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Study of complexes of a tryptophan-free mutant of *E. coli trp* aporepressor with tryptophan analogues using optically detected magnetic resonance (ODMR)

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Abstract Phosphorescence and optically detected magnetic resonance (ODMR) spectra of tryptophan (W) and several of its analogues (4-, 5-, 6-methyltryptophan (MeW); 4-, 5-, 6-fluorotryptophan (FW); 5-bromotryptophan) are compared with those of complexes formed with the W-free *trp* aporepressor from *Escherichia coli* (W19,99F). W19,99F binds W and each analogue except 4-FW with an estimated $K_D \leq 30$ μM; triplet state spectroscopic and kinetic effects that accompany binding at the corepressor site are reported. ODMR data for the MeW isomers are presented for the first time. No binding of 7-azaW is observed, in agreement with the low affinity found by previous workers.

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Key words: Phosphorescence; Ootically defined magnetic resonance spectroscopy; *Escherichia coli trp* repressor; Tryptophan analogue

1. Introduction

The *trp* repressor protein of *Escherichia coli* (apo-trpR) is involved in the regulation of tryptophan biosynthesis. Apo-trpR is a symmetrical dimeric protein containing two tryptophan residues (Trp-19 and Trp-99) in each 107 amino acid subunit [1]. The active holoprotein, trpR, is formed upon non-cooperative binding of two molecules of the corepressor, L-tryptophan, to apo-trpR. TrpR binds selectively to at least four sites of the *E. coli* genome, the *trp*, *aroH*, *mtr*, and *trpR* operons, to regulate the expression of genes involved in tryptophan synthesis and transport [2–7]. L-Tryptophan binds independently to each subunit in a hydrophobic pocket located between the core and the DNA binding domain [8], presumably inducing a conformational change in apo-trpR such that the reading heads of the dimer can penetrate two successive grooves of the operator [9].

Fluorescence measurements of the binding of L-tryptophan to apo-trpR [10] revealed a large blue shift, consistent with the hydrophobic, non-polar nature of the binding site as deduced from the X-ray crystallography results [8]. Millar et al. [11] isolated the fluorescence of the corepressor site in trpR by using a tryptophan-free mutant of apo-trpR, W19L,W99M, further confirming the earlier work [8,10]. We have carried out fluorescence, phosphorescence and ODMR studies [12,13] of the single tryptophan mutants of apo-trpR, W19F and W99F. Because of the highly resolved nature of tryptophan phosphorescence and ODMR spectra, we were able to discriminate between bound corepressor and each intrinsic tryptophan residue using wavelength-selection methods. The phosphorescence of L-tryptophan is found to become more highly resolved and to be red-shifted upon binding to apo-trpR. The ODMR bands become narrower, and the D-E transition is shifted to a lower frequency. Each of these observations is consistent with the binding of the corepressor at a non-polar, polarizable site. More recently, we have extended the phosphorescence and ODMR measurements to a tryptophan-free apo-trpR (W19,99F) [14]. These results confirmed those of the earlier studies on the single tryptophan proteins. We also found that when holo-W19,99F was complexed with the self-complementary *trp* operator mimic, 5′-CGTACTAGT-TAACTAGTACG-3′, further linewidth reduction and changes in the tryptophan zero-field splittings (zfs) are produced, demonstrating that DNA binding affects the bound corepressor triplet state properties.

