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SSB Binding to ssDNA Using Isothermal Titration Calorimetry

Alexander G. Kozlov and Timothy M. Lohman

Abstract

Isothermal titration calorimetry (ITC) is a powerful method for studying protein–DNA interactions in solution. As long as binding is accompanied by an appreciable enthalpy change, ITC studies can yield quantitative information on stoichiometries, binding energetics (affinity, binding enthalpy and entropy) and potential site–site interactions (cooperativity). This can provide a full thermodynamic description of an interacting system which is necessary to understand the stability and specificity of protein–DNA interactions and to correlate the activities or functions of different species. Here we describe procedures to perform and analyze ITC studies using as examples, the *E. coli* SSB (homotetramer with 4 OB-folds) and *D. radiodurans* SSB (homodimer with 4 OB-folds). For oligomeric protein systems such as these, we emphasize the need to be aware of the likelihood that solution conditions will influence not only the affinity and enthalpy of binding but also the mode by which the SSB oligomer binds ssDNA.

Keywords

EcoSSB; DrSSB; ssDNA binding; ITC; SSB-ssDNA thermodynamics

1. Introduction

Isothermal titration calorimetry (ITC) is the only method that allows for simultaneous determination of binding affinity (K_{eq}), and binding enthalpy (ΔH°) for an interacting system from a single equilibrium titration experiment. As a result one ITC experiment can provide a nearly complete thermodynamic characterization of an interacting system under one set of conditions, i.e., standard state binding free energy (ΔG°), binding enthalpy (ΔH°), and binding entropy (ΔS°), since these functions are related through Eq. 1,

$$\Delta G^\circ = -RT \ln K_{eq} = \Delta H^\circ - T \Delta S^\circ \quad (1)$$

where R is the gas constant (cal/mol deg) and T is the temperature (K°). Additional experiments performed at different temperatures allow for the determination of another important thermodynamic quantity, the heat capacity change

$\left(\Delta C_p = \left(\frac{\partial \Delta H}{\partial T} \right)_p = T \left(\frac{\partial \Delta S}{\partial T} \right)_p \right)$. Further experiments performed as a function of pH, salt concentration and type or another second ligand that may bind any of the primary species under study (protein, DNA, etc.) will also provide information on any heterotropic linkage

that affects binding (1). Such thermodynamic information is necessary to understand stability and specificity of protein–DNA complexes and their mechanisms of interactions. The thermodynamics of *Eco*SSB binding to ssDNA has been extensively studied using ITC (2–7). More limited examples of ITC experiments are also available for *Dr*SSB (8) and yeast *sc*RPA (9).

The *Escherichia coli* ssDNA binding protein (*Eco*SSB) is the most studied representative of the large class of bacterial SSB proteins (10). It is a stable homotetramer in solution containing four OB folds (one per subunit) that each provide a site for binding ssDNA (11). Due to the presence of these four potential ssDNA binding sites, the *Eco*SSB tetramer can bind to long, polymeric ssDNA in multiple binding modes that differ by the amount of ssDNA that is wrapped around the tetramer. Two major binding modes, denoted (SSB)₃₅ and (SSB)₆₅, differ by the number of subunits, either two or four, respectively, that interact with ssDNA with corresponding occluded site sizes of ~35 and 65 nucleotides (10). The stabilities of these binding modes are influenced by salt concentration, type and valence and protein binding density (10, 12). The four C-termini of the SSB tetramer (residues 112–177) are unstructured even when SSB is bound to ssDNA (13). The last 8–10 amino acids in these C-termini serve as the primary sites to which more than a dozen other proteins bind to SSB (14). The binding of *Eco*SSB tetramer to oligodeoxythymidylates that are close in size to the occluded site size (65 nucleotides) is of such high affinity (i.e., stoichiometric) under most solution conditions, even at very high salt concentrations, that the affinity is difficult to measure directly. Binding of *Eco*SSB to (dT)₇₀ is characterized by an extremely large and negative enthalpy change (up to –160 kcal/mol at low salt conditions (3)), and a large and negative heat capacity change, $C_{p,obs}$ (up to –1.2 kcal/mol °K (6)). In fact, this is the largest binding enthalpy yet reported for a protein–DNA interaction. The thermodynamics of *Eco*SSB–ssDNA binding has contributions from the binding of multiple other small molecule species that accompany SSB–DNA binding. These so-called “linked equilibria” or linkage effects include the binding of cations and anions (2, 3, 6, 7), and protonation (5, 15). Conformational transitions within the DNA (4) and SSB (6, 7) have also been documented and quantified.

