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Received 29 September 1999; accepted 14 December 1999

The Dissociation Rate of the EcoRI–DNA-Specific Complex is Linked to Water Activity

In many respects, the dissociation rate constant of a DNA–protein or protein–protein complex is as important a physical parameter as the equilibrium constant. The regulation of most cellular activities and developmental control are dynamic rather than static processes. With many techniques, the successful physicochemical characterization of a complex depends critically on the lifetime of the complex during isolation or measurement. With present technologies, the very powerful, single molecule methods used for mapping the kinetic barriers of complex dissociation reactions require lifetimes on the order of minutes.[1–3] We report here that the dissociation rate of the specific complex between the restriction nuclease EcoRI and its recognition DNA sequence is strongly dependent on water activity (in addition to its known dependence on salt activity[4–7]). This observation means that the dissociation rate of complexes in the crowded conditions found within cells cannot be straightforwardly predicted from dilute solution measurements, even though salt, temperature, and pH conditions are fixed to those found in vivo. In addition, these results suggest a practical method to extend the lifetime of "weak" complexes sufficiently to perform biophysical and biochemical characterizations.

The thermodynamic analysis of protein, peptide, and drug interactions with DNA has focused on the sensitivity of free energies to temperature, pH, and salt concentration (reviewed in Refs. 8–11). However, the displacement of water that should accompany specific complex formation as direct DNA–protein contacts replace DNA–water and protein–water interactions (reviewed in Refs. 12 and 13) means that binding energies will also depend on water activity. The number of water molecules released to the bulk solution in the process of DNA–protein complex formation can be measured from the sensitivity of the binding constant to bulk solution water activity. This procedure is analogous to measuring ion release through the dependence of binding constant on salt activity, or protonation through pH sensitivity. Water activity can be varied by adding neutral solutes that do not themselves directly affect the DNA–protein binding.[14–17] This approach has been used to measure changes in hydration accompanying the DNA binding of several proteins: *Escherichia coli* gal repressor,[15] *E. coli* CAP protein,[18] Hin recombinase,[19] Ultrabithorax and Deformed homeodomains,[20] *E. coli* tyr repressor,[21] EcoRI,[16,17,22] and the Sso7d protein.[23]

Using an equilibrium competition approach, we showed previously[16,17] that the free energy difference between complexes of the restriction nuclease EcoRI with nonspecific DNA and with the enzyme's recognition sequence is linearly dependent on the change in water chemical potential of the solution with added osmolyte. This dependence translates into an additional $\sim$ 110 waters that are sequestered by the nonspecific complex relative to the specific complex at 20°C[16] and $\sim$ 70 more waters at 4°C.[17] This significant difference in retained waters between specific and nonspecific complexes is accompanied by a difference of $\sim 10^4$ between the specific and nonspecific EcoRI DNA binding constants.[24] The difference in hydration additionally was seen to be insensitive to the size and chemical nature of the solute used to change water activity[16] for a wide variety of osmolytes. This result most probably implies that the water retained by the nonspecific complex is sequestered in a cavity at the DNA–protein interface that is sterically inaccessible to solutes.

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Biopolymers, Vol. 53, 363–368 (2000)

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We now show that the observed dependence of the equilibrium binding constant on water activity (or solute concentration) is reflected in dissociation rate constant of the EcoRI from its canonical site on DNA as well. In the presence of the osmolytes betaine and sucrose, the stability of the specific EcoRI–DNA complex increases dramatically. The half-life of the complex in 3 molal betaine is about 20 times longer than in 1.6 molal betaine, and is estimated as about 260 times longer than with no added osmolyte. The observed dependence of the dissociation rate constant on water activity translates into an uptake of about 70 water molecules for the dissociation of the EcoRI from its specific site at 4°C. This number of waters is practically identical to the difference in waters between specific and nonspecific binding derived from the equilibrium competition measurements.

