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Chemically Modified ATP Derivatives for the Study of Aminoacyl-tRNA Synthetases from Baker's Yeast: ATP Analogs with Fixed Conformations or Modified Triphosphate Chains in the Aminoacylation Reaction

WOLFGANG FREIST, HARALD WIEDNER, AND FRIEDRICH CRAMER

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-34 Göttingen, Federal Republic of Germany

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The systematic investigation of substrate specificity of aminoacyl-tRNA synthetases from yeast is completed by tests of ATP analogs with fixed conformation about the glycosidic bond and with modifications in the triphosphate chain as substrate analogs in the aminoacylation reaction. Two analogs with fixed high *anti* (8,2′-*O*-cyclo-ATP, 8,2′-*S*-cyclo-ATP) and two with fixed *anti* (8,3′-*O*-cyclo-ATP, 8,3′-*S*-cyclo-ATP) conformation have been tested in the esterification reaction of phenylalanyl-, seryl-, lysyl-, valyl-, isoleucyl-, arginyl-, and tyrosyl-tRNA synthetases from baker's yeast. None of the compounds was a substrate, whereas 11 K_i values could be determined. 8,2′-*S*-cyclo-ATP, remarkably, is the only analog which inhibits all these synthetases. Each compound with a fixed *anti* conformation inhibits two enzymes. Among 11 analogs with modifications in the triphosphate chain, four were substrates for phenylalanyl-, three for seryl-, one for lysyl-, three for valyl-, one for isoleucyl-, and none for arginyl- and for tyrosyl-tRNA synthetases. Two compounds were inhibitors of different types for phenylalanyl-, two for seryl-, seven for lysyl-, six for valyl-, nine for isoleucyl-, seven for arginyl-, and two for tyrosyl-tRNA synthetases. Their K_m, V, and K_i values have been determined. In the general picture of substrate specificity the subunit enzymes can tolerate substitutions in position 2, 2′, at the α-phosphorus, at the β,γ-P–X–P bridge and at the γ-phosphorus atom. The single chain enzymes tolerate substitutions in position 7 and at the γ-phosphorus. All seven synthetases from yeast need an intact NH_2 group in position 6 and an oxygen atom in position 3′.

INTRODUCTION

Aminoacyl-tRNA synthetases play an important role in the translation of the genetic code into protein structures. These enzymes catalyze the esterification reaction of a particular tRNA with the corresponding amino acid and show high specificity for their substrates.

During our recent investigations of substrate specificity of aminoacyl-tRNA synthetases from baker's yeast with regard to ATP analogs having modifications at the base and at the sugar moiety, we found considerable differences in the behavior of the individual enzymes (*1–3*). Eight of these enzymes, those specific for phenylalanine, serine, threonine, lysine, valine, isoleucine, tyrosine, and arginine could roughly be divided into two groups according to the ATP analogs

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which were accepted as substrates or were active as inhibitors in the aminoacylation reaction. In a preliminary interpretation of the results it was proposed that the first four enzymes react with an ATP–magnesium complex with an *anti* conformation of the nucleoside moiety (*1, 3*), whereas the other enzymes perhaps accept a complex with a *syn* conformation (*2, 3*). For methionyl-tRNA synthetase from *Escherichia coli* it was also supposed that ATP is accepted with a *syn* conformation in the ATP-PP_i exchange reaction (*4, 5*). To get some more information on the conformation of ATP in the enzyme–substrate complex we prepared some ATP analogs with fixed conformations and investigated their behavior in the aminoacylation reaction of the seven aminoacyl-tRNA synthetases.

Because up until now there has been no information about the influence of modifications in the triphosphate chain on these enzymes, the behavior of analogs with sulfur atoms as well as with fluorine, with azido, with methylene, and with alkylester groups in the phosphate moiety was also investigated. These results allow one to provide a more nearly complete and general picture of the ATP sites of the different aminoacyl-tRNA synthetases from yeast and give the necessary prerequisites for determining the best modification sites for affinity labels at the ATP molecule.

EXPERIMENTAL PROCEDURES

Materials. Phenylalanyl- (*6*), seryl- (*6*), valyl- (*6*), isoleucyl- (*6*), tyrosyl- (*7*), lysyl- (*3*), and arginyl- (*3*) tRNA synthetases were purified from baker's yeast, as described previously (EC 6.1.1.20, 6.1.1.11, 6.1.1.9, 6.1.1.5, 6.1.1.1, 6.1.1.6, 6.1.1.19). The preparations had specific activities of 1240, 135, 3990, 552, 12000, 560, and 2440 units/mg protein in the aminoacylation reaction. ^{14}C-labeled amino acids of Stanstar grade were purchased from Schwarz Bioresearch (Orangeburg, N.J.). In the aminoacylation reaction unfractionated tRNA from Boehringer (Mannheim, West Germany) was used.

Triphosphates. α,β-Methylene-adenosine 5′-triphosphate (**5**) (AMPCPP) and β,γ-methylene-adenosine 5′-triphosphate (**6**) (AMPPCP) were purchased from P-L Biochemicals (Milwaukee, Wisc.), β,γ-imido-adenosine 5′-triphosphate (**7**) (AMPPNP) from Boehringer (Mannheim, West Germany).

