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RESEARCH ARTICLES

Configurational Effects in Antibody–Antigen Interactions Studied by Microcalorimetry

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ABSTRACT **In this paper we study the binding of two monoclonal antibodies, E3 and E8, to cytochrome *c* using high-sensitivity isothermal titration calorimetry. We combine the calorimetric results with empirical calculations which relate changes in heat capacity to changes in entropy which arise from the hydrophobic effect. The change in heat capacity for binding E3 is -350 ± 60 cal K^{-1} mol^{-1} while for E8 it is -165 ± 40 cal K^{-1} mol^{-1}. This result indicates that the hydrophobic effect makes a much larger contribution for E3 than for E8. Since the total entropy change at 25°C is very similar for both antibodies, it follows that the configurational entropy cost for binding E3 is much larger than for binding E8 (-77 ± 15 vs. -34 ± 11 cal K^{-1} mol^{-1}). These results illustrate a case of entropy compensation in which the cost of restricting conformational degrees of freedom is to a large extent compensated by solvent release. We also show that the thermodynamic data can be used to make estimates of the surface area changes that occur upon binding. The results of the present study are consistent with previous hydrogen–deuterium exchange data, detected using 2D NMR, on the two antibody–antigen interactions. The NMR study indicated that protection from exchange is limited to the binding epitope for E8, but extends beyond the epitope for E3. These results were interpreted as suggesting that a larger surface area was buried on cytochrome *c* upon binding to E3 than to E8, and that larger changes in configurational entropy occur upon binding of E3 than E8. These findings are confirmed by the present study using isothermal titration calorimetry.**

Key words: cytochrome *c*, thermodynamics, antibody binding, microcalorimetry

INTRODUCTION

Studies on the recognition of protein antigens by B cells have been facilitated in the past decade by the availability of epitope specific monoclonal antibodies (mAbs) produced by hybridoma technology which has allowed a detailed molecular analysis of the interface between the antibody combining site and antigenic sites on proteins using X-ray crystallography[1–7] and 2D NMR.[8–10] The published X-ray crystallographic studies of mAbs complexed with neuraminidase and hen egg lysozyme (HEL) reveal that an extensive surface of the protein antigen, about 700–900 Å^2, interacts with the antibody and that this surface is comprised of two to five discontiguous stretches of polypeptide backbone.[1–7] However, a major question has emerged from the ability to study antibody–antigen complexes at the atomic level which concerns the extent of conformational change that takes place in either the antibody or the antigen upon complex formation. Since both neuraminidase[11] and HEL[12] have known crystal structures direct comparisons have been made between the antibody bound and free forms or these antigens. Differences in the degree of complementarity have been observed in these existing antibody–antigen X-ray structures and indicate that in some of these complexes small changes can take place in the three-dimensional fold of the antigen that improves the complementarity between the interaction sites.

Abbreviations: BCA, bicinchonicic acid; cyt *c*, cytochrome *c*; ITC, isothermal titration calorimetry; mAb(s), monoclonal antibodie(s); tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PBS, phosphate buffered saline.

