Anilinonaphthalenesulfonate (ANS) and tryptophan compete for binding to the trp repressor protein; thus, the fluorescence decrease associated with ANS dissociation can be used as a fluorometric marker for tryptophan binding to the protein. Using this approach, the tryptophan equilibrium dissociation constant was measured at 25 °C to be 3.7 (±1.2) × 10⁻³ M, a value which compares favorably with that obtained by other methods for determining the affinity of this ligand.

The presence of nonspecific DNA had no effect on the yield of ANS displacement. The kinetics of tryptophan binding to the aporepressor were monitored directly and by ANS displacement. The association rate constant was ~4 × 10⁴ M⁻¹ s⁻¹, and the dissociation rate constant was ~60 s⁻¹. The ratio of these values agrees with the binding constant determined by equilibrium dialysis at this temperature. Using the gel retardation method (Carey, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 975–979), the dissociation rate constant for the 40-base pair operator fragment was estimated to be 2 × 10⁻³ s⁻¹, which combines with the measured Kd of 0.3 nm to yield an association rate constant comparable to other DNA binding proteins (~10⁶ M⁻¹ s⁻¹).

Changes in the orientation and position of the D and E helices facilitate DNA recognition (Otwinski et al., 1988; Zhang et al., 1987). The active conformation is thus supported by bound tryptophan, and this corepressor participates in DNA binding (Marmorstein and Sigler, 1989; Otwinski et al., 1988); removal of the tryptophan yields a stable aporepressor conformation that does not bind operator (Zhang et al., 1987).

Two identical and independent ligand sites have been found in the native trp aporepressor dimer, and binding constants for L-tryptophan have been studied by several methods (Arvidson et al., 1986; Lane, 1986; Marmorstein et al., 1987). The α-amino group of tryptophan contributes relatively little to the observed binding energy but is essential for corepressor function, whereas the indole function and α-carboxylate contribute primarily to binding energy, and the indole ring nitrogen makes a direct DNA contact (Marmorstein et al., 1987; Marmorstein and Sigler, 1989). The α-amino group contributes to operator DNA binding by orienting the α-carboxylate to participate in a hydrogen bond network and by neutralizing the negative pole of the B-helix dipole (Lawson and Sigler, 1988; Otwinski et al., 1988). The method of fluorescence spectroscopy is widely used to study the interaction of proteins with small molecules. Using this method to measure directly the binding of trp aporepressor with tryptophan is complicated by the fluorescence of the extrinsic tryptophan ligand overwhelming that from intrinsic tryptophan (Lane, 1986). To avoid the interference from extrinsic tryptophan, an environmentally sensitive fluorescent probe, 8-anilino-1-naphthalenesulfonate (ANS), was utilized in this study.

The trp repressor binds its cognate operator with an apparent dissociation constant of 2 × 10⁻⁷ M as first measured by restriction site protection (Gunsalus and Yanofsky, 1980; Joachimiak et al., 1983). The nitrocellulose filter assay developed by Klug et al. (1987) found a similar Kd of 2.6 × 10⁻⁸ M. An electrophoretic gel retardation method developed by Carey (1988) to determine the affinity and stoichiometry of trp repressor and operator DNA interaction showed the apparent dissociation constant for the trp repressor-operator DNA complex to be 5 × 10⁻¹⁰ M at pH 6 with an average stoichiometry of one trp repressor dimer/DNA. We have used this method to measure the dissociation rate of repressor from operator DNA.

**MATERIALS AND METHODS**

**RESULTS AND DISCUSSION**

ANS Binding—ANS is a fluorescent probe which binds to proteins, due at least in part to its conjugated ring structure.

1 The abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; bp, base pair.

2 Portions of this paper (including "Materials and Methods" and Figs. 2, 4, and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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and is sensitive to changes in its environment (reviewed by Slavik, 1982). Since its fluorescence quantum yield increases in an apolar environment, ANS binding to trp aporepressor can be monitored fluorometrically (Fig. 1). Equilibrium dialysis was also utilized to measure the binding of ANS to trp repressor by monitoring the absorbance of ANS in the dialysate and in the protein solution (Fig. 2, Miniprint Supplement). Analysis of the ANS fluorescence titration and equilibrium dialysis data yielded a $K_d$ for aporepressor binding to ANS at 25 °C of 5.0 (±2.4) × 10^{-8} M. This value is similar to the binding constant observed for tryptophan to trp repressor (see below), and it is noteworthy that tryptophan and ANS share both aromatic and acidic properties.

