A Preferential Role for Lysyl-tRNA$_4$ in the Synthesis of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate by an Arginyl-tRNA Synthetase–Lysyl-tRNA Synthetase Complex from Rat Liver†

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ABSTRACT: The synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) can be catalyzed in vitro by a tetrameric tRNA synthetase complex from rat liver containing two lysyl-tRNA synthetase and two arginyl-tRNA synthetase subunits. This reaction required ATP, AMP, 50–100 μM zinc, and inorganic pyrophosphatase. We show here that AMP can be omitted from the reaction and that the zinc levels can be markedly reduced provided catalytic amounts of $tRNA^{Lys}$ are added to the reaction mixture. Ap_4A synthesis with purified $tRNA^{Lys}$ isoacceptors showed that the minor species, $tRNA_4^{Lys}$, was 3-fold more active than either of the two major $tRNA^{Lys}$ species, $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$. No activity could be demonstrated with $tRNA^{Lys}$ from *Escherichia coli* or with $tRNA^{Lys}$ or $tRNA^{Phe}$ from yeast. Aminoacylation of $tRNA_4^{Lys}$ was strictly required as determined by the fact that (1) Ap_4A synthesis was not observed until aminoacylation was nearly complete, (2) inhibitors of aminoacylation blocked Ap_4A synthesis, and (3) there was a strict requirement for added lysine. None of the above observations could be demonstrated, however, when lysyl-$tRNA^{Lys}$ was directly supplied to the reaction mixture. Optimum Ap_4A synthesis was obtained by the addition of 1 mol of $tRNA^{Lys}$/mol of the synthetase complex. This reaction is unique because it does not require the prior formation of an aminoacyl-AMP intermediate and because it can actively synthesize Ap_4A at physiological zinc concentrations. The preferential role for $tRNA_4^{Lys}$ in Ap_4A synthesis is consistent with its prior implication in cell division.

In the past few years evidence has accumulated that suggests both $tRNA_4^{Lys}$ and diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) are intracellular elements that may have a role in controlling cell division. The levels of $tRNA_4^{Lys}$ correlate directly with the growth rate of cells in culture (Conlon-Hollingshead & Ortwerth, 1980). The synthesis of $tRNA_4^{Lys}$ is carried out by a series of modification reactions (Ortwerth et al., 1984), and these modification reactions appear to be controlled by serum and purified growth factors (Ortwerth et al., 1982; Lin & Ortwerth, 1983). Furthermore the level of $tRNA_4^{Lys}$ remains high when cells are rapidly dividing, but when cell division is inhibited, there is a decrease in $tRNA_4^{Lys}$ prior to the inhibition of proliferation (Ortwerth et al., 1982; Conlon-Hollingshead & Ortwerth, 1980).

Ap_4A may help trigger cell division by stimulating DNA synthesis (Zamecnik, 1983). Ap_4A is barely detectable in quiescent cells but increases several orders of magnitude when cells are stimulated to divide (Weinmann-Dorsch et al., 1984). This behavior has been shown in a variety of cells in culture and in proliferating tissues (Rapaport & Zamecnik, 1976; Zamecnik, 1983; Weinmann-Dorsch et al., 1984). The hypothesis that Ap_4A stimulates DNA synthesis has been supported by the observation that there is an Ap_4A binding site on eukaryotic DNA polymerase α (Grummt et al., 1979) and by experiments with permeabilized cells, which showed that the addition of Ap_4A to the culture medium stimulated DNA synthesis (Grummt, 1978). In addition, there is evidence that suggests that Ap_4A can serve as an initiator of nascent DNA chains (Zamecnik et al., 1982).

Recently Grummt et al. (1986) have shown a role for zinc in the control of Ap_4A synthesis. The addition of ethylenediaminetetraacetic acid (EDTA) to cultures of BHK cells inhibited Ap_4A synthesis, but Ap_4A levels were returned to normal by the addition of zinc. Ap_4A synthesis in vitro has been demonstrated with both lysyl-tRNA synthetase and phenylalanyl-tRNA synthetases isolated from *Escherichia coli*, yeast, sheep liver, and rat liver (Plateau et al., 1981; Brevet et al., 1982; Hilderman, 1983; Wahab & Yang, 1985). In these experiments optimum Ap_4A synthesis required the absence of tRNA and the presence of 80–100 μM $ZnCl_2$. The reaction appears to proceed by the transfer of AMP from aminoacyl-AMP to ATP bound at the pyrophosphate site. The levels of zinc required for this reaction, however, are clearly unphysiological, since little or no free zinc can be detected in rat liver extracts (Ludany et al., 1978; Norton & Heaton, 1980). We report here that a tetrameric lysyl-tRNA synthetase–arginyl-tRNA synthetase complex is capable of synthesizing Ap_4A from ATP in the almost complete absence of free zinc provided Lys-tRNA is supplied in catalytic amounts.