Adenosine 5′-(*O*-1-thiotriphosphates) A (**8**) and B (**9**) (ATPαS (A) and ATPαS (B)), adenosine 5′-(*O*-2-thiotriphosphates) A (**10**) and B (**11**) (ATPβS (A) and ATPβS (B)), adenosine 5′-(*O*-3-thiotriphosphate) (**12**) (ATPγS), *P*-3-fluoro *P*-1-adenosine 5′-triphosphate (**13**) (ATPγF), *P*-3-methyl *P*-1-adenosine 5′-triphosphate (**15**) (ATPγOCH$_3$), and *P*-3-phenyl *P*-1-adenosine 5′-triphosphate (**16**) (ATPγOPhe) were gifts from Dr. F. Eckstein, Göttingen, West Germany.

Thin-layer electrophoreses (TLE) of the synthesized mono- and triphosphates were carried out on Silicagel (F 254) from Woelm (Eschwege, West Germany) in 1 *M* citrate buffer, pH 6.5. The R_x values are related to AMP = 1.00 or ATP = 1.00, respectively. All compounds were found to be pure in electrophoresis.

*8,2′-Anhydro-8-oxy-9-β-*D*-arabinofuranosyladenine 5′-monophosphate.* Twenty five milligrams (0.0939 mmol) of 8,2′-anhydro-8-oxy-9-β-D-

arabinofuranosyladenine (**8**) was dissolved in 1 ml triethylphosphate and phosphorylated according to (*9*) by addition of 0.1 ml $POCl_3$. The mixture was kept at room temperature for 24 hr and after addition of 1 ml water neutralized with triethylamine. The monophosphate was isolated by chromatography on Sephadex A 25 with a linear gradient of 0.05–0.4 *M* triethylammonium hydrogen carbonate buffer, pH 8. Yield 532 OD units (λ_{max} = 260 nm) ≙ 0.0484 mmol = 51%. TLE R_x = 1.3.

*8,2'-Anhydro-8-oxy-9-β-*D*-arabinofuranosyladenine 5'-triphosphate* (**1**) (*8,2'-O-cyclo-ATP*). Five hundred and thirty-two OD units (0.0482 mmol) of 8,2'-anhydro-8-oxy-9-β-D-arabinofuranosyl-adenine 5'-monophosphate was dissolved in 1 ml dry dimethylformamide as the tri-*n*-butylammonium salt (*10*) and a solution of 46 mg carbonyldiimidazole in 0.5 ml dimethylformamide was added. A thin-layer chromatogram (cellulose F, Merck, and isopropanol/concentrated ammonia/water, 6 : 1 : 3) showed the total conversion of the monophosphate to the imidazolide. After addition of 0.01 ml methanol, 250 mg tri-*n*-butylammonium pyrophosphate (*10*) dissolved in 1 ml dimethylformamide was added. The mixture was kept for 24 hr at room temperature and evaporated to dryness. The residue was suspended in 3 ml water and the triphosphate was isolated by chromatography on a Sephadex A 25 column with a linear gradient of 0.05–0.6 *M* triethylammonium hydrogen carbonate, pH 8. Yield 103 OD units (λ_{max} = 260 nm) ≙ 0.0094 mmol ≙ 20%. TLE R_x = 1.00.

*8,3'-Anhydro-8-oxy-9-β-*D*-xylofuranosyladenine 5'-monophosphate.* Fifteen milligrams (0.057 mmol) of 8.3'-anhydro-8-oxy-9-β-D-xylofuranosyladenine (*11*) was phosphorylated as above. Yield 474 OD units (λ_{max} = 262 nm) = 0.0318 mmol ≙ 55%. TLE R_x = 0.98.

*8,3'-Anhydro-8-oxy-9-β-*D*-xylofuranosyladenine 5'-triphosphate* (**3**) (*8,3'-O-cyclo-ATP*). Four hundred seventy-four OD units = 0.0318 mmol 8,3'-anhydro-8-oxy-9-β-D-xylofuranosyladenine 5'-monophosphate was converted to the triphosphate as above. Yield 312 OD units (λ_{max} = 262 nm) = 0.0209 mmol = 66%. TLE R_x = 0.90.

*8,2'-Anhydro-8-mercapto-9-β-*D*-arabinofuranosyladenine 5'-monophosphate.* Ten milligrams (0.035 mmol) of 8,2'-anhydro-8-mercapto-9-β-D-arabinofuranosyladenine (*12, 13*) was phosphorylated as above. Yield 190 OD units (λ_{max} = 277 nm) = 0.0098 mmol = 28%. TLE R_x = 0.97.

*8,2'-Anhydro-8-mercapto-9-β-*D*-arabinofuranosyladenine 5'-triphosphate* (**2**) (*8,2'-S-cyclo-ATP*). One hundred ninety units (0.0098 mmol) of 8,2'-anhydro-8-mercapto-9-β-D-arabinofuranosyladenine 5'-monophosphate was converted to the triphosphate as described above. Yield 56 OD units (λ_{max} = 277 nm) ≙ 0.0029 mmol = 30%. TLE R_x = 0.67.

*8,3'-Anhydro-8-mercapto-9-β-*D*-xylofuranosyladenine 5'-monophosphate.* Fourteen milligrams (0.0495 mmol) of 8,3'-anhydro-8-mercapto-9-β-D-xylofuranosyladenine (*12, 13*) was phosphorylated in the same way as already described. Yield 380 OD units (λ_{max} = 283 nm) = 0.0187 mmol = 38%. TLE R_x = 1.00.

