The properties of ATP-analogs in initiation of RNA synthesis catalyzed by RNA polymerase from E. coli

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SUMMARY

Various base and sugar modified derivatives of ATP and UTP were used as substrate analogs for the steady state initiation reaction ATP+UTP=pppApU and the single step addition reaction ApC+ATP=ApCpA.These reactions were carried out by E.coli RNA polymerase on T7 DNA in the presence of rifampicin.The steady state kinetic parameters of the analogs, either as substrates or inhibitors, were determined.On the basis of the obtained results it is concluded that purine NTP s in initiation require anti-conformation about the glycosidic bonds as well as gauche-gauche conformation of the C(4')-C(5') bonds.The latter conformation is also a prerequisite for substrates in elongation, whereas strict anti-conformation of glycosidic bonds is not.

INTRODUCTION

Various attempts have been undertaken to unravel the stereochemical requirements of substrates for bacterial RNA polymerases by means of modified nucleotides (for a review see the literature cited) [1-3]. The general picture which emerged was that the stereochemical requirements for substrates seemed to be more strict during initiation than in the elongation phase of RNA synthesis. Ribose configuration of the sugar part as well as anti-conformation of the glycosidic bonds were apparently required at the initiation but not at the elongation or substrate site (for a definition of these two sites see the literature cited [4]).However the initiation of RNA chains was experimentally difficult to assess and differentiation between inhibitory effects of purine NTP's during elongation and initiation was impossible. Only scarce information is available concerning the effect of the nuceotides at the 3 -terminal end of a growing RNA chain upon the incorporation of the next incoming substrate.Recently McClure et al.[5,6] discovered promoter specific

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abortive initiation by RNA polymerase from E.coli.Abortive initiation of transcription is observed, when only a limited number of substrates, leading to the synthesis of the 5'-terminal dinucleotide ppp(Pur)p(Pyr) of the RNA molecule , is available. The presence of rifampicin with all substrates allows only synthesis of ppp(Pur)p(Pyr). This abortive reaction presents a genuine steady state enzymatic reaction. Its kinetic analysis provides kinetic parameters, which allow insight into the mechanism of formation of the first phosphodiester bond. The steady state kinetic analysis of abortive and primed abortive initiation of transcription of λ-DNA [6] and T7 DNA [7] has been reported. We anticipated that abortive initiation should be ideally suited to study the stereochemical requirements of primer [8] and substrate sites of RNA polymerase from E.coli by means of NTP analogs.Abortive initiation on promoters A1 and A3 of T7 DNA leads to synthesis of pppApU [5-8]. A series of ATP analogs was employed to probe the stereochemical basis of ATP recognition by RNA polymerase in initiation. A small selection of UTP analogues was also included in this study. Primed abortive initiation allows the specific synthesis of ApCpA at promoter A3 according to the following scheme: ApC+ATP=ApCpA+pp [8].We exploited this reaction for an investigation of the stereochemical basis of ATP recognition by RNA polymerase in elongation.

MATERIALS AND METHODS

Enzymes

RNA polymerase from E.coli has been purified according to Zillig [9] through the DEAE-cellulose step and then by affinity chromatography on heparin-sepharose as described by Sternbach et al.[10].The isolated holoenzyme had a specific activity of 16000 units/mg assayed on T7 DNA and contained one equivalent of sigma subunit, as determined by the rifampicin challenge assay [11]. The polymerase preparation was judged 95% pure by SDS-polyacryamide gel electrophoresis. Alkaline phosphatase and phosphodiesterase were commercial preparations obtained from Boehringer (Mannheim).

Chemicals

ATP, dATP, UTP and dTTP were purchased from Boehringer (Mannheim), radioactively labelled substrates (^{14}C)UTP,(^{14}C)dATP,(^{14}C)ATP (spec.activities 530 mCi/mmole) from Amersham-Buchler (Braunschweig).rTTP ,3'dATP and n^2h^6ATP were synthesized as described elsewhere[3,14]. The 8-and 2-substituted analogs of ATP and 3'd,3'-aminoATP were generously supplied by Dr.W.Freist (Goettingen)[12].The chemical purity of substrates was such that contamination by other nucleotides, which might have effected the experiments, could not be detected.T7 DNA was isolated and purified from phage as described[13].

