Biophysical Chemistry, 49 (1994) 233–239
Elsevier Science B.V., Amsterdam

BIOCHE 01819

Thermodynamic studies of the interaction of trp aporepressor with tryptophan analogs

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(Received 24 March 1993; accepted in revised form 30 August 1993)

Abstract

The association of L-tryptophan and some of its analogs, including three conformationally restricted analogs, with trp aporepressor (apo trpR) was studied by isothermal titration microcalorimetry. Contributions of the functional groups of a ligand to the free energy change, $\Delta G^{\circ\prime}$, and enthalpy change, $\Delta H^{\circ\prime}$, of the interaction were evaluated on a molecular basis. Analogs without the α-amino group (i.e. desamino analogs) bind with a slightly higher affinity to the protein. On the other hand, descarboxy analogs show weaker binding to the apo trpR. In addition, it is found that there exists enthalpy-entropy compensation for the association of the congener series of ligands with the protein. The entropy change, $\Delta S^{\circ\prime}$, appears to play a more important role in the binding of the conformationally restricted analogs than in the binding of L-tryptophan and the unlocked ligands.

Keywords: Tryptophan aporepressor; Thermodynamics of ligand binding; Titration microcalorimetry; Tryptophan; Conformationally restricted analogs

1. Introduction

Tryptophan aporepressor (apo trpR) from *E. coli* is a homodimeric protein with subunits of 107 amino acids. The two subunits are interlocked with each other and have helix–turn–helix domains [1–4]. This regulatory protein is generated from the trpR gene of *E. coli*. It controls the expression of several genes, such as trpR, trpEDCBA, and aroHa [5–11]. The biological function of apo trpR has been extensively investigated. Association of L-tryptophan (also referred to as the corepressor) with apo trpR generates an active binary complex, trp repressor, which can further interact with its cognate operator DNA. The ternary complex formed between L-tryptophan, apo trpR, and the operator DNA can block transcription of the gene trpEDCBA and thus cease biosynthesis of L-tryptophan.

Understanding the mode of interaction between L-tryptophan and apo trpR is crucial for understanding the mechanism of the activation of the apo trpR and the interaction between the activated trp repressor and the operator DNA. X-ray crystallography studies have shown that the binding of L-tryptophan causes a repositioning and bracing of the two helix–turn–helix domains

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SSDI 0301-4622(93)E0073-E

of the protein in a manner that can better interact with the major grooves of dsDNA [12]. There is no obvious change in the subunit interface upon the association of L-tryptophan [2,13]. Consistent with this is that the binding of two L-tryptophan ligands to the two binding sites of apo trpR has been found to occur independently [14,15]. Equilibrium dialysis binding experiments with a series of analogs of tryptophan, performed by Sigler and co-workers [14,16], have shown that the indole ring and the α-carboxy group of L-tryptophan are important for high affinity between the ligand and protein. However, the complex between a desamino analog (i.e. one lacking the α-amino group) of L-tryptophan, indole-3-propionic acid (IPA), and apo trpR is an inactive pseudorepressor, i.e. this binary complex has very low affinity for the operator DNA as compared to the binary complex with L-tryptophan. Thus, the α-amino group was found to be important for the formation of the ternary complex with operator DNA.

Although the system of apo trpR has been examined by many researchers in great detail in the past decade, many of the biophysical and biochemical properties of this protein have yet to be investigated. In this article, we focus on the thermodynamic aspects of the formation of the protein-ligand binary complex. We report free energy, $\Delta G^{\circ\prime}$, enthalpy change, $\Delta H^{\circ\prime}$, and entropy change, $\Delta S^{\circ\prime}$, data for the association of a series of analogs of L-tryptophan, among which are three conformationally restricted tryptophan analogs (see fig. 1). It is our interest to understand the roles of the functional groups in determining the thermodynamic changes for the protein–ligand interaction.

