Phytochemistry, 1975, Vol. 14, pp. 1701–1706. Pergamon Press. Printed in England.

STABILIZATION OF AMINOACYL-*t*RNA SYNTHETASES BY SEPHADEX AND POLYACRYLAMIDE GELS

ROGER D. NORRIS

Department of Biochemistry, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC 1 M6 BQ, England

and

JERZY PAWEŁKIEWICZ

Institute of Biochemistry, University of Agriculture, Wołyńska 35, 60–637, Poznań, Poland

(*Revised Received* 12 *February* 1975)

Key Word Index—*Lupinus luteus*; leguminosae; lupin; aminoacyl-*t*RNA synthetases; enzyme stabilization; thermal stabilization; Sephadex and Biogel entrapment.

Abstract—Several aminoacyl-*t*RNA synthetases from the yellow lupin (*Lupinus luteus*) were stabilized against inactivation during storage at 0–4°, by entrapment in Sephadex or Biogel matrices and drying over P_2O_5. The degree of stabilization depended on the rate of drying of the gel and the pH of the medium and to a lesser extent on the ionic strength and protein concentration. With the exception of prolyl-*t*RNA synthetase, a greater stability was achieved with those enzymes which were relatively stable to thermal denaturation. Aminoacyl-*t*RNA synthetases for glutamic acid, glutamine, methionine and arginine, which become inactivated during purification, were considerably stabilized by this procedure.

INTRODUCTION

Many aminoacyl-*t*RNA synthetases from higher plants are unstable under certain conditions [1–4], and although some of these enzymes have recently been obtained in high purity [4,5], loss of ATP-$^{32}PP_i$ exchange or *t*RNA esterification activity often occurs on storage at 0–4°. Glycerol and other polyols may protect synthetases against such activity losses [3,6,7] presumably by maintaining hydrogen bonding between the polypeptide chains. Many enzymes form insoluble complexes in networks of DEAE cellulose, Sephadex or polyacrylamide gel [8,9]. Some enzymes entrapped within the pores of such matrices are well protected against thermal denaturation [10]. Previous work in this laboratory has indicated that the entrapment of glycerol dehydratase in Sephadex gels followed by drying over P_2O_5, results in a decreased lability of the enzyme to storage at low temperatures [9]. The present paper assesses the value of this method for stabilizing the aminoacyl-*t*RNA synthetases from the seeds of yellow lupin and compares it with other methods of storing these enzymes.

RESULTS

Effect of rate of drying, temperature and protein concentration on the stabilization of synthetases entrapped in gels

At 0–4°, maximum stabilization was achieved when the gels were taken to dryness over P_2O_5 within 12–18 hr. Samples taken to dryness within 2–3 days often retained only 30–70% of their activity compared with those taken to dryness within 15 hr. The data (Fig. 1) show that the increased loss of activity of 2 synthetases entrapped in Sephadex G-25 with a slower rate of drying was parallel with the loss of activity of the enzymes in the absence of the gel. The rate of drying was increased at room temperature although the lysyl- and histidyl-*t*RNA synthetases were unstable under these conditions. The rate of loss of activity

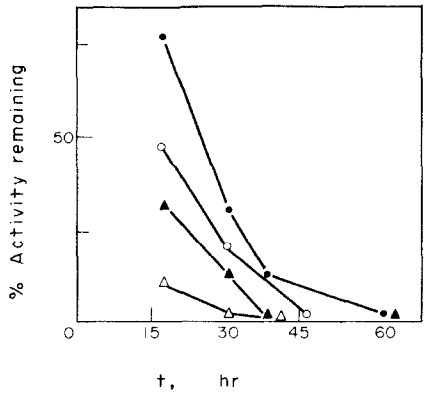

Fig. 1. Influence of drying time on activity of prolyl- and lysyl-*t*RNA synthetases. Enzyme (fraction D) was equilibrated with 0·02 M Tris–HCl buffer, pH 7·5, absorbed in Sephadex G-25, then taken to dryness over P_2O_5 at 4° within the times indicated on the graph. Enzyme was then eluted and the activity remaining assayed and compared with the unabsorbed control and with samples of enzyme stored over P_2O_5 in the absence of Sephadex. ●——● lysyl enzyme dried in Sephadex; ○——○ lysyl enzyme dried in absence of Sephadex; ▲——▲ prolyl enzyme dried in Sephadex; △——△ prolyl enzyme dried in absence of Sephadex.

of prolyl-*t*RNA synthetase absorbed in Sephadex in the presence of mercaptoethanol was slower at 20 than at 0°. However, when drying the gels at room temp, precautions had to be taken to prevent microbial contamination by including sodium azide (0·02%) or streptomycin in the solution to be absorbed to the gel. The optimum concentration of enzyme required for maximum stabilization was about 1–3 mg/ml for fractions A–D (see Experimental) and most DEAE cellulose fractions. When protein concentrations of above 10 mg/ml were absorbed in the gel, several synthetases were less efficiently stabilized e.g. those specific for glutamine, threonine, isoleucine, leucine, proline and phenylalanine.