Experimental conditions for abortive initiation:

The enzyme-DNA complex was prepared in a total volume of 0.1 ml by adding 9 pmoles holoenzyme and 3 pmoles T7 DNA to buffer containing: 0.04 M Tris-HCl,pH 7.9, 0.05 M NaCl, .1 mM EDTA, 0.01 M $MgCl_2$ and 1 mM mercaptoethanol.After 10 minutes at 37°C, rifampicin was added to a final concentration of 10 uM and the mixture further incubated for 10 minutes.The reaction was started by addition of substrates or analogs in concentrations as described in legends to tables and figures.After 30 minutes at 37°C, aliquots of the reaction mixtures were chromatographed and product formation determined as described earlier . Inhibition studies were performed with those analogs which proved to be no substrates. In order to verify the structure of ppp(dA)pU, the reaction mixture was treated with alkaline phosphatase. The resulting mixture of dinucleoside monophosphate and nucleoside was separated by paper chromatography in solvent n-propanol:$NH_4OH_{conc.}$:water=7:1:2. The dinucleoside monophosphate was eluted from paper and hydrolyzed further by snake venom phosphodiesterase.Deoxynucleosides were identified chromatographically using borate buffer (70% ethanol, 30% 1M ammonium acetate pH 9 saturated with sodium tetraborate.

RESULTS

The adenine nucleotides employed can be grouped into the following categories: 1) adenine nucleotides with a varying number of phosphate moieties at 5'-position: ATP, ADP and AMP; 2) nucleoside 5'-triphosphates with modified sugar phosphates: 2'dATP, 3'dATP, 3'd,3'-aminoATP; 3) nucleoside 5'-triphosphates

with substituents in 8-position: br^8ATP, $azido^8ATP$,sh^8ATP,n^8ATP; 4) nucleoside 5'-triphosphates with altered base pairing properties: cl^2ATP,n^2h^6ATP.

The behaviour of ATP analogs as primers in abortive synthesis of pppApU is depicted in Table I.It can be seen that ADP and AMP, although with an efficiency reduced by an order of magnitude, replace ATP as primer. Interestingly, this decrease in efficiency is not gradual, but occurs between ATP and ADP.All ATP derivatives bearing substituents at the adenine moiety in either position 8 or 2 do not function as primers.Inhibition studies revealed that the transcriptional complex had no affinity for 8-substituted ATP analogs. The results obtained with two representative examples, namely br^8ATP and n^8ATP are shown in Fig.1.

The analog cl^2ATP, likewise not utilized as primer in initiation behaved differently.At low concentrations of UTP, cl^2ATP functioned as competitive inhibitor of ATP with a K_i-value of 0.25 mM(data not shown). If the rates of pppApU synthesis were plotted vs UTP concentrations at constant ATP or cl^2ATP, UTP evidently inhibits at concentrations above 1 mM (Fig.2).

This is in agreement with a mode of inhibition, in which cl^2ATP functions as substrate analog that promotes partial reaction in an ordered sequence[14]; i.e. it binds to the transcriptional complex, opens the binding site for UTP, but the resulting complex E-cl^2ATP-UTP is catalytically inactive. Apparently, the affinity of UTP to the complex E-cl^2ATP is much lower (K_m=1mM) than to the complex E-ATP.In spite of that, with increasing UTP concentrations, an increasing fraction of UTP binds unproductively, thus inhibiting the reaction in the region of higher UTP concentrations. We conclude that cl^2ATP binds with relatively high affinity to the primer site and renders the substrate site less accessible to UTP, hence reducing the affinity of UTP for this site.

