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Aminoacyl-tRNA Synthetase Stimulatory Factors and Inorganic Pyrophosphatase†

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ABSTRACT: A protein was purified from rat liver which stimulated a number of liver aminoacyl-tRNA synthetases. This stimulatory factor was identical with the "tRNA activator" of Dickman & Boll [(1976) *Biochemistry 15*, 3925] in its mechanism of action and chemical properties, although it was considerably more purified. The two preparations stimulated synthetases by virtue of their pyrophosphatase activity which destroyed the potent inhibitor, PP_i, that was present in the reaction mixtures. This PP_i was either generated during the reaction or was introduced by contamination of the tRNA or ATP preparations. The degree of inhibition of PP_i was strongly influenced by assay conditions, being most effective at low amino acid concentrations, at low pH, and in the presence of heterologous tRNAs. By use of certain assay conditions, PP_i concentrations as low as 2 μM could inhibit some synthetases close to 50%. The pitfalls associated with some assay conditions commonly used for aminoacyl-tRNA synthetases are discussed. These studies raise questions about the physiological significance of many previously described aminoacyl-tRNA synthetase stimulatory factors.

Since aminoacyl-tRNA synthetases catalyze the first committed step in a biosynthetic pathway, namely, protein synthesis, the possibility that the activity of these enzymes is subject to regulation must be considered. Over the years a number of laboratories have isolated factors which stimulate the activity of aminoacyl-tRNA synthetases and could thus serve some regulatory function. Boman & Svensson (1961) described a "regenerating enzyme" which increased the incorporation of methionine into *Escherichia coli* tRNA using yeast methionyl-tRNA synthetase. Makman & Cantoni (1966) isolated an "enhancing factor" which stimulated yeast seryl-tRNA synthetase when heterologous *E. coli* tRNA was the substrate, but which had little effect with yeast tRNA.

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Pearlman & Bloch (1967) partially purified a factor from liver which greatly stimulated the ability of liver tyrosyl-tRNA synthetase to utilize yeast tRNA, while having no effect on its reaction with liver tRNA. Davies & Marshall (1975) also identified a factor from liver which stimulated asparaginyl-tRNA synthetase using yeast tRNA as substrate, although its effect with liver tRNA was not examined. None of these factors was well characterized, and their mechanisms of stimulation remained obscure. However, Kull et al. (1969), who were studying the potent inhibition of heterologous misacylation reactions by inorganic pyrophosphate, noted a similarity between the effect of inorganic pyrophosphatase on their reactions and the stimulations by the various factors. They suggested that some of the factors described earlier might, in fact, represent inorganic pyrophosphatase activity that was relieving inhibition by PP_i.

More recently, Dickman & Boll (1976) partially purified and studied a protein from liver which stimulated a number of mammalian aminoacyl-tRNA synthetases when yeast tRNA was the substrate, but only slightly when liver tRNA was used. These authors suggested that this protein was a "tRNA activator" which catalytically increased the capability of tRNA to accept certain amino acids. During the course of our studies of liver aminoacyl-tRNA synthetases, we separated a factor which stimulated aminoacyl-tRNA formation with liver tRNA, but which otherwise appeared similar to the protein described by Dickman & Boll. Because of the possible importance of such a stimulatory factor and the paucity of information on the mechanism of action of any of these factors, we undertook a more detailed study of this system.

In this paper we will describe the purification and properties of our stimulatory factor and its relation to the "tRNA activator" of Dickman & Boll. Our data indicate that both factors represent inorganic pyrophosphatase activity which stimulates aminoacyl-tRNA formation by removal of PP_i present in the reagents or generated during the reaction. The pitfalls associated with the use of certain assay conditions of aminoacyl-tRNA synthetases that lead to potent inhibition by PP_i will also be discussed. These results cast doubt on the physiological significance of the aminoacyl-tRNA synthetase stimulatory factors previously described.

