On the promoter complex formation rate of E.coli RNA polymerases with T7 phage DNA

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ABSTRACT

Influence of ionic strength on the kinetics of the promoter complex formation between E. coli RNA polymerase and T7 phage DNA was investigated using a membrane filter assay. The enzyme-promoter association rate constant was determined. It varies from $10^9$ to $3 \cdot 10^8 \text{ M}^{-1}\text{sec}^{-1}$ when the ionic strength is changed from zero to 0.15 M NaCl. Basing on the theoretical analysis of experimental data obtained the model for the promoter site selection assuming the enzyme sliding along the DNA is discussed.

INTRODUCTION

One of the possible ways of the transcription regulation can be realized on the level of RNA synthesis initiation. The initiation process includes a set of subsequent steps: the promoter selection, the promoter opening, the substrate binding, the formation of the first phosphodiester bond [1]. The estimation of the time scales for each step permits determination the rate limiting one. The transcription initiation process as a whole can be regulated most effectively by the control of this step. The estimates for the rate constants and appropriate time scales of the transcription initiation intermediate steps are now available [2-7].

The study of the promoter selection step is of special interest in particular from physico-chemical point of view. Indeed at this stage the physico-chemical aspects of DNA-recognizing protein interaction are the most pronounced. The understanding of the promoter site selection mechanism is rather poor at present. The study of the promoter-RNA polymerase complex formation kinetics was fulfilled mainly by the assay of the complex retention on the nitrocellulose filters [2,3,5,8]. The selection kinetics was shown to depend strongly on the environment and concentration of reagents [2,5,8]. In our previous work [5] we have shown that the ionic strength affects significantly the promoter selection time, this time being nonmonotonic function of ionic
strength with a minimum at 50-75 mM NaCl. Here we are considering this kinetics in more details and determine the rate constant for the association reaction of RNA polymerase with T7 DNA promoters.

Based on the theoretical analysis of experimental data obtained the model for the promoter site selection involving the enzyme sliding along the template is suggested.

MATERIALS AND METHODS

$\sigma$-saturated RNA polymerase holoenzyme was isolated from E. coli MRE600 by the modified [9] method of Burgess and Jendrisak [10].

$^{3}$H thymidine labeled T7 phages were grown on E. coli C in 500 ml as described in [11]. 15 mCi of $^{3}$H thymidine (13.5 Ci/mmol) was added to the culture simultaneously with the T7 phage (multiplicity of infection 0.1). The phage DNA was isolated as in [12]. The alkali sucrose gradient analysis of DNA showed that ~85% of DNA sedimented as intact single strands. The specific activity of DNA was $10^5$ cpm/µg. T7 DNA concentration was estimated using a molar extinction coefficient at 260 nm of 6750 [13].

AM* ("Serva") concentration in solution was determined using extinction at 440 nm of 24800 [14]. The bound to DNA AM concentration was determined at 425 nm using the extinction coefficients 23300 for the free and 12400 for the bound dye as in [15].

Poly(rI) ("Miles") was additionally purified by the phenol extraction and dialysed against 10 mM Tris (pH 8), 5 mM NaCl, $10^{-4}$ M EDTA.

The kinetics of the promoter-enzyme complex formation were measured using a membrane filter assay described by Hinkle and Chamberlin [16]. The binding reaction was carried out at 25° in 1 ml of incubation mixture containing 10 mM Tris (pH 8), 10 mM MgCl$_2$, $10^{-3}$ M EDTA, $2\cdot10^{-4}$ M DTT (buffer A), 6 µg/ml $^{3}$H T7 DNA, 500 µg/ml BSA ("Sigma") and NaCl at the indicated concentration. The binding reaction was started by addition of 0.25 µg of RNA polymerase (2 enzyme molecules per one DNA molecule) in 5 µl of buffer A containing 2.5 µg BSA and 50 mM NaCl. At the indicated time intervals the 100 µl samples were removed and mixed with 150 µl of a solution containing 10 µg of poly(I) and NaCl in a concentration which yields the final ionic strength of 50 mM. After 30 min incubation at 25° (no stable complex dissociation was observed [5]) each sample was deluted to 1 ml with the buffer A and filtered.

* Abbreviations used: AM, actinomycin D; BSA, bovine serum albumin.
through a 24 mm "Millipore" PHWP filter with a gentle suction, then dried and radioactivity was determined in a toluene-based solvent in a liquid-scintillation counter.

The control experiment showed that the bound AM even at saturating concentrations does not affect the DNA retention on the filter.

