Roles 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+} †

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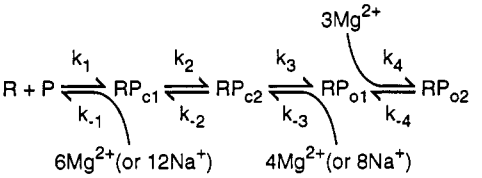
ABSTRACT: Comparative studies of the effects of Mg^{2+} vs Na^+ and of acetate (OAc^-) vs Cl^- on the kinetics of formation and dissociation of *E. coli* RNA polymerase ($E\sigma^{70}$)–λP_R promoter open complexes have been used to probe the mechanism of this interaction. Composite second-order association rate constants k_a and first-order dissociation rate constants k_d, and their power dependences on salt concentration Sk_a ($Sk_a \equiv d \log k_a/d \log [salt]$) and Sk_d ($Sk_d \equiv d \log k_d/d \log [salt]$), were determined in $MgCl_2$ and NaOAc to compare with the results of Roe and Record (1985) in NaCl. Replacement of NaCl by $MgCl_2$ reduces the magnitude of Sk_a 2-fold ($Sk_a = -11.9 \pm 1.1$ in NaCl; $Sk_a = -5.2 \pm 0.3$ in $MgCl_2$) and (by extrapolation) drastically reduces the magnitude of k_a at any specified salt concentration (e.g., $\sim 10^6$-fold at 0.2 M). Replacement of NaCl by NaOAc does not significantly affect Sk_a ($Sk_a = -12.0 \pm 0.7$ in NaOAc) and (by extrapolation) increases k_a by ~80-fold at any fixed $[Na^+]$. In the absence of Mg^{2+}, replacement of NaCl by NaOAc is found to increase the half-life of the open complex by ~560-fold at fixed $[Na^+]$ without affecting Sk_d [$Sk_d = 7.6 \pm 0.1$ in NaOAc; in NaCl, $Sk_d = 7.7 \pm 0.2$ (Roe & Record, 1985)]. Replacement of NaCl by $MgCl_2$ drastically reduces both Sk_d and the half-life of the open complex at any salt concentration below ~0.2 M. Strikingly, $Sk_d = 0.4 \pm 0.1$ in $MgCl_2$, indicating that the net uptake of Mg^{2+} ions in the kinetically significant steps in dissociation of the open complex is much smaller than that expected by analogy with the uptake of ~8 Na^+ ions in the corresponding steps in NaCl. In NaCl/$MgCl_2$ mixtures, at a constant [NaCl] in the range 0.1–0.2 M, initial addition of $MgCl_2$ (0.5 mM $\leq [MgCl_2] \leq$ 1 mM) increases the half-life of the open complex; further addition of $MgCl_2$ causes the half-life to decrease, though the effect of $[MgCl_2]$ on k_d is always less than that predicted by a simple competitive model. The observed effects of $MgCl_2$ on Sk_d and k_d differ profoundly from those expected from the behavior of k_d and Sk_d in NaCl and NaOAc and indicate that the role of Mg^{2+} in dissociation is not merely that of a nonspecific divalent competitor with RNAP for interactions with DNA phosphates and of a DNA helix-stabilizer, both of which should cause k_d to increase monotonically with increasing $[Mg^{2+}]$. These observations provide evidence for differences in the nature of the open complex at the λP_R promoter in the presence and absence of Mg^{2+} and lead us to propose the existence of at least two mechanistically significant forms of the open complex in the presence of Mg^{2+} (RP_{o1}, RP_{o2}). We deduce that the conversion of RP_{o1} to RP_{o2} requires uptake of ~3 Mg^{2+} ions, presumably at specific sites on RNA polymerase, and does not occur in the absence of Mg^{2+} (i.e., in NaCl or NaOAc). We therefore propose a minimal four-step mechanism in which the specific uptake of Mg^{2+} upon conversion of RP_{o1} to RP_{o2} contrasts with nonspecific cation accumulation (either Na^+ or Mg^{2+}, as shown) in the dissociation of the initial closed complex (RP_{c1}) to free promoter DNA (P) and RNA polymerase (R), and in the closing (DNA helix formation) of the initial open complex (RP_{o1}) to form RP_{c2}, the intermediate closed complex which differs from RP_{c1} as the result of a large conformational change in RNA polymerase.



According to this mechanism, the open complex RP_{o1} (formed in the absence or presence of Mg^{2+}) is an obligate intermediate in formation of RP_{o2}, the open complex which initiates transcription upon binding nucleotides.

Univalent and divalent cations are important probes of both the thermodynamics and mechanisms of site-specific protein–DNA interactions. At a thermodynamic level, information about the contribution of ionic interactions and cation release from the DNA polyion to the binding free energy under specified conditions is obtained from analysis of the dependences of apparent equilibrium constants on concentrations of salts of univalent and divalent cations. This is analogous to the use of temperature to dissect enthalpic and entropic contributions to the binding free energy, or to the use of pH to analyze protonation effects. At a kinetic level, the division of individual ion effects between the association and disso-

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ciation rate constants indicates whether the mechanism is elementary or multistep in each direction and provides information about the nature of the steps and the important intermediates. For reviews of these analytical strategies, see Record et al. (1978, 1991), Lohman (1985), and Lohman and Mascotti (1991).

Roe and Record (1985) investigated the effects of NaCl concentration on the kinetics of formation (k_a) and dissociation (k_d) of "open" complexes (RP_o) between *E. coli* RNA polymerase $E\sigma^{70}$ (designated RNAP in the text and by the symbol R in formulas) and the λP_R promoter. In the absence of other cations, the overall second-order association rate constant $k_a \propto [NaCl]^{-12}$ and the dissociation rate constant $k_d \propto [NaCl]^{8}$, so that the overall equilibrium constant for association to form open complexes $K_{obs} \equiv k_a/k_d \propto [NaCl]^{-20}$. These strong dependences of k_a and k_d on salt concentration, along with the unusual temperature dependences of these rate constants [a negative activation energy of dissociation and large magnitudes of the activation heat capacities of both association and dissociation (Roe et al., 1984, 1985)], provided strong supporting evidence for a three-step mechanism (summarized below) in which the initial step in both the association and dissociation directions rapidly equilibrates on the time scale of the second step on the pathway, and in which this second step (in both directions) involves a large conformational change in RNAP as part of the interconversion of two "closed" complexes (RP_{c1} and RP_{c2}, also designated I_1 and I_2 or RP_c and RP_i by various investigators) [cf. reviews by Buc (1989), Leirmo and Record (1990), and Record et al. (1991)].

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RP_{c1} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RP_{c2} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} RP_o \qquad (1)$$

In the *minimal* mechanism of eq 1, R is RNA polymerase $E\sigma^{70}$ (RNAP), P is the promoter DNA, and RP_{c1} is the initial closed complex. The species RP_{c2} is a second (intermediate) closed complex (Roe et al., 1984; Buc & McClure, 1985). In several polymerase–promoter systems RP_{c2} has been found to protect a greater length of promoter DNA from DNase digestion than RP_{c1} (Spassky et al., 1985; Cowing et al., 1989). The interconversion of RP_{c1} and RP_{c2} is thought to involve a large conformational change in RNAP which alters the amount of nonpolar surface exposed to water (Roe et al., 1985; Record, 1988; Ha et al., 1989). In mechanism 1, RP_o refers generally to open complexes in which the DNA strands typically are denatured over a region of 11–16 bp between the −10 region and the vicinity of the start site of transcription (Siebenlist et al., 1980; Kirkegaard et al., 1983; O'Halloran et al., 1989; Newlands et al., 1991). The kinetic studies presented here lead us to propose that the nature of the open complex depends on the presence or absence of Mg^{2+} ion.

In the present work, we use Mg^{2+}, Na^+, Cl^-, and acetate (OAc^-) to probe the steps of open complex formation involving RNAP $E\sigma^{70}$ and the λP_R promoter. Our primary method of obtaining kinetic data is the fluorescence-detected abortive initiation (FDAI) assay (Yarbrough et al., 1979; Bertrand-Burggraf et al., 1984). Interpretation of the effects of $[Mg^{2+}]$ on the overall rate constants k_a and k_d provides evidence for two mechanistically significant open complexes (RP_{o1}, RP_{o2}). In the absence of Mg^{2+}, the open complex at the λP_R is designated RP_{o1}; we propose that, in the presence of Mg^{2+}, RP_{o1} isomerizes to RP_{o2}, the transcriptionally competent open complex. Both kinetic data and $KMnO_4$ chemical probing of the open complex (Suh, W. C., Ross, W., and Record, M. T. Jr., submitted) are consistent with these minimal mechanisms in the absence and presence of Mg^{2+}.

