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Evaluation of mixed-salt effects on thermodynamic and kinetic parameters of RNA polymerase–promoter DNA complexes in terms of equivalent salt concentrations. General applicability to DNA complexes

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Facile evaluation of mixed-salt effect on the strongly salt-dependent thermodynamic and kinetic parameters of protein–DNA complexes is of importance for relevant biochemical and biophysical studies. In pursuit of this aim, binding isotherms for open transcription complex (RPo) of *Escherichia coli* RNA polymerase (R) at λP_R promoter DNA (P) were determined as a function of salt concentration in pure NaCl and Tris/HCl solutions, and as a function of [NaCl] in the presence of fixed concentrations of $MgCl_2$ and Tris/HCl. A concept of equivalent salt concentrations, i.e. concentrations at which the binding equilibrium constant is the same, was introduced and applied for prediction of binding isotherms in mixed salt solutions. Full coincidence between the experimental and predicted isotherms indicated that individual contributions of salts to the global salt-effect are additive in a broad range of salt concentrations. A generalized formula for calculation of salt equivalents characteristic for any of the thermodynamic or kinetic parameters of a complex (e.g., free energy, binding equilibrium and association/dissociation kinetic rate constants) is presented and its applicability to a number of protein–DNA complexes and dsDNA melting demonstrated using authors' own and literature data.

Keywords: protein–DNA complexes, salt-dependence of equilibrium and kinetic constants, mixed salts effects, equivalent salt concentrations

INTRODUCTION

Protein–DNA binding equilibria in aqueous salt solutions are entropy driven by removal/reassociation of electrolyte ions from/with surfaces of the complex components (Record *et al.*, 1991). The thermodynamic stability of protein–DNA complexes is thus strongly dependent both on the concentration and nature of electrolytes. Model experimental studies on monovalent salt-dependence of the equilibrium constant for binding of Z-valent oligolysines and oligoarginines to double- and single-stranded nucleic acids (Lohman *et al.*, 1980; Mascotti & Lohman, 1993; 1997) have demonstrated that thermodynamic properties of such systems are primarily governed by the polyelectrolyte properties of DNA. Upon complex formation, competition between Z-valent peptide and monovalent salt cations for the vicinity of the DNA polyanion leads to release of approximately Z electrolyte cations thermodynamically associated with DNA phosphates. The number of ions released can be determined from the dependence of equilibrium binding constant (K_{obs}) on the concentration of monovalent salt. In these systems, different anions (fluoride, acetate, chloride) contribute negligibly to the salt effect the magnitude of which is correlated with the anions' position in the Hoffmeister series (Leirmo & Record, 1990). In

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Abbrevations: BB, binding buffer; BSA, bovine serum albumin; DAPI, 4′,6′-diamidino-2-phenylindole; dsDNA, double-stranded DNA; DTT, dithiothreitol; MFDAI, modified fluorescence detected abortive initiation assay; NLPB, non linear Poisson-Boltzman model; RNAP, R, *Escherichia coli* RNA polymerase; RPo, open transcription complex; ss, single-stranded DNA; TB, transcription buffer.

mixed salt solutions containing two cationic species of different valence, binding of a protein to DNA is expected to be accompanied by cations' release in the $Z_1:Z_2$ ratio. A simple system of λ phage *lac* repressor–operator DNA conforms to the polyelectrolyte interpretation since formation of this complex is accompanied by the release of Na^+ and Mg^{2+} ions in a 2:1 stoichiometry (deHaseth *et al.*, 1977; Capp *et al.*, 1996). Also formation of open transcription complex (RPo) by *Escherichia coli* RNA polymerase has long been believed to depend primarily on the polyelectrolyte properties of DNA (Leirmo & Record, 1990; Record *et al.*, 1991; 1996). Our recent thorough studies (Łoziński *et al.*, 2009) have clearly demonstrated, however, that this interpretation does not apply to this complex since Na^+ and Mg^{2+} ions proved to be equivalent in driving the [salt]-dependent binding equilibrium of RPo. Non linear Poisson-Boltzman (NLPB) calculations of the salt-dependent contributions to the electrostatic binding free energy of the λcI repressor and *Eco*RI endonuclease to cognate DNA sequences (Misra *et al.*, 1994a; 1994b) have shown that the observed salt effects in these systems are due to compensating changes in the global pattern of cation and anion distribution in the solvation layers of both components. The open transcription complex seems to adhere to this interpretation (Łoziński *et al.*, 2009). Experimental studies on equilibrium binding of *E. coli* single-strand binding protein (SSB) to single-stranded nucleic acids in the $(SSB)_{65}$ mode (Overman *et al.*, 1988) have documented a net release of both electrolyte co-ions with a stoichiometry dependent on the electrolyte nature. Also experimental and NLPB studies of β hairpin RNA box—N-peptide λ antitermination complex (Cuauhtemoc & Draper, 2003) have demonstrated a contribution of anions to salt-control of this system. In proteins having buried specific binding sites for DNA of polycationic character, complex formation requires their opening by competitive interactions with both electrolyte cations and anions, as it has been amply demonstrated to be the case for binding of T4 gene 32 and T7 gene 2.5 proteins to ssDNA and/or dsDNA (Rouzina *et al.*, 2005; Shokri *et al.*, 2006; 2008).

The enthalpic contribution to the overall free energy of protein–nucleic acid complexes may depend significantly also on the interaction of electrolyte species with the complex components. For instance, the stability of RPo in $MgCl_2$ solution has been found to be three-fold lower than in NaCl (Łoziński *et al.*, 2009) due to a much higher unfavorable overall enthalpy of the complex in the former salt. In contrast, the stability of this complex in monovalent acetate and glutamate salt solutions was much higher than in NaCl (KCl), presumably due to a weaker interaction with RNAP cationic groups of the respective anions relative to the chloride (Roe & Record, 1985; Leirmo *et al.*, 1987; Suh *et al.*, 1992).

