¹H-NMR characterization of L-tryptophan binding to TRAP, the *trp* RNA-binding attenuation protein of *Bacillus subtilis*

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A ¹H-NMR study of the binding of L-tryptophan to the *trp* RNA-binding attenuation protein of *Bacillus subtilis* (TRAP), an ondecamer (91.6 kDa), has been implemented. The assignment of the aromatic indole ring proton resonances of the bound tryptophan ligand has been successfully carried out by two-dimensional chemical exchange experiments. The observation of only a single set of chemical shifts of the bound ligand demonstrates that the tryptophan binding site is identical in all the 11 subunits of the protein. Further, the large change in ligand chemical shifts suggests that the conformation of tryptophan

ligand undergoes a significant rearrangement after complex formation with TRAP. This is further substantiated by the extensive ligand-induced chemical shift changes observed to the protein resonances and identification of several strong ligand–protein intermolecular nuclear Overhauser effects. A correlation of these preliminary NMR data with the X-ray crystal structure of the TRAP–tryptophan complex also suggests, tentatively, that the observed changes to the NMR spectra of the protein might correspond to changes associated with residues surrounding the tryptophan binding pocket owing to complex formation.

INTRODUCTION

The regulation of transcription of the *trp* operon of *Bacillus subtilis* involves the selection between alternative RNA secondary structures in the leader region of the *trp* mRNA, which act as ‘anti-terminator’ and ‘terminator’ signals for the transcribing RNA polymerase [1]. This selection is mediated by an RNA-binding protein, TRAP (*trp* RNA-binding attenuation protein), which when activated binds to the 5′ segment of the ‘anti-terminator’ sequence, preventing formation of the ‘anti-terminator’ secondary structure [2–4]. This facilitates the formation of the ‘terminator’ structure, which acts as a signal for RNA polymerase to halt transcription. However, one of the salient features of this mode of transcriptional regulation, termed ‘transcription attenuation’, is that TRAP requires L-tryptophan as an essential cofactor for its activation and its subsequent binding to the RNA [2,3]. When the concentration of tryptophan in the cell is low, TRAP remains in the unactivated form and, as a consequence, formation of the ‘anti-terminator’ secondary structure is favoured, acting as a signal for RNA polymerase to continue with transcriptional read-through. Further, some of the analogues of tryptophan, although exhibiting similar (or higher) affinity for the protein, fail to activate the latter [5].

In *Escherichia coli*, regulation of the expression of the *trp* operon takes place by both repression and a variation of the transcription attenuation mechanism described above, accounting for about 80-fold and 6-fold variation in the range of expression of the operon respectively [6]. The initiation of transcription is regulated by the *trp* repressor protein, after its activation by the co-repressor ligand L-tryptophan, binding to the operator DNA target site within the promoter region of the operon [7] and references therein). In contrast, transcription attenuation (termination) takes place within the 162 base pair transcribed mRNA leader region, again involving the selection

between alternative secondary structures [6]. Near the 5′-end of the leader transcript there is a coding region for a short leader peptide (14-mer) that has two tryptophans occurring in tandem. Depending on the levels of charged tRNA^{Trp}, which in turn depends on the concentration of tryptophan in the cell, translation of these regulatory codons will or will not proceed. This event determines whether transcription will terminate in the leader region, because translation of this coding region is proposed to allow formation of a secondary structure in the mRNA that causes termination of transcription at the *trp* attenuator. An initial NMR study of a synthetic oligoribonucleotide (21-mer) has provided qualitative evidence for the stem-loop secondary structure of the terminator sequence [8].

Recently, the X-ray crystal structure of TRAP complexed to L-tryptophan at 1.8 Å has been reported [9,10]. The results show that the protein is an ondecamer (91.6 kDa), made up of 11 symmetrically related subunits arranged in a circular manner with a wheel-like structure. Each subunit has a mass of 8.3 kDa, and 56% of its residues reside in the seven-stranded antiparallel β-sheet. Each tryptophan molecule binds in a hydrophobic cleft formed between β-sheets of adjacent subunits and the ligand is rendered almost inaccessible to solvent after being buried by extensive interactions with surrounding residues. The α-amino and carboxy groups of tryptophan make eight hydrogen bond contacts with residues from the two adjacent subunits, and the indole ring nitrogen is hydrogen bonded to the carbonyl oxygen of Gln-47. On the surface of the molecule there are two histidine residues in tandem, His-33 and His-34, whose imidazole carbons point towards the indole ring of the ligand and form part of one of the two loops that wrap around the amino and carboxy groups of the bound tryptophan ligand. The conformation of the two loops, formed by residues 25–33 and 49–52, is significant as it is maintained solely by the bound tryptophan and is devoid of

Abbreviations used: 1-D, one-dimensional; 2-D, two-dimensional; FID, free induction decay; NOE, nuclear Overhauser effect; TRAP, *trp* RNA-binding attenuation protein of *Bacillus subtilis*.

