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SUBSTRATE SELECTION BY RNA POLYMERASE FROM E. coli. THE ROLE OF RIBOSE AND 5'-TRIPHOSPHATE FRAGMENTS, AND NUCLEOTIDES INTERACTION

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Steady-state kinetic studies of the rifampicin-effected abortive initiation of transcription by E. coli RNA polymerase (EC 2.7.7.6) on the A1 T7 phage promoter were carried out with the use of ATP, UTP and a number of their appropriately modified analogues. The kinetic parameters $K_A$, $K_{MB}$, $K_I$ and $K_{AB}$ characterizing the affinity of the substrates and inhibitors of the reaction to the initiation and elongation sites of the enzyme:promoter and the enzyme:promoter:nucleoside triphosphate complexes were determined therefrom. Their comparative analysis indicated that 1) the triphosphate chain of the initiating purine nucleoside triphosphate interacts with some protein acceptor groups through the $\alpha$- and $\beta$-phosphate residues; the phosphates are engaged in binding of nucleoside triphosphates at the elongation site in the absence of the primer nucleotide; 2) the ribose 2'-OH of the elongating nucleotide, but neither of the ribose hydroxyl groups of the initiating nucleotide, participate in substrate recognition by protein receptors; 3) either substrate, ATP or UTP, bound to the initiation complex increases by about the same factor ($\geq 10$) the affinity of the other to its binding site; 4) the 3'-OH of the primer nucleotide and the $\gamma$-phosphate of the elongating nucleotide are involved in the synergistic interaction of the substrates; $\alpha$- and $\beta$-phosphates of the elongating nucleotide, bound to some protein receptors, also contribute to this process. It is postulated that the interaction of substrates is mediated through an Mg$^{2+}$ ion, known to be required for binding of the substrates in the elongation site, and a minimal molecular model of a PuoTP:Mg (II): nucleoside triphosphate chelate complex in the catalytic centre of the transcription initiation open complex is proposed.

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Stereochemical constraints imposed by the catalytic centre of *E. coli* RNA polymerase (EC 2.7.7.6) on the substrates have been studied extensively with the use of variously modified DNA templates, substrates and inhibitors of RNA synthesis (cf review articles by Krakow *et al.*, 1976; Scheit, 1980; Kumar, 1981; Wu & Tweedy, 1982). Results of these studies clearly indicate that the topology of the active site of the enzyme: DNA template complex is precisely adapted to (i) the geometry of Watson-Crick base pairs formed between the template DNA and nascent RNA chains (Rackwitz & Scheit, 1977), and (ii) some general conformational pattern characteristic of the ribonucleoside 5'-triphosphates: anti conformation about the glycosidic bond, a gauche, gauche one about the C(4')-C(5') bond of the ribose moiety (Stütz & Scheit, 1975; Smagowicz & Scheit, 1981a). These structural and conformational requirements towards substrates were shown to be more stringent during initiation than in the elongation phase of RNA synthesis.

The role played by 2'- and 3'-hydroxyl groups of ribose and by the 5'-triphosphophate chain has been also probed through the use of appropriately altered substrate analogues. Studies with 3'-dATP indicate that, while a free 3'-OH group is indispensable for phosphodiester bond formation, it is not required for binding of a nucleotide at either binding site of the catalytic centre (Smagowicz & Scheit, 1981a). Inhibition studies of RNA synthesis with the use of various pentose and pentose nucleoside 5'-triphosphates (Sylvester & Dennis, 1977; Dennis *et al.*, 1980) led to the conclusion that binding of substrates at the elongation site and productive catalysis requires probably a 3'-endo conformation of the pentose moiety and presence of the 2'-OH group in axial position. It has also been observed that binding of purine nucleotides of the initiation site in the absence of template decreases in the order PuoTP > PuoDP > PuoMP (Wu & Goldthwait, 1969) but in intact 5'-triphosphophate fragment is not necessary for initiation of RNA synthesis (McClure *et al.*, 1978; McClure & Cecch, 1978; Hansen & McClure, 1979; Smagowicz *et al.*, 1981). Obviously the latter condition is absolutely essential for elongating substrates. Moreover, the enzyme is able to discriminate particular diastereoisomers of substrate-metal complexes (Armstrong *et al.*, 1979). Little is known, however, which particular phosphate residues are involved in binding of substrates at the elongation site. No experimental information is available either concerning groups involved in mutual interaction between the initiating nucleotide (or the one at the 3'-terminal end of the growing RNA chain) and the next incoming substrate. Such an interaction can only be anticipated from the mechanism of phosphodiester bond formation as occurring between the 3'-OH of the initiating nucleotide and the γ-phosphate residue of the elongating nucleotide.

In view of the incomplete and largely qualitative character of most
of the kinetic data concerning the recognition pattern of the ribose 5'-triphosphate fragment of the substrates, as obtained from transcription of different templates and promoters with the use of various assay methods (Wu & Tweedy, 1982), it seemed worthwhile to carry out a comparative study on the structural determinants of this fragment recognition upon binding of nucleotides to a particular enzyme: promoter complex. Rifampicin-effected abortive initiation of transcription by *E. coli* RNA polymerase on the A1 promoter of T7 phage D111 deletion mutant (ATP + UTP = pppApU + pp) was investigated with a number of phosphate and sugar modified analogues of ATP and UTP as substrates or inhibitors. This equilibrium ordered reaction with ATP binding as the first nucleotide (Smagowicz & Scheit, 1977; McClure et al., 1978) and pppApU released before pyrophosphate (Smagowicz & Scheit, 1981b), has been shown to be ideally suited to studies of stereochemical requirements of the initiation and elongation sites of the enzyme towards substrates. In this work we report results of these studies and discuss the kinetic constants derived therefrom from the point of view of involvement of particular groups and local conformations in binding of substrates and in their interaction.