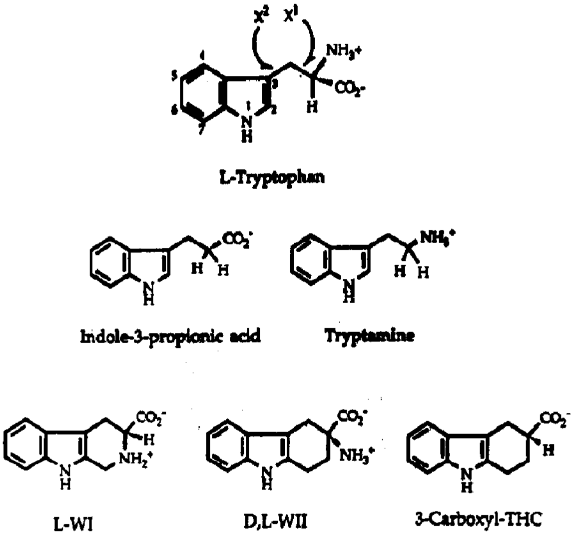

Fig. 1. Structures of L-tryptophan (Trp), tryptamine (TAM), and indole-3-carboxylic acid (IPA) and three conformationally restricted analogs, L-W(I), D,L-W(II), and 3-carboxyTHC.

2. Experimental section

Materials: The following materials were purchased from Sigma Chemical Company: L-tryptophan (Trp), tryptamine (TAM), indole-3-propionic acid (IPA), TRIZMA base, potassium phosphate, potassium chloride, EDTA (disodium salt), streptomycin sulfate, ampicillin (anhydrous), and isopropyl-β-D-thiogalactopyranoside (IPTG). The following materials were obtained from Aldrich Chemical Company: triethylamine, phenyl hydrazine, and toluene-*p*-sulfonic acid. The following chemicals were bought from Fisher Scientific Company: ammonium carbonate, potassium cyanide, and zinc chloride. Formaldehyde solution and acetic anhydride were obtained from J.T. Baker Chemical Company. Chromatographic phosphocellulose media was purchased from Whatman Company.

L-2,3,4,9-tetrahydro-1H-β-carboline-3-carboxylic acid (L-W(I)): This restricted tryptophan analog was prepared according to Harvey and co-workers [17]. The compound was purified by recrystallization from hot water twice. The purity was checked with a silica get TLC plate (*n*-butanol : acetic acid : H_2O, 5 : 1 : 1, single spot). The compound (mp. 284°C) was confirmed by IR and ^{1}H NMR; the results were identical to those in the literature [17,18].

3-amino-1,2,3,4-tetrahydrocarbazole-3-carboxylic acid (D,L-W(II)): The synthesis of this compound was performed according to Britten and Lockwood [19] and Maki et al. [20]. The final product was recrystallized from H_2O : acetic acid (1 : 1) solution to yield light yellow crystals having a melting point of 320°C. TLC, developed with *n*-butanol:acetic acid:water (4:1:1), showed a single spot upon exposure to UV light. The compound was confirmed by means of UV (H_2O, $\lambda_{max} = 280$ nm) and ^1H NMR.

3-carboxy-tetrahydrocarbazole (3-carboxy-THC): The preparation of 3-carboxyTHC was carried out according to Perkin [21] with minor alterations. The product was purified by recrystallization from benzene (mp. 190°C) and was checked with UV (methanol, $\lambda_{max} = 280$ nm), MS (m/z 215 molecular ion) and ^1H NMR.

Purification of apo trpR: Plasmid pJPR2-containing *E. coli* cells, CY15070, which overproduce apo trpR, were generously provided by Dr. Kathleen S. Matthews (Rice University). The procedure for cell growth and overproduction was as described by Joachimiak et al. [1] and Matthews and co-workers [15]. The purity of the protein was examined by gel electrophoresis (1% SDS:15% polyacrylamide). The L-trp binding affinity of the protein was tested by the radioactive ammonium sulfate assay [15,22]. The trpEDCBA operator binding specificity of the protein was checked using a DNA gel retardation method [23]. Quantitation of the developed film was done by using a scanning densitometer (E-C Aparatures Corp.).

The concentration of apo trpR was determined by measuring the absorbance of protein solutions at 280 nm using a molar extinction coefficient of 1.50×10^4 M^{-1} cm^1 per monomeric subunit [1].

Methods. Isothermal titration microcalorimetry: A differential power compensation isothermal titration calorimeter (ITC) was used for conducting the thermodynamic studies. The instrument was designed by Freire et al. [24]. ITC directly measures the heat associated with a biochemical reaction as a constant temperature. When a protein solution is titrated with a ligand solution, the $\Delta H^{\circ\prime}$, $\Delta G^{\circ\prime}$ and $\Delta S^{\circ\prime}$ can be determined from a single titration. The ITC which we used has a sensitivity of approximately one microcalorie [24]. We have employed protein concentrations in the few micromolar range, which enables association constants above 10^7 to be determined. The ITC has a dual injection system (i.e. injection into both the sample and the reference cell) to compensate for any mechanical and dilution heat effects. Also, a differential compensation circuit reduces the response time to less than a minute for each injection. This rapid response minimizes problems of baseline drift and noise.

