \times 10^{-5}$	$1{\cdot}4 \times 10^{-4}$
L-Azetidine-2-carboxylic acid	3×10^{-4}	3×10^{-5}	$1{\cdot}4 \times 10^{-3}$
L-Thiazolidine-4-carboxylic acid	4×10^{-3}	$1–5 \times 10^{-2}$	2×10^{-2}
N-Methylglycine	3×10^{-2}	$7–8 \times 10^{-4}$	7×10^{-2}
D-Proline	$\sim 7 \times 10^{-3}$	$\sim 0{\cdot}7–1{\cdot}3 \times 10^{-4}$	∞

* See text for details.
† Substrate binding constant (see Ref. 5).
‡ K_m measured for Pro- or analogue-dependent ATP-^{32}PPi exchange.

DISCUSSION

The sharp inflection in the pH-activity profile of the *Delonix* and *Phaseolus* enzymes below pH 6·7 may reflect either; (a) changes in the ionization of a substrate molecule or of a catalytic group on the enzyme, or (b) dissociation of the enzyme into inactive subunits. A rapid decrease in ATP-^{32}PPi exchange below pH 6·5 has been reported for Gly-*t*RNA synthetase from rat liver[10] and Val-*t*RNA synthetase from *E. coli*.[12] Many other aminoacyl-*t*RNA synthetases from *E. coli*[13] and wheat germ[14] are unstable under acid conditions, and the Pro-*t*RNA synthetase from *Delonix* is almost instantaneously inactivated at pH 6 (Norris and Fowden, unpublished results). The rapid loss of exchange activity catalyzed by the Pro-*t*RNA synthetase from *Delonix* on decreasing the pH, compared with that of *Phaseolus*, is consistent with the idea that dissociation of the *Delonix* enzyme into subunits occurs below pH 6·7 and leads to an inactive form of the enzyme. Such a situation has been described for aldolase.[15]

The effect of pH on the K_m of substrates of the Pro-*t*RNA synthetase may provide information concerning the type of ionizing groups present at the active site.[16] The inflection in the pH vs log K_m(Pro) graph (Fig. 2) indicated that a change in ionization associated with the Pro-binding site occurs at pH 6·2. The pK_a for the COO^- group of Pro is 1·92 and the pK_a for the imino group is 9·73.[17] Even when Pro is complexed with the enzyme, it is unlikely that either of these groups would undergo an ionization change at pH 6·3, since the pK ranges of individual groups are not generally shifted more than 2 units by interaction with neighbouring groups.[18] The pK_a values for ATP (1, 4 and 6·0–6·9),[17] do not rule

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out the possibility that a change in ionization of ATP is partly responsible for the inflection in the pH-activity curve. However, since ATP binds to the synthetase in the form of various ATP–Mg–PPi complexes (chiefly as the ATP-monomagnesium complex),[19] the β- and γ-PO_4^{3-} groups are likely to be neutralized under most conditions, leading to pK_a values for ATP–Mg–PPi complexes of less than 4.[20–22] The constant K_m values for ATP over the pH ranges tested for the *Phaseolus* and *Delonix* enzyme supports the view that a change in ionization of a group at the ATP-binding site is not responsible for the decrease in ATP-^{32}PPi exchange activity below pH 6·7. Since a change in ionization of substrate molecules may be ruled out, the inflection in the pH vs log K_m graph must correspond to an ionization change of a group in the enzyme. The group most likely to undergo such a change at pH 6·2 is the imidazole ring of His which as a pK_a of 6·09[23] and the slope of the pH vs K_m graph below pH 6·7 (1·15) indicates that only a single group is involved.[24] The inflection at pH 6·2 in the pH vs log K_m (imino acid) profiles for the Pro-enzyme from *Phaseolus* (measured by ATP-^{32}PPi exchange) contrasts with that reported for the Arg-, Lys-, $GluNH_2$- and Pro-enzymes from *E. coli* (measured by the aminoacylation assay), where a steep inflexion was observed at pH 7.[25]

Supporting evidence for a His residue at the active site of the Pro-enzyme was provided by photoinactivation studies. Photoinactivation of His residues in peptides and enzymes in the presence of methylene blue is more rapid than the photoinactivation of other residues.[26–28] Loss of enzyme activity on photoinactivation of lysozyme,[28] ribonuclease,[29] dextransucrase[30] and chymotrypsin[31] has been correlated with the destruction of His residue. The pH curve for methylene blue or rose bengal-mediated photoinactivation fellowed that expected from the ionization of a His residue.

