Acta Biochimica Polonica

Vol. 48 No. 4/2001

985–994

QUARTERLY

Mg^{2+} Does not induce isomerization of the open transcription complex of *Escherichia coli* RNA polymerase at the model P*a* promoter bearing consensus –10 and –35 hexamers✪

Iwona K. Kolasa, Tomasz Łoziński and Kazimierz L. Wierzchowski✉

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5a, 02-106 Warszawa, Poland

Received: 14 November, 2001; accepted: 27 November, 2001

Key words: transcription open complex, *Escherichia coli* RNA polymerase, consensus-like P*a* promoter, kinetics and thermodynamics of transcription initiation, effect of Mg^{2+}

The kinetics and thermodynamics of the formation of the transcriptional open complex (RPo) by *Escherichia coli* RNA polymerase at the synthetic P*a* promoter bearing consensus –10 and –35 recognition hexamers were studied *in vitro*. Previously, this promoter was used as a control one in studies on the effect of DNA bending by $A_n \cdot T_n$ sequences on transcription initiation and shown to be fully functional in *E. coli* (Łoziński *et al.*, 1991, *Nucleic Acids Res.* 19, 2947; Łoziński & Wierzchowski, 1996, *Acta Biochim. Polon.* 43, 265). The data now obtained demonstrate that the mechanism of P*a*-RPo formation and dissociation conforms to the three-step reaction model: bind-nucleate-melt, commonly accepted for natural promoters. Measurements of the dissociation rate constant of P*a*-RPo as a function of $MgCl_2$ concentration allowed us to determine the number of Mg^{2+} ions, $n_{Mg} \approx 4$, being bound to the RPo in the course of renaturation of the melted DNA region. This number was found constant in the temperature range of 25–37°C, which indicates that under these conditions the complex remaines fully open. This observation, taken together with the recent evidence from $KMnO_4$ footprinting studies that the length of the melted region in P*a*-RPo at 37°C is independent of the presence of Mg^{2+} ions (Łoziński & Wierzchowski, 2001, *Acta Biochim. Polon.* 48, 495), testifies that binding of Mg^{2+} to RPo does not induce its further isomerization, which has been postulated for the λP_R–RPo complex (Suh *et al.*, 1992, *Biochemistry* 31, 7815; 1993, *Science* 259, 358).

✪This work was in part supported by the State Committee for Scientific Research (KBN, Poland), grant 6P30202406 to K.L.Wierzchowski.

✉Corresponding author, e-mail: klw@ibb.waw.pl

Abbreviations: $E\sigma^{70}$ or R, *Escherichia coli* RNA polymerase; RPo, open transcription complex; P, promoter; FDIA, fluorescence-detected abortive initiation assay.

Kinetic-mechanistic and structural studies on the initiation of transcription by *Escherichia coli* RNA polymerase ($E\sigma^{70}$) at a number of cognate promoters: *lac*UV5 (Buc & McClure, 1985; λP_R (Roe *et al.* 1984; 1985), *T7*A1 (Rosenberg *et al.*, 1982) and *tetR* (Duval-Valentin & Ehrlich, 1987) showed that this process involves at least two kinetically-significant intermediate "closed" complexes, I_1 and I_2; their interconversion connected with a large conformational change in $E\sigma^{70}$ is the rate-limiting step, followed by strand separation of promoter DNA downstream of the –11 base pair and formation of the so called "open complex", RPo, (reviewed by deHaseth *et al.*, 1998; Helmann & deHaseth, 1999). Kinetic studies on the effect of Mg^{2+} on the formation/dissociation of RPo at the λP_R promoter (Suh *et al.*, 1992) and $KMnO_4$ footprinting patterns (Suh *et al.*, 1993) led the authors to postulate that in this system in the absence of Mg^{2+} an incompetent transcriptionally open complex RPo1 is formed which upon binding of two Mg^{2+} ions to the active site of $E\sigma^{70}$ undergoes further isomerization manifesting itself by an extension of the melted region to include the transcription start-site. Mg^{2+}-Induced extension of the melted region in RPo has been postulated also for other bacterial RNA polymerases (Chen & Helmann, 1997; Zaychikov *et al.*, 1997).

Recently we have determined by $KMnO_4$ footprinting under $\pm Mg^{2+}$ conditions the rate constants of oxidation of susceptible thymines in the bubble region of RPo formed by $E\sigma^{70}$ at the model P*a* promoter and found that at 10 mM Mg^{2+} their inherent reactivity was merely enhanced but the length of the melted region at 37°C remained unchanged (Łoziński & Wierzchowski, 2001). This finding prompted us to study the effect of Mg^{2+} on the kinetics of formation/dissociation of RPo at this promoter in order to see if in this case the postulated fourth step on the pathway to RPo can be also observed.

