Volume 220, number 2, 347-352 FEB 05023 August 1987

Tryptophan 54 and phenylalanine 60 are involved synergistically in the binding of *E. coli* SSB protein to single-stranded polynucleotides

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Received 17 June 1987

The binding of both wild-type and point-mutated *E. coli* single-stranded DNA-binding (SSB) protein to poly(deoxythymidylic acid) has been studied by fluorescence and optical detection of triplet state magnetic resonance spectroscopy. Involvement of tryptophan residues 40 and 54 in stacking interactions with nucleotide bases has been inferred earlier from such studies. Investigation of a point mutation in the *E. coli SSB* gene product obtained by site specific oligonucleotide mutagenesis in which Phe-60 is replaced by alanine strongly suggests the participation of Phe-60 in the binding process, possibly by the formation of an extended stacking structure by Trp-54, thymine and Phe-60. This hypothesis is supported by results on the point mutations in which His-55 is replaced by either leucine or tyrosine.

ODMR spectroscopy; single-stranded DNA-binding protein; Site-specific mutagenesis; Stacking interaction; Zero-field splitting

1. INTRODUCTION

It is known from previous studies [1-3] on Eco SSB, a well-characterized single-stranded DNA-binding protein from *E. coli* (review [4]), that two of its four tryptophan residues undergo stacking interactions with nucleotide bases upon binding. Fluorescence and optically detected triplet state magnetic resonance (ODMR) spectroscopy on wild-type and point-mutated Eco SSB proteins prepared by site-directed oligonucleotide mutagenesis shows that Trp-40 and Trp-54 are involved in hydrophobic interactions with the nucleic acid bases and contribute significantly to the stability of the complexes [2,3]. The contribution of the free energy of binding is larger from Trp-54 than for Trp-40 [3]. Trp-54 also exhibits an unprecedented reversal in the polarity of its $|D| - |E|$ and $2|E|$ ODMR signals in the Eco SSB/poly(dT) complex [2,3]. Based on the predicted secondary structure [5] Trp-54 is contained in an extended α-helical region which includes Phe-60, a residue known to be the protein site of photocrosslinking to thymine oligonucleotides [6]. Since both Trp-54 and Phe-60 are in close proximity to the bound polynucleotide and may be facing in the same direction (they would be separated by two α-helix turns [3,5]), Phe-60 could also participate in the binding process through hydrophobic interactions with nucleotide bases. To test this possibility, we have investigated the poly(dT) complex of a point-mutated Eco SSB which has a Phe → Ala-60 substitution introduced by site-directed oligonucleotide mutagenesis. Point mutations containing substitutions at His-55 have been studied to assess the effect of modifying the local environment of Trp-54.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

The Eco SSB proteins used in this study were prepared according to published procedures [7,8]. Details of the construction of the gene encoding the SSB protein in which Phe-60 is substituted by alanine (Eco SSB-Ala-60) or in which His-55 is substituted by leucine (Eco SSB-Leu-55) will be published elsewhere. Amplification of the *ssb-1* gene encoding a mutant *E. coli* SSB protein (His-55 → Tyr) has been described earlier [9].

Poly(deoxythymidylic acid) was obtained from P.L. Biochemicals and was used without further purification. The procedures for obtaining phosphorescence spectra, triplet state lifetimes, ODMR spectra and fluorescence titrations have been described ([1] and references therein).

3. RESULTS AND DISCUSSION

3.1. *Equilibrium binding isotherms*

Table 1 shows the binding constants (K_w), limiting fluorescence quenching (Q_{max}) and salt-back midpoints of several Eco SSB proteins complexed with poly(dT). It is noteworthy that Eco SSB-Phe-54 and Eco SSB-Ala-60 have practically indistinguishable binding constants and salt-back midpoints, which suggests that Trp-54 and Phe-60 make a similar contribution to the stability of the complex. This result also suggests that Phe-60 undergoes a stacking interaction as does Trp-54. The affinity of both proteins for poly(dT) is decreased by three orders of magnitude relative to that of wild-type Eco SSB (table 1). On the other hand, substitutions at position 55 (Eco SSB-Leu-55 and Eco SSB-1 proteins) result in binding constants an order of magnitude higher than those for Eco SSB-Phe-54 or Eco SSB-Ala-60. The same relative affinity has been found from thermal melting studies of poly[d(A-T)] in the presence of Eco SSB, Eco SSB-1 and Eco SSB-Ala-60 [4,10]. While in 30 mM NaCl Eco SSB lowers the T_m of poly[d(A-T)] by 33°C [10], from 53°C to 20°C, Eco SSB-1 induces a T_m depression of only 22.5°C [10] and Eco SSB-Ala-60 decreases the T_m of this polynucleotide by 21°C [4]. We can calculate a minimum association constant based on the melting temperature of the poly[d(A-T)] from the expression [11]