Experimental Procedures

Materials. Alumina C_γ gel, calcium phosphate gel, PMSF,[1] phosphoglucomutase, UDP-glucose pyrophosphorylase, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. DEAE-cellulose (DE-52) was from Whatman, and Sephadex G-25 and G-100 were from Pharmacia. Bovine serum albumin (A grade) was purchased from Calbiochem. Rabbit liver tRNA was prepared as described previously (Deutscher, 1972), and yeast tRNA was obtained from Schwarz/Mann. In later experiments both tRNAs were purified on Sephadex G-25 in 1 M NaCl to remove low molecular weight contaminants.

Assays. Aminoacyl-tRNA synthetases were generally assayed in 0.1-mL reaction mixtures containing 0.25 M Tris–acetate, pH 7.4, 5 mM ATP, 5 mM $MgCl_2$, 0.2 mM EDTA, 250 μM ^{14}C-labeled amino acid ($\sim$10 cpm/pmol) and the other 19 unlabeled amino acids, and 200 μg of liver tRNA and enzyme. For study of the stimulatory factor, the amino acid concentration was lowered to 2.5 μM ($\sim$500 cpm/pmol), the unlabeled amino acids were removed, and 200 μg of yeast tRNA was substituted for the liver tRNA. Stimulatory factor, or the buffer in which it was dissolved, was added as described in the legends to the figures. Reaction mixtures were incubated for 15 min at 37 °C and stopped by the addition of 3 mL of cold 10% Cl_3AcOH containing 0.02 M $NaPP_i$. After 10 min in ice the precipitate was collected and processed as described by Bandyopadhyay & Deutscher (1971).

Pyrophosphatase was assayed in 0.1-mL reaction mixtures containing 50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, and 10 mM $NaPP_i$. After incubation for 15 min at 37 °C, reactions were stopped by the addition of 0.2 mL of 10% Cl_3AcOH containing 5 mM $CuSO_4$. P_i was determined colorimetrically after addition of 0.7 mL of the reagent described by Chen et al. (1956). One unit of pyrophosphatase activity catalyzes the hydrolysis of 1 μmol of PP_i in 15 min.

Pyrophosphate was determined by measuring NADPH production in a coupled assay system as described by Johnson et al. (1968).

Preparation of Aminoacyl-tRNA Synthetases. All procedures were carried out at 0–4 °C. Fresh rat liver (200 g) was homogenized in 2.5 vol of 0.1 M Tris–acetate, pH 7.4, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM PMSF, and 10% glycerol with 10 strokes of a loose-fitting Dounce homogenizer and 3 strokes of a tight-fitting one. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 21500*g* for 20 min. The supernatant fluid was then centrifuged for 90 min at 100000*g*. Alumina C_γ gel (0.4 mg/mg of protein) was added to the supernatant fraction and stirred for 20 min. The gel was collected by centrifugation and washed three times with 200-mL portions of 0.08 M sodium phosphate, pH 7.4, 0.1 mM DTT, 0.1 mM EDTA, and 10% glycerol. Synthetases were eluted with three 60-mL portions of the same solution containing 0.2 M sodium phosphate and 10% ammonium sulfate (adjusted to pH 7.4). The combined eluates (180 mL) were dialyzed against the same solution containing 0.05 M sodium phosphate buffer. Calcium phosphate gel (5 mg/mg of protein) was added to the dialyzed material and stirred for 30 min. The gel was collected by centrifugation and washed with four 80-mL portions of the same solution containing 0.13 M sodium phosphate. Synthetases were eluted with two 50-mL portions of solution containing 0.1 M sodium phosphate and 10% ammonium sulfate. The combined eluates were dialyzed against the same solution containing 0.02 M sodium phosphate and stored frozen at −70 °C. The synthetases were stable for at least 8 months under these conditions. Aminoacyl-tRNA synthetases prepared by this procedure were active for most amino acids, but were devoid of tRNA nucleotidyltransferase activity. This preparation was used for all of the experiments reported here.

