Cation binding linked to a sequence-specific CAP–DNA interaction

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Abstract

The equilibrium association constant observed for many DNA–protein interactions in vitro (K_{obs}) is strongly dependent on the salt concentration of the reaction buffer ([MX]). This dependence is often used to estimate the number of ionic contacts between protein and DNA by assuming that release of cations from the DNA is the dominant involvement of ions in the binding reaction. With this assumption, the graph of $\log K_{obs}$ versus $\log[MX]$ is predicted to have a constant slope proportional to the number of ions released from the DNA upon protein binding. However, experimental data often deviate from log-linearity at low salt concentrations. Here we show that for the sequence-specific interaction of CAP with its primary site in the lactose promoter, ionic stoichiometries depend strongly on cation identity and weakly on anion identity. This outcome is consistent with a simple linkage model in which cation binding by the protein accompanies its association with DNA. The order of ion affinities deduced from analysis of DNA binding is the same as that inferred from urea-denaturation experiments performed in the absence of DNA, suggesting that ion binding to free CAP contributes significantly to the ionic stoichiometry of DNA binding. In living cells, the coupling of ion-uptake and DNA binding mechanisms could reduce the sensitivity of gene-regulatory interactions to changes in environmental salt concentration.
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1. Introduction

The *Escherichia coli* cyclic AMP receptor protein (CAP) regulates the transcription of a large network of genes [1–3]. CAP is a stable dimer of identical subunits of molecular weight 23,619, each of which is capable of binding a single molecule of cAMP [4,5]. CAP dimers bind to duplex DNA in sequence specific and nonspecific modes that differ markedly in affinity and magnitude of nearest-neighbor cooperativity (reviewed in [1]). The apparent equilibrium constants (K_{obs}) for the nonspecific and specific DNA interactions of CAP are strongly dependent on salt concentration [6–9]. These effects can be interpreted in terms of the direct stoichiometric participation of ions in the DNA-binding reaction [10,11].

At constant temperature and pH, the association of CAP (C) and DNA (D) to form CAP–DNA complexes (C·D) in a salt solution containing a single type of monovalent cation (M$^+$) and a single type of monovalent anion (X$^-$) may be represented by

$$C \cdot M_{m_1}^+ \cdot X_{n_1}^- + D \cdot M_{q_1}^+ \rightleftharpoons (C \cdot M_{m_2}^+ \cdot X_{n_2}^-) \cdot (D \cdot M_{q_2}^+) + (m_1 - m_2 + q_1 - q_2)M^+ + (n_1 - n_2)X^-. \quad (1)$$

Here m, n and q represent numbers of ions associated (in the thermodynamic sense) with C, D and C·D before and after binding and we count separately the changes in the numbers of cations associated with the protein ($m_1 - m_2$), anions associated with the protein ($n_1 - n_2$) and cations associated with the DNA ($q_1 - q_2$) [9, 10]. With appropriate assumptions about macromolecular hydration and ion activities, the composite cation and anion stoichiometries of this reaction can be estimated from the dependence of $\log K_{obs}$ on $\log[MX]$ [10–13]. Eq. (2) is a version

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of the linkage relation that counts changes in cations associated with protein and DNA separately.

$$\frac{\partial \log K_{obs}}{\partial \log[MX]} = -(\Delta m + \Delta n + \Delta q). \tag{2}$$

Here $\Delta m = m_1 - m_2$, $\Delta n = n_1 - n_2$ and $\Delta q = q_1 - q_2$. Because the charge density of DNA generally exceeds that of protein, it is often assumed that Δm and Δn are negligible, i.e., that $\partial \log K_{obs}/\partial \log[MX] = -\Delta q$. When this is the case, a graph of $\log K_{obs}$ versus $\log[MX]$ is linear, with a slope equal to $-\Delta q$, the change in the number of DNA-associated cations [11,14]. Since monovalent cations associate with duplex DNA to an extent equal to 0.88/phosphate over a wide range of salt concentrations [10], the value $Z = -\Delta q/0.88$ has been interpreted as the number of ion pairs formed between protein and DNA (cf., [7,13,15–17]). However, $\partial \log K_{obs}/\partial \log[MX]$ is not always constant over the experimental range of salt concentrations. For many well-characterized systems, $\partial \log K_{obs}/\partial \log[MX]$ becomes less negative with decreasing [salt] [7,9,17–24] and for three that have been studied at sufficiently low salt concentrations $\partial \log K_{obs}/\partial \log[MX]$ becomes positive [9,18,23].

What is the source of the ion uptake implied by positive values of $\partial \log K_{obs}/\partial \log[MX]$? Cation release from the DNA and anion release from the protein may account for some nonlinearity in the dependence of $\log K_{obs}$ on $\log[MX]$ [10,12,25] but these processes alone cannot produce net ion uptake. As a working hypothesis, we have proposed that the protein binds cations as it associates with DNA [9,23]. Several features of this idea can be tested. If cation binding is specific, changes in the identity of the dominant solvent cation should lead to changes in the affinity and possibly the stoichiometry of the ion-binding reaction that accompanies protein–DNA interaction. On the other hand, if anions are preferentially bound, anion substitution should lead to these changes. If ion substitution changes the mechanism of the protein–DNA interaction (by mediating for example a conformational change in protein or DNA), this may be accompanied by changes in the cation-release stoichiometry (Δq) or by changes in the non-electrostatic component of the binding free energy [10]. Finally, if the same ensemble of protein sites is involved in ion binding in free solution and during the formation of the protein–DNA complex, ion substitution may affect DNA binding and urea denaturation in similar ways. The results of experiments designed to test these predictions are presented below.

2. Materials and methods

2.1. Reagents

Acrylamide (ultra-pure grade) and urea were purchased from Boehringer Mannheim. Cyclic AMP, bovine serum albumin and N,N'-methylene bisacrylamide were purchased from Sigma. Cesium chloride, lithium chloride, potassium hydroxide, potassium chloride, potassium acetate and potassium phosphate were purchased from Malinckrodt. L-Glutamic acid and ultra-pure urea were from Schwartz-Mann. [γ-^{32}P]-ATP was

purchased from Du Pont-New England Nuclear. Endonucleases f1 and HinHII and T_4 polynucleotide kinase were purchased from New England Biolabs. Bacterial alkaline phosphatase was from Pharmacia.

