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 Pa promoter. A quantitative KMnO$_4$ footprinting study

Tomasz Łoziński and Kazimierz L. Wierzchowski

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland

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Footprinting studies of prokaryotic open transcription complexes (RP$_O$), based on oxidation of pyrimidine residues by KMnO$_4$ and/or OsO$_4$ at a single oxidant dose, have suggested that the extent of DNA melting in the transcription bubble region increases in the presence of Mg$^{2+}$. In this work, quantitative KMnO$_4$ footprinting in function of the oxidant dose of RP$_O$, using *Escherichia coli* RNA polymerase (E$_{70}$) at a fully functional synthetic promoter Pa having –35 and –10 consensus hexamers, has been used to determine the dependence of oxidation on DNA binding. By using a method of quantification of end-labeled DNA fragments corresponding to oxidized Ts, the extent of DNA melting was determined at 37°C in the absence of Mg$^{2+}$ and in the presence of 10 mM MgCl$_2$, and to evaluate the effect of Mg$^{2+}$ on the extent of DNA melting. Population distributions of end-labeled DNA fragments corresponding to oxidized Ts were quantified and analyzed according to the single-hit kinetic model. Pseudo-first-or der activity rate constants, $k_p$, thus obtained demonstrate that Mg$^{2+}$ ions bound to RP$_O$ merely enhanced the reactivity of all 11 oxidizable thymines with an average of 3 to 4 for those located close to the transcription start point +1 in either DNA strand, and about 1.6 for those located more distantly therefrom. On the basis of these observations, we conclude that Mg$^{2+}$ ions bound to RP$_O$ at Pa do not influence the length of the melted DNA region and propose that the higher reactivity of thymines results mainly from lower local repulsive electrostatic barriers to MnO$_4^-$ diffusion around carboxylate binding sites in the catalytic center of RP$_O$ and promoter DNA phosphates.

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Corresponding author: Kazimierz L. Wierzchowski, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawinińskiego 5a, 02-106 Warszawa, Poland; e-mail address: klw@ibb.waw.pl

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; E$_{70}$ or RNAP, *Escherichia coli* RNA polymerase; nt, non-template DNA strand; t, template DNA strand; RP$_O$, open complex; T, thymin residue.
It has long been known that Echerichia coli RNA polymerase (ε70) in the process of RNA synthesis exhibits an absolute requirement for Mg$^{2+}$ (or another diivalent cation) [1, 2], related to the binding of metal chelates of the ribonucleoside 5’-triphosphates to the elongation site of the transcriptional complex [3] and to the involvement of Mg$^{2+}$ in the catalysis of internucleotide phosphodiester bond formation [4–9]. More recently it has been surmised that Mg$^{2+}$ ions participate also in enlargement of the transcription bubble [10–15]. Magnesium ions at 10 mM concentration led to a several-fold increase in the number of KMnO$_4$-oxidized pyrimidine residues in the λPR promoter open complex within the melted region, therefore propagation of DNA strands opening from the center of the transcription bubble outwards was proposed [11]. Also backbone reactivity of the λPR promoter template strand in the open complex toward HO$^\cdot$ radicals, generated locally in the reaction of Fe(EDTA)$^{2–}$ with H$_2$O$_2$, was found significantly enhanced by 10 mM MgCl$_2$ in the single stranded region of the template strand at positions from –4 to +1 [12]. Studies on the dependence of the rate of dissociation of the binary open complex at λPR promoter on MgCl$_2$ concentration have suggested that this process can be accompanied by the release of about 3 Mg$^{2+}$ ions [10]. All these findings together with the evidence on the existence in a number of DNA and RNA polymerases of two crucially important carboxylate Mg$^{2+}$-binding sites near the catalytic site [8], led to the proposal that 2 of the 3 Mg$^{2+}$ ions bound specifically to the RPO$_2$ form of the Eo$^{70}$–λPR open complex are located near the transcription start site [12]. Unidirectional expansion of the bubble towards the transcription start point as a result of Mg$^{2+}$ binding has also been postulated for E. coli RNAP–T7A1 promoter complex [13] as well as for promoter complexes formed by Bacillus subtilis [16] and Thermatoga maritima [17] RNA polymerases.