*8,3'-Anhydro-8-mercapto-9-β-*D*-xylofuranosyladenine 5'-triphosphate* (**4**) (*8,3'-S-cyclo-ATP*). Three hundred eighty OD units (0.0187 mmol) of 8,3'-anhydro-8-

mercapto-9-β-D-xylofuranosyladenine 5'-monophosphate was converted to the triphosphate as above. Yield 179 OD units (λ_{max} = 283 nm) = 0.00882 mmol = 47%. TLE R_x = 0.90.

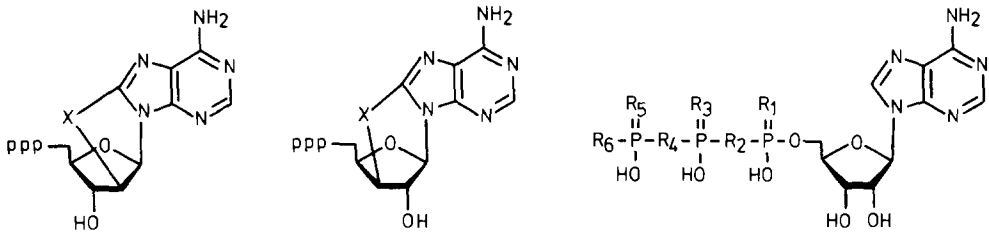
γ-Azidoadenosine 5'-triphosphate (14) (*ATPγN*$_3$). Nine thousand OD_{260} units (0.6 mmol) of ATP (disodium salt) was converted to the triethylammonium salt by passing it through a column (2 × 20 cm) of DOWEX 50 (triethylammonium form) followed by evaporation. The residue was dried by coevaporation with three 15-ml portions of dry dimethylformamide, and 1236 mg (6 mmol) of dicyclohexylcarbodiimide suspended in 25 ml of dry dimethylformamide with the aid of a sonicator was added with stirring. After stirring for 2 hr at room temperature a solution of 1176 mg (24 mmol) LiN_3 (dried as described above for the ATP) was added in 15 ml of dry dimethylformamide to the clear reaction mixture and stirred for another 18 hr at room temperature. The mixture was evaporated to dryness and then dissolved in 3 ml of H_2O. After 1 hr the precipitated dicyclohexyl urea was filtered off and to the filtrate were added 45 ml of a 1% $NaClO_4$ solution in acetone. The pellet formed by centrifugation at 2500*g* for 10 min was redissolved in 3 ml of H_2O and again treated with 30 ml of 1% $NaClO_4$ solution followed by centrifugation. The pellet was washed with 10 ml of acetone and dried. The residue was dissolved in 3 ml 0.1 *N* Tris–HCl, pH 7.9, and incubated with 1.5 mg of alkaline phosphatase for 3 hr at 37°C. The reaction mixture was diluted with H_2O to a volume of 10 ml and applied to a column (3 × 50 cm) of Sephadex-DEAE-A-25. Elution was performed with a linear gradient of 0.05–0.6 *M* triethylammonium hydrogencarbonate in 3 liters and appropriate fractions (elution volume 1750–2200 ml) were pooled and evaporated. The residue was coevaporated twice with 30 ml of methanol and then dissolved to give 1735 OD_{260} units (19%) of γ-azido-ATP. TLE R_x = 1.6. This preparation is related to that described by Knorre *et al.* (*14*). The nmr spectrum of the compound was identical to that reported by Chladek *et al.* (*15*). The compound was not a substrate for alkaline phosphatase but was a substrate for snake venom phosphodiesterase.

Aminoacylation assay. The reaction mixture (0.1 ml) contained 0.1 *M* KCl, 0.01 *M* $MgSO_4$, 20 μ*M* ^{14}C-labeled amino acid, and 1 mg unfractionated tRNA in 0.15 *M* Tris–HCl buffer with a pH of 7.65. The concentration of ATP and ATP analogs was 0.2 m*M*. The temperature was kept at 37°C. A compound was considered to be an inhibitor if in these tests a concentration of 0.2 m*M* of the analog caused more than a 20% decrease in ^{14}C incorporated. In some cases the K_i value of less active compounds is given in order to compare it with the K_i values for the other enzymes. Inhibition constants (K_i) were determined from Lineweaver–Burk plots (*15*).

RESULTS AND DISCUSSION

Structures of the ATP Analogs

In contrast to the naturally occurring nucleoside triphosphates, in the first four synthesized ATP analogs (**1–4**) (Fig. 1) the torsion about the glycosyl bond is



	X
8,2'-O-cyclo-ATP (1)	O
8,2'-S-cyclo-ATP (2)	S

	X
8,3'-O-cyclo-ATP (3)	O
8,3'-S-cyclo-ATP (4)	S

		R_1	R_2	R_3	R_4	R_5	R_6
AMPCPP	(5)	O	CH_2	O	O	O	OH
AMPPCP	(6)	O	O	O	CH_2	O	OH
AMPPNP	(7)	O	O	O	NH	O	OH
ATPαS (A)	(8)	S	O	O	O	O	OH
ATPαS (B)	(9)	S	O	O	O	O	OH
ATPβS (A)	(10)	O	O	S	O	O	OH
ATPβS (B)	(11)	O	O	S	O	O	OH
ATPγS	(12)	O	O	O	O	S	OH
ATPγF	(13)	O	O	O	O	O	F
ATPγN_3	(14)	O	O	O	O	O	N_3
ATPγOCH_3	(15)	O	O	O	O	O	OCH_3
ATPγOPhe	(16)	O	O	O	O	O	OC_6H_5