2.2. CAP

The E. coli cyclic AMP receptor protein was isolated from strain pp47 containing plasmid pHA5 (the kind gift of Dr. H. Aiba), using previously-described methods [26]. The isolated protein was more than 95% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically, using $\varepsilon_{280} = 3.5 \times 10^4$ $M^{-1}cm^{-1}$ per CAP dimer [27]. The preparations used in this study were 50%–60% active in cAMP-dependent binding to the lac promoter, according to the method of Fried and Crothers [28]. Both cAMP- and DNA-binding activities were unchanged over the salt- and protein-concentration ranges used in this study. Since the cAMP- and DNA-binding activities of monomeric CAP differ significantly from those of the dimer [29,30], these results are inconsistent with large changes in mole fraction of CAP dimer over the range of solution conditions investigated. In the binding studies presented below, the CAP concentrations given refer to the species active in cAMP-dependent sequence-specific DNA binding. In the fluorescence experiments, the CAP concentrations given refer to total protein, determined by absorbance at 280 nm.

2.3. DNA

Plasmid pMM02 has been previously described [23]. A 219 base pair lactose promoter fragment (Fig. 1) was isolated from this plasmid by HinHII endonuclease digestion followed by chromatography on Sepharose CL-4B as previously described [31]. In a few experiments, a 214 bp lactose promoter fragment isolated from endonuclease Hinf1 digests of pUC19 DNA [32] was employed. With the exception of their ends, these fragments span the same DNA sequence as the 203 bp lac

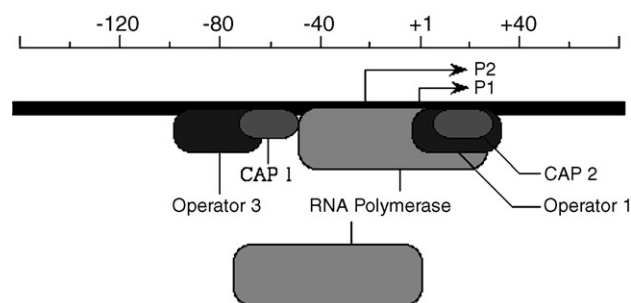Fig. 1. Map of regulatory and promoter binding sites in the lac regulatory region. Redrawn from [8]. Residues are numbered from the start-point of transcription of the primary in vivo promoter, P1. There are two high-affinity binding sites for CAP, two for lac repressor and two for RNA polymerase (one has been displaced for clarity). The site designated CAP1 is the genetically-defined locus of gene activation. For the range of binding conditions used in this study, $K_{obs}^{site\ 1} > 20\ K_{obs}^{site\ 2}$ [6,28,36]. Accordingly, CAP is predominantly bound at site 1 in the 1:1 complexes considered here.

promoter fragment employed in previous studies [28,33]. DNA fragments were labeled at 5′ termini with ^{32}P according to the method of Maxam and Gilbert [34].

2.4. Binding assays

Electrophoresis mobility shift assays were carried out as described by Fried and Crothers [28] with the following modifications. Polyacrylamide slabs (9.86% acrylamide, 0.14% bisacrylamide) were cast in buffer containing 45 mM Tris-borate, 1 mM EDTA (pH 8.0 at 20±1 °C) and equilibrated with 20 μM cAMP. The binding buffer was either 10 mM Tris (pH 8.0 at 20±1 °C), 1 mM EDTA or 1 mM Tris (pH 8.0 at 20±1°C), 0.1 mM EDTA as indicated; these buffers were supplemented with 20 μM cAMP, 25 μg/ml BSA, 5% glycerol and potassium chloride, lithium chloride or cesium chloride to obtain the desired final concentrations. Reaction mixtures were equilibrated at 20±1 °C for 1 h to ensure attainment of binding equilibrium.[1] Samples were mixed with 1/20 volume loading dye (0.01% bromophenol blue, 0.01% xylene cyanol, 50% glycerol, 10 mM Tris (pH 8.0 at 20±1 °C), 1 mM EDTA) and applied immediately to the gel.[2] Electrophoresis was carried out at 8 V/cm for 45 min. Autoradiographs of developed gels were obtained with Kodak XAR-5 film, exposed at 4 °C. These were used to guide the excision of gel sections containing the individual electrophoretic species and the interband regions in each lane. The ^{32}P-DNA present in each gel slice was quantitated by scintillation counting. Gel slices of similar size containing no ^{32}P-labeled species were excised from the margins of each gel for use as scintillation counting controls.

Nitrocellulose filter-binding assays were carried out as described by Koop et al. [35], with the following modifications. The filters used were type HAWP, 0.45 mM, from Millipore. The binding buffer consisted of either 10 mM Tris (pH 8.0 at 20±1 °C), 1 mM EDTA or 1 mM Tris (pH 8.0 at 20±1 °C), 0.1 mM EDTA as indicated; these buffers were supplemented with 20 μM cAMP, 25 μg/ml BSA, 5% glycerol and potassium chloride, lithium chloride or cesium chloride to obtain the desired final concentrations. Equilibration of samples was for 1 h at 20±1 °C. The filter manifold pressure was controlled so that filtration of samples (500 μl) required 30 s. Filters were washed at the same filtration rate with 500 μl of binding buffer, air dried and counted in 5 ml of Beckman Ready Value® scintillation cocktail in a Beckman LS 5000 scintillation counter.

2.5. Binding analyses

Within the range of CAP concentrations examined here, only one complex formed with *lac* promoter DNA is detected by native gel electrophoresis (Fig. 2A). This complex contains one equivalent of CAP (dimer) per DNA, occupying predominantly
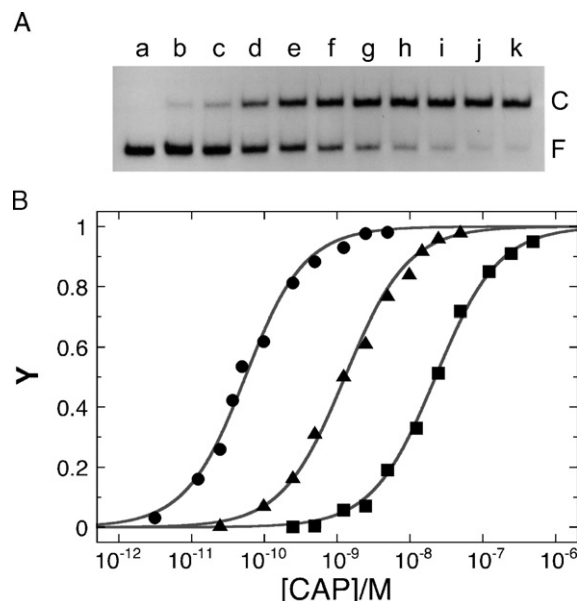

