Isothermal Titration Calorimetric Study of the Association of Hen Egg Lysozyme and the Anti-Lysozyme Antibody HyHEL-5†

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Received October 15, 1993; Revised Manuscript Received January 18, 1994®

ABSTRACT: The thermodynamics of association of hen egg lysozyme and the antibody HyHEL-5 was characterized by isothermal titration calorimetry. The structure of this complex has been determined to 2.8-Å resolution by Sheriff et al. [Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C., & Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84,* 8075–8079]. The calorimetric enthalpy of association is negative and declines linearly with temperature in the range 10–37 °C (ΔC_p = −340 ± 40 cal mol^{-1} K^{-1}). Entropic contributions calculated using previously determined values of the affinity of association are negative (unfavorable) in this temperature range. This result is consistent with the loss of mobility upon association of the unusually mobile segments of HEL which form the HyHEL-5 epitope. As the affinity of association is approximately constant in this temperature range, an enthalpy–entropy compensation effect is implied. The hydrophobic and vibrational contributions to ΔS and ΔC_p are estimated using the method of Sturtevant [Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2236–2240]. The experimental value of ΔC_p is in rather close agreement with the ΔC_p estimated from the polar and nonpolar surface areas buried upon association.

Molecular recognition by proteins of the immunoglobulin family is at the heart of the immune system, and serves as the basis of therapeutic agents (Pastan & FitzGerald, 1991; Kim et al., 1993), purification methods (Bailon & Roy, 1990), and immunoassays. Immune recognition has long been an important focus of studies of molecular recognition and association. Recent crystallographic studies of antibody/protein antigen complexes have opened the way to the application of molecular simulation and directed mutagenesis to the problem (Sheriff et al., 1987; Padlan et al., 1989; Bentley et al., 1990; Bhat et al., 1990; Fischmann et al., 1991; Tulip et al., 1992a,b; Chitarra et al., 1993; Prasad et al., 1993). As structures have become available, biophysical methods for characterization of intermolecular association have also advanced, particularly direct measurement of thermodynamic parameters by calorimetry. To date, however, only a few studies have combined thermodynamic characterization with structural perturbations by mutagenesis or ligand substitution (Kelley et al., 1992, 1993; Brummell et al., 1993).

Much of the recent work in structural immunochemistry has involved hen egg lysozyme (HEL).[1] HEL was the first enzyme to be subjected to X-ray crystallography (Blake et al., 1965), and further work has produced high-resolution structures of alternate crystalline forms of HEL (Kundrot & Richards, 1987; Ramanadham et al., 1990). A variety of avian lysozymes have been characterized and used in mapping the antigenic surface of HEL (Jólles & Jólles, 1984; Smith-Gill et al., 1982; Darsley & Rees, 1985). Many of the known antibody/protein complex structures involve anti-lysozyme Fab fragments in complex with an avian lysozyme (Sheriff et al., 1987; Padlan et al., 1989; Bhat et al., 1990; Fischmann et al., 1991). The availability of these structures has stimulated considerable experimental and theoretical interest in these Fab/lysozyme complexes.

We have focused our attention on the complex of the murine IgG1κ antibody HyHEL-5 with hen egg lysozyme. The HyHEL-5 epitope on HEL was originally mapped using avian species variants by Smith-Gill et al. (1982, 1984a,b). Their identification of the HyHEL-5 epitope region was confirmed when the structure of the complex was determined to 2.8-Å resolution by Sheriff et al. (1989). The HyHEL-5/HEL complex was used to test an empirical method of predicting the Gibbs free energy of association of complexes of known structure by Novotny et al. (1989). While the method accurately predicted the affinities of two other antibody/antigen complexes, it overestimated HyHEL-5/HEL affinity, possibly due to the difficulty of accurately treating the electrostatic interactions which play an unusually large role in HyHEL-5/HEL association. The relative contributions of Arg 45 and Arg 68, however, were more accurately predicted. Brownian dynamics simulations of HyHEL-5/HEL association by Kozack and Subramanian (1993) showed that the charged residues closest to the binding site exert the greatest influence in steering the antigen into positions and orientations favorable for association. Pellegrini and Doniach (1993) tested a novel molecular docking algorithm using the HyHEL-5/HEL complex; the native orientation was found to be the state of lowest energy. Two-dimensional ^{1}H NMR amide-exchange measurements by Benjamin et al. (1992) showed that complex formation changes the exchange behavior of the antigen at sites far removed from the epitope, probably due to dynamic effects. Affinities of HyHEL-5 association with avian variants and with directed mutant forms of HEL

† This work was supported by NIH Grant GM44344 and by the Office of Naval Research under the Accelerated Research Initiative in Molecular Recognition.

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® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: HEL, hen egg lysozyme; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fab, antigen-binding fragment of antibody; PCR, polymerase chain reaction.