THEORETICAL

The molecular scheme of the enzyme-DNA interaction can be represented as follows:

$$E + D \xrightarrow{k_a} ED$$  \hspace{1cm} (1)

$$E + P \xrightarrow{k_p} EP$$  \hspace{1cm} (2)

Here $E$ is the enzyme, $D$ is the nonspecific binding site, $P$ is the specific binding site (promoter), $k_a$, $k_d$ and $k_p$ are the rate constants of appropriate reactions. Being stable the EP complexes are considered to be nondissociating during observation of binding reaction. The reaction scheme is independent of the promoter site selection mechanism. The latter influences only the values of reaction constant.

The experimental conditions specify the initial ($t=0$) enzyme concentration $E_0$, the total DNA concentration $N_t$ and the number of promoters per one DNA molecule ($m$). Besides we use the data of DeHaseth et al. |17| for the equilibrium constant values for the nonspecific binding, $K_{eq}$. The parameter we are going to determine is the rate constant $k_p$. In order to connect the experimental kinetic data with this parameter we present the following analysis.

Let the volume concentrations at time $t$ be: $P(t)$ for the not occupied by $E$ promoters, $E(t)$ for the free enzyme, $D(t)$ for the nonspecific DNA binding sites, $N_f$ for DNA molecules no one promoter of which is occupied by enzyme. It should be noted that $D(t)$ is equal to the volume concentration of base pairs outside the promoter sequences because the number of potential nonspecific sites coincides with the number of base pairs in the nonpromoter region |18|. Because of the promoter length is only a small part of the whole DNA length, $D_0 \approx L \cdot N_t$, where $L$ is the DNA molecule length expressed in the number of base pairs. Under the ratios $r=|E|/|DNA|$ used in the experiment a condition $E \ll D_0$ is always satisfied. Therefore it is clearly that $D_0 \approx D(t)$.

The kinetic system based on the reaction scheme (1,2) takes the form:
Besides the balance condition should be satisfied:

\[ E + |ED| + |EP| = E_0 \]  
\[ P + |EP| = P_0 \]  

(4)  
(5)

In view of relation \( D_0 \gg P_0 \) the relaxation time to the equilibrium in the reaction (1) is much less than the time of accumulation of the promoter complexes. Thus at the time-interval of experiment (of the order of reaction (2) time) it is reasonable to consider the quasiequilibrium for the reaction (1) to be attained. This specifies \( E \) which is to be introduced into the equation (3b). From equations (4), (5) and the condition \( dE/dt = 0 \) one obtains for the \( E(P) \):

\[ E = \frac{k_d(E_0 - P_0 + P)}{k_dD_0 + k_d} \]  
(6)

Substituting \( E \) in the equation (3b) by \( E \) one gets:

\[ \frac{dP}{dt} = \frac{d|EP|}{dt} = - \frac{k_p(E_0 - P_0 + P)P}{k_{eq}D_0 + 1} \]  
(7)

The integration of equation (7) yields the dependence \( P(t) \):

\[ P(t) = \frac{(P_0 - E_0)P_0}{P_0 - E_0 \exp\left(-\frac{P_0 - E_0}{E_0} \frac{t}{\tau}\right)} \]  
(8)

Or for the kinetics of accumulation of the promoter complexes we find from (8) and (5)

\[ |EP| = P_0 - P(t) = P_0\left(1 - \frac{P_0 - E_0}{P_0 - E_0 \exp\left(-\frac{P_0 - E_0}{E_0} \frac{t}{\tau}\right)}\right) \]  
(9)

Based on expression (9) it is easy to describe the time dependence of the number of DNA molecules retained on the filters. Indeed the ratio of mole-
cules passing the filters, \( N_f \), to the total number of DNA molecules, \( N_t \) is expressed in the form:

\[
\frac{N_f}{N_t} = \left| \frac{P(t)}{P_0} \right|^m
\]  

(10)

Equations (10) and (8) yield the time dependence of the number of retained DNA molecules \( N_r \):

\[
N_r(t) = N_t - N_f(t) = (1 - \left| \frac{P(t)}{P_0} \right|^m)N_t
\]  

(11)

It is seen that in order to find the evolution of \( N_r \) with time upon \( |EP|(t) \) one does not need in the combinatorics. It is of value since otherwise the approximation of the sum of binomial distribution by the Poisson function is required \([8, 16]\) what is justified at the limit \( m \to \infty \).