**MATERIALS AND METHODS**

*Enzyme.* RNA polymerase (EC 2.7.7.6) was purified from late log phase *E. coli* MRE 600 culture according to the procedure of Burgess (1969) up to the DEAE-cellulose step and then by affinity chromatography on heparin-Sepharose as described by Sternbach et al. (1975). The specific activities of two holoenzyme preparations obtained were 180 and 301 milliunits/mg protein, where 1 unit equals 1 μmol of UMP incorporated in 1 minute with T7 DNA as template under standard assay conditions (Chamberlin *et al.*, 1979); this corresponds to 33 and 56% of active *E. coli* RNA polymerase molecules, respectively. The preparations were judged as 95% pure by SDS-polyacrylamide gel electrophoresis and had a sigma factor content of over 80% as determined by the rifampicin challenge assay (Mangel & Chamberlin, 1974). Both preparations showed identical steady-state kinetic characteristics of the abortive initiation.

*DNA.* Template DNA was isolated from purified T7 and ΔD111 (a T7 deletion mutant which lacks the A2, A3 and B promoters) phages by phenol extraction (Thomas & Abelson, 1966).

*Chemicals.* AdoP[CH₂]PP1 and AdoPP[CH₂]P were from Miles; 2',3'-dATP was from P-L Biochemicals. The other nucleotides were Sigma products.

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1 Abbreviations used: AdoPPPP, adenosine 5'-tetraphosphate; AdoP[CH₂]PP, adenosine 5'-(x,β-methylene)-triphasphate; AdoP[CH₂]P adenosine 5'-(β,γ-methylene)-triphasphate; aATP, arabinoadenosine 5'-triphosphate; 2',3'-dATP, 2',3'-dideoxyadenosine 5'-triphosphate; APS, adenosine 5'-phosphosulphate; EP, RNA polymerase: promoter complex; TLC, thin layer chromatography.
All nucleotides were checked qualitatively for the degree of decomposition by the polyethyleneimine TLC method with 1 M-LiCl as solvent. \([^{14}C]UTP\) of specific activity 447 mCi/mmol was purchased from Amersham. Rifampicin was from Boehringer.

*Abortive initiation assay.* The reaction mixture consisted of 0.04 M-Tris/HCl (pH 7.9), 0.05 M-NaCl, 0.01 mM-EDTA, 0.01 M-MgCl\(_2\), 1 mM-2-mercaptoethanol, 3 nM-T7 D111 DNA, 18 nM-RNA polymerase holoenzyme (2 nM when pppApU was synthesized), 10 \(\mu\)M-rifampicin, nucleotides at concentrations indicated in the next paragraph and \([^{14}C]UTP\) added to specific activity of 5 to 50 cpm/pmol, in final volume of 50 \(\mu\)l. After a 10-min preincubation of enzyme and DNA at 37°C, rifampicin was added and incubated for another 10 min. The reaction was started by addition of the nucleotides. After incubation for 60 min at 37°C (corresponding to about 2-10% of substrate utilization), aliquots of the reaction mixture were analysed by chromatography on Whatman 3MM paper with water-saturated ammonium sulphate - isopropyl alcohol (40:80:2, by vol.). The chromatograms were cut into strips and measured for radioactivity in toluene-based scintillation fluid. The structure of dinucleotide products was verified according to Johnston & McClure (1976).

RESULTS AND DISCUSSION

*Kinetic experiments and determination of kinetic and dissociation constants*

Synthesis of pppApU and dinucleotides \(p^*Ap^*pU\) with the use of ATP or its analogues \(p^*A\): AdoPPP, AdoP\([CH_2]PP\), AdoPP\([CH_2]P\), ADP, APS and pppA*: 2'-dATP and aATP. The initial velocities \((v)\) of the dinucleotide product formation were measured in function of one substrate (UTP) concentration (0.1 - 1.0 mM) at a number of fixed concentrations of the other one ([A], in the range of 0.1 - 1.0 mM) and analysed according to Cleland (1970) using the initial velocity rate equation (1) for the steady-state two-substrate equilibrium ordered reaction:

\[
v = \frac{[A] [UTP]}{K_{mA} + K_{mA} [A] + K_{mB} [UTP] + [A] [UTP]} \tag{1}
\]

where \(K_{mA}\) is the dissociation constant of ATP or of an analogue (A) from the initiation site of the EF^\*ATP (ATP analogue) complexes, \(K_{mB}\) is the Michaelis constant of UTP binding to the elongation site of these complexes and \(K_{mA}\) is the Michaelis constant of ATP or its analogue. The kinetic constants were determined from replots of the slopes and intercepts of the previous double reciprocal plots: \(v^{-1}\) versus \([UTP]^{-1}\) and \([A]^{-1}\),
as illustrated for the ATP+UTP reaction in Fig. 1. They are listed in Tables 1 and 2. In all cases, except for ATP and AdoPPPP, $K_{mA}$ was equal to zero.