The P*a* promoter, bearing two consensus –10 and –35 recognition hexamers, was constructed as a control one for *in vivo* studies on the effect of promoter DNA bending by $A_n \cdot T_n$ sequences on transcription initiation and shown to initiate correctly the transcription of the *dhfr* gene cloned into the pDS3 plasmid in *E. coli* (Łoziński *at al.*, 1989; 1991; Łoziński & Wierzchowski, 1996). More recently we studied this effect on the formation/dissociation of RPo under *in vitro* conditions (Kolasa, 2001; Kolasa, I.K., Łoziński, T. & Wierzchowski, K.L. in preparation). It was thus necessary to characterize the kinetics and thermodynamics of RPo formation/dissociation at this control promoter. Here we report the results of these investigations and show that the three-step pathway to RPo is fully applicable also to the P*a*–$E\sigma^{70}$ system but the claimed fourth Mg^{2+}-dependent step (Suh *et al.*, 1992; 1993) does not occur.

MATERIALS AND METHODS

***E. coli* RNA polymerase ($E\sigma^{70}$).** RNA polymerase was prepared from the *E. coli* C600 strain according to the method described by (Burgess *et al.*, 1975) except that Sephacryl S300 was used instead of Bio-Gel A5m. The enzyme was at least 90% pure as judged by SDS/PAGE. The results of quantitative activity measurements (Chamberlin *et al.*, 1983) showed that 50% of the RNA polymerase holoenzyme was active. The enzyme concentrations reported here refer to its active form. All enzyme dilutions were made at 0°C with the RNA polymerase storage buffer.

Promoter. The *E. coli* P*a* promoter made of the consensus –35 and –10 hexamers separated by a 17 bp spacer (Fig. 1) was synthesized by the solid phosphoramidite method, purified by PAGE and cloned into pDS3 as described earlier (Łoziński *et al.*, 1989; 1991). For studies on the open complex formation, a 226 bp pDS3 DNA fragment, containing in its

middle P*a*, was obtained by PCR amplification with appropriately designed primers and an Ampligene thermocycler. The concentration of PAGE purified fragment was determined spectrophotometrically.

Reagents and chemicals. γ-ANS-UTP (γ-aminonaphtalene-sulfonate-UTP) was prepared and purified as described (Yarbrough *et al.*, 1979). ANS (1-naphtylamine-5-sulfonic acid) and carbodiimide were from Fluka. UTP, ApA and heparin were from Sigma. All other chemicals were of reagent grade. Magnesium chloride was dried to constant weight before preparation of stock solution and concentration of the latter was measured refractrometrically.

Fluorescence-detected abortive initiation assay (FDAI) of association kinetics. Association kinetic experiments were performed according to the abortive initiation assay with γ-ANS-UTP as the elongating NTP and continuous spectrofluorimetric detection of the amount of fluorescent ANS-pyrophosphate liberated in the course of the reaction (Bertrand-Burggraf *et al.,* 1984; Suh *et al.,* 1992). ApA was used as the initiating nucleotide so that $E\sigma^{70}$ transcribed ApApUpU as the only abortive transcription product from the promoter studied (cf. Fig. 1). A laboratory-made double-monochromator ratio-recording and computer controlled spectrofluorimeter equipped with a thermostated cell compartment was used to monitor at 500 nm fluorescence intensity of ANS excited at 360 nm. The reactions were initiated by addition of $E\sigma^{70}$ solution at 35 (±0.1)℃ to the reaction mixture held at the same temperature in the fluorimetric cuvette and mixing for approximately 15 s with a Pasteur capillary pipette. The final concentrations of the reaction mixture (250 μl) components were as follow: 100, 115 or 125 mM $MgCl_2$, 25 mM Hepes buffer (pH adjusted to 8.0 with NaOH; 15 mM Na^+), 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 0.45 mM ApA, 0.1 mM γ-ANS-UTP, 5 nM promoter DNA, 25–200 nM active $E\sigma^{70}$ plus 10 mM NaCl and 5% glycerol from the RNA polymerase storage buffer. Fluorescence intensities were measured every 1–10 seconds with a maximum 500 points per sample for a period of time corresponding to at least seven time constants (τ_{obs}) of the reaction and stored on an IBM PC. Data from 3–6 independent reactions at every $E\sigma^{70}$ concentration were analyzed simultaneously by a nonlinear least- squares weighted (fluorescence intensity fluctuations as weighting factors) fit to the function:

$$N = N_0 + Vt - V\tau_{obs}\,(1 - e^{-t/\tau obs}) \quad (1),$$

where N and N_0 are the product per promoter at time t and $t = 0$, proportional to the fluorescence intensity, V the product per promoter per second, t the time (s), and $\tau_{obs} = 1/k_{obs}$, where k_{obs} (s^{-1}) is the observed first-order rate constant. Standard errors in τ_{obs} and V are those returned by the Marquardt algorithm used for minimization of χ^2. Steady-state rates (V) obtained in lag assays at different $E\sigma^{70}$ concentrations agreed within 10% with those determined in control reactions initiated by addition of ApA and γ-ANS-UTP to preformed open complex under the same solution conditions.

```
          -35                          -10        +1
5′-CTCGAGTTA TTGACA ATTATTTATTTATTATT TATAAT TATTT AA TTGAATTC-3′
```

Figure 1. Sequence (non-template DNA strand) of the synthetic *Escherichia coli* promoter P*a*.

