Effects of Molecular Crowding on the Interaction between DNA and the *Escherichia coli* Regulatory Protein TyrR

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ABSTRACT Fluorescence quenching has been used to measure quantitatively the effects of sucrose and triethylene glycol on the interaction between the *Escherichia coli* regulatory protein TyrR and a 30-basepair oligonucleotide containing the strong TyrR box of the *TyrR* operon. It was observed that the apparent binding constant increased in the presence of co-solutes, the dependence of the logarithm of the apparent binding constant on molar concentration being indistinguishable and essentially linear for both co-solutes. This activation of the TyrR-oligonucleotide interaction is attributed to thermodynamic nonideality arising from molecular crowding, an interpretation which is supported by the reasonable agreement observed between the experimental extent of reaction enhancement and that predicted on the statistical-mechanical basis of excluded volume.

INTRODUCTION

The control of gene expression is mediated by numerous DNA-binding proteins—polymerases, helicases, activators, and repressors. The in vivo action of these proteins is determined largely by the magnitudes of the binding constants governing their interaction with DNA. Most in vitro assays for the quantitative characterization of interactions between proteins and DNA are carried out in relatively dilute solutions, and hence under conditions where the reactants behave in a thermodynamically ideal manner. However, the occupation of cell space by subcellular organelles, cytoskeletal networks, and other solutes (small as well as macromolecular) has the potential to create a situation wherein the thermodynamic concentration (activity) of DNA and binding proteins in vivo may well be considerably higher than their actual concentrations. There are estimates that 20–30% of the total cell volume is occupied by proteins and nucleic acids (Minton, 1983, 1995; Zimmerman and Minton, 1993). The resultant molecular crowding or volume exclusion in the cellular environment acts to decrease the conformational entropy of the confined macromolecules and thus to increase their chemical potential.

Because of its ability to provide estimates of activity coefficients, a standard statistical mechanical treatment of excluded volume can be used to predict the magnitude of the effect of molecular crowding on protein interactions (Edmond and Ogston, 1968; Wills et al., 1980; Hermans, 1982; Winzor and Wills, 1995a). Furthermore, several experimental studies have demonstrated that these effects can be substantial. For example, the effective binding constant for the interaction of aldolase with muscle myofibrils is enhanced by 35–40% in the presence of 1.4% albumin (Harris and Winzor, 1985). An even more dramatic effect is observed in studies of the interaction between bacteriophage T4 gene 45 protein with gene 44/62 proteins, for which the inclusion of 7.5% polyethylene glycol 12,000 gives rise to a 50-fold increase in the binding constant for replication complex formation (Jarvis et al., 1990). That small solute molecules can also participate in excluded volume effects is illustrated by the threefold enhancement of the dimerization constant for α-chymotrypsin in the presence of 0.15 M sucrose (Shearwin and Winzor, 1988). Similar effects of small solutes on DNA-protein equilibria have also been noted. For example, Robinson and Sligar (1993) reported that sucrose increased the binding of *Eco*R1 to sites of secondary specificity on DNA (star activity), and Garner and Rau (1995) found a 10-fold increase in the association constant for the binding of the Gal repressor to DNA in the presence of sucrose, betaine, or triethylene glycol. Though not quantitative, a case for the operation of molecular crowding effects in other protein-DNA interactions can be inferred from the enhanced enzyme activities observed for DNA polymerase (Zimmerman and Trach, 1988), DNA ligase (Teraoka and Tsukada, 1987), and topoisomerase (Forterre et al., 1985) that are observed in the presence of co-solutes.

The TyrR regulatory protein from *Escherichia coli* controls the expression of eight genes that code for proteins required for the synthesis of aromatic amino acids and their transport into the cell—a topic reviewed by Pittard and Davidson (1991). Interaction occurs between the dimeric TyrR protein and 22-basepair sequences known as TyrR boxes, the TyrR activity being modified by the binding of ATP and aromatic amino acids (Argaet et al., 1994). Repression, which is usually induced by the binding of tyrosine in the presence of ATP, is affiliated with self-association of the TyrR dimer to form a hexamer (Wilson et al.,

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1994a,b). This interplay of macromolecular interactions responsible for control of TyrR activity is governed by equilibrium constants that are susceptible to modification by effects of thermodynamic nonideality in the crowded cellular environment. To that end, Zimmerman and Trach (1991) have estimated that 34–44% of the cytoplasmic volume of *E. coli* is occupied by macromolecules, and that the excluded volume effects may be even greater in the vicinity of the cell nucleoid.