The *Deinococcus radiodurans* SSB (*Dr*SSB) belongs to the *Deinococcus*–*Thermus* group and functions as a dimer; however, each monomer contains two OB-folds linked by a conserved spacer sequence (16). Therefore, the functional form of this protein also is composed of four OB-folds, although the sequence differences between the two OB-folds within each dimer impose an asymmetry that affects its DNA binding properties (8). The occluded site size of this protein is also dependent on salt concentration, although not as dramatically as for the *Eco*SSB tetramer, changing from 50 to 53 nucleotides at moderate and high salt concentrations to 45 nucleotides at lower salt concentrations (8). ITC measurements (8) showed that *Dr*SSB binding to oligo(dT)s with lengths close to the occluded site size (45–55 nucleotides) is stoichiometric at low and moderate salt concentrations (0.02–0.2 M NaCl), although in contrast to *Eco*SSB the binding is weaker at higher salt concentrations (1 M NaCl), such that an affinity can be directly measured. The $H_{obs} = -94$ kcal/mol determined at 0.2 M NaCl salt conditions is somewhat smaller than observed for *Eco*SSB (approximately –130 kcal/mol) under the same conditions. However,

the observed binding enthalpy shows a large sensitivity to NaCl concentration, similar to that observed for *EcoSSB*, but with a smaller $C_{p,obs}$.

In the following sections we describe the procedures for ITC titration experiments performed using VP-ITC instrument from MicroCal (GE Healthcare) for *EcoSSB* and *DrSSB* binding to oligo (dT)s of different lengths that exemplify the effects of salt concentration on both stoichiometry, affinity and binding enthalpy.

2. Materials

2.1. Buffer Solutions

All solutions were prepared with reagent grade chemicals and glass distilled water that was subsequently treated with a Milli Q (Millipore, Bedford, MA) water purification system. Buffer T is 10 mM Tris (tris(hydroxymethyl) aminomethane), pH 8.1 and buffer C is 10 mM Cacodylate, pH 7.0, 25 % (V/V) glycerol. Both buffers also contained NaCl at the indicated concentration (either 0.20 and 1.00 M for Buffer T or 0.02 and 0.20M for Buffer C) and 0.1 mM Na₃EDTA (ethylenediaminetetraacetic acid sodium salt). We emphasize that these buffers do not contain additional reducing agents (e.g., 2-mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP)) (see Note 1).

1. Buffer T (1 l). Dissolve 1.211 g of Trizma-base and either 11.67 g or 58.44 g of NaCl (for 0.2 and 1 M final concentration of NaCl, respectively) in ~900–950 mL of MilliQ deionized water in a 1 l glass beaker under constant stirring with the magnetic stirrer bar on a stirrer plate (see Note 2). Add 200 μ l of 0.5 M EDTA stock solution (pH 8.1–8.3). Adjust the pH to 8.10 (25 °C) with ~5 M HCl stock solution (2–2.2 ml will be required) as monitored with a glass electrode pH meter. Pour the solution into a volumetric flask (1 l) being careful to rinse several times with a few mL of MilliQ water to ensure all components are transferred to the volumetric flask, then add sufficient MilliQ water to adjust the volume to 1 l. Be sure that the contents are mixed thoroughly by wrapping with Parafilm around the top of the volumetric flask to make a leak proof seal. Filter the solution through a disposable NALGENE filter unit (0.22 μ m nitrocellulose) using a standard laboratory bench vacuum outlet and collect the filtrate in the plastic bottle that comes with the unit. Cap and store at 4 °C.

¹Neither *DrSSB* nor *EcoSSB* contains cysteines and, therefore, do not require reducing agents to prevent inter and intra protein disulfide bonds formation. Otherwise, 1–2 mM concentrations of BME may be necessary. TCEP proved to be a more effective reducing agent being odorless, more powerful, hydrophilic, and more resistant to oxidation in air. It also can be used at lower concentrations—0.5–1.0 mM. However, it is more expensive and should be added to the buffer solution before pH adjustment (in Subheading 1, step 1 or Subheading 1, step 2), since it is highly acidic and will affect the pH of the buffer even at low concentrations. DTT (dithiothreitol) is not recommended for ITC experiments since it can affect the stability of the baseline, presumably due to oxidation of the DTT. Due to the fact that these reducing agents (particularly 2-ME) are oxidized rapidly, buffers containing these should be used within a day after preparation.

²Stock solution of 5 M NaCl in water can also be used for buffer preparations. For buffer T it is required to add 40 and 200 mL of, such stock to obtain final concentrations of 0.2 and 1 M, respectively. The precise concentration of the stock buffer can be checked using refractive index $n = 1.3770$ (20 °C) (23). This becomes particularly important when using such hygroscopic salts as MgCl₂ (usually exists as a hexahydrate in solid state). In the latter case preparation of approximately 2 M MgCl₂ $\times$ 6H₂O stock solution in water with subsequent correction of concentration using refractive index for 2 M concentration ($n = 1.3766$ (20 °C)) (23) is highly recommended for further use.

2. Buffer C (1 l). Dissolve 1.38 g of cacodylic acid and the required amount of NaCl as above in ~500 ml of MilliQ deionized water in a 1 l glass beaker and then add 250 ml of glycerol (see Note 3). Add 200 μ l of 0.5M EDTA, mix and add water again up to ~950 ml total volume, mix and adjust the pH to 7.00 with ~5M NaOH (3–3.5 ml will be required). Proceed with filtering and storage as described above.

