Equilibrium Binding of Single-stranded DNA with Herpes Simplex Virus Type I-coded Single-stranded DNA-binding Protein, ICP8*

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We have carried out solution equilibrium binding studies of ICP8, the major single-stranded DNA (ssDNA)-binding protein of herpes simplex virus type I, in order to determine the thermodynamic parameters for its interaction with ssDNA. Fluorescence anisotropy measurements of a 5'-fluorescein-labeled 32-mer oligonucleotide revealed that ICP8 formed a nucleoprotein filament on ssDNA with a binding site size of 10 nucleotides/ICP8 monomer, an association constant at 25°C, K = 0.55 ± 0.05 × 10^6 M^{-1}, and a cooperativity parameter, α = 15 ± 3. The equilibrium constant was largely independent of salt, δlog(K)δlog([NaCl]) = −2.4 ± 0.4. Comparison of these parameters with other ssDNA-binding proteins showed that ICP8 reacted with an unusual mechanism characterized by low cooperativity and weak binding. In addition, the reaction product was more stable at high salt concentrations, and fluorescence enhancement of etheno-ssDNA by ICP8 was higher than for other ssDNA-binding proteins. These last two characteristics are also found for protein-DNA complexes formed by recombinases in their active conformation. Given the proposed role of ICP8 in promoting strand transfer reactions, they suggest that ICP8 and recombinase proteins may catalyze homologous recombination by a similar mechanism.

ICP8 is 128-kDa zinc metalloprotein coded by the UL29 gene of herpes simplex virus type 1 (HSV-1). It is one of seven virus-encoded proteins necessary for HSV-1 origin-dependent DNA replication (1). ICP8 forms an extended nucleoprotein filament with ssDNA in vitro; it binds more readily to ssDNA than to double-stranded DNA (1–6), and DNA binding may involve contacts by free sulfhydryl groups and surface lysine and tyrosine residues (6, 7). In addition to its role as an ssDNA-binding protein, complexes of ICP8 with other proteins may act during viral replication. ICP8 physically interacts via the UL8 protein with the HSV-1 helicase primase heterotrimer thereby stimulating unwinding of intact and damaged duplex DNA (8, 9); likewise a physical interaction between ICP8 and the herpes UL9 helicase has also been shown (10). Specific protein-protein interactions between ICP8, UL9 protein, and subunits of the DNA helicase primase are required for the assembly of these proteins into prereplicative sites, and recruitment of the DNA polymerase into this complex is mediated by the UL42 subunit (11, 12).

Cells infected with HSV-1 display a high frequency of homologous recombination (13). In vitro experiments suggest that ICP8 may be responsible for homologous recombination in vivo. ICP8 exhibits helix destabilizing and DNA renaturing activities (2, 14). The protein also catalyzes homologous pairing and strand exchange activity in vitro (15) where it is able to transfer a DNA strand from a linear duplex to a complementary single-stranded circular DNA. The reaction requires MgCl2 but not (d)NTPs, and strand exchange is limited to a few 100 base pairs.

Understanding the role of ICP8 in the DNA replication and recombination reactions of HSV-1 will require knowledge of the thermodynamics and kinetics of the various macromolecular interactions involved. Currently, the thermodynamic characteristics of the simple reaction between ICP8 and ssDNA are largely unknown compared with other ssDNA-binding proteins such as gp32 from phage T4 (16, 17), Escherichia coli SSB (18), or eucaryote RPA (19, 20). In order to address this question, we have undertaken solution equilibrium binding studies of the reaction of ICP8 with ssDNA using fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Chemicals and Reaction Conditions—ICP8 was purified as described (8). DNA was synthesized by published methods (21). 5'-Fluorescein (F)-labeled 32-mer F-5CATC5C5AAAATGACCTTATCTAAGGA and the corresponding unlabeled oligonucleotide were synthesized by Genosys. Concentration units of ICP8 and DNA are M protein and M nucleotide, respectively.