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any type of interaction with the rest of the protein. Thus it will be interesting to determine the conformations of these two loops in the absence of tryptophan ligand, including the new interactions governing them, by techniques such as NMR, to permit a structural comparison with the former. The crystal structure of the apo form of the TRAP protein has not been reported so far.

Although *E. coli* *trp* repressor and *B. subtilis* TRAP share similar functional features, such as in their requirement of L-tryptophan for activation, their three-dimensional crystal structures are quite different, with the former being a homodimer (25 kDa) and is mostly α -helical (80%), including the architecture of the tryptophan binding site [7]. We have successfully applied NMR spectroscopy to characterize the mechanism of activation of the *E. coli* *trp* repressor by L-tryptophan [11,12] and have recently determined the mode of binding of several co-repressor and inducer ligand analogues of tryptophan in structural terms [13]. It is proposed to apply similar NMR methodology to elucidate the mechanism of activation of TRAP by L-tryptophan, including the determination of the mode of binding of several ligand analogues of the latter. The result of this NMR study in solution should be important and provides a unique opportunity to compare the mechanism of activation of the two different regulatory proteins by the same cofactor, L-tryptophan, in a structural context. In this paper we describe the results of our NMR study of the binding of L-tryptophan to TRAP, which provide a firm basis for a detailed investigation.

For large proteins such as TRAP with short spin-spin relaxation times (T_2), the prevailing multidimensional NMR techniques [14] are not readily amenable to fulfilling the spectral assignment prerequisites [15]. However, if a high-resolution crystal structure of the protein is available, a judicious combination of 'residue type' isotopic labelling (^1H , ^{13}C , ^{15}N) [12,16,17] and PCR-based mutagenesis protocols [18] offers a practical alternative route to achieving as many resonance assignments as possible. A successful illustration of this approach is provided by the mapping of residues involved in antigen binding of larger antigen-Fab complexes (approx. 100 kDa), by using the carbonyl ^{13}C resonances as sensitive spectroscopic probes [19]. NMR characterization of ligand binding to protein is generally performed by measuring the following [20,21]: (1) chemical shifts of bound ligand proton resonances; (2) chemical shift changes to assigned protein resonances, especially those corresponding to residues at the ligand binding site; and (3) unambiguous identification and assignment of intermolecular ligand-protein nuclear Overhauser effects (NOEs), including intersubunit NOEs. The measurement of even a limited number of intermolecular ligand-protein ^1H - ^1H NOE distance constraints should allow energetically favourable docking of the ligand (and its analogues) into the binding site of the protein [13], provided that the crystal structure of the protein known *a priori*.

EXPERIMENTAL

Materials and methods

TRAP was isolated from *E. coli* cells grown by using the strain CY15251 containing the plasmids pGP1-2 and pTZmtrAB (kindly provided by Professor Charles Yanofsky, Stanford, CA, U.S.A.) [2]. A single colony of the strain containing the plasmids was picked from an LB-Amp-Kan plate and inoculated into 50 ml of Luria broth starter culture medium. After overnight growth at 30 °C, 10 ml of the cell culture was inoculated into 1 litre of super-rich medium [2% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% NaCl, 0.2% (v/v) glycerol, 50 mM potassium phosphate, pH 7.2] with selection (100 $\mu\text{g}/\text{ml}$ ampicillin and 50 $\mu\text{g}/\text{ml}$ kanamycin) and incubated at 30 °C.

The cells were allowed to grow (approx. 10 h) to an attenuation, D_{595} , of 1.5 and expression of the protein was then induced by shifting the temperature to 42 °C for 25 min. Rifampicin was added to a final concentration of 100 mg per ml of cell culture, the temperature was shifted to 37 °C and the cells were allowed to grow for a further period of 2 h. The cells were harvested by spinning the culture at 7000 rev./min (15050 *g*) for 10 min at 4 °C (JA-10 rotor in a Beckman centrifuge) to yield 8.9 g wet wt. of cells per litre of culture. The cells were lysed with a French press and the debris was removed by ultracentrifugation at 45000 rev./min (285000 *g*) for 20 min in a TFT 50.38 rotor. The supernatant was brought to 70% saturation with ammonium sulphate, stirred for 60 min to ensure an even distribution, then dialysed overnight in Spectra-por MWCO 8000 dialysis tubing against 2 litres of buffer B [10 mM sodium phosphate, pH 7.6, containing 0.1 mM EDTA and 15% (v/v) glycerol] containing 50 mM NaCl. The dialysate was loaded onto a phosphocellulose column (1.6 cm $\times$ 40 cm) equilibrated with buffer B and 50 mM NaCl, and the proteins were eluted successively with 100 ml of buffer B with 350 mM NaCl and 50 ml of buffer B with 1 M NaCl. The TRAP protein was recovered in the 1 M NaCl wash in a highly pure state, and fractions containing TRAP were pooled, concentrated by freeze-drying and dialysed against NMR buffer (50 mM sodium phosphate, pH 7.6, containing 100 mM NaCl). The purified protein was characterized by SDS/PAGE and N-terminal (five residues) sequencing; the final yield of TRAP was 12 mg per litre of cell culture.

