I. ENVIRONMENT OF TYROSIINES IN TERMS OF COMPLEX FORMATION WITH DNA*

Structural Features of φ29 Single-stranded DNA-binding Protein

The single-stranded DNA-binding protein (SSB) of *Bacillus subtilis* phage φ29 is absolutely required for viral DNA replication in vivo. About -95% of the intrinsic tyrosine fluorescence of φ29 SSB is quenched upon binding to ssDNA, making tyrosine residues strong candidates to be directly involved in complex formation with ssDNA. Thus, we have studied the spectroscopic properties of the φ29 SSB tyrosines (Tyr-50, Tyr-57, and Tyr-76) using steady-state and time-resolved fluorescence measurements. φ29 SSB tyrosines do not seem to be highly restricted by strong interactions with neighboring residues, as suggested by (i) the high value of the average quantum yield of the φ29 SSB fluorescence emission (Φφ = 0.067 ± 0.010), (ii) the fast motions of the tyrosine side chains (φαsorth = 0.14 ± 0.06 ns), and (iii) the lack of tyrosinate emission at neutral pH. Stern-Volmer analysis of the quenching by acrylamide and I- indicates that φ29 SSB tyrosines are surrounded by a negatively charged environment and located in a relatively exposed protein domain, accessible to the solvent and, likely, to ssDNA. Changes in the intrinsic fluorescence upon ssDNA binding allowed us to determine that temperature has an opposite effect on the thermodynamic parameters K (intrinsic binding constant) and ω (cooperativity) defining φ29 SSB-poly(dT) interaction, the effective DNA binding constant, Kφeff = Kωω, being largely independent of temperature. Altogether, the fluorescent properties of φ29 SSB tyrosines are consistent with a direct participation in complex formation with ssDNA.

Interactions of nucleic acids and proteins provide the structural framework for a wide variety of biological processes. Among them, complexes formed by the association of single-stranded DNA-binding proteins (SSBs)1 with single-stranded DNA (ssDNA) are of particular interest. First, they modulate parameters that have an opposite effect on the thermodynamic parameters K (intrinsic binding constant) and ω (cooperativity) defining φ29 SSB-poly(dT) interaction, the effective DNA binding constant, Kφeff = Kωω, being largely independent of temperature. Altogether, the fluorescent properties of φ29 SSB tyrosines are consistent with a direct participation in complex formation with ssDNA.

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**Instituto de Química Física Rocasolano (CSIC), Serrano 119, 28006 Madrid, Spain

†To whom correspondence should be addressed. Tel.: 1-397-8435; Fax: 1-397-4799.

1 The abbreviations used are: SSB(s), single-stranded DNA-binding protein(s); 5-MeOL, 5-methoxyindole; NaClTyrA, N-acetyl-l-tyrosine amide; nt, nucleotide(s); ssDNA, single-stranded DNA; Pf3 phage, *Pseudomonas aeruginosa* filamentous bacteriophage.

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Intrinsic Tyrosine Fluorescence of $\Delta29$ SSB

The domain directly involved in ssDNA complex organization, they should not be highly restricted by interactions with other neighboring residues and, likely, placed in an accessible domain of the protein. To test this possibility, we have characterized the global spectroscopic properties of the tyrosines of $\Delta29$ SSB by using complementary approaches: (i) steady-state and time-resolved measurements of the total emission and anisotropy of $\Delta29$ SSB fluorescence, (ii) analysis of the resistance of $\Delta29$ SSB tyrosines to undergo ionization by pH increase, and (iii) selective attenuation of the tyrosine fluorescence by small diffusible quenchers. In addition, the fluorescence properties of $\Delta29$ SSB were also considered to study ssDNA binding under different temperatures. Our results regarding the mobility and accessibility of $\Delta29$ SSB tyrosines strongly suggest a direct role of these residues in complex formation. In the accompanying paper (23), some of the fluorescence features of $\Delta29$ SSB tyrosines are combined with other spectroscopic and hydrodynamic studies, to model the conformational properties of $\Delta29$ SSB and the effect of complex formation on both $\Delta29$ SSB and ssDNA structure.

**MATERIALS AND METHODS**

**Buffers, Reagents, and Proteins**—All the solutions were made with distilled, deionized (MilliQ) and sterilized water, and finally filtered through 0.45-$\mu$m filters using a Milllex-HA Filter Unit (Millipore). The buffer used in time-resolved fluorescence analysis was 5 mM Tris-HCl, pH 7.5 (20°C) (buffer A), while the buffer used in absorption and steady-state fluorescence studies was 50 mM Tris-HCl, pH 7.5 (20°C), 4% glycerol (buffer B). Poly(dT) was from Pharmacia Biotech Inc. $\Delta29$ SSB was purified by differential ammonium sulfate fractionation of $\Delta29$-infected cellular extracts followed by phosphocellulose and DEAE-cellulose chromatography, essentially as described (20). Protein concentration was estimated by the Lowry (24) method, using bovine serum albumin as a standard.

