NON-PARTICIPATION OF AMINOACYL ADENYLATES IN THE SPERMINE CATALYZED AMINOACYLATION OF TRANSFER-RNA

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SUMMARY: Igarashi et. al. (1) have reported that spermine catalyzes the overall reaction of aminoacylation of tRNA without catalyzing ATP:PPi exchange. Quantitation now shows that the rate of spermine catalyzed ATP:PPi exchange is only one twentieth that of spermine catalyzed esterification (and one ten thousandths of the Mg^{2+} catalyzed ATP:PPi exchange). This evidence is incompatible with the obligatory intermediate formation of Enz·(AA~AMP) and PPi in the biosynthesis of AA-tRNA and is compatible with a concerted reaction of the three substrates and the enzyme to form three products including AA-tRNA.

It has been generally accepted that the first step in protein synthesis is the reaction between an amino acid:tRNA ligase, ATP and the amino acid to form an enzyme bound aminoacyl adenylate (Enz·AA~AMP) which reacts subsequently with the cognate tRNA to form aminoacyl tRNA (AA-tRNA). Numerous investigators have succeeded in carrying out the first reaction and in isolating, usually by Sephadex chromatography, an enzyme bound aminoacyl adenylate. In many cases this complex has transferred the amino acid moiety to tRNA with yields that range from poor to good (see review by Allende and Allende, 2). We do not argue with the experimental observations that these reactions are possible and do occur in the test tube; we have argued that under physiological conditions the aminoacylation of tRNA proceeds largely or exclusively through a concerted reaction mechanism in which Enz·(AA~AMP) is neither a necessary or probable intermediate (3,4).

Igarashi, Matsuyaki and Takeda (5) have been examining the natural organic cationic compounds, spermine, spermidine and putrescin as possible substitutes for Mg^{2+} in the overall formation of AA-tRNA. As with several inorganic cations, the polyamines appear to be capable of replacing the ordinarily necessary Mg^{2+}.

Recently Igarashi et. al. (1) have observed that not only do polyamines replace Mg^{2+} in the overall reaction, but they fail to catalyze ATP:PPi exchange. The exchange of [^{32}P] PPi into ATP in the presence of amino acid:tRNA ligase and amino acid provided the original evidence that the first reaction is formation of aminoacyl adenylate. If, indeed, spermine catalyzes the aminoacylation of tRNA without catalyzing the synthesis of aminoacyl adenylate, aminoacyl adenylates cannot be obligatory intermediates in the esterification. The report by Igarashi et. al. (1) certainly showed that spermine catalyzed exchange of PPi into ATP is no more than one-hundreth as fast as the comparable exchange catalyzed by Mg^{2+} in the case of 20 different amino acids and, in the case of the isoleucine system of E. coli, they have eliminated sources of doubt such as the inhibitory properties of spermine, denaturation of enzyme, destruction of substrates etc. However, there remain some alternative interpretations of their work as reported.

The ATP:PPi exchange is usually one hundred to one thousand times as fast as the rate of tRNA esterification. Thus, if the first reaction is (a)

$$AA + Enz + ATP \rightleftharpoons Enz\cdot(AA\sim AMP)\cdot(PPi) \rightleftharpoons Enz\cdot(AA\sim AMP) + PPi \qquad (a)$$

and the second reaction is (b), and if (a) is one thousand times faster than (b),

$$Enz\cdot(AA\sim AMP) + tRNA \rightleftharpoons Enz + AA\text{-}tRNA + AMP \qquad (b)$$

it is necessary that spermine catalyzed ATP:PPi exchange be less than one one-thousandths of the Mg^{2+} catalyzed rate before reaction (a) becomes rate-limiting. Examination of the report by Igarashi et. al. (1) shows that spermine catalyzed ATP:PPi exchange is very slow, the data do not preclude the possibility that there is some ATP:PPi exchange and that it may occur at a rate sufficiently high to be greater than the rate of esterification. We have used the E. coli valine system to determine whether spermine fails to catalyze ATP:PPi exchange at a rate comparable to the rate of spermine catalyzed Val-tRNA formation. Because our conclusions depend on the sensitivity with which we can determine very slow rates, we provide a detailed description of the assays and report the experimental observations without "corrections".

