Phytochemistry, 1974, Vol. 13, pp. 1677 to 1687. Pergamon Press. Printed in England.

# COLD-LABILITY OF PROLYL-*t*RNA SYNTHETASE FROM HIGHER PLANTS

ROGER D. NORRIS

Department of Botany and Microbiology, University College, London

and

LESLIE FOWDEN

Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ

(*Received* 23 *January* 1974)

**Key Word Index**—*Delonix regia*; *Phaseolus aureus*; Leguminosae; Pro-*t*RNA Synthetase; cold-lability; substrate protection; enzyme reactivation; subunit structure; imino acid analogues.

**Abstract**—Pro-*t*RNA synthetase from *D. regia* and *P. aureus* lost enzymic activity more rapidly at 0° than at room temperature. The enzyme from a number of higher plants that produce azetidine-2-carboxylic acid (A2C) was more rapidly inactivated in the cold than the enzyme from plants which do not contain A2C. The rate of cold inactivation was dependent on temperature and on the concentration of glycerol, protein and sulphydryl-reducing reagents. Substrates of Pro-*t*RNA synthetase also stabilized the enzyme against cold inactivation. Enzyme which had been completely inactivated by storage in the cold, could be reactivated by warming in the presence of a sulphydryl-reducing reagent. The rate of reactivation was dependent on temperature, pH and the concentration of sulphydryl-reducing reagent. Kinetic analysis indicated the existence of more than one molecular form of the enzyme. It is suggested that the cold-lability of Pro-*t*RNA synthetase may be due to dissociation of the active enzyme molecule into inactive subunits.

## INTRODUCTION

RECENT investigations in the authors' laboratories has provided considerable information concerning the properties and substrate specificity of Pro-*t*RNA synthetase extracted from higher plants.[1–4] The enzyme from species which synthesize A2C e.g. *Delonix regia*, is able to discriminate more efficiently against imino acid analogues which are less bulky than Pro, than the enzyme from species which lack A2C e.g. *Phaseolus aureus*.[2,3] The efficiency of such discrimination may partly depend on the presence of ATP.[3] Many plants synthesize non-protein amino acids which are capable of acting as analogue molecules. The ability of specific aminoacyl-*t*RNA synthetases from such plants to discriminate against these potentially toxic compounds has been discussed elsewhere.[5–8] In some cases these synthetases are unstable during purification[2,3,6–9] and more sensitive to salts[2,7] and heat [2,3] than

[1] PETERSON, P. J. and FOWDEN, L. (1965) *Biochem. J.* **97,** 112.
[2] NORRIS, R. D. and FOWDEN, L. (1972) *Phytochemistry* **11,** 2921.
[3] NORRIS, R. D. and FOWDEN, L. (1973) *Phytochemistry* **12,** 2109.
[4] NORRIS, R. D. and FOWDEN, L. (1973) *Phytochemistry* **12,** 2829.
[5] LEA, P. J. and NORRIS, R. D. (1972) *Phytochemistry* **11,** 2897.
[6] LEA, P. J. and FOWDEN, L. (1972) *Phytochemistry* **11,** 2129.
[7] ANDERSON, J. W. and FOWDEN, L. (1970) *Biochem. J.* **119,** 677.
[8] SMITH, I. K. and FOWDEN, L. (1968) *Phytochemistry* **7,** 1065.
[9] LEA, P. J. and FOWDEN, L. (1972) *Phytochemistry* **12,** 1903.

the corresponding enzymes from plants which do not contain amino acid analogues. Pro-*t*RNA synthetase from *D. regia* is particularly heat-labile in the absence of substrates[2,3] and becomes inactivated very rapidly during purification, especially at low temperatures.[3,4] Although the Pro-enzyme is the most heat-stable of the aminoacyl-*t*RNA synthetases from *P. aureus*,[10] it also becomes inactivated during lengthy purification procedures.[2–4] We previously reported that enzymically active preparations of Pro-*t*RNA synthetase from *D. regia* often could only be obtained by preheating the enzyme for several minutes prior to assay.[2,3] This reactivation was dependent on the presence of a sulphydryl-reducing reagent.[3,4] Papas and Mehler[11] have suggested that the Pro-*t*RNA synthetase from *Escherichia coli* undergoes modification at low temperatures. Lee and Muench[12] have proposed that this is due to a dissociation of the active enzyme into inactive subunits. We have shown that the rate of ATP-$^{32}$PPi exchange catalysed by Pro-*t*RNA synthetase from both *D. regia* and *P. aureus* shows a complex dependence on temperature.[3] This was thought to be due in part to the existence of different molecular forms of the enzyme. In this paper we explore more fully the nature of the cold-lability of Pro-*t*RNA synthetase from both A2C-producer and non-producer plants and present further data concerning the reactivation of these enzymes.

