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Replacement of Potassium Chloride by Potassium Glutamate Dramatically Enhances Protein–DNA Interactions in Vitro†

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ABSTRACT: Although protein–nucleic acid interactions exhibit dramatic dependences on both ion concentration and type in vitro, large variations in intracellular ion concentrations can occur in *Escherichia coli* and other organisms without apparent effects on gene expression in vivo. *E. coli* accumulates K^+ and glutamate as cytoplasmic osmolytes. The cytoplasmic K^+ concentration in *E. coli* varies from <0.2 to >0.9 *m* as a function of external osmolarity; corresponding cytoplasmic glutamate concentrations range from <0.03 to >0.25 *m*. Only low levels of chloride occur in the cytoplasm of *E. coli* at all osmotic conditions. Since most in vitro studies have been performed in chloride salts, whereas glutamate is the more relevant physiological anion, we have measured the effects of the substitution of potassium glutamate (KGlu) for KCl on the kinetics and equilibria of a variety of site-specific protein–DNA interactions in vitro. Both the interaction of *E. coli* RNA polymerase with two phage λ promoters and the interactions of various restriction enzymes with their DNA cleavage sites are enhanced by this substitution. Using the abortive initiation assay, we find a greater than 30-fold increase in the second-order rate constant for open complex formation at the λP_R promoter and a 10-fold increase at the $\lambda P_R'$ promoter, when KGlu is substituted for KCl. Replacement of KCl by KGlu does not affect the strong salt dependences of these interactions; increasing either KCl or KGlu concentrations decreases both reaction rates and extents. Substitution of glutamate for chloride does, however, shift the range of salt concentrations over which these interactions are observable to higher K^+ concentrations. This higher range of accessible K^+ concentrations in KGlu is in closer correspondence with the physiological range of K^+ concentrations in *E. coli*.

Protein–nucleic acid interactions in vitro are extraordinarily sensitive to the concentrations and the types of electrolyte ions in solution. Most in vitro studies of protein–nucleic acid interactions are carried out in an electrolyte mixture containing either NaCl or KCl (typically at concentrations in the range 0.05–0.15 M) and often $MgCl_2$ (at 0.003–0.01 M). Although one purpose of such a mixed electrolyte is presumably to mimic the intracellular ionic environment, in fact this may not be the case. Chloride is present only at low concentration in the cytoplasm of *Escherichia coli*. [Early determinations of the cytoplasmic chloride content of *E. coli* were biased by its presence in the periplasm. The results of Stock et al. (1977) and Castle et al. (1986) suggest that when *E. coli* is grown in a chloride-containing medium, the intracellular chloride is localized in the periplasm, although no direct measurements have been made.] In fact, *E. coli* can be grown without the addition of chloride to its growth medium (Miller, 1972; D. S. Cayley, unpublished results). Other examples of eubacteria and eukaryotic cells are known in which chloride is not the major univalent anion or is not the only univalent anion present in significant concentrations (Measures, 1975; Gerald & Gilles, 1972; Gilles, 1980; Rankin & Davenport, 1981). In *E. coli*, as well as in some other eubacteria, the anion glutamate (Glu^-) not only is a major member of the amino acid pool but also constitutes a significant fraction of the total concentration of univalent anions (Measures, 1975). Other carboxylic acid anions [lactate, acetate (Ac^-), etc.] may also contribute. At moderate-to-high external osmolarities, potassium and glutamate ions are concentrated in the cytoplasm of *E. coli* to prevent dehydration and maintain turgor pressure. The K^+ concentration in *E. coli* can range from ~0.2 to ~0.9 *m* or higher; the corresponding range of glutamate concentrations is from ~0.03 to ~0.25 *m* (Richey et al., 1987). Investigations of protein–nucleic acid interactions in vitro are typically performed in chloride-containing buffers at K^+ concentrations that are at the lower end of or below this physiological range.

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Since most protein–nucleic acid interactions are extraordinarily salt sensitive in chloride salts in vitro, we have examined the effects of replacing chloride by glutamate in vitro on the kinetics and equilibria of various site-specific protein–DNA interactions (at constant K^+ concentration and as a function of K^+ concentration) in order to mimic more closely the range of physiological solute environments in which these noncovalent interactions occur in *E. coli*.

Materials and Methods

Enzymes and DNA. *E. coli* K12 RNA polymerase holoenzyme ($E\sigma^{70}$) was purified as previously described (Lowe et al., 1979). It was 80 ± 10% σ saturated and 35 ± 5% active in promoter binding (Roe et al., 1984). Active enzyme concentrations are reported in the text. All restriction enzymes were purchased from New England Biolabs except *Hph*I, which was obtained from Bethesda Research Laboratories.

Plasmid DNA was isolated by the boiling lysis method and purified on a CsCl gradient (Maniatis et al., 1982). Restriction fragments containing the λP_R and $\lambda P_R'$ promoters were isolated from pGR40 (Roe et al., 1984) and pD12 (Luk & Szybalski, 1982), respectively. They were purified by separation on polyacrylamide gels, followed by electroelution and chromatography on benzoylated, naphthoylated DEAE-cellulose.[1] Concentrations of promoter fragments were determined spectrophotometrically with ϵ_{260} = 13 000 M^{-1} bp^{-1} cm^{-1}.