Spectroscopic Measurements—Fluorescence measurements were performed using a model MOS-400 spectrophotometer from Bio-Logic (Claix, France). Samples were stirred continuously at 25 ± 1°C in a 1.0 × 0.4-cm thermostated cuvette with the short path in the direction of incident light in order to reduce inner filter effects. Results were corrected for dilution and inner filter effects as described previously (21). pH was checked before and after the reaction and did not change during the titration.

In experiments with DNA, samples were excited at 305 nm, and fluorescence intensity was measured using a 345 nm cut-off filter. Fluorescence signals of DNA and of protein were linear functions of concentration in the concentration ranges used, which is consistent with the absence of aggregation. Fluorescence signal from protein alone was subtracted and data reported as fluorescence enhancement, (Flucplx − Flucplx)/Flucplx, where Flucplx is the fluorescence of the protein-DNA complex and Flucplx is the fluorescence of ssDNA alone.

In experiments using 5'-fluorescein-labeled oligonucleotide, samples were excited at 490 nm, and fluorescence intensity was measured through a 550-nm cut-off filter. Vertically and horizontally polarized fluorescence were measured simultaneously by a photoelastic modula-
tor PEM-90 (Hinds Instruments) and used to calculate fluorescence and anisotropy (23). Fluorescence anisotropy is reported either as $A_{\text{obs}}$ or $A = A_{\text{obs}} - A_{0}$, where $A_{\text{obs}}$ is the observed anisotropy, and $A_{0}$ is the signal of the oligonucleotide alone.

RESULTS

Binding of ICP8 to eDNA—Etheno-DNA is a fluorescent derivative of M13 ssDNA frequently used to measure protein binding to ssDNA in nucleoprotein filaments (17, 21, 24–27); conformational changes in the filament that stretch binding to ssDNA in nucleoprotein filaments (17, 21, 24–27); derivative of M13 ssDNA frequently used to measure protein molar ratios of ICP8 and 

7.4, data not shown), indicating that the reaction is stoichiometric. The stoichiometry of the reaction was $10 \pm 1$ nt/ICP8 molecule.

The ICP8-oligonucleotide complex could be dissociated with NaCl (Fig. 2b). The anisotropy of the oligonucleotide alone increased with salt concentration (data not shown) and equaled the slightly elevated anisotropy values observed after dissociation of the complex. This effect was taken into account in order to determine the salt titration midpoint (STMP), the salt concentration necessary to decrease anisotropy by 50%. Monovalent cations Na$^+$ and K$^+$ destabilized the protein-DNA complex with equal efficiency; the STMPs of NaCl and KCl were 0.35 ± 0.03 mM and 0.31 ± 0.02 mM, respectively. In contrast the destabilizing efficiency was sensitive to anion, STMP NaCH$_3$COO$^- = 0.76 \pm 0.02$ mM, and the divalent cation was more potent than monovalent cations, STMP MgCl$_2$ = 0.08 ± 0.01 mM. It should be noted that data in Fig. 2b are salt titrations of complexes formed in the presence of 150 mM NaCl which was required to maintain the integrity of ICP8.

Unlike most spectroscopic measurements, fluorescence anisotropy is independent of the concentration of fluorophore (23). Therefore, in order to study ICP8 binding to ssDNA using this method, it was important to experimentally determine the relationship between anisotropy and the concentration of bound fluorescent oligonucleotide. This can be accomplished using the macromolecular binding density function method of Lohman and co-workers (29, 30). Briefly, several concentrations of oligonucleotide are titrated by protein (Fig. 3). All titrations with a particular anisotropy, $A$, have the same binding density $\nu = L_f/M_p$, which is determined by the chemical potential of the free ligand, approximated by $L_f$; here $L_f$ is bound ligand concentration, $L_f$ is free ligand concentration (M ICP8), and $M_p$ the total macromolecule concentration (M nt). The set of $[L_f]$, $M_p$ which gives a particular value of $A$ obeys conservation of mass, $L_f = L_o + L_f = v M_f + L_f$, where $L_f$ is the total ICP8 concentration (M protein). A plot of $L_f$ versus $M_p$ for the set of values $[L_f]$, $M_p$ corresponding to a particular anisotropy should be linear with a slope of $\nu$ and an intercept of $L_f$. Fractional saturation, $\theta$, can be calculated from binding density by $\theta = n \nu$ where $n$ is the binding site size (nt/protein molecule).