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have been reported (Smith-Gill et al., 1982; Kirsch et al., 1989; Lavoie et al.,1989; Malcolm et al., 1989), and structure determinations of HyHEL-5 complexes with other lysozyme forms are in progress. In addition, the cDNA encoding the HyHEL-5 variable regions has been isolated by PCR amplification, and experiments to express the antibody as chimeric Fab fragments in *Escherichia coli* are in progress (D. Newman, J. A. Wibbenmeyer, K. I. Maillard, U. Prasad, P. Patel, S. J. Smith-Gill, and R. C. Willson, unpublished results). This expression system will facilitate studies of HyHEL-5/HEL association by mutagenesis of antibody contact residues.

In this work we report measurement by isothermal titration calorimetry of the enthalpy of association of HyHEL-5 with HEL under a variety of conditions, allowing further characterization of the thermodynamic forces driving association. We have found that ΔH is relatively large and negative and decreases linearly over the temperature range from 10 to 37 °C, giving a negative calculated value of ΔC_p. ΔH was found to be independent of pH and buffer enthalpy of ionization, implying the absence of proton liberation or uptake upon association. Values of ΔS calculated using literature values for the affinity of association are negative over the entire temperature range. This unfavorable entropic contribution may arise in part from the loss of vibrational degrees of freedom of the HEL domains comprising the HyHEL-5 epitope, which are known to be unusually mobile in the uncomplexed molecule.

MATERIALS AND METHODS

Cell Culture. The hybridoma cell line producing the HyHEL-5 antibody (Smith-Gill et al., 1982) was provided by Dr. S. Smith-Gill and cultivated in a hollow fiber reactor at the National Cell Culture Center (Minneapolis, MN). Culture fluid was centrifuged to remove cells and shipped on dry ice. Samples were stored at −80 °C until antibody purification.

Protein Purification. HyHEL-5 was purified by sequential anion-exchange, size-exclusion, and hydroxylapatite chromatography. Anion-exchange chromatography used a 2.5-cm-diameter by 25-cm column of Q Sepharose Fast Flow (Pharmacia). The column was equilibrated with 50 mM Tris and 0.1 mM EDTA, pH 8.0 (buffer A). After being loaded, the column was washed with buffer A and then eluted with a gradient of NaCl in buffer A from 0 to 150 mM over 10 column volumes. Peaks containing antibody were identified by silver-stained SDS–PAGE on 8–25% polyacrylamide gradient gels (PhastSystem, Pharmacia), pooled, and concentrated to a final volume of *ca.* 25 mL using a stirred ultrafiltration cell (Spectrum, Los Angeles, CA).

The concentrated ion-exchange pool was further purified by size-exclusion chromatography on a 2.5-cm-diameter by 65-cm column of Sephacryl S-300 HR (Pharmacia). Loading volume was *ca.* 5 mL. Elution was with 10 mM sodium phosphate, pH 6.8, to exchange proteins into a buffer suitable for the hydroxylapatite chromatography which followed.

Final purification was achieved with a column of hydroxylapatite (Bio-Gel HT, BioRad, 2.5 cm diameter by 60 cm). HyHEL-5 antibody was eluted using a gradient of sodium phosphate at pH 6.8 from 10 to 300 mM over 5 column volumes. Fractions containing HyHEL-5 were pooled and concentrated to greater than 1 mg/mL using a stirred ultrafiltration cell (Spectrum). Upon further concentration and analysis by silver-stained SDS–PAGE, the final HyHEL-5 antibody preparation normally showed only bands corresponding to the antibody heavy and light chains. BSA, the primary contaminant remaining after the second chromatographic step, was sometimes present at levels estimated to be less than 3%. The binding activity of the final HyHEL-5 preparation was assayed by dot-blot immunoassays based on the binding of HyHEL-5 to HEL adsorbed on nitrocellulose membranes (Pierce). Membranes were blocked with 3% nonfat dry milk before HyHEL-5 binding, and bound antibody was detected with protein G–alkaline phosphatase conjugate (Pierce) using the BCIP/NBT (Pierce) chromogenic substrate system as described by the manufacturer.

Hen egg lysozyme (2× crystallized) was obtained from Worthington (Freehold, NJ). Size-exclusion chromatography and silver-stained SDS–PAGE were used to establish that the HEL used (lot no. 32C875) was at least 99% pure and free of aggregates. The HEL activity was also tested using the *Micrococcus lysodeikticus* lysis assay of Shugar (1952). HEL was used as received for calorimetric experiments, except as noted below.