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The mAbs to horse cytochrome *c* (cyt *c*) which we have created and characterized provide another system in which to examine this question.[13] We have studied the interaction between three mAbs and their antigen, horse cyt *c*, using ^{1}H NMR spectroscopy, a technique which, in general, is not directly applicable to antibody–protein antigen complexes due to their large molecular structure. We solved this problem by immobilizing the antibody on a solid support which allows for rapid dissociation of the antigen, allowing us to measure the rate of hydrogen–deuterium (H–D) exchange of the amide protons in horse cyt *c*–antibody complexes and to analyze those protons on the antigen protected by complex formation using 2D-COSY NMR.[8,14] We found that for the surface on cyt *c* bound by one antibody, E8, the protected residues, 36–38, 59–67, and 100–101, and their H-bond acceptors, are brought together in the three-dimensional structure to form a contiguous, largely exposed protein surface.[8] The interaction site defined in this way is consistent with prior epitope mapping studies on E8[13,15–18] and the size of the apparent epitopic surface, about 750 Å^2, is comparable to that determined by the X-ray crystallographic analyses described above of other mAbs complexed with small protein antigens. Further studies, on other anti-cyt *c* antibodies, E3 and C3, which bind to a different surface of cyt *c*, however, showed somewhat different effects on the kinetics of H–D exchange on complex formation and indicated that the binding of these antibodies to horse cyt *c* not only affects the hydrogen exchange behavior of regions within the epitope but also beyond the immediate binding site.[9] The differing effects of these antibodies on H–D exchange kinetics can be accounted for by considering the local segmental flexibility of the surfaces of cyt *c* to which they bind. In the case of E8 which binds to a relatively immobile surface on cyt *c*, there appears to be little conformational reorientation of the antigen on antibody binding. E3 and C3, however, bind to a highly flexible region, the 37–59 Ω loop,[19] and in so doing appear to stabilize a network of hydrogen bonds which restricts the conformational flexibility of regions remote from the epitope.[9]

Microcalorimetry has recently been applied to determining the thermodynamic parameters of antibody–antigen interactions.[20–22] In this study we have investigated the energetics of binding of the two mAbs, E3 and E8, using isothermal titration calorimetry (ITC). By combining calorimetric measurements with structural energetic calculations, we show that, although the total entropy change at 26°C is similar for the two mAbs, the cost in terms of configurational entropy for binding of cyt *c* to E3 is significantly greater than for E8. We also show that the cost of restricting conformational degrees of freedom on complex formation with E3 is to a large extent compensated by solvent release due to a larger buried surface area for E3 than for E8. The results of the present study are discussed in the context of the previous hydrogen–deuterium exchange data described above and shown to be consistent with the interpretation in the NMR study that a larger surface area was buried on cytochrome *c* by E3 than by E8 and that larger changes in configurational entropy occur upon binding of E3 than E8.

MATERIALS AND METHODS

Monoclonal antibodies, E3 and E8 specific for horse cytochrome *c* (cyt *c*), were prepared and affinity purified as previously described.[23] Horse cyt *c* grade VI was purchased from Sigma (St. Louis, MO) and used without further purification. All buffers were prepared using distilled-deionized water and protein solutions were dialyzed extensively before use in PBS (20 mM Na_2H/KH_2PO_4, 0.8% NaCl, 0.02% KCl, pH 7.2). Stock solutions of E3 (6.5 mg ml^{-1}), E8 (6.7 mg ml^{-1}), and cyt *c* (9.3 mg ml^{-1}) were diluted with dialysis buffer as needed. The concentration of mAb solutions was determined using E_{280} = 1.44 for a 1 mg ml^{-1} solution, while the concentration of cyt *c* solutions was determined using E_{409} = 7.43 for a 1 mg ml^{-1} solution after transfer to the Biocalorimetry Center. Prior to shipping, concentrations of IgG solutions were determined both by A_{280} and BCA assay (Pierce Chemical Co. Rockford, IL) as per the manufacturer's instructions. For E8 the ratio of BCA to A_{280} determination was 1.15 and for E3 it is 0.87. In all cases, the concentrations determined after shipping and high speed centrifugation, to remove aggregated IgG, were lower than the initial concentrations.

Isothermal titration calorimetry (ITC) experiments were performed in an Omega microcalorimeter (Microcal, Inc., Northhampton, MA) interfaced to a microcomputer using an A/D converter board (Data Translation DT-2801) for automatic instrument control and data collection. All solutions were degassed by water aspiration prior to loading. The sample cell (1.36 ml) was filled with mAb solution of approximately 0.02 mM concentration and a 250 μl syringe was filled with cyt *c* solution at approximately 0.5 mM concentration. A series of 10 μl injections were made until subsequent injections resulted in no further change in the heat effect, indicating that all the binding sites were saturated. Injections of cyt *c* into buffer were performed to correct for any heat of dilution of the cyt *c* solution.

