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THE SPECIFICITY OF AMINOACYL-sRNA SYNTHETASES WITH SPECIAL REFERENCE TO ARGININE ACTIVATION

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Abstract—The amino acid substrate specificities and the stabilities of the arginyl- and lysyl-sRNA synthetases prepared from seed of *Canavalia ensiformis* and cells of *Staphylococcus aureus* have been examined. The lysyl-sRNA synthetase of both species appears to be able to activate arginine and homoarginine to a limited extent, but not canavanine. The arginyl-sRNA synthetase of *Canavalia* could not activate either homoarginine or canavanine, but homoarginine competitively inhibited the enzyme's ability to utilize arginine. Whereas the lysyl enzyme could be stored with little loss of activity, arginyl-sRNA synthetase preparation quickly lost activity on storage at either 0 or −30°.

INTRODUCTION

THE AMINOACYL-sRNA synthetases are responsible for the activation and transfer of the protein amino acids to their specific transfer ribonucleic acid (tRNA) molecules in the initial steps involved in amino acid incorporation into proteins. The reactions catalysed may be summarized as:

(1) amino acid + ATP + enzyme $\rightleftarrows$ [aminoacyl-AMP-enzyme] + PP_i

(2) [aminoacyl-AMP-enzyme] + tRNA $\rightarrow$ aminoacyl-tRNA + AMP + enzyme

This selection of substrates by this group of enzymes from among the protein amino acids is exacting, but occasional reports of non-specific activation appear in the literature. For instance, the isoleucyl-sRNA synthetase not only catalysed the formation of an isoleucyl-adenylate, but also a valyladenylate.[1, 2] The K_m values observed for the two substrates, when reaction was assayed by the ATP-$^{32}PP_i$ exchange procedure (reaction 1 in reverse) were 5×10^{-6} M and $3{\cdot}9 \times 10^{-4}$ M respectively. Valine inhibited the use of isoleucine in reaction 1, and also the transfer of the isoleucyl moiety to tRNA in reaction 2. However, the valyladenylate formed in reaction 1 is a relatively poor substrate for reaction 2; $tRNA^{isol}$ plays a direct role in limiting transfer of the aminoacyl group for its presence induces the breakdown of the enzyme-bound valyladenylate complex. It is perhaps particularly significant that $tRNA^{isol}$, alone among the various types of tRNA molecules, effects this breakdown, although the underlying mechanism is largely unknown.[3–7]

A similar situation may exist in relation to 4-hydroxyproline and proline. Although 4-hydroxyproline forms an important constituent of collagen and of the protein tightly

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associated with plant cell walls, no hydroxyproline-specific activating enzyme is known. Indeed, there is fairly general agreement that free hydroxyproline is not incorporated into protein, and that protein-bound hydroxyproline arise by hydroxylation of proline residues present in nascent protein molecules probably whilst they are still ribosomal-bound.[8] However, in some studies with partially-purified prolyl-sRNA synthetase preparations, 4-hydroxy-L-proline was shown to stimulate ATP-$^{32}PP_i$ exchange (reaction 1); these instances included enzymes from rat liver,[9] *Escherichia coli*,[10] and chick tibia.[11] Transfer of the hydroxyprolyl residue to $tRNA^{pro}$ (reaction 2) was not demonstrated. Possibly, as with the example of valyladenylate above, the hydroxyprolyladenylate-enzyme complex undergoes a facile breakdown in the presence of $tRNA^{pro}$.

The exacting nature of amino acid selection by aminoacyl-sRNA synthetases generally does not exclude the use as substrates of certain naturally-occurring compounds included among a large group of non-protein amino acids produced by plants. Among such compounds are azetidine-2-carboxylic acid, ethionine, and mimosine which can be utilized as substrates in reaction 1 by prolyl, methionyl, and phenylalanyl activating enzymes respectively.[12]

