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Role of tryptophan 54 in the binding of *E. coli* single-stranded DNA-binding protein to single-stranded polynucleotides

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Fluorescence and optical detection of triplet state magnetic resonance spectroscopy have been employed to study the complexes formed by single-stranded polynucleotides with both *E. coli* single-stranded DNA-binding protein and an *E. coli ssb* gene product in which Trp-54 is replaced by phenylalanine using site specific oligonucleotide mutagenesis. Our results strongly suggest the involvement of Trp-54 in stabilizing the protein-nucleic acid complexes via stacking interactions of the aromatic residue with the nucleotide bases.

ODMR spectroscopy; Single-stranded DNA binding protein; Stacking interaction; Zero field splitting; Heavy atom effect

1. INTRODUCTION

In earlier studies [1,2], stacking interactions between at least one Trp residue in the single-stranded DNA-binding protein from *E. coli* (*Eco* SSB) and nucleotide bases in single-stranded polynucleotides were demonstrated using ODMR spectroscopy.

The amino acid sequence of *Eco* SSB reveals the presence of 4 Trp residues [3]. In order to characterize the Trp residue(s) that are undergoing stacking interactions with the nucleotide bases, a series of experiments has been conducted recently in our laboratory on plasmid-encoded single-stranded DNA-binding proteins that have extensive amino acid sequence homology with *Eco* SSB in the putative DNA-binding (N-terminal) domain but lack Trp-135, which is located in a distinct (C-terminal) domain (Khamis, M.I. et al., unpublished). Those results demonstrate that Trp-135 is involved neither in the stabilization of the protein-nucleic acid complexes nor in a stacking interaction with the nucleotide bases.

In this communication we report our initial results from combining site specific oligonucleotide mutagenesis to selectively replace Trp-54 by phenylalanine in the *E. coli ssb* gene product, together with fluorescence and ODMR spectroscopy to study the relevance of this particular residue in the binding process and its involvement in stacking interactions with single-stranded polynucleotides.

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Abbreviations: Eco SSB, single-stranded DNA-binding protein encoded by *E. coli*; *Eco* SSB-Phe 54, single-stranded DNA-binding protein with Trp-54 replaced by Phe, encoded by a cloned *ssb* gene modified by site specific oligonucleotide mutagenesis; ODMR, optically detected triplet state magnetic resonance; poly(dT), polydeoxythymidylic acid; poly(5-BrU), brominated polyuridylic acid; poly(5-HgU), mercurated polyuridylic acid

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2. MATERIALS AND METHODS

The SSB protein used in this study was prepared by published procedures [4,5]. Details of the construction of the gene encoding the SSB protein in which Trp-54 is substituted by phenylalanine (*Eco* SSB-Phe 54) will be described elsewhere.

Polydeoxythymidylic acid and mercurated polyuridylic acid were obtained from P.L. Biochemicals and were used without further purification. Brominated polyuridylic acid was prepared as described earlier [6]. The procedures for obtaining phosphorescence spectra, triplet state lifetimes, ODMR spectra and the fluorimetric titrations have been described [2,6].

3. RESULTS AND DISCUSSION

3.1. *Equilibrium binding isotherms*

Table 1 shows a comparison of the binding constants of *Eco* SSB and *Eco* SSB-Phe 54 with various single-stranded polynucleotides. Of particular relevance to this study is the finding that there is a decrease of about an order of magnitude in the binding constant upon replacing Trp-54 by Phe. Furthermore, the complexes of *Eco* SSB-Phe 54 with single-stranded polynucleotides exhibit a higher dependence of the affinity constant on the salt concentration than do the complexes of the *Eco* SSB protein, as evidenced by the salt-back midpoints (table 1). These results suggest that Trp-54 in *Eco* SSB contributes to the stability of the complexes with single-stranded polynucleotides by means of hydrophobic interactions, and hence is involved in the binding process.