In view of the complicated molecular mechanisms of electrolyte control underlying protein–nucleic acids interactions, for practical purposes it should be sufficient to use salt concentration as a variable for calculation of salt-dependent parameters. The more so that investigations of physical-chemical and/or biochemical properties of protein–DNA complexes are rarely carried out in solutions containing a single salt species. In most cases, besides the principal supporting electrolyte (NaCl, KCl) the presence of other salts, *viz*. acting as buffers or cationic cofactors of enzyme activity like Mg^{2+}, is required. In such mixed salt solutions, the single-exponential dependence of thermodynamic and/or kinetic observables on the concentration of the principal electrolyte breaks down leading to a curvature in otherwise linear double-logarithmic plots. Therefore, it is important to know how to evaluate contribution of each of the electrolytes present in mixed salt solutions to the salt-dependent equilibrium and kinetic parameters of complex formation. For instance, the effect of Mg^{2+} on *lac* repressor-operator DNA (Record *et al.*, 1977) and λP_R RPo (Suh *et al.*, 1992) has been accounted for with the help of an apparent binding constant of these ions to dsDNA. Here, we propose a more general approach based on the concept of equivalent salt concentrations, at which a parameter of interest attains the same value, and demonstrate its applicability to the evaluation of effects of added salts ($MgCl_2$ and Tris/HCl) on the binding equilibrium of RPo in NaCl solution, and on the thermodynamic and/or kinetic parameters of selected protein–DNA and other DNA complexes for which pertinent literature data are available.

MATERIALS AND METHODS

RNA polymerase. RNA polymerase (EC 2.7.7.6) was prepared according to (Burgess & Jendrisek, 1975) as described previously (Łoziński *et al.*, 1989; Kolasa *et al.*, 2001; Łoziński & Wierzchowski, 2005). 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 (Chamberlin *et al.*, 1983) at about 50%. The enzyme concentrations reported here refer to its active form.

Promoter. λP_R promoter, encompassing positions –59 to +21 with respect to the transcription start-site, was obtained by PCR (Łoziński & Wierzchowski, 2005) and cloned into pDS3 plasmid, as described in detail previously (Łoziński *et al.*, 1989; http://genome-www.stanford.edu/vectordb/vector_descrip/PDS3.html). A 264 bp long DNA fragment

bearing the promoter sequence was prepared by PCR. Concentration of PAGE-purified DNA fragments was determined spectrophotometrically.

Reagents and chemicals. γ-ANS-UTP was prepared according to (Yarborough *et al.*, 1979). ANS was from Fluka. UTP, CpA, heparin, Tris and 1.0 M solution of $MgCl_2$ were from Sigma. NaCl stock solution was prepared from dried salt (CHEMPUR).

Buffers. Binding buffer (BB) used for RPo formation/equilibration contained: 0.01 M Tris/HCl, pH 7.9, 0.001 M DTT, 0.01% BSA, and varying concentration of NaCl. 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 $MgCl_2$, 10 μg/ml of heparin and substrates for abortive transcription initiation (final concentration: 0.2 mM in initiating CpA and 0.1 mM in γ-ANS-UTP). TB buffer containing 0.03 M Tris was used for quantification of RPo in equilibration experiments conducted in NaCl at constant 0.1 M Tris/HCl, pH 7.9, while in experiments carried out in pure Tris/HCl, pH 7.9, TB buffer was 0.15 M in Tris.

RPo formation. RPo/λP_R complex was formed by incubating DNA fragment containing λP_R promoter (4 nM) and RNAP (40 nM) in the BB buffer with 0.15 M NaCl for 2 h at 37°C. For experiments in pure Tris/HCl buffer Tris concentration was 0.225 M. These conditions (Łoziński *et al.*, 2009) proved to be optimal for full occupancy of promoter DNA. Higher concentrations of NaCl in BB were used to set the binding equilibrium at a lower level of promoter occupancy.

Determination of equilibrium fraction of RPo as a function of salt concentration. Measurements of RPo content at equilibrium were performed using the modified fluorescence detected abortive initiation assay (MFDAI) (Łoziński *et al.*, 2009). Aliquots of equal volume taken from a bulk solution containing preformed RPo at the optimal salt condition were mixed with a series of BB solutions to set the final salt concentration (NaCl or Tris/HCl) at successively higher values, and equilibrated at 25°C for 6 h. Thereafter, samples were diluted with salt solutions containing substrates (initiating CpA and γ-ANS-UTP) to establish in all the samples of a given series the same standard conditions for transcription initiation. Abortive transcription was carried out at 37°C in TB buffer up to about 20% of consumption of the ANS-UTP substrate and then stopped by several-fold dilution with 0.02 M EDTA. Finally, fluorescence intensity of accumulated fluorescent 5′-pyrophosphate-ANS product, proportional to the content of RPo, was measured using a microplate Fluorecence Reader (FLx Biotek). Sigmoid plots of fluorescence intensity *versus* ln[salt] were first fitted to a Boltzman function (using Origin 4.1) and then normalized yielding the sought f_{RPo}(ln[salt]) binding isotherms.

RESULTS AND DISCUSSION

The effect of added salt(s) on the binding equilibrium of the open transcription complex in NaCl solution was studied by the method of binding isotherms (Łoziński *et al.*, 2009) allowing determination of the fraction of the complex formed, f_{RPo}, as a function of salt concentration. The $f_{RPo} = F\{\ln([NaCl]),T\}$ binding isotherms were determined at 25°C for RPo–λP_R in pure NaCl solution and in NaCl at two fixed $MgCl_2$ concentrations: 10 and 30 mM. $MgCl_2$ has been selected as the added salt since it is known to lower profoundly the thermodynamic stability of RPo (Łoziński *et al.*, 2009) and because Mg^{2+} ions serve as a catalytic cofactor in the transcription reaction (Sosunov *et al.*, 2003). As shown in Fig. 1, the obtained isotherms are sigmoid. The strong effect of $MgCl_2$ on RPo manifests itself in a shift of the binding isotherms to lower [NaCl] values even in the presence of small amounts of the added salt (cf. Fig. 1). The most apparent measure of this shift is the salt concentration x_0 at which $f_{RPo} = 0.5$. This parameter can be evaluated by fitting the experimental $f_{RPo} = F\{\ln([NaCl]),T\}$ data to the sigmoid Boltzman function (or appropriately parameterized one, as shown below). The x_0 values calculated in this way, listed in the legend to Fig. 1, indicate that $x_0 = 0.247$ M for the binding isotherm in pure NaCl, decreases to 0.221 M and 0.146 M for the isotherms measured in the presence of 0.01 M and 0.03 M $MgCl_2$, respectively.