Restriction Enzyme Digestions. *Eco*RI digestions contained 10 mM Tris (pH 7.4 at 25 °C), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 100 μg/mL bovine serum albumin (BSA). Digestions of plasmid DNA by other restriction enzymes were performed under recommended assay conditions (New England Biolabs). Typical reaction mixtures contained 0.5 μg of pBR322, 1 unit of restriction enzyme, and variable concentrations of KCl or KGlu in a volume of 30 μL. Digestions were terminated between 45 and 120 min by bringing the solution to 0.1% SDS and 40 mM EDTA. Digestion products were then loaded on 1% agarose gels, electrophoresed in 40 mM Tris–acetate and 1 mM EDTA, and visualized by UV illumination after staining with ethidium bromide.

Abortive Initiation Assay for RNA Polymerase–Promoter Association Kinetics. Promoter-specific open complexes were detected by the abortive initiation assay developed by McClure and co-workers (McClure et al., 1978; Hawley & McClure, 1980). Unless otherwise noted, the standard reaction buffer contained 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH adjusted to 7.5 at 25 °C with a freshly prepared KOH solution), 10 mM $MgCl_2$, 100 μg/mL BSA, 1 mM DTT, and the indicated amounts of KCl or KGlu. For the λP_R promoter, reactions contained 0.5 mM CpA, 50 μM UTP ([α-^{32}P]UTP added to a specific activity of 600 cpm/pmol), 0.5 nM promoter-containing restriction fragment, and 2.5–100 nM RNAP. Corresponding conditions for the $\lambda P_R'$ promoter were 0.5 mM ApA, 25 μM CTP (1200 cpm/pmol), 1 nM restriction fragment, and 5–90 nM RNAP. All experiments were performed at 37 °C. Abortive products (CpApU or ApApC) were separated from unincorporated α-^{32}P-labeled nucleoside triphosphates by paper chromatography (McClure et al., 1978).

The data were analyzed by a weighted nonlinear least-squares fit to the equation $N = Vt - V\tau_{obsd}(1 - e^{-t/\tau_{obsd}})$, where N = product × promoter^{-1}, V = product × promoter^{-1} s^{-1}, t = time (s), and τ_{obsd} = reciprocal of the pseudo-first-order rate constant k_{obsd} (s) (Hawley & McClure, 1982). Weighting factors for the individual time points were taken to be the reciprocal of the sum of the variances of the background (determined in the absence of protein or DNA) and of the amount of product formation at a given time. The relative error of product formation was determined to be about 5%. Standard errors in τ_{obsd} and V were estimated by the Marquardt algorithm (Bevington, 1969).

The concentration dependence of τ_{obsd} was analyzed by using the rapid-equilibrium mechanism $R + P \rightleftarrows RP_c \rightarrow\rightarrow RP_o$, where RP_c is the intermediate closed complex and where the isomerization process forming the transcriptionally active open complex (RP_o) is kinetically complex (Roe et al., 1985). For this rapid-equilibrium mechanism in excess RNAP

$$\tau_{obsd} = k_{obsd}^{-1} = (k_a[R])^{-1} + k_i^{-1} \quad (1)$$

A plot of τ_{obsd} vs. $[R]^{-1}$ gives a slope of k_a^{-1} (where k_a is the second-order association rate constant) and an intercept of k_i^{-1} (where k_i is a composite isomerization rate constant).

Results and Discussion

Replacement of Cl^- by Glu^- Shifts the Salt Optimum for Site-Specific Cleavage by a Variety of Restriction Endonucleases. Salt concentration is an important factor influencing restriction enzyme activity. Although recommended assay buffers differ widely in their pH and specific ion requirements, KCl concentrations do not exceed 0.15 M, which is well below the physiological range of K^+ concentrations and above the physiological range of Cl^- concentrations in *E. coli* (Richey et al., 1987). Figure 1 illustrates the effect of substitution of KGlu for KCl on *Eco*RI and several other restriction enzymes, selected because their assay buffers differ from one another in the recommended concentration of salt. In all cases, the range of salt concentrations over which site-specific DNA cleavage occurs is markedly shifted to higher K^+ concentrations when Cl^- is replaced by Glu^- in digestions performed with a fixed, limiting enzyme concentration and a fixed time of digestion. For example, Figure 1A shows nearly complete digestion of pBR322 after 120 min of incubation with *Bam*HI in a reaction containing 0.45 M KGlu, a $[K^+]$ at which cleavage activity in KCl is essentially eliminated.

We find that the optimal salt concentration for DNA cleavage is significantly increased upon substitution of Glu^- for Cl^-. (The optimal salt concentration is defined as that yielding the most rapid initial rate of DNA cleavage for a given concentration of enzyme and of DNA sites.) For a given anion, both the initial rate of digestion and the time required for complete reaction are optimized at the same $[K^+]$. Figure 1 shows the results of reactions allowed to proceed for a time sufficient to yield complete cleavage only at the optimal level of salt. Since a limited number of salt concentrations were examined, these studies only provide estimates of the KCl and KGlu optima but clearly indicate the shift in the optimal $[K^+]$ upon replacing Cl^- by Glu^-. For *Eco*RV (Figure 1B) the optimal $[K^+]$ is shifted from ~0.15 M KCl to ~0.25 M KGlu. For *Eco*RI (Figure 1C) the optimum in Cl^- occurs at ~0.2 M K^+, consistent with previous results (Poliskey et al., 1975), whereas in KGlu the optimum is centered at ~0.3 M, with optimal activity maintained from ~0.2 to ~0.4 M KGlu. Generally the range of salt concentrations over which significant endonucleolytic activity occurs is broadened in KGlu relative to KCl. *Cla*I is an extreme example, where the broad

[1] Abbreviations: DEAE-cellulose, (diethylaminoethyl)cellulose; KGlu, potassium glutamate; bp, base pairs; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RNAP, RNA polymerase.