EXPERIMENTAL

MATERIALS:

^{14}C-L-Valine was obtained from Amersham/Searle Corp., (^{32}P) Pyrophosphate from New England Nuclear, tRNA from Schwarz-Mann Bioresearch Inc., Spermine and ATP from Calbiochem, Escherichia coli cells from Miles Laboratories, Inc.

The Valyl-tRNA ligase was prepared according to the procedure described by Yaniv and Gros (6) with omission of the calcium phosphate gel fractionation. In this way prepared enzyme had a specific activity of 1820 U/mg for esterification and 8700 U/mg for ATP-PPi exchange. Activity of enzyme stored in 50% glycerol (.1 m TRIS, pH 716, 1mM Glutathione) at -25 °C dropped to 30% of the original after 2 months.

TECHNIQUES:

Assay for Valyl-tRNA Ligase Activity: The reaction mixture contained the following in .4 ml: TRIS buffer, pH 7.6, 40 μmoles; ATP, .8 μmoles; $MgCl_2$, .88 μmoles; bovine albumin, .4 mg; tRNA, .5 mg; reduced glutathione, 1.0 μmole; ^{14}C-L-Valine, .022 μmoles; and enzyme. The mixture was incubated at 26 °C. 75λ aliquots were taken and expressed into 5 ml 5% ice-cold Trichloro-Acetic Acid (TCA), mixed and allowed to stand for 10 min. The precipitate was filtered onto a No. AAWG 02400 Millipore filter and washed twice with 5 ml. portions of cold 5% TCA. After removing the filter chimney, the disks were washed further in order to elute the last trace of free ^{14}C-Valine. After oven drying at 90 °C the radioactivity (of precipitate) was determined on a low background-end window Geiger counter (7). One unit of enzyme activity was defined as the esterification of 1 nMole Valine to tRNA in one minute at 26 °C.

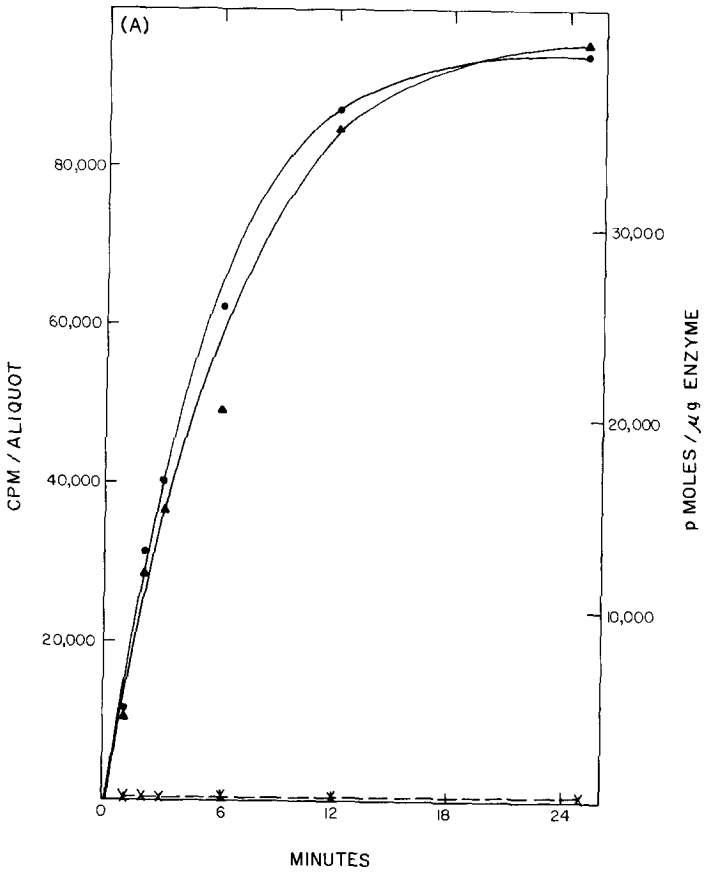
Assay for Amino Dependent ATP-PPi Exchange: The reaction mixture contained the following in .2 ml: TRIS buffer, pH 7.6, 20 μmoles; ATP .4 μmoles; $MgCl_2$, 44 μmoles; bovine albumin, .2 mg; tRNA, .25 mg; ^{12}C Valine, .011 μmoles, (^{32}P) pyrophosphate, .013 μmoles; reduced glutathione, .5 μmoles and enzyme. 25μl aliquots of reaction mixture were pipetted onto the origin line of a

