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Characterization of the role of side-chain interactions in the binding of ligands to apo trp repressor: pH dependence studies

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Abstract

The pH dependence of the association of apo trp repressor with the series of ligands, tryptophan, tryptamine, indole propionic acid (IPA), and *trans*-β-indole acrylic acid (IAA), has been studied using fluorescence titrations and isothermal titration microcalorimetry (ITC). The purpose of such a comparison of ligands and the pH dependency studies is to reveal the role played by the side-chain functional groups in the energetics of the binding of the ligands to the protein. We find that, whereas the binding of tryptamine and IPA have essentially no pH dependence between pH 6 and 10, the binding of tryptophan and IAA depends on pH. For IAA, the affinity drops between pH 6 and 10, consistent with a shift in pK_a of some group on the protein from a value of pK_a 7.4 to 7.9 upon binding of this ligand. The affinity of IAA also drops below pH 5, but shows saturable binding at pH 2–3, where the protein has previously been found to exist as a partially folded monomeric state. For tryptophan, the pH dependence data indicate that the equilibrium is complicated. We present a model to describe the data in which the α-ammonium group of tryptophan has its pK_a shifted upward upon binding (i.e. preferential binding of the protonated form of this functional group) and in which the pK_a of an unknown group on the protein also has its pK_a increased. © 1997 Published by Elsevier Science B.V.

Keywords: Tryptophan; Trp repressor; Isothermal titration microcalorimetry; Indole propionic acid; Tryptamine; *trans*-β-Indole acrylic acid; Thermodynamics of binding

1. Introduction

Trp repressor (*trpR*) is a prototypical helix–turn–helix homo-dimeric DNA binding protein and has the biological function of repressing the transcription of genes involved in the biosynthesis of tryptophan [1]. Specific binding to the DNA operator occurs when the corepressor, tryptophan, is also bound to the protein [2–4]. When tryptophan binds to the apo*trpR*, there is a repositioning and bracing of the helix–turn–helix "reading heads", so that the binary complex (called holo*trpR*) can then more optimally fit within two adjacent major grooves of the operator sequence [5–10].

Abbreviations: apo*trpR*, apo form of trp repressor; holo*trpR*, binary complex of trp repressor and tryptophan; IAA, *trans*-β-indole acrylic acid; IPA, indole propionic acid; ITC, isothermal titration microcalorimetry; TAM, tryptamine

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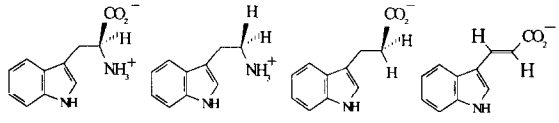
There has been much interest in the interaction of the holo*trpR* with its operator. In this paper we focus on the thermodynamics of the formation of the binary complex between the apo*trpR* and tryptophan. There have been a number of studies of the binding of tryptophan and a variety of its analogs to apo*trpR* [2,11–17]. Indole propionic acid (IPA) and *trans*-β-indole acrylic acid (IAA), which lack an α-NH_3^+ group, are found to bind better to the protein than does tryptophan. Conversely, tryptamine (TAM), which lacks an α-CO_2^- group, is found to bind more weakly than does tryptophan. The indole ring seems to be the primary determinate for binding, since poor binding is obtained with an *N*-methylindole, phenyl, or indoline ring [12]. (Some modifications of the benzene part of the indole ring are tolerated.) This information suggests that the indole ring and α-CO_2^- group contribute favorably to binding, and that the α-NH_3^+ group disfavors binding.

To confirm the above suggested roles of the various functional groups in determining the affinity and specificity of the interaction of the corepressor with apo*trpR*, we have studied the pH dependence of the binding of tryptophan, IPA, IAA, and TAM, using a combination of isothermal titration microcalorimetry (ITC) and fluorometric titration methods. There have been only limited, previous studies of the pH dependence of interactions of tryptophan with this protein [18], and no systematic pH dependence studies with analogs. From the above presumed unfavorable contribution of the α-NH_3^+ group, we expected to find that the affinity of tryptophan and TAM would increase as pH is increased and the ammonium group is deprotonated. However, we will see that the data are not as expected from this simple model.



2. Materials and methods

2.1. Materials

L-Tryptophan, IPA, IAA, and TAM were obtained from Sigma Chemical Company. Trp aporepressor was isolated from an overexpressing strain of E. coli, as described by Joachimiak et al. [19] and then Chou et al. [14]. Based on SDS-PAGE experiments, the protein preparation was found to be more than 90% pure. Protein samples were stored at −70°C at a concentration of about 50 μM in buffer composed of 0.1 M KCl, 1.5 mM EDTA, and 0.01 M potassium phosphate at pH 7.5. Prior to a fluorescence or ITC study, the frozen sample was thawed, dialyzed versus the appropriate buffer, and the protein concentration was determined by measuring the absorbance at 280 nm, using a molar extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} per mole of subunit [19]. Each buffer contained 0.1 M KCl and 1.5 mM EDTA and the following buffer components for the pH range indicated: 10 mM sodium acetate (pH 2–5.5); 10 mM potassium phosphate (pH 6–8, with some measurements made at pH 5, 9, and 10.1); 10 mM sodium tetraborate (pH 8.5–10.5, for ITC studies); 10 mM sodium pyrophosphate (pH 8.5–12.5, for fluorescence studies). Some ITC studies were performed at pH 7.5 with different buffers: 10 mM HEPES, Tris, or potassium phosphate.

