Enthalpy of Antibody−Cytochrome *c* Binding†

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ABSTRACT: High-sensitivity titration calorimetry is used to measure changes in enthalpy, heat capacity, and protonation for binding of two monoclonal antibodies (MAbs) to topologically distinct surfaces of cytochrome *c*. MAb 2B5 binds near the exposed heme crevice in a reaction involving proton uptake, while there is no change in protonation for MAb 5F8 binding to the opposite side of the molecule. Both antibodies have association rate constants with the activation enthalpy and viscosity dependence expected of diffusion-limited reactions [Raman et al. (1992) *Biochemistry 31*, 10370−10379], and bind with high affinity ($\Delta G°_b = -12.6$ kcal mol^{-1} for MAb 2B5 and −13.9 kcal mol^{-1} for MAb 5F8, at pH 7, 25 °C). At 25 °C, the equilibrium enthalpy and entropy contributions to the free energy of binding are negative for both antibodies ($\Delta H°_b = -21.0$ kcal mol^{-1}, $\Delta S°_b = -28.2$ cal mol^{-1} K^{-1} for MAb 2B5; and $\Delta H°_b = -21.7$ kcal mol^{-1}, $\Delta S°_b = -26.3$ cal mol^{-1} K^{-1} for MAb 5F8). The enthalpy of MAb 2B5−cytochrome *c* association exhibits a marked temperature dependence ($\Delta C_p = -580$ cal mol^{-1} K^{-1}), but the enthalpy for MAb 5F8 binding is much less dependent on temperature ($\Delta C_p = -172$ cal mol^{-1} K^{-1}). The large differences in ΔC_p for binding of the two antibodies suggest corresponding differences in the mode of binding, or in the molecular surfaces buried in the binding reactions. In particular, factors other than hydrophobic effects may be significant contributors to the thermodynamics of antibody−cytochrome *c* binding, especially when ΔC_p is small (MAb 5F8).

Binding of an antibody to a protein antigen is an unparalleled example of molecular recognition. The uniqueness of this process is highlighted by the fact that antibodies use a common molecular framework for creating a variety of binding sites which accommodate a plethora of antigens. Crystal structures of several protein antigens complexed with antibody fragments identify some common features of the complexes (Davies et al., 1990). Properties shared by most antibody−protein complexes are as follows: (1) the protein antigen and the antibody associate to give rise to a complementary interfacial surface of 650−916 Å^2; (2) the antigenic face of this surface, the epitope, usually comprises 14−21 residues arranged discontinuously on the surface of the protein antigen; (3) irrespective of the antigen size, the epitope interacts with 4−6 hypervariable loops on the antibody via H-bonds, electrostatic, or van der Waals interactions; (4) antibody combining sites have flat or concave shapes; (5) the epitope is fully exposed to the solvent prior to complex formation, suggesting that water molecules are expelled from the interface on binding; (6) there is little or no conformational change on binding (no movement >2 Å); and (7) the complementarity determining region (CDR)[1] contains a preponderance of aromatic residues (Padlan, 1990).

Some aspects of antibody−protein interactions can be viewed as being analogous to protein folding in that both processes involve a competition between protein−protein and protein−solvent interactions. Both are desolvation processes. Nevertheless, there are fundamental differences. Folding is dominated by the sequestering of apolar groups to the interior of the molecule, while antibody−protein interaction involves burial of antigenic protein surfaces, which are often highly polar and designed to interact favorably with water. Are the fundamental differences between protein folding and antibody−protein binding discernible from their respective thermodynamic properties? For protein folding, heat capacity changes have been shown to be closely correlated with the change in exposure of hydrophobic groups to water. Although there are heat capacity effects on solvating polar groups (Makhatadze & Privalov, 1990), the heat capacity change is considered primarily an attribute of the hydrophobic effect (Edsall, 1935; Makhatadze & Privalov, 1990; Baldwin & Muller, 1992). This arises from the unfavorable free energies of transfer of nonpolar solutes to water, and reflects the large cohesive energy of water, which would rather bind to itself than to a nonpolar surface. The transfer of nonpolar solutes to water is opposed by the entropy needed to order the solvent around the solute. Thus, the large positive heat capacity change that accompanies the dissolution of apolar solutes has been rationalized in terms of the extra energy required to "melt" the ordered solvent [for review, see Tanford (1980)].

For small nonpolar solutes, the hydrophobic contribution to the free energy of solution is believed to be proportional to the change in the water-accessible surface area on transfer

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[1] Abbreviations: MAb, monoclonal antibody; FAb, antigen binding fragment of an MAb; nm, nanometers; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; THAM, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; CDR, complementary determining region of an MAb; ASA, solvent-accessible surface area.

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from a nonaqueous solvent to water, with values of the proportionality constant of 20−43 cal mol^{-1} Å^{-2} (Creighton, 1993). Recent calculations include a volume correction suggesting a higher value of ~47 cal mol^{-1} Å^{-2} (Sharp et al., 1991). Changes in solvation of nonpolar side chains are among the dominant thermodynamic effects for protein folding, so assessments of the contributions of individual side chains are based on estimates of the solvent accessibility of the side chain in the unfolded protein and the remaining solvent accessibility in the native state. Studies of mutant proteins suggest that the volume-corrected values provide good estimates of observed stability differences for protein folding (Shirley et al., 1992). However, the theoretical basis for the volume correction has been questioned (Holtzer, 1992, 1994; Lee, 1993; Chan & Dill, 1994).

Recently, antibodies have been used to study folding of cytochrome *c* (Allen et al., 1994). Antibody-detected protein folding measures the time at which the antigenic site regains sufficient native-like structure to become a competent antigen. But, how much structure is needed for successful binding? The structural prerequisites for binding are unknown, but need to be defined to interpret the results of antibody-detected folding experiments. Structural insight into the binding process can be obtained from studies of both the kinetics and thermodynamics of antibody−cytochrome *c* association. The cytochrome *c* binding kinetics for the MAbs used for our calorimetric experiments are diffusion-limited (Raman et al., 1992), suggesting that there are no significant energetic barriers to MAb binding.