Since each value of $\theta$ corresponds to a unique value of the experimental signal, it is possible to determine the relationship between anisotropy and density of protein on the oligonucleotide (Fig. 4a). Each value of $\theta$ also corresponds to a particular free ligand concentration ($L_o$) which allows one to construct a binding isotherm (Fig. 4b) and to determine $K$ and $\omega$ independently of the relation between experimental signal and binding density.

The intrinsic anisotropy of the 5′-fluorescein-labeled 32-mer oligonucleotide was $0.02 \pm 0.0007$ (Fig. 2a) which is characteristic of mobile fluorophore (23). Titration of oligonucleotide with ICP8 increased anisotropy to 0.16–0.18 reflecting lower mobility as a result of protein binding. No fluorescence quenching occurred during the titration (data not shown). The stoichiometry of the reaction was $10 \pm 1$ nt/ICP8 molecule.

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On the basis of the salt-dependent stability of the protein-DNA complex (Fig. 2b), we chose to study equilibrium binding in the presence of 300 mM NaCl. Fluorescence anisotropy of 5–25 μM oligonucleotide titrated with ICP8 in these conditions is shown in Fig. 3. Intrinsic anisotropy anisotropy has been

### Table I

| Complex Formed                       | Stoichiometry (nt/ICP8) | Fluorescence enhancement$^a$ |
|--------------------------------------|-------------------------|-----------------------------|
| ICP8 titrated with eDNA              | 10.3 ± 0.7              | 3.1 ± 0.1                   |
| eDNA titrated with ICP8              | 11.5 ± 1.0              | 2.9 ± 0.2                   |

$^a$ (Fluorescence of complex − fluorescence of eDNA)/fluorescence of eDNA.
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idea of the robustness of the fit. These parameters were then used to fit experimental binding isotherms (Fig. 3). Theoretical curves were constructed assuming $A_{\text{max}} = 0.165$ and a linear relationship between anisotropy and fractional saturation. They agree well with experimental data at anisotropy values in the range 0.06–0.125 which corresponds to 40–80% saturation. However, at lower anisotropy values the effect of the nonlinear relation between anisotropy and fractional saturation (Fig. 4a) becomes apparent. These differences between experimental and theoretical curves can be corrected by taking into account experimental relationship between $A$ and $\theta$ in Fig. 4a (not shown). However, it should be recalled that $K$ and $\omega$ determined in Fig. 4b are independent of this relationship.

The salt dependence of the binding parameters was then determined. 5 $\mu$M oligonucleotide was titrated with ICP8 in 20 mM Tris, pH 7.4, at 25°C in the presence of 200–400 mM NaCl. $K$ and $\omega$ were calculated from these titration curves as above assuming $n = 10$ (Table II). Values of the cooperativity constant which gave best fits were in the range of $\omega = 5$–15 and did not significantly depend on salt concentration. A plot of log(KNaCl) versus log([NaCl]) was linear (Fig. 5). The slope of this plot, $-2.4 \pm 0.4$, is a function of the number of electrostatic interactions per protein monomer contributing to the stability of the nucleoprotein filament (33). These results, together with the dependence of the reaction on the nature of the anion (Fig. 2b), show that equilibrium binding of ICP8 with ssDNA is not simply entropically driven displacement of cations from polynucleotide as reported for simple peptide-DNA complexes (33–35).

