Involvement of Water Molecules in the Association of Monoclonal Antibody HyHEL-5 with Bobwhite Quail Lysozyme

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ABSTRACT Fluorescence polarization spectroscopy and isothermal titration calorimetry were used to study the influence of osmolytes on the association of the anti-hen egg lysozyme (HEL) monoclonal antibody HyHEL-5 with bobwhite quail lysozyme (BWQL). BWQL is an avian species variant with an Arg→Lys mutation in the HyHEL-5 epitope, as well as three other mutations outside the HyHEL-5 structural epitope. This mutation decreases the equilibrium association constant of HyHEL-5 for BWQL by over 1000-fold as compared to HEL. The three-dimensional structure of this complex has been obtained recently. Fluorescein-labeled BWQL, obtained by labeling at pH 7.5 and purified by hydrophobic interaction chromatograpy, bound HyHEL-5 with an equilibrium association constant close to that determined for unlabeled BWQL by isothermal titration calorimetry. Fluorescence titration, stopped-flow kinetics, and isothermal titration calorimetry experiments using various concentrations of the osmolytes glycerol, ethylene glycol, and betaine to perturb binding gave a lower limit of the uptake of ~6–12 water molecules upon formation of the HyHEL-5/BWQL complex.

INTRODUCTION

The immune system frequently encounters proteins as antigens; hence a detailed analysis of antibody/protein antigen interaction is essential for understanding important aspects of the immune response. HyHEL-5 is a murine IgG_1 κ antibody whose epitope on hen egg lysozyme (HEL) was mapped using avian species variants by Smith-Gill et al. (1982, 1984a,b). Bobwhite quail lysozyme (BWQL) is an avian species variant with a HyHEL-5 association constant over three orders of magnitude lower than that of HEL; the only change in the HyHEL-5 structural epitope is an Arg68→Lys mutation (there are three other mutations that are buried and are not part of the HyHEL-5 structural epitope). In the HyHEL-5/HEL complex, HEL residues Arg45 and Arg68 form a ridge on the surface of HEL that interacts with a groove on the HyHEL-5 surface containing the acidic residues Glu-H35 and Glu-H50 (Sheriff et al., 1987). Arg68 forms a salt link and numerous hydrogen bonds with Glu-H35 and Glu-H50 (Cohen et al., 1996). In the HyHEL-5/R68K-HEL complex, no global structural changes were observed, but it was found that a new water molecule mediated the interactions between Lys68 and the Glu-H50 (Chacko et al., 1995). The HyHEL-5/BWQL complex showed hydrogen bonding in the antibody/antigen interface similar to that in the HyHEL-5/R68K-HEL complex including the additional bridging water molecule (Chacko et al., 1996). Association of HyHEL-5 with HEL or BWQL is enthalpically driven, with unfavorable entropic contributions in the temperature range 10–37°C (Hibbits et al., 1994; Shick et al., 1997).

The importance of water molecules in antibody/lysozyme and other association processes has been established by previous studies. Comparison of the three-dimensional structure of the antibody variable fragment (Fv) of D1.3, free and bound to HEL (Bhat et al., 1994), revealed that water molecules are involved in complex formation. In a recent analysis using more highly refined structures of HEL, 25 conserved waters were observed to be involved in the Fv D1.3/HEL interaction, either through direct hydrogen bonding to both proteins or through water bridges, contributing a net 10 hydrogen bonds to complex stability (Braden et al., 1995). Furthermore, the water molecules reduced the formation of cavities between the antibody and antigen surfaces. The three-dimensional structures of the Trp-L92→Asp (Ysern et al., 1994), Tyr-L50→Ser, and Tyr-H32→Ala (Fields et al., 1996) mutants of D1.3 in complex with HEL and the Arg68→Lys mutant of HEL in complex with HyHEL-5 (Chacko et al., 1995) reveal solvent rearrangements at the antibody/antigen interface. Based on the crystal structures of 4-4-20 antigen binding fragment (Fab) in complex with fluorescein in 2-methyl-2,4-pentanediol and in poly(ethylene glycol) (PEG), it was concluded that the lower affinity of 4-4-20 with fluorescein in 2-methyl-2,4-pentanediol was due to depletion of the hydration shell of the antigen-combining site (Herron et al., 1994).

Decreasing the water activity of the bulk solution by using osmolytes is a method for probing the role of waters of hydration in various phenomena involving proteins (Parsegian et al., 1986, 1995). The involvement of water molecules has been demonstrated for the oxygenation of hemoglobin (Colombo et al., 1992, 1994), the binding of glucose to hexokinase (Rand et al., 1993), the interaction of calf

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intestinal adenosine deaminase with substrates and competitive inhibitors (Dzingeleski and Wolfenden, 1993), internal electron transfer in cytochrome *c* oxidase (Kornblatt and Hui Bon Hoa, 1990), the association of cytochrome *c* with cytochrome b_5 and cytochrome *c* oxidase (Kornblatt et al., 1988, 1993; Rodgers et al., 1988), and the association of ferredoxin with ferredoxin:$NADP^+$ reductase (Jelesarov and Bosshard, 1994). Experiments using osmotic pressure and hydrostatic pressure indicated that site-specific recognition of DNA by the restriction enzymes *Eco*RI, *Bam*HI, and *Pvu*II is mediated by water (Robinson and Sligar, 1993, 1994, 1995).

The lower association constant of HyHEL-5 for BWQL compared to HEL and the crystallographic evidence of solvent rearrangement at the antibody/antigen interface on association makes the HyHEL-5/BWQL system ideal for investigating the role of water in macromolecular association. The involvement of water molecules in the HyHEL-5/BWQL interaction has been shown by titration experiments in the presence of glycerol, ethylene glycol, and betaine by both fluorescence polarization spectroscopy and isothermal titration calorimetry and by association constants derived from kinetic measurements.

