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Aminoacyl-tRNA synthetases catalyze AMP → ADP → ATP exchange reactions, indicating labile covalent enzyme-amino acid intermediates

(protein synthesis/aminoacyl adenylates/aminoacylation)

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ABSTRACT **Aminoacyl-tRNA synthetases (amino acid–tRNA ligases, EC 6.1.1.-) catalyze the aminoacylation of specific amino acids onto their cognate tRNAs with extraordinary accuracy. Recent reports, however, indicate that this class of enzymes may play other roles in cellular metabolism. Several aminoacyl-tRNA synthetases are herein shown to catalyze the AMP → ADP and ADP → ATP exchange reactions (in the absence of tRNAs) by utilizing a transfer of the γ-phosphate of ATP to reactive AMP and ADP intermediates that are probably the mixed anhydrides of the nucleotide and the corresponding amino acid. AMP and ADP produce active intermediates with amino acids by entering the back-reaction of amino acid activation, reacting with labile covalent amino acid-enzyme intermediates. Gramicidin synthetases 1 and 2, which are known to activate certain amino acids through the formation of intermediate thiol-esters of the amino acids and the enzymes, catalyze the same set of reactions with similar characteristics. Several lines of evidence suggest that these activities are an inherent part of the enzymatic reactions catalyzed by the aminoacyl-tRNA synthetases and gramicidin synthetases and are not due to impurities of adenylate kinase, NDP kinase, or low levels of tRNAs bound to the enzymes. The covalent amino acid-enzyme adducts are likely intermediates in the aminoacylation of their cognate tRNAs. The use of gramicidin synthetases has thus helped to illuminate mechanistic details of amino acid activation catalyzed by the aminoacyl-tRNA synthetases.**

The mechanism of aminoacylation catalyzed by aminoacyl-tRNA synthetases (amino acid–tRNA ligases, EC 6.1.1.-) is generally accepted as involving the formation of enzyme-bound aminoacyl adenylates followed by transfer (charging) of the corresponding amino acid onto its cognate tRNA (1–4). The sensitivity of synthetases to sulfhydryl reagents, which in most cases affect the aminoacylation activities to a much higher degree than their inhibition of the ATP/pyrophosphate exchange activities, prompted suggestions of labile acyl-enzyme adducts as intermediates between enzyme-bound aminoacyl adenylates and aminoacylated tRNAs (5–8). Free-energy considerations favor the relative stability and low reactivity of enzyme-bound acyl adenylates (9, 10), which led to suggestions of an alternative concerted mechanism for aminoacylation (11). Many kinetic and mechanistic studies, however, have established the aminoacyl adenylates as obligatory intermediates in the overall reaction (4). Several recent reports demonstrate the likely involvement of some aminoacyl-tRNA synthetases in biological processes that are not directly related to the first step in protein synthesis (12–14). Especially intriguing are the reports demonstrating dramatic variations in levels and specific activities among aminoacyl-tRNA synthetases that do not correlate with rates of protein synthesis but are dependent on age, tissue, and degree of development (15, 16). Tryptophanyl-tRNA synthetase constitutes 2% of the soluble proteins in adult bovine pancreas, whereas $tRNA^{Trp}$ levels are similar to those found in other tissues where the level of the synthetase is lower by a factor of 10–100 (15). Establishing the intermediates in the aminoacylation reaction along with the various catalytic activities of the synthetases is likely to point to the secondary metabolic activities of synthetases and to suggest the type of agents capable of inhibiting the activation of specific amino acids. The studies outlined here show that a variety of aminoacyl-tRNA synthetases have the ability to catalyze AMP → ADP → ATP exchange reactions. The significance of these reactions in relation to the presence of labile covalent amino acid-enzyme intermediates and the molecular mechanism of amino acid activation and aminoacylation of tRNA are discussed.

MATERIALS AND METHODS

Proteins. Phenylalanyl-tRNA synthetase (PheRS) (17), valyl-tRNA synthetase (ValRS) (18), arginyl-tRNA synthetase (ArgRS) (19), and aspartyl-tRNA synthetase (AspRS) (20) were purified to electrophoretic homogeneity by published procedures. All synthetases were kept at −20°C at a concentration of 12 mg/ml in 20 mM phosphate buffer (pH 7.2) containing 0.1 mM dithiothreitol, 0.1 M EDTA, and 50% (vol/vol) glycerol. Gramicidin S synthetases 1 and 2 (GS1 and GS2) were purified as described (21).