In bold font –10 and –35 hexamers and the adenine bases at which transcription reaction initiates.

FDAI fixed-time assay of dissociation kinetics. To determine the rate constant of dissociation of the open complexes, k_d, the decrease in their original concentration was measured with the *FDAI* assay at various times after addition of an excess of the

polyanionic competitor heparin. The enzyme (50 nM) and promoter (10 nM) were preincubated in the reaction buffer (25 mM Hepes, pH 8.0, 0.1 mg/ml BSA, 1 mM DTT), containing 60–90 mM $MgCl_2$, for 30 min at 35°C. Heparin was added to a final concentration of 25 μg/ml, at which the reaction proved to be independent of further increase in the competitor content. Aliquots (200 μl) were removed before and at various times after heparin addition and placed in a temperature-equilibrated fluorescence cuvette. FDAI steady-state reactions were initiated at 35°C by addition of the substrates in the Hepes buffer (50 μl) to the final concentrations of 0.45 mM ApA, 0.1 mM γ-ANS-UTP and the fluorescence intensity was measured as described above. For determination of k_d at temperatures lower than 35°C, solutions containing complexes preformed at 35°C were transferred to a water bath at a desired temperature and equilibrated for 10 min, then heparin was added and the aliquots removed before and at various times after heparin addition assayed as described above when the temperature of the reaction mixture again reached 35°C. The fraction θ of open complexes remaining at time t after heparin addition is equal to the ratio of the steady-state rate of abortive product synthesis at this time divided by the steady-state rate measured prior to addition of heparin (t = 0). The first-order dissociation rate constants k_d were determined from the linear least-squares fit of the function: $\ln(\theta) = A + k_d t$ to the experimental $\theta(t)$ data.

RESULTS AND DISCUSSION

The main aim of this study was to find if (i) the open complex formation at the P*a* promoter follows the minimal three-step pathway (Scheme 1) and (ii) the claimed (Suh *et al.*, 1992; 1993) fourth step consisting of the binding of Mg^{2+} to the active center followed by enzyme isomerization and an extension of the transcription bubble occurs also in this case. The experimental conditions were thus selected so as to make the results of the present study comparable with those used for the λP_R promoter (Suh *et al.*, 1992). Therefore, $MgCl_2$ was used as the sole salt since Mg^{2+} has been shown to act as a divalent nonspecific cation competitor with $E\sigma^{70}$ at the levels of the closed and open complexes (Leirmo *et al*, 1990; Suh *et al*. 1992). This was advantageous in view of the complexity of the ionic equilibria in mixed salt solutions (Record *et al.*, 1977; Suh *et al.*, 1992).

Kinetics of the open complex formation

To evaluate the number of Mg^{2+} ions involved in the open complex formation at P*a*, the kinetics of the abortive transcription reaction was studied as a function of $E\sigma^{70}$ concentration ([R]) at three selected $MgCl_2$ concentrations: 100, 115 and 125 mM. The association reactions initiated by the addition of an excess of $E\sigma^{70}$ to the reaction mixture containing promoter DNA (P) tended to attain steady-state with a lag-time, τ_{obs}, dependent on the enzyme concentration, as illustrated by a representative FDAI assay record, shown in panel (a) of Fig. 2. The values of $\tau_{obs} = 1/k_{obs}$ at various $E\sigma^{70}$ concentrations were derived with a reasonable accuracy from the non-linear weighted least-squares fit of eqn. 1 to the experimental N(t) data.

According to the minimal three-step mechanism of the open complex (RPo) formation (Scheme 1) involving two kinetically significant intermediates (I_1, I_2) and two long-lived complexes (I_2, RPo), expected to be independent of the promoter sequence (Tsodikov & Record, 1999):

$$\mathrm{R + P} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} \mathrm{I_1} \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} \mathrm{I_2} \underset{k_{-3}}{\overset{k_3}{\Leftrightarrow}} \mathrm{RPo}$$

Scheme 1.

$1/\tau_{obs} \equiv k_{obs}$ is related to the composite overall second-order association rate k_a and the composite first-order isomerization rate constant k_i (see below) by the following equation:

$$k_{obs} \equiv 1/\tau_{obs} = 1/k_a[R]_T + 1/k_i \qquad (2),$$

where $[R]_T$ is the total concentration of $E\sigma^{70}$. Thus, the plot of τ_{obs} *vs* $1/[R]_T$ allows one to determine both rate constants k_a and k_i.