The studies concerning Mg$^{2+}$-induced expansion of the transcription bubble were based on comparative analyses of KMnO$_4$ footprint of open complex obtained under a selected single oxidant concentration and time of exposure (a single oxidant dose) under Mg$^{2+}$ conditions. In most of these reports, however, neither the experimental methods used nor the data obtained were presented in sufficient detail to assure the readers that the conditions of single oxidation event per DNA molecule were rigorously controlled (single-hit footprinting). Such conditions should be fulfilled to make possible a reliable interpretation of footprints in terms of relative oxidizabilities of bases in DNA–protein complexes. Only recently it has been shown [18] how to extract these data quantitatively from footprints obtained under multiple-hit conditions in function of the oxidant dose.

We have therefore studied quantitative KMnO$_4$ footprinting as a function of the oxidant dose on the open transcription complex formed at a synthetic consensus-like E. coli promoter Pa by the cognate RNA polymerase, in order to determine individual reactivity rate constants for T residues within the transcription bubble region, in the absence and in the presence of 10 mM MgCl$_2$, and to evaluate on this basis the effect of Mg$^{2+}$ ions on the extent of DNA melting. The functional, kinetic and thermodynamic properties of the open complex at this promoter [19–22] were shown to be generally similar to those of the complexes at natural promoters thus far studied [22–26]. A great advantage of the use of promoter Pa in footprinting of the open complex was the presence within the melted DNA region of as much as 14 AT base pairs. We have demonstrated that binding of Mg$^{2+}$ ions to the open transcription complex is not accompanied by an extension of the melted DNA region. This binding merely duces a characteristic change in the oxidizability pattern of T residues within the transcription bubble: a 3–4 fold increase of reactivity rate constants
of those located close to the transcription start point and a smaller, about 1.6-fold one, in the rate constants of those lying further upstream thereof. In a separate study [27], we have also investigated quantitatively the effect of 10 mM Mg\(^{2+}\) on KMnO\(_4\)-oxidation of pyrimidines in double-stranded DNA, and observed a similar increase in their reactivity. Most likely all these effects are due to the shielding by Mg\(^{2+}\) ions of negatively charged groups on DNA and RNAP surfaces lowering thereby repulsive interactions with MnO\(_4^-\) anions.

**MATERIALS AND METHODS**

**Pa promoter DNA and E\(_{70}\) RNA polymerase.** Promoter Pa was obtained, cloned into the pDS3 plasmid and DNA was purified as described earlier [19–21]. RNA polymerase was prepared from E. coli K12 according to [28, 29], using Sephacryl S300 instead of Bio-Gel. Klenow fragment of DNA polymerase I and polynucleotide kinase were from Boehringer (Mannheim), and \([\gamma-\text{32P}])\text{ATP}\) was from Amersham. Two DNA primers, Pr(t) and Pr(nt), complementary to the template (from –138 to –117) and non-template (from +78 to +99) DNA strand, respectively, were synthesized by the solid phase phosphoramidite method, and purified by denaturing PAGE followed by DEAE-Sephacl column chromatography and ethanol precipitation. The 5′-end of the primer was phosphorylated with a 2-fold molar excess of \([\gamma-\text{32P}])\text{ATP}\) by polynucleotide kinase. All other chemicals were molecular biology grade products.

**Open complex formation and KMnO\(_4\) footprinting.** Modification of nucleotides in DNA by KMnO\(_4\) oxidation as a function of the oxidant dose applied and their detection by primer extension were performed according to [30, 31]. To minimize scatter of data, we used premixes that were aliquoted into a series of reactions and volumes that could be accurately pipetted.

A buffered solution of RNA polymerase and supercoiled plasmid pDS3 carrying the investigated promoter Pa was divided into halves, to one MgCl\(_2\) was added to a final concentration of 10 mM and the other supplemented appropriately with pure water; then each solution was aliquoted into 50\(\mu\)l samples containing: 1 pmol of pDS3, 2 pmoles of RNA polymerase, 25 mM Tris/HCl, pH 7.0, 100 mM KCl, 0.2 mM EDTA. For formation of the open complex each sample was placed at constant time intervals in a water bath at 37°C for 15 min. Then 5\(\mu\)l of a KMnO\(_4\) solution (freshly diluted from 0.3 M stock) was added to obtain the desired final concentration of oxidant (0.05, 0.1, and 0.2 mM), and the reaction mixture was incubated for precisely 3 min at 37°C. The reaction was quenched by addition of 150\(\mu\)l of stop solution (containing: 2 mM EDTA, 1% of \(\beta\)-mercaptoethanol and 5 nmoles of marker DNA — the Sall–HindIII fragment of the same plasmid). The samples were deproteinized by phenol/chloroform extraction, the water phase collected and the oxidized DNA purified using a Sephadex G50 spin column.