As can be seen from Table I, n^2h^6ATP substituted ATP as primer in abortive initiation, however n^2h^6ATP functions poorly as a substrate in elongation[15].

As expected, 3'dATP and 3'd,3'-aminoATP were found not to be primers, but competitive inhibitors in abortive initiation.

Table I Substrate Analogs in Initiation

First Nucleotide	Second Nucleotide	Synthesis (pmoles/min)
ATP	UTP	114
ADP	UTP	8
AMP	UTP	6
2 dATP	UTP	56
n^2h^6ATP	UTP	22
3 dATP	UTP	- K_i=0.64 mM[1]
3 d,3 -aminoATP	UTP	- K_i=0.52 mM
br^8ATP	UTP	-
$azido^8$ATP	UTP	- [2]
n^8ATP	UTP	-
sh^8ATP	UTP	-
cl^2ATP	UTP	-
ATP	s^4UTP	42
ATP	rTTP	100
ATP	2'dTTP	41
2'dATP	2'dTTP	-
2'dATP	UTP,2'dTTP	56[3),4)]

The experimental conditions for the abortive initiation reactions were as described in section Material and Methods.
The concentrations of the first nucleotide was 1 mM that of the second nucleotide (^{14}C)UTP (4300 cpm/nmole) or UTP analogs 0.15 mM throughout.In expriments with ATP analogs,(^{14}C)ATP (4500 cpm/nmole) was used.
1) Competitive inhibition;2) no inhibition;3) the reaction mixture contained both substrates in concentrations of 0.15mM.
4)Abbreviations:s^4UTP,4-thiouridine 5'-triphosphate;br^8ATP, 8-bromoadenosine 5'-triphosphate;n^2h^6ATP,2-aminopurine 5'-triphosphate;sh^8ATP,8-mercaptoadenosine 5'-triphosphate;cl^2ATP, 2-chloro-adenosine5 -triphosphate;n^2ATP,2-aminoadenosine 5'-triphosphate;3'd,3'aminoATP,3'-deoxy,3'-aminoadenosine 5'-triphosphate;$azido^8$ATP,8-azidoadenosine 5'-triphosphate;n^2ATP, 2-aminoadenosine 5'-triphosphate;8-azaGTP,8-azaguanosine 5'-triphosphate;o^8GTP,8-oxoguanosine 5'-triphosphate;br^8GTP,8-bromoguanosine 5'-triphosphate;n^8AMP,8-aminoadenosine 5'-phosphate;mn^8AMP,8-methylaminoadenosine 5'-phosphate.

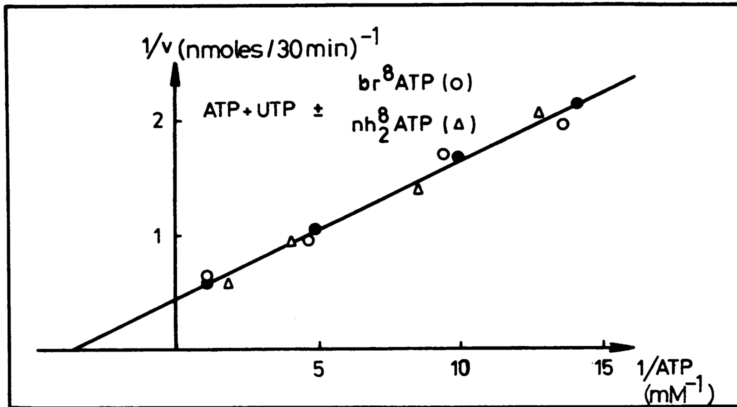

Fig.1 Synthesis of pppApU in presence of br^{8}ATP and n^{8}ATP. The concentration of (^{14}C) UTP (4300 cpm/nmole) was 0.15 mM, that of br^{8}ATP and n^{8}ATP 1 mM.