In Fig. 2b there are shown tau-plots for the association of $E\sigma^{70}$ with P*a* at 100, 115 and 125 mM $MgCl_2$ and 35°C. Linear weighted least-squares fit of eqn. 2 to the experimental data yielded k_a and k_i parameters collected in Table 1. As shown recently (Tsodikov & Record, 1999), when single-exponentiality of the association reaction is observed at $[R]_T \geq 0.3$ k_i/k_a and the fraction of long-lived complexes approaches unity in the range of temperatures investigated, then $k_i \approx k_2 << k_{-1}$ and $k_a = K_1 k_2$. These conditions were fulfilled, as can be judged from the experimental data and the fitted kinetic parameters. Thus, using the k_a and k_i parameters, corresponding K_1 equilibrium constants were calculated (Table 1), except for 115 mM $MgCl_2$, because in this case due to a large error in the intercept of the tau-plot, k_i could not be determined.

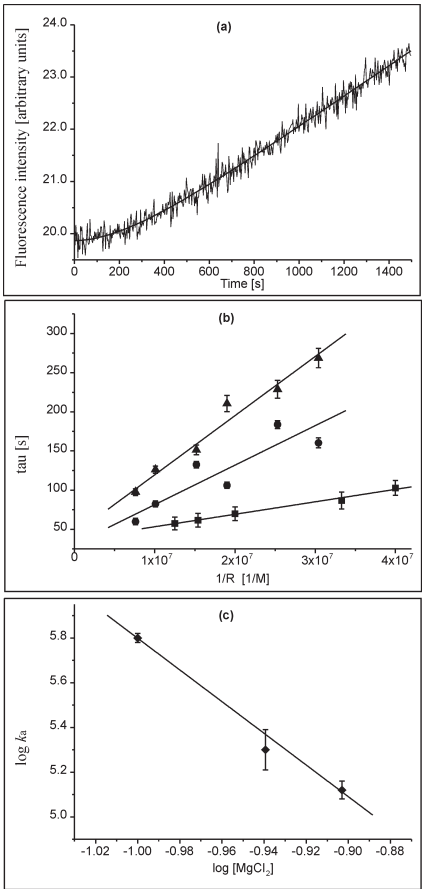

Figure 2. Kinetics of the open complex formation at P*a* promoter.

(a) A representative record of FDAI assay (RNA polymerase concentration: [R] = 25 nM, τ_{obs} = 269 ± 12 s), (b) tau-plot analysis (eqn. 2) of experimental data at 35°C and different $MgCl_2$ concentrations: 100 mM (squares), 115 mM (circles) and 125 mM (triangles); [R] – molar concentration of $E\sigma^{70}$, (c) double-logarithmic plot of k_a *vs* $MgCl_2$ molar concentration, k_a values calculated from the slopes of tau-plots shown in Fig. 2b; the slope of the plot Sk_a = –7.1 (± 0.4).

All the rate and equilibrium parameters depend exponentially on the number of Mg^{2+} ions, acting as non-specific competitors, in equilibrium with the initiation complex at various steps of its formation. Therefore, the slope Sk_a = –7.1 ± 0.4 of the double logarithmic plot of k_a *vs* $[MgCl_2]$ (Fig. 2c) measures the number of Mg^{2+} ions released upon open complex formation at P*a*. The rate $k_i \cong k_2$ of the $E\sigma^{70}$ isomerization step is considered to be salt-independent (Leirmo *et al.*, 1990). This observation is supported by the similarity of the k_i values determined at 100 and 125 mM Mg^{2+} for P*a*–$E\sigma^{70}$ (Table 1). The measured Sk_a value corresponds to the number of Mg^{2+} ions removed from $E\sigma^{70}$–P*a* interface upon formation of the first closed complex I_1. According to a recent theoretical interpretation of Mg^{2+} binding isotherms for nucleic acids (Misra & Draper, 1999), 1.8 Na^+ ions are displaced for every Mg^{2+} ion bound, so that $Sk_a \approx -7$ would correspond roughly to 12 monovalent ions. For the formation of the first closed complex at the λP_R promoter in NaCl solution a similar value of Sk_a = –11.9 ± 1.1 has been determined experimentally (Roe *et al.*, 1985), while that found (Suh *et al.*, 1992) in $MgCl_2$, Sk_a = –5.2 ± 0.3, is somewhat lower than the one measured for P*a*. The general similarity of the Sk_a values for the P*a* and λP_R promoters demonstrates that the closed com-

Table 1. Rate and equilibrium constants for the formation/dissociation of the open complex of the P*a* promoter at various $MgCl_2$ concentration (25 mM Hepes buffer, pH 8, at 35°C).