Experimental Procedures

Materials. [γ-^{32}P]ATP, [^{3}H]AMP, and Aquasol-2 were obtained from New England Nuclear. Uniformly labeled [^{14}C]lysine was purchased from Schwarz/Mann. Precoated plastic thin-layer sheets of (PEI)–cellulose F poly(ethylenimine) were purchased from Brinkmann Instruments Co. Naphthoxyacetic acid *N*-hydroxysuccinimide was obtained from Aldrich Chemical Co. and recrystallized from hot 2-

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propanol prior to use. Benzoylated DEAE-cellulose was obtained from Bio-Rad under the trade name Cellex BD. This material was extensively washed with 1.0 M NaCl in 40% ethanol before chromatography. The Partisil PXS 10/25 SAX high-performance liquid chromatography (HPLC) column was purchased from Whatman. All nucleotides and the yeast pyrophosphatase were obtained from Sigma Chemical Co., and all other reagents were of analytical reagent grade or better.

Isolation of the Synthetase Complex and tRNA^{Lys}. The procedure for the isolation of the arginyl-tRNA synthetase–lysyl-tRNA synthetase complex was identical with that described previously (Dang et al., 1982). The isolation of crude tRNA from both rat liver and mouse leukemic cells 5178Y (MLC) was carried out by phenol extraction and DEAE-cellulose chromatography (Ortwerth & Liu, 1973). These tRNAs were fractionated by RPC5 chromatography, and the individual isoacceptor species were further purified by derivatization of aminoacylated lysyl-tRNA with naphthoxyacetic acid *N*-hydroxysuccinimide with the method of Demushkin et al. (1971), followed by chromatography over BD-cellulose as originally described by Roy et al. (1971). Two peaks of derivatized tRNA were eluted during the ethanol gradient with each isoacceptor. The second peak, which was presumably derivatized on both lysine amino groups, was pooled and either was used directly or was deacylated by an incubation in 1.0 M tris(hydroxymethyl)aminomethane (Tris), pH 9,0, for 1 h in ice.

Ap_4A Synthesis Assays. Ap_4A synthesis was measured in 25-μL reaction mixtures by two different assay systems. The first method employed the optimized conditions for aminoacylation as established previously (Dang et al., 1982), but modified for Ap_4A synthesis. Each reaction mixture contained 250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 8.0, 15 mM $MgCl_2$, 15 mM [γ-^{32}P]ATP (1000–3000 cpm/nmol), 5 mM KCl, 0.2 mM Na_2EDTA, 2.5 μM $ZnCl_2$, 0.25 mL lysine, 10 μg/mL bovine serum albumin, 50 μg of crude tRNA or 0.2 μg of purified tRNA^{Lys}, and 2 μg of yeast pyrophosphatase. The second assay was carried out without chelating agents and contained 250 mM Hepes buffer, pH 8.0, 5 mM $MgCl_2$, 5 mM ATP (treated with dithizone to remove heavy metal ions), [γ-^{32}P]ATP to a final specific activity of 1000–3000 cpm/nmol, 5 mM KCl, 0.2 μg of derivatized lysyl-tRNA^{Lys}, and 2 μg of yeast pyrophosphatase. Both assays were initiated by the addition of 0.65 μg of the tetrameric synthetase complex. Reaction mixtures were incubated at 37 °C, and 1–3-μL aliquots were removed and spotted on PEI–cellulose thin-layer plates along with known standards. The plates were developed with 1.0 M LiCl; the Ap_4A spot was cut out, eluted, and counted in a Beckman LS-3100 liquid scintillation counter as described (Hilderman, 1983). In the initial experiments the reaction products were confirmed by HPLC (Hilderman, 1983).

Zinc Determinations. Zinc levels in a total reaction mixture and in the individual reaction components were determined by graphite-furnace atomic absorption analysis. Analysis of the total reaction mixture without added zinc showed an endogenous zinc level of 3.5 μM. This was contributed equally by ATP, the synthetase preparation, and the commercial pyrophosphatase ($\sim$1.2 μM each). Assays carried out in the absence of chelating agents required the prior chelation of ATP for maximum activity. This was accomplished by extracting a 0.1 M ATP solution with an equal volume of a 1.0 mg percent solution of dithizone (diphenylthiocarbazone) in carbon tetrachloride. This extraction was repeated 5 times, followed by five extractions with carbon tetrachloride to remove any dithizone from the ATP solution. The final solution was left at room tempoerature for 30 min to remove any residual carbon tetrachloride by evaporation.

Protein and tRNA Assays. Synthetase protein was measured on freshly dialized preparations with the fluorescamine assay described by Bohlen et al. (1973), and the moles of enzyme were calculated by use of a molecular weight of 285 000 (Dang et al., 1982). tRNA^{Lys} levels were measured by the increase in fluorescence (546/590) due to the addition of an aliquot of the tRNA sample (0–200 mg) to a 0.5 μg/mL solution of ethidium bromide in 0.1 M Tris buffer, pH 7.5. These values were compared to a standard curve prepared with commercial yeast tRNA^{Phe}.

