# Fluorescence Anisotropy Assays Implicate Protein–Protein Interactions in Regulating *trp* Repressor DNA Binding†

Veronique LeTilly and Catherine A. Royer*

*School of Pharmacy, University of Wisconsin—Madison, Madison, Wisconsin 53706*

*Received October 29, 1992; Revised Manuscript Received March 29, 1993*

ABSTRACT: The study of interactions between proteins and nucleic acids is central to the understanding of the control of genetic expression. Fluorescence anisotropy has been used to measure, in solution, the equilibrium binding profiles of a bacterial repressor protein, the tryptophan repressor (TR), to a fluorescently labeled oligonucleotide containing one of its target operator sequences. Investigation of the effects of changing concentrations of corepressor, operator DNA, and protein implicate TR oligomers in the regulation of DNA binding. These studies also demonstrate that the relatively straightforward technique of fluorescence anisotropy can be applied to the study of the interactions between proteins and nucleic acids. The fluorescence technique exhibits sufficient sensitivity to replace radioactive methods of detection in most cases. In addition, since it is a solution-based methodology, it offers a true equilibrium measure of the protein–nucleic acid equilibria, and the effects of changes in solution conditions such as salt and ligand concentration, pH, and temperature can be readily evaluated. Data acquisition is relatively simple and rapid, and the data are of sufficient quality for detailed thermodynamic analyses of complex systems. Given these attributes, fluorescence anisotropy will find multiple applications in the area of genetic regulation.

We report a novel quantitative methodology for monitoring interactions between proteins and nucleic acids. The technique is based upon the observation of changes in the anisotropy of fluorescence of a fluorescein (or other fluorescent) moiety covalently bound to the 5′-end of an oligonucleotide, which in the present study contains the target operator site for the bacterial repressor protein, *trp* repressor (TR).[1] Binding of the protein to the oligonucleotide causes an increase in the rotational correlation time and, thus, the anisotropy of fluorescence of the fluorescein moiety due to an increase in the size of the oligonucleotide–protein complex as compared to that of the free oligonucleotide. The anisotropy of fluorescence ($A$) is defined as the difference between the vertical and horizontal emission components ($I_{\parallel}$ and $I_{\perp}$) with respect to the total intensity when vertically polarized excitation is used.

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

It is related to the correlation time ($\tau_c$) of the fluorophore through the Perrin equation (Perrin, 1926)

$$\frac{A_0}{A} - 1 = \frac{\tau}{\tau_c} \tag{2}$$

where $A_0$ is the limiting anistropy of the fluorophore, a known constant, and $\tau$ is the fluorescence lifetime. An increase in the measured anistropy as a function of the concentration of macromolecule, although reporting on an average between global particle tumbling and local fluorophore rotations, is nonetheless a measure of macromolecular binding.

† These studies were carried out with support from the National Institutes of Health, GM39969, and the University of Wisconsin Graduate School to C.A.R.

[1] Abbreviations: TR, *trp* repressor; F-25-mer,

```
5 FATCGAACTAGTTAACTAGTACGCAA3
  |||||||||||||||||||||||||
3  TAGCTTGATCAATTGATCATGCGTTS
```

Recently, several investigators have used fluorescence anisotropy as a means of monitoring protein binding to DNA (Heyduk & Lee, 1990; Guest et al., 1990; Royer et al., 1992). Due to sensitivity limitations, however, none of these previous experiments were performed at concentrations such that binding could be observed in the equilibrium, rather than the stoichiometric limit. Thus, reliable values for the dissociation constants for high-affinity specific DNA binding could not be extracted from those studies. We demonstrate here that with appropriate optical arrangement fluorescent anisotropy assays can be of sufficient sensitivity and precision not only to allow the detection of the low concentrations necessary to study high-affinity protein–DNA binding but also to yield information concerning both the size and number of protein–DNA complexes. Since it is a solution methodology, the effects of changing solution conditions such as ligand concentration and oligonucleotide concentration, as well as pH, temperature, and salt concentration, can be readily ascertained. This method provides a very rapid, reproducible, and relatively inexpensive technique for detecting, characterizing, and quantitating protein binding to nucleic acids. One broad area of application is the study of transcriptional regulation including the linked interactions of specific and general transcription factors with their target DNA sequences, ligands, and each other.