Promoter: $MgCl_2$	$k_a = k_i K_1$ $10^5\ M^{-1}s^{-1}$	k_i $10^{-2}\ s^{-1}$	k_1 $10^7\ M^{-1}$	k_d* $10^{-3}\ s^{-1}$	$k_{eq} = k_a / k_d$ $10^8\ M^{-1}$
P*a*: 100 mM	6.3 (0.3)	2.7 (0.2)	2.3 (0.1)	1.4 (0.3)	4.5
115 mM	2.0 (0.5)	nd	nd	2.3 (0.3)	0.9
125 mM	1.3 (0.1)	2.3 (0.5)	0.6 (0.1)	3.1 (0.3)	0.4
λP_R**: 100 mM	0.065	nd	nd	0.026	2.5

*Obtained by linear extrapolation from a lower range (30–90 mM) of $MgCl_2$ concentrations using the following fitted function: $\log k_d = 0.69(\pm 0.36) + 3.55(\pm 0.3) \times \log[MgCl_2]$. **For the λP_R–Eσ^{70} complex the parameters were obtained by extrapolation to 100 mM $MgCl_2$ and to 35°C by using the experimental data reported for the 5–60 mM range of [$MgCl_2$] at 25°C (Suh *et al.*, 1992) and corresponding Arrhenius functions (Roe *et al.*, 1984; 1985).

plex formation in both cases is entropically controlled (K_1, cf. Scheme 1) at the first step by the release of a similar number of cations from the enzyme/promoter DNA interface.

Kinetics of dissociation of the open complex at the P*a* promoter

The kinetics of dissociation of RPo at P*a* was studied in the presence of an excess of the polyanionic competitor heparin to make the dissociation reaction irreversible. Under these experimental, rapid equilibrium ($k_2 << k_{-1}$), conditions, the first-order rate constant of dissociation, k_d, was shown to be related to the pertinent microscopic parameters (cf. reaction scheme, eqn. 2) as follows (Tsodikov & Record, 1999):

$$k_d = k_{-2}/(1+ K_3) \qquad (3),$$

where $K_3 = k_{-3}/k_3$. Monophasic first-order decay kinetics were observed for the open complex at P*a* at all temperatures in the range 15–35°C. Consequently, values of k_d were obtained from the experimentally determined fractions, Θ, of the open complexes (cf. Methods) left at time t after heparin addition, by linear weighted least-squares fit of the $\ln\theta = A - k_d t$ function to the $\theta(t)$ data.

Temperature dependence of k_d for open complex dissociation

In the whole range of temperatures of interest (15–35°C), the values of k_d were determined in 60 mM $MgCl_2$ in Hepes buffer. The Arrhenius plot of k_d shown in Fig. 3 indicates that the activation energy of dissociation, $E_{a,d}$, is temperature dependent and negative

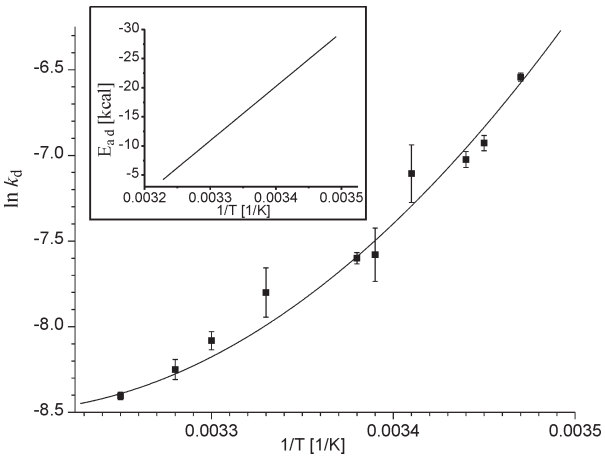

Figure 3. Arrhenius plot of the temperature dependence of k_d for the open complex P*a*–Eσ^{70} dissociation.

Experimental data points at 60 mM $MgCl_2$ (squares); solid line represents the fitted function (eqn. 4) of the form: $\ln k_d = -3.29\ (\pm 0.66)\ 10^3 + 1.53\ (\pm 0.29)\ 10^5\ T^{-1} + 4.86\ (0.98) 10^2\ \ln T$; inset – calculated $\partial \ln k_d / \partial\ (1/T)$ derivative of the fitted function showing temperature dependence of $E_{a,d}$.

throughout the whole temperature range investigated. The calculated derivative of the experimental Arrhenius function: $\partial k_d/_\partial(1/T)$ (cf. inset in Fig. 3) shows that $E_{a,obs}$ varies from the highest to the lowest temperature from about –5 to about –26 kcal, respectively. From the theoretical fit of the Arrhenius function of the form consistent with eqn. 3:

$$\ln k_d = A + (E_{a,d} - \Delta H^0_3)/RT + [(\partial E_{a,d}/\partial T)/R]\ln T \quad (4),$$

(where ΔH^0_3 is the vant'Hoff enthalpy at the melting step: $K_3 = k_{-3}/k_3$) to the experimental k_d (1/T) data, $C_p^0 \equiv \partial E_{a,d}/\partial T = +0.9$ kcal/deg was estimated (cf. legend to Fig. 3 for the values of the fitted parameters). The general form of the Arrhenius plot and the values of the fitted parameters are similar to those obtained for the λP_R (Roe *et al.*, 1985), *lac*UV5 (Buc & McClure, 1985) and *tet*R (Duval-Valentin & Eherlich, 1987) promoter complexes. We can thus conclude that dissociation of the P*a*-RNAP open complex follows the minimal three-step mechanism of the open complex formation/dissociation (Scheme 1).

