Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 975–979, February 1988
Biochemistry

Gel retardation at low pH resolves *trp* repressor–DNA complexes for quantitative study

(gel pH effects/specific and nonspecific binding/apparent dissociation constant/stoichiometry/cooperativity)

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Communicated by Robert L. Baldwin, September 28, 1987

ABSTRACT **The affinity and stoichiometry of DNA binding by *Escherichia coli trp* repressor were studied by electrophoresis in nondenaturing gels. The ability of *trp* repressor to retard the electrophoretic mobility of an operator DNA fragment depends on the pH of the gel system. Above the pI of the protein, little retardation of DNA is observed, although complex formation can be detected by other assays. As the pH of the gel is lowered, retardation is enhanced. The apparent dissociation constant for the interaction between *trp* repressor and *trpEDCBA* operator fragments is 0.5 nM under the conditions used here. Nonspecific binding occurs with only about 200-fold weaker affinity. The stoichiometries of specific and nonspecific complexes were determined directly by using *trp* repressor labeled *in vivo*. High-affinity operator binding requires a single dimer of *trp* repressor. DNase I-protection analysis ("footprinting") was used to confirm the dissociation constants and to locate the binding site.**

Escherichia coli trp repressor (TrpR) regulates transcription initiation at three operons involved in tryptophan biosynthesis: *trpEDCBA* (1), *aroH* (2), and *trpR* (3). Operator sites have been identified near the transcription initiation points by homology of the three DNA sequences. Repressor is a dimer of identical 12.5-kDa subunits (4). The crystal structure shows that the protein is all α-helical (5) and each subunit contains a helix-turn-helix supersecondary structure (6), which mutational analysis (7) suggests is the site of DNA binding. The subunits are in intimate contact over a large area and are nearly interwound (5). Unlike many repressors, TrpR has an acidic isoelectric point (4), has fewer polar and positively charged amino acids in the DNA-binding domain (5), and requires the binding of two molecules of L-tryptophan per dimer for operator-specific DNA binding (8). Each tryptophan binding site comprises residues from both subunits (5). Binding of L-tryptophan induces a local conformational change that repositions the two DNA-binding domains of the dimer so that they exactly align with two successive major grooves of the DNA (9). DNA binding by TrpR has been studied primarily by an indirect assay (3, 4) in which bound protein prevents the restriction enzyme *Rsa* I from cleaving a site in the operator. To begin to understand the biochemical basis for DNA binding to TrpR, I have used a direct quantitative assay to determine the binding constants and stoichiometry of the interaction.

Electrophoresis in nondenaturing gels has been used for nearly two decades to study ribonucleoproteins (10). The current popularity of the technique dates from 1981, when Garner and Revzin (11) and Fried and Crothers (12) applied it to detect protein–DNA interactions. The method is based on the observation that the electrophoretic mobility of a polynucleotide is reduced when a protein is bound to it. Binding proteins can be detected in and purified from crude extracts by their retardation activity. The potential to separate complexes that differ only by their stoichiometries (12) is a major advantage of the gel method over other common assays, particularly nitrocellulose filter-binding. In spite of the wide use the gel method enjoys, the explanation for the retardation effect has not been studied experimentally. Systematic study of gel conditions in the course of optimizing the method for TrpR has led to a clearer understanding of certain aspects of the gel method, and these results are also presented here.

MATERIALS AND METHODS

Proteins and DNAs. Aporepressor (ApoR) was purified as described (13). Purity was >99% as estimated by silver staining (14) of overloaded 20% acrylamide/NaDodSO$_4$ gels (15). Protein was stored directly from the phosphocellulose column pool in aliquots at −80°C. DNA fragments were purified from agarose gels by electrophoresis onto NA45 paper (Schleicher & Schuell) and recovered by elution with 1 M NaCl. DNAs were labeled using the large fragment of *E. coli* DNA polymerase I.