2.2. Methods

Steady-state fluorescence measurements were made with a Perkin-Elmer MPF44A spectrophotofluorometer, equipped with a thermojacketed cell holder. IAA binding studies were performed as described previously [15],

with excitation at 295 nm and emission at 340 nm. Briefly, fluorescence titrations were performed by adding aliquots of a concentrated solution of IAA ($(0.1–2.0) \times 10^{-3}$ M) to a standard 1 × 1 cm cuvette containing 2.0 ml of an apo*trpR* solution (at a concentration of about $(2–5) \times 10^{-6}$ M). As described in our earlier work, care was taken to minimize photolysis of IAA by closing the shutter when not making measurements and by using an excitation bandwidth of 2 nm. The fluorescence signal was corrected for dilution and for absorptive screening [15]. The data were then fitted to a model for the binding of IAA to two independent and identical binding sites. As we have shown, the binding of the first IAA molecule causes a quenching of essentially all of the tryptophanyl fluorescence of both subunits by what is most likely a resonance energy transfer mechanism. The model described in Hu and Eftink [15] was fitted to the IAA titration data using a non-linear least-squares program [20].

ITC binding studies were performed with an instrument designed by Freire et al. [21], as described previously [16]. This instrument has a feedback compensation feature, a twin cell design, and a sensitivity of about one microcalorie. In a typical experiment, 5 ml of a protein, at a concentration of 10–15 μM in subunits, was loaded into the microcalorimeter. After thermal equilibration, 15–20 aliquots of a concentrated ligand solution (tryptophan, IPA, or TAM, at concentrations that were approximately 50 times the reciprocal of the association constant) were injected into the calorimeter and the heat effect was integrated. The instrument has dual cells; injection of the ligand into a buffer blank enables direct subtraction of any heat effect due to dilution of the ligand. The data (cumulative heat, Q, versus total ligand concentration) were analyzed via a non-linear least-squares program to a general independent site model (Eq. (1)) with fitting parameters: K, the association constant for ligand; and ΔH^{α}, the apparent molar enthalpy change.

$$Q = \frac{n\Delta H^{\alpha}[\text{sites}]_0 K[L]}{1 + K[L]} \tag{1}$$

The number of binding sites per mole of protein, n, (expressed as subunit concentration) was fixed at $n = 1.0$ in the analysis, since the total site concentration, $[\text{sites}]_0$, (10–15 μM) was approximately equal to or lower than the reciprocal of the association constant; when n is floated in the analysis, values between 0.8–1.2 are frequently recovered, but the high correlation between n and ΔH^{α} leads to large uncertainties when both are floated at such relatively low ratios of $[\text{sites}]_0/K$. The apparent ΔH^{α} values were not corrected for proton transfer from the buffer. The buffers employed have relatively small heats of proton dissociation ($\Delta H_{\text{H dissoc}} = 1.0$ kcal mol^{-1} for the secondary pK_a of phosphate and 3.36 kcal mol^{-1} for borate), so that corrections would be small. Whether or not such a correction is made does not affect the value of K, which is the parameter of primary interest in this study. We have, however, performed ITC measurements of tryptophan binding in a set of buffers having different heats of proton dissociation at pH 7.5 (and having adequate buffering capacity at this pH) in order to estimate the magnitude of the proton transfer effect. These buffers were phosphate, HEPES ($\Delta H_{\text{H dissoc}} = 5.0$ kcal mol^{-1}), and Tris ($\Delta H_{\text{H dissoc}} = 8.0$ kcal mol^{-1}).

3. Results

3.1. IAA binding by fluorescence titration: pH dependence

Shown in Fig. 1(A) are typical data for the binding of IAA to apo*trpR*, as determined by quenching of the protein's fluorescence. Data and fits to an independent site model are shown for pH 2.5, 6.0 and 11.9. Shown in Fig. 2 is a plot of the apparent free energy change for binding as a function of pH from 2 to 12.5 at 25°C. To obtain fluorescence data between pH 3 and 6 we worked with protein concentrations of less than 1.0×10^{-6} M. This was necessary to minimize the turbidity of the protein solution. Apparently, apo*trpR* undergoes a self-association that results in light scattering in this pH range. Since the isoelectric point of apo*trpR* is 5.9 [18], this self-association is not surprising. At pH 2–3 and above pH 6 the solutions are clear. The strongest binding

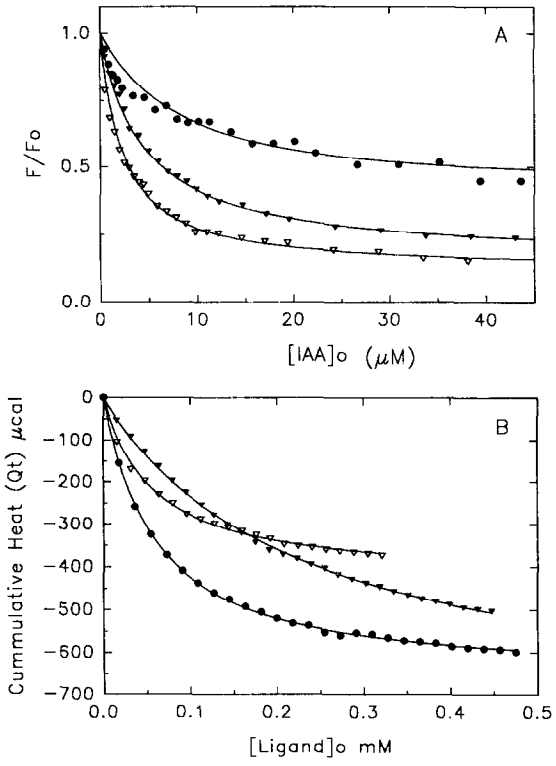


Fig. 1. (A) Typical fluorescence quenching binding data and fits for the interaction of IAA with apo*trpR* at pH 2.5 (●), 6.0 (▽), and 11.9 (▼). The association constants used to fit the data are given in Fig. 2. (B) Typical ITC binding isotherm data and fits for the association of tryptophan (●), IPA (▽), and TAM (▼) with apo*trpR* at pH 7.5. The associations constants and $\Delta H^{o\prime}$ used in the fits are given in Table 1.

is observed around pH 6, with a larger drop in affinity at lower pH and a smaller drop at higher pH. The association constant decreases by a factor of about 3–4 from pH 6 to pH 10–12.