There has been interest recently in the use of tryptophan analogues as spectroscopic probes of biomolecular structure introduced either by biosynthetic incorporation at tryptophan sites in proteins, or as substrate analogues [15–20]. Fluorine-substituted analogues are primarily useful as NMR probes, while analogues such as 5-hydroxytryptophan (5-HW) and 7-azatryptophan (7-AW) have red-shifted absorption bands which allow their luminescence to be excited selectively in the presence of tryptophan and/or nucleic acids. We have measured recently the triplet state properties of 5-HW, 7-AW [21,22] and 4-, 5- and 6-fluorotryptophans (Ozarowski, A., Wu, J.Q., Davis, S.K., Wong, C.-Y., Eftink, M.R. and Maki, A.H., submitted for publication) using phosphorescence and ODMR spectroscopy. We report here on the interaction of several tryptophan analogues with W19,99F using the photoexcited triplet state of the analogue as a probe. In earlier studies of the structural basis for the interaction between tryptophan and apo-trpR, the binding affinities of tryptophan and a number of its analogues were investigated [7]. We find good general agreement with the earlier studies [7]. Complex formation of 4-, 5-, and 6-MeW, 5- and 6-FW, and 5-BrW with W19,99F is observed, indicating that these tryptophan analogues bind with quite high affinity. We detect no binding of 4-FW or 7-AW with W19,99F, however. ODMR

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Abbreviations: 4-, 5-, 6-FW, 4-, 5-, and 6-fluorotryptophan; 4-, 5-, and 6-MeW, 4-, 5-, and 6- methyltryptophan; 5-BrW, 5-bromotryptophan; 5-HW, 5-hydroxytryptophan; 7-AW, 7-azatryptophan; apo-trpR, wild type *trp* aporepressor from *Escherichia coli*; EEDOR, electron-electron double resonance; EG, ethylene glycol; HAE, heavy atom effect; MIDP, microwave-induced delayed phosphorescence; ODMR, optical detection of magnetic resonance; SLR, spin-lattice relaxation; T_i (i = x, y or z), a triplet state sublevel; trpR, wild type *trp* holorepressor from *Escherichia coli*; W19,99F, tryptophan-free mutant of apo-trpR; zfs, zero-field splittings

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of the methyl tryptophan isomers is reported in this paper for the first time.

2. Materials and methods

The equipment for obtaining phosphorescence and ODMR spectra has been described recently [22,23]. Phosphorescence and ODMR measurements were carried out in pumped liquid He at 1.2 K. Data analysis algorithms have been presented previously that compensate for fast passage transient effects in slow passage steady state ODMR [23] and in slow passage delay ODMR [21], enabling accurate determination of the band center frequency, ν_0, and bandwidth, $\nu_{1/2}$ (half-width at half-maximum). Global analysis of microwave-induced delayed phosphorescence (MIDP) [24] responses, which results in reliable values for the triplet sublevel decay constants, k_i, and radiative rate constant ratios, $R_{ji} = k_j^{(r)}/k_i^{(r)}$, i = x, y, z, in the presence of non-negligible spin-lattice relaxation (SLR) also has been described [22].

Apo-trpR and W19,99F were generous gifts from Dr. Maurice Eftink; preparation of the latter by site-directed mutagenesis has been described earlier [25]. 4-, 5-, 6-FW, 4- and 6-MeW were obtained from ICN, while 5-BrW and 5-MeW were supplied by Aldrich. L-Tryptophan was obtained from Fluka and 7-AW was from Sigma.

L-Tryptophan or its analogue (each was a racemic mixture) was dissolved in 0.01 M aqueous phosphate buffer, pH 7, containing 0.075 M NaCl. Ethylene glycol (EG) was added to 40% of final volume as cryosolvent; the concentration of the analogue was 10^{-3} M. This solution was used for low temperature phosphorescence and ODMR measurements of the free amino acid.

The binding was carried out as follows. 50 μl of stock W19,99F (8×10^{-4} M in monomer) was added to 500 μl of the buffer described above. 80 μl of the tryptophan analogue, or 40 μl of L-tryptophan (10^{-3} M in EG-free buffer) was added, and the mixture was allowed to stand for 1 h at 37°C. At this point, the L-isomer, D-isomer (absent for L-tryptophan), and W19,99F (monomer) are present in equimolar amounts. The sample was filtered through a Centricon filter (10 kDa cutoff) to a final volume of 47 μl. Addition of 31 μl EG produced a solution containing about 5×10^{-4} M W19,99F (monomer basis) in 40% (v/v) EG/buffer. We assume that the D-isomer binds with much less affinity than L, and thus binding of the D-isomer is negligible; in previous binding studies [7] it was found that the K_D of D-tryptophan is 25 times larger than that of L-tryptophan. For ideal isomer-selective stoichiometric binding in which effectively all of the L-isomer binds to W19,99F, while the D-isomer passes through the filter, the concentrations of unbound D-isomer, and that of holo-W19,99F would be about 3.8×10^{-5} M (0 for L-tryptophan), and 5×10^{-4} M, respectively. Using the dissociation constants measured by Marmorstein et al. [7] for 5-MeW (3.25 μM) and L-tryptophan (14.6 μM), we can estimate that holo-W19,99F would be less than 1% and ca. 3.5% dissociated respectively, under the conditions of our experiment. On the other hand, we would not expect to observe binding of 7-AW (K_D = 466 μM) [7].