We have used here, as a model DNA binding protein, the *trp* repressor of *Escherichia coli* (TR). In the presence of its corepressor, tryptophan, this protein binds with high affinity to a number of sites in the *E. coli* genome, one of which is the operator sequence found within the promoter region of the *trpEDCBA* operon. When TR binds this site, it represses transcription of those genes whose protein products are responsible for the synthesis of tryptophan. The repressor protein has been extensively studied from a genetic as well as a structural standpoint (Kelley & Yanofsky, 1985; Klig et al., 1988; Hurlburt & Yanofsky, 1990; Bass et al., 1988; Schevitz et al., 1985; Zhang et al., 1987; Otwinowski et al., 1988), although the mechanism of action of the corepressor

0006-2960/93/0432-7753$04.00/0 

in inducing specific DNA binding is still not well understood (Carey et al., 1991; Fernando & Royer, 1992).

## MATERIALS AND METHODS

The target DNA used in the following studies was a 25 base-pair oligonucleotide containing the *trp* operator sequence and 5'-labeled with fluorescein (F-25-mer).

```
5 F A T C G A A C T A G T T A A C T A G T A C G C A A 3
  | | | | | | | | | | | | | | | | | | | | | | | |
3   T A G C T T G A T C A A T T G A T C A T G C G T T 5
```

It was obtained in HPLC purified form from Promega, Inc. (Madison, WI). Annealing of the sense and anti-sense strands was performed in 1 M NaCl, 10 mM phosphate, and 0.1 mM EDTA, pH 7.6. The solution was heated to 80 °C for 10 min and allowed to cool slowly on the bench. Because of the propensity to form single-stranded hairpin, approximately two-thirds of the oligonucleotide was found to be in the double-stranded form by comparison of ethidium stained gels with fluorescence 2-D scans of gel retardation assays carried out using a prototype fluorimager available at Promega. No binding to the single-stranded hairpin was observed even at extremely high protein concentrations. Double-stranded DNA concentrations were estimated from the optical density at 260 nm (corrected 50% for hypochromic effects) and the estimated fraction of double-stranded sites.

*Protein Purification.* An *E. coli* strain, CY15071, which overproduces the *trp* repressor (Paluh & Yanofsky, 1986), was obtained from Dr. Kathleen Matthews, Rice University. TR was purified according to the method of Paluh and Yanofsky (1986), Joachimiak et al. (1983), or He and Matthews (1989) with the following changes. Cells were suspended in 100 mL of 0.1 M Tris-HCl, pH 7.5, for each 60 g of cells. They were broken by three successive passages through a pressurized extruder. The extract was centrifuged for 20 min at 10 000 rpm on a Sorvall RC-5B centrifuge with a GS3 rotor at 4 °C. Streptomycin sulfate was added while stirring to the supernatant to a final concentration of 1%. The solution was stirred at 4 °C for 60 min and then heated to 62 °C for 5 min. A large amount of precipitate formed at this step. The mixture was cooled on ice to 15 °C and then centrifuged for 20 min at 10 000 rpm. The supernatant was precipitated with 45% ammonium sulfate, stirred in the cold room for 45 min, centrifuged for 20 min at 10 000 rpm, and reprecipitated with 75% ammonium sulfate. After stirring in the cold for 60 min, the solution was centrifuged for 20 min at 10 000 rpm. The pellet was then resuspended in 10 mM potassium phosphate, 0.1 M KCl, and 0.1 mM EDTA, pH 7.6. A 400-mL phosphocellulose column was equilibrated with 10 mM potassium phosphate, 0.2 M KCl, and 0.1 M EDTA, pH 7.6, until the elution buffer had the same pH and conductivity as the starting buffer. The dissolved pellet was loaded onto the column and a 250 mL against 250 mL, 0.2–0.5 M KCl gradient was run. The repressor eluted after the gradient, and a wash of 300 mL of 0.5 M KCl was applied. The purified repressor was >95% pure as estimated from silver staining of an SDS–polyacrylamide gel. Each fraction containing TR was divided into 200-$\mu$L aliquots which were stored at −70 °C in the elution buffer (10 mM potassium phosphate, pH 7.6, with 0.5 M KCl). Protein concentrations were determined using an extinction coefficient of $1.45 \times 10^4$ $cm^{-1}$ $M^{-1}$ per monomeric subunit (Joachimiak et al., 1983).