MATERIALS AND METHODS

Materials

The plasmid pUC19 and restriction enzyme PvuII were purchased from New England Biolabs and used without further purification. A 322 base pair (bp) fragment carrying one EcoRI specific site, GAATTC, was isolated from the PvuII digestion products of pUC19 using standard techniques. The self-complementary oligonucleotide ggcgatcgaGAATTCtcgatcgcc carrying one specific site (shown in capital letters) was purchased from Gibco BRL, dissolved in 10-1 TE buffer (10 m*M* Tris Cl, 1 m*M* EDTA), purified using P6 Bio-Spin columns at room temperature, and self-annealed. The double-stranded character of the oligonucleotides was confirmed by electrophoresis on 20% polyacrylamide gel. The concentrations of the DNA fragment and the oligonucleotide were determined spectrophotometrically, using an extinction coefficient of 0.013 (m*M* base pairs)$^{-1}$ cm^{-1} at 260 nm. Absorption spectra were obtained with a Shimadzu UV-2101 PC spectrophotometer.

EcoRI restriction nuclease was purchased from New England Biolabs and used without further purification (as in Refs. 16 and 17). Active protein concentrations were determined by direct titration with the fragment containing respective recognition sequence under conditions of stoichiometric binding. Control equilibrium experiments with highly purified EcoRI (a generous gift of Dr. L. Jen-Jacobson) were performed previously.[17] In agreement with others,[22,25] it was confirmed that there is no significant difference in the binding properties or osmotic dependence between the two enzyme preparations.

Betaine was purchased from United States Biochemical, sucrose was purchased from Mallinckrodt. Both were used without further purification. Osmolal concentrations of betaine and sucrose were determined by direct measurement using a vapor pressure osmometer operating at room temperature (Wescor, Logan, UT; model 5100).

Dissociation Kinetics and Gel Mobility-Shift Experiments

Solution conditions for all kinetic experiments were 25 m*M* TrisCl (pH 7.5 at 20°C), 90 m*M* NaCl, 2.5 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 mg/mL bovine serum albumin, and 2.5% ficoll. The total reaction volume was 25 μL. These conditions were the same as in the equilibrium competition experiments previously reported[16,17] in order to compare directly differences in numbers of sequestered waters measured by two different methods.

Sufficient EcoRI restriction nuclease was added to the 322 bp fragment at 0.58 μg/mL (2.5 n*M* binding sites) to give about 40–60% of stoichiometrically bound fragment without added competitor. Stoichiometric binding of EcoRI with DNA was confirmed by the titration both of the protein with the specific DNA fragment and of the DNA fragment with the protein as described previously.[16] There was no observable cleavage of the EcoRI in the absence of Mg^{2+}, as expected.[24,26,27] An excess concentration of the oligonucleotide containing the recognition sequence was then added to the reaction mixture of EcoRI and 322 bp fragment, and incubated on ice for various times.

The loss of specific binding to the 322 bp fragment as the complex dissociates and binds to the oligonucleotide was monitored by the gel mobility shift assay. Reaction mixtures were electrophoresed in a 1.5% agarose gel, 0.5 × TBE buffer, at 120 V, at ~ 4°C for 2 h to separate free DNA fragment and EcoRI-bound complex. As was previously verified,[28] EcoRI-specific DNA fragment complexes are remarkably stable in the gel. No change in the fraction of complex could be observed between 30 min and 2 h of electrophoresis.

The observed dissociation rates of the EcoRI–DNA fragment complex were independent of the ratio of the concentration of specific binding sites on the oligonucleotide to the 322 bp fragment concentration for 20-, 200-, and 2000-fold excess of oligonucleotide specific sites. In order to verify that the loss of the specific fragment complex with time was not due to a loss of active protein, complexes of the 322 bp fragment with EcoRI were incubated for the same time intervals in the absence of specific site oligonucleotide. No loss in complex was observed with no oligonucleotide added.

Quantitation and Data Analysis

Electrophoretic bands containing free DNA fragment and DNA–protein complex were stained with SYBR Green I (Molecular Probes) and quantitated using fluorescent intensities as described previously.[16] The linearity of fluorescent intensity vs DNA amount per band over the range of concentrations studied was confirmed using pBR322 DNA fragments generated by MspI digestion. The fraction of specific fragment with bound EcoRI, F_b (= $[(DNA_b]/[DNA_{total}])$, is experimentally determined from the gel mobility-shift assay.