MATERIALS AND METHODS

Glycerol, ethylene glycol, betaine, sucrose, PEG-300, and *N*-methylformamide were obtained from Fluka and were of the highest available purity. All other chemicals were of analytical grade. The osmotic pressures of osmolyte solutions were determined with a Wescor 5100B vapor pressure osmometer up to 4 MPa; these agreed with published values (Wolf et al., 1988) within experimental error (~3%). The osmotic pressure of 50% (w/w) glycerol solution was calculated as

$$\pi \Delta V_{water} = -RT \ln X_{water} \quad (1)$$

where ΔV_{water} is the partial molal volume of water (estimated using the density of 50% (w/w) glycerol solution) and X_{water} is the mole fraction of water (Atkins, 1994). The viscosities of osmolyte solutions were determined using a Brookfield digital viscometer (model DV-II+) and agreed with published values (Wolf et al., 1988) to within 5%.

Cell culture and antibody purification

The hybridoma cell line producing the HyHEL-5 antibody was cultivated in a hollow fiber reactor at the National Cell Culture Center (Minneapolis, MN). Supernatant was stored at −80°C until antibody purification. The protocol for HyHEL-5 purification was modified from that previously employed (Hibbits et al., 1994) and involved sequential anion exchange, hydrophobic interaction chromatography, and cation-exchange chromatography. The cell culture supernatant was loaded on a Q Sepharose Fast Flow (Pharmacia) anion-exchange column equilibrated with 10 mM sodium phosphate (pH 8.0), and the antibody was eluted by NaCl gradient at ~175 mM NaCl. Appropriate fractions were pooled and concentrated using a stirred ultrafiltration cell (Amicon 8200). The antibody was further purified by flow-through on a Phenyl Sepharose (Pharmacia) column equilibrated with 150 mM sodium phosphate and 1.0 M NaCl (pH 8.0). After dialysis into 10 mM sodium phosphate (pH 6.0), the pooled Phenyl Sepharose fractions were loaded on an SP Sepharose Fast Flow (Pharmacia) cation-exchange column. HyHEL-5 was collected in the flow-through and concentrated to ~2.0 mg/ml with a stirred ultrafiltration cell. Occasionally, a final Q Sepharose column was used (run using the same conditions as the first column). The final purity of HyHEL-5 was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using silver-stained 8–25% polyacrylamide gradient gels (PhastSystem, Pharmacia), and in some cases trace amounts of a 66-kDa protein (believed to be bovine serum albumin) were found. Hibbits et al. (1994) have shown that the presence of even much higher concentrations of bovine serum albumin does not alter the energetics of antibody/antigen association. HyHEL-5 was stored at 4°C in the presence of 0.05% sodium azide, in 100 mM sodium phosphate and 150 mM NaCl (pH 8.0).

BWQL purification

Bobwhite quail eggs were obtained from Stevenson Game Bird Farm (Riverside, TX). The egg white was separated from the yolk and stored at −80°C. BWQL was purified by the protocol of Arnheim et al. (1969) as modified by Shick et al. (1997).

Labeling and characterization of fluoresceinated BWQL

Fluorescein-5-isothiocyanate (Molecular Probes) was used at pH 7.5 to preferentially label the amino-terminus of BWQL, which is outside the HyHEL-5 structural epitope. Fluoresceinated BWQL purified using only size exclusion chromatography had a fluorescein/protein ratio greater than unity, and gave irreproducible results in association experiments. Fluoresceinated BWQL was therefore fractionated on a Phenyl Sepharose (Pharmacia) column after extensive dialysis. A decreasing NaCl gradient from 1000 mM to 0 mM in 100 mM sodium phosphate (pH 8.0) was used for elution. Fluoresceinated BWQL with a fluorescein/protein ratio of 1.0 ± 0.1 eluted toward the end of this gradient. Single photon-counting lifetime fluorescence spectroscopy (performed by D. O'Connor, Center for Fast Kinetics Research, University of Texas, Austin, TX) showed that the lifetimes of fluorescein in free BWQL (5.1 ns) and BWQL complexed with HyHEL-5 (5.3 ns) are similar, suggesting that the fluorescein label is covalently bound away from the antibody epitope. The results were best fit by a single exponential lifetime in each case, suggesting that the fluoresceinated sample is homogeneous. Furthermore, the close agreement between the affinity determined by fluorescence polarization spectroscopy (FPS) and by isothermal titration calorimetry (ITC) (discussed below) for HyHEL-5/BWQL association indicates that the association is not affected by fluoresceination of BWQL.

Sample preparation

Both calorimetry and FPS equilibrium experiments were performed in 100 mM sodium phosphate and 150 mM NaCl adjusted to pH 8.0. Stopped-flow measurements were made in 10 mM sodium phosphate adjusted to pH 8.0. Osmolyte solutions were prepared gravimetrically. In FPS equilibrium experiments, the solutions were made by adding previously prepared buffer to an appropriate quantity of osmolyte, after which the pH was adjusted to 8.0. Control FPS experiments using buffer solution diluted by 25% with no osmolyte and 25% (w/w) glycerol solution containing 100 mM sodium phosphate and 150 mM NaCl (pH 8.0) gave association constants that were within 5% of those reported in Table 1, indicating that buffer dilution did not affect the results. HyHEL-5 and BWQL samples were prepared by codialysis and ultracentrifugation as described previously (Hibbits et al., 1994). After ultracentrifugation the final concentrations of HyHEL-5 and BWQL were determined spectrophotometrically, using $E_{280} = 1.49$ (calculated using the constant-region sequences of the murine plasmacytoma MOPC-21 (Kabat et al., 1991) and the known HyHEL-5 Fv sequence by the method of Gill and von Hippel (1989)) and $E_{281.5} = 2.64$, respectively, for 1 mg/ml solutions (Hibbits et al., 1994). The molecular mass of HyHEL-5 was taken to be 150,000 Da (the mass determined in a single matrix-assisted laser desorption/ionization-TOF mass spectrometry run was within 3% of this; J. M. Peltier and K. Waddell, PerSeptive Biosystems, Framingham, MA), and that of BWQL was taken to be 14,263 Da

TABLE 1 Association constants for the HyHEL-5/BWQL system in the presence of various osmolytes determined by FPS

Condition*	$K_a \times 10^{-6}$ (M^{-1})#
No added osmolyte	5.9 ± 0.1
10% (w/w) glycerol	5.2 ± 0.1
25% (w/w) glycerol	4.0 ± 0.1
10% (w/w) ethylene glycol	4.3 ± 0.1
25% (w/w) ethylene glycol	3.4 ± 0.3
25% (w/w) betaine	4.3 ± 0.5

*In ~100 mM sodium phosphate and 150 mM NaCl; at 25 ± 0.2°C and pH 8.0 ± 0.1.
#Standard deviations calculated from triplicate data.

from its amino acid sequence (the mass determined by electrospray ionization mass spectrometry was 14,261, as previously described by Shick et al. (1997)).