Background: Kinetics of RNA Polymerase–Promoter Interactions; Analysis of Ion Effects on Rate Constants. Experimental studies of the kinetics of *formation* of open complexes between RNAP and promoters *in excess RNAP* yield first-order time constants τ_{obs} for formation of an open complex which in general are composed of additive contributions from [RNAP]-dependent (pseudo-first-order) and [RNAP]-independent (first-order) terms:

$$-\mathrm{d} \ln ([P]_T - [RP_o])/\mathrm{d}t \equiv k_{obs} \equiv \tau_{obs}^{-1} = [(k_a[R]_T)^{-1} + k_i^{-1}]^{-1} \qquad (2)$$

where $[P]_T$ and $[R]_T$ are the total concentrations of promoter and RNAP, respectively, k_{obs} is the observed first-order rate constant, k_a is a composite second-order association rate constant, and k_i is a composite first-order (isomerization) rate constant [McClure (1980); cf. Leirmo and Record (1990) and Record et al. (1991) for reviews]. [In this section, no distinction is made between the two forms of the open complex proposed below. Strictly speaking, the subsequent analysis is therefore applicable to the first open complex (RP_{o1}) which is formed in the absence of Mg^{2+}. Mechanism 1 is modified to include RP_{o2} in the Discussion.]

Both filter binding and abortive initiation assays of the association kinetics are performed in excess protein (pseudo-first-order conditions) and under solution conditions where open complex formation is essentially irreversible. The time constant τ_{obs} is determined from the lag in the approach to a steady-state rate of abortive product synthesis. The dependence of τ_{obs} on protein concentration is typically displayed in a double-reciprocal plot [called a τ-plot; McClure (1980), cf. Figure 2 below], in which $k_{obs}^{-1} = \tau_{obs}$ is plotted as a function of the reciprocal of the total active RNAP concentration ($[R]_T^{-1}$). From eq 2, the slope of the τ-plot is k_a^{-1} and the intercept is k_i^{-1}. It is important to distinguish between the *observed* composite rate constants that are obtained directly from the data (here designated k_a and k_i) and the mechanism-dependent equilibrium constants and elementary or composite rate constants that are deduced from the observed rate constants using assumptions regarding the relative rates of various steps.

In the *association* reaction of RNAP with the λP_R promoter, the kinetically significant steps under the conditions investigated appear to be

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RP_{c1} \overset{k_2}{\rightarrow} RP_{c2} \overset{k_3}{\rightarrow} RP_o \qquad (3)$$

Furthermore, under the conditions investigated for the λP_R promoter, it appears valid to assume that $k_2, k_3 \gg k_{-2}$ and $k_{-1} \gg k_2$ (Roe et al. 1984, 1985; Record, et al., 1991), so that the general steady-state analysis of the mechanism yields the relationships

$$k_a \simeq k_1k_2/k_{-1} = K_1\,k_2 \qquad (4)$$

$$k_i \simeq k_2k_3/(k_2 + k_3) = (k_2^{-1} + k_3^{-1})^{-1} \qquad (5)$$

In the present work it is found that only the composite second-order rate constant k_a (and not the composite isomerization rate constant k_i) can be determined from manual-mixing

studies on the λP_R promoter at or above 25 °C. (Under these conditions k_i^{-1} is zero within experimental uncertainty.)

For the *dissociation* of RP_o, the mechanism is proposed to be

$$RP_o \underset{k_3}{\overset{k_{-3}}{\rightleftarrows}} RP_{c2} \underset{k_2}{\overset{k_{-2}}{\rightleftarrows}} RP_{c1} \overset{k_{-1}}{\rightarrow} R + P \quad (6)$$

The final dissociation step is rendered irreversible via the presence of a competitor. Applying the assumptions k_2, $k_3 \gg k_{-2}$ and $k_{-1} \gg k_2$ (which were used to simplify the expressions for k_a and k_i in eqs 4 and 5), the general steady-state analysis of this mechanism yields

$$k_d = K_3^{-1} k_{-2} \quad (7)$$

At conditions where the above rapid equilibrium approximations (eqs 4 and 7) hold for the association and dissociation reactions, the power dependences on salt concentration of k_a ($Sk_a \equiv$ d log k_a/d log [salt]) and k_d ($Sk_d \equiv$ d log k_d/d log [salt]) are given by

$$Sk_a = SK_1 + Sk_2 \quad (\text{where } SK_1 \equiv \text{d log } K_1/\text{d log [salt]} = Sk_1 - Sk_{-1}) \quad (8)$$

$$Sk_d = -SK_3 + Sk_{-2} \quad (\text{where } SK_3 \equiv \text{d log } K_3/\text{d log [salt]} = Sk_3 - Sk_{-3}) \quad (9)$$

The most significant contribution to Sk_a is thought to be from Sk_{-1} (the salt concentration dependence of the elementary dissociation rate constant of the closed complex), because the reassociation of counterions contributes to the entropic activation barrier for dissociation of RP_{c1} (Lohman et al., 1978; Leirmo & Record, 1990). Neither Sk_1 nor Sk_2 is expected to be large in magnitude for this mechanism on theoretical grounds (Leirmo & Record, 1990). The most significant contribution to Sk_d is expected to be from Sk_{-3}, resulting from reassociation of counterions when the DNA renatures and when the surface area involved in protein–DNA contacts is reduced upon conversion of the open complex to the closed intermediate RP_{c2}. Record et al. (1991) and Lohman and Mascotti (1991) reviewed general strategies for using ions and other physical variables as probes of the kinetics and thermodynamics of protein–DNA interactions.

MATERIALS AND METHODS

Enzymes and DNA. Restriction enzymes, calf intestinal alkaline phosphatase, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). *E. coli* K12 RNA polymerase holoenzyme ($E\sigma^{70}$, 80 ± 10% σ-saturated) was purified as described previously (Hager et al., 1990) and was a gift of Dr. Richard Burgess. The $E\sigma^{70}$ used in this work was 40 ± 10% active as assayed both by filter binding to the λP_R promoter under stoichiometric binding conditions [by the method of Roe et al. (1984)] and by the abortive initiation method (McClure et al., 1978). Concentrations reported in the text represent active enzyme concentrations. For measurement of binding activity and kinetic constants, dilutions of the enzyme were made in reaction buffer as described by Chamberlin et al. (1983) and stored on ice. Before the reaction was initiated, enzyme and DNA were incubated separately at the reaction temperature for 5 min.

An 890-bp *Pvu*II restriction fragment containing the λP_R promoter was isolated from pGR40 as previously described (Leirmo et al., 1987) and used for all assays. DNA for filter binding assays was labeled at both ends using T4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). This restriction fragment contains the wild-type λ control region, including the λP_{RM} promoter. Open complexes formed specifically at the λP_R promoter were assayed by inclusion of the promoter-specific initiating dinucleotide CpA in both filter binding and abortive initiation assays. (Abortive products formed at the λP_{RM} promoter are initiated with UpA.) Cleavage of the fragments with *Hinc*II, which cuts at –38 in the λP_R sequence, eliminated CpApU synthesis in abortive initiation assays. Substitution of UpA for CpA in filter binding assays reduced filter retention to background (minus NTP) levels. These observations were independent of the salt used in the reaction mixtures.

Reagents and Chemicals. All salts used were of the highest grade available and were obtained from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), and Fluka (Ronkonkoma, NY), with no source-dependent differences noted. Magnesium salts were dried to constant weight before stock solutions were prepared. Total Mg^{2+} concentrations in stock solutions were determined by a calorimetric calmagite binding assay available in kit form from Sigma (Procedure No. 595). Ribonucleotides UTP and GTP were ultrapure grade from Pharmacia (Piscataway, NJ). CpA was from Sigma and ICN (Costa Mesa, CA). HEPES, BSA, DTT, and heparin (176 units/mg) were from Sigma.

The synthesis and purification of γ-aminonaphthalene-sulfonate-UTP (γ-ANS-UTP) (the fluorescent UTP analog used in the abortive initiation experiments) were performed as described by Yarbrough et al. (1979). UTP was from Sigma, 5-aminonaphthalene-1-sulfonate (ANS) was from Fluka, and carbodiimide was from Pierce (Rockford, IL). We have observed some variation in the stability of the fluorescence emission of γ-ANS-UTP in the form of nonsystematic signal drift. This drift is typically on the order of 10–15% of the overall signal change in a lag assay. This drift appears to be random in character and is reflected in the deviation of τ-plot data from perfect linearity and in the errors reported for the association and dissociation rate constants.

FDAI Assay of Association Rate Constants (k_a). Association kinetic studies in $MgCl_2$ were performed at 25 °C with the fluorescence-detected abortive initiation (FDAI) assay (Yarbrough et al., 1979; Bertrand-Burggraf et al., 1984) at a promoter DNA concentration of 1 nM over a range of RNAP concentrations from 5 to 200 nM in 0.025 M HEPES buffer (pH 7.5 at 25 °C; 0.012 M K^+), 10^{-3} M DTT, 0.1 mg/mL BSA, and 0.03–0.05 M $MgCl_2$. An SLM Aminco 8000C spectrofluorometer was used to monitor γ-ANS-UTP fluorescence intensities at 500 nm with excitation at 360 nm. Reactions were initiated by addition of DNA to the fluorometer cuvette, using a plastic plunger to manually mix the solution in approximately 20–25 s. Data were taken every 1–10 s with a maximum of 1000 points per sample for a period of time ≥7 time constants (τ_{obs}) for the reaction. Fluorescence intensity measurements are expressed as ratios of the fluorescence of the reaction cell to that of a reference cell containing 5 mg/mL rhodamine B. Data were stored on an IBM PC and transferred to a VAX 8650 for analysis by a nonlinear least-squares fit to

$$N = Vt - V\tau_{obs}(1 - e^{-t/\tau_{obs}}) \quad (10)$$

where N is the product per promoter, 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 (Hawley & McClure, 1982). No weighting factors were used. Standard errors in τ_{obs} and V as estimated by the Marquardt algorithm

(Bevington, 1969) were typically less than standard errors obtained from repeated measurements. Steady-state rates (V) obtained by computer fits of lag assays agree with those obtained in control reactions initiated by addition of nucleotides to preformed complexes (±10%). Steady-state rates determined in lag assays at different protein concentrations under the same set of solution conditions also met this criterion.