**Optical Absorption Measurements**—Absorption spectra were carried out in a CARY 219 (Varian) at a constant temperature of 22°C with cells of path-lengths from 2 to 10 mm.

**Steady-state Fluorescence Spectroscopy**—Measurements of the intrinsic tyrosine fluorescence of $\Delta29$ SSB were recorded, unless otherwise stated, at 25°C. Fluorescence excitation and emission spectra, solvent quenching experiments, as well as ssDNA binding assays were performed in a Schoeffel Instrument Corp. RRS100 spectrophuorimeter (slits set to 2 nm), with a 2-mm path-length cell, and corrected for residual emission of the buffers. Fluorescence quantum yield and fluorescence anisotropy measurements were carried out in a SLM-8000D photon counting fluorimeter fitted with Glan-Thompson polarization optics, and emission slits were 4 and 8 nm, respectively. When necessary, corrections for wavelength dependence of the instrument response were taken into account as described elsewhere (25).

The steady-state average fluorescence quantum yield ($\Phi_F^0$) of the protein was calculated as in Demas and Crosby (26) using an aqueous solution of 5-methoxy-indole (5-MeOInd) as reference ($\Phi_{F,5MeOInd} = 0.28 \pm 0.01$; Ref. 27). Magic angle orientation of the polarizers was selected to avoid anisotropic effects (28).

The accessibility to solvent and theionic environment of the fluorescent tyrosines of $\Delta29$ SSB were analyzed by monitoring the fluorescence quenching ($\lambda_{exc} = 276$ nm, $\lambda_{em} = 308$ nm) induced by adding increasing amounts of stock solutions (2.5 M) of neutral (acrylamide) or charged (asKI) and CsCl to the protein sample (7–11 mg/ml) with pH variation (from pH 7.0 to 7.6) does not alter the global structural properties of $\Delta29$ SSB (see accompanying paper (23)); no pH readjustments were made during the binding measurements. The thermodynamic parameters, nucleotide site size, n, intrinsic affinity constant, $K_n$ and cooperativity, $\omega$, were obtained by fitting the experimental data to the theoretical ones given by the McGhee and von Hippel’s (35) equations for binding of proteins to large lattices with overlapping binding sites (see Ref. 21, for further details). The enthalpy and entropy of the binding process were approximated from the slope and the y intercept, respectively, of the best fit of van’t Hoff plots for binding of poly(dT) to $\Delta29$ SSB tyrosines to undergo ionization by pH increase, and (iii) selective attenuation of the tyrosine fluorescence by small diffusible quenchers. In addition, the fluorescent properties of $\Delta29$ SSB strongly suggest a direct role of these residues in complex formation. In the accompanying paper (23), some of the fluorescence features of $\Delta29$ SSB tyrosines are combined with other spectroscopic and hydrodynamic studies, to model the conformational properties of $\Delta29$ SSB and the effect of complex formation on both $\Delta29$ SSB and ssDNA structure.

**Time-resolved Fluorescence Spectroscopy**—The decay of total fluorescence intensity, measured at a magic angle orientation of the polarizers, and those of the parallel and perpendicular components were recorded at 22°C in a time-correlated single-photon counting spectrometer, as described (36), for a protein concentration ranging from 10 to 30 $\mu$M. The samples were excited at 290 ± 4 nm with vertically polarized light pulses from a thyratron-gated nanosecond flash lamp (Edinburgh Instr. El 199) filled with N2. The emission was isolated with a cut-off 310 nm (Schott KV) and a band-pass U-54 (Schott) filters, and detected with a Phillips XP2020Q photomultiplier.

The kinetic parameters (lifetimes $\tau_i$ and amplitudes $a_i$) of the decay of the fluorescence intensity were determined by iterative convolution of the signal recorded at magic angle, $I_{m}(t)$. The fitting routines were based on non-linear least-squares techniques (37, 38), using standard weighting factors (39). Wavelength-dependent timing effects in the photodetection were corrected as indicated (36). The weighted average lifetime $\bar{\tau}$, $\tau$, was computed from,

$$\bar{\tau} = \sum a_i \tau_i$$

(Eq. 1)

The fluorescence quantum yield of $\Delta29$ SSB was also estimated by time-resolved techniques as the ratio $\Phi_T$, where $\tau_T$ stands for the radiative lifetime of tyrosine, $27 \pm 2$ ns (33, 40).

The fluorescence anisotropy decay parameters (rotational correlation times, $\phi_i$, and amplitudes, $b_i$) were determined by simultaneously fitting the measured intensities $I_{1/2}$, $I_0$ to a sum of exponentials using a non-linear least-squares global analysis method as detailed elsewhere (36). The experimental decay of the tyrosine fluorescence anisotropy of $\Delta29$ SSB was best fitted to a biexponential function,

$$r_\beta \delta \rho_\beta^{\text{obs}} + \beta (r_\delta + \delta + \delta)^\text{obs} = \sum b_i - 1$$

(Eq. 2)

where $r_\beta$ is the anisotropy of the tyrosine emission at $t = 0$.