Before NMR measurements, the protein sample was dissolved in $^2\text{H}_2\text{O}$ and incubated at pH 7.6 and 300 K for several hours to pre-exchange TRAP with deuterium, followed by freeze-drying three times in 100% $^2\text{H}_2\text{O}$ solvent. For NMR experiments the sample consisted of 0.35 mM TRAP (ondecamer) in 0.4 ml solution containing 250 mM NaCl, 50 mM sodium phosphate, pH 7.6, at 298 K. L-Tryptophan was purchased from Sigma and was used as a ligand to titrate TRAP. The titration was carried out by the addition of microlitre volumes of a concentrated solution of the ligand to the protein sample inside the NMR tube.

NMR spectroscopy

^1H -NMR spectra were recorded at 298 K with a Bruker AMX 600 MHz spectrometer. Routinely, both one-dimensional (1-D) and two-dimensional (2-D) spectra of TRAP in 100% $^2\text{H}_2\text{O}$ and in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1) were acquired with spectral widths of 12 and 14 p.p.m. respectively. Suppression of the proton resonance of solvent water in both 1-D and 2-D experiments was achieved by gated irradiation of its signal during the relaxation delay (1.2 s) between successive transients. Typically, 400 transients for 1-D experiments and 128 transients per incremental free induction decay (FID) for 2-D experiments were collected to achieve satisfactory signal-to-noise ratios. Chemical shifts (p.p.m.) are reported with reference to dioxan, used as an internal standard, whose protons resonate as a sharp peak at 3.766 p.p.m. [15].

Phase-sensitive NOESY spectra, with time-proportional phase incrementation phase cycling, were acquired by using standard pulse schemes, with a mixing time of 50 ms. Usually 256 time incremental FIDs (t_1) were measured and each FID was sampled over 2000 data points. The data were processed with Bruker UXNMR software. Before Fourier transformation, the 2-D time-domain data matrices were zero-filled once along F1 and multiplied by a Gaussian window function (LB = 15, GB 0.08) along both F1 and F2 dimensions to generate frequency-domain 2-D spectra. The 2-D spectra were baseline corrected only along F2 for display and plotting purposes.

RESULTS AND DISCUSSION

Figure 1(A) shows the one-dimensional ^1H -NMR spectrum of apo TRAP protein, measured in $^2\text{H}_2\text{O}$ at pH 7.6 and 298 K. The spectrum of the protein remains invariant during prolonged periods (several days) of NMR data acquisition, manifesting the general stability of the protein under the chosen experimental conditions. However, the protein in solution turns opalescent and tends to become insoluble when the pH is lowered to less than 6.0. The large line widths of the resonances observed in the spectrum clearly demonstrate the high molecular mass of the

protein (91.6 kDa) [3,10], and attempts to sharpen the lines by the addition of 200 mM NaCl to prevent any aggregation had no effect. Further, as the spectra of apo TRAP and its complex with tryptophan have very similar line widths (discussed below), this suggests that the protein functions with the same oligomeric composition (i.e. as an ondecamer) in both states in solution.

A striking feature of the spectrum (Figure 1A) is the observation of a broad envelope of resonances in the low-field 8–10 p.p.m. range, corresponding to the exchange-retarded residual amide NH protons of the protein, which persist even after 14 days of incubation in $^2\text{H}_2\text{O}$ at 300 K. The same spectrum also

Figure 1 600 MHz 1-D ^1H -NMR spectra of the titration of apo TRAP (0.35 mM, ondecamer) with L-tryptophan, measured in $^2\text{H}_2\text{O}$ at pH 7.6 and 298 K

(A) Apo TRAP; (B) TRAP complexed with tryptophan, molar ratio of ligand to protein 0.6; (C) TRAP complexed with saturating amounts of tryptophan, molar ratio of ligand to protein 1.1; (D) TRAP complexed with excess of tryptophan, molar ratio of ligand to protein 1.8; (E) same sample as in (D) but measured in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1) at a lower pH of 6.2. The six well resolved sharp resonances due to the aromatic indole ring protons of the 'free' tryptophan ligand of the complex have been labelled at the top of spectrum; the broad signal at the low-field end (10.53 p.p.m.) due to the 'bound' indole NH proton is labelled as NH(b).