Gel Electrophoresis. Assay gels contained 10% acrylamide, 0.27% *N,N'*-methylenebisacrylamide, 10 mM NaH_2PO_4 (pH 6.0), 0.1 mM L-tryptophan, 0.14% ammonium persulfate, and 0.028% *N,N,N',N'*-tetramethylethylenediamine and were "cured" for 2 hr. The gels were preelectrophoresed to constant current at 100 V with recirculation of the 10 mM NaH_2PO_4, pH 6.0/0.1 mM L-tryptophan buffer. Reaction mixtures were loaded onto gels running at 300 V by use of a siliconized capillary. As soon as the dyes had completely entered the gel, the voltage was reduced to 100 V until the xylene cyanol had migrated 1 inch. Gels were dried on Whatman no. 1 paper under vacuum at 80°C and then were exposed to preflashed Kodak X-AR film at −80°C with one DuPont Cronex Lightning Plus intensifying screen. Film was developed in a Konica processor. Quantitation was done with a Hoefer GS300 scanning densitometer with a Hewlett–Packard 3390A integrator or by excising bands for scintillation counting in toluene containing 2,5-diphenyloxazole. Fragments protected from digestion with DNase I ("footprints") were electrophoresed in 8% acrylamide/8 M urea gels (16).

RESULTS

Effect of Gel pH. In an attempt to develop a gel-based assay for TrpR binding to DNA, conditions similar to those used for other proteins (17) were chosen, except that L-tryptophan was added to the pH 8.3 Tris borate/EDTA gel and running buffer. Reaction mixtures were as for the *Rsa* I

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Abbreviations: TrpR, *trp* repressor protein; ApoR, aporepressor (TrpR without L-tryptophan bound); K_d(app), apparent dissociation constant.

assay (4) but without enzyme. Retardation of a 90-base-pair (bp) fragment from the *trpEDCBA* operator region of *Serratia marcescens* was observed only at protein concentrations in the micromolar range. However, protection of the operator site from *Rsa* I digestion, assayed in parallel, showed that binding occurred in the nanomolar range. Protein was detected by silver staining (14) in only one band, slightly above the position of the DNA in the assay gels. This result suggested that these gels failed to resolve complexes from free DNA because the electrophoretic mobility of TrpR is very similar to that of the DNA; thus the protein provides little retardation effect.

To reduce the mobility of the protein, the pH of the gel buffer was reduced to 6.0, very near the isoelectric point of TrpR (4). Under these conditions the separation between free and bound DNA was markedly improved and the TrpR concentration at which binding could be detected was reduced to the nanomolar range. The effect of gel buffer pH on the mobility of complexes and free TrpR relative to free DNA is shown in Fig. 1. As the pH is lowered from 8.3 to near 4.0, the mobility of both free TrpR and complexes declines steadily and roughly in parallel. At pH 8.3, where complexes (□) are not resolved from free DNA, free TrpR (■) migrates 70% as far as free DNA. At pH 4, complexes migrate only about half as far as free DNA, and free TrpR does not enter the gel. Therefore the charge on the protein is an important factor that exerts a similar effect on the mobilities of free protein and complexes.

Operator Binding Affinity. To estimate the apparent dissociation constant [K_d(app)] for TrpR binding to operator DNA, standard conditions were adopted for the gel assay (Fig. 2 legend). These conditions represent a compromise between the need to resolve the complexes and the desire to deviate minimally from physiological conditions. The gel bands were excised and ^{32}P was determined by scintillation counting, or the autoradiogram (Fig. 2 *A* and *B*) was scanned by densitometry, with comparable results. Because the concentration of DNA is <10 pM in these reaction mixtures, $[TrpR]_{total} \approx [TrpR]_{free}$, so the protein concentration required for half-maximal binding is very close to K_d(app). The half-maximal binding point was determined by measuring the decrease in free DNA rather than the increase in complexes (Fig. 2*C*). For the 90-bp *S. marcescens trpEDCBA* operator fragment (Fig. 2*A*), the K_d(app) at pH 6.0 and 25°C is 0.5 nM. This value is one-fourth the upper limit estimated previously by the restriction site protection assay (4) or a filter-binding assay (19). At 100 and 250 nM TrpR, severely retarded complexes are formed. At comparable TrpR concentrations in the *Rsa* I assay, plasmid pRK9 cannot be linearized even though several *Rsa* I sites are present at locations far removed from the one in the operator region (data not shown), indicating extensive nonspecific DNA binding.