Two monoclonal antibodies (MAbs) that bind to distinct epitopes on diametrically opposite sides of horse heart cytochrome *c* have been used for the titration calorimetry measurements. MAb 2B5 binding to cytochrome *c* requires the presence of Pro 44 (Figure 1A,B) which is within an Ω loop on the protein surface (Leszczynski & Rose, 1986; Fetrow & Rose, 1990), while MAb 5F8 binding requires the presence of Lys 60 (Figure 2A,B) on a relatively flat back surface at the N-terminal end of an α helix (Goshorn et al., 1991). The latter epitope is within a region of the protein in which the backbone folding differs for different members of the cytochrome *c* family (Bushnell et al., 1990). High-sensitivity titration microcalorimetry is used to measure the enthalpy change on association at several temperatures to obtain estimates of the change in heat capacity. The results show that the changes in enthalpy and entropy on binding are comparable for the two MAbs, but that there are large differences in the change in heat capacity (ΔC_p of binding). The differences in ΔC_p for the two MAbs are discussed in terms of possible differences in the interactions formed at the interface between the antibody and the protein antigen.

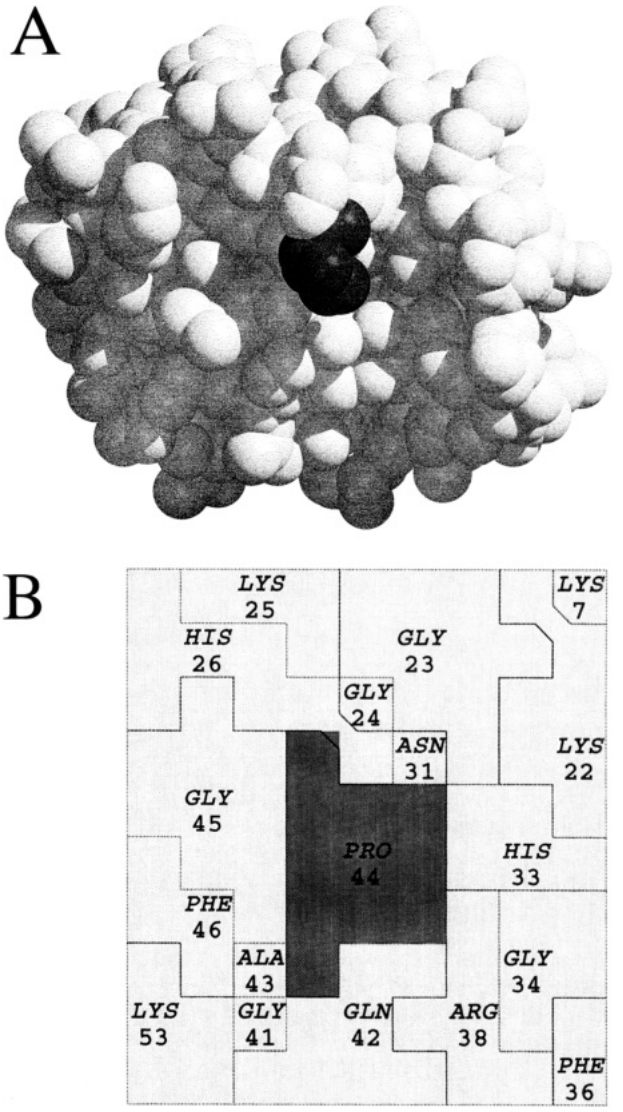

FIGURE 1: Antigenic surface of cytochrome *c* recognized by monoclonal antibody MAb 2B5. MAb 2B5 binding requires the presence of Pro 44 which is assumed to be part of the antigenic site. (A) A Corey−Pauling−Koltun (CPK) model of horse heart cytochrome *c* in which Pro 44 is heavily shaded. The model, based on the X-ray structure of Bushnell et al. (1990) (PDB Accession Code: 1HRC), was prepared using the programs MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Merritt & Murphy, 1994). (B) A schematic diagram of the antigenic region recognized by MAb 2B5 constructed using a modified version of ROADMAP (Chapman, 1993). The surface area (ASA) of cytochrome *c* accessible to a sphere with $r = 1.7$ Å was projected in a parallel fashion. The area shaded in black corresponds to Pro 44.

MATERIALS AND METHODS

Tris(hydroxymethyl)aminomethane (THAM) was obtained from the Natinal Bureau of Standards (Washington, D.C.; reference material no. 724a). Affinity-purified MAb, FAb, and horse heart cytochrome *c* were prepared and characterized as described earlier (Raman et al., 1992). FAb fragments were obtained from intact antibodies, and their concentration was measured spectrophotometrically using $\epsilon_{280} = 0.75 \times 10^5$ M^{-1} cm^{-1} (Fasman, 1976) assuming a molecular weight of 50 000. MAb concentrations were measured using an extinction coefficient of $\epsilon_{280} = 210\,000$ M^{-1} cm^{-1} (Corradin et al., 1984) assuming an MAb molecular weight of 150 000. Concentrations of MAbs and FAbs determined spectrophotometrically were considered to be estimates. Thus, binding site concentrations were determined by titration with cytochrome *c*, for which the molar extinction coefficient is well established. Cytochrome *c* concentrations were measured spectrophotometrically using $\epsilon_{410} = 106\,100$ M^{-1} cm^{-1} (Margoliash & Frohwirt, 1959). Unless otherwise stated, 0.1 M sodium phosphate buffer, pH 7.0, was used for all experiments: (i) to be consistent with our earlier kinetic measurements and (ii) to minimize contributions from buffer protonation to the observed enthalpy.

Titration Calorimetry. Titrations were performed using the OMEGA high-sensitivity microcalorimeter manufactured