Table 1

Binding constants obtained from fluorescence binding isotherms for the complexes of *Eco* SSB and *Eco* SSB-Phe 54 with various single-stranded polynucleotides

Sample	K_ω (M^{-1})	Q_{max}[a]	Salt-back midpoint	
			NaCl (M)	$MgCl_2$ (M)
Eco SSB				
+ poly(5-HgU)	1.9×10^7	0.56	0.34	–
+ poly(5-BrU)	6.8×10^7	0.88	1.35	–
+ poly(dT)	10^8–10^{10}[b]	0.88	>3	>2
Eco SSB-Phe 54				
+ poly(5-HgU)	2.6×10^6	0.58	0.28	–
+ poly(5-BrU)	1.9×10^7	0.74	0.70	–
+ poly(dT)	3.2×10^7	0.74	>3	0.65

[a] Limiting fluorescence quenching
[b] From [9]

Fluorescence measurements are conducted as described in section 2. The protein concentration is ~3×10^{-7} M (monomer) in cacodylate buffer (20 mM, pH 7.0) containing 150 mM NaCl and 0.1 mM EDTA

3.2. *Phosphorescence spectra and lifetime*

The phosphorescence spectra of *Eco* SSB and *Eco* SSB-Phe 54 are presented in fig.1A and C, respectively. The 0,0-band of the Trp residues in both proteins is well resolved and peaks at 411.8 and 412.6 nm, respectively (see table 2). It has been found previously [2] that there are two distinct Trp environments in *Eco* SSB: a spectrally blue-shifted environment emitting at $\lambda < 409$ nm, and a red-shifted environment with $\lambda > 410$ nm. The red shift of the *Eco* SSB-Phe 54 0,0-band relative to that of *Eco* SSB indicates that Trp-54 resides in a blue-shifted site and is probably influenced by polar interactions.

Lifetime measurements on these proteins reveal a normal Trp lifetime of ~6 s in both samples.

Upon complexing *Eco* SSB with poly(dT) a significant red shift in the phosphorescence 0,0-band of ~4 nm is observed (fig.1B, table 2). We attribute this red shift to the presence of stacking interactions between Trp residue(s) and thymine bases in the complex. Upon complexing *Eco* SSB-Phe 54 to poly(dT), however, a less pronounced red shift of only 0.9 nm is seen (fig.1D, table 2). These results indicate that the extent of

Fig.1. Phosphorescence spectra of (A) *Eco* SSB (1×10^{-4} M), (B) *Eco* SSB (8.6×10^{-5} M) complexed with poly(dT) (1.6×10^{-3} M), (C) *Eco* SSB-Phe 54 (1×10^{-4} M), and (D) *Eco* SSB-Phe 54 (8.6×10^{-5} M) complexed with poly(dT) (1.6×10^{-3} M). Excitation is at 295 nm with 16 nm band pass and the emission slits are set at 3 nm. Temperature is 4.2 K. The samples are prepared in 20 mM cacodylate buffer, pH 7.0, containing 1 mM EDTA, 10 mM β-mercaptoethanol, 30% glycerol and 0.3 M NaCl.