Nitrocellulose Filter Binding Assay for Association Rate Constants (k_a). The kinetics of association in NaOAc at 25 °C to form promoter-specific (open) complexes were observed by a specific filter binding assay utilizing NTP stabilization and a high-salt challenge, as described in Roe et al. (1984). Binding studies were performed as a function of NaOAc concentration (0.30–0.37 M) in a buffer containing 0.04 M HEPES (pH 7.5 at 25 °C; 0.02 M Na^+), 10^{-3} M DTT, and 0.1 mg/mL BSA. Reactions were initiated by the addition of RNAP. Aliquots (usually 25 μL) were removed as a function of time and rapidly mixed with a 10-fold greater volume of initiation buffer containing 0.025 M HEPES (pH 7.5), 0.01 M $MgCl_2$, and 2×10^{-4} M CpA, UTP, and GTP for 10 s at 25 °C before filtration. Filters were rinsed with 1 mL of a wash buffer containing 0.025 M HEPES (pH 7.5), 1 M NaCl, and 10^{-4} M EDTA. Background filter retention was measured as a function of time in each reaction by mixing with initiation buffer which did not contain NTPs; this background depended upon the quality of the DNA sample, was constant for a given sample, and was always in the range from 10 to 20% of the total input counts. Filter retention at the plateau in the time course ranged from 60 to 80% of the total input counts. Dried filters were counted by Cerenkov radiation. Analysis of individual measurements of k_{obs} was performed as described by Roe et al. (1984).

FDAI Assay of Dissociation Rate Constants (k_d). Dissociation kinetics in NaOAc, $MgCl_2$, and mixed $MgCl_2$/NaCl were measured using a "fixed-time" FDAI sampling assay to detect the decay of the concentration of promoter-specific open complexes with time after addition of an excess of the competitor heparin. Typically RNAP (20 nM) and DNA (2 nM) were preincubated in binding buffer containing varying concentrations of NaOAc, $MgCl_2$, or $MgCl_2$/NaCl for 30 min at 25.0 ± 0.1 °C. For experiments in $MgCl_2$ and $MgCl_2$/NaCl mixtures, the buffer was 0.01 M HEPES (pH 7.5; 0.005 M K^+), 10^{-3} M DTT, and 0.1 mg/mL BSA. For experiments in NaOAc, the buffer was 0.04 M HEPES (pH 7.5; 0.02 M Na^+), 10^{-3} M DTT, and 0.1 mg/mL BSA. Heparin was added to a final concentration of 5–15 μg/mL in $MgCl_2$ or $MgCl_2$/NaCl and 60–100 μg/mL in NaOAc. Aliquots were removed before heparin addition and at various times after heparin addition and mixed with an assay solution at 25 °C containing a final concentration of 10^{-4} M γ-ANS-UTP, 10^{-3} M CpA, and 5×10^{-3} M $MgCl_2$. The solution was added to the temperature-equilibrated cuvette, and the fluorescence intensity was monitored as described above.

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 time t divided by the steady-state rate prior to addition of heparin ($t = 0$). The first-order dissociation rate constant k_d was determined from a plot of log θ vs t. In order to examine the effectiveness of heparin competition at the concentrations of heparin and electrolyte ions investigated here, order-of-addition control experiments were performed over the range 0.01–0.06 M [$MgCl_2$] by addition of 20 nM RNAP to the appropriate binding buffer containing 2 nM DNA, CpA, γ-ANS-UTP, and heparin. The steady-state rate observed in these control experiments was less than 5% of the zero-time steady-state rate in the corresponding dissociation experiments, indicating that heparin is an effective competitor under our conditions. Dissociation rate constants were found to be independent of heparin concentration over the range investigated.

Calculation of Mg^{2+} Binding Density (ν_{Mg}) on DNA and the Effect of Mg^{2+} Competition on k_d of RP_o Complexes in Mixed $MgCl_2$/NaCl Salts. Binding densities of Mg^{2+} on DNA (ν_{Mg}, in moles of DNA-associated Mg^{2+} per mole of DNA phosphate) were calculated using the binding model of ligands to a one-dimensional homogeneous lattice (McGhee & von Hippel, 1974). All the anticooperativity of binding of Mg^{2+} to DNA is parameterized using the overlap parameter $n = 2$ (i.e., each phosphate is considered to be a potential binding "site", but one Mg^{2+} "overlaps" two phosphates on binding). For this case, the Mg^{2+}–DNA binding isotherm is

$$\nu_{Mg}/[Mg^{2+}] = K_{obs}^{Mg}(1 - 2\nu_{Mg})^2(1 - \nu_{Mg})^{-1} \quad (11)$$

The applicability of this equation to Mg^{2+} effects on DNA conformational transitions and on binding of other ligands to DNA has been examined (Record, 1975; Record et al., 1976, 1977). The equilibrium constant K_{obs}^{Mg} for the Mg^{2+}–DNA interaction is strongly dependent on the [Na^+] in the mixed Na^+/Mg^{2+} salt and may exhibit a small dependence on the buffer. In HEPES buffer (the buffer used in the present experiments), Shaner et al. (1983) found that

$$\log K_{obs}^{Mg} = -(1.58 \pm 0.46) \log [Na^+] + (0.52 \pm 0.38) \quad (12)$$

Record et al. (1977) found that the effects of Mg^{2+} on specific and nonspecific binding of *lac* repressor are quantitatively interpreted as a three-way competition between repressor, Mg^{2+}, and Na^+ for the vicinity of DNA phosphates. If the effect of Mg^{2+} on the rate constant k_d is modeled as competition at the level of the equilibrium constant K_3, then we predict that k_d for dissociation of open complexes will vary with [Mg^{2+}] and [Na^+] according to the equation

$$\log k_d = \log k_o + 0.88z \log [Na^+] + z \log \{0.5[1 + (1 + 4K_{obs}^{Mg}[Mg^{2+}])^{0.5}]\} \quad (13)$$

where k_d is the observed dissociation rate constant in mixed $MgCl_2$/NaCl buffer and k_o is the extrapolated value of k_d at 1 M Na^+ in the absence of Mg^{2+}. From eq 13, the competitive effect of Mg^{2+} is predicted to increase k_d to an extent which increases with increasing [Mg^{2+}] and with decreasing [Na^+]. In general, log k_d varies nonlinearly with log [Na^+] in the presence of a fixed [Mg^{2+}].

RESULTS

Fluorescence-Detected Abortive Initiation Assay for Association Kinetics. Bertrand-Burggraf et al. (1984) modified the radiolabel-detected (discrete-time) abortive initiation assay developed by McClure (1980) to use continuous spectrophotometric detection of abortive product synthesis. The fluorescence of ANS is quenched when it is covalently attached to the γ-phosphate of UTP. As UTP is hydrolyzed in abortive product synthesis, ANS-pyrophosphate is generated and fluorescence intensity increases. We have utilized fluorescence-detected abortive initiation whenever possible to investigate the kinetics of association since the continuous assay allows for more efficient data collection. Abortive initiation is not suitable for all applications, however, since it requires the continuous presence of Mg^{2+} and the initiating nucleotides during the course of the association reaction.

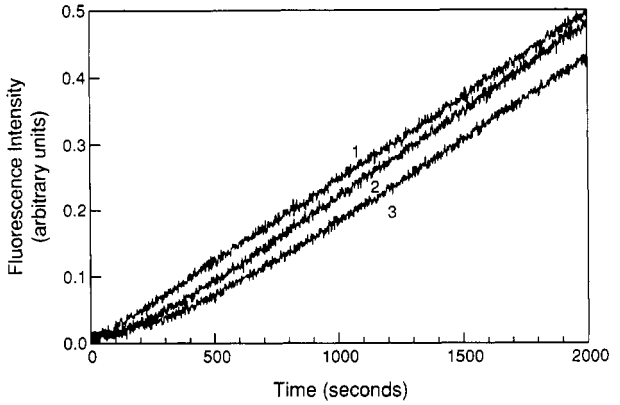

FIGURE 1: Representative FDAI lag assays for the kinetics of association of RNAP with the λP_R promoter in 40 mM $MgCl_2$ (HEPES buffer, pH 7.5). The assays shown were performed at 25 °C; reaction conditions are given in the text. Fluorescence intensities are in arbitrary units. Line 1 is the steady-state rate (0.24×10^{-3} s^{-1}) of abortive product synthesis in a reaction initiated by addition of CpA and γ-ANS-UTP to preformed complexes ([RNAP] = 100 nM, [DNA] = 1 nM). Curve 2 is the lag plot obtained when abortive product synthesis was initiated by addition of 33 nM RNAP to DNA (1 nM), CpA, and γ-ANS-UTP; nonlinear least-squares analysis of this lag plot by eq 10 yields $\tau_{obs} = 181 \pm 3$ s and a steady-state rate of 0.26×10^{-3} s^{-1}. Curve 3 is the lag plot obtained by addition of 20 nM RNAP to DNA (1 nM), CpA, and γ-ANS-UTP; in this case $\tau_{obs} = 346 \pm 5$ s and the steady-state rate is 0.26×10^{-3} s^{-1}. These τ_{obs} are plotted in Figure 2.