Changes in heat capacity were determined by performing ITC experiments over a range of temperature from 25 to 45°C, and plotting the fitted ΔH° of binding versus temperature. In order to determine if the binding reactions were accompanied by any change in protonation, experiments were also performed in tricine buffer (20 mM tricine, 0.8% NaCl, 0.02% KCl, pH 7.2) which has an ionization enthalpy change, ΔH°_{ion}, of 7.52 kcal mol^{-1} at 25°C.[24]

Calorimetric titration curves were fitted to an identical, independent sites model by nonlinear least squares minimization using software developed at the Biocalorimetry Center.[25] According to this model, the parameters to be determined are N, the number of binding sites, $\Delta H°$, the binding enthalpy change per site, and K, the binding constant. While the number of binding sites on an IgG molecule is known to be two, this parameter was adjusted in the fit in order to account for uncertainties in the concentration of mAb in solution. Confidence intervals of the fitted parameters were determined using a support plane analysis.[26]

RESULTS

Determination of the Thermodynamic Parameters of Binding

Representative data from a titration experiment are given in Figure 1a. The area under each peak is the heat, q, for that injection. The positive deflections observed at the end of the titration reflect the enthalpy of dilution of the cyt c solution and are subtracted from the binding data. The calorimetric titration curve obtained after subtracting the heat of dilution of cyt c is shown in Figure 1b. The curvature evident in Figure 1b indicates that the experiment has been performed in a concentration range which permits resolution of the binding constant from the titration data.

The thermodynamic parameters obtained by nonlinear least squares fitting of the titration data for the two antibodies are given in Table I. For E8, the fitted number of binding sites is somewhat greater than the theoretically expected value of two, suggesting an underestimation of the concentration by use of the average extinction coefficient (see Materials and Methods). On the other hand, the lower than expected value of N for E3 may reflect either an error in the concentration estimation, or else the presence of protein which is incompetent for binding. The parameters in Table I are based on IgG concentrations determined by A_{280}. While the deviation of N from two apparently results from variation in the IgG concentration determinations, this deviation has little effect on the fitted value of $\Delta H°$ as indicated by the confidence intervals.

For both antibodies, $\Delta H°$ decreases with increasing temperature which indicates a negative ΔC_p upon binding (Fig. 2). The binding entropy change, $\Delta S°$, is determined from the standard relationship:

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (1)$$

where T is the absolute temperature and $\Delta G°$ is evaluated from the binding constant, K, using a 1 M standard state as

$$\Delta G° = -RT \ln K \quad (2)$$

where R is the gas constant.