The decay of the fluorescence intensity and anisotropy contains sub-nanosecond components that were not well resolved in the flash lamp spectrometer. To have a better definition of these parameters, a set of both kinds of measurements were also carried out at the Laboratoire of...
The theoretical features of 29 SSB structure deduced from its primary sequence. A, the regions with a higher probability of being in the protein surface (Chou-Fasman predictions, Ref. 43) are boxed. The three tyrosines, at positions 50, 57, and 76 are indicated with asterisks. B, hydropathy profiles (Kyte-Doolittle index; 45) of 29 SSB. The location of residues limiting the regions with high hydrophilicity (shadowed) is indicated with numbers.

Biochimie Moleculaire et Cellulaire (Orsay, Paris) with a Ti:sapphire (Spectra Physics) ps laser spectrometer, tuned at the third harmonic (B). The three tyrosines, at positions 50, 57, and 76 are indicated with asterisks. The fluorescence spectra of 29 SSB and that of a stock solution of 2.5 M NaOH were added to a solution containing the indicated concentrations of protein in buffer B, the change in volume at the end of the titration being <2.5%. The pH increase was recorded simultaneously with the decrease in the fluorescence at 308 nm. Free tyrosine was taken as a model since its fluorescent properties depending on the ionic environment in response to pH increase are well documented (41–43) and no equivalent data were, to our knowledge, available for NAcTyRA. Corrections for dilution and filter effects were made as described (21).

**RESULTS**

**Theoretical Analysis Based on the 29 SSB Primary Structure**—The amino acid sequence of 29 SSB (Fig. 1A) was analyzed with currently available computer programs to assess its basic theoretical structural features. According to Chou-Fasman secondary structure predictions (44), the region around tyrosines 50 and 57 (see Fig. 1A) showed a high probability of being in the protein surface. Moreover, analysis of the sequence-related hydropathy (45) indicated that this same region (from residues 49 to 69), based on the negative values of the Kyte-Doolittle index (Fig. 1B), could not be highly hydrophobic.

In order to have a more reliable picture on both, the accessibility of the tyrosines of 29 SSB to the solvent, and potentially to DNA, as well as their ionic environment, we analyzed the parameters defining the steady-state and the time-resolved intrinsic fluorescence properties of the 29 SSB. The results obtained are summarized in Table I.

**Fluorescent Tyrosines of 29 SSB Are Not Ionized at Neutral pH**—The fluorescence spectra of 29 SSB free in solution, pH 7.5, and complexed with ssDNA are depicted in Fig. 2. The area under the excitation and emission spectra is reduced by 90% upon binding. Although the same excitation and emission maxima, \( \lambda_{ex} = 276 \) nm and \( \lambda_{em} = 308 \) nm, respectively, were observed in both cases. Moreover, the emission spectra did not show any shoulder around 340 nm and, therefore, ionization or strong hydrogen bonding of the phenol hydroxyl group of tyrosine does not seem to take place (28, 46) either for free or complexed 29 SSB.

As expected for A-type proteins, that is, proteins lacking tryptophan in their primary structure (28, 47), no changes in either the shape of the excitation or the emission spectra were observed for the denatured 29 SSB (4 M guanidinium chloride) (not shown).

**Fluorescence Emission of 29 SSB Is Polarized**—The anisotropy of 29 SSB fluorescence monitored by continuous excitation with polarized light gave a value of 0.173 ± 0.010 (at 22 °C), much smaller than the maximum (0.32) corresponding to immobilized tyrosines (48, 49). Different extrinsic mechanisms (once discarded light scattering and other artificial effects, see “Materials and Methods”), like radiationless energy transfer among the residues or rotational mobility of the fluorophore and/or the protein, could account for this depolarization. The former is very unlikely since it would require spectral overlap among the excitation and emission spectra that is not observed (Fig. 2) and a much higher concentration of the residues (28). To distinguish between localized motions of the tyrosine residues (that would be indicative of their rotational mobility) and global motions of the protein, the time-resolved fluorescence anisotropy of 29 SSB was analyzed.