These examples raise the question whether such variable substrate specificity is more common among amino acid activating enzymes. We have attempted to test this concept by investigating arginyl-sRNA synthetase preparations from different sources being concerned especially with the enzyme's ability to utilize either canavanine or homoarginine as substrates. Both these amino acids have been isolated from members of the family Leguminosae and both have been reported to be growth-inhibitory to micro-organisms. Canavanine, but as yet not homoarginine, has been reported as a constituent of protein formed by cells of canavanine- or homoarginine-treated cultures of *Staphylococcus aureus*, *E. coli*, Walker carcinoma 256, and rat liver.[12] The ability of both canavanine and homoarginine to form either substrates for, or inhibitors of, arginyl-sRNA synthetase has been tested with enzymes from rat liver[13] and *E. coli*.[14] Work with the more highly purified mammalian enzyme confirmed the earlier observation, made with the bacterial enzyme, that the presence of canavanine caused a marked competitive inhibition of the rate of ^{14}C-arginine transfer to tRNA. In consequence of these findings, it seemed pertinent to learn whether the substrate specificity of arginyl-sRNA synthetase present in jack bean (*Canavalia ensiformis*), the original legume source of canavanine, differed from that of enzyme present in other higher plants or micro-organisms that lacked the ability to synthesize canavanine. The answers obtained in our investigations are somewhat indefinite, but the results provide new information concerning the properties of the arginyl- and, incidentally, of the lysyl-sRNA synthetases of plants and micro-organisms.

RESULTS

Originally, it was hoped to compare the substrate specificities of the arginyl-sRNA synthetases from two legume species, i.e. *Canavalia* which produces canavanine, but does

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not incorporate it into protein molecules, and *Phaseolus aureus* which lacks canavanine. However, every attempt to obtain an active arginine enzyme by fractionation of extracts from mung bean failed, and *Staphylococcus aureus* (Copenhagen strain) was substituted as the non-producer of canavanine. A second, arginine-dependent, strain (524 SC) of this organism was also used which incorporates canavanine into its cell protein when culture media are supplemented with the guanidinoxy-amino acid.[15]

Enzyme preparations catalysing an arginine-stimulated ATP–$^{32}PP_i$ exchange were prepared from powdered *Canavalia* seed by a three-stage procedure (see Methods): step C1 involved pH fractionation, step C2 ammonium sulphate fractionation, while the third stage involved absorption of the enzyme on to calcium phosphate gel (step C3a) or separation on DEAE-cellulose (step C3b). The nucleotide content of enzyme obtained after step C3a was about 4 per cent, and that after step C3b about 1·2 per cent.

To obtain corresponding enzyme preparations from *S. aureus*, cells were disintegrated by mixing with an equal quantity of ballotini glass beads and homogenizing in a Virtis blendor. Purification involved pH fractionation (step S1), a modified ammonium sulphate treatment (step S2) and finally separation on a DEAE-cellulose column (step S3).

Enzyme preparations from cells of mutant strain 524 SC were prepared by similar techniques, except that a direct ammonium sulphate fractionation procedure, comparable to that in step C2, was used.

All operations involved in the above fractionation steps were performed at 0–4°. Enzyme activities were assayed at 37° by measuring ATP–$^{32}PP_i$ exchange occurring in appropriate reaction mixtures (see Tables and Figures) during 15 min with *Canavalia* preparations and 30 min with bacterial enzymes. The general experimental and assay procedures adopted represented a slight modification of those described by DeMoss and Novelli[16] and Peterson

TABLE 1. THE PROGRESSIVE ENHANCEMENT OF THE ARGININE- OR LYSINE-STIMULATED ATP–$^{32}PP_i$ EXCHANGE REACTION DURING THE FRACTIONATION OF *Canavalia* AND *Staphylococcus* ENZYMES

	Enzyme fraction	Substrate	Specific activity§	Protein (mg)	% Yield
A. *Canavalia**	Step C1	5 mM-arginine	0·018	7,300	100‡
	Step C2	5 mM-arginine	0·042	528	17·1
	Step C3a	5 mM-arginine	0·084	49	3·2
B. *Staphylococcus*†	Step S1	100 mM-arginine	0·22	331	100‡
	Step S2	100 mM-arginine	2·33	73·3	234
	Step S3	100 mM-arginine	7·36	21·3	215
C. *Canavalia*†	Crude homogenate	5 mM-lysine	0·023	12,000	100‡
	Step C1	5 mM-lysine	0·014	4,960	25·9
	Step C2	5 mM-lysine	0·088	367	11·7
	Step C3b	5 mM-lysine	0·403	10·5	1·5

All assay mixtures contained in addition to amino acid substrate, 4 mM-ATP, 4 mM-pyrophosphate, 10* or 20† mM-$MgCl_2$, and 100 mM-tris-HCl. Assays in sections A and C were performed at pH 6·8, and those in B at pH 8·3.