FIG. 1. Structure of ATP analogs.

prevented. The adenine base is fixed in two different conformations: for the 8,3'-anhydro compounds (**3, 4**) the torsion angle χ lies in the *anti* range (*17, 18*) (Fig. 2), for the 8,2'-anhydro compounds (**1, 2**) the value of χ belongs to that part of the *syn* range which is referred to as high *anti* (*17–20*) (Fig. 2). The corresponding

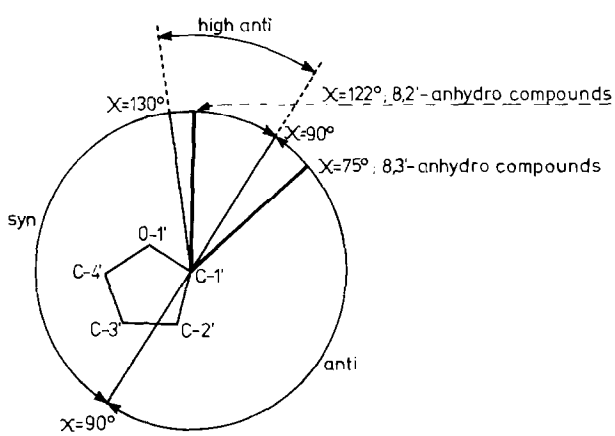


FIG. 2. Schematic representation of the position of the base in the ATP analogs **1–4.** In the conformation circle the range of the torsion angle $\chi = 0° \pm 90°$ is referred to as *anti* and the range $\chi = 180° \pm 90°$ as *syn* (*18*). The range of glycosyl torsions between $\chi = 90°$ and 130° is also referred to as high *anti* (*18*). χ values for ATP · Na_2 are 69° and 39° (*anti*), for formycin, 109.8° (high *anti*), 8-bromoadenosine, 119° (high *anti*), and 8-azaadenosine, 103.6° (high *anti*) (*18*). For 8,2'-and 8,3'-anhydro compounds two known values are given in the circle: $\chi = 75°$ for 8,3'-*S*-cycloadenosine and $\chi = 122°$ for the 3,5'-cyclic monophosphate of 8,2'-*S*-cycloadenosine (*17*).

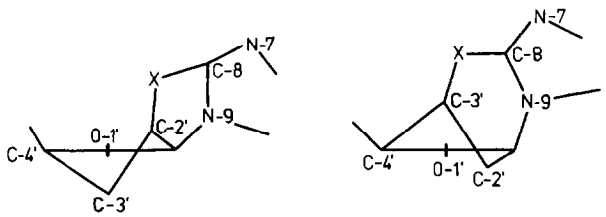


FIG. 3. Schematic representation of the sugar pucker in analogs 1–4 (left, 8,2'-bridging; right, 8,3'-bridging).

sugar conformations are of the N type (C-3'endo) for the 8,3'-anhydro compounds and of the S type (C-2' endo) for the 8,2'-anhydro compounds (*17, 19–21*) (Fig. 3). From model building it is obvious that, due to the greater bond length, the sulfur atom is more suitable for bridges from position 8 to positions 2' and 3'. Therefore the two compounds with sulfur bridges should exhibit less deformation of the molecule than the analogs containing oxygen bridges.

The natural substrate of the aminoacyl-tRNA synthetases, ATP, and also the nucleoside, adenosine, exhibit an *anti* conformation of the base and both types of the sugar pucker, the N and the S type, as determined by X-ray diffraction (*18, 22–25*). Among the synthesized analogs the 8,3'-anhydro compounds (**3, 4**) with their fixed *anti* conformation and the N type of the sugar are similar to the normal substrate ATP.

The analogs with oxygen or sulfur bridges from position 8 to 2' (**1, 2**) with their fixed high *anti* conformation and the S type of the sugar are similar to some ATP analogs such as the triphosphates of formycin, 8-azaadenosine, and 8-bromoadenosine (*18, 26–28*) (Fig. 2), which are active as poor substrates or as inhibitors of the aminoacyl-tRNA synthetases from yeast (*1–3*).

The three analogs in which the oxygen of the P–O–P linkage is substituted by an imido or a methylene group (**5–7**) differ from the P–X bond length and in ATP in the P–X–P bond angles (*29*). The bond length increases from P–O to P–N and to P–C (1.61, 1.68, and 1.79 Å), the bond angles of the P–O–P and the P–N–P group are only slightly different (130° and 127°) whereas the P–C–P angle is much smaller (117°) (*29*).