In a typical ITC titration, 5 ml of a sample of apo trpR ($(2\text{–}5) \times 10^{-6}$ M in binding sites, dialyzed versus 10 mM phosphate buffer, 0.1 M KCl, 1.5 mM EDTA, pH 7.5) was placed in the calorimeter to equilibrate for 1–2 h at 25°C. L-tryptophan (or the analogs TAM or IPA) was dissolved in the same buffer to a concentration of 20–40 mM. The pH of the protein, ligand and buffer solution to be used in the measurement was carefully adjusted to within ±0.01 using an Orion 720 pH meter. Each solution was also degassed using an ultrasonic bath (Branson, SmithKline Co.) under reduced pressure and was incubated in a 25°C water bath for 30 min before being loaded into the calorimeter.

The ligand was titrated into the sample in 5 microliter injections. The heat generated from each injection was obtained by integration of the thermal power output versus time. A plot of cumulative heat, Q, versus total ligand concentration, $[L]_0$, was produced. The Q versus $[L]_0$ data sets were fitted by the following equation:

$$Q = Q_{max}K[L]/(1 + K[L]), \tag{1}$$

where K is the association constant, $[L]$ is the free ligand concentration. Q_{max}, the maximum heat effect, is related to $\Delta H^{\circ\prime}$ by $Q_{max} = \Delta H^{\circ\prime}[M]_0$, where $[M]_0$ is the total concentration of protein binding sites. The free ligand concentration is related to the total ligand concentration by $[L] = [L]_0 - Q[M]_0/Q_{max}$. The latter expression was inserted into eq. (1) and the resulting equation was fitted to Q versus $[L]_0$ data (obtained at a particular $[M]_0$) by a non-linear least-squares program. The program was kindly provided by M. Straume (University of Virginia) and E. Freire (Johns Hopkins University). This model assumes a single type of binding site.

For ligands such as L-W(I), D,L-W(II), and 3-carboxyTHC, which have low solubility, a displacement titration method was developed to determine the thermodynamic parameters for their binding to the protein. (To perform direct titration measurements it is generally necessary to prepare a ligand/titrant concentration that is at least 50 times the dissociation constant, K^{-1}, for the protein–ligand interaction. Some of the above ligands are insufficiently soluble to do this.) In this displacement method the poorly soluble ligand, L_2, is added to the protein solution to a concentration of approximately $[L_2] = K_2^{-1}$. The protein–L_2 mixture is then titrated with the displacing ligand, L_1, for which the K and $\Delta H^{\circ\prime}$ are known (in the absence of L_2). The apparent association constant for binding L_1 in the presence of L_2 is given by

$$K_1(\text{app}) = K_1/(1 + K_2[L_2]) \qquad (2)$$

and the apparent enthalpy change for the binding of L_1 in the presence of L_2 is given by

$$\Delta H_1^{\circ\prime}(\text{app}) = \Delta H_1^{\circ\prime} - \Delta H_2^{\circ\prime} K_2[L_2]/(1 + K_2[L_2]). \qquad (3)$$

From a determination of $K_1(\text{app})$ and $\Delta H_1^{\circ\prime}(\text{app})$ at a known $[L_2]$ (and with independently determined values of K_1 and $\Delta H_1^{\circ\prime}$), values of K_2 and $\Delta H_2^{\circ\prime}$, the thermodynamic parameters for the association of L_2, can be calculated. (The above equations also assume that $[L_2] \approx K_2^{-1} > [\text{protein sites}]_0$, which is the case for the ligands studied here.) For this displacement method to work it is necessary to have $[L_2] \approx K_2^{-1}$. To obtain an estimate of K_2 we used the ANS fluorescence displacement method of Chou et al. [15].

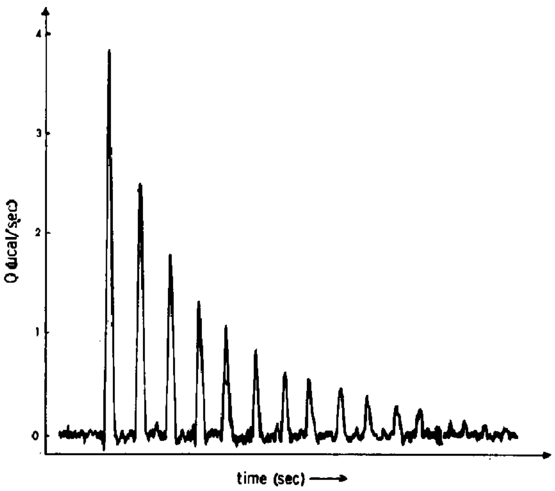

Fig. 2. Heat effect (differential thermal power) for mixing 5 microliter aliquots of L-trp solution (0.01 M) with apo trpR (8.2×10^{-6} M monomer) at 25°C, pH 7.5.