The binding isotherms shown in Fig. 1 were obtained in the presence of 10 mM Tris/HCl buffer at pH 7.9, required for effective complex formation. It was thus necessary to evaluate also the effect of this salt on RPo equilibrium in NaCl. Therefore binding isotherms for RPo–λP_R in pure Tris/HCl solution at pH 7.9 ($x_0 = 0.259$ M) and in NaCl containing 0.1 M Tris ($x_0 = 0.189$ M) were also measured at 25°C (cf. Fig. 2 and legend). The latter is much less shifted to lower [NaCl] than that in pure NaCl ($x_0 = 0.247$ M). The effect of 0.1 M Tris on x_0 is much smaller than that of 0.03 M $MgCl_2$ (Fig. 1).

We have shown previously (Łoziński *et al.*, 2009) that the binding isotherm conforms to appropriately parameterized sigmoid Boltzman function:

$$f_{RPo} = \frac{1}{1 + e^{(n_{obs} \times \ln[salt] - \ln K)}} \quad (1),$$

where $\ln K = \ln K_{eq} + \ln[RNAP]$, $K_{eq} \equiv K_{obs,1.0M}$ is a [salt]-dependent equilibrium binding constant extrapolated to 1.0 M salt and n_{obs} is the net change

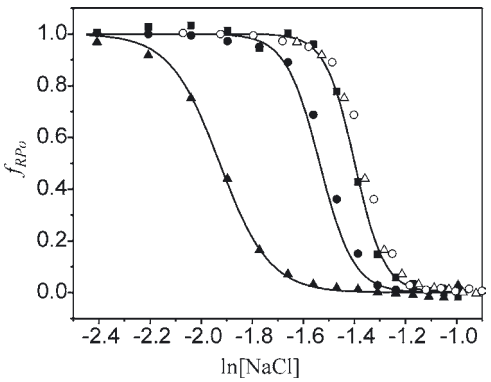

Figure 1. Binding isotherms at 25°C for RPo formation by *E. coli* RNA polymerase at λP_R promoter in NaCl solution and in the presence of a fixed concentration of $MgCl_2$.

Experimental data points obtained in NaCl are shown as rectangles, in NaCl at 0.01 M $MgCl_2$ as circles (apparent fitted parameters: n_{obs} = 14.4, x_0 = 0.221 and ln*K* = −21.74) and in NaCl at 0.03 M $MgCl_2$ as triangles (apparent fitted parameters: n_{obs} = 9.6, x_0 = 0.146 and ln*K* = −18.47). The line for the isotherm in pure NaCl was drawn using Eqn. 1 and the fitted parameters: n = 17.3 and lnK = −24.2 (Table 1); those for the isotherms in mixed salts were drawn similarly using the apparent fitted parameters (in brackets above). Open symbols along the control isotherm correspond to data points with X axis values calculated according to Eqn. 1a using $[NaCl]_{eqv,salt}$ equivalents of the added salt: 0.036 and 0.107 M NaCl for 0.01 and 0.03 M $MgCl_2$, respectively.

in the number of electrolyte ions associated with the free and bound complex components accompanying RPo formation/dissociation. Under conditions of [RNAP]/[DNA] = 10 used in this work, the concentration of the enzyme in the salt-titration experiments can be assumed to be practically constant (Łoziński *et al.*, 2009). By fitting of Eqn. 1 to the experimental (f_{RPo}, ln([salt])) data sets for the pure NaCl and Tris/HCl solutions all the sought parameters were obtained and collected in Table 1. Values of the parameters for RPo in pure NaCl solution included in Table 1 agree with those obtained previously (Łoziński *et al.*, 2009). Salt concentrations x_0, calculated from the relationship $\ln(x_0) = \ln K/n_{obs}$, are also included in Table 1.

Table 1. Binding equilibrium parameters for RPo–λP_R complex at 25°C in pure NaCl and Tris/HCl solutions.

The parameters were calculated by fitting of experimental data sets corresponding to isotherms shown in Figs. 1 and 2 to Eqn. 1; those for the complex in $MgCl_2$ are from (Łoziński *et al.*, 2009). In brackets standard errors returned from the fits using Marquard minimization algorithm are shown.

Parameter	NaCl	$MgCl_2$	$Tris^+Cl^-$
x_0, (M)	0.247	0.0694	0.259
n_{obs}	17.30 (0.39)	18.34 (0.80)	21.75 (0.41)
$\ln K \equiv \ln K_{eq}$+ln[RNAP]	−24.20 (0.55)	−48.92 (2.11)	−29.46 (0.63)
$\ln K_{eq} \equiv \ln K_{obs,1.0\ M}$	−7.16	−31.88	−12.43

In single-species salt solutions, the value of n_{obs} has been shown to be practically independent of the electrolyte nature (Leirmo *et al.*, 1987; Suh *et al.*, 1992; Łoziński *et al.*, 2009). In mixed salts, however, the calculated values of n_{obs} are no longer constant and vary with [$MgCl_2$] and [Tris/HC] in NaCl titration experiments (cf. legend to Figs. 1 and 2). Both the shift of the isotherms to lower [NaCl] values and the variation of n_{obs} with the amount of added salts are only apparent, however. The two effects are due to a failure to include the effect of the added electrolyte in plotting (f_{RPo}, ln([NaCl]); $[MgCl_2]_{const}$) or (f_{RPo}, ln([NaCl]); $[Tris/HCl]_{const}$) data *versus* ln[NaCl]. The contribution of the added electrolyte to the overall salt concentration can be evaluated by introducing a concept of equivalent salt concentrations of two or more salts, at which a given thermodynamic or kinetic observable attains an identical value.