The purpose of this communication is to demonstrate the consequence of molecular crowding on TyrR-DNA interactions by examining the effect of two small solutes, sucrose and triethylene glycol, on the binding of TyrR to a 30-basepair oligonucleotide containing a TyrR box. To establish that the resulting increase in the measured binding constant is, indeed, the consequence of molecular crowding rather than specific binding of the co-solute, the magnitude of the enhancement is shown to be consistent with that predicted on the statistical-mechanical basis of excluded volume (McMillan and Mayer, 1945; Hill, 1960; Winzor and Wills, 1995a).

MATERIALS AND METHODS

Materials

Adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS) was purchased from Boehringer Mannheim (Mannheim, Germany), fluorescein-5-isothiocyanate (FITC) from Molecular Probes Inc. (Eugene, Oregon), and β-cyanoethyl phosphoramidites from Applied Biosystems (Foster City, California). Reagent grade sucrose and triethylene glycol were products of Mallinckrodt (Paris, Kentucky) and Aldrich (St. Louis, Missouri), respectively. TyrR was prepared as described previously (Argaet et al., 1994); and its concentration, expressed in terms of dimer, was determined spectrophotometrically at 280 nm on the basis of a molar absorption coefficient of 34,500 M^{-1} cm^{-1} for monomer (Hagmar et al., 1995).

Oligonucleotide synthesis

Single-stranded oligonucleotides with the sequence shown in Fig. 1 were synthesized by standard β-cyanoethyl phosphoramidite chemistry and purified by trityl-on reverse-phase HPLC. Oligonucleotide concentrations were determined spectrophotometrically at 260 nm on the basis of respective molar absorption coefficients of 323,000 and 336,000 for the primary and complementary strands. The primary oligonucleotide was synthesized with an amino-uridine base, 5-(3-aminoprop-1-yl-2′-deoxyuridine, substituted for thymidine at position 9 from the 5′ end (Hagmar et al., 1995). The resultant oligonucleotide was labeled with FITC and purified by HPLC (Hagmar et al., 1995). Purified oligonucleotides were dried and dissolved in phosphate-chloride buffer (25 mM K_2HPO_4, 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 0.02% NaN_3), pH 7.4. Molar concentrations of oligonucleotides are expressed on the basis of the double-stranded species.

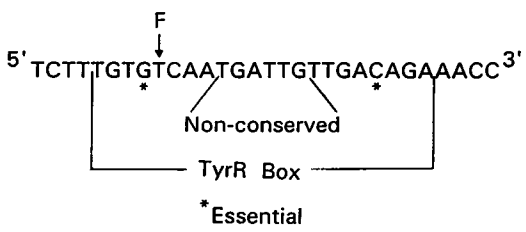

FIGURE 1 Sequence of the primary oligonucleotide strand used to study the interaction of TyrR with its 22-basepair palindromic consensus sequence (the TyrR box). Also indicated is the site for attachment of fluorescein (*F*), the probe inserted to allow characterization of the interaction by fluorescence quenching.

Fluorescence titrations

Steady-state fluorescence measurements were made at 20°C in a Perkin-Elmer LS-5 fluorometer equipped with a thermostatically maintained cell block. Titrations of DNA with TyrR were made in the above phosphate-chloride buffer using a 10-mm fluorescence cuvette and respective excitation and emission wavelengths of 495 and 520 nm. In a typical titration, aliquots of TyrR (5 μl, 3.0 μM) were added to a solution of 32 nM DNA containing 200 mM ATPγS, 10 mM $MgCl_2$, and a specified concentration (0–0.5 M) of inert co-solute (sucrose or triethylene glycol) in the phosphate-chloride buffer. The cell contents were mixed by gentle inversion and the fluorescence intensity measured 10 min after each addition. The co-solvents did not affect the fluorescence of the fluorescein-labeled oligonucleotide. ATPγS was substituted for ATP in these fluorescence titrations to avoid complications emanating from the very weak ATPase activity of TyrR (Cui et al., 1993). The quenching of fluorescence resulting from the interaction of TyrR with the labeled DNA was analyzed by curve-fitting the titration results to Eq. 1 in order to obtain the apparent binding constant using the nonlinear least-squares program available on SigmaPlot.