## RESULTS

### *Enzyme fractionation*

Crude homogenates or $(NH_4)_2SO_4$ fractions of *D. regia* containing Pro-*t*RNA synthetase activity were unstable to fractionation on Sephadex G100 at 4°, even in the presence of 15% glycerol. Passage through Sephadex G75 in the absence of mercaptoethanol often caused partial inactivation of the enzyme (10–40%). However, at room temperature in the presence of 40 mM mercaptoethanol, no loss of enzyme activity was observed following passage through Sephadex G75, although a decrease in activity accompanied fractionation of Sephadex G100 or G200. By carrying out enzyme extraction and $(NH_4)_2SO_4$ fractionation at room temperature in the presence of mercaptoethanol a slightly higher specific activity of Pro-*t*RNA synthetase was obtained than at 4°. Desalted $(NH_4)_2SO_4$ fractions of *Delonix* enzyme were extremely unstable on DEAE-cellulose at 0–4°C and active enzyme preparations could only be obtained with a fast flow rate and high glycerol concentration (> 15%) in the elution buffer. Fractionation on DEAE-cellulose at 30° gave better yields of active enzyme but further purification on Sephadex was impossible even in the presence of 30% glycerol and 40 mM mercaptoethanol. Pro-*t*RNA synthetase from *Parkinsonia aculeata* and *Convallaria majalis* (both A2C-producer plants) exhibited similar fractionation characteristics to the *Delonix* enzyme. The enzyme from *P. aureus* was less stable to fractionation on DEAE-cellulose at 4° than at room temperature and further fractionation on hydroxyapatite resulted in considerable loss of ATP-$^{32}$PPi exchange activity. Elution profiles from hydroxyapatite columns were variable, even under identical running conditions and there was often a complete inactivation of the enzyme on columns run at 4°.

### *Storage properties*

$(NH_4)_2SO_4$ fractions of Pro-*t*RNA synthetase from *P. aureus* were fully stable in solution for several days at 2° in the presence of 15% glycerol and 40 mM mercaptoethanol,

[10] NORRIS, R. D. and FOWDEN, L. (1973) *Biochim. Biophys. Acta* **312**, 695.
[11] PAPAS, T. S. and MEHLER, A. H. (1968) *J. Biol. Chem.* **243**, 3767.
[12] LEE, M.-L. and MUENCH, K. H. (1969) *J. Biol. Chem.* **244**, 223.

but lost most of their ATP–$^{32}$PPi exchange activity within a few hours in the absence of these reagents. $(NH_4)_2SO_4$ precipitates were considerably more stable at −20° than the redissolved pellet. Although some variation in the rate of cold-inactivation of DEAE-cellulose fractions was observed, these were always more labile than $(NH_4)_2SO_4$ preparations.

TABLE 1. EFFECT OF IONIC STRENGTH, pH AND PROTEIN CONCENTRATION ON THE STORAGE PROPERTIES AT 2° OF PROLYL-*t*RNA SYNTHETASE FROM *Delonix regia*

| Storage conditions* | % ATP–$^{32}$PPi exchange activity remaining after 5 min (duplicate results) | |
|---|---|---|
| 0·1 M Tris–maleate KOH buffer, pH 6·5 | 50 | 25 |
| 0·1 M Tris–maleate KOH buffer, pH 7·0 | 20 | 19 |
| 0·1 M Tris–maleate KOH buffer, pH 7·5 | 37 | 26 |
| 0·1 M Tris–maleate KOH buffer, pH 8·0 | 15 | 14 |
| 0·1 M Tris–maleate KOH buffer, pH 9·0 | 20 | 27 |
| 0·1 M Tris–HCl buffer, pH 8·0, protein conc. 50–100 μg/ml | 32 | 35 |
| 0·02 M Tris–HCl buffer, pH 8·0, protein conc. 50–100 μg/ml | 30 | 22 |
| 0·002 M Tris–HCl buffer, pH 8·0, protein conc. 50–100 μg/ml | 18 | 11 |
| 0·02 M Tris–HCl buffer, pH 8·0, protein conc. 500 μg/ml† | 42 | 30 |
| 0·02 M Tris–HCl buffer, pH 8·0, protein conc. 30 μg/ml | 27 | 22 |

* $(NH_4)_2SO_4$ fractions desalted on Sephadex G75 were used for these experiments.
† Protein concentration diluted to 30 μg/ml prior to assay.

The ionic strength and concentration of protein during storage also appeared to influence the rate of inactivation of Pro-*t*RNA synthetase at low temperatures (Table 1). Under all storage conditions, the enzyme from *Ranunculus bulbosa* and *Hemerocallis fulva* (plants which do not produce A2C) appeared to be marginally less stable than that from *P. aureus* (also a non-producer of A2C). Preparations of Pro-*t*RNA synthetase from A2C-producer plants were extremely unstable to storage at low temperature, complete loss of activity being observed within 30 min at 2° (in the absence of glycerol and mercaptoethanol). The rate of inactivation of these enzymes at 2° was reduced by including a sulphydryl-reducing