Equilibrium measurements by fluorescence polarization spectroscopy

A SPEX Fluorolog 212 fluorometer (Instruments SA, Edison, NJ) with Glan-Thompson polarizers was used for polarization experiments. The polarizers were aligned and the G-factor of the instrument was determined as recommended by the manufacturer. A manual shutter positioned after the excitation monochromator was used to expose the sample to the excitation light only during the course of a measurement. Steady-state polarization measurements were done in the L-format, exciting at 490.0 nm with a 1.7-nm bandpass. To maximize sensitivity, a 520-nm cut-on filter (Omega Optical, Brattleboro, VT) was used on the emission side. In preliminary experiments fluorescence was observed to decrease during the course of an experiment (~2 h). This was ascribed to adsorption of dilute, cationic lysozyme on the quartz cuvette and was largely overcome by coating the cuvette with Gel Slick (AT Biochem) and by adding 30 μg ml^{-1} of the weakly fluorescent protein ubiquitin (Foote and Winter, 1992) to the solution. A typical experiment involved 2.5 ml of fluoresceinated BWQL (either 50.0 nM or 75.0 nM) in a quartz cuvette (Starna Cells, Atascadero, CA), to which concentrated HyHEL-5 solution was added in 20-μl aliquots. After each addition, the contents of the cuvette were allowed to equilibrate for 10 min (a time determined by control experiments to be sufficient for equilibration). In all titration experiments the cumulative titrating volume added was less than 6% of the total sample volume. To avoid excessive adsorption and photobleaching of the proteins and to limit the extent of antigen dilution, two or three separate titration experiments with antibody stock solutions of varying concentrations were performed for each titration isotherm. The protein solution was mixed with a microsubmersible magnetic stirrer, and its temperature was controlled with a circulating water bath (Haake model A82). At subambient temperatures, a dry nitrogen gas sweep was used to prevent condensation on the cuvette. A mock titration of buffer into a cuvette containing fluoresceinated BWQL showed that the anisotropy was stable to within ±0.002 for over 2 h.

When fluoresceinated BWQL binds to HyHEL-5 its anisotropy increases, and this change in anisotropy with association can be used to determine the equilibrium association constant (K_a). The anisotropy was calculated from the measured intensities I_{VH} and I_{VV} (where H and V refer to horizontal and vertical positions of the polarizer respectively), using the separately determined G-factor for the instrument (Lakowicz, 1983). The anisotropy was used to determine the fraction of the fluoresceinated BWQL bound to HyHEL-5 (F_b):

$$F_b = \frac{(A - A_f)}{(A_b - A_f)} \tag{2}$$

where A, A_b, and A_f are the anisotropy of the sample, bound antigen, and free antigen. A_b was determined by using a double-reciprocal plot of $1/(A - A_f)$ versus $1/P_o$, where P_o is the total antibody concentration. F_b is corrected for quenching using Q_m, the maximum quenching/enhancement factor found by using a double-reciprocal plot of $1/Q$ versus $1/P_o$, where Q is the normalized decrease in fluorescence with each addition of antibody. The corrected fraction of antigen bound f_b is given by

$$f_b = \frac{F_b}{1 + Q_m(1 - F_b)} \tag{3}$$

K_a was determined by nonlinear regression of total antibody concentration P_o versus the fraction of antigen bound f_b, using the equation

$$2P_o = \frac{f_b K_a^{-1}}{(1 - f_b)} + L_o f_b \tag{4}$$

where L_o is the total antigen concentration (Wei and Herron, 1993). Linear regression of the same data by Stinson-Holbrook analysis (Stinson and Holbrook, 1973; Gabler et al., 1992) gave K_a values typically within 10–15% of the values obtained by nonlinear regression. Standard errors reported are based on triplicate experiments, and any effect of photodestruction or adsorption is neglected, as the anisotropy was stable to within ±0.002 during the course of an experiment. Fluorescence polarization experiments involving osmolytes were done at 10% (w/w) and 25% (w/w) osmolyte concentrations only, because the increased viscosity of the solution precluded accurate determination of K_a at higher osmolyte concentrations.

Kinetic measurements

The kinetics of HyHEL-5/BWQL association was studied with an SFA-20 stopped-flow kinetics accessory (Hi-Tech, Salisbury, England) in conjunction with the Fluorolog 212 fluorometer. The protein samples were taken in two 1.0-ml sample syringes, and the concentration of antibody binding sites was usually 10 or 20 times the concentration of BWQL. It was verified that the antibody/antigen interaction followed pseudo-first-order kinetics (Xavier and Willson, unpublished results). The contents of the syringes were mixed rapidly with a pneumatic drive system at a mixing ratio of 1:1 (the dead time of the accessory is estimated by the manufacturer to be less than 20 ms). The computer controlling the fluorometer was used to acquire the kinetic data, using an external trigger interface (Instruments SA). The temperature of the protein solutions being mixed and of the cuvette were controlled with circulating water baths (Haake model A82). In kinetic experiments the temperature was controlled to within ±0.8°C. It was verified that the presence of slight antibody aggregates (less than 5%) did not affect the kinetics of association, presumably because of the large excess of antibody-binding sites. Therefore, antibody and antigen solutions were not ultracentrifuged before use in kinetic experiments. All experiments were done in the L-format, exciting at 490.0 nm with 3.4-nm bandpass. The kinetic traces of five different experiments for each polarizer position were averaged to reduce the noise associated with the measurement. Readings corresponding to the deadtime of the instrument were not deleted, as they constituted a small fraction (less than 1%) of the whole. The data were converted into a ASCII file and modified by using Lotus for analysis by KINFIT (OLIS, Bogart, GA). The data were fitted to the single-exponential equation,

$$A = (A_f - A_b) \times \exp(-k_{obs}t) + A_b \tag{5}$$

where k_{obs} is the first-order rate constant, using a successive integration algorithm as implemented in KINFIT. The goodness of fit was judged by using the residuals and the autocorrelation function. Typically, experiments with four different combinations of concentrations of the antibody and the antigen were studied (under pseudo-first-order conditions), and the pseudo-first-order rate constant (k_{obs}) was determined with KINFIT. The second-order rate constant for association was determined by dividing the k_{obs} by

the antibody concentration and averaging four second-order rate constants (k_{assn}).