$$K_{app} = (1/a)\,\{[e^{-(T_m - T'_m)\Delta H/(B_c R T_m T'_m)}] - 1\} \quad (1)$$

where T'_m and T_m are the melting temperatures of poly[d(A-T)] in the presence and absence of SSB protein, respectively; ΔH for melting poly[d(A-T)] is about -8 kcal/mol of base pairs; R is the gas constant; B_c is the reciprocal of the protein binding site size expressed in base pairs of the double-stranded polynucleotide; and a is the free SSB concentration at T'_m. At 30 mM NaCl Eco SSB is in a metastable state characterized by high coopera-

Table 1

Binding constants obtained from fluorescence binding isotherms for the complexes of wild-type and point-mutated Eco SSB proteins with poly(deoxythymidylic acid)[a]

Sample	K_w (M^{-1})	Q_{max}[b]	Salt-back midpoint[c] $MgCl_2$ (M)
Poly(dT)			
+ Eco SBB	$>10^{10}$[d]	0.88	>2
+ Eco SSB-Phe-54	3.2×10^7	0.74	0.65
+ Eco SSB-Ala-60	3.5×10^7	0.81	0.66
+ Eco SSB-Leu-55	1.9×10^8	0.78	0.76
+ Eco SSB-1	4.5×10^8	0.78	0.45

[a]Fluorescence measurements were conducted as described in section 2. The protein concentration was approx. 3×10^{-7} M (monomer) in cacodylate buffer (20 mM, pH = 7.0) containing 150 mM NaCl and 0.1 mM EDTA
[b]Limiting fluorescence quenching
[c]Salt-back midpoint is the molar concentration of salt which induces a relative fluorescence recovery equal to half of the maximal recovery
[d]From [4]

tivity and a low binding site size [12,13]: comparable information is not available, however, for the point-mutated SSB proteins. Since the binding constants at low ionic strength (calculated from the above equation) do not correspond to the thermodynamical state of the SSB proteins under the conditions of our fluorimetric titrations, we have calculated the relative binding constants assuming the same binding site size for the wild-type and point-mutated SSB proteins. We find, using eqn 1, that substitution of His-55 by Tyr (Eco SSB vs Eco SSB-1) results in a reduction by approx. 2000-fold of the binding constant, while Eco SSB-Ala-60 is predicted to have a 3-fold larger reduction of its affinity for the polynucleotide than Eco SSB-1. The relative magnitudes of the binding constants are in reasonable agreement with the binding constants obtained at high ionic strength from fluorimetric titrations with poly(dT) (see table 1).

It is interesting to point out that none of the point-mutated Eco SSB proteins produces a limiting fluorescence quenching as high as the wild-type protein (table 1). Substitution of Trp-54 induces the lowest Q_{max}, since it removes the contribution of this chromophore to the total intrinsic fluorescence emission and its subsequent quenching upon binding (the fluorescence quantum yield of the stacked Trps is likely to be near zero). Still, substitution of Phe-60 by Ala affects noticeably the Q_{max} of the Eco SSB-Ala-60/poly(dT) complex (table 1). Since Phe is not excited under our experimental conditions, this effect is not due to removal of an energy transfer mechanism. Therefore, removal of Phe-60 reduces the degree of fluorescence quenching of other residues, possibly including Trp-54, thereby suggesting that both residues may be structurally 'connected': further evidence for this hypothesis will be presented in section 3.3. Such a connection obviously exists for the neighboring residues of Trp-54: substitution of either Leu or Tyr for His-55 produces significant changes in Q_{max} observed for the poly(dT) complexes of the respective mutants. It is assumed that the effects of the amino acid substitutions are direct effects on DNA binding, rather than effects on conformation or oligomerisation, and that the effects of substitution at position 55 are mainly due to microenvironmental effects of Trp-54.