The decay of the anisotropy was best fitted (\( \chi^2 = 1.17 \)) to biexponential functions (see Equation 2 and Fig. 3) with a \( r_c \) value (0.28 ± 0.01) close to the maximum expected for the tyrosine chromophore (see above), and two well separated correlation times. The fast one, \( \phi_{short} = 0.14 \pm 0.06 \) ns, accounts for approximately one-fourth of the depolarization. The remaining anisotropy decays almost completely through the slow rotational component, \( \phi_{long} = 7.0 \pm 0.5 \) ns. Since \( \phi_{short} \ll \phi_{long} \) the decay of the total anisotropy can be expressed as a product of two depolarizing processes, one due to fast movements of the protein segment containing the tyrosine residues (\( r'(t) \)), and another related to the global rotational motion of the whole protein (for reviews, see Refs. 50 and 51), as,

\[
\tau(t) = \tau'(t) e^{-t/\phi_{short}} - \tau'(t) e^{-t/\phi_{long}}
\]

where \( \tau'(t) = (r_c - r_m) e^{-t/\phi_{short}} + r_m \) being the limiting anisotropy that would be attained if the tumbling of the whole protein was arrested. According to the parameters of the anisotropy fitting (Table I), \( r_m \) was found to be 0.21.

On the other hand, since

\[
\phi_{short} = \phi_{segmental} + \phi_{global} \quad \text{and} \quad \phi_{long} = \phi_{global}^{-1}
\]

the short correlation time can be assigned to fast segmental motions of the tyrosine residues, and the long correlation time to the global motion of the entire protein (see Ref. 50 for review).

**The Efficiency of the Fluorescence Emission of 29 SSB Is High**—The efficiency of the tyrosine emission of 29 SSB was assessed by measuring the quantum yield of its global fluorescence emission (\( \Phi_{ST} \)) and steady-state (\( \Phi_{TR} \))

measurements.

The experimental decay of the total protein fluorescence was best fitted (\( \chi^2 = 1.1 \)) to triexponential functions that gave the lifetimes \( \tau_1 = 0.4 \pm 0.1 \) ns, \( \tau_2 = 1.5 \pm 0.1 \) ns, and \( \tau_3 = 3.8 \pm 0.1 \) ns, the pre-exponential terms (amplitudes) being \( a_1 = 0.39 \pm 0.03 \), \( a_2 = 0.27 \pm 0.03 \), and \( a_3 = 0.34 \pm 0.03 \), respectively. Since the fluorescence decay kinetics of polypeptides and proteins, even with a single tyrosyl residue, is usually complex (22, 46, 49), the results presented here should be taken as a numerical approximation to a more complex kinetics. However, the averaged lifetime of the protein fluorescence (\( \bar{\tau} = 1.8 \pm 0.1 \) ns) is a valid parameter to estimate the global quantum yield of the fluorescence emission of 29 SSB. Thus, the ratio between and the radiative lifetime of the tyrosine \( \tau_R \) (see “Materials and Methods”) gave a \( (\Phi_{TR}) \) of 0.067 ± 0.010. A similar value (see
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Table I  Parameters of the tyrosine-dependent fluorescence of φ29 SSB

| Steady state<sup>a</sup> | Time-resolved<sup>b</sup> |
|------------------------|-------------------------|
| λ<sub>max</sub>         | 276 nm                  |
| λ<sub>em</sub>         | 298 nm                  |
| F<sub>em</sub>         | 0.65 ± 0.010            |
| N<sub>310</sub>       | 3.1 ± 0.3               |
| Acryl                  |                         |
| k<sub>e</sub>         | (3.82 ± 0.50) × 10<sup>9</sup> m<sup>-1</sup> s<sup>-1</sup> |
| A                      | 4 π                     |
| f<sub>sec</sub>       | 1                       |
| KI                     |                         |
| k<sub>e</sub>         | (2.33 ± 0.20) × 10<sup>9</sup> m<sup>-1</sup> s<sup>-1</sup> |
| A                      | 3.3 π                   |
| f<sub>sec</sub>       | 1                       |
| τ<sub>310</sub>       | 1.8 ± 0.1 ns            |
| τ<sub>298</sub>       | 0.067 ± 0.010           |
| τ<sub>A</sub>        | α<sub>1</sub>           |
| τ<sub>B</sub>        | α<sub>2</sub>           |
| τ<sub>C</sub>        | α<sub>3</sub>           |
| φ<sub>exc</sub>      | 0.28 ± 0.01             |
| φ<sub>em</sub>       | 0.21 ± 0.05             |
| φ<sub>exc</sub><sup>c</sup> | 0.27 ± 0.03              |
| φ<sub>em</sub><sup>c</sup> | 0.28 ± 0.03              |
| φ<sub>exc</sub><sup>d</sup> | 0.14 ± 0.06              |
| φ<sub>em</sub><sup>d</sup> | 0.70 ± 0.05              |
| φ<sub>exc</sub><sup>e</sup> | 0.03                      |
| φ<sub>em</sub><sup>e</sup> | 0.76 ± 0.03              |

<sup>a</sup> All the measurements were done in buffer B at 25 °C.

<sup>b</sup> These measurements were done in buffer A, at 25 °C for λ<sub>max</sub> 294 nm and λ<sub>em</sub> 310 nm.

<sup>c</sup> Expected limiting anisotropy if the protein was immobile (see Equation 3).