Substituting (10) into (11) and conserving only the first member in the set over the small value

\[
\frac{E_o}{P_0} \exp \left| - \frac{P_0 - E_o}{E_o} \frac{t}{\tau} \right|
\]

we get

\[
1 - \frac{N_r}{N_t} = \left| \frac{P_0 - E_o}{P_0} \right|^m (1 + \frac{mE_o}{E_o} \exp \left| - \frac{P_0 - E_o}{E_o} \frac{t}{\tau} \right|)
\]  

(12)

or

\[
\ln \left( \frac{1 - (N_r/N_t)}{1 - (E_0/P_0)^m} - 1 \right) = - \frac{P_0 - E_o}{E_o} \frac{t}{\tau} + \ln \frac{mE_o}{P_0}
\]  

(13)

where

\[
\tau = \frac{K_{eq}D_o + 1}{E_0 k_p}
\]  

(14)

The expression (13) allows to find the value of the enzyme-promoter association rate constant \( k_p \) from the experimentaly obtained kinetics for the retained DNA molecules \( N_r(t) \).
Fig. 1 shows the kinetics of promoter complex formation at different ionic strengths. This kinetics plotted in coordinates $\ln\left(\frac{1 - (N_t/N_e)}{1 - (E_0/P_0)}\right)$ are redrawn in Fig. 2. According to the formula (13) the slope of these curves gives the value of the following combination of parameters:

$$B = \frac{(P_0 - E_0)k_p}{K_{eq}D_0 + 1} \text{ (sec}^{-1}\text{)}$$

(15)

$B$ characterizes the time scale of the specific complex formation kinetics. One can see that: (i) the intersection point of the curves in Fig. 2 does not lie on the ordinate axis as it is predicted by the formula (13), (ii) the intersection point has an ordinate magnitude larger than that predicted by the formula (13) ($\ln\left(\frac{1}{m_E/P_0}\right)_{t=0} = \ln \frac{mE_0}{P_0}$). For the explanation of these disagreements see Discussion.

The values of the essential kinetic parameters are summarized in Table I.
The kinetics of enzyme-promoter complex formation presented in Fig. 1 and replotted in coordinates $\ln\left(\frac{1-N_r/N_t}{1-(E_0/P_0)^m}\right)$, $t; m=7, P_0=1.4 \times 10^{-9} M, E_0=4 \times 10^{-10} M$.

The dependence of $1/B = T$ on the ionic strength is shown on Fig. 3. The characteristic feature of this dependence is its nonmonotonic character.

It is expected that both the specific and nonspecific binding reactions are controlled by the three dimensional diffusion [19]. In this case according to [19, 20] the dependence of $k_p$ on I due to the effects of electrostatic attraction between negatively charged DNA and positively charged enzyme molecules must be weak. At the same time the ionic strength dependence of the equilibrium nonspecific binding constant $K_{eq}$ according to [17] should be monotonic. Based upon only this one would conclude the $T(I)$ dependence to be monotonic too. The reason of the nonmonotonic character of the curve $T(I)$ (Fig. 3) is in significant ionic strength dependence of the $k_p$ (see Table I). This dependence is to be explained and its understanding will throw the light on the mechanism of the promoter site selection.

A natural explanation of the strong dependence $k_p(I)$ noted above is in suggestion that a linear diffusion (sliding) of the enzyme along the DNA molecule can take place in the process of promoter site selection. The charac-
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Fig. 3. The dependence of the characteristic time of the specific complex formation, \( T = 1/B^7 \text{ sec} \), on the ionic strength.

The characteristic diffusion length, \( \ell \), is determined by the lifetime of the nonspecific complex:

\[
\ell = \sqrt{2D_1 \tau_{\text{diss}}} = \sqrt{2D_1/k_d}
\]

The sliding results in reaction radius enhancement of the enzyme-promoter interaction [19]. This makes it clear the dependence of \( k_p \) on \( I \) observed (Table 1). The occurrence of the one-dimensional diffusion of the enzyme in the promoter selection process can be checked experimentally. To study this one can introduce into the template the agents preventing the proposed enzyme sliding. If the sliding takes place indeed one would assume those agents to decrease the rate of the complex formation. We used actinomycin D (AM) as the agent hindering one-dimensional diffusion. AM is known to inhibit the transcription but not the specific binding [15, 21]. The lifetime of the AM-DNA complex is of the order of hundred sec [14], i.e. greater than the time of kinetics observation. Therefore the complex AM-DNA is believed to be non-dissociating during the reaction time.

The influence of AM on the promoter selection rate at the different ionic strengths is exhibited in Fig. 4. At \( r = 2P/|A_{\text{bound}}| = 300 \) AM slows down the specific complex formation rate at \( I = 50,75 \text{ mM} \) only. At \( r = 100 \) AM affects the kinetics in the whole range of the ionic strength we used, excluding \( I = 150 \text{ mM} \). At \( r > 500 \) AM has no reliable effect in the whole range of ionic strengths. At large AM concentrations \( (r \sim 10) \) a significant decrease in the complex formation rate occurs independently of ionic strength.