Purification of the Aminoacyl-tRNA Synthetase Stimulatory Factor. The material in the 100000*g* supernatant fraction from the synthetase preparation that did not bind to alumina C_γ gel was used as the initial source of the stimulatory factor. Purification of the stimulatory factor was followed by its effect on leucyl-tRNA formation assayed as described in Experimental Procedures. One unit of stimulatory activity is the amount required to give a twofold stimulation of leucyl-tRNA synthetase. Solid ammonium sulfate was added to this fraction to 45% saturation. The solution was stirred for 1 h and centrifuged, and the supernatant fraction was brought to 75% saturation. The 45–75% precipitate was dissolved in 0.05 M sodium phosphate, pH 7.0, 10% glycerol, and 0.1 mM DTT. This fraction was then precipitated with an equal

[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; Cl_3AcOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

Table I: Purification of Aminoacyl-tRNA Synthetase Stimulatory Factor

step	activity (units × 10^{-5})	specific activity (units/mg × 10^{-2})	relative purification	stimulatory factor/ pyrophosphate
alumina C_γ treated supernatant	163	16		800
ammonium sulfate (45–75%)	174	27	1.7	762
trichloroacetic acid precipitation	29	108	6.8	629
DEAE-cellulose	15	2900	180	734
Sephadex G-100	9.5	3650	230	649
"tRNA activator" (Dickman & Boll procedure)		73		580

volume of 10% Cl_3AcOH. The precipitated protein was collected by centrifugation and extracted by homogenization with 70 mL of 0.25 M sodium phosphate, pH 6.5, 1 mM $MgCl_2$, 10% glycerol, and 0.1 mM DTT. After being centrifuged, the precipitate was reextracted with 70 mL of the same solution. The combined extracts were dialyzed against the same solution containing 0.025 M sodium phosphate, pH 7.4.

The dialyzed material was applied to a DEAE-cellulose column (2.5 × 26 cm) equilibrated with the same solution. After washing with this solution until the A_{280} was at base line, we eluted the stimulatory activity factor with a 1-L linear gradient (0.025–0.25 M sodium phosphate in the equilibration solution). Fractions containing stimulatory activity were combined and concentrated in an Amicon ultrafiltration apparatus with a PM-10 membrane. The concentrated material was applied to Sephadex G-100 (1.5 × 86 cm) equilibrated with 50 mM Tris–acetate, pH 7.5, 1 mM $MgCl_2$, 0.1 mM DTT, and 10% glycerol. Active fractions were stored at −70 °C and were stable for at least 6 months under these conditions. The purified stimulatory factor was devoid of aminoacyl-tRNA synthetase and tRNA nucleotidyltransferase activity.

The tRNA activator described by Dickman & Boll (1976) was purified according to their procedure and stored at −70 °C.

Results

Identification and Purification of an Aminoacyl-tRNA Synthetase Stimulatory Factor. In the course of our studies of mammalian aminoacyl-tRNA synthetases, we repeatedly observed a dramatic loss of activity upon treatment of the high-speed supernatant fraction of rat liver with alumina C_γ gel. Readdition of the material that did not stick to the gel revealed the existence of a factor that stimulated many of the aminoacyl-tRNA synthetases to their original levels. This factor was purified several hundredfold as described in Experimental Procedures and summarized in Table I. The purified factor had a molecular weight of about 42000 as determined by chromatography on Sephadex G-100. Although the purified factor retained its ability to stimulate aminoacyl-tRNA synthetases, it contained no amino acid incorporating activity itself.

A surprising feature of the action of both the purified and the crude stimulatory factor was that only certain aminoacyl-tRNA synthetases were affected, and these were the same ones that lost activity during purification with alumina C_γ. This observation became understandable when we discovered that the stimulatory factor was inorganic pyrophosphatase (see below), so that only those synthetases inhibited by PP_i under our assay conditions were stimulated by addition of the factor.

Relation of the Stimulatory Factor to the "tRNA Activator". Early in our studies we suspected that the stimulatory factor we had identified might be related to the "tRNA activator" described by Dickman & Boll (1976). This was suggested to us by the similarity in molecular weights and the observation that our stimulatory factor was also relatively stable to precipitation with trichloroacetic acid. In fact, this latter property was incorporated into the purification scheme for the stimulatory factor (see Table I). In order to compare our factor to the "tRNA activator" directly, we purified the latter as described by Dickman & Boll (1976) and tested each preparation for its ability to stimulate the activity of seven different aminoacyl-tRNA synthetases. For these studies we lowered the concentration of amino acid to 2.5 μM and substituted yeast tRNA for liver tRNA (see Experimental Procedures) since these assay conditions led to increased levels of stimulation and more closely resembled those employed by Dickman & Boll (1976). The data in Table II show that the maximum stimulations obtainable by titration with either our stimulatory factor or the "tRNA activator" were identical for each of the aminoacyl-tRNA synthetases tested. Included in these data were some synthetases which were stimulated and others which were not. These results strongly suggested that the two factors were stimulating by the same mechanism and were probably identical. This conclusion was confirmed by the finding that the "tRNA activator" preparation also contained inorganic pyrophosphatase activity (see Table I and below).