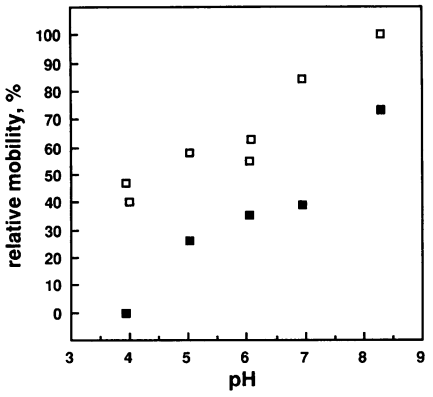


FIG. 1. Electrophoretic mobility of complexes (□) and free TrpR (■) as a function of gel pH. Gel conditions were as described in *Materials and Methods*, using the 90-bp DNA described in the legend to Fig. 2*A*. Mobility is expressed relative to that of free DNA; i.e., % relative mobility = (distance migrated by complexes or free TrpR/distance migrated by free DNA) × 100.

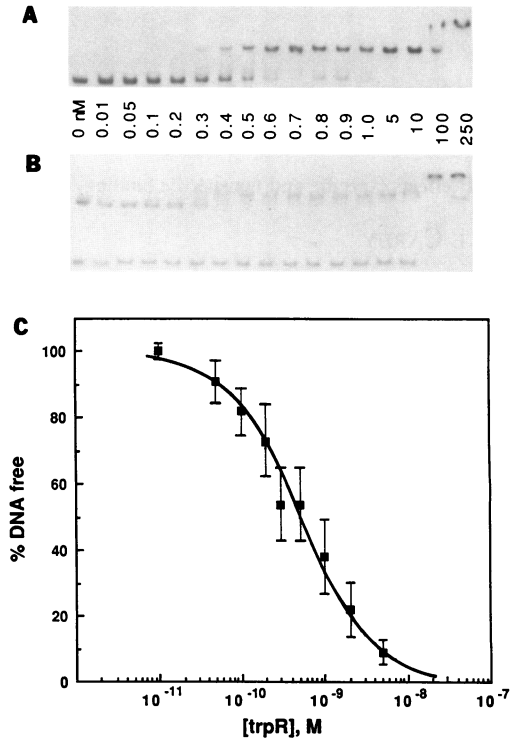


FIG. 2. Gel assay of TrpR–DNA binding. Protein solution was thawed and diluted freshly on ice into 12.5 mM NaH_2PO_4, pH 6.0/125 mM NaCl. As each dilution was made, 0.25 volume of diluted protein solution was added immediately to the DNA mixture at room temperature. Final concentrations after addition of the protein were 12.5 mM NaH_2PO_4 (pH 6.0), 25 mM NaCl, 0.4 mM L-tryptophan, <10 pM labeled DNA, 16% (vol/vol) glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue. Reaction mixtures were equilibrated for 15 min. These standard binding conditions gave reproducibly lower K_d(app) values than when the diluted protein was prepared from refrozen aliquots or exposed to variable times or temperatures before addition of DNA. Electrophoresis conditions are described in *Materials and Methods*. (*A*) Ninety-base-pair *S. marcescens trpEDCBA* operator DNA fragment derived from plasmid pRK9 (18) by *Eco*RI and *Bam*HI cleavage. (*B*) *E. coli trpEDCBA* operator region DNAs derived from the labeled *Sau*3A 490-bp fragment of plasmid pBN60 (18) by cleavage with *Hin*fI. The first lane in *B* contains more labeled DNA than subsequent lanes. TrpR concentrations given apply to both *A* and *B*. (*C*) Quantitation of gel assay. Squares, average (±SD) of three gels each of the two DNAs described in *A* and *B*; solid line, theoretical curve calculated for the relationship $K_d = 0.5$ nM $= [TrpR_{free}] \cdot [DNA_{free}]/[complexes]$.