<sup>d</sup> Effective quenching rate constant of the fluorescent tyrosines of φ29 SSB. In the same experimental conditions, k<sub>e</sub> of Acryl and KI for NAcTyrA were 5.1 × 10<sup>9</sup> m<sup>-1</sup> s<sup>-1</sup>, and 5.3 × 10<sup>9</sup> m<sup>-1</sup> s<sup>-1</sup>, respectively (for these calculations, τ of NAcTyrA was considered as 1.6 ns; 52).

<sup>e</sup> Anisotropy at t = 0.

<sup>f</sup> Expected limiting anisotropy if the protein was immobile (see Equation 3).

Fig. 2. Corrected fluorescence spectra of φ29 SSB. Data were obtained at a final concentration of 9 μM in buffer B at 25 °C in the absence (thick curves) and presence (thin curves) of saturating amounts of ssDNA (300 μM poly(dT)). The excitation (λ<sub>exc</sub> = 308 nm) and emission spectra (λ<sub>em</sub> = 276 nm) were corrected for background emission, inner filter effect of DNA absorption and wavelength dependence of the fluorimeter response as indicated under “Materials and Methods.”

Table I was obtained when the quantum yield was estimated in steady-state measurements (Φ<sub>em</sub><sup>ST</sup>) when the area of the emission spectra of φ29 SSB was compared to that of the reference emitter, the well characterized 5-MeO1 (not shown). Therefore, the φ29 SSB tyrosine-dependent fluorescence is not being quenched by ground-state mechanisms. In contrast to the situation found in most proteins, the value of Φ<sub>em</sub><sup>ST</sup> did not increase upon denaturation (results not shown).

The intensity of φ29 SSB fluorescence was directly dependent on the temperature of the solution, this dependence being biphasic with a clear slope change at 45 °C (Fig. 4). This transition could reflect conformational changes in the environment of the fluorescent tyrosines, rather than on the global conformation of φ29 SSB, which seems to be stable up to 50 °C (23).

All Tyrosine Residues of φ29 SSB Are Fluorescent—If there is no energy transfer among the tyrosines (see below), it is possible to estimate whether or not a tyrosine(s) contributes appreciably to the observed emission by combining the estimated global Φ<sub>exc</sub> and of the protein fluorescence. This is based on the fact that the pre-exponential terms of the function that fits the experimental fluorescence decay data are related to the fractional concentration of the emitting species. Thus, the number of emitting fluorophores (N<sub>E</sub>) can be related to that of the excited residues by the expression N<sub>E</sub> = N<sub>T</sub>Φ<sub>em</sub><sup>ST</sup>/(τ<sub>τ<sub>E</sub></sub>), where N<sub>T</sub> is the total number of fluorophores (25). In our case, N<sub>E</sub> was calculated to be ~3. Therefore, all tyrosines contribute to φ29 SSB, although they may not be necessarily mechanistically identical.

Fluorescent Tyrosines of φ29 SSB Are Equally Accessible to Solvent and Placed in an Exposed and Negatively Charged Microenvironment—It is well known (41, 42) that positive charges in the surroundings of a tyrosine facilitate ionization, allowing tyrosine to appear below pH 10 for free tyrosine. On the contrary, in the presence of a negative environment or for buried tyrosines, a higher pH is required for effective ionization.

I ionization of φ29 SSB tyrosines was followed by the extent of fluorescence quenching at 308 nm as a function of pH increase, since the quantum yield of tyrosine emission is highly reduced upon ionization (47). The results, compared with those of the free tyrosine are shown in Fig. 5. From pH 7.5 (the standard during the functional assays of φ29 SSB) to pH 9, the quenching pattern of φ29 SSB closely resembles that of the exposed tyrosine in solution. However, both ionization patterns became different at higher pH. The initial fluorescence was reduced by 50% at pH 10.2 for free tyrosine, as expected, and at pH 10.9 for...
SSB fluorescence emission. A solution containing 12 μM 29 SSB buffer B at 22 °C was excited (λex = 276 nm) with a continuous light source (see “Materials and Methods”). Fluorescence emission (Iem) was recorded at 308 nm with magic angle orientation of the polarizers (excitation 0°, emission 54°) to avoid anisotropic effects. Samples were kept 5 min at the indicated temperatures before carrying out the measurements. The estimated errors were <2%.

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Therefore, either (i) the 29 SSB tyrosines are located in heterogeneous regions and they are differentially ionized at different pH or (ii) they are exposed in the protein surface, and as a consequence of denaturing effects became protected from ionization (by being included within the protein bulk and/or by being surrounded by negatively charged residues). More precise information could not be obtained by this technique, since the protein structure, as indicated by CD spectra, was significantly affected at pH > 9 (23). Further insight on the tyrosine microenvironment, in terms of solvent accessibility and neighbor ionic residues, was obtained by measuring the quenching of the protein fluorescence by small and highly diffusible quencher agents: acrylamide, iodide, and cesium ions.