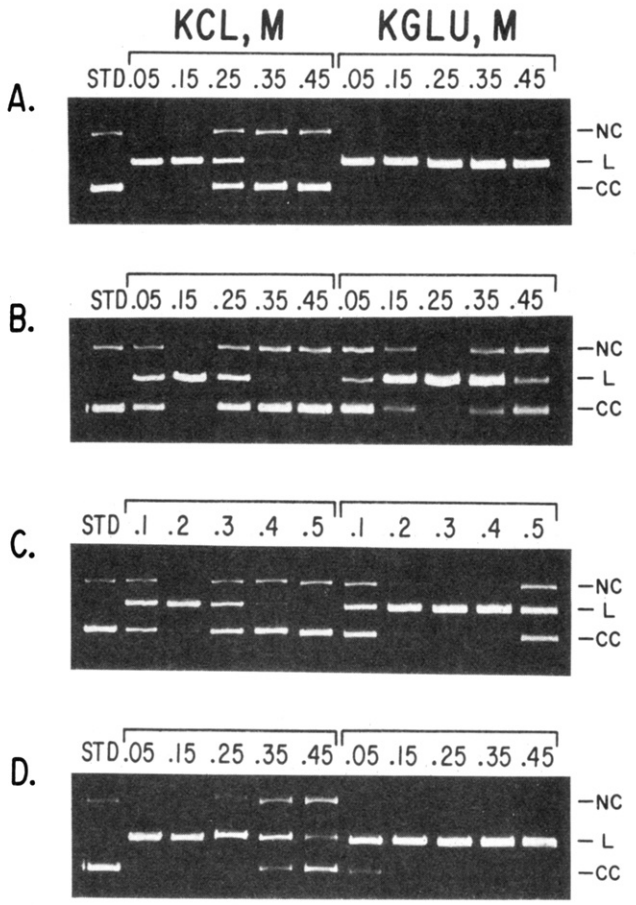

FIGURE 1: Comparison of cleavage of pBR322 by restriction enzymes in KCl and KGlu as a function of salt concentration. Reactions were performed as specified under Materials and Methods, with variable KCl and KGlu concentrations. Digestions were terminated after 45 min for *Eco*RI and *Eco*RV or 120 min for *Bam*HI and *Cla*I. Panel A, *Bam*HI; panel B, *Eco*RV; panel C, *Eco*RI; panel D, *Cla*I. NC, nicked circular; CC, closed circular; L, linear.

optimum in [KGlu] (0.05–0.45 M, Figure 1D) overlaps and extends well beyond the optimum in KCl (0.05–0.15 M).

When the kinetics of digestion of pBR322 are compared at the concentrations of KCl and KGlu that are optimal for that enzyme, the rate of cleavage is found to be anion independent. Substitution of Glu^- for Cl^- does not significantly alter the maximal rate of cleavage of DNA by the endonucleases examined here but rather shifts to higher $[K^+]$ and broadens the range of salt concentrations over which efficient cleavage occurs. All restriction endonucleases examined so far (including *Acc*I, *Ava*II, *Hph*I, *Nde*I, *Sal*I, and *Sma*I) respond similarly to replacement of KCl by KGlu. KGlu may therefore be useful in obtaining simultaneous cutting of DNA by restriction enzymes with salt optima that are incompatible with codigestion in KCl solutions.

Substitution of Glu^- for Cl^- Greatly Increases the Rate and Extent of Open Complex Formation at Promoters. Salt concentration is also an important variable in in vitro transcription studies. For example, Kajitani and Ishihama (1983) have shown that salt concentration affects relative promoter strengths measured by a runoff transcription assay. Both the kinetics and equilibrium of open complex formation at the λP_R promoter are salt dependent (Roe et al., 1984). Most studies of salt effects on RNAP–promoter interactions have been performed in buffers containing NaCl or KCl at concentrations of less than 0.2 M and often containing 0.01 M $MgCl_2$.

The kinetics of the binding of *E. coli* RNA polymerase (RNAP) to the two λ promoters P_R and P_R' were measured