The samples prepared as above were subjected to quantitative analysis for the extent of oxidation of thymine residues within the transcription bubble located both in the non-template and template DNA strands. For this analysis the same ples were divided into halves and the primer extension reaction was carried out separately for each half with the appropriately designed \(32\text{P}\)-end-labeled primer, Pr(nt) or Pr(t). Reaction conditions were optimized for each strand separately to ensure the specificity of primer’s hybridization. In the case of the non-template strand, 35\(\mu\)l of the purified DNA was mixed with 4\(\mu\)l of 0.01 M NaOH containing 2 pmoles of \(32\text{P}\)-end-labeled primer and denatured at 80°C for 3 min. After cooling to 0°C it was neutralized with 8\(\mu\)l of Klenow reaction buffer (containing 250 mM Tris/HCl, pH 7.2, 50 mM MgSO\(_4\), 1 mM DTT and 2.5 mM of each dNTP) and hybridized by incubation at 45°C for 15 min. Then 2 units of
Klenow fragment of DNA polymerase I enzyme in 2 μl of the enzyme diluent (containing 25% glycerol, 25 mM KH$_2$PO$_4$, pH 7.0, 1 mM DTT and 50 mg/ml BSA) were added and primer extension performed at the same temperature for 10 min. The reaction was quenched by addition of 18 μl of a stop solution (containing 3 M ammonium acetate, 20 mM EDTA and 2 μg tRNA). Products of the Klenow reaction were ethanol precipitated, rinsed with 70% ethanol, dried and dissolved in 10 μl of gel loading buffer (containing 70% freshly deionized formamide, 7 M urea, 3 mM NaOH, 0.1 mM EDTA and 0.02% Bromophenol blue and Xylene cyanol). After denaturing the products at 95°C for 2 min, 3 μl of single-stranded DNA solution were resolved in duplicate on 6% polyacrylamide sequencing gels in TBE buffer (containing 0.089 M Tris/borate and 2 mM EDTA, pH 8.3). Dried gels were exposed to storage phosphor screens from Molecular Dynamics. With respect to the template strand, all the reaction conditions were the same except that neutralization, hybridization and primer extension steps were carried out at 60°C, directly after denaturation at 80°C, with out the cooling step at 0°C.

Each footprinting experiment was duplicated and products of each of the two reactions resolved on 2 footprinting gels, so that on the whole 4 footprints at each oxidant concentration were obtained and analyzed, as described below.

**Phosphorimager analysis and quantification of band intensities.** Images of footprints were obtained using the Molecular Dynamics Phosphorimager. Integrated intensities of bands (or groups of bands) were obtained using the “ImageQuant” software, by the volume integration and area integration options, respectively. For area integration, the lowest intensity point in the graph was used as the horizontal baseline (background). For volume integration, local background was used for bands corresponding to promoter bubble DNA fragments, whereas for the whole lanes gel with out any radioactivity (outside the lanes). These data were analyzed further with software: “Origin™” from MicroCal Software and “Quattro Pro 4.0” from Borland.

The scans were analyzed in three steps. First, the integrated intensity (IQ volume inegration) of the group of bands corresponding to DNA fragments terminated at all oxidized bases within the transcription bubble was measured and normalized to the integrated intensity of the whole lane. Then the distribution of the integrated intensity within the bubble along the lane, an intensity profile, was obtained using the IQ area integration function. Integrated intensities of particular bands were evaluated by deconvolution of the intensity profiles assuming Gaussian distribution of the intensity within the bands and a constant halfwidth for all the bands.