RESULTS

The tetrameric arginyl-tRNA synthetase–lysyl-tRNA synthetase complex from rat liver has been shown to be capable of synthesizing Ap_4A (Hilderman, 1983). This synthesis required the presence of AMP and high levels of zinc but did not require either lysine or tRNA. The high levels of zinc employed in the assay completely inhibited the normal aminoacylation reaction and, therefore, were probably not relevant to the in vivo situation. In this work, Ap_4A synthesis was measured by the assay system that had previously been optimized for the aminoacylation of tRNA^{Lys} with this synthetase complex (Dang et al., 1982). Ap_4A synthesis was measured at various zinc levels both in the presence and in the absence of added tRNA. We observed that in the presence of tRNA there was a markedly reduced zinc requirement for Ap_4A synthesis. The addition of only 2.5 μM zinc was able to cause the same extent of Ap_4A synthesis as seen at high zinc levels in the absence of tRNA.[1] Also at 2.5 μM zinc, no inhibition of tRNA aminoacylation was observed in this assay (data not shown).

Using this assay system we measured the time course for both aminoacylation of tRNA^{Lys} and Ap_4A synthesis. This was accomplished with identical reaction mixtures, each containing 2.5 μM added zinc, 2 μg of pyrophosphatase, and 50 μg of crude tRNA isolated from either rat liver or mouse leukemic cells (MLC). The time courses for these two synthetase activities are shown in Figure 1. Aminoacylation was initiated immediately upon addition of the enzyme (Figure 1A) and reached plateau values after 10 min. Little or no Ap_4A synthesis, however, was detected at the earlier times (Figure 1B), but rather displayed a lag period of at least 10 min. These data suggested that aminoacylation may be required before Ap_4A synthesis can be initiated. The rate and extent of Ap_4A synthesis with the MLC tRNA was severalfold greater than with the rat liver tRNA. This was true in spite of the fact that the rat liver tRNA was aminoacylated to a greater extent than the MLC tRNA. These data suggested that individual tRNA^{Lys} isoacceptors may differ in their ability to stimulate Ap_4A synthesis. The RPC5 chromatographic profiles of rat liver and MLC tRNA are known to be very different (Ortwerth & Liu, 1973). Most striking is the fact that MLC tRNA contains 5–10-fold more tRNA$_4^{Lys}$ than does rat liver tRNA, suggesting that tRNA$_4^{Lys}$ may be the principal isoacceptor for Ap_4A synthesis.

Individual lysyl-tRNA^{Lys} species were fractionated by RPC5 chromatography and purified by derivatization and BD-cel-

[1] In the paper by Hilderman (1983), Ap_4A synthesis was reported as micromoles of Ap_4A per 100 μL of reaction mixture. This was in error and should have been nanomoles per 100 μL of reaction mixture. Likewise, the specific activity of the ATP should have been 1000–3000 cpm/nmol.

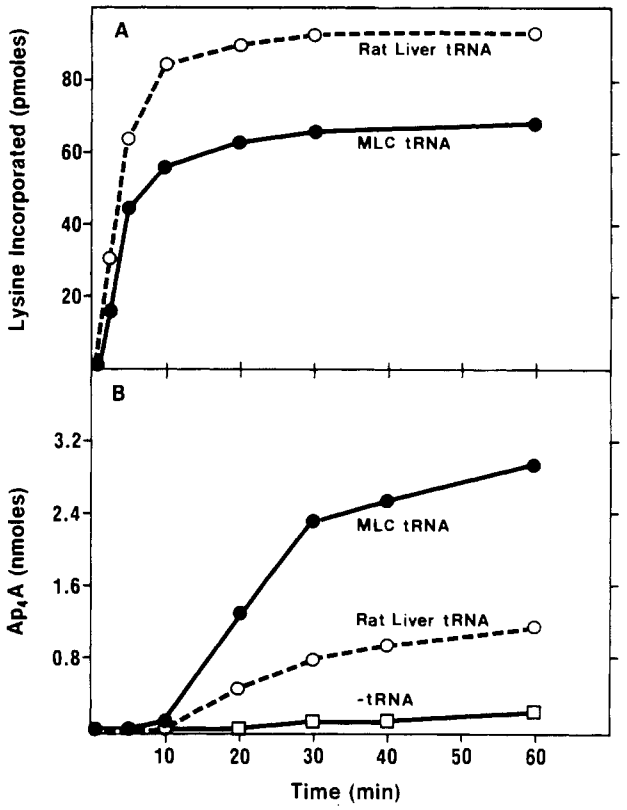

FIGURE 1: Correlation between aminoacylation and Ap_4A synthesis by the tetrameric synthetase complex. (Panel A) time course for the aminoacylation of rat liver and MLC tRNA with [^{3}H]lysine. (Panel B) time course for Ap_4A synthesis in the presence of rat liver and MLC tRNA. Both assays were carried out with the first reaction mixture described under Experimental Procedures except that each 100-μL reaction mixture contained 50 μg of tRNA and 1.6 μg of purified synthetase. Rat liver tRNA (○); MCL tRNA (●); no added tRNA (□).