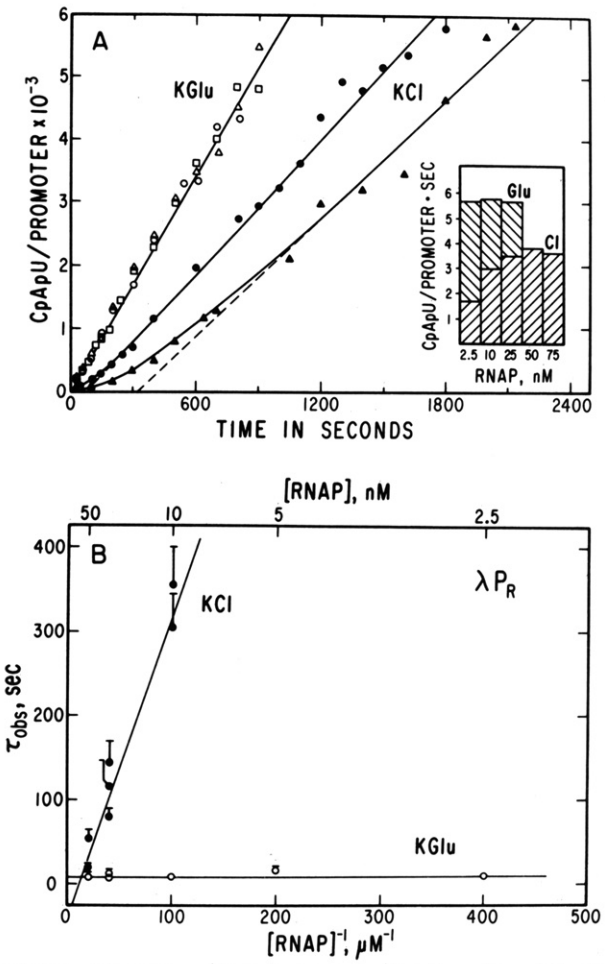

FIGURE 2: (A) Time required for open complex formation at the λP_R promoter. All reactions were initiated by the addition of RNAP to the assay mixture containing either 0.2 M KGlu (open symbols) or 0.2 M KCl (closed symbols). RNAP concentrations were 25 (○, ●), 10 (△, ▲), and 2.5 nM (□). Standard reaction conditions were used except that the buffer was 40 mM Tris base titrated with HCl to pH 8.0 at 37 °C. Solid lines show the computer fit to the data; dotted lines extrapolate the steady-state rate (V) to the time axis to give τ_{obsd}. Inset: V (proportional to promoter occupancy) as a function of RNAP concentration for data obtained in this experiment. (B) τ plot for formation of open complexes at the λP_R promoter in 0.2 M KGlu (○) and 0.2 M KCl (●). The pseudo-first-order time constant τ_{obsd} is plotted as a function of the reciprocal of the active RNAP concentration. Error bars are standard errors of the computer fit. At 0.20 M KCl, the weighted linear least-squares fit to the data is $\tau_{obsd} = -(50 \pm 20) + [(2.7 \pm 0.5) \times 10^5]^{-1}[RNAP]^{-1}$ s, where $\tau_{obsd} = k_i^{-1} + k_a^{-1}[RNAP]^{-1}$. In 0.20 M KGlu, where τ_{obsd} is approximately equal to the mixing time, we estimate $k_a \geq 10^7$ M^{-1} s^{-1}.

under pseudo-first-order conditions of RNAP excess by the abortive initiation assay (McClure, 1980). This assay is based on the observation that the steady-state rate of promoter-specific oligonucleotide synthesis, which occurs upon addition of ribonucleoside triphosphates to preformed RNAP–promoter complexes, is proportional to the amount of open complex present. In a reaction initiated by addition of RNAP, the lag in the approach to the final rate of oligonucleotide synthesis is τ_{obsd}, the inverse of the pseudo-first-order rate constant (k_{obsd}) for open complex formation (cf. eq 1).

The effects of replacing Cl^- by Glu^- on kinetics of association of RNAP with the λP_R promoter at 0.20 M K^+ are shown in Figure 2. In 0.20 M KGlu, no lag can be detected over a 10-fold range in [RNAP] (2.5–25 nM active holoenzyme; cf. Figure 2A), indicating that kinetics of binding are too fast to measure in this concentration interval ($\tau_{obsd} < 10$ s; cf. Figure 2B). On the other hand, in 0.20 M KCl τ_{obsd} ranges from

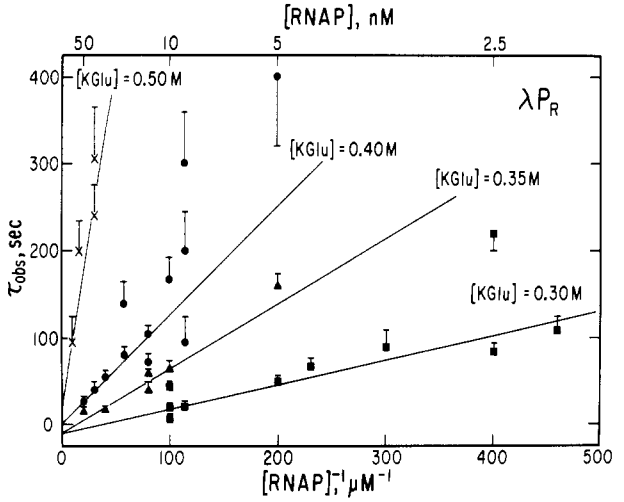

FIGURE 3: τ plots for open complex formation at the λP_R promoter as a function of [KGlu]. The reaction buffer was that of Figure 1. Weighted least-squares fits to the data give the following values for k_i^{-1} (s) and k_a (M^{-1} s^{-1}): 0.30 M KGlu (■), -9 ± 11 and $(3.5 \pm 1.0) \times 10^6$; 0.35 M KGlu (▲), -8 ± 7 and $(1.3 \pm 0.3) \times 10^6$; 0.40 M KGlu (●), 2 ± 11 and $(0.8 \pm 0.2) \times 10^6$; 0.50 M KGlu (×), 27 ± 56 and $(0.12 \pm 0.06) \times 10^6$.

approximately 40 ± 20 s at 50 nM RNAP to 330 ± 70 s at 10 nM RNAP. These are represented on the τ plot of Figure 2B, which yields a second-order rate constant $k_a = (2.7 \pm 0.5) \times 10^5$ M^{-1} s^{-1} in KCl and $k_a \gg 10^7$ M^{-1} s^{-1} in KGlu. Consequently, the association rate constant for formation of the transcriptionally competent open complex is increased more than 30-fold by the replacement of KCl by KGlu.

As shown in the inset to Figure 2A, replacing Cl^- by Glu^- at a constant $[K^+]$ of 0.20 M affects not only the rate but also the extent of open complex formation. Constant steady-state rates of oligonucleotide synthesis are observed in KGlu over the [RNAP] range 2.5–25 nM. In KCl, however, a 2-fold decrease in steady-state rate is observed over the same range of [RNAP], indicating a 2-fold decrease in the equilibrium extent of promoter occupancy. (A secondary effect of the nature of the anion on the steady-state rate is also observed; under conditions of promoter saturation the maximum steady-state rate in KCl is ~65–75% of that in KGlu at this $[K^+]$.)