The [salt]-dependence of the binding equilibrium constant K_{obs} for entropy-driven protein–nucleic acid complexes is usually expressed in the form of a double-logarithmic linear plot (Record *et al.*, 1991) the slope of which is $d\ln K_{obs}/d\ln[salt] = n_{obs}$. Hence,

$$\ln K_{obs} = \ln K_{obs,1.0M} - n_{obs} \times \ln[salt] \qquad (2).$$

From the equality of the left-hand sides of Eqn. 2 written for salt1 and salt2, an expression for the equivalent concentration of salt2 in terms of [salt1], $[salt1]_{eqv,salt2}$, is obtained (subscript *obs* omitted for clarity of notations):

$$\ln[salt1]_{eqv,salt2} = \frac{n_{salt2}}{n_{salt1}} \times \ln[salt2] + \frac{\ln K_{1.0M,salt1} - \ln K_{1.0M,salt2}}{n_{salt1}} \qquad (3).$$

Hence, the thermodynamically effective concentration of salt1 should be expressed as [salt1] + $[salt1]_{eqv,salt2}$ and the binding isotherm given by Eqn. 1 reformulated accordingly to:

$$f_{RPo} = \frac{1}{1 + e^{\{n_{salt1} \times \ln([salt1] + [salt1]_{eqv,salt2}) - \ln K_{salt1}\}}} \qquad (1a).$$

Plots of the experimental (f_{RPo}, ln[NaCl]) titration data at a constant [$MgCl_2$] (or [Tris/HCl]) *versus* (ln([NaCl] + $[NaCl]_{eqv,MgCl_2}$) (or ln([NaCl] + $[NaCl]_{eqv,Tris/HCl}$)) should thus yield according to Eqn. 1a an isotherm identical with that obtained in the absence of the added $MgCl_2$. Indeed, the experimental isotherms for RPo–λP_R in mixed salts replotted accordingly transform to the one predicted by Eqn. 1 (cf. filled and open symbols in Figs. 1 and 2). This is an important finding showing that the quantitatively greatly different contributions of both electrolytes to the salt-de-

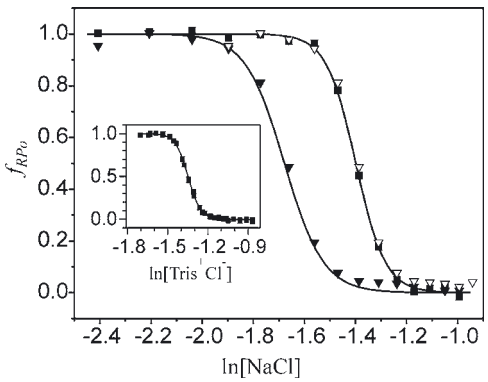

Figure 2. Binding isotherms at 25°C for RPo formation by *E. coli* RNA polymerase at λP_R promoter in NaCl, in the presence of a fixed concentration of 0.1 M Tris/HCl, pH 7.9, and in pure Tris/HCl buffer pH 7.9 (inset).
Experimental data points obtained in NaCl (BB buffer) are represented as rectangles, at 0.1 M Tris/HCl, pH 7.9 as filled triangles. Solid line for the isotherm in NaCl was calculated using Eqn. 1 and the fitted parameters: n = 17.3 and ln*K* = −24.2 (Table 1), that for the isotherm in mixed salt was calculated similarly using the apparent fitted parameters: n_{obs} = 14.5, x_0 = 0.189 and ln*K* = −24.16. Open symbols along the control isotherm correspond to the data points with X axis values calculated according to Eqn. 1a using $[NaCl]_{eqv,salt}$ = 0.060 M as the equivalent of the added 0.1 M Tris/HCl. The fitted parameters of the isotherm in pure Tris buffer are collected in Table 1.

pendence of RPo binding equilibrium are additive in the studied range of electrolyte concentrations. These contributions are only slightly concentration interdependent, as governed by the $n_{obs,MgCl_2}/n_{obs,NaCl}$ ratio in Eqn. 3, close to unity. In practical calculation of salt equivalents for RPo, this ratio can be set equal to 1 since the values thus calculated would remain within experimental error limits.

As in the case of (f_{RPo},ln[NaCl], $[MgCl_2]$) data, a good fit of the Boltzman function to the f_{RPo} ([NaCl], 0.1 M Tris) data (Fig. 2) was likewise obtained using the calculated equivalent $[NaCl]_{eqv,Tris}$ = 0.06 M NaCl of the added Tris/HCl (corresponding to 0.09 M Tris consisting in 70% of the acidic $Tris^+Cl^-$ form). Since in all the experiments shown in Fig. 1 a tenfold lower concentration of this buffer (0.01 M) was used, its effect on the salt dependence of RPo could be neglected. It is worth stressing in this context, that since both salts have a common Cl^- anion the Na^+ and $Tris^+$ ($^+N(CH_2OH)_3$) cations contribute almost equally to RPo stability, in spite of their very different sizes.

As for the equilibrium constant of RPo, the effects of mixed salts on composite kinetic rate constants of the complex formation, k_a, or dissociation, k_d, ($K_{obs} = k_a/k_d$) can be described in terms of equivalent salt concentrations at which these parameters assume equal values. The equivalent salt concentrations can be calculated similarly from experimental $d\ln(k_a, k_d)/d\ln[salt]$ data for any selected pair of salts using the corresponding fitted parameters and a generalized form of Eqn. 3:

$$\ln[salt1]_{eqv,salt2} = \frac{n_{salt2}}{n_{salt1}} \times \ln[salt2] + \frac{\ln X_{1.0M,salt1} - \ln X_{1.0M,salt2}}{n_{salt1}} \quad (3a),$$

where *X* stands for any experimentally determined equilibrium or kinetic constant or theoretically calculated free energy of binding.