*Fluorescence Measurements.* Fluorescence anisotropy titration measurements were carried out using an ISS KOALA fluorometer (ISS, Inc., Champaign, IL). The fluorescein-labeled oligonucleotide was diluted to the desired concentration in a total volume of 700 $\mu$L in a reduced volume fused silica cuvette, 10 mm × 4 mm i.d. (Uvonic Instruments, Plainview, NJ). The assay buffer was 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.6, and 16% glycerol. The protein was diluted appropriately, in stepwise dilutions (to reduce instability; Carey, 1988), and 2-$\mu$L aliquots were successively added to the 700-$\mu$L total volume. In this manner, the total volume varied less than 10% in each assay, and thus the concentration of oligonucleotide can be assumed to have remained constant. Parallel and perpendicular emission components were measured in L-format by excitation at 488 nm from a 300-W xenon arc lamp (ISS) coupled to the optical chamber through an Oriel (Stratford, CT) rectangular to circular optical fiber coupled to the monochromator output slit. The excitation bandpass was 8 nm, limited by the 1-mm width of the rectangular fiber optic. To achieve the sensitivity limit of 200 pM in fluorescein-labeled oligonucleotide, the emission monochromator was removed from the light path and emission was monitored through a 530 cuton filter (Oriel). The high quantum yield of fluorescein is key in determining the sensitivity limits. Other high quantum yield probes, such as rhodamine and eosine, would also make good labels, although the prelabeled phosphoramidites are not available with these probes and thus the DNA must be labeled subsequent to synthesis. In each anisotropy measurement the parallel and perpendicular intensity of the background buffer solution was subtracted from the measured values of the sample and the anisotropy was calculated. Anisotropy was measured four times at each titration point with an integration time of 10 s for each intensity measurement, and the resulting anisotropy values were averaged. Following this procedure, each anisotropy point in the titration required approximately 5 min to acquire. Standard deviations were ±0.004 anisotropy unit or less.

## RESULTS

To verify the validity of our technique, fluorescence anisotropy DNA binding profiles for the specific equilibrium binding of the *trp* repressor to the F-25-mer under conditions comparable to previously reported gel retardation assays (Carey, 1988) were performed with an assay buffer containing 0.4 mM L-tryptophan. The anisotropy of the fluorescein emission was measured for each added aliquot resulting in a binding profile such as that shown in Figure 1. Since the dissociation constant is on the order of the F-25-mer concentration, 0.2 nM, it cannot be extracted from the visual inspection of the titration curve. However, nonlinear least-squares analysis of the data using a binding data analysis program will yield a reliable value for the dissociation constant under these concentration conditions. The data in Figure 1 were analyzed with a numerically based equilibrium binding program which allows for analysis of models which can include multiple protein subunit equilibria as well as the protein–DNA binding equilibrium (Royer et al., 1991). Two models were tested: a simple dimer–DNA equilibrium and a linked dimer–monomer and dimer–DNA system. The result of the fit in terms of a simple protein–DNA binding model is described by the dashed line through the data in Figure 1. The TR–DNA dissociation constant was found to be 0.73 nM. The full line in Figure 1 demonstrates that when the data are fit in terms of a monomer–dimer equilibrium, as well as a dimer–operator equilibrium, the quality of the fit improves significantly, particularly at low protein concentrations, with the $X^2$ decreasing from 2.1 to 1.3. With the linked binding

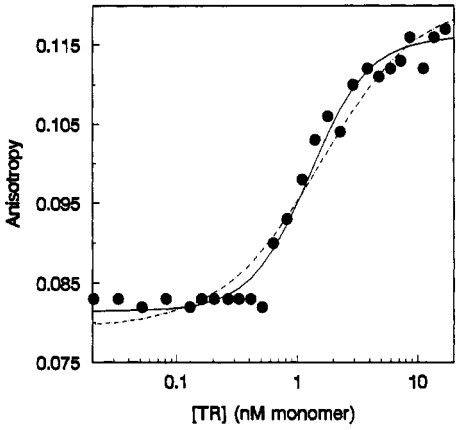


FIGURE 1: Titration of a fluorescein-labeled 25-mer containing the *trp* operator sequence at a concentration of 300 pM with *trp* repressor. Assay conditions were 16% glycerol, 0.4 mM L-tryptophan, 10 mM potassium phosphate, and 0.1 mM EDTA, pH 7.6 at 21 °C. The F-25-mer was a gift from Promega. (···) Results of the fit with a simple binding model; (—) results of the fit including a free monomer species. Note that for the full line the fit is particularly improved at low protein concentrations. No change in fluorescence intensity was observed for any of the profiles, indicating that the anisotropy values were not influenced by lifetime effects.