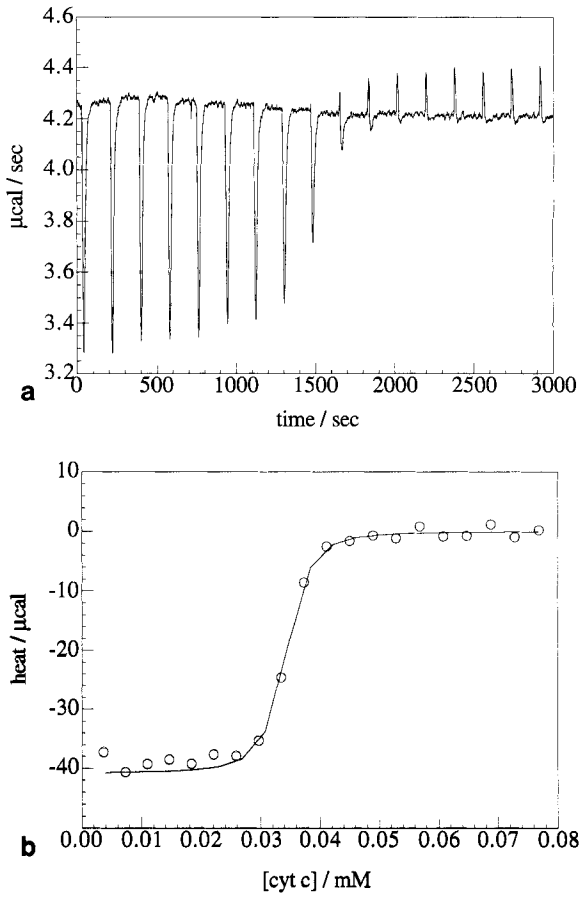

Fig. 1. (**a**) Calorimetric trace of the titration of E3 with cytochrome c at 25°C. The area under each peak is the heat, q, for that injection. The positive deflections towards the end of the titration represent the dilution of the cytochrome c solution. (**b**) Heat effect (corrected for dilution) versus concentration of titrant added to the cell. The open circles represent the integrated heats from **a**, corrected for cyt c dilution and the solid line is the theoretical fit of the data using the parameters from Table I.

The thermodynamic parameters at 25°C for the binding of cyt c to the two antibodies are given in Table II. The binding parameters were not changed in the presence of tricine buffer, indicating that no change in protonation occurs upon binding at pH 7.2. In both cases the binding is favored both enthalpically and entropically, although the major contribution to the binding free energy is enthalpic in origin. Similar features are observed in the binding of the extracellular domain of $p185^{HER2}$ to humanized murine mAbs[20] and in the binding of the octapeptide hormone angiotensin II to an mAb.[21]

The binding constant for E8 at 25°C cannot be reliably obtained from the data because the titration occurs under stoichiometric conditions. This is apparent in that an upper limit on log K cannot be determined as indicated in the table. Values of $\Delta G°$ at 25°C can be estimated using the better determined values of K at 35 and 45°C using the Gibbs–

TABLE I. Fitted Parameters for the Binding of Antibodies E8 and E3 to Horse Cyt *c

Antibody	Temperature (°C)	N	$\Delta H°$ (kcal mol^{-1})	log K
E8	25	2.2 (2.1,2.3)	−9.5 (−10.4,−8.8)	7.8 (7.0,*)
E8	35	2.2 (2.1,2.3)	−10.5 (−11.0,10.0)	6.7 (6.4,7.1)
E8	45	2.1 (2.0,2.2)	−12.8 (−14.0,−11.8)	6.5 (6.1,7.1)
E3	25	1.7 (1.6,1.7)	−7.3 (−7.7,−7.0)	7.1 (6.8,7.3)
E3	35	1.8 (1.7,1.9)	−9.8 (−10.2,−9.2)	7.1 (6.8,7.8)
E3	45	1.7 (1.6,1.8)	−14.3 (−15.4,−13.4)	6.7 (6.4,7.6)

*The one standard deviation confidence limits as determined by F-testing are given in parentheses. An asterisk indicates that no upper limit could be determined.

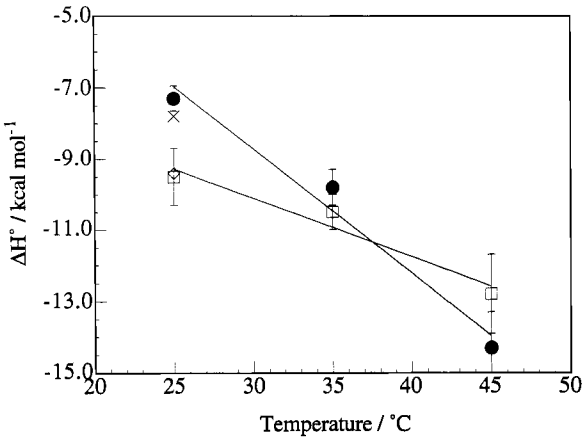

Fig. 2. The $\Delta H°$ of binding cytochrome *c* to E3 (filled circles) and E8 (squares) as a function of temperature in phosphate buffer. The slope of the line gives the heat capacity change, ΔC_p. Values at 25°C in tricine buffer are shown for comparison: E3 (×); E8 (◇).

Helmholtz equation and the determined values of $\Delta H°$ and ΔC_p. The average of the values determined from these two temperatures is indicated parenthetically in the table. The value of $\Delta S°$ at 25°C calculated using this value is also indicated.