The data given agree satisfactorily with the concept of the particular role of enzyme sliding in the promoter selection process.
DISCUSSION

The data about the AM influence on the promoter complex formation kinetics allow estimating the average distance of the enzyme sliding. The rate constant of irreversible recombination of two types of particles when the reaction is
limited by the three dimensional diffusion as was shown by von Smoluchowski [22], to be

\[
k_p = \frac{2\pi(D_a + D_b)b \cdot N_A}{1000}
\]  

where \(D_a\) and \(D_b\) are the coefficients of the three dimensional diffusion of the reacting particles; \(b\) is the reaction radius which is equal to the sum of the interacting particles radii and \(N_A\) is the Avogadro number.

The existence of sliding during the nonspecific complex life time results in the increase in reaction radius for it is sufficiently to enzyme to get the template region adjacent the promoter at the distance not exceeding the diffusion length to find the promoter before the dissociation occurs. The influence of AM on the rate constant \(k_p\) becomes observable at such large AM concentration that the distance between the two neighbouring dye molecules does not exceed the enzyme diffusion length. Thus the threshold concentration of the bounded AM permits estimating the average diffusion length. From the data of the preceding section (Fig. 4) the following estimations are obtained: \(\ell = 0\) at 150 mM Na\(^+\); \(\ell = 100\) b.p. at 100 mM Na\(^+\); \(\ell = 500\) b.p. at 75 ± 50 mM Na\(^+\) and \(\ell \geq 500\) b.p. at 0 mM Na\(^+\). The T7 DNA promoter for the host RNA-polymerase are mapped precisely [23]. Three strong promoters (\(A_1\), \(A_2\), \(A_3\)) are located inside ~ a 400 b.p. region of the genom. Comparing this with the \(\ell\) estimations given above one concludes that diffusion length overlaps the regions between \(A_1-A_2\) and \(A_2-A_3\) promoters.

So the acceleration of the \(A_2\) promoter - enzyme complex formation rate caused by linear diffusion of the enzyme along the nonspecific DNA sequences adjacent to the \(A_2\) promoter play a less role than for the sufficiently distant promoters (B - E) [23]. By other words the \(A_1-A_3\) promoter cluster is recognized by the enzyme as one binding site under the conditions when sliding controls the selection rate (low ionic strength). This is why the number of promoters which is effectively recognized on the T7 DNA is considered to be \(m=5\). The experimental data plotted in coordinates \(\ln(1-(N_f/N_0))(m - 1)\), \(t\) for \(m=5\) are given on the Fig. 5. The corresponding promoter association rate constants \(k_p\) obtained from the slopes of the curves in Fig. 5 are summarised in Table I. Now in agreement with formula (13) the curves intersection point finds itself on the ordinate axis. However as in the case \(m=7\), the magnitude of the intercept appears to be overestimated as compared to those predicted by expression (13). This disagreement can be explained by the fact of not
Fig. 5. The same as in Fig. 2 calculated for \( m=5 \).

100% retention on the filter on the DNAs with only one bounded enzyme [16].

It should be noted that at ionic strengths less 0.1 M NaCl the enzyme dimerization is possible [24]. This must be accounted by the enzyme concentration reducing and would be seen in the decrease in the saturation level of kinetic curves in Fig. 1. At the same time the observed saturation level is close to those predicted when the possibility of enzyme dimerization is neglected.

The nonmonotonic behavior of the curve \( T(I) \) (Fig. 3) can be explained in terms of the sliding model. Qualitatively such behavior is clear from the following. The enzyme-DNA interaction leading to the specific complex formation can be characterized by two time scales [5] - \( \tau_d \) is the life time of the nonspecific complex and \( \tau_0 \) is the time enzyme spent in the solution between dissociation and subsequent attachment to DNA. \( \tau_0 \) is shown to depend weakly on the ionic strength [19] while \( \tau_d \) decrease in \( \tau_d \) results in the increase of \( \ell \) and from the other hand the number of the reassociation acts per time unit decreases. Therefore while the effective target size, i.e. \( \ell \) is large at low ionic strengths the time spent by the enzyme on the DNA sequences distant from promoter is also large. In these conditions the reassociations are rela-
tively rare and the enzyme sliding determines mainly the promoter selection. Because of frequent reassociations at high ionic strength the probability of the enzyme getting the DNA regions adjacent to the promoters is raised although the size of the target decreases. So it becomes clear that the optimum of the promoter selection rate is attained when $\tau_0 \approx \tau_d$. This conclusion can be done by a more rigorous consideration. Based on the sliding model,

$$k_p = \ell = \sqrt{1/k_d}$$

Now we rewrite the formula (14):

$$B = \text{Const} \frac{\sqrt{1/k_d}}{1 + D_0 k_a/k_d}$$

(17)