RESULTS

The binding of TyrR to the fluorescein-labeled oligonucleotide whose structure is shown in Fig. 1 results in a quenching of fluorescein fluorescence that can be used to quantify the equilibrium interaction. Results of titrations in the presence of various concentrations of sucrose and triethylene glycol are shown in Fig. 2, *A* and *B*, respectively. Also shown are the best-fit descriptions of the experimental results obtained at a given co-solute concentration in terms of the relationship

$$(\Delta F)^2 K_{app}(C_D)_t - \Delta F \Delta F_m[1 + K_{app}\{(C_P)_t + (C_D)_t\}] + (\Delta F_m)^2 K_{app}(C_P)_t = 0 \quad (1)$$

for a TyrR-oligonucleotide interaction governed by 1:1 stoichiometry (Bailey et al., 1995). ΔF and ΔF_m are the measured and maximal extents of fluorescence quenching, respectively, in reaction mixtures with $(C_P)_t$ and $(C_D)_t$ the respective total concentrations of TyrR and oligonucleotide, the latter being held essentially constant at 31.7 nM. Nonlinear least-squares fitting of ΔF (the dependent variable) as a function of the independent variable, $(C_P)_t$, was used to deduce the magnitudes of K_{app} and ΔF_m that best describe the dependence for a given co-solute concentration. In the absence of co-solute the measured equilibrium constant should approximate the thermodynamic association constant, *K*, because of the dilute nature of the reaction mixture. Gel shift assays and competitive binding assays using labeled and unlabeled oligonucleotide (data not shown) show that the presence of the label reduces to association constant by a factor of ~30.