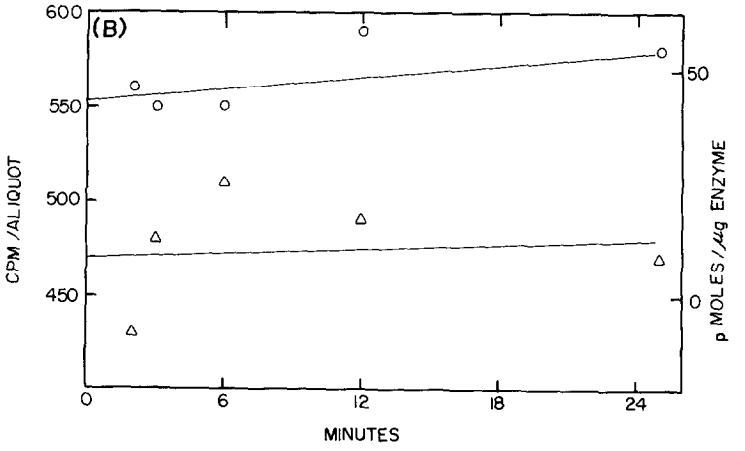
14 x 2.5 cm. strip of anion exchange paper, pyrophosphate form pH 6.5 (7) (Reeve Angel SB-2 paper soaked in .25M $Na_2H_2P_2O_7$ at pH 6.5 for 15 min., washed once in water and air dried). The strip was held in a jet of steam for 15 sec. to destroy the enzyme. The paper strips were developed by ascending chromatography with .06 M $Na_2H_2P_2O_7$ pH 6.5 solution (usually 45-55 min.), the lid was removed from the tank and strips are allowed additional 50 min. developing. The air dried strips were cut in 1 cm. pieces and radioactivity distribution estimated on a low background counter. ATP remains near the origin. One unit of enzyme activity was defined as the incorporation of 1 nMole ^{32}PPi into ATP in 1 minute at 26 °C.

For the comparison of Valyl-tRNA-ligase activity in aminoacylation and ATP-PPi exchange in the presence of Mg^{2+} or Spermine it was necessary to modify the above assays. 1 ml. of the reaction mixture contained .1 mmoles TRIS buffer, pH 7.6; 2 μmoles ATP; .01 mg. bovine albumin; 2.5 μmoles reduced glutathione; .1 μmoles EDTA; .065 μmoles pyrophosphate; enzyme solution 1.4 μg; with additions as required, tRNA (dialyzed against .1 M MaCl and 10^{-4} M EDTA, 10^{-4} M EDTA, distilled H_2O) $16A_{260}$ units; valine, .075 μmoles; Mg^{2+}, 2.0 μmoles; Spermine 1.0 μmoles. For the esterification assay ^{14}C-L-Valine and nonradioactive pyrophosphate was used, for ATP-PPi exchange, ^{32}P-pyrophosphate and nonradioactive L-valine. The assay for enzyme activity in aminoacylation and ATP-PPi exchange was further conducted as separately described above.