Salt dependence of k_d and the effect of temperature on Sk_d

To evaluate the overall equilibrium constant, $K_{eq} = k_a/k_d$, for the open complex under the salt conditions used for the determination of the kinetics of the forward reaction, k_d should be measured under the same salt conditions. Unfortunately, the dissociation reaction at $[MgCl_2] \geq 100$ mM and 35°C proved to be too fast to be accurately determined with the method employed. Therefore, k_d was measured as a function of $MgCl_2$ concentration in a lower range thereof, usually 60–90 mM, and from linear plots of $\log k_d$ *vs* $\log[MgCl_2]$ the sought values of k_d were obtained by extrapolation to a higher $[MgCl_2]$.

The slope Sk_d of the double-logarithmic plot of k_d *vs* $[MgCl_2]$ was interpreted in connection with eqn. 3 as:

$$Sk_d = -SK_3 + Sk_{-2} \quad (5).$$

As discussed earlier, the isomerization step of Eσ^{70}-promoter (k_2, k_{-2}) on the pathway to open complex formation is considered independent of counterions concentration, hence the most significant contribution to Sk_d is thought to arise from Sk_{-3}, resulting from the reassociation of counterions in the process of the bubble DNA renaturation and reduction of the surface area involved in Eσ^{70}-promoter DNA interaction upon conversion of RPo to I_2 (Roe *et al.*, 1984; 1985; Suh *et al.*, 1992). The slope of this plot for the open complex at P*a* at 35°C (cf. Fig. 4) was found to be $Sk_d \approx 4$, and thus equivalent to $Sk_d \approx 7$ for Na^+ or K^+ ions, according to (Misra & Draper, 1999). For the Eσ^{70}–λP_R system (Suh *et al.*, 1992), $Sk_d \approx 0.4$ (± 0.2) was found in $MgCl_2$ at 25°C, in disagreement with expectations based on $Sk_d \approx 7.7$ (±0.2) for univalent NaCl at the same temperature. This observation led the authors to the conclusion that Sk_d "is determined primarily by the net cation stoichiometry, which results from uptake of univalent cations and/or Mg^{2+}, and, in addition, release of specifically bound Mg^{2+} in dissociation of the open complex RPo2". In terms of this interpretation it was difficult to rationalize the $Sk_d \approx 4$ found in our system. Therefore, Sk_d was determined as a function of temperature and found constant at 3.6 (±0.9) in the range 25–37°C, as shown in Fig. 4. This means that in this temperature interval the size of the transcription bubble in RPo, estimated equal to about 14 bp from $KMnO_4$ footprinting data at 37°C (Łoziński & Wierzchowski, 2001), is maximal and independent of temperature. The calculated overall equilibrium constant K_{eq} for RPo of P*a* (Table 1) at 35°C depends exponentially on $n_{Mg^{2+}} \approx 11$(not shown) in agreement with the number of Mg^{2+} ions in

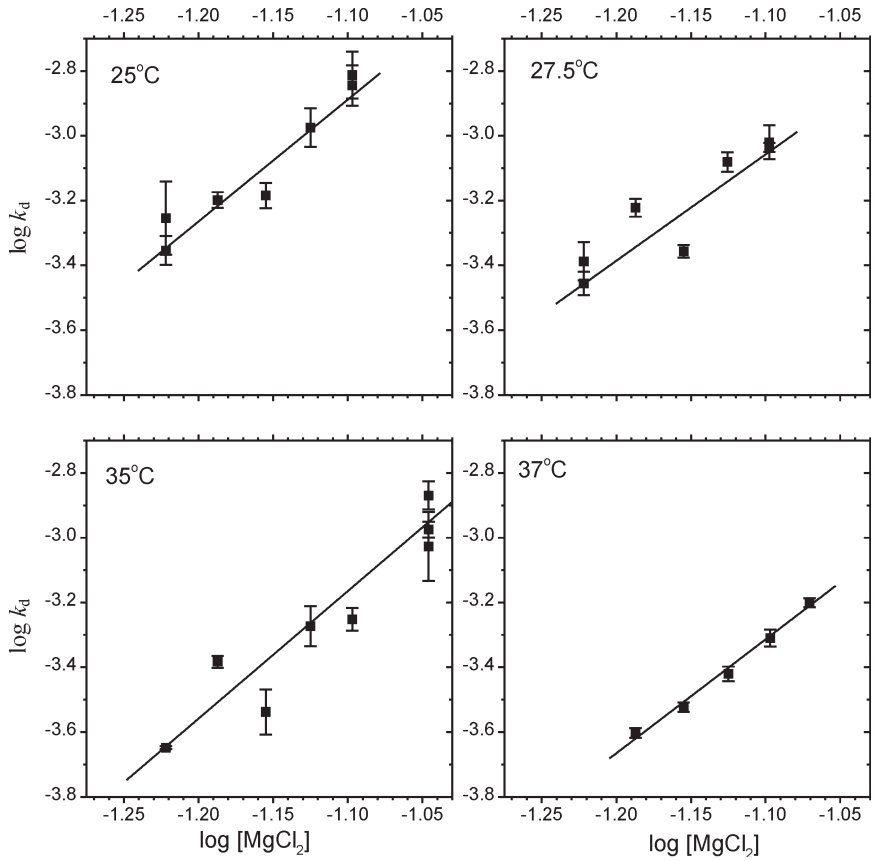

Figure 4. Double-logarithmic plots of k_d *vs* $MgCl_2$ molar concentration for the open complex formed by $E\sigma^{70}$ at P*a* promoter at 25, 27.5, 35 and 37°C.