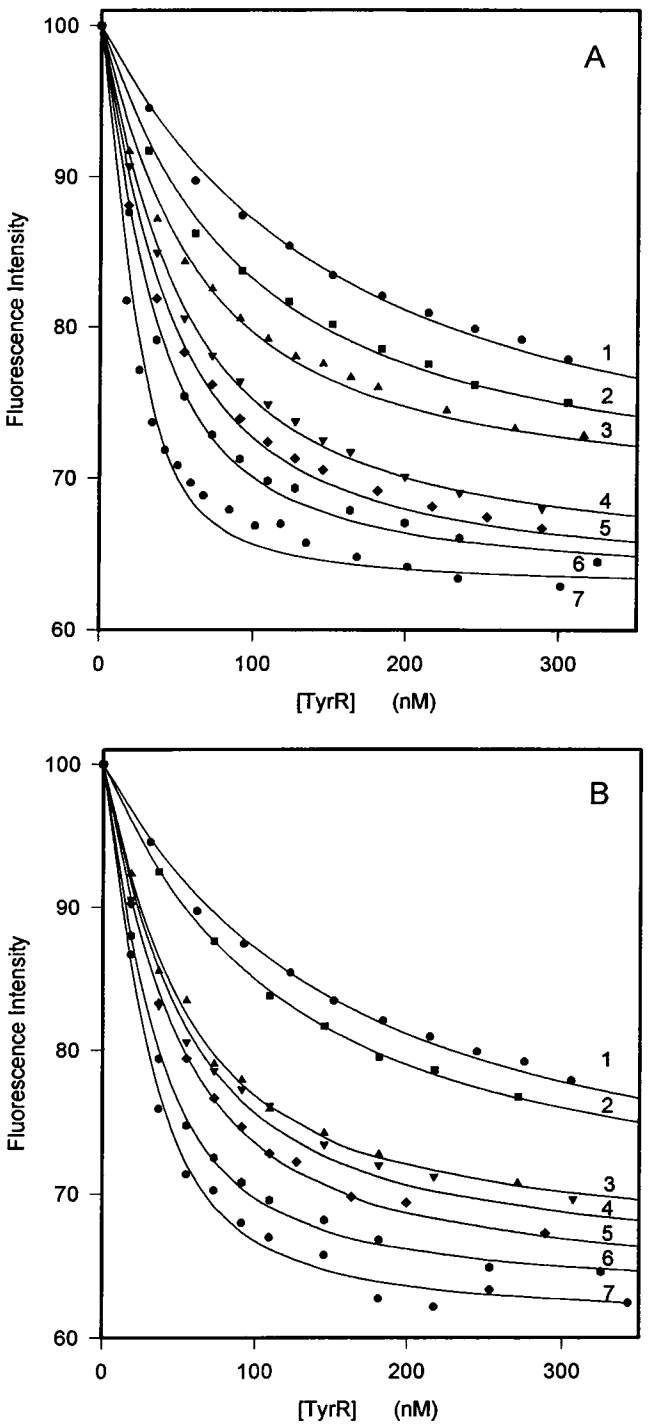

FIGURE 2 Dependence of the relative fluorescence intensity upon the total concentrations of tyrR, $(C_P)_t$, present during stepwise titrations of labeled oligonucleotide (32 nM initially) in phosphate-chloride buffer (pH 7.4) supplemented with the following concentrations of (*A*) sucrose and (*B*) triethylene glycol. Curves 1 to 6 denote concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 sucrose or triethylene glycol, respectively. Lines denote the best-fit descriptions of results for a given cosolute concentration obtained by nonlinear regression analysis in terms of Eq. 1, the resulting binding constants (K_{app}) being reported in Table 1.

Apparent equilibrium constants obtained by this means are listed in Table 1, which signifies progressive enhancement of the interaction with increasing concentrations of either sucrose or triethylene glycol. The concentration dependence of the logarithm of the apparent equilibrium constant is essentially linear, being described by the relationship $\ln [(K')_{app}/K'] = 1 + bC_M$ with $b = 4.7$ ($\pm$ 0.3) l/mol (Fig. 3). Although this result could be taken to signify preferential interaction of co-solute *M* with the TyrR-oligonucleotide complex, such interpretation seems unlikely in that the superimposition of the two dependencies in Fig. 3 would require the complex to exhibit identical affinities for sucrose and triethylene glycol. We now show that this extent of enhancement of the TyrR-oligonucleotide interaction may be rationalized as a consequence of thermodynamic nonideality on the statistical-mechanical basis of molecular crowding.

Interpretation in terms of thermodynamic nonideality

For a 1:1 interaction between protein P and oligonucleotide D to form complex C, the thermodynamic association constant *K* is most appropriately defined as

$$K = a_C/(a_P a_D) \qquad (2)$$

where a_i denotes the thermodynamic activity of species *i* expressed on a molal basis because its chemical potential is being defined under conditions of constant pressure (Wills et al., 1993). Although molality (m_i) is therefore the more direct concentration scale to employ experimentally under such circumstances, we can take into account the fact that the usual practice of using molar concentration (C_i) has been adopted in the fluorescence studies. In the present design of experiments a very dilute solution of reactants has been supplemented with a moderately high concentration of small co-solute, *M*; and hence any thermodynamic nonideality is essentially the consequence of the effects of *M* on the activities of the three reactants. Because $C_M \gg C_P +$

TABLE 1 Effect of cosolute concentration on the measured binding constants $(K')_{app}$ for the interaction of TyrR with a 30-basepair oligonucleotide containing the consensus sequence of the TyrR box

Co-solute Concentration (M)	Binding Constant (μM^{-1})*	
	Sucrose	Triethylene glycol
0	7.2 (±0.4)	7.2 (±0.4)
0.1	13.6 (±0.8)	10.0 (±0.3)
0.2	22.8 (±1.7)	26.1 (±1.9)
0.2	—	28.0 (±2.3)
0.3	28.6 (±1.5)	28.8 (±2.2)
0.3	—	32.3 (±2.0)
0.4	43.3 (±3.7)	33.5 (±1.7)
0.4	68.8 (±5.3)	—
0.5	70.3 (±4.6)	59.3 (±3.4)
0.6	165 (±7)	77.9 (±4.9)

* Numbers in parentheses denote the uncertainty (±SD) of the estimate.

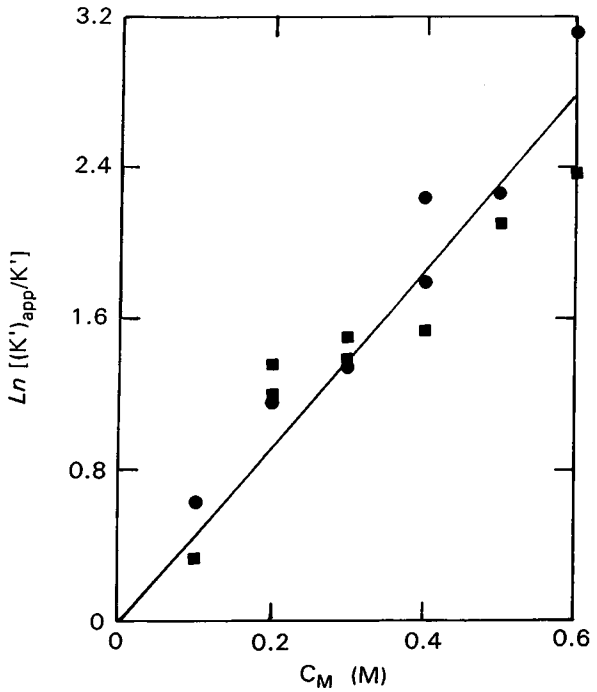

FIGURE 3 Dependence of the apparent binding constant for the TyrR-oligonucleotide interaction upon the concentration (C_M) of either sucrose (●) or triethylene glycol (■) included in reaction mixtures, the solid line being the best-fit linear description in terms of Eq. 5.