Stabilization of synthetases against activity loss by storage and thermal denaturation

Table 1 shows the percentage aminoacylation activity remaining compared with the zero time controls for several aminoacyl-*t*RNA synthetases absorbed in Sephadex G-50 or Biogel P-60 and stored for 2 weeks at 0°. Most of these enzymes when entrapped in gels lost only 10–20% of their aminoacylation activity within 2 weeks (Table 1) whereas in the absence of gel many became rapidly inactivated (see below). In most cases the stabilization of synthetases in Sephadex G-25 and G-75 was similar to that observed in Sephadex G-25, although Sephadex G-75 appeared to stabilize the lysyl and histidyl enzymes less efficiently. Biogels P-100 and P-150 gave results similar to those observed for Biogel P-60. However, isoleucyl-*t*RNA synthetase was significantly better stabilized by the former two gels.

Table 1. Percentage synthetase activity remaining following entrapment of the enzymes in Sephadex or Biogel*

Synthetase	Type of enzyme preparation†	Sephadex G-50		Biogel P-60	
		Not heated	Heated	Not heated	Heated
Alanyl	A	84 ± 7	61 ± 8	92 ± 3	50 ± 1
Arginyl	A	89 ± 3	84 ± 4	93 ± 2	81 ± 1
Aspartyl	A	87 ± 5	74 ± 4	86 ± 5	78 ± 4
Glutaminyl	A	54 ± 1	24 ± 6		
Glutamyl	A	82 ± 1	79 ± 2	63 ± 10	58 ± 3
Histidyl	D	80 ± 2	79 ± 2	59 ± 19	58 ± 16
Isoleucyl	B	82 ± 8	73 ± 7	57 ± 7	40 ± 3
Leucyl	B	85 ± 1	85 ± 2		
Lysyl	D	73 ± 2	44 ± 2	81 ± 5	40 ± 1
Methionyl	C	85 ± 3	67 ± 2	95 ± 2	37 ± 3
Phenylalanyl	C	87 ± 5	80 ± 3	83 ± 2	48 ± 2
Prolyl	D	57 ± 5	43 ± 7	70 ± 1	38 ± 2
Seryl	A	80 ± 2	75 ± 1	83 ± 3	75 ± 1
Threonyl	C	67 ± 13		84 ± 7	51 ± 1
% Protein recovered from gels		91 ± 2	85 ± 3	72 ± 4	64 ± 5

* Various synthetase preparations were entrapped and eluted from gels as described in Experimental and residual activity determined after 2 weeks storage at 0°. Figures in "heated" column represent activity remaining in gels heated as described in the text. The standard error follows each percentage.

† See Experimental section.

Table 2. Effect of pH on stability of synthetases stored on gels*

Synthetase	pH	% Activity remaining compared with zero-time control 5	6	7	8	8·9
Glutamyl		36 (30)	87 (82)	76 (55)	85 (62)	46 (40)
Histidyl		10 (0)	63 (25)	74	90	82
Isoleucyl		66 (51)	84 (69)	89 (81)	78 (60)	70 (58)
Methionyl		29	52	75	72	62
Phenylalanyl		33		94	73	71

* DEAE-cellulose fractions desalted on Sephadex G-25 in 0·05 M phosphate buffer were absorbed in dry Sephadex G-25 at different pHs.

Following drying over P_2O_5 and storage for 3 weeks at 0°, enzyme was eluted and adjusted to pH 7·4. Figures in parentheses represent the activity of samples heated for 15 min at 110° (see text).

The stability of aminoacyl-tRNA synthetases entrapped in gel matrices was further investigated by heating the dry gels in an oven at 100° for 10 min. Such a procedure rapidly inactivates all synthetases in simple solution [2]. The gels appeared to protect most synthetases against thermal denaturation, the extent of protection varying with the enzyme concerned (Table 1).

