Thermodynamics of Tryptophan-Mediated Activation of the trp RNA-Binding Attenuation Protein (TRAP)

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Summary

The *trp* RNA-binding attenuation protein (TRAP) functions in many Bacilli to control the expression of the tryptophan biosynthesis genes. Transcription of the *trp* operon is controlled by TRAP through an attenuation mechanism, in which competition between two alternative secondary structural elements in the 5´ leader sequence of the nascent mRNA is influenced by tryptophan-dependent binding of TRAP to the RNA. Previously, NMR studies of the undecamer (11-mer) suggested that tryptophan-dependent control of RNA binding by TRAP is accomplished through local ligand-induced protein folding. We now present further insights into this ligand-coupled event from hydrogen/deuterium (H/D) exchange analysis, circular dichroism (CD) spectroscopy, differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC). Scanning calorimetry showed tryptophan dissociation to be independent of global protein unfolding, while analysis of the temperature dependence of the binding enthalpy by ITC revealed an excess negative heat capacity change, a hallmark of binding-coupled processes. Analysis of the data using empirically determined parameters estimates that 16-19 residues per monomer of TRAP fold upon tryptophan binding, in agreement with qualitative analysis of residue-specific broadening in TROSY NMR spectra of the 91 kDa oligomer. The data presented provide new qualitative and quantitative insights into the mechanism of ligand-mediated TRAP activation.

Keywords

Isothermal titration calorimetry; binding-coupled protein folding; allosteric regulation; oligomer; *trp* RNA-binding attenuation protein

Introduction
The undecameric (11-mer) trp RNA-binding attenuation protein (TRAP) is responsible for controlling the transcription\textsuperscript{1; 2; 3; 4; 5}, and in some cases the translation\textsuperscript{6; 7; 8; 9; 10; 11}, of the genes responsible for tryptophan biosynthesis in many Bacilli. Transcriptional regulation of the trp operon in these Bacilli is achieved through attenuation, in which competing secondary-structural elements in the 5′ leader region of the nascent mRNA control the extent of transcriptional read-through of the structural genes\textsuperscript{3; 4; 5}. TRAP exercises transcriptional control by influencing the formation of these secondary-structural elements through tryptophan-dependent binding to the RNA. When tryptophan is limiting, TRAP is inactive and does not bind to the RNA. This allows a stable anti-terminator hairpin to form, promoting transcriptional read-through of the entire operon. However, when the intracellular tryptophan level is sufficient, TRAP binds to tryptophan and becomes activated to bind to eleven triplet repeats consisting of (G/U)AG’s in the 5′ leader region of the mRNA. When TRAP is bound, the anti-terminator RNA structure cannot form, allowing preferential formation of the terminator hairpin, thereby halting transcription in the leader region prior to the structural genes\textsuperscript{3; 4; 5}.

The crystal structures of tryptophan-activated Bacillus stearothermophilus TRAP in binary complex with tryptophan (1QAW)\textsuperscript{12} and in ternary complex with tryptophan and RNA (1C9S)\textsuperscript{13; 14} reveal an oligomer of eleven identical subunits related by an eleven-fold rotational axis of symmetry. The structure is comprised of eleven anti-parallel beta-sheets, with each sheet consisting of three strands from one subunit and four strands from the neighboring subunit (Figure 1). The tryptophan ligand is completely buried between the beta-sheets at the interface between subunits in a cavity that is largely apolar with a few polar side-chain and backbone moieties making hydrogen bond contacts to the sidechain amide and backbone amino and

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carboxyl groups of the tryptophan \(^{12; 14; 15; 16}\). The bound RNA wraps around the protein with conserved bases making specific contacts with TRAP residues \(^{13; 16; 17; 18; 19}\).

Until recently, limited data were available on the structure of inactivate, apo-TRAP; therefore, little was known about the allosteric mechanism of tryptophan activation of TRAP for RNA-binding. The oligomeric state of TRAP (11-mer) is not altered in the absence of tryptophan \(^{20; 21; 22}\), ruling out assembly as an activation mechanism. TROSY NMR studies of the 91-kilodalton TRAP protein both free and bound to tryptophan revealed severe exchange broadening for the backbone amide resonances of 19 residues in the tryptophan and RNA binding regions of the protein, while limited proteolysis revealed a ligand-dependent change in proteolytic accessibility for these residues \(^{21}\). Analytical ultra-centrifugation studies of apo- and holo-TRAP \(^{22}\) also suggest that holo-TRAP is more tightly packed than apo-TRAP, consistent with the protein becoming more structured upon tryptophan binding. These findings are consistent with the proposal that tryptophan activates TRAP to bind to its RNA target through binding-coupled local folding of the RNA binding surface of TRAP (Figure 2)\(^{21}\).

Here we report results aimed at providing a quantitative structural and thermodynamic description of the tryptophan binding-coupled transition that leads to TRAP activation. Hydrogen/deuterium (H/D) exchange experiments revealed increased protection of amide resonances upon binding tryptophan. Circular dichroism (CD) spectra of apo-TRAP are consistent with the proposal that in the absence of the ligand the oligomer is less structured than was observed in the crystal structure of holo-TRAP. A binding titration monitored by intrinsic tryptophan fluorescence indicated that the 11 tryptophan ligands bind to TRAP non-cooperatively. Scanning calorimetry showed that ligand dissociation involves a large thermal event separate from global protein unfolding. Measurement of the binding-associated change in
heat capacity ($\Delta C_p$) by isothermal titration calorimetry (ITC) revealed a larger change than expected from burial of solvent accessible surface area. The qualitative and quantitative data presented provide new insights into the structural and dynamic changes that accompany ligand-mediated activation of TRAP (Figure 2).