The data obtained from methylene blue photoinactivation experiments (Table 2) supported the view that the readily photoinactivated residue was associated with the Pro-binding site rather than the ATP-binding site. The degree of protection conferred by A2C and thiazolidine-4-carboxylic acid against photoinactivation of the *Phaseolus* enzyme was related to the ability of these compounds to stimulate ATP-^{32}PPi exchange.[3] The failure of pyrrolidine and 3-pyrroline to protect the *Phaseolus* enzyme from photoinactivation even in the presence of ATP indicates that the His residue is associated with the position occupied by the carboxyl group of Pro when the imino acid binds to the enzyme. It is difficult to interpret the protection against photoinactivation conferred by *t*RNA as being due to the –CCA end of the polynucleotide because of the bulky nature of this molecule. However, it seems plausible that the –CCA terminus of *t*RNA should be associated more closely with the imino acid site than with the ATP site to facilitate aminoacylation.[6,32] The inability of ATP to reduce the protection conferred by Pro against photoinactivation to any significant extent

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contrasts markedly with the results of Boyko and Fraser[10] for the Gly-*t*RNA synthetase from rat liver, where the addition of ATP abolished the ability of Gly to protect the enzyme against inactivation. The inability of ATP to affect photoinactivation in the presence of Pro, reported here, is consistent with the idea that Pro and ATP are strongly bound to their respective sites.[5,33] If the amino acid substrate did move to another site in the presence of ATP as suggested by Boyko and Fraser, there must be a rapid equilibrium in the present instance between Pro bound to the Pro site and Pro-adenylate bound to the aminoacyl site. The methylene blue-mediated photoinactivation data for the Pro-*t*RNA synthetase from *Delonix* suggests that a His group might be present at the active site of this enzyme. Although methylene blue-mediated photoinactivation is not entirely specific for His,[34–36] inactivation of the SH group at the active site was discounted by the observation that ATP protected both the *Phaseolus* and *Delonix* enzymes against *p*CMB inhibition but not against photoinactivation. Photoinactivation in the presence of rose bengal is more specific for His residues than is methylene blue-mediated photoinactivation.[37] The similarity of both photoinactivation systems suggests that the same residue in the protein is undergoing photoinactivation in both cases. However, the conclusions reached from photoinactivation results alone should be interpreted with care.[36]

The reversible photoinactivation of the *Delonix* enzyme in the absence of methylene blue may reflect a dissociation of the synthetase into inactive subunits and their recombination in the presence of mercaptoethanol. The protection against photoinactivation afforded by ethylene glycol is consistent with this since such polyols are known to prevent subunit dissociation of aminoacyl-*t*RNA synthetases.[38,39]

The slight decrease in the V_m of Pro for the ATP-^{32}PPi exchange in D_2O compared with H_2O is consistent with the idea that general base catalysis is involved in the mechanism of the exchange reaction. However, the magnitude of this effect is not sufficient to support the idea that proton transfer from water represents the rate determining step as found in the chymotrypsin catalytic mechanism.[40] Nucleophilic catalysis has been implicated in aminoacyl-*t*RNA synthetase mechanisms by several workers,[41–43] and the observed inhibition of ATP-^{32}PPi exchange by D_2O may reflect an inhibitory effect upon the base catalytic action of the imidazole and sulphydryl groups.

Under certain conditions, sodium azide acts as a specific inhibitor of Met residues.[44,45] The lack of inhibition of ATP-^{32}PPi exchange after treatment of either the *Phaseolus* or *Delonix* enzyme with 10^{-2} M NaN_3 suggests that a Met residue is not implicated in the catalytic mechanism, although confirmatory evidence is required.