The simplified, first-order scheme for the binding reaction is

$$\mathrm{DNA_f} + \mathrm{P_f} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{DNA_b} \tag{1}$$

where DNA_f and P_f represent free DNA and protein, and DNA_b corresponds to the complex; k_{on} and k_{off} are the simplified reaction rate constants. Since the probability of a dissociated protein rebinding with the 322 bp fragment in the presence of a high excess of competitor-specific site oligonucleotide is negligible, the kinetic equation can be well approximated by the irreversible first-order rate equation[27]:

$$\frac{d[\mathrm{DNA_b}]}{dt} = -k_{off}[\mathrm{DNA}]_b \quad \text{or} \qquad \ln\left(\frac{\mathrm{F_b}}{\mathrm{F_{b,0}}}\right) = -k_{off}t \tag{2}$$

The parameters $[DNA_b]$ and F_b are the concentration and fraction, respectively, of DNA–EcoRI complex at time t; $[DNA_{b,0}]$ and $F_{b,0}$ are these values immediately after the addition of an excess of specific oligonucleotide ($t = 0$).

Analogous to osmotic dependence of the equilibrium binding constant,[14] the dependence of k_{off} on water activity can be calculated as change in the number of waters that exclude solute coupled to the dissociation of the specific complex,

$$\frac{d[\ln(k_{off})]}{d[\mathrm{osmolal}]} = -\frac{\Delta N_w}{55.6} \tag{3}$$

Water activity and solute osmolal concentration are linked through: $d[\mathrm{osmolal}] = -55.6\ d[\ln(a_w)]$.

RESULTS

Dissociation rates of a complex between the restriction nuclease EcoRI and a 322 bp fragment containing its recognition sequence were measured using a standard technique.[29,30] An excess of oligonucleotide containing a specific recognition site was added to preformed 322 bp complexes and the mixtures allowed to incubate on ice for varying times. Under these conditions, dissociation of EcoRI from the 322 bp fragment is essentially irreversible process. The loss of the EcoRI–322 bp fragment complex with time was measured using the gel mobility shift assay.[30,31] Typical experimental results are illustrated in Figure 1 for 2 and 3 molal betaine added to the standard reaction mixture.

Figure 2 shows kinetic curves for the dissociation of EcoRI for several betaine concentrations as prescribed by Eq. (2) (similar curves were obtained for sucrose, data not shown). In all cases, the kinetics can be well described by a simple, single exponential process. Dissociation rate constants k_{off} determined from the slopes vary from (0.091 $\pm$ 0.001) min^{-1} at 1.6 molal betaine to (0.0044 $\pm$ 0.0004) min^{-1} at 3 molal. At constant osmolyte concentration the dissociation rate of EcoRI from the 322 bp was independent of oligonucleotide concentration for 20-, 200-, and 2000-fold excess of added recognition sequences (data not shown).

The effect of osmolytes on the equilibrium binding of EcoRI[16,17,22] and of other DNA binding proteins[15,18–21,23] has been instructively analyzed through their effect on water activity. In analogy to plots of binding free energy vs osmotic stress, Figure 3 shows the dependence of $\ln(k_{off})$ on osmolal concentration for both betaine and sucrose. The apparent activation free energy for dissociation varies linearly with osmolal concentration for both solutes over a 20-fold change in rate constant. The dissociation rate in the absence of osmolytes can be estimated from extrapolation as $\sim$ 1.15 min^{-1} or $1.9 \cdot 10^{-2}\ \mathrm{s}^{-1}$. The different experimental conditions (length of the DNA fragment, temperature and buffer composition) make comparison with values obtained by others[6] problematic.

Both the linearity of plots shown on Figure 3 and their insensitivity to solute identity indicate that betaine and sucrose are indeed acting on the dissociation rate indirectly through their effect on water activity. The change in the number of solute-excluding water molecules associated with the EcoRI dissociation process can be determined from linear fits to the data in Figure 3 as given in Eq. (3). The slopes translate into an uptake of (65 $\pm$ 8) and (69 $\pm$ 6) water molecules during dissociation of the EcoRI from its specific site at 4°C for the sucrose and betaine, respectively. We have used the same experimental conditions used by us previously for the equilibrium experiments[17] in order to compare directly the numbers of waters measured by two different approaches. Under the same salt, pH, and temperature conditions, the competition equilibrium measurements gave $\Delta N_w = 71 \pm 8$ for the difference between specific and nonspecific binding of EcoRI.