The binding is also accompanied by a sizable ΔC_p in both cases as is typical of binding processes in biological systems. A negative ΔC_p is also observed in the other antibody–antigen interactions mentioned previously[20,21] and is indicative of the burial of hydrophobic surface upon binding.[27–30] Finally, the difference in $\Delta G°$ between the binding of the two antibodies is less than the $\Delta H°$ or $T\Delta S°$ differences, reflecting the existence of enthalpy–entropy compensation.[31,32]

Entropic Effects in Antibody/ Antigen Association

There are several contributions to $\Delta S°$ in a biological binding process,[33] namely the entropy change from the release of solvent, ΔS_{solv}, and the entropy change from the restriction of amino acid side chains and the polypeptide backbone, ΔS_{conf}. Additionally, there is a term which reflects the loss of translational and rotational degrees of freedom.[33–36] It has been argued that this term can be assessed by assuming that the protein in solution behaves as a particle in a box and hence one can apply the well known Sakur–Tetrode equation.[35] However, empirical and theoretical considerations[33] suggest that this term might best be treated as a cratic entropy which, for a bimolecular binding process, contributes −8 cal K^{-1} mol^{-1} to the binding entropy. Entropy changes arising from protonation are also possible, but need not be considered here since no protonation changes are observed upon binding (see below).

Calculation of ΔS_{solv} and ΔS_{conf} for the Antibody–Antigen Interactions

The total $\Delta S°$ of binding, given the considerations just outlined, is given as

$$\Delta S° = \Delta S°_{solv} + \Delta S°_{conf} + \Delta S°_{crat} \quad (3)$$

where $\Delta S°_{solv}$ is the change in entropy resulting from solvent release upon binding, $\Delta S°_{conf}$ is the change in entropy resulting from configurational changes in the antigen and antibody, and $\Delta S°_{crat}$ is the cratic entropy change. The changes in water structure around the apolar surface (i.e., the hydrophobic effect) as well as any changes in water structure around polar groups will contribute to ΔS_{solv} while differences in configurational degrees of freedom in amino acid side chains and backbone of both the antigen and the antibodies will contribute to $\Delta S°_{conf}$.

The entropy of aqueous dissolution of apolar compounds has been found to be independent of the apolar surface area when extrapolated to 112°C (using a constant ΔC_p).[30,37] This lack of apolar dependence indicates that the hydrophobic contribution to $\Delta S°_{solv}$, denoted by ΔS_{hyd}, must be zero or negligibly small at this temperature and can be calculated as

$$\Delta S_{hyd} = \Delta C_{p,hyd} \ln(T/T_S*) \quad (4)$$

where T_S* is 385 K (112°C) and $\Delta C_{p,hyd}$ is the hydrophobic contribution to ΔC_p.

TABLE II. Thermodynamics of Binding of Antibodies E8 and E3 to Horse Cyt *c* at 25°C

Antibody/buffer	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal K^{-1} mol^{-1})	ΔC_p (cal K^{-1} mol^{-1})
E8/phosphate	−10.6 (−9.5) ± 0.7	−9.5 ± 0.9	3.7 (0.3) ± 3.8	−165 ± 38
E8/tricine	−10.2 ± 0.8	−9.4 ± 0.5	2.7 ± 3.2	
E3/phosphate	−9.7 ± 0.6	−7.3 ± 0.4	8.8 ± 2.4	−350 ± 58
E3/tricine	−9.4 ± 0.5	−7.8 ± 0.3	5.4 ± 1.9	