Despite having a similar binding constant for

Table 2

Tryptophan zero field ODMR frequencies and zero field splitting parameters of wild-type and point-mutated Eco SSB proteins and their complexes with poly(dT)[a]

Sample	$\lambda_{0,0}$ (nm)	$\|D\| - \|E\|$ (GHz)	$2\|E\|$ (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
Eco SSB	411.8	1.71(+)	2.57(+)	2.99	1.28
Eco SBB + poly(dT)	415.8	1.64(−)	2.43(−)	2.86	1.21
Eco SSB-Phe-54	412.6	1.70(+)	2.58(+)	2.99	1.29
Eco SSB-Phe-54 + poly(dT)	413.5	1.71(+)	2.62(+)	3.02	1.31
Eco SSB-Ala-60	412.2	1.70(+)	2.56(+)	2.98	1.28
Eco SSB-Ala-60 + poly(dT)	415.2	1.70(+)	2.42(−)	2.91	1.21
Eco SSB-Leu-55	412.6	1.71(+)	2.56(+)	2.99	1.28
Eco SSB-Leu-55 + poly(dT)	414.8	1.65(−)	2.44(−)	2.87	1.22
Eco SSB-1	412.6	1.71(+)	2.57(+)	2.99	1.29
Eco SSB-1 + poly(dT)	416.4	1.66(−)	2.45(−)	2.89	1.22

[a]ODMR measurements were made at 1.2 K with monochromator at 3 nm resolution and set at $\lambda_{0,0}$, the peak of the phosphorescence 0,0-band. The error in the tabulated wavelength of the 0,0-band maxima is ± 0.2 nm. Estimated uncertainty in the ODMR frequencies is ± 0.01 GHz. The signal polarity (increase or decrease in phosphorescence intensity) is given in parentheses

poly(dT), Eco SSB-1 exhibits a salt-back midpoint much lower than Eco SSB-Leu-55 (table 1). Since the *ssb-1* mutation is known to weaken the SSB tetramer stability as a function of ionic strength, even at permissive temperatures [10], we attribute the anomalously low salt-back midpoint to disruption of the tetramer structure. The free Eco SSB-1 protein will be predominantly a monomer at the concentration used in our fluorescence titrations [10], which is similar to its in vivo concentration in non-overproducing cells [10]. If the tetramer reassembles during the process of binding to poly(dT), however, its disruption will result in the loss of the moderate cooperativity of the binding and, hence, produce a lower salt-back midpoint.

3.2. *Phosphorescence spectra and lifetime*

The phosphorescence spectra of Eco SSB-Ala-60, Eco SSB-1 and their complexes with poly(dT) show a well resolved 0,0-band which is red-shifted upon binding of SSB to poly(dT). The phosphorescence 0,0-band maxima of different Eco SSB proteins and their poly(dT) complexes are given in table 2. Eco SSB-Phe-54 exhibits the smallest red-shift (~1 nm) while all the other systems have a red-shift of 2–4 nm upon binding to poly(dT). Most of the observed red-shift is therefore induced by the stacking of Trp-54.

Lifetime measurements on the above systems are presented in table 3. The free SSB proteins have a major component with a normal Trp lifetime of ~6 s. Binding to poly(dT) induces a shortening of the long component as well as an increase of the contribution of the short component. The reduction of the phosphorescence lifetime relative to the free protein is about 21% for the wild-type SSB/poly(dT) complex, 5% for Eco SSB-Phe-54/poly(DT) complex, and ~12% for the other systems. Again, Trp-54 seems to be responsible for most of the reduction in the phosphorescence lifetime (the 5% reduction observed for the Eco SSB-Phe-54/poly(dT) complex probably is induced by stacking of the second interacting tryptophan, Trp-40). A change of the residue adjacent to Trp-54 also affects the extent of lifetime reduction. Since one observes the largest red-shift and phosphorescence lifetime reduction only when Trp-54 is not replaced by Phe, these results provide strong evidence for a unique interaction of Trp-54 with thymine bases in the complexes.

3.3. *ODMR spectra*

The peak ODMR frequencies, together with the zero field splitting parameters of several Eco SSB

Table 3

Phosphorescence lifetimes of tryptophan in wild-type and point-mutated Eco SSB proteins and their complexes with poly(dT)[a]