Fig. 2. Binding of CAP to *lac* promoter fragments. (A) Mobility shift analysis. Reactions were carried out at 21±1 °C in 10 mM Tris, 1 mM EDTA, 100 mM KCl, 50 μg/ml bovine serum albumin, 5% glycerol and 20 μM cAMP. Each reaction contained the 219 bp *lac* promoter fragment at a final concentration of 5.1×10^{-11} M. CAP concentrations in samples a–k are 0 M, 2.9×10^{-10} M, 5.9×10^{-10} M, 1.2×10^{-9} M, 1.8×10^{-9} M, 2.4×10^{-9} M, 3.2×10^{-9} M, 4.5×10^{-9} M, 5.9×10^{-9} M, 1.2×10^{-8} M and 2.1×10^{-8} M, respectively. The complex (C) contains one CAP dimer per DNA fragment bound to CAP site 1 [9,28,36]. Free DNA is designated by F. (B) Representative isotherms. EMSA and nitrocellulose filtration assays were carried out at 21±1 °C. Reaction mixtures contained 219 bp *lac* promoter fragment (1.3×10^{-11} M) in 10 mM Tris, 1 mM EDTA, 20 μM cAMP, 5% glycerol, 25 μg/ml bovine serum albumin, supplemented with (●) 0.02 M CsCl, (▲) 0.1 M KCl or (■) 0.32 M LiCl. Reactions with CsCl and LiCl were assayed by nitrocellulose filtration; that with KCl was assayed by EMSA. The smooth curves are fits of Eq. (4) to the data, returning $K_{obs} = 2.1 \pm 0.1 \times 10^{10}$ M^{-1} for samples in 0.02 M CsCl, $7.9 \pm 0.4 \times 10^{8}$ M^{-1} for samples in 0.1 M KCl and $4.5 \pm 0.2 \times 10^{7}$ M^{-1} for samples in 0.32 M LiCl.

the genetically defined regulatory binding site, CAP site 1 (Fig. 1) [8,28,36]. At the concentration of cAMP employed (20 μM), one molecule of cAMP is bound per CAP dimer both free in solution and in the CAP–promoter complex [28,37]. The formation of the 1:1:1 CAP–cAMP–*lac* promoter complex can be represented by

$$CA + D \underset{}{\overset{K_{obs}}{\rightleftarrows}} CAD. \tag{3}$$

In which CA represents the CAP–cAMP complex, D the *lac* promoter restriction fragment, CAD the CAP–cAMP–DNA complex and $K_{obs} = [CAD]/[CA][D]$. Values of K_{obs} were obtained by fitting Eq. (4) to the binding data.

$$Y = E\left(\frac{a - (a^2 - 4b)^{1/2}}{2}\right). \tag{4}$$

Here $Y = [CAD]/([D] + [CAD])$, $a = 1 + ([CA]_{tot} + 1/K_{obs})/[D]_{tot}$, $b = [CA]_{tot}/[D]_{tot}$ and $[CA]_{tot}$ and $[D]_{tot}$ are the total concentrations of CAP–cAMP complex and DNA molecules in

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[1] Verified by incubating selected duplicate samples for longer intervals.
[2] Control experiments in which samples were applied directly to gels without the aid of the dye-glycerol buffer gave equivalent results, indicating that this addition does not perturb binding to a detectable degree (results not shown).

the sample, respectively [23]. The concentrations [D] and [CAD] were determined from the known specific activity of the *lac* promoter fragment, while [CA] was calculated from [CA] = [CA]$_{input}$ − [CAD]. The parameter E is the efficiency with which radioactive DNA is recovered from reaction mixtures. For mobility shift assays, E represents the fraction of sample counts recovered within the gel (typically ≥ 0.97). For filter binding assays E is the fraction of counts retained by the filter at protein saturation. Over the range of conditions considered here, $E = 0.6 \pm 0.1$. This value compares well with ones previously reported for CAP [9] and for other proteins (cf., [38]). In control filter-binding assays, the value of E showed no obvious systematic dependence on salt concentration or identity of the dominant buffer cation or anion (result not shown).

2.6. Linkage of ion binding and DNA binding

A simple model in which the ion stoichiometry of the protein–DNA interaction is the sum of contributions from cation binding by the protein and cation dissociation from the DNA predicts positive values of $\partial \log K_{obs}/\partial \log[MX]$ at low [salt] and negative values at high [salt] [9]. If m_{tot} identical, independent cation binding sites on the protein become occupied as CAP binds DNA, $\partial \log K_{obs}/\partial \log[MX]$ becomes

$$\frac{\partial \log K_{obs}}{\partial \log[MX]} = -\left(m_{tot}\left(1 - \frac{K_a^{M^+}[MX]}{1 + K_a^{M^+}[MX]}\right) + \Delta t\right). \quad (5)$$

Here $K_a^{M^+}$ is the association constant for ion binding to protein and $\Delta t = \Delta n + \Delta q$, the sum of cation- and anion-release stoichiometries. In this context, the dependence of K_{obs} on [salt] can be approximated by

$$\log K_{obs} = \log K_T$$
$$= -\left(m_{tot}\left(1 - \frac{K_a^{M^+}[MX]}{1 + K_a^{M^+}[MX]}\right) + \Delta t\right)\log[MX]. \quad (6)$$

Here K_T is the equilibrium constant for formation of the CAP–DNA complex in a standard state at 1 M salt, extrapolated from the limiting low-salt behavior. Because salts are osmolytes and since these expressions neglect preferential hydration, the parameters derived from fits to the data should be considered "apparent" rather than true thermodynamic quantities. Similar relationships were first derived by Record et al. (cf. [11,14,39]).

2.7. Urea denaturation detected by fluorescence anisotropy

Fluorescence spectra and anisotropy measurements were obtained at 20 ± 0.1 °C, as described [8]. For a two-state denaturation reaction, the observed anisotropy r_{obs} is given by

$$r_{obs} = f_n r_n + f_d r_d. \quad (7)$$

Here f_n and F_d are the fractional contributions to the total fluorescence of native and denatured states and r_n and r_d are the anisotropies of native and denatured states, respectively.

If no change in quantum yield occurs on denaturation, f_n and f_d are equal to the mole fractions of the corresponding states and are related to experimentally observed anisotropy according to

$$f_d = (r_{obs-}r_n)/(r_d - r_n)$$
$$f_n = 1 - f_d. \quad (8)$$

Samples were adjusted to the desired final concentration of urea and equilibrated at 20 °C until no further change in anisotropy was evident. This required ≤ 2 h, judged by comparison with samples equilibrated overnight. The same anisotropy values were obtained regardless of whether the final concentration of urea was reached by addition of urea to native CAP solution dilution or urea-denatured CAP with buffer (result not shown). This path independence is evidence that denaturation of CAP is reversible and that equilibrium was attained under the conditions reported here.