Sample Preparation. Experiments were carried out (except where noted) in 10 mM sodium phosphate adjusted to pH 8.0 at the intended experimental temperature in an environmental room (NorLake Scientific). HyHEL-5 and HEL samples of *ca.* 5 mL each were codialyzed overnight at 4 °C against the same 4-L volume of buffer to ensure precise matching of buffer concentration and pH. After dialysis, the concentrations of HEL and HyHEL-5 solutions were determined by A_{280} measurements using a Beckman DU-64 spectrophotometer. The extinction coefficient used for HEL was $E_{281.5} = 2.64$ (Aune & Tanford, 1969), and the molecular weight used for HEL was 14 388 (Jólles, 1969). The molecular weight of HyHEL-5 was taken as 150 000, and its extinction coefficient was estimated as $E_{280} = 1.49$ (K. A. Xavier, unpublished results) by the method of Gill and von Hippel (1989) using the known HyHEL-5 Fv sequence and the constant-region sequences of the murine (Balb/c) plasmacytoma MOPC-21 (Kabat, 1991). The HEL concentration was adjusted by the addition of dialysis buffer to a concentration which (after centrifugation as described below) would saturate all of the antibody binding sites near the midpoint of the calorimetric titration. Spectra of the dialyzed material sometimes displayed significant absorbance at wavelengths greater than 350 nm which was interpreted as scattering due to aggregation. All samples were therefore centrifuged at 300000*g* in a Beckman TL-100 ultracentrifuge for 30 min immediately before use. After centrifugation the final concentrations of antibody and HEL samples were determined spectrophotometrically. The A_{280} of protein samples was reduced up to 25% after centrifugation, and the samples were free of any detectable absorbance at wavelengths above 350 nm.

Isothermal Titration Calorimetry. An OMEGA isothermal titration calorimeter (Microcal, Northampton, MA) interfaced with a 386/25 personal computer was used for all experiments. The design and operation of the instrument have been previously described by Wiseman et al. (1989). A voltage conditioner (Tripp Lite) and a ferroresonant transformer (General Signal) were connected in series for power stabilization, and a circulating water bath (Haake Model A81) was used to help stabilize the experimental temperature. The calorimeter was calibrated with electrically generated heat pulses as suggested by the manufacturer. The instrument was equilibrated overnight with the circulator set *ca.* 5 °C below the desired experimental temperature. The sample cell was cleaned prior to use with 500 mL of 2% Tween-20 (Bio-Rad) in deionized water and then rinsed with 2 L of deionized water. The sample cell, filling syringe, and injection syringe were each rinsed three times with deionized water and with

dialysis buffer before being filled with protein solution. The reference cell was filled with 0.02% sodium azide, the 1.275-mL sample cell with HyHEL-5 solution (typically 1.0 mg/mL), and the 100 μL (nominal volume) syringe with HEL solution (typically 4 mg/mL). We titrated the antibody with antigen rather than the reverse, because the concentration of HEL was known more accurately than that of HyHEL-5. For operation at subambient temperatures, a partial vacuum (residual pressure *ca.* 100 Torr) was applied to the compartment around the sample cell to eliminate the possibility of condensation. During equilibration the stirrer was set to rotate at 400 rpm. After equilibration to baseline stability (rms noise < 15 ncal/s) a 1-μL preinjection (accounted for in the data analysis) was made to correct for any exchange of the syringe and cell contents during equilibration and to ensure that the first experimental injection made was 10 μL. Omission of the preinjection resulted in the heat effect for the first experimental injection being observably smaller than those for later injections. Experiments involved 11 injections of 10 μL each. Injections were separated by 4 min, and each occurred over 15 s. More rapid injections, or injections of larger volume, could produce artifacts due to incomplete thermal equilibration of the entering titrant. Analysis of the cell contents by silver-stained native PAGE (8–25% acrylamide gradient gel) after titration confirmed the presence of antibody/antigen complex and excess HEL and the absence of free antibody.

Data Analysis. Origin, the data analysis software provided with the calorimeter, was used to analyze all results. Manual peak-by-peak integration was found to yield better representations of the data than did the automatic baseline determination provided by the software. Integrated areas for injections prior to saturation typically varied by <4% and were averaged to obtain the apparent binding enthalpy. To obtain the reported enthalpies, the apparent binding enthalpy values were corrected for the HEL enthalpy of dilution, determined by averaging the integrated areas obtained for injections after saturation.

RESULTS

Figure 1 shows data obtained from a typical calorimetric titration experiment. Panel A shows the raw titration data, and panel B shows the integrated areas for each injection, normalized by the moles of lysozyme injected. The high affinity of HyHEL-5/HEL association precludes the determination of affinity by calorimetric titration as described by Wiseman et al. (1989). Experiments were designed, therefore, to facilitate accurate measurement of the enthalpy and stoichiometry of HyHEL-5/HEL binding and the apparent HEL heat of dilution. The heat effects observed during the first five injections represent the sum of the heat of HEL dilution (see below) and the heat of complex formation for all HEL injected. The HEL injected during the sixth injection exceeds the number of free antibody combining sites remaining in the sample cell. The observed signal arises from the heat of HEL dilution and from HyHEL-5 binding to a fraction of the HEL injected. The heat released during the last five injections represents the HEL heat of dilution into a solution of HyHEL-5/HEL complex.