Figure 1 illustrates representative data from FDAI assays of the kinetics of association of RNAP with the λP_R promoter in 40 mM $MgCl_2$ at 25 °C. Fluorescence intensity is plotted as a function of time and is directly proportional to the amount of γ-ANS-UTP hydrolyzed and thus to the amount of CpApU produced. When protein and DNA are preincubated to allow the full extent of open complex formation and the reaction is initiated by the addition of CpA and γ-ANS-UTP, line 1 (Figure 1) is obtained. This line passes through the origin and gives the steady-state rate of CpApU synthesis from preformed complexes. In contrast, when the reaction is initiated by the addition of promoter DNA to a cuvette containing RNAP and nucleotide substrates, a lag is observed before the full steady-state rate of CpApU synthesis is reached. This lag is a function of protein concentration: in Figure 1, the lag is approximately 180 s at 33 nM RNAP (curve 2) and 350 s at 20 nM RNAP (curve 3). The data of Figure 1 are included in the τ-plot obtained at 40 mM $MgCl_2$ in Figure 2.

Comparison of Effects of Na^+ and Mg^{2+}: Addition of Mg^{2+} Reduces k_a and Sk_a. The kinetics of association of RNAP with the λP_R promoter were investigated using the FDAI assay as a function of [$MgCl_2$] to compare with the value of Sk_a determined in NaCl. This comparison allows us to assess the role of Mg^{2+} in association and to dissect the cation contribution to Sk_a. In Figure 2, lag times (τ_{obs}) for open complex formation at the λP_R promoter at three $MgCl_2$ concentrations at 25 °C are plotted vs reciprocal total polymerase concentration ($[R]_T^{-1}$). The slopes of the individual τ-plots increase with increasing [$MgCl_2$], indicating that k_a decreases as [$MgCl_2$] increases from 30 to 50 mM. Qualitatively this is the result expected if Mg^{2+} competes with RNA polymerase at the level of closed complex (RP_c) formation, as is observed with univalent cations at higher salt concentration (Roe & Record, 1985; Leirmo et al., 1987). Intercepts of these plots are zero within experimental uncertainty. Therefore, second-order rate constants (k_a) were calculated by assuming a τ-intercept of zero [$k_a = (\tau_{obs}[R]_T)^{-1}$] and taking the mean of the values of k_a calculated at each polymerase concentration (cf. Table I). (Values of k_a determined from the nonweighted least-squares slope of the τ-plots are systematically lower than those tabulated in Table I by an amount which in general does not exceed the experimental uncertainty. Systematic use of these values does not significantly affect Sk_a).

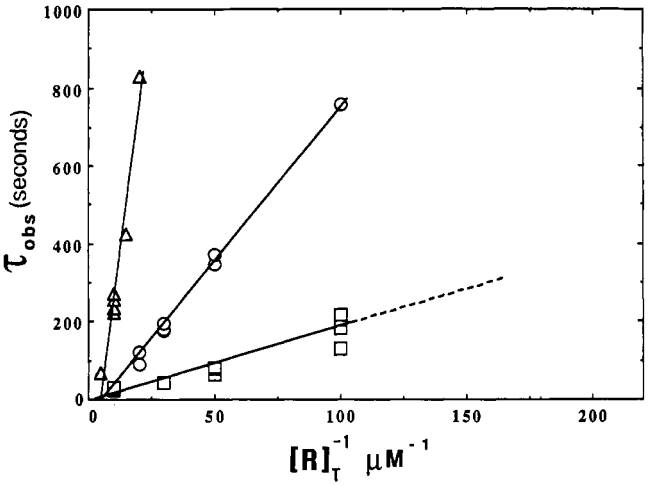

FIGURE 2: Double-reciprocal plots (τ-plots) of $\tau_{obs} = k_{obs}^{-1}$ (in seconds) vs $[R]_T^{-1}$ [the concentration (μM) of RNAP, in excess over promoter DNA] at 50 mM $MgCl_2$ (△), 40 mM $MgCl_2$ (○), and 30 mM $MgCl_2$ (□) demonstrating the effect of [$MgCl_2$] on kinetics of association. The lines represent least-squares fits to eq 1. Within experimental uncertainty, values of k_i^{-1} determined from intercepts of these τ-plots are equal to zero, and hence association rate constants k_a are calculated directly from $k_a = (\tau_{obs}[R]_T)^{-1}$. Average values of k_a at each [$MgCl_2$] are listed in Table I and plotted in Figure 3A.

Table I: Dependence of Rate and Equilibrium Constants of RNAP–λP_R Interaction on [$MgCl_2$] at 25 °C

[$MgCl_2$] (mM)	k_a (M^{-1} s^{-1})[a]	k_d (s^{-1})[b]	$K_{obs}^{RP_{o2}} = k_a/k_d$ (M^{-1})
5		$5.2 (\pm 0.7) \times 10^{-5}$	
10		$5.2 (\pm 0.6) \times 10^{-5}$	
20		$9.0 (\pm 0.8) \times 10^{-5}$	
30	$5.7 (\pm 1.5) \times 10^5$	$9.9 (\pm 0.9) \times 10^{-5}$	$5.8 (\pm 1.6) \times 10^9$
40	$1.5 (\pm 0.2) \times 10^5$	$1.0 (\pm 0.1) \times 10^{-4}$	$1.5 (\pm 0.3) \times 10^9$
50	$3.5 (\pm 1.0) \times 10^4$	$1.2 (\pm 0.1) \times 10^{-4}$	$2.9 (\pm 0.9) \times 10^8$
60		$1.4 (\pm 0.1) \times 10^{-4}$	

[a] Average of 6–20 determinations over a range of $[R]_T$, calculated by assuming $k_i = 0$ and $k_a \cong (\tau_{obs}[R_T])^{-1}$. [b] k_d (±1 standard deviation) from analysis of 4–20 fixed-time FDAI measurements of the fraction of open complexes remaining (θ) in an excess of the competitor heparin.

Figure 3 represents the association kinetic data of Table I as a log–log plot of k_a vs [$MgCl_2$]. The least-squares slope of the linear plot is the power dependence of k_a on [$MgCl_2$]. The corresponding dependence of k_a on [NaCl] is shown for comparison (Roe & Record, 1985). The value of Sk_a in $MgCl_2$ ($Sk_a = -5.2 \pm 0.3$) is approximately half of that in NaCl ($Sk_a = -11.9 \pm 1.1$). This result is consistent with that expected if primarily cations (and not anions) act as stoichiometric participants in the kinetically significant association steps [cf. Record et al. (1977, 1991)] and suggests that Mg^{2+} is acting as a divalent (otherwise nonspecific) cation competitor with RNAP at the level of the first closed complex (RP_{c1}).

Linear extrapolation of the data of Figure 3A to 0.2 M salt concentration (cf. Figure 3B) indicates that k_a increases by ~10^6-fold when 0.2 M $MgCl_2$ is replaced by 0.2 M NaCl. Equivalently, the same absolute value of k_a is obtained in solutions of $MgCl_2$ and NaCl that differ in cation concentration by ~1 order of magnitude. For example, we find that $k_a = 6 \times 10^5$ M^{-1} s^{-1} at ~0.03 M Mg^{2+} (in $MgCl_2$) and at ~0.30

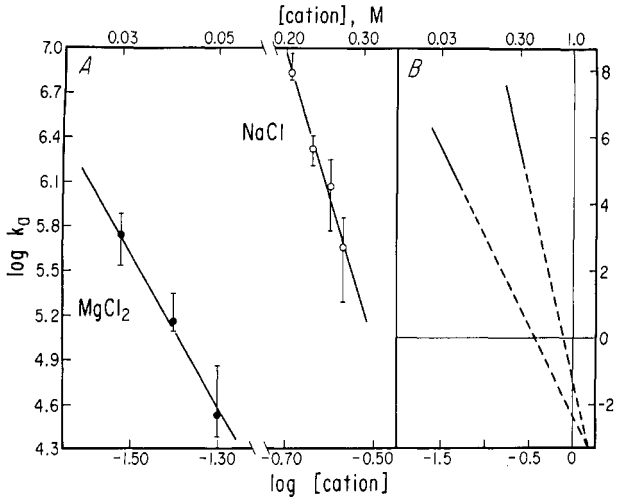

FIGURE 3: Dependence of k_a on [$MgCl_2$] and comparison with behavior in NaCl. (A) log–log plot of association rate constants $k_a \equiv (\tau_{obs}[R]_T)^{-1}$ from Figure 2 vs [$MgCl_2$]. Linear least-squares fit is shown: $\log k_a = -(2.2 \pm 0.4) - (5.2 \pm 0.3) \log [MgCl_2]$. For comparison, the data of Roe and Record (1985) for NaCl are plotted: $\log k_a = -(1.2 \pm 0.7) - (11.9 \pm 1.1) \log [NaCl]$. (B) Extrapolation of data in (A) to 1 M [salt]. Solid lines indicate regions defined by data of (A).