Results and Discussion

Tryptophan binding to *B. stearothermophilus* TRAP is non-cooperative

Prior to detailed analysis of the mechanism of TRAP activation we examined whether tryptophan binding to *B. stearothermophilus* TRAP is cooperative. We monitored binding of TRAP to tryptophan using the change in intrinsic fluorescence of the latter upon binding (Figure 3). The wavelength for the emission maximum of tryptophan fluorescence shows the typical blue shift (from 357 nm to 332 nm) and increase in intensity (~6-fold) that is expected upon transfer of tryptophan from a polar to nonpolar environment. The data were well described by a single-site binding quadratic (Figure 3, bottom), and analysis of the binding data using a Hill plot (Figure 3, inset) yielded highly linear data with a Hill coefficient of 1.02. Thus, although there are 11 tryptophan-binding sites in each TRAP oligomer, tryptophan binding to *B. stearothermophilus* TRAP appears to be non-cooperative. This behavior simplifies further analysis and allows the binding to be treated as identical and independent instead of requiring the use of more complicated partition functions.

Structural changes upon tryptophan binding: H/D exchange and CD

On the basis of site-specific NMR resonance linebroadening we previously proposed that ligand binding to TRAP involved a ligand-mediated change in protein dynamics – coupled folding. If folding were coupled to ligand binding, upon tryptophan addition to TRAP the amide hydrogens of residues involved in the folding transition should show increased protection
from exchange with solvent. Unfortunately, the majority of the TRAP residues of most interest (i.e., those in the tryptophan and RNA-binding regions; colored red and green in Figure 2) are broadened beyond detection in the apo-protein $^{21}$, preventing NMR-based measurement of the hydrogen/deuterium (H/D) exchange rates of these residues. Nevertheless, H/D exchange experiments (Figure 4) provided important insight into the ligand induced structural changes in TRAP. Although many of the amides show no measurable exchange even after a week, reflecting the presence of an extremely stable structured core in both apo- and holo-TRAP, increased protection from exchange in holo-TRAP is evident for several residues, including Ala12, Val19, Ile20, Leu36, Asp37, Glu40 and Ile61. Most of these are located distant from the site of tryptophan binding and thus protection cannot simply be a consequence of surface burial by the ligand. The increased protection of amides from Asp37 and Glu40 (Figure 4) is particularly telling, since these residues are involved in direct or water-mediated RNA base recognition $^{13}$, reflecting the mechanism by which ligand binding is transmitted to the RNA binding residues.

The structure of holo-TRAP reveals an all beta sheet protein $^{12; 14}$; therefore, if tryptophan binding leads to stabilization of secondary structural elements, one might expect a concomitant increase in beta-sheet signature in the circular dichroism (CD) spectrum. As expected, the CD spectrum of apo-TRAP has features characteristic of a protein composed of beta strands, beta turns and unstructured regions (Figure 5). Deconvolution of the apo-TRAP spectrum indicates ~12% less beta sheet than seen in the crystal structure of holo-TRAP $^{14}$, supporting the proposal that some of the beta strands are not well formed in the apo-protein. However, quantitation of secondary structure is less accurate for beta sheets than for alpha helices $^{33; 34}$, and the observed difference may be within the error of estimation. In addition, upon titrating tryptophan into TRAP, strong signals are evident in the CD spectrum (Figure 5) that result from interactions
between the tryptophan ligands \(35; 36\). The overlap of these signals with those regions of the spectrum that are typically diagnostic for beta structure complicates the use of CD spectroscopy to quantitate ligand-induced secondary structural changes in TRAP because the spectral changes report on ligand binding without quantitatively describing secondary structure.

**Thermodynamics of tryptophan binding to TRAP**

Difficulties in characterizing the ligand-induced structural/dynamic changes in TRAP using spectroscopic techniques led us to use changes in thermodynamic heat capacity (\(\Delta C_p\)) \(^{23}; 24; 25; 26; 27; 28; 30\) to characterize binding-coupled changes in protein structure using isothermal titration calorimetry (ITC). Excellent quality ITC data were obtained (Figure 6), with high signal-to-noise over a range of temperatures, enabling accurate determination of association constants (\(K_A\)), enthalpy changes (\(\Delta H\)), and stoichiometry of binding (\(n\)) (Table 1). Consistent with the fluorescence titration, the ITC data fit well to a one-site model allowing the sites to be treated as identical and independent, simplifying further analysis.

ITC data were recorded at five-degree intervals between 25 and 50 °C. Strong enthalpy/entropy compensation was observed, making the Gibbs free energy of binding (\(\Delta G\)) nearly independent of temperature with a change of only 22 cal mol\(^{-1}\) K\(^{-1}\) (Figure 7). Tryptophan binding was found to be slightly temperature dependent as noted previously \(^{21}\), with \(K_A\) values ranging from \(9.75 \times 10^6\) at 25 °C to \(1.17 \times 10^6\) at 50 °C.\(^{†}\) Using a linear fit of \(\partial T\Delta S/\partial T\), the characteristic temperature (\(T_s\)) \(^{28}\) at which the entropic contribution to binding (\(\Delta S_{assoc}\)) is zero was found to be 287.1 K. The temperature dependence of the binding enthalpy was found to be

\(^{†}\) In these studies the temperature dependence of binding was found to be in the opposite direction from the previous studies. Although the origin of this discrepancy is unknown, it is small, and may be a consequence of the differing conditions compared to the previous NMR-based experiment where the protein concentration (between 11-12 mM) was much higher than the \(K_d\) (\(\sim 1\) µM at 50 °C, Table 1).
linear in the measured temperature range (see below), and a large negative change in heat capacity ($\Delta C_p$) of $-366$ cal mol$^{-1}$ K$^{-1}$ was obtained from a linear fit of $\partial \Delta H / \partial T$ (Figure 7). This large heat capacity change is much larger than what would be expected from burial of hydrophobic surface area (see below).