The equivalent salt concentrations calculated according to Eqn. 3a can be used in reformulated Eqn. 2a:

$$\ln X_{salt1,salt2} = \ln X_{1.0M,salt1} - n_{salt1} \times \ln([salt1] + [salt1]_{eqv,salt2}) \quad (2a),$$

allowing explanation of the non-linearity of double-logarithmic plots of thermodynamic or kinetic parameters determined at a given [salt2] as a function of [salt1], and to predict the effects of salt mixtures on these parameters.

Using the available data for K_{obs}, k_a and/or k_d of RPo in NaCl, Tris/HCl, $MgCl_2$ and NaOAc (Roe & Record, 1985; Suh *et al.*, 1992; Łoziński *et al.*, 2009) we have evaluated with the help of Eqn. 3a the constant ratios of equivalent concentrations for selected pairs of salts (Table 2). In global calculations, the values of corresponding coefficients were set as a shared parameter. Inspection of Table 2 shows that the ratios of salt-equivalents for a given pair of salts in RPo formation (k_a) and dissociation (k_d) reac-

Table 2. Ratios of equivalent salt concentrations for RPo and selected pairs of salts.

The ratios were calculated using Eqn. 3a and thermodynamic parameters of RPo measured at identical conditions for each pair. Parameters in (A) are from Table 1 and (Łoziński *et al.*, 2009); those for k_a were calculated from the relationship: $\ln k_a = \ln K + \ln k_d$ using following relationships: $\ln K = -24.20 - 17.30 \times \ln[NaCl] = -48.92 - 18.34 \times \ln[MgCl_2]$; $-\ln k_d = -6.16 - 8.52 \times \ln[NaCl] = -16.62 - 9.85 \times \ln[MgCl_2]$; $\ln k_a = -18.04 - 8.78 \times \ln[NaCl] = -32.30 - 8.49 \times \ln[MgCl_2]$; $\ln K = -24.20 - 17.30 \times \ln[NaCl] = -29.46 - 21.75 \times \ln[Tris^+Cl^-]$; $\ln K = -29.46 - 21.75 \times \ln[Tris^+Cl^-] = -48.92 - 18.34 \times \ln[MgCl_2]$. Parameters in (B) are from (Roe & Record, 1985; Suh *et al.*, 1992): $\ln K_{eq} = -6.21 - 19.60 \times \ln[NaCl] = 4.50 - 19.60 \times \ln[NaOAc]$; $-\ln k_d = -3.45 - 7.70 \times \ln[NaCl] = 3.00 - 7.60 \times \ln[NaOAc]$; $\ln k_a = -2.76 - 11.9 \times \ln[NaCl] = 1.5 - 12.00 \times \ln[NaOAc]$.

Pairs of salts	k_a	k_d	K_{eq}	Data from
$[MgCl_2]_{eqv,NaCl}/[NaCl]$	0.17	0.38	0.28	(A)
$[NaCl]_{eqv,TrisCl}/[Tris^+Cl^-]$			0.96	(A)
$[MgCl_2]_{eqv,NaCl}/[Tris^+Cl^-]$			0.27	(A)
$[NaCl]_{eqv,NaOAc}/[NaOAc]$	0.70	0.43	0.58	(B)

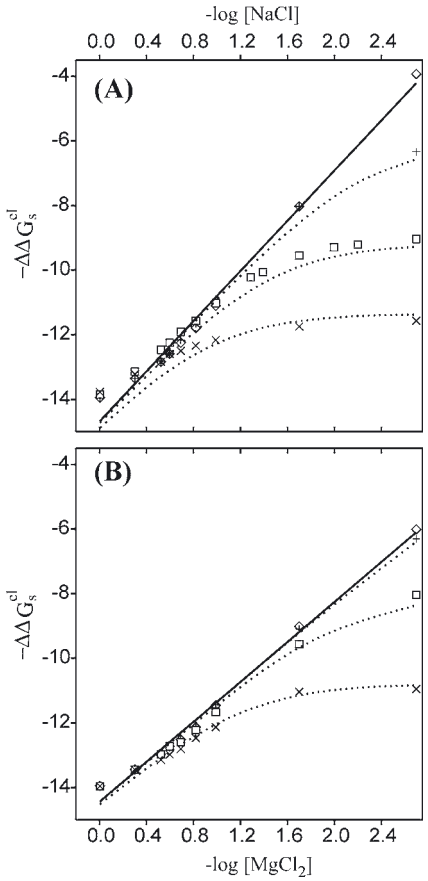

Figure 3. Comparison of NLPB-calculated electrostatic binding free energies of the λcI repressor–DNA complex in mixed NaCl + $MgCl_2$ salts (Chen & Honig, 1997) with those predicted using the respective calculated salt equivalents.

(A) ($\Delta\Delta G^{el}_s$, log[cation]) data points reproduced from Fig. 4 of (Chen & Honig, 1997) plotted as a function of log[Na^+]: (diamonds) [Mg^{2+}] = 0.0 M; (+) [Mg^{2+}] = 0.002 M; (rectangles) [Mg^{2+}] = 0.02 M; (×) [Mg^{2+}] = 0.1 M. Solid line is the linear least squares fit of the data in a pure NaCl salt; (B) ($\Delta\Delta G^{el}_s$, log[cation]) data points plotted as a function of log [Mg^{2+}] in the presence of NaCl: (diamonds) [Na^+] = 0.0 M; (+) [Na^+] = 0.002 M; (rectangle) [Na^+] = 0.02 M; (×) [Na^+] = 0.1 M. Solid line is the linear least squares fit of the data in pure $MgCl_2$. Numerical equations describing the solid lines in both panels are collected in legend to Fig. 4. Dotted lines in both panels represent respective $\Delta\Delta G^{el}_s$ dependencies on log[Na^+] and log[Mg^{2+}] predicted by Eqn. 2a using equivalent salt concentrations in pure NaCl and $MgCl_2$, calculated with help of Eqn. 3a. For parameters see the numerical equations in legend to Fig. 4.

tions and at equilibrium (K_{obs}) differ from one another. Because of the small differences between n_{obs} for association and dissociation reactions the averaged sum of these ratios for k_a and k_d is close to that calculated for K_{obs}.

