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Equivalence of Mg^{2+} and Na^{+} ions in salt dependence of the equilibrium binding and dissociation rate constants of *Escherichia coli* RNA polymerase open complex

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ABSTRACT

Conflicting experimental data on the influence of Mg^{2+} ions on the salt dependence of formation/dissociation of open transcription complex (RPo) of *Escherichia coli* RNA polymerase led us to carry systematic measurements of the dissociation rate constant (k_d) and thermodynamic stability of complexes at λP_R and Pa promoters in a broad range of [NaCl] and [$MgCl_2$] at 25, 31 and 37 °C, using fluorescence detected abortive transcription assay. Values of k_d determined in $MgCl_2$ in the presence of heparin, as a commonly used anionic competitor, were shown to depend on heparin concentration whereas in NaCl this effect was not observed. Kinetics of dissociation was therefore determined in the course of salt-induced down-shift of the binding equilibrium. Salt derivatives of k_d's (n_d) appeared to be similar in NaCl (~8.5) and $MgCl_2$ (~10) for both complexes. Isotherms of fractional occupancy of promoters by RNAP as a function of ln [salt] were shown to conform to a sigmoid Boltzman function parameterized to include binding constant of RPo and a net change (n_{obs}) in the number of electrolyte ions associated with complex components upon its formation/dissociation. The fitted values of n_{obs} appeared also similar in NaCl and in $MgCl_2$: ~18 for RPo/λP_R and ~20 for RPo/Pa, respectively. Overall unfavorable vant'Hoff enthalpy (ΔH_{obs}) of RPo proved to be much higher in $MgCl_2$ than in NaCl by ca. 20 kcal/mol for both complexes, rendering them profoundly less stable in the former salt. In both salts, ΔH_{obs} was higher by ~30 kcal/mol for RPo/Pa relative to RPo/λP_R. Similarity of n_{obs} and n_d values for the two salts indicates thermodynamic equivalence of Mg^{2+} and Na^{+} in [salt]-controlled binding equilibrium of RPo. This finding remains in disagreement with earlier data and suggests that salt effects on open complex stability should be sought in global compensating changes in distribution of all ionic species around the interacting complex components.

1. Introduction

The divalent magnesium cation is an essential element for the growth and maintenance of living cells, whose transport and role in cell homeostasis are extensively studied [1,2]. It is an essential cofactor required for catalytic function of a variety of enzymes, in particular for DNA and RNA polymerases [3,4]. However, the role of Mg^{2+} ions in salt-controlled kinetics of initiation of transcription by bacterial RNA polymerase and in stability of open transcription complex is not well understood.

The major contribution to the free energy driving formation of transcription competent complexes by *Escherichia coli* RNA polymerase at cognate promoters in dilute salt solutions is of entropic origin connected primarily with a net change in the number of cations associated with negatively charged DNA phosphates [5–7]. The corresponding equilibrium binding constants of these complexes are thus strongly dependent on salt concentration. Kinetic experiments in NaCl have revealed that both the association (k_a) and dissociation (k_d) rate constants for the open transcription complex (RPo) of *E. coli* RNA polymerase (R) at the λP_R promoter (P) vary as $[Na^+]^{-12}$ and $[Na^+]^8$, respectively. Consequently, the corresponding equilibrium constant, $K_p = k_a / k_d$, varies with $[Na^+]^{-20}$ [8]. In dilute $MgCl_2$ solutions, this dependency has been reported to be very different for the association and dissociation reactions [6,9]: while k_a varied as $[Mg^{2+}]^{-5}$, the value of k_d appeared only very weakly [salt]-dependent, with an exponent of ca. 0.4. The latter observation has been interpreted as a net result of (i) reassociation of ca. 4 Mg^{2+} ions (equivalent to 8 Na^+) upon renaturation of the melted promoter DNA to double-stranded form during RPo dissociation, and (ii) concomitant release of 3Mg^{2+} ions from a postulated Mg-induced form of the complex called RPo2. In a similar study for RPo at a model Pa promoter [10], shown to conform like λP_R to the three-step mechanism of the complex formation [5,6,8], we have demonstrated that k_d varies as $[Mg^{2+}]^4$. To resolve the apparent discrepancy between the two works, we extended our investigations on [salt]-dependence of the kinetics of dissociation of RPo at both λP_R and Pa

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promoters to a broader range of [$MgCl_2$]. Parallel reference experiments were conducted in NaCl solutions. The reported herewith results of these studies demonstrate that: (i) heparin in the presence of Mg^{2+} inactivates RPo in a bimolecular reaction so that it does not behave as a true polyanionic competitor that explains the discrepancy between the earlier measured values of [salt]-derivatives of k_d, and (ii) salt-derivatives of heparin-independent k_d's in $MgCl_2$ and in NaCl are similar one with another. Subsequent determination of binding isotherms for RPo in the two salts at a number of temperatures allowed confirmation of the latter finding by showing that also [salt]-dependence of the binding equilibrium constant in the two salts is similar. Thermodynamic stability of RPo at both promoters appeared to much lower in $MgCl_2$ than in NaCl, however, owing to a higher unfavorable overall enthalpy of the complexes in the former solvent.

2. Experimental

2.1. RNA polymerase

RNA polymerase (EC 2.7.7.6) was prepared according to [11] as described previously [10,12,13]. The enzyme was stored at −20 °C in buffer S (50% glycerol, 100 mM NaCl, 10 mM Tris–HCl pH 7.9, 0.1 mM DTT); its activity was estimated according to [14] at ca. 50%. The enzyme concentrations reported here refer to its active form.