Amino Acid Activation and Exchange Reactions. Reaction mixtures (50 μl) included 0.1 M Hepes (pH 7.8), 20 mM $MgCl_2$, 5 mM ATP, 150 mM KCl, 10 μM amino acid, 0.5 mM EDTA or 100 μM Zn^{2+}, with or without 2.5 μg of yeast inorganic pyrophosphatase (0.05 mg/ml). Reactions were initiated by the addition of 4 μg of GS1 or GS2 or 12 μg of PheRS, ValRS, ArgRS, or AspRS in 0.1 M Hepes, pH 7.8/150 mM KCl. Incubations were at 37°C unless otherwise stated. Aliquots (5 μl) were removed at 0, 1, 5, 10, and 15 min into 2 μl of ice-cold 10 mM AMP/10 mM ADP/10 mM ATP. The combined solution was analyzed by thin-layer chromatography on polyethyleneimine (PEI)-cellulose plastic thin-

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Abbreviations: PheRS, phenylalanyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; GS1, gramicidin S synthetase 1; GS2, gramicidin S synthetase 2; pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate.

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layer plates, using elution with water followed by 1 M LiCl. Spots were visualized by ultraviolet light and excised, and radioactive material eluted with 4 M ammonium hydroxide was quantitated by liquid scintillation counting. For each set of reactions one time point was also analyzed by two-dimensional thin-layer chromatography (22) in order to ascertain that the resolutions obtained by the one- and two-dimensional thin-layer separations were similar. The rates of exchange reactions were linear during the first 15 min. Substrates for the exchange reactions were 0.05–0.5 mM [^{3}H]- or [^{14}C]AMP, [^{3}H]- or [^{14}C]ADP, and [^{3}H,γ-^{32}P]ATP.

Aminoacylation. Aminoacylation was assayed in enzymatic reaction mixtures as described above. Yeast mixed tRNA (5 mg/ml) was present in the aminoacyl-tRNA synthetase reaction mixtures, and acid-precipitable radioactivity was determined. Gramicidin synthetases were aminoacylated with [^{3}H]phenylalanine (GS1) or [^{3}H]valine (GS2). A mixture of L-proline, L-valine, L-ornithine, and L-leucine (each at 5 μM) was used in the GS2 incubation mixture. Adenosine 5'-[α,β-methylene]triphosphate (pp[CH_2]pA) and adenosine 5'-[β,γ-methylene]triphosphate (p[CH_2]ppA) (Sigma) contained about 1–2% ATP as an impurity. These ATP levels did not affect the aminoacylation reactions supported by pp[CH_2]pA or p[CH_2]ppA, since the ATP impurities were completely consumed by the reaction within 1 min. Relatively high levels of aminoacyl-tRNA synthetases were utilized in order to eliminate the effects of small impurities of ATP. High-pressure liquid chromatography on Partisil-10 SAX columns (Whatman) was used for assay of the ATP content. Rates of aminoacylation supported by pp[CH_2]pA or p[CH_2]ppA were calculated from the levels of aminoacylated tRNA between 5 and 10 min after initiation of the reactions.

RESULTS AND DISCUSSION

We will show that electrophoretically homogeneous aminoacyl-tRNA synthetases from yeast (PheRS, ValRS, ArgRS, and AspRS) and the two gramicidin synthetases from *Bacillus brevis* (GS1 and GS2) allow entry of AMP and ADP into the back-reaction of amino acid activation in the absence (or presence) of tRNA. The mechanism of amino acid activation catalyzed by GS1 and GS2 has been established (23, 24) and involves the transfer of enzyme-bound aminoacyl adenylates to intermediate aminoacyl thiol-enzyme, leading to the synthesis of gramicidin S. These two enzymes thus serve as models for the type of reactions that are plausible through a covalent aminoacyl thiol-enzyme. Fig. 1 demonstrates that all six enzymes catalyze the synthesis of ADP from AMP and the formation of ATP from ADP in amino acid-activating systems. The reactions are strictly dependent on the presence of ATP (Table 1) and are stimulated by inorganic pyrophosphatase. The possibility of adenylate kinase contaminations in these enzyme preparations is thus eliminated, since adenylate kinase catalyzes the back-reaction 2ADP → AMP + ATP without the initial presence of ATP, and its activity is not stimulated by inorganic pyrophosphatase (unpublished data). The significant stimulation of both AMP → ADP and ADP → ATP steps by inorganic pyrophosphatase suggests that both reactions are dependent on the initial formation of aminoacyl adenylates and cannot be attributed to an NDP kinase-type activity. The presence of 100 μM Zn^{2+} affects only the reactions catalyzed by PheRS, decreasing the rate of AMP → ADP and ADP → ATP exchange reactions (Fig. 1). The rate of AMP → ADP exchange (Fig. 1*A*) is expressed by the initial rate of ADP and ATP formation from AMP, since ADP is partially converted