To see whether the proposed approach for evaluation of thermodynamic and/or kinetic parameters in mixed salt solutions can have a more general analytical applicability to nucleic acids complexes we compared published experimental data for a number

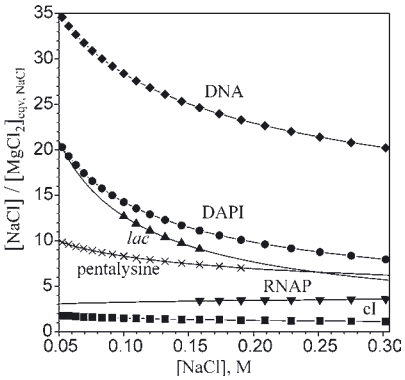

Figure 4. Ratio of equivalent NaCl and $MgCl_2$ concentrations as a function of [NaCl] for various dsDNA binding systems.

Data points, restricted to the original experimental range of salts, are reproduced from the cited papers and equivalent salt concentrations calculated using linear logarithmic correlation between the studied parameters and [salt] (shown in brackets): (diamonds) DNA duplex melting (Nakano *et al.*, 1999) (T_m = 39.0 + 6.43×log[NaCl] = 44.6 + 4.93×log[$MgCl_2$]); (circles) electrostatic binding free energy of the DAPI–DNA complex (Chen & Honig, 1997) ($-\Delta\Delta G$ = –8.67–2.35×log[NaCl] = –9.47–1.48×log [$MgCl_2$]); (up triangles) K_{obs} for *lac* repressor–operator DNA binding (deHaseth *et al.*, 1977) (logK_{obs} = –3.7–10.0×log[NaCl] = –5.9–5.8×log[$MgCl_2$]); (crosses)K_{obs} for pentalysine —T7 DNA binding (Lohman *et al.*, 1980) (logK_{obs} = –0.92–5.3×log[NaCl] = –3.7–4.2×log [$MgCl_2$]); (down triangles) K_{eq} for RPo–λP_R (see Table 1); (rectangles) $\Delta\Delta G^{el}_s$, of the λcI repressor–DNA complex (Chen & Honig, 1997) ($-\Delta\Delta G^{el}_s$ = –14.7–3.89×log[NaCl] = –14.4–3.09×log[$MgCl_2$]).

of selected DNA complexes with those predicted using salt equivalents. For instance, good agreement was obtained between the experimental (Leirmo *et al.*, 1987) and predicted association rate constants for *E. coli* RNA polymerase with λP_R promoter in KCl + KGlu solutions kept at constant [K^+] (cf. Table 3). This approach also proved to be applicable to NLPB calculated salt-dependence of electrostatic binding free energy, $\Delta\Delta G^{el}_s$, of λcl repressor and the antibiotic DAPI to dsDNA in mixed NaCl/$MgCl_2$ solutions (Chen & Honig, 1997). Good agreement was obtained between the literature $\Delta\Delta G^{el}_s$ *vs.* log[salt] plots and those predicted according to Eqn. 2a, as illustrated in Fig. 3 for λcl repressor. The demonstrated applicability of this method also to NLPB-calculated free electrostatic energies of binding seems to be of particular importance because the NLPB model breaks down at high salt concentrations due to a neglect of short-range interactions (Gavryushov & Zielenkiewicz, 1998; Misra & Daper, 1999). A similar quantitative agreement between published data and those predicted from salt equivalents was found for a number of other systems in mixed NaCl and $MgCl_2$ solutions. Pertinent data for dsDNA melting (Nakano *et al.*, 1999) are tabulated in Table 4. Those for *lac* repressor (Record *et al.*, 1977) and pentalysine (Lohman *et al.*, 1980) binding to dsDNA also indicat-

Table 3. Comparison of experimental and calculated association rate constants (k_a) for λP_R promoter and RNA polymerase in Cl^-/Glu^- mixtures at constant 0.2 M K^+.

Experimental k_a values are from (Leirmo *et al.*, 1987), those calculated were obtained with the help of Eqn. 2a using salt equivalents calculated according to Eqn. 3a. Fitted parameters from (Leirmo *et al.*, 1987): $\ln k_a = 4.55–4.80\times\ln[KCl] = 7.14 –6.50\times\ln[KGlu]$.

Fraction of Glu^-	Experimental k_a	Predicted k_a
	(M^{-1} s^{-1})	
0.00	$(1.7 \pm 0.1) \times 10^5$	2.1×10^5
0.18	$(5.6 \pm 3.1) \times 10^5$	4.6×10^5
0.36	$(1.2 \pm 0.6) \times 10^6$	1.0×10^6
0.54	$(2.9 \pm 1.2) \times 10^6$	2.4×10^6
0.90	$> 10^7$	2.1×10^7

ed a general agreement between experimental and predicted functional plots (not shown owing to single data series and difference in experimental conditions between particular data sets).