3. Results

3.1. Binding affinities and $\partial \log K_{obs}/\partial \log[MX]$ depend on cation concentration and identity

A prediction of models that couple cation- and DNA-binding is that substitution of the dominant solution cation (at constant pH and temperature) should change $\partial \log K_{obs}/\partial \log[MX]$.[3] This is because changing the identity of the dominant cation should alter the population-average association constant of cation–protein interactions ($K_a^{M^+}$) and possibly the number of ion binding sites that change occupancy (m_{tot}). Cation substitution should, in addition, alter the strength of the cation–DNA interaction [25,40,41] and hence the free energy change associated with the cation release from the DNA that accompanies protein binding.

To test these predictions, we examined the effect of cation substitution on the stability of the CAP complex with lactose promoter CAP site 1 [33]. Fig. 2A shows a typical electrophoresis mobility shift assay (EMSA), allowing measurement of free and bound DNA concentrations as a function of [CAP]. The complex designated C consists of CAP and DNA in a 1:1 molar ratio, with CAP bound predominantly at CAP site 1 [33,36]. Shown in Fig. 2B are representative binding isotherms determined by EMSA and filter binding in the presence of CsCl, KCl or LiCl. Similar results were obtained when both assays were used in parallel (see below), indicating that assay-dependent errors in the measurement of K_{obs} are likely to be small.

At salt concentrations greater than 0.1 M (log[KCl] > −1.0), the graph of $\log K_{obs}$ *versus* log[KCl] is nearly linear and has a negative slope indicative of net ion release [11,42]. Linear regression on this portion of the data gives $\partial \log K_{obs}/\partial \log[KCl] = -3.2 \pm 0.1$. This value is comparable to earlier measurements

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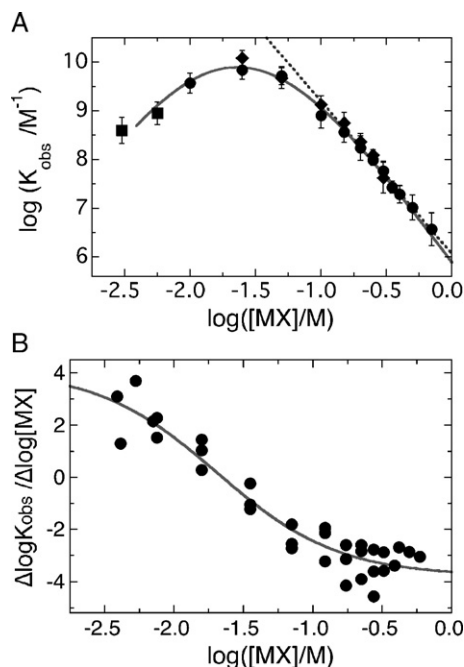[3] Except for the trivial case in which the cation stoichiometry difference for the protein (Δm) is zero.

Fig. 3. KCl concentration dependence of CAP binding to CAP site 1. (A) Relation of $\log K_{obs}$ to $\log[KCl]$. Reaction mixtures contained (■) 6.26×10^{-10} M *lac* promoter DNA fragment, suspended in 1 mM Tris (pH 8.0 at 20 ± 1 °C), 0.1 mM EDTA, 20 µM cAMP, 25 µg/ml BSA, 5% glycerol, plus KCl as indicated, or (●, ◆) 1.3×10^{-11} M *lac* promoter DNA fragment, suspended in 10 mM Tris (pH 8.0 at 21 °C), 1 mM EDTA, 20 µM cAMP, 25 µg/ml BSA, 5% glycerol, plus KCl as indicated. Binding was carried out at 20 ± 1 °C. Samples were assayed by filtration (■, ●) or by EMSA (◆). The error bars represent 95% confidence limits for the individual measurements. A subset of this data has been previously published [9]. The dashed line corresponds to a linear fit to the data for which $\log[KCl] \geq -0.69$. The solid curve was obtained by fitting Eq. (6) to the entire data set using values of cation stoichiometry (m_{tot}, Δq) and affinity ($K_a^{M^+}$) derived from the slope analysis shown in panel B. (B) Change in slope ($\partial \log K_{obs}/\partial \log[MX]$) with $\log[MX]$ for data shown in panel A. Differences ($\Delta \log K_{obs}/\Delta \log[MX]$) were calculated for pairs of contiguous points ($i, i+1$) and for pairs of points separated by one ($i, i+2$). Points are plotted at the average log [MX] value for each interval. The curve is a least-squares fit to these data with $\partial \log K_{obs}/\partial \log[MX]$ given by Eq. (5). This fit returned cation stoichiometries of $\Delta q = -3.8 \pm 0.3$ and $m_{tot} = 7.9 \pm 0.6$ and the apparent association constant $K_a^{M^+} = 48.6 \pm 11.2$ M^{-1}.

obtained by Takahashi et al. [6] and by Fried and Stickle [9], but is smaller than that found by Ebright et al. for the binding of CAP to a 40 bp fragment containing the CAP site 1 sequence ($\partial \log K_{obs}/\partial \log[KCl] = -5.2$; [7]). This may reflect differences in DNA templates used in the binding assays or in the cation composition of binding buffers, as described below. When

the salt concentration is less than 0.2 M ($\log[KCl] < -0.69$), $\partial \log K_{obs}/\partial \log[KCl]$ becomes less negative and below log $[KCl] \leq -1.6$ (25 mM KCl) it becomes positive, consistent with the operation of an additional mechanism involving net ion uptake that becomes dominant at low [salt]. The smooth curves shown in Fig. 3 demonstrate that the simple model embodied in Eqs. (5) and (6) can account for the binding data over a wide range of [KCl]. The apparent ion stoichiometries (m_{tot}, Δt) and association constants $K_a^{M^+}$ and K_T obtained by fitting Eqs. (5) and (6) to the data are summarized in Table 1.

Experiments carried out with solutions containing LiCl and CsCl gave results that were qualitatively similar to those obtained with KCl solutions (Fig. 4). Positive values of $\partial \log K_{obs}/\partial \log[MX]$ at low [salt] demonstrate that net ion uptake is not a unique property of the molecular system in KCl-containing buffers, while the fits of Eqs. (5) and (6) to the data show that the cation-binding model can account for the dependence of K_{obs} on [salt] when Li$^+$ and Cs$^+$ are the dominant cations. Intriguingly, these fits show that the limiting number of cations bound (m_{tot}) is similar in Li$^+$, Cs$^+$ and K$^+$ solutions (Table 1), suggesting that similar ensembles of binding sites are available to these cations. On the other hand, the association constants that characterize binding ($K_a^{M^+}$) differ significantly, as do values of the aggregate ion-release stoichiometry (Δt). Change in $K_a^{M^+}$ with cation substitution is a prediction of the cation-uptake model (see above). Differences in the ion-release term Δt with cation substitution may reflect changes in the numbers of cations released from the DNA (Δq) or changes in the number of anions released from CAP as it associates with the DNA (Δn), or both. If anions are bound specifically, we might expect anion substitution to influence $\partial \log K_{obs}/\partial \log[MX]$. A test of this possibility is described below.

