Association and Dissociation Kinetics of Anti-Hen Egg Lysozyme Monoclonal Antibodies HyHEL-5 and HyHEL-10

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ABSTRACT The immunoglobulin G1 (IgG_1) κ antibodies HyHEL-5 and HyHEL-10 interact with nonoverlapping epitopes on hen egg lysozyme (HEL); the HyHEL-5/HEL interface has two energetically and structurally important salt links, whereas the HyHEL-10/HEL interface involves predominantly hydrogen bonds and van der Waals interactions. The kinetics of association and dissociation of antibodies HyHEL-5 and HyHEL-10 with HEL under a variety of conditions were investigated in this study. The association of each antibody with HEL follows second-order kinetics. The association process is significantly diffusion-limited, as indicated by the viscosity dependence of the interaction of both antibodies with HEL, although detailed energetics suggest that the association process may be more complex. The association rate constant for the HyHEL-5/HEL system is within a factor of 2 of the modified Smoluchowski estimate for proteins of this size, whereas HyHEL-10 interacts with HEL with an association rate an order of magnitude lower. The association reactions are insensitive to ionic strength, showing only a twofold decrease in the association rate constant when the ionic strength was increased from 27 mM to 500 mM. Interestingly, the association rate constant for the interaction of HyHEL-5 with HEL varies with pH in the range 6.0–10.0, whereas HyHEL-10/HEL association is not affected by pH in the same range. The dissociation of the HyHEL-5/HEL and HyHEL-10/HEL complexes follow first-order kinetics with half-lives at 25°C of ~3,150 s and ~21,660 s, respectively.

INTRODUCTION

Hen egg lysozyme (HEL) is a well-studied model for investigating antibody/protein antigen interactions (reviewed in Davies and Cohen, 1996). The resources available for work with HEL as an antigen include crystal structures (both free and in complex with several different antibodies), avian species variants of lysozyme with known sequences, and systems for heterologous expression and site-directed mutagenesis (Kumagai et al., 1987; Kam-Morgan et al., 1993). The three-dimensional crystal structures of the HyHEL-5/HEL (Sheriff et al., 1987; Cohen et al., 1996) and HyHEL-10/HEL (Padlan et al., 1989) complexes have been determined. HyHEL-5 binds to a HEL epitope centered on Arg^{45} and Arg^{68} (Smith-Gill et al., 1982; Lavoie et al., 1989; Shick et al., 1997), which are in close contact with the HyHEL-5 residues Glu-H35 and Glu-H50; the two arginines form salt links with Glu-H50 (Sheriff et al., 1987; Cohen et al., 1996; Table 1). In contrast, the interaction between HyHEL-10 and HEL involves predominantly hydrogen bonds and van der Waals interactions, with the antibody binding site containing a disproportionate number of aromatic residues (Padlan et al., 1989; Table 2).

To date, the only antibody/protein antigen system of known structure extensively studied in kinetic terms is the D1.3/HEL complex (reviewed in Braden et al., 1995a). The association follows a bimolecular mechanism, and the predominant effect of D1.3 mutations is on the dissociation rate constant (Ito et al., 1995). An extensive study of the association and dissociation kinetics of anti-cytochrome *c* antibodies 2B5 and 5F8 has been made by Raman et al. (1992); their principal conclusion was that the association is diffusion-controlled and follows a bimolecular mechanism.

In this study we have investigated the association and dissociation kinetics of HyHEL-5 and HyHEL-10 with HEL by stopped-flow fluorescence polarization. Sucrose and glycerol have been used as viscosity modifiers to study the viscosity dependence of the association process. Calculation of pK_a values of residues in HEL, HyHEL-5, and the HyHEL-5/HEL complex (McDonald et al., 1995) has shown that pK_a values of several residues in the contact region, including those involved in the Arg-Glu salt links, change significantly upon association. Computational investigation of the role of electrostatics in the HyHEL-5/HEL complex (Slagle et al., 1994) revealed that neutralization or charge reversal of any of the residues involved in intermolecular salt links resulted in decreased binding affinities. Charged residues were observed to exert considerable influence on steering of the two proteins into a favorable configuration for binding in Brownian dynamics simulations (Kozack and Subramaniam, 1993; Kozack et al., 1995). Therefore the association rate constant for both antibodies interacting with HEL was studied as a function of pH and ionic strength. For complete characterization of the antibody/antigen interaction and further understanding of the mechanism of binding, the dissociation rate constants of HyHEL-5/HEL and HyHEL-10/HEL complexes were also determined.