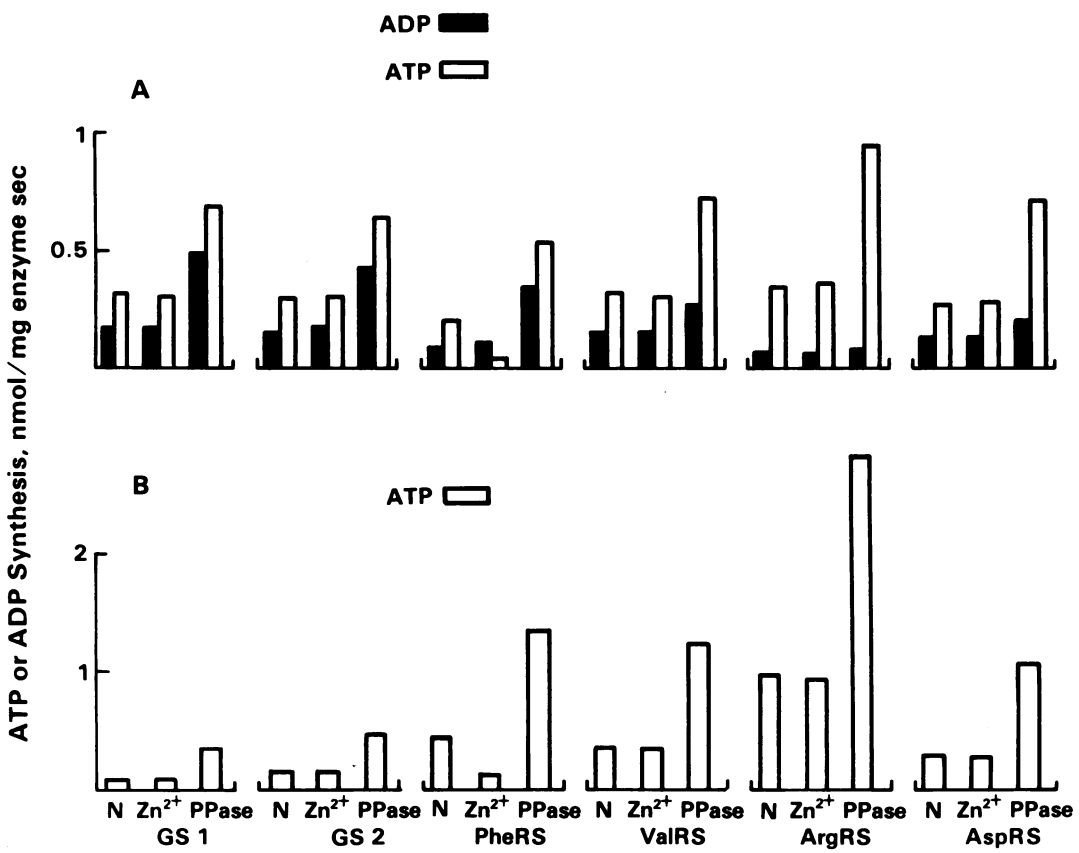

FIG. 1. AMP → ADP and ADP → ATP exchange reactions in amino acid-activating systems. Labeled substrates were 0.1 mM [^{14}C]AMP (*A*) and 0.1 mM [^{14}C]ADP (*B*). N, normal reaction mixture; Zn^{2+}, reaction in the presence of 100 μM $ZnSO_4$; PPase, reaction in the presence of inorganic pyrophosphatase (yeast) at 0.05 mg/ml.