3.2. The dependence of $\log K_{obs}$ on $\log[MX]$ is little changed by anion substitution

If Δn is significant compared to Δq, changes in the occupancy of anion binding sites on the protein should result in measurable changes in the aggregate ion-release stoichiometry, Δt. Since anion substitution has the potential to alter the affinity and/or number of anion sites contributing to Δn, we examined the effects of anion substitution on $\partial \log K_{obs}/\partial \log[MX]$. Shown in Fig. 5 are graphs of $\log K_{obs}$ as a function of $\log[MX]$ for solutions in which the dominant

Table 1
Estimates of cation stoichiometries and affinities [a]

Cation	m_{tot} [b]	$K_a^{M^+}$ (M^{-1}) [b]	Δt [b]	Z [c]	$\log K_T$ [d]	ΔG_T^0 (kcal/mol)	$\Delta G_{0.1\ M}^0$ (kcal/mol)
K$^+$	7.9 ± 0.6	48.6 ± 11.2	-3.8 ± 0.3	4.3 ± 0.3	5.89 ± 0.21	-7.9 ± 0.3	-12.1 ± 0.2
Cs$^+$	8.5 ± 0.7	8.26 ± 0.19	-6.2 ± 0.9	7.0 ± 1.0	6.52 ± 0.25	-8.7 ± 0.3	-13.6 ± 0.3
Li$^+$	8.3 ± 0.5	24.4 ± 6.1	-4.9 ± 0.6	5.6 ± 0.6	5.56 ± 0.28	-7.5 ± 0.4	-12.5 ± 0.2

[a] The error ranges are 95% confidence limits.

[b] From fitting Eq. (5) to the dependence of $\partial \log K_{obs}/\partial \log[MX]$ on $\log[MX]$.

[c] Ion pairs formed between DNA and CAP estimated from $Z = -\Delta q/0.88$ [15].

[d] From fitting Eq. (6) to the dependence of $\log K_{obs}$ on $\log[MX]$.

cation is potassium and the dominant anion is glutamate (Glu), acetate (Ac) or phosphate (Pi). Data for chloride have already been presented above. The smooth curves are fits to the data using Eq. (6), with values of $\partial \log K_{obs}/\partial \log[MX]$ determined at small intervals in log[MX] using Eq. (5). The correspondence of the curves to the data indicates that Eq. (6) is a reasonable model for the dependence of $\log K_{obs}$ on log [MX]. As summarized in Table 2, the ion-release and ion-uptake stoichiometries and the most probable values of $K_a^{M^+}$ do not differ significantly with anion substitution. The small differences in $\log K_T$, estimated by extrapolation to 1 M salt, suggest that anion substitution is accompanied by small changes in the non-electrostatic component of binding affinity. The absence of a significant anion-substitution effect suggests that the ion-release stoichiometry is dominated by cation release from DNA, i.e., that $\Delta t \sim \Delta q$ and argues that [salt]-dependent changes in anion binding (or release) do not account for the changes of the slope function $\partial \log K_{obs}/\partial \log[MX]$ with salt concentration.
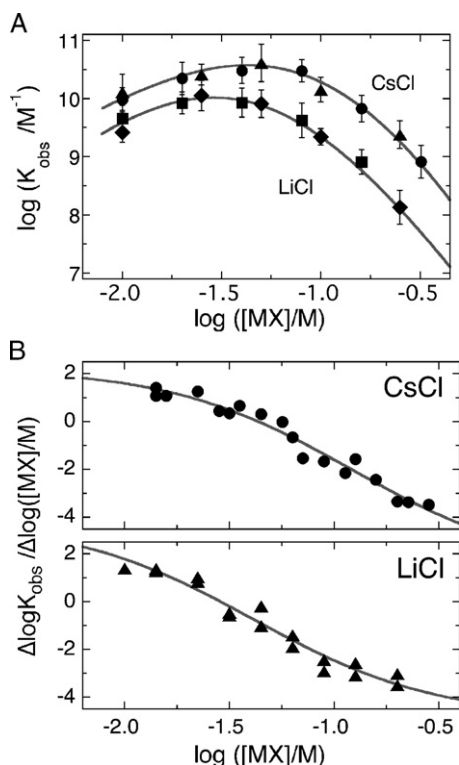

Fig. 4. Dependence of CAP binding on CsCl and LiCl concentrations. (A) Relation of $\log K_{obs}$ to log[MX]. Reaction mixtures contained 1.3×10^{-11} M *lac* promoter DNA fragment, in 1 mM Tris (pH 8.0 at 21 °C), 0.1 mM EDTA, 20 µM cAMP, 25 µg/ml BSA, 5% glycerol, plus CsCl or LiCl as indicated. Samples were assayed by nitrocellulose filtration (●, ■) or by EMSA (▲, ♦). The error bars represent the 95% confidence limits for the individual measurements. The solid curve was obtained by fitting Eq. (6) to the entire data set using values of cation stoichiometry (m_{tot}, Δq) and affinity ($K_a^{M^+}$) derived from the slope analyses shown in panel B. (B) Dependence of $\Delta \log K_{obs}/\Delta \log[MX]$ on log[MX] for reactions carried out in CsCl and LiCl-containing buffers. Differences ($\Delta \log K_{obs}/\Delta \log[MX]$) were calculated for pairs of contiguous points (i, $i+1$) and for pairs of points separated by one (i, $i+2$). Points are plotted at the average log[MX] value for each interval. The curves are least-squares fit to these data with $\partial \log K_{obs}/\partial \log[MX]$ given by Eq. (5). The parameters obtained by fitting are summarized in Table 1.
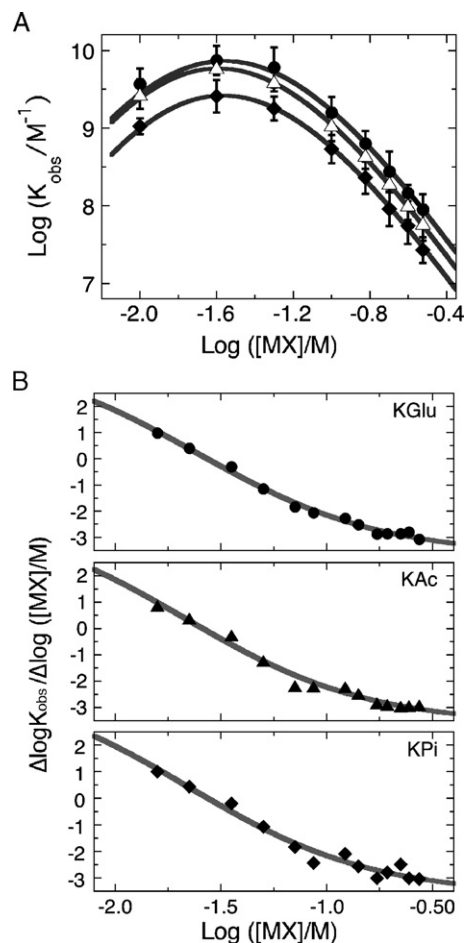

