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Synthesis of Modified Tryptophanyl-Adenylates and of Modified Adenosine-Triphosphates and their Use as Tools for Elucidation of the Mechanism of Tryptophanyl-tRNA Synthetase from Yeast

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Six analogs of tryptophanyl-adenylate, which is an important intermediate in the enzymatic synthesis of Trp-tRNA^{Trp}, have been prepared. Four compounds, tryptophanyl-8-bromoadenylate, tryptophanyl-2-chloroadenylate, tryptophanyl-7-deazaadenylate and tryptophanyl-(N^6-methyl)adenylate, contain modifications in the nucleobase moiety, while tryptophanyl-2′deoxyadenylate and tryptophanyl-3′-deoxyadenylate were modified in the carbohydrate part of the molecule. Three of these analogs (2-chloro, 7-deaza, 2′-deoxy analogs) as well as ATP analogs with the same modifications were substrates in the aminoacylation reaction; three analogs (8-bromo, N^6-methyl, 3′-deoxy analogs) were inactive as well as the corresponding ATP analogs. In contrast, in the ATP/*PP*$_i$ pyrophosphate exchange in the absence of tRNA all ATP analogs except 8-bromo-ATP were substrates. However, the presence of tRNA reduced the number of ATP analogs being substrates to that number of substrates observed in the aminoacylation. Therefore, it can be concluded that the presence of tRNA is responsible for an increase of specificity. The diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS), adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS), and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were tested with various divalent metals as substrates in the pyrophosphate exchange reaction. The S_p diastereomer of ATPαS is a substrate with Mg^{2+}, whereas the R_p diastereomer is inactive. Both diastereomers are inactive in the presence of Zn^{2+}. Since Zn^{2+} binds preferentially to the sulfur atom, an explanation of these results is that the Mg^{2+} ion is not bound to the α-phosphate. Only the S_p isomer of the diastereomers of ATPβS acts as substrate in the presence of Mg^{2+}. The stereospecificity becomes reversed in the presence of Zn^{2+}. ATPγS acts as substrate with both Mg^{2+} and Zn^{2+}. These results suggest that the Δ isomer of the β,γ-bidentate ATP–Mg^{2+} complex is the substrate for this enzyme. From these results a molecular model of the ATP–Mg^{2+} complex in the active site can be derived in which the nucleotide is attached to the enzyme by interactions in which the 3′-OH and 6-NH_2 group, one oxygen atom of the α-phosphorus atom, and the coordinated magnesium cation are all involved.

INTRODUCTION

Aminoacyl-tRNA synthetases catalyze the esterification of a particular tRNA with its corresponding amino acid. For this reaction two mechanisms have been proposed; a concerted process in which ATP, tRNA, and the amino acid react simultaneously to give aminoacyl-tRNA, AMP and pyrophosphate (*1, 2*)

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$$\text{ATP} + \text{aa} + \text{tRNA} \overset{E}{\rightleftharpoons} \text{aa-tRNA} + \text{AMP} + PP_i, \quad [1]$$

and second, a two-step mechanism involving the formation of an aminoacyl-adenylate intermediate from which the aminoacyl moiety is transferred to the tRNA. It is the second of these mechanisms which is generally preferred to be the method of tRNA aminoacylation (*3*).

$$E + \text{ATP} + \text{aa} \rightleftharpoons E \cdot \text{aa-AMP} + PP_i \quad [2]$$

$$E \cdot \text{aa-AMP} + \text{tRNA} \rightleftharpoons E + \text{aa-tRNA} + \text{AMP} \quad [3]$$

As one approach for distinction between the mechanisms, several authors have studied the substrate specificities of aminoacyl-tRNA synthetases with regard to analogs of ATP or amino acids in the ATP/PP_i pyrophosphate exchange (this is Reaction [2] running in reverse direction) and in the aminoacylation reaction; generally, the activation step was found to be less specific than the overall aminoacylation (*1, 4, 5*), an observation which favors the two-step aminoacylation mechanism. However, no chemically synthesized analogs of aminoacyl-adenylates have yet been tested as substrates in the aminoacylation reaction; such a strategy would provide a better differentiation of the two reaction steps, since the activities of the adenylate and similarly modified ATP analogs could be compared directly. Furthermore, a concerted mechanism could be implicated directly by an adenylate analog which was inactive and a corresponding ATP analog that was an active substrate in the aminoacylation. Therefore we have prepared analogs of tryptophanyl-adenylate and tested their properties in the esterification of $tRNA^{Trp}$ catalyzed by tryptophanyl-tRNA synthetase from baker's yeast. Additionally, experiments with ATP and tryptophanyl-adenylates modified in the sugar and adenine moiety provide clues to the structural features required for ATP and aminoacyl-adenylate to bind to the active site of the enzyme.

To provide a more complete picture of the ATP site on the tryptophanyl-tRNA synthetase from baker's yeast, analogs of ATP with modifications of the triphosphate moiety were tested. Up until now only analogs with substituents on the γ-phosphorus atom have been used (*6*); therefore, we also investigated the behavior of the phosphorothioate analogs of ATP in which a non-bridging oxygen on either the α-, β-, or γ-phosphorus is replaced by sulfur. The α- and β-thio analogs are chiral and therefore enable us to discover which stereoisomer is used by the enzyme; additionally, the phosphate groups to which Mg^{2+} is coordinated in the ATP–Mg^{2+} complex can be identified by replacing ATP by ATPS and Mg^{2+} by Zn^{2+} (*7–9*).