Effect of pH

The ability of Sephadex G-25 to stabilize certain synthetases at different pH's was determined using P_i buffers (Table 2). Stabilization was relatively insensitive to changes in pH above 6, but entrapment of the enzymes in the gel at pH values lower than this decreased the stability of many synthetases. This was probably due to the inherent instability of individual synthetases in solution at acid pH values, since the prolyl-tRNA synthetase exhibited similar pH-activity curves following storage in the presence or absence of Sephadex G-25. Different buffer ions only slightly altered the protective effect of gels upon aminoacyl-tRNA synthetases. An ionic strength of 0·05 (P_i buffer, pH 7·5) was superior to an ionic strength of 0·01 in stabilizing both the alanyl- and isoleucyl-tRNA synthetases on Sephadex G-25. The percentage synthetase activity remaining compared with the zero time controls was 93 and 86 for the isoleucyl enzyme and 69 and 63 for the alanyl enzyme (ionic strength = 0·05 and 0·01 respectively in both cases).

Effect of $MgCl_2$ and substrates

At molarities up to 0·2, $MgCl_2$ had little effect on the stability of the histidyl-, isoleucyl-, leucyl- or methionyl-tRNA synthetases absorbed by Sephadex G-25. The data indicated that the presence of Mg ions was not strictly necessary for the stabilization reaction.

Although the presence of ATP decreased the activity loss of the enzymes on storage compared with preparations lacking this substrate, specific amino acids had little effect (Table 3).

Comparison with other methods of stabilizing aminoacyl-tRNA synthetases

An enzyme preparation containing several aminoacyl-tRNA synthetases (fraction A) was divided into four equal portions which were stored at −20° as: (i) concentrated $(NH_4)_2SO_4$ pellet; (ii) soln in 40% glycerol; (iii) frozen soln

Table 3. Effect of substrates on stability of synthetases to storage on gels*

Synthetase	% Activity remaining compared with zero-time control No substrate	+ATP	+Specific amino acid
Glutaminyl	51 ± 3	63 ± 4	
Histidyl	60 ± 2	66 ± 2	61 ± 2
Isoleucyl	75 ± 3	86 ± 2	73 ± 3
Leucyl	71 ± 4	96 ± 5	
Lysyl	63 ± 2	68 ± 3	63 ± 2
Methionyl	47 ± 4	63 ± 5	51 ± 4

* Aliquots of desalted enzyme (fraction A) were entrapped in Sephadex G-25 in the presence of 0·05 M P_i buffer, pH 7·2 containing 10 mM $MgCl_2$ and either ATP (2 mM) or specific amino acid (40 mM) or no substrate. Enzyme was eluted after storage of the dry gels for 2 weeks and dialysed or passed through Sephadex G-25 to remove substrates.

Table 4. Comparison of stability of synthetases to storage for 5 weeks at −20° by various methods

Synthetase	% Activity remaining compared with zero-time control			
	$(NH_4)_2SO_4$ pellet	40% Glycerol	7% Glycerol	Entrapped in Sephadex G-25
Alanyl	60		0	85
Arginyl	0	50	0	78
Glutamyl	46	69	0	68
Histidyl	43	40	0	70
Isoleucyl	69	95	7	87
Lysyl		90	0	77
Methionyl	17	50	0	35
Phenylalanyl	76	87	17	80
Prolyl	15		0	40

in 7% glycerol; (iv) enzyme entrapped in Sephadex G-25. After storage for 4 weeks the enzyme preparations were suitably diluted and assayed for synthetase activity. Data (Table 4) indicate that the storage of synthetases entrapped in Sephadex G-25 compares well with other methods of storing these enzymes. However, this method is best used for storing relatively small amounts of enzyme since a rapid drying time of the gel over P_2O_5 is required. Furthermore, the elution of enzyme from the gel should be performed as rapidly as possible in a small column to reduce the loss of activity of unstable synthetases. Pilot experiments indicated that the elution of certain synthetases, e.g. prolyl, lysyl, histidyl, from gels with a low flow rate such as Sephadex G-200 sometimes resulted in the loss of activity of these enzymes.