Analogs **8–12** contain a thiophosphate group in which one oxygen of the normal substrate is substituted by a sulfur atom. The P–S distance in thiophosphates determined by X-ray structure analysis (*30, 31*) indicates a P–S double bond which may be polarized to some extent and a localization of the negative charge at the oxygen atom. The analogs with sulfur substitutions at the α-phosphorus and at the β-phosphorus atom exist as two pairs of diastereomers (ATPαS (A) (**8**), ATPαS (B) (**9**), and ATPβS (A) (**10**), ATPβS (B) (**11**). The absolute configurations of the diastereomers have been established as having the S configuration for the A isomers and R configuration for the B isomers (*32*).

In analogs **13** and **14** one of the oxygen atoms at the γ-phosphorus is substituted by a fluorine atom or an azido group. These compounds have nearly the same molecular size as ATP, but one of the acid–base dissociation possibilities is lost. In the γ-methyl and in the γ-phenyl esters of ATP (**15, 16**) the number of oxygen

atoms is preserved, but the possibility of gaining one negative charge is lost and the molecules contain bulky groups at the end of the phosphate chain.

Activity of Compounds **1–4** *in Aminoacylation Reaction*

None of the first four synthesized ATP analogs with a fixed conformation (**1–4**) was a substrate for the seven tested aminoacyl-tRNA synthetases. This result can be explained by our previous work, in which we found arabinofuranosyladenine 5′-triphosphate to be an inactive compound for the same enzymes (*1–3*). In the anhydro compounds **1–4** a similar modification as in the arabino compound appears, i.e., the 2′ oxygen (or the 3′ oxygen) is orientated above the sugar plane. Additionally, the purine base has a fixed position, and the resulting doubly modified molecules may become even more unsuitable as substrates.

More activity of the anhydro compounds was found in inhibition tests. The results are listed in Table 1. All enzymes are inhibited by the sulfur bridged 8,2′-anhydro compound **2**. This inhibition is mostly competitive; only for isoleucyl- and phenylalanyl-tRNA synthetases is a noncompetitive pattern observed. The K_i values are in the same range as the K_m value of ATP (*1–3*), except the one for phenylalanyl-tRNA synthetase, which is about four times lower. Lysyl-tRNA synthetase is the only enzyme which is also inhibited by the oxygen bridged 8,2′-anhydro compound **1**. The K_i value is again in the range of the K_m of ATP. Three of the enzymes, arginyl-, seryl-, and phenylalanyl-tRNA synthetases, are also inhibited by 8,3′-anhydro compounds (**3, 4**). All K_i values are again similar to the K_m of ATP.

Considering these results it seems to be remarkable that all enzymes are inhibited by compound **2**, 8,2′-*S*-cyclo-ATP, which exhibits a high *anti* conformation. This observation may be understood following a suggestion made by Miles *et al.* for the action of adenosine kinase (*33*). These authors point out that adenosine kinase may require a high *anti* conformation of its substrate during enzymatic reactions. Therefore, nucleosides which are hindered by the 2′OH group above the sugar plane cannot act as substrates with this enzyme. Similarly for all aminoacyl-tRNA synthetases tested, the substrate analog with an arabinofuranosyl moiety is inactive as substrate (*1–3*); whereas formycin 5′-triphosphate, one of the ATP analogs which exhibit a high *anti* conformation, is a substrate for all synthetases tested (*1–3*). A reason for the lower activity of the oxygen bridged isomer **1** may be the greater deformation of the molecule due to the shorter bond length of the oxygen atom.

For phenylalanyl- and seryl-tRNA synthetases there are also strong inhibitors (**4** and **3, 4,** respectively) with a fixed *anti* conformation. For the first enzyme compound **4** (*anti* conformation) is a much stronger inhibitor than compound **2** (high *anti* conformation). This fact seems to support our previous observation that these two enzymes accept those ATP analogs as substrate which are in an *anti* conformation (*1*). Similarly, arginyl-tRNA synthetase is also inhibited by a compound with an *anti* conformation (**3**), in contrast to our previous assumption (*3*) that this enzyme acts with an ATP–metal ion complex with a *syn* conformation.

TABLE 1

STRUCTURAL ANALOGS OF ADENOSINE 5'-TRIPHOSPHATE IN THE AMINOACYLATION REACTION BY SEVEN AMINOACYL-tRNA SYNTHETASES[a]

Synthetase for:	Phenylalanine			Serine			Lysine		
Subunit structure:	A_2B_2			A_2			A_2		
Molecular weight ($\times 10^3$ daltons):	$2 \times 50 + 2 \times 60$			2×60			2×52		
Kinetic data:	K_m (mM)	V rel.	K_i (mM)	K_m (mM)	V rel.	K_i (mM)	K_m (mM)	V rel.	K_i (mM)
8,2'-*O*-cyclo-ATP (1)	—	—	—	—	—	—	—	—	0.4 (c)
8,2'-*S*-cyclo-ATP (2)	—	—	1.4 (n)	—	—	0.1 (c)	—	—	0.1 (c)
8,3'-*O*-cyclo-ATP (3)	—	—	—	—	—	0.4 (c)	—	—	—
8,3'-*S*-cyclo-ATP (4)	—	—	0.7 (c)	—	—	0.8 (c)	—	—	—
AMPCPP (5)	—	—	0.4 (c)	—	—	—	—	—	0.7 (c)
AMPPCP (6)	0.05	0.17	—	—	—	0.03 (nc)	0.03	0.13	—
AMPPNP (7)	0.7	0.20	—	—	—	Slope, 0.06; intercept, 0.9 (mtnc)	—	—	—
ATPαS (A) (8)	2.5	0.28	—	0.2	0.16	—	—	—	2.0 (c)
ATPαS (B) (9)	—	—	—	—	—	—	—	—	0.6 (c)
ATPβS (A) (10)	—	—	—	—	—	—	—	—	3.0 (nc)
ATPβS (B) (11)	—	—	—	—	—	—	—	—	—
ATPγS (12)	—	—	—	—	—	—	—	—	—
ATPγF (13)	—	—	—	—	—	—	—	—	Slope, 1.0; intercept, 9.0 (mtnc)
ATPγN_3 (14)	—	—	—	—	—	—	—	—	—
ATPγOCH_3 (15)	0.03	0.15	—	0.02	0.17	—	—	—	2.0 (nc)
ATPγOPhe (16)	—	—	Slope, 0.09; intercept 0.2 (mtnc)	3.0	0.14	—	—	—	6.0 (nc)
ATP (17)	0.4	1.00	—	0.06	1.00	—	0.2	1.00	—