M Na^+ (in NaCl). These effects of replacement of $MgCl_2$ by NaCl are similar to those observed for equilibrium constants for the nonspecific association of *lac* repressor with helical DNA (deHaseth et al., 1977) and are a consequence of the large cation stoichiometries and the relatively greater effectiveness of Mg^{2+} than Na^+ as a competitor with RNAP at the level of formation of the first closed complex RP_{c1}. If the data are linearly extrapolated to 1 M salt (cf. Figure 3B), the higher intercept obtained in NaCl than in $MgCl_2$ is also consistent with this interpretation, though the error ranges marginally overlap. Since the intrinsic affinity of Mg^{2+} for DNA is higher than that of Na^+, as measured both by equilibrium dialysis (Braunlin et al., 1982) and by competitive effects of [Mg^{2+}] on binding of oligocations and proteins to DNA as a function of [Na^+] (Lohman et al., 1980, Shaner et al., 1983), closed complex formation is less favorable under standard-state conditions (i.e., 1 M salt) in the presence of Mg^{2+} than in Na^+, and hence the extrapolated k_a at 1 M salt is expected to be smaller in Mg^{2+}. The large magnitudes of Sk_a in NaCl and in $MgCl_2$ justify the use of the rapid equilibrium approximation for formation of RP_{c1} [as opposed to an irreversible reaction step; cf. Record et al. (1991)] in analysis of the mechanism in NaCl and in $MgCl_2$ (i.e., $k_a \cong K_1k_2$; cf. eq 4).

Anomalous Reduction in Sk_d on Replacement of NaCl by $MgCl_2$. The effect of Mg^{2+} on the first-order rate constant k_d for dissociation of the open complex between *E. coli* RNA polymerase and λP_R promoter was investigated using the FDA assay in $MgCl_2$ (without added NaCl) at 25 °C. A typical set of steady-state rate measurements taken as a function of time after addition of heparin are shown in Figure 4. The steady-state rate is directly proportional to the amount of active open complexes remaining in the overall population. Figure 5 demonstrates that log k_d is a linear function of log [$MgCl_2$] over the range 0.005–0.06 M. From the slope, $Sk_d \equiv d \log k_d / d \log [MgCl_2] = 0.4 \pm 0.1$, indicating that a net 0.4 Mg^{2+} ions are taken up upon dissociation of the open complex, if the Cl^- stoichiometry is negligible. This result appears inconsistent with that obtained in univalent salt ($Sk_d = 7.7 \pm 0.2$), which indicates a net uptake of 7.7 ± 0.2 univalent ions in the kinetically significant steps of the dissociation

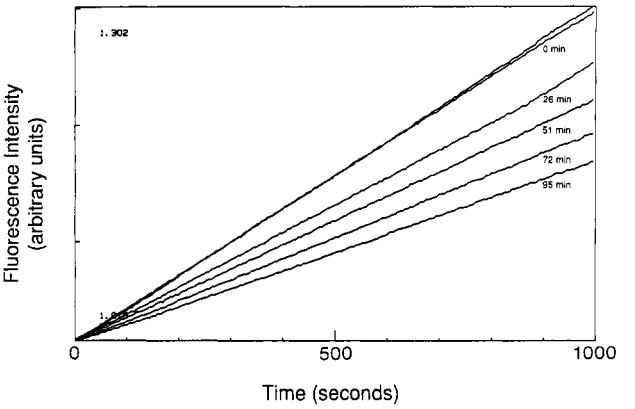

FIGURE 4: Representative FDAI assays for the kinetics of irreversible dissociation of open complexes in 50 mM $MgCl_2$, HEPES buffer, 25 °C, with excess heparin (7.5 μg/mL) as competitor. After preincubation of RNAP (20 nM) and DNA (2 nM) for 30 min, heparin was added ($t = 0$). Aliquots were removed as a function of time, and CpApU formation was monitored by the FDAI assay. Upper line: before heparin addition. Lower lines: 0, 26, 51, 72, and 95 min after heparin addition.

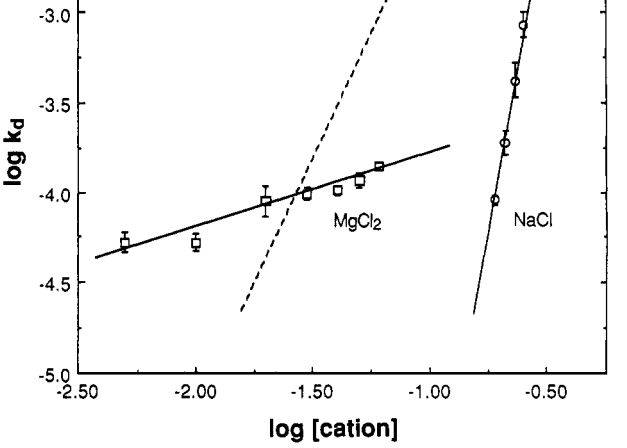

FIGURE 5: Dependence of k_d on [$MgCl_2$] and comparison with behavior in NaCl. log–log plot of dissociation rate constants k_d vs [$MgCl_2$], including data of Figure 4. The dashed line is the curve predicted by the competitive model, assuming $Sk_d(MgCl_2) \equiv d \log k_d / d \log [MgCl_2] = 3.8$ [i.e., half the value of $Sk_d(NaCl) \equiv d \log k_d / d \log [NaCl] = 7.7$]. The linear least-squares fit is shown: $\log k_d = -(3.4 \pm 0.1) + (0.4 \pm 0.1) \log [MgCl_2]$. For comparison, the data of Roe and Record (1985) in NaCl are plotted: $\log k_d = (1.5 \pm 0.1) + (7.7 \pm 0.2) \log [NaCl]$.

reaction [Roe and Record (1985); cf. Figure 5]. This large stoichiometry is believed to result in part from uptake of cations accompanying the renaturation (closing) of the locally denatured region of DNA in the open complex, which occurs in the initial step of the dissociation reaction (Strauss et al., 1980; Shaner et al., 1983; Roe et al., 1984, 1985). Replacement of protein–phosphate contacts with cation–phosphate contacts in conversion of the open complex to RP_{c2} may also be involved. If one assumes that the [salt] dependence of k_d (Sk_d) is primarily due to cation reassociation, the expected value of Sk_d in Mg^{2+} (without added Na^+) would be approximately 4 (i.e., approximately half the Sk_d in NaCl) (Record et al., 1977). The dashed line in Figure 5 shows the anticipated dependence of k_d on the concentration of a truly nonspecific divalent cationic competitor. The large difference between the expected (+4) and observed (+0.4) values of Sk_d suggests that a specific effect of Mg^{2+} (relative to Na^+) is being observed here, presumably superimposed on its predicted

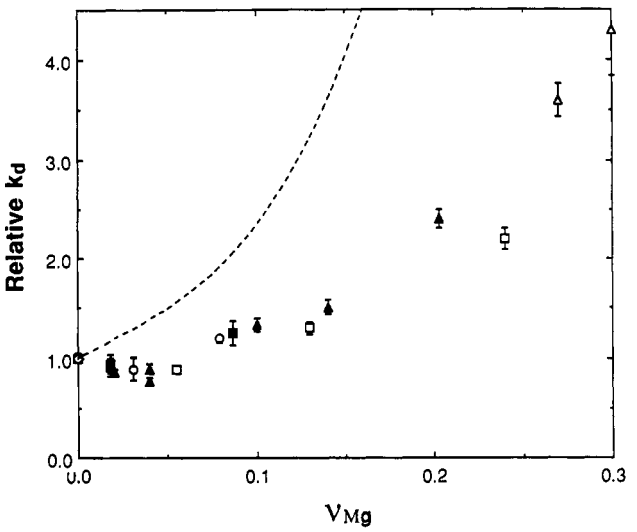

FIGURE 6: Relative values of k_d (normalized to the k_d in NaCl) as a function of the calculated nonspecific binding density of Mg^{2+} on DNA (ν_{Mg}) in mixed Mg^{2+}/Na^+ solutions at 25 °C. The dashed line is the theoretical curve predicted by the Mg^{2+} competitive model (eq 13). Data correspond to measurements of k_d as a function of $[MgCl_2]$ (5×10^{-4}–10^{-2} M) at a series of fixed [NaCl] (0.10–0.23 M). Key: 10^{-2} M Mg^{2+}/0.10 M Na^+ and 10^{-2} M Mg^{2+}/0.12 M Na^+ (△); 10^{-3}–10^{-2} M Mg^{2+}/0.15 M Na^+ (□); 5×10^{-4}–10^{-2} M Mg^{2+}/0.19 M Na^+ (▲); 5×10^{-4}–3×10^{-2} M Mg^{2+}/0.21 M Na^+ (■); 10^{-3}–3×10^{-3} M Mg^{2+}/0.23 M Na^+ (○).

effect as a competitor (or DNA helix-stabilizing) divalent cation.