$C_D + C_C$, the relationships for the composition dependence of the thermodynamic activities of the reacting species (Wills et al., 1993) simplify to

$$\ln a_P = \ln C_P - \ln \rho_s + (B_{PM} - M_M\bar{\nu}_M - M_P\bar{\nu}_P)C_M + .. \quad (3a)$$

$$\ln a_D = \ln C_D - \ln \rho_s + (B_{DM} - M_M\bar{\nu}_M - M_D\bar{\nu}_D)C_M + .. \quad (3b)$$

$$\ln a_C = \ln C_C - \ln \rho_s + (B_{CM} - M_M\bar{\nu}_M - M_C\bar{\nu}_C)C_M + .. \quad (3c)$$

where ρ_s is the solvent density and B_{iM} is the second virial coefficient describing nearest-neighbor interactions between i and M: partial molar volumes are expressed as the product of molecular weight (M_i), and partial specific volume ($\bar{\nu}_i$) of the species. This treatment assumes that the co-solute does not perturb the structure of either reactant. Substitution of these expressions into Eq. 2 then gives

$$K/\rho_s = (C_C/C_PC_D) \cdot \exp[(B_{CM} - B_{PM} - B_{DM} + M_M\bar{\nu}_M)C_M + ..] \quad (4)$$

where the ratio K/ρ_s converts the thermodynamic association constant (molal^{-1}) into units of reciprocal molar concentration, and hence allows its comparison with K', the experimentally determined equilibrium constant (M^{-1}) in the absence of co-solute; and where $(K')_{app}$ may be substituted for the ratio of reactant concentrations. With these substitutions Eq. 4a becomes

$$K' = (K')_{app}\exp[(B_{CM} - B_{PM} - B_{DM} + M_M\bar{\nu}_M)C_M + ..] \quad (5a)$$

$$\ln[(K')_{app}/K'] = [(B_{CM} - B_{PM} - B_{DM} + M_M\bar{\nu}_M)C_M + ..] \quad (5b)$$

where $M_M\bar{\nu}_M$, the partial molar volume of sucrose, is 0.2 l/mol (Lee and Timasheff, 1981). A linear dependence of the logarithm of the ratio of equilibrium constants is thus predicted over the range of co-solute concentration for which the virial expansion truncated at the linear term suffices to provide an adequate description of the thermodynamic nonideality.

The above considerations establish the feasibility of interpreting the linear dependence of the equilibrium constant ratio for the TyrR-oligonucleotide system upon co-solute concentration (Fig. 3) as a consequence of thermodynamic nonideality. That conclusion is now accorded even greater credibility by estimating the magnitude of the linear coefficient in Eq. 5 that is predicted by rationalization of the thermodynamic nonideality in terms of the statistical mechanical interpretation of a second virial coefficient, B_{iM}, as an excluded volume (McMillan and Mayer, 1945; Hill, 1960; Winzor and Wills, 1995a), which becomes synonymous with the co-volume, U_{iM}, in the absence of charge on the co-solute. The co-volume describes the volume from which the centers of two molecules are mutually excluded (Fig. 4).

Quantitative prediction of the linear coefficient

In order to estimate the magnitudes of the co-volumes U_{iM}, the co-solute has been modeled as a sphere, the radius r_s of which has been taken as 0.3 nm—the effective thermodynamic radius of sucrose deduced from statistical mechanical considerations of isopiestic (Wills et al., 1993), freezing point depression (Winzor and Wills, 1995a) and gel chromatographic (Shearwin and Winzor, 1988) measurements. A comparable (slightly smaller) value should apply to triethylene glycol, but quantitative confirmation of its magnitude by the same approaches is precluded because the negative deviations from Raoult's law as the result of binary system formation between triethylene glycol and water (Wise et al., 1950) counter the positive deviations emanating from self-self excluded volume effects. The oligonucleotide has been modeled as a rod with a length (l) of 10.5 nm and a radius (r_r) of 1.1 nm, whereas the dimeric TyrR has been regarded as a prolate ellipsoid with semi-major (a) and semi-minor (b) axes of 6.2 and 3.1 nm, respectively: the latter value is the estimated Stokes radius of a TyrR monomer (mol wt 58,000). Because the length of the tyrR ellipsoid exceeds that of the oligonucleotide rod, the complex has also been modeled as a prolate ellipsoid with a semi-major axis (a) of 6.2 nm and a semi-minor axis (b) of 3.34 nm—the value calculated from the expression $V = 4\pi ab^2/3$,