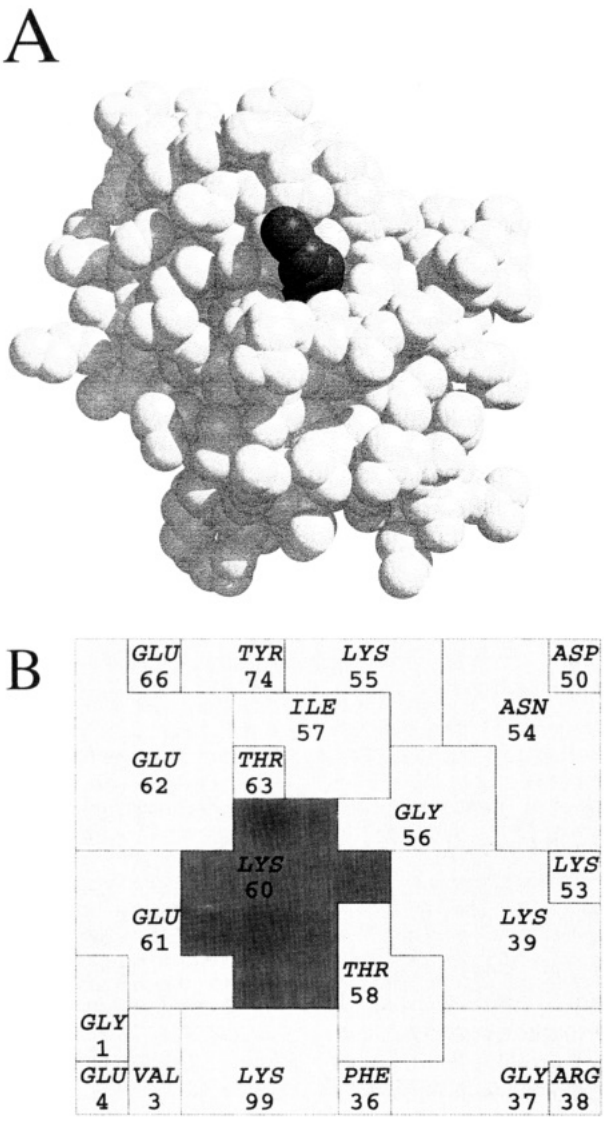

FIGURE 2: Antigenic surface of cytochrome *c* recognized by monoclonal antibody MAb 5F8. MAb 5F8 binding requires the presence of Lys 60 which is at the amino-terminal end of an α-helical segment. (A) A Corey−Pauling−Koltun (CPK) model of horse heart cytochrome *c* in which Lys 60 is heavily shaded. The model, based on the X-ray structure of Bushnell et al. (1990) (PDB Accession Code: 1HRC), was prepared using the programs MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Merritt & Murphy, 1994). (B) A schematic diagram of the antigenic region recognized by MAb 5F8 constructed using a modified version of ROADMAP (Chapman, 1993). The surface area (ASA) of cytochrome *c* accessible to a sphere with $r = 1.7$ Å was projected in a parallel fashion. The area shaded in black corresponds to Lys 60.

by MicroCal Inc. (Northampton, MA). A detailed description of this instrument has been provided (Wiseman et al., 1989). The instrument was calibrated by (i) measuring the area under a test electrical heat pulse and comparing it with the heat input and (ii) measuring the heat of a standard chemical reaction. For the latter, the heat of protonation of THAM by HCl was measured (Hopkins et al., 1992). The two calibration methods were in excellent agreement. Test runs were repeated periodically to ensure continued accuracy in the performance of the calorimeter. To avoid sample-related artifacts, extensive care was taken with sample preparation for the titration experiments. Both the MAb and cytochrome *c* were dialyzed exhaustively in the same flask to minimize thermal effects arising from differences in solution components. The final dialysate was used for concentration adjustments and blank titrations (see below). Protein samples were clarified by passage through a sterile 0.22 or 0.45 μm filter (Gellman Sciences). For enthalpy measurements, the antibody was loaded into the sample cell of the calorimeter (volume = 1.38 mL), and the reference cell was filled with water containing 0.01% sodium azide. Next, cytochrome *c* was placed in the syringe at a concentration 20-fold higher than that of the antibody combining sites. The system was allowed to equilibrate and a stable base line recorded prior to initiating an automated titration of the antibody with cytochrome *c*. A typical titration sequence involved 20 injections of 5 μL aliquots of cytochrome *c* into the sample cell. Throughout the titration, the cell was stirred continuously at 400 rpm. Each injection was made over a 9 s interval, and the base line was allowed to restabilize for 2 min prior to the subsequent addition. The calorimeter includes an electrical compensation circuit for a rapid return to base-line level after each injection. The heat change accompanying every injection is displayed as a peak on the output, and the area under each peak represents the heat evolved on MAb binding to the cytochrome *c*. To measure the heat of dilution of cytochrome *c* in the absence of MAb, control experiments were performed in which the antibody solution in the sample cell was replaced by the dialysate buffer. Heats of dilution for cytochrome *c* in the presence of the MAb−cytochrome *c* complex were estimated from the enthalpies for injections of cytochrome *c* measured after all MAb binding sites had been saturated (posttransition base line). Heats of dilution in the presence of complex obtained from the posttransition base line were used to correct the binding enthalpy for the heat of dilution. After correction for the heat of dilution, the heat of binding for each injection was obtained by integrating the area under the peak using ORIGIN (version 2.3, Microcal Inc.). To obtain the apparent molar enthalpy of binding (ΔH_b^{app}), the enthalpy per mole of cytochrome *c* injected for all individual injections was fit to an independent binding site model using software provided by MicroCal. No attempt was made to obtain binding constants from the calorimetric data, since the antibodies used in this study bind to cytochrome *c* with binding constants that are too large to be measured by titration calorimetry.

Proton Linkage. We have used the method of Sturtevant (Beres & Sturtevant, 1971) to measure the protons taken up or released on binding. Both the antibody and cytochrome *c* were dissolved in a buffer with a small ionization enthalpy and of sufficient strength that essentially all the binding-induced protonation changes in the MAb or cytochrome *c* result in complementary changes in buffer ionization (0.1 M sodium phosphate, pH 7.0). Therefore, the observed heat of binding is the sum of the intrinsic heat of binding and the heat of transferring protons to the buffer. We repeated this measurement in a buffer with a large enthalpy of ionization (0.1 M Tris, pH 7.0). The pH of the Tris buffer was measured using a calomel electrode to account for the change in junction potential. The titration data together with the published values of enthalpies of ionization (Beres & Sturtevant, 1971) were used to calculate the intrinsic heat of binding and the number of protons exchanged in the reaction using the equation:

$$\Delta H_b^{app} = \Delta H_b + n_{H^+}\Delta H_i \qquad (1)$$

where ΔH_b^{app} is the observed enthalpy change, ΔH_b is the intrinsic binding enthalpy, ΔH_i is the enthalpy of buffer ionization, and $^nH^+$ is the number of protons taken up by the complex during the binding reaction. Notice that while the binding enthalpy has been corrected for the enthalpy of buffer ionization, ΔH_b retains contributions from the ionization enthalpies of groups on the proteins.