EXPERIMENTAL PROCEDURES

Materials. Analogs of adenosine 5′-monophosphate and 5′-triphosphate were prepared as described by Freist (*10*); phosphorothioate analogs of ATP were a gift from Dr. F. Eckstein and Dr. B. A. Connolly (Göttingen). All synthesized 5′-monophosphates and 5′-triphosphates were purified by chromatography on Sephadex A25 columns and analyzed by their UV spectra and paper or thin-layer electrophoresis. All triphosphates were free of ATP. The 5′-monophosphates and 5′-triphosphates of adenosine and 2′-deoxyadenosine were obtained from

Boehringer (Mannheim, Germany). Purified $tRNA^{Trp}$ with an acceptor activity of 1580 pmol tryptophan per A_{260} unit was obtained after chromatography of bulk tRNA from baker's yeast which was regenerated by incorporation of AMP into the 3'end using ATP(CTP):tRNA nucleotidyl transferase from yeast (EC 2.7.7.25) according to a procedure published elsewhere (Piel and Cramer, in preparation). [^{14}C]Tryptophan and [^{32}P]pyrophosphate were products of the Radiochemical Centre (Amersham, England). Infrared spectra were taken on a Perkin–Elmer spectrophotometer, Model 325, and nmr spectra were performed on a 60 MHz CU Bruker HX60. HPLC analyses were performed on a Dupont 850 4C liquid chromatograph (Bad Nauheim, Germany) equipped with column compartments thermostated at 35°C and variable wavelength detector set at 260 nm. The separation was performed on a 250 × 4.6-mm C_{18} silica column (ODS-Hypersil, 5-μm particles, purchased from Shandon Southern Ltd., Runcorn, England) at a flow rate of 1.5 ml/min using buffer A: 20 m*M* KH_2PO_4/K_2HPO_4, pH 5.5, and buffer B: 20 m*M* KH_2PO_4/K_2HPO_4, pH 5.5, containing 70% methanol (v/v). The following gradient was employed: 0 to 100% buffer B, mixed with buffer A during 60 min.

Syntheses of tryptophanyl-adenylates. In general, the preparation of tryptophanyl-adenylates followed the procedure described by Armstrong *et al.* (*11*). For the preparation of *N-tert*-butoxycarbonyl-tryptophanyl-adenylates, 0.5 mmol *N-tert*-butoxy-carbonyl-tryptophan (*N*-boc-tryptophan) was dissolved in anhydrous dioxane and the solution evaporated. This process was repeated three times and then the compound was dissolved in 7 ml anhydrous dioxane. To the cooled solution 0.5 mmol tri-*n*-butylamine and 0.6 mmol isobutylchloroformate were added dropwise. After stirring for 30 min at 0°C, 0.25 mmol of the tri-*n*-ethylammonium or the tri-*n*-octylammonium salt of adenosine 5'-monophosphate, or one of its analogs, dissolved in 3 ml dry dimethylformamide, was added. The mixture was stirred at 0°C for 2 hr and then at room temperature for 2 hr. The product precipitated after addition of 30 ml dry diethylether. After centrifugation the residue was washed three times with diethylether and lyophilized.

The *N-tert*-butoxycarbonyl group was removed by dissolving the lyophilized compound in a mixture of 1 ml trifluoroacetic acid/ethylacetate 95/5 (v/v) and keeping the solution for 10 min under nitrogen at room temperature. The product was precipitated by addition of 10 ml dry diethylether. After collection by centrifugation, the product was washed two times with dry diethylether, twice with dry dichloromethane, again with ether, and then finally dried *in vacuo* at 25°C. Yields are given in Table 2.

Characterization of tryptophanyl-adenylates. Tryptophanyl-adenylates were analyzed by IR, nmr and HPLC (compare Table 2). In addition, the hydroxamic acid tests were performed using the procedure of Moldave (*12*) and the products separated by paper chromatography (ascending) using the solvent mixture isobutanol/formic acid/water 15/3/2 (v/v). A brown spot appeared immediately upon spraying the dried chromatogram with a solution of 0.067 *N* $FeCl_3$ in 1 *M* HCl, indicating a tryptophanyl-hydroxamate (compare Table 2). The absence of a 2' or 3' amino acid ester of 5'-AMP was demonstrated by snake venom phosphodies-

terase (EC 3.1.4.1) digest of the adenylates: 0.5 mg of tryptophanyl-adenylates was dissolved in 100 μl 10 m*M* KH_2PO_4/K_2HPO_4, pH 5.5, and 17 μg snake venom phosphodiesterase was added. The mixture was incubated for 2 min at room temperature and used for analysis by HPLC. No detectable cleavage of adenylate was observed in controls by HPLC. Infrared spectra show a strong stretching of the phosphoanhydride bond (P—O—C) at 1040–1090 cm^{-1}. The 60-MHz pmr spectra, for example, of tryptophanyl-adenylate in 1 *M* DCl show the following signals: (δ from trimethyl propionic acid) 3.4 (t, 2H), 4.1–4.6 (m, 3), 4.7–5.1 (m + HOD), 6.2 (d, 1H), 7.1–7.3 (m, 3), 7.5 (d, 1H), 7.6 (d, 1H), 8.4 (s, 1H), 8.6 (s, 1H).