2.2. Proteins and ssDNA

1. The *Eco*SSB and *Dr*SSB proteins used in the ITC experiments were expressed and purified as described ((17) and (18), respectively). Protein stock solutions in storage buffer (20 mM Tris (pH 8.3), 50 % (V/V) glycerol, 0.50 M NaCl, 1 mM EDTA, and 1 mM BME) are usually (4–6) mg/ml (50–80 μ M *Eco*SSB tetramer) and 10 mg/ml (~160 μ M *Dr*SSB dimer). Proteins are stored in 0.5–1.0 mL aliquots in 2 mL cryogenic vials in storage buffer at –80 °C (see Note 4). Protein concentrations are determined spectrophotometrically after dialysis in buffer T containing 0.20 M NaCl using the extinction coefficients, $\epsilon_{280} = 1.13 \times 10^5 \text{ M}^{-1}$ (tetramer) cm^{-1} for *Eco*SSB and $\epsilon_{280} = 8.2 \times 10^4 \text{ M}^{-1}$ (dimer) cm^{-1} for *Dr*SSB (see Note 5).
2. The single stranded oligodeoxythymidylates, (dT)₇₀ and (dT)₄₅, used in the ITC experiments were synthesized in our lab using an Applied Biosystems DNA synthesizer and purified by gel electrophoresis as described (19). The DNA is 98% pure as judged by denaturing gel electrophoresis and autoradiography of a sample that were 5' end-labeled with ³²P using polynucleotide kinase (20). The aliquots (~0.5 ml) of synthesized oligodeoxynucleotides (0.5–0.7 mM (molecule concentration)) are usually stored in water or Tris buffer at –20 °C. Concentrations of oligo(dT) are determined spectrophotometrically by UV absorbance in buffer T (pH 8.1), 0.1 M NaCl using the extinction coefficient, $\epsilon_{260} = 8.1 \times 10^3 \text{ M}^{-1}$ (nucleotide) cm^{-1} or $5.67 \times 10^5 \text{ M}^{-1}$ ((dT)₇₀ molecule) cm^{-1} and $3.65 \times 10^5 \text{ M}^{-1}$ ((dT)₄₅ molecule) cm^{-1} (see Note 5).

³We recommend to use 99.5 % spectrophotometric grade glycerol from “Sigma-Aldrich.” If the glycerol is first placed in an intermediate vessel (e.g., a graduated cylinder), it is essential that after pouring the glycerol into the beaker, the graduated cylinder is rinsed several times with MilliQ water and the rinse added to the beaker so that all of the glycerol is transferred. If this is not done, then it will be impossible to ensure reproducibility of the glycerol content in future Buffers.

⁴*Eco*SSB protein can be stored at –20 °C in storage buffer up to a few years. Due to the presence of the 50 % (V/V) glycerol in the storage buffer it does not freeze at this temperature, which allows one to remove aliquots and avoid freeze–thaw cycles.

⁵Precise values of extinction coefficients for proteins and ssDNA are required for determination of their concentrations. An accurate value of the extinction coefficient (usually at 280 nm) for an unfolded protein in 6.0 M Guanidine HCl can be calculated based on its amino acid composition (24). Various calculators are available on the Internet to assist this (for example, one is available in “Sednterp” software: <http://www.jphilo.mailway.com/download.htm>). One can obtain the extinction coefficient of the fully folded protein in the buffer of interest by measuring absorbance spectra (230–320 nm) of the same amount of the protein in 6.0 M Guanidine HCl and in the particular buffer (X) of interest. The value of the extinction coefficient in the buffer is obtained from the calculated value in 6 M Guanidine HCl, multiplied by the ratio of the absorbance values in the native buffer and in 6 M Guanidine HCl ($\epsilon_{\lambda, X} = \epsilon_{\lambda, \text{GHCl}} \times (A_{\lambda, X}/A_{\lambda, \text{GHCl}})$). Free calculators are also available for calculating extinction coefficients of ssDNA of any sequence and length (see for example from “Integrated DNA Technologies” (IDT) Web site: <http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). These calculations are based on the nearest-neighbor model (25, 26). Reliable estimates of extinction coefficients can also be obtained by degrading the DNA to its mononucleotides using Phosphodiesterase I digestion and calculating the extinction coefficient from the known mononucleotide extinction coefficients and the DNA composition (27). For both protein and DNA a full UV spectrum must be taken from 240 to 350 nm and at least 3 determinations at different concentrations should be made and a plot of the absorbance versus concentration should follow the linear Beer’s law relationship with a zero intercept. Make sure that the absorbance between 310 and 350 is zero after subtraction of the absorbance of the buffer. Any signal in this range above 10 % of the maximum absorbance at 280 (or 260 nm for DNA) is due to light scattering and is likely the result of some protein insolubility problems (usually not the case for DNA) and this sample may need to be discarded.