Tryptophan Competition with ANS—The fluorescence emission intensity of ANS bound to trp aporepressor decreases upon addition of tryptophan so that tryptophan binding can be monitored free of spectral overlap. This decrease may result from either a change of hydrophobicity of the ANS binding site upon L-tryptophan binding or displacement of ANS by L-tryptophan. To distinguish between these explanations, a series of titrations of tryptophan-trp aporepressor complex with ANS was performed at different fixed concentrations of L-tryptophan (Fig. 1A). Double reciprocal Line-Weaver-Burke type plots of these data (Fig. 1B) indicate that ANS and tryptophan bind to trp aporepressor competitively.

Either the ANS and tryptophan binding sites on the aporepressor overlap, or occupation of the respective sites is mutually exclusive. Equilibrium dialysis of ANS binding, measuring absorbance of ANS to determine concentration, was carried out in the presence and absence of tryptophan and confirmed that the effect of tryptophan was on ANS binding and not simply ANS fluorescence properties (Fig. 2, Miniprint Supplement). The similarity in structural features of ANS and trypophan is consistent with this interpretation. Marmorstein et al. (1987) concluded that tryptophan binding to aporepressor relies primarily on the hydrophobicity of the indole ring and the electrostatic nature of the α-carboxyl group. The anilinophthalene ring is aromatic, and the sulfonate gives a negative charge comparable to the carboxylate.

These results which indicate the geometrical overlapping of tryptophan and ANS binding sites can be rationalized based on the structure of the trp repressor (Schevitz et al., 1985; Zhang et al., 1987; Otwinowski et al., 1988). The two subunits interweave in a manner such that the subunit interface is quite large compared to other oligomeric proteins. Because of this unique tertiary/quaternary structure, the tryptophan binding site appears to be the only possible hydrophobic pocket where ANS can bind. Although ANS, like indole-3-propionic acid (Marmorstein et al., 1987), can bind to trp aporepressor with tryptophan affinity, neither molecule activates the protein to bind with operator DNA. This difference derives from the crucial role of the α-amino group in positioning the contacts correctly for trp operator binding (Lawson and Sigler, 1988; Otwinowski et al., 1988; Marmorstein and Sigler, 1989). The indole ring of tryptophan may bind to trp aporepressor first and then serve to guide the α-carboxyl and α-amino groups into the right position to form hydrogen bonds with aporepressor. The fused rings of ANS may serve an analogous role. This hypothesis is similar to the conceptual model for protein association proposed by Ross and Subramanian (1981). The first step of their model consisted of hydrophobic association between protein and ligand. In the second step, ionic interactions, hydrogen bonds, and van der Waals interactions stabilize the complex.

Tryptophan Equilibrium Constant—This competition between ANS and tryptophan can be utilized to determine binding constants for tryptophan and trp repressor. The equilibrium constant of trp aporepressor-tryptophan was calculated from the displacement curve of ANS bound to trp aporepressor by the method of Horovitz and Levitzki (1987) to be 3.7 (±1.2) × 10^{-8} M at 25 °C. This value agrees with results obtained by Arvidson et al. (1986) using equilibrium dialysis and flow dialysis methods that yielded an equilibrium dissociation constant of 4.8 × 10^{-8} M at 25 °C. The titration of aporepressor with tryptophan has been monitored by difference ultraviolet absorption, fluorescence, and circular dichroism which demonstrated a dissociation constant of 1.8 × 10^{-8} M at 25 °C (Lane, 1986). The results of Arvidson et al. (1986) demonstrate a temperature dependence for tryptophan binding (2.8 × 10^{-8} M at 6.5 °C, 4.8 × 10^{-8} M at 25 °C, and 2.2 × 10^{-4} M at 40 °C), but the minimal effects of salt concentration and pH variation observed (see also Lane, 1986) account for the similarity in $K_d$ values measured in different buffer systems. Marmorstein et al. (1987) carried out equilibrium dialysis experiments at lower temperature (4 °C) and higher pH and obtained a binding constant for tryptophan and aporepressor of 1.5 × 10^{-8} M. The concordance between the ANS displacement method and other measurements of tryptophan binding validates the ANS assay as a nonperturbing means of monitoring tryptophan binding.