Substitution of Glu^- for Cl^- Allows Open Complex Formation at High K^+ Concentrations. The τ plot of Figure 3 contains data collected over the range of KGlu concentrations for which kinetics of RNAP–λP_R open complex formation can be conveniently measured by this assay. Values of k_a decrease more than 10-fold from $(3.5 \pm 1.0) \times 10^6$ M^{-1} s^{-1} at 0.3 M KGlu to $(1.2 \pm 0.6) \times 10^5$ M^{-1} s^{-1} at 0.5 M KGlu. Only at 0.5 M K^+ (in KGlu) does k_a become comparable to that observed at 0.2 M K^+ (in KCl; cf. Figure 2B). While k_a varies strongly with $[K^+]$ and the nature of the anion, the τ intercept (k_i^{-1}) is zero within experimental error (±10–60 s) at all K^+ concentrations examined with either Cl^- or Glu^-.

Similarly large effects of the substitution of Glu^- for Cl^- are observed on the kinetics of the interaction of RNAP with the $\lambda P_R'$ promoter. Table I summarizes second-order rate constants k_a as a function of $[K^+]$ in both KCl and KGlu. At 0.35 M K^+, k_a increases by more than 10-fold when Cl^- is replaced by Glu^-. These data also demonstrate that the same range of values of k_a is observed between 0.20 and 0.35 M KCl as between 0.35 and 0.475 M KGlu. No systematic variation in the τ intercept (k_i^{-1}) with salt concentration could be detected in either KCl or KGlu, though the large experimental error associated with the assay would obscure a small effect.

Table I: Association Rate Constants for the $\lambda P_R'$ Promoter in KCl and KGlu[a]

[KCl] (M)	$k_a \times 10^{-6}$ (M^{-1} s^{-1})	k_i (s)	[KGlu] (M)	$k_a \times 10^{-6}$ (M^{-1} s^{-1})	k_i (s)
0.20	4 (3–7)[b]	−1 ± 7[b]	0.35	5 (3–10)[b]	35 ± 12[b]
0.25	1 (0.8–2)	9 ± 11	0.40	6 (5–7)	22 ± 6
0.28	2 (1–10)	24 ± 8	0.425	2 (1–3)	2 ± 7
0.35	0.4 (0.3–0.5)	11 ± 13	0.45	1 (0.8–2)	39 ± 15
			0.475	0.5 (0.4–0.9)	19 ± 23

[a] k_a and k_i are defined in eq 1. [b] Standard errors of the weighted linear least-squares fit determined from three to seven measurements of τ_{obsd}.

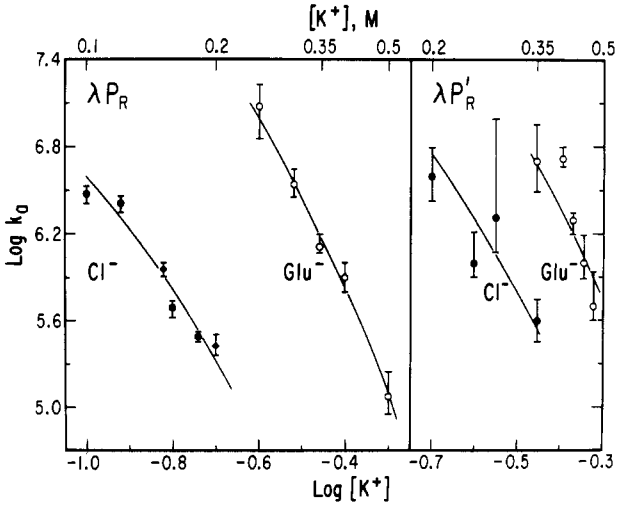

FIGURE 4: Dependence of association rate constants for promoter open complex formation on $[K^+]$ in KCl (closed symbols) and KGlu (open symbols). λP_R promoter in KCl: (●) data of Roe et al. (1984); (◆) this work. The curves were calculated as described in the text.

RNAP–Promoter Interactions Have Similar K^+ Concentration Dependences in both KCl and KGlu. As seen in Figure 4, the dramatic shift in RNAP–promoter association rate constants to higher K^+ concentrations in KGlu occurs without a marked change in the $[K^+]$ dependence of the rate constant. The slope of a plot of log k_a vs. log [KX] yields a quantitative estimate of the net number of univalent ions (K^+ and X^-, where X^- is Cl^- or Glu^-) released before the rate-limiting step of open complex formation (Roe et al., 1984). For the λP_R promoter, Roe et al. proposed that the salt dependence of k_a arises primarily from the release of ions in the initial rapid binding equilibrium of closed complex (RP_c) formation. This simplifying assumption was used in analyzing the salt dependence of k_a.