Thermal (DSC) scans of apo- and holo-TRAP (Figure 8) showed the partial specific heat capacity to be linear in the pre-transition temperature range where ITC experiments were carried out. In some cases, coupling of binding to global unfolding has been shown to result in a non-linear temperature dependence of $\Delta H$, making quantitative analysis of the heat capacity changes by ITC more difficult$^{37}$; such nonlinearity results when measurements encompass the transition temperature of the coupled folding event. On the other hand, linear temperature-dependence of heat capacity terms is generally observed for a wide range of binding-coupled processes$^{25; 27; 28; 38; 39; 40; 41}$ as long as measurements are outside of the thermal transition region, as is the case here.

The independence of global and local unfolding was also revealed by the DSC analysis of apo- and holo-TRAP (Figure 8). The data showed the transition for ligand dissociation to be separate from that of global unfolding, occurring between 70 and 100 °C, with a midpoint temperature $T_m$ of 85 °C. Further, the high thermal stability for global unfolding of the oligomeric protein (> 100 °C) is evident from the data. Non-reversibility of the high-temperature transition and absence of a post-transition baseline precluded detailed deconvolution analysis of the traces. The fact that there are two thermal transitions in addition to that associated with ligand binding merits more careful investigation, but indicate that thermal disassembly and denaturation of the oligomeric complex are not tightly coupled.
Solvent accessible surface area and the expected $\Delta C_p$

The heat capacity change upon protein folding or protein-ligand binding is best understood to result from changes in solvent accessible surface area (ASA), with the largest contributions attributable to the hydrophobic effect \(23; 24; 25; 26; 27; 28; 29; 30\). Coupling of conformational changes \(30; 42\), including local protein folding \(28; 43\), to ligand binding leads to a greater heat capacity change than that expected from the burial of accessible surface area ($\Delta ASA$) upon binding. The heat capacity change expected from the burial of polar and nonpolar surface area ($\Delta C_p^{\text{calc}}$) has been parametrized \(29; 44; 45; 46\) and can be calculated from the three-dimensional structure of the complex. The change in polar and non-polar ASA due to burial of the tryptophan in the tryptophan-binding cavity was determined from the crystal structure of \(B.\) \textit{stearothermophilus} TRAP in complex with tryptophan (1QAW) \(14\) using the program STC \(47\) (Table 2). From the change in ASA, and the parameters of Spolar & Record \(28\) we calculated an expected change in heat capacity upon association ($\Delta C_p^{\text{calc}}$) in the absence of protein folding of -88 cal M\(^{-1}\) K\(^{-1}\); similar values are obtained with alternate parametrizations \(29; 44; 45; 46\), including when aromatic and aliphatic carbons are treated differently \(29\). The difference between the calculated value and that measured by ITC (-366 cal M\(^{-1}\) K\(^{-1}\)) demonstrates that the large negative $\Delta C_p$ is poorly accounted for by surface burial in the absence of some other binding-coupled event.

Contributions to $\Delta C_B$

Binding-associated changes in $\Delta C_p$ can have a number of origins \(23; 30\): burial of solvent accessible surface area, a binding-coupled change in the protonation state of either binding partner (proton linkage) \(48\), release of ions due to salt bridge formation during binding (ion
linkage) \(^{49}\), burial of tightly bound water molecules \(^{50};^{51}\), loss of translational and rotational degrees of freedom, changes in conformational entropy, and an induced fit conformational change \(^{23};^{24};^{25};^{26};^{27};^{28};^{42}\). In order to understand the nature of the binding event the magnitudes of possible contributors to \(\Delta C_p\) should be considered.

The intermolecular contacts between TRAP and bound tryptophan involve mainly hydrophobic contacts and polar interactions between groups that ionize outside of physiological pH ranges\(^{14}\). This suggested that proton or ion linkage were unlikely to contribute to the excess change in heat capacity; nonetheless, potential contributions from these sources were explored by performing titrations of the binding partners in buffers with different enthalpies of ionization \(^{48}\) and salt concentrations \(^{49};^{52}\). The change in enthalpy (\(\Delta H\)) of tryptophan binding to TRAP at 25 °C was measured in buffers with ionization enthalpies spanning 10 kcal mol\(^{-1}\) (Figure 9). The enthalpy of tryptophan binding was found to be nearly independent of the heat of ionization of the buffer. The binding affinity of TRAP for tryptophan was similarly found to be largely independent of salt concentration (Figure 9). Therefore, the contributions to the observed \(\Delta C_p\) from proton and ion linkage are very small.

Examination of the structure of the tryptophan-binding site in \(B.\ stearothermophilus\) holo-TRAP alone \(^{14}\) and bound to RNA \(^{13}\) reveals a surface-exposed water molecule bound between the carboxylate group of the tryptophan ligand and the sidechain of His49 in the majority of the binding pockets. Burial of water molecules in a molecular interface has been implicated in contributing to the measured \(\Delta C_p\) \(^{50};^{51}\), so possible contribution from this surface-exposed bound water molecule was considered. Estimates for the contribution of burying a water molecule in an interface (i.e., transfer of a water molecule from solution to the protein) have
been obtained from sorption isotherms\textsuperscript{53}, the standard enthalpies of anhydrous and hydrated inorganic salts\textsuperscript{54}, and from high-resolution structural data\textsuperscript{51}. These analyses arrived at an upper limit of -12 cal mol\(^{-1}\) K\(^{-1}\) for the contribution to \(\Delta C_p\) of burying a water molecule in an interface. Use of these estimates suggests that the contribution to the observed \(\Delta C_p\) of crystallographically-observed water molecules in the binding cavity is small compared to the observed \(\Delta C_p\) (–366 cal K\(^{-1}\) mole\(^{-1}\)). It should be noted, however, that the nature of these bound water-mediated contributions to \(\Delta C_p\) are not yet well understood and it has been proposed they can be insignificant\textsuperscript{54} or even significantly larger\textsuperscript{50}. Because we judge local ordering of one water molecule per binding site as unlikely to contribute significantly to the observed NMR resonance broadening\textsuperscript{21}, a maximal contribution of -12 cal K\(^{-1}\) mol\(^{-1}\) is assumed.