The quenching of intrinsic apo*trpR* fluorescence provides a convenient way to monitor the binding of IAA, since this ligand is non-fluorescent and since its binding quantitatively quenches the tryptophanyl fluorescence of the protein. For other ligands, however, the fluorescence titration method is not amenable, since the ligands are fluorescent themselves. For tryptophan, IPA, and TAM, we have therefore used a second method (ITC) for studying the binding process.

3.2. ITC studies of the binding of tryptophan, IPA, and TAM: pH dependence

Shown in Fig. 1(B) are typical ITC enthalpic binding isotherms and fits for the titration of apo*trpR* with tryptophan, IPA, and TAM at pH 7.5. For each ligand, the binding process is exothermic. In fact, the binding of each ligand is exothermic over the pH range 5–10 (10.5 for tryptophan). The apparent $\Delta G^{o\prime}$ and $\Delta H^{o\prime}$ values given in Table 1 are for an independent site binding model. The enthalpic titrations were well described by the independent site model (i.e. the binding isotherms were hyperbolic, with no indication of cooperative or anti-cooperative binding). The entries in Table 1 are an average of 2–8 replicate experiments. The pH range was limited at low pH by the above-mentioned tendency of the protein to self-associate, although we did push a measurement to pH 5 for tryptophan. The binding of tryptophan and TAM was not extended to higher pH values since the weaker binding requires higher stock concentrations of the ligands; the solubility of TAM diminishes

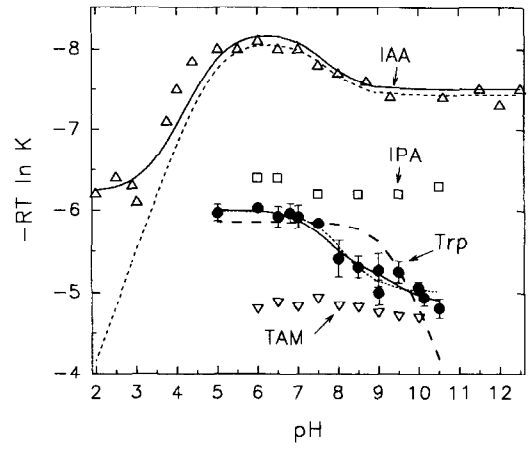


Fig. 2. Plot of $\Delta G^{o'}$ for the binding of IAA (△), IPA (□), tryptophan (●), and TAM (▽) to apo*trpR* as a function of pH at 25°C. The IAA binding data were obtained by fluorescence titrations; the IPA, tryptophan, and TAM binding data were obtained by ITC measurements. The dotted line through the IAA data is a fit to a model in which only the unprotonated (carboxylate) form of IAA binds (fixing the $pK_{a,LH}$ of free IAA as 4.9), with a group on the protein shifting its $pK_{a,MH}$ from 7.48 ± 0.25 (free protein) to $pK_{a,MHL} = 7.98 \pm 0.20$ for the complex; the association constant, $K_{L,MH}$ for the IAA carboxylate and the protonated form of the protein is $(9.3 \pm 0.8) \times 10^5$ M^{-1}. The solid line is a fit with a model in which both the protonated and unprotonated forms of IAA can bind. The fit is with $pK_{a,LH} = 4.9$ (fixed) for free IAA, $pK_{a,LHMH} = 3.61 \pm 0.07$ for bound IAA, $pK_{a,MH} = 7.52 \pm 0.25$ for the group on the free protein, $pK_{a,MHL} = 8.00 \pm 0.23$ for the group on the complex, and $K_{L,MH} = (9.1 \pm 1.0) \times 10^5$ M^{-1} for the association constant of unprotonated IAA with the fully protonated form of the protein.

Three fits are shown through the tryptophan data. *Model 1*: The long-dashed line (— — —) through the tryptophan data is for a model in which only a ligand with its α-NH_3^+ protonated can bind. This fit is with $pK_{a,LH} = 9.3$ (fixed) for free tryptophan and the association constant of this protonated ligand, K_L, equal to $(2.0 \pm 0.8) \times 10^4$ M^{-1}. *Model 2*: The short-dashed line (- - -) is a fit for a model in which either the protonated or unprotonated α-NH_3^+ forms of tryptophan can bind equally well, and with the pH dependence due to a shift in the pK_a of a group on the protein. The fit is with $pK_{a,MH} = 7.75 \pm 0.27$ for the free protein, $pK_{a,MHLH} = pK_{a,MHL} = 8.47 \pm 0.27$, and the association constant of the ligand to the protonated form of the protein, $K_{L,MH} = (2.57 \pm 0.37) \times 10^4$ M^{-1}. *Model 3*: The solid line (————) is a fit to a model in which both the pK_a of the α-NH_3^+ group of tryptophan and the pK_a of the group on the protein are perturbed upon binding. The fit is with the perturbation of the pK_a of the free ligand from $pK_{a,LH} = 9.3$ (fixed) to the bound state value of $pK_{a,LHMH} = pK_{a,LHM} = 9.62 \pm 0.07$, and with the perturbation of the pK_a of the group on the free protein from $pK_{a,MH} = 7.5$ (fixed) to the bound complex value of $pK_{a,MHLH} = 8.0$ (fixed), with the association constant of $K_{LH,MH} = (2.63 \pm 0.16) \times 10^4$ M^{-1} for binding the protonated form of the ligand to the protonated form of the protein.

greatly above pH 10 as its ammonium group becomes deprotonated. The binding of IPA was studied less extensively; data for this ligand (at least the $\Delta H^{o'}$) were found to be less reproducible, possibly due to the lower solubility of the ligand (and possible adsorption onto surfaces) of the higher affinity (and associated errors due to seepage from the tip of the injection syringe into the cell).