Solutes could also affect the dissociation rate through their effect on solution viscosity. Even if the rate-limiting dissociation step is assumed to be entirely coupled to bulk solution viscosity with kinetics that vary inversely with viscosity, however, the observed slowing in dissociation rate with increasing solute concentration is much larger than can be accounted for by viscosity. In particular, the change in viscosity with betaine concentration would predict a change in dissociation rate that is, at maximum, only $\sim$ 10% of that observed in Figure 3. Since sucrose solutions are more viscous than betaine solutions (a 3 osmolal sucrose solution is $\sim$ 6 times more viscous than a 3 osmolal betaine solution), the possible contribution from viscosity to the decrease in dissociation rate would be much larger for sucrose than for betaine. Given, however, the very similar effect of sucrose and betaine on the dissociation kinetics and the very close correspondence of ΔN_w for dissociation and for the specific–nonspecific equilibrium reaction, we strongly suspect that the critical step in dissociation is insensitive to solution viscosity.

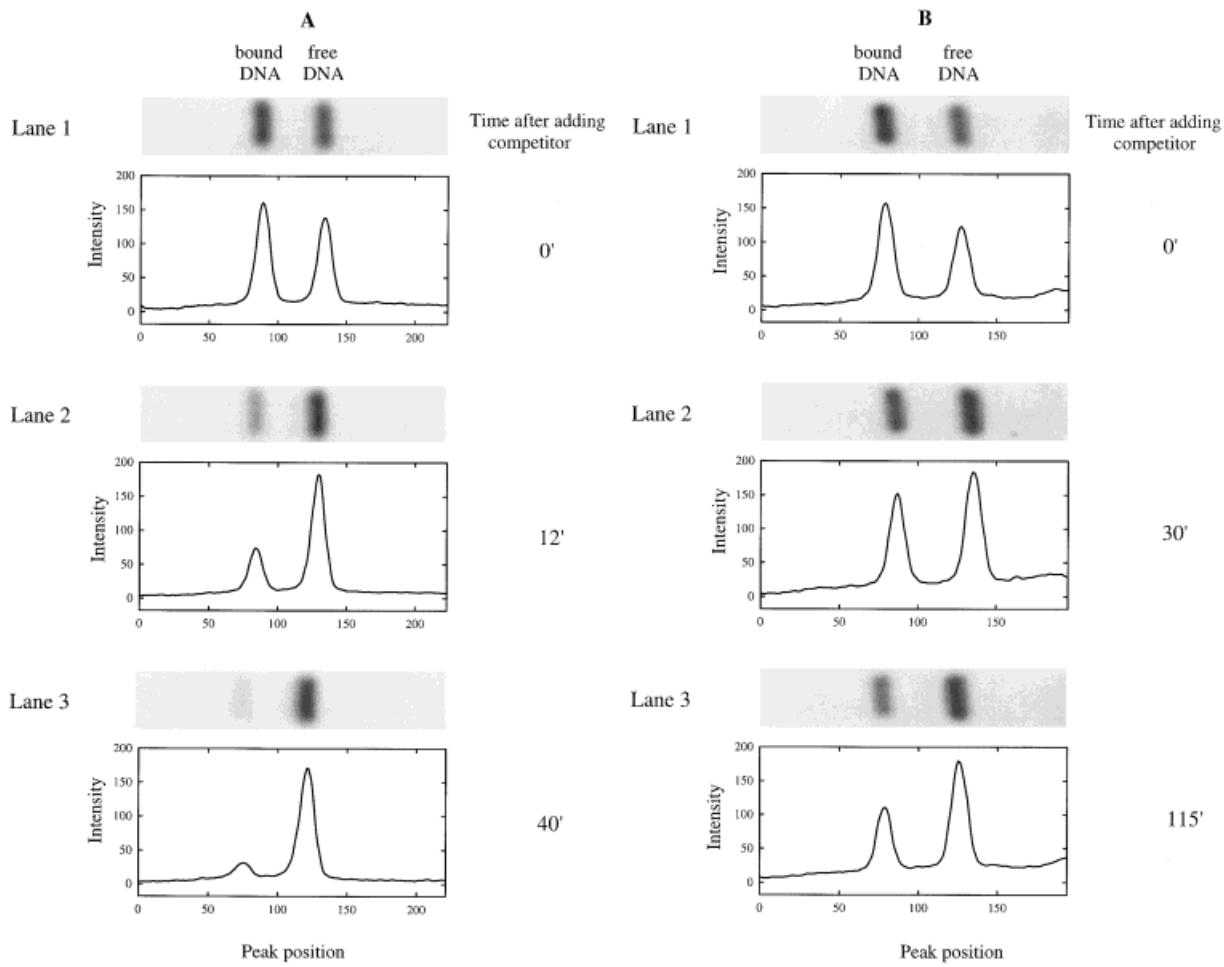

FIGURE 1 Dissociation rates of EcoRI from its specific site on a 322 bp fragment are significantly slowed by increasing concentrations of betaine. Gel lanes and their corresponding density profiles illustrating the kinetics of EcoRI dissociation are shown for (A) 2 molal betaine and (B) 3 molal betaine. A 20-fold excess of competitor oligonucleotide containing a specific EcoRI recognition sequence was added to the preformed complex between EcoRI and the 322 bp fragment. The reaction mixtures were incubated on ice for the indicated time intervals and gel electrophoresed in the cold room. Buffer conditions were 25 m*M* Tris Cl (pH 7.5 at 20°C), 90 m*M* NaCl.

DISCUSSION