Steady state kinetic analysis revealed the competitive nature of this inhibition and furnished the K_i-values of 0.64 mM and 0.52 mM respectively.These K_i-values are very close to the K_m-value for ATP in abortive initiation.The kinetic data for 3'dATP are given in Fig.3. The replot of slopes, shown as insert in Fig.3

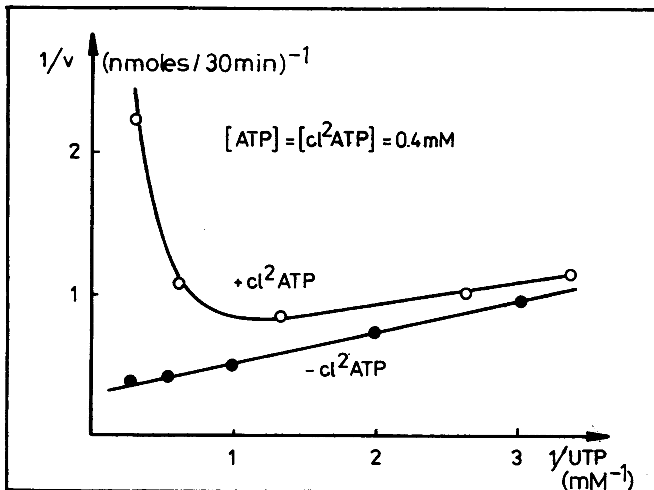

Fig.2 Kinetics of pppApU-synthesis in presence of cl^{2}ATP. The radioactive substrate was (^{14}C)UTP (4300 cpm/nmole).

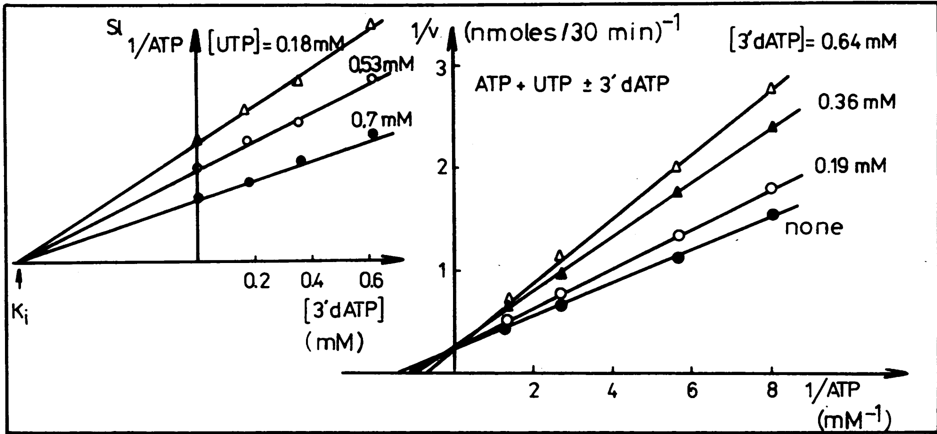

Fig.3 Kinetics of pppApU-synthesis in presence of 3'dATP. The concentration of UTP was kept constant at 0.18 mM; those of ATP and 3'dATP were varied as indicated in the figure.The insert depicts a replot of slopes vs.3'dATP concentrations.The radioactive substrate was (^{14}C)UTP (4300 cpm/nmole).

yielded a K_i-value which is independent of UTP concentration.This is in agreement with a dead-end mode of 3'dATP binding: the inhibitor excludes the binding of the next incoming substrate UTP.Thus the 3'-OH group is not important for the binding of the primer nucleotide, although it might be critical with respect to opening of the substrate site.

We have recently studied the steady state kinetics of primed abortive initiation on promoter A3 of T7 DNA[8].The reaction ApC+ATP=ApCpA+pp occuring at promoter A3 was selected to investigate the properties of ATP analogs as elongation substrates in single step addition.The two analogs studied, namely br^8ATP and $azido^8$ATP, proved to be substrates. However, the rate of synthesis of the respective trinucleoside diphosphates was in the order of 5% of that observed for the formation of ApCpA under identical conditions.The analog br ATP effectively inhibited the synthesis of ApCpA in a competitive manner. The respective kinetic data are depicted in Fig.4.