Fig. 1. Initial velocity pattern for pppApU synthesis: A, Reciprocal initial velocity ($v^{-1}$) plotted versus reciprocal ATP concentration at UTP concentrations indicated; B, Reciprocal initial velocity plotted versus reciprocal UTP concentration at ATP concentrations indicated; C, The slopes ($\alpha$) and intercepts ($\Delta$) of the linear functions from Fig. 1A replotted versus the reciprocal of UTP concentration; D, The slopes ($\alpha$) and intercepts ($\Delta$) of the linear functions from Fig. 1B replotted versus the reciprocal of ATP concentration. The kinetic constants obtained, $K_{IA}$ and $K_{MB}$, are shown in Table 1; $K_{mA} = 0.41$ mM.

Numerical values of the $K_{IA}$ and $K_{MB}$ constants for the ATP+UTP reaction (Table 1) are somewhat different from those obtained previously (Smagowicz & Scheit, 1977) for the reaction initiated at A1 and A3 T7 phage
promoters and at about 70 times higher EP concentration. While the present value of $K_{iA} = 1.0$ mm ($\sigma = 0.3$) resembles within the experimental error $K_{iA} = 0.7$ mm found in the study referred to above, the Michaelis constant for UTP, $K_{mB} = 0.31$ mm, is distinctly higher than $K_{mB} = 0.12$ mm ($\sigma = 0.04$) found presently. This difference could be probably due to the change in the reaction model from steady-state ($K_{mB} = 0.41$ mm) to rapid equilibrium.

### Table 1

Dissociation constants ($K_{iA}$) of ATP and of its phosphate modified analogues ($p^*_n A$) from the initiation site of E. coli RNA polymerase: A1 T7 promoter: ATP ($p^*_n A$) complexes and Michaelis constants ($K_{mB}$) of UTP

In this and the following Tables mean square errors, $\sigma$, are given in brackets.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Phosphate group configuration</th>
<th>$K_i$ (mm)</th>
<th>$K_{iA}$ (mm)</th>
<th>$K_{mB}$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoPPPP</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>0.17 (0.04)</td>
<td>0.011 (0.001)</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>1.0 (0.3)</td>
<td>0.12 (0.04)</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>1.1 (0.2)</td>
<td>0.34 (0.07)</td>
<td></td>
</tr>
<tr>
<td>APS</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>0.95 (0.03)</td>
<td>0.46 (0.13)</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>3.7 (1.2)</td>
<td>0.17 (0.12)</td>
<td></td>
</tr>
<tr>
<td>AdoP(CH$_2$)PP</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>1.1 (0.2)</td>
<td>0.38 (0.12)</td>
<td></td>
</tr>
<tr>
<td>AdoPP(CH$_2$)P</td>
<td><img src="image" alt="Phosphate Structure" /></td>
<td>1.6 (0.1)</td>
<td>0.23 (0.05)</td>
<td></td>
</tr>
</tbody>
</table>
(\(K_{m_A} = 0\)) at higher EP concentration. It is worth noting in this connection that a negative effect of high EP concentration on productive initiation of transcription observed recently by Shimamoto \textit{et al.} (1981) was attributed to the enhancement of abortive initiation at higher enzyme concentrations. Inhibition of dinucleotide product release under these conditions could thus explain the change in the reaction model.

\textit{Synthesis of pApU from AMP and UTP.} A reversed order of substrates binding, with UTP binding before AMP to the EP complex was found for this reaction (Szafranski, 1983). The initial velocity data were therefore analysed (Fig. 2) as in the former case using an, analogous to eq. 1, initial velocity rate equation (2):

\[
v = \frac{[\text{AMP}][\text{UTP}]}{K_{i_A} K_{m_B} + K_{m_B} [\text{UTP}] + [\text{AMP}][\text{UTP}]}
\]  

(2),

where \(K_{i_A}\), because of the reversed order of binding of the substrates, corresponds now to the dissociation constant of UTP from the elongation site of the EP:UTP complex and \(K_{m_B}\) is the Michaelis constant for AMP binding to the initiation site of this complex. \(K_{m_A}\) is equal to zero in this case.

\textit{Inhibition of pppApU synthesis by AMP.} In the presence of ATP, AMP and UTP two dinucleotides pppApU and pApU are formed simultaneously. AMP behaves in this system as a competitive inhibitor of pppApU synthesis. Investigations of the initial velocity of pppApU formation in function of [ATP] and [AMP], as shown in Fig. 3, at two UTP concentrations (0.1 and 0.2 mm) according to the rate equation (3) derived elsewhere (Szafranski, 1983), permitted determination of the dissociation constant \(K_{i}\) of AMP from the initiation site of the EP:AMP complex and the Michaelis constant \(K_{m^*_B}\) of UTP binding to this complex (Table 1).

\[
\frac{1}{v} = \left(\frac{K_{i_A} K_{m_B} + K_{m_A} [\text{UTP}]}{V}\right) \frac{1}{[\text{UTP}]} + \\
+ \left(\frac{[\text{AMP}]}{K_{i} [\text{UTP}]} + \frac{[\text{AMP}]}{K_{i} K_{m^*_B}}\right) \frac{1}{[\text{ATP}]} + \frac{1}{V} \left(1 + \frac{K_{m_B}}{[\text{UTP}]}\right)
\]

(3).