Fig. 2*B* shows an identical assay using *E. coli trpEDCBA* operon DNA. A 490-bp fragment from the operator region was labeled at both ends and then cut to produce an operator-containing 415-bp piece and a 75-bp non-operator piece. The smaller fragment provides an internal standard for quantitation and allows independent observation of binding at nonoperator sites. As the input concentration of TrpR is increased, the larger fragment forms complexes with K_d(app) = 0.5 nM, while the smaller fragment remains unbound until 100 nM TrpR is added. At this protein concentration both fragments form complexes with severely retarded gel mobilities. If L-tryptophan is omitted from the binding reaction mixtures or gels (data not shown), the high-affinity complex on the larger fragment is not observed, and both fragments bind simultaneously near 100 nM TrpR to form severely retarded complexes.

DNase Footprinting. To locate the sites on the DNA where TrpR is bound, and to confirm the K_d(app) independently, DNase I footprinting of the *E. coli trpEDCBA* operator region was carried out at a range of TrpR concentrations. At low concentrations of TrpR, protection occurs only in the operator region extending from bp −25 to +4 near the start of transcription (Fig. 3 *Left*), in agreement with the site size determined by protection from dimethyl sulfate (21). At TrpR concentrations above 50–100 nM, binding occurs over much of the fragment (Fig. 3 *Right*). The protein concentration at which half-maximal protection of the operator site occurred was estimated visually from several independent DNase-protection experiments in which the TrpR concentration was varied from 0.1 to 1.0 nM in 0.1-nM increments; a value near 0.5 nM was obtained as was found in the gel assay. Because the DNase experiments were performed under conditions that did not perturb the equilibrium for the TrpR–DNA interaction (ref. 16; data not shown), the agreement between the two methods means that the gel assay gives a true measure of the binding constant for TrpR–DNA interaction. For other interactions, this conclusion must be verified independently.

Stoichiometry of Complexes. The stoichiometries of the complexes observed in the gels were determined directly in double-label experiments using the gel assay. Labeled TrpR was purified from cells grown on [^{3}H]leucine. The 90-bp *S. marcescens trp* operator DNA was titrated with ^{3}H-labeled TrpR; to increase the ^{3}H signal in the complex bands, the DNA concentration was raised, causing a shift in the midpoint for complex formation (Fig. 4*A*). The molar ratios of protein dimers to DNA are shown in Fig. 4*B*. The high-affinity complexes present between 5 and 160 nM TrpR have an average stoichiometry of 1.0 ± 0.10 TrpR dimer per DNA molecule. The TrpR/DNA stoichiometries of the more retarded complexes range from 2.8 at 160 nM to 17 at 2 μM. The effect on mobility of adding one or more additional dimers to the 1:1 complex is much less than the effect of adding the first dimer to naked DNA. Consequently, higher-order complexes are not resolved from one another, and the measured stoichiometry reflects the average of the population in the band. This 90-bp DNA can accommodate along its length up to four TrpR dimers occupying 28 bp. Because intermediate TrpR/DNA stoichiometries of approximately 1, 2, and 3 are observed, coating of the nonspecific sites cannot be highly cooperative.