The temperature-dependent reactivation of the *p*CMB-inhibited enzyme from *Delonix*

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may be explained by a dissociation of the synthetase into inactive subunits on addition of *p*CMB and temperature-dependent reassociation of these to form the active enzyme. The reassociation of inactive subunits of the Pro-*t*RNA synthetase from *Delonix* is dependent on mercaptoethanol concentration, temperature and incubation time (Norris and Fowden, unpublished results) and this is reflected by the reactivation kinetics observed for the *p*CMB-inhibited enzyme (Fig. 5). Thus reassociation of subunits of the *p*CMB inhibited enzyme occurs most rapidly at about 30° and is slower at lower temperatures.

Partial protection against *p*CMB inhibition by substrates may suggest a role of the SH group in the binding of these compounds to the active site of the synthetase, although organic mercurials at high concentrations can inhibit enzyme reactions not involving SH groups.[46] The concentrations of *p*CMB used in the experiments reported here for the Pro-*t*RNA synthetase rule out this possibility. The data relating to substrate protection against *p*CMB inhibition suggest that the carboxyl group of Pro is required for this reaction, since Pro and its analogues, but not pyrrolidine or 3-pyrroline (in the presence or absence of ATP), protected the enzyme against the mercurial. The protection of the *Phaseolus* enzyme by D-Pro and the *Delonix* enzyme by A2C, together with the $P^{\frac{1}{2}}$ values for several Pro analogues, indicated that the efficiency of protection of individual compounds against *p*CMB inhibition relative to Pro was lower than that expected from the relative values of the binding constants (see Ref. 5), but greater than that expected from the K_ms for the ATP-^{32}PPi exchange reaction (Table 6). This suggests that the degree of protection conferred by analogues of Pro against *p*CMB inhibition is related to; (a) initial binding of the compound to the enzyme, and (b) carboxyl group orientation with respect to the SH group. Compounds smaller than Pro, e.g. A2C, protected the *Delonix* enzyme less efficiently relative to Pro against *p*CMB inhibition than the *Phaseolus* enzyme. This is consistent with the idea that the *Delonix* enzyme has a larger active site than the *Phaseolus* enzyme. ATP, Pro and *t*RNA protected Pro-*t*RNA synthetase against *p*CMB inhibition but no significant increase over the level of protection exhibited by Pro alone was observed on the addition of both ATP and Pro. This suggested that a single SH group was involved in the ATP–^{32}PPi exchange reaction. The ability of a combination of ATP and Pro to protect the enzyme from *Delonix* against *p*CMB inhibition to a greater extent than either substrate added alone may merely reflect the use of non-saturating protective concentrations of substrates or the rapid oxidation of the SH group in the absence of mercaptoethanol.

The role of the His and Cys (SH) groups at the active site remains to be clarified. However, nucleophilic attack by the imidazole N of the His group on the carbonyl oxygen of the carboxyl group of Pro may be responsible for the formation of an acyl bond with the α-PO_4^{3-} group of ATP. Additional nucleophilic catalysis could also be provided by the SH group. The ability of the *Delonix* enzyme to discriminate against A2C may be related to the positions of the His and Cys groups relative to the imino binding site. The conformational change elicited on the *Delonix* enzyme by the binding of A2C may be different from that elicited by Pro.[5] Thus it may be envisaged that the alignment of the carboxyl group of the analogue with the SH or His groups (or both) may be such as to minimize nucleophilic catalytic action of these groups on A2C, whilst the alignment of the carboxyl group of Pro with these residues may be such that efficient ATP-^{32}PPi exchange can be promoted.

EXPERIMENTAL

Plant materials. Seeds of *P. aureus* were obtained from the Bombay Emporium, London. *D. regia* seeds were the gift of Prof. L. B. Thrower (Hong Kong).

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Imino acid analogues. All samples were obtained from sources previously described[5] except for proline amide (Sigma) and proline methyl ester (Sigma). All samples were tested for purity by PC.

Radioisotopic chemicals. [^{32}P]Pyrophosphate was prepared as previously described[5] to give a product of specific activity 0·3–0·5 μCi/μmol.