It was necessary to correct the apparent heat of HyHEL-5/HEL association calculated from the heat effects observed during the early injections by the heat of HEL dilution calculated from the results of injections after antibody saturation. The absolute value of this correction (which could be positive or negative) was typically less than 1.5 kcal mol^{-1},

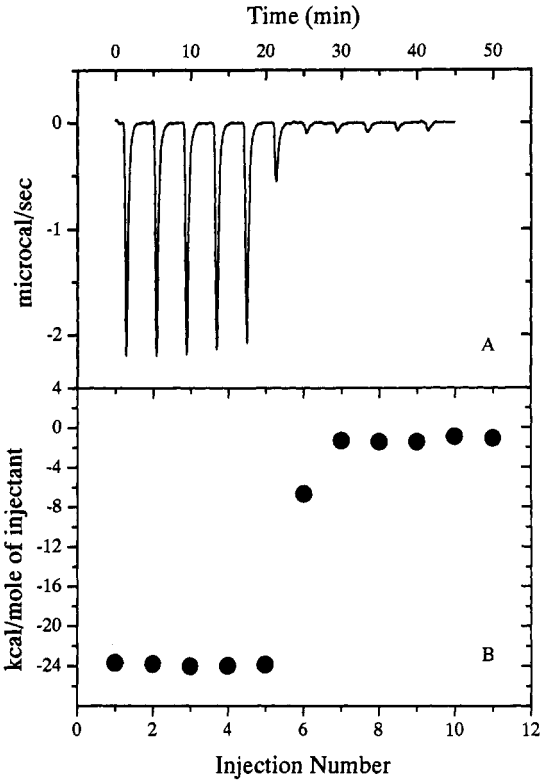

FIGURE 1: Typical isothermal titration calorimetric profiles of HyHEL-5/HEL association. (A) Incremental heat liberation upon titration of HEL into HyHEL-5 at 25.2 °C. Eleven 10-μL injections of HEL (0.279 mM in 10 mM sodium phosphate, pH 8.0) were made into 6.0 μM HyHEL-5 codialyzed into the same buffer. Injections occurred over 15 s at 4.0-min intervals. (B) Integrated areas for the above peaks plotted against the injection number. Analysis of this data set gave corrected $\Delta H = -22.63$ kcal mol^{-1}.

or <10% of the value of ΔH. The heat of dilution was found to be a strong function of HEL concentration, in agreement with the results obtained by Banerjee et al. (1975) under slightly different conditions (30 °C; 10 mM sodium phosphate, pH 7; 0.1 M ionic strength by the addition of NaCl). These and other workers have found the self-association of HEL to be best characterized by a value of K_a in the range 200–700 M^{-1} depending upon the self-association model used (Sophianopoulos & Van Holde, 1964; Bruzzesi et al., 1965; Deonier & Williams, 1970). If the affinity of association is similar under our experimental conditions, these results imply that only a small fraction of HEL injected in our experiments was self-associated. The HEL heat of dilution into the HEL-saturated HyHEL-5 solution in the later injections was found to differ by ±1.5 kcal mol^{-1} from the heat of HEL dilution into protein-free buffer measured under the same conditions, possibly due to nonspecific interactions between HEL and the HyHEL-5/HEL complex and/or heat of complex dilution. The former values were used for correction of the apparent enthalpies of association. Corrected heats of association are shown in Table 1, along with the number of replicate experiments and the standard deviations associated with the stated values of ΔH.

Table 1 also presents the experimental equivalence ratio determined by dividing the number of moles of lysozyme present at the estimated point of stoichiometric equivalence by the calculated number of moles of antibody combining sites present. Experimental equivalence ratios systematically lie *ca.* 15% below the theoretical value of unity. This deviation does not affect the accuracy of the given enthalpies of association, which are based on the injection of an accurately

Table 1: Titration Calorimetry Results

buffer	T (°C)	no. of expts	ΔH (kcal mol^{-1})	equivalence[a] ratio
10 mM sodium phosphate, pH 8.0	37.1	4	−28.2 ± 0.93	0.90 ± 0.04
10 mM sodium phosphate, pH 8.0	30.0	2	−25.5 ± 1.25	0.86 ± 0.03
10 mM sodium phosphate, pH 8.0	25.0	3	−22.6 ± 0.44	0.90 ± 0.15
10 mM sodium phosphate, pH 8.0	17.5	2	−21.7 ± 0.33	0.77 ± 0.06
10 mM sodium phosphate, pH 8.0	10.2	3	−18.7 ± 0.62	0.82 ± 0.03
10 mM Tris, pH 8.0	37.0	3	−27.2 ± 0.92	0.80 ± 0.08
10 mM Tris, pH 8.0	10.1	3	−19.1 ± 0.64	0.88 ± 0.12
10 mM sodium phosphate + 100 mM NaCl, pH 8.0	25.4	2	−22.6 ± 0.28	0.80 ± 0.02
10 mM sodium phosphate, pH 7.0	24.9	2	−23.4 ± 0.50	0.88 ± 0.04