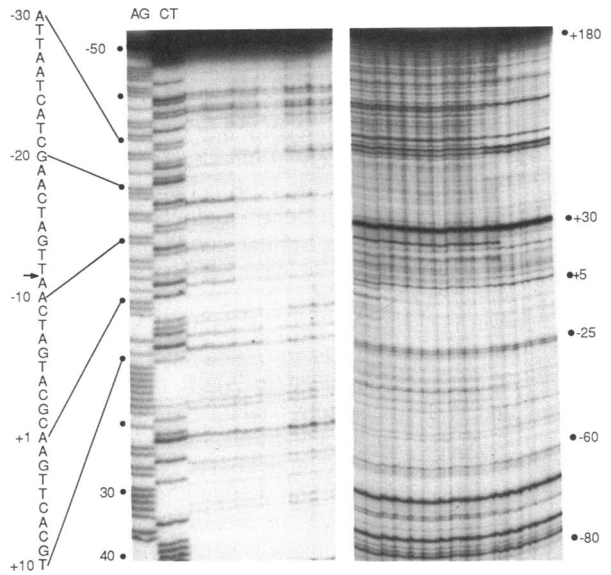


FIG. 3. DNase I footprinting of *E. coli trpEDCBA* operator DNA. The general procedures (except for quantitation) suggested by Brenowitz *et al.* (16) were followed. Reaction mixtures were as in Fig. 2 but also contained 5 mM sodium acetate (pH 4.5), 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.0002% DNase I (Worthington) for 15 sec. (*Left*) Limits of the specific operator site. Plasmid pBN60 (18) was labeled on the noncoding strand at the single *Bam*HI site and then cut with *Ssp* I, which generates a 308-bp fragment with the operator centered 267 bp from the labeled end. The first two lanes show chemical modification with formic acid for purines and hydrazine for pyrimidines (20), and the sequence of the fragment is given at the left; numbers indicate position relative to +1, the start of transcription. TrpR concentrations, left to right: 0, 0.1, 1.0, 10, 100, and 500 nM. (*Right*) Overview of specific and nonspecific binding to a long DNA fragment. The 415-bp *Sau*3A–*Hin*fI fragment described for Fig. 2*B* was used; the labeled end is on the coding strand about 200 bp from the operator region. TrpR concentrations, left to right: 0, 0.05, 0.2, 0.4, 0.6, 0.8, 1.0, 5, 10, 50, 100, 250, 700, 1400, and 2800 nM. Position marker +30 is approximate.

The double-label experiments were also performed using the 415- and 75-bp *E. coli* fragments. The high-affinity operator complex has an average stoichiometry of 1.0 ± 0.18 TrpR dimer per DNA molecule (Fig. 4 *C* and *D*). Nonspecific complexes with higher ratios of protein to DNA are observed on both fragments at protein concentrations ≥100 nM. At 250 nM TrpR, the large fragment has bound enough dimers to coat the length of the DNA, while the small fragment has twice as many as needed to coat it. The coexistence of these two stoichiometries suggests that the effect of nucleation at the operator site is small.

DISCUSSION

The results indicate that protein charge is an important factor influencing the retardation effect observed in gels used to study protein–nucleic acid interactions. Although TrpR is larger than several other proteins that give gel retardation effects at pH 8.3 (17), TrpR does not retard DNAs under these conditions, presumably due to its slightly acidic pI. When the pH of the gel system is reduced to the pI of TrpR, resolution of complexes from free DNA is enhanced. Thus the retardation effect can be experimentally controlled through the pH of the gel system. This is an important consideration in studies with previously uncharacterized proteins. The present results reveal that some binding proteins may go undetected under the usual gel conditions. On the other hand, *lac* repressor retards DNA fragments at pH 7.4 (12) even though it has an acidic pI; however, *lac* repressor is a tetramer of 150 kDa. A further indication that pH alone does not explain the retardation effect completely is the observation that, at high acrylamide concentrations, specific operator binding by TrpR does not require L-tryptophan in the gels (data not shown) if the amino acid is present in the binding-reaction mixtures. There has been a previous report of gel retardation by TrpR at pH 7.4 (22).

Quantitation of the gel assay results was accomplished by measuring the decrease in free DNA concentration as a function of input TrpR concentration. Because complexes can and do dissociate during running of the gels, the disappearance of the free DNA band is a more reliable indicator of complex formation than is the appearance of complex bands (23). In principle, it is not strictly necessary to observe complex bands to detect and quantitate complex formation, because once all the free DNA has entered the gel, there can be no further change in the amount of material in the free DNA band. In practice, accurate measurements require that the free DNA band be resolved from the smear above it that results when complexes dissociate during running of the gel. Thus the characteristic dead-time of the gel method is the time for free DNA to leave the sample well completely. This time is experimentally controllable by the loading voltage, within the limits of heating of the complexes in the gel. During the time required for free DNA to enter the gel, perturbation of the preexisting solution equilibrium is ex-