The two neutral solutes used here, betaine and sucrose, have a dramatic effect in slowing the dissociation rate of the specific EcoRI–DNA complex that is much larger than can be rationalized by solution viscosity changes. The apparent activation free energy, $RT\ln(k_{\mathrm{off}})$, varies linearly and identically with changes in water chemical potential for the two solutes. Lastly, the number of waters coupled to dissociation (~ 70 at 4°C), calculated from the slope of the lines in Figure 3, is the same within experimental error as the number of waters coupled to the specific–nonspecific binding equilibrium measured under the same conditions of salt, temperature, and pH.[17] We take this close correspondence to indicate that the osmotic dependence of the dissociation rate reflects the transition from the specific binding mode of EcoRI either to the nonspecific binding mode or to a transition state with hydration properties that closely resemble the nonspecific binding mode.

That both sucrose and betaine give the same ΔN_{w} linked to the dissociation rate is also consistent with the very weak dependence on osmolyte nature of ΔN_{w} coupled with the equilibrium between specific and nonspecific EcoRI binding. Previous equilibrium competition measurements at room temperature[16] showed that a variety of osmolytes, betaine, glycine, sucrose, methylglucoside, triethylene glycol, and glycerol all affect the difference in specific and nonspecific binding free energies equally, within experimental error. Since a change in solute accessible surface area seems to result in ΔN_{w} values that are dependent on solute identity,[15] this insensitivity to solute nature indicates that the extra waters sequestered by the nonspecific complex

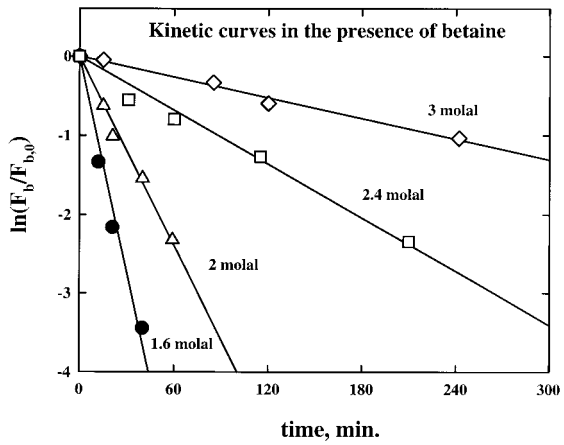

FIGURE 2 Kinetic curves calculated from experiments analogous to the one described in the legend to Figure 1. The fraction of DNA bound measured at different times F_b, was normalized by $F_{b,0}$ measured immediately after 20-fold excess of specific oligonucleotide was added to the preformed EcoRI–322 bp fragment complex. The concentrations of solute in the reaction mixture were (●) 1.6 molal betaine, (△) 2 molal betaine, (□) 2.4 molal betaine, and (◇) 3 molal betaine.

are in a volume sterically inaccessible to these osmolytes, probably at the DNA–protein interface.