Heat Capacity Measurements. Heat capacities of binding were obtained by measuring the enthalpies of association at various temperatures between 8 and 32 °C. Differential scanning calorimetric experiments were conducted using an MC-2 instrument manufactured by Microcal Inc. A scan rate of 1 K min^{-1} and protein concentrations of 3 mg mL^{-1} were employed. Sample preparation was essentially identical to that used in the titration experiments.

Equilibrium Fluorescence Quench Titration. Fluorescence titrations of antibody with cytochrome *c* were performed with an SLM 500C spectrofluorometer (SLM-Aminco, Urbana, IL). The excitation wavelength was 285 nm (5 nm bandpass) and the emission wavelength 350 nm (7.5 nm bandpass). The cell block temperature was regulated at 25 ± 0.1 °C with a Neslab RTE-100 programmable water bath. A typical titration involved up to 30 injections of 3 μL samples of cytochrome *c* (concentration = 1.2 μM) into a 3 mL quartz cuvette containing 10−50 nM antibody combining sites. Ubiquitin (Sigma), a protein lacking significant fluorescence, was added to the solutions at a concentration of 30 μg/mL as a carrier protein to block MAb or cytochrome *c* adsorption to the quartz cuvettes. The lack of tryptophan, which results in a very low fluorescence, makes ubiquitin well suited to this purpose (Foote & Winter, 1992). Fluorescence readings were averaged for a 10 s period at a time 3 min after each addition of cytochrome *c*. No photobleaching was detectable. The inner filter corrections were assumed to be negligible, since the absorbance at 285 nm was less than 0.01. Cytochrome *c* contains a single tryptophan but has less than 2% of the fluorescence of the model compound *N*-acetyltryptophanamide, because in native cytochrome *c* the heme quenches the intrinsic tryptophan fluorescence. After correction for dilution, the data in the form of the observed fluorescence intensity, F_{obs}, at a particular total cytochrome *c* concentration, $[C]_{tot}$, were fit to the following equation using the MINSQ software (Micromath Software, Saltlake City, UT):

$$F_{obs} = \{f_{ab}[Ab]_{tot} + f_c[C]_{tot}\} - (f_{abc}/2)\{([C]_{tot} + [Ab]_{tot} + 1/K_a) - \{([C]_{tot} + [Ab]_{tot} + 1/K_a)^2 - 4[C]_{tot}[Ab]_{tot}\}^{1/2}\} \quad (2)$$

The values of K_a, f_{ab}, f_c, f_{abc}, and $[Ab]_{tot}$ were varied to obtain the best fit of the data to eq 2. $[Ab]_{tot}$ is the total MAb binding site concentration; K_a is the equilibrium binding constant for MAb−cytochrome *c* binding; f_{ab} and f_c are the molar fluorescence of free antibody binding sites and cytochrome *c*, respectively; and f_{abc} is the molar fluorescence of the MAb−cytochrome *c* complex.

RESULTS

Heats of Binding of Antibody to Cytochrome c. Figure 3A shows a typical calorimetric titration of MAb 5F8 with cytochrome *c*. The area of each peak corresponds to the heat released upon addition of a single aliquot of cytochrome *c* into a solution of MAb 5F8. Figure 3B shows the titration data plotted as the enthalpy per mole of cytochrome *c* injected vs injection number. The data indicate saturation of the MAb binding sites with a stoichiometry of approximately 1 mol of cytochrome *c* per mole of antibody binding sites. No attempt has been made to obtain equilibrium binding constants from the calorimetric titration data, since the binding constants are too large to be measured by the titration calorimeter. The magnitude of the binding enthalpy per mole of binding sites is slightly smaller for intact 2B5 MAb than for the 2B5 FAb fragment (Table 1). For FAb 2B5, the binding enthalpy is largely independent of the redox state of cytochrome *c* (Table 1). The interactions of both MAb 2B5 and MAb 5F8 with cytochrome *c* are strongly exothermic in nature. Under standard conditions (T = 25 °C, 0.1 M sodium phosphate, pH 7.0), binding of MAb 2B5 to cytochrome *c* gives $\Delta H°_b = -21.0$ kcal mol^{-1} while for MAb 5F8 $\Delta H°_b = -21.7$ kcal mol^{-1} (Table 2). For all temperatures at which measurements have been made, the binding enthalpy contributes favorably to the free energy of binding. As temperature increases, the binding enthalpy becomes more exothermic (more favorable).

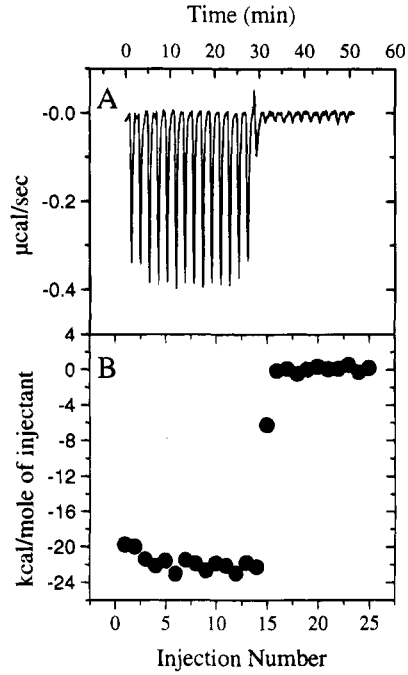

FIGURE 3: Calorimetric titration of MAb 5F8 with oxidized cytochrome *c*. The programmed sequence consisted of 25 injections of 5 μL each of a 96 μM cytochrome *c* stock solution. The cytochrome *c* solution was injected into a sample cell (volume = 1.38 mL) containing 6 μM antibody combining sites at a temperature of 25 °C. Each injection was made over a 9 s time interval with a 2 min pause between additions. The sample cell was continuously stirred at 400 revolutions per minute throughout the experiment. The daa shown in (A) are differences between the sample cell and a reference cell filled with water containing 0.01% sodium azide, and have been corrected for the heats of dilution. (B) The enthalpy per mole of cytochrome *c* injected time into the cell is plotted vs injection number for MAb 5F8. Conditions are 0.1 M sodium phosphate buffer, pH 7.0.