3. Results and discussion

Shown in fig. 2 are raw data for an ITC titration of apo trpR with L-trp. The thermal signals were integrated and summed to produce a binding isotherm. Shown in fig. 3 are ITC binding isotherms for L-trp, IPA, and TAM with apo trpR at pH 7.5 and 25°C. The solid lines are theoretical fits with eq. (1). Note that the fits are adequate for a single type of binding site. (That is, there is no significant improvement in the fit if two K_i and two $\Delta H_i^{\circ\prime}$, are used to fit the data.) This is consistent with previous studies [14,15] having found no interaction between the two subunits of apo trpR in the binding of small ligands. For each of the ligands studied, the binding is an exothermic process. Shown in fig. 4 are ITC displacement binding curves in which the protein is titrated with L-trp in the presence of L-W(I), D,L-W(II), or 3-carboxyTHC. Thermodynamic parameters for the interaction of these ligands with apo trpR are summarized in table I.

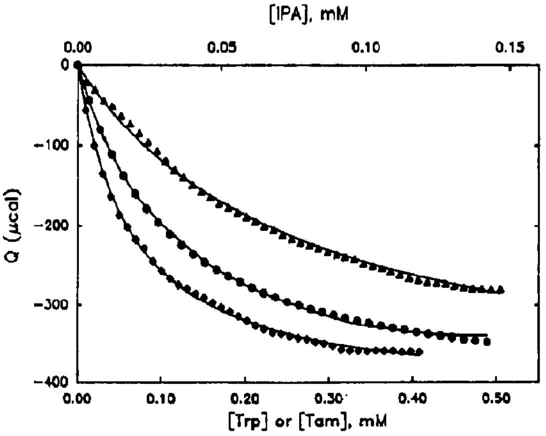

Fig. 3. ITC enthalpic binding isotherms for trp (♦), TAM (▲), and IPA (●) to apo trpR at 25°C, pH 7.5. The solid lines are theoretical fits to equation 1 with $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ values given in table 1.

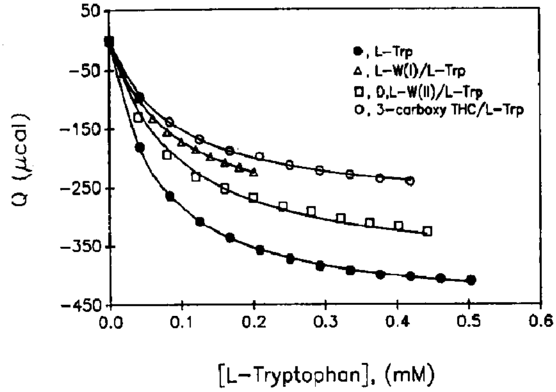

Fig. 4. Displacement ITC binding isotherm of trp to apo trpR in the absence (●) and presence of L-W(I) (△), D,L-W(II) (□), and 3-carboxyTHC (○). Final concentrations of L-W(I), D,L-W(II), and 3-carboxyTHC were 4.0×10^{-4} M, 1.8×10^{-3} M, and 2.8×10^{-5} M, respectively. The solid lines are theoretical fits to apparent K an $\Delta H^{\circ\prime}$ values.

(As a test of the displacement method, thermodynamic parameters were also determined for the binding of TAM by this indirect method.)

Binding Affinities: Comparing the association constants of L-trp, IPA and TAM, it is found that the removal of the α-ammonium group increases the binding affinity of a ligand for the protein. In contrast, removal of the α-carboxylate group reduces the affinity. It appears that the α-ammonium group makes an unfavorable contribution to the free energy of formation of the binary complex, whereas the α-carboxylate group makes a favorable contribution to the interaction. These observations are consistent with the results of Marmorstein et al. [14].