\textit{Inhibition of pppApU synthesis by 2',3'-dATP.} Because of the lack of the 3'-OH group, 2',3'-dATP cannot enter the reaction with UTP and behaves as a competitive inhibitor with respect to ATP. The dissociation constant \(K_i\) of this nucleotide from the initiation site of the EP:2',3'-dATP complex and the dissociation constant of UTP, \(K_{i_B}\), from the elongation site of the EP:2',3'-dATP:UTP complex (Table 2), were estimated using the initial velocity data of pppApU formation obtained in function of [ATP] at several fixed concentrations of the inhibitor (Fig. 4) and at two different UTP concentrations (0.1 and 0.3 mm) according to the rate equation (4):
Fig. 2. Initial velocity pattern for pApU synthesis: A, Reciprocal initial velocity plotted versus reciprocal AMP concentration at UTP concentrations indicated; B, Reciprocal initial velocity plotted versus reciprocal UTP concentration at AMP concentrations indicated; C, The slopes of the linear functions from Fig. 2A replotted versus the reciprocal of UTP concentration; D, The slopes (o) and intercepts (△) of the linear functions from Fig. 2B replotted versus the reciprocal of AMP concentration. The kinetic constants obtained, $K_{IA}$ and $K_{IB}$, are shown in Table 1.

\[
\frac{1}{v} = \left( \frac{K_{IA} K_{MB} + K_{MA} [UTP]}{V} \right) \frac{1}{[UTP]} + \\
+ \left( \frac{[2',3'-dATP]}{K_i[UTP]} + \frac{[2',3'-dATP]}{K_i K_{IB}} \right) \frac{1}{[ATP]} + \frac{1}{V} \left( 1 + \frac{K_{MB}}{[UTP]} \right)
\]

(4).

Inhibition of pppApU synthesis by UTP analogues: UDP, UMP and 2'-dUTP. Neither of these analogues is able to enter the reaction with ATP but
Table 2

Dissociation constants ($K_{iA}$, $K_i$) of sugar-modified ATP analogues (ATP*) from the initiation site of RNA polymerase: A1 T7 promoter: ATP* ternary complexes, dissociation constants ($K_{iB}$) of UTP from the enzyme: A1 T7 promoter: ATP*: UTP quaternary complexes and Michaelis constants ($K_{mB}$) for UTP

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Pentose configuration</th>
<th>$K_i$</th>
<th>$K_{iA}$</th>
<th>$K_{iB}$</th>
<th>$K_{mB}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td></td>
<td>1.0</td>
<td>(0.3)</td>
<td>0.12</td>
<td>(0.04)</td>
</tr>
<tr>
<td>2'-dATP</td>
<td></td>
<td>1.2</td>
<td>(0.2)</td>
<td>0.18</td>
<td>(0.04)</td>
</tr>
<tr>
<td>3'-dATP</td>
<td></td>
<td>0.73*</td>
<td></td>
<td>3.3*</td>
<td></td>
</tr>
<tr>
<td>2',3'-dATP</td>
<td></td>
<td>1.2</td>
<td>(0.1)</td>
<td>2.8</td>
<td>(0.6)</td>
</tr>
<tr>
<td>aATP</td>
<td></td>
<td>0.46</td>
<td>(0.08)</td>
<td>0.80</td>
<td>(0.19)</td>
</tr>
</tbody>
</table>

* Recalculated on the basis of earlier data (Smagowicz & Scheit, 1981a).

Fig. 3. Inhibition of pppApU synthesis by AMP: A, Reciprocal initial velocity plotted versus reciprocal ATP concentration at 0.2 mM-UTP and AMP concentrations indicated in the plot; B, The slopes of the linear functions from Fig. 3A replotted versus AMP concentration. The kinetic constant obtained $K_i = 1.7$ mM.
Fig. 4. Inhibition of pppApU synthesis by 2',3'-dATP: A, Reciprocal initial velocity plotted versus reciprocal ATP concentration at 0.1 mM-UTP and several 2',3'-dATP concentrations indicated in the plot; B, The slopes of the linear functions from Fig. 4A replotted versus 2',3'-dATP concentration. The kinetic constant obtained $K_i = 1.1$ mm.

all form dead-end complexes with the EP:ATP initiation complex and thus inhibit synthesis of pppApU. The dissociation constants of these analogues, $K_i$, from the elongation site of the respective EP:ATP:UTP analogue complexes (Table 3) were obtained by measurements of the initial velocity