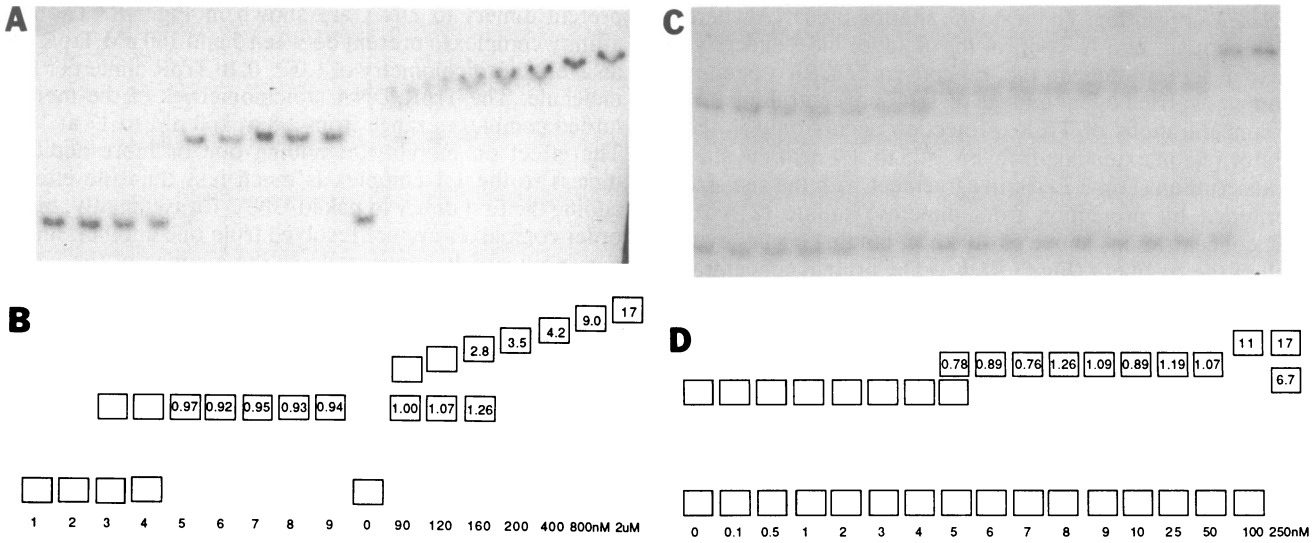


FIG. 4. Stoichiometry of complexes. Reaction mixtures and gels were as for Fig. 2, except ^{3}H-labeled TrpR was used and DNAs were at 6 nM. Labeled protein was obtained from 100 ml of cell culture grown with the addition of 5 mCi (185 MBq) of L-[4,5-^{3}H]leucine and without acid casein hydrolyzate, and the purification procedures were scaled down by a factor of 10. The autoradiograms of the ^{32}P-labeled DNAs (shown) were used as templates to excise the bands for scintillation counting. Gel slices were oxidized overnight at 60°C in 21% H_2O_2/17% $HClO_4$ in tightly closed vials to dissolve polyacrylamide. Vials were cooled, 14 volumes of Aquasolve (New England Nuclear) was added, and the vials were stored in the dark for 2 days at 10°C. Liquid scintillation counting was done in a Beckman LS230 counter using windows preset for ^{3}H and for ^{14}C + ^{32}P above ^{3}H, with a gain setting of 700. Background was determined by counting of control gel slices from unused regions of the gels. Spillover between channels was determined using control gel slices to which were added only ^{32}P or ^{3}H samples; 10% of the total net ^{32}P counts appeared in the ^{3}H channel, and no ^{3}H counts appeared in the ^{14}C + ^{32}P channel. Each set of samples was recounted after a 10- to 14-day decay period to verify that the background and spillover corrections were appropriate. Specific radioactivity was determined by measuring the concentration of DNA or TrpR spectrophotometrically, and by scintillation counting of a measured amount of the pure samples added to control gel slices. (*A*) Ninety-base-pair *S. marcescens* DNA. (*B*) Tracing of *A*. Boxes correspond to bands in *A*; numbers inside are molar ratios of TrpR dimers to DNA for complexes that had enough radioactivity to allow reliable determinations. Input TrpR concentration is shown below each lane. (*C*) Four-hundred-fifteen- and 75-bp *E. coli* DNAs. (*D*) Tracing of *C*.