Table II: Comparison of Stimulation by the Stimulatory Factor and the "tRNA Activator"[a]

aminoacyl-tRNA synthetase	stimulation (x-fold)	
	stimulatory factor	"tRNA activator"
leucine	3.3	3.3
threonine	4.4	4.6
proline	3.1	3.1
lysine	1.3	1.2
asparagine	3.4	3.6
isoleucine	1.1	1.1
methionine	3.4	3.4

[a] Aminoacyl-tRNA synthetases were assayed as described in Experimental Procedures by using 2.5 μM ^{14}C-labeled amino acid and yeast tRNA. Increasing amounts of purified stimulatory factor or "tRNA activator" were added until a plateau of stimulation was observed. The data presented are the maximum stimulation observed at the plateau.

Identification of the Stimulatory Factor as Inorganic Pyrophosphatase. Our initial studies of the mechanism of stimulation by the factor suggested that at least part of the effect was due to the removal of an inhibitor from the liver tRNA preparation. This inhibitor could be removed by dialysis or chromatography on Sephadex G-25, and it contained no material absorbing ultraviolet light at 260 nm. These properties raised the possibility that the inhibitor was PP_i. Accordingly, we tested the purified stimulatory factor for pyrophosphatase activity, and the results were positive. In addition, commercial yeast inorganic pyrophosphatase stimulated both leucyl- and methionyl-tRNA synthetases to the same level as was found with the stimulatory factor (data

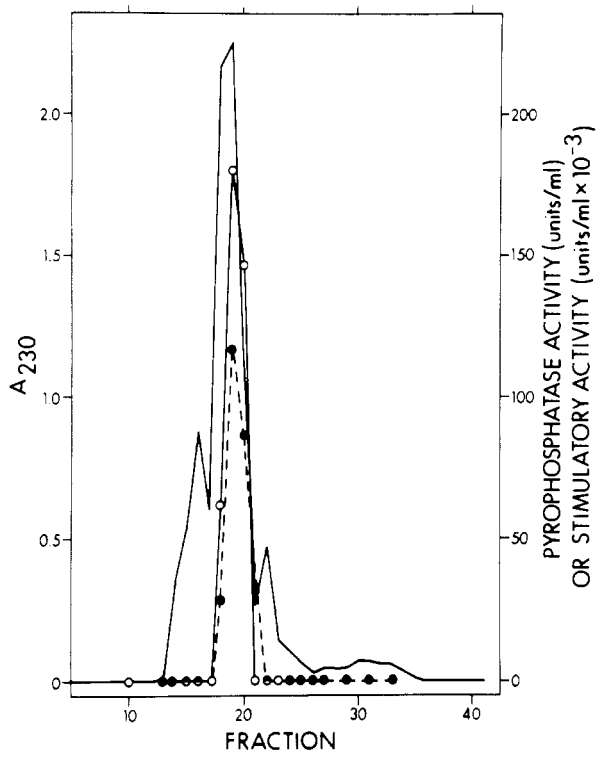

FIGURE 1: Copurification of stimulatory factor and pyrophosphatase on Sephadex G-100. The concentrated DEAE-cellulose fraction was applied to a column of Sephadex G-100 (1.5 × 86 cm) as described in Experimental Procedures. Fractions of 3.7 mL were collected and assayed for stimulatory factor and pyrophosphatase as described in Experimental Procedures. Protein was determined by absorbance at 230 nm (—). Stimulatory activity (●); pyrophosphatase (○).

not shown). The identity of the stimulatory factor with pyrophosphatase was also shown by the constant ratio of the two activities throughout the purification (Table I). In addition, the peaks of the two activities were superimposable in the last step of purification, Sephadex G-100 chromatography (Figure 1).