The dissociation rate constant was determined by adding unlabeled BWQL to a solution of preformed 1:2 antibody:fluorescein-labeled BWQL complex. Triplicate experiments were done with 5- and 10-fold excesses of unlabeled BWQL. It was verified with 20-, 30-, and 60-fold excesses of unlabeled BWQL that the dissociation followed first-order kinetics (Xavier and Willson, unpublished results). The dissociation reaction was studied by using the stopped-flow accessory due to the relatively short lifetime of the HyHEL-5/BWQL complex. All experiments were done at least in triplicate, and the data were fit to Eq. 5.

Isothermal titration calorimetry

A MCS isothermal titration calorimeter (MicroCal, Northampton, MA) interfaced with a 486/66 personal computer was used for all experiments. A similar instrument has been described in detail 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 at 25°C (the water bath had a set point 4°C lower than the experimental temperature). The instrument was calibrated by using known test electrical heat pulses, as suggested by the manufacturer. The reference cell was filled with 0.02% sodium azide solution, the 1.375-ml sample cell with HyHEL-5 solution (typically ~1.5 mg/ml), and the 250-μl (nominal volume) syringe with BWQL solution (typically >2.5 mg/ml). Known volumes of antigen solution were titrated into the antibody solution, because the antigen concentration was known more accurately than the antibody concentration. The contents of the cell were stirred continuously at 400 rpm during the course of the titration. After equilibration to baseline stability (rms noise < 15 ncal s^{-1}), a 2-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 was exactly 10 μl. Experiments involved 18 injections of 10 μl each, separated by 4 min, with each occurring over 12.6 s. Origin, the data analysis software provided with the calorimeter, was used to analyze all results. Manual peak-by-peak integration was found to yield a better representation of the data than did the automatic baseline determination provided by the software. Heat of dilution in the presence of complex was calculated from the data after complete titration of the antibody combining sites by averaging the integrated areas and was used to correct the binding enthalpy. In this particular case, prior experiments have shown the lack of any significant proton linkage (Shick et al., 1997), and hence the apparent enthalpy of binding obtained is equal to the molar enthalpy of binding. To obtain the molar enthalpy of binding and the association constant for the antibody/antigen interaction, the data were fit to a model for a single set of identical sites (with association constant K_a, molar enthalpy of association ΔH, and the number of binding sites as variable parameters), using Marquardt methods as implemented in Origin. Although the calorimetrically determined equivalence ratio was ~20% below the theoretical value, the association constant determined by ITC is in very good agreement with that obtained by FPS (the fluorescence data were analyzed assuming the number of binding sites to be 2). The observed deviation could be due to impurities in protein preparation, the presence of nonbinding antibody, or inaccuracy in the calculated extinction coefficient of HyHEL-5 (Gill and von Hippel (1989) estimated the error in their method of calculating extinction coefficient at 5–10%). The equivalence ratio was worst (typically 25% below the theoretical value) at 50% (w/w) osmolyte concentration, possibly because of aggregation enhanced by dehydration. Isotherms obtained in the presence of osmolytes in some cases did not have as many leading points as a similar isotherm in the absence of the osmolyte. This could increase the uncertainty in the nonlinear fit for the isotherm; however, there is good agreement between K_a obtained by FPS and ITC for 25% (w/w) of glycerol and ethylene glycol, suggesting that the nonlinear fitting method is quite robust.

Analysis of binding experiments in the presence of osmolytes

Decreasing the water activity in the bulk solution by the addition of osmolytes perturbs the proteins' hydration shell(s). Solvent rearrangement creates an osmotic work contribution to the overall free energy of binding of two proteins (Rand et al., 1993):

$$\Delta G = -RT \ln K_a + RT \ln W = \text{constant} \qquad (6)$$

where W is the water activity. The osmotic work term is related to the osmotic pressure π and the volume of osmotically labile water ΔV_w by

$$RT \ln W = \pi \Delta V_w \qquad (7)$$

If the association constant for protein-protein association is determined in the absence (K_a^o) and in the presence (K_a^π) of osmolyte, it follows that

$$\Delta\pi \Delta V_w = RT \ln(K_a^\pi / K_a^o) \qquad (8)$$

where $\Delta\pi$ is the change in osmotic pressure due to the addition of osmolyte.

RESULTS

The association of HyHEL-5 with BWQL was studied by using fluoresceinated BWQL because no significant change was observed in the intrinsic fluorescence of the proteins on association. The top panel of Fig. 1 shows the typical change in anisotropy observed when increasing amounts of HyHEL-5 solution are added to a cuvette containing fluoresceinated BWQL. Using the known anisotropy of free antigen in solution (A_f), the calculated anisotropy of completely bound antigen from a double-reciprocal plot (A_b), and the quenching constant (Q_m), the fraction of antigen bound at any given antibody concentration can be determined as shown in the bottom panel of Fig. 1. These data can be fit by nonlinear regression to obtain the association constant for the antibody/antigen interaction. Fig. 1 shows that the association constant for HyHEL-5/BWQL association decreases with increasing temperature.