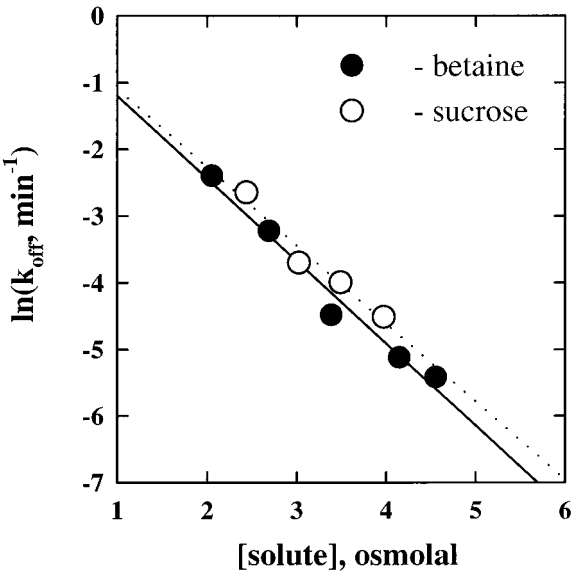

FIGURE 3 The dependence of EcoRI dissociation rate constant on solute osmolal concentration is shown for the two solutes: (●) betaine and (○) sucrose. Each point is the average of 2–3 independent experiments. The average errors for each $\ln(k_{off})$ value did not exceed 10%. The slopes of the lines translate into an uptake of about 69 waters for betaine (solid line) and about 65 waters for sucrose (dotted line) for the dissociation of EcoRI from its specific site on the 322 bp fragment.

The kinetic scheme shown in Eq. (1) is, of course, a simplification of the actual dissociation process. It has been long known that EcoRI locates and leaves its recognition sequence through a facilitated diffusion or sliding mechanism (Ref. 4, also reviewed in Refs. 11 and 32). In addition to a transition between specific and nonspecific binding modes at the specific site, a more precise kinetics scheme should also include a sliding rate and the kinetics for rebinding to the specific site and for dissociation of the nonspecifically bound protein from the DNA.[5] The dissociation of protein from the DNA is accompanied by a large change in solute-accessible surface area that might be expected to contribute to an additional, solute-dependent osmotic effect that is not observed. The nearly identical ΔN_w values for the relative nonspecific–specific equilibrium constant and for the dissociation rate implies that the dissociation rate of nonspecifically bound EcoRI to solution either is very fast relative to the kinetics of the specific to nonspecific transition or has a very small osmotic dependence. In the first case, neither the dissociation rate of a nonspecifically bound protein nor its sensitivity to water activity would contribute to the kinetics of loss of specific fragment complex. In the latter case, it would be the association rate that would be expected to show an osmotic sensitivity that is dependent on solute identity.

The effect of osmolytes on the dissociation rate of EcoRI from its specific site is quite dramatic and suggests a possibly effective way to stabilize other complexes. The dissociation half-life of the specific EcoRI complex with 322 bp DNA fragment is $\sim$ 8 min at 1.6 molal betaine and $\sim$ 160 min at 3 molal (Figure 2). At 4 molal betaine, the dissociation kinetics is simply too slow to measure accurately ($<$ 15% dissociation after 4 h, data not shown). This is in comparison with a dissociation half-life of the specific EcoRI complex with no added solute of $\sim$ 0.6 min (estimated from extrapolating the data in Figure 2). Any complex that has fewer solute excluding waters than its dissociated components will show, of course, qualitatively similar behavior. In these cases, application of osmotic stress will allow one to manipulate dissociation rates controllably and to measure complex properties on the time scale of the experiment. Although both salt activity and pH can be varied to control dissociation rates, both are only practical over a limited range of concentration. The effectiveness of the osmolytes examined here seems limited only by their solubilities. Even complexes showing a much weaker osmotic sensitivity than EcoRI, such as the CAP complex,[33] could still be stabilized by osmolytes, but of course, much higher osmotic stresses would be required.

Unlike EcoRI, the reaction of many proteins with their recognition sequences couples folding or structuring of the protein with DNA binding. The measured ΔN_w for the equilibrium binding of these proteins or their competitive binding to different sequences will necessarily include a contribution from changes in protein conformation as well as differences in water at the protein–DNA interface. Depending on the relative rates of breaking specific DNA–protein contacts and replacing them with hydration waters

and of the consequent unfolding of the protein, the osmotic dependence of the dissociation rate may not be sensitive to the folding reaction. Probing different aspects of the binding reaction through equilibrium and kinetic measurement of ΔN_w may allow one to separate protein conformational changes from protein–DNA hydration.