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Initiation complex</th>
<th>$K_i$ (mm)</th>
<th>$K_{iA}$ (mm)</th>
<th>$K_{mB}$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>EP:UTP</td>
<td>3.3 (0.6)</td>
<td></td>
<td>0.12 (0.04)</td>
</tr>
<tr>
<td></td>
<td>EP:ATP:UTP</td>
<td>2.8 (0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP:UDP</td>
<td>0.79 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP</td>
<td>EP:ATP:UDP</td>
<td>18 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td>EP:UMP</td>
<td>4.0 (1.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP:ATP:UMP</td>
<td>2.9 (0.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP:2'-dUTP</td>
<td>0.98 (0.27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-dUTP</td>
<td>EP:ATP:2'-dUTP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of the dinucleotide synthesis at a constant ATP concentration (1.0 mm) and varying concentrations of UTP (0.1 - 1.0 mm) and of an inhibitor ([U], 0 - 7 mm), in agreement with the rate equation (5):
\[
\frac{1}{v} = \frac{K_{mB}}{V} \left( 1 + \frac{K_{iA}}{[ATP]} + \frac{[U]}{K_i} \right) \left( \frac{1}{[UTP]} + \frac{1}{V} \left( 1 + \frac{K_{mA}}{[ATP]} \right) \right)
\]  

(5).

Inhibition of pApU synthesis by UTP analogues: UDP, UMP and 2'-dUTP. These analogues, like UTP, bind before AMP to the EP complex (Szafranski, 1983). In agreement with eq. 6, two series of measurements of \(v\) for pApU formation at 0.1 and 0.2 mm-AMP concentration in function of UTP (0.2 - 1.6 mm) and of an inhibitor ([U], 0 - 4 mm) concentrations allowed determination of the dissociation constants: \(K_i\) of the analogues from the elongation site of the EP:UTP analogue complexes (Table 3) and \(K_{mA}\) of AMP from the initiation site of the EP:AMP:(UTP analogue) complexes (Table 4).

Table 4

Dissociation constants (\(K_{mA}\)) for AMP from the initiation site of the RNA polymerase: A1 T7 promoter: UTP (UTP analogue) complex

<table>
<thead>
<tr>
<th>Elongating nucleotide</th>
<th>(K_{mA})</th>
<th>(K_{mB})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>2.1 (1.2)</td>
<td>0.18 (0.02)</td>
</tr>
<tr>
<td>UDP</td>
<td>3.9 (1.4)</td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td>0.90 (0.51)</td>
<td></td>
</tr>
<tr>
<td>2'-dUTP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
\frac{1}{v} = \frac{K_{mB} K_{iA}}{V} \left( \frac{1}{[AMP]} + \frac{[U]}{K_i [AMP]} + \frac{[U]}{K_i K_{mA}} \right) \left( \frac{1}{[UTP]} + \frac{1}{V} \left( 1 + \frac{K_{mA}}{[AMP]} \right) \right)
\]  

(6).

The initial velocities of the reaction studied were measured at at least four concentrations of substrates or inhibitors, and the series of experiments often repeated. In all cases the experimental data were fitted by the method of non-weighted least squares to the analytical linear functions, and the mean square errors involved in determination of the kinetic constants in eqs. 1 - 6 were estimated (Table 1 - 4, in brackets).

Substrate selection at the initiation site

Comparison of dissociation constants of ATP and its analogues from the initiation site of the EP:ATP (ATP analogue) complexes (Tables 1 and 2) provides clues as to the involvement of particular groups of atoms within the two building blocks in recognition of the primer nucleotide.

Recognition of the 5'-triphosphate chain. In view of possible involvement of electric field effects in binding of charged substrates to enzymes (Neumann,
1981), ionic forms of adenine nucleotides in solution under the experimental conditions used should first be considered. By using the known stability constants of 1:1 chelate complexes of Mg$^{2+}$ with ATP, ADP and AMP (Izatt et al., 1977), corrected for the actual ionic strength (Adoltsch & Mourdrianakis, 1978), it can be shown that MgATP$^{2-}$, MgADP$^{-}$ and AMP$^{2-}$ were dominant species in the reaction mixtures. Although stability constants for Mg$^{2+}$ complexes with other adenine nucleotides investigated are not known, it seems reasonable to assume that the dominant forms thereof in solution were also 1:1 chelate complexes MgAdoP[CH]PP$^{2-}$, MgAdoPP[CH]P$^{2-}$ and AgAPS$^{0}$.

Close similarity of $K_{iA}$ values of MgATP$^{2-}$, MgADP$^{-}$ and MgAPS$^{0}$, in spite of differences in electric charge between these species, allows us to conclude that (i) electric field effects do not contribute significantly to the free energy of their binding to the enzyme surface and (ii) that the γ-phosphate group is not involved in ATP binding. Consequently, the large difference in binding affinities between MgATP$^{2-}$ and MgADP$^{-}$, on one hand, and AMP$^{2-}$ on the other, together with the fact that Mg$^{2+}$ ions are not required for binding of the purine nucleotides at the initiation site (Wu & Goldthwait, 1969), provide convincing evidence that the β-phosphate residue is indispensable for the triphosphate chain recognition. The same concerns most probably the α-phosphate residue because the apparent $K_{iA}$ constant of adenosine in ApU synthesis was found (McClure et al., 1978) to be fourfold higher than that of AMP at the same UTP concentration. The similarity of $K_{iA}$ values of MgATP$^{2-}$ and of MgAdoP-[CH$_2$]PP$^{2-}$ clearly indicates that the α,β bridge oxygen atom of ATP is not involved in binding. The somewhat larger $K_{iA}$ value of MgAdoPP-[CH$_2$]P$^{2-}$ may point to the participation of the β,γ oxygen in ATP binding. Since this oxygen atom is absent in ADP, which exhibits similar affinity to the initiation site as ATP, other reasons like different bond lengths and angles for O and CH$_2$ can also be held responsible for the observed effect on $K_{iA}$.