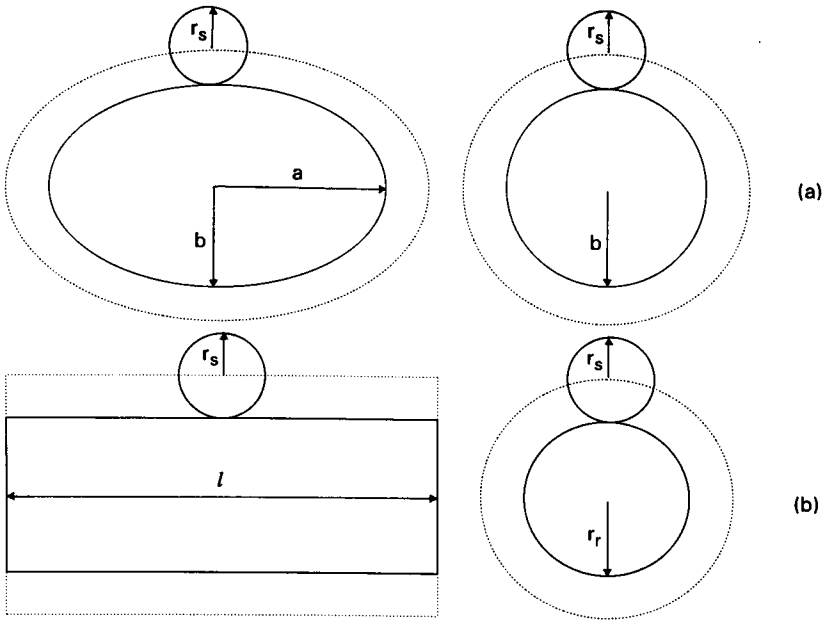

FIGURE 4 Schematic representations of the mutually excluded volumes (co-volumes) for sphere-ellipsoid and sphere-rod combinations. (*a*) Side and end views of the co-volume (····) for a sphere, radius r_s, and a prolate ellipsoid of revolution with semi-major and semi-minor axes a and b, respectively (Eq. 6). (*b*) Corresponding views of the co-volume (····) for a sphere, radius r_s, and a rod with length l and radius r_r (Eq. 7).

with V taken as the combined volumes of the TyrR ellipsoid and oligonucleotide rod.

For the excluded volume interaction between a sphere with radius r and a prolate ellipsoid of revolution with semi-major and semi-minor axes a and b respectively, the co-volume U is

$$U = N[4\pi r_s^3/3 + 4\pi ab^2/3 + 2\pi abr_s\{(1 - \epsilon^2)^{1/2} + (\sin^{-1}\epsilon)/\epsilon\} + 2\pi ar_s^2\{1 + [(1 - \epsilon^2)/2\epsilon]\ln[(1 + \epsilon)/(1 - \epsilon)]\}] \quad (6)$$

where $\epsilon^2 = 1 - b^2/a^2$ (Ogston and Winzor, 1975; Nichol et al., 1976), whereas that for the sphere and a rod is

$$U = N[\pi l(r_s + r_r)^2] \quad (7)$$

where l denotes the length of the rod with radius r_r (Edmond and Ogston, 1968). Avogadro's number (N) is introduced into these expressions to express the co-volume on a molar rather than a molecular basis.

Calculations of co-volumes on the above bases leads to the following estimates of parameters required for evaluating the magnitude of the linear coefficient in Eq. 5: U_{PM} = 186 l/mol, U_{DM} = 39 l/mol, U_{CM} = 213 l/mol. The predicted value of ($U_{PM} + U_{DM} - U_{CM}$) is thus 12 l/mol, which is only marginally larger than the experimentally determined slope (5 l/mol) of the linear dependence of $\ln[(K')_{app}/K']$ upon co-solute concentration (Fig. 3). In view of the geometric oversimplification of the actual shapes of the molecules, we conclude that the activating effect of sucrose and triethylene glycol on the TyrR-oligonucleotide interaction may be attributed to thermodynamic nonideality emanating from molecular crowding in the more concentrated environment created by the presence of these co-solutes.