3. Methods

3.1. Estimates of Amounts of SSB and DNA Required for ITC Experiment

An estimate of the amount of SSB and DNA needed for an ITC experiment is facilitated if some initial information on the system is known. For example, in the experiments presented in Figs. 1a and 2a, b the affinity of the SSB-(dT)_L complexes is so high that binding is “stoichiometric” and the reaction is complete at total DNA to protein ratio $R = 1$ (mole (dT)_L/mole SSB). As used here, the term “stoichiometric” means that essentially all of the titrant being added (ligand) becomes bound to the species in the cell (macromolecule), so that there is essentially no free ligand in solution up until the “stoichiometric point” of $R = 1$. The results in the experiment presented in Fig. 1b indicates that this titration is not stoichiometric due to the weaker binding of (dT)₄₅ to *Dr*SSB under these conditions, hence saturation of the SSB requires a ratio, $R = [(dT)_{45}]_{\text{tot}}/[\text{SSB}]_{\text{tot}} \approx 4$. In any case, required concentrations of species along with the volume and number of experimental injections can be roughly calculated using simple formula in Eq. 2:

$$R \cdot V_{\text{cell}} \cdot C_{\text{cell}} = n_{\text{inj}} \cdot V_{\text{inj}} \cdot C_{\text{syr}} \quad (2)$$

Where R is the desirable total DNA to total protein ratio in the calorimetric cell at the end of the titration, where $V_{\text{cell}} \approx 1.45$ ml is the volume of calorimetric cell, C_{cell} is the concentration of the SSB in the cell, C_{syr} is concentration of DNA in the syringe, n_{inj} is number of injections, and V_{inj} is injection volume. Note that the two latter parameters are constrained by the syringe volume $V_{\text{syr}} = n_{\text{inj}} \cdot v_{\text{inj}} < 300$ μL . Therefore, for example, to reach $R = 4$ (as in Fig. 1b) by performing 19 injections (15 μL each) at the concentration of SSB in the cell 0.6 μM , one needs a (dT)₄₅ concentration in the syringe near 12 μM . The following procedures describe the preparation of SSB and DNA samples used in performing 2–3 ITC experiments (including reference titrations) for 0.5–1.0 μM of SSB in the cell and 10–20 μM of DNA in the syringe (see Note 6).

3.2. Dialysis of Proteins and ssDNA

Extensive dialysis of the protein and DNA solutions is required to exactly match buffer conditions for titrant in the syringe ((dT)_L) and sample in the calorimetric cell (SSB). This is particularly important for interactions that have small binding enthalpies, where the reference heats of dilution are comparable to the experimental heats (for example, see Fig. 1b).

1. Prepare ~3–4 mL of an approximately 2 μM solution of either *Dr*SSB or *Eco*SSB by adding some of the concentrated SSB stocks to dialysis buffer.
2. Prepare ~1 mL of an approximately 50 μM (dT)_L solution by adding calculated aliquot of concentrated DNA stock (usually 70–100 μL) to dialysis buffer.
3. Place the SSB and DNA solutions in dialysis bags of corresponding size (MWCO: 10,000 Spectra/Por) and secure the contents with plastic clamps (see Note 7).

⁶A 10–30-fold difference between the concentrations of the species in the cell and in the syringe is typical for ITC experiments.

⁷Prior to use wash the dialysis membrane with distilled water and let it soak for 5–10 min in dialysis buffer. Put one plastic clamp on one end of the bag first, then fill the bag with sample and then secure the other end with the second clamp.

4. Dialyze protein and DNA in separate beakers containing 300 ml of dialysis buffer at 4 °C with constant stirring of the buffer. Change dialysis buffer two times every 3–4 h.
5. Remove SSB solution after dialysis, distribute in Eppendorf tubes and centrifuge at 10,000 rpm (~7000 g) for 15 min in a cold room. Pipette out the protein solution from each Eppendorf tube avoiding 5–10 µl at the bottom (to avoid any possible precipitated protein) and combine them in a larger 5–10 mL plastic tube. Remove DNA from dialysis bag and place in Eppendorf tube for further use.

3.3. Preparing for ITC Experiment and Performing the Titrations

Here we describe the experimental procedure for a titration of SSB in the cell with DNA in the syringe, followed by a reference titration of buffer in the cell with DNA in the syringe. We assume that the experimenter is familiar with the basics of instrument operation and software. Otherwise, we recommend reading the VP-ITC Micro-calorimeter user's manual first.

1. Turn on the instrument and leave it to equilibrate for 1 h.
2. While waiting determine the concentrations of dialyzed samples (see Note 5).
3. Use dialysis buffer to dilute the dialyzed SSB and DNA samples to the concentrations calculated previously using Eq. 2 for any experiment of your choice (see Fig. 1 or Fig. 2). Prepare 2.6 mL of protein solution and 1 mL of DNA solution to fill the calorimetric cell and the syringe (see Note 8).
4. Prepare the cell. The cell should have been cleaned after the previous run and filled with distilled water. Remove the water and fill the cell with dialysis buffer using a disposable plastic syringe attached to a long stainless steel needle (see Note 9). Remove the buffer and using a fresh syringe fill the cell with protein solution. It is important to avoid introducing bubbles into the cell. Save the rest of the protein solution (~500 µl) for a redetermination of its concentration.
5. While waiting for the instrument equilibration to be completed (15–20 min) (see Note 10), fill the syringe with the DNA solution using a plastic syringe connected to the filling port (~500 µl required) and carefully introduce syringe assembly into the cell.
6. Allow for equilibration (another 10–15 min). Using the instrument computer software set all parameters for the titration (number and volume of the injections, injections intervals, reference power, filter period, etc., see manual for details) and start the run (see Note 11).