The ITC measurements were all made within a protein concentration range of 10–15 μM (expressed as monomer). This is a lower concentration range than used by other groups in ITC studies. We have used this relatively low concentration range, and have added 0.1 M KCl to the buffer, to minimize the self-association of the dimeric protein to form higher-order aggregates [22,23]. With the conditions employed we should be studying binding primarily to the dimeric form of the protein. Note also that our previous studies of the pH dependence of the urea-induced unfolding of the protein indicate that the protein is dimeric and native-like up to a pH of 12 [24].

The $\Delta H^{o'}$ values are uncorrected for protonic equilibria. The heats of proton dissociation of phosphate and borate buffer are fairly small, making such correction likely to be less than about 1 kcal mol^{-1} for $\Delta H^{o'}$ values at most pHs. The pH dependence of the binding process (for tryptophan, at least) indicates that there is a transfer of protons from the buffer to the protein–ligand complex upon binding (see the model presented in

Table 1
Thermodynamic parameters for the binding of ligands to apo*trpR* as a function of pH [a]

pH	L-Tryptophan		IPA		TAM	
	$\Delta G^{o\prime}$	$\Delta H^{o\prime}$	$\Delta G^{o\prime}$	$\Delta H^{o\prime}$	$\Delta G^{o\prime}$	$\Delta H^{o\prime}$
5.0 [b]	−5.97±0.10	−9.2±1.2	ND	ND	ND	ND
6.0 [b]	−6.02±0.02	−10.0±0.9	−6.40±0.02	−9.7±1.3	−4.81±0.04	−14.7±0.6
6.5 [b]	−5.92±0.13	−10.6±2.4	−6.40±0.08	−14.0±1.9	−4.89±0.04	−14.40±0.05
6.8 [b]	−5.97±0.12	−11.7±1.5	ND	ND	ND	ND
7.0 [b]	−5.92±0.14	−12.4±1.7	ND	ND	−4.84±0.08	−14.9±2.4
7.5 [b]	−5.84±0.06	−12.2±1.5	−6.20±0.14	−14.3±3.9	−4.94±0.11	−15.6±2.5
8.0 [b]	−5.41±0.22	−15.7±0.45	ND	ND	−4.85±0.05	−18.1±1.0
8.5 [c]	−5.31±0.13	−16.1±0.39	−6.20±0.15	−14.1±0.5	−4.83±0.06	−18.1±0.35
9.0 [c]	−5.27±0.20	−16.9±0.15	ND	ND	−4.76±0.01	−16.5±0.5
9.0 [b]	−4.99±0.15	−18.5±1.2	ND	ND	ND	ND
9.5 [c]	−5.25±0.14	−17.7±2.8	−6.20±0.01	−14.4±2.5	−4.72±0.12	−18.7±2.6
10.0 [c]	−5.06±0.07	−19.7±1.35	ND	ND	−4.70±0.07	−24.1±4.1
10.1 [b]	−4.94±0.10	−19.8±1.5	ND	ND	ND	ND
10.5 [c]	−4.81±0.12	−20.2±4.1	−6.30±0.1	−10.9±1.6	ND	ND

[a] All measurements were made at 25°C and are in units of kcal mol^{-1}. Values are an average of 2–8 replicates. ND = not determined. The protein concentrations were 10–15 μM in binding sites. We have also made a single ITC measurement of the binding of IAA to apo*trpR* and find a $\Delta G^{o\prime}$ association constant of −7.5 kcal mol^{-1}, in reasonably good agreement with the values determined by fluorometric titration.
[b] Sodium phosphate buffer (10 mM), with 0.1 M KCl and 1.5 mM EDTA.
[c] Sodium borate buffer (10 mM), with 0.1 M KCl and 1.5 mM EDTA.

Section 4, in which the pK_as of groups on the ligand and/or protein are shifted upwards upon binding). To independently assess the magnitude of this proton transfer effect, we have measured the apparent $\Delta H^{o\prime}$ for tryptophan binding to apo*trpR* at pH 7.5 in three different buffers (phosphate, HEPES, Tris) of known heats of proton dissociation, $\Delta H_{\text{H dissoc}}$. Shown in Fig. 3 is a plot of these apparent $\Delta H^{o\prime}$ values for tryptophan binding versus the $\Delta H_{\text{H dissoc}}$ of the buffers. (The association constants for tryptophan were found to be $(1.8 \pm 0.4) \times 10^4$ M^{-1} for each buffer.) The slope of such a plot gives, Δn, the number of protons transferred from the buffer to the protein–ligand complex upon binding.

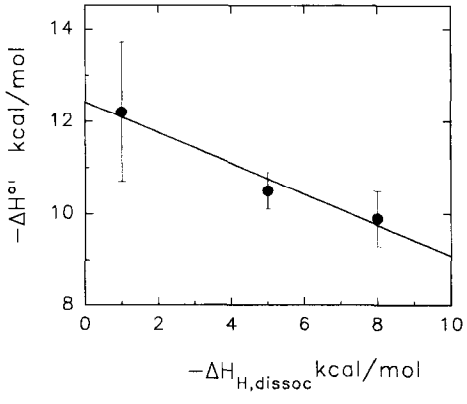


Fig. 3. Plot of the apparent $\Delta H^{o\prime}$ for the binding of tryptophan to apo*trpR* at 25°C and pH 7.5 in Tris buffer, HEPES buffer, and phosphate buffer (left to right) versus the heat of proton dissociation of each buffer. The slope is $\Delta n = 0.33$, the proton transfer from buffer to the protein–ligand complex upon binding; the value of $\Delta H^{o\prime}$ extrapolated to zero heat of proton dissociation is the value of $\Delta H^{o\prime}$ for the binding reaction that is "corrected" for the proton transfer contribution.