‡ First fractions possessing sufficient activity to assay and so taken to represent 100 per cent yield.

§ The specific activity is expressed as μmoles of PP_i exchanged/hr/mg protein after subtraction of endogenous exchange occurring in absence of added substrate.

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and Fowden.[17] For instance it was found essential to maintain constant concentrations of K^+, Na^+, SO_4^{2-} and Cl^- in reaction mixtures, and generally a high concentration of Mg^{2+} (20 mM) was required for optimal enzyme activity.

The apparent progress of enzyme fractionation of different extracts is illustrated in Table 1. The purification of the arginine-activating enzyme from *Canavalia* after step C3a was approximately five-fold, whereas that from *S. aureus* (Copenhagen strain) was over 30-fold. This difference may reflect in part the greater lability of the higher plant enzyme (see later). However, the figures should be accepted with caution, because the specific activity calculated for each enzyme after the pH precipitation step is subject to considerable error due to the high ratios of endogenous to arginine-stimulated exchange rates associated with early fractionation steps. This inability to determine accurate specific activities for initial enzyme fractions also may explain the impossibly high yields calculated for later fractionation steps using the bacterial enzyme. The significance of lysine-stimulated ATP–$^{32}PP_i$ exchange rates for the *Canavalia* fractionation procedure terminating in DEAE-cellulose chromatography is discussed below.

Properties of the Enzyme Preparations

Canavalia *step C3a enzyme.* The enzyme as recovered from calcium phosphate gel was quite labile, becoming totally inactive when stored in 0·02 M-tris-HCl buffer (pH 7·9) for 48 hr at 0°. Only 18 per cent activity remained after 24 hr at −30° when it was kept as a precipitate under saturated ammonium sulphate. If incubated under the usual assay conditions, but in the absence of arginine, the enzyme was totally or largely inactivated in 15 min at 37°.

Enzyme activities were measured normally using tris-HCl buffers, although the optimum pH seemed to lie beyond the lower pH end of its useful buffering range. Phosphate was tried as an alternative buffer, but phosphate ion became increasingly inhibitory at higher pH values and caused total inhibition above pH 7·2. Using tris-HCl the optimum pH apparently was below 6·5, but pH 6·8 was generally used for assay purposes: at this pH the enzyme exhibited about 85 per cent of the activity measured at pH 6·5.

ATP–$^{32}PP_i$ exchange rates measured for different arginine concentrations gave linear Hofstee[18] plots indicating K_m (arginine) values of $7–18 \times 10^{-5}$ M for the enzyme. When 20 mM-canavanine was supplied as a substrate, stimulation of ATP–$^{32}PP_i$ exchange was between 0–10 per cent (in different extracts) of the maximum rate measured with arginine. When 20 mM-canavanine was incubated with various concentrations of arginine, there was no suggestion of competition between the two substrates for the same enzyme, i.e. exchange attributable to canavanine represented a constant increment over the exchange induced by arginine alone. Homoarginine (20 mM) was not activated by this type of enzyme preparation but it exerted competitive inhibition on arginine activation. In a particular experiment the normal K_m value for arginine was $10·3 \times 10^{-5}$ M, but in the presence of 20 mM-homoarginine it was increased to 27×10^{-5} M. Assuming homoarginine binds at the active site of the enzyme, then the K_i (homoarginine) value was about $1·2 \times 10^{-2}$ M. Two lower homologues of arginine, α-amino-β-guanidinopropionic and α-amino-γ-guanidinobutyric acids, were activated at about 3 per cent of the maximum rate measured for arginine.