[a] A dash (—) means the substance does not act as substrate or inhibitor. The V values are related to that of ATP = 1.00 (17); c, competitive; uc, uncompetitive; nc, noncompetitive (classic type); mtnc, mixed type noncompetitive; nLi, nonlinear plot.

However, the results obtained with the ATP analogs with fixed conformations show that the question of ATP conformation is not as simple as supposed in our preliminary interpretations (*1–3*), in which we assumed that some synthetases take an ATP–metal ion complex with *anti* conformation as substrate and another group of synthetases a complex with *syn* conformation. One can at this stage make the following proposals: The synthetases form an enzyme–substrate complex with an ATP–metal ion complex which is available in the reaction mixture in an *anti* or perhaps in a *syn* conformation (*34–38*). Following a conformational change the ATP develops a high *anti* conformation, and the products can then be formed. Some enzymes, however, can also react with ATP in an *anti* conformation,

TABLE 1—*Continued*

Valine A_1 122			Isoleucine A_1 112			Arginine A_1 75			Tyrosine A_1 40		
K_m (mM)	V rel.	K_i (mM)	K_m (mM)	V rel.	K_i (mM)	K_m (mM)	V rel.	K_i (mM)	K_m (mM)	V rel.	K_i (mM)
—	—	—	—	—	—	—	—	—	—	—	—
—	—	0.9 (c)	—	—	0.09 (n)	—	—	0.2 (c)	—	—	0.7 (c)
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	0.3 (c)	—	—	0.6 (nc)	—	—	1.5 (c)	—	—	0.3 (c)
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	2.0 (c)	—	—	2.0 (nc)	—	—	2.0 (c)	—	—	—
—	—	0.9 (c)	—	—	1.0 (nc)	—	—	0.8 (c)	—	—	—
—	—	0.2 (c)	—	—	0.5 (nc)	—	—	1.0 (c)	—	—	—
—	—	0.9 (c)	—	—	4.0 (nc)	—	—	2.0 (c)	—	—	—
0.01	0.76	—	0.01	0.64	—	—	—	—	0.03	0.60	—
—	—	2.0 (nc)	—	—	nli	—	—	Slope, 1.0; intercept, 4.0 (mtnc)	—	—	—
—	—	—	—	—	1.0 (uc)	—	—	—	—	—	—
1.0	0.09	—	—	—	1.0 (nc)	—	—	0.05 (c)	—	—	Slope 1.0; intercept 4.0 (mtnc)
0.4	0.11	—	—	—	5.0 (uc)	—	—	—	—	—	—
0.1	1.00	—	0.4	1.00	—	0.4	1.00	—	0.4	1.00	—

whereas we have no indication for the maintenance of a *syn* conformation (outside the high *anti* sector) during catalysis. The present study cannot distinguish between these possibilities and is to be seen as the only available indication for complicated conformational changes at the glycosidic bond during catalytic activity of the enzymes.

Whereas none of the ATP analogs with a cyclo-anhydro structure can act as substrate and only inhibitors were found, among the analogs with modifications in the triphosphate chain there are not only inhibitors but also substrates (Table 1). α,β-Methylene-adenosine 5′-triphosphate (**5**) is an inhibitor for all synthetases tested except seryl-tRNA synthetase (for which it is inactive). K_i values are in the

range of the K_m of ATP. Inhibition is mostly of the competitive type, and only isoleucyl-tRNA synthetase shows a noncompetitive pattern. Because the activation reaction of the amino acid takes place at the α-phosphorus atom and because a P–C bond—in contrast to the P–O bond—is more difficult to cleave, behavior of the analog as an inhibitor or complete inactivity of the compound is to be expected. β,γ-Methylene-(**6**) and β,γ-imido-adenosine 5′-triphosphates (**7**) are substrates for phenylalanyl-tRNA synthetase, the first compound even with a K_m value nearly 10 times lower than that of ATP, whereas that of the second compound is in the same range as ATP. The V values of both are about a fifth of the value of the natural substrate. Seryl-tRNA synthetase is inhibited noncompetitively with relative low inhibition constants. β,γ-Methylene-adenosine 5′-triphosphate (**6**) is also a substrate for lysyl-tRNA synthetase with K_m and V values nearly the same as for phenylalanyl-tRNA synthetase, whereas the imido-substituted compound did not show any activity as substrate or as inhibitor.