[a] The equivalence ratio is determined by dividing the number of moles of lysozyme present at stoichiometric equivalence (judged by the abrupt reduction in heat liberation upon addition of HEL) by the number of moles of antibody combining sites present. The number of moles of lysozyme present was taken as the sum of the contributions of the 1-μL preinjection, the injections completed before stoichiometric equivalence and the number of moles of lysozyme bound during the injection in which saturation occurred. The latter was calculated as the number of moles injected, scaled by the ratio of the heat liberated during this injection (corrected for average heat of dilution) to the average heat liberated during previous injections (also corrected for heat of dilution).

known quantity of HEL into an excess of HyHEL-5. Possible sources of the deviation from unity are the presence of non-antibody proteins or nonbinding antibody molecules (inactive HyHEL-5 and bovine IgG) in the HyHEL-5 preparations and inaccuracy in the assumed molecular weight or calculated molar extinction coefficient of HyHEL-5. The level of non-immunoglobulin proteins is believed to be less than a few percent, as discussed above. The concentration of bovine immunoglobulins was reduced by the use of fetal bovine serum, which is low in immunoglobulins. The possibility of a systematic error in the value of the extinction coefficient used for HyHEL-5 is increased by the absence of direct information on the sequences of the constant domains of the antibody. In addition, the errors reported by Gill and von Hippel (1989) for calculation of molar absorbances by their method are commonly 5–10%. Identification of the abrupt reduction in heat liberation upon HEL injection (Figure 1, injection 6) as the point of complete occupation of antibody combining sites is supported by native PAGE analysis of the cell contents after titration experiments (results not shown). Independent fluorescence titration experiments (performed by K. A. Xavier) also reveal a break near the calculated point of stoichiometric equivalence (results not shown).

Table 1 lists values of HyHEL-5/HEL association enthalpies in 10 mM sodium phosphate, pH 8.0, at temperatures ranging from 10 to 37 °C. As a control for proton liberation or uptake upon binding, experiments were also performed in 10 mM Tris, pH 8.0, at the maximum (37 °C) and the minimum (10 °C) temperatures studied. The values of ΔH measured in Tris buffer (enthalpy of ionization, 11.51 kcal mol^{-1} at 25 °C; Morin & Freire, 1991) did not differ significantly from those measured in phosphate buffer (1.22 kcal mol^{-1} at 25 °C; Morin & Freire, 1991), showing that the contribution of buffer titration to the observed ΔH is negligible. As shown in Table 1, ΔH does not vary greatly upon changing the pH from 8.0 to 7.0 or upon addition of 100 mM NaCl. Finally, the possible influence of contaminating proteins was tested in a control experiment with a HyHEL-5 preparation containing 80 wt % BSA, the contaminant most abundant during intermediate stages of antibody purification. The enthalpy obtained in the presence of BSA agreed with the result for purified antibody within the stated precision (results not shown). We believe, therefore, that the calorimetric enthalpies were not significantly influenced by the presence of traces of BSA in the HyHEL-5 preparations.

The ΔH of association depends strongly on temperature (Figure 2), declining linearly from −18.7 kcal mol^{-1} at 10 °C to −28.2 kcal mol^{-1} at 37 °C. The slope of this line yields a value of ΔC_p of −340 ± 40 cal mol^{-1} K^{-1}. While the high

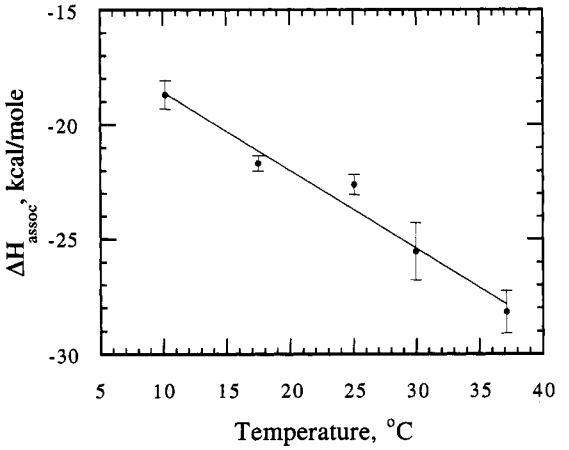

FIGURE 2: Enthalpy of HyHEL-5/HEL association as a function of temperature. All experiments were performed in 10 mM sodium phosphate adjusted to pH 8.0 at the experimental temperature. The solid line is a linear least-squares regression of the form ΔH(kcal mol^{-1}) = (−15.2 ± 0.9) − (0.34 ± 0.04)T(°C), r = 0.984. ΔC_p is calculated from the slope of the regression line.