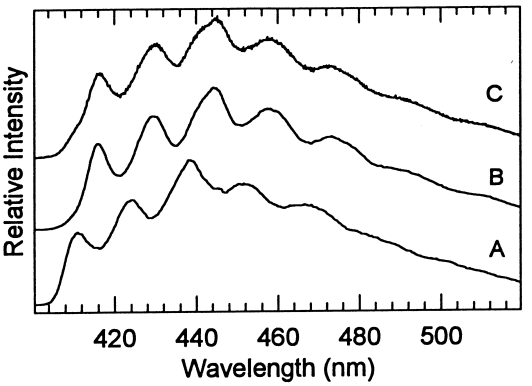

Fig. 1. Phosphorescence of (A) 6-FW, (B) 6-FW complexed with W19,99F with uncomplexed 6-FW reduced by Centricon filtration, and (C) control (see text). Spectrum (C) can be accurately represented by adding 0.3 (A) and 1.0 (B). This ratio does not reflect the relative abundance of species (A) and (B) since the phosphorescence quantum yields are unknown.

To prepare controls, dilution of the stock W19,99F with buffer was carried out as described above, and the sample was concentrated by Centricon filtration. The racemic tryptophan analogue was added at this point, followed by incubation for 1 h. EG was added to 40% (v/v) and the phosphorescence of this sample was measured. The sample would contain approximately equimolar amounts of the holorepressor and the free D-tryptophan analogue under ideal stoichiometric isomer-selective binding conditions.

Samples were excited using a 100 W high pressure mercury arc passed through a monochromator set at 16 nm bandpass and a glass cutoff filter.

3. Results

The phosphorescence of free 6-FW is compared with its W19,99F complex in Fig. 1. The narrowing of the vibronic bands and a 0-0 band red shift of 5 nm are clearly indicative of complex formation. Emission resulting from unbound 6-FW makes only a minor contribution to the spectrum of the complex. This is supported by the third spectrum in Fig. 1,

Table 1
ODMR frequencies and linewidths of tryptophan analogues and their complexes with W19,99F

Sample	λ_{0-0} (nm)	D-E (GHz)[a,b]	2E (GHz)[a,b]	D+E (GHz)[a,b]	D (GHz)	E (GHz)
W	407	1.763 (58)[c]	2.514 (146)[c]	4.250 (81)[c]	3.007	1.248
W+W19,99F	410	1.684 (34)[c]	2.538 (83)[c]	–	2.953	1.269
4-MeW	411.4	1.768 (66)	2.438 (110)	4.183 (66)[d]	2.976	1.213
4-MeW+W19,99F	412.9	1.703 (39)	2.475 (76)	4.172 (50)[d]	2.937	1.236
5-MeW	414	2.03 (118)[c]	–	3.93 (107)[c]	2.98	0.95
5-MeW+W19,99F	416	1.937 (59)[c]	–	3.932 (89)[c]	2.935	0.998
6-MeW	418	1.621 (52)	2.627 (84)	4.253 (66)[d]	2.937	1.315
6-MeW+W19,99F	423	1.558 (31)	2.662 (65)	4.235 (53)[d]	2.896	1.335
4-FW	399	1.765 (56)	2.75 (98)	4.51 (66)[d]	3.14	1.37
5-FW	408.5	1.924 (73)	2.250 (133)	4.163 (83)[d]	3.043	1.122
5-FW+W19,99F	412	–	2.235 (88)	4.11 (84)[d]	2.99	1.118
6-FW	411	1.654 (44)	2.635 (87)	4.289 (57)[d]	2.97	1.32
6-FW+W19,99F	416	1.574 (34)	2.674 (84)	4.24 (48)[d]	2.91	1.33
5-BrW	~410	1.77 (72)[c]	2.61 (115)[c]	–	3.07	1.30
5-BrW+Apo-TrpR[e]	414	1.720 (64)[c]	2.583 (83)[c]	4.285 (61)[c]	3.002	1.287