DISCUSSION

Little is known about the mechanism by which enzymes are entrapped and insolubilized in polysaccharide gel matrices. In the present study we have used the term entrapment in the wider sense; that enzymes have penetrated the pores of polysaccharide or polyacrylamide gel beads, but are still capable of diffusing out from the beads when placed in an aqueous medium. That the synthetases are easily eluted from the gel beads following swelling is demonstrated by the data (Fig. 1 and Table 1), which indicate that very little enzyme activity is lost by the absorption and drying process if this is performed rapidly. However, some proteins were found to adsorb more strongly to the gels than did synthetases as indicated by the yields of protein obtained following elution (Table 1). In this respect, Biogels appeared to adsorb more protein than Sephadex. It is thought that hydrophobic reactions may be important for stabilizing the conformation of aminoacyl-*t*RNA synthetases [4] thus promoting internal hydrogen bond formation. Hydrogen bond stabilization of synthetases is also produced by various polyols [3,6]. The protection of these enzymes by Sephadex and Biogels may therefore partly depend on the OH groups in the dextran network of the former and NH and CO groups in the polyacrylamide chains of the latter which stabilize the synthetases by hydrogen bonding. The rapidity with which the gels can be dried and reswollen makes them particularly suitable for these studies. Although some variations in the ability of different types of Sephadex to stabilize synthetases was observed, Sephadex G-50 usually gave the best results due to its rapid drying time. Although Biogels were also efficient in stabilizing individual synthetases, the results were rather variable and these gels did not stabilize the enzymes against thermal denaturation to as great as extent as did Sephadex G-50. This may be due to differences in the nature of the matrix of these 2 types of gel.

The relative stability of individual synthetases entrapped in gels was generally similar to the stabilities of these enzymes toward storage in the cold in the absence of gel (Fig. 1) and their stability toward thermal denaturation (Tables 1 and 4, see also Ref. [2]). However several synthetases were anomalous in this respect; histidyl- and glutamyl-*t*RNA synthetases were more stable in the gels than expected whilst prolyl-*t*RNA synthetase was unstable, presumably due to its cold lability [3].

The observation that a rapid rate of drying over P_2O_5 was essential for maximum stabilization may indicate that (i) with the relatively impure enzyme preparations used, proteases may inactivate the enzymes (ii) loss of synthetase activity may occur in a similar manner as in solution in the absence of gel (Fig. 1). Once the enzymes have been entrapped in the gel in the dry state, they appear to be very stable. Experiments not reported here indicate that some synthetases e.g. leucyl, phenylalanyl, could be stored in the dry gels for at least 2–3 months with only a small activity loss. The thermostability of synthetases in the dry state is in marked contrast to their instability in solution. This is probably due to the fact that the enzymes are rigidly held within the gel particles, thus reducing the vibrational (thermal) energy responsible for the unfolding of peptide chains when the temperature is raised.

Since ATP and specific amino acids are known to stabilize synthetases against thermal denaturation [11–13] the effect of these substrates on the synthetases entrapped in gels was investigated. Although it appeared that some stabilization by ATP was achieved, this was not as significant as expected from thermal denaturation data [2]. The reason for this is not clear.

The stabilization of aminoacyl-tRNA synthetases on dry gels may be a useful method for reducing loss of enzyme activity during storage in the cold (Table 4) and is more efficient in this respect than storage as $(NH_4)_2SO_4$ pellets though less efficient than storage in 40% glycerol.

EXPERIMENTAL

Determination of protein and nucleic acids. Protein was calculated from A measured at 260 and 280 nm [14]. RNA in the synthetase preparations was measured as described in Ref. [15] with yeast RNA as a standard.

Enzyme purification. Yellow lupin seed meal (8 g) was extracted for 15 min with 50 ml of a buffer containing 0·1 M Tris–HCl, pH 7·5, 0·03 M $MgCl_2$, 10 mM mercaptoethanol or dithiothreitol and 15% glycerol. After centrifugation at 28000 *g* for 20 min, solid $(NH_4)_2SO_4$ was added and the following fractions used for enzymes studies: (A) 11–22 g $(NH_4)_2SO_4$/50 ml supernatant; (B) 11–14 g $(NH_4)_2SO_4$/50 ml supernatant; (C) 14–16·5 g $(NH_4)_2SO_4$/50 ml supernatant; (D) 16–22·5 g $(NH_4)_2SO_4$/50 ml supernatant. Fractions were equilibrated on a small column of Sephadex G-25 (with a running buffer which varied according to the conditions to be used for the entrapment of enzyme in the gels) prior to either absorption in the gels or further purification. Additional purification of selected enzyme fractions was carried out by batchwise DEAE-cellulose chromatography: following 2× equilibration on a column of Sephadex G-25 containing a running buffer of 15% glycerol, 0·05 M Tris–HCl pH 7·5, 0·03 M $MgCl_2$ and 10 mM mercaptoethanol, the $(NH_4)_2SO_4$ fraction was adsorbed on DEAE-cellulose and eluted stepwise with solns of NaCl until the final concn of salt was 0·32 M. Enzyme fractions eluted from DEAE-cellulose were desalted on Sephadex G-25 as before and then used for gel entrapment.