Table 1. Characterization of AMP → ADP → ATP exchange reactions catalyzed by GS1, GS2, PheRS, ValRS, ArgRS, and AspRS

Conditions	Reaction rate*	
	AMP → ADP	ADP → ATP
− ATP, + AMP	NR	NR
− ATP, + ADP	NR	NR
+ K_2HPO_4 (25 mM, pH 7.8)	+	+
+ tRNA (mixed)	+	+
+ Ap_4A (1 mM)	+	+
+ Ap_5A (1 mM)	−	+
+ Ap_6A (1 mM)	+	+

*NR, no reaction; +, no inhibition (90–110% of control); −, substantial inhibition (10–20% of control). Reactions were assayed by the [^{14}C]AMP → [^{14}C]ADP and [^{14}C]ADP → [^{14}C]ATP exchanges as well as the transfer of γ-^{32}P from ATP to AMP and ADP under the conditions described in the text.

to ATP (Fig. 1*B*). The rate of phenylalanyl adenylate synthesis, catalyzed by PheRS, is not affected by Zn^{2+} (8), but the rate of aminoacylation of $tRNA^{Phe}$ is significantly reduced (25). Since the decrease in AMP → ADP and ADP → ATP exchanges and the inhibition of aminoacylation catalyzed by PheRS in the presence of Zn^{2+} are of similar magnitudes, both sets of reactions may proceed through a common labile intermediate. We have previously shown (8) that, in the presence of 100 μM Zn^{2+}, PheRS undergoes an autoaminoacylation reaction, yielding a stable covalent amino acid-enzyme adduct as a final product. This adduct is a result of the formation of an isopeptide linkage between phenylalanine and the ε-amino group of a single lysine residue on the β subunit of PheRS (8).

The AMP → ADP and ADP → ATP exchange reactions involve the phosphorylation of active intermediates of AMP and ADP by transfer of the γ-phosphate of ATP to yield ADP and ATP, respectively (Fig. 2). The ADP → ATP exchange reaction, resulting from γ-^{32}P transfer from ATP to an active ADP intermediate, was assayed by the formation of [^{3}H]ADP from doubly labeled [^{3}H,γ-^{32}P]ATP in the presence of unlabeled ADP (Fig. 2*B*). No direct exchange of AMP to ATP could be demonstrated in the presence of pyrophosphatase (which eliminates the pyrophosphate exchange). In the presence of excess unlabeled ADP, the formation of any labeled ATP from labeled AMP is prevented. The exchange reactions are dependent on the presence of ATP and are not affected by tRNA (Table 1). The reactions are not affected by large amounts of inorganic phosphate (Table 1) and would thus involve a direct transfer of the γ-phosphate of ATP to reactive AMP and ADP intermediates rather than initial hydrolysis of ATP followed by phosphorolysis of the reactive intermediates. Ap_5A significantly inhibits the AMP → ADP exchange and not the ADP → ATP exchange. Ap_4A and Ap_6A do not affect either catalytic reaction (Table 1). The specific inhibition of AMP → ADP exchange by Ap_5A provides further support for the proposed mechanism. Ap_4A and Ap_5A inhibit a variety of kinases where the transition states involving two substrates resemble the structures of Ap_4A or Ap_5A (26, 27). The lack of inhibition of ADP → ATP