Other chemicals. Methylene blue was obtained from A. Gurr Ltd., sodium azide from Sigma Chemicals and *p*-chloromercuribenzoate from British Drug Houses Ltd.

Determination of protein and nucleic acids. Protein was determined by the Lowry method[47] or from extinction values measured at 260 and 280 nm[48] with bovine serum albumin as a standard. The amount of RNA in the synthetase preparations was determined by the method of Key and Shannon[49] or by the orcinol method[50] using yeast nucleic acid as a standard.

Enzyme fractionation. Pro-*t*RNA synthetase from *Delonix* and *Phaseolus* was purified as described in a previous paper.[5] DEAE-cellulose fractions containing Pro-*t*RNA synthetase activity were used for most experiments after removal of salts and *t*RNA on a column of Sephadex G75, equilibrated with 0·02 M Tris-HCl buffer, pH 8, containing 10 mM $MgCl_2$. Sephadex G75 chromatography was performed at room temp. (20–23°) to prevent cold inactivation of the Pro-enzyme.

tRNA isolation. The modification of the general phenol method as described by Vanderhoef *et al.*[51] was used to isolate *t*RNA from *Phaseolus*. Carbohydrates were removed from *Delonix t*RNA preparations by the methoxyethanol method[52] and from *Phaseolus* preparations by DEAE-cellulose chromatography.

Photoinactivation procedure. A modification of the method described by Weil *et al.*[26] was used. Purified enzyme preparations were photoinactivated, in the presence or absence of 0·01% methylene blue (or 0·01% rose bengal) by irridation with a 500 W tungsten lamp placed 75 cm vertically above aliquots (2 ml) of the solution. Enzyme photoinactivation in the absence of dye was also performed using UV and IR light sources (Philips 150 W) placed 52 and 42 cm respectively above the surface of the enzyme solution. The light intensity at the surface of the enzyme solution was 150 lx for the tungsten lamp and 40 lx for both the UV and IR lamps. The solution to be photoinactivated contained Tris-HCl buffer, pH 8·7, (50 mM); $MgCl_2$ (10 mM); enzyme preparation (200–800 μg protein) and methylene blue or rose bengal (0·01%). Dark controls and controls in the absence of methylene blue were run concomitantly with the experimental tubes. Substrates and inhibitors of the Pro-*t*RNA synthetase (when present in the photoinactivation system) were added so that they were generally present at a final concentration of 2, 4 or 40 mM. The tubes were kept well shaken (generally at 23°). Aliquots of 0·1 ml were withdrawn periodically as the photoinactivation reaction proceeded, and stored in the dark until required for assay.

Azide and pCMB preincubation. Preincubation solutions contained Tris-HCl buffer, *p*H 8 (50 mM), $MgCl_2$ (10 mM), enzyme preparation (200–800 μg protein), protecting substrates (0–40 mM) and either sodium azide (10 mM) or *p*CMB (0·1–100 μM); total vol. 1 ml. Enzyme was added last and the tubes were kept well shaken at room temp. and aliquots of 0·1 ml withdrawn after 10 min and assayed for residual enzyme activity.

Assay procedure. (*a*) *ATP-^{32}PPi exchange.* Incubation mixtures generally contained: Tris-HCl buffer pH 8 (100 μmol), $MgCl_2$ (14 μmol), ATP (2 μmol), ^{32}PPi (2 μmol), Pro (10 μmol) and enzyme preparation ($\sim$100 μg); total vol. 1 ml. The concentration of ATP and Pro added to make up the assay mixture in individual experiments were related to the concentration of substrates or inhibitors (when present) in the aliquots of photoinactivated or *p*CMB-inhibited enzyme. The assay procedure followed that previously described.[3] Residual activity of methylene blue (or rose bengal) photoinactivated enzyme was assayed in the dark. The concentration of methylene blue in the assay medium did not affect the ATP-^{32}PPi exchange reaction nor affect the adsorption of ATP onto charcoal. (*b*) *Aminoacylation reaction:* This was performed at 25° as described in a previous paper.[3] The initial rate of charging was calculated after aliquots of the reaction mixture were withdrawn after 1, 2 or 5 min.

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