The slopes, Sk_d, correspond to n_{Mg}: 3.8 (±0.5), 3.4 (±0.7), 3.6(±0.3) and 3.5(±0.2), respectively.

equilibrium with the complex at the first and third steps of the pathway to RPo.

The standard free energy of melting of a 14 bp bubble DNA of the P*a* promoter can be calculated as ΔG^0 = 17.4 kcal, on the basis of fractional values of thermodynamic functions for all the possible base steps in this DNA sequence (Breslauer *et al.*, 1986). For the λP_R promoter the calculated value is distinctly larger ΔG^0 = 21.0 kcal, so that at 25°C the bubble region might not be fully melted (Suh *et al.*, 1992). This would explain the low value of $Sk_d \approx$ 0.4 found at 25°C for this promoter, as corresponding to some 10% of melting, on the assumption of the discrete-steps model of reversible DNA melting in the formation/dissociation of the open complex (Helmann & deHaseth, 1999), based on chemical probing experiments carried out on complexes detected at different temperatures (Buc & McClure, 1985; Chen & Helmann, 1997; Zaychikov *et al.*, 1997) and recent fast-kinetic studies (Strainic *et al.*, 1998).

The demonstrated temperature independence of Sk_d, taken together with the results of permanganate footprinting of the P*a* open complex showing that Mg^{2+} ions do not induce extension of the transcription bubble (Łoziński & Wierzchowski, 2001), makes the hypothesis stating that binding of Mg^{2+} at the catalytic center of $E\sigma^{70}$ triggers a structural transformation in the open complex from its magnesium free form RPo1 to the transcription competitive RPo2 form (Suh *et al.*, 1992; 1993; deHaseth *et al.*, 1998) inapplicable to the system studied here. Binding of Mg^{2+} in the catalytic center may only induce local conformational changes around this site.

It seemed worth to compare the kinetic parameters for the open complex at the P*a* promoter with those for the $E\sigma^{70}$–λP_R complex. The latter were evaluated by linear extrapola-

tion to 100 mM $MgCl_2$ and 35°C of the experimental data measured at 5–60 mM $MgCl_2$ and 25°C (Suh *et al.*, 1992) and using corresponding Arrhenius functions (Roe *at al.*, 1984; 1985). The comparison (cf. Table 1) indicates that RPo forms slower at λP_R than at P*a* by about two orders of magnitude but is more stable by a similar factor, so that the overall equilibrium constants remain similar. In view of these rather large differences between the two promoters in the kinetics of RPo formation/dissociation and the number of Mg^{2+} ions in control of the stability of RPo, the latter parameter should be determined for λP_R as a function of temperature in order to verify the validity of the postulated fourth step in the pathway to RPo formation.

The authors thank Dr. K. Bolewska and T. Rak for preparation of RNA polymerase.

REFERENCES

Bertrand-Burggraf, E., Lefévre, J.F. & Daune, M. (1984) A new experimental approach for studying the association between RNA polymerase and the *tet* promoter of pBR322. *Nucleic Acids Res.* **12**, 1697–1706.

Buc, H. & McClure, W.R. (1985) Kinetics of open complex formation between *Escherichia coli* RNA polymerase and the *lac* UV5 promoter. Evidence for a sequential mechanism involving three steps. *Biochemistry* **24**, 2712–2723.

Breslauer, K.J., Frank, R., Blocker, H. & Marky, L.A. (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3746–3750.

Burgess, R.R. & Jendrisak, J.J. (1975) A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. *Biochemistry* **14**, 4634–4638.

Chamberlin, M., Kingston, R., Gilman, M., Wiggs, J. & deVera, A. (1983) Isolation of bacterial and bacteriophage RNA polymerases and their use in synthesis of RNA *in vitro*. *Methods Enzymol.* **101**, 540–568.

Chen, Y.-F. & Helmann, J.D. (1997) DNA melting at the *Bacillus subtilis* flagellin promoter nucleates near –10 and expands unidirectionally. *J. Mol. Biol.* **267**, 47–59.

DeHaseth, P.L., Zupancic, M.L. & Record, M.T., Jr. (1998) RNA polymerase-promoter interactions: The comings and goings of RNA polymerase. *J. Bacteriol.* **180**, 3019–3025.

Duval-Valentin, G. & Ehrlich, R. (1987) Dynamic and structural characterization of multiple steps during complex formation between *E. coli* RNA polymerase and *tetR* promoter from pSC101. *Nucleic Acids Res.* **15**, 575–594.