Table I: Amino Acid Acceptance Activity and Ap_4A Synthetase Activity of Different $tRNA^{Lys}$ Isoacceptors[a]

$tRNA^{Lys}$ species	pmol of lysine accepted/ A_{260} unit of tRNA	pmol of Ap_4A synthesized/ A_{260} unit of tRNA	pmol of Ap_4A/pmol of aminoacylated tRNA
$tRNA_4^{Lys}$	1000	2.2×10^6	2.2×10^3
$tRNA_2^{Lys}$	640	0.4×10^5	0.6×10^3
$tRNA_5^{Lys}$	1300	0.8×10^5	0.7×10^3

[a] Both aminoacylation and Ap_4A synthesis were carried out with the first assay described under Experimental Procedures. The reactions contained 2.5 mM added zinc, 0.01 A_{260} unit of $tRNA^{Lys}$, and 1.6 μg of enzyme. Each 100-μL reaction mixture was incubated at 37 °C for 30 min and assayed in duplicate. Blank values minus enzyme were subtracted in each case.

lulose chromatography. These purified tRNAs were deacylated and assayed individually for Ap_4A synthesis. Table I shows that all three $tRNA^{Lys}$ species were active in Ap_4A synthesis but that $tRNA_4^{Lys}$ was severalfold more active than the other isoacceptors. Each isoacceptor was assayed for aminoacylation activity in the same assay mixture, and these data were used to calculate the picomoles of Ap_4A synthesized per picomole of aminoacyl tRNA. This correction failed to alter the preference for $tRNA_4^{Lys}$, as this species was still 3-fold more active in the synthesis of Ap_4A than the two major $tRNA^{Lys}$ isoacceptors ($tRNA_5^{Lys}$ and $tRNA_5^{Lys}$). This difference was observed with several different $tRNA_4^{Lys}$ preparations from rat liver and with $tRNA^{Lys}$ isoacceptors isolated from both rat liver and mouse leukemic cells. The time course for

Table II: Requirements for Ap_4A Synthesis in the tRNA-Dependent Assay[a]

condition	nmol of Ap_4A
(I) complete	6.6
minus $tRNA_4^{Lys}$	<0.1
minus zinc	<0.1
minus lysine	<0.1
minus pyrophosphatase	<0.1
(II) complete	6.2
minus $tRNA_4^{Lys}$	0.1
minus $tRNA_4^{Lys}$, plus 10 μg of yeast $tRNA^{Phe}$	<0.1
minus $tRNA_4^{Lys}$, plus 20 μg of *E. coli* $tRNA^{Lys}$	0.1
minus $tRNA_4^{Lys}$, plus 25 μg of crude yeast tRNA	<0.1

[a] All assays were carried out with the first assay described under Experimental Procedures. The complete reaction mixture contained 2.5 μM added zinc, 0.65 μg of enzyme, and 200 ng of $tRNA_4^{Lys}$. Each value represents the average of duplicate determinations following a 30-min incubation period at 37 °C. Blank values minus enzyme (~ 400 cpm) were subtracted in each case. Values reported as <0.1 were less than 100 cpm over blank values.

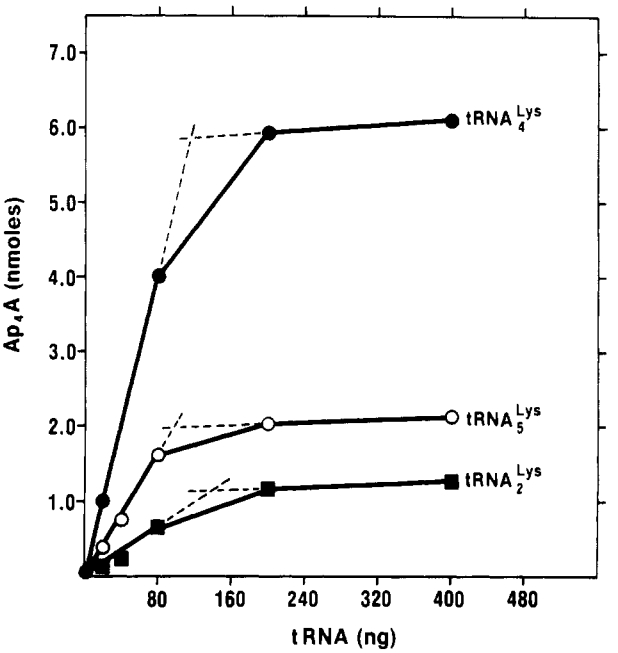

FIGURE 2: Effect of increasing $tRNA^{Lys}$ on the synthesis of Ap_4A. Each reaction was carried out for 30 min at 37 °C exactly as described for the first assay under Experimental Procedures.

aminoacylation was identical with each isoacceptor, suggesting no preference for aminoacylation of $tRNA_4^{Lys}$ by the synthetase (data not shown). The picomoles of Ap_4A formed per picomole of $tRNA^{Lys}$ show that the tRNA is only required in catalytic amounts.