In principle, both cation release from the DNA and anion release from the protein can contribute to the salt dependence of an equilibrium constant or a rate constant. When cation release from the DNA determines the salt dependence of k_a, the slope of a plot of log k_a vs. log $[K^+]$ (in the absence of competitive cations) is independent of $[K^+]$ (Lohman et al., 1978). However, in a cation mixture (K^+, Mg^{2+}) this slope is no longer constant. Because the binding of RNAP displaces less K^+ from DNA when the competitive cation Mg^{2+} is present, displacing instead some more strongly associated Mg^{2+}, the presence of Mg^{2+} reduces both the magnitude of k_a at a given $[K^+]$ and the dependence of log k_a on log $[K^+]$. These competitive effects of Mg^{2+} are especially pronounced at low $[K^+]$ where the extent of association of Mg^{2+} is large relative to K^+. In particular, the competitive effects of Mg^{2+} are predicted to be more important in the range of KCl concentrations investigated than in the range of KGlu concentrations investigated. We have analyzed the different com-

petitive effects of Mg^{2+} at different K^+ concentrations.[2] The fact that the experimental dependences of log k_a on log [K^+] appear steeper in KGlu than in KCl (cf. Figure 4) can be accounted for entirely by the fact that Mg^{2+} competition is more effective at lower [K^+] (in KCl) than at higher [K^+] (in KGlu). For λP_R the curves drawn through the data in both Cl^- and Glu^- were calculated on the assumption that nine phosphates are neutralized in the DNA binding reaction (n = 9 in eq 2) and that the only effect of Glu^- is to increase the salt-independent rate constant, k_0, by a factor of 100 over that in Cl^- at the same [K^+]. For $\lambda P_R'$, the corresponding parameters are eight phosphates neutralized and a 10-fold shift in k_0. For both promoters comparable fits are obtained over a small range of n (e.g., $n = 9 \pm 1$ for λP_R). Small extents of anion release, or small differences in the extent of anion release accompanying complex formation in KCl and in KGlu, would therefore not be detected because of this uncertainty in the fit.

What is the origin of the 10–100-fold shift in k_0 and in k_a at a fixed [K^+] for these promoters? Since we cannot rule out a small amount of anion release in closed complex formation, the anion effect on k_a could result from the free energy difference between displacement of Cl^- and of Glu^- from the DNA binding site of the protein during this step. Alternatively, a [K^+]-independent term in k_a could be affected by the substitution of Glu^- for Cl^-. In the three-step mechanism postulated by Roe et al. (1985), an isomerization step thought to involve a protein conformation change enters into the expression for k_a. Since anion-linked conformational changes in RNAP have also been postulated (Shaner et al., 1982), replacement of Cl^- by Glu^- could increase the rate constant for open complex formation by stabilizing a particular promoter-binding conformation of RNAP. No unique explanation of the large shift in values of k_a to higher [K^+] in Glu^- can be offered. However, the characteristic K^+ concentration dependence of k_a seen in both KCl and KGlu argues that cation release from the DNA is the major contributor to the salt dependence and that differences in the extent of displacement of Cl^- and Glu^- are not the origin of the large anion effect seen in these interactions. Similar observations have been made for the effects of Ac^- on the binding of *lac* repressor to both operator and nonspecific DNA (Barkley et al., 1981; deHaseth et al., 1977) and on the binding of ribosomal protein S8 to 16S rRNA (Mougel et al., 1986).

Effects of Cl^-/Glu^- Mixtures on Protein–DNA Interactions. To determine whether these large anion effects result from binding of a small number of Cl^- or Glu^- anions to a high-affinity site (or sites) on the protein, or from more diffuse preferential interaction effects, we measured k_a for the RNAP–λP_R interaction in Cl^-/Glu^- mixtures at constant [K^+]. These experiments also test whether small amounts of Cl^- (from $MgCl_2$ and, in some cases, the buffer) were of consequence in experiments in which Glu^- effects were examined. The results at two different K^+ concentrations are shown in Table II. At 0.20 M K^+, under conditions where the only anion present was Cl^-, replacement of 20% of the KCl (0.04 M) by KGlu had a detectable effect on k_a. However, the effect was small (a 3-fold increase) relative to the greater than 50-fold increase seen when 90% of Cl^- was replaced by Glu^- at this [K^+]. Similarly, at 0.4 M K^+, under conditions where the only anion present was Glu^-, replacement of 10% of the KGlu (0.04 M) by KCl produced a relatively small decrease in the rate constant. These results rule out a high-affinity, strong-binding mode of action of either anion. At all [K^+] examined for both λP_R and $\lambda P_R'$ promoters, the association rate constant varied monotonically between the extremes observed in pure KCl and pure KGlu. While these data are not consistent with the existence of a single high-affinity (>200 M^{-1}) site for Cl^- to which Glu^- does not bind, they are consistent with a model where Cl^- and Glu^- compete with one another (and with the DNA) for one or more weaker binding sites on the protein (B. Richey, unpublished results).

Table II: λP_R–RNA Polymerase Association Rate Constants in Cl^-/Glu^- Mixtures[a]

0.2 M K^+		0.4 M K^+[b]	
fraction of Glu^-	k_a (M^{-1} s^{-1})	fraction of Glu^-	k_a (M^{-1} s^{-1})
0.00	$(1.7 \pm 0.1) \times 10^5$	0.00	$<10^4$[c]
0.18	$(5.6 \pm 3.1) \times 10^5$	0.80	$(6.0 \pm 0.1) \times 10^5$
0.36	$(1.2 \pm 0.6) \times 10^6$	0.90	$(1.0 \pm 0.3) \times 10^6$
0.54	$(2.9 \pm 1.2) \times 10^6$	1.00	$(3.1 \pm 1.7) \times 10^6$
0.90	$>10^7$		

[a] k_a calculated from the average of two to three determinations at different protein concentrations by using the relationship $k_a = (\tau_{obsd} \times [RNAP])^{-1}$. [b] Standard reaction conditions except that $MgGlu_2$ was substituted for $MgCl_2$. [c] Extrapolated from the data of Figure 4.