TABLE 2. ABILITY OF POLYOLS AND SULPHYDRYL-REDUCING REAGENTS TO PROTECT PRO-*t*RNA SYNTHETASE AGAINST INACTIVATION ON STORAGE AT 2°

| Additions to storage medium* | % ATP–$^{32}$PPi activity remaining after storage for 20 min at 2° | |
|---|---|---|
| | *Delonix regia* | *Phaseolus aureus* |
| None | 8 | 15 |
| Glycerol, 15%† | 20 | 52 |
| Glycerol, 25% | 46 | 80 |
| Glycerol, 50% | 79 | 95 |
| Ethylene glycol, 15% | 15 | |
| Ethylene glycol, 25% | 41 | |
| Ethylene glycol, 50% | 63 | 87 |
| Sucrose, 15% | 12 | |
| Sucrose, 25% | 20 | 27 |
| Mercaptoethanol, 40 mM | 10 | 17 |
| Dithiothreitol, 40 mM | 10 | 18 |
| Thioglycollic acid, 40 mM | 8 | 15 |

* The storage medium always contained 0·05 M Tris–HCl buffer pH 8 and 0·05 M $MgCl_2$.
† All percentages based on w/v.

reagent or a polyol, e.g. glycerol, in the storage medium. The degree to which such compounds protected the synthetase against cold-inactivation was dependent on their nature and concentration (Table 2). Although sulphydryl-reducing reagents protected the enzyme only marginally, the inclusion of a polyol together with the sulphydryl-reducing reagent appeared to synergistically enhance the stability of the enzyme on storage at 2° (Fig. 1).

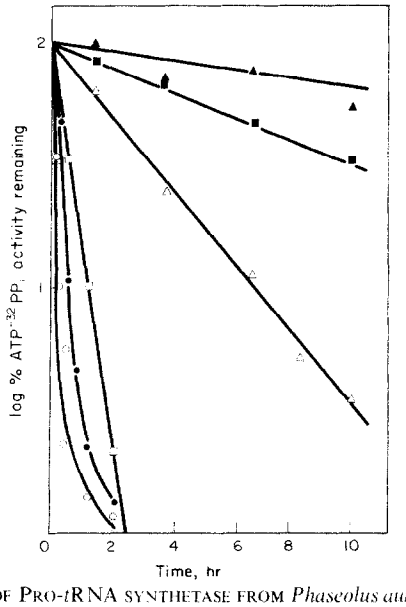


FIG. 1. STORAGE PROPERTIES OF PRO-*t*RNA SYNTHETASE FROM *Phaseolus aureus* AND *Delonix regia* AT 2°. Storage conditions; *Delonix* enzyme in 0·02 M Tris–HCl buffer, pH 8·0: ○—○, no additions; ●—●, +40 mM mercaptoethanol; △—△, +40% glycerol; ▲—▲, +40% glycerol + 40 mM mercaptoethanol. *Phaseolus* enzyme in 0·02 M Tris–HCl buffer, pH 8·0: □—□, no additions; ■—■, + 15% glycerol.

Similar patterns of stabilization were observed with DEAE-cellulose fractions from those plants which do not contain A2C. ATP, Pro and several Pro analogues also prevented inactivation of the enzyme from both *Delonix* and *Phaseolus* at 0° (Table 3). The *Phaseolus* enzyme appeared to be more efficiently protected by A2C than that from *Delonix*. Enzyme inactivation at 0–5° in the presence or absence of substrates followed approximately first-order kinetics although the Pro-*t*RNA synthetase from A2C-producer plants exhibited slight deviations from this pattern (Fig. 1). Experiments conducted at pH values from 6·5–9 (using Tris–maleate–KOH and Tris–HCl buffers) were generally inconclusive; large variations in the rate of decay of similar enzyme preparations were observed under the same conditions of pH, ionic strength and protein concentration.

When freshly prepared enzyme from either *Delonix* or *Phaseolus* was stored at room temperature or at 37° in the presence of mercaptoethanol, very little inactivation (< 10%) was observed within 20 min. The presence of glycerol was not essential to maintain enzyme stability at these temperatures.

*Urea inactivation*

At room temperature in the presence of mercaptoethanol, glycerol protected Pro-*t*RNA synthetase from both *Phaseolus* and *Delonix* against inactivation by urea. The time taken

for urea (4 M) to reduce enzyme activity by 50% was increased in the presence of 15% glycerol from 0·7 to 7 and from 0·3 to 1·5 min for the *Phaseolus* and *Delonix* enzymes respectively.

TABLE 3. ABILITY OF SUBSTRATES OF PRO-*t*RNA SYNTHETASE TO PROTECT THE ENZYME AGAINST INACTIVATION AT 0°

| Substrate | % ATP-$^{32}$PPi exchange activity remaining after 20 min at 0° | |
|---|---|---|
| | *Delonix regia* | *Phaseolus aureus* |
| None | 9 | 15 |
| L-Proline (4 mM) | 96 | 98 |
| L-Proline (0·4 mM) | 80 | 91 |
| L-Azetidine-2-carboxylic acid (20 mM) | 45 | 84 |
| L-Azetidine-2-carboxylic acid (4 mM) | 18 | 80 |
| 3,4-Dehydro-DL-proline (20 mM) | 70 | |
| 3,4-Dehydro-DL-proline (4 mM) | 39 | |
| L-Thiazolidine-4-carboxylic acid (20 mM) | 14 | 18 |
| ATP (2 mM) | 99 | 99 |

* Enzyme was always dissolved in 0·01 M Tris–HCl buffer containing 0·05 M $MgCl_2$.