Sample	λ_{em} (nm)	Lifetime components
Eco SSB	412.0	2.93 s(16%), 6.20 s(84%)
Eco SSB + poly(dT)	416.2	1.82 s(31%), 4.87 s(69%)
Eco SSB-Phe-54	412.7	1.90 s(8%), 5.90 s(92%)
Eco SSB-Phe-54 + poly(dT)	413.5	1.75 s(18%), 5.63 s(82%)
Eco SSB-Ala-60	412.4	2.41 s(20%), 6.06 s(80%)
Eco SSB-Ala-60 + poly (dT)	415.5	2.28 s(37%), 5.35 s(63%)
Eco SSB-Leu-55	412.5	1.50 s(9%), 5.74 s(90%)
Eco SSB-Leu-55 + poly(dT)	415.5	2.40 s(33%), 5.10 s(67%)
Eco SSB-1	412.9	1.90 s(11%), 5.99 s(89%)
Eco SSB-1 + poly(dT)	416.9	2.24 s(40%), 5.23 s(60%)

[a]Measurements were made at 77 K, the excitation wavelength was 295 nm and the emission was monitored with monochromator slits set at 3 nm bandpass. The phosphorescence decay was fitted arbitrarily to two exponential components

proteins and their poly(dT) complexes are presented in table 2. All systems containing Trp-54 give a reversal of the ODMR signal polarity reported [2,3]. Eco SSB-Phe-54 produces ODMR signals of positive polarity, while Eco SSB-Ala-60 displays a negative $2|E|$ signal and a $|D|-|E|$ transition of positive polarity when complexed with poly(dT). The reduction in the $|D|$ parameter of Trp-54 (which is induced by stacking interactions [14]) in the poly(dT) complex relative to the free protein is reduced from 135 MHz in Eco SSB to 73 MHz in Eco SSB-Ala-60. The reduction of the $|E|$ parameter induced upon binding to poly(dT) is the same for both proteins (~70 MHz). It is interesting to note that substitution of Phe-60 affects the zero field splitting parameters of the stacked Trp-54, suggesting a synergistic effect or 'connection' of these two residues in the protein binding to the polynucleotide. If one assumes an α-helical conformation for the region Trp[54]-His-Arg-Val-Val-Leu-Phe[60], space-filling models reveal a cavity between the two aromatic residues which can accommodate 1–2 stacked thymine rings (the rise per thymine base is 3.5 Å [15], the aromatic residues in a putative α-helix would be 10.8 Å apart, and the closest approach of the aromatic side chain and the

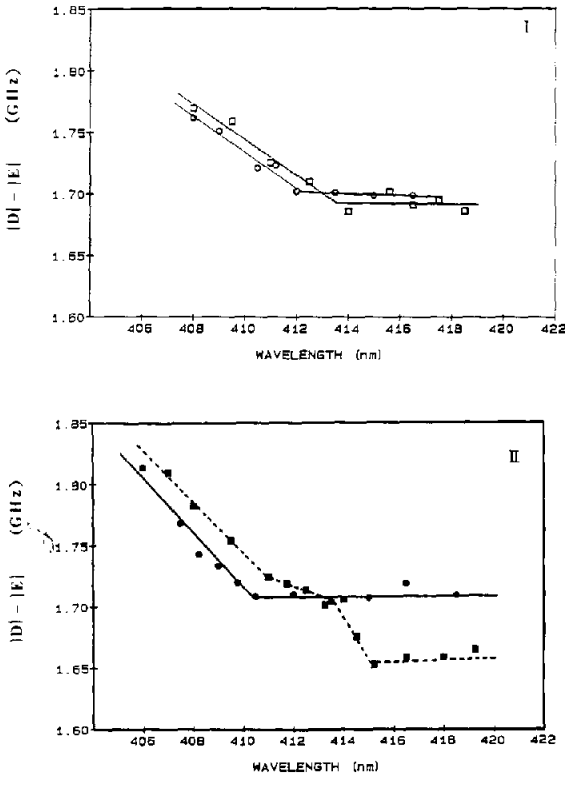

Fig.1. Plot of the |D| – |E| ODMR frequency vs emission wavelength through the 0,0-band in: (I) Eco SSB-Ala-60 (1×10^{-4} M) (open circles), and Eco SSB-Ala-60 (8.7×10^{-5} M) complexed with poly(dT) (1.6×10^{-3} M) (open squares); (II) Eco SSB (1×10^{-4} M) (full circles), and Eco SSB (8.6×10^{-5} M) complexed with poly(dT) (1.6×10^{-3} M) (full squares). The samples were prepared in 20 mM cacodylate buffer, pH 7.0, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 30% glycerol and 0.3 M NaCl. The temperature was 1.2 K, the excitation was at 295 nm with 16 nm bandpass and the emission slits were set at 1.5 nm resolution. For the Eco SSB-poly(dT) sample a 10% neutral density filter was present in the excitation path.