Origin of the Glutamate Effect. Replacement of Cl^- with Glu^- greatly enhances the stability of protein–DNA interactions at a constant [K^+]. Clearly, the large effects of the nature of the anion on these site-specific protein–DNA interactions arise from a general mechanism involving neither a large anion stoichiometry nor high-affinity interactions. These effects must result from differences in the preferential interactions of Glu^- and Cl^- with the proteins involved, since anion-specific effects on DNA denaturation are not observed at the electrolyte concentrations investigated here (von Hippel & Schleich, 1969, and references cited therein). We have found that substitution of KGlu for KCl has no effect on the thermal denaturation profile of poly(dAT·dAT) over a range of [K^+] from 0.01 to 0.9 M (G. Bills and C. Harrison, unpublished results). Furthermore, anion-specific effects are not observed on the binding of simple ligands such as oligolysine to DNA (Lohman et al., 1980).

What is the molecular nature of the protein–anion interactions responsible for large anion effects on protein–DNA complex formation? Perhaps the simplest picture is that of a favorable binding interaction of anions (relative to H_2O) with the positively charged DNA binding site, so that anion displacement accompanies complex formation. Evidence exists for weak binding of Cl^- to a number of proteins, with affinities ranging from $\sim 10^{-1}$ to ≥ 100 M^{-1} [reviewed by Record et al. (1978)]. For example, Cl^- binds to the diphosphoglycerate effector site of deoxyhemoglobin with an affinity of ~ 400 M^{-1} ($\sim$10-fold lower than the affinity of ATP for the same site). We are not aware of any evidence for weak site binding of glutamate to proteins.

[2] Plots of log k_a vs. log [K^+] in the presence of Mg^{2+} were analyzed to correct for competitive, salt-dependent effects of Mg^{2+} by using the relationship (Lohman et al., 1978)

$$\log k_a = \log k_0 - n \log S - n\psi \log [K^+] \quad (2)$$

where k_a is the observed second-order association rate constant, k_0 is the salt-independent (nonelectrostatic) component of k_a, S is the Mg^{2+} binding polynomial (which is determined by the observed binding constant K_{obsd}^{Mg} for the Mg–DNA interaction, itself a function of [K^+]), n is the number of phosphates neutralized in the binding reaction, and ψ is the thermodynamic fraction of a counterion released per phosphate neutralized (ψ = 0.88 for double-stranded DNA). K_{obsd}^{Mg} was evaluated as a function of [K^+] from the expression (Shaner et al., 1982) $\log K_{obsd}^{Mg} = -1.57 \log [K^+] + 0.59$. Equation 2 is strictly applicable only to the situation in which no anions are released from the protein in the DNA binding interaction.

Alternatively, anions may be preferentially *excluded* from the protein surface, as has been observed by Timasheff and co-workers for a number of solutes including glycerol, salts, neutral amino acids, and other neutral osmolytes (Arakawa & Timasheff, 1984a, and references cited therein). Exclusion occurs when a relatively *unfavorable* interaction between protein and solute gives rise to a lower local concentration of solute, and thus a higher concentration of water, at the surface of the protein than in the bulk solution. The entropy increase that accompanies the formation of a more random distribution of anions will drive processes in which the solvent-exposed surface area of the protein is reduced, as is the case upon binding to DNA or assuming a more compact comformation. Accordingly, solutions of KGlu would stabilize protein–DNA complexes more than KCl if Glu^- were more excluded than Cl^-. Support for this view comes from a study by densimetry of the relative extents of interaction of NaCl and NaGlu with BSA, which found that Glu^-, at salt concentrations near or above 1 M, is more excluded from the domain of BSA than Cl^- (Arakawa & Timasheff, 1982, 1984b). Arakawa and Timasheff (1984b) also observed preferential hydration of tubulin in the presence of NaGlu and suggested that Glu^- exclusion is the mechanism by which NaGlu stabilizes tubulin–colchicine complexes (at 0.1–0.2 M NaGlu) and enhances microtubule formation (at 1 M NaGlu). Whatever the detailed molecular origin of the difference between Cl^- and Glu^- interactions with proteins, we believe it is the rearrangement of local concentration gradients of anions and water during complex formation that produces the large effects we observe on the kinetics and stability of these complexes.

Independent of the molecular picture of the protein–anion interactions involved, anions are known to affect a wide variety of processes involving proteins. At high (>1 M) concentrations, anion effects on protein solubility follow the Hofmeister or lyotropic series, a series that is mirrored at lower concentrations in effects of anions on enzymatic activity (Jencks, 1969). The ranking of different anions, based upon the $[K^+]$ at which a significant reduction in the activity of *Eco*RI is observed in salt titrations similar to Figure 1, follows the lyotropic series: $Glu^- > Ac^- > Cl^- > Br^- \simeq NO_3^- > I > ClO_4^-$ (D. S. Cayley, unpublished results). A similar ranking of anions is observed when the rate constants for open complex formation between RNA polymerase and the λP_R promoter are compared (M. Mansfield and S. Leirmo, unpublished results): $k_{Glu} > k_{Ac} \gg k_{Cl} > k_I$. At 0.3 M K^+, the second-order rate constant k_a is decreased 4-fold by replacing KGlu with KAc, which is a small effect compared to the 30-fold effect of replacing KGlu by KCl (cf. Figure 4). Our results with glutamate ($Glu^- > Ac^-$) indicate that it should be ranked in the lyotropic series with anions regarded as weakly interacting with a variety of proteins. Similar qualitative observations have been made for the effect of anions on complexes of single-stranded DNA with *E. coli* recA protein (Roman & Kowalczykowski, 1986) and single-stranded DNA binding protein (T. Lohman, personal communication).