**Binding-coupled folding of TRAP**

When the large negative change in heat capacity cannot be accounted for by changes in solvent accessible surface area, changes in protonation state, ion release, or the burial of water molecules in the protein-ligand interface, it has been inferred that much of the excess heat capacity change results from changes in conformational mobility that accompany binding-coupled induced fit/local protein-folding\textsuperscript{23; 28; 30}. We followed the approach of Spolar and Record\textsuperscript{28}, which estimates the number of residues involved in coupled folding \((\mathcal{R})\) by dividing the experimentally measured excess \(\Delta C_p\), minus the estimated contributions from loss of translational and rotational entropy upon binding \(\Delta S_{rt}\), by an empirical value obtained from studies of protein folding of –5.6 cal mol\(^{-1}\) K\(^{-1}\). Using this approach, after correction for contributions from trapped interfacial water (-12 cal mol\(^{-1}\) K\(^{-1}\)), we estimate that upon tryptophan binding to TRAP, 16 residues fold per monomer (**Table 2**) (equation 7, methods). An alternative
to using a generic value of $-50 \text{ cal mol}^{-1} \text{ K}^{-1}$ for $\Delta S_{tr}$ is to use the available structural data to estimate the $\Delta S_{tr}$, which at the $T_s$ and in the absence of folding or conformational changes is equal to $\Delta S_{HE}$ (equations 6 and 8, methods)\textsuperscript{28}; from the calculated $\Delta \text{ASA}$, in the absence of folding we would predict a value of $-36$ for $\Delta S_{tr}$. (We note, however, that there remains considerable disagreement in the literature over the magnitude of the contribution of $\Delta S_{tr}$ to the overall entropy of association and has been proposed to be much smaller\textsuperscript{55}. ) Thus, from the resulting excess $\Delta C_p$ we conclude that the ligand-coupled loss of conformational mobility corresponds to the folding of 19 residues ($\Re$) per monomer of TRAP (Table 2).

The results of these calorimetric estimations (16-19 residues folded) are in remarkable agreement with NMR studies which showed that 19 residues in the tryptophan and RNA binding regions of apo-TRAP were severely exchange broadened\textsuperscript{21} (shown in green in Figure 2). As a check on the validity of the underlying approximations, we computed the $\Delta \text{ASA}$ for binding-coupled folding of the 19 residues identified by NMR, and obtained a $\Delta C_p$ for the binding-coupled folding event of $-427 \text{ cal mol}^{-1} \text{ K}^{-1}$ (Table 2). Because the unfolded residues are in loops between regions that remain structured even in apo-TRAP, this number is clearly an over-estimate of the possible change in folding, but agrees quite well with the experimentally measured value of $-366 \text{ cal mol}^{-1} \text{ K}^{-1}$. The agreement between these quantitative and qualitative analyses suggest that much of the excess $\Delta C_p$ measured by ITC directly reflects the changes in protein structure (folding) that lead to TRAP activation and to severe exchange-broadening in the apo-TRAP NMR spectrum.

Binding-coupled folding and a model for allosteric control of TRAP
The data presented lend strong support for a model for allosteric control of TRAP in which tryptophan-dependent coupled folding of the tryptophan and RNA-binding residues control RNA-binding (Figure 2)\textsuperscript{21}. In this model, the tryptophan and RNA-binding residues are dynamically disordered in apo-TRAP, preventing RNA-binding and allowing entry of the tryptophan to its binding-site. Tryptophan binding induces local folding or ordering of the RNA and tryptophan-binding residues, thereby forming the RNA-binding interface and activating TRAP to bind to its RNA target. We propose that the loss of conformational mobility\textsuperscript{28} (reduction in “soft vibrational modes”\textsuperscript{23}) in this RNA binding surface upon ligand binding is responsible for much of the large excess negative heat capacity detected in the calorimetric experiments.

Since the tryptophan is completely buried in tryptophan-activated TRAP, it seems reasonable that some of the residues in the tryptophan-binding site must remain dynamic even in the RNA-bound state in order to allow TRAP to remain sensitive to the levels of intracellular tryptophan. This prediction is in agreement with the observation that NMR resonances from the tryptophan-binding region remain exchange-broadened in tryptophan-activated TRAP (regions in red, Figure 2)\textsuperscript{21}. This observation is also consistent with the observed lack of cooperativity in tryptophan binding, as the persistent dynamics would result in, at best, weak coupling between adjacent binding sites. If, on the contrary, ligand binding were to lead to rigidification of one interprotomer interface, conventional allostery models\textsuperscript{32; 56; 57; 58; 59} would predict strong coupling and lead to undesirably high affinity tryptophan binding and loss of regulation.

The thermodynamic separation of ligand binding and protein denaturation (Figure 8) merits a final point of discussion. Tight coupling between ligand binding and global unfolding of
proteins has been widely observed, and deconvolution of thermograms from such systems has been used to obtain the relevant thermodynamic parameters. In contrast, the observed uncoupling of local and global unfolding in holo-TRAP is more reminiscent of the thermal unfolding behavior of proteins with independently folding domains. The thermodynamic separation of the two binding events permits detailed studies of the thermodynamics of TRAP activation by tryptophan independently of global unfolding. The fascinating and complex mystery of how TRAP regulates transcription is hereby further accentuated by the observation that TRAP activation seems to involve the discrete stabilization of an RNA binding “domain” that is thermodynamically distinct from the underlying oligomeric protein scaffold that supports it.