model, the dissociation constant between the TR dimer and DNA was found to be 0.1 nM, with a dimer dissociation constant of 4 nM. The monomer–dimer equilibrium, although important for *in vitro* assays of specific DNA binding, is of such high affinity that it should not be a factor at the TR concentrations observed *in vivo* (Gunsalus et al., 1986). Rigorous confidence limit testing (Royer et al., 1991) of the recovered free energy for the dimer–DNA interaction in the linked binding model yielded deep, reasonably symmetric $X^2$ profiles with 67% confidence limits for dissociation constants between 0.04 and 0.16 nM. The dimerization constant was not well-resolved since no data containing direct dimerization information were analyzed. Repetition of the titration yielded very similar results for the TR–DNA dissociation constant: 0.85 nM with the simple binding model and 0.25 nM when the dimer–monomer equilibrium was included. These constants are in good agreement with those reported by Carey, 0.5 nM, using gel retardation assays at pH 6.0 on a 90-mer (Carey, 1988) and Hurlburt and Yanofsky (1990), 0.2 nM, using filter binding assays on a 46-mer. In the analysis of all repetitions of this experiment, the simple binding model did not adequately describe the cooperative character of the binding profiles. These results demonstrate that the assay exhibits sufficient reproducibility and precision to allow for the differentiation between alternate thermodynamic models.

We have used the fluorescence anisotropy assay to evaluate the effects of changing the concentration of protein, DNA, and corepressor, since the conditions for measuring the specific affinity of TR for its target site are rather far removed from the concentration conditions relevant to its function. The intracellular TR concentration oscillates between near 0.85 $\mu$M in the absence of corepressor and 0.2 $\mu$M in its presence (Gunsalus et al., 1986). Tryptophan concentration depends upon the extracellular conditions as well as the levels of protein synthesis and degradation. Titrations of three concentrations of F-25-mer (0.3, 1.5, and 30 nM) in presence of 4.0 mM corepressor with TR at concentrations extending up to 3 $\mu$M in dimer are shown in Figure 2a.

For the anisotropy profile observed with 300 pM F-25-mer, there is a relatively sharp increase in the anisotropy from near 0.08 to near 0.110 between 0.1 and 1 nM in TR monomer, followed by a more gradual increase in anisotropy up to near

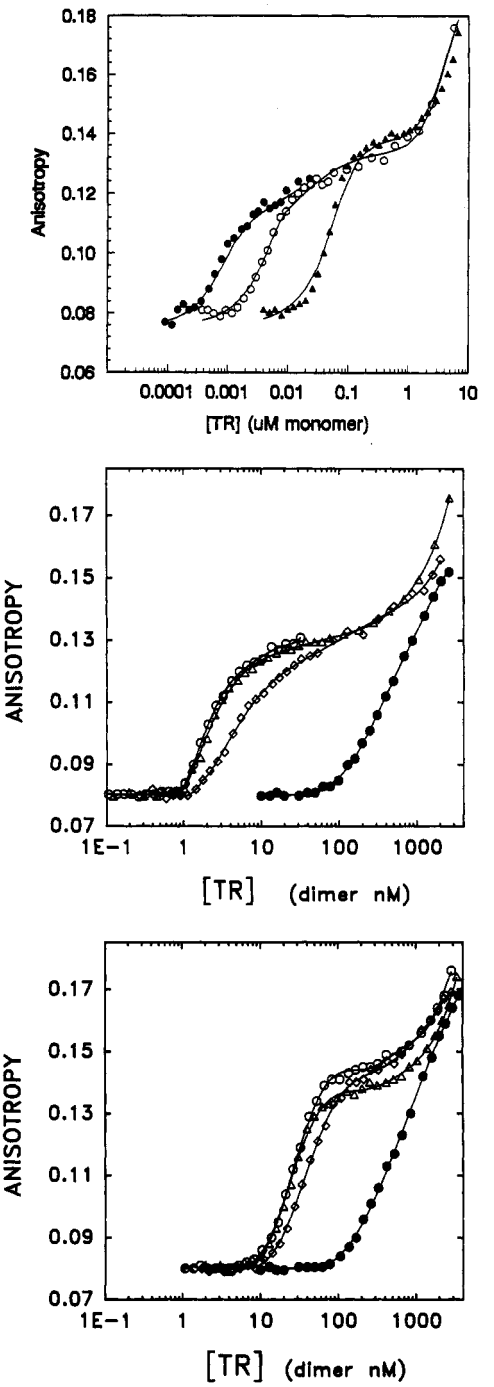


FIGURE 2: (a, top) Titration of (●) 300 pM, (○) 1.5 nM, and (▲) 30 nM fluorescein-labeled 25-mer containing the *trp* operator sequence with *trp* repressor. Lines through the data points are the results of a global analysis of the data in terms of the model described under Results. The anisotropy value for the dimer-bound F-25-mer was fixed at 0.115, that of the tetramer-bound F-25-mer was 0.134, and that of the octamer-bound F-25-mer was 0.195. The anisotropy of the free F-25-mer was not fixed in the fit, and the value recovered was 0.076. (b, middle) Titration of 1.5 nM F-25-mer and (c, bottom) 30 nM F-25-mer, with (●) 0, (◇) 0.04, (△) 0.4, and (○) 4.0 mM L-tryptophan. Conditions otherwise were the same as in Figure 1. Lines through the data are simply to help distinguish among the data sets.