Table I lists average values of k_a and k_d, as well as estimates of the equilibrium constant $K_{obs}^{RP_o} \equiv k_a/k_d$, as a function of $[MgCl_2]$. In summary, the magnitude of Sk_a is approximately twice as large in NaCl as in $MgCl_2$, indicating that the effect of Mg^{2+} on the formation of the initial closed complex RP_{cl} (which appears in $k_a = K_1k_2$) is quantitatively consistent with a simple competitive model. However, the effect of Mg^{2+} on dissociation of open complexes cannot simply be explained by an analogous competitive model at the level of the step $RP_o \rightleftarrows RP_{c2}$ in mechanism 1, because the observed magnitude of Sk_d in $MgCl_2$ is significantly less than that predicted by the competitive model.

Behavior of k_d in Mg^{2+}/Na^+ Mixtures. To investigate further the nonspecific competitive role and possible specific, facilitating role of Mg^{2+} in the open complex, the rate of dissociation of open complexes was determined by the fixed-time FDAI assay as a function of $[MgCl_2]$ (5×10^{-4}–10^{-2} M) at a series of fixed [NaCl] (0.1–0.23 M). Figure 6 plots relative values of the first-order dissociation rate constant k_d in mixed Mg^{2+}/Na^+ salts (normalized by the corresponding values of k_d at the same $[Na^+]$ in the absence of Mg^{2+}) as a function of the calculated binding density of Mg^{2+} on double-helical DNA, ν_{Mg} (cf. eqs 11–13), which provides a normalization of the data with respect to the nonspecific competitive effect of Mg^{2+}. A simple competitive model for the effect of Mg^{2+} on k_d (eq 13) predicts that the k_d of the open complex should increase monotonically with increasing Mg^{2+} binding density (dashed line, Figure 6). This behavior is not observed. At low ν_{Mg} ($\nu_{Mg} \lesssim 0.06$), the data of Figure 6 show that k_d remains constant or decreases with increasing ν_{Mg}, thereby demonstrating that the effect of Mg^{2+} on k_d is not simply a competitive interaction with DNA phosphates. Instead, Figure 6 indicates that, under conditions where the predicted competitive effect of Mg^{2+} (determined by ν_{Mg}) is small (i.e., at low ν_{Mg}), a facilitating effect of Mg^{2+} is equally or more important and causes the lifetime of the open complex to be relatively invariant

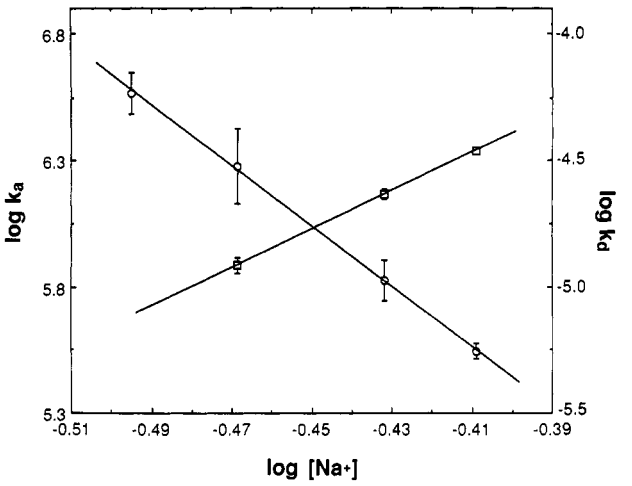

FIGURE 7: log–log plots of dependences of k_a (○) and k_d (□) on $[Na^+]$ in NaOAc solutions (0.30–0.37 M) at 25 °C. The concentration of Na^+ plotted includes the contribution from the buffer (0.02 M Na^+). Lines are linear least-squares fits: $\log k_a = -(12.0 \pm 0.7) \log [Na^+] + (0.65 \pm 0.30)$; $\log k_d = (7.6 \pm 0.1) \log [Na^+] - (1.3 \pm 0.1)$.

to $[Mg^{2+}]$. At higher ν_{Mg} (i.e., higher $[Mg^{2+}]$ at each fixed $[Na^+]$), k_d is found to increase with increasing ν_{Mg}, indicating that the competitive effect becomes dominant at higher $[Mg^{2+}]$. Even at calculated nonspecific binding densities of Mg^{2+} above 0.06, the increase in the relative value of k_d is smaller than predicted by the competitive model. Similar behavior in response to additions of Mg^{2+} ($[Mg^{2+}]$-invariance or slight decrease in k_d at calculated nonspecific $\nu_{Mg} < 0.06$, with a subsequent increase in k_d at higher ν_{Mg}) has been observed for the dissociation of the RNAP $E\sigma^{32}$–groE promoter open complex in mixed Mg^{2+}/Na^+ salts (Fisher, 1990).

Magnesium ions thus appear to exhibit dual and opposing modes of action in the process of dissociation of open complexes, serving both as a competitor with RNAP for binding to DNA and/or a helix-stabilizer (which favors conversion of open complexes to RP_{c2} and therefore increases the rate of dissociation of the open complex) and as a stoichiometric participant in the open complex (see below), which decreases the dissociation rate. Since the stabilizing effect of Mg^{2+} is most evident at low $[Mg^{2+}]$ ($\lesssim 10^{-3}$ M), where the competitive effect (estimated by ν_{Mg}) is small, it is probable that relatively strong specific interactions of Mg^{2+} (binding constants $\gtrsim 10^3$ M^{-1}) are important in the open complex formed in $MgCl_2$ or $NaCl/MgCl_2$ mixtures, and not nonspecific (i.e., screening) effects of Mg^{2+}.

If the role of Mg^{2+} as a DNA helix-stabilizer [cf. Dove and Davidson (1962) and Record (1975)] is important and is not adequately parameterized by the simple competition model, the effect of Mg^{2+} on k_d predicted by eq 13 may be systematically in error. However, we see no simple way in which any combination of DNA helix-stabilizing and phosphate-neutralizing (competitive) roles of Mg^{2+} can yield the behavior of k_d exhibited in Figure 5 and in Figure 6 at low Mg^{2+}.

Comparison of Effects of [NaOAc] and of [NaCl] on k_a and k_d. To determine the effect of the nature of the anion on the kinetics of association of RNAP with the λP_R promoter, association rate constants k_a and dissociation rate constants k_d in NaOAc (0.30–0.37 M) were measured for comparison with previous results of Roe and Record (1985) in NaCl (0.19–0.27 M). Figure 7 shows that both k_a and k_d are strongly dependent on $[Na^+]$.

Table II: Dependence of Rate and Equilibrium Constants of RNAP–λP_R Interaction on [NaOAc] at 25 °C

[NaOAc] (M)	k_a (M^{-1} s^{-1})[a]	k_d (s^{-1})[b]	$K_{obs}^{RP_{o2}} = k_a/k_d$ (M^{-1})
0.30	3.7 (±0.7) × 10^6		
0.32	1.9 (±0.6) × 10^6	1.4 (±0.2) × 10^{-5}	1.4 (±0.5) × 10^{11}
0.35	6.7 (±1.2) × 10^5	2.6 (±0.1) × 10^{-5}	2.6 (±0.6) × 10^{10}
0.37	3.5 (±0.2) × 10^5	3.9 (±0.1) × 10^{-5}	9.0 (±0.6) × 10^9

[a] Nucleotide-stabilization filter binding assay. [b] Fluorescence-detected abortive initiation assay.

Though the ranges of salt concentration investigated in NaOAc and NaCl do not overlap, linear extrapolation of the log–log plots indicates that replacement of Cl^- by OAc^- increases k_a by ~80-fold and reduces k_d by 560-fold (near 0.3 M salt). In the ranges of salt concentration examined, substitution of OAc^- for Cl^- does not significantly affect either Sk_a or Sk_d and hence does not affect the thermodynamically derived stoichiometry of participation of univalent ions (Na^+, Cl^-, or OAc^-) in the kinetically significant steps of the mechanism of open complex formation. Values of $-Sk_a$ (±1 SD) determined from the slopes of the weighted linear least-squares fits are 12.0 ± 0.7 in NaOAc and 11.9 ± 1.1 in NaCl (Roe & Record, 1985); values of Sk_d are 7.6 ± 0.1 in NaOAc and 7.7 ± 0.2 in NaCl (Roe & Record, 1985). Hence, overall equilibrium constants for open complex formation (evaluated from ratios k_a/k_d) are estimated to be ~4 × 10^4 times larger in NaOAc than in NaCl at any [Na^+]! Since the large effects of replacement of Cl^- by OAc^- on k_a and k_d are not accompanied by detectable effects on Sk_a, Sk_d, and SK_{obs}, one must conclude that these effects do not correspond to a detectable difference in the stoichiometry of anion participation. Likewise, Leirmo et al. (1987) found that replacement of KCl by potassium glutamate (in the presence of $MgCl_2$) increased k_a by approximately 30-fold at fixed salt concentration but did not detectably affect Sk_a.