Pyrophosphate exchange. The reaction was monitored by incorporation of [^{32}P]pyrophosphate into ATP (*13*). A 100-μl assay mixture contained 100 m*M* Tris/HCl, pH 7.25, 100 m*M* KCl, 100 μ*M* tryptophan, 2 m*M* potassium pyrophosphate, 0.1–2 m*M* ATP or one of its analogs, and 10 m*M* $MgCl_2$. The amount of enzyme varied from 0.7 to 5 n*M* on the particular ATP analog and was chosen to give linear reaction rates. The incubation was carried out at 37·C. In experiments with phosphorothioate analogs of ATP, Mg^{2+} was substituted by Zn^{2+}, at a concentration equal to that of the nucleotides. After 1, 2, 3, and 5 min, 10-μl samples were spotted onto charcoal filter discs (Cn 69/K, 2.5 cm diameter, J. C. Binzer, Hatzfeld/Eder, Germany) and washed as described in Ref. (*13*), and the radioactivity was counted in a liquid scintillation counter. In experiments in presence of $tRNA^{Trp}$ the tRNA was added to a concentration of 36 μ*M*, and 10-μl samples were removed at 0.5, 1, and 1.5 min after the start of incubation. Under these experimental conditions less than 5% aminoacylation of the tRNA occurred.

Aminoacylation. The aminoacylation was monitored by the formation of [^{14}C]Trp-$tRNA^{Trp}$ from [^{14}C]tryptophan (*13*). The reaction was performed at 37°C in a 100-μl assay mixture containing 100 m*M* Tris/HCl, pH 7.25, 100 m*M* KCl, 60 μ*M* [^{14}C]tryptophan (56 μCi/mmol), 10 m*M* $MgCl_2$, 0.1–2 m*M* ATP or one of its analogs, and 32 μ*M* $tRNA^{Trp}$. The amount of enzyme varied from 0.7 to 5 n*M* on the particular ATP analog and was chosen to give linear reaction rates. Usually, after 1, 2, 3, and 5 min intervals, 10-μl aliquots were streaked onto Whatman No. 3 MM filters. Samples were prepared for liquid scintillation counting as described in Ref. (*13*).

Aminoacylation with tryptophanyl-adenylates. Transfer of unlabeled aminoacyl moieties from aminoacyl-adenylate to $tRNA^{Trp}$ was measured using the method of back titration (*13*): 3 nmol $tRNA^{Trp}$ was preincubated in a total volume of 100 μl with 100 m*M* Tris/HCl, pH 7.25, 100 m*M* KCl, 10 m*M* $MgCl_2$, and 100 m*M* aminoacyl-adenylate in the presence of 0.7 μ*M* enzyme for 3 min at 32°C. At the end of this period 20-μl samples were transfered to a 100-μl mixture containing, in the same buffer as before, 2 m*M* ATP, 80 μ*M* [^{14}C]tryptophan, 20 μg inorganic pyrophosphatase (EC 3.6.1.1), and 0.7 μ*M* tryptophanyl-tRNA synthetase from baker's yeast. A 10-μl aliquot was withdrawn after 30 sec, and a further aliquot was taken after 5 min. Samples were prepared for liquid scintillation counting as described above.

Purification of tryptophanyl-tRNA synthetase (EC 6.1.1.2). Starting from 12 kg baker's yeast, the procedure described by F. von der Haar (*14*) was followed up to step 8. The following additional steps were then applied: CM-Sephadex C50 was equilibrated with 30 m*M* potassium phosphate, pH 7.2, containing 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, 10% v/v glycerol, and 0.01 m*M* phenylmethylsulfonyl fluoride (PMSF) (buffer C) and poured in a 60 × 6-cm column. The dialysate obtained in step 8 was diluted 1 : 1.5 with buffer C and applied to the column. The column was extensively washed with buffer C containing 0.1 *M* KCl, and the tryptophanyl-tRNA synthetase activity was eluted with buffer C containing 0.2 *M* KCl. Fractions with the highest enzyme activity were pooled and saturated with ammonium sulfate. The precipitate was collected by centrifugation (30 min at 17,000*g*) and dissolved in a minimum quantity of buffer C. After addition of diisopropylfluorophosphate up to a concentration of 10^{-5} *M*, the clear solution was applied to a 30 × 3-cm column filled with Sepharose 4B and equilibrated with buffer C containing 390 g ammonium sulfate per liter. The column was washed with buffer C containing 390 g ammonium sulfate per liter and then developed with a reversed concentration gradient from 390 to 275 g of ammonium sulfate per liter in buffer C. Tryptophanyl-tRNA synthetase was eluted at 45% ammonium sulfate. The active fractions were pooled and the protein was precipitated by addition of an equal volume of buffer C saturated with ammonium sulfate. The precipitate was collected by centrifugation (30 min at 17,000*g*), dissolved in a small amount of buffer A, and dialyzed over night against buffer C.

The clear dialysate was diluted with the same amount of buffer C and applied to a CM-Sephadex C50 column (20 × 3 cm) equilibrated with the same buffer. Tryptophanyl-tRNA synthetase was eluted with a solution of bulk tRNA from brewer's yeast (10 A_{280} units/ml) in buffer C containing 0.1 *M* KCl.