0.133 at 10 nM. The shape of the profile obtained using 1.5 nM F-25-mer is similar to the previous one, but shifted to higher concentrations of TR because the concentration of F-25-mer is above the dissociation constant for dimer–F-25-mer interactions, and thus the binding begins to take on a stoichiometric, rather than equilibrium, character. However,

the sharp increase, followed by a more gradual change in anisotropy, is again apparent. At high protein concentrations, above 1 $\mu$M, another increase in anisotropy is observed. The titration at 30 nM in F-25-mer is clearly within the stoichiometric binding limit. A sharp transition to near 0.14 in anisotropy unit is observed followed by a transition, as in the previous case, above 1 $\mu$M in TR monomer.

These data were fit simultaneously to a model in which titration of the F-25-mer results sequentially in the binding of one, two, and four dimers of TR. The first phase of binding (up to 0.11 in anisotropy unit) was taken to correspond to binding of one dimer of TR. The gradual increase that follows up to near 0.135–0.14 was taken to correspond to the binding of the second dimer. The final increase in anisotropy was assumed to be nonspecific binding of higher order species, which we arbitrarily chose as octamer. Since there was no plateau reached for this final phase, the actual stoichiometry and affinity were not recovered with precision, anyway. We assumed that this last phase was nonspecific binding and were content to observe that it occurred in the micromolar range. There exists in the literature ample evidence for the model presented here. Kumamoto et al. (1987) demonstrated by footprinting techniques that multiple TR dimers could bind to the *trp*EDCBA operator site, from which the present F-25-mer is derived. Staake et al. (1990), Carey et al. (1991), and Haran et al. (1992) observed tetramers bound to a 19-mer of different symmetry than the classical target site yet also derived from the *trp*EDCBA sequence. Filter binding results of Hurlburt and Yanofsky (1992) were also indicative of the formation of higher order species. Liu (1992) and Liu and Matthews (1993) have observed both dimers and tetramers of TR bound to 40-mer target sequences also derived from the same operator sequence. These investigators have demonstrated that the relative population of the dimer and tetramer complexes with DNA depends upon the length of the target sequence as well as the sequence composition. As for the higher order, nonspecific TR–DNA complexes, we have observed multiple bands above the dimer in gel retardation experiments upon addition of micromolar TR (data not shown). These multiple higher order bands were first observed by Carey (1988), and the stoichiometries deduced from those double-radiolabeling experiments yielded TR dimer/DNA site ratios that would coat the entire sequence at least twice with protein, implicating protein–protein interactions in these complexes.

While the gel retardation, filter binding, and footprinting techniques clearly show the existence of multiple forms of TR–DNA complexes, they do not provide data of sufficient quality for a detailed analysis in terms of the free energies of the various species. The lines through the data points in Figure 2a correspond to the fit of the anisotropy data using the above model and the BIOEQS program (Royer et al., 1991; Royer, 1993).[2] This program fits the relative free energies of formation of each of the species in the model, relative to zero values for the free protein and DNA. The individual binding constants are deduced from these values from the principle of conservation of free energy. The free energy for binding of the first dimer was found to be −13.4 kcal/mol of complex, or a $K_d$ of 0.13 nM. This is in good agreement with the values found for the data in Figure 1, in which only the first portion of the titration curve was considered. The binding of the second dimer was significantly less favorable, with a free energy change of −10.7 kcal/mol of complex, $K_d$ = 12.5 nM. This

[2] The model also includes free monomer, dimer, and tetramer, although the free energies for the protein oligomerization equilibria were again not uniquely recovered.