The reaction requirements for Ap_4A synthesis were determined with a purified preparation of $tRNA_4^{Lys}$, and the results are shown in Table II. The reaction requirements support a role for aminoacylation in Ap_4A synthesis, since both tRNA and lysine were required. This is clearly distinct from the assay at high zinc levels, where both tRNA and lysine could be omitted with no loss in activity (Hilderman, 1983). Zinc and pyrophosphatase, however, were still required in the tRNA-dependent assay. Attempts to replace $tRNA_4^{Lys}$ with purified $tRNA^{Phe}$ from yeast or $tRNA^{Lys}$ from *E. coli* were unsuccessful; however, it should be noted that neither of these purified tRNA species was capable of being aminoacylated by the purified rat liver synthetase (data no shown). Crude yeast tRNA was capable of being partially aminoacylated by the purified rat liver synthetase but did not stimulate Ap_4A synthesis in the tRNA-dependent assay.

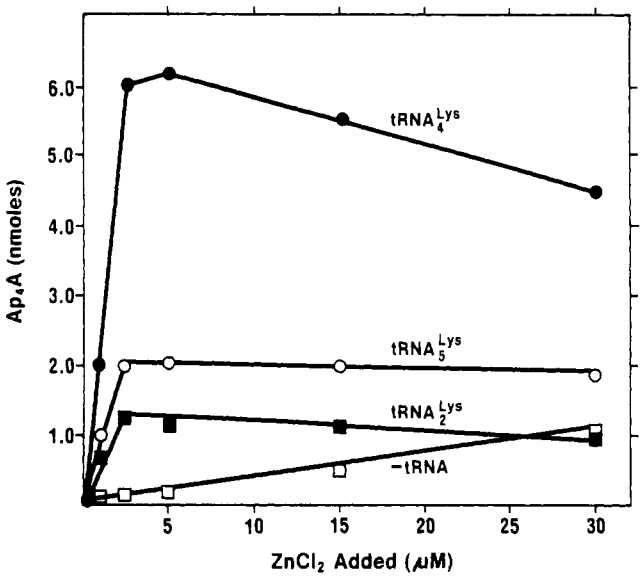

FIGURE 3: Effect of increasing zinc on the synthesis of Ap_4A by three different $tRNA^{Lys}$ species. Each reaction was carried out for 30 min at 37 °C exactly as described for the first assay under Experimental Procedures.

The effect of tRNA concentration on Ap_4A synthesis was measured with each of the purified $tRNA^{Lys}$ isoacceptors. Figure 2 shows the nanomoles of Ap_4A obtained after a 30-min incubation period. Increasing amounts of $tRNA^{Lys}$ produced a linear increase in Ap_4A synthesis, reaching a plateau value at higher $tRNA^{Lys}$ concentrations. Optimum $tRNA^{Lys}$ levels were determined graphically as shown in Figure 2 and multiplied by the acceptance activity previously determined in Table I. These calculations gave values of 2.4, 2.6, and 2.0 pmol/assay for $tRNA_4^{Lys}$, $tRNA_5^{Lys}$, and $tRNA_2^{Lys}$, respectively. Since each reaction mixture contained 2.3 pmol of the tetrameric enzyme complex, maximum Ap_4A synthesis occurs at a ratio of 1 mol of tRNA per mole of synthetase. Even at saturating tRNA levels, the rate of Ap_4A synthesis was severalfold greater with $tRNA_4^{Lys}$ than with the other $tRNA^{Lys}$ species. A time course for Ap_4A synthesis was carried out with saturating levels of $tRNA_4^{Lys}$ and $tRNA_2^{Lys}$ (data not shown). A lag phase of 15 min was seen with $tRNA_2^{Lys}$, while that with $tRNA_4^{Lys}$ was only 5 min. After the lag phase, the synthesis of Ap_4A was linear with both $tRNA^{Lys}$ species. The catalytic rate constants were calculated from these straight lines and were 1.7 s^{-1} for $tRNA_4^{Lys}$ and 0.56 s^{-1} for $tRNA_2^{Lys}$.

Assays at increasing zinc levels (Figure 3) showed that each $tRNA^{Lys}$ isoacceptor required 2–3 μM added zinc for optimum Ap_4A synthesis. Decreased synthesis was seen at higher zinc levels, possibly reflecting the ability of zinc at these concentrations to inhibit aminoacylation. In the absence of tRNA, the synthesis of Ap_4A was directly proportional to the amount of zinc added as seen previously (Hilderman, 1983). Even at the highest zinc level tested, however, the extent of Ap_4A synthesis was not equivalent to that seen with $tRNA_4^{Lys}$ present. At 2.0 μM zinc, the reaction was essentially $tRNA^{Lys}$ dependent. The total zinc present in these assays was determined by furnace atomic absorption analysis. The assay reagents contained 3.5 μM zinc. Therefore, optimum Ap_4A synthesis required a total of 6.0 μM zinc in the presence of $tRNA^{Lys}$. This low level of total zinc was quite surprising, considering the fact that the assay mixture contained 200 μM EDTA. Under these conditions, almost no free zinc could have been present in the assay.