To dissociate the enzyme–tRNA complex, the solution was passed through a DEAE-Sephadex A25 column (15 × 2 cm) equilibrated with buffer C containing 0.1 *M* KCl. tRNA was bound quantitatively while the enzyme was eluted. The enzyme was applied to a CM-Sephadex C50 column (20 × 3 cm) equilibrated with the same buffer, and the protein eluted by running a gradient from 0.1 to 0.3 *M* KCl in buffer C. Fractions with highest enzyme activity were pooled, diluted with buffer C 1 : 1 (v/v), and passed through a Blue-Sepharose column (10 × 1.5 cm) equilibrated with buffer C.

In order to concentrate the tryptophanyl-tRNA synthetase, the filtration was repeated on a (10 × 1.5 cm) CM-Sepharose C50 column equilibrated with buffer C. 18 mg (13% yield) of tryptophanyl-tRNA synthetase were eluted with buffer C containing 0.5 *M* KCl. For storage at −20°C an equal volume of glycerol was added to this enzyme solution. Under these storage conditions tryptophanyl-tRNA synthetase was stable for 6 months without loss of activity. The inspection of purity by sodium dodecyl sulfate electrophoresis showed that the preparation was more than 95% homogeneous. No ATPase activity was detected. Tryptophanyl-tRNA synthetase isolated by this procedure exhibited a specific activity of 8570 units per A_{280} unit (one unit is defined as the capacity to aminoacylate 1 nmol of tRNA per minute under standard assay conditions).

RESULTS AND DISCUSSION

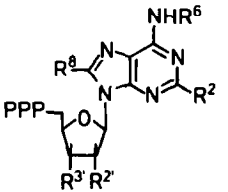
Substrate Specificity with Regard to ATP Analogs Modified at the Adenine and Ribose Moieties

Structure of ATP Analogs. To test the influence of substituents at the adenine and at the ribose part of the ATP molecule on the activity of this substrate in the pyrophosphate exchange reaction and in the aminoacylation of tRNA, analogs of ATP were chosen which had shown characteristic results in the aminoacylation reaction with some other enzymes investigated previously (*4, 10, 15–17*). N^6-Methyladenosine 5′-triphosphate is modified at the amino group of the base by the substitution of a hydrogen atom for a methyl group. 8-Bromoadenosine 5′-triphosphate differs from the natural substrate ATP in the conformation at the glycosidic bond which is changed to a syn conformation in which the base moiety is located above the sugar plane (*18*), whereas 2-chloroadenosine 5′-triphosphate is fixed in an anti conformation in which the adenine base is turned away from the ribose moiety (*18*). Tubercidin 5′-triphosphate is a base-modified compound lacking the N7 of the heterocyclic system, which may be important for complex formation with metal ions (*19*). By using 2′-deoxyadenosine and 3′-deoxyadenosine 5′-triphosphates the importance of the 2′ and 3′ hydroxyl is demonstrated.

Activity of ATP Analogs in the Pyrophosphate Exchange and Aminoacylation. The results obtained with the six ATP analogs in the pyrophosphate exchange are listed together with those of the aminoacylation in Table 1. N^6-Methyladenosine 5′-triphosphate showed a different behaviour in the two reactions. In the pyrophosphate exchange this analog was tolerated as a substrate with a K_m value in the same range as ATP, although the k_{cat} value is reduced about 20-fold as compared to ATP. Interestingly, this compound is inactive in the aminoacylation. The simplest explanation for this observation is that the enzyme is more specific in the aminoacylation than in the pyrophosphate exchange. The main difference between these two reactions is the presence of tRNA in the aminoacylation and its absence in the pyrophosphate exchange. To measure the influence of tRNA on the aminoacyl-adenylate formation directly, the pyrophosphate exchange was also performed in the presence of $tRNA^{Trp}$. In this case N^6-methyladenosine 5′-triphosphate did not show any activity which demonstrates that the tRNA enhances the specificity of the ATP binding site on the enzyme (Table 1). Similar results with this analog were previously obtained with lysyl-tRNA synthetase (*4*). For tryptophanyl-tRNA synthetase from beef pancreas it was also shown that the presence of a tRNA molecule bound to the enzyme improves its substrate specificity (*20, 21*). The enhancement of specificity in the presence of tRNA shows that it is not the second step in the aminoacylation reaction which is more specific than the amino acid activation as proposed for several aminoacyl-tRNA synthetases from baker's yeast (*4*), but the enzyme–tRNA complex itself which improves the specificity for adenylate formation. In contrast to this observation the specificity for tryptophan binding is not influenced by the tRNA (Piel and Cramer, in preparation).

TABLE I

KINETIC CONSTANTS OF STRUCTURAL ANALOGS OF ATP IN THE PYROPHOSPHATE EXCHANGE AND IN THE AMINOACYLATION

Substance		Substituents					Pyrophosphate exchange		Aminoacylation	
		$R^{2'}$	$R^{3'}$	R^2	R^6	R^8	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
ATP	ATP	OH	OH	H	H	H	0.8(0.67)[a]	7800(3200)[a]	0.78	1510
N^6-Methyl-ATP	N^6-CH_3-ATP	OH	OH	H	CH_3	H	0.68 (−)[a,b]	480 (−)	—	—
2-Chloro-ATP	2-Cl-ATP	OH	OH	Cl	H	H	1	2770	0.77	590
8-Bromo-ATP	8-Br-ATP	OH	OH	H	H	Br	—	—	—	—
Tubercidin-TP[c]	TuTp	OH	OH	H	H	H	1.3	2700	1	440
2′-Deoxy-ATP	2′-dATP	H	OH	H	H	H	1	150	1.2	70
3′-Deoxy-ATP	3′-dATP	OH	H	H	H	H	1.5 (−)	106 (−)	—	—

[a] In parentheses: ATP/PP_i exchange measured in presence of tRNA.