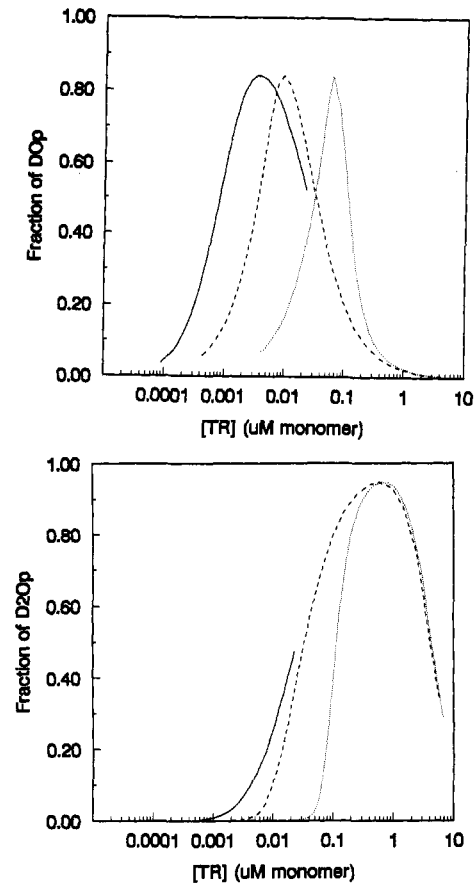


FIGURE 3: Simulations of fractional species populations using the free energies recovered from the fit of the data in Figure 2a. (a, top) Fractional population of the TR–DNA complex containing 1 mol of TR dimer/mol of F-25-mer target DNA (Dop). (b, bottom) Fractional population of TR–DNA complex containing 2 mol of dimer/mol of F-25-mer (D2op). Lines correspond to (—) 300 pM F-25-mer, (- - -) 1.5 nM F-25-mer, and (···) 30 nM F-25-mer.

could be interpreted as antagonistic binding. However, examination of the F-25-mer sequence reveals that if one DNA binding domain from each dimer shares the central major groove half-site on the DNA, as suggested by Kumamoto et al. (1987), then there is a second half-site with the correct spacing, although it is three (CAA) rather than four (CTAG) bases long. This imperfection in the half-site could account for the lower affinity of binding of the second dimer to the F-25-mer. Such a proposal is in agreement with the sequence length dependence of the affinity of the tetrameric complex as observed by Liu and Matthews (1993). Finally, the proposed octamer affinity is −7.7 kcal/mol of dimer (apparent $K_d$ near 2 $\mu$M), although again the assignment of octamer as the nonspecific complex stoichiometry was arbitrary, and this value is only indicative that nonspecific binding occurs in the micromolar range. A certain degree of uncertainty in the free energy values arises from the assignment of the anisotropy values of the dimer and tetrameric species; i.e., these are fixed parameters in the fit. Nonetheless, the shape of the curves, with the sharp increase followed by a more gradual one, indicates a lower affinity for the binding of the second dimer than for the first, even in the absence of detailed data analysis. It is interesting to explore the degree to which the dimeric and tetrameric DNA bound species are populated over the entire TR concentration range for these three titrations, given the recovered free energy values. The results of such a simulation are shown in Figure 3. In all three curves the dimeric species is populated to a level of 80% before yielding to the tetrameric form. The dimer peak becomes sharper as the F-25-mer

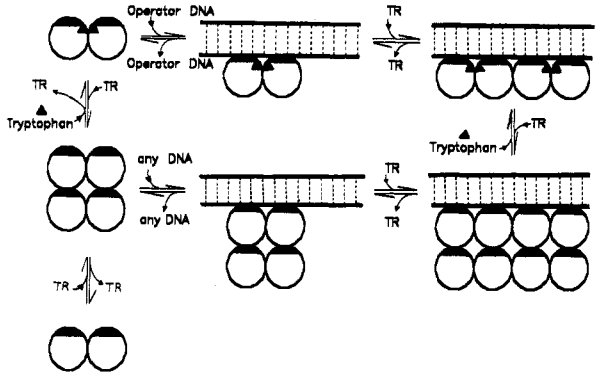


FIGURE 4: Model for the role of the different oligomeric species in controlling the binding of *trp* repressor to DNA. Circles represent the TR protein monomer, with the shaded regions corresponding to the HTH DNA binding domains and the black triangles representing the corepressor, L-tryptophan. See text for explanation.

concentration increases and one enters the stoichiometric rather than equilibrium limits of binding.

In parts b and c of Figure 2 are shown the effects of varying the L-tryptophan concentration on the profiles obtained with 1.5 and 30 nM in F-25-mer. In the absence of tryptophan, only the nonspecific binding observed above 100 nM in TR dimer is apparent. At high tryptophan concentrations, the curves are shifted to their low-concentration limit. At intermediate tryptophan concentration [40 $\mu$M or approximately the $K_d$ for tryptophan binding (Chou & Matthews, 1990)], the anisotropy curves appear to have both specific and nonspecific binding characteristics.