Variation of osmotic pressure by the addition of osmolytes was used to probe the involvement of water molecules in the HyHEL-5/BWQL association process. Affinity was measured in the presence of varying concentrations of glycerol, ethylene glycol, and betaine by both FPS and ITC (Tables 1, 2, and 3). (In the presence of PEG-300 and *N*-methylformamide, the proteins produced time-varying anisotropies; hence neither PEG-300 nor *N*-methylformamide was considered further. Sucrose at high concentrations and the lysozyme substrate analogs tri-*N*-acetylchitotriose and hexa-*N*-acetylchitohexose appear to exhibit specific interactions (Xavier and Willson, unpublished results); therefore sucrose was not pursued further as an osmolyte.)

The change in association constant in the presence of osmolytes (glycerol, ethylene glycol, and betaine) did not correlate with changes in the viscosity or the dielectric constant. Whereas the association constant decreases in the presence of all three osmolytes, the dielectric constants of glycerol and ethylene glycol solutions are lower than that of

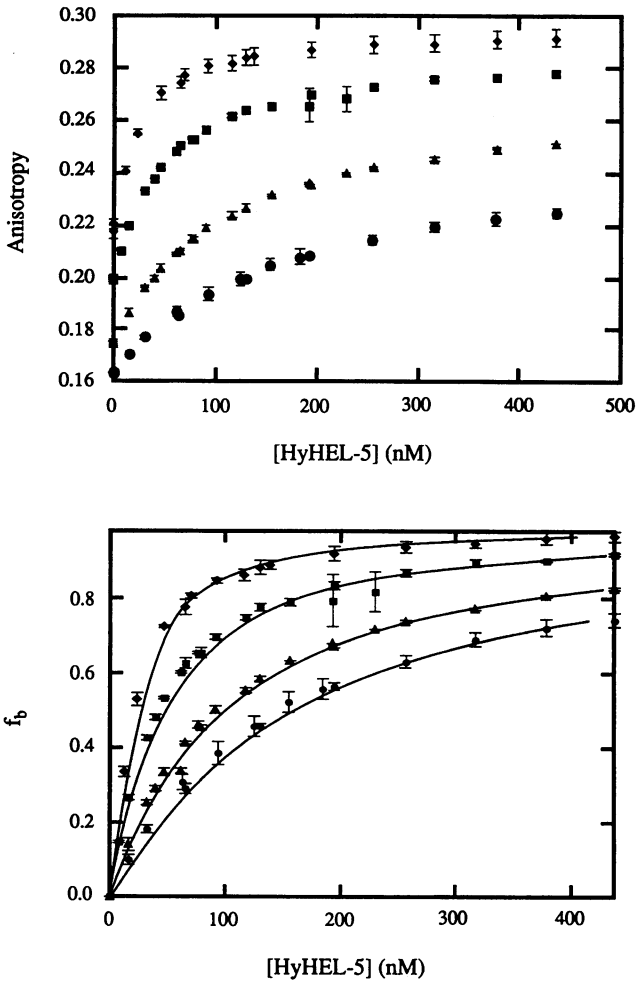

FIGURE 1 Fluorescence polarization data used to characterize binding of BWQL with HyHEL-5. (*Top*) Anisotropy as a function of HyHEL-5 concentration at four different temperatures: 5°C (♦), 15°C (■), 25°C (▲), and 30°C (●) (error bars represent ± one standard deviation from triplicate data). (*Bottom*) Fraction of antigen bound as function of antibody concentration. Symbols as in top panel.

pure water, whereas betaine solutions have an increased dielectric constant (Sidorova and Rau, 1995). The best correlation was with osmotic pressure (Figs. 2, 3, and 4); the

TABLE 2 Effect of osmotic pressure on the kinetics of HyHEL-5/BWQL interaction

Condition*	k_{assn} # ($M^{-1}\,s^{-1}$)	k_{diss} § (s^{-1})	$K_a = \frac{k_{assn}}{k_{diss}}$ (M^{-1})
No osmolyte	$1.8 \pm 0.3 \times 10^7$	0.96 ± 0.06	1.9×10^7
10% (w/w) glycerol	$1.1 \pm 0.2 \times 10^7$	0.80 ± 0.05	1.4×10^7
20% (w/w) glycerol	$6.1 \pm 1.2 \times 10^6$	0.60 ± 0.08	1.0×10^7
30% (w/w) glycerol	$3.1 \pm 0.9 \times 10^6$	0.51 ± 0.06	6.1×10^6

*In 10 mM sodium phosphate; 25 ± 0.8°C and pH 8.0 ± 0.1.
#Standard deviations calculated from association rate constants determined for four different combinations of concentrations of antigen and antibody.
§Standard deviations calculated from three experiments each at 5- and 10-fold excess of unlabeled BWQL.

TABLE 3 Calorimetric association constants and association enthalpies for the HyHEL-5/BWQL system determined in the presence of various osmolytes

Condition*	$K_a \times 10^{-6}$ (M^{-1})#	ΔH (kcal mol^{-1})#
No added osmolyte	5.5 ± 0.8	−19.2 ± 0.9
25% (w/w) glycerol	4.1 ± 0.4	−19.1 ± 0.1
37% (w/w) glycerol	2.1 ± 0.4	−18.2 ± 0.4
50% (w/w) glycerol	0.85 ± 0.01	−18.9 ± 0.2
25% (w/w) ethylene glycol	2.1 ± 0.1	−22.7 ± 0.1
37% (w/w) ethylene glycol	1.3 ± 0.3	−14.3 ± 0.4
50% (w/w) ethylene glycol	0.53 ± 0.1	−8.9 ± 0.1
25% (w/w) betaine	2.3 ± 0.1	−14.7 ± 0.4
50% (w/w) betaine	0.21 ± 0.03	−10.4 ± 0.4

*In 100 mM sodium phosphate and 150 mM NaCl; at 25 ± 0.2°C and pH 8.0 ± 0.1.
#Standard deviations calculated from results of two or more separate experiments.

results are fit reasonably well by Eq. 8. Lower affinity at lower water activity is consistent with the uptake of water molecules in the association process.