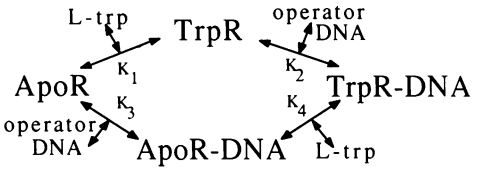
pected to occur. Thus, if meaningful values of K_d(app) are to be obtained, it is essential to verify independently that the K_d(app) determined by the gel assay is in agreement with that derived from a method that does not perturb the equilibrium.

In view of the potential for the gel method to perturb the equilibrium of complex formation, it is surprising that for TrpR the K_d(app) is in close agreement with that determined by several other methods. This result may be explained if the rate constants are such that perturbations in the gel dead-time are minimal or are mutually offset. An attempt to determine the association and dissociation rates showed that both were too fast to measure under conditions of the gel assay, leading to limits of $>2 \times 10^8$ $M^{-1} \cdot sec^{-1}$ for the association rate constant and >0.05 sec^{-1} for the dissociation rate constant. The slight difference between the K_d(app) from the gel assay and the published value from the *Rsa* I assay (4) is not due to the lower pH in the gel. When the two assays are performed in parallel using the same carefully prepared dilutions of TrpR (see Fig. 2 legend), K_d(app) = 0.5 nM by both methods. TrpR is a very thermostable and protease-resistant protein (unpublished observations), yet its DNA binding activity is apparently rather sensitive to seemingly innocuous procedures. Thus it is possible that the 0.5 nM K_d determined here still underestimates the actual affinity. However, TrpR preparations from four different laboratories have been used in the gel assay, and all gave this same value. Further, the stoichiometric titrations show that 100% of the TrpR molecules are active in DNA binding.

TrpR forms a specific high-affinity complex at its operator site in the presence of L-tryptophan and forms nonspecific complexes in the absence of L-tryptophan or at random DNA sequences. K_d(nonspecific) could not be determined exactly from the gel studies because individual complexes with discrete stoichiometries were not resolved, but nonspecific binding is first detected at TrpR concentrations of 50–100 nM whether or not L-tryptophan or an operator site is present. Thus the specificity ratio [K_a(specific)/K_a(nonspecific), where $K_a = 1/K_d$] for TrpR is remarkably low, on the order of 200 or less. In contrast, *lac* repressor has a specificity ratio of 10^6 under similar conditions (24). There may be additional factors that increase the specificity ratio for TrpR *in vivo*.

The 200-fold increase in operator affinity in the presence of L-tryptophan corresponds to 3 kcal/mol at 25°C. The conformational change induced in ApoR upon binding of L-tryptophan has been characterized by comparing the x-ray crystal structures of liganded and unliganded forms of TrpR (9) and consists of a local movement of the two DNA-binding domains away from each other so that they exactly align with successive major grooves of the DNA. The energetic cost of this conformational change is paid from the energy of L-tryptophan binding. Thus it is likely that the increase in operator binding affinity observed with liganded TrpR results because this form of the protein expends little or no energy for conformational change in the DNA-binding step. It may become possible to determine the energetic cost of the L-tryptophan-induced conformational change, depending on the structures of liganded and unliganded forms of the protein when bound to DNA. If x-ray analysis of DNA cocrystals shows that the conformation responsible for DNA binding is identical for the two forms of TrpR, then the reason their DNA-binding energies differ by 3 kcal/mol is that this is the energetic cost of the required conformational transition, which in the case of ApoR is paid entirely from the energy of DNA binding. If the structures of ApoR and liganded TrpR differ when bound to DNA, then the energetic cost of the L-tryptophan-induced conformational change may not be related in a simple way to the difference in their DNA-binding energies.