⁸The minimum volume to fill the cell is 2.0–2.1 mL. The rest of the solution is used to redetermine the protein concentration. The remaining 500 µl of DNA will be enough to refill the syringe for the reference titration and for a redetermination of the DNA concentration. We have not found it necessary to degas our SSB and DNA solutions, since controls with degassed samples showed no difference.

⁹We recommend to use disposable plastic syringes (slip tip) of 3 or 5 mL with the set of 3 attachable long stainless steel needles (9 in. long from “Hamilton”) instead of the syringes provided with the instrument since it facilitate handling and reduce time required for cleaning.

¹⁰Equilibration can be considered as completed when the DP (Differential Power) signal is about 0 ± 0.2 µcal/s. We have noticed that equilibration of the system with the syringe introduced immediately after filling the cell requires much longer times.

7. After completion of the run carefully remove the syringe assembly to avoid bending the syringe needle and place it in the holder, carefully rinse the needle with distilled water and wipe it with “Kimwipes.”
8. To prepare for reference titration, remove the contents of the cell using a disposable syringe, then rinse the cell with distilled water a few times and once with dialysis buffer (see Note 12). Fill the cell with dialysis buffer and allow it to equilibrate as before.
9. Fill the syringe with the rest of the DNA solution manually manipulating the plunger position in the Auto-Pipette window. This is different from what is described in step 5 above and serves to save up to 200 μ l of ligand solution (see Note 13).
10. Put the syringe assembly back into the cell, allow equilibration, and run a reference titration keeping all the settings identical to those used in the actual titration performed above.
11. While performing the titrations redetermine the concentrations of SSB and ssDNA used in the experiment as exactly as possible (see Notes 5 and ¹⁴).

3.4. Data Analysis

The analysis of ITC titration data and the subsequent fit of the data to an appropriate binding model can be performed using the software provided by the instrument manufacturer or using Micro-Math “Scientist” (St. Louis, MO), a software package specifically designed for data fitting (see Note 15). The results of ITC titrations of *Dr*SSB with (dT)₄₅ and *Eco*SSB with (dT)₇₀ at different NaCl concentrations are presented in Figs. 1 and 2, respectively. The solution conditions and concentrations of SSB and ssDNA for each particular experiment are specified in the figure legends along with the experimental design and fitted parameters. The upper panels show raw titration data representing the heat signal (microcalories per sec)

¹¹Remember, when you press the “START” button, the syringe starts to rotate (we usually use 300 rpm) and some time is required for the DP signal to reach the preset reference plateau value (we usually use 2 μ cal/s). We do not recommend using the automatic option to start the titration but rather waiting to make sure that the baseline is stable, indicating that the protein solution remains stable during rotation of the injection syringe. Do not leave until the end of the titration, so that you can check the progress of the run from time to time. Make sure that injection intervals are long enough for heat signal to return to the baseline (not waiting for baseline return is often a mistake made in ITC experiments). Peak equilibration times can increase during the course of the titration (see Fig. 1a for example) and may not fit within the injection time intervals that were initially set. Note that the parameters of the titration, such as injection intervals and number, duration and volume of the injections can be corrected during the run.

¹²It is desirable to run the reference titration (if necessary) immediately after the experimental one. Since SSB–ssDNA complexes are quite soluble and show no aggregation or precipitation, there is no need to subject the ITC cell to thorough cleaning with the detergent after each run. However, if you study a new protein–DNA system for which you have not performed previous ITC experiments, we recommend running a reference titration(s) first.

¹³After the first titration the syringe can be refilled fully or partially with the same solution without opening the filling port by using buttons “distance,” “up,” and “down” in the Auto-Pipette window. To make sure there is no air in the needle, first, set short distance (~0.02 in.) and move plunger down until solution appears at the end of the needle. Place the syringe in the tube containing the DNA solution, set appropriate distance and move the plunger up until the syringe is filled to the desired level. Do Purge/Refill.

¹⁴It is important to know the precise values of the concentrations of SSB and DNA used in the experiment for meaningful quantitative analyses of the data. Mistakes can be made when diluting the samples to the experimental concentrations. Up to 5–7 % of SSB can be lost if the protein solution is degassed. An accurate determination of the concentration of the species in the syringe (DNA) is of particular importance under stoichiometric binding conditions (see Figs. 1a and 2) since it is used when the observed heats are normalized.

¹⁵Recent upgrades of the Origin 5 ITC analysis software provide the following models for data analysis: “One set of sites,” “Two set of sites,” “Sequential binding sites,” “Competitive binding,” and “Dissociation.” As an alternative we also use the data analysis software “Scientist” (St. Louis, MO), particularly when a more complex equilibrium model is needed that is not available in Origin.

versus time (minutes). Each injection causes a negative deviation of the signal from the baseline indicating the release of heat (exothermic reaction, $H < 0$) which returns back to baseline as the injected amount of DNA reaches equilibrium with the protein in the cell. As the titration progresses the SSB in the cell becomes saturated with DNA and at the end of the titration the only heats observed reflect those due to DNA dilution (reference heats). Reference titrations of the DNA into the buffer (see inserts in Fig. 1a, b) are required (see Note 16) to be certain that saturation is attained and to correct experimental heats for use in subsequent analyses.