**Recognition of the ribose moiety.** The initiation site is quite tolerant towards structural modifications in the ribose moiety because removal of either hydroxyl group, 2'- or 3'-, or both simultaneously, changes only slightly the dissociation constant of the respective nucleotides: 2'-dATP, 3'-dATP and 2',3'-dATP, as compared with that of ATP (Table 2). This means that neither 2'-OH nor 3'-OH participate in binding of ATP to this site. The lack of interaction between the 3'-OH group of the primer nucleotide and the enzyme has been already suggested by Smagowicz & Scheit (1981a). The twofold higher affinity of aATP ($K_{iA} = 0.46$ mm) as compared with that of ATP ($K_{iA} = 1.0$ mm) indicates that the configuration of the 2'-OH group in arabinose different from that in ribose, and thus also somewhat different conformational properties of the two groups of
nucleotides (Yathindra & Sundaralingam, 1979) may be the reason for tighter binding of aATP.

*Activation of the elongation site by ATP*

The Michaelis constants of UTP (\(K_{mb}\), Tables 1 and 2) can be regarded in the first approximation as a measure of the affinity of this nucleotide to the elongation site of various EP:ATP (ATP analogue) complexes. They do not vary significantly with modification of the 5'-triphosphate chain in ATP (Table 1) which testifies to a distant location of this ATP fragment from the elongation site. Neither has replacement of the 2'-OH group of ATP with a hydrogen atom any significant effect on binding of UTP since \(K_{mb}\) values (Table 2) measured in reactions primed by 2'-dATP and ATP are very similar. On the other hand, closely similar dissociation constants, \(K_{mb}\), of UTP from the EP:3'-dATP:UTP and EP:2',3'-dATP:UTP complexes (3.3 and 2.8 mM, respectively), similar also to that of UTP dissociation from the EP:UTP complex (\(K_A = 3.3\) mM) are by one order of magnitude larger than \(K_{mb}\) of UTP in the presence of ATP (0.12 mM). This clearly indicates that the 3'-OH group of ATP occupying the initiation site is responsible for this large activating effect on UTP binding to the elongation site. The activation ability of aATP (\(K_{mb} = 0.80\) mM), which also possesses a 3'-OH group of the same configuration as in ribose, is, however, distinctly lower than that of ATP. It is known that the close proximity of a base residue and of a 2'-OH group, as well as repulsive interactions between the O(5') and axially oriented O(2') oxygen atoms, induce strong preference for the 3'-endo (N) type sugar puckering in 5'-arabinonucleotides (Yathindra & Sundaralingam, 1979; Doornbos et al., 1983). Therefore, binding of aATP in this conformation seems very probable. If so, one may conclude that the N conformation of the furanose ring does not allow for proper spatial exposure of the 3'-OH group towards incoming UTP, resulting in a smaller activating effect of aATP. In ribonucleotides the N/S conformational equilibrium is easily shifted to either side as the two conformers have similar energies and are separated by a low energy barrier of pseudorotation (Saenger, 1984). Thus, ATP can be bound in a more favourable 2'-endo (S) conformation, not so easily accessible to aATP. In this conformation the 3'-OH group of ATP may attain an optimal spatial position in the ternary initiation complex. A similar change in ribose conformation from 3'-endo to 2'-endo, accompanied by insertion of the 3'OH into the catalytic locus of the transcribing complex, has been proposed recently by Dennis & Sylvester (1981) for the 3'-end of the growing RNA; chain, occupying the initiation site.
Substrate selection at the elongation site

It has long been known that binding of substrates to the elongation site requires Mg\(^{2+}\) ions (Krakow et al., 1976) and that one tight binding site for Mg\(^{2+}\) (\(K_{	ext{diss}} = 10\) \(\mu\)M) occurs within this region (Koren & Mildvan, 1977). Under the ionic conditions of the abortive initiation reaction investigated, this site should be completely occupied. It seems thus reasonable to assume that it is this tightly bound Mg\(^{2+}\) ion which is involved in binding of nucleotides through interaction with their phosphate groups. The affinity of nucleotides to the elongation site should therefore be independent of whether they are or are not pre-chelated to Mg\(^{2+}\), because of rapid exchange of ligands between nucleotide Mg\(^{2+}\) complexes in solution and protein-bound magnesium cation. Indeed, a stable, substitution inert \(\alpha,\beta,\gamma\)-tridentate CrATP does not replace MgATP as a substrate in the elongation reaction catalysed by RNA polymerase (Stein & Mildvan, 1978).

The similarity of dissociation constants for UTP and UDP from the elongation site of the EP:UTP and EP:UDP complexes (Table 3), in contrast to very large difference in the stability constants between MgUTP\(^{2-}\) and MgUDP\(^{2-}\) (Izatt et al., 1977), suggests that, upon binding of the two nucleotides to the elongation site, their chelation modes occurring in solution are not retained. One may thus conclude that the elongation site in the absence of a primer nucleotide in the initiation site does not differentiate between di- and triphosphate fragments of the bound nucleotides. In other words, the \(\gamma\)-phosphate residue is not required for binding of nucleoside 5'-triphosphates.