We then investigated the effect of temperature on the reaction. We first tried to determine $K$ and $\omega$ at several temperatures between 20 and 40 °C. In this approach, the percent anisotropy increase during titration was assumed to be proportional to DNA binding at all temperatures as is approximately the case at 25 °C (Fig. 4a). In order to determine this parameter, the maximum anisotropy, $A_{\text{max}}$, which depends on temperature according to the Perrin equation (23), must be evaluated at each temperature. Preliminary experiments showed that plateau values of the binding isotherm were not sufficiently precise to determine $A_{\text{max}}$. We therefore measured binding parameters at 37 °C using the macromolecular binding density function methods as above (29–31). Four concentrations of oligonucleotide 10–25 $\mu$M were titrated with ICP8 at 37 ± 0.5 °C in 20 mM Tris acetate, 300 mM NaCl, pH 7.4. A slightly

subtracted from these data. Plots of $L_T$ versus $M_F$ for $L_T$, $M_F$ corresponding to anisotropies between 0.01 and 0.125 were linear (not shown); nonlinear curves were observed at larger values that may indicate aggregation at higher protein concentrations. The relationship between $A$ and $\theta$ was determined from the slopes of these plots and the binding site size $n = 10$ nt/protein (Fig. 4a). Small positive curvature was observed at low binding density. The simplest explanation of this observation is that initial bound protein inhibited mobility less than protein bound at high density. Maximum anisotropy was extrapolated from data at high fractional saturation, $A_{\text{max}} = 0.165 \pm 0.005$.

We fit the model-free binding isotherm (Fig. 4b) using the method of Epstein (31) for a protein ligand binding to a 32-mer oligonucleotide macromolecule and $n = 10$. This approach assumes that monomeric ICP8 binds to DNA; ICP8 sediments in a glycerol gradient as a monomer (32) which is consistent with the assumption. The best visual fit was found to be association constant $K = 0.55 \pm 0.05 \times 10^6$ M$^{-1}$ and cooperativity parameter $\omega = 15 \pm 3$. Visual fit was confirmed by examining the residuals between experimental and theoretical points for $K$ and $\omega$ near these values. Identical results were found with two independent titrations of 5–25 $\mu$M oligonucleotide with ICP8. The effects of variations of $K$ and $\omega$ illustrated in Fig. 4b give an
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**Table II**

**Binding parameters for the reaction of ICP8 with 32-mer oligonucleotide**

| Temperature | NaCl (mM) | $K_u$ (M) | $\omega$ |
|-------------|-----------|------------|----------|
| 25 °C       | 200       | $1.5 \times 10^6$ | 9        |
| 250         | 1.2 $\times 10^4$ | 8        |
| 300         | 0.6 $\times 10^4$ | 15       |
| 350         | 0.36 $\times 10^4$ | 13       |
| 400         | 0.16 $\times 10^4$ | 15       |
| 37 °C       | 300       | 0.22 $\pm 0.04 \times 10^6$ | 20–35    |

**Fig. 5. Salt dependence of the binding constant.** 5 μM oligonucleotide was titrated with ICP8 in the presence of 200–400 mM NaCl, and equilibrium constants and cooperativity parameters were calculated as in Fig. 3. The best straight line fit was calculated by linear regression, slope $= -2.4 \pm 0.4$, intercept $= 5.5 \pm 0.2$.

non-linear relation between anisotropy and $\theta$ was observed at 37 °C similar to results at 25 °C (Fig. 4c). The model free binding isotherm was fit using $n = 10$ (Table II). Enthalpy and entropy were assumed to be independent of temperature, and these were estimated from a van’t Hoff plot using $K_\omega$ at the 25 °C and 37 °C, $\Delta H = -5.6 \pm 1.3$ kcal/(mM$^{-1}$), $\Delta S = 12.6 \pm 4.4$ cal/(Kμ) $^{-1}$ (K is degrees Kelvin) where the uncertainties are from the variations of $K_\omega$ which fit the binding isotherms. These results indicate that entropy and enthalpy contributed about equally to the free energy of ICP8 binding to ssDNA.