It was also of interest to know whether the salt equivalents determined for protein–DNA complexes of different structure differ from one another. For this purpose, we have compared equivalents of NaCl and $NaOOCCH_3$ for the binding equilibrium of $(SSB)_{65}$–poly(U) complex, calculated from published data: $\log K_{obsd} = –0.2–7.0\times\log[NaCl] = 1.9–6.6\times\log[NaOAc]$ (Overman *et al.*, 1988), with the corresponding one (Table 2) for the binding equilibrium of RPo. In spite of the widely different structure of the two complexes the $[NaCl]_{eqv,NaOOCCH3}/[NaOAc]$ ratios proved to be quite similar in the 0.05–0.2 M range of NaCl concentration, 0.58 and 0.56, respectively, for RPo and $(SSB)_{65}$–poly(U). Similar, as to the order of magnitude, $[MgCl_2]_{eqv,NaCl}$ values were also found for RPo and λcI–operator DNA complex (evaluated from NLPB calculations of the electrostatic free energy (Chen & Honig, 1997), as it can be inferred from the plots of $[NaCl]/[MgCl_2]_{eqv,NaCl}$ *vs.* [NaCl] shown in Fig. 4.

Table 4. Comparison of the experimental and predicted T_m values for d(GCCAGTTAA)/d(TTAACTGGC) formation as a function of $[MgCl_2]$ in the presence of 0.1 M NaCl.

Experimental T_m data are from Table 2 in (Nakano *et al.*, 1999). T_m values were predicted by Eqn. 2a using salt equivalents calculated according to Eqn. 3a; for the parameters see legend to Fig. 4.

Salt concentration (M)		Melting temperature (°C)		
NaCl	$MgCl_2$	T_m[b]	T_m[a]	Predicted T_m
0.1	0.1	38.5	38.7	39.7
0.1	0.01	35.1	35.6	35.4
0.1	0.001	31.8	32.1	33.0
0.1	0	32.0	31.9	32.6

[a]Experimental data (curve fit parameter); [b]Experimental data (T_m^{-1} *vs* log C_t parameter).

The n_{salt2}/n_{salt1} ratio dictates the dependence of $[salt1]_{eqv,salt2}$ on [salt2] (cf. Eqn. 3a). For binding of RNAP to λP_R and Pa promoters (Łoziński *et al.*, 2009) and of λcI repressor to its cognate dsDNA (Chen & Honig, 1997) this ratio depends only weakly on [NaCl] because in these systems $n_{MgCl_2}/n_{NaCl} \approx 1$. In contrast, in systems controlled mainly by competition between cationic species of different valence for dsDNA vicinity, this ratio reflects the stoichiometry of electrolyte cation binding. In mixed $NaCl/MgCl_2$ solutions, for systems characterized by $n_{salt2}/n_{salt1} \approx 2$, the ratio of $[NaCl]/[MgCl_2]_{eqv,NaCl}$ quickly decreases as a function of [NaCl]. Plots of the latter quotient in the 0.05–0.3 M NaCl range for a number of systems referred to above, calculated according to Eqn. 3a, have a pseudo-exponential form and differ characteristically from one another (Fig. 4). At 0.05 M NaCl, the $[NaCl]/[MgCl_2]_{eqv,NaCl}$ ratio attains the highest value for dsDNA melting (Nakano *et al.*, 1999) and consecutively lower values for dsDNA complexes with DAPI (Chen & Honig, 1997), λlac repressor (deHaseth *et al.*, 1977) and pentalysine (Lohman *et al.*, 1980). On the other hand, for RPo/ λP_R, RPo/ Pa and λcI repressor-operator complexes this ratio varies but weakly with [NaCl], as discussed above. In this way Mg^{2+} ions can be used as a probe to differentiate DNA–protein systems governed mainly by polyelectrolyte effect of DNA and those dependent mostly on the overall ionic compensation.

CONCLUDING REMARKS

The most important conclusion of this work is that in mixed solutions of commonly used salts their individual contributions to the cumulative salt-effect on the thermodynamic and kinetic *in vitro* observables for DNA–protein complexes, expressed as thermodynamically equivalent salt concentrations, are additive in the whole range of salt concentrations within which the complexes are stable. Based on this finding a simple method for practical evaluation of these observables in mixed salt solution was developed and its applicability to a number of different protein–DNA complexes and dsDNA melting demonstrated. It can be thus recommended for a more general use in biochemical and biophysical studies for facile assessment of the sought optimal mixed salt conditions. Salt concentrations considered as equivalent to those found in *E. coli* cells, *viz.* 0.15 M KCl/NaCl and 0.005 M $MgCl_2$ (Record *et al.*, 1996), remain within the range of concentrations used in the *in vitro* experiments analyzed in this work. The method of salt equivalents can not be applied, however, to *in vivo* systems since electrolytes in living cells do not form true aqueous solutions, as for a large part they are dynamically associated with macromolecular polyionic cell components.

REFERENCES

Burgess RR, Jendrisek JJ (1975) A procedure for the rapid, large scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polyamine P precipitation and DNA-cellulose chromatography. *Biochemistry* **14**: 4634–4638.

Capp MW, Cayley DS, Zhang W, Guttman HJ, Melcher SE, Saecker RM, Anderson CF, Record MT Jr (1996) Compensation effects of opposing changes in putrescine (2+) and K^+ concentrations on *lac* repressor–*lac* operator binding: *in vitro* thermodynamic analysis and *in vivo* relevance. *J Mol Biol* **258:** 25–36.

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

Chen SW, Honig B (1997) Monovalent and divalent salt effects on electrostatic free energies defined by the nonlinear Poisson-Boltzmann equation: application to DNA binding reactions. *J Phys Chem B* **101**: 9113–9118.

Cuauhtemoc G-G, Draper DE (2003) Electrostatic interactions in a peptide-RNA complex. *J Mol Biol* **331**: 75–88.

deHaseth PL, Lohman TM, Record MT Jr (1977) Nonspecific interaction of lac repressor with DNA: an association reaction driven by counterion release. *Biochemistry* **16**: 4783–4790.

Gavryushov S, Zielenkiewicz P (1998) Electrostatic potential of B-DNA: effect of interionic correlations. *Biophys J* **75**: 2732–2742.

Kolasa IK, Łoziński T, Wierzchowski KL (2001) Mg^{2+} does not induce isomerization of the open transcription complex of *Escherichia coli* RNA polymerase at the model Pa promoter bearing consensus –10 and –35 hexamers. *Acta Biochim Polon* **48**: 985–994.