Standard ITC titration isotherms (known as Wiseman isotherms (21)) are shown in the lower panels of Figs. 1 and 2. These are constructed from the raw data in the upper panels. In these isotherms the integrated heat responses are normalized per amount of injected DNA (after subtraction of corresponding heats of dilution) and plotted versus the mole ratio of total DNA to total protein. This so called differential representation of the ITC data is more informative than the integral representation (see Note 17), particularly for the case of very tight (stoichiometric) binding since it provides a visual estimate of the stoichiometry and binding enthalpy (from the flat portions of the isotherms) for complex formation (see Figs. 1a and 2a, b). For a system with weaker binding affinity (nonstoichiometric) (see Fig. 1b) it is necessary to fit these data to a model to obtain estimates of the stoichiometry, binding enthalpy, and equilibrium binding constant(s).

The data in Figs. 1a, b and 2a can be fit to an n -independent and identical sites model using the instrument software and described elsewhere (21). This model describes binding of a ligand (X) to a macromolecule (M) possessing n independent and identical sites for the ligand with macroscopic affinity, $K = [MX]/[X][M]$, and enthalpy, H per binding site. Here we provide basic equations for analysis and simulation of the ITC data for this model.

The integral or cumulative heat (see Note 17) is defined by Eq. 3

$$Q = V_{\text{cell}} \Delta H [M]_{\text{tot}} \langle X \rangle = V_{\text{cell}} \Delta H [M]_{\text{tot}} \frac{nK[X]}{1 + K[X]} \quad (3)$$

It is assumed to be directly proportional (see Note 18) to the binding density $\langle X \rangle = nK[X]/(1 + K[X])$, with the calorimetric cell volume (V_{cell}), enthalpy change (H), and total

¹⁶Titration of the buffer in the cell with ligand in the syringe is the most effective reference titration, since this corresponds to more than a 100-fold dilution of the ligand into the cell, resulting in the largest effects due to heat of dilution. Another useful control is to have buffer in the syringe and titrate this into the protein in the cell. This is useful to determine whether the protein solution in the cell is stable to rotation of the injection syringe. A mismatch of the heats at the end of titration to those obtained from the reference titration should not be ignored, since this may result from the following: (1) The reaction is not completed. (2) Syringe and cell buffers do not match. (3) Oligomerization state of the species in the syringe changes upon dilution in the cell.

¹⁷The data can also be presented in integral form, where at each titration point the heat is calculated as a sum of the heats for the current and all previous injections and is plotted versus the total titrant concentration. This form corresponds to a general representation of titration data where the integral response of the system is measured as a function of saturation of the macromolecule with the ligand (for example, absorbance or fluorescence).

¹⁸As discussed previously (28) for a macromolecule that binds multiple ligands, the change in the experimentally observed property of the system that is being monitored to follow binding (e.g., absorbance or fluorescence or heat) is not always directly proportional to the average extent of ligand binding to the macromolecule (binding density). If this is not the case then incorrect isotherms will be obtained if direct proportionality is assumed. Rather than making this assumption a model independent analysis of a number of experimental titrations performed at different macromolecule concentrations should be performed and analyzed as described to obtain the actual relationship between the signal change (Q) and the average extent of ligand binding (28).

macromolecule concentration in the cell ($[M]_{\text{tot}}$) being proportionality constants. An expression for free ligand concentration ($[X]$) can be found by solving the mass conservation Eq. 4

$$[X]_{\text{tot}} = [X] + \langle X \rangle [M]_{\text{tot}} = [X] + \frac{nK[X]}{1+K[X]} [M]_{\text{tot}} \quad (4)$$

where $[X]_{\text{tot}}$ is total concentration of the ligand in the cell. The analytical expression for the differential heat, $q_n = 1/V_{\text{cell}} dQ/d[X]_{\text{tot}}$ (heat of injection normalized per amount of injected ligand), can be obtained differentiating Eqs. 3 and 4 with respect to the free ligand concentration to yield Eq. 5

$$q_n = \frac{1}{V_{\text{cell}}} \frac{dQ}{d[X]_{\text{tot}}} = \frac{1}{V_{\text{cell}}} \frac{dQ/[d[X]]}{[dX]_{\text{tot}}/d[X]} = \Delta H \frac{nK[M]_{\text{tot}}}{(1+K[X])^2 + nK[M]_{\text{tot}}} \quad (5)$$

Where $[X]$ is determined from Eq. 4. Equations 3 for the integral heat and 5 for the differential heat are functions of the total ligand and total macromolecule concentrations and can be used to predict and fit the experimental ITC isotherms (see Note 19).