The relative contribution of the polar groups can be estimated by considering protein unfolding data. In proteins, the residual $\Delta S°$ of unfolding at 112°C averages 4.3 cal K^{-1} (mol-res)$^{-1}$.[30,37,38] This value is very close to that expected for the configurational entropy change alone. For example, the configurational entropy change per residue for the polypeptide backbone without sidechains is estimated as approximately 5 cal K^{-1} mol^{-1}.[39] The presence of a side chain restricts the flexibility of the peptide backbone, decreasing its entropy by 2.4–4.8 cal K^{-1} mol^{-1}.[40] Thus for a protein with no disulfide links or other covalent restrictions, the backbone configurational entropy is expected to be on the order of 1.5–2.5 cal K^{-1} (mol-res)$^{-1}$. The ΔS for unfolding a sidechain that is buried in the interior of a protein has an average value estimated between 1.7 and 3.8 cal K^{-1} mol^{-1} [41–44] (B.W. Matthews, personal communication). Finally, the unfolding of an exposed side chain contributes in the neighborhood of 0.5–0.6 cal K^{-1} mol^{-1} [41] (B.W. Matthews, personal communication). These values lead to overall configurational entropy changes near the value of 4.3 cal K^{-1} (mol-res)$^{-1}$ observed at 112°C indicating that the polar contribution to the $\Delta S°$ at this temperature should be small.

In contrast, some authors have proposed a relatively large polar contribution to $\Delta S°_{solv}$ [45,46] based on the observation that the entropy of aqueous dissolution of gaseous polar compounds is more negative than that observed for the corresponding alkanes. If this contribution is used in the analysis of experimental data, configurational entropy values per residue between 9.3[45] and 19[46] cal K^{-1} mol^{-1} are obtained. These values are considerably larger than the independent estimates described above. However, nearly the same entropic difference between polar compounds and alkanes is observed in the condensation of these compounds as in the dissolution so that it appears that the difference may be largely attributable to internal degrees of freedom rather than hydration.[47]

Given that the assumption of a significant polar solvation entropy results in an overestimation of the configurational entropy in proteins relative to the theoretically expected value, and that a significant polar solvation entropy does not appear to be necessary to understand the model compound data, the $\Delta S°$ at 112°C can be considered as lacking significant solvation contributions and ΔS_{solv} can then be calculated according to

$$\Delta S°_{solv} = \Delta C_p \ln(T/T_S*) \quad (5)$$

where ΔC_p now includes both the apolar and polar contributions as opposed to Eq. (4).

At 25°C, the estimated solvent contibution to $\Delta S°$ is 42 ± 10 cal K^{-1} mol^{-1} for E8 and 90 ± 15 cal K^{-1} mol^{-1} for E3. The difference between the overall $\Delta S°$ and ΔS_{solv} is the sum of the cratic and configurational contributions and is −34 ± 10 cal K^{-1} mol^{-1} for E8 and −77 ± 15 cal K^{-1} mol^{-1} for E3. The cost in terms of configurational entropy for binding cyt *c* to E3 is thus considerably greater than for E8, even though the overall $\Delta S°$ values at 25°C are rather similar.

These results also indicate that the overall small, favorable $\Delta S°$ of binding arises from the compensating contributions of solvent release, cratic, and configurational entropy. The gain in entropy from solvent release seems to be a common feature of biological systems by which they overcome the high free energy cost of inducing order. It is also seen in the interaction of an mAb with angiotensin II, a flexible peptide hormone,[21] as well as in the folding of globular proteins. It must be noted, however, that not all binding processes of this type need be accompanied by an overall positive entropy change at 25°C. Since the overall value results from different contributions, its value will depend on the relative magnitude of each contribution. For example, if the binding involves the apposition of predominantly polar surfaces, the dominant term will be the configurational entropy resulting in an overall negative entropy of binding.

Estimation of the Antigen and Antibody Surfaces Buried by Complex Formation

We have previously shown that structural data, in conjunction with semiempirically derived fundamental thermodynamic parameters, can be used to make accurate estimates of the thermodynamics of protein folding/unfolding transitions,[48,49] and peptide–protein interactions.[21] The same fundamental thermodynamic parameters can be used to provide estimates of structural features from thermodynamic data as has recently been shown for the compact denatured state of the protein III^{Glc}.[50]