RESULTS

Figure 1 is representative of a number of experiments. The scatter of points in Figures 1B and 1C is typical of the data obtained in this ATP:PPi exchange assay in which more than 99.5% of the [^{32}P] radioactivity is being removed from the origin and the presumptive ATP. Despite the scatter, rates can be estimated by Least Squares Approximation with confidence. These rates are listed in the first column of Table I. For this enzyme and under these conditions, Mg^{2+} catalyzed ATP:PPi, exchange is 11,000 times faster than spermine catalyzed ATP:PPi exchange. (It will be noted that in the absence of added cations there


(A)
80,000
60,000
40,000
20,000
CPM / ALIQUOT
30,000
20,000
10,000
p MOLES / μg ENZYME
0
6
12
18
24
MINUTES


(B)
600
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CPM / ALIQUOT
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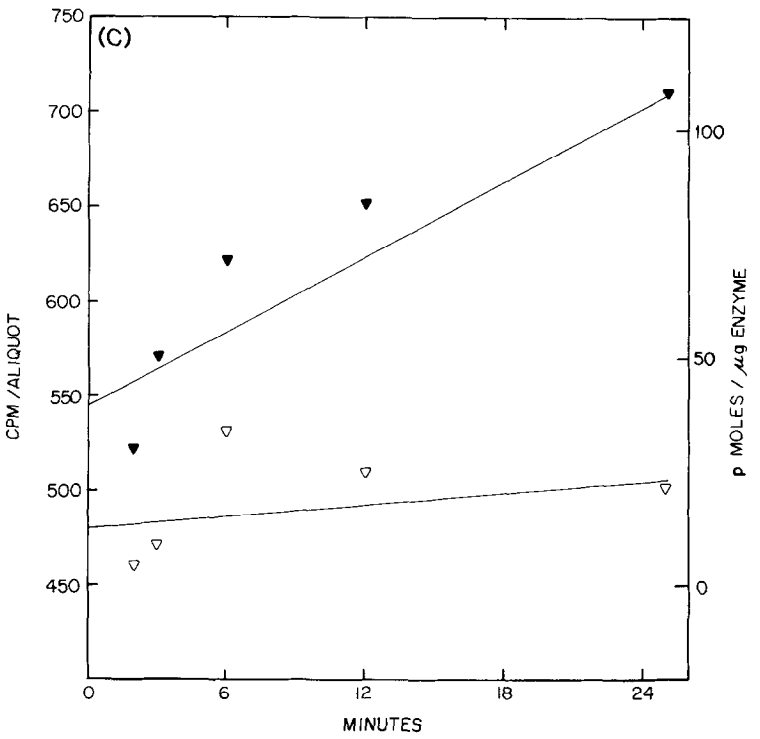

Figure 1. Assays were conducted as described in the Experimental Section. Each incubation flask had exactly the same amount of enzyme, buffer, salt, ATP, PPi and (unless otherwise indicated) valine and tRNA. At least five aliquots were removed between 1 minute and 25 minutes, placed on the origin of a strip of anion exchange paper and eluted. Unreacted [^{32}P] PPi moves from the origin leaving newly formed [^{32}P] ATP together with a small amount of undefined phosphate compounds. The left hand scale indicates the number of counts actually observed at the origin with a 25 μl aliquot (120,000 counts of [^{32}P] PPi had moved from the origin with the solvent front). The right hand scale is a measure of the conversion in picomoles per microgram of enzyme in the 25 μl aliquot. 1A, 2 mM Mg^{2+}, ●—● complete, ▲—▲ less tRNA, X—X less valine; 1B, 1 mM spermine, O—O complete, Δ—Δ less valine; 1C, no polyvalent cations, ▼—▼ complete, ▽—▽ less valine.

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is about six times more ATP:PPi exchange than with 1 mM spermine present. It may be that the enzyme preparation still contains traces of Mg^{2+} or some other effective cation and that this is displaced or otherwise inactivated by spermine.)

The second column of Table 1 shows the rates of aminoacylation under the same conditions. Though much slower than the Mg^{2+} catalyzed esterification, the spermine catalyzed synthesis of valyl tRNA is 20 times faster than the spermine catalyzed ATP:PPi exchange. The rapidity of the Mg^{2+} catalyzed ATP:PPi exchange provided evidence that, if Enz·(AA~AMP) is formed, these experimental conditions

Table I

Units are pmoles per minute per ug of enzyme. The figures for ATP:PPi exchange are taken directly from the Least Squares Approximations of the five points shown in Figures 1B and 1C and from the first 3 points of Figure 1A. The esterification figures are from a similar experiment with [^{14}C] valine conducted under identical conditions.