Acrylamide, a highly efficient quencher of the tyrosine fluorescence (52), which does not interact with proteins and is not influenced by nearby charged residues (20, 31), was used to analyze the exposure of the emitting residues of 29 SSB to solvent. Stern-Volmer plots for the acrylamide quenching of 29 SSB fluorescence (Fig. 6A) were linearly dependent on the quencher concentration, the fractional accessibility being 1 (Fig. 6B). The rate constant of the global quenching process, \( k_{q, eff} \) (acryl) was (3.8 ± 0.5) × 10^9 M^-1 s^-1, 75% of the value measured for the fully accessible NAcTyrA under the same conditions. This difference may be related to the difference in the diffusion coefficients of the tyrosyl residues of the protein relative to free NAcTyrA, since the solid angle \( \Omega \) under which the acrylamide can approach these fluorophores was estimated as 4\( \pi \) for both, the set of tyrosyl residues of 29 SSB and the NAcTyrA. Therefore, these results are consistent with a situation in which the fluorescent tyrosines of 29 SSB are equally accessible to the solvent, and, apparently, located in non-buried regions of the protein.

The ionic environment of the 29 SSB tyrosine residues was assessed by using \( I^- \) (as KI) and Cs\(^+\) (as CsCl) as quenchers. Fig. 6C shows the results obtained in titrations of 29 SSB and NAcTyrA with KI. Again, the plots were linear, with all of the fluorophores being accessible (Fig. 6D). However, the quenching was less efficient, \( k_{q, eff} \) (KI) being (2.3 ± 0.2) × 10^9 M^-1 s^-1, ~46% of NAcTyrA, and the angle \( \Omega = 3.3\pi \) (see Table I). Thus, it seems that although all of the fluorescent tyrosines of 29 SSB are homogeneously quenched by I\(^-\), they seem to be somehow shielded by negative charges that would make the interaction with the quencher difficult. We also used a positively charged quencher (Cs\(^+\)), but the results were erratic. Due to its low quenching efficiency (33), high amounts of salt (more than 0.6 M) had to be added to significantly quench the 29 SSB fluorescence, giving rise to important changes in the ionic strength of the solution and, most likely, in the protein organization. Similar undesired effects of CsCl have been previously reported for other A-type proteins, e.g. ribosomal proteins S\(_p\) and S\(_{15}\) (33) or calmodulin (52).

Temperature Has an Opposite Effect on the Thermodynamic Parameters \( K \) and \( \omega \) of 29 SSB-ssDNA Complex—Changes in the intensity of 29 SSB intrinsic fluorescence induced upon ssDNA interaction at different temperatures were studied to determine the relative contribution of the different thermodynamic parameters on 29 SSB-ssDNA complex formation. The experimental temperature range was 10–42 °C, where the protein conformation is stable (23) and the intensity of the fluorescence emission is linearly dependent on temperature (Fig. 4). Data from direct titrations of poly(dT) with increasing amounts of 29 SSB were processed according to the “unlimited” cooperative model of McGhee and von Hippel (35) as outlined under “Materials and Methods” (see Ref. 21, for further details). Representative examples of the experimental assays at 15 and 42 °C and the fitting procedure are shown in Fig. 7, A and B, respectively. The value of the nucleotide site size, \( n \), the intrinsic affinity \( K \), and the cooperativity, \( \omega \), are indicated in Table II. \( Q_{max} \) and \( n \) were found to be relatively independent on the temperature of the binding solution. On the contrary, \( K \) was highly affected by temperature increase, diminishing from 7100 M^-1 (10 °C) to 700 M^-1 (42 °C). Interestingly, the cooperativity parameter \( \omega \) increased with temperature, being ~4 times higher at 42 °C (\( \omega = 220 \)) than at 10 °C (\( \omega = 56 \)). The result of these compensating effects was a slight (2.5-fold) reduction of the effective DNA binding constant \( K_0 \). The van’t Hoff plot of \( K_0 \) versus 1/T was linear within the temperature range used (Fig. 8), indicating that \( \Delta C_p^0 = 0 \) and, therefore, that the enthalpy (\( \Delta H^f \)) is independent of temperature (53).

Approximated values of the enthalpy (\( \Delta H_{obs}^f \)) and the entropy (\( \Delta S_{obs}^f \)) of the 29 SSB-ssDNA complex formation were obtained from the y axis intercept and the slope of the plot, respectively. Thus, \( \Delta H_{obs}^f = -5 \) kcal mol^-1 and \( \Delta S_{obs}^f = -8 \) cal K^-1 mol^-1.