The drastically higher dissociation constant of UMP (\(K_{i_A} = 18\) mm) and uridine (\(K_{i_A}\) too high to be properly estimated) can consequently be taken as evidence for involvement of both \(\alpha\)- and \(\beta\)-phosphate residues in UTP recognition.

Comparison of \(K_{i_A}\) and \(K_{mB}\) values of UTP (Table 3), characterizing dissociation of the substrate from the EP:UTP and EP:ATP: :UTP complexes, respectively, shows that in the presence of bound ATP the affinity of UTP to the elongation site is increased by about one order of magnitude. The dissociation constants of UDP and UMP from the initiation complexes including ATP are also 3 - 5 times smaller than those corresponding to dissociation from the respective EP:UDP and EP:UMP complexes (\(K_{i}\) in Table 3). They remain, however, several-fold larger than \(K_{mB}\) of UTP. The activation ability of ATP decreases thus in the order UTP > UDP > UMP. It can be concluded therefrom that all three phosphate residues of the 5'-triphosphate chain must be in some way engaged in binding of substrates in the elongation site activated by ATP.

The dissociation constants of 2'-dUTP and UTP from the EP:2'-dUTP and EP:UTP complexes were found closely similar (2.9 and 3.3 mm.
respectively, Table 3), in spite of the fact that 2'-dUTP does not function as a substrate in the abortive initiation reaction with ATP. With ATP present in the initiation site, binding of 2'-dUTP ($K_s = 0.98$ mm) is less favourable than that of UTP ($K_{mb} = 0.12$ mm) and correlates well with the inability of this nucleotide to enter the initiation reaction. Consequently, also the ability of ATP to induce stronger binding of 2'-dUTP is much less pronounced as compared with that towards UTP. The same concerns activation of AMP binding in the initiation site by 2'-dUTP (Table 4), which is by far smaller than that effected by UTP. All these findings can be rationalized in connection with the results of earlier studies (Sylvester & Dennis, 1977; Dennis et al., 1980) on transcription with the use of various pentose and nucleopentose 5'-triphosphates. They suggest that tight binding of substrates and inhibitors in the elongation site requires either a 3'-OH of the 2'-endo conformation (S) or a 2'-OH of the 3'-endo conformation (N), while for productive catalysis the bound pentose moiety should be in the 3'-endo conformation. We are thus tempted to suggest that 2'-dUTP is bound through its 3'-hydroxyl in an S-type conformation in which this group may assume a similar spatial position as the 2'-OH of normal substrate (UTP) in an N type conformation. Such an N to S change in the sugar pucker correlated with changes in torsional angles about the glycosidic and C(4')-C(5') bonds (Saenger, 1984), apparently does not allow for proper mutual alignment of the two substrates in the catalytic centre, as evidenced by their dissociation constants from the initiation complex, so that a phosphodiester linkage between them cannot be formed.

**Activation of the initiation site by UTP**

Inspection of the dissociation constants of AMP various ternary EP:AMP:UTP (UTP analogue) complexes (Table 4) in connection with that of AMP dissociation from the EP:AMP complex (Table 1) shows that the observed strengthening of nucleotide binding to the elongation site by the primer ATP is mutual. Affinity of the incoming AMP to the initiation site, with the elongation site already occupied by UTP ($K_{mb} = 0.18$ mm), is at least by one order of magnitude larger than in the absence of UTP in the elongation site ($K_i = 3.7$ mm). A similar enhancement factor has been found also for UTP binding activated by ATP.

$K_{mb}$ of AMP in the presence of UDP (2.1 mm) is of the same order of magnitude as the dissociation constant of AMP from the EP:AMP complex (3.7 mm). One can thus conclude that it is the γ-phosphate residue of UTP bound in the elongation site which should be primarily held responsible for the enhanced binding of the primer nucleotide. Whether
α- and β- residues also contribute to this effect cannot be inferred from the $K_{ab}$ value for the EP:AMP:UDP(UMP) complexes (Table 4) because of large experimental errors involved in their determination. Involvement of these phosphate residues was postulated earlier (vide infra) from the analysis of the dissociation constants of uridine nucleotides from initiation complexes.

Model of interaction and alignment of substrates in the catalytic centre

Conclusions drawn from the analysis of kinetic constants in relation to the pronounced synergism observed in binding of substrates to the initiation complex can be summarized as follows: (i) either substrate, ATP or UTP, bound in the initiation complex increases by approximately the same factor of about 10 the affinity of the other one to its binding site, (ii) the 3'-OH group of the primer nucleotide, left free in the absence of the elongating nucleotide, is involved in the activation process, and (iii) the γ-phosphate residue of the elongating 5'-triphosphate nucleotide, left free in the absence of the primer, is involved in the activation; the other two phosphate residues, α- and β-, engaged in binding to some protein receptors and/or to the protein-bound Mg$^{2+}$ ion, also contribute to this process.