To test the effect of the 5′-fluorescein on the reaction, we performed competitive binding experiments between labeled and unlabeled 32-mer in stoichiometric reaction conditions (20 mM Tris, 150 mM NaCl, pH 7.4, 25 °C). Fluorescence anisotropy was measured for complexes formed with 0.5 μM ICP8 and 10 μM of both labeled and unlabeled oligonucleotide. Assuming a binding site size of 10 nt, ICP8 should bind 5 μM nucleotides in these conditions and, if the fluorescent label has no effect, 25% of the fluorescent molecule would be bound to protein. Anisotropy, corrected for the intrinsic signal of unbound oligonucleotide, was 0.042 $\pm 0.005$, independently of the order of addition of labeled and unlabeled molecule. This value is in good agreement with the anisotropy expected for fractional saturation $\theta = 0.25$ (Fig. 4g). Hence, these competition experiments show that the fluorescent label did not interfere with DNA binding. Likewise, the absence of fluorescence quenching during titration suggests an absence of interaction between protein and the fluorescein moiety.

Binding site size for the oligonucleotide and for polynucleotide eDNA were the same (Table I and Figs. 1 and 2) which suggests that the product of the reaction between ICP8 and ssDNA is independent of the length of DNA. The binding parameters determined using a 32-mer oligonucleotide can probably be considered a good estimate for those of the reaction with ssDNA. We have not investigated the effect of DNA sequence which is known to influence the association constants of ssDNA-binding proteins. Nevertheless, our results give a qualitative description of the reaction between ICP8 and ssDNA which can be compared with DNA binding studies reported for ICP8 and other proteins.

**DISCUSSION**

DNA-binding site sizes of 12–22 nt/ICP8 monomer have been previously determined from electron microscope studies (36) or from indirect measurements based on the concentration of ICP8 required to optimize protein activity or nuclease protection (2, 14, 32, 37). Here we report direct measurement of the DNA-binding site size of ICP8 in solution using spectroscopic techniques, $n = 10 \pm 1$ nt/ICP8 monomer (Table I). Identical results were observed for binding to ss eDNA (Fig. 1) with NaCl concentrations from 150 to 500 mM or without NaCl in the presence of 10% glycerol as well as for the reaction with a single-stranded 32-mer oligonucleotide (Fig. 2). ICP8 binding site size for the reaction with eDNA was independent of salt concentration, 0–500 mM NaCl, indicating a single DNA binding mode as a function of this salt, unlike the case of E. coli SSB (18).

Binding of ICP8 to ssDNA stretched the DNA judging from enhanced fluorescence of etheno-modified DNA (Fig. 1). These spectroscopic results are in agreement with electron microscopy experiments showing that ICP8 interacts preferentially with ssDNA to form regular repeating structures that hold DNA in an extended conformation (4, 36). The induction of a stretched DNA conformation is found in nucleoprotein filaments of ssDNA-binding proteins gp32 from phage T4 (17), SSB from E. coli (38), and RPA from yeast (24). This extended conformation is a property shared with protein-DNA filaments formed by recombinases such as bacterial RecA (39), UvsX from phage T4 (25), and eucaryote Rad51 (27). Although this fluorescence increase does not depend in a quantitative fashion on DNA length, it is nevertheless a useful qualitative manner to compare conformational changes brought about by various ssDNA-binding proteins. The maximum fluorescence enhancement factor at 25°C of ICP8 (2.9 ± 0.2) was larger than the value reported for ssDNA-binding proteins gp32 (2.0 (17)), yeast RPA (1.7 (24)), and for inactive RecA protein in the absence of cofactor or in the presence of ADP (2.0 (26)). Larger fluorescence enhancement values are reported for active recombinases in the presence of ATP cofactor at 25°C: UvsX protein (2.0–2.5 (25)); RecA protein (2.8 (21, 26)); and Rad51 at 30°C (5.0 (27)). There have been reports that the fluorescence enhancement of eDNA varies with the quantity and quality of etheno modification (25). We therefore compared the fluorescence enhancement of ICP8 and RecA using the same eDNA substrate. Fluorescence increase by ICP8 was indistinguishable from the signal with RecA in the presence of ATP/Pi/S (not shown).