[b] − means substance is neither substrate nor inhibitor.

[c] Tubercidin triphosphate = 7-deazaadenosine triphosphate.

2-Chloroadenosine 5′-triphosphate is a good substrate with high K_m and k_{cat} values in both reactions; in contrast, 8-bromoadenosine 5′-triphosphate is inactive. These results indicate that ATP, which is bound to the enzyme in an ATP–Mg^{2+} complex as the true substrate, is bound with an anti conformation. Studies on the substrate specificities of phenylalanyl-, seryl-, threonyl-, and lysyl-tRNA synthetases from yeast with regard to ATP analogs revealed that these enzymes also favor an anti conformation of the ATP–Mg^{2+} complex (*10, 16*). All these enzymes as well as tryptophanyl-tRNA synthetase belong to that particular group of aminoacyl-tRNA synthetases which consist of subunits (*17*).

Tubercidin 5′-triphosphate is also accepted as a good substrate with relatively high K_m and k_{cat} values in both pyrophosphate exchange and aminoacylation by tryptophanyl-tRNA synthetase. The K_m and the k_{cat} values appear lower in the aminoacylation reaction, as is also observed in similar ratio for ATP. Many of the investigated subunit aminoacyl-tRNA synthetases do not tolerate this analog (*10, 16*) and it was therefore assumed that the N7 of the heterocyclic system of ATP could be involved in complex formation with Mg^{2+} ions (*19*). This does not seem to be the case for the ATP metal complex in the active site of the tryptophanyl-tRNA synthetase.

For 2′-deoxyadenosine and 3′-deoxyadenosine 5′-triphosphates K_m values as for ATP and 50-fold lower k_{cat} values were obtained in the pyrophosphate exchange. However, while 2′-deoxadenosine is also a poor substrate in the aminoacylation (K_m similar to ATP, k_{cat} 20-fold lower), 3′-deoxyadenosine is completely inactive. Similarly as with N^6-methyladenosine 5′-triphosphate, no activity can be observed with 3′-deoxyadenosine 5′-triphosphate in the pyrophosphate exchange in the presence of $tRNA^{Trp}$. This supports the assumption that the tRNA confers additional selectivity on the ATP binding site, thus resulting in a better fit for the natural substrate ATP. Furthermore, it can be concluded that the 3′hydroxyl group of the ribose sugar is not necessary for adenylate formation in the

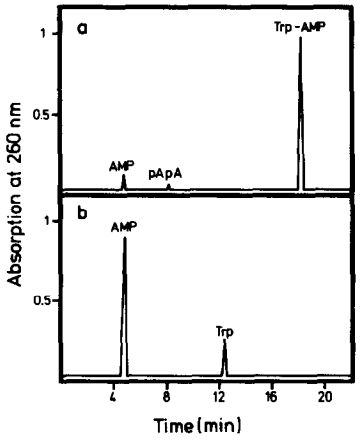


FIG. 1. Analysis of tryptophanyl-adenylate by HPLC before (above) and after (below) digestion with snake venom phosphodiesterase. Chromatography was carried out on a reversed phase column as described under "Materials and Methods."

absence of the tRNA, but in its presence it is important for binding of ATP, formation of adenylate, binding of adenylate or for the transfer step.

Considering all results obtained one can envisage the following picture of essential parts of the ATP molecule: (i) In position 6 of the adenine base an intact amino group is required. The enzyme does not accept analogs without intact NH_2 group in this position. The simplest explanation is an interaction between this NH_2 group and a functional group on the enzyme. (ii) The enzyme accepts substituents in position 2 of the base which do not change the anti conformation. 8-Bromoadenosine 5′-triphosphate, which exhibits a syn conformation, is not recognized by the enzyme. Therefore, it is reasonable to assume that the ATP–Mg^{2+} complex must adopt the anti conformation at the active site. However, tryptophanyl-tRNA synthetase does not seem to require an ATP–Mg^{2+} complex with anti conformation in which the cation is coordinated to N7 of the base. (iii) Lack of the 3′-hydroxyl group of the normal substrate leads to a completely inactive compound; however, such analogs do not act as inhibitors. One can assume that this hydroxyl group is necessary for binding on the enzyme, probably by an interaction with an H-bonding function on the enzyme.

Substrate Specificity with Regard to Tryptophanyl-Adenylate Analogs Modified at the Adenine and Ribose Moiety

Chemical synthesis of tryptophanyl-adenylates. To investigate the topography of the tryptophanyl-adenylate binding site, analogs of this compound were synthesized which were modified in the same positions of the adenine and of the ribose moiety as the ATP analogs tested in the pyrophosphate exchange and in the aminoacylation reaction. Syntheses were carried out using the methods published by Armstrong *et al.* (*11*) for the preparation of aminoacyl-adenylates. Relative pure products were obtained in sufficient yields (Table 2). In the first step of these syntheses, the N-boc-protected aminoacyl-adenylate is prepared by condensing the isobutylcarbonate anhydride of the amino acid, which is formed *in situ* with adenosine 5′-monophosphate. In the second step, the N-boc protecting group is quantitatively removed from the aminoacyl moiety by treatment with trifluoroacetic acid.