Table 2: Thermodynamic Parameters for HyHEL-5/HEL Association

T (°C)	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
37.1	−14.0	−28.2	−45.7
30.0	−14.3	−25.5	−37.0
25.0	−14.5	−22.6	−27.2
17.5	−14.7	−21.7	−24.0
10.2	−14.8	−18.7	−13.7

affinity of HyHEL-5/HEL association is difficult to measure precisely, it is believed to vary by less than 1 order of magnitude from 4 × 10^{10} M^{-1} over the temperature range studied in this work (D. Newman and S. J. Smith-Gill, personal communication; Benjamin et al., 1992; Denton et al., 1991; Lavoie et al., 1989). The association constant at 25 °C (taken as 4 × 10^{10} M^{-1}) and the experimentally determined values of ΔH as a function of temperature (ΔC_p) were used to calculate values of K_a (and ΔG) over the temperature range of interest using the relation $\partial(\ln K_a)/\partial T = \Delta H/RT^2$ (the range of calculated values of K_a is smaller than the estimated uncertainty in the literature data). Values of ΔS as a function of temperature were calculated from the experimental values of ΔH and the calculated values of ΔG (Table 2). It is apparent that HyHEL-5/HEL association is accompanied by a favorable enthalpy change and an unfavorable entropy change at all temperatures examined. This conclusion would remain valid even in the face of an uncertainty of several orders of magnitude in the affinity of association.

Table 3: Hydrophobic and Vibrational Contributions to HyHEL-5/HEL Association[a]

T (°C)	ΔG_u[b]	ΔH	ΔS	ΔS_u[b]	$\Delta S_{u(v)}$	$\Delta S_{u(h)}$	$\Delta C_{p(v)}$	$\Delta C_{p(h)}$	$\Delta H_{(v)}$	$\Delta G_{u(v)}$
10.2	−16.5	−18.7	−13.7	−5.7	−82	76	−78	−262	−11.7	11.5
17.5	−16.7	−21.7	−24.0	−16.0	−84	68	−80	−260	−12.3	12.1
25.0	−16.9	−22.6	−27.2	−19.2	−86	67	−82	−258	−13.0	12.7
30.0	−17.0	−25.5	−37.0	−29.0	−88	59	−83	−257	−13.4	13.1
37.1	−17.1	−28.2	−45.7	−37.7	−90	52	−85	−255	−14.0	13.8

[a] Calculated using the method of Sturtevant (1977). Subscripts denote hydrophobic and vibrational components. ΔG and ΔH are in kcal mol^{-1}, and ΔC_p and ΔS are in cal mol^{-1} K^{-1}. [b] ΔS_u and ΔG_u are unitary free energy and entropy changes calculated assuming a standard state of unit mole fraction. ΔS_u was calculated by adding the cratic entropy change ΔS_c = +8 cal mol^{-1} K^{-1} (Kauzmann, 1959; Flogel & Biltonen, 1975; Kelley, 1992).

Table 4: Calorimetrically Derived Thermodynamic Data for Protein/Protein Associations at 25 °C

system	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔC_p (cal mol^{-1} K^{-1})	ref[a]
trypsin + soybean inhibitor	−12.3	8.6	69.8	−442	1
trypsin + modified soybean inhibitor[b]	−10.8	12.6	78.6	−387	1
trypsin + ovomucoid	−10.2	5.6	53.1	−270	1
trypsin + lima bean inhibitor	−12.7	2.1	49.7	−430	1
subtilisin inhibitor + α-chymotrypsin	−7.1	4.5	38.2	−260	2
subtilisin inhibitor + subtilisin	−13.8	−4.7	31.1	−240	3
calmodulinCa^{2+} + myosin light chain kinase	−11.5	−20.3	−29.2		4
calmodulinCa^{2+} + seminalplasmin	−12.0	−12.0	0		4
calmodulinCa^{2+} + melittin	−11.7	7.2	63.3		4
calmodulin + myosin light chain kinase	−7.2	0	24.1		4
calmodulin + seminalplasmin	−8.1	0	−27.2		4
calmodulin + melittin	−8.1	4.8	43.3		4
ch4D5 Fab + p185HER2-ECD	−13.5	−17.2	−12	−400	5
hu4D5-5 + p185HER2-ECD	−12.8	−10	9	−320	5
hu4D5-6 + p185HER2-ECD	−13.4	−11.4	7	−400	5
hu4D5-8 + p185HER2-ECD	−13.7	−12.9	3	−370	5
angiotensin II + Ab 131[c]	−11	−8.9	6.9	−240	6
HyHEL-5 + HEL	−14.5[d]	−22.6	−27.2[d]	−340	this work

[a] References are as follows: (1) Baugh and Trowbridge (1972); (2) Fukada et al. (1985); (3) Takahashi and Fukada (1985); (4) Milos et al. (1988); (5) Kelley et al. (1992); (6) Murphy et al. (1993). [b] Arg 64-Ile 65 peptide bond cleaved. [c] Temperature is 30 °C. [d] Values for ΔG and ΔS are approximate.