The affinity of the ApoR–DNA complex for L-tryptophan is predicted to be 200-fold greater than that of ApoR alone. This prediction can be made because the equilibria for L-tryptophan and operator binding to TrpR are linked.



The linkage relationship among these equilibria means that $K_2/K_3 = K_4/K_1$. From the gel data, $K_2 = 0.5$ nM and $K_3 \approx 100$ nM, and K_1 from equilibrium dialysis is 16 μM (4); thus K_4 is predicted to be $\approx$80 nM. Preliminary measurement of K_4 supports this prediction (W.-Y. Chou and K. S. Matthews, personal communication).

The stoichiometry measurements show that the high-affinity operator complex consists of one TrpR dimer per operator. Multiple higher-order complexes are formed at only slightly higher TrpR concentrations until the DNA is coated with TrpR. The coating reaction is not highly cooperative, and the effect of nucleation at the operator site is too small to be detected. Some complexes are detected that contain more TrpR dimers than the number that fills all the binding sites along the length of the DNA. This finding suggests that protein–protein interactions may occur without direct DNA binding. The variety of complexes observed indicates that many kinds of intermolecular interactions occur within a narrow range of affinities.

Nonspecific DNA binding by TrpR is probably essential to its regulatory behavior. When intracellular L-tryptophan levels are reduced, the affinity of TrpR for its operator targets is reduced by a factor of at least 200. If ApoR was not "sopped up" by formation of nonspecific complexes, the concentration of free ApoR in the cell might be high enough to allow the protein to partially occupy its operator sites even with its lower affinity. Although the concentration of TrpR in the cell has been determined under conditions of repression and induction (25), it does not seem reasonable to try to rationalize those values with the affinities determined here because there is no way to correlate the two sets of conditions.

Both specific and nonspecific TrpR–DNA complexes are remarkably salt-resistant (ref. 19 and data not shown). However, the gel method is not well-suited to measuring the effects of variables such as increased salt concentration or temperature because under these conditions even the free DNA band can become very diffuse. It was possible to compare 0 and 10 mM $MgCl_2$, and both give the same K_d(app) and specificity ratio. This result is consistent with the observations that specific binding of TrpR to DNA is detected by filter-binding even in the presence of rather high salt and that the optimal salt concentration is dependent on DNA length (19). These results suggest that counterion release from the DNA does not dominate the energetics of complex formation as it does for many other protein–DNA interactions (24). One explanation for the small energy contribution of counterion release is that few ion pairs are formed between TrpR and DNA in either the specific or nonspecific binding mode. This interpretation is consistent with the x-ray crystal structure of TrpR, which shows few positively charged amino acids on the surface thought to contact the DNA (5). On the other hand, resistance to moderate salt concentrations is also consistent with an interaction in which ions are displaced from the DNA and taken up by the protein simultaneously. The sensitivity of TrpR activity to salt concentration during storage and handling (ref. 26 and unpublished observations) suggests the protein may bind ions.

The three TrpR regulons are differentially regulated by TrpR: *trpEDCBA* expression decreases by a factor of $\approx$70 in response to TrpR binding when L-tryptophan levels are high, whereas *aroH* and *trpR* expression decreases by a factor of only about 3–5 (27). One explanation for tighter regulation of *trpEDCBA* is that more high-affinity binding sites are present in its operator than in the other two operators, as suggested by inspection of the DNA sequences of the three operator regions (3). The stoichiometry measurements and DNase footprint experiments show that this is not the case. Preliminary determination of the binding constants for the three operators by the gel assay indicates that repressor affinity does not explain the induction levels.

It is a pleasure to acknowledge the encouragement and generosity of Charley Yanofsky and Buzz Baldwin. I thank Mitch Gittelman, Lisa Klig, and Thomas Wagner for gifts of protein and Tania Baker, Rob Fairman, Ben Glick, Susan Marqusee, and Kevin Shoemaker for discussion. This work was supported by National Institutes of Health Research Grant GM19988 to R. L. Baldwin. J.C. is supported by a research award from the Arthritis Foundation.

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