**RESULTS**

Our main goal in this study was to determine the rate constants of oxidation by KMnO$_4$ of individual thymine residues within the melted DNA region of the open complex and evaluation on this basis of the effect of Mg$^{2+}$ ions on the reactivity of these bases and the length of the transcription bubble. For this purpose, the open complex formed by Eσ70 at a strong synthetic promoter Pa, containing –10 and –35 consensus recognition hexamers and the bubble region made exclusively of AT base pairs (Fig. 1), was subjected to increasing concentrations of KMnO$_4$ at a constant exposure time (dose: $x = [\text{KMnO}_4] \times 180$ s) at 37°C in the absence and presence of 10 mM MgCl$_2$ in the reaction buffer. Conditions of the oxidation reaction were chosen according to the commonly used recommendations [31], including KMnO$_4$ concentration high enough to satisfy conditions for pseudo first-order oxidation reaction with pyrimidines, a proper time of exposure to the oxidant and close to physiological
logical 10 mM MgCl$_2$ concentration. Thymine residues oxidized to corresponding thymine glycols [32] were localized on both the nontemplate (nt) and template (t) DNA strands by the technique of DNA primer extension with Klenow fragment of DNA polymerase I, and appropriately designed 5'-32P-labeled DNA primers [31]. This allowed to compare directly the reactivity of the two DNA strands within the melted DNA region of the open complex. End-labeled DNA products, viz. DNA fragments terminated at individual oxidized thymine residues in a given strand within the transcription bubble, were separated on a polyacrylamide sequencing gel yielding footprints exemplified in panels (a) and (c) of Fig. 2. The integrated areas of groups of bands corresponding to oxidized bases within the bubble region, corrected for local background radioactivity and normalized to the integrated intensity of the whole lane, were deconvoluted into individual components assuming Gaussian distribution of the intensity within bands (cf. panels b and d of Fig. 2). Note that due to the termination of the primer extension reaction at a base preceding T$_i$ when the glycol form of the latter was hydrolyzed to an urea derivative during alkaline denaturation [33, 34], some oxidized Ts appear as doublets of bands while those corresponding to T$_n$ runs were unresolved n +1 multiplets (cf. bands in di cated by ar rows in pan els b and d of Fig. 2).Areas un der in di vid ual bands were used to cal cu late fractions $f_i$ of DNA fragments corresponding to particular oxidized T$_i$ residues. Partition of DNA fragments between twin bands due to termination of the primer extension reaction both at a thymine glycol (Tg) and at a preceding base when the glycol form was hydrolyzed to urea (Tu),

$$5'\text{CTGAGTTATTGCAATTATTATATTATTATATTATAATTATTTA-3'}$$
$$\text{nontemplate strand}$$

$$3'\text{TATTTAATTAAATTGAAATTG-5'}$$
$$\text{template strand}$$

direction of Pr(t)(–138...–117) extension

direction of Pr(nt)(+78...+99) extension

**Fig 1. Sequence of the synthetic promoter Pa cloned into pDS3 plasmid.**

Transcription start site (+1) indicated by kinked arrow, 14 AT bp long transcription bubble in bold font, arrows in di cated di rection of ex ten sion of end-labeled prim ers Pr(t) and Pr(nt) by Klenow en zyme (in brackets location relative to the start site).

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Figure 2. KMnO$_4$ footprinting of E$_{70}$–Pa promoter complexes.

Footprinting as a function of KMnO$_4$ concentration in the absence and in the presence of 10 mM MgCl$_2$ was performed as described in Materials and Methods. Panels (a) and (c): autoradiograms of PAGE resolved $^{32}$P-end-labeled DNA products of Klenow primer extension reaction carried out on the non-template and template DNA strands, respectively; KMnO$_4$ concentrations (in mM) indicated at the top of lanes 1–6, “–” and “+” signs indicate the absence and presence of 10 mM MgCl$_2$ in footprinting reactions; along the rightmost lanes are indicated DNA bands corresponding to markers DNA’s (329 nt and 833 nt long in (a) and (c), respectively) and to individual bases of Pa within the bubble region. Panels (b) and (d): selected lanes 4 integrated intensity profiles of Pa bubble (solid lines), deconvoluted into Gaussian components P1–P10 (dotted lines) corresponding to individual oxidized thymines (marked by arrows).
lower than that found for nt strand thymines: T+3, T+2, T–2, T–3 and T–4 in ssDNA form in the RPO. Thus, it can be safely concluded that the transcription bubble region spans at least 14 bp between positions +3 and -11 of the promoter Pa.