DISCUSSION

Specific and Nonspecific Effects of Mg^{2+} on the Steps of Open Complex Formation: Evidence for a Second Open Complex in Mg^{2+}. Comparison of values of Sk_a (the power dependence of the composite second-order association rate constant k_a) in NaCl and $MgCl_2$ indicates that Sk_a is determined by a cation competition effect which is valence-dependent. We believe that Sk_a is determined primarily by the stoichiometry of cation uptake by the DNA in the dissociation of the initial closed complex RP_{c1}. The dominant effects of univalent (Na^+) and divalent (Mg^{2+}) cations on k_a are as competitors for DNA binding sites. This competitive effect is expected to be valence-dependent but otherwise nonspecific and should contribute strongly to k_a if the initial binding step ($R + P \rightleftarrows RP_{c1}$) is in rapid equilibrium on the time scale of the subsequent isomerization step. In contrast, comparison of values of Sk_d (the power dependence of the composite first-order dissociation rate constant) in the presence and absence of Mg^{2+} indicates that the effect of Mg^{2+} on the steps detected by Sk_d cannot be interpreted simply in terms of competition between Mg^{2+} and RNAP for phosphate groups on the DNA, or as the effect of Mg^{2+} as a helix-stabilizing cation (favoring renaturation of the nonhelical region of promoter DNA in closing the open complex).

Measurements of k_d in mixtures of $MgCl_2$ and NaCl provide additional evidence for dual, opposing effects of Mg^{2+} which act at the level of open complexes: (1) a nonspecific competitive role of Mg^{2+}, which increases the rate of open complex dissociation and (2) a specific stoichiometric role of Mg^{2+}, which decreases the rate of open complex dissociation. The nonspecific competitive effect of Mg^{2+} is well-known in other ligand–DNA interactions, including pentalysine binding to DNA (Lohman et al., 1980) and nonspecific binding of RNA polymerase (deHaseth et al., 1978; Shaner et al., 1983), as well as in the composite second-order association rate constant k_a of open complex formation (Figure 3). The two opposing effects of Mg^{2+} appear to compensate approximately at the level of k_d, resulting in a relative insensitivity of k_d to [$MgCl_2$]. (The net effect is a small competitive one.) Although the kinetic data by themselves do not permit an unambiguous interpretation of the functional and structural roles of Mg^{2+} in stabilizing and destabilizing open complexes, these data provide strong evidence for differences between open complexes formed in the absence and presence of Mg^{2+} and for a mechanism in which the open complex formed in the absence of Mg^{2+} [here designated RP_{o1}; described by Roe and Record (1985)] is an intermediate on the pathway to formation of the open complex formed in the presence of Mg^{2+} (here designated RP_{o2}). RP_{o1} is an open complex, on the basis of conventional criteria of stability (heparin-resistance) and rapid conversion to an initiated complex upon addition of nucleotides and Mg^{2+}, as well as its large Sk_d in NaCl and NaOAc, which is attributed to cation uptake accompanying closing the DNA (Roe & Record, 1985; Leirmo & Record, 1990). Results of $KMnO_4$ reactivity experiments (Suh, W. C., Ross, W., and Record, M. T., Jr., submitted) which are specific for pyrimidine residues in the open region (Sasse-Dwight & Gralla, 1989; O'Halloran et al., 1989) provide a structural basis for the differences in Sk_d in NaCl and in $MgCl_2$. The stable complex at the λP_R promoter formed in the absence of Mg^{2+} (RP_{o1}) exhibits less $KMnO_4$ reactivity of T (and C) residues at both ends of the open region, as compared to the stable complex formed in the presence of Mg^{2+} (RP_{o2}).

The observed differences in Sk_d (and in $KMnO_4$ reactivity) indicate that the Mg^{2+}-induced open complex (RP_{o2}) is different from the open complex (RP_{o1}) formed in the absence of Mg^{2+} as result of the specific binding of approximately three Mg^{2+} ions under the conditions examined. However, these differences in Sk_d do not by themselves demonstrate that RP_{o1} is an intermediate on the pathway to formation of the initiation-competent complex RP_{o2}. In NaCl, in the absence of Mg^{2+}, Roe and Record (1985) deduced that the mechanism was

$$R + P \underset{12Na^+}{\rightleftharpoons} RP_{c1} \rightleftharpoons RP_{c2} \underset{8Na^+}{\rightleftharpoons} RP_{o1} \qquad (14)$$

In $MgCl_2$, in the absence of NaCl, our current kinetic data indicate that either

$$R + P \underset{6Mg^{2+}}{\rightleftharpoons} RP_{c1} \rightleftharpoons RP_{c2} \underset{4Mg^{2+}}{\rightleftharpoons} RP_{o1} \overset{3Mg^{2+}}{\rightleftharpoons} RP_{o2} \qquad (15)$$

or

$$R + P \underset{6Mg^{2+}}{\rightleftharpoons} RP_{c1} \rightleftharpoons RP_{c2} \overset{3Mg^{2+}}{\underset{4Mg^{2+}}{\rightleftharpoons}} RP_{o2} \qquad (16)$$

If mechanism 16 were correct in $MgCl_2$, so that RP_{o1} were not an intermediate in formation of RP_{o2}, then the process of forming an initiated complex by addition of Mg^{2+} and nucleotides to RP_{o1} (formed in the absence of Mg^{2+}) would

require passage through RP_{c2} (i.e., closing and then reopening the complex). This appears unlikely, both because of the rapid formation of the initiated complex from RP_{o1} [within the 10-s mixing time; Roe & Record (1985)] and because the same core of $KMnO_4$-accessible bases is observed in the open regions of RP_{o2} (Suh et al., submitted). We therefore strongly favor mechanism 15, in which RP_{o1} is an intermediate on the pathway to formation of RP_{o2}. Cation participation in the steps of mechanisms 14–16 is indicated in the direction of uptake, in order to indicate the rate constant which is dependent on ion concentration because ions behave as reactants [cf. Record et al. (1991) for a review]. The same number of ions is released in the opposing step, but the corresponding rate constant is not a function of salt concentration because ions are products of that step. (Ion stoichiometries in mechanisms 14–16 are rounded to the nearest integer.)

The analysis of this section is based on the assumption (consistent with our data but unproven) that the step $RP_{o2} \rightleftarrows RP_{o1}$ rapidly equilibrates on the time scale of subsequent steps in the dissociation mechanism. In this situation eq 4 for k_a is unaffected, so the only effect of Mg^{2+} on k_a is its competitive role on k_{-1}, as observed in Figure 3. However, eq 7 for k_d is replaced in the presence of Mg^{2+} by

$$k_d \cong K_4^{-1}K_3^{-1}k_{-2} \qquad (7')$$

In the absence of Mg^{2+}, only RP_{o1} is formed, and from eq 7, $Sk_d = -SK_3 + Sk_{-2} = 7.7 \pm 0.2$ (in NaCl). In the presence of Mg^{2+} (no NaCl), RP_{o2} is formed, and from eq 7', $Sk_d = -SK_4 - SK_3 + Sk_{-2}$. In $MgCl_2$ the value of $-SK_3 + Sk_{-2}$ (which we attribute to cation uptake in the step $RP_{o1} \rightarrow RP_{c2}$ above) should be half as large in magnitude as in NaCl (i.e., uptake of ~ 4 Mg^{2+} ions in the step $RP_{o1} \rightarrow RP_{c2}$). Consequently in $MgCl_2$, the step $RP_{o1} \rightarrow RP_{o2}$ must be accompanied by uptake of approximately $-(0.4 \pm 0.1) + (3.8 \pm 0.1) = 3.4 \pm 0.1$ Mg^{2+} ions (which is rounded to 3 in mechanisms 15 and 16).

An alternative explanation of the relative insensitivity of k_d to $[MgCl_2]$, which is highly unlikely although not impossible based on the data of Figures 3, 5, and 7, would propose anion uptake as the primary contributor to Sk_d. If anion uptake were a major determinant of the ion stoichiometry (Sk_d) in dissociation of RP_o, by analogy to anion effects on SK_{obs} for binding of proteins to single-stranded nucleic acids (Kowalczykowski et al., 1981; Overman et al., 1988), and if this anion (Cl^-) effect on the dissociation rate constant were much less significant in the lower range of $[Cl^-]$ examined in $MgCl_2$, then the observed relative independence of k_d on $[MgCl_2]$ could be explained. However, since replacement of NaCl by $MgCl_2$ dramatically increases k_d at fixed salt concentration and since replacement of Cl^- by OAc^- does not significantly affect Sk_d, we conclude 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 RP_{o2}.

Effects of Univalent Ions (Na^+,Cl^-,OAc^-) on the Steps of Open Complex Formation. A comparison of the kinetics of RNAP–λP_R promoter interactions in NaCl and NaOAc [also sodium glutamate (Leirmo et al., 1987)] shows that both association and dissociation rate constants are *strongly* dependent on both the concentration of the salt and the chemical identity of the anion. The effect of univalent salt concentration is moderately well understood, and results from the requirement for cation uptake in the steps of the mechanism where the DNA axial charge density increases [i.e., $RP_{c1} \rightarrow R + P$ and $RP_{o1} \rightarrow RP_{c2}$ in mechanisms 14 and 15; cf. Leirmo and Record (1990) and Record et al. (1991)].