Fig. 5. Dependence of CAP binding on potassium glutamate, acetate and phosphate concentrations. (A) Relation of $\log K_{obs}$ to log[MX]. Reaction mixtures contained 6.2×10^{-10} M *lac* promoter DNA fragment, in 1 mM Tris (pH 8.0 at 21 °C), 0.1 mM EDTA, 20 µM cAMP, 25 µg/ml BSA, 5% glycerol, plus potassium glutamate (●), acetate (△) or phosphate (♦). Samples were assayed by EMSA. The error bars represent the 95% confidence limits for the individual measurements. The solid curve was obtained by fitting Eq. (6) to the entire data set using values of cation stoichiometry (m_{tot}, Δq) and affinity ($K_a^{M^+}$) derived from the slope analyses shown in panel B. (B) Dependence of $\Delta \log K_{obs}/\Delta \log[MX]$ on log[MX] for reactions carried out in potassium glutamate (KGlu), acetate (KAc) and phosphate (KPi) buffers. Differences ($\Delta \log K_{obs}/\Delta \log[MX]$) were calculated for pairs of contiguous points (i, $i+1$) and for pairs of points separated by one (i, $i+2$). Points are plotted at the average log[MX] value for each interval. The curves are least-squares fit to these data with $\partial \log K_{obs}/\partial \log[MX]$ given by Eq. (5). The parameters obtained by fitting are summarized in Table 2.

3.3. Salt concentration-dependent and -independent contributions to binding

The association constant K_T, obtained by extrapolation of $\log K_{obs}$ to log[MX]=0, contains contributions that are independent of ion binding or release.[4] Estimation of the residual free energy using $\Delta G_T^0 = -RT \ln K_T$ allows experimental binding free energies (ΔG_{obs}^0) to be parsed into electrolyte-dependent (E) and -independent (T) contributions according to $\Delta G_{obs}^0 =$

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[4] For example, terms in Eqs. (5) and (6) that depend on log[MX] vanish at log [MX]=0.

Table 2
Effects of anion substitution on apparent ion stoichiometries and cation affinities [a]

Salt	m_{tot} [b]	$K_a^{M^+}$ (M^{-1}) [b]	Δt [b]	Z [c]	$\log K_T$ [d]	ΔG_T^0 (kcal/mol)	$\Delta G_{0.1\ M}^0$ (kcal/mol)
KGlu	7.8±0.6	41.0±6.4	−3.7±0.1	4.2±0.1	6.21±0.26	−8.3±0.3	−12.3±0.3
KAc	8.05±1.1	46.9±11.9	−3.6±0.2	4.1±0.2	6.01±0.18	−8.1±0.2	−12.1±0.3
KPi	8.1±1.3	44.9±13.1	−3.6±0.2	4.1±0.2	5.74±0.29	−7.7±0.4	−11.7±0.2
KCl	7.9±0.6	48.6±11.2	−3.8±0.3	4.3±0.3	5.89±0.21	−7.9±0.3	−12.1±0.2

[a] The error ranges are 95% confidence limits.
[b] From fitting Eq. (5) to the dependence of $\partial \log K_{obs}/\partial \log[MX]$ on $\log[MX]$.
[c] Ion pairs formed between DNA and CAP estimated from $Z = -\Delta q/0.88$ [15].
[d] From fitting Eq. (6) to the dependence of $\log K_{obs}$ on $\log[MX]$.

$\Delta G_E^0 + \Delta G_T^0$. A comparison of ΔG_T^0 and ΔG_{obs}^0 can provide insight into the chemical processes that contribute to the stability of protein–DNA complexes at low- and moderate salt concentrations (cf., [10, 43]). Values of ΔG_T^0 and $\Delta G_{obs,\ 0.1\ M}^0$ (Tables 1 and 2) show that at moderate salt concentrations (0.1 M), approximately 35% of the binding free energy is contributed by salt-concentration dependent processes, while the balance is from [salt]-independent contributions. In comparison ~50% of the interaction free energies *lac* repressor with its operator[5] and *Eco*R1 with its cognate site are due to [salt]-dependent processes [18,44]. The relatively large non-electrostatic contribution to the stability of the CAP complex may reflect the conformational changes that CAP and DNA undergo in forming the sequence-specific complex [45,46].

3.4. Cation substitution changes the stability of free CAP

In the model described above, m_{tot} cation binding sites become fully-occupied when CAP binds DNA and it is the fractional occupancy of these sites in the free protein that determines the number of cations that can be taken up on DNA binding. If the same ensemble of cation sites is lost on denaturation and if anion binding does not contribute strongly to stability of the native form of the protein, [salt]-dependent stabilization of CAP should depend on $K_a^{M^+}$ in the same way that values of Δm derived from DNA binding do. To determine whether cation binding to the free form of CAP follows the same relative order of affinity deduced from $\partial \log K_{obs}/\partial \log[MX]$, we carried out urea-denaturation experiments in the presence of low (50 mM) and moderate (300 mM) concentrations of KCl, LiCl and CsCl (Fig. 6A). A characteristic change in fluorescence[6] anisotropy [8] was used to determine mole fractions of native and denatured forms according to a two-state model (Eqs. (7) and (8)). The reasonable fit indicates that the two-state model is consistent with the denaturation data.

CAP becomes more resistant to urea denaturation as [salt] increases, with relative stabilities at 50 mM salt in the order KCl>LiCl>CsCl. This is the same order as the cation affinities deduced from DNA-binding analyses ($K_a^{K^+} > K_a^{Li^+} > K_a^{Cs^+}$). At

300 mM salt, CAP samples in KCl, LiCl and CsCl buffers are almost equally stable. This pattern would be expected if K^+-, Li^+- and Cs^+-forms of the protein have similar stabilities and if the cation sites important for stability in urea are occupied at 300 mM salt.[7] The values of $K_a^{M^+}$ inferred from DNA binding predict that the fractional occupancies of Cs^+, Li^+ and K^+ sites should be 0.71, 0.88 and 0.94, respectively in solutions containing 300 mM of the corresponding chloride salts. These fractional occupancies are consistent with the near-saturation of the stabilization of CAP at 300 mM [salt].