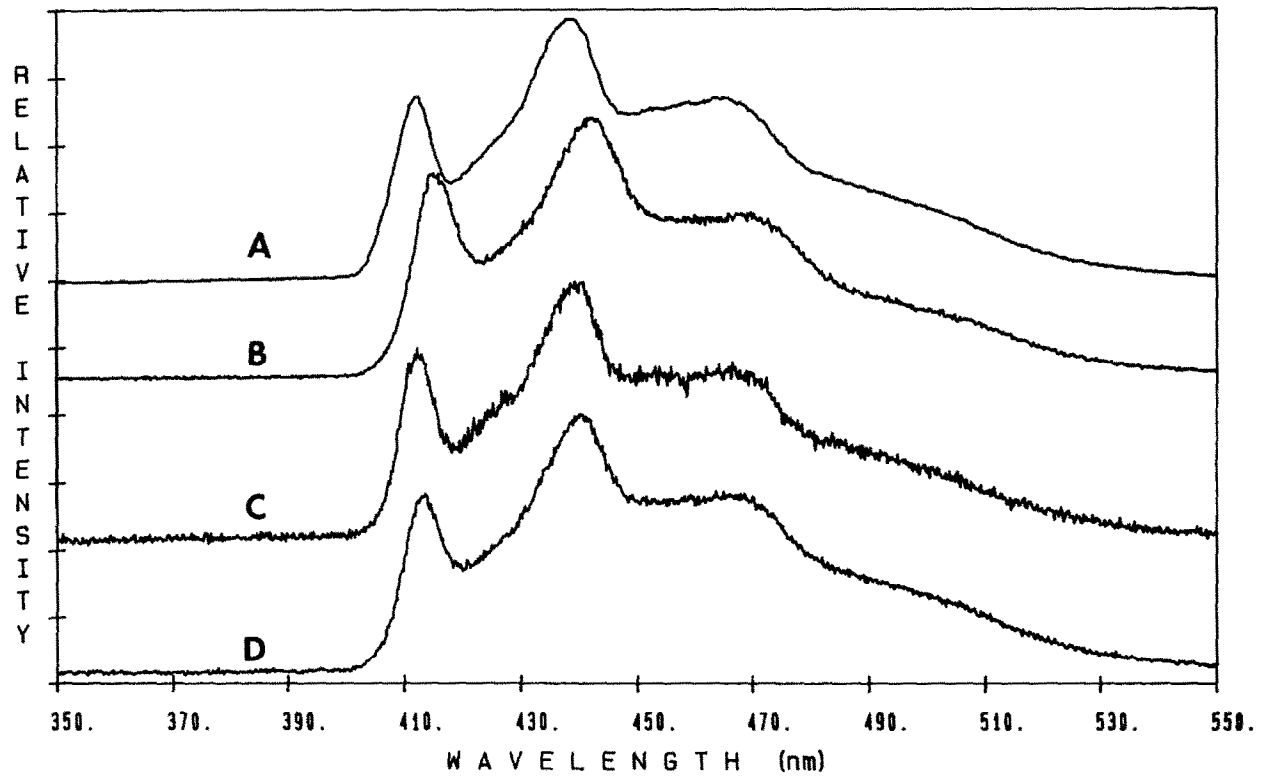
Table 2

Tryptophan zero field ODMR frequencies and zero field splitting parameters in complexes of *Eco* SSB and *Eco* SSB-Phe 54 with various single-stranded polynucleotides

Sample	$\lambda_{0,0}$ (nm)	$\|D\| - \|E\|$ (GHz)	$2\|E\|$ (GHz)	$\|D\| + \|E\|$ (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
Eco SSB	411.8	1.708	2.568	–[a]	2.992	1.284
Eco SSB-Phe 54	412.6	1.702	2.577	–[a]	2.991	1.289
Eco SSB + poly(5-HgU)	413.6	1.780	2.551	4.346	3.063	1.276
Eco SSB-Phe 54 + poly(5-HgU)	412.5	1.723	2.592	4.326	3.019	1.296
Eco SSB + poly(5-BrU)	415.2	1.655	2.610	4.275	2.965	1.305
Eco SSB-Phe 54 + poly(5-BrU)	415.2	1.657	2.576	4.201	2.945	1.288
Eco SSB + poly(dT)	415.8	1.643	2.428	–[a]	2.857	1.214
Eco SSB-Phe 54 + poly(dT)	413.5	1.709	2.619	–[a]	3.019	1.310

[a] Signal is not observed

Measurements are made at 1.2 K with monochromator at 3 nm resolution, set at $\lambda_{0,0}$



stacking interactions between Trp residues and thymine bases in the complex of *Eco* SSB-Phe 54 with poly(dT) is less than that of *Eco* SSB. Furthermore, the lifetime of Trp in the *Eco* SSB/poly(dT) complex is reduced by 20–30% relative to the uncomplexed protein, while the reduction of the Trp lifetime in *Eco* SSB-Phe 54/poly(dT) complex is only 5–10%. The lifetime results give additional evidence for a unique interaction of Trp-54 with thymine bases in the complexes.