**DISCUSSION**

SSBs share two main functional properties: several orders of magnitude more efficient binding to ssDNA than to dsDNA, and critical roles in the maintenance and expression of the
genetic information. *B. subtilis* phage φ29 encodes its own SSB, which is absolutely required for viral DNA replication *in vivo*. φ29 SSB plays its role(s) by binding to the ssDNA regions of the replicative intermediates produced during strand-displacement φ29 DNA replication (18–20). Therefore, the molecular basis of the contacts responsible for the formation and stability of this complex must be determined to properly understand the functional significance of φ29 SSB during phage φ29 infective cycle.

Towards the Identification of Residues Involved in Complex Formation—Steady-state fluorescence studies have served to determine that the interaction between φ29 SSB and ssDNA is characterized by an unlimited cooperative DNA binding mode (ω = 50–80 at 25 °C), a relatively low binding affinity (K<sub>eff</sub> = 1–3 × 10<sup>5</sup> M<sup>−1</sup> at 25 °C), a small nucleotide binding size (n = 3–4 nt), and a net release of a very low number of cations (21). The lack of a clear sequence homology or defined patterns in protein motifs involved in ssDNA binding made difficult the identification of the φ29 SSB residues interacting with ssDNA. One feature of the φ29 SSB-DNA interaction that could give a hint on a potential DNA binding domain is the high reduction of the intrinsic tyrosine fluorescence of φ29 SSB upon complex formation, the maximal quenching (Q<sub>max</sub> > 95%) being one of the highest values reported for a SSB-ssDNA interaction (21). Among the several mechanisms described to account for tyrosine quenching in proteins that bind to DNA (22, 46, 54), those leading to the highest reduction in fluorescence intensity have been related to direct interactions between tyrosines and DNA (22, 46). It should be kept in mind that since φ29 SSB binding is cooperative, the possibility that quenching occurs as a consequence of protein-protein interactions must be also taken into account. In any case, a detailed knowledge of the tyrosine environment and accessibility in the φ29 SSB molecule is necessary to understand the molecular nature of the interactions that contribute to φ29 SSB-DNA complex formation.

The average quantum yield Φ<sub>f</sub> of the intrinsic φ29 SSB fluorescence emission, analyzed by steady-state and time-resolved measurements, gave a value of 0.065 ± 0.010. The fact that both techniques gave the same Φ<sub>f</sub> value can be considered as indicating that none of the three possible emitters has been eliminated by ground-state deactivating processes. In other words, the three tyrosines of φ29 SSB contribute to its intrinsic

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**Fig. 6.** Stern-Volmer analysis of the solvent accessibility and ionic environment of the fluorescent tyrosines of φ29 SSB. Fluorescence quenching of φ29 SSB (11.7 μM, closed symbols) upon addition of increasing amounts of acrylamide (A) or KI (C) was compared to that of NAcTyrA (20.9 μM, open symbols). In both cases, solutions were made in buffer B and kept at 25 °C. The slopes of the plots (K<sub>sv,eff</sub>) allow the calculation of the bimolecular rate constant, k<sub>b,sv</sub>, as indicated in the text. The fraction of tyrosines accessible to acrylamide (B) and I<sup>−</sup> (D) were obtained from the y axis intercept of double-reciprocal Lehrer-modified Stern-Volmer plots. The values of these parameters are listed in Table I.

![Stern-Volmer analysis of the solvent accessibility and ionic environment of the fluorescent tyrosines of φ29 SSB.](image)

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**Fig. 7.** Temperature effect on φ29 SSB-ssDNA complex formation. A, direct titrations of poly(dT) with increasing amounts of φ29 SSB at 15 °C (open symbols) and 42 °C (closed symbols). The broken line at the beginning of the titration represents the increase in fluorescence resulting from the absence of DNA binding. The emission constant of the free protein, f<sub>f</sub>, was estimated from the slope of this line. The same slope was achieved at the end of the titration, indicating that all of the present DNA has been saturated. B, Scatchard plot of the best fit (lines) of the experimental data (points) corresponding to the experiments shown in A. The fluorescence intensity at each protein concentration [SSB]<sub>T</sub> together with the f<sub>f</sub> and Q<sub>max</sub> allowed the determination of the amount of free [SSB]<sub>f</sub> and bound [SSB]<sub>B</sub> protein. From these values, the binding density (ν = [SSB]<sub>B</sub>/[DNA]) corresponding to the experimental data was estimated and compared with theoretical ones computed according to the McGhee and von Hippel’s unlimited cooperative model for binding of proteins to large lattices (32) as described previously (21). The calculated values for the thermodynamic parameters of φ29 SSB-ssDNA for these and other temperatures are shown in Table II.
Theoretical values giving the best fit to experimental data corresponding to direct titrations of ssDNA (30–40 μM) in buffer B, analyzed according to the unlimited cooperative model of McGehee and von Hippel (35) as outlined before (see Ref. 21). The maximal quenching ($Q_{\text{max}}$) values upon complex formation were determined as (96 ± 3%) from dilution experiments (21, 66) carried out in the same conditions. Poly(dT) was chosen as a DNA binding lattice to ensure homogeneous binding and therefore to avoid undesired sequence-dependent effects (53).