Conclusions

We believe that use of KGlu in place of KCl in buffers used to investigate protein–DNA interactions in vitro provides a better approximation to the in vivo ionic environment. Because the levels of K^+ and Glu^- in *E. coli* vary widely in response to variations in the external osmotic strength, one cannot specify a unique [KGlu] that is "physiological". Compounding this problem is the fact that the total $[K^+]$ in *E. coli* is a factor of 3–6 greater than the corresponding $[Glu^-]$ at any osmolarity, due to the high concentration of polyanion charge and the contribution of other small anions (phosphate, acetate, etc.). In spite of the difficulty in defining a physiologically relevant [KGlu], we expect that the Glu^-/Cl^- difference is responsible for the general observation that optimal ionic conditions in KCl for protein–DNA interactions or function in vitro lie at or below the low-salt end of the physiological range. Use of KGlu in place of KCl appears to shift the range of ionic conditions useful in vitro into better correspondence with the physiological range.

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Transverse Nuclear Spin Relaxation in Phospholipid Bilayer Membranes†

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ABSTRACT: Experimental proof is presented that some of the motions responsible for transverse relaxation (T_2) in deuterium magnetic resonance (^{2}H NMR) experiments on acyl chains of a model membrane in the liquid-crystalline phase are extremely slow on the ^{2}H NMR time scale being characterized by a correlation time $\tau_2 \gg 10^{-5}$ s. The experiments used to investigate these slow motions involve a form of the Carr–Purcell–Meiboom–Gill pulse sequence modified so as to be suitable for ^{2}H NMR. The most plausible mechanism responsible for T_2 relaxation is the gradual change in the average molecular orientation due to lateral diffusion of the phospholipid molecules along curved membrane surfaces. A procedure for separating contributions to T_2 relaxation due to slow and fast motions is described.

The technique of deuterium nuclear magnetic resonance (^{2}H NMR) has provided important information on structure and molecular motion in model and biological membranes (Seelig & Seelig, 1980; Jacobs & Oldfield, 1981; Davis, 1983; Devaux, 1983; Bloom & Smith, 1985). Most ^{2}H NMR studies have concentrated on the determination of local orientational order from the spectrum of quadrupolar splittings. In this paper we are particularly concerned with the dynamic properties of these systems as determined from measurements of ^{2}H NMR longitudinal (T_1) and transverse (T_2) spin relaxation times. Interpretation of T_1 and T_2 measurements requires a knowledge of the correlation times for those molecular motions that modulate the quadrupolar interactions. The T_1 relaxation processes in ^{2}H NMR are only sensitive to "spectral densities", $J(\omega)$, of the fluctuating quadrupolar interactions at $\omega = \omega_0$ and $\omega = 2\omega_0$, where $f_0 = \omega_0/2\pi$ is the nuclear Larmor frequency, while the T_2 relaxation processes are also affected by $J(0)$. This dependence of T_2 on the low-frequency components of the spectral density means that T_2 is very sensitive to slow motions. As emphasized by Davis (1979), the observation that $T_1 \gg T_2$ for ^{2}H nuclei in the acyl chains of phospholipid molecules implies that there must exist motions with correlation times $\tau_1 \lesssim \omega_0^{-1}$ that are responsible for T_1 relaxation and other motions with correlation times $\tau_2 \gg \omega_0^{-1}$ that are dominant in T_2 relaxation.

It has been implicitly assumed up to now that the motions responsible for ^{2}H NMR relaxation in the liquid-crystalline phase of lipid bilayers are associated with local chain reorientation, either collective or noncollective (Brown, 1982, 1983; Kimmich et al., 1983; Bloom & Smith, 1985, p 70), and are characterized by correlation times that are short "on the NMR time scale for motional averaging", τ_M (Seelig & Seelig, 1980). If the relaxation is produced by a fluctuating interaction, which accounts for ΔM_2 of the ^{2}H NMR second moment (Davis, 1979, 1983), this would require that its correlation time $\tau_c \ll \tau_M = (\Delta M_2)^{-1/2}$. The hierarchy of correlation times implied by this interpretation is

$$\tau_1 \lesssim \omega_0^{-1} \ll \tau_2 \ll \tau_M$$

Such an interpretation of T_1 and T_2 is at the present time consistent with all published experimental data. Measured values of T_1 lead to $\tau_1 \leq 10^{-10}$ s while a typical value of the Larmor frequency in ^{2}H NMR, $f_0 \approx 35$ MHz, gives $\omega_0^{-1} \simeq 5 \times 10^{-9}$ s. Since the total second moment of ^{2}H spins on acyl chains is $M_2 \simeq 1.1 \times 10^{11}$ s^{-2}, a lower limit for τ_M is given by $\tau_M \gtrsim 3 \times 10^{-6}$ s and a reasonable estimate of τ_M corresponds to $\tau_M \simeq 10^{-5}$ s (Seelig & Seelig, 1980). The relatively shorter T_2 values in the liquid-crystalline phase are compatible with values of τ_2 in the range 10^{-7}–10^{-8} s. Indeed, with this interpretation of T_2, the reduction of T_2 always observed upon addition of proteins to phospholipid bilayer membranes may be interpreted as indicating a significant slowing down of the acyl chains at the lipid–protein interface (Paddy et al., 1981; Bienvenue et al., 1982).

Although the interpretation of T_2 described above is internally consistent with published experimental T_2 data, none of the experiments performed thus far rules out the possibility that $\tau_2 \gg \tau_M$. For this reason, we undertook an investigation

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