The ITC displacement method (see fig. 4) enables thermodynamic information to be obtained for the poorly soluble, conformationally restricted trp analogs in fig. 1. It is found that all three conformationally restricted analogs, L-W(I), D,L-W(II), and 3-carboxyTHC, are able to bind to the protein, suggesting that the aromatic portion in these compounds interacts at the same position as the indole ring of L-trp in the binding site of apo trpR. Nevertheless, the association constants of L-W(I) and D,L-W(II) to the protein are between 10 to 40 times smaller than is the association constant for L-trp. However, the desamino analog, 3-carboxyTHC, binds with slightly higher affinity than does L-trp. The behavior of the latter restricted analog is similar to that of IPA.

Enthalpy and Entropy Changes: The enthalpy changes (see table 1) for the ligand–protein interactions are exothermic for each of the ligands studied and the $\Delta H^{\circ\prime}$ range from −16 kcal/mol for TAM to −3.5 kcal/mol for 3-carboxyTHC. A striking finding is that the $\Delta H^{\circ\prime}$ for the conformationally restricted analogs are much less negative (−3.5 to −4.3 kcal/mol) than are the $\Delta H^{\circ\prime}$ for the flexible tryptophan analogs (−12.3 to −16.3 kcal/mol). The entropy changes, $\Delta S^{\circ\prime}$, for the binding of L-trp, TAM and IPA are large and negative, whereas the $\Delta S^{\circ\prime}$ for the conformationally restricted analogs are near zero or positive. For example, the $\Delta S^{\circ\prime}$ for 3-carboxyTHC is +8.4 cal/mol K.

The $\Delta H^{\circ\prime}$ value of −12.3 kcal/mol for L-trp is in disagreement with the van't Hoff ΔH value of +10 kcal/mol reported previously by Lane [25], using a difference spectroscopy method. We have no explanation for the discrepancy. Our $\Delta H^{\circ\prime}$ value for the binding of L-trp to apo trpR is, however, in reasonably good agreement with the calorimetric $\Delta H^{\circ\prime}$ of −14.7 kcal/mol recently reported by Jin et al. [26] for similar conditions.

The values of $\Delta H^{\circ\prime}$ for each ligand are apparent values in phosphate buffer at 25°C. In work to be published, we have found a pH dependence to the $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ for the binding of L-trp (there is a proton uptake of approximately 0.3 H^+/(bound ligand) at pH 7.5). Thus the $\Delta H^{\circ\prime}$ values for the ligands will contain a contribution from deprotonation of the buffer. However, phosphate buffer has a very small heat of protonation (−1.2 kcal/mol); the contribution from proton shifts will be very small in this buffer and should not be important for the comparisons in this work.

We have also performed ITC titrations of L-trp binding over a range of protein concentrations from 2.8×10^{-6} M to 1.2×10^{-5} M. We find no significant change in the $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ over this range, i.e. $\Delta G^{\circ\prime}$ changes by less than 0.1 kcal/mol over this range and $\Delta H^{\circ\prime}$ is found to be −14.4 kcal/mol at the lower protein concentration and −12.6 kcal/mol at the higher concentration, values that differ by slightly more than the standard deviation (≈ 1.5 kcal/mol) of each $\Delta H^{\circ\prime}$ value.

Additionally, we have measured $\Delta H^{\circ\prime}$ for L-trp binding to be −10.0 kcal/mol at 15°C, from which a value of the heat capacity change, $\Delta C_p^{\circ\prime}$, for binding was calculated to be −230 cal/mol deg.

The conformationally restricted ligands have one or two additional methylene groups, as compared to L-trp. Consequently, one would expect these ligands to have a greater hydrophobic tendency. We have determined the aqueous solubility of L-W(I), D,L-W(II), and 3-carboxyTHC to be 2.8×10^{-3} *M*, 2.2×10^{-2} *M*, and 1.55×10^{-4} *M* at 25°C, as compared to a solubility of 5.58×10^{-2} *M* for L-trp. The more positive $\Delta S^{\circ\prime}$ found for the restricted analogs is consistent with a larger hydrophobic contribution to the binding of these ligands. Except for 3-carboxyTHC, however, the affinity of the restricted analogs for apo trpR is lower than the affinity of L-trp. For L-W(I) and D,L-W(II) the more favorable hydrophobic tendency must not be enough to outweigh less favorable interactions of these ligands with the protein. The latter ligands must be restricted into a conformation which does not optimize interactions between the side chain groups (ammonium and carboxylate) with the protein. 3-carboxyTHC must be restricted into a conformation that is nearly as optimal as that adopted by the flexible IPA.