Assay of the "tRNA activator" preparation of Dickman & Boll revealed that it also contained pyrophosphatase activity in the same ratio to stimulatory activity as our purified factor (Table I). Since the "tRNA activator" and the stimulatory factor appeared to be identical and stimulating by virtue of their pyrophosphatase activity, the latter preparation was used in all subsequent studies due to its higher degree of purity (see Table I).

Source of PP_i in the Reaction Mixture. Since the mode of action of the stimulatory factor presumably was to remove the inhibitor, PP_i, it was important to determine the source of this PP_i and whether its removal was sufficient to account for the stimulations observed. Under our usual conditions of assay for liver aminoacyl-tRNA synthetases (250 μM each amino acid, liver tRNA), in which stimulation by the factor was up to twofold for some amino acids, part of the inhibition was due to PP_i in the tRNA preparation. Direct assay of the tRNA for PP_i indicated that ~10 μM PP_i was added to the reaction mixture from this source. Removal of this PP_i by dialysis or chromatography on Sephadex G-25 increased the basal rate of aminoacyl-tRNA formation, but further stimulation by the factor to the maximum level was still obtained. Preincubation of the other components of the reaction mixture with the factor did not identify any other major source of PP_i contamination in the reagents. This suggested that the PP_i was being generated during the reaction, undoubtedly because of the presence of the amino acid mixture and the use of a

Table III: Identification of Source of PP_i by Preincubation with Stimulatory Factor[a]

components in preincubation	stimulatory factor in preincubation	relative activity
A. complete	–	1.1
–tRNA	–	1.1
–ATP	–	1.1
B. complete	+	2.8
–tRNA	+	2.8
–ATP	+	0.9

[a] Aminoacyl-tRNA synthetase assay mixtures were set up as described in Experimental Procedures by using 2.5 μM [^{14}C]leucine and yeast tRNA. The preincubation for 30 min at 37 °C was in the absence of aminoacyl-tRNA synthetase and contained the components as indicated. After the preincubation, the missing component, if any, was added and the reaction was initiated by the addition of aminoacyl-tRNA synthetase. In part A, the stimulatory factor was also added just prior to assay. The assay for leucyl-tRNA formation was for 3 min at 37 °C. The amount of stimulatory factor present, when added, was 6 ng and was chosen to give almost no stimulation when added just prior to assay. The data presented show activity relative to a sample (set at 1.0) treated in an identical manner, but which contained no stimulatory factor.

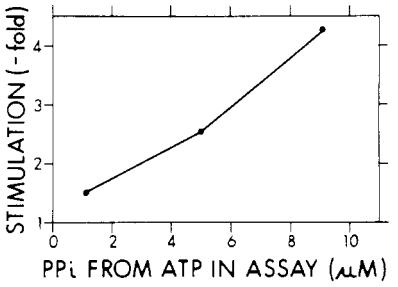

FIGURE 2: Comparison of stimulation of leucyl-tRNA formation with PP_i in the assay contributed from ATP. Leucyl-tRNA formation was measured as described in Experimental Procedures by using 2.5 μM [^{14}C]leucine, yeast tRNA, and three different preparations of ATP. The stimulation of leucyl-tRNA formation in the presence of 0.3 μg of stimulatory factor is plotted against the amount of PP_i in the assay determined by analysis of the ATP preparations.

partially purified synthetase preparation with specificity for many amino acids. This idea was shown to be correct since removal of the 19 unlabeled amino acids essentially eliminated the remaining effect of the stimulatory factor (data not shown).