*Enzyme reactivation*

Pro-*t*RNA synthetase from *D. regia*, following loss of ATP-$^{32}$PPi exchange activity, appeared to be gradually reactivated during several days storage at 0–2° in the presence of mercaptoethanol or dithiothreitol. Enzyme preparations from *D. regia* and *P. aculeata*, inactivated at 0–2°, could also be reactivated by incubation at 37° in the presence of a sulphydryl-reducing reagent. The extent of reactivation was dependent on the concentration of sulphydryl-reducing reagent, preincubation time and pH (Figs. 2–4). No reactivation

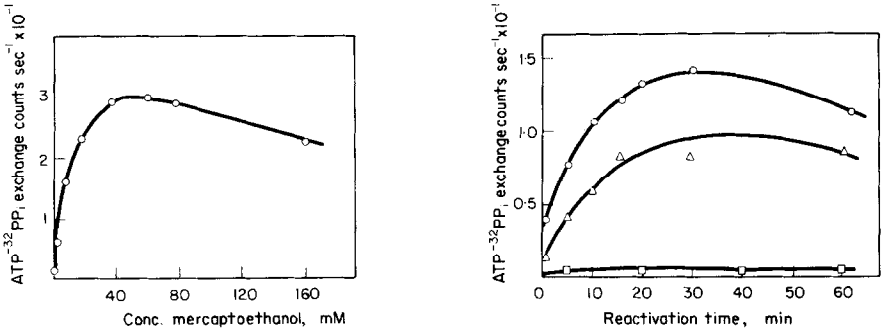


FIG. 2. EFFECT OF MERCAPTOETHANOL CONCENTRATION ON REACTIVATION OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.
Inactive enzyme in 0·02 M Tris–HCl buffer, pH 8·0 was preincubated for 15 min in the presence of various concentrations of mercaptoethanol, then diluted so that the assay tubes contained 2 mM mercaptoethanol. Results corrected for control containing mercaptoethanol but not preincubated.

FIG. 3. TIME COURSE OF REACTIVATION OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.
Inactive enzyme in 0·02 M Tris–HCl buffer, pH 8·0 was preincubated at 37° for various lengths of time prior to assay for ATP-$^{32}$PPi exchange activity. Preincubation and assay conditions; □——□, no dithiothreitol; △——△, +5 mM dithiothreitol; ○——○, +40 mM dithiothreitol.

was observed in the absence of sulphydryl-reducing reagents and Pro, ATP and glycerol had no effect on this reaction. Dithiothreitol (40 mM) was 15% more efficient than the same concentration of mercaptoethanol in promoting reactivation but cysteine, thioglycollic acid and reduced glutathione were far less effective. A similar reactivation of cold-treated Pro-*t*RNA synthetase from *P. aureus* could also be demonstrated although the extent of reactivation was comparatively less. The *Delonix* enzyme could be cycled through active and inactive forms in the absence of glycerol by allowing the enzyme to decay at 0° in the presence of mercaptoethanol followed by heating at 37° (Fig. 5). However, a variable loss of ATP–$^{32}$PPi exchange activity (5–20%) accompanied each cycle of activation and reactivation of the *Delonix* enzyme, whilst only 2 or 3 cycles of reactivation could be obtained with the enzyme from *Phaseolus*. The addition of 25% glycerol, Pro or ATP effectively prevented this cycle of decay and reactivation by maintaining the enzyme in the active form. Cold-inactivated enzyme from both *Phaseolus* and *Delonix* could be reactivated even after storage for 1 week at 0–2° but preparations left for longer periods became increasingly refractory. When the *Delonix* enzyme had completely lost its ATP–$^{32}$PPi exchange activity by storage at 2° in the presence of glycerol and mercaptoethanol (15–25 days), no reactivation of the enzyme could be demonstrated.