It is apparent that while all Ts of the t strand within the transcription bubble did appear oxidizable, T–6, T–7 and T–10 in the –10 region of the nt strand were inaccessible to the oxidant under the single-hit regime. Faint bands due to respective DNA fragments were too weak to be quantified reliably; moreover, they came up more clearly only at higher oxidant doses (not shown) corresponding to multiple-hit reaction, accompanied by simultaneous oxidation of RNAP and severe perturbation of the open complex structure (T. £oziñski, unpublished observation).

The data determined as a function of the oxidant dose (x = ct, [M]) plotted in Figs. 3 and 4, were subjected to kinetic analysis. The plots clearly show the strong and greatly differentiated effect of Mg2+ ions on the oxidizability of particular thymines within the melted DNA region. The range of oxidant doses within which the single-hit footprinting regime could be regarded to prevail was evaluated according to the proposed criteria [18]; in most cases the upper limit was found between 0.018 and 0.036 Ms. The fi(x) data as well as fractions fu(x) of unoxidized DNA corresponding to these conditions conformed to a single exponential functions:

\[ f_i = 1 - \exp(-k_i x) \]  
(1)

\[ f_u = \exp(-\Sigma k_i x) \]  
(2),

where x = ct is the oxidant dose, \( \Sigma k_i \) is the pseudo-first order rate constant of oxidation of an i-th thymine, and \( \Sigma k_i \) a sum taken over all i. At x \( \geq 0.036 \) Ms, \( f \) did not obey a pseudo-first order relationship with the oxidant dose (not shown except for x = 0.036 Ms, cf. data points falling down from the fitted curve in some panels of Figs. 3 and 4; these points were not included in fitting), i.e. the footprinting conditions corresponded to the multiple-hit oxidation regime [18].

The rate constants \( k_i \) were derived with a reasonable accuracy from the global non-linear least squares analysis of all the experimental \( f_i(x) \) and \( f_u(x) \) data according to equations (1) and (2). Inpection of the kinetic data column plotted in Fig. 5a shows that, in the absence of Mg2+ ions, values of the measured \( k_i \) vary in a characteristic way with the location of corresponding T with respect to the +1 transcription start point and greatly differ one from another by a factor up to approx. 20. Thymines located close to the transcription start point: T+1, T–1 of the t strand and T+2 of the nt strand proved to be weakly reactive. The most reactive were T–11, T–9 and T–8 of the t strand and T–3 of its complementary counterpart of the nt strand. In the presence of Mg2+, the reactivity of all Ts in both strands was strongly enhanced (cf. plot of \( k_{i,Mg}/k_i \) values in Fig. 5b). The largest increase in reactivity was exhibited by weakly reactive thymines, especially by T+1 and T–1 of the t strand by a factor of about 3.7 and 4.3, respectively, and T+3 and T+2 of the nt strand by a smaller factor of approx. 2.6. The reactivity of all the remaining Ts, located in either DNA strand more distantly from the transcription start point, was increased by a similar factor of approx. 1.6. Thymines residues within the bubble region of the Pa promoter can be thus divided into two classes of distinctly different reactivity and magnitude of Mg2+ effect: (i) located close to the active center of RPo and (ii) upstream therefrom.

**DISCUSSION**

The presented results of KMnO4 footprinting of the open complex formed by Eσ70 holoenzyme at the cognate Pa promoter clearly demonstrate that, in the absence of
Mg$^{2+}$, the upstream end of the melted DNA domain is defined by the most reactive T-11 in the t strand and the downstream end thereof by T+3 in the nt strand. Since these two thymines are 3'-flanked by unreactive purines, the length of the transcription bubble domain is at least 14 bp long. In the presence of bound Mg$^{2+}$ (at 10 mM MgCl$_2$), the position-dependent reactivity of all the oxidizable Ts is increased in a characteristic manner: strongly of these located in either DNA strand close to the +1 position, and uniformly to a smaller extent of those located more distantly therefrom.

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Figure 3. Kinetics of oxidation of thymines in the bubble region of the non-template DNA strand of the Pa promoter in the open transcription complex at 37°C.