These results created a dilemma in trying to understand the data of Dickman & Boll (1976) since under their assay conditions (one amino acid at 2.5 μM, yeast tRNA) negligible amounts of PP_i were generated in the reaction, and the commercial yeast tRNA we used was essentially devoid of PP_i. Nevertheless, the purified stimulatory factor increased aminoacyl-tRNA formation as much as three- to fourfold (Table II). Preincubation of the various components of the reaction mixture with the factor revealed that the component required for stimulation was the ATP (Table III). In contrast to the results of Dickman & Boll (1976), no effect of the factor on the tRNA could be demonstrated. Likewise, since the stimulatory effect could be potentiated by preincubation in the absence of the aminoacyl-tRNA synthetase, a direct effect of the factor on the synthetase was eliminated. In further support of the involvement of ATP in the stimulation was the finding that the use of different ATP preparations led to stimulations of leucyl-tRNA formation by a factor which varied from 1.5- to 4.3-fold and that the magnitude of stimulation was directly related to the amount of PP_i contributed to the reaction mixture as a contaminant of the ATP

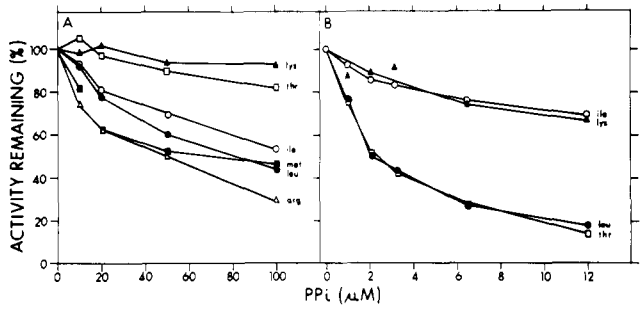

FIGURE 3: Effect of added PP_i on aminoacyl-tRNA synthetase activity using different assay conditions. (A) 250 μM amino acid, liver tRNA; (B) 2.5 μM amino acid, yeast tRNA. The other conditions of the assay are described in Experimental Procedures. The PP_i concentration represents the total PP_i in the reaction mixture, including 1 μM contributed by the ATP preparation. Activity in the absence of PP_i was determined from a reaction mixture containing added pyrophosphatase.

(Figure 2). These results demonstrate that PP_i contamination of ATP amounting to as little as 0.1% (i.e., 5 μM) can have profound effects on aminoacyl-tRNA synthetases. (In the following section we will show the importance of assay conditions for these effects.) Furthermore, extrapolation of the curve in Figure 2 suggests that essentially no stimulation would be observed if the ATP were free of PP_i. Thus, the stimulatory effect under these assay conditions could be accounted for totally by the contamination of ATP with PP_i.[2]

Effect of Assay Conditions on Inhibition by PP_i. The previous results suggested that the effect of the stimulatory factor by use of the two synthetase assay conditions (i.e., 250 μM amino acid and liver tRNA compared to 2.5 μM amino acid and yeast tRNA) was quite different. Thus, when the former conditions were used the maximum stimulation seen for any aminoacyl-tRNA synthetase was about twofold, whereas for lower amino acid concentrations and yeast tRNA stimulations of fourfold were common for many synthetases. In addition, when the latter assay conditions were used the small amount of PP_i contaminating the ATP was found to be the source of the inhibitory effect, whereas with our original assay conditions the PP_i in the ATP played no role in the inhibition since purification of the liver tRNA and removal of the amino acid mixture eliminated essentially all stimulation by the factor. These results suggested that the actual inhibition of PP_i would be quite different on use of the two assay conditions. In order to test this idea directly, we examined the effect of increasing concentrations of PP_i on aminoacyl-tRNA synthetase activity. The data in Figure 3 show that PP_i is an unusually potent inhibitor of many synthetases on use of either assay condition but that it is considerably more effective with a low amino acid concentration and yeast tRNA (Figure 3B). In fact, some synthetases, such as those specific for leucine and theonine, could be 50% inhibited by ~2 μM PP_i when these latter conditions are used.

The sensitivity of different aminoacyl-tRNA synthetases to inhibition by PP_i varied widely on use of either assay system, but in each case the relative degree of inhibition correlated exactly with the stimulation by the factor. Those synthetases least affected by PP_i under a given set of conditions were least

[2] One additional source of PP_i, which was not a consideration in these experiments, but which could have major effects when crude synthetase preparations are used, is the presence of tRNA nucleotidyltransferase. Commercial yeast tRNA is predominantly tRNA-C-C, and in the presence of ATP in the synthetase assay system large amounts of PP_i would be generated due to the incorporation of AMP into these tRNAs by tRNA nucleotidyltransferase.