Equations 4 and 5 can be rewritten in the form of the Wiseman isotherm (21) introducing the variable $R = [X]_{\text{tot}}/[M]_{\text{tot}}$ and a new parameter $r = 1/C$ (where $C = K[M]_{\text{tot}}$) as shown in Eq. 6

$$q_n = \frac{1}{V_{\text{cell}}} \frac{dQ}{d[X]_{\text{tot}}} = \Delta H \left(\frac{1}{2} + \frac{1 - (1+r)/2 - R/2}{(R^2 - 2R(1-r) + (1+r)^2)^{1/2}} \right) \quad (6)$$

This equation predicts that the optimal range for performing an ITC experiment in which the binding constant and enthalpy change can both be determined accurately is when the product of the total macromolecule concentration and binding constant ($C = K[M]_{\text{tot}}$) is in the range between 1 and 1,000 (21). If C exceeds 1,000, then stoichiometric binding is observed and only H , but not K can be determined accurately. When $C < 1$ the observed isotherm becomes too shallow to allow determinations of either K or H reliably. If C is outside the appropriate range, one can increase or decrease the concentration of macromolecule ($[M]_{\text{tot}}$) to shift the value of C into the appropriate range. One can also modify the solution conditions (e.g., salt, pH, or temperature) to change the value of K (see below). This should be taken into account when planning the experiment.

The ITC titrations of *DrSSB* with (dT)₄₅ at two NaCl concentrations shown in Fig. 1 illustrate two cases of strong “stoichiometric” binding at 0.2 M NaCl, when the value of C exceeds 1,000 (Fig. 1a), and weaker binding ($C = K[M]_{\text{tot}} = 10$) at the much higher NaCl concentration of 1 M (Fig. 1b). In the first case, one cannot estimate K with any accuracy, only a lower limit of $K > 1,000/[M]_{\text{tot}} = 1.7 \times 10^9 \text{ M}^{-1}$ can be determined. However, a very

¹⁹When analyzing and fitting experimental data the corrections for heat displacement effects and ligand and macromolecule dilutions in the calorimetric cell during the titration should be applied as described in the ITC Data Analysis in Origin: Tutorial Guide (MicroCal, LLC) and in ref. (3).

precise estimate of $H = -79.9 \pm 1.3$ kcal/mol can be obtained from these data from the flat portion of the isotherm in the R range from 0 to 1, since for this conditions $q_n \rightarrow H$ when $[X] \rightarrow 0$ in Eq. 5 or when $r = 1/C$ is very small in Eq. 6. As the salt concentration increases to 1 M NaCl both K and H can be determined from a fit of the data to the n -independent and identical sites model described above (see Fig. 2b).

The results of two ITC titrations of *Eco*SSB with (dT)₇₀ at moderate (0.2 M NaCl) and low (20 mM NaCl) salt concentrations are presented in Fig. 2a, b, respectively, and demonstrate differences between two proteins when bound to oligonucleotides of approximately the length of the occluded site size. At 0.2 M NaCl *Eco*SSB binds (dT)₇₀ stoichiometrically, similarly to *Dr*SSB (Fig. 1a), but the binding enthalpies differ significantly (1.7 fold more negative (favorable) for *Eco*SSB). As the salt concentration increases the affinity of *Dr*SSB to ssDNA decreases and the binding enthalpy becomes less favorable (see Fig. 1b). The same trend in the binding enthalpy is observed for *Eco*SSB (3) (data not shown), and is due to the linkage of a release of anions upon formation of the SSB–DNA complex, although the binding of *Eco*SSB to ssDNA remains stoichiometric up to 3.0 M NaCl (22).

Upon decreasing the salt concentration to 20 mM NaCl the *Eco*SSB switches its mode of interaction with (dT)₇₀ from its (SSB)₆₅ mode (a 1:1 complex in which 65 nucleotides of ssDNA are fully wrapped around the tetramer) to its (SSB)₃₅ binding mode in which two SSB tetramers bind to (dT)₇₀, both in a partially wrapped state in which only two subunits of each tetramer (on average) interact with ssDNA). In contrast, *Dr*SSB still forms a 1:1 complex with ssDNA at this lower NaCl concentration (8). The data in Fig. 2b for the titration of *Eco*SSB with (dT)₇₀ at low salt demonstrate that although (dT)₇₀ binding to *Eco*SSB remains stoichiometric, it undergoes the transition from the 2:1 (SSB)₃₅ mode to 1:1 (SSB)₆₅ mode upon increasing the (dT)₇₀ concentration so that the protein to DNA ratio decreases (see Note 20).

At the beginning of the titration when a small amount of (dT)₇₀ is added to a large excess of *Eco*SSB the 1:2 complex forms stoichiometrically and the overall heat per injection corresponds to overall binding enthalpy for formation of a 2 SSB per (dT)₇₀ complex (≈ -135 kcal/mol). This complex becomes fully populated at $R = [(dT)_{70}]_{\text{tot}}/[EcoSSB]_{\text{tot}} = 1/2$. Further addition of (dT)₇₀ causes a redistribution of the 2:1 complexes to form two fully wrapped 1:1 (SSB)₆₅ complexes (see Scheme in Fig. 2b). This process is accompanied by a more favorable overall heat $q_n \approx -160$ kcal/mol. Finally, at $R = 1$ the transition is complete and only the 1:1 (SSB)₆₅ complex exists after this point (see Note 21).

²⁰This titration is an example of a so-called “reverse titration,” in which the ligand in the cell (*Eco*SSB) is titrated with the macromolecule in the syringe (dT)₇₀. (The macromolecule is defined as the species that binds multiple ligands.) When existing models incorporated in the ITC software, such as “two independent nonidentical sites” or “two sequential sites” models are used, you should switch options in the main menu from “macromolecule in the cell” to “ligand in the cell” to analyze these data properly.