We find a value of $\Delta n = 0.33$ protons absorbed by the protein–ligand complex (per mole of site filled). The intercept (at buffer $\Delta H_{\text{H dissoc}} = 0$) is the intrinsic $\Delta H^{\alpha} = -12.6$ kcal mol^{-1} for the ligand binding reaction at pH 7.5, corrected for the identity of the buffer (assuming that the buffer only contributes via this heat of proton dissociation effect). This intrinsic ΔH^{α} may itself depend on pH due to contributions from heat of protonation of protein or ligand functional groups. The value of Δn is likely to be smaller at pH below 7, where the ΔG^{α} versus pH profile is flat; we expect the Δn values to be non-zero and positive from pH 7.5 to 10.5. Although it is clear that there is a non-zero Δn value for tryptophan binding, and that the magnitude of this Δn value should depend on pH, we have not corrected our data for this proton transfer effect, since our primary interest is in the pH dependence of the association constants. To give an example of the expected magnitude of the proton transfer correction, the apparent ΔH^{α} for a ligand binding reaction is equal to the sum of the intrinsic ΔH° of binding plus a contribution of $\Delta n \times \Delta H_{\text{H dissoc}}$. At pH 7.5 with phosphate buffer, the magnitude of the term for proton transfer from buffer should be only about 0.33 kcal mol^{-1}, so that the actual intrinsic ΔH° is 0.33 kcal mol^{-1} more negative than the apparent ΔH^{α} of 12.2 kcal mol^{-1} at this pH. For the pH range where borate buffer was used (pH 8.5–10.5), the correction is expected to be larger, but still less than 10% of the magnitude of the apparent ΔH^{α}.

The entropy change values are not listed in Table 1, but they are large and negative for each ligand, since the binding is exothermically driven. Jin et al. [17] and Hu and Eftink [16] have previously determined the ΔC_p value for tryptophan binding (at pH $\approx$ 7.5) to be about -230 cal mol^{-1} deg^{-1}.

In Fig. 2 is shown the pH dependence of the apparent ΔG^{α} for the binding of tryptophan, IPA, and TAM to apo*trpR*. Whereas the affinity of IPA and TAM shows little change with pH, the affinity of the natural ligand, tryptophan, shows the largest variation with pH, having an affinity that is nearly equal to that of IPA at pH 6–7 and an affinity that approaches that of TAM at pH 10–10.5. The apparent ΔH^{α} for ligand binding (see Table 1) has a tendency to become more exothermic as pH is increased for tryptophan and TAM, whereas the ΔH^{α} seems to be relatively independent of pH for IPA.

4. Discussion

An interesting feature of the binding of ligands to apo*trpR* is that there apparently is no interaction between the two binding sites [11]. That is, the binding of ligands can be described by the existence of a pair of identical, non-interacting sites on this dimeric protein. X-ray crystallographic studies show that each of the two identical binding sites of apo*trpR* is formed by residue side chains of the C and E helixes of one subunit and the turn between the B and C helixes of the second subunit. In addition, the two subunits are intertwined. It is rather surprising that the individual sites can be filled with ligand without altering the affinity of the second site. A possible molecular explanation for this observation is that the inter-subunit interface region forms a very rigid central core [12], such that there is little or no thermodynamic communication between the two sites.

Since the goal of this study is to explore the various elementary interactions involved in forming the binary complex, we will briefly discuss what is already known about these interactions. Equilibrium binding studies by Sigler and co-workers [12] and others [2,11,14,17,25] have shown the importance of three moieties of tryptophan, that is, the indole ring, the α-CO_2^- group, and the α-NH_3^+ group. Indole itself is a relatively good ligand for apo*trpR*, with a ΔG° of binding of about -5.8 kcal mol^{-1} at pH 8.1 and 4°C [12]. Some substitutions at the 5, 6, and 7 positions on the indole ring are tolerated, but the interaction is reduced by other substitutions and by changing the indole ring to 7-azaindole or a phenyl ring (i.e. over a 1000-fold reduction in affinity in the latter case) [12]. Addition of the α-CO_2^- group (to form IPA) results in about a threefold improvement in the association constant (or an improvement in the ΔG° value of about 0.5 kcal mol^{-1}). Formation of a favorable electrostatic interaction between this ligand CO_2^- group and the positively charged guanido group of Arg-84 provides an explanation for this improvement in binding [5].

If a side-chain α-NH_3^+ group is added to the ethylindole nucleus, the binding is weakened at neutral pH (an unfavorable ΔG contribution of 0.3–0.5 kcal mol^{-1}, which will depend on temperature, pH and possibly ionic strength) [12,25]. The logical interpretation of this observation, as stated in Section 1, is that there is some type of unfavorable interaction involving the α-NH_3^+ group. This leads to the prediction (found not to be correct in this study, however) that deprotonation of this group to the free α-NH_2 form should result in an improvement of binding. Starting with these presumed roles of the three moieties of tryptophan, we now consider our pH dependence studies.

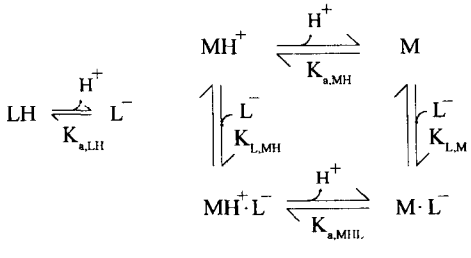
The pH dependence of the binding of IAA shows a multi-phasic pattern. The pattern can be explained by the simple thermodynamic model shown in Scheme 1.