Relationship of the present treatment to that based on osmotic stress

The present treatment of co-solute effects on DNA-protein interactions in terms of thermodynamic nonideality is seemingly redundant in the sense that comparable observations have been attributed to the consequences of osmotic stress (Robinson and Sligar, 1993; Garner and Rau, 1995). However, on the grounds that osmotic pressure is a phenomenon for which confinement of a macromolecular solute to one phase gives rise to a flow of solvent from another phase to maintain constancy of solvent chemical potential throughout both phases, it seems odd to find the osmotic stress concept being invoked to account for co-solute effects in single-phase systems (Rand et al., 1993; Garner and Rau, 1995; Reid and Rand, 1997). Adoption of the osmotic stress strategy entails the assumption that the macromolecular species (DNA, protein, and DNA-protein complex) may be considered to be surrounded by a membrane that is permeable to solvent but impermeable to the macromolecular species and co-solute. Subject to the validity of that proviso, solvent activity in the vicinity of macromolecular species may be varied by the addition of co-solute to the bulk solvent phase. Such addition of co-solute lowers the chemical potential of solvent in the bulk phase and hence leads to a flow of solvent from the macromolecular solute microphase to reestablish constant chemical potential of solvent. In terms of this treatment, the counterpart of Eq. 5b becomes (Rand et al., 1993; Garner and Rau, 1995; Reid and Rand, 1997)

$$\ln[(K')_{app}/K'] = \Pi(\Delta V)/(RT) \quad (8)$$

where ΔV is the volume change in response to the osmotic pressure (Π) of co-solute-supplemented solvent phase. The physicochemical principles of osmometry require this vol-

ume change to be identified as the change in volume of the putative macromolecule-containing phase as the result of DNA-protein complex formation.

Although there is clearly no membrane surrounding the macromolecular reactants, the basic tenets of the above argument could still be considered to apply to a microenvironment within the solution that contains macromolecular solutes and solvent, but from which co-solute is excluded. A prerequisite for interpretation of the inferred volume change ΔV is, thus, location of the putative membrane of the model. Surprisingly, the originators of the osmotic stress concept have not done so.

Inspection of Fig. 4 indicates that there is, indeed, a distance of closest approach for the center of a co-solute molecule and a macromolecule, whereupon there is a region in which solvent but not co-solute molecules can be located. Each macromolecule could therefore be considered to exist in a co-solute-free microenvironment with a volume equal to the co-volume (excluded volume): this is the volume that would be subjected to osmotic stress. Consequently, the volume change determined by adoption of the osmotic stress strategy (Eq. 8) is now recognized as the change in reactant-co-solute co-volume as the result of complex formation—the conclusion reached by classical interpretation of the co-solute effect in terms of composition-dependent activity coefficients (Winzor and Wills, 1995a,b). This exposure of the osmotic stress strategy to more stringent physicochemical scrutiny has thus pinpointed the incorrect interpretation being placed on the volume change (ΔV) evaluated by the application of Eq. 8, and thereby reconciled the consequent findings with those based on the more rigorous analysis in terms of thermodynamic nonideality.

DISCUSSION

Wilson et al. (1994b) proposed that the dimer/hexamer self-association of TyrR that is promoted by tyrosine in the presence of ATP (or ATPγS) is an essential feature of tyrosine-mediated repression by this protein in *E. coli*. They postulated that at low intracellular tyrosine concentrations, TyrR dimers bind to the strong TyrR box(es) in the operator DNA of genes repressed by TyrR, and that an increase in the in vivo tyrosine concentration alters the nature of the TyrR-operator complex, because TyrR dimers hexamerize in situ on the DNA. The new complex would exclude RNA polymerase more effectively from the promoter and thereby repress the level of transcription initiation of the gene. The experiments reported in this paper were carried out under conditions where TyrR is a dimer, and hence the results indicate that the interaction of TyrR dimers with strong TyrR boxes are enhanced by molecular crowding effects. It is reasonable to expect that the interaction of TyrR hexamers with TyrR boxes would be similarly affected. Furthermore, as suggested previously (Wilson et al., 1994b), the dimer/hexamer self-association is also likely to be displaced toward the larger species by the thermodynamic nonideality prevailing in the intracellular milieu compared with that in vitro. We conclude, therefore, that the phenomenon of molecular crowding in vivo will influence the interaction of TyrR with DNA in a number of ways, although at this stage it is not possible to make a quantitative assessment of the overall contribution that it makes to the affinity of TyrR-DNA interactions in the cell.