The lag period prior to Ap_4A synthesis suggested that aminoacylation may be required before Ap_4A synthesis can be initiated. One possible explanation of these data is that the $tRNA^{Lys}$ must be aminoacylated to function in Ap_4A synthesis. To test this hypothesis, Ap_4A synthesis was carried out both with $tRNA_4^{Lys}$ and with a sample of $tRNA_4^{Lys}$ that had been aminoacylated and derivatized with naphthoxyacetic acid to stabilize the aminoacyl bond from hydrolysis during the assay. The time course for Ap_4A synthesis with these two $tRNA_4^{Lys}$ species was measured and is shown in Figure 4. The typical lag period was observed with the unaminoacylated species, but no lag period was present with the aminoacylated tRNA. The rates of both reactions were similar after the initial 5-min lag period. A requirement for aminoacylation was also shown by experiments conducted with dead-end inhibitors of aminoacylation. Table III shows that Ap_4A, a competitive inhibitor for ATP, and lysine hydroxamate, a competitive inhibitor for lysine, blocked the synthesis of Ap_4A with $tRNA_4^{Lys}$. These inhibitors, however, were ineffective with the derivatized lysyl-$tRNA_4$. The aminoacylation reaction, therefore, appeared

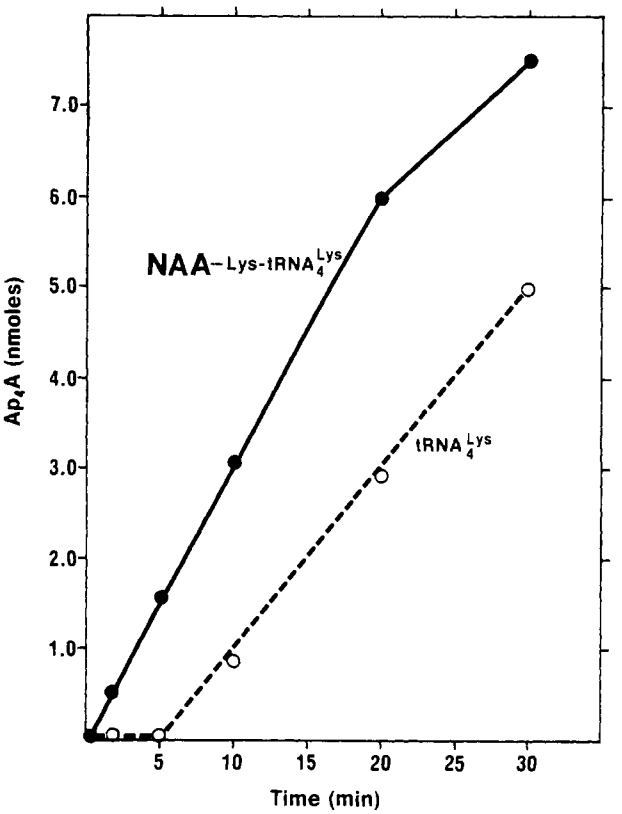

FIGURE 4: Time course for the synthesis of Ap_4A using either $tRNA_4^{Lys}$ or derivatized lysyl-$tRNA_4$. Each reaction was carried out at 37 °C exactly as described for the first assay under Experimental Procedures except that lysine was omitted from the reaction with derivatized lysyl-$tRNA_4$.

Table III: Effect of Inhibitors of Aminoacylation on Ap_4A Synthesis[a]

assay	nmol of Ap_4A
$tRNA^{Lys}$	3.3
$tRNA^{Lys}$ plus 10 μM Ap_4A[b]	0.5
$tRNA^{Lys}$ plus 9 μM lysine hydroxamate	0.4
lysyl-$tRNA^{Lys}$	6.8
lysyl-$tRNA^{Lys}$ plus 10 μM Ap_4A	6.6
lysyl-$tRNA^{Lys}$ plus 9 μM lysine hydroxamate	7.2

[a] Ap_4A synthesis was carried out according to the first assay described under Experimental Procedures. Each reaction mixture contained 2.5 μM added zinc, 200 ng of tRNA, and 0.65 μg of enzyme. Ap_4A synthesis was measured after 20 min at 37 °C. [b] The K_i for Ap_4A is 2.5 μM, and the K_i for lysine hydroxamate is 1.0 μM (Hilderman et al., 1983).

Table IV: Reaction Requirements for Ap_4A Synthesis[a]

condition	nmol of Ap_4A
complete	5.2
minus lysyl-$tRNA_4^{Lys}$	<0.1
minus pyrophosphatase	<0.1
plus 5 mM AMP	4.5
plus 0.2 mM EDTA	<0.1
plus 6.2 μM 1,10-phenanthroline	<0.1
complete	6.0
complete plus 3 μg of lysine decarboxylase	5.4

[a] Ap_4A synthesis was measured with the second assay described under Experimental Procedures. Each reaction mixture contained 67 ng of lysyl-$tRNA_4$ and 0.65 μg of enzyme and was carried out for 30 min at 37 °C.

to have no role in Ap_4A synthesis other than the production of lysyl-tRNA. This was confirmed by determining the reaction requirements for Ap_4A synthesis with derivatized lysyl-$tRNA_4$. The requirements were the same as those shown in Table II except that lysine was no longer required for Ap_4A synthesis (data not shown).