The purities of the resulting aminoacyl-adenylates were checked by HPLC (Table 2, Fig. 1). Only small amounts of AMP were present in these preparations, and tryptophan was not detectable. In order to demonstrate the absence of 2′ or 3′ esters, tryptophanyl-adenylate was digested with snake venom phosphodies-

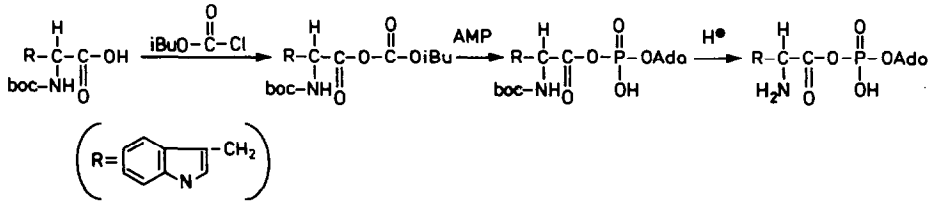
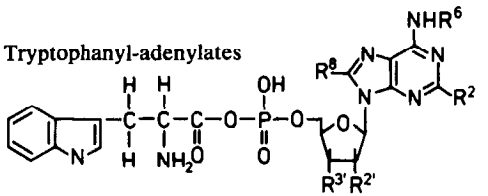


TABLE 2

PREPARATION, ANALYSIS, AND PROPERTIES OF TRYPTOPHANYL-ADENYLATES

Tryptophanyl-adenylates 	Yield (%)	Infrared absorption (cm^{-1})	HPLC Analysis			
			R_f values of hydroxamates	Retention time (min)	Retention time after phosphodiesterase cleavage (min)	Aminoacylation (% of Trp-AMP)
Trp-AMP	42	1040–1090	0.43	18.4	4.8; 12	100
Trp-N^6-CH_3-AMP	38	1040–1090	0.43	21.2	7.0; 12	0
Trp-2-Cl-AMP	37	1040–1090	0.43	20.4	7.1; 12	57
Trp-8-Br-AMP	32	1040–1090	0.43	19.7	6.9; 12	0
Trp-TuMP	52	1040–1090	0.43	19.8	5.2; 12	70
Trp-2′-dAMP	41	1040–1090	0.43	18.2	5.9; 12	48
Trp-3′-dAMP	37	1040–1090	0.43	18.4	5.8; 12	0

terase; only AMP and tryptophan were detected in a ratio of 1 : 1 (Fig. 1). Jaeger *et al.* (*22*) mentioned that tryptophan with its indole substituted by butyl, N^{in}-butyltryptophan, is formed upon deblocking the α-NH_2 group of N-boctryptophan with trifluoroacetic acid. However, in contrast to this report, nmr spectroscopy showed that no N^{in}-butyltryptophan was formed when the deblocking was carried out in 95% trifluoroacetic acid in ethylacetate under a nitrogen atmosphere (Fig. 2).

Aminoacylation with tryptophanyl-adenylates. When aminoacylation of $tRNA^{Trp}$ was carried out with tryptophanyl-adenylate as substrate instead of ATP and tryptophan, quantitative formation of Trp-$tRNA^{Trp}$ was obtained (Table 2). However, no transfer of the tryptophanyl part to the tRNA occurs with tryptophanyl-(N^6-methyl)adenylate (Trp-N^6-CH_3-AMP) (Table 2). Similarly as in the pyrophosphate exchange, the presence of tRNA influences not only the ATP-binding site but also the adenylate site of the enzyme. Additionally, the amino group in position 6 of the base is necessary for binding of ATP and the subsequent aminoacyl-adenylate.

The aminoacylation of the tRNA is reduced by 40% when tryptophanyl-adenylate is substituted by tryptophanyl-2-chloroadenylate (Trp-2-Cl-AMP) (Table 2). However, the activity of this analog supports the idea that an anti conformation of

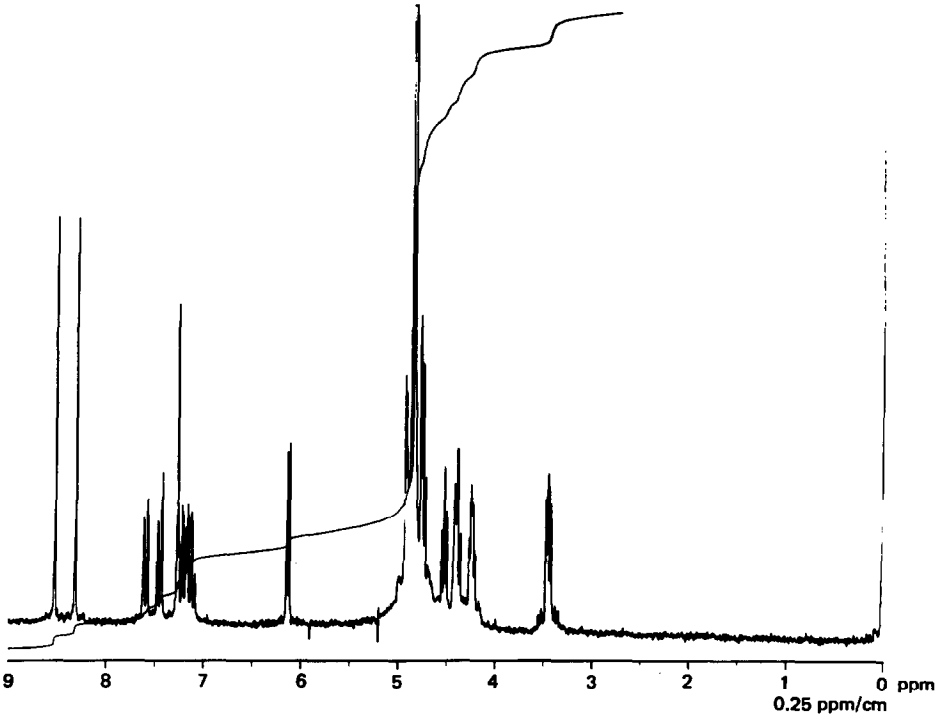


FIG. 2. NMR spectrum of tryptophanyl-adenylate in 1 *M* DCl, reference: trimethylpropionic acid.