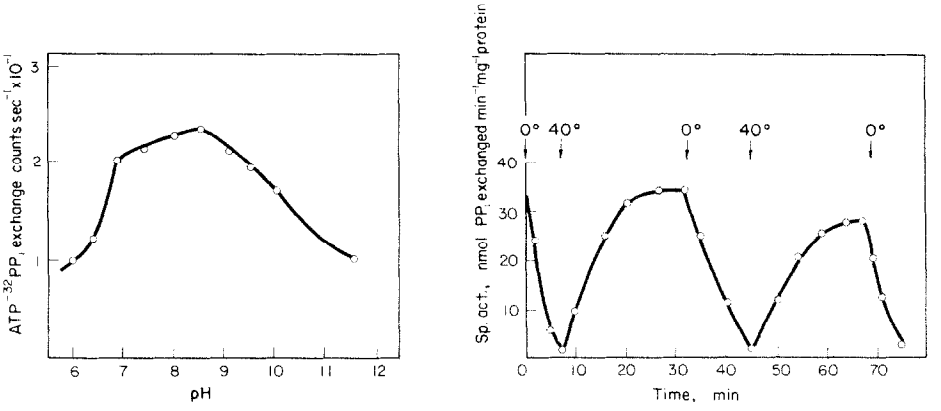


FIG. 4. EFFECT OF pH ON REACTIVATION OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.
Inactive enzyme was preincubated for 15 min at various pH's in the presence of 40 mM mercaptoethanol, then diluted so that the assay tubes contained 1 mM mercaptoethanol and assayed at pH 8 (adjusted to this value with Tris-HCl buffer). Preincubation buffers: pH 5–6·8, maleic acid–KOH; pH 6–10, Tris–maleate–KOH; pH 12, hydroxide-chloride.

FIG. 5. REVERSIBLE REACTIVATION OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.
An enzyme preparation in 0·02 M Tris–HCl buffer, pH 8·0 containing 20 mM mercaptoethanol was either plunged into an ice bucket at 0° or heated at 40° at the times marked by the arrows. Aliquots were taken at various time intervals and diluted to contain 1 mM mercaptoethanol before being assayed.

The kinetics of reactivation were investigated using two methods (see Methods section). (a) Assay at 4°; if it is assumed that the enzyme exists in an inactive form at low temperatures and an active form at high temperatures, assay of the amount of active enzyme at any particular time will depend on the equilibrium, active form $\rightleftharpoons$ inactive form.[12] (b) Assay at 37°; this method is based on the observation that at low mercaptoethanol concentrations there is little reactivation of Pro-*t*RNA synthetase. Both assay methods pro-

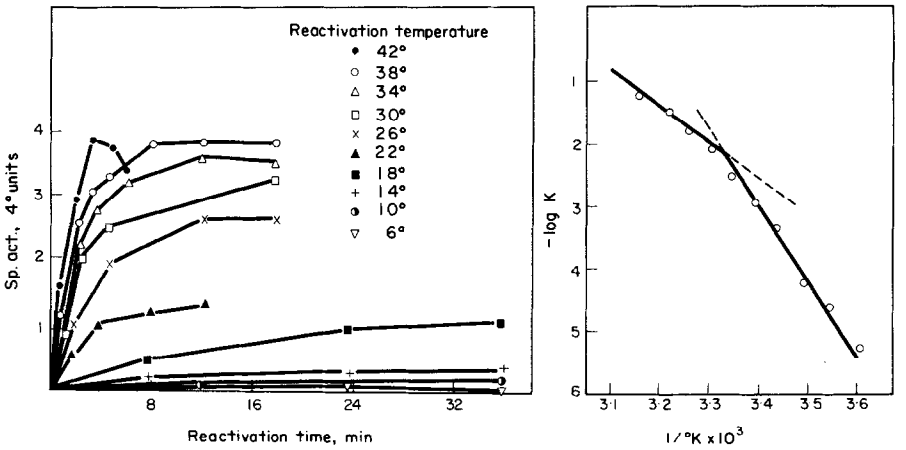


FIG. 6. KINETICS OF REACTIVATION OF PRO-*t*RNA SYNTHETASE FROM *Delonix*.
Inactive enzyme (prepared by storage for 9 hr at 0°) was preincubated in the presence of a buffer containing 0·02 M Tris–HCl pH 8 and 40 mM dithiothreitol at a range of temperatures. Aliquots were removed at various times for assay at 4°.

FIG. 7. ARRHENIUS PLOT OF REACTIVATION KINETICS OF *Delonix* ENZYME.

duced similar results and the time course for both types of assay were shown to be linear. The reactivation kinetics for the *Delonix* enzyme (Fig. 6) could be expressed in terms of the second order rate constants of reactivation using the formula:

$$k = \frac{1}{t}\frac{x}{a(a-x)}$$

where $t$ = time in min; $a$ = original concentration of "monomer" [$=2C_T$, where $C_T$ is the total amount of enzyme present assuming that it is a dimer in its active form i.e. in this case 4 "4° units" (see Fig. 6)]; $x$ = concentration of "monomer" reacting in time $t$ [$= 2C_A$, where $C_A$ is the amount of active enzyme present at any particular time]; $k$ = second order rate constant of reactivation. The initial rates of reaction were calculated from Fig. 6 and plotted in the form of an Arrhenius plot (Fig. 7). The equilibrium constants were also calculated by using the formula:

$$K_{eq} = \frac{C_A}{[2(C_T - C_A)]^2}$$

(see Ref. 12) and plotted against the reciprocal of the absolute temperature (Fig. 8). In both plots, a break in the line was observed at a temperature corresponding to about 22–25°C. In other experiments the breaks occurred at 18–22° and 19–24°. Similar kinetics were obtained for the *Phaseolus* enzyme but the breaks in the Arrhenius plots were not adequately defined because of the lower efficiency of enzyme reactivation. However, a slight change in the slope of the plots was observed between 16 and 25°.