For a two-state unfolding mechanism, the experimental free energy of denaturation ΔG_{ex}^0 is linearly-dependent on [urea] [48].

$$\Delta G_{ex}^0 = \Delta G_{N-D}^0 + m[\text{urea}]. \tag{9}$$

Here ΔG_{N-D}^0 is the free energy of the transition in urea-free solution. The value of m has been correlated with change in solvent-accessible surface area (ASA) of the protein [49] and change in fractional exposure of buried residues [50,51] and with corresponding changes in the protein interactions of urea and solvent [52–54]. If CAP undergoes significant conformation change with changes in cation-binding status, we would expect to see different values of m for samples denatured in low and high [salt] or in buffers with different dominant cations. As shown in Fig. 6B and summarized in Table 3, the dependence of ΔG_{ex}^0 on [salt] is linear and the values of m are very similar for all conditions tested. This argues against large differences in conformation as consequences of changes in [salt] or dominant cation. The value of ΔG_{N-D}^0 obtained by extrapolating ΔG_{ex}^0 to [urea]=0 follow the order ΔG_{N-D}^0 (K^+)> ΔG_{N-D}^0 (Li^+)> ΔG_{N-D}^0 (Cs^+) consistent with the sequence of cation affinities $K_a^{K^+} > K_a^{Li^+} > K_a^{Cs^+}$ obtained from analysis of DNA binding.

4. Discussion

The affinity of CAP for its regulatory site in the lactose promoter depends strongly on the type and concentration of ions in the solution [8,9]. To account for this dependence we have proposed a model in which DNA binding is thermodynamically-linked to cation binding by the protein and cation

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[5] Calculated using the relation $\log K_{obs} = -7.04 \log[K^+] + 6.9$, obtained by Record et al. [44].

[6] Cs^+ is an inefficient quencher of tryptophan fluorescence [47]. At the highest [CsCl] used here, (300 mM) both native and denatured CAP retained >85% of the fluorescence intensity observed in the absence of CsCl.

[7] This does not require that all sites in the ensemble are saturated but only that ones that affect the stability of CAP in urea solutions.
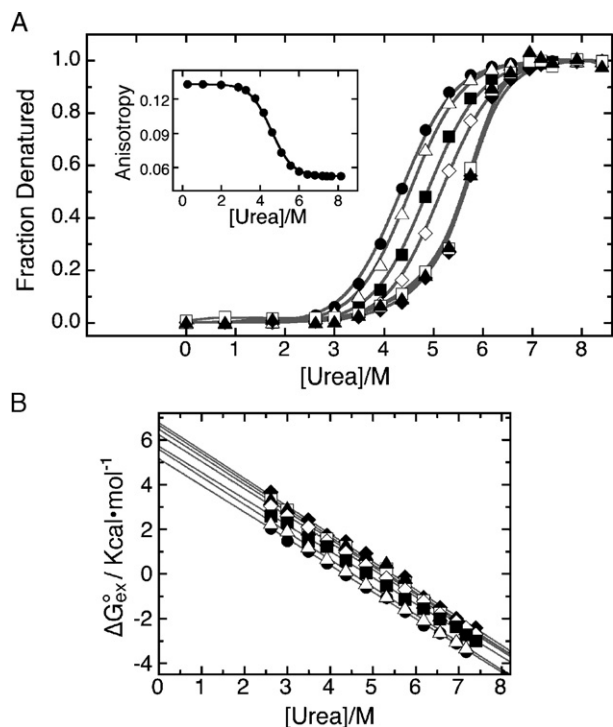
Fig. 6. Urea denaturation of free CAP monitored by fluorescence anisotropy. (A) CAP protein (~ 0.8 μM) in 10 mM Tris (pH 8.0 at 20 °C), 0.1 mM EDTA (●) or 10 mM Tris (pH 8.0 at 20 °C), 0.1 mM EDTA containing (△) 50 mM CsCl, (■) 50 mM LiCl, (◇) 50 mM KCl, (▲) 300 mM CsCl, (□) 300 mM LiCl or (◆) 300 mM KCl. Samples were adjusted to indicate final concentrations of urea using stock solutions made in the corresponding salt solutions. Anisotropy was measured at 20 ± 1 °C using excitation and emission wavelengths of 295 nm and 350 nm, respectively. Normalized mole fraction of denatured form (determined as described in Materials and methods) is graphed. Inset: anisotropy as a function of [urea] for CAP in 10 mM Tris (pH 8.0 at 20 °C), 0.1 mM EDTA containing (●) 50 mM CsCl. (B) Dependence of ΔG_{ex}^0 on [urea] for the data shown in panel A. The lines are least-squares fits to the data. The fit parameters are summarized in Table 3.

displacement from the DNA. This model is similar to one described earlier [9,23] in that it explicitly treats the protein–cation component of the interaction, but it differs in the postulated mechanism of cation uptake. The earlier model relied on the difference in cation concentrations in bulk solution and in the vicinity of the DNA to drive cation binding. In consequence, it suffered from the limitation that neither the concentrations nor the activity coefficients of cations near the DNA could be specified with certainty (both are subjects of ongoing theoretical and experimental research; cf., [55–61]). The current model posits that cation sites become occupied as the protein associates with DNA. Possible mechanisms include (i) increased cation affinity of anionic residues transferred from the bulk solution environment to the vicinity of negatively-charged DNA and (ii) allosteric transition that increases the affinity of existing cation binding sites on the protein as it associates with DNA. In the current model, the observed dependence of Δm on log[MX] is due to changes in fractional occupancy of cation sites on the *free* protein in response to changes in [MX] in the bulk solution. As shown above, the simplest ion-binding model, with m_{tot} identical, independent

sites accounts well for experimentally observed change in $\partial \log K_{obs}/\partial \log[MX]$ with log[MX].

Several other mechanisms have been proposed to account for changes in $\partial \log K_{obs}/\partial \log[MX]$ with [salt]. These include (iii) competition between mono- and divalent cations for interaction with DNA [44], (iv) [salt]-dependent binding of anions to free protein with subsequent release on DNA binding (cf., [12,39]) and (v) formation of salt bridges on the protein surface that are disrupted on DNA binding [24,62]. Mechanisms (iii) and (iv) produce differences in $\partial \log K_{obs}/\partial \log[MX]$ through [salt]-dependent changes in the stoichiometry of ion release. Because ion release contributes negatively to $\partial \log K_{obs}/\partial \log[MX]$, these mechanisms cannot account for the positive values of $\partial \log K_{obs}/\partial \log[MX]$ observed with CAP. In mechanism (v), salt bridges form on the protein surface under low-salt buffer conditions. On DNA binding, these salt bridges are disrupted. Unmasked cationic residues associate with DNA phosphates, releasing buffer cations from the vicinity of the DNA. Unmasked anionic residues, brought into the vicinity of DNA, associate with buffer cations, reducing the net cation-release stoichiometry. At higher salt concentrations, salt bridges are destabilized and counterions from buffer associate to a greater extent with the protein. This has the potential to increase anion release and decrease cation uptake on DNA binding. Both processes make $\partial \log K_{obs}/\partial \log[MX]$ more negative with increasing [salt], but as formulated, they do not result in net ion uptake at low [salt]. On the other hand, if the DNA-binding surface of the protein contains an excess of anionic residues over cationic ones, DNA binding might drive a net uptake of cations at low [salt]. With this modification, mechanism (v) becomes a special case of mechanism (i).