Binding-Induced Changes in Protonation. To measure protonation changes on binding, the apparent binding enthalpies have been measured in two buffers with very different enthalpies of protonation. At 24.8 °C in 0.1 M sodium phosphate, MAb 2B5 binding to cytochrome *c* yields $\Delta H_b^{app}(P_i) = -19.3$ kcal mol^{-1}. The same reaction in 0.1

Table 1: Titration Calorimetry Results[a]

binding reaction (buffer)	temp (°C)	ΔH_b^{app} [b] (kcal/mol)	ΔH_b[c] (kcal/mol)
MAb 2B5 + oxidized Cyt *c* (phosphate)	8.5	−10.7	−11.4
MAb 2B5 + oxidized Cyt *c* (phosphate)	16.4	−16.6	−17.3
MAb 2B5 + oxidized Cyt *c* (phosphate)	24.8	−19.3	−20.0
MAb 2B5 + oxidized Cyt *c* phosphate	32	−25.1	−25.8
FAb 2B5 + oxidized Cyt *c* (phosphate)	24.8	−21.7	−22.4
FAb 2B5 + reduced Cyt *c* (phosphate)	24.8	−22.1	−22.8
MAb 2B5 + oxidized Cyt *c* (Tris)	24.8	−11.7	
MAb 5F8 + oxidized Cyt *c* (phosphate)	8.5	−18.9	−18.9
MAb 5F8 + oxidized Cyt *c* (phosphate)	16.6	−20.2	−20.2
MAb 5F8 + oxidized Cyt *c* (phosphate)	24.8	−21.7	−21.7
MAb 5F8 + oxidized Cyt *c* (Tris)	24.8	−21.5	

[a] A typical titration consists of 20 injections of cytochrome *c* solution into the MAb-containing calorimetry cell of volume 1.38 mL. Each injection consists of 5 μL of a 90−100 μM cytochrome *c* solution injected into an antibody solution at a concentration of 6 μM binding sites. [b] ΔH_b^{app} is the apparent enthalpy of binding per mole of cytochrome *c* bound. ΔH_b^{app} is obtained by using an independent binding site model to fit the injection heat titration data. Base-line corrections are carried out prior to fitting by subtracting the average value of the injection heat after saturation from the directly measured heats of injection. This process corrects for the heat of dilution of cytochrome *c* in the presence of antibody−cytochrome *c* complex. The values of ΔH_b^{app} are not corrected for ionization of the buffer. [c] ΔH_b is the enthalpy of binding corrected for the enthalpy of buffer ionization (see Materials and Methods). This correction requires knowledge of the number of protons (n_{H^+}) taken up or released on complex formation (see Table 2). All calculations of ΔH_b have used the value of n_{H^+} determined at 24.8 °C, since this is the only temperature for which n_{H^+} has been measured. The value of n_{H^+} determined for MAb 2B5 has been used to calculate ΔH_b for FAb 2B5.

M Tris results in $\Delta H_b^{app}(\text{Tris}) = -11.7$ kcal mol^{-1} (Table 1). Since the apparent heat of binding of MAb 2B5 to cytochrome *c* is less exothermic in Tris buffer than in phosphate buffer, we conclude that there is a net proton uptake during the binding process. Using an enthalpy of ionization for Tris buffer of $\Delta H_i^{Tris} = 11.35$ kcal mol^{-1}, and an enthalpy of ionization for phosphate buffer of $\Delta H_i^{P_i} = 1$ kcal mol^{-1} (Beres & Sturtevant, 1971), the number of protons taken up on MAb 2B5−cytochrome *c* binding can be calculated using the relation:

$$n_{H^+} = \frac{\Delta H_b^{app}(P_i) - \Delta H_b^{app}(\text{Tris})}{\Delta H_i^{P_i} - \Delta H_i^{Tris}} \quad (3)$$

where n_{H^+} is the number of protons taken up. The value of $n_{H^+} = +0.73$ for MAb 2B5−cytochrome *c* binding indicates that protons are being taken up on complex formation (Table 2). In contrast, the enthalpy of binding of MAb 5F8 with cytochrome *c* is essentially the same in Tris and in phosphate buffer, and, hence, there is no significant change in protonation on binding ($n_{H^+} = +0.02$; Table 2). The corrected enthalpies of binding, ΔH_b (Tables 1 and 2), have been corrected for the enthalpy of buffer protonation using the procedure described under Materials and Methods. Most of the experiments were carried out in sodium phosphate buffer, which has a small enthalpy of ionization, so only small corrections were required. The values of ΔH_b, although corrected for the enthalpy of buffer ionization, contain contributions from ionization of groups on the proteins (the MAbs, cytochrome *c*, or both). This is a formal possibility for binding of both MAb 2B5 and MAb 5F8 to cytochrome *c*. While there are no net changes in protonation in the case of MAb 5F8−cytochrome *c* binding, compensating ionizations may occur. Correction of the binding enthalpies for protein ionization requires assignment of the ionization reactions to particular groups on the proteins, and estimation of the ionization enthalpies of these groups.

Equilibrium Constant for Antibody−Cytochrome c Association. Previous measurements of the rates of association and dissociation (Raman et al., 1992) have been used to estimate a value for $K_a = 1.4 \times 10^{10}$ M for the interaction of MAb 5F8 with cytochrome *c* at 25 °C. Measurements for the MAb 2B5−cytochrome *c* complex suggest a similar value for K_a for binding of this MAb to its antigenic site, but are less reliable (Raman et al., 1992). The direct measurement of very high values of K_a from calorimetric titrations is not possible, so an equilibrium titration monitored by quenching of MAb fluorescence has been used to obtain an improved value for the MAb 2B5−cytochrome *c* binding constant of $K_a = 2 \times 10^9$ M^{-1} (Figure 4). The errors in K_a are large because the high value of K_a together with the sensitivity of the fluorescence measurements requires the use of higher than optimal concentrations of MAb 2B5 and cytochrome *c*.

Heat Capacity Change. The enthalpies of binding were measured at various temperatures between 8 and 32 °C. Figure 5A shows that MAb 2B5 binding to cytochrome *c* displays a substantial variation of the enthalpy with temperature, and, hence, the process involves a large change in the heat capacity, $\Delta C_p = -580$ cal mol^{-1} K^{-1} (Figure 5A; Table 2). The enthalpy of binding of MAb 5F8 to cytochrome *c* is less dependent on temperature, and results in a smaller $\Delta C_p = -172$ cal mol^{-1} K^{-1} (Figure 5B; Table 2). For both MAb 2B5 and MAb 5F8, there is insufficient data to determine whether ΔC_p depends on temperature. Thus, the values of ΔC_p have been obtained from the slopes of a linear regression analysis of the ΔH_b vs T data.