Data points $f_i(x)$ corresponding to DNA fractions of unoxidized DNA ($f_u$, panel a) and oxidized thymines ($f_i$, marked $T_i$ in panels b–f) at different KMnO$_4$ doses ($x$) in the absence (solid squares) and in the presence (solid circles) of 10 mM MgCl$_2$ were obtained by quantification of footprints (exampled in Fig. 2, panels a and c) as described in Materials and Methods; calculated mean standard deviation ($n=4$) was in the range of 5–20% relative to the $f_i(x)$ value. The solid lines correspond to fitted single-exponential functions (eqns. 1 or 2).
Figure 4. Kinetics of oxidation of thymines in the bubble region of the template DNA strand of the Pa promoter in the open transcription complex at 37°C.

Data points $f_i(x)$ corresponding to DNA fractions of unoxidized DNA ($f_u$, panel a) and oxidized thymines ($f_i$, marked T$_i$ in panels b–g) at different KMnO$_4$ doses ($x$) in the absence (solid squares) and in the presence (solid circles) of 10 mM MgCl$_2$ were obtained by quantification of footprints (exemplified in Fig. 2) as described in Materials and Methods. For further explanations see the legend to Fig. 3.
The approx. 14-bp length of the melted DNA domain in the open complex formed by $E\sigma^{70}$ at $Pa$ lies well within the limits of 12–15 bp found to become accessible to footprinting agents in a number of other cognate promoter complexes thus far studied [15, 35]. The length of the melted DNA region is most probably predetermined by location of the −10 region and the +1 site within the structure of $E\sigma^{70}$ [9, 36–39]. According to the recent model of RP0 [39], based on crystal structure of $T$. aquaticus core RNA polymerase at 3.3 Å resolution [9] and over 100 UV-induced crosslinks mapped between individual phosphates of lacUV5 promoter DNA and individual segments of $E\sigma^{70}$ subunits, the extreme downstream end of the transcription bubble including positions +4 to +1 of the $t$ strand and position +4 of the nt strand, binds deep within the active-center cleft while the remainder of the transcription bubble up to position −11 of the $t$ strand rises from the floor of the active-center cleft along an axis perpendicular to the helix axis of the downstream duplex. So the limits of the bubble region found for RP0 at the Pa promoter are in agreement with this model.

The model [39] provides also a structural framework for interpretation of the observed oxidizability pattern of Ts in both single-stranded DNA strands within the bubble region of the open $E\sigma^{70}$–Pa complex. The $t$ strand of the transcription bubble is held within an apparent channel formed by $\beta$ and $\beta'$ subunits leading from the extreme downstream end surrounding position +1 at the floor of the active-center cleft, about 20 Å from the active-center Mg$^{2+}$, to wards the edge of the holoenzyme. This channel is large enough to accommodate the A-form DNA:RNA hybrid formed during initial abortive synthesis of RNA, so that the reactive 5,6 double bond of all the unpaired Ts at and up stream of position +1 should be sterically accessible to MnO$_4^-$ anions (similar in size and shape to orthophosphate). The apparent reactivity of Ts can be expected to depend, how ever, both on

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**Figure 5.** Rate constants of oxidation by KMnO$_4$ of thy min residues in the transcription bubble of the open complex formed at promoter Pa by $E$. coli RNA polymerase.

Panel (a): white and gray columns correspond to $k_i$ determined in the absence of Mg$^{2+}$ and in the presence of 10 mM MgCl$_2$, respectively; panel (b): plot of $k_{i,Mg} / k_i$ ratios. Sequence above the numerical scale, indicating positions of the bases in Pa promoter DNA, corresponds to the non-template strand, sequence below this scale — to the template strand.
sterical and electrostatic accessibility of MnO₄⁻ anions to the reaction center. The low reactivity of Ts at or close to the +1 promoter region, being fully accessible to specific base-pairing with initiating NTPs, together with its marked enhancement in the presence of Mg²⁺ strongly suggest that kinetics of oxidation of these bases is primarily under the control of a high electrostatic barrier. Binding sites for Mg²⁺ within the active-center of RPo are most likely aspartates in the conserved motif NADFDGD of the β' subunit [8, 36]. They were implicated in chelating catalytically active Mg²⁺ ions [8] since their replacement by Fe²⁺ in the open complex of Eσ₇₀ at T7A1 promoter led to cleavage of the template DNA strand around +1 position in the Fenton reaction, whereas Eσ₇₀ carrying the triple D → A mutant in the NADFDGD motif failed to cleave this DNA strand in the presence of Fe²⁺. Inspection of the footprints of RPo formed at the A1T7 promoter by the mutant Eσ₇₀ lacking the specific binding sites for Mg²⁺ in the catalytic center (cf. Fig. 3 of ref. [8]) shows that in the presence of 10 mM MgCl₂ some thymines close to +1 position were weakly oxidized. The anionic binding sites in this region of the latter complex screened by Mg²⁺ are, of course, DNA phosphates. The increased reactivity of Ts residues located in the strand close to the catalytic center of RPo formed by wild Eσ₇₀ can thus be explained reasonably by electrostatic shielding by Mg²⁺ ions of negative charges of aspartates of the NADFDGD motif and of ssDNA phosphates.