the base is necessary for both the activation step and aminoacyl transfer to the tRNA. For phenylalanyl- and seryl-tRNA synthetases from yeast, it was assumed that rotation of the base around the glycosidic bond is necessary during aminoacylation of tRNA (*23*). This seems to be less important for tryptophanyl-tRNA synthetase for which the anti conformation seems to be kept constant during the whole process. This observation was also indicated for lysyl- and arginyl-tRNA synthetase from yeast (*23*).

As was also observed in the activation step, tryptophanyl-8-bromoadenylate (Trp-8-Br-AMP) (Table 2) showed no substrate activity, and it is thus concluded that the syn conformation is not tolerated. This result again supports our suggestion that ATP and the corresponding adenylate have an anti conformation in the active site on the enzyme.

As expected from the K_m and k_{cat} values of tubercidin 5′-triphosphate in the aminoacylation, tryptophanyl-tubercidin 5′-monophosphate (Trp-TuMP) (Table 2) is an active analog in the aminoacyl transfer reaction, and a relative aminoacylation of 70% is obtained with this compound.

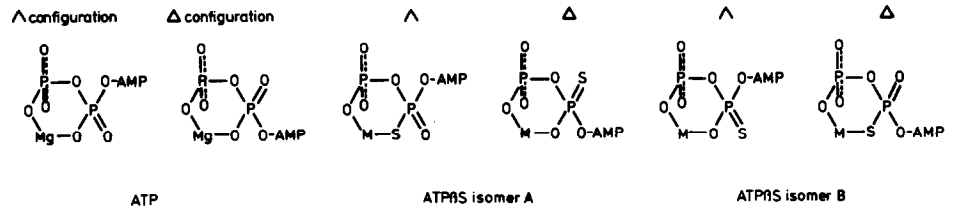
Tryptophanyl-2′-deoxyadenylate (Trp-2′-dAMP) (Table 2) was accepted as substrate by the enzyme with 48% relative aminoacylation whereas tryptophanyl-3′-deoxyadenylate (Trp-3′-dAMP) was inactive. Thus the 3′ hydroxyl group appears to be necessary for binding of ATP or adenylate formation and also for binding of the adenylate or for the transfer step itself.

From these results the observation can be made that tryptophanyl-adenylate analogs are substrates in the aminoacylation reaction if the ATP analog which is modified in the same position is also a substrate in the aminoacylation (Tables 1, 2). For both substrates the same parts of the adenine and the ribose moieties are essential. It may be assumed that in the binding site of ATP and tryptophanyl-adenylate the topography of the nucleotide pocket is identical and that the enzyme uses the same binding site for the nucleotide moiety of ATP and the adenylate.

The identical substrate specificity of tryptophanyl-tRNA synthetase with regard to ATP analogs and analogs of tryptophanyl-adenylate favors a two-step mechanism for the aminoacylation reaction by which an adenylate is formed, followed by transfer of the aminoacyl part to the tRNA. Only different substrate specificities could indicate different mechanisms for the pyrophosphate exchange reaction and the aminoacylation, an observation which could not be made with our compounds.

Substrate Specificity with Regard to ATP Analogs Modified in the Triphosphate Moiety

Structure of phosphorothioate analogs. To get information about the structure of the triphosphate moiety of the ATP–Mg^{2+} complex and the binding pocket belonging to this part of the ATP molecule, some studies with phosphorothioate analogs of ATP were carried out. To elucidate which oxygen atoms of the triphosphate chain metal cations may be coordinated, two approaches had been developed. Cornelius and Cleland (*24*) and Dunaway-Mariano (*25*) have prepared inert



Co^{2+} and Cr^{2+} complexes of ATP, Jaffe and Cohn (*7*) and Eckstein (*8, 9, 26*) used the phosphorothioate analogs of ATP. The phosphorothioate analogs of ATP, in which non-bridging oxygen on either the α- or β-phosphorus is replaced by sulfur, consist normally of a pair of diastereomers for which the absolute configurations are known: ATPαS A and ATPβS A both have the S_p configuration and ATPαS B and ATPβS B the R_p configuration (*8*).

The observation made by Jaffe and Cohn (*7*) that Mg^{2+} coordinates preferentially to oxygen, whereas Cd^{2+} and Zn^{2+} coordinate to sulfur, allows one to identify the diastereomers which are accepted as substrates (*9*). Reversal of the specificity against diastereomers, caused by changes of the Mg^{2+} cation against Cd^{2+} or Zn^{2+} ions, indicates to which phosphate moiety of the triphosphate the oxygen belongs which complexes the magnesium ion.