Leirmo S, Harrison C, Cayley DS, Burgess RR, Record MT Jr (1987) Replacement of potassium chloride by potassium glutamate dramatically enhances protein–DNA interactions *in vitro*. *Biochemistry* **26**: 2095–2101.

Leirmo S, Record MT Jr (1990) Structural, thermodynamic and kinetic studies of the interaction of $E\sigma^{70}$ RNA polymerase with promoter DNA. In *Nucleic acids and molecular biology*. Eckstein F, Lilley DMJ, eds. vol 4, pp 123–151. Springer-Verlag, Berlin, Heidelberg.

Lohman TM, deHaseth PL, Record Jr MT (1980) Pentalysine-deoxyribonucleic acid interaction. A model for the general effects of ion concentrations on the interaction of proteins with nucleic acids. *Biochemistry* **19**: 3522–3530.

Łoziński T, Wierzchowski KL (2005) Mg^{2+} modulated $KMnO_4$ reactivity of thymines in the open transcription complex reflects variation in the negative electrostatic potential along the separated DNA strands. Footprinting of *Escherichia coli* RNA polymerase complex at the λP_R promoter revisited. *FEBS J* **272**: 2838–2853.

Łoziński T, Markiewicz WT, Wyrzykiewicz TK, Wierzchowski KL (1989) Effect of the sequence-dependent structure of the 17 bp AT spacer on the strength of consensus-like *E.coli* promoters *in vivo*. *Nucleic Acids Res* **17**: 3855–3863.

Łoziński T, Bolewska K, Wierzchowski KL (2009) Equivalence of Mg2+ and Na+ ions in salt dependence of the equilibrium binding and dissociation rate constants of *Escherichia coli* RNA polymerase open complex. *Biophys Chem* **142**: 65–75.

Mascotti DP, Lohman TM (1993) Thermodynamics of single-stranded RNA and DNA interactions with oligolysines containing tryptophan. Effects of base composition. *Biochemistry* **32**: 10568–10579.

Mascotti DP, Lohman TM (1997) Thermodynamics of oligoarginines binding to RNA and DNA. *Biochemistry* **36**: 7272–7279.

Misra VK, Draper DE (1999) The interpretation of Mg(2+) binding isotherms for nucleic acids using Poisson-Boltzman theory. *J Mol Biol* **294**: 1135–1147.

Misra VK, Sharp KA, Friedman RA, Honig B (1994a) Salt effects on ligand-DNA binding; Minor groove binding antibiotics. *J Mol Biol* **238**: 245–263.

Misra VK, Hecht J, Sharp K, Friedman RA, Honig B (1994b) Salt effects on protein–DNA interactions. The lambda cI repressor and *Eco*R1 endonuclease. *J Mol Biol* **238**: 264–280.

Nakano S, Fujimoto M, Hara H, Sugimoto N (1999) Nucleic acid duplex stability: influence of base composition on cation effects. *Nucleic Acids Res* **27**: 2957–2965.

Overman LB, Bujalowski W, Lohman TM (1988) Equilibrium binding of *Escherichia coli* single-strand binding protein to single-stranded nucleic acids in the (SSB)65 binding mode. Cation and anion effects and polynucleotide specificity. *Biochemistry* **27**: 456–471.

Record Jr MT, deHaseth PL, Lohman TM (1977) Interpretation of monovalent and divalent cation effects on the *lac* repressor-operator interaction. *Biochemistry* **16**: 4783–4791.

Record Jr MT, Ha J-H, Fisher MA (1991) Analysis of equilibrium and kinetic measurements to determine thermodynamic origins of stability and specificity and mechanism of formation of site-specific complexes between proteins and helical DNA. *Methods Enzymol* **208**: 291–343.

Record Jr MT, Reznikoff WS, Craig ML, McQuade KL, Schlaz PJ (1996) *Escherichia coli* RNA polymerase ($E\sigma^{70}$), promoters, and the kinetics of the steps of transcription initiation. In *Escherichia coli and Salmonella: cellular and molecular biology*. Neidhardt FC, ed, pp 792–820. ASM Press, Washington D.C.

Roe J-H, Record Jr MT (1985) Regulation of the kinetics of the interaction of *Escherichia coli* RNA polymerase with the λP_R promoter by salt concentration. *Biochemistry* **24**: 4721–4726.

Rouzina I, Pant K, Karpel RL, Williams MC (2005) Theory of electrostatically regulated binding of T4 gene 32 protein to single- and double-stranded DNA. *Biophys J* **89**: 1941–1956.

Shokri L, Marintcheva B, Richardson CC, Rouzina I, Williams MC (2006) Single molecule force spectroscopy of salt-dependent bacteriophage T7 gene 2.5 protein binding to single-stranded DNA. *J Biol Chem* **281**: 38689–38696.

Shokri L, Marintcheva B, Eldib M, Hanke A, Rouzina I, Williams MC (2008) Kinetics and thermodynamics of salt-dependent T7 gene 2.5 protein binding to single- and double-stranded DNA. *Nucleic Acids Res* **36**: 5668–5677.

Sosunov V, Sosunova E, Mustaev A, Bass I, Nikiforov V, Goldfarb A (2003) Unified two-metal mechanism of RNA synthesis and degradation by RNA polymerase. *EMBO J* **22**: 2234–2244.

Suh WC, Leirmo S, Record Jr MT (1992) Roles of Mg^{2+} in the mechanisms of formation and dissociation of open complex between *Escherichia coli* RNA polymerase and the λP_R promoter; kinetic evidence for a second open complex requiring Mg^{2+}. *Biochemistry* **31**: 7815–7825.

Yarborough LR, Schlageck JG, Baughman M (1979) Synthesis and properties of fluorescent nucleotide substrates for DNA-dependent RNA polymerases. *J Biol Chem* **254**: 12069–12073.