The relation between the thermodynamic parameters for ligand binding can be illustrated by the so-called entropy–enthalpy compensation plot in fig. 5. A linear plot is observed. The flexible and conformationally restricted tryptophan analogs cluster into two regions. The binding of tryptophan and its flexible analogs is driven by a large exothermic $\Delta H^{\circ\prime}$; the binding of the conformationally restricted analogs is characterized by a more favorable $\Delta S^{\circ\prime}$, although their $\Delta H^{\circ\prime}$ are still exothermic. The molecular explanation for the large exothermic $\Delta H^{\circ\prime}$ for L-trp, TAM and IPA is not obvious. Until the pH dependence is fully understood, we refrain from discussing the contributing forces for the binding of these ligands. Binding of the restricted analogs seem to be driven, to a greater relative extent, by a hydrophobic tendency, but in some cases (i.e. L-W(I) and D,L-W(II)) the orientation of the side chain functional groups must not be optimal for interacting with complementary functional groups in the binding sites. A possible view is that the

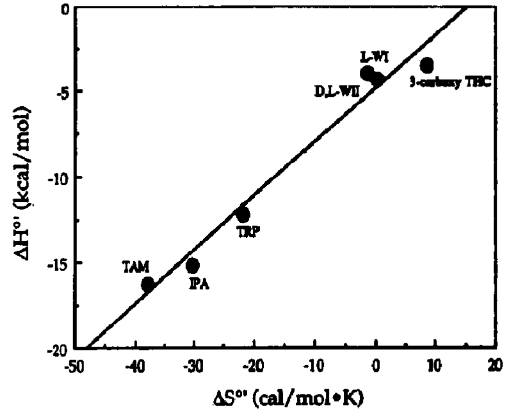

Fig. 5. Plot of the $\Delta H^{\circ\prime}$ versus $\Delta S^{\circ\prime}$ for the binding of the congener series of tryptophan analogs to apo trpR.

Table 1

Thermodynamic parameters for the interaction of ligands with apo trpR [a]

Compound	K_a (M^{-1})	$\Delta G^{\circ\prime}$ (kcal/mol)	$\Delta H^{\circ\prime}$ (kcal mol)	$\Delta S^{\circ\prime}$ (cal/mol K)
IPA [b]	3.4×10^4	−6.2	−15.2	−30.2
3-carboxyTHC [c]	2.4×10^4	−6.0	−3.5	+8.4
L-trp [b]	1.8×10^4	−5.8	−12.3 [d]	−21.8
TAM [b]	4.3×10^3	−5.0	−16.3	−37.9
L-W(I) [c,e]	1.7×10^3	−4.4	−4.3	+0.3
D,L-W(II) [c]	4.5×10^2	−3.6	−4.0	−1.3

[a] From ITC titration experiments at pH 7.5 (10 mM phosphate, 0.1 M KCl, 1 m*M* EDTA), 25°C. All values are the average of at least triplicate measurements. The standard deviation of the $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ values are approximately 0.18 kcal/mol and 1.5 kcal/mol, respectively, for 16 determinations of L-trp. For the other ligands the standard deviations are similar. The concentration of apo trpR was $(3\text{–}8)\times10^{-6}$ *M*. The values of $\Delta G^{\circ\prime}$ were calculated as $\Delta G^{\circ\prime} = -RT \ln K$. The values of $\Delta S^{\circ\prime}$ were calculated as $-(\Delta G^{\circ\prime} - \Delta H^{\circ\prime})/T$.

[b] Obtained via a direct ITC titration.

[c] Obtained by a displacement ITC titration, with L-trp used as the displacing ligand.

[d] The $\Delta H^{\circ\prime}$ for L-trp was also determined to be −10.0 kcal/mol at 15°C. From this value and the $\Delta H^{\circ\prime}$ at 25°C we calculate the heat capacity change, $\Delta C_p^{\circ\prime}$, for binding to be −230 cal/mol deg.

[e] The alternate enantiomer, D-W(I), was also prepared. It showed an association constant to apo trpR that is 9-fold lower than that for L-W(I) and was not studied further.

restricted analogs do not bind in exactly the same manner as does L-trp, but that their indole ring slips into the aromatic binding site with the cyclic side chain making poor interactions with the protein. We have no information as to whether the restricted analogs act as co-repressors or pseudo-repressors.

4. Acknowledgement

This research was supported by NSF Grant DMB 91-06377. We thank Dr. Kathleen S. Matthews, Rice University, for providing us with plasmid pJPR2-containing *E. coli*. We also thank Dr. Marty Straume, University of Virginia, for providing advice on using the titration microcalorimeter.

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