The possibility of salt (ion) inhibition of this enzyme preparation was not directly investigated, but certain observations suggested that sulphate ion exerted a non-competitive in-

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hibitory action upon arginine-stimulated ATP–$^{32}PP_i$ exchange: chloride ion appeared less inhibitory while partial alleviation of ionic inhibition could be effected by increasing the magnesium concentration in the assay mixtures (cf. below the more detailed observations made with the bacterial enzymes).

Canavalia *step C3b enzyme.* In contrast to the preceding preparation, the freshly prepared enzyme obtained after DEAE-cellulose fractionation exhibited a low affinity for arginine (K_m $3{\cdot}8 \times 10^{-3}$ M) at its optimum pH of 6·8, and was very much more stable. For instance, when stored for 4 days at 0–4° it retained 91 per cent of its original activity, while 93 per cent was retained when kept for 8 days at −30° under saturated ammonium sulphate. This difference of behaviour led us to examine the properties, particularly the K_m (arginine) value, of enzyme obtained in the previous purification stage, i.e. step C2. The Hofstee-type kinetic plot obtained was non-linear and strongly suggested the presence at this stage of two distinct enzymes each capable of catalysing arginine-stimulated ATP–$^{32}PP_i$ exchange: one had the high K_m (arginine) value mentioned above, while the other showed a low K_m of the same order as that obtained for enzyme after calcium phosphate gel treatment. During 24 hr storage, or during DEAE-cellulose fractionation, the low K_m enzyme activity was apparently lost. These observations indicate that during the gel treatment, the high K_m enzyme was discarded.

A survey of the amino acid substrate specificity of the more stable enzyme revealed that lysine was activated much more readily than arginine: the K_m value determined for lysine with freshly prepared enzyme was $2{\cdot}5 \times 10^{-5}$ M (cf. the value of $3{\cdot}8 \times 10^{-3}$ M for arginine). Homoarginine also was utilized as a substrate and K_m values determined for an enzyme preparation stored for 7 days at −30° were $7{\cdot}3 \times 10^{-5}$ M, $3{\cdot}1 \times 10^{-3}$ M, and $1{\cdot}1 \times 10^{-2}$ M for lysine, homoarginine, and arginine respectively. The maximum rate of ATP–$^{32}PP_i$ exchange observed for both homoarginine and arginine in the presence of this stored enzyme was 65 per cent of that measured with lysine. The enzyme was saturated by 5 mM-lysine and the further addition of either 20 mM-homoarginine or 20 mM-arginine did not lead to an increased rate of exchange. Likewise when 20 mM-arginine was supplied together with 20 mM-homoarginine, the exchange measured was equal to that obtained with homoarginine alone. These experiments provide strong evidence for the view that a single enzyme,

TABLE 2. THE INHIBITORY EFFECT OF ADDITIONAL SALT UPON THE RATES OF ARGININE- AND LYSINE-STIMULATED ATP–$^{32}PP_i$ EXCHANGE CATALYSED BY THE HIGH K_m *Canavalia* ENZYME*

Salt	Concentration (mM)	Rate of ATP–$^{32}PP_i$ exchange*	
		Arginine-stimulated	Lysine-stimulated
NaCl	100	87·5	—
	200	61	95
KCl	50	—	99
	100	108	103
	200	73	108
Na_2SO_4	100	55	85

* Exchange rates are reported as percentages of the rate measured using the following mixtures: 4 mM-ATP, 4 mM-pyrophosphate, 20 mM-$MgCl_2$, 100 mM-tris-HCl (pH 6·8), and 5 mM-arginine HCl or 2 mM-lysine.

which we regard as a lysyl-sRNA synthetase, can activate all three basic amino acids. However, the enzyme was unable to activate canavanine.

Addition of certain salts partially inhibited the activity of the high K_m enzyme (Table 2), but the degree of inhibition was considerably less than that observed under comparable conditions with enzyme preparations from *Staphylococcus*. The presence of additional salts caused greater inhibition of the arginine-stimulated ATP–$^{32}PP_i$ exchange than of the lysine-dependent reaction.