By differentiating over $k_d$ (the only I depending parameter) one makes sure the maximum of (17) to be reached at $I = I_0$ such that

$$k_d D_0 = k_d (I_0) \quad \text{or} \quad K_{eq}(I_0) = 1/D_0$$

(18)

In confirmation of this result the data of DeHaseth et al. |17| can be applied (see Table 1). The condition (18) is seen to be realized at $I = 0.05 - 0.1 \text{ M NaCl}$ that is in a qualitative agreement with our observation (see Fig. 3).

From the specific association rate constant and estimates of the diffusion lengths the rate constant of the nonspecific complex formation can be found. Assuming the enzyme to DNA association to be controlled by the three-dimensional diffusion we get:

$$\frac{k_p}{k_a} = \frac{\ell + R}{R}$$

(19)

Where $R$ is the nonspecific association reaction radius. Its value is close to the size of the DNA region covered by the enzyme: $R = 50 \text{ b.p.}$ Then one gets for $k_a$

$$k_a = 3 \cdot 10^7 \text{ M}^{-1}\text{sec}^{-1}$$

Note this value is different from one obtained by Giacomoni |8|, $k_a \sim 3 \cdot 10^8 \text{ M}^{-1}\text{sec}^{-1}$. That work dealt with low DNA concentration and therefore the influence of nonspecific binding on the kinetics of the specific complex formation was not considered. To understand the reason of this disagreement it would be appropriate to make the experiments similar to the presented in
TABLE I

<table>
<thead>
<tr>
<th>Ionic strength mM NaCl</th>
<th>0</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{k_a}{k_d} )</td>
<td>8.10^6</td>
<td>1.6.10^6</td>
<td>5.6.10^5</td>
<td>1.8.10^5</td>
<td>2.2.10^4</td>
</tr>
<tr>
<td>( B^7 ) ((s^{-1}))</td>
<td>1.1.10^{-2}</td>
<td>5.6.10^{-2}</td>
<td>7.10^{-2}</td>
<td>3.4.10^{-2}</td>
<td>1.8.10^{-2}</td>
</tr>
<tr>
<td>( k_p ) ((M^{-1}s^{-1}))</td>
<td>7.10^8</td>
<td>8.10^8</td>
<td>3.8.10^8</td>
<td>8.3.10^7</td>
<td>1.8.10^7</td>
</tr>
<tr>
<td>( b_s ) ((s^{-1}))</td>
<td>10^{-2}</td>
<td>4.9.10^{-2}</td>
<td>6.2.10^{-2}</td>
<td>3.10^{-2}</td>
<td>1.6.10^{-2}</td>
</tr>
<tr>
<td>( k_p ) ((M^{-1}s^{-1}))</td>
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</tr>
<tr>
<td>( k_d ) ((s^{-1}))</td>
<td>3.8</td>
<td>2.10^1</td>
<td>5.4.10^1</td>
<td>1.7.10^2</td>
<td>1.4.10^3</td>
</tr>
</tbody>
</table>

a the data from the work |17|

b \( B^7 = \frac{d}{d t} \left[ \ln \left( \frac{1-(N_r/N_e)}{(1-E_o/P_o)^m} \right) - 1 \right] \); \( m=7\) (the initial slope of the curves in Fig. 2)

c \( k_p = \frac{K_{eq}D_0 + 1}{P_o - E_o} \cdot B^7 \) (see formula (15))

d the same as (b) recalculated for \( m=5 \) (corresponds to the slopes of the curves in Fig. 5.)

e \( k_p = \frac{K_{eq}D_0 + 1}{P_o - E_o} \cdot B^5 \)

this work at different DNA concentrations.

Using the \( k_a \) values obtained and the equilibrium constants from |17| it is easy to find the nonspecific complex dissociation rate constant (see Table 1) and the one-dimensional dimensional diffusion coefficient \( D_1 \):

\[
D_1 = \frac{\xi^2 k_d}{2} \sim 10^{-9} \text{ cm}^2 \text{s}^{-1}
\]

This value is determined with accuracy up to the order of magnitude and varies slightly with the ionic strength. The value of \( D_1 \) obtained appears to
be close in magnitude to the diffusion coefficient for the lac-repressor protein [25].

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REFERENCES