Materials and Methods

Preparation of apo-TRAP

*B. stearothermophilus* TRAP was expressed in *E. coli* and purified as described previously. Although tryptophan binding is relatively weak (~1 µM), complete removal of the tryptophan from TRAP by dialysis is inefficient. Therefore, to remove the tryptophan that co-purifies with the protein, TRAP was purified by reverse phase HPLC on a 1 x 25 cm C4 column (Vydac model 214TP) at a flow rate of 3 ml/min using a gradient elution with 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma) in water (buffer A) and 0.1% TFA in acetonitrile (buffer B). The gradient elution consisted of a 15 ml wash with 5% buffer B, a 30 ml gradient from 5-20% buffer B, a 30 ml wash with 20% buffer B (during which the tryptophan eluted), and a 90 ml gradient from 20-70% buffer B, where TRAP eluted between 40 and 60% acetonitrile. The
fractions containing TRAP were then lyophilized and the powder (~32 mg) was dissolved in 3 ml of 6 M guanidine hydrochloride (GdnHCl), 50 mM sodium phosphate at pH 8.0, and 100 mM sodium chloride (NaCl). The re-suspended TRAP was placed in a 3500 Da cutoff dialysis cassette (Pierce) and dialyzed against 0.5 L of 3 M GdnHCl, 50 mM sodium phosphate at pH 8.0, 100 mM NaCl for 12 hours at 55 ºC. The GdnHCl was then diluted to 1.5 M with 50 mM sodium phosphate at pH 8.0 and 100 mM NaCl (buffer C) and the protein was dialyzed for an additional 12 hours at 55 ºC. The protein was then dialyzed against 4 L of buffer C for 24 hours at 55 ºC, followed by an additional dialysis against 4 L of buffer C for 48 hours at 4 ºC. Previous studies have shown that TRAP can be denatured into unfolded monomers by GdnHCl and then renatured into fully functional 11-mers by simply removing the denaturant²⁰. The efficacy of the refolding procedure was confirmed by the ITC experiments, which yielded an n-value close to 11 (10.16-11.07, Table 1) for each oligomer.

H/D exchange of TRAP

Apo and holo-TRAP were exchanged into 90/10 (²H₂O/¹H₂O) by a ten-fold dilution into buffer C only in 99.9% ²H₂O appropriately adjusting the pH to account for the pH/pD difference. TRAP was then concentrated using an ultra-filtration centrifugal concentrator (Amicon). The first time point was taken approximately 1 hour after exchanging into ²H₂O. Spectra were recorded every hour for the first 24 hours with additional time points taken after 48 hours and one week.

CD of TRAP

All CD data were collected at 25 ºC from 180 to 300 nm at 1 nm intervals with a 2 nm bandwidth and 2.5 second averaging on an AVIV 202 circular dichroism spectrometer in a Starna Cells quartz cuvette with a 1 mm pathlength. The protein was prepared by diluting 560
µM apo-TRAP monomer in 50 mM Borate pH 8.0 with 100 mM NaCl with water to a final concentration of 19 µM apo-TRAP monomer. The holo-TRAP (1:1) complex was formed by adding 53 mM tryptophan to the apo-TRAP solution to a final concentration of 19.5 µM. Blank runs with buffer at the appropriate dilution or buffer and tryptophan were performed and subtracted from the final data. Data were normalized to the concentration of TRAP to give the molar ellipticity. Deconvolution of the apo-TRAP spectrum was performed using the self-consistent method 64 with multiple reference data sets as implemented in Dichroprot v2.6 33 and Contin-LL 34. The definition of the secondary structural elements present in the crystal structure was obtained using DSSP 65.

**Tryptophan fluorescence**

Protein concentration was estimated using the native extinction coefficient, 2798.6 M$^{-1}$ cm$^{-1}$ at 276 nm, determined using the theoretical extinction coefficient, 2900 M$^{-1}$ cm$^{-1}$ at 276 nm, and comparing the absorbance under denaturing (6 M GdnHCl and 100 mM sodium phosphate at pH 6.5) and native conditions (50 mM sodium phosphate at pH 8.0, 100 mM NaCl) 66. The tryptophan concentration was determined using the extinction coefficient 5579 M$^{-1}$ cm$^{-1}$ at 278 nm 67. For the concentrated solutions of protein or tryptophan, errors in the estimation of the concentrations were obtained from two different dilutions; for the dilute solutions, errors are the deviation of two measurements taken on the same solution. In order to ensure that the buffer conditions of the protein and ligand were identical, tryptophan (Sigma) was dissolved in the dialysate from the last dialysis of TRAP. All fluorescence experiments were performed on a Fluoromax-3 fluorometer with an excitation wavelength of 295 nm, an integration time of 0.1 s, 5 scan signal averaging, a slit width of 2 nm, and scans taken from 300-450 nm at 1 nm.
increments. The cell contained 2.8 ± 0.2 µM tryptophan and was titrated with 46.3 ± 0.3 µM apo-TRAP oligomer. Binding was monitored from the emission intensity at 332 nm after each addition of TRAP and the fraction of bound tryptophan was calculated from:

\[ r_i = \frac{(I_i - I_f)}{(I_b - I_f)} \]  

(1)

where \( r_i \) is the fraction of bound tryptophan after the \( i^{th} \) addition of TRAP, \( I_i \) is the signal intensity at 332 nm after the \( i^{th} \) addition of TRAP, \( I_f \) is the intensity at 332 nm of free tryptophan, and \( I_b \) is the intensity at 332 nm of fully bound tryptophan. The resultant binding curves were fit to a standard binding quadratic:

\[ r_i = 1 - \frac{([\text{Trp}] + [\text{TRAP}] \cdot n + K_d) - \sqrt{([\text{Trp}] + [\text{TRAP}] \cdot n + K_d)^2 - 4 \cdot n \cdot [\text{Trp}] \cdot [\text{TRAP}]}}{2 \cdot [\text{Trp}]} \]  

(2)

where \( r_i \) is the fraction of bound tryptophan, \( n \) is the number of binding sites, \( K_d \) is the dissociation constant, \([\text{Trp}]\) is the total tryptophan concentration, and \([\text{TRAP}]\) is the total TRAP oligomer concentration. The Hill coefficient was determined by taking the slope of the best fit line from the plot of the log \( (r_i/(1-r_i)) \) versus the log \([\text{TRAP}]_f \) (the free TRAP concentration).