Kinetic Measurements—The kinetics of tryptophan binding
were examined using fluorometric stopped-flow spectrometry. Aporepressor was mixed with varying concentrations of tryptophan, and the fluorescence change was monitored over time (Fig. 3). The change of tryptophan fluorescence upon binding to repressor was too rapid (\( t_{1/2} < 3 \text{ ms} \)) and too small to measure at room temperature. Thus, all the experiments were performed at 4°C, and even under these conditions the signal to noise ratio was small. However, the data were well described by a single exponential decay. A plot of the fitted rate constant versus [trp] allowed an estimation of association and dissociation rate constants, \( k_a \) and \( k_d \), based on the equation \( k_{obs} = k_a + k_d \). The \( k_a \) for tryptophan was obtained from the slope of the plot, \( \sim 4 \times 10^{7} \text{M}^{-1} \text{s}^{-1} \); the y axis intercept yielded a \( k_d \) of \( \sim 60 \text{ s}^{-1} \). The rate constant for the dissociation of tryptophan from repressor was determined directly by displacing the bound amino acid with ANS (Fig. 4, Miniprint Supplement). At high concentrations of ANS, the rate-limiting step for ANS binding should be dissociation of prebound tryptophan. The observed replacement rate should be equal to the dissociation rate constant for tryptophan and reached a limiting value of \( \sim 50 \text{ s}^{-1} \), in good agreement with the rate determined from the data in Fig. 3. The \( K_d \) calculated from these values \( (k_d/k_a) \) was \( 1.5 \times 10^{-5} \text{ M} \), in concordance with the results obtained from equilibrium dialysis at 5°C in our laboratory \((2.0 \times 10^{-5} \text{ M})\) and those derived from equilibrium flow dialysis at 6.5°C \((2.8 \times 10^{-5} \text{ M}; \text{Arvidson et al., 1986})\).

Further evidence for the validity of the replacement method is shown in Fig. 4B (Miniprint Supplement). ANS was displaced by a high concentration of tryptophan, and the fluorescence change observed was in the opposite sense. At high tryptophan concentrations, the observed replacement rate should equal the ANS dissociation rate constant and was observed to be \( \sim 35 \text{ s}^{-1} \). The binding reactions of ANS and tryptophan are apparently governed by comparable rate as well as equilibrium constants. Lane (1986) estimated the rate constant for tryptophan dissociation at 25°C by analyzing the lineshapes of NMR spectra to be in the range 400–600 \text{ s}^{-1} \). Although temperature effects (4 versus 25°C) may account in part for the observed difference, the direct fluorescence measurements should yield more accurate results than estimates based on NMR lineshape analyses (Lane, 1986).

Effects of DNA on Tryptophan Binding—The ANS competition method was utilized to determine the \( K_d \) of tryptophan for aporepressor in the presence of trp or lac 40-bp operator DNA fragments. These data are important for understanding the general binding scheme for the repressor, illustrated in Table I. Based on the conservation of energy, \( 2 \Delta G_1 + \Delta G_2 = 2 \Delta G_3 + \Delta G_4 \). Holorepressor has a higher affinity for operator DNA than does aporepressor (Carey, 1988, 1989), and tryptophan would be expected to exhibit a higher affinity for aporepressor-operator DNA complex than for aporepressor alone. This effect was observed but not quantitated by Joachimiak et al. (1983) in equilibrium dialysis experiments. Using the ANS displacement method, the \( K_d \) of tryptophan for aporepressor in the presence of operator DNA is about 6-fold lower \((6.3 (\pm 1.0) \times 10^{-8} \text{ M})\) than that observed without operator DNA (Fig. 5, Miniprint Supplement). Under the same conditions, the \( K_d \) of tryptophan binding to trp aporepressor was found to be 3.2 (\( \pm 0.6 \)) \times 10^{-5} \text{ M} \) in the presence of lac operator DNA instead of trp operator DNA. These results support the observations that nonspecific DNA binding of this repressor is about 50- to 200-fold weaker than specific DNA binding (Carey, 1988, 1989). The agreement between \( \Delta G_1 \) and \( \Delta G_2 \) (Table I) indicates that the binding constants measured for the reactions are consistent with the binding scheme.

Operator Binding—Operator binding assays were per-

![Fig. 3. Determination of kinetic constants for tryptophan. Tryptophan (5 \times 10^{-5} \text{ M as dimer}) in 10 mM potassium phosphate, pH 7.5, 0.1 mM KCl, 1 mM EDTA was mixed with varying concentrations of tryptophan in a stopped-flow spectrometer at 4°C. Fluorescence changes were measured at wavelengths greater than 350 nm (Corning 0-52 filter) using an excitation wavelength of 285 nm. The fluorescence traces were analyzed by computer (A), and \( K_{obs} \) was plotted versus tryptophan concentration to determine the kinetic constants (B). The smooth line in A represents fit to a single exponential.](image-url)
formed by the gel retardation method at pH 6.0 developed by Carey (1988). A 90-bp operator-containing DNA used by Carey (1988) and a synthetic trpEDCBA operator fragment consisting of 40 bp were used for the assay. The apparent equilibrium dissociation constant for trp repressor and 40-bp operator DNA is $3.33 \pm 0.16 \text{nM}$, a value similar to the results observed previously by Carey (1988) using the 90-bp DNA fragment. No difference in $K_d$ was noted between these two DNAs in our experiments. The 40-bp fragment encompassed the trpEDCBA regulatory sequences indicated by Kumamoto et al. (1987) to be protected from methylation and DNase digestion by trp repressor binding (i.e. $-25$ to $+10$ on the top strand and $-27$ to $+7$ on the bottom strand); thus, all direct determinants for binding are present in this sequence. The flanking sequences found in the 90-bp fragment do not appear to exert significant effects on repressor affinity.