Staphylococcus aureus *enzymes*. A high K_m (arginine) value was determined for the bacterial enzyme irrespective of the strain used or the type of preparation (step S2 or S3) employed. For instance, with step S3 type enzyme from a culture of the Copenhagen strain, K_m values of $1{\cdot}1 \times 10^{-2}$ M and $2{\cdot}2 \times 10^{-3}$ M were calculated for arginine and homoarginine respectively. At saturating substrate concentrations the exchange rate measured in the presence of homoarginine was 1·07 times that determined with arginine. A step S2 type enzyme from strain 524 SC cells exhibited K_m values of $1{\cdot}6 \times 10^{-5}$ M, $2{\cdot}4 \times 10^{-3}$ M, and $4{\cdot}6 \times 10^{-3}$ M for lysine, homoarginine and arginine respectively. Addition of 20 mM-arginine did not increase the exchange rate measured with 5 mM-lysine and at saturating substrate concentrations the arginine-stimulated exchange was 76 per cent of the corresponding rate determined for lysine. The latter enzyme retained 88 per cent of its activity after storage for 3 weeks at $-30°$. These properties strongly resemble those described for the high K_m *Canavalia* enzyme and equally suggest that the enzyme should be regarded as a lysyl-sRNA synthetase. Neither type of bacterial enzyme utilized canavanine as a substrate.

The *Staphylococcus* enzymes however differed from the higher plant ones in (*a*) their higher optimum pH values of about 8·5, and (*b*) the stronger inhibition of the arginine-stimulated exchange caused by the presence of additional salts in reaction mixtures. Increased concentrations of Mg^{2+} could partially alleviate the inhibitions caused by a number of salts (see Tables 3 and 4), and since a large number of individual experiments indicated that 20 mM-$MgCl_2$ was approximately optimal (see Table 3), this concentration was routinely added to reaction mixtures. The data in Table 4 suggest that at equivalent concentrations Na^+ is more inhibitory than K^+; NH_4^+ was probably more inhibitory than either. Among anions, SO_4^{2-} appeared to inhibit more strongly than Cl^-. The extent of these salt inhibitions

TABLE 3. THE EFFECT OF VARYING THE CONCENTRATION OF Mg^{2+} UPON ARGININE-STIMULATED ATP–$^{32}PP_i$ EXCHANGE IN THE PRESENCE AND ABSENCE OF 120 mM-NaCl

Concentration of $MgCl_2$ (mM)	Concentration of NaCl (mM)	Percentage exchange rate*
8	120	58
9	120	79
10	120	100
16	120	166
20	120	161
24	120	136
10	0	214
20	0	210

Reaction mixture contained *Staphylococcus* enzyme plus 4 mM-ATP, 4 mM-pyrophosphate, 5 mM-arginine-HCl, and 100 mM-tris-HCl (pH 7·9).

* Rates of exchange are expressed as percentages of that measured in the presence of 10 mM-$MgCl_2$ and 120 mM-Nacl.

TABLE 4. THE EFFECT OF VARIOUS SALTS UPON ARGININE-STIMULATED ATP–$^{32}PP_i$ EXCHANGE AT TWO $MgCl_2$ CONCENTRATIONS

Concentration of $MgCl_2$ (mM)	Additional salt		Percentage exchange rate*
	Type	Conc. (mM)	
10	—	—	100
10	NaCl	100	56
10	NaCl	200	33
10	KCl	100	66
10	KCl	200	45
20	KCl	200	68
20	NH_4Cl	200	48
10	Na_2SO_4	100	26
10	K_2SO_4	100	32
20	$(NH_4)_2SO_4$	100	49

Reaction mixtures contained *Staphylococcus* enzyme plus 4 mM-ATP, 4 mM-pyrophosphate, 5 mM-arginine-HCl and 100 mM-tris-HCl (pH 8·3).

* Rates of exchange are expressed as percentages of that measured with 10 mM-$MgCl_2$ and no added salts.

made it essential that reaction mixtures should have similar ionic compositions and that the Cl^- and SO_4^{2-} introduced into some mixtures as arginine hydrochloride or canavanine sulphate should be balanced by corresponding additions of anions to other assay mixtures.