	ATP:PPi exchange	tRNA Val esterification
2 mM Mg^{2+} + Val + tRNA	6100	162
Same less tRNA	5200	
Same less valine	2.8	
1 mM spermine + Val + tRNA	0.55	10.5
Same less valine	0.17	
No polyvalent cations, + Val + tRNA	3.0	0.3
Same less valine	0.5	

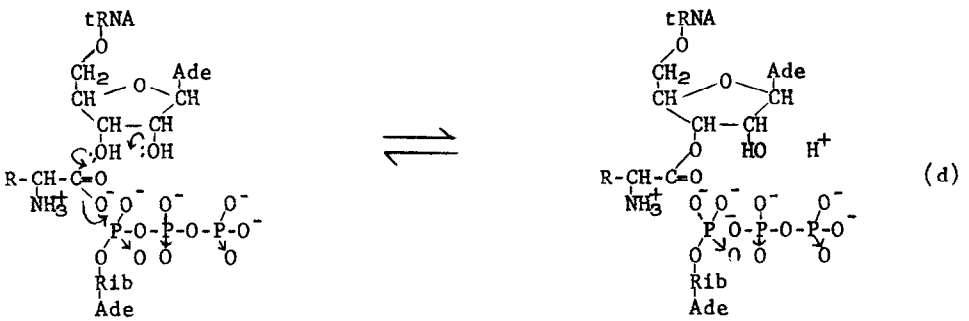
provide sufficient PPi to reverse the synthesis and release [^{32}P] ATP at a rate comparable to the rate of synthesis of Enz·(AA~AMP).

CONCLUSION

Thus we have evidence that reaction (a), as written, proceeds much too slowly to be an intermediate step in the spermine catalyzed synthesis of valyl tRNA. There must be another path, (c), in which free PPi and Enz·(AA~AMP) do not appear. An acceptable interpretation of the present experimental data is that Enz·(AA~AMP)·(PPi) does form under the influence of either spermine or Mg^{2+} (first half of reaction [a]), but that PPi cannot dissociate from the enzyme in the absence of Mg^{2+} unless the complex reacts with tRNA. Numerous other lines of evidence have led us to doubt the role of amino-acyl adenylates as obligate intermediates in protein biosynthesis. These other experimental data involve the ineffectiveness of d ATP in the exchange reaction relative to its effectiveness

in supporting esterification (8), the opposing effects of several bases on the two reactions (3), the total inability of several tRNA ligases to support ATP:PPi exchange except in the presence of intact tRNA (9, 10, 11), kinetic studies showing unordered addition of the three substrates and kinetic evidence that transfer of amino acid from Enz·(AA~AMP) to tRNA is very slow compared to the over all synthesis of AA-tRNA from ATP, AA and tRNA (4).

Since none of these latter lines of evidence is complicated by reservations concerning the dissociation of PPi from enzyme, we propose that our failure to observe a rapid spermine catalyzed ATP:PPi exchange is not due to a tight Enz·PPi association but is a result of a failure to form Enz·(AA~AMP). These present data like the others previously cited (3, 4, 8 - 11) are consistent with a concerted mechanism in which all three substrates (bound to enzyme) react concurrently to produce the three product molecules--no intermediate compounds existing along the pathway (d)



(d)

Contrary to the report of Igarashi et. al. (1) who find approximately equal rates of tRNA esterification whether Mg^{2+} or spermine is the catalyst, we find the spermine catalyzed rate to be only one-sixteenth that of the Mg^{2+} catalyzed rate. If we use undialyzed tRNA, we observe that the spermine catalyzed rate is about 20% greater than the Mg^{2+} catalyzed rate. Since the former is reduced twenty fold by ethylene-diamine-tetra-acetate, we suspect that a small residue of Mg^{2+} or other cation is present in the tRNA and can contribute very actively to the esterification in a way not done by relatively high concentrations of Mg^{2+} or spermine. EDTA does not reduce further the role of esterification of dialyzed tRNA in the presence of spermine.

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