In terms of our general cation-uptake model, possible contributors to change in $\partial \log K_{obs}/\partial \log[MX]$ with cation substitution include changes in the number of cation binding sites (m_{tot}), changes in the affinity with which cations are bound ($K_a^{M^+}$) and changes in the cation-release stoichiometry, Δq. Very similar values of m_{tot} were obtained in KCl, LiCl and CsCl buffers (summarized in Table 1), consistent with the notion that the same set of binding sites is available to all cations. On the other hand, cation binding was selective, with $K_a^{K^+} > K_a^{Li^+} > K_a^{Cs^+}$. This order is different from those of electronic polarizability ($P_{Cs+} > P_{K+} > P_{Li+}$ [63]), crystal radii ($r_{Cs+} > r_{K+} > r_{Li+}$ [64]), hydrated stokes radii ($r_{Li+} > r_{K+} > r_{Cs+}$ [65]) or cation-to-water oxygen internuclear distances ($d_{Cs+} >$

Table 3
Effects of cation substitution on urea denaturation of CAP

Buffer	ΔG_{N-D}^0 (kcal/mol) [a]	m-value[a]
10 mM Tris (pH 8.0), 1 mM EDTA (TE)	5.16 ± 0.06	-1.19 ± 0.04
TE + 50 mM CsCl	5.58 ± 0.03	-1.23 ± 0.03
TE + 50 mM LiCl	5.72 ± 0.08	-1.17 ± 0.05
TE + 50 mM KCl	6.23 ± 0.03	-1.21 ± 0.04
TE + 300 mM CsCl	6.66 ± 0.28	-1.24 ± 0.05
TE + 300 mM LiCl	6.48 ± 0.20	-1.23 ± 0.04
TE + 300 mM KCl	6.77 ± 0.17	-1.24 ± 0.03

[a] The error ranges are 95% confidence limits.

$d_{K+} > d_{Li+}$ [64]), but it is the same as the order of cation effects on CAP stability in urea solutions ($K^+ > Li^+ > Cs^+$ summarized in Table 3). This result is consistent with the model embodied in Eqs. (5) and (6), in which the fractional occupancy of cation binding sites on the free protein determines the cation-uptake stoichiometry of DNA binding. It is intriguing that $K_a^{M^+}$ follows the same order as the Hofmeister series ($K^+ > Li^+ > Cs^+$ [63]). Although there are other possible interpretations, an attractive one is that cation substitution may alter the relative stabilities of compact proteins, among which are ones that are active in the linked binding of cations and DNA.

The observation that the ion-release stoichiometry (Δt) changes with cation type (Table 1) is an unexpected result that raises significant questions for further study. As discussed below, anion substitution results suggest that anion release is not a significant contributor to $\partial \log K_{obs} / \partial \log[MX]$. If this is correct, Δt is dominated by cation release from the DNA. Since the fractional neutralization of DNA charge is effectively independent of monovalent cation type over a wide concentration range [40,55,66,67], cation substitution should not change the number of cations displaced from each DNA phosphate that forms an ion pair with CAP. On this basis, the more negative values of Δt found when Li^+–or Cs^+–is substituted for K^+ can be interpreted as evidence for an increase in the number of ionic contacts (Z) between CAP and DNA. Such differences might be detected as changes in nuclease- or chemical-protection patterns in CAP–DNA complexes, or possibly as differences in the degree of protein-induced DNA binding.

Is there a specific role for anions in the CAP–DNA interaction? Anion substitution has been correlated with large changes in the DNA affinities of *lac* repressor and *E. coli* SSB proteins [12,39,68] and more modest ones for CAP [8]. As shown above, nearly identical values of $\partial \log K_{obs} / \partial \log[MX]$ were obtained over the experimental range of [salt] for CAP–DNA association carried out in buffers containing glutamate or acetate or chloride or phosphate as the dominant anion (Fig 5, Table 2). This outcome is incompatible with models in which specifically-bound anions are released on DNA binding. On the other hand, differences in K_T suggest that anion substitution affects [salt]-independent components of the interaction. The sequence of K_T values (glutamate > acetate > chloride > phosphate) is similar to that observed for *lac* repressor [12,68] and follows the Hofmeister series for anions [63]. Thus, changes in binding affinity with anion substitution may reflect the preferential exclusion of anions in favor of water, near protein and/or nucleic acid surfaces.

Urea denaturation provides a convenient, quantitative test for the effects of cations on the stability of free CAP protein. The modest differences in ΔG_{N-D}^0 and nearly identical m-values obtained in solutions containing 300 mM LiCl, KCl and CsCl argue against models in which cation substitution results in large differences in protein structure. It may be significant that the order $\Delta G_{N-D}^0 (K^+) > \Delta G_{N-D}^0 (Li^+) > \Delta G_{N-D}^0 (Cs^+)$ is the same as that of cation affinities ($K_a^{M^+}$) obtained for DNA binding but different from the orders of physical properties of the individual cations (see above). The simplest models that account for these features are ones in which the same population of cation

binding sites contributes to the stability of the folded protein and modulates its DNA-binding affinity. Based on these results, our working hypothesis is that the cation binding sites that we can detect are located on the exterior of the folded protein, where occupancy affects the electrostatic component of ΔG_{N-D}^0 with minimal perturbation of the native conformational ensemble (hence little change in m-value). Anionic groups within or near the DNA binding surface of the protein represent a class of potential cation binding sites that may become occupied as CAP associates with DNA. A testable prediction of this model is that m_{tot} values should be reduced by mutations that substitute uncharged residues for anionic ones located within or near the DNA binding surfaces of CAP.

The cation-uptake mechanism proposed here is one of several that may contribute to the nonlinear dependence of $\log K_{obs}$ on $\log[MX]$. Such nonlinearity is encountered frequently enough to justify the suggestion that it may be of adaptive value. A gradual change of $\partial \log K_{obs} / \partial \log[MX]$ from positive values (or zero) at low [MX] to negative values at high [MX] has the effect of keeping K_{obs} nearly constant over an extended salt concentration range. Since the salt concentration of *E. coli* cytoplasm varies with external osmolarity [69,70], such mechanisms may help to maintain normal patterns of gene expression as the extracellular environment changes.

Acknowledgements

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