*Attempts to separate active and inactive forms of Pro-tRNA Synthetase*

To test whether the cold-inactivation of Pro-*t*RNA synthetase was due to a dissociation of the active enzyme into inactive subunits, the behaviour of the molecule on Sephadex G200 and on sucrose or glycerol density gradients was observed under a variety of conditions. Chromatography of either the *Delonix* and *Phaseolus* enzyme on Sephadex G200

in the presence of dithiothreitol resulted in indistinct elution profiles and large losses of enzyme activity. The enzyme from both plants appeared to elute at a position corresponding to a MW of $1{\cdot}2$–$1{\cdot}6 \times 10^5$. When chromatography was performed at a series of constant temperatures between 2 and 30°, Pro-*t*RNA synthetase appeared to elute as a broad peak corresponding to a MW of $7$–$14 \times 10^4$. However, no definite correlation was obtained between the running temperature and the position of the peak of enzyme activity.

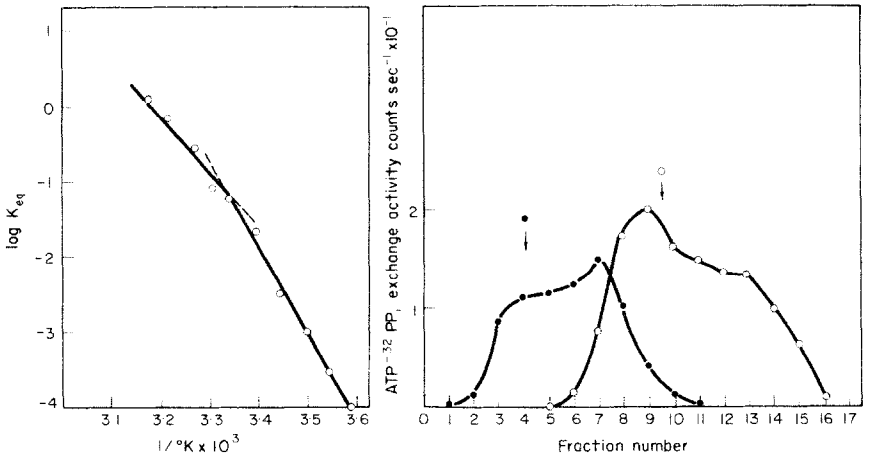


FIG. 8. VAN'T HOFF PLOT OF REACTIVATION KINETICS OF *Delonix* ENZYME.

FIG. 9. SUCROSE DENSITY GRADIENT PATTERNS OF PRO-*t*RNA SYNTHETASE FROM *Delonix* RUN AT TWO DIFFERENT TEMPERATURES.

Gradients were run in the presence of dithiothreitol (40 mM) and fractions preincubated for 15 min at 37° prior to assay. ○——○, run at 0·4°; ●——●, run at 23·28°. Arrows indicate the position of the marker enzyme, alcohol dehydrogenase.

Attempts were made to distinguish between the active and inactive forms of the enzyme by incubating fractions obtained from a column of Sephadex G200 (run at 2° in the presence of dithiothreitol) at 37° for 15 min prior to assay, in order to convert inactive enzyme into the active form. (If the active form of the enzyme was composed of two or more identical subunits which dissociate into an inactive form the latter would be retarded on Sephadex G200 relative to the former). However, this method was unable to resolve two forms of enzyme.

Similar experiments using sucrose or glycerol density gradients were conducted at several temperatures in the presence or absence of dithiothreitol. In general, a better resolution of Pro-*t*RNA synthetase activity was obtained with glycerol gradients than with sucrose gradients. The results were variable, but when gradients were run in the presence of dithiothreitol, two peaks of Pro-dependent ATP–$^{32}$PPi exchange activity were occasionally observed following a preincubation treatment at 37° in the presence of a sulphydryl-reducing reagent. Although these peaks were not well defined, there appeared to be a greater amount of a lighter species of Pro-*t*RNA synthetase in gradients run at 0·8° compared with those run at higher temperatures (Fig. 9). However, the temperature control of the centrifuge was not stable at higher temperatures, thus precluding any accurate measurement of MW.

## DISCUSSION

The lability of aminoacyl-*t*RNA synthetases under a variety of conditions is well documented.[10,13,14] However, reports that some synthetases are more unstable at low temperatures (0–10°) than at room temperature are rare. Lee and Muench[12] have demonstrated that Pro-*t*RNA synthetase from *E. coli* is cold-labile, whilst an unconfirmed report of Attwood and Cocking has suggested that the purified Ala-*t*RNA synthetase from tomato roots is more stable to storage at 37° than at lower temperatures.[15] However, with the exception of the Pro-enzyme all the synthetases from *P. aureus* appeared to be more stable at 4° than at 25° (Norris and Fowden, unpublished results). The increased specific activity of Pro-*t*RNA synthetase from higher plants obtained by carrying out enzyme purification at room temperature rather than in the cold, suggests (by analogy with the *E. coli* enzyme) that the synthetase may dissociate into inactive subunits at low temperatures.