The replot of slopes versus inhibitor concentration furnished K_i=0.59 mM. The K_m-value for ATP in unhibited reaction was determined to 0.26 mM. The analog cl^2ATP did not replace ATP as

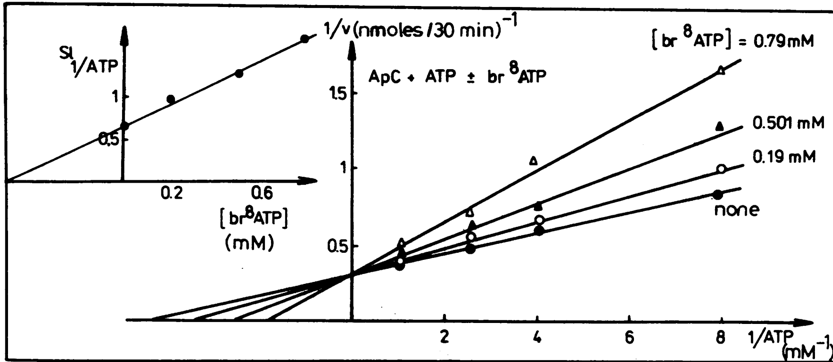

Fig.4 Kinetics of ApCpA synthesis in presence of br^{8}ATP. (^{14}C)ATP (4500 cpm/nmole) was employed. Insert: Replot of slopes vs. inhibitor concentration.

substrate in the above single step addition reaction, but instead inhibited this reaction.The replot of kinetic data, as shown in Fig.5 yielded a non linear, parabolic, relationship. This is

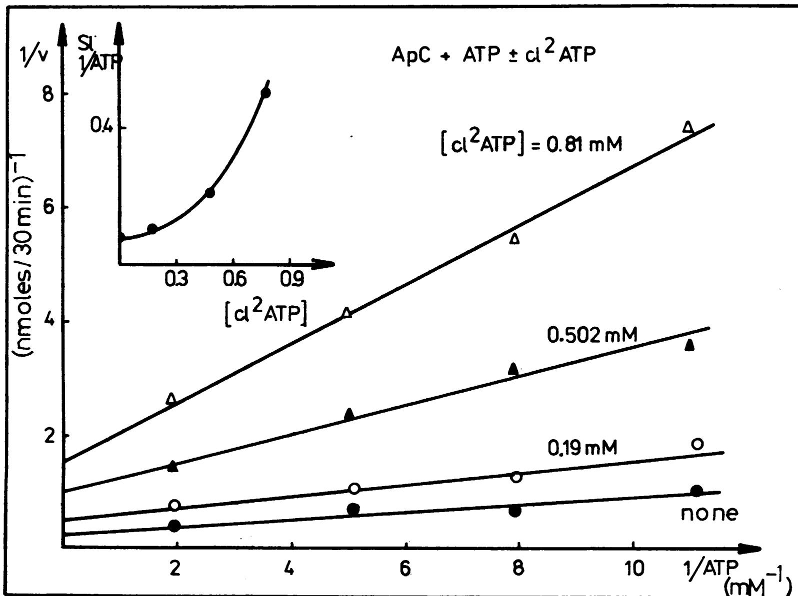

Fig.5 Kinetics of primed abortive initiation in presence of cl^{2}ATP. (^{14}C)ATP (4500 cpm/nmole) was employed. Insert:Replot of slopes versus cl^{2}ATP concentration.

indicative of two binding sites for the above inhibitor at the transcriptional complex.As a consequence of a limited movement of the enzyme at a given promoter[7,8], cl^2ATP can be either bound as primer or as substrate, because there exists a dynamic equilibrium between promoter bound enzyme molecules, ready to carry out the reactions ATP+UTP=pppApU or ApC+ATP=ApCpA.