²¹A more detailed interpretation of the data shown in Fig. 2b and fitting of this data to an appropriate model is complicated by the fact that in both complexes *Eco*SSB binds to (dT)₇₀ with very high affinity (stoichiometrically), which makes it difficult to distinguish between different models, as for example, a simple model of two independent sites, from models that include cooperativity between sites or in more complicated cases, when intermediate species are populated significantly.

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Fig. 1.

ITC isotherms for the binding of $(dT)_{45}$ to *DrSSB* to form a 1:1 molar complex at two different NaCl concentrations. (a) Calorimetric titration of $(dT)_{45}$ into *DrSSB* in the presence of 0.2 M NaCl (Tris buffer (pH 8.1), 0.2 M NaCl, 25 °C). *Upper panel*: Raw titration data, plotted as the heat signal (microcalories per second) versus time (minutes), obtained for 19 injections (15 μL each) of $(dT)_{45}$ (8.6 μM , syringe) into a solution containing *DrSSB* protein (0.6 μM dimer, cell). *Lower panel*: the ITC data from the upper panel in the form of titration isotherm, where the integrated heat responses per injection, after subtraction of the heats of dilution obtained from a blank titration of $(dT)_{45}$ into buffer (shown in insert), were normalized to the moles of injected ligand ($(dT)_{45}$) and plotted

versus total DNA to protein ratio. The continuous curve shows the best fit of the data to a 1:1 binding model with $N = 1.02 \pm 0.01$, $H_{\text{obs}} = -80.0 \pm 1.3$ kcal/mol. The affinity of (dT)₄₅ is too high to measure under these “stoichiometric” conditions. A minimum estimate of $K_{\text{obs}} = 2 \times 10^{10} \text{ M}^{-1}$ was used for the simulation. (b) Calorimetric titration of (dT)₄₅ (10.5 μM) into DrSSB (0.6 μM dimer) in the presence of 1.0 M NaCl (experimental setup and buffer conditions are the same as in Fig. 1a). The raw data and corresponding titration isotherm are presented in *upper* and *lower panels*, respectively. The continuous curve in the lower panel shows the best fit of the data to a 1:1 binding model with $N = 1.02 \pm 0.01$, $K_{\text{obs}} = (1.69 \pm 0.08) \times 10^7 \text{ M}^{-1}$, $H_{\text{obs}} = -62.5 \pm 0.9$ kcal/mol.

Fig. 2.

The stoichiometry of *EcoSSB*:(dT)₇₀ complexes depends on salt and protein concentration reflecting its salt-dependent binding mode transition. (a) ITC titration of (dT)₇₀ into *EcoSSB* at moderate salt (0.2 M NaCl) reveals the formation of only the fully wrapped 1:1 molar complex under high affinity “stoichiometric” conditions. *Upper panel*: The raw titration data, plotted as heat signal (microcalories per second) versus time (minutes), was obtained for 26 injections (10 μl each) of (dT)₇₀ (17 μM) into a solution containing *EcoSSB* protein (1.0 μM tetramer) (Cacodylate buffer (pH 7.0), 25 % Glycerol, 0.2 M NaCl, 25 °C). *Lower panel*: standard titration isotherm of normalized heats versus total DNA to protein ratio as described in Fig. 1a. The continuous curve shows the best fit of the data to a 1:1 binding model with $N = 1.03 \pm 0.01$, $H_{\text{obs}} = -136.5 \pm 0.7$ kcal/mol. The affinity of (dT)₇₀ is too high to measure under these conditions. A minimum estimate of $K_{\text{obs}} = 1 \times$

10^{10} M^{-1} was used for the simulation. (b) ITC titration of (dT)₇₀ (10.0 μM , syringe) into *EcoSSB* (1.0 μM , cell) at low salt (0.02 M NaCl) reveals a transition from a 2:1 (2 SSB per DNA) complex to a 1:1 complex as the DNA to protein ratio increases (experimental setup and buffer conditions are the same as in Fig. 2a). This data represents an example of a reverse titration (macromolecule ((dT)₇₀) in the syringe and ligand (*EcoSSB*) in the cell). The raw data and corresponding titration isotherm are presented in the *upper* and *lower panels*, respectively. In the DNA to protein ratio range from 0 to $\frac{1}{2}$ (from very large to twofold excess of *EcoSSB* over DNA) only the 2:1 *EcoSSB*: (dT)₇₀ complex is formed, whereas as more free ssDNA is added the transition to the 1:1 complex occurs, which is completed at $[(\text{dT})_{70}]_{\text{tot}}:[\text{EcoSSB}]_{\text{tot}} = 1$. The fitting of these data to obtain binding parameters (affinities, enthalpies, cooperativity) will depend on the type of the model (see text), but in any case will be difficult due to the very high affinities of SSB to DNA for both complexes reflected by the very sharp transitions within the titration isotherm. Estimates of the enthalpies of formation of these complexes can be obtained from analysis of the flat portions of the titration isotherm (see text and Note 21).