An alternate assay system was established that contained aminoacyl-tRNA and dithizone-treated ATP but did not contain any added chelating agent. The reaction requirements in this assay system are shown in Table IV. The complete reaction mixture did not contain either added zinc or added lysine, and neither was required for Ap_4A synthesis. Both EDTA and phenanthroline inhibited the reaction, confirming a zinc requirement. AMP did not stimulate the reaction, and in similar experiments we have been unsuccessful in demonstrating the incorporation of [^{3}H]AMP into Ap_4A. The synthesis of Ap_4A, therefore, requires the addition of only lysyl-$tRNA_4$, ATP, and pyrophosphatase. The possibility that a small amount of lysine could be present in our assay reagents was eliminated by showing that the addition of lysine decarboxylase failed to inhibit Ap_4A synthesis.

The time course for Ap_4A synthesis in this assay was no different than that shown previously for lysyl-$tRNA_4$ (Figure 4). The catalytic rate constant was 3.3 s^{-1} during the initial 10 min of the reaction. The optimum level of lysyl-$tRNA_4$ for Ap_4A synthesis was determined in this assay system, and the results are shown in Figure 5. Plateau levels of Ap_4A synthesis were observed by the addition of 60 ng of lysyl-$tRNA_4$. The lysyl-tRNA preparation was assumed to be completely aminoacylated since it represented a derivatized peak of purified lysyl-$tRNA_4$. Maximum Ap_4A synthesis was, therefore, obtained with 2.4 pmol of lysyl-$tRNA_4$ or 1 mol of tRNA/mol of synthetase tetramer.

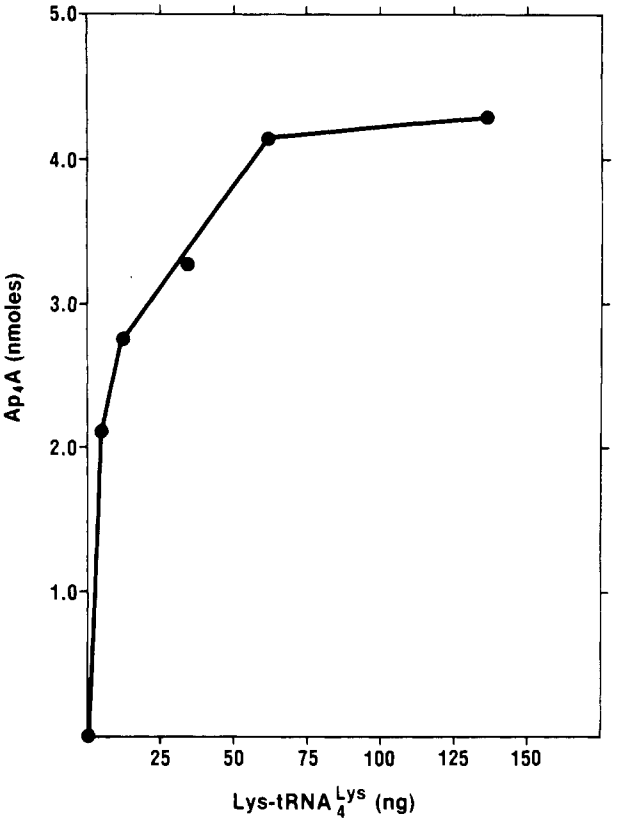

FIGURE 5: Effect of increasing derivatized lysyl-$tRNA_4$ on the synthesis of Ap_4A. Each reaction was carried out for 10 min at 37 °C exactly as described for the second assay under Experimental Procedures.

Discussion

The mechanism responsible for the synthesis of Ap_4A in vivo is unknown; however, synthesis can be obtained in vitro by a reversal of aminoacylation. This reaction is readily catalyzed by either lysine or phenyalanine tRNA synthetases (Zamecnik et al., 1966; Brevet et al., 1982; Plateau et al., 1982). Three different forms of lysyl-tRNA synthetase have been isolated from rat liver, and all three have been shown to be capable of Ap_4A synthesis (Hilderman, 1983; Wahab & Yang, 1985a,b). The synthetase monomer and a multiple synthetase aggregate both catalyze the condensation of lysyl-AMP and ATP, but with different kinetic mechanisms (Wahab & Yang, 1985a,b). The tetrameric complex composed of two arginine and two lysine tRNA synthetases converts ATP and AMP into Ap_4A without a lysyl-AMP intermediate (Hilderman, 1983). All of these mechanisms require the absence of tRNA and the presence of 50–100 μM zinc. Neither situation, however, exists in vivo. The work reported here demonstrates that the tetrameric arginyl-tRNA synthetase–lysyl-tRNA synthetase complex catalyses the synthesis of Ap_4A in the presence of lysyl-tRNA and in the almost complete absence of free zinc. As little as 6.0 μM zinc was sufficient to maximally stimulate the synthesis of Ap_4A even in the presence of 0.2 mM EDTA. Therefore, the synthetase–lysyl-tRNA complex was capable of successfully competing with EDTA for zinc binding. This supports a strong binding site for zinc on the enzyme. Calculations, which were based upon the computer program developed by O'Sullivan and Smithers (1979) and which included all the reaction mixture components, estimated that the synthetase–tRNA complex must contain a zinc binding site with an affinity constant of at least 10^{13}. The decreased zinc requirement due to the presence of $tRNA_4^{Lys}$ can be clearly seen in Figure 3. At 2.5 μM zinc the addition of $tRNA_4^{Lys}$ caused a 30-fold increase in Ap_4A synthesis. Because of the presence of EDTA, these assay conditions were analogous to physiological conditions, since only traces of free zinc were present. If EDTA was omitted from the assay, the endogenous zinc in the enzyme preparations was sufficient to give maximum Ap_4A synthesis.