Both, 3'dATP and 3'd,3'-aminoATP were found to be substrates in single step elongation with rates beeing 50% that of the normal reaction under identical conditions (data not shown).

In abortive synthesis of pppApU, ATP could be replaced by 2'dATP (see Table I).Under standard conditions of synthesis, the rate of reaction with dATP was approximately half that of the normal reaction. However, dTTP could not replace UTP in abortive initiation, although it inhibited the utilization of the latter in the formation of pppApU. When primed abortive initiation on promoter A1, the reaction CpA+UTP=CpApU, was investigated, it turned out that dTTP indeed replaced UTP as substrate.Thus, ribo-configuration of sugar moieties of substrates is no requirement for function at neither primer nor substrate site of the transcriptional complex.All attempts to achieve abortive initiation with both dATP and dTTP, substituting for the respective normal ribo- substrates failed.Furthermore, the reaction dATP+UTP=ppp(dA)pU was not inhibited by comparable concentrations of dTTP.These results indicate that simultaneous binding of two deoxynucleotides to the transcriptional complex does not take place. Under normal reaction conditions, the synthesis of even shortest deoxyoligonucleotides on DNA templates by RNA polymerase seems to be precluded.

DISCUSSION

We can principally expect three classes of ATP analogs: 1) ATP analogs which function both in initiation and elongation; 2) ATP analogs which function in initiation, but not in elongation, and 3) ATP analogs which only function in elongation.

Inspection of the properties of ATP analogs in abortive initiation as depicted in Table II tells us that analogs defined under 2) might not exist.A potential candidate would be n^2h^6ATP, which works well in initiation, but is a rather bad substrate in

Table II The Properties of ATP Analogs in Abortive Initiation

Nucleotide	Initiation	Elongation a)
n^8ATP	-	+
br^8ATP	-	+
azido^{8}ATP	-	+
sh^8ATP	-	+
cl^2ATP	+(i)	+(i)
n^2h^6ATP	+	(+)
3 dATP	+(i)	+
3 d,3 -aminoATP	+(i)	+
2 dATP	+	+

a)The (-) sign indicates that the nucleotide is not recognized by the transcriptional complex neither as substrate, nor as inhibitor. The (+) sign indicates substrate properties.The +(i) sign indicates affinity as inhibitor.

elongation. However, under special circumstances, this analog is utilized as elongation substrate and therefore in a true sense is not a representative of category 2).Analogs, active in initiation, likewise function in elongation, whereas ATP analogs which proved to be substrates in elongation not neccessarily are able to start a RNA chain as primer.

Substitution of position 8 in ATP in general destroys the primer function and yields derivatives belonging to category 3).This is in agreement with findings reported by Kapuler and Reich [16].Substitution of position 2 in ATP abolishes both primer and substrate function, however cl^2ATP retains the affinity for both sites at the transcriptional complex (see Table II).It appears that the stereochemical requirements for purine substrates with respect to the heterocyclic moiety are different for initiation and elongation.It is without doubt more strict in initiation.Since both an oxygen (as in o^8GTP) and bromine as in br^8ATP,br^8GTP) as substituents have qualitatively the same effect, we conclude that 8-substitution of purine NTP's in general conflicts with the topography of the active site in initiation.This cannot hold for 2-substitution of purine NTP's, because the natural GTP and the well utilized ATP analog n^2ATP [15] bear substituents in this position.However, a chlorine

substituent, which exceeds the size of a NH_2-group by approximately 30% already drastically impairs primer function (see also Table III). It is known that a CH_3-group in position 2 interfers with base pairing between adenine and uracil.One assumes the formation of Hoogsteen base pairs between 2-chloroadenine and uracil instead of normal Watson-Crick base pairing [17].Most likely, 2-chloroadenine does not form normal base pairs with the template bases thymine either.However, besides the formation of Hoogsteen base pairs,we would like to emphasize that also the formation of non-planar Watson-Crick base pairs with distorted geometry between 2-chloroadenine and thymine must be considered . The observed inhibition by cl^2ATP could be the result of improperly positioned functional groups involved in catalysis caused by the formation of a distorted base pair, 2-chloroadenine-thymine, at the transcribing complex.