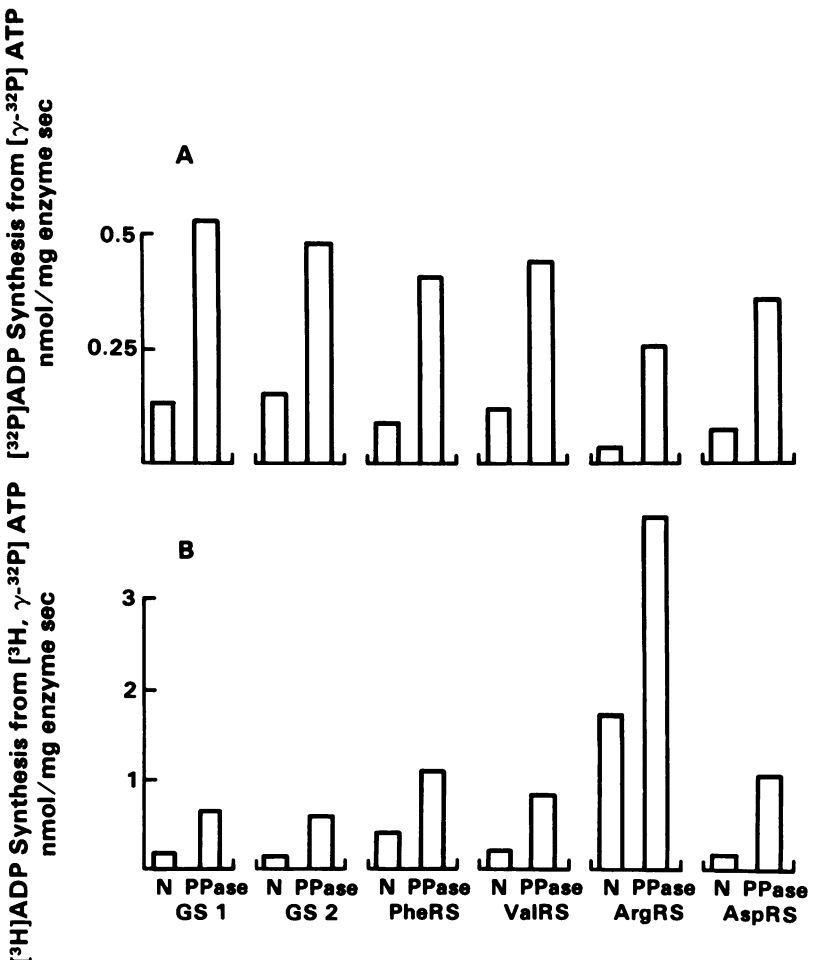

FIG. 2. Rates of transfer of γ-^{32}P from [γ-^{32}P]ATP to AMP (*A*) and the rate of formation of [^{3}H]ADP, which serves as an assay for the transfer of γ-^{32}P from [^{3}H,γ-^{32}P]ATP to ADP (*B*). Substrates were 0.5 mM AMP (*A*) and 0.5 mM ADP (*B*) along with [^{3}H]ATP and [γ-^{32}P]ATP under reaction conditions described in the text.

catalyzed exchange by Ap_6A is attributed to the greater degree of flexibility of Ap_6A when compared to Ap_4A and Ap_5A. Considering the nature of the amino acid activation reactions catalyzed by the two gramicidin synthetases and the four aminoacyl-tRNA synthetases, the plausible reactive intermediates in the exchange reactions are aa~AMP (aminoacyl adenylate) and aa~ADP. These intermediates are in turn produced by the back-reaction of AMP and ADP with a labile aminoacyl enzyme, presumably an aminoacyl thiol-enzyme.