What rate constants characterizing the binding and isomerization steps of the mechanism of open complex formation are most affected by the nature of the anion? Are anions net stoichiometric participants in mechanisms 14 and 15 for the processes of open complex formation in the absence amd presence of Mg^{2+}? Unfortunately, none of these key questions can yet be answered. Since neither Sk_a nor Sk_d is affected (within ± 1 SD) by substituting OAc^- for Cl^-, the stoichiometries of participation of Cl^- and OAc^- are not detectably different. Within error, these stoichiometries Sk_a, Sk_d do not vary with salt concentration. Comparison of Sk_a in $MgCl_2$ and in NaCl suggests that the cation stoichiometry is the dominant contributor to Sk_a and that the anion stoichiometry is small. An analogous deduction regarding a possible stoichiometric contribution of anions to dissociation is impossible, as a result of the apparent change in the nature of the open complex (from RP_{o1} to RP_{o2}) when Mg^{2+} is added. However, the large increase in k_d when NaCl is replaced by $MgCl_2$ at fixed salt concentration and the insensitivity of Sk_d to the nature of the anion (Cl^-, OAc^-) suggest that Sk_d in the absence of Mg^{2+} is determined primarily by the amount of cation uptake accompanying renaturation ("closing") of the open region of the promoter DNA in the step $RP_{o1} \rightarrow RP_{c2}$. The nature of the anion may affect either of these steps as a result of strong site binding interactions with a small stoichiometry and/or may affect the protein conformational change in the isomerization step ($RP_{c1} \rightarrow RP_{c2}$).

Effects of Mg^{2+} and other Divalent Cations on Site-Specific Binding of Proteins to DNA and on the Steps of Transcription. We assert that the specific role of Mg^{2+} in the process $RP_{o1} \rightarrow RP_{o2}$ at the λP_R promoter is superimposed on its role as a nonspecific competitor in the processes $RP_c \rightarrow R + P$ and $RP_{o1} \rightarrow RP_{c2}$. The net effect of $[MgCl_2]$ on the overall thermodynamics of open complex formation in $MgCl_2$ solutions (without added NaCl) is a competitive one: an increase in $[MgCl_2]$ destabilizes RP_{o2} relative to R + P, as demonstrated by the observation (Table I) that the estimated equilibrium constant $K_{obs}^{RP_{o2}} \equiv k_a/k_d$ in $MgCl_2$ decreases with increasing $[MgCl_2]$. In mixed salt ($MgCl_2$/NaCl) solutions, where the competitive effect of Mg^+ is reduced, addition of low concentrations of $MgCl_2$ permits formation of RP_{o2} from RP_{o1} and hence is observed to increase the half-life of the open complex slightly (cf. Figure 6). At higher $[MgCl_2]$ this specific effect of $MgCl_2$ is apparently counterbalanced by the nonspecific effect of Mg^{2+} on $RP_{o1} \rightarrow RP_{c2}$, and further addition of $MgCl_2$ therefore reduces the half-life of the open complex to an extent which correlates with the predicted Mg^{2+}–DNA binding density. We have not yet determined k_a under these conditions and hence cannot estimate $K_{obs}^{RP_{o2}}$. It will be of interest to determing the balance between specific and competitive effects of Mg^{2+} on k_a, k_d, and $K_{obs}^{RP_{o2}}$ in NaCl/$MgCl_2$ mixtures as a function of $[MgCl_2]$, especially at lower $[MgCl_2]$ where the competitive effect of Mg^{2+} should be less important and the specific effect on $RP_{o1} \rightarrow RP_{o2}$ can be more clearly observed.

In our studies of the λP_R promoter, effects of Mg^{2+} on k_a appear to be purely competitive, by comparison of Sk_a in NaCl and $MgCl_2$ (Figure 3). The competitive effect of Mg^{2+} on k_a is presumably general for all promoters in which a rapid equilibrium occurs between RP_c and reactants so that the step $RP_c \rightarrow R + P$ and the rate constant k_{-1} (which increases as a large power of the cation concentration) are important determinants of the composite rate constant k_a (cf. eqs 4 and

8). Nakanishi et al. (1975) used a transcription assay to investigate the extent of inhibition of transcript production as a function of [$MgCl_2$] (0–0.02 M) at constant [KCl]. They found that increasing [Mg^{2+}] "strongly interferes" with the kinetics of open complex formation at the *lac* and *gal* promoters, "partially inhibits" the λP_R promoter, and is without effect on the λP_L promoter. We interpret these results as a simple competitive effect of Mg^{2+} at the level of the initial closed complex (RP_{c1}). At the λP_L promoter, this step may not rapidly equilibrate on the time scale of subsequent isomerization steps ($RP_{c1} \rightarrow RP_{c2}$, etc.) at the ionic conditions investigated. If so, the [Mg^{2+}]-dependent step k_{-1} would not be expected to contribute to k_a.

Limited evidence from structural and functional studies on RNAP and its complexes with promoters is consistent with our proposal of a specific role of Mg^{2+} at the level of the open complex which is distinct from the requirement for Mg^{2+} in nucleotide binding. Perhaps most relevant is the observation of Woody and co-workers (Shimer et al., 1988) that the extent of DNA denaturation (estimated by UV hyperchromicity) in a nonspecific open RNAP–poly[d(I-C)] complex increased from 16 ± 2 bp in the absence of Mg^{2+} to 20 ± 2 bp upon addition of 5×10^{-3} M $MgCl_2$. However, addition of $MgCl_2$ had no effect on the extent of DNA denaturation in a nonspecific RNAP–poly[d(A-T)] open complex (16 ± 2 bp).

Kuwabara and Sigman (1987) used footprinting with 1,10-phenanthroline–copper of complexes formed in the presence of 0.01 M Mg^{2+} and separated on acrylamide gels (in the absence of Mg^{2+}) to deduce a specific role of Mg^{2+} in formation of the RNAP–*lac* UV5 open complex. Under the conditions examined, they found that Mg^{2+} is required for the maintenance of the region of single-stranded DNA characteristic of the open complex at the *lac* UV5 promoter. The apparent inability of RNAP to exist in an open complex at the *lac* UV5 promoter in the absence of Mg^{2+} (under the conditions examined) contrasts with the behavior of the wild-type *lac* promoter and the λP_R promoter, where Mg^{2+} is not required to form an open complex (Nakanishi et al., 1975; Roe & Record, 1975). In particular, for λP_R, Mg^{2+} is not required to form a heparin-resistant complex (which we designate RP_{o1}) which can initiate rapidly upon addition of nucleotides and Mg^{2+} (presumably by isomerization to RP_{o2}), and which is rapidly and selectively stabilized by nucleotides and Mg^{2+} against dissociation in a high-salt challenge experiment (Roe & Record, 1985).

Koren and Mildvan (1977) used Mn^{2+} paramagnetic resonance to determine that there are approximately seven binding sites for divalent cations on $E\sigma^{70}$ RNAP, including one tight binding site with a binding constant greater than 10^5 M^{-1} and 6 ± 1 weaker binding sites (binding constants $\sim 10^3$ M^{-1}). The tight-binding site is thought to function as the catalytic site for RNA chain elongation. Arndt and Chamberlin (1990) observed that the elongation ternary transcription complex designated the "release complex" is stabilized by millimolar concentrations of Mg^{2+}, while an increase in concentration of K^+ destabilizes the complex. Dissociation of the elongation "release complex" results in release of the nascent RNA chain.

Another example of a divalent cation which appears to exhibit both competitive and facilitating effects on site-specific protein–DNA interactions is Ba^{2+}, an ion thought to promote DNA bending (Laundon & Griffith, 1987). Flashner and Gralla (1988) showed that the substitution of $BaCl_2$ (0.05 M) for NaCl (0.05 M) in their binding buffer increased the extent of binding of *lac* repressor and CAP protein to their recognition sites but reduced the extent of binding of *trp* repressor to its specific site. In all cases, Ba^{2+} (as a divalent cation) should exert a net competitive effect (relative to Na^+ at the same concentration) on protein binding. Presumably this competitive effect is completely compensated by a favorable effect resulting in net uptake of Ba^{2+} in site-specific binding of *lac* repressor and CAP protein, but not in binding of *trp* repressor.

CONCLUSIONS

Mg^{2+} concentration is demonstrated to be a useful probe of the kinetics and mechanism of RNA polymerase–promoter interactions. It is noteworthy that the second form of the open complex (RP_{o2}, requiring Mg^{2+}) is detected by the behavior of the composite dissociation rate constant k_d as a function of [Mg^{2+}]. Most studies of the kinetics of RNA polymerase–promoter interactions have been conducted at a fixed set of ionic conditions and have emphasized the kinetics of the association process; the present study emphasizes the importance of ionic variables and the analysis of the dissociation kinetics. Currently we are comparing the dual (competitive, facilitating) roles of Mg^{2+} in open complex formation with the behavior of other divalent cations (Ca^{2+}, Ba^{2+}), as well as characterizing the structural basis of the differences between RP_{o1} and RP_{o2}.

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