What then is the explanation for the observed effect of 8-substitution in ATP? It is assumed that substitutions at the 8-position affects the conformation about glycosidic bonds, forcing the nucleosides or nucleotides to adopt syn-conformation [18]. ^{1}H-nmr spectroscopy, however, reveals that 8-substitutions do not neccessarily result in a rigid conformation about the glycosidic bond in solution, but equilibria exist between syn-and anti-conformations with preference for either syn-or anti-form.Evans and Kaplan [19]

Table III Maximal Space Requirements of Certain Substituents [a)]

Substituent	d(Å)
NH_2	2
CH_3	2
Cl	2.64
Br	2.88
F	1.84
O	1.84

a) The substituent is in first approximation regarded as a sphere with diameter d.The diameters have been determined from CPK-models.

demonstrated that n^8AMP and mn^8AMP prefer the anti-conformation in solution, whereas substitution at position 8 by the significantly smaller fluoro-or oxo-groups cause preference for the syn conformation [20,21].One might conclude that 8-substitution does not a priori prohibit rotation about glycosidic bonds.Hence, if RNA polymerase requires purine NTP's in the anti-conformation, it could select the correct conformation of the ATP analog from the equilibrium mixture.Since n^8AMP exists preferentially in the anti-conformation, this may hold for n^8ATP as well.This implies however that another structural abnormality in n^8ATP abolishes the primer function of this ATP analog and not the lack of anti-conformation. Recently, we established that gauche-gauche conformation of C(4')-C(5') bonds in NTP's is an essential prerequisit for substrates of RNA polymerase [22]. A consequence of this conformation, as demonstrated in the crystal structure of ATP, is the location of the C(5')-O-P bond above the ribose ring and the orientation of the α-phosphate moiety towards the imidazole part of the adenine ring . Inspection of CPK-models reveals that 8-substitution interfers with normal gauche-gauche conformation as well as the interaction of the tripolyphosphate chain with the imidazole ring when the ATP analog adopts anti-conformation about the glycosidic bond. It is known that gauche-gauche conformation of the C(5')-C(4') bond can already be perturbed by repulsion because of a neighbouring electronegative nitrogen ring atom as in 6-azauridine 5'-phosphate [24]. A similar situation exists in 8-azaGTP and formycin 5'-triphosphate [25]. If these analogs are in anti-conformation, and there are no objections for this assumption on steric grounds, the ring-nitrogen atoms in position 8 will repel the α-phosphate moieties and hence force these nucleotides to adopt a conformation about C(5')-C(4') bond different from gauche-gauche. This could explain the differential behaviour of both analogs in initiation and elongation.

An alternative or additional explanation for the observed lack of affinity of 8-substituted ATP analogs could be a close contact between C(8) of the purine ring and functional groups at the nucleotide binding site of RNA polymerase in intitiation.This is, however, contradicted by the observation that Cp(br^8A)

effectively competes with CpA as primer in primed abortive initiation on promoter A3 of T7 DNA [26]. Thus the distortions of structural features in Cp(br^8A) seem to be less drastic and tolerated by the transcriptional complex than those imposed by 8-substitution in br^8ATP. We would therefore like to conclude that the minimal stereochemical requirements for purine NTP's in initiation are: anti-conformation about glycosidic bonds; gauche-gauche conformation of C(5')-C(4') bonds and interaction of tripolyphosphate chains with the imidazole parts of purine rings. In 8-substituted purine NTP's these requirements are not met. Since we know that 8-substituted purine NTP's function as substrates in elongation and gauche-gauche conformation is likewise important for elongation substrates we conclude that strict anti-conformation is not a stringent requirement for NTP's to act as substrates in elongation.

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