The empirical method of Sturtevant (1977) was used to calculate the hydrophobic and intramolecular vibrational contributions to the unitary entropy ($\Delta S_{u(h)}$ and $\Delta S_{u(v)}$) and the heat capacity change ($\Delta C_{p(h)}$ and $\Delta C_{p(v)}$) as well as the vibrational contributions to ΔH and ΔG. These results are given in Table 3. The method also predicts the loss of 46 mol of water per mole of HEL upon binding, from the calculated value of $\Delta C_{p(h)}$.

DISCUSSION

HyHEL-5/HEL association is enthalpically driven at all temperatures examined. ΔH declines linearly with temperature, yielding a value of ΔC_p of −340 cal mol^{-1} K^{-1}. Along with the relatively constant value of ΔG between 10 and 37 °C, these results imply that an enthalpy–entropy compensation occurs in this system. Table 4 shows calorimetrically determined thermodynamic parameters for protein/protein associations taken from the literature. These results show that the value of ΔC_p of HyHEL-5/HEL is comparable to the values seen in previously studied systems. Negative values of ΔC_p are usually interpreted as arising from hydrophobic interactions (Kauzmann, 1959; Brandts, 1964; Formisano et al., 1977; Waelbroeck et al., 1979; Sigurskjold & Bundle, 1992). Such interactions would be expected to occur due to the large surface area buried upon association (1800 Å^2). The changes in nonpolar and polar solvent-accessible areas for HyHEL-5/HEL association were calculated to be ΔA_{np} = −1158 Å^2 and ΔA_p = −634 Å^2 by Dr. J. Novotny (Bristol-Myers Squibb) (Novotny et al., 1989) using the method of Lee and Richards (1971) (probe radius, 1.4 Å) as implemented in the software package CONGEN (Bruccoleri & Karplus, 1987). The method of Murphy and Freire (1992) was used to estimate the value of ΔC_p from the buried polar and nonpolar surface areas as −356 cal mol^{-1} K^{-1}, in rather close agreement with the experimentally determined value of −340 cal mol^{-1} K^{-1}.

The negative value of ΔS at 25 °C is unusual among protein/protein systems, as illustrated by Table 4. For HyHEL-5/HEL association, ΔS is negative (unfavorable) at all temperatures studied. Because of the large negative values of ΔH, the extrapolated temperature at which ΔS becomes positive (for assumed constant ΔG and constant ΔC_p) is −2 °C. This temperature was also calculated using experimental data (assuming constant ΔG) for other systems for which sufficient data are available (Aune et al., 1971; Baugh & Trowbridge, 1972; Fukada et al., 1985; Takahashi & Fukada, 1985; Varadarajan et al., 1992; Jin et al., 1993; Murphy et al., 1993). The temperature at which ΔS becomes positive is below 0 °C only for the HyHEL-5/HEL system and the systems involving smaller molecules (Trp + Trp repressor, ribonuclease S protein + peptide). The Ab 131/angiotensin II complex gives a temperature of *ca.* 7 °C, and the other protein/protein systems give inversion temperatures above 25 °C.

The unusual thermodynamic characteristics of HyHEL-5/HEL association may arise in part from the importance of electrostatic interactions in this system. In the complex, Glu 50 of H2 forms salt bridges to both Arg 45 and Arg 68 of HEL, and Glu 35 of H1 is salt-linked to Arg 68. A 6500-fold excess of bobwhite quail lysozyme, in which Arg 68 is replaced by Lys, is required to competitively inhibit binding of HyHEL-5 to HEL by 50% (Smith-Gill et al., 1982). The same degree of inhibition is achieved using recombinant hen lysozymes with Arg 68 or Arg 45 mutated to Lys at 10 000 and 250 times the HEL concentration, respectively (Lavoie et al., 1989). Salt links normally make positive contributions

to ΔS and ΔC_p, but their influence on ΔH is small and can be either positive or negative (Eftink & Biltonen, 1980; Ross & Subramanian, 1981). Negative contributions to ΔS, ΔH, and ΔC_p can be made by hydrogen bonds and van der Waals contacts (Eftink & Biltonen, 1980; Ross & Subramanian, 1981; Connelly et al., 1993). The energetic contribution of hydrogen bonds is enhanced in an environment of low dielectric constant, such as the desolvated HyHEL-5/HEL interface (Ross & Subramanian, 1981). There are 10 hydrogen bonds and 74 van der Waals contacts formed upon HyHEL-5/HEL association (Sheriff et al., 1987). While we believe that these interactions make the main contributions to the favorable value of ΔH as well as contributing to the unfavorable ΔS and negative ΔC_p, the assignment of thermodynamic effects to particular intermolecular contacts is presently uncertain, as discussed by Connelly et al. (1993) and below.