We are deeply grateful for V. A. Parsegian for the fruitful discussions and to L. Jen-Jacobson for the kind gift of highly purified EcoRI.

REFERENCES

1. Balsera, M.; Stepaniants, S.; Izrailev, S.; Oono, Y.; Schulten, K. Biophys J 1997, 73, 1281–1287.
2. Evans, E.; Ritchie, K. Biophys J 1997, 72, 1541–1555.
3. Izrailev, S.; Stepaniants, S.; Balsera, M.; Oono, Y.; Schulten, K. Biophysical J 1997, 72, 1568–1581.
4. Jack, W. E.; Terry, B. J.; Modrich, P. Proc Natl Acad Sci USA 1982, 79, 4010–4014.
5. Lohman, T. M. CRC Crit Rev Biochem 1985, 19, 191–245.
6. Jen-Jacobson, L.; Lesser, D.; Kurpiewski, M. Cell (Cambridge, Mass) 1986, 45, 619–629.
7. Lesser, D. R.; Kurpiewski, M. R.; Waters, T.; Connolly, B. A.; Jen-Jacobson, L. Proc Natl Acad Sci USA 1993, 90, 7548–7552.
8. Record, M. T., Jr.; Ha, J.-H.; Fisher, M. Methods Enzymol 1991, 208, 291–343.
9. Jen-Jacobson, L. Methods Enzymol 1995, 259, 305–344.
10. Hard, T.; Lundback, T. Biophys Chem 1996, 62, 121–139.
11. Jen-Jacobson, L. Biopolymers 1997, 44, 153–180.
12. Pabo, C. O. Annu Rev Biochem 1992, 61, 1053–1095.
13. Steitz, T. A. Quart Rev Biophys 1990, 23, 205–280.
14. Parsegian, V. A.; Rand, R. P.; Rau, D. C. Methods Enzymol 1995, 259, 43–94.
15. Garner, M. M.; Rau, D. C. EMBO J 1995, 14, 1257–1263.
16. Sidorova, N. Y.; Rau, D. C. Proc Natl Acad Sci USA 1996, 93, 12272–12277.
17. Sidorova, N. Y.; Rau, D. C. J Biomol Struct Dynam 1999, 17, 19–31.
18. Vossen, K. M.; Wolz, R.; Daugherty, M. A.; Fried, M. G. Biochemistry 1997, 36, 11640–11647.
19. Robinson, C. R.; Sligar, S. G. Protein Sci 1996, 5, 2119–2124.
20. Li, L.; Matthews, K. S. Biochemistry 1997, 36, 7003–7011.
21. Poon, J.; Bailey, M.; Winzor, B. E.; Davidson, B. E.; Sawyer, W. H. Biophys J 1997, 73, 3257–3264.
22. Robinson, C. R.; Sligar, S. G. Proc Natl Acad Sci USA 1998, 95, 2186–2191.
23. Lundback, T.; Hansson, H.; Knapp, S.; Ladenstein, R.; Hard, T. J Mol Biol 276, 775–786.
24. Lesser, D. R.; Kurpiewski, M. R.; Jen-Jacobsen, L. Science 1990, 250, 776–786.
25. Venditti, S.; Wells, R. D. J Biol Chem 1991, 266, 16786–16790.
26. Terry, B. J.; Jack, W. E.; Rubin, R. A.; Modrich, P. J Biol Chem 1983, 258, 9820–9825.
27. Halford, S. E.; Johnson, N. P. Biochem J 1983, 211, 405–415.
28. Sidorova, N. Y.; Gazoni, P.; Rau, D. C. J Biomol Struct Dynam 1995, 13, 367–385.
29. Riggs, A. D.; Bourgeois, S.; Cohn, M. J Mol Biol 1970, 53, 401–417.
30. Garner, M. M.; Revzin, A. Nucleic Acids Res 1981, 9, 3047–3060.
31. Fried, M. G.; Crothers, D. M. Nucleic Acids Res 1981, 9, 6505–6525.
32. Pingoud, A.; Jeltsch, A. Eur J Biochem 1997, 246, 1–22.
33. Fried, M.; Bromberg, J. L. Electrophoresis 1997, 18, 1–6.