Entrapment of enzyme fractions in gels. ca 2–3 mg protein of $(NH_4)_2SO_4$ or DEAE-cellulose fractions or 10–20 mg of crude homogenate were desalted and equilibrated on a small column of Sephadex G-25. After elution from the column, 0·5 ml aliquots of the protein were absorbed in the powdered dry gels spread out in thin layers on the bottom of wide-bottomed vials; dry gel used/0·5 ml aliquot were as follows: Sephadex G-25, 0·4 g; Sephadex G-50, 0·25 g; Sephadex G-75 and G-100, 0·2 g; Biogel P-60, 0·2 g; Biogel P-100, 0·17 g; Biogel P-150, 0·15 g. The entrapment conditions for the enzymes were defined by the nature of the equilibration buffer used with the Sephadex G-25 columns. Gels containing the entrapped enzymes were then dried as rapidly as possible over P_2O_5 *in vacuo* at either room temp. or at 0–4°. A sample of the enzyme was also taken for the zero-time (unabsorbed) control.

Elution of synthetases from the dried gels. Dry gels with entrapped enzymes were crushed into a fine powder and allowed to swell in H_2O at 4° until their H_2O regain values had been reached. The gels were then packed into a small column (4 × 0·5 cm) and protein eluted with dil. buffer (0·05 M Tris–HCl, 0·05 M $MgCl_2$, 2 mM mercaptoethanol and 5% glycerol). It was more convenient to allow the gels to swell directly in the column with the column placed horizontally. Eluted protein was collected in a vol of 1–2 ml and adjusted with respect to pH and/or molarity of buffer ions by equilibration on Sephadex G-25 to maintain constant assay conditions.

t-*RNA isolation.* The modification of the general PhOH method [16,17] was used as described in Ref. [18]. Carbohydrates were removed from lupin tRNA preparations by DEAE cellulose chromatography.

Assay. Mixtures contained 0·1 ml enzyme soln (30–150 μg protein) and 0·2 ml containing ATP (1 μmol); $MgCl_2$ (4 μmol), tRNA (0·035–0·15 mg) and radioactive amino acid (20 nmol). After 10 min at 35° reactions were terminated by addition of 5% TCA (0·02 ml) at 0°. The ppt's. were collected on filter paper discs and treated as previously described [4].

Acknowledgements—R. D. N. thanks the Polish Ministry of Higher Education, Science and Technology and London University for grants made in order to carry out this work in Poznań. This work was supported by the Polish Academy of Sciences within project 09.3.1.

REFERENCES

1. Lea, P. J. and Fowden, L. (1972) *Phytochemistry* **11,** 2129–2138.
2. Norris, R. D. and Fowden, L. (1973) *Biochim. Biophys. Acta* **312,** 695–707.
3. Norris, R. D. and Fowden, L. (1974) *Phytochemistry* **13,** 1677–1687.
4. Jakubowski, H. and Pawełkiewicz, J. (1973) *FEBS Letters* **34,** 150–154.
5. Jakubowski, H. and Pawełkiewicz, J. Submitted to *European J. Biochem.*
6. 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.

7. Lee, M.-L. and Muench, K. H. (1969) *J. Biol. Chem.* **244**, 223–230.
8. Betnfeld, P. and Wan, J. (1964) *Science* **142**, 678–679.
9. Schneider, Z., Stroiński, A. and Pawełkiewicz, J. (1968) *Bull. Acad. Polon. Sci. Ser. Sci. Biol.* **16**, 203–204.
10. Chappelle, E. W., Rich, E. Jr. and Macleod, N. H. (1967) *Science* **155**, 1287–1288.
11. Baldwin, A. N. and Berg, P. (1966) *J. Biol. Chem.* **241**, 831–838.
12. Chuang, H. Y. K., Atherly, A. G. and Bell, F. E. (1967) *Biochem. Biophys. Res. Commun.* **28**, 1013–1018.
13. Norris, R. D. and Fowden, L. (1973) *Phytochemistry* **12**, 2109–2121.
14. Layne, E. (1957) *In Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.), Vol. **3**, p. 447. Academic Press, New York.
15. Key, J. and Shannon, J. C. (1964) *Plant Physiol.* **39**, 365–370.
16. Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marim, M. A. and Merrill, S. H. (1961) *J. Biol. Chem.* **236**, 200–202.
17. Vanderhoef, L. N., Bohannon, R. F. and Key, J. L. (1970) *Phytochemistry* **9**, 2291–2304.
18. Norris, R. D. and Fowden, L. (1972) *Phytochemistry* **11**, 2921–2935.