Table 1 Change in chemical shifts ($\Delta\delta$), p.p.m., of aromatic indole ring proton resonances of L-tryptophan after binding to TRAP, the *trp* RNA-binding attenuation protein, recorded in $^1\text{H}_2\text{O}$ at pH 6.2 and 298 K

A (—) sign indicates a shift of the respective resonance to high-field.

Proton	L-Tryptophan		
	δ (bound)	δ (free)	$\Delta\delta$
H2	7.10	7.33	(—) 0.23
H4	7.04	7.73	(—) 0.69
H5	6.80	7.21	(—) 0.41
H6	6.63	7.29	(—) 0.66
H7	6.95	7.55	(—) 0.60
NH	10.53	10.19	(+) 0.34

shows a number of low-field shifted C α H resonances in the 5.0–5.5 p.p.m. region (results not shown), suggesting that the protein is endowed with β -sheet-like structure [15]. However, though there are eight aromatic residues (four His, three Phe and one Tyr) out of a total of 75 amino acid residues per subunit of TRAP [2], there are no aromatic ring current-shifted resonances in the high-field methyl region (i.e. lower than 0.0 p.p.m.) of the spectrum.

After the addition of L-tryptophan to TRAP in $^2\text{H}_2\text{O}$, a number of significant changes are evident in the spectrum of the apo protein (Figure 1A), both in chemical shifts and intensities of resonances (Figures 1B–1D). Noteworthy among these are the progressive changes observed in both the aromatic and aliphatic regions, especially to the intense peaks at 7.7 and 1.2 p.p.m., and these changes are indicated by broken lines in the spectrum (Figure 1A) of the apo protein. The former may correspond to the imidazole ring H2 proton resonances of some of the histidine residues of the protein, such as His-33 and His-34, which have been reported to reside on the surface of the protein and act as a gate for bound tryptophan ligand in the crystal structure of the complex [10].

In addition to these changes, another characteristic feature of tryptophan binding is the appearance and progressive increase in intensity of two well resolved resonances at 7.73 and 7.55 p.p.m., after the addition of saturating amounts of the ligand (Figure 1C). They undergo a continuous increase in intensity with further additions of the titrating ligand and eventually appear as resolved doublets in the presence of excess tryptophan ligand (Figure 1D). This kind of titration behaviour of a resonance is characteristic of a slowly exchanging ligand proton between ‘protein-bound’ complexed and ‘protein-free’ uncomplexed states, on the NMR chemical shift time scale [20]. Under such conditions, and with reference to standard chemical shifts, the two resonances originating at 7.73 and 7.55 p.p.m. can be reliably assigned to the aromatic H4 and H7 protons of free tryptophan ligand. Analogously, recording the spectrum of the sample (Figure 1D) in $^1\text{H}_2\text{O}$ at a lower pH of 6.2 results in the observation of two well resolved resonances in the low-field end at 10.53 and 10.19 p.p.m. (Figure 1E), corresponding to the bound and free indole ring NH protons respectively of the tryptophan ligand. Lowering the pH helped to decrease the rate of exchange of the labile indole ring NH proton with solvent water and yielded the observation of the indole NH resonance with enhanced intensity, including the chemical exchange cross-peak of the same ligand proton between free and protein-bound states (see below). As the indole NH protons resonate in a well resolved, non-overlapping low-field

region of the spectrum, their assignment and NOEs can be reliably carried out. In TRAP, this is further facilitated by the absence of tryptophan residues in its composition, thereby making the assignment unambiguous. Similar titration characteristics have been observed for other aromatic ring protons, namely H2, H6, H5, and their resonances identified (Figure 1E). The chemical shifts of the bound aromatic proton resonances of tryptophan ligand were determined separately by measuring the 2-D chemical exchange spectrum, recorded with the NOESY pulse sequence, of TRAP complexed with an excess of tryptophan in $^1\text{H}_2\text{O}$ (discussed below); the results are tabulated in Table 1. An important feature of the binding is that all the protons of the indole ring exhibit large change in chemical shifts ($\Delta\delta = 0.2$ –0.6 p.p.m.). Further, it is significant that except for the indole NH proton, the resonances of all the other ring protons undergo shifts to high-field.