[a]ODMR frequencies and linewidths were found by fitting slow passage ODMR [23] unless otherwise stated.
[b]Linewidths (HWHM) in MHz are given in parentheses.
[c]Values are from delay ODMR analysis [21].
[d]Obtained from analysis of electron-electron double resonance, saturating the D-E transition.
[e]Wild type Apo-trpR was used.

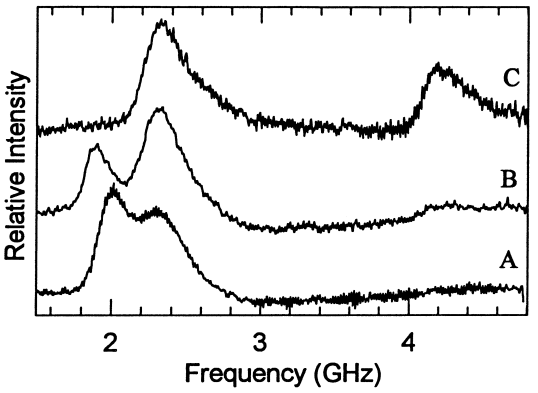

Fig. 2. Slow passage steady state ODMR spectra of (A) 5-FW, (B) 5-FW complexed with W19,99F and filtered (see text), and (C) sample in (B) observed using EEDOR, saturating the D-E (low frequency) transition, to enhance the D+E (high frequency) band. The transition at intermediate frequency is 2E.

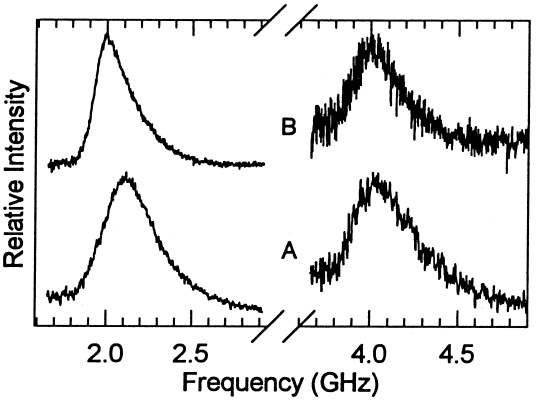

Fig. 3. Delay ODMR spectra of (A) 5-MeW with emission monitored at 414 nm, and (B) 5-MeW complexed with W19,99F and filtered (see text), with emission monitored at 416 nm. Low frequency band is D-E; high frequency band is D+E. Band center frequencies and band widths are listed in Table 1.

obtained from the control that contains equimolar amounts of both 6-FW isomers, and W19,99F without separation of bound and unbound analogue. The significant contribution of bound analogue to the emission of this control demonstrates that binding is nearly stoichiometric. The ODMR spectra of free and bound 6-FW also reveal differences induced by binding. The ODMR frequencies and band widths obtained from ODMR analysis are presented in Table 1, along with those of the other samples studied. Fig. 2 shows a comparison of the slow passage steady state ODMR spectra of free and repressor-bound 5-FW that illustrates the type of changes that typically are induced in these analogues by binding to W19,99F. Typical slow passage delay ODMR responses (analyzed [21] for each sample to determine the zfs and ODMR bandwidths) are shown in Fig. 3 for free 5-MeW and its W19,99F complex. The narrowing of the ODMR bands and of the phosphorescence spectrum (not shown) is apparent. No effect on the spectra of adding W19,99F to 7-AW or 4-FW was observed under our experimental conditions indicating that the binding affinity is low.