## DISCUSSION

The results of the present anisotropy titrations of fluorescein-labeled oligonucleotide containing the *trp* operator binding site are in good agreement with many previous studies which demonstrated that TR could interact with DNA in a variety of modes, exhibiting different protein/DNA stoichiometries. In addition to supporting these previous studies, the present work demonstrates for the first time that the tetrameric complexes with operator DNA are dependent upon the corepressor, L-tryptophan. Since corepressor binding confers specificity (which is simply the relative affinity compared to binding to nonspecific DNA), then these complexes, like those containing only one dimer, can be termed specific TR–DNA complexes.

It has been shown previously (Fernando & Royer, 1992; Martin et al., 1993) that tryptophan binding destabilizes tetramers and higher order oligomers of TR in the solution in the absence of DNA, whereas in the present work we see that TR–DNA complexes containing two dimers are stabilized by tryptophan. One explanation for this apparent contradiction is that the protein–protein interactions in the two cases are fundamentally different. Footprinting techniques (Kumamoto et al., 1987; Carey et al., 1992) indicate that, as one would expect, multiple dimers of TR are arranged in a linear fashion on the DNA and that they may share major groove half-sites. We have no physical data as yet concerning the structure of TR oligomers in solution in the absence of DNA. One possibility is that rather than a linear or side-by-side interaction, apo-TR dimers form front-to-back stacked oligomers. Such a model is presented in Figure 4. It is consistent with the overall kidney bean shape of the repressor (Zhang et al., 1987) and the electrostatic potential gradient from the back side (negative) to the front or DNA binding side (positive) of the repressor (Otwinowski et al., 1988). In this framework, shown schematically in Figure 4, two tryptophan and two DNA binding domains would be obscured per tetramer of aporepressor and it would be logical that tryptophan binding would destabilize such oligomers. Likewise, since some of the DNA binding domains would be unavailable for interaction with DNA, such an oligomer could contribute to lowering the apparent affinity of aporepressor for its target DNA. In other words, protein–protein interactions would compete for protein–DNA interactions in the absence of tryptophan. Experiments are currently under way to further characterize the TR oligomers in the absence and presence of corepressor and DNA.

The results of the present study demonstrate that both the dimeric and tetrameric TR–operator complexes are specific (i.e., induced by tryptophan). These 2:1 complexes have generally been considered to be nonspecific in nature. Our results also suggest that in addition to sequence effects, the differences in the reported TR–DNA complex stoichiometries and affinities stem, in large part, from differences in the operator concentrations employed in the various assays. At higher operator concentrations, more protein is required before binding is detected, and at the higher concentrations of protein, the tetrameric complex is favored. Because fluorescence anisotropy is a simple solution-based assay, we have been able to obtain binding profiles under a number of different conditions and to assess their effect on the relative population of the dimeric and higher order protein–DNA complexes. By involving protein–protein, as well as protein–ligand, interactions in transcriptional regulatory mechanisms, the cell affords itself an additional level of control, since changes in the polypeptide concentration will also affect the levels of transcription. TR is autogenously regulated (Kelley & Yanofsky, 1982), such that its intracellular concentration drops by nearly a factor of 5 in the repressed state (Gunsalus et al., 1986). This may serve to favor the high-affinity dimeric species, as well as to control tandem binding and thus the differential repression of the various target operons containing varying numbers of sites (Kumamoto et al., 1987). Proposing *in vivo* significance from *in vitro* assays always requires a great deal of caution. Nonetheless, the complexity in the number and stoichiometry of the protein–protein and protein–DNA complexes which we have observed and the effect of tryptophan on these complexes indicate that the mechanism of action of tryptophan in regulating TR target operon transcription involves the modulation of the interaction between TR dimers, as well as between TR and its operator sites.

In addition to their relevance to a deeper understanding of regulatory mechanisms in the *trp* repressor system, the present studies demonstrate that fluorescence anisotropy provides a rapid, reliable solution-based methodology for monitoring protein binding to DNA. Simple detection of binding factors in cell extract or purification fractions requires a few minutes and a complete equilibrium binding profile, approximately 2 h to acquire. Others have used fluorescence anisotropy to monitor protein–DNA interactions (Heyduk & Lee, 1990; Guest et al., 1990), but none have demonstrated the subnanomolar sensitivity required for the study of specific protein–DNA interactions. The sensitivity of the technique, as currently practiced in our laboratory, is now less than 100 pM in fluorescein-labeled oligonucleotide. Since the technique is currently limited by scattered exciting light, we estimate that the detection limit could be further decrease to 1 pM by appropriate use of laser excitation and optical filters.