Differences in the elution profile of the *E. coli* enzyme on hydroxyapatite columns have been observed when different concentrations of glycerol are present in the elution buffer. The instability and variability of the elution profiles of the plant enzymes on these and DEAE-cellulose columns may be due to dissociation of the enzyme into inactive subunits.

The results of the reactivation experiments, when plotted in the form of Arrhenius or Van't Hoff plots, indicate that there is a change in the type of subunit structure of the enzyme as the temperature is increased from 6° to 42°. The breaks in these plots may indicate that at temperatures below about 18–24°, the enzyme is predominantly in the form of subunits (monomers) which exhibit no catalytic activity whilst at temperatures above this, catalytic dimers may predominate. Enzyme reactivation may thus be due to the association of subunits to form the active enzyme. The calculations used in the above kinetics have assumed that the MW of the inactive form of the enzyme is half that of the active form. The possibility that the plant enzyme is able to dissociate into more than two subunits on cold inactivation cannot be ruled out. However, since the deactivation of freshly prepared enzyme obeys first order kinetics (which reflects a simple dissociation of the protein into two inactive subunits), it is assumed that if there are more than two subunits, some of these are more strongly bound together leading to an initial fast split into two portions of the molecule. The low temperature modification of the Pro-*t*RNA synthetase from higher plants differs from that of the bacterial enzyme in that (a) the reactivation of the latter is not dependent on the presence of a sulphydryl-reducing reagent (b) deactivation of the plant enzyme, especially with purer preparations, is generally more rapid.

The possible identification of two molecular forms of the Pro-*t*RNA synthetase with different MW (the inactive form exhibiting a lower MW) must be regarded with care because some variability occurred in the positions (volumes) at which characteristic marker proteins were eluted under identical conditions. However, on the basis of the position of the marker enzyme, alcohol dehydrogenase, it would seem that the MW of the enzyme from both *Phaseolus* and *Delonix* is slightly higher than that of the bacterial Pro-*t*RNA synthetase. The indefinite position of peaks during centrifugations (see Fig. 9) also indicated that the active form of the enzyme was dissociating as it moved down the density gradients. However, the relative levels of enzyme in the two peaks in gradients run at different temperatures appeared to substantiate the data concerning the kinetics of thermal reactiva-

[13] LOFTFIELD, R. B. (1971) in *Protein Synthesis* (MCCONKEY, E., ed.) Vol. 1, p. 1, Marcel Dekker, New York.
[14] REZNIKOVA, M. B. (1965) *Biokhimiya* **30**, 727.
[15] ATTWOOD, M. and COCKING, E. C. (1965) *Biochem. J.* **96**, 616.

tion. The variable results obtained by chromatography of the enzyme on Sephadex G200 also may reflect the dissociation of the enzyme as it moves down the coloumn. Such variable profiles for Pro-*t*RNA synthetase have been noted for the bacterial enzyme.[11,16]

It is well known that glycerol stabilizes aminoacyl-*t*RNA synthetases against loss of activity on storage[17] and against heat denaturation.[10] This action is probably due to the ability of this compound to protect hydrogen bonding (and hence tertiary structure) in protein molecules. The comparative storage properties of Pro-*t*RNA synthetase from various higher plants suggests that the enzyme from those plants which contain A2C dissociates more readily into subunits than the enzyme from plants which do not contain the analogue. This is supported by the results of the urea denaturation experiments. The apparent difference in the ability of various types of polyols to protect either type of enzyme against cold inactivation (see Table 2) may merely reflect the differences in the MW of these compounds.

The ability of sulphydryl-reducing reagents to marginally protect the Pro-*t*RNA synthetase against cold inactivation may reflect a role of the sulphydryl group in maintaining the tertiary structure of the enzyme. The enhanced storage ability of 2° obtained by including both glycerol and a sulphydryl-reducing reagent in the medium also suggests an important role of this group in preventing enzyme dissociation. The ability of ATP, Pro and several Pro-analogues to protect the synthetase against inactivation at low temperatures indicates that these compounds may alter the conformation of the enzyme so preventing unfolding of the polypeptide chain about the active site. In this respect we have already shown that the binding of substrates causes the active site of the enzyme to become more rigid.[3] The ability of A2C to protect the *Phaseolus* enzyme against cold-inactivation more efficiently than it did the *Delonix* enzyme may be related to the differences in the active sites of these proteins.[2–4] These observations of substrate protection may also indicate that the active site of the enzyme bridges the subunit.

The ability of sulphydryl-reducing reagents to markedly decrease the rate of enzyme decay at 37° in the absence of glycerol suggests that this elevated temperature is fulfilling a role equivalent to that of glycerol in maintaining the enzyme conformation required for catalytic activity. The observation that the enzyme could not be reactivated after complete decay at 0° in the presence of glycerol and dithiothreitol also supports this conclusion. Thus it appears that the enzyme exists in the active form at relatively higher temperatures in the presence of sulphydryl-reducing reagents although at low temperatures (0–10°) it becomes inactivated rapidly even in the presence of sulphydryl-reducing reagents. The ability of the enzyme to undergo reversible changes between active and inactive forms merely by alteration of temperature is of considerable interest with regard to aminoacylation *in vivo*. Whether or not the dissociation of Pro-*t*RNA synthetase into inactive subunits at low temperatures is able to control the level of protein synthesis *in vivo* by reducing the level of aminoacylated-Pro-*t*RNA in the cell remains an intriguing question.