**ITC of TRAP**

Protein and tryptophan concentrations were estimated as described above. All ITC data were obtained on a MicroCal VP-ITC with a 3 µl first injection followed by 5 µl injections with a spacing of 220 seconds between injections. The cell contained TRAP at a concentration of 5.1 ± 0.6 µM oligomer (the relatively large error in the estimation in concentration is due to the extremely low extinction coefficient of TRAP) and the syringe contained 662.1 ± 12.0 µM tryptophan. The exothermic heat pulse detected after each injection was integrated, the heat of
dilution subtracted from the integrated value, and the corrected heat value divided by the total moles of tryptophan injected. The resulting values were plotted as a function of the molar ratio of TRAP oligomer and fit to a one binding site model using a non-linear least squares method (Figure 6). All experiments were performed in triplicate and all data were fit to a one binding-site model using Origin v. 7 (MicroCal). Reported errors are the standard deviation in the values between the three data sets. In order to subtract the heat of dilution for both ligand and protein simultaneously, baseline runs were performed using the fully titrated TRAP (after removing the excess volume and refilling the syringe with ligand).

**Differential scanning calorimetry**

Protein and tryptophan concentrations were estimated as described above. All DSC data were acquired on a MicroCal VP-DSC with a scan rate of 60 ºC/hour and scans taken from 25 to 130 ºC at a starting pressure of 29 psi and cells were refilled at 27 ºC. The protein concentration for apo-TRAP was 562.3 µM TRAP monomer and for holo-TRAP was 550.5 µM (due to dilution from the addition of tryptophan). 26.6 mM tryptophan was added to a final concentration of 560 µM in the holo-TRAP sample. Data were normalized to the protein concentration and the holo-TRAP data were y-axis translated to make the initial baselines for the apo and holo-TRAP data coincident using Origin v. 7 (MicroCal).

**Solvent accessible surface area, \( \Delta C_{p}^{\text{calc}} \), and \( \Delta S_{\text{HE}} \)**

Solvent accessible surface areas (ASA) of both the tryptophan ligand and the tryptophan-binding cavities of TRAP were determined using STC v. 5.0 with extended atom radii and a probe radius of 1.4 Å. To measure the change in solvent accessible surface area upon tryptophan binding, separate PDB files were constructed containing one protomer of TRAP and one
tryptophan ligand from the crystal structure of the binary complex of tryptophan with *Bacillus stearothermophilus* TRAP (1QAW)\(^{14}\). In all, 23 files were generated, two for each protomer (one with each of the two tryptophan ligands that are contacted by that protomer). The average polar and non-polar solvent accessible surface areas of the tryptophan ligands were obtained from the STC calculated values for the free tryptophans. The average change in polar and non-polar solvent accessible surface area of the tryptophan binding pockets was calculated by adding the change in solvent accessible surface area for the two protomers that contact the same tryptophan. The overall change in polar and non-polar solvent accessible surface is the sum of the solvent accessible surface area of the tryptophan and the solvent accessible surface area of the binding pocket. The heat capacity change expected from the structural data (\(\Delta C_p^{\text{calc}}\)) was calculated from the change in ASA using\(^{28}\):

\[
\Delta C_p = 0.32(\Delta A_{np}) - 0.14(\Delta A_p)
\]

where \(\Delta A_p\) is the change in polar solvent accessible surface area, \(\Delta A_{np}\) is the change in non-polar solvent accessible surface area, and \(\Delta C_p\) is the change in heat capacity. The change in entropy due to the hydrophobic effect (\(\Delta S_{HE}\)) in the absence of a change in conformation or folding was calculated from\(^{28}\):

\[
\Delta S_{HE}(T_s) = 0.32(\Delta A_{np}) \ln(T_s / 386)
\]

where \(T_s\) is the temperature at which the entropy of association is equal to zero.

**Proton and ion linkage effects**

For the study of proton and ion linkage in tryptophan binding by TRAP, apo-TRAP was prepared as described above, but with one additional dialysis step. After refolding, the resultant protein was split into seven aliquots, which were placed into separate 3500 Da cutoff dialysis...
cassettes and dialyzed into the appropriate buffers. Buffers use to test for ion linkage were 50 mM sodium phosphate at pH 8.0 with no NaCl, 100 mM NaCl, 250 mM NaCl, and 500 mM NaCl. The buffers used for analysis of proton-linkage contributions were 50 mM sodium phosphate at pH 8.0, 100 mM NaCl; 20 mM Tris at pH 8.0, 100 mM NaCl; 20 mM HEPES at pH 8.0, 100 mM NaCl; ionization enthalpies were used as reported.