Neither compound (**6, 7**) was a substrate for valyl-, isoleucyl-, arginyl-, and tyrosyl-tRNA synthetases and also did not inhibit the aminoacylation reaction catalyzed by these enzymes. This observation can be correlated with structural differences between the enzymes. Phenylalanyl-, seryl-, and lysyl-tRNA synthetases are composed of subunits, whereas valyl-, isoleucyl-, arginyl-, and tyrosyl-tRNA synthetases consist of single peptide chains (*39, 40*). A difference in substrate specificity of aminoacyl-tRNA synthetases from baker's yeast correlated with the subunit structure was also observed in experiments with ATP analogs modified at the base and at the sugar moiety (*1–3*), and it was supposed that the enzymes may take different ATP–metal ion complexes as substrates. The results obtained with the β,γ-substituted analogs confirm the assumption of differences in the active sites of aminoacyl-tRNA synthetases between those composed of subunits and of single chains. They also substantiate the observation made by Papas *et al.* that these analogs can be substrates or inhibitors for aminoacyl-tRNA synthetases from *E. coli* (*41*). Some of the subunit enzymes can tolerate the different P–X–P bond angles and the different distances of the α- and β-phosphorus atoms.

Among the analogs with thiophosphate groups, isomer A of ATPαS (**8**) is a substrate for phenylalanyl- and seryl-tRNA synthetases. In the first case the K_m is relatively high, the V value is about a quarter of the ATP value. The second enzyme exhibits a K_m in the range of the one for ATP, while V is about a fifth of the ATP value. This property of ATPαS (A) has already been used for investigations of the stereochemistry of activation of phenylalanine by phenylalanyl-tRNA synthetase (*42*). This compound has now additionally been found to be a competitive inhibitor for lysyl-, valyl- and arginyl-, and a noncompetitive inhibitor for isoleucyl-tRNA synthetases; whereas practically no activity with tyrosyl-tRNA synthetase was observed. K_i values are in the range of the K_m of ATP. Isomer B of ATPαS (**9**) is not a substrate for any of the seven enzymes but is an inhibitor for the same enzymes as is isomer A. The type of inhibition is also the same whereas K_i values are lowered by a factor of 2–3. Similar results are obtained with isomer A of ATPβS (**10**). It does not show any activity as a

substrate but is an inhibitor for the same enzymes as ATPαS (A) (**8**) and ATPαS (B) (**9**). The inhibition patterns are the same except in the case of lysyl-tRNA synthetase for which now a noncompetitive pattern is obtained. The inhibition constant is about 10-fold higher than in the case of lysyl-tRNA synthetase, while for the other enzymes it is in the range of the K_m for ATP. Isomer B of ATPβS (**11**) is not a substrate for the seven enzymes and shows activity as an inhibitor only for valyl-, isoleucyl-, and arginyl-tRNA synthetases. The K_i values are higher by a factor of 2–8 than for isomer A. ATPγS (**12**) shows a quite different behavior. It is a good substrate for valyl-, isoleucyl-, and tyrosyl-tRNA synthetases with a low K_m and a high V value. Activity as inhibitor is not observed.

In the experiments with the thiophosphate-containing analogs, again a difference in the substrate specificity of the subunit and the single chain enzymes is to be seen. In the first enzyme group one compound can act as substrate or some can act as inhibitors. In the second group the two isomers of ATPαS and of ATPβS are inhibitors. The K_i values of ATPαS (B) and of ATPβS (A) are systematically lower than those of the corresponding other isomer. Although the difference is in some cases not very significant, this may perhaps be an indication for a better binding of the R configuration of ATPαS and of the S configuration of ATPβS. All sulfur-containing analogs except ATPγS are inactive with tyrosyl-tRNA synthetase. The high specificity of this enzyme was also observed in our previous work with base- and sugar-modified analogs (*2*) and may be due to unusual properties of this enzyme which are also indicated by its low molecular weight (*39*).

Substitution of one oxygen atom at the γ-phosphorus in analogs **13** and **14** results in loss of activity as substrate with all seven enzymes. ATPγF (**13**) is a noncompetitive inhibitor with relatively high inhibition constants for lysyl-, valyl-, and arginyl-tRNA synthetases, whereas with the enzyme specific for isoleucine nonlinear Lineweaver–Burk plots are obtained and no simple inhibition type can be determined. ATPγN_3 (**14**) is only an uncompetitive inhibitor with a relatively high K_i value for isoleucyl-tRNA synthetase, an enzyme which is inhibited by many ATP analogs with different inhibition patterns (*2*).

If the number of oxygen atoms at the γ-phosphorus atom is not changed and the phosphate chain is lengthened by esterification with a methyl or a phenyl group (analogs **15** and **16**), activity as substrate is not lost in all cases. ATPγOCH_3 is a substrate with low K_m and low V values for phenylalanyl-, seryl-, and valyl-tRNA synthetases. It is a noncompetitive inhibitor with relatively high inhibition constants for lysyl-, isoleucyl-, and tyrosyl-tRNA synthetases, and a competitive inhibitor with low K_i value for arginyl-tRNA synthetase. The phenyl ester of ATP (**16**) is a poor substrate for seryl- and valyl-tRNA synthetases with high and low K_m, respectively, and low V values. Noncompetitive inhibition with low and high inhibition constants, respectively, is obtained with phenylalanyl- and lysyl-tRNA synthetases. For isoleucyl-tRNA synthetase this compound is a noncompetitive inhibitor with a high K_i value. The results obtained with analogs **15** and **16** show that esterification does not necessarily lead to loss of substrate activity and that there is in some cases enough space in the active center of the enzyme for bulky groups at the end of the phosphate chain.