The observation that the triple D → A mutant in the NADFDGD motif of Eσ₇₀ is able to form RPo in the absence of Mg²⁺ [8] supports our conclusion that these ions are not required for the open complex formation by wild Eσ₇₀ at the Pa. Recent stopped-flow spectrofluorometric investigations on the kinetics of open complex formation have also indicated that DNA opening is not affected by Mg²⁺ ions [40].

The gradually increasing reactivity of Ts in the t strand with their distance from the +1 promoter site, together with the unimolecular and smaller effect of Mg²⁺ (ki,Mg²⁺/ki = 1.6) for T-5, T-8, T-9 and T-11, suggest that the observed variation in ki may be due to a parallel decrease of the steric cal bar rier for the oxidation towards the edge of the t strand channel, at a constant height of the electrostatic barrier associated with ssDNA phosphates. The non-template strand along the entire length of the bubble domain of RPo, upstream the tightly constrained extreme downstream end within the active-center cleft (see infra), lies in a deep narrow groove the walls of which are made by a fragment of the β subunit, and in the –10 region contacts σ²₀ region 2 “capping” DNA in the active-center cleft [39]. There are several lines of evidence that strongly conserved nt strand adenines: –11, –9 and –8 are involved in specific interactions with residues Y425, Y430, W433 and W434 in the sequence YKFSTYATWW of the conserved subregion 2.3 of σ²₀ forming the ssDNA binding site on the pathway to RPo formation and play an important role in the nucleation and maintenance of promoter melting [35, 39, 41]. All these residues are aligned along one face of the amphipathic helix 14 in the crystal structure of σ²₀ [42] which contains also Q437 implicated in recognition of the conserved -12 A–T base pair, unmelted in most promoters. The very low if any oxidizability of Ts residues in the –10 region of RPo at the Pa promoter: T–10 and T–7 and T–6 of the nt strand of promoter Pa, can be thus explained by their steric cal inaccessibility to MnO₄⁻. Relatively high reactivity of T–4, T–3 and T–2 (cf. Fig. 5) indicates that these bases are similarly accessible to oxidants as the distantly located Ts in the t strand and thus are probably not involved in specific interactions with amino acid side chains of Eσ²₀ do mains in contact with DNA back bone phosphates. The magnitude of the Mg²⁺ effect for these bases, ki,Mg²⁺/ki = 1.6, is the same as for
their distantly located counterparts in the t strand; this indicates that the electrostatic barrier to oxidation is also associated with negative charges of ssDNA phosphates. It is apparent from the RP0 model [39] that thymines T+2 and T+3 are located further apart from the Mg2+ locus within the active-center than T+1 and T-1 of the t strand. This explains why the magnitude of the Mg2+ effect for these bases, \( k_{i,Mg}/k_i = 2.6 \), is distinctly smaller than for those in the t strand.

Reactivity of sterically accessible Ts depends on local effective concentration of MnO4\(^-\), which in turn depends on the extent of condensation of counterions on negatively charged DNA phosphates. Preferential diffusive condensation of Mg2+ at 10 mM concentration in the presence of 100 mM monovalent electrolyte [43] lowers the height of a negative electrostatic barrier making thus thymines residues apparently more reactive towards the oxidant.