**Binding-coupled folding of TRAP**

As proposed by Spolar and Record, excess heat capacity changes can be used to obtain an estimate of the number of residues that fold upon ligand binding using the following procedure: Once the contributions to $\Delta C_p$ from other sources are determined, the remaining heat capacity is used to calculate the change in entropy due to the hydrophobic effect ($\Delta S_{HE}$):

$$\Delta S_{HE} = 1.35 \Delta C_p \ln(T / 386)$$  \hspace{1cm} (5)

where $T$ is temperature and the factor 1.35 assumes a $\Delta A_p/\Delta A_{ap} = 0.59$ (which is approximately the case in the folding of globular proteins). The entropy of association ($\Delta S_{assoc}$) can be further dissected at the characteristic temperature ($T_s$) where the overall entropy change is zero:

$$\Delta S_{assoc} (T_s) = \Delta S_{HE} + \Delta S_{rt} + \Delta S_{other} = 0$$  \hspace{1cm} (6)

where $\Delta S_{rt}$ is the change in entropy due to loss of rotational and translational freedom and $\Delta S_{other}$ is the change in entropy due to other events attributed predominantly to the loss of conformational mobility upon protein folding. Since at the $T_s$, the change in entropy of molecular association is zero, the magnitude of $\Delta S_{HE}$ must equal the sum of $\Delta S_{rt}$ and $\Delta S_{other}$. For molecular associations in which no conformational rearrangements of the ligand or receptor occur (rigid-body or lock-and-key mechanisms), $\Delta S_{other}$ should be negligible and $\Delta S_{HE}$ should be entirely balanced by $\Delta S_{rt}$. Analysis of a number of proteins for which structural information
suggests that there are no binding-coupled conformational rearrangements or folding suggests an empirical value for $\Delta S_{rt}$ of $-50 \pm 10$ cal K$^{-1}$ mol$^{-1}$. Analysis of the thermodynamics of protein folding suggests that the unfavorable entropic cost of protein folding is relatively constant $-5.6 \pm 0.5$ cal K$^{-1}$ mol$^{-1}$ when expressed per residue. Therefore an estimation of the number of residues that become folded upon binding can be obtained at the $T_s$ and is given by:

$$\mathcal{R} = \frac{1.35 \Delta C_p \ln(T_s / 386) - \Delta S_{rt}}{5.6}$$

where $\mathcal{R}$ is the number of residues that fold upon binding. In the absence of folding where $\Delta S_{other}$ is assumed to be zero, $\Delta S_{rt}$ is calculated from the structural data using:

$$\Delta S_{HE} = 0.32 \Delta A_{np} \ln(T_s / 386) = \Delta S_{rt} \text{ (at } T_s)$$

where $\Delta A_{np}$ is the change in non-polar surface area.

**Estimation of $\Delta C_p$ for binding-coupled folding from structural data:**

The 19 residues in the tryptophan and RNA binding sites that become exchange broadened upon removal of ligand, and thus may be involved in the coupled folding event (21-25, 31-35, 45-46, and 48-54) were modeled in an extended structure using MOLMOL$^68$ and ASA calculations were performed using STC v. 5.0$^47$ with both the extended structures and the tryptophan treated as the “ligand” for the calculations. The ASA buried by the linkage between the structured and unstructured regions was subtracted from the ASA and the resulting value was used to calculate the $\Delta C_p$ for binding-coupled folding using equation 3.

**Acknowledgements**

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**Figure legends:**

Figure 1: Ribbon diagram of the tertiary and quaternary structure of the undecameric TRAP in ternary (bottom; 1C9S) complex with tryptophan and RNA. Each subunit is displayed in a different color with the tryptophan residue shown in CPK and RNA in yellow. The tryptophan ligands are buried in the interface between subunits and the RNA wraps around the perimeter of the TRAP oligomer making contacts to beta sheet residues.

Figure 2: Schematic model for TRAP activation via ligand-dependent stabilization (folding) of the RNA binding surface of the oligomeric protein. Top and side views of the crystal structures of *B. stearothermophilus* TRAP in binary complex with tryptophan (1QAW) and in ternary complex with tryptophan and RNA (1C9S) are shown in the center and right, respectively. On the left is a model derived from NMR and proteolytic accessibility data, in which the RNA-binding residues of TRAP are poorly structured in the absence of tryptophan. Residues in green exhibited severe exchange broadening in NMR spectra of apo-TRAP, while residues in red were exchange broadened in both apo- and holo-TRAP. TRAP residues in blue could be assigned in both NMR spectra. Tryptophan ligands are shown as CPK models; for simplicity, green/red color-coding is omitted for the ternary complex.

Figure 3: Non-cooperative binding of tryptophan to TRAP. (a) Titration of tryptophan with TRAP monitored by tryptophan fluorescence (25 °C). (b) A typical binding isotherm for TRAP binding tryptophan (squares) with a non-linear fit to equation 2 (line); at 25 °C the dissociation constant $K_d$ was $0.16 \pm 0.01 \, \mu$M, with a best-fit stoichiometry of $10.6 \pm 0.9$ tryptophans per TRAP 11-mer. Inset, Hill plot demonstrating that tryptophan binds TRAP in a non-cooperative manner with a Hill coefficient of $1.02 \pm 0.01$ (slope of the best fit line).
Figure 4: Hydrogen/deuterium exchange in apo- and holo-TRAP. An expanded region of $^1$H-$^{15}$N correlation spectra of apo- (bottom) and holo- (top) TRAP recorded before and at a series of time intervals (left to right: 0, 1, 5, 9 and 15 hours) after exchange into 90/10 $^2$H$_2$O/$^1$H$_2$O.

Figure 5: Circular dichroism spectra of apo-TRAP (solid line) and holo-TRAP (dashed line). The holo-TRAP spectrum shows large contributions from the bound tryptophan, as illustrated by the positive peak at 225 nm $^{35}$.

Figure 6: ITC of TRAP. (a) Thermogram from a titration of tryptophan into TRAP (25 ºC) showing the change in power required to maintain a constant temperature difference between the sample and reference cells upon injections of tryptophan. (b) Time-integrated heat data normalized per mole of injectant (squares) with nonlinear fit to identical and independent sites model (line). Best fit parameters are shown in Table 1.