CONCLUSIONS

The results obtained in this work complete our investigations on the substrate specificity of aminoacyl—tRNA synthetases from yeast which were done with regard to systematic modifications of all parts of the ATP molecule. Considering all results obtained with the seven tested enzymes one can for the subunit enzymes and for the single chain enzymes envisage two general pictures concerning tolerated modifications of the ATP molecule and modifications which cause inhibitory activity (Figs. 4 and 5). In the group of the subunit enzymes (Fig. 4) one specific property is the toleration of substituents in position 2 of the base (*1, 3*). These compounds exhibit the *anti* conformation (*1, 3*) and are good substrates for the enzymes. Two of the enzymes can accept as substrates the ATP molecule without the 2′-OH group (*1*), an analog with a substitution of one oxygen atom at the α-phosphorus by sulfur, and compounds with substitution of the oxygen connecting the β- and the γ-phosphorus by a methylene or an imido group.

The single-chain enzymes (Fig. 5) do not need a nitrogen atom in position 7 of the purine ring system, as is indicated by tubercidin 5′-triphosphate acting as substrate (*2, 3*); it is obvious that these enzymes do not need an ATP–Mg^{2+} complex with a coordination of the cation to the N(7). Substitution of an oxygen atom at the γ-phosphorus by sulfur can also be tolerated. Substitution of the oxygen atoms in the other positions of the phosphate chain leads to good inhibitors of the aminoacylation reaction.

From the results obtained with the analogs which have a fixed conformation at the glycosidic bond it can be assumed that free rotation is necessary to some extent for all the enzymes except perhaps some of the subunit enzymes.

An important part of the ATP molecule is the amino group in position 6 of the base. None of the tested enzymes can accept an analog without an intact NH_2 group in this position.

Another part of the molecule which is important for the seven aminoacyl-tRNA synthetases from yeast is position 3′ at the sugar moiety. Lack of the OH group of

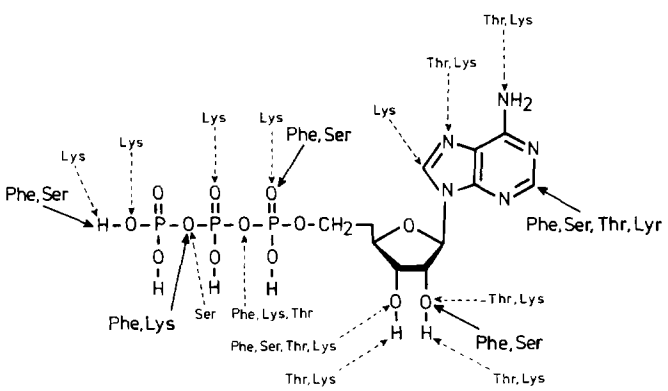


FIG. 4. Modified positions in the ATP molecule with "subunit" aminoacyl-tRNA synthetases from yeast: →, where the modified ATP keeps being a substrate; ⇢, where the modified ATP is converted to an inhibitor. (The three-letter abbreviation designates the aminoacyl-tRNA synthetase in question.)

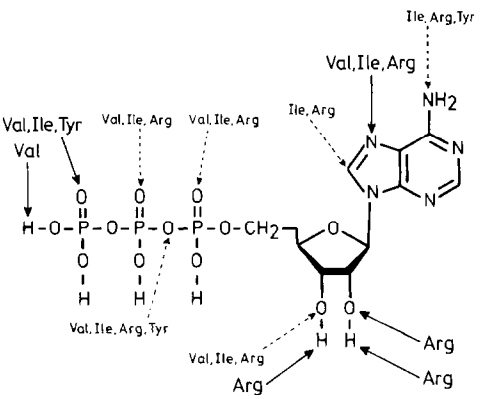


FIG. 5. Modified positions in the ATP molecule with "single-chain" aminoacyl-tRNA synthetases from yeast: →, where the modified ATP keeps being a substrate; ⇢ where the modified ATP is converted to an inhibitor. (The three-letter abbreviation designates the aminoacyl-tRNA synthetase in question.)

the normal substrate leads to inhibition (*1–3*), but in the case of the arginyl-tRNA synthetase 3′-*O*-methyl-ATP is a substrate (*3*). One can postulate that in position 3′ an oxygen atom is necessary.

A third common property of the synthetases can be seen from this work. Elongation of the phosphate chain of ATP by esterification with methanol or phenol results either in substrates or inhibitors for both enzyme groups. The phosphate chain may therefore project from the binding pocket of the enzyme permitting such substitutions.

In general one can say that aminoacyl-tRNA synthetases from yeast show an individual behavior concerning their substrate specificity with regard to ATP analogs but there are strong indications such as those mentioned above and in our previous work (*1–3*) for the existence of enzyme groups having a similar structure of the active sites.

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