Another potential source of unfavorable entropy changes upon association is constraint of vibrational motions. As noted by Sheriff et al. (1987), HEL residues 41–53 and 65–72, which comprise the majority of the HyHEL-5 epitope, are among the most highly mobile residues in uncomplexed HEL as judged from crystallographic mean square displacements (Artymiuk et al., 1979). Benjamin et al. (1992) found that complex formation results in perturbations in the hydrogen-exchange behavior of HEL residues far removed from the epitope, lending further support to the idea of dynamical changes in HEL upon association.

The empirical method of Sturtevant (1977) was applied to our data to estimate the hydrophobic and vibrational contributions to the enthalpy, entropy, and heat capacity of binding (Table 3). This method neglects the potential contributions of electrostatic interactions and hydrogen bonds and also makes the assumption that changes in entropy associated with conformational changes are equal to zero ($\Delta S_{u(conform)} = 0$). The calculated hydrophobic contribution to ΔC_p is large and negative. Although the mobility of the HEL epitope in the uncomplexed molecule suggests that vibrational constraints make an important contribution to the unfavorable ΔS, the predicted $\Delta S_{u(v)}$ for HyHEL-5/HEL association is not significantly larger than those found for other antibody/antigen complexes either in absolute terms or relative to $\Delta S_{u(h)}$. Sturtevant's method also predicts the loss of *ca.* 46 mol of water lost per mole of HEL upon association. This is of the same order as would be predicted from the buried surface area (Arakawa & Timasheff, 1982) and is subject to direct confirmation through osmotic stress experiments (Colombo et al., 1992; Rand et al., 1993).

Calorimetric data are available for antibody complexes with three types of antigens: oligosaccharides (Sigurskjold et al., 1991; Sigurskjold & Bundle, 1992; Brummell et al., 1993), human epidermal growth factor receptor 2 (Kelley et al., 1992, 1993), and the octapeptide hormone angiotensin II (Murphy et al., 1993). The enthalpic contribution to binding is favorable in each of these cases, but the entropic contributions are of either sign. Most of these associations show enthalpy–entropy compensation, with the enthalpy of association being favorable and the entropy being favorable or unfavorable depending on the temperature. Murphy et al. (1993) found that both enthalpy and entropy were favorable at 30 °C for Ab 131/angiotensin II association. In the study of the anti-p185^{HER2} antibody Fab fragment/antigen complex by Kelley et al. (1992), an unfavorable entropy was found for the complex formed with the chimeric Fab fragment, but favorable entropies were observed with all of the humanized Fab fragments, even those containing the same combining site residues as the original chimeric Fab. These results suggest that the interpretation and rational manipulation of the structural bases of antibody/antigen affinity will continue to pose important challenges.

We have presented isothermal titration calorimetric data for an antibody/protein (as opposed to peptide) system of known structure. Like other protein/protein complexes, the HyHEL-5/HEL association shows a favorable ΔH at 25 °C. An enthalpy–entropy compensation effect is also observed, although the precise characterization of this effect will require additional affinity data. The value of ΔC_p is comparable to that of other complexes. The thermodynamics of HyHEL-5/HEL association are distinguished by unfavorable values of ΔS at all temperatures studied. This effect may arise to some extent from loss of vibrational freedom in the highly mobile HEL epitope region. The determination of the structure of the complex by Sheriff et al. (1987) and the availability of heterologous expression systems for both HEL (Kumagi & Miura, 1989; Kirsch et al., 1989) and HyHEL-5 (D. Newman, J. A. Wibbenmeyer, K. I. Maillard, U. Prasad, P. Patel, S. J. Smith-Gill and R. C. Willson, unpublished results) will allow directed mutagenesis to be used to explore the structural basis of molecular recognition in this system.

NOTE ADDED IN PROOF

After the completion of this work, Tello et al. [Tello, D., Goldbaum, F. A., Mariuzza, R. A., Ysern, X., Schwarz, F. P., & Poljak, R. J. (1993) *Biochem. Soc. Trans. 21* (4), 943–946] presented thermodynamic data measured at 20–25 °C for other antibody/lysozyme complexes, including several of known structure. At this temperature, these complexes also involve dominant favorable enthalpic contributions to association.

ACKNOWLEDGMENT

We thank Drs. Don Marcus, Andrew McCammon, Sandra Smith-Gill, Steven Sheriff, James Snyder, and Shankar Subramanian for helpful discussions; Dr. Sandra Smith-Gill for providing the HyHEL-5 cell line; and Dr. Mark Hirschel for advice on cell culture. We thank Dr. John Brandts and the late Thomas Wiseman of Microcal, Inc., for advice on calorimetric experiments. We are grateful to Dr. Jiri Novotny for surface area calculations. We acknowledge the skilled technical assistance of Colin Johnston and Rene Ochoa.

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