Figure 7: Temperature dependence of tryptophan binding to TRAP. The error bars represent the standard deviation in three repeat measurements at each temperature. Lines represent the best linear fit to the data. The data show the temperature dependence of the binding enthalpy ($\Delta H_{app}$, circles), entropy ($T\Delta S_{app}$, triangles), and free energy change ($\Delta G_{app}$, squares). A linear fit of the temperature dependence of the enthalpy yields a heat capacity change ($\Delta C_p$) of –366 cal mol$^{-1}$ K$^{-1}$.

Figure 8: DSC of apo- and holo-TRAP. Scanning thermograms of apo (solid line) and holo-TRAP (dashed line) reveal (1) the hyperthermal stability of the oligomeric protein, (2) the thermal separation between tryptophan dissociation and global unfolding of the protein, and (3) the linearity of the partial specific heat capacity in the temperature range studied by ITC (25-50 ºC). Quantitative deconvolution analyses of these traces is not possible however, due to the
absence of a post-transition baseline and by lack of reversibility of the high temperature global unfolding (as illustrated by the re-scan of holo-TRAP, dotted line).

Figure 9: Proton and ion linkage. (a) There is very little change in the binding enthalpy in titrations of tryptophan into TRAP in the presence of buffers with different enthalpies of ionization (a slope of $-0.08$ compared to a slope of $-1$ in the case of a single proton uptake $^{48,69}$), demonstrating that changes in protonation state upon binding do not contribute to the observed heat capacity change. (b) Changes in salt concentration show little effect on the association constant (a slope of $-0.17$), demonstrating that uptake or release of ions also does not contribute to the observed heat capacity change $^{49,52}$.

REFERENCES


Table 1: Temperature dependent thermodynamics for the titration of tryptophan into TRAP\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>(n)</th>
<th>(\Delta G)</th>
<th>(\Delta)</th>
<th>(T\Delta S)</th>
<th>(K_A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>10.97±0.03</td>
<td>-9.55±0.06</td>
<td>-13.877</td>
<td>4.331±0.075</td>
<td>9.75e6±1.57e5</td>
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<tr>
<td>303</td>
<td>11.07±0.09</td>
<td>-9.43±0.01</td>
<td>-15.603</td>
<td>6.171±0.070</td>
<td>6.28e6±2.35e5</td>
</tr>
<tr>
<td>308</td>
<td>10.77±0.29</td>
<td>-9.35±0.01</td>
<td>-17.370</td>
<td>8.018±0.071</td>
<td>4.24e6±5.03e4</td>
</tr>
<tr>
<td>313</td>
<td>10.40±0.10</td>
<td>-9.22±0.32</td>
<td>-19.133</td>
<td>9.912±0.284</td>
<td>2.72e6±1.65e5</td>
</tr>
<tr>
<td>318</td>
<td>10.16±0.23</td>
<td>-9.13±0.01</td>
<td>-21.093</td>
<td>11.967±0.420</td>
<td>1.83e6±3.61e4</td>
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<tr>
<td>323</td>
<td>10.53±0.24</td>
<td>-8.98±0.02</td>
<td>-23.027</td>
<td>14.051±0.056</td>
<td>1.17e6±4.51e4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reported errors are the standard deviation of three repeat data sets at each temperature. Values of \(\Delta H\), \(T\Delta S\), and \(\Delta G\) are in kcal mol\(^{-1}\), \(T\) is in Kelvin, \(K_A\) is in M\(^{-1}\), and \(n\) is the number of tryptophan binding sites per TRAP oligomer.
Table 2: Thermodynamics of the binding-coupled folding of TRAP.

Change in Solvent Accessible Surface Area ($\Delta\Delta\Delta\Delta$ASA):

<table>
<thead>
<tr>
<th></th>
<th>Polar</th>
<th>Nonpolar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of folding $^a$</td>
<td>239 Å$^2$</td>
<td>378 Å$^2$</td>
</tr>
<tr>
<td>Presence of folding $^b$</td>
<td>1328 Å$^2$</td>
<td>1916 Å$^2$</td>
</tr>
</tbody>
</table>

Change in Heat Capacity ($\Delta\Delta\Delta\Delta$C$_p$):

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Expected for tryptophan burial $^c$</td>
<td>-88 cal mol$^{-1}$ K$^{-1}$</td>
</tr>
<tr>
<td>Experimental</td>
<td>-366 cal mol$^{-1}$ K$^{-1}$</td>
</tr>
<tr>
<td>Upper limit for folding $^d$</td>
<td>-427 cal mol$^{-1}$ K$^{-1}$</td>
</tr>
</tbody>
</table>

Change in Entropy from the Hydrophobic effect ($\Delta S_{HE}$):

<p>| | |</p>
<table>
<thead>
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</thead>
<tbody>
<tr>
<td>Expected for tryptophan burial</td>
<td>35 e.u.</td>
</tr>
<tr>
<td>Experimental</td>
<td>146 e.u.</td>
</tr>
</tbody>
</table>

Binding-Coupled Folding ($\mathcal{R}$):

<table>
<thead>
<tr>
<th></th>
<th>17-20 residues/monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC</td>
<td></td>
</tr>
<tr>
<td>NMR $^e$</td>
<td>19 residues/monomer</td>
</tr>
</tbody>
</table>

---

$^a$ $\Delta$ASA for the burial of tryptophan measured from the structure 1QAW$^{11}$ see methods for details

$^b$ $\Delta$ASA for the burial of tryptophan and the 19 residues that are believed to be folding measured from the structure 1QAW$^{11}$ see methods for details

$^c$ Expected heat capacity change in the absence of folding calculated from $\Delta$ASA $^a$ using equation 1

$^d$ Upper limit for the heat capacity change in the presence of folding if all the residues go from an extended structure to a folded structure calculated from $\Delta$ASA $^b$ using equation 1

$^e$ Residues for which the NMR resonances are exchange-broadened beyond detection in apo-TRAP but are present in holo-TRAP and are located in the tryptophan or RNA-binding sites
Figure 6

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