DISCUSSION

The labile nature of the presumed arginyl-sRNA synthetase from *Canavalia*, studied after elution from calcium phosphate gel, suggests that all the true arginine-activating enzyme present in cells of *Staphylococcus aureus* may have been inactivated during the initial, harsh homogenizing procedures. If this were so, and if arginine (and homoarginine) activation were effected non-specifically by the bacterium's lysyl-sRNA synthetase, then an explanation exists for the failure to observe activation of canavanine by enzyme preparations from mutant 524 SC, although this strain can incorporate canavanine into its cell protein. Canavanine activation by the calcium phosphate gel fraction from *Canavalia* containing the arginyl-sRNA synthetase was never more than a few percent of the related arginine value, and often it was hardly discernible. A single experiment showing that arginine and canavanine did not behave as competitive substrates suggested that the small activation of canavanine may be effected by an enzyme, different from arginyl-sRNA synthetase, but also present in the eluate from the calcium phosphate gel.

If the idea is accepted that the more stable enzyme preparations exhibiting a high K_m value for arginine are in fact lysyl-sRNA synthetases, then the range of substrates studied enable interesting conclusions to be reached concerning the specificity of the enzyme. The pK value of the ϵ-amino group of lysine is about 10·5, and that of the guanidino groups in arginine and homoarginine is about 12·5. In contrast, the guanidinoxy group of canavanine exhibits a much lower pK of about 8·0. Thus at the assay pH's commonly used for bacterial enzyme preparations, the guanidinoxy group of canavanine would be largely unprotonated, whereas the terminal basic groups of the other amino acids would carry a proton. This difference may explain why canavanine cannot become attached at the active centre of the lysyl-sRNA synthetase. If the size of the terminal basic group is less important than the

need for it to be protonated, then the resemblance between lysine and homoarginine, in terms of stereochemical and ionization characteristics, may be closer than that between lysine and arginine: an equal number of methylene groups separate the polar groups in the former pair of amino acids.

Previous investigations of the lysyl-sRNA synthetases from rat liver[19] and from *Escherichia coli*[20] have indicated somewhat lower K_m (lysine) values of 5×10^{-6} M and $5{\cdot}7 \times 10^{-7}$ M respectively. The possibility that homoarginine or arginine might act as substrates for these two enzymes apparently was not tested.

The activation of homoarginine by what are regarded as lysyl-sRNA synthetases from both *Canavalia* and *Staphylococcus* is reminiscent of the relationship found earlier between valine and the isoleucyl-sRNA synthetase. At present the similarity extends only to the ability of homoarginine to participate as an anomalous amino acid in reaction 1, as measured by ATP–$^{32}PP_i$ exchange, and it will be of interest to learn whether it can be readily transferred to $tRNA^{lys}$ (in reaction 2) or whether this specific tRNA molecule can discriminate against homoarginyladenylate to reduce such transfer to a minimum (cf. the action of $tRNA^{isol}$ upon valyladenylate). Homoarginine exhibited a different behaviour in regard to the arginyl-sRNA synthetase for although it showed competitive inhibition of arginine-stimulated ATP–$^{32}PP_i$ exchange, it was not itself activated.

Homoarginine can be used in place of arginine as a substrate for arginase, when lysine forms the end-product. Therefore it was important to establish that homoarginine activation by preparations of lysyl-sRNA synthetase was not due to the presence of arginase in these preparations. Each type of enzyme preparation was assayed for arginase activity and, although the cruder preparations obtained at earlier stages of the fractionation procedure exhibited some activity, no such contamination could be detected in the DEAE-cellulose fractions containing the lysyl-sRNA synthetases of *Canavalia* or *Staphylococcus*. Some arginase activity was associated with the calcium phosphate gel fraction of *Canavalia* which exhibits arginyl-sRNA synthetase activity, but this fraction did not activate homoarginine.