The equilibrium constants obtained by gel retardation are 10-fold lower than the data obtained from restriction enzyme protection and filter binding assays (Joachimiak et al., 1983; Klig et al., 1987). The size of the operator-containing fragment does not appear to account for these differences. The restriction enzyme assay involves a competing reaction for the same site, and the apparent binding constants may be influenced by the presence of the enzyme. Filter binding assays (Klig et al., 1987) were performed under high salt conditions to maximize adherence to the filter and utilized a 280-bp DNA fragment 0.33 nm, a value similar to the results observed previously by Carey (1988) using the 90-bp DNA fragment. The holorepressor-operator dissociation rate was determined by mixing unlabeled 40-bp trp operator DNA with preincubated holorepressor-$^{32}$P-labeled operator complex at time 0 and monitoring an increase in radioactivity of free DNA on the gel over time ($t_{1/2} \approx 30 \text{s}$). This value is similar to data from the nitrocellulose filter binding assay under quite different buffer conditions (Klig et al., 1987) and yields a $k_d$ of $2 \times 10^3 \text{s}^{-1}$. The association rate constant of $-10 \text{s}^{-1}$ calculated from these data is at the upper limit of a diffusion-controlled reaction (Berg et al., 1981) and is in the lower range of values found for the lac repressor binding to 40-bp lac operator (Whitson et al., 1986).

In summary, competitive binding of ANS to the trp repressor can be rationalized by the nature of the tryptophan binding site in the protein and provides a fluorometric method for measuring tryptophan affinity and assessing kinetic constants. The equilibrium dissociation constant for operator DNA binding measured by gel retardation (Carey, 1988) is similar for 40- and 90-bp operator-containing DNAs and is approximately 10-fold lower than observed by other assay methods, whereas the dissociation rate from the operator is rapid and consistent with measurements from filter binding (Klig et al., 1987). These various affinity and rate constants are concordant with the requirements for tryptophan repressor to fulfill its cellular role of regulating tryptophan availability for protein synthesis (Arvidson et al., 1986).

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Binding Parameters for Tryptophan and Operator DNA Binding

MATERIALS AND METHODS


discussion of Experimental System

DNA with Operator Suppressor

The binding parameters for the repression of a single operator DNA with Tryptophan were determined. The experiments were performed with a wild-type E. coli strain (K-12) harboring the plasmid pBR322. The operator DNA was isolated from the plasmid and purified as described elsewhere.


time-of-event

RESULTS

The fluorescence spectra of DNA-DNA complexes were recorded in the presence of Tryptophan. The fluorescence spectra were recorded at different Tryptophan concentrations and at different temperatures. The spectra were characterized by a maximum at 340 nm and a shoulder at 360 nm. The emission spectra were recorded using a spectrofluorimeter and the emission was excited using a 280 nm light source. The emission intensities at 340 nm were plotted against the Tryptophan concentration, and the results were fitted to a non-linear regression model. The dissociation constant, 1.0 x 10^-6 M, was determined using the mass action law. The results were interpreted in terms of the thermodynamic parameters of the interaction.


diagram

Figure 1. Fluorescence spectra of DNA-DNA complexes in the presence of Tryptophan at different temperatures. The spectra were recorded at different Tryptophan concentrations and at different temperatures. The spectra were characterized by a maximum at 340 nm and a shoulder at 360 nm. The emission spectra were recorded using a spectrofluorimeter and the emission was excited using a 280 nm light source. The emission intensities at 340 nm were plotted against the Tryptophan concentration, and the results were fitted to a non-linear regression model. The dissociation constant, 1.0 x 10^-6 M, was determined using the mass action law. The results were interpreted in terms of the thermodynamic parameters of the interaction.


diagram

Figure 2. Graphical presentation of the binding parameters for Tryptophan and Operator DNA Binding. The graph shows the relationship between the binding affinity (Kd) and the concentration of Tryptophan (C_T). The binding affinity (Kd) was determined from the dissociation constant, 1.0 x 10^-6 M, using the mass action law. The binding parameters were fitted to a non-linear regression model and the results were interpreted in terms of the thermodynamic parameters of the interaction.


diagram

Figure 3. Flow chart of the experimental procedure for the determination of binding parameters. The flow chart includes the following steps: a) preparation of the DNA-DNA complex, b) measurement of the fluorescence spectra, c) determination of the dissociation constant, and d) interpretation of the results in terms of the thermodynamic parameters of the interaction.