Fluorescence anisotropy, being a solution-based methodology, is a true equilibrium measurement that does not require

separation of free and bound species. As such, it offers the possibility of characterizing the effects of pH, salt concentration, temperature, and effector ligand or competing or enhancing polypeptide on protein–DNA or protein–RNA interactions. The anisotropy assay eliminates the practical constraints of solid support-based assays such as complex instability during electrophoresis or lack of total retention on nitrocellulose filters. However, like filter binding, our method yields quantitative results and, like gel retardation, it also contains some information concerning the number and sizes of the protein–DNA complexes. Nonetheless, when applied to systems as complex as the *trp* repressor, independent verification of complex stoichiometries is needed for a detailed analysis of the anisotropy data in terms of the various stoichiometric species which can be populated. Given its speed, sensitivity, precision, and ease of use, this approach should find a wide range of applications. For example, in the field of transcriptional control, it could be used both in the purification of transcription factors and in the more detailed study of interactions between the many specific or general transcription factors and their target sequences.

## ACKNOWLEDGMENT

We express our gratitude to Promega, Inc., for the gift of the fluorescent oligonucleotide.

## REFERENCES

Arvidson, D. N., Bruce, C., & Gunsalus, R. P. (1986) *J. Biol. Chem. 261*, 238–243.

Bass, S., Sorrells, V., & Youderian, P. (1988) *Science 242*, 240–245.

Carey, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 975–979.

Carey, J., Lewis, D. E., Lavoie, T. A., & Yang, J. (1991) *J. Biol. Chem. 266*, 24509–24513.

Chou, W.-Y., & Matthews, K. S. (1989) *J. Biol. Chem. 264*, 18314–18319.

Fernando, T., & Royer, C. A. (1992) *Biochemistry 31*, 3429–3441.

Guest, C. R., Hochstrasser, R. A., Dupuy, C. G., Allen, D. J., Benkovic, S. J., & Millar, D. P. (1991) *Biochemistry 30*, 8759–8770.

Gunsalus, R. P., Miguel, A. G., & Gunsalus, G. L. (1986) *J. Bacteriol. 167*, 272–278.

Haran, T. E., Joachimiak, A., & Sigler, P. B. (1992) *EMBO J. 11*, 3021–3030.

He, J.-j., & Matthews, K. S. (1990) *J. Biol. Chem. 265*, 731–737.

Heyduk, T., & Lee, J. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1744–1748.

Hurlburt, B. K., & Yanofsky, C. (1990) *J. Biol. Chem. 265*, 7853–7858.

Hurlburt, B. K., & Yanofsky, C. (1992) *J. Biol. Chem. 267*, 16783–16789.

Joachimiak, A., Kelley, R. L., Gunsalus, R. P., Yanofsky, C., & Sigler, P. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 668–672.

Kelley, R. L., & Yanofsky, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3120–3124.

Kelley, R. L., & Yanofsky, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 483–487.

Klig, L. S., Oxender, D. L., & Yanofsky, C. (1988) *Genetics 120*, 651–655.

Kumamoto, A. A., Miller, W. G., & Gunsalus, R. P. (1987) *Genes Dev. 1*, 556–564.

Liu, Y.-C. (1992) Ph.D. thesis, Rice University.

Liu, Y.-C., & Matthews, K. (1993) (submitted for publication).

Martin et al. (1993) *Biophys. J.* (submitted for publication).

Otwinowsky, Z., Schevitz, R. W., Zhang, R.-g., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., & Sigler, P. B. (1988) *Nature 335*, 321–329.

Paluh, J. L., & Yanofsky, C. (1986) *Nucleic Acids Res. 14*, 7851–7860.

Perrin, F. (1926) *J. Phys. Rad. 1*, 390–401.

Royer, C. A. (1993) *Anal. Biochem.* (in press).

Royer, C. A., Smith, W. R., & Beechem, J. M. (1992) *Anal. Biochem. 191*, 287–294.

Royer, C. A., Ropp, T., & Scarlata, S. F. (1992) *Biophys. Chem. 43*, 197–211.

Schevitz, R. W., Otwinowski, Z., Joachimiak, A., Lawson, C. L., & Sigler, P. B. (1985) *Nature 317*, 782–786.

Staacke, D., Walter, B., Kisters-Woike, B., Wilcken-Bergmann, B.-v., & Muller-Hill, B. (1990) *EMBO J. 9*, 1963–1967.

Zhang, R.-g., Joachimiak, A., Lawson, C. L., Schevitz, R. W., Otwinowski, Z., & Sigler, P. B. (1987) *Nature 327*, 591–597.