Differential scanning calorimetric measurements (DSC) of the temperature dependence of the heat capacity were made for free MAb, free cytochrome *c*, and the MAb 2B5−cytochrome *c* complex as a test for temperature-induced transitions over the temperature range used for the titration calorimetry experiments (data not shown). The results show that there are no detectable temperature-induced transitions in the range of 10−60 °C. Above 60 °C, free MAb 2B5, cytochrome *c*, and the MAb 2B5−cytochrome *c* complex are all irreversibly denatured in processes involving endothermic changes in C_p followed by sharp exotherms.

DISCUSSION

Thermodynamics of Antibody Binding to Cytochrome c. Figure 5A,B shows the temperature dependence of ΔH_b, $-T\Delta S_b$, and ΔG_b for (A) MAb 2B5 and (B) MAb 5F8 binding to cytochrome *c*. For both antibodies, there is little change in the ΔG_b with temperature, but for different reasons. MAb 2B5 exhibits a strong temperature dependence of both ΔH_b and $-T\Delta S_b$, and the value of ΔG_b remains relatively constant because of significant enthalpy−entropy compensation (Figure 5A). There is some enthalpy−entropy compensation for MAb 5F8 too, but the primary reason ΔG_b for MAb 5F8−cytochrome *c* binding shows so little temperature dependence is that neither ΔH_b nor $-T\Delta S_b$ changes appreciably with temperature (Figure 5B). Such distinctly different thermodynamic behavior suggests fundamental

Table 2: Thermodynamic Parameters for the Association of Monoclonal Antibodies and Oxidized Cytochrome *c*[a]

reaction	K_a[b] (M^{-1})	ΔC_p[c] (cal mol^{-1} K^{-1})	$\Delta G°_b$[d] (kcal mol^{-1})	$\Delta S°_b$[d] (cal mol^{-1} K^{-1})	$\Delta H°_b$[e] (kcal mol^{-1})	n_{H^+}[f]
MAb 2B5 + cytochrome *c*	2×10^9	−580	−12.6	−28.2	−21.0	+0.73
MAb 5F8 + cytochrome *c*	1.4×10^{10}	−172	−13.9	−26.3	−21.7	+0.02

[a] Values are for 0.1 M sodium phosphate, pH 7, 25 °C. [b] For the MAb 2B5, K_a was obtained by fluorescence titration and curve fitting (see Figure 4). For the MAb 5F8, K_a is taken from Raman et al. (1992) where K_a was calculated from $K_a = k_{on}/k_{off}$ at 25 °C. [c] ΔC_p is obtained from the slope of least-squares fits to the data given in Figure 5A,B. [d] Calculated from $\Delta G°_b = -RT \ln K_a$ and $\Delta S°_b = (\Delta H°_b - \Delta G°_b)/298$. [e] The intrinsic heat of binding, ΔH_b, is the binding enthalpy corrected for buffer ionization by the method of Beres and Sturtevant (1971). $\Delta H°_b$ is the value of ΔH_b under the standard conditions of 0.1 M sodium phosphate, pH 7.0, 25 °C. $\Delta H°_b$ is the value of the function $\Delta H_b(T)$ at 25 °C, where $\Delta H_b(T)$ is obtained as a least-squares fit of the data given in Table 1 to a straight line. [f] The parameter n_{H^+} is the apparent number of moles of protons taken up (+) or released (−) per mole of complex formed. The moles of complex are expressed in terms of the moles of MAb binding sites. The values of ΔH_b^{app} are measured in both phosphate and Tris buffers (Table 1), and the value of n_{H^+} is calculated from $n_{H^+} = [\Delta H_b^{app}(P_i) - \Delta H_b^{app}(\text{Tris})]/[\Delta H_i^{P_i} - \Delta H_i^{Tris}]$, where $\Delta H_i^{P_i}$ and ΔH_i^{Tris} are the ionization enthalpies of phosphate and Tris buffers, respectively (see Materials and Methods).

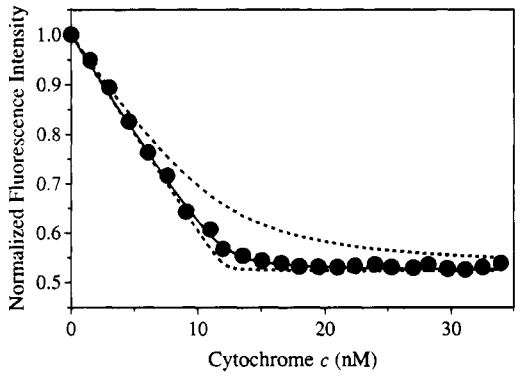

FIGURE 4: Equilibrium fluorescence quench titration of MAb 2B5 with oxidized cytochrome *c*. Each data point corresponds to the quenching observed on the addition of 3 μL of cytochrome *c* (1.2×10^{-6} M) to 2800 μL of antibody combining sites at a concentration of 12×10^{-9} M. The fluorescence intensity of the antibody solution has been corrected for dilution errors and normalized to a fluorescence emission of free antibody sites of 1.0. The line through the points is a nonlinear least-squares fit to a binding model that assumes independent binding sites. The solid line gives the best fit to the data with $K_a = 2 \times 10^9$ M^{-1}. The dashed lines above and below the solid line are simulated binding curves for $K_a = 2 \times 10^8$ M^{-1} and $K_a = 2 \times 10^{10}$ M^{-1}, respectively. Excitation was at 285 nm, and the fluorescence emission was observed at 350 nm. Conditions are: 25 °C, 0.1 M sodium phosphate buffer, pH 7. The buffer contains 30 μg mL^{-1} ubiquitin, a low fluorescence carrier protein, to block adsorption of the antibody or cytochrome *c* to the cuvette walls.