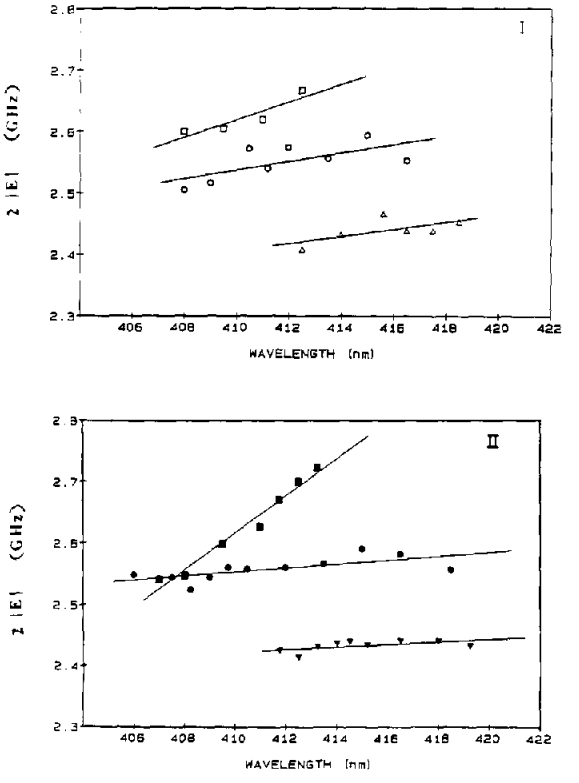

Fig.2. Plot of the 2|E| transition frequencies of Trp vs emission wavelength for (I) Eco SSB-Ala-60 (open circles), Eco SSB-Ala-60 complexed with poly(dT) (positive signals, open squares; negative signals, open triangles), and (II) Eco SSB (full circles), and Eco SSB complexed with poly(dT) (positive signals, full squares; negative signals, full triangles). Conditions are given in fig.1.

stacked pyrimidine base is 3.4 Å [16]). An extended stacked structure containing two thymines between Trp-54 and Phe-60 can be envisioned. It is noteworthy that an increase in the interelectron distance of the triplet pair along the z (stacking) axis as a result of stacking will cause the observed reduction of the $|D|$ parameter. Removal of Phe-60 could decrease the stacking interaction of Trp-54 and, hence, the extent of delocalization along z, thus inducing a smaller change of $|D|$. For Eco SSB-Leu-55 and Eco SSB-1, on the other hand, there is no direct effect on the mutual interaction of Trp-54 and Phe-60 in the complex; consequently, the reduction of the $|D|$ value on binding to poly(dT) is similar to that of the wild-type SSB.

Wavelength-selected ODMR of Eco SSB, Eco SSB-Ala-60 and their poly(dT) complexes are presented in figs 1 and 2. Two distinct Trp sites are present, as shown by a discontinuity in the plot of $|D| - |E|$ vs wavelength (fig.1). The red-shifted site, with lower $|D| - |E|$ values in the complex relative to the free protein is assigned to Trp-54 [2,3]. The extent of reduction in the $|D|$-$|E|$ peak frequencies and the degree of red-shift of the discontinuity in the plot of $|D|$-$|E|$ vs wavelength induced by the binding to poly(dT) are much smaller in the Eco SSB-Ala-60 mutant (fig.1I) than in the wild-type protein (fig.1II). The blue-shifted site (which has contributions from the stacked Trp-40 as well as from non-interacting Trp residues [2,3]) is not significantly altered (cf. fig.1I and II).

Two Trp sites are also observed in the plots of 2 $|E|$ vs wavelength (fig.2): upon binding to poly(dT), the normally occurring ODMR transition of the free protein (circles) splits into two transitions of opposite polarity (+, squares; −, triangles). Substitution of Phe-60 by Ala does not affect the splitting of the2 $|E|$ ODMR transition (cf. fig.2I and II). The change in the frequency of the Trp-54 2$|E|$ ODMR signals induced upon binding poly(dT) is similar for both Eco SSB and Eco SSB-Ala-60 (table 2). Only perturbations of the electronic distribution in the Trp xy plane (the indole plane) would result in a change of $|E|$. The observation that only the $|D|$ parameter is affected differently in the two proteins upon forming poly(dT) complexes gives additional evidence for the existence of an extended stacked structure of Trp-54, Phe-60 and thymine bases.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Institutes of Health ES-02662 (to A.H.M), GM11301 and CA13330 (to J.W.C.), as well as a fellowship from Amideast (to M.I.K.).

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