Here the protein is considered to have a proton dissociating group, such that both a protonated (MH^+) and an unprotonated (M) form of the protein exist. It is possible that there is more than one group on the protein having a pK_a coupled to ligand binding; we present here the simplest model to account for the linked binding. The pK_a of this group is allowed to change upon binding of the ligand IAA (with $pK_{a,MH}$ for the free protein and $pK_{a,MHL}$ for the binary complex). Free IAA has a pK_a of 4.9 (determined in our laboratory from absorbance change measurements). In the simplest version of this model, only the anionic form, L^-, of IAA is assumed to bind to the protein. With these assumptions, the pH-dependent association constant is

$$K(\mathrm{pH}) = \frac{K_{L,ML}(1 + K_{a,MHL}/[H^+])}{(1 + K_{a,MH}/[H^+])} \frac{1}{(1 + [H^+]/K_{a,LH})} \tag{2}$$

The dotted line in Fig. 2 is a fit of this equation (as $\Delta G^{\circ}(\mathrm{pH}) = -RT \ln K(\mathrm{pH})$) with $pK_{a,LH} = 4.9$ (fixed), $pK_{a,MH} = 7.48 \pm 0.25$, $pK_{a,MHL} = 7.98 \pm 0.20$, and $K_{L,MH} = (9.3 \pm 0.8) \times 10^5\ M^{-1}$. In other words, there is an increase from 7.5 to 8.0 in the pK_a of some group (or groups) on the protein upon binding L^-. This model fits the data relatively well from pH 6 to 12.5. To fit the data below pH 6, we have extended this model to consider the binding of both the protonated, LH, and unprotonated, L^-, forms of IAA to the protein. Essentially, this model has an extra factor of $(1 + K_{a,LHMH}/[H^+])$ in the numerator, where $pK_{a,LHMH}$ is the pK_a of LH when it is bound to the protein. The solid line though the IAA data in Fig. 2 is a fit with this second model, with $pK_{a,LH} = 4.9$ (fixed), $pK_{a,MH} = 7.5 \pm 0.3$, $pK_{a,MHL} = 8.0 \pm 0.2$, $pK_{a,LHMH} = 3.6 \pm 0.1$, and $K_{L,MH} = (9 \pm 1) \times 10^5\ M^{-1}$.

According to this model, the neutral LH form of IAA can bind to the fully protonated form of the protein with an association constant of about $4 \times 10^4\ M^{-1}$, 25 times lower than the association constant of the anionic form of IAA. That is, there is an appreciable affinity of the neutral ligand for the protein. An interesting point about the binding of IAA at low pH is that we have previously shown, based on the thermodynamics of urea-induced unfolding of the protein, that dimeric apo*trpR* dissociates at low pH and behaves as a folded monomer at pH 2–3 [24]. Such a monomeric form of apo*trpR* shows some loss of secondary structure and an expanded hydrodynamic volume, characteristic of a molten globule-like state. The present results seem to indicate that this partially folded, monomeric form of apo*trpR* is capable of binding IAA in a saturable manner



Scheme 1.

with modest affinity. (In view of this consideration that the protein undergoes a dimer $\rightleftarrows$ 2 monomers equilibrium at low pH, the above thermodynamic model and fit are oversimplified.)

Following from the results with IAA, one might have expected that the binding of IPA would show the same affinity and pH dependence, since the two ligands are chemically similar. The association constant for IPA is about a factor of 20 weaker at neutral pH, as compared to that for IAA. However, what is more interesting is that the binding of IPA does not show a pH dependence for its apparent K from pH 6 to 10. (Note that the $\Delta G^{o'}$ for IPA binding varies by only about 0.2 kcal mol^{-1} over the pH range 6–10, which is almost within the standard deviation of the determinations at an individual pH; by comparison, the $\Delta G^{o'}$ for IAA binding varies by 0.5–0.7 kcal mol^{-1} from pH 6 to 10. We did not do the ITC experiments for IPA binding below pH 6, due to the aggregation of the protein.) Whereas the binding of IAA is weakened by the acid dissociation of some group on the protein having $pK_a = 7.5$ (on the unligated protein), the binding of IPA does not show demonstrable evidence of being linked to this group. What can account for this difference between IAA and IPA? Although they both have an indole ring and a $-CO_2^-$ group, they differ in that the side chain of IAA is rigid and is fully extended in a trans conformation. The $-CH_2-CH_2-CO_2^-$ group of IPA has conformational flexibility.