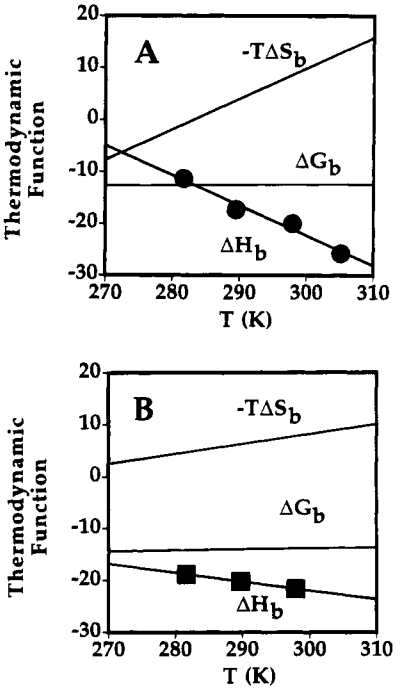

FIGURE 5: Temperature dependence of the thermodynamic parameters for binding of cytochrome *c* to (A) MAb 2B5 and (B) MAb 5F8. The parameters have been corrected for the effects of buffer ionization. Data points measured by titration calorimetry (Table 1) are included for ΔH_b. The heat capacity change associated with antibody binding to oxidized cytochrome *c* (Table 2) was determined by linear regression analysis as the slope of the plot of ΔH_b vs temperature. Values of $\Delta G_b(T)$ are calculated from the thermodynamic parameters given in Table 2 using the equation: $\Delta G_b(T) = (T/298)\Delta G°_b + [1 - (T/298)]\Delta H_b - \Delta C_p[298 - T + T \ln(T/298)]$ where $\Delta G°_b$ and $\Delta H°_b$ are the thermodynamic parameters (Table 2) under standard conditions: 0.1 M sodium phosphate, pH 7.0, 25 °C. ΔC_p is assumed to be independent of temperature. The values of $-T\Delta S_b(T)$ are calculated from $-T\Delta S_b(T) = \Delta G_b(T) - \Delta H_b(T)$.

differences in the underlying physical features of binding for the two antibodies.

The sign of the entropy change on binding provides some clues to the kinds of physical processes involved. Above 10 °C, the entropy changes on binding to cytochrome *c* are negative for both antibodies (Figure 5A,B; Table 2). Processes for which a negative entropy change is expected are hydrogen bond formation, a decrease in the number of isoenergetic conformations, and a decrease in soft internal vibrational modes (Sturtevant, 1977). For MAb 5F8, the binding entropy remains negative at all temperatures above the freezing point of water, but for MAb 2B5 the entropy change is increasingly positive at temperatures below 283 K. Processes for which a positive entropy change is expected are the burial of electrostatic charges or hydrophobic groups (Sturtevant, 1977).

There is a small increase in the magnitude of the enthalpy of binding of FAb 2B5 to oxidized cytochrome *c* compared to binding of intact MAb 2B5 (Table 1). This suggests that separation of the two binding sites into separate molecules enhances the magnitude of the binding enthalpy. It is difficult, however, to rule out systematic errors related to sample preparation, or the higher total protein concentration (in milligrams per milliliter) for MAb titrations compared to FAb titrations. Interestingly, the enthalpy of binding of 2B5 FAb to cytochrome *c* is independent of the redox state of cytochrome *c* (Table 1). This contrasts with a slight (~20%) decrease in the bimolecular association rate constant obtained from studies of the kinetics of binding of 2B5 MAb to reduced cytochrome *c* compared to oxidized cytochrome *c* (Raman et al., 1992).

Proton Linkage in Antibody−Cytochrome c Association. The results show that 0.73 proton is taken up on binding of MAb 2B5 to cytochrome *c*, while there is little or no change in protonation ($n_{H^+} = +0.02$) for binding of MAb 5F8 to cytochrome *c* (Table 2). Thus, protonation changes coupled to binding (Ross & Subramanian, 1981) are likely contributors to the overall thermodynamic parameters for binding of MAb 2B5 to cytochrome *c*. Assuming that there are no significant compensating protonation changes, protonation does not contribute to the thermodynamics of MAb 5F8−cytochrome *c* binding. The protonation changes for MAb 2B5−cytochrome *c* binding can be viewed as arising from a change in the pK of one or more groups on complex formation. The ionizable groups may be those of the antibody, cytochrome *c*, or both. While an unambiguous assignment of the specific residue(s) responsible for the binding-induced uptake of protons is not possible, His side chains are likely candidates for ionizing groups near neutral pH. His 33 for horse cytochrome *c* has a p$K = 6.54$ (Cohen & Hayes, 1974) which is near the pH used for the antibody binding studies. Moreover, His 33 of cytochrome *c* is a likely component of the antigenic surface recognized by MAb 2B5 (Figure 1A,B). If a His side chain is assumed to be the only ionizing residue, then the contribution to the overall enthalpy of binding from protonation can be estimated by multiplying the negative of the enthalpy of ionization for a His side chain by the number of protons taken up on complex formation. Taking the enthalpy of ionization of His to be +6.0 kcal/mol (Shiao & Sturtevant, 1976), the protonation contribution to $\Delta H°_b$ for formation of the MAb 2B5−cytochrome *c* complex is −4.4 kcal mol^{-1}. Presumably, the contributions to $\Delta H°_b$ from side chain protonation changes are negligible for formation of the MAb 5F8−cytochrome *c* complex. Thus, the contributions to $\Delta H°_b$ from sources other than changes in protonation are likely to differ for formation of MAb 2B5 and MAb 5F8 complexes with cytochrome *c*, despite the fact that the overall values of $\Delta H°_b$ are similar.

Binding-induced changes in protonation may provide a partial explanation for the differences in ΔC_p for the two antibodies. Assuming that a His side chain is responsible for all of the proton uptake for MAb 2B5−cytochrome *c* binding, the contributions to ΔC_p resulting from binding-induced protonation changes can be estimated to be of the order of −40 cal mol^{-1} K^{-1} for MAb 2B5−cytochrome *c* binding. This is a relatively small contribution, similar in magnitude to the errors in ΔC_p.