When 18 ml mol^{-1} is used as the partial molal volume of water, the FPS and ITC equilibrium data give ~6.0 ± 1.0 and 8.0 ± 1.0 water molecules, respectively, taken up on complex formation (Figs. 2 and 4). Using the calculated K_a obtained from the association and dissociation rate constants determined in the presence of varying concentrations of glycerol, the kinetics data give ~12.0 ± 3.0 water molecules taken up on association of BWQL with HyHEL-5 (Fig. 3). It is very likely that some bound waters are perturbed or released when proteins are transferred from buffer to osmolyte/buffer solutions; the effect of these water molecules on the binding process will be difficult to assess. Furthermore, different osmolytes could perturb the hydra-

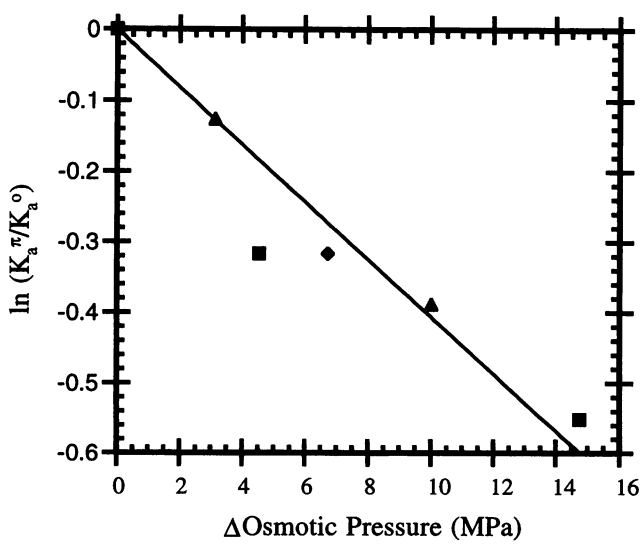

FIGURE 2 Normalized association constant obtained from equilibrium measurements by fluorescence polarization as a function of osmotic pressure for the three osmolytes glycerol (▲), ethylene glycol (■), and betaine (♦), fitted to $\Delta\pi\Delta V_w = RT \ln(K_a^{\pi}/K_a^{o})$. Linear regression gives ~6.0 ± 1.0 water molecules taken up on complex formation.

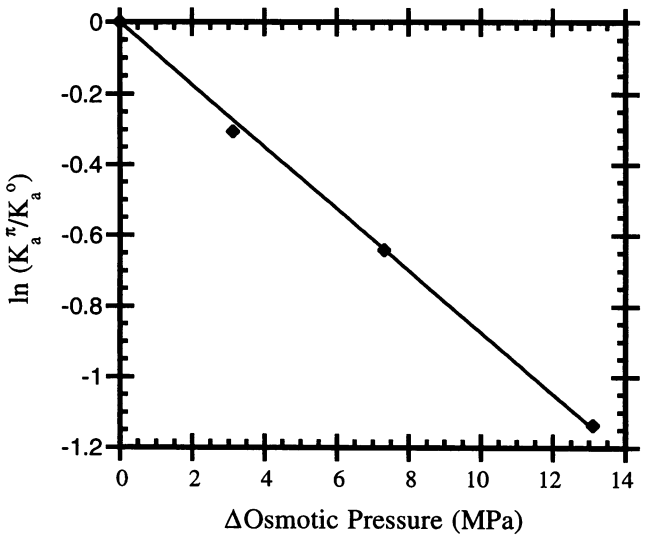

FIGURE 3 Normalized association constant calculated from kinetic measurements as a function of osmotic pressure for glycerol. Linear regression of the data gives ~12.0 ± 3.0 water molecules taken up in the HyHEL-5/BWQL association.

tion layer waters differently, that is, show different solvation effects, contrary to the assumption of ideality made in the derivation of Eq. 8. Because of these factors, the estimate of 6–12 water molecules taken up on complex formation is a lower bound, and the interpretation of the enthalpy of association in various osmolytes is difficult. Betaine is chemically distinct from glycerol and ethylene glycol, but the three osmolytes show similar effects on the association process; hence to a first approximation the binding process is not affected by the chemical nature of the osmolyte used. It has been suggested that at concentrations similar to those used in this study, lysozyme exhibits a "hydration shell" that excludes sugars (lactose and glucose; Arakawa and Timasheff, 1982) and glycerol (Gekko and Timasheff, 1981). Infrared spectra of lysozyme in 10–30% (v/v) glycerol and ethylene glycol solutions show secondary structural changes but no evidence for denaturation (Huang et al., 1995). It was suggested that these changes may result from changes in water structure that perturb the protein structure and/or from direct osmolyte-protein interaction. Ethylene glycol solutions up to 60% (v/v) cause no conformational changes in γ-globulins (Tanford et al., 1962). As shown in Table 3, the variation of enthalpy of association with osmolyte concentration depends on the nature of the osmolyte. It is noteworthy, however, that the exothermicity of association declines with increasing osmolyte concentration in almost all cases. This is consistent with the sequestration of water molecules from the osmolyte solution upon formation of the antibody-antigen complex, as the addition of water to ethylene glycol or to glycerol is an exothermic process (Newman, 1968; Christensen et al., 1982). A dominant osmotic effect is suggested by the enthalpy-entropy compensation observed in this system (Fig. 5). Enthalpy-entropy compensation has been proposed to be a consequence of the properties of water, and its presence is often taken as diagnostic of coupling of the process under study to processes involving the solvent (Lumry and Rajender, 1970), probably due to hydrogen bonding interactions (Gilli et al., 1994; Dunitz, 1995).