Binding poly(5-HgU) to *Eco* SSB produces a red shift of ~1.8 nm in the Trp 0,0-band and the appearance of a very short lived component in the decay profile [2] (see also table 2). On the other hand, binding poly(5-HgU) to *Eco* SSB-Phe 54, produces no shift in the 0,0-band (table 2). However, a short component in the decay profile (~10 ms) is still observable, indicating interaction of a Trp residue other than Trp-54 with the bases.

3.3. *ODMR spectra*

The $|D| - |E|$ and $2|E|$ ODMR transitions of the Trp residues in *Eco* SSB and *Eco* SSB-Phe 54, monitored at the peak wavelength of their 0,0-band, are shown in fig.2A and C, respectively. The peak frequencies, together with the zero field splitting parameters are presented in table 2. Upon binding *Eco* SSB to poly(dT), a drastic effect on the zero field splitting parameters of Trp and on the polarity of its ODMR signals is observed (fig.2B, table 2). These effects consist of a decrease in the values of $|D|$ and $|E|$ and a reversal of the ODMR signal polarity. Binding of poly(dT) to *Eco* SSB-Phe 54 produces ODMR signals with normal positive polarity, however, and a relatively small increase of $|D|$ and $|E|$ (table 2). This result suggests that Trp-54 undergoes special interaction with thymine bases leading to a reversal of the usual positive ODMR signal polarity and the