Inhibition of aminoacyl-sRNA synthetases by ions has been observed only on a few occasions. Schweet, Holley and Allen[21] have noted that a Na^+ inhibition of the tyrosyl-sRNA synthetase of hog pancreas could be partially relieved by increased K^+ concentrations. More recently the transfer of ^{14}C-leucine to yeast tRNA catalysed by the leucyl-sRNA synthetase from *E. coli* (a heterologous reaction) has been shown to be inhibited by NaCl: no salt inhibition was observed when a homologous system containing yeast enzyme and yeast tRNA were used.[22] Our studies, particularly with the lysyl-sRNA synthetase from *Staphylococcus*, have shown the more general nature of ion inhibition, both cation and anion, although it must be noted that the results recorded in Tables 3 and 4 relate to arginine-stimulated exchange catalysed by this enzyme. When the normal substrate lysine was used, ion inhibition was not so marked. Increased concentration of Mg^{2+} also served to reduce the degree of inhibition produced by a fixed concentration of salt (see Table 3). Although perhaps merely coincidental, it is nevertheless interesting to note that, in the presence of 0·12 M-NaCl, optimal protective effect is shown by 16 mM-$MgCl_2$, i.e. a concentration of Mg^{2+} that is just sufficient to both fully chelate with the ATP and complex (and largely precipitate at the alkaline assay pH) all the pyrophosphate in the reaction mixture. In the

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absence of additional NaCl, 10 mM-$MgCl_2$ facilitates maximum exchange rates: under these circumstances a high proportion of the pyrophosphate remained in solution and the extent to which precipitation occurred did not affect the measured rate of reaction. Soluble pyrophosphate then may inhibit the reaction by some mechanism, but only when salts are present. Although the mechanism of ion inhibition is at present little understood, the results themselves indicate that caution must be exercised when this group of enzymes are under investigation for quite unwittingly normal buffer ions may cause enzyme inhibition. In a recent review, Novelli[23] has stressed the need to ascertain the optimal Mg^{2+}/ATP concentration ratio for aminoacyl group transfer since the requirements may differ widely for the various aminoacyl-sRNA synthetases, and even for the same enzyme obtained from different species.

EXPERIMENTAL

Chemicals

Amino acids were obtained as follows: L-canavanine sulphate (Mann Research Laboratories, New York), L-homoarginine (Koch-Light, Colnebrook, Bucks.), and γ-guanidino-L-α-aminobutyric acid and β-guanidino-L-α-aminopropionic acid (Calbiochem., Los Angeles). ATP (disodium salt) was obtained from Sigma Chemical Corporation and the ^{32}P-pyrophosphate was prepared from ^{32}P-orthophosphate (Radiochemical Centre, Amersham) by pyrolizing at 500° to give material of specific activity 1–1·5 μc/μmole pyrophosphate.

Calcium phosphate gel was prepared by the method of Tiselius *et al.*[24] and stored at 1° under mM-phosphate buffer, pH 6·8. The DEAE-cellulose used was Whatman Chromedia DE 11.

Assay Methods

Protein was determined generally using the Folin reagent as described by Lowry *et al.*,[25] bovine serum albumin being used as a standard. Protein present in eluates from DEAE-cellulose columns was calculated from extinction values measured at 260 and 280 nm by the method of Layne.[26]

ATP-$^{32}PP_i$ exchange was normally assayed in reaction mixtures containing the following components in a final volume of 1 ml.: ATP (4 μmoles), ^{32}P-pyrophosphate (4 μmoles), $MgCl_2$ (10 μmoles), tris-HCl buffer, pH 7·9 (100 μmoles), amino acid (5 μmoles), and enzyme preparation (0·4 or 0·5 ml). Modification of this basic mixture in respect of Mg^{2+} and amino acid concentrations, and of pH were occasioned during the study of the properties of certain enzyme preparations. After reaction, labelled ATP was separated from unchanged ^{32}P-pyrophosphate by absorbing the nucleotide on Darco G-60 charcoal.[16,17] The specific activities of enzyme preparations are expressed as μmole PP_i exchanged/hr/mg protein (see Davie *et al.*[27]).