Intrinsic Tyrosine Fluorescence of φ29 SSB—

The calculated $K_d$ values of φ29 SSB-ssDNA complex (see Table II) were plotted against the inverse of the solution temperature (Kelvin). The best fit to the data was achieved for linear expressions, which gave $K_d$ and $T$ against the inverse of the temperature ($T$).

Further information regarding the environment of the φ29 SSB tyrosines was obtained from the analysis of the rotational depolarization of the intrinsic protein fluorescence. As shown above, time-resolved anisotropy revealed two depolarizing motions with clearly different correlation times. In the absence of resonance energy transfer, the short correlation time, $\phi_{\text{short}}$, of φ29 SSB-ssDNA complex formation ($T$ = 0.13 ns; 60) and, therefore, can be considered as indicating absence of restrictive interactions among tyrosine side-chains and other residues. The long correlation time ($\phi_{\text{long}} = 7.0 ± 0.5$ ns) describes a slower motion of the whole protein. According to the parameters of the anisotropy fitting (Table I), the limiting anisotropy, $r_0$, that would be attained if the tumbling of the whole protein was arrested, was found to be 0.21. From this value, the average angular oscillations of the emitting residues of φ29 SSB were estimated (50) as spanning about 50 °C. In summary, φ29 SSB tyrosines are able to undergo fast subnanosecond oscillations as if they were not blocked, although some steric hindrance, likely due to the presence of the rest of the protein bulk, partially limits the amplitude of these motions.

Thermodynamic Aspects of φ29 SSB-ssDNA Complex—The large quenching of the intrinsic fluorescence of φ29 SSB upon complex formation ($Q_{\text{max}} = 95%$; 21) precluded the analysis of the effect of DNA interaction on the rotational correlation times of the anisotropy, the lifetimes of the total fluorescence or the Stern-Volmer constants. However, the changes in the intrinsic fluorescence intensity as a result of DNA binding, monitored at different experimental temperatures, can be used to determine the individual contributions of the intrinsic affinity and the cooperativity.
parameters to the global binding process, as well as to obtain a rough estimation of the enthalpy and entropy values, as it has been described for other SSBs (52, 61–63). The increase of the reaction temperature in the range in which the global protein conformation is stable (see “Results” and Ref. 23) has opposite effects on the intrinsic binding constant, $K$, and on the cooperative parameter, $\omega$, the effective binding constant ($K_{\text{eff}} = K\omega$) being largely independent of temperature. Thus, while a $10^\circ$C decrease of $K$ was estimated from 10 to 42 $\circ$C, an interesting simultaneous 4-fold increase is observed for $\omega$ and, as a result, $K_{\text{eff}}$ is reduced only by $2^\circ$C. Therefore, thermally-induced brownian motions seem to favor a more accessible conformation of the protein domain involved in monomer-monomer interactions. The van’t Hoff analysis of temperature effect on $K\omega$ allows a gross estimation of the energies driving the global ssDNA binding process. The linearity of the van’t Hoff plots, characteristic of non-site specific DNA interactions (53, 64) indicated that, at low salt concentration, $\Delta G_{\text{obs}}$ ($=-5$ kcal mol$^{-1}$) was temperature independent ($\Delta C_p \Delta = 0$), allowing the estimation of $\Delta G_{\text{obs}}$ as $-8$ cal K$^{-1}$ mol$^{-1}$.

Altogether, our results are consistent with fluorescent tyrosines being located either on the surface of the protein or in a pocket whose size and shape allow the quencher to gain access to the tyrosine residues. The fact that the nucleotide site size has a relatively small value (3–4 nt are covered by protein monomer; 21) supports the possibility of a discrete domain involved in complex formation with ssDNA. Furthermore, tyrosine mobility does not seem to be highly restricted by interactions with neighboring residues. This localization might contribute to complex formation, either indirectly, by establishing protein contacts between contiguous monomers, or through direct interactions with the DNA bases. Preliminary results support the last hypothesis, since $\leq$29 SSB point mutants in each of the three tyrosines have an impaired ability to bind ssDNA. In this sense, the presence of a net negative charge surrounding the tyrosines could be the reason for the relatively low affinity of this protein for ssDNA, a property which may be directly related to its functional roles during $\phi 29$ DNA replication (20, 21). In addition, the relative independence of the effective DNA binding constant of $\leq$29 SSB of the solution temperature could provide an adaptive advantage for phage $\phi 29$ development in the very different habitats, e.g., soil, field crops, different foodstuffs, seawater (see Ref. 65, for a review), in which the cellular host, $B. subtilis$, is able to grow.

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Structural Features of φ29 Single-stranded DNA-binding Protein: I.
ENVIRONMENT OF TYROSINES IN TERMS OF COMPLEX FORMATION
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