Binding-Induced Heat Capacity Changes. The binding to cytochrome *c* of MAb 2B5 and MAb 5F8 involves substantially different changes in the heat capacity (ΔC_p of binding). While the MAb 2B5−cytochrome *c* interaction results in a ΔC_p of −580 cal mol^{-1} K^{-1}, the interaction involving MAb 5F8 yields only −172 cal mol^{-1} K^{-1}. This suggests qualitative differences in the binding process for MAb 5F8 compared to MAb 2B5. The small magnitude of ΔC_p for MAb 5F8 binding is especially interesting. Ladbury et al. (1994) have studied heat capacity changes for binding of Trp repressor to DNA and find that a large negative ΔC_p of binding occurs for both high-affinity ($\Delta G° = -12.1$ kcal mol^{-1}; $\Delta C_p = -950$ cal mol^{-1} K^{-1}) and low-affinity ($\Delta G° = -7.6$ kcal mol^{-1}; $\Delta C_p = -880$ cal mol^{-1} K^{-1}) repressor−DNA interactions. Ladbury et al. conclude that high-affinity binding and a large negative ΔC_p are not necessarily correlated. The results for MAb 5F8−cytochrome *c* binding complement the DNA binding studies and support their conclusion by showing that high-affinity binding can also occur with relatively modest changes in heat capacity: $\Delta G° = -13.9$ kcal mol^{-1} and $\Delta C_p = -172$ cal mol^{-1} K^{-1} for MAb 5F8 binding to cytochrome *c* (Table 2). Ladbury et al. suggest that a large negative ΔC_p may be a consequence of high specificity binding, whether at high or low affinity. They propose that a large negative heat capacity change results from the formation of an "intimate complementary interface of a 'specific' complex" and is "not a consequence of a high affinity reaction *per se*". The hypothesis relating large negative values of ΔC_p to high specificity may not hold for antibody−protein antigen interactions. Assuming that binding of an antibody to a protein antigen always involves high specificity, then the differences in the magnitudes of ΔC_p for MAb 5F8 and MAb 2B5 binding to cytochrome *c* indicate that high-specificity binding of a protein antigen by an antibody can occur with both large and small changes in heat capacity.

The observed values of ΔC_p can be used to estimate the amount of both polar and apolar surfaces buried on binding. For both MAb 2B5 and MAb 5F8, we assume that approximately 1600 Å^2 of surface is buried in the interface (Janin & Chothia, 1990). The following relations from the literature are used to calculate the amount of polar and apolar interfacial surface necessary to account for the observed ΔC_p values. Note that these relationships are intended to relate ΔC_p for protein folding transitions to the loss of solvent-accessible surface and, thus, may or may not apply to antibody binding to protein antigens.

Method I is that of Murphy and Freire (1992):

$$\Delta C_p = 0.45\Delta A_{np} - 0.26\Delta A_p \quad (4)$$

A second relationship, method II, has been proposed by Spolar and Record (1994):

$$\Delta C_p = 0.32\Delta A_{np} - 0.14\Delta A_p \quad (5)$$

For both equations, ΔA_{np} is the change in nonpolar solvent-accessible surface area and ΔA_p is the change in polar solvent-accessible surface area. Note that the changes in area are defined in such a manner that they have negative signs when the surface area is buried. For MAb 2B5 with $\Delta C_p = -580$ cal mol^{-1} K^{-1}, method I indicates that the surface area buried on complex formation is 88% nonpolar surface and 12% polar surface. Using method II for MAb 2B5 indicates that essentially all (100%) of the surface buried on complex formation is nonpolar (Table 3). In the case of MAb 5F8 with a $\Delta C_p = -172$ cal mol^{-1} K^{-1}, method I suggests that the surface buried is 52% nonpolar and 48% polar. Method II gives very similar values for the type of surface buried (54% nonpolar and 46% polar).

While the heat capacity change for protein folding often correlates well with the burial of nonpolar surface, a similar correlation may not apply to antibody−protein interactions. Binding of variant S-peptides by the S-protein for RNase S shows little, if any, correlation between ΔC_p and the nonpolar surface area buried on binding (Connelly et al., 1990; Varadarajan et al., 1992). On the other hand, Hibbits et al. (1994) have reported a titration calorimetry study of the binding of an antibody to lysozyme that suggests that relationships like those of eqs 4 and 5 may be applicable to

Table 3: Estimates of the Amount and Type of Surface Area Buried on Complex Formation[a]

complex	method	surface area buried (Å²)	
		nonpolar ($-\Delta A_{np}$)	polar ($-\Delta A_p$)
MAb 2B5	I	1403 ± 110	197 ± 190
MAb 2B5	II	(1748 ± 91)[b]	(−148 ± 209)[b]
MAb 5F8	I	828 ± 110	772 ± 190
MAb 5F8	II	861 ± 91	739 ± 209

[a] Estimates of the polar and the nonpolar surface area that is buried when the monoclonal antibody listed in column 1 forms a complex with cytochrome *c*. ΔA_{np} and ΔA_p are, respectively, the changes in nonpolar and polar solvent-accessible surface area. Changes in solvent-accessible surface area are defined to have a negative sign when surface is buried. Two different methods are used to estimate the characteristics of the buried surfaces from the value of ΔC_p for binding. Method I is that of Murphy and Freire (1992), and method II is that of Spolar and Record (1994). The total surface area buried is assumed to be equal to 1600 Å² (i.e., $\Delta A_{np} + \Delta A_p = -1600$ Å²). Error estimates are made by recalculating ΔA_{np} and ΔA_p for the case (lower limit) where $\Delta A_{np} + \Delta A_p = -1300$ Å², and for (upper limit) $\Delta A_{np} + \Delta A_p = -1900$ Å². [b] The numbers obtained for MAb 2B5 by method II are given in parentheses, since for $\Delta A_{np} + \Delta A_p = -1600$ Å² the values for ΔA_{np} and ΔA_p are not physically reasonable. Method II predicts burial of an amount of nonpolar surface greater than the total surface area (1600 Å²), and burial of a negative amount of polar surface (i.e., exposure of polar surface). Note, however, that physically reasonable values of ΔA_{np} and ΔA_p are obtained at the upper limit of the range where $\Delta A_{np} + \Delta A_p = -1900$ Å².

some antibody−protein antigen binding reactions. Hibbits et al. estimated the amount of buried polar and apolar surface area from the X-ray structure (Sheriff et al., 1987) and calculated a value for ΔC_p using method I (Murphy & Freire, 1992). The calculated value for ΔC_p was in excellent agreement with that measured by titration calorimetry. However, Bhat et al. (1994) also find good agreement between the calorimetrically determined value for ΔC_p and that estimated from the burial of hydrophobic surface for an Fv-lysozyme binding reaction. This is despite the fact that the X-ray structure of the complex shows significant hydration of the interacting surfaces. They conclude that the relationship between ΔC_p and the burial of hydrophobic surface may be fortuitous.

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