Our preliminary footprinting experiments aimed at determination of \( k_i \) in function of Mg2+ concentration indicate that the two classes of Mg-binding sites, aspartates and phosphates, differ in the magnitude of the respective dissociation constant, \( K_d \), being about 3-fold smaller for aspartates within the catalytic center of the open complex. Thus, the different magnitude of the effect of Mg2+ observed for the two groups of Ts seems to be due primarily to different occupancy of binding sites at a given MgCl2 concentration.

The interpretation of the effect of Mg2+ ions on the reactivity of thymines residues outside the catalytic center of the open complex in terms of electrostatic interactions is strongly supported by the results of our parallel studies on their effect on permanganate oxidation of pyrimidines in double-stranded pDS3 plasmid DNA [27]. Reaction rate constants of oxidation of the majority of pyrimidines of the Pa promoter in the double-stranded DNA form, were generally 2–3 orders of magnitude lower than those measured for the same Ts in the single-stranded DNA domain of the open complex, while in the presence of 10 mM Mg2+ were found larger by a factor varying in the range of 2–3 in a sequence-dependent way. A similar effect of Mg2+ on oxidizability of pyrimidines in free dsDNA and within the open transcription complex in ssDNA embedded in protein matrix [39] strongly suggests that in both cases the main mechanism of the enhancement of reactivity of thymines towards MnO4\(^-\) anions is the electrostatic shielding of negative charges of phosphates by counterions condensed on DNA surface.

The reduction by bound Mg2+ of negative charge density due to the E\(\alpha\)^70 carboxylates and DNA phosphates, leading to an increase in local concentrations of MnO4\(^-\) and Fe(EDTA)2\(^-\) anions, has been proposed previously [12] as a possible explanation of the increased reactivity of DNA bases and back bone close the start site of \( \lambda P_R \) promoter in RP0. A similar, though smaller, effect of Mg2+ on modification of thymines by the highly polarized hexacoordinate OsO4–2,2′-bipyridine complex [13, 41] in our opinion can be analogously rationalized. In view of the multistep mechanism of thymine glycolation in the latter reaction [44], the effect of Mg2+ on the reactivity of Ts is probably more complex.

The claimed smaller length of this region in the open complex formed at the \( \lambda P_R \) promoter in the absence of MgCl2, called RP0, and its extension to the full 14 bp length in the transcriptionally competent RP02 form upon binding of Mg2+[11] can be due to insufficient sensitivity of detection of weakly oxidizable Ts residues located close to the +1 position on either DNA strand at the selected single oxidant dose, as they come up more clearly in footprints only in the presence of bound Mg2+ ions. Similar remark seems to apply to “Mg-induced extension” of T7A1 promoter [13] probed by OsO4–2,2’-bipyridine at a very low level of DNA oxidation (estimated as \( \leq 10\% \)), under these conditions DNA bands due
to T+1 and T+2 might have not appeared in the absence of Mg^{2+} owing to the very low reactivity of the two bases.

**CONCLUDING REMARKS**

The presented analysis of kinetics of oxidation by KMnO₄ of thymine residues under controlled single-hit conditions in the open promoter complex formed by E. coli RNA polymerase at the Pa promoter has unequivocally demonstrated that binding of Mg^{2+} ions does not influence the length of the melted DNA region. In the light of the most recent structural model of RP₀ [39], for motion of the open complexes at other cognate promoters may appear also to be independent of Mg^{2+}, because the up per length of the melted DNA region seems to be predetermined by the structure of Eσ^{70}. The obtained distribution pattern of the oxidation rate constants of thymines within the transcription bubble provides a measure of their relative sterical accessibility within each of ssDNA strands in the open complex structure.

Earlier claims that binding of Mg^{2+} ions to the open complex is required for extension of the melted DNA region to its full length, from the nucleation site around –10 position down-stream to the region of the transcription start-point, were based on the single-dose footprinting experiments. In such experiments, if the oxidant dose is too low, oxidation of weakly reactive pyrimidine residues around the +1 region can easily be over looked. The extent of the promoter DNA melting can be, of course, affected by sequence-dependent thermodynamic stability of the bubble region and thus also by temperature at which a footprinting experiment is carried out. A meaningful comparison of the melting properties of open complexes formed at different promoters can be thus made only on the basis of pyrimidine reactivity rate constants towards a given oxidant measured under similar experimental conditions.

**REFERENCES**