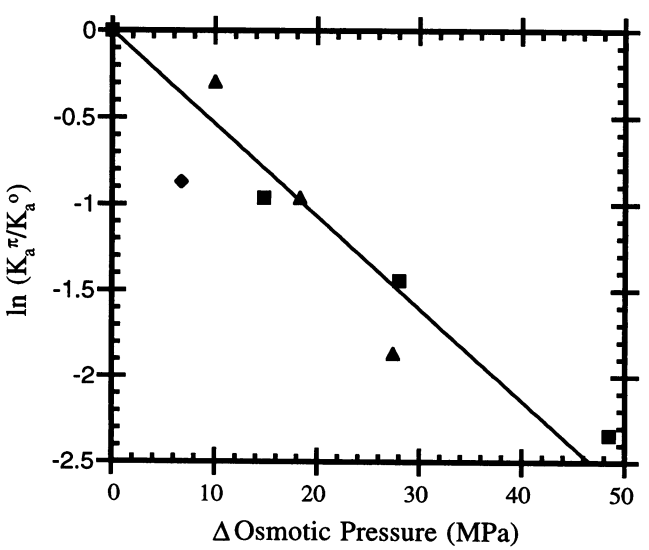

FIGURE 4 Normalized association constant obtained from equilibrium measurements by titration calorimetry as a function of osmotic pressure for the three osmolytes glycerol (▲), ethylene glycol (■), and betaine (◆), fitted to $\Delta\pi\Delta V_w = RT \ln(K_a^{\pi}/K_a^{\circ})$. The data point corresponding to 50% (w/w) betaine is not shown, because of nonavailability of osmotic pressure for betaine solution of this concentration. Linear regression gives ~8.0 ± 1.0 molecules taken up on complex formation.

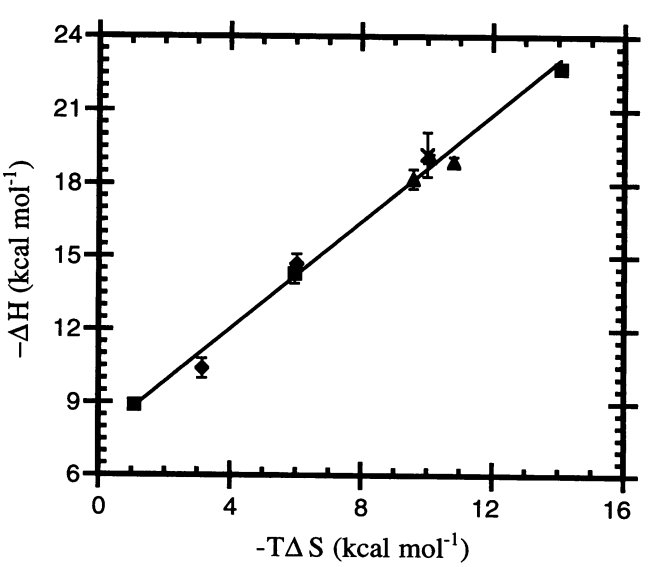

FIGURE 5 Enthalpy-entropy compensation plot (based on the data presented in Table 3) for the association of HyHEL-5 with BWQL in the presence of osmolytes (glycerol (▲), ethylene glycol (■), and betaine (◆)) or without osmolyte (×). The error in $-T\Delta S$ is less than about ±0.3 kcal mol^{-1}.

DISCUSSION

Fluoresceination of BWQL at the amino-terminus, which is not part of the HyHEL-5 structural epitope, was suggested by fluorescence lifetime experiments not to interfere with HyHEL-5 binding. Furthermore, there is good agreement between the association constants determined by fluorescence polarization experiments with labeled BWQL (ΔG varies from -9.7 kcal mol^{-1} to -9.1 kcal mol^{-1} in the temperature range 5–30°C) and ITC experiments with unlabeled BWQL (ΔG varies from -9.7 kcal mol^{-1} to -8.7 kcal mol^{-1} in the temperature range 12.9–36.4°C) (Shick et al., 1997). The association constants determined by FPS (in equilibrium measurements) (Table 1) and ITC (Table 3) were somewhat lower than those reported previously (Lavoie et al., 1989) for particle concentration fluorescence immunoassay. The association constant calculated using the association and dissociation rate constants (Table 2) is in better agreement with the values obtained by Lavoie et al. (1989). Hence the data have been analyzed separately for each method used to determine the association constant (Figs. 2–4), and the separate estimates of the number of water molecules involved in HyHEL-5/BWQL association are in reasonable agreement, as discussed below.

Experiments in the presence of glycerol, ethylene glycol, and betaine give a lower bound estimate of 6–12 additional water molecules taken up upon association. An independent estimate of the number of water molecules involved can be made by using the calculated entropy change of HyHEL-5/BWQL association and the relationships described by Spolar and Record (1994). It is observed by calorimetry that HyHEL-5/BWQL association is enthalpically driven and entropically unfavorable at 25°C (Shick et al., 1997). The negative entropy change arises from loss of protein translational, rotational, and conformational degrees of freedom on complex formation (including immobilization of the mobile loops around epitope residues 45 and 70 of BWQL; Chacko et al., 1996) and decreased solvent entropy due to ordering of water molecules in and around the antibody/antigen interface, and is opposed by any increase in solvent entropy due to burial of hydrophobic surfaces. Following the method of Spolar and Record (1994), the entropy change can be delineated as

$$\Delta S = \Delta S_{\text{hydr}} + \Delta S_{\text{trans}} + \Delta S_{\text{other}} \quad (9)$$

where ΔS_{hydr} is due to the hydrophobic effect, ΔS_{trans} accounts for the decrease in the rotational and translational degrees of freedom and for the immobilization of amino acid side groups as a result of complex formation, and ΔS_{other} is due to contributions not accounted for by the first two terms in Eq. 9. ΔS_{hydr} can be estimated by using the following relationship:

$$\Delta S_{\text{hydr}} = 1.35 \Delta C_{\text{P}} \ln(T/386) \quad (10)$$

where ΔC_{P} is the measured heat capacity change, T is the absolute temperature, and 386 K is the reference temperature at which the transfer entropy of nonpolar liquids to water vanishes (Spolar and Record, 1994). From Table 3, ΔS is -33.6 cal mol^{-1} K^{-1} and ΔC_{P} is -430 cal mol^{-1} K^{-1} (Shick et al., 1997); hence at 298 K for the HyHEL-5/BWQL system we calculate $\Delta S_{\text{hydr}} = 150.2$ cal mol^{-1} K^{-1}, which gives $\Delta S_{\text{trans}} + \Delta S_{\text{other}}$ ($= \Delta S - \Delta s_{\text{hydr}}$) $= -183.8$ cal mol^{-1} K^{-1}. If an estimate of -50 cal mol^{-1} K^{-1} is used for ΔS_{trans} (Spolar and Record, 1994), then we obtain a value for ΔS_{other} of -133.8 cal mol^{-1} K^{-1}. The unfavorable entropy change of water taken up in the association of BWQL with HyHEL-5 is not reflected in ΔS_{hydr}, but rather in ΔS_{other}. Dunitz (1994) has used the entropy of water in ice and hydrates of various salts (at 298 K) to arrive at an upper limit for the entropy of water tightly bound to the surface of a protein as 7 cal mol^{-1} K^{-1}; using this value and assuming that all of ΔS_{other} is due to water uptake, an estimate of ~19 water molecules is obtained. This is an overestimate because ΔS_{other} should also include a number of other system-specific contributions such as local folding, and this value should be compared with the lower limit of 6–12 waters obtained by osmotic pressure experiments. Jelesarov and Bosshard (1994) used the above approach to determine the number of water molecules involved in the association of ferredoxin with ferredoxin:NADP$^+$ reductase and obtained estimates in good agreement with their experimental results with osmolytes. Both osmotic pressure experiments and the correlations of Spolar and Record (1994), therefore, indicate the important contribution of water molecules to the HyHEL-5/BWQL interaction. Calorimetric studies of antibody/protein antigen interactions have found most to be enthalpically driven at 25°C, whereas entropic contributions are either slightly favorable or unfavorable (Tello et al., 1993, 1994; Hibbits et al., 1994; Tsumoto et al., 1994a,b, 1995; Murphy et al., 1995; Raman et al., 1995; Schwarz et al., 1995; Shick et al., 1997), and some of this unfavorable entropy could be contributed by ordering of additional water molecules in and around the antibody/antigen interface upon complex formation. In contrast to this, antibody association with haptens (Herron et al., 1986), peptides (Murphy et al., 1993), and oligosaccharide antigens (Sigurskjold et al., 1991; Sigurskjold and Bundle, 1992) is in many cases entropically favored, probably because of solvent release associated with hydrophobic interactions. This difference presumably also reflects the difference in the interaction of proteins with antibodies as compared to small antigens; the protein and antibody surfaces that interact are relatively flat (reviewed in Davies and Cohen, 1996), whereas small antigens often insert into grooves or pockets in the antibody combining site (discussed in Wilson and Stanfield, 1993).

There are few protein-protein complexes for which estimates of the number of water molecules involved in complex formation have been obtained experimentally. For the cytochrome c–cytochrome b_5 system, about three water molecules are liberated on complex formation; whereas for the association of cytochrome c and cytochrome c oxidase, probably ~12 water molecules are taken up on protein-protein association (Kornblatt et al., 1988, 1993). Exclusion

of four to seven water molecules was observed for the association of ferredoxin with ferredoxin:$NADP^+$ reductase (Jelesarov and Bosshard, 1994). In the D1.3/HEL system, 25 well-ordered water molecules were observed in the antibody/lysozyme interface, and these waters were conserved among the wild-type and five mutant Fv D1.3/HEL complexes (Braden et al., 1995). This was further confirmed by studying the antibody/antigen interaction under conditions of reduced water activity, and it was observed that ~13 water molecules were bound upon complex formation (Goldbaum et al., 1996). Goldbaum et al. (1996) also observed the release of a very small number of water molecules when antibody D44.1 interacted with HEL. They observed that for the D1.3/HEL system, the negative binding enthalpies and free energies of association decreased with increasing concentrations of glycerol, whereas a smaller and opposite effect was shown by the D44.1/HEL system.

The resolution of the available crystal structures of free BWQL and the HyHEL-5/BWQL complex precludes a direct analysis of the total number of water molecules involved in complex formation (Chacko et al., 1996); however, two water molecules were observed to make bridging contacts between the HyHEL-5 Fab and BWQL. Similarly, the further refined three-dimensional structure of the HyHEL-5/HEL complex shows the presence of three water molecules at the interface (Cohen et al., 1996) at a resolution of 2.65 Å. The close shape complementarity between the BWQL and HyHEL-5 protein surfaces (Chacko et al., 1996) suggests that some of the osmotically sensitive water molecules may mediate antibody/antigen interactions at the edge of the interface. These water molecules could contribute favorably to the enthalpy of association by formation of water-mediated hydrogen bonds (under favorable conditions the net free energy gain of formation of a hydrogen bond is 0.5–1.5 kcal mol^{-1}; Fersht et al., 1985), while reducing enthalpically unfavorable desolvation of polar groups at the edge of the interface (Connelly et al., 1994). Molecular modeling analysis of the HyHEL-5/BWQL complex shows the formation of four cavities large enough to accommodate water molecule(s) upon complexation of HyHEL-5 with BWQL (J. Liang and S. Subramaniam, University of Illinois at Urbana-Champaign, personal communication). Their volumes according to a molecular surface model are 185.9 $Å^3$, 69.1 $Å^3$, 44.3 $Å^3$, and 14.6 $Å^3$. If each water molecule occupies 30 $Å^3$, as in the 55 M liquid state (corresponding to the partial molar volume of water used earlier), there could be ~6.2 + 2.3 + 1.5 + 1.0 ~ 10 water molecules taken up by the cavities on antibody/antigen complex formation. It has been observed for the D1.3/HEL system that antibody-bound water molecules form, with antibody residues, a structure that is more closely complementary to the antigen; some of these water molecules were also seen in the structure of the free antibody D1.3 Fv fragment (Bhat et al., 1994).

In conclusion, we have shown by osmotic pressure experiments using three different methods that there is uptake of ~6–12 water molecules when the HyHEL-5/BWQL complex is formed, demonstrating the importance of water molecules in this protein-protein association. This is compatible with the upper bound estimate of 19 water molecules calculated by using the entropy and heat capacity change on association of BWQL with HyHEL-5. Along with the crystallographic and biophysical results for the anti-HEL antibodies D1.3 and D44.1 and modeling results for the HyHEL-5/BWQL complex, these results suggest that water molecules may be important in many antibody/antigen systems in increasing the shape complementarity between antibody and protein antigen surfaces, and probably contribute favorably to the enthalpy of association by the formation of water-mediated hydrogen bonds.

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