In the light of these conclusions a direct, or mediated by a common receptor, interaction of the two substrates with use of the 3'-OH of ATP and 5'-triphosphate of UTP can be postulated. Direct interaction between the 3'-OH and the terminal γ-phosphate as the only close contact between the two substrates would not however, explain, activation of UDP and UMP binding by ATP. Furthermore, formation of the phosphodiester bond between the substrates according to an $S_n2$ in-line mechanism (Burgess & Eckstein, 1978) requires a close juxtaposition of the attacking nucleophile, i.e. 3'-OH, and the α-phosphorus atom at which displacement of the leaving pyrophosphate would have to occur. In view of the well documented occurrence of one tight binding site for Mg$^{2+}$ within the substrate (elongation) site of E. coli RNA polymerase (Koren & Mildvan, 1977), requirement for Mg$^{2+}$ in binding of substrates therein (Wu & Goldthwait, 1969) and involvement of divalent metal ions in almost all enzyme catalysed nucleophilic displacements on phosphorus (Mildwan & Grisham, 1974), we are tempted to postulate interaction through a central Mg$^{2+}$ ion of the two groups involved in synergistic binding of substrates and in formation of the phosphodiester bond.

To better verify the reliability of this proposed model, molecular building and computer drawing studies were carried out on the possible mutual alignment of the substrates in the initiation complex, assuming
(i) the anti conformation about the glycosidic bond and the gauche, gauche one about the C(4\textsuperscript{'})-C(5\textsuperscript{'}) bond for both nucleotides (Stütz & Scheit, 1975; Smagowicz & Scheit, 1981a), (ii) S and N type ribose conformation of the initiating and elongating nucleotide, respectively, (iii) in-line disposition of the 3\textsuperscript{'}-OH oxygen atom of the entering nucleophile and the P(\(\alpha\))-O(\(\alpha,\beta\)) bond of the leaving phosphate, both occupying apical positions of the trigonal bipyramid at the \(\beta\)-phosphate (Burgess & Eckstein, 1978), and (iv) hexacoordinate Mg(II) ion with about 2.2 Å distance between the metal ion and ligand oxygen atoms (Poonia & Bajaj, 1979).

On the basis of these studies and conclusions concerning groups involved in recognition of the substrates by RNA polymerase a minimal molecular model of ATP and UTP bound in the catalytic site of the enzyme in the form of a chelate complex ATP:Mg(II):UTP was elaborated (Fig. 5). The most plausible arrangement of the ligand groups around the central Mg(II) ion was obtained with a puckered triphosphate chain at the \(\beta\)-phosphorus atom. It allows for inner-sphere in-plane coordination through oxygen atoms of the \(\alpha\)- and \(\gamma\)-phosphate residues of UTP (nucleoside 5\textsuperscript{'}-triphosphate, in general) and the 3\textsuperscript{'}-OH group of ATP (a purine nucleoside 5\textsuperscript{'}-triphosphates). Such a puckered conformation of the triphosphate chain has been suggested for 2\textsuperscript{'}-dTTP in the DNA polymerase I:Mn\(^{2+}\):2\textsuperscript{'}-dTTP complex (Sloan et al., 1975). The three remaining inner-sphere coordination sites of Mg(II) are left in our model for binding to some protein acceptor groups or other ligands like water and counterions (Fig. 1A).

The model satisfies quite naturally the S\(_{22}\) mechanism of the reaction in that the Mg\(^{2+}\) ion would activate the \(\alpha\)-phosphorus atom for the nucleophilic attack by the 3\textsuperscript{'}-OH group and increase the nucleophilicity of the latter. It helps also to rationalize the molecular mechanism of synergistic binding of the substrates. This would involve induced-fit formation of the Mg(II) chelated complex upon binding of the next substrate when the first is bound in its binding site within initiation complex. Since the 3\textsuperscript{'}-OH of the primer nucleotide, as well as the \(\gamma\)-phosphate of the elongating one, are left free until the next nucleotide is accepted in the active site, formation of the chelated complex should be connected with a local relaxation of the protein conformation, bringing the receptor group(s) to which Mg\(^{2+}\) ion is attached into a proper spatial disposition in respect to the other ligands.

In view of the attractive simplicity and internal consistency of the proposed model, further experimental studies of initiation complexes containing appropriate transition state substrate analogues, with the use of more direct spectroscopic method, may prove worth attempting. Studies in this direction (Sloan et al., 1975; Bean et al., 1977; Stein & Mildvan, 1978) with the use of \(^{1}\text{H}\) and \(^{32}\text{P}\) NMR or Mn(II) and Cr(III) ESR methods point to the general feasibility of such an approach. The RNA:polymerase:
Fig. 5. Model of catalytic centre of the RNA polymerase: promoter:ATP:UTP quaternary complex. A, Perspective view along an axis at 80° angle to the plane determined by Mg(II), O(α), O(γ) and O(3'-OH) atoms of the Mg(II) chelated substrates; the dashed area illustrates the arbitrarily drawn protein surface of the enzyme. I and I', 5'-triphosphate and purine binding sites, respectively, for the initiating nucleotide; E and E', 5'-triphosphate and ribose protein binding sites for the elongating nucleotide, respectively; T, promoter binding site. B, Computer drawing of the ATP:Mg(II):UTP complex, disposition of the bases one to another and in respect to the sugar-phosphate skeleton close to that in an A type double helix (coordinates of the atoms are available from the authors)
substrate complexes investigated so far did not contain a DNA template, so that conclusions drawn from these studies are not directly relevant to the present work.

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