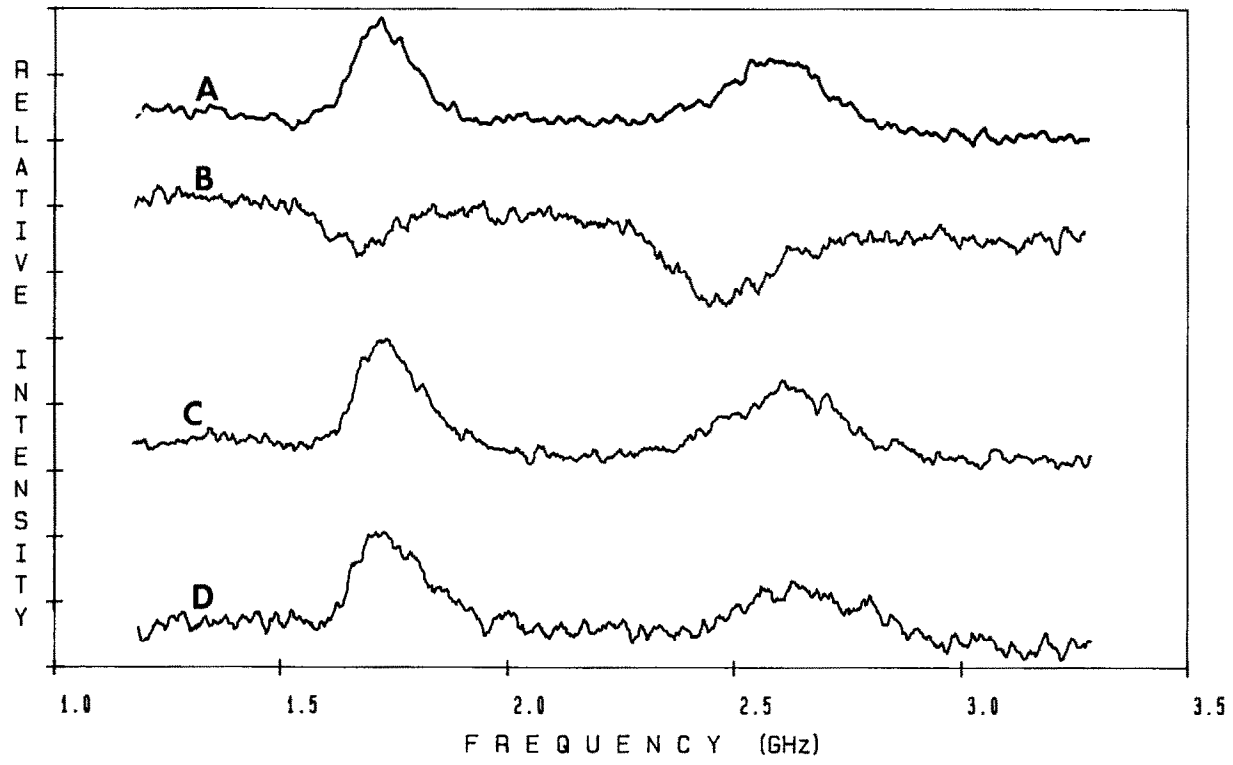


Fig.2. $|D| - |E|$ and $2|E|$ transitions of Trp in (A) *Eco* SSB, (B) *Eco* SSB complexed with poly(dT), (C) *Eco* SSB-Phe 54, and (D) *Eco* SSB-Phe 54 complexed with poly(dT). Sample concentration is the same as given for fig.1. Excitation is at 295 nm using 16 nm band pass. The temperature is 1.2 K, and the phosphorescence is monitored at the 0,0-band peak of each sample with 3 nm slits.

reduction of the zero field splitting parameters. Since stacking interactions of Trp are known to produce a reduction in the $|D|$ value [7], the above results are consistent with the presence of stacking interactions between Trp-54 and thymine bases in the complex. We believe that this unprecedented reversal of the ODMR signal polarity of Trp-54 upon complexing with poly(dT) results from the introduction of triplet-triplet energy transfer from the thymine residue which is stacked with it. Altered sublevel populating rates then lead to a situation where the most radiative Trp sublevel has a larger steady-state population than the less radiative ones, which is a reversal of the normal situation arising from intramolecular intersystem crossing. For the validity of this hypothesis it is necessary to postulate that the thymine energy donor has a significant triplet yield. It could acquire this by means of a specific interaction (unspecified at present) with the protein. Normally, thymine has a vanishingly small triplet quantum yield [8].

Binding poly(5-BrU) or poly(5-HgU) to *Eco* SSB-Phe 54 produces an external heavy atom effect on at least one Trp residue in the complex, as is evident from the optical appearance of the otherwise dark $|D| + |E|$ transition (table 2). The fast passage transient through this signal indicates the presence of a short lived triplet state in the complex. Analysis of this fast passage response reveals a lifetime the order of 1 ms in the case of the poly(5-HgU) complex, and of ~100 ms for the poly(5-BrU) complex. These results are similar to those observed previously for *Eco* SSB complexed with poly(5-HgU) and poly(5-BrU) [2] and confirm the presence of at least one Trp residue, other than Trp-54, which undergoes stacking interactions with nucleotide bases. Complexing of *Eco* SSB-Phe 54 with poly(dT) leads to a small increase in the zero field splitting parameters, as seen in table 2. This would seem at first to be inconsistent with stacking interactions. We are, however, observing ODMR signals simultaneously from all three Trp residues in this complex. If only one of these undergoes a stacking interaction, its decrease in zero field splitting parameters may be overwhelmed by increases resulting from Stark effects on one or both unstacked Trp residues. We have independent evidence from wavelength-selected ODMR measurements for the occurrence of such an increase in the zero field splittings of a Trp site in *Eco* SSB upon complexing with poly(dT) (Casas-Finet, J.R. et al., unpublished).

In conclusion, we have provided evidence for the involvement of Trp-54 in stacking interactions with nucleotide bases in complexes formed between *Eco* SSB and single-stranded polynucleotides. In addition, there is at least one other Trp residue undergoing such interactions. Further studies involving site specific oligonucleotide mutagenesis of the cloned *E. coli ssb* gene, in which Trp-40 or Trp-88 are replaced by phenylalanine are currently in progress to identify the role of these residues in the binding process.

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