The sublevel decay constants and relative radiative rate constants obtained from global analysis of MIDP responses [22] are presented in Table 2. Also presented in Table 2 are the wavelengths of the phosphorescence 0-0 band maxima (λ_{0-0}) of the samples measured in this work.

4. Discussion

No quantitative measurements of binding affinity were carried out. For each of the tryptophan analogues for which binding was observed (5- and 6-FW; 4-, 5-, and 6-MeW; and 5-BrW), however, in comparing the phosphorescence spectrum of the free ligand with that reacted with apo-W19,99F (e.g. Fig. 1), the binding was found to be fairly stoichiometric. The fraction of unbound L-isomer could be estimated as no greater than 20% in any case, and is far less in many of them (i.e. 6-FW in Fig. 1). This estimate assumes that the phosphorescence quantum yield of bound and free ligand are comparable; lack of relative quantum yields makes a more quantitative measurement of binding affinity impossible by this method. However, a limiting value of $K_D \leq 30$ μM can be set for each of the L-isomers listed above.

Substituent effects on the zfs parameters of L-tryptophan are interesting. We reinvestigated L-tryptophan binding in

Table 2
Kinetic and relative radiative parameters of tryptophan analogues and their complexes with W19,99F

Sample	λ_{0-0} (nm)	k_x (s^{-1})[a,b]	k_y (s^{-1})[a,b]	k_z (s^{-1})[a,b]	R_{yx}[a,b]	R_{zx}[a,b]
W	407	0.306(9)	0.102(5)	0.000(1)	0.128(7)	0.000(1)
W+W19,99F	410	0.33(1)	0.090(5)	0.008(3)	0.02(1)	0.02(1)
4-MeW	411.4	0.298(6)	0.096(3)	0.000(3)	0.125(7)	0.00(1)
4-MeW+W19,99F	412.9	0.29(2)	0.082(6)	0.007(3)	0.059(8)	0.08(1)
5-MeW	414	0.27(1)	0.13(1)	0.008(6)	0.22(2)	0.02(2)
5-MeW+W19,99F	416	0.28(6)	0.11(4)	0.02(3)	0.24(6)	0.14(6)
6-MeW	418	0.324(1)	0.108(1)	0.000(2)	0.076(1)	0.000(1)
6-MeW+W19,99F	423	0.32(1)	0.111(6)	0.000(3)	0.089(8)	0.00(1)
4-FW	399	0.483(6)	0.147(3)	0.000(3)	0.058(4)	0.000(6)
5-FW	408.5	0.36(1)	0.122(5)	0.000(3)	0.126(6)	0.08(1)
5-FW+W19,99F	412	0.36(2)	0.10(1)	0.00(1)	0.20(3)	0.00(3)
6-FW	411	0.419(2)	0.193(1)	0.000(2)	0.148(5)	0.000(2)
6-FW+W19,99F	416	0.40(2)	0.22(2)	0.00(1)	0.30(1)	0.00(3)
5-BrW	~410	35	38	1.5	0.4	0.02
5-BrW+Apo-TrpR[c]	414	25.6(7)	34.5(6)	1(1)	1.41(2)	0.04(5)

[a]Obtained from global analysis of MIDP data [22]. Standard error in last digit is given in parentheses.
[b]z-axis is out of plane. x-axis is approximately normal to the ethylenic double bond in W, but not determined for derivatives.
[c]Wild type Apo-trpR used.