Activity of phosphorothioate analogs of ATP in the pyrophosphate exchange reaction. In the pyrophosphate exchange reaction catalyzed by tryptophanyl-tRNA synthetase Mg^{2+} cations could only be replaced by Zn^{2+} ions but not by Cd^{2+} (Table 3). In the case of Mg^{2+} optimal activity was gained with a fivefold excess of Mg^{2+} over the triphosphate whereas in the presence of Zn^{2+} equimolar amounts of Zn^{2+} and the triphosphate caused maximal reaction rates. Similar effects with Mg^{2+} and Zn^{2+} have been observed with phenylalanyl- and methionyl-

TABLE 3

KINETIC CONSTANTS FOR PHOSPHOROTHIOATE ANALOGS OF ATP IN THE PYROPHOSPHATE EXCHANGE

	In presence of Mg^{2+}		In presence of Zn^{2+}	
Substance	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
ATP	0.8	7900	0.9	5700
ATPαS A	1	240	—[a]	—
ATPαS B	—	—	—	—
ATPβS A	1	3.8	—	—
ATPβS B	—	—	1.2	3.1
ATPγS	0.9	2800	1.1	1.4

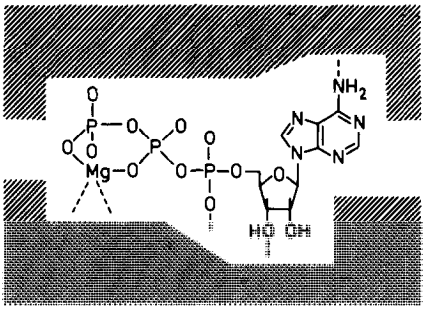
[a] Neither substrate nor inhibitor.

tRNA synthetases from *Escherichia coli* and with phenylalanyl-tRNA synthetase from yeast (*27, 28*). K_m and V values of ATP and the phosphorothioate analogs obtained in the presence of Mg^{2+} and Zn^{2+} are summarized in Table 3. ATP showed very similar substrate activities in the presence of both metal ions. ATPαS A is only a substrate if it is complexed with Mg^{2+} exhibiting similar K_m but 30-fold lower k_{cat} values than ATP. ATPαS B is completely inactive with both cations and also did not act as an inhibitor although it was added in 20-fold excess over ATP.

The lack of activity of the ATPαS isomers in the presence of Zn^{2+} suggests that the Mg^{2+} of the ATP–Mg^{2+} complex is not coordinated to the α-phosphate moiety. If such a complex is formed by ATPαS and Zn^{2+} ions the compound is not accepted as substrate. On the other hand, the difference between the properties of ATPαS A and ATPαS B in presence of Mg^{2+} implies that although the metal ion does not seem to be coordinated to the α-phosphate part, this position may be important for binding of the ATP–Mg^{2+} complex to the enzyme, most probably by a positive charge in the binding pocket. Only ATPαS A may be able to fit the binding site in a suitable way. As another possibility a simple steric effect caused by the bulky sulfur atom may perhaps hinder binding of the B isomer.

ATPβS A is a poor substrate only if Mg^{2+} ions are present in the reaction mixture, the k_{cat} value is 2000-fold lower than that one obtained with ATP, and no activity is observed with Zn^{2+} cations. In contrast to this observation ATPβS B is inactive in presence of Mg^{2+}, it does not act as substrate or inhibitor. However, the complex of this compound with Zn^{2+} ions is a poor substrate. Within the limits of our detection methods the ratio $V_{ATP\beta S\ A}/V_{ATP\beta S\ B}$ is greater than 2000 with Mg^{2+} and with Zn^{2+} equal to 0.002. This reversal of stereoselectivity is only possible if the metal ion is bound to the β-phosphorothioate group of the substrate (*7*).

The third one of the phosphorothioate analogs, ATPγS, acts as good substrate complexed with Mg^{2+} ions and as a poor one in presence of Zn^{2+}. Because the zinc ion prefers coordination to sulfur and the resulting complex is accepted as a substrate as well as the complex consisting of ATPβS B and Zn^{2+}, one can conclude that the Mg^{2+} cation of the ATP–Mg^{2+} complex is coordinated to the β- and γ-phosphate moieties of the triphosphate chain and that this complex exhibits the Δ configuration (*29*).



Summarizing our results obtained in studies on the substrate specificity with regard to ATP analogs, it can be postulated that the ATP–Mg^{2+} complex as a β,γ-bidentate complex with Δ configuration is most probably fixed in its binding pocket by interaction of the amino group in position 6 of the adenine base, the OH group in 3'position, one oxygen of the α-phosphate moiety, and perhaps of the Mg^{2+} ion with the enzyme surface. Our data do not favor an ATP–metal ion complex in which the metal ion is also coordinated to the nitrogen in position 7 of the purine system as proposed by Sundaralingam (*19*), but they also cannot exclude this possibility. For binding of tryptophanyl-adenylate the same positions of the nucleoside part of the molecule are important.

Furthermore, our results favor a two-step mechanism for the aminoacylation reaction over a concerted one-step pathway. However, a covalent enzyme–tryptophan intermediate as described by Kovaleva *et al.* (*30*) can occur prior to formation of an adenylate or the high chemical energy of the aminoacyl-adenylate may allow its production after adenylate formation, as also proposed by Graves *et al.* (*5*). Thus, it cannot be ruled out that a covalent enzyme–tryptophan intermediate is formed as a further step in the aminoacylation process after adenylate formation.

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