In the absence of protonation effects, the $\Delta H°$ of binding has contributions from the burial of apolar surface (i.e., hydrophobic and van der Waals interactions) and polar surface (i.e., hydrogen bonds and electrostatic interactions), and can be expressed as

$$\Delta H^\circ = \Delta H^* + \Delta C_p\,(T - T_H^*) \quad (6)$$

where T_H^* is the temperature where the burial of apolar surface makes no contribution to ΔH° and ΔH^* is the polar contribution to ΔH° at the temperature T_H^*. From the analysis of model compound and protein unfolding data, the value of T_H^* has been estimated to be 100°C.[51] At this temperature it has been shown that the unfolding ΔH° of proteins is proportional to the buried polar surface area and is given as[48,49]

$$\Delta H^* = 35\ \Delta A_p \quad (7)$$

where ΔA_p is the change in the polar accessible surface area and is negative for a binding reaction.

The change in heat capacity is also related to changes in buried surface area.[28,29,48,49,51,52] Considering changes in both apolar and polar surface, ΔC_p is given as[48,49]

$$\Delta C_p = 0.45\ \Delta A_{ap} - 0.26\ \Delta A_p \quad (8)$$

where ΔA_{ap} is the change in the apolar accessible surface area. We have shown that Eq. (6)–(8), in conjunction with changes in polar and apolar surface areas determined from crystallographic structures,[53] can be used to predict ΔH° and ΔC_p for protein unfolding within 9 and 12%, respectively, of the experimentally determined values.[48,49]

Given that Eq. (6)–(8) can be used to estimate the thermodynamic quantities of ΔH° and ΔC_p if structural data are available, the reverse should also be possible. That is, given accurate thermodynamic data it should be possible to estimate values for ΔA_{ap} and ΔA_p from Eq. (6)–(8). Using the experimentally determined values of ΔH and ΔC_p from Table II, we calculate that the binding of E8 to cyt *c* buries 625 Å^2 of polar surface and 730 Å^2 of apolar surface for a total of 1350 Å^2 of surface area buried. For E3, the calculated values are 960 Å^2 of polar surface area buried and 1330 Å^2 of apolar surface area buried for a total of 2300 Å^2 of surface area buried.

DISCUSSION

The Estimated Antigen and Antibody Surfaces Buried by Complex Formation Agree With Independent Estimates

In known crystal structures of antibodies complexed with protein antigens, the antigen and antibody each contributes about half of the total buried area.[54] Thus, as a first approximation, the empirical calculations above would predict that E8 buries 675 Å^2 of cyt *c* surface while E3 buries 1150 Å^2. These values compare quite favorably with the values estimated from the H–D exchange and epitope mapping studies of 750 Å^2 for E8[8] and 1200 Å^2 for E3.[9] While these results on a single system must be viewed with caution, they suggest that estimates of structural changes might be possible for accurate thermodynamic data.

Comparison of H–D Exchange Kinetics and Thermodynamics of E3 and E8–Cyt *c* Interactions

As noted above, the combination of calorimetric measurements and structural energetic calculations indicates a significant difference in the configurational entropy associated with the binding of cyt *c* to the two different antibodies, but they do not indicate whether this difference arises from restrictions in the antibodies or the antigen. Some insight into this question can be gained from structural studies previously performed on these systems using H–D exchange detected by 2D-NMR.[8,9]

The H–D exchange experiments performed on E8[8] and E3[9] indicated a much larger area of protection for E3 than for E8. Amide protons in an antibody–antigen complex were considered to be significantly protected if the ratio of the H–D exchange rate for the free antigen to that for the bound antigen is ≥ 3. For E8 the amide protons of 12 residues are significantly protected, all of which are located in a contiguous surface on cyt *c* of about 750 Å^2 and appear to comprise the epitope recognized on cyt *c* by the antibody.[8] In the case of E3, a similar number of residues, 14, have slower exchange kinetics in the complex; however these residues are distributed over about half the total surface area of the antigen.[9] By comparing the H–D exchange data with previous epitope mapping studies by Paterson and co-workers[13,16–18] it was concluded that the epitope on cyt *c* bound by E3 is about 1200 Å^2,[9] still somewhat larger than the crystallographically measured epitopes for protein antigens of similar size.[54] The remainder of the residues influenced by E3 binding appear to be slowed by indirect cooperative effects.