2.2. Promoters

Model promoter Pa was synthesized and cloned into pDS3 plasmid as described previously [12]; λP_R promoter, encompassing positions from −59 to +21 with respect to its transcription start site, was obtained by PCR [13] and cloned similarly into pDS3 plasmid. DNA fragments bearing promoter sequences: Pa (226 bp long) and λP_R (264 bp long) were prepared by PCR. Concentration of PAGE purified DNA fragments was determined spectrophotometrically. Sequences of the two promoters are shown in Scheme 1.

2.3. Reagents and chemicals

γ-ANS-UTP was prepared according to Yarbrough et al. [15]. ANS was from Fluka. UTP, ApA, CpA, heparin, Tris and 1.0 M solution of $MgCl_2$ were from Sigma. Poly(dT) was from Miles Laboratories. NaCl stock solution was prepared from dried salt (Chempur).

Buffers. Binding buffer (BB) used for RPo formation contained: 0.01 M Tris–HCl/pH 7.9, 0.001 M DTT, 0.01% BSA, and varying concentration of either NaCl (0.15–0.45 M) or $MgCl_2$ (0.05–0.13 M). For experiments in pure Tris–HCl buffer, pH 7.9, [Tris] was set at 0.225 M. Transcription buffer (TB) used for quantification of RPo in experiments carried out in the presence of NaCl contained: 0.01 M Tris–HCl/pH 7.9, 0.001 M DTT, 0.01% BSA 0.1 M NaCl, 0.01 M $MgCl_2$ and 10 μg/ml of heparin; in experiments conducted in $MgCl_2$ concentration of the latter salt in TB was 0.04 M, while when in pure Tris–HCl/pH 7.9 buffer–[Tris] was set at 0.15 M.

2.4. RPo formation

RPo complexes for equilibrium binding experiments were formed by incubating DNA fragment containing λP_R or Pa promoter (4 nM) and RNAP (40 nM) in the BB buffer 0.15 M in NaCl or 0.05 M in $MgCl_2$ for 2 h at 37 °C. These conditions were established with a reference to the published data [8,9], our laboratory experience [10] and results of the present work. They proved to be optimal for full occupancy of promoter DNA in the formation of both RPo/λP_R and RPo/Pa complexes. Higher [salt]'s in BB were used to set the binding equilibrium at a lower level of promoter occupancy. For kinetic studies the complexes were formed at a lower [RNAP]/[DNA] ratio of 4–5 and a smaller reactants concentration.

2.5. Modified fluorescence detected abortive initiation assay (MFDAI)

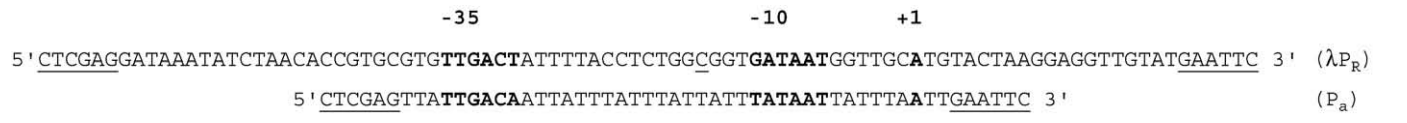
In order to be able to measure fast rates of RPo dissociation at high salt concentrations and to increase general accuracy of fluorescence detected abortive initiation assay (FDAI) [9,10], used for quantification of RPo at equilibrium, we modified this assay in the following way. A series of equilibrated RPo formation or dissociation reactions in BB buffer were sampled and immediately brought to TB buffer containing a lower [salt] and heparin (10 μg/ml), to prevent any changes in the original binding equilibrium, and substrates for abortive transcription initiation (final concentration: 0.2 mM in initiating dinucleotide, ApA or CpA respectively for RPo/Pa and RPo/λP_R, and 0.1 mM in γ-ANS-UTP). The thus started steady-state transcription in the whole series of samples was carried out at 37 °C up to ca. 20% of consumption of the ANS-UTP substrate and then stopped by several-fold dilution with 0.02 M of EDTA solution. Finally, fluorescence intensity of accumulated fluorescent 5′-pyrophosphate-ANS product, proportional to content of RPo, was measured using earlier described dedicated spectrofluorimeter [10] and/or a microplate Fluorescence Reader (FLx Biotek). Control experiments showed that during the long lasting transcription reactions the extent of inactivation of RPo was very small and similar within the whole series of samples, so that relative content of RPo therein could be accurately determined.

2.6. Kinetics of RPo dissociation as a function of salt concentration

Dissociation of preformed open complexes in BB solution, equilibrated at a selected [salt] and temperature (25 or 37 °C, for RPo/λP_R and RPo/Pa, respectively), was initiated by the addition of an equal volume: (1) of a concentrated $MgCl_2$ or NaCl solution to a desired final [salt], to shift the binding equilibrium to a lower level (called salt-shift method), or/and (2) of a solution containing DNA competitor (heparin: 10–60 μg/mL, or poly(dT): 0.8 mg/mL), to make the reaction irreversible [9,10]. Fractions f_{RPo} of open complexes remaining at time t after initiation of dissociation process were determined using the MFDAI assay described in the preceding paragraph. The first-order dissociation rate constants k_d were evaluated from linear least-squares fit (Origin 4.1) of the function: $\ln(f_{RPo}) = A + k_d \times t$ to the experimental f_{RPo}, (õ[salt], t) data points.

2.7. Determination of equilibrium fraction of RPo as a function of salt concentration

The amount of complexes formed in BB solution at a number of [salt]'s, or attained after shifting down the binding equilibrium by the addition of a concentrated salt solution to the binding reaction



Scheme 1. Sequences (nontemplate strand) of the λP_R and P_a promoters; within restriction sites (underlined) used for cloning into pDS3 plasmid. Transcription start site, −10 and −35 recognition hexamers are in bold font.