Helmann, J.D. & deHaseth, P.L. (1999) Protein-nucleic acid interactions during open complex formation investigated by systematic alteration of the protein and DNA binding partners. *Biochemistry* **38**, 5959–5967.

Kolasa, I.K. (2001) Wpływ sekwencji $A_n \cdot T_n$ zaginających DNA na moc promotora *Escherichia coli in vitro*. *Ph.D. Thesis*, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland (in Polish).

Leirmo, S. & Record, M.T., Jr. (1990) Structural, thermodynamic and kinetic studies of the interaction of E^{70} RNA polymerase with promoter DNA; in *Nucleic Acids and Molecular Biology* (Eckstein, F. & Lilley, D.M.J., eds.) vol 4, pp. 123–151, Springer-Verlag, Heidelberg.

Łoziński, T., Markiewicz, W.T., Wykrzykiewicz, T.K. & Wierzchowski, K.L. (1989) Effect of the sequence-dependent structure of the 17 bp AT spacer on the strength of consensus-like *E. coli* promoters *in vivo*. *Nucleic Acids Res.* **17**, 3855–3863.

Łoziński, T., Adrych-Roek, K., Markiewicz, W.T. & Wierzchowski, K.L. (1991) Effect of DNA bending in various regions of the consensus-like *Escherichia coli* promoter on its strength *in vivo* and structure of the open complex *in vitro*. *Nucleic Acids Res.* **19**, 2947–2953.

Łoziński, T. & Wierzchowski,K.L. (1996) Effect of reversed orientation and length of $A_n \cdot T_n$ DNA bending sequences in the –35 and spacer domains of a consensus-like *Escherichia coli*

promoter on its strength *in vivo* and gross structure of the open complex *in vitro*. *Acta Biochim. Polon*. **43**, 265–280.

Łoziński, T. & Wierzchowski, K.L. (2001) Mg^{2+} ions do not induce expansion of the melted DNA region in the open complex formed by *Escherichia coli* RNA polymerase at a cognate synthetic P*a* promoter. A quantitative $KMnO_4$ footprinting study. *Acta Biochim. Polon*. **48**, 495–510.

Misra, V.K. & Draper, D.E. (1999) The interpretation of Mg^{2+} binding isotherms for nucleic acids using Poisson-Boltzmann theory. *J. Mol. Biol.* **294**, 1135–1147.

Record, M.T., Jr., deHaseth, P.L. & Lohman, T.M. (1977) Interpretation of monovalent and divalent cation effects on the *lac* repressor-operator interaction. *Biochemistry* **16**, 4792–4796.

Roe, J.-H., Burgess, R.R. & Record, M.T., Jr. (1984) Kinetics and mechanism of the interaction of *Escherichia coli* RNA polymerase with the λP_R promoter. *J. Mol. Biol.* **176**, 495–521.

Roe, J.H., Burgess, R.R. & Record, M.T., Jr. (1985) Temperature dependence of the rate constants of the *Escherichia coli* RNA polymerase-λP_R promoter interaction. Assignment of the kinetic steps corresponding to protein conformational change and DNA opening. *J. Mol. Biol.* **184**, 441–453.

Rosenberg, S., Kadesch, T.R. & Chamberlin, M.J. (1982) Binding of *Escherichia coli* RNA polymerase holoenzyme to bacteriophage T7 DNA. Measurements of the rate of open complex formation at T7 promoter A1. *J. Mol. Biol*. **155**, 31–51.

Strainic, M.G., Jr., Sullivan, J.J., Vevelis, A. & deHaseth, P.L. (1998) Promoter recognition by *Escherichia coli* RNA polymerase: Effects of the UP element on open complex formation and promoter clearance. *Biochemistry* **37,** 18074–18080.

Suh, W.C., Leirmo, S. & Record, M.T., Jr. (1992) Role of Mg^{2+} in the mechanism of formation and dissociation of open complexes between *Escherichia coli* RNA polymerase and the λP_R promoter: Kinetic evidence for a second open complex requiring Mg^{2+}. *Biochemistry* **31**, 7815–7825.

Suh, W.C., Ross, W. & Record, M.T., Jr. (1993) Two open complexes and a requirement for Mg^{2+} to open the λP_R transcription start site. *Science* **259**, 358–361.

Tsodikov, O.V. & Record, M.T., Jr. (1999) General method of analysis of kinetic equations for multistep reversible mechanisms in the single-exponential regime: Application to kinetics of open complex formation between Eσ^{70} RNA polymerase and λP_R promoter DNA. *Biophysical J.* **76**, 1320–1329.

Yarbrough, L.R., Schlageck, J.G. & Baughman, M. (1979) Synthesis and properties of fluorescent nucleotide substrates for DNA-dependent RNA polymerases. *J. Biol. Chem*. **254**, 12069–12073.

Zaychikov, E., Denissova, L., Meier, T., Gotte, M. & Heumann, H. (1997) Influence of Mg^{2+} and temperature on formation of the transcription bubble. *J. Biol. Chem.* **272**, 2259–2267.