The differences in the H–D kinetics for the two antibody cyt *c* complexes could be the result of the relative mobility of the two surfaces on cyt *c* engaged by the antibodies. The E8 epitope is centered on two relatively rigid α-helical regions. For E3, however, the central part of the epitope is the 38–59 Ω loop which has high segmental flexibility.[19] When this region of cyt *c* is immobilized by antibody binding, it may stabilize a network of hydrogen bonds which cannect it to the rest of the molecule. Thermodynamically, the overall stability of the cyt *c* molecule must be increased upon binding of the antibody. It is interesting to note that this stabilization appears to be cooperative, since no amide protons in the molecule have the significantly faster exchange kinetics which would result if hydrogen bonds were broken upon antibody binding. This type of cooperative stabilization might not be expected if the binding required an induced fit mechanism. In such a case it is inconceivable that some regions of the cyt

c might be locally destabilized so long as the overall complex is thermodynamically stable.

The above interpretation of the H–D exchange experiments[8,9] performed on E3 and E8 cyt *c* complexes would predict a larger configurational entropy change in the antigen for the binding of E3 than for E8. This is precisely what is indicated by the results presented in this paper. Overall, the combination of the energetic results presented here with the structural information obtained from the H–D exchange experiments suggests that the difference in configurational entropy is largely due to differences in the antigen rather than the antibodies. It is worth reemphasizing that this difference is not observed in the overall $\Delta S°$ at 25°C because the additional loss of configurational entropy is compensated by additional gain of solvent entropy.

CONCLUSIONS

The binding of the two mAbs, E3 and E8, to cyt *c* has been found to be favored both enthalpically and entropically. In both cases the predominant contribution to the favorable $\Delta G°$ is enthalpic. Both binding events are accompanied by significant ΔC_p values which indicates significant burial of apolar surface. At pH 7.2 the binding is free of protonation events (and thus of pH) as indicated by the observation that ΔH_{bind} is independent of the buffer.

Although the overall entropic changes for E3 and E8 binding to horse cyt *c* are quite similar at 25°C ($\Delta S°_{total}$/cal K^{-1} mol^{-1} for E8 $\cong$ 4 and for E3 $\cong$ 9), the solvation and configurational components differ significantly for the two antibodies as indicated by the measured ΔC_p values. Thus for E3 there is a greater entropic gain from loss of solvent on complex formation (ΔS_{solv}/cal K^{-1} mol^{-1} = 90) compared to E8 (ΔS_{solv}/cal K^{-1} mol^{-1} = 42) but this is offset by the loss in configurational entropy (ΔS_{conf}/cal K^{-1} mol^{-1} = −77) compared to E8 (ΔS_{conf}/cal K^{-1} mol^{-1} = −34). These examples illustrate what appears to be a general entropy compensation for biological assembly processes in which the cost of restricting conformational degrees of freedom is largely paid for by solvant release. Previous results of H–D exchange experiments on these two systems is consistent with the configurational entropy difference primarily resulting from restriction of the antigen.

Thus, the thermodynamic measurements on the interactions of E3 and E8 with horse cyt *c* support our interpretation of the H–D exchange data from these two antibody–antigen complexes and together with those findings suggest that varying conformational changes may take place in the antigen on antibody binding and that the degree of conformational change may be related to the relative flexibility of the surface of the antigen to which the antibody is directed.

We have also estimated the total buried surfaces on the interacting antibodies and antigen which would account for the energetics that we have measured for E3 and E8 on complex formation. The values obtained for the antibody binding site on cyt *c* for E8 (675 Å^2) and for E3 (1150 Å^2) are consistent with the size of the epitopes recognized by these antibodies determined by prior epitope mapping studies[13] and NMR studies,[8,9] but further examples are necessary to generalize this approach.

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