Lysine was required for Ap_4A synthesis only to promote the aminoacylation of the $tRNA^{Lys}$. In the presence of (naphthoxyacetyl)lysyl-tRNA, lysine could be omitted from the assay, and the addition of lysine hydroxamate had no inhibitory effect. Measurements of (naphthoxyacetyl)[^{3}H]lysyl-tRNA showed that a constant, catalytic amount remained throughout the assay period. Since the lysyl-tRNA was derivatized, any deacylation would have released (naphthoxyacetyl)lysine, not free lysine. The removal of any lysine that could have been present in our reagents with lysine decarboxylase failed to significantly diminish Ap_4A synthesis. We cannot eliminate the possibility, however, that a lysine residue could be covalently bound to the synthetase and participate in the reaction

as an AMP acceptor. Ap_4A is a competitive inhibitor of ATP binding for aminoacylation (Hilderman et al., 1983). Since this molecule failed to inhibit Ap_4A synthesis in the presence of lysyl-tRNA, an ATP binding site other than that used for aminoacylation must be required for Ap_4A synthesis. AMP is required in the high zinc assay Hilderman, 1983) and becomes incorporated into Ap_4A. No such requirement could be demonstrated in the tRNA-dependent reaction. The addition of AMP did not stimulate Ap_4A synthesis, and no incorporation of [^{3}H]AMP into Ap_4A was observed. The fact that [γ-^{32}P]ATP was incorporated into Ap_4A, however, suggests a condensation between ATP and AMP. These observations are consistent with an ATP hydrolysis producing a bound AMP that can be transferred to ATP.

A lag period was always observed with $tRNA^{Lys}$ prior to Ap_4A synthesis, which correlated with the time required to completely aminoacylate the $tRNA^{Lys}$ added. The kinetics of aminoacylation by the tetrameric synthetase (Hilderman et al., 1983) showed that $tRNA^{Lys}$ was the first substrate to bind and lysyl-tRNA was the last product to leave the enzyme surface. In this mechanism the lysyl-tRNA product may be displaced by the incoming $tRNA^{Lys}$ substrate. This displacement would prevent Ap_4A synthesis since the $tRNA^{Lys}$ must be aminoacylated to carry out Ap_4A synthesis. Such a mechanism would explain the lag period before Ap_4A synthesis and would be consistent with the observed stoichiometry of one lysyl-tRNA per synthetase tetramer. This mechanism is very different from that reported by Wahab and Yang (1985a,b) foreither the lysyl-tRNA synthetase monomer or the high molecular weight synthetase complex. These enzyme forms synthetize Ap_4A by a reversal of the amino acid activation reaction. The tetrameric complex, on the other hand, appears to function by a reversal of the transfer reaction. In this sequence, lysyl-tRNA binds to the enzyme. ATP hydrolysis introduces a bound AMP, and then ATP binds at the pyrophosphate site. Condensation of the AMP and ATP would then produce the Ap_4A product. This mechanism, while consistent with the kinetics of Dang et al. (1982) for the tetrameric synthetase complex, remains to be established.

Rapidly proliferating cells exhibit high levels of both Ap_4A and $tRNA_4^{Lys}$. The synthesis of $tRNA_4^{Lys}$ occurs via a sequence of modification reactions (Ortwerth et al., 1984), which are under the control of growth factors (Lin & Ortwerth, 1983). We show here that $tRNA^{Lys}$ is the preferred $tRNA^{Lys}$ species for the synthesis of Ap_4A by the tetrameric synthetase. This preference was seen even at saturating levels of each isoacceptor. In each case, maximum Ap_4A synthesis occurred at 1 mol of $tRNA^{Lys}$/mol of synthetase. These data suggest that tRNA modification can effect the ability of the enzyme to catalytically synthesize Ap_4A. In the presence of lysyl-$tRNA^{Lys}$, the catalytic rate constant was similar to that reported by Wahab and Yang (1985a) for the isolated lysyl-tRNA synthetase monomer and was 4–5 times more rapid than that observed by Hilderman (1983). The function of lysyl-$tRNA_4$ is 2-fold in Ap_4A synthesis. It reduces the zinc requirement for the enzyme and eliminates the requirement for AMP. In this regard, it may stimulate ATP hydrolysis and provide a binding site for the AMP intermediate. The preference shown for $tRNA_4^{Lys}$ in these in vitro experiments, however, is as yet insufficient to explain the rigid requirement for $tRNA_4^{Lys}$ before a cell can enter S phase. Additional studies on the mechanism and control of this lysyl-tRNA-dependent reaction may indicate a more exclusive role for lysyl-$tRNA_4$ in Ap_4A synthesis in vivo.

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