In Fig. 4 we show a schematic of the binding site of *trpR*, with the different ligands inserted. Looking first at the drawings for the IAA and IPA complexes, the schematics show the indole ring to be inserted in its binding pocket and that the $-CO_2^-$ groups are interacting electrostatically with Arg-84. To account for the pH dependence of IAA binding, we propose above that there is some unidentified proton dissociating group (with $pK_a = 7.5$), which also stabilizes the IAA complex when this group is protonated. The identity of this putative group is unknown; none of the amino acid side chains in the immediate vicinity of the bound tryptophan appear to be likely candidates [5,27]. With a pK_a of about 7, one would think of a histidine residue, of which there are two, His-16 and His-35, on each subunit. From inspection of the crystal structure of the binary complex, His-16 is in the middle of the A-helix, which forms the rigid interfacial core of the protein. The imidazole group of His-16 is about 14 Å from the carboxylate of tryptophan bound to the same subunit, so any interaction between this histidine residue and the binding site must be indirect and not obvious from the crystal structure. Likewise, the other histidine residue, His-35, is located at the N-terminal end of the B-helix and its imidazole side chain is approximately 18 Å from the carboxylate of tryptophan bound to the second subunit. To account for the observation of a coupling to some proton dissociating group with IAA, but not with IPA, we must speculate that the fully extended trans conformation of IAA places its $-CO_2^-$ group in an optimal position for interacting (directly or indirectly) with the unknown proton dissociating group, whatever the identity of this group. IPA should be able to potentially adopt the same trans conformation as IAA. However, doing so would lock IPA into one particular rotamer about its side chain. Assuming that there are six equi-energetic rotamers about the χ_1 and χ_2 bonds of IPA, the loss of entropy due to locking in one particular rotamer would be $\Delta S = R \ln 6 = 3.5$ cal mol^{-1} K^{-1}, which at 298 K corresponds to a free energy penalty of about 1.0 kcal mol^{-1}. Our interpretation of the lack of a pH dependence for the IPA data is that there is a trade-off, for this ligand, between the favorable interaction with the protonated unknown group on the protein and the loss of entropy for locking in the trans conformation. Consequently, the interaction with the protonated unknown group may be greatly diminished or sacrificed in favor of retention of rotational freedom of the side chain on binding. Another possible explanation is that the side chain of IPA is oriented so as to interact maximally with Arg-84, whereas the trans conformation of IAA extends its $-CO_2^-$ away from Arg-84, allowing an interaction with the unknown group to come into play. We should also point out that a crystal structure of the binary complex between the protein and IPA shows its indole ring to be flipped 180° from the orientation of the indole ring for bound tryptophan or TAM [8]. We are uncertain as to the thermodynamic consequences of this flip; perhaps it is minimal, since the $\Delta H^{o'}$ for IPA binding is exothermic, as is that for tryptophan and TAM. In addition, we do not know whether the indole ring of bound IAA is flipped or oriented like that of tryptophan and TAM.

A surprising finding was that the affinity of TAM shows little dependence on pH. Since free TAM has a pK_a for its α-NH_3^+ group of 10.2 [28], we expected to find the affinity of this ligand to increase as we deprotonate

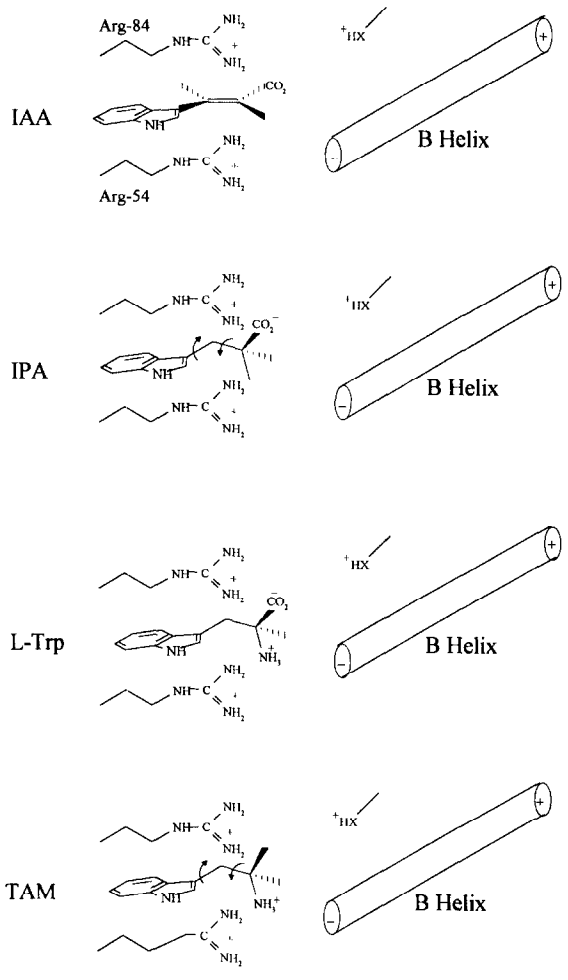


Fig. 4. Schematic representation of the binding site, filled with the various ligands. Redrawn from the figure shown in Joachimiak et al. [26].

this α-NH_3^+ group. In contrast, we find a slight decrease in affinity as we go from pH 6 to 10 (we are limited from studying higher pH due to the poor solubility of deprotonated TAM and the weak binding constant). We note that the protein is quite stable at high pH. In our previous urea-induced unfolding studies we found apo*trpR* to remain a native-like dimer (in the μM concentration range) from pH 4.5 to 12, so we do not think that the pH-dependent patterns, above pH 6, in Fig. 2 reflect subunit dissociation or unfolding.

The poorer binding of TAM at neutral pH is thought to be due to unfavorable interactions between its positively charged α-NH_3^+ group and positively charged amino acid side chains at the binding site, particularly those of Arg-54 and Arg-84. It has also been suggested that the α-NH_3^+ group interacts favorably with the negative end of the B-helix's intrinsic dipole, through hydrogen bonding with carbonyl oxygens of Leu-41 and Leu-43 of the same subunit and Ser-88 of the opposite subunit [5]. The lack of a significant pH dependence of the affinity of TAM suggests that there again is a trade-off between unfavorable and favorable interactions involving the α-NH_3^+ group, with the result being that the state of protonation of this group is not significantly perturbed by binding. Like IPA, the side chain of TAM is flexible and locking it into place, for example to interact maximally with carbonyl oxygens at the end of the B-helix would require a loss of conformational

entropy (again amounting to a free energy penalty of about 1.0 kcal mol^{-1}). Our interpretation is that the side chain of TAM remains fairly flexible when it binds and that the above-mentioned electrostatic interactions with the α-NH_3^+ group are roughly balanced.