Enzyme Preparations

(*a*) Canavalia *fractionation. Canavalia* seed meal (40–80 g) was ground for 10 min in a mortar with one-fifth its weight of sand and double its weight of a grinding solution containing 0·1 M-tris-HCl (pH 7·9), 0·4 M-sucrose, 10 mM-$MgCl_2$, 1 mM-thioglycollate and 0·5 mM-EDTA. After addition of further grinding solution, the macerate was expressed through muslin and the extract centrifuged for 20 min at 20,000 *g* to remove mitochondria and other larger particles.

The clarified extract was brought to pH 4·6 by careful addition of 0·2 N-acetic acid, and the precipitated protein was redissolved in a solution of 0·1 M-tris-HCl (pH 7·9), 1 mM-thioglycollate and 0·5 mM-EDTA (step C1). Protein present in this solution was fractionally precipitated by gradual addition of ammonium sulphate. Although arginine-activating enzyme was widely distributed among these fractions, the major amount of enzyme was confined to the 50–70 per cent ammonium sulphate saturation precipitate (step C2). This fraction was selected for further purification, either by treatment with calcium phosphate gel or by gradient elution from a DEAE-cellulose column as below:

Calcium phosphate gel treatment (step C3a)—after dialysis to remove ammonium sulphate, step C2 enzyme (containing up to 600 mg protein) was treated with a suspension of calcium phosphate gel (2·3 g

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dry wt.), equilibrated to 0·02 M-tris-HCl (pH 7·9) buffer, for 20 min. The recovered gel was washed with more buffer, and then stepwise elution of absorbed protein was effected with 20 mM-phosphate (discarded) followed by 70 mM-phosphate (retained). The enzyme present in the latter eluate was concentrated by precipitation with saturated ammonium sulphate: it was sedimented, redissolved in appropriate buffer and dialysed free from ammonium sulphate before assay.

DEAE-cellulose treatment (step C3b)—enzyme from step C2 was applied to a DEAE-cellulose column (bed volume 50 ml), and fractionally eluted using a linear gradient from 0–0·3 M-NaCl in 0·02 M-tris-HCl (pH 7·9) buffer. The eluted peak corresponding to arginine-activating enzyme appeared at about 0·13 M-NaCl, and pooled fractions representing the major proportion of the enzyme were concentrated before use as in step C3a.

(*b*) Staphylococcus aureus *enzyme fractionation.* The bacterial cells (strains Copenhagen and 524 SC) were grown in shake culture at 37° in a medium containing yeast extract (0·5 per cent), peptone (0·5 per cent), K_2HPO_4 (0·1 per cent) and glucose (1 per cent), final pH 7·1. After harvesting and washing the cells by centrifuging, they were suspended with an equal weight of ballotini beads (dia. 0·2 mm) and broken in a solution containing 0·1 M-tris-HCl (pH 7·9), 1 mM-thioglycollate, and 0·5 mM-EDTA using a Virtis blendor (three periods of 4 min each at 45,000 rev/min). After centrifuging, the residue was homogenized a second time, and the two clarified extracts were combined.

Enzyme purification from Copenhagen strain extracts first involved acid fractionation, the pH 4·7–5·2 protein precipitate being retained and redissolved in 0·1 M-tris-HCl (pH 7·9), 1 mM-thioglycollate and 0·5 mM-EDTA (step S1). This solution was saturated with ammonium sulphate. The precipitated protein next was stirred with a series of ammonium sulphate solutions of successively diminishing degrees of saturation. Preliminary assay indicated that about 70 per cent of the total arginine-activating enzyme activity was dissolved at the 50–40 per cent saturation stage. Step S2 enzyme was obtained by dialysing this solution overnight against 0·02 M-tris-HCl (pH 7·9) buffer containing 1 mM-thioglycollate and 0·2 mM-EDTA. Further purification on a DEAE-cellulose column using a linear gradient of 0·1–0·7 M-NaCl in 0·02 M-tris-HCl (pH 7·9) yielded a protein peak at about 0·3 M-NaCl possessing arginyl-sRNA synthetase activity (step S3).