To determine the mode of interaction of L-tryptophan with TRAP, NOESY spectra of the complex were measured both in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$; the spectrum of the complex in $^1\text{H}_2\text{O}$ is shown in Figure 2. Three kinds of cross-peak are expected for tryptophan ligand protons in the NOESY spectrum.

(1) Intra-molecular NOEs between the aromatic protons of bound tryptophan: the NOEs of ‘free’ tryptophan protons are positive, but if bound to a protein with a long correlation time they become negative, having the same phase as the diagonal peaks, and hence give rise to dipolar correlated crosspeaks.

(2) Crosspeaks due to chemical exchange of the ligand protons between the bound (δ_b) and free (δ_f) states: when the rate of ligand exchange, k_{off} , is ‘slow’ on the NMR chemical shift time scale ($k_{\text{off}} \ll |\delta_b - \delta_f|$), separate peaks for a proton in each state are observed.

(3) Inter-molecular NOEs between the ligand and protein in the bound state: owing to chemical exchange, these NOEs are observed at the free-ligand chemical shift positions.

To facilitate the analysis of NOESY data (Figure 2), cross-sections of the 2-D spectrum along the column F1 dimension, corresponding to the chemical shift of each of the aromatic protons of ‘free’ tryptophan, were taken; the projection plots are shown in Figure 3. As an example, the cross-section taken at the free H2 proton chemical shift (7.33 p.p.m.) shows a number of correlated peaks; their assignments have been labelled (Figure 3D). The strongest correlation is shown by its partner in the bound state, peak H2(b) appearing with large intensity at 7.10 p.p.m., arising from chemical exchange. The two low-field peaks at 10.53 and 10.19 p.p.m. arise owing to intramolecular NOEs, via exchange, to the adjacent indole NH proton in the bound and free states respectively. Similar intramolecular NOEs are also observed to the side chain C β H $_2$ protons at 3.47 and 3.30 p.p.m. which appear as broad peaks (however, their cross-peaks are clearly seen in the NOESY spectrum of Figure 2). Further, the indole H2 proton shows strong intermolecular NOEs to the high-field aliphatic proton resonances of the protein at 1.54 and 0.51 p.p.m. In the crystal structure, the binding site is lined by several aliphatic residues in close proximity to the indole ring protons of the ligand, thus creating the potential for strong NOEs to be observed. The source of NOEs and their identification, arising from each aromatic proton of the ligand, are labelled in the respective plots (Figures 3A–3F).

In summary, the NMR data presented above provide strong support for the binding of L-tryptophan to TRAP in solution. As the binding gives rise to only one set of chemical shifts for its bound aromatic indole-ring protons, this demonstrates that the tryptophan binding site is identical in all the subunits and is endowed with a unique conformation in solution. Further, the large chemical shift changes induced to the tryptophan ligand

Figure 2 600 MHz ^1H - ^1H NOESY spectrum (F2 6.0–8.5, 9.5–11.0 p.p.m.; and F1 11.0 to –1.0 p.p.m.) of TRAP (0.35 mM, ondecamer) complexed with excess of tryptophan (molar ratio of ligand to protein 1.8), measured in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1) at pH 6.2 and 298 K

Mixing time was 50 ms.

Figure 3 Projection plots (A–F) of the columns of the NOESY spectrum (Figure 2), taken along the chemical shifts of the 'free' aromatic indole ring protons, resonating in the following order: NH, H4, H7, H2, H6, H5

The respective correlated peaks due to intramolecular and intermolecular NOEs and chemical exchange seen in each plot have been labelled. Peaks arising due to chemical exchange from bound aromatic ligand protons have been labelled with a suffix (b), such as H2(b). Peaks labelled with a dot signify strong intermolecular ligand–protein NOEs (D, E). The signal at 4.8 p.p.m. labelled with asterisks (B–F) corresponds to a baseline artefact from the intense residual protons of water remaining after presaturation.

resonances highlight the significant conformational rearrangement of the ligand taking place owing to complex formation. Additional support for this is provided by the extensive ligand-induced chemical shift changes to protein resonances and observation of several strong intermolecular ligand–protein NOEs, targeted to the same aliphatic protons affected by the above chemical shift changes. Thus the preliminary NMR results suggest, tentatively, that the ligand makes close dipolar contacts with the surrounding residues of the protein at the tryptophan binding pocket. These results provide a strong basis for further NMR studies to characterize the binary TRAP–tryptophan and ternary TRAP–tryptophan–RNA complexes by the application of isotopic labelling (^2H , ^{13}C , ^{15}N) and NMR editing/filtration techniques [22], which are in progress.

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