## EXPERIMENTAL

*Plant materials and amino acid analogues.* All materials were obtained from sources previously described.[2] Amino acids were tested for purity by PC.

[16] NASS, G. and STÖFFLER, G. (1967) *Mol. Gen. Genet.* **100**, 378.
[17] MUENCH, K. H. and BERG, P. (1966) in *Procedures in Nucleic Acid Research* (CANTONI, G. L. and DAVIS, D. R., eds.), p. 375. Harper-Row, New York.

*Radioisotopic chemicals.* Sodium ($^{32}$P)orthophosphate was obtained from the Radiochemical Centre, Amersham. ($^{32}$P)Pyrophosphate was prepared by the method of Bell[18] to give a product of specific activity 0·2–0·4 μCi/μmol.

*Determination of protein.* Protein was determined by the Lowry method[19] with bovine serum albumin as a standard.

*Enzyme fractionation.* Pro-tRNA synthetase was fractionated by methods previously described.[2–4]

*Assay procedure by ATP–$^{32}$PPi exchange.* Incubation mixtures generally contained: Tris–HCl buffer pH 8 (200 μmol), $MgCl_2$ (400 μmol), ATP (4 μmol), $^{32}$PPi (4 μmol), Pro (100 μmol) and enzyme preparation; total volume 1 ml. Reactions were allowed to proceed at 37° for 16 min then terminated by the addition of 5% (w/v) TCA. Labelled ATP was separated from uncharged $^{32}$PPi by absorbing the nucleotide triphosphate on to charcoal.[20] A unit of enzyme activity is defined as the number of nmol PPi exchanged $mg^{-1}$ protein $min^{-1}$ at 37°. Occasionally, the assay was conducted at 4° (see below) and the 4° unit of activity was then defined as the number of nmol PPi exchanged $mg^{-1}$ protein $min^{-1}$.

*Enzyme reactivation.* Inactive enzyme (prepared by storage of the active enzyme for 9 hr at 0°) was preincubated at various temperatures in the presence of mercaptoethanol or dithiothreitol (40 mM). Aliquots were taken at different time intervals in order to assay the amount of reactivated enzyme. The amount of reactivated enzyme was measured by 2 methods (i) assay at 4°: If ATP–PPi exchange is carried out at high temp, considerable amounts of active enzyme would be formed from inactive enzyme during the assay period, thus leading to spurious results. Therefore, the whole assay procedure was carried out at 4° where little spontaneous reactivation would occur. This, however, leads to the difficulty that inactivation of the enzyme at this low temp might be encountered. This was previously shown not to occur in the presence of the substrates required for the ATP–$^{32}$PPi exchange reaction. In this experiment the assay time was extended to 1 hr and high sp. act. $^{32}$PPi was used. (ii) assay at 37°: Aliquots of reactivated enzyme were diluted so that the assay mixture contained less than 1 mM sulphydryl-reducing reagent to prevent further reactivation during assay. The assay was then conducted at 37° as described for the standard ATP–$^{32}$PPi exchange procedure. For both these types of assay it was essential to use enzyme preparations with low endogenous ATP–$^{32}$PPi exchange activity in order to eliminate any stabilizing effect due to the liberation of Pro by proteolytic activity.

*Density gradient centrifugation.* The procedure followed that of Martin and Ames[21] and Britten and Roberts.[22] A linear gradient of 5–20% sucrose or 10–35% glycerol was used, containing 0·05 M Tris–HCl buffer pH 8, with or without mercaptoethanol (40 mM). An enzyme suspension (0·2 ml) was placed on top of the gradient and centrifuged in a MSE superspeed 50 at 130000 *g* for 10 hr using a 3 × 10 ml swing out rotor. Yeast alcohol dehydrogenase was used as a marker enzyme and assayed by the method of Racker.[23]

*Sephadex G200 chromatography.* The method followed that outlined by Fischer.[24] The column (60 × 2 cm) was calibrated for MW determination with cytochrome *c*, ribonuclease, yeast alcohol dehydrogenase, bovine serum albumin, catalase and urease (all obtained from Sigma).

[18] Bell, R. N. (1950) in *Inorganic Synthesis* (Audrieth, L. F., ed) Vol. 3, p. 93, McGraw–Hill, New York.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[20] De Moss, J. A. and Novelli, G. D. (1965) *Biochim. Biophys. Acta* **18,** 592.
[21] Martin, R. G. and Ames, E. W. (1961) *J. Biol. Chem.* **263,** 1372.
[22] Britten, R. J. and Roberts, R. B. (1960) *Science* **131,** 32.
[23] Racker, E. (1950) *J. Biol. Chem.* **184,** 313.
[24] Fischer, L. (1969) *An Introduction to Gel Chromatography,* North Holland, Amsterdam.