It is yet too early to conclude, based on the results reported here, that the kinase action of ATP during amino acid activation reactions is coupled to the formation of the amino acid-enzyme adducts and ultimately to aminoacylation of tRNAs. On a molar basis, the exchange reactions occur at 10-fold higher rates than aminoacylation of tRNAs or formation of aminoacyl thiolenzymes (in GS1 or GS2). To assess whether a hydrolyzable α,β-pyrophosphate moiety or a hydrolyzable β,γ-moiety is sufficient to support aminoacylation in the absence of ATP, we studied the aminoacylation reactions in the presence of $p[CH_2]ppA$ and $pp[CH_2]pA$, respectively. The results (Table 2) show that $p[CH_2]ppA$ is sufficient for supporting aminoacylation when catalyzed by PheRS. $pp[CH_2]pA$ is inactive in this case. $pp[CH_2]pA$ is capable of supporting ArgRS-catalyzed aminoacylation to a much higher degree than $p[CH_2]ppA$. Since ArgRS is also known not to catalyze pyrophosphate exchange in the absence of tRNA (28, 29) and not to yield an isolable enzyme-bound arginyl adenylate (29), it is a distinct possibility that the major intermediate in the forward reaction of arginine activation is Arg~ADP. As is illustrated in Figs. 1 and 2, the ADP → ATP exchange reaction is most pronounced when catalyzed by ArgRS, in comparison with the other synthetases. The AMP → ADP exchange reaction catalyzed by ArgRS is the slowest among all the enzymes studied (Fig. 2*A*). Thus, in the case of ArgRS, the formation of Arg~ADP may be favored in the forward as well as backward reactions. Only in the case of AspRS did the combination of $p[CH_2]ppA$ and $pp[CH_2]pA$ support aminoacylation in the absence of ATP to a much higher degree than either $p[CH_2]ppA$ or $pp[CH_2]pA$ alone. This type of result would indicate a participation of γ-phosphate transfer onto aminoacyl adenylate as one of the pathways leading to the active aminoacyl-enzyme adduct and aminoacylation of tRNAs. It is important to note that certain enzyme-bound aminoacyl adenylates (aa~AMP) were shown to be sufficient for the aminoacylation of tRNAs (4) and that, in the case of PheRS, the sole presence of the α,β-pyrophosphate moiety of ATP is sufficient to support the formation of Phe~tRNA (Table 2). These observations are by no means general, as is illustrated by the substrate specificities of ArgRS and AspRS. Naturally, the ability of $p[CH_2]ppA$ or $pp[CH_2]pA$ to support aminoacylation in the absence of ATP depends to a large extent on the active-site topography of the enzymes and the free-energy state of enzyme-bound ATP or its analogues. The affinity of synthetases for their corresponding aminoacyl adenylates may contribute to the ability of $pp[CH_2]pA$ to promote aminoacylation from an enzyme-bound aminoacyl adenylate, which was in turn produced by reaction with $p[CH_2]ppA$ in the absence of ATP. AspRS has been reported to have a very poor affinity for aspartyl adenylate (30), whereas ValRS was shown to have a remarkably high affinity for valyl adenylate (31). The unexpectedly high stability of ValRS-bound valyl adenylate was reported to be significantly reduced upon addition of ATP (31).

Table 2. Ability of $pp[CH_2]pA$ and $p[CH_2]ppA$ to substitute for ATP in support of the aminoacylation of GS1 and GS2 and tRNA aminoacylation catalyzed by PheRS, ValRS, ArgRS, and AspRS

	Aminoacylation, % of control*		
Enzyme	$pp[CH_2]pA$ (5 mM)	$p[CH_2]ppA$ (5 mM)	$pp[CH_2]pA$ + $p[CH_2]ppA$ (2.5 mM each)
GS1	0	6 ± 2	8 ± 2
GS2	0	0	0
PheRS	5 ± 1	77 ± 10	81 ± 6
ValRS	0	0	0
ArgRS	71 ± 6	21 ± 4	69 ± 7
AspRS	8 ± 2	19 ± 3	36 ± 5

Enzymatic reactions were assayed as described in the text; 4 μg of GS1 or GS2 or 12 μg of aminoacyl-tRNA synthetases was added per 50-μl incubation mixture. Data represent means ± SD of four separate experiments.
*Control aminocylation is that supported by 5 mM ATP.

The data reported here indicate that the exchange reactions, which comprise the entry of exogenous AMP or ADP into the back-reaction of amino acid activation followed by γ-phosphate transfer from ATP, are an inherent part of the catalytic activities of the synthetases. The possibility that these exchange reactions are catalyzed by adenylate kinase present as a contaminant is eliminated by the absolute requirement for ATP, by the effects of Zn^{2+} on the exchange reactions catalyzed exclusively by PheRS (which is the only enzyme, out of the six studied here, whose aminoacylation activity is also inhibited by Zn^{2+}), and by the marked stimulation of the exchange reactions in the presence of inorganic pyrophosphatase. The Zn^{2+} and inorganic pyrophosphatase effects also exclude the possible catalysis by impurities of NDP kinase. The ability of impurities of tRNA to account for the exchange reactions can be discounted, since low levels of tRNA are completely aminoacylated (charged) whether inorganic pyrophosphatase is present or not, which would not be commensurable with the marked stimulation of the exchange reactions by inorganic pyrophosphatase.

The reactions reported here could also be related to a mechanism designed to prevent the buildup of enzyme-bound or free aminoacyl adenylates under conditions where protein synthesis is stalled. We (8) and others (30) have shown that under certain conditions, either enzyme-bound (8) or free (30) aminoacyl adenylates can react with ε-amino groups of lysine residues to produce aminoacylated N^{ε}-isopeptidyl proteins. Such aminoacylated proteins are likely to be susceptible to rapid cellular proteolytic degradation (32).

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