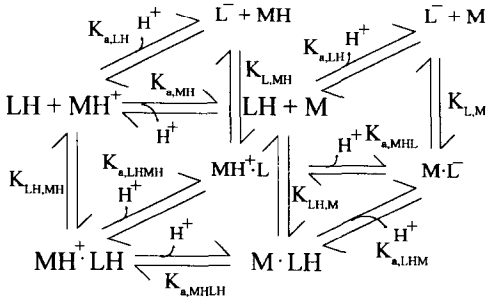
Tryptophan shows the largest change in affinity with pH. Tryptophan has both side-chain functional groups to consider. We assume that the most favorable interaction of the α-CO_2^- group will be with Arg-84 and that the most favorable interaction of the α-NH_3^+ group will be with the carbonyl groups at the end of the B-helix. A difference between tryptophan and the other ligands is that the entropy loss for forming these interactions only has to be counted once. That is, even if there is a free energy penalty of about 1 kcal mol^{-1} for locking the side chain into its optimal position, this penalty can be overcome by the favorable interactions of both the α-CO_2^- and α-NH_3^+ groups. The fact that the binding of tryptophan at neutral pH is not as strong as that of IPA indicates that the summed optimal interactions are not quite as favorable as that with the $-CO_2^-$ alone of IPA, which may mean that some compromise in positioning must occur when both functional groups are present on the side chain.

With the schematic in Fig. 4 in mind, one would expect the affinity of tryptophan to depend on the state of protonation of its α-NH_3^+ group, which is 9.3 for free tryptophan [28]. The postulated interaction with the B-helix would favor the protonated form of this functional group. NMR studies by Evans et al. [25] suggest that the α-NH_3^+ group of bound tryptophan remains protonated over the range of pH 8–10, consistent with the above suggestion that the protonated form of the ligand preferentially binds. The observed pH dependence of the ΔG° for tryptophan binding indicates that a shift in the pK_a of the α-NH_3^+ alone cannot account for the data. The long-dashed line in Fig. 2 through the tryptophan data is an attempted fit with Eq. (3), in which $pK_{a,NH}$ is the pK_a of the free ligand and only the protonated form of the α-NH_3^+ is considered to bind (i.e. the pK_a of this group shifts to an infinitely high value upon binding).

$$\Delta G^{\circ} = -RT\ln\left(\frac{K}{1 + K_{a,NH_3^+}/[H^+]}\right) \tag{3}$$

The actual pH dependence for tryptophan binding is more gradual than predicted by this equation. (Extension of this model to include a non-infinite shift in pK_a of the α-NH_3^+ group also was not able to describe the data.) The drop in affinity between pH 6 and 9 suggests that the unknown group on the protein, which is linked to IAA binding, also is linked to the binding of tryptophan. To test this hypothesis, we fitted the general model shown in Scheme 2 to the data.

This scheme includes a $pK_{a,MH}$ for some group on the free protein, which is shifted to $pK_{a,MHLH}$ when the LH form of the ligand binds (and shifts to $pK_{a,MHL}$ when the L^- form of the ligand binds). This model also includes the $pK_{a,LH}$ for the α-NH_3^+ group of free tryptophan (which was fixed at the known value of 9.3),



Scheme 2.

which shifts to a value of $pK_{a,LHMH}$ upon binding to the MH form of the protein (and shifts to $pK_{a,LHM}$ upon binding to the M form of the protein). The resulting general equation for this model is

$$\Delta G^{\alpha} = -RT\ln\left(\frac{K_{LH,MH}\left(1+[H^+]/K_{a,MHLH}+[H^+]/K_{a,LHMH}+[H^+]^2/(K_{a,MHLH}K_{a,LHM})\right)}{(1+[H^+]/K_{a,MH})(1+[H^+]/K_{a,LH})}\right)$$

The dotted curve in Fig. 2 is a fit of this model with the assumption that the pK_a of the ligand is not perturbed upon binding (i.e. $pK_{a,LHMH} = pK_{a,LHM} = pK_{a,LH}$, in which case all of the pH dependence is due to perturbation of the pK_a of an amino acid side chain(s)). The fit (dotted curve) captures the trend of the data, but it is not completely satisfactory. If we further consider both the pK_a of the amino acid side chain(s) and the pK_a of the α-NH_3^+ of the ligand to be perturbed upon binding (by floating the value of $pK_{a,LHM}$ and fixing $pK_{a,LH}$ to be that for free tryptophan) we obtain the solid line fit. This is arguably the best fit of the data.

The purpose of these fits with the above model is not to try to recover with confidence the thermodynamic parameters for the model. Instead, we present these fits to show that the above model can adequately describe the observed pH dependence of tryptophan binding. The essence of the interpretation is that the unexpected pH dependence for tryptophan binding can be accounted for in terms of the perturbation of at least two protonic groups, the α-NH_3^+ group of tryptophan and some unknown group on the protein. The α-NH_3^+ group is described as contributing modestly to the affinity of the protein via interactions with the carbonyl groups at the end of the B-helix (this interaction being stronger than that in TAM, since the entropy penalty for restriction of the side chain only has to be paid once).

The above model and the schematic drawings in Fig. 4 suggest that the positioning of the functional groups about the α-carbon are important. It is interesting in this context that D-tryptophan is found to bind about 20 times more weakly than does L-tryptophan [25]. Also we note that the above discussion has not focused on the positioning of the indole ring. It is possible that the degree of insertion of this ring within its binding pocket is altered for some of the ligands. The present data do not address this issue, although the importance of interactions with the indole ring is shown by previous works [12].

The pH dependence of the ΔH^{α} values should also be interpretable in terms of the above models. However, there are too many variables (e.g. the ΔH for each vertical binding step and horizontal proton dissociation step) to attempt a fit of the data, which for tryptophan shows only a gradual increase in the magnitude of $-\Delta H^{\alpha}$ with increasing pH (and, as mentioned above, small corrections to the $-\Delta H^{\alpha}$ for proton transfer effects have not been made)

Acknowledgements

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