In a more general context it should be noted that the present interpretation of enhanced protein-DNA interaction in the presence of co-solutes contrasts with earlier considerations of similar phenomena in terms of osmotic stress (Robinson and Sligar, 1993; Garner and Rau, 1995). Indeed, the results of the latter study (Garner and Rau, 1995) are of particular relevance because they concerned the activating effect of sucrose (and other small co-solutes) on the interaction between a dimeric repressor protein and linear double-stranded DNA segments from the *Eco*R1-*Pst*I fragment of a plasmid (pSA509). In present terms the indistinguishable linear dependencies of $\ln[(K')_{app}/K']$ upon osmolarity of the sucrose-supplemented buffer for the O_E (181 basepairs) and O_I (103 basepairs) fragments translate into a value of 3 l/mol for $(B_{PM} + B_{NM} - B_{CM} + M_M\bar{\nu}_M)$: a transformation based on the reported relationship between osmolality and molar concentration (Garner and Rau, 1995) and Eq. 9 of Wills et al. (1993) for the interconversion between molal and molar concentration scales. Because, in present terminology, a decrease in asymmetry is manifested as a change in co-volume, the slightly smaller magnitude of the co-solute dependence for the enhancement of the gal repressor interaction than that for the tyrR system finds rational explanation in terms of the greater lengths of the O_E and O_I fragments (181 and 103 basepairs, respectively) compared with the 30-basepair fragment used here: for the gal repressor systems the complex retains an asymmetry in closer conformity with that of the uncomplexed DNA.

Our reasons for favoring the molecular crowding interpretation of the enhancing effect of co-solutes on protein-DNA interactions are twofold. First, the interpretation adheres to a classical treatment of thermodynamic nonideality on the statistical-mechanical basis of excluded volume. Secondly, from the above considerations it is evident that the concept of osmotic stress has been introduced without sufficient consideration being given to 1) the justification of describing the behavior of a single-phase system in terms of theory based on the existence of two phases, and 2) the actual location of the postulated phase barrier in the event that such arbitrary division of the single-phase system can be justified. More rigorous considerations of thermodynamic nonideality (Winzor and Wills, 1995a,b) have indicated that the volume change (and hence change in number of water molecules) refers to variation in the co-volume—not protein volume—as asserted by proponents of the osmotic stress concept (Parsegian et al., 1979, 1986; Rau et al., 1984; Rand et al., 1993; Robinson and Sligar, 1993; Garner and Rau, 1995; Reid and Rand, 1997).

Finally, it is also noted that many DNA-protein interactions are reported to occur at reaction rates greater than

those seemingly commensurate with diffusion control, a phenomenon that is usually attributed to linear diffusion of the protein along the DNA strands (Richter and Eigen, 1974; Berg and von Hippel, 1985; Kabata et al., 1994; Jeltsch et al., 1996). This particular means of reaction enhancement relies upon the existence of weak, nonspecific binding of the protein to the DNA chain to increase the effective local concentration of protein in the immediate vicinity of the specific oligonucleotide sequence with high affinity for the protein (Richter and Eigen, 1974). The present illustration that thermodynamic nonideality in a crowded molecular environment can also increase the effective reactant concentrations (thermodynamic activities) provides an additional means of increasing the operative rate constants for complex formation based on bulk concentrations: that operative rate constant may well exceed the magnitude inferred from in vitro studies. Whereas the linear diffusion model is a specific mechanism of activation that relies upon the existence of a second chemical interaction between the protein and DNA, thermodynamic nonideality is a completely general phenomenon reflecting the inability of molecules to encroach upon space already occupied by another molecule. Molecular crowding therefore provides an alternative means of enhancing the strength of interactions for which the linear diffusion model is inappropriate, and an additional means of enhancement in situations where the linear diffusion model already operates. Finally, this investigation serves not only to emphasize the fact that binding constants under conditions pertaining in the physiological environment may well be significantly larger than their in vitro counterparts as the result of molecular crowding, but also to emphasize the redundancy of the osmotic stress concept (Parsegian et al., 1979, 1986; Rau et al., 1984) as a means of interpreting the enhanced extent of interaction (Winzor and Wills, 1995b).

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