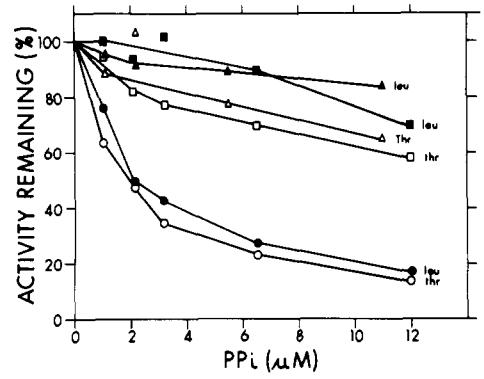

FIGURE 4: Effect of added PP_i on aminoacyl-tRNA synthetase activity at high amino acid concentration or with liver tRNA. Leucyl- and threonyl-tRNA synthetase activities were measured in the presence of increasing PP_i as described in Experimental Procedures with 2.5 μM amino acid and yeast tRNA (●, ○), 50 μM amino acid and yeast tRNA (■, □), or 2.5 μM amino acid and liver tRNA (▲, △). Correction for the PP_i present in the ATP preparation (1 μM) was done as in Figure 3.

stimulated by the factor, and vice versa. Furthermore, addition of stimulatory factor to assays containing added PP_i reversed the effect of the PP_i.

In order to understand the difference in PP_i sensitivity in the two assay systems, we examined the effect of independently increasing the amino acid concentration or changing the tRNA by using two of the most sensitive synthetases, those specific for leucine and threonine. The data in Figure 4 indicate that increasing the amino acid concentration from 2.5 to 50 μM greatly decreases inhibition by PP_i of both leucyl- and threonyl-tRNA synthetase. Similarly, substitution of liver tRNA for yeast tRNA relieves most of the inhibition by PP_i (Figure 4). In the latter experiment the amount of liver tRNA added was equivalent to the acceptor tRNA in the yeast tRNA preparation. Since yeast tRNA also contains substantial amounts of tRNA-C-C and tRNA-C, we also examined the effect of PP_i on leucyl-tRNA formation by using an artificial mixture of liver tRNAs containing 5% tRNA-C-C-A, 70% tRNA-C-C, and 25% tRNA-C. However, the presence of the partially degraded tRNAs did not affect the level of inhibition by PP_i (data now shown). These results indicate that the difference between the two assay systems is due to both the amino acid concentration and the type of tRNA used and that the greater inhibition with yeast tRNA is an intrinsic property of this heterologous acceptor and not due to the presence of nonacceptor molecules in the preparation.

In addition to the effect of amino acid concentration and the type of tRNA used, the inhibition of aminoacyl-tRNA synthetases by PP_i is also influenced by the pH of the reaction mixture. We have not examined this effect in detail, but stimulation by the factor is greater at pH 6.2 than at pH 7.4 (data not shown).

Discussion

The data presented in this paper indicate that the "tRNA activator" described by Dickman & Boll (1976) is identical with an aminoacyl-tRNA synthetase stimulatory factor isolated in this laboratory and that both of these proteins are actually inorganic pyrophosphatase, which stimulates aminoacyl-tRNA formation by removal of the potent inhibitor PP_i. This conclusion is supported by the following evidence: (a) the stimulatory factor and the "RNA activator" stimulate a number of aminoacyl-tRNA synthetases in an identical manner, and the same as yeast pyrophosphatase; (b) purified stimulatory factor and "tRNA activator" purified according

to Dickman & Boll (1976) have the same molecular weight; (c) the ratio of stimulatory factor to pyrophosphatase activity remains constant throughout an extensive purification, and a similar ratio was obtained with the "tRNA activator"; (d) PP_i is a potent inhibitor of aminoacyl-tRNA synthetases, and this inhibition can be overcome by the addition of the stimulatory factor; and (e) neither the stimulatory factor nor the "tRNA activator" has any effect on aminoacyl-tRNA formation if care is taken to remove all sources of PP_i.