The ICP8-eDNA complex was stable in the presence of high concentrations of NaCl (Fig. 1). This stability was observed for complexes formed by both titration of protein with DNA and titration of DNA with protein. Furthermore, titrations of eDNA with protein in the presence of 200–500 mM NaCl gave similar binding isotherms as titration of DNA in 150 mM NaCl or in the presence of 10% glycerol without NaCl. We conclude that equilibrium binding occurs in these reaction conditions, and the protein-ssDNA complex cannot be dissociated by high NaCl concentrations.
Homologous recombination is carried out by recombinases in nucleoprotein filaments by a mechanism that has been found in all organisms from phage to man (42). Furthermore, recombinases are the only DNA repair proteins with a common domain arrangement in bacteria, archea, and eucaryotes (43). This conservation across evolution is likely the result of requirements of the homologous recombination reaction. If this argument is correct, ICP8 which catalyzes strand transfer in vitro (15), might likewise be expected to share some common properties with recombinases that are not found in other ssDNA-binding proteins.

We have observed that the ICP8-ssDNA nucleoprotein filament produces higher fluorescence enhancement of eDNA, is more stable in the presence of high concentrations of NaCl (Fig. 1), and exhibits smaller values of $\log(K_{o})/(\log([NaCl]))$ than other well studied ssDNA-binding proteins, gp32, ecSSB, and RPA (Table III). The fluorescence enhancement of ICP8 resembles that reported for active recombinases RecA and Rad51 and indicates that DNA may assume a similar conformation in these filaments. Stability at high NaCl concentrations is likewise a characteristic of both the ICP8 nucleoprotein filament and recombinase filaments in their active conformation.

Table III

| Nucleic acid substrate | $\log(K_{o}/[\log([NaCl])])$ | Fluorescence enhancement $K_{o}$ (0.2 M NaCl, 25 °C) |
|------------------------|-----------------------------|-----------------------------------------------|
| Poly(dA)              | $-5.9^a$                    | $2.0^b$                                        |
| dX ssDNA$^c$          | $-6.3^a$                    | $1.0 \times 10^{6}$                            |
| (SSB)$_{35}$          | $-6.1$                      | $2.6 \times 10^{6}$                            |
| (SSB)$_{65}$          | $-4.8$                      | $1.1 \times 10^{6}$                            |
| RPA                   | $1.71$                      | $10.20$                                        |
| ICP8$^d$              | $-2.4 \pm 0.4$              | $2.9 \pm 0.2$                                  |
| 32-mer ssDNA          | $2.9 \pm 0.2$               | $1.35 \times 10^{7}$                          |

$^a$ See Ref. 16.
$^b$ Poly(6A) (17).
$^c$ ecSSB in low salt binding mode, $n = 35$ nt/protein monomer (18).
$^d$ ecSSB in high salt binding mode ($\geq 0.2$ M NaCl), $n = 65$ nt/protonomer protein (18, 45).

Of course bona fide recombinases also have general characteristics that are not found for ICP8. They form long nucleofilaments with well defined right-handed helical structures (42, 44), whereas ICP8 nucleoprotein filaments are left-handed and twice as thick as RecA filaments, suggesting that more than one ICP8 molecule contribute to its width (32). Recombinases but not ICP8 have ATPase activity (1, 42). However, despite these and other differences, the protein-DNA complexes of ICP8 and recombinases appear to share some structural and biochemical similarities. Further comparison of ICP8 and recombinases should give important insights into the chemistry of homologous recombination.

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