this paper since the new ODMR analysis methods [21–23] were not available for the previous study [14]. The unusually large value of E is the result of localization of considerable spin density in the ethylenic double bond of the five-membered ring of indole. Either fluorine or methyl substitution at the 5-position leads to a large decrease of E, while substitution by either at the 6-position has the opposite effect. These zfs changes probably are the result of spin density shifts from and to the ethylenic bond, respectively. These substituents have opposite effects when introduced at the 4-position, however. E is subject to a large increase with fluorine substitution, but decreases slightly with methyl substitution. It may be significant that widely different affinities for apo-trpR are manifested only when these substituents are in the 4-position. Bromine substitution at the 5-position produces an increase in E, in contrast with methyl or fluorine substitution at this position. The bromine may interact with indole in a unique manner, however, because of its large polarizability. The major effect of 5-bromo substitution is an internal HAE on the sublevel decay constants, manifested selectively in k_x and k_y (Table 2). This HAE pattern is predicted for an in-plane perturbing atom [26]. Although methyl substitution leads to negligible effects on the decay constants (Table 2), fluorine substitution at the 4- and 6-position leads to small but significant increases in both k_x and k_y. Fluorine substitution at the 5-position has less effect on the decay constants (Table 2). The relatively light (relative to Br) F atom appears to introduce a minor HAE, most noticeably when present at the 4- or 6-positions.

The effects of binding on the phosphorescence and ODMR of the tryptophan analogues, with the exception of 5-BrW, can be compared with those observed for L-tryptophan. Upon binding to W19,99F, the phosphorescence 0-0 band of L-tryptophan undergoes a red shift of 3 nm and narrows significantly. The ODMR bands become significantly narrower; the D-E transition shifts lower while the 2E transition shifts higher in frequency, the net effect being an increase in the zfs E parameter, and a decrease in the D parameter (Table 2). Each of these effects is associated with a change of the environment from polar to polarizable and hydrophobic when the analogue binds in the corepressor site [27]. These effects are also exhibited by the three MeWs and by 6-FW. 5-FW behaves similarly, except that E decreases slightly, rather than increases upon binding. There are no observable effects of binding on the kinetic rate constants outside of experimental error, with the exception of 5-BrW (Table 2). In this case, binding to apo-trpR results in a 28% reduction of k_x, accompanied by a smaller reduction of k_y. There also is a large decrease in the radiative rate constant of T_x relative to that of T_y that accompanies binding. These effects can be attributed to the influence of binding to the corepressor site on the operation of the internal HAE of 5-BrW which dominates the kinetics.

What have we learned from this investigation regarding the structural basis for the interaction between L-tryptophan analogues, and the corepressor binding site in trpR? We have confirmed the conclusion of a previous investigation [7] that introduction of polar residues into indole (7-AW) severely reduces binding affinity, and that binding affinity is retained if the group substituted at the 5-position is non-polar (5-MeW, 5-FW, 5-BrW). Thus, steric constraints are not the cause of the low binding affinity of 5-HW observed earlier [7]. We have found, in addition, that introduction of non-polar substituents at the 6-position (6-MeW, 6-FW) does not inhibit binding (although we did not quantitate the effect on K_D within its upper limit of ca. 30 μM), and thus there are no serious steric constraints introduced by substituents at that position, either. The effect of substitution at the 4-position is less straightforward, however. Methyl substitution retains the binding affinity of the analogue, while fluorine substitution reduces its affinity to a limit undetectable by our assay ($K_D >$ ca. 200 μM). Modification at the 4-position brings the substituent close to C3, and interactions with the peptide linkage could introduce intramolecular distortions to account for the observed differences in accommodation by the corepressor site.

To summarize, we have used phosphorescence and ODMR spectroscopy to characterize the photoexcited triplet state of a set of tryptophan analogues, and the changes induced by binding to the corepressor site of W19,99F. Effects of binding were observed in L-tryptophan and in each of the analogues investigated with the exception of 7-AW and 4-FW. 7-AW was found to exhibit very low binding affinity in previous work [7]. New tryptophan analogues with relatively high binding affinity ($K_D \leq 30$ μM) have been identified in this work, i.e. 4-, 5-, and 6-MeW, 5- and 6-FW, and 5-BrW. The triplet state of each responds to binding in a manner similar to L-tryptophan, except for 5-BrW, whose triplet state is influenced strongly by Br atom polarizability and its HAE, and 5-FW, whose E parameter is less sensitive to binding than that of L-tryptophan.

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