However, there are some differences between our results and those reported by Dickman & Boll. Generally, the degree of stimulation by the "tRNA activator" was higher than we have observed. This difference is probably attributable to a greater contamination by PP_i in their assays, or to greater sensitivity to PP_i in their assay conditions. One possible source of greater contamination could be the presence of tRNA nucleotidyltransferase in their synthetase preparation since when we purified synthetases by their procedure tRNA nucleotidyltransferase was present. The presence of this enzyme and commercial yeast tRNA would be expected to generate significant levels of PP_i. The assay conditions of Dickman & Boll (1976) would also lead to greater sensitivity to any PP_i that was present since these workers used 5 mM Tris-HCl, pH 7.05, in their reaction mixtures. At this concentration and pH, Tris has very little buffering capacity and the measured pH of their reaction mixture was actually pH 6.2. At the lower pH the sensitivity of synthetases to PP_i is increased (Lui et al., 1978), and, as expected, we have observed greater stimulations when the assays were run under their conditions. Dickman & Boll (1976) also showed that preincubation of yeast tRNA with the "tRNA activator" led to stimulation even if the activator were subsequently removed. This finding could be explained if their yeast tRNA contained any PP_i. In addition, we were able to show that about 10% of the activator remained with the tRNA after ethanol precipitation. If they added the activator in sufficient excess, the remaining 10% could account for the whole effect. One difference between their results and ours, which we have not been able to explain, is their finding that the "tRNA activator" is stable to heating for 2 min at 93 °C in the presence of tRNA. However, despite this difference the available evidence strongly suggests that the "tRNA activator" is inorganic pyrophosphatase.

Contamination of synthetase assay mixtures by PP_i can come from any one of a number of sources, and the final level of inhibition observed will be greatly affected by the actual conditions of assay. In the studies reported here significant PP_i was contributed by liver tRNA and ATP and also generated during the reaction. This latter source may be particularly important if relatively crude synthetases are used in combination with an amino acid mixture to eliminate nonspecific incorporation, or with any tRNA that contains defective -C-C-A termini, such as commercial yeast tRNA. Additional problems may be encountered by use of low amino acid concentrations or by use of heterologous tRNAs since, under these conditions, PP_i is an extremely effective inhibitor. Concentrations as low as 2 μM, which could be added with even the best ATP preparations, inhibit some synthetases close to 50%. All of these conditions are commonly employed in studies of aminoacyl-tRNA synthetases and could lead to gross underestimation of synthetase activities, as well as to the "discovery" of factors that stimulate synthetase activity. We suspect that many studies in the literature may have been affected by these considerations. Simple solutions to these problems would be the addition of pyrophosphatase when relatively crude synthetase fractions are under study and the use of higher amino acid concentrations for study of purified enzymes. The potent inhibition of synthetases by PP_i has been pointed out previously by Kull et al. (1969), but generally has not been considered in many studies of synthetases.

The mechanism of PP_i inhibition has been considered previously by other workers (Kull et al., 1969; Kim et al., 1977; Lui et al., 1978). Their work suggests that even low concentrations of PP_i can react effectively with aminoacyl-AMP to degrade this intermediate and prevent transfer of the amino acid to tRNA. In this model PP_i would not compete directly with tRNA for binding to the enzyme, but would compete with tRNA for reaction with the aminoacyl–AMP intermediate. Presumably, PP_i competes more effectively when heterologous tRNA is the substrate because of slower transfer of the amino acid and/or poorer binding of the tRNA.

The studies presented here raise questions about previously described aminoacyl–tRNA synthetase stimulatory factors (Boman & Svensson, 1961; Makman & Cantoni, 1966; Pearlman & Bloch, 1967; Davies & Marshall, 1975). The properties of these factors, as well as the assay conditions employed, suggest strong similarities with the studies reported here. We wish to point out, however, that the existence of factors which could regulate aminoacyl-tRNA synthetase activities is still an attractive idea, but stimulatory factors should be viewed cautiously until one excludes the possibility that they are merely enzymes which destroy inhibitors. In fact, the specific activator of alanyl-tRNA synthetase recently described by Hilderman (1977) appears to differ from previously described stimulatory factors.

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