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TABLE 1 Hydrogen bonds and salt links in the HyHEL-5 Fab/HEL interface

Fab V_L	HEL	Fab V_H	HEL
Arg^{93} $N^{\eta 2}$	Asn^{44} $O^{\delta 1}$	Gly^{55} O	Gln^{41} $N^{\varepsilon 2}$
Trp^{91} O	Arg^{45} $N^{\eta 2}$	Ser^{56} O^{γ}	Gln^{41} O
Gly^{92} O	Arg^{45} $N^{\eta 2}$	Thr^{57} O	Thr^{43} $O^{\gamma 1}$
Gly^{92} O	Arg^{45} N^{ε}	Asn^{58} $N^{\delta 2}$	Thr^{43} $O^{\gamma 1}$
Arg^{93} O	Arg^{45} N^{ε}	Asn^{58} $N^{\delta 2}$	Thr^{43} O
Arg^{93} N^{ε}	Arg^{45} O	Glu^{50} $O^{\varepsilon 2}$	Arg^{45} $N^{\eta 1}$
Arg^{93} $N^{\eta 2}$	Arg^{45} O	Trp^{33} $N^{\varepsilon 1}$	Tyr^{53} O^{η}
Arg^{93} $N^{\eta 2}$	Asn^{46} $O^{\delta 1}$	Tyr^{97} N	Gly^{67} O
		Glu^{50} $O^{\varepsilon 1}$	Arg^{68} $N^{\eta 1}$
		Glu^{50} $O^{\varepsilon 2}$	Arg^{68} $N^{\eta 2}$

From Cohen et al. (1996).

MATERIALS AND METHODS

Labeling of HEL

HEL (2× crystallized) was purchased from Worthington (Freehold, NJ) and was used as received. The change in intrinsic fluorescence of either protein on association was insufficient to characterize the binding interaction. Therefore, fluorescein isothiocyanate (Molecular Probes, Eugene, OR) was used at pH 7.5 in 100 mM sodium bicarbonate to label HEL preferentially at the N-terminus. A fraction having a lysozyme/fluorescein ratio of 1.0 ± 0.1 was isolated by fractionation on a phenyl Sepharose column, as described previously (Xavier et al., 1997). Labeling and fractionation of fluoresceinated HEL produces protein that appears to be predominantly labeled at the N-terminus, which is not part of the structural epitope of either antibody. For interaction of HyHEL-5 with an avian species variant lysozyme (bobwhite quail lysozyme, BWQL), it was previously shown that the fluorescent tag did not interfere with binding and that the lifetime of the fluorescein label is the same in free and HyHEL-5-bound BWQL (Xavier et al., 1997).

Antibody purification

HyHEL-5 and HyHEL-10 were purified by sequential anion-exchange, hydrophobic interaction, and cation-exchange chromatography as described previously (Xavier et al., 1997). The purities of both antibodies were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using silver-stained 8–25% polyacrylamide gradient gels (PhastSystem, Pharmacia). In some cases, a minor amount (less than ~5%) of a 66-kDa protein (believed to be bovine serum albumin) was present as an impurity. Previously Hibbits et al. (1994) have shown that even the presence of much higher concentrations of bovine serum albumin does not alter the energetics of antibody/antigen association. All proteins were concentrated by using a stirred ultrafiltration cell (Amicon 8200) and stored at 4°C in 100 mM sodium phosphate and 150 mM NaCl, pH 8.0.

TABLE 2 Hydrogen bonds in the HyHEL-10 Fab/HEL interface

Fab V_L	HEL	Fab V_H	HEL
Asn^{31} $O^{\delta 1}$	Lys^{96} N^{ζ}	Thr^{30} O	Arg^{73} $N^{\eta 1}$
Asn^{32} $N^{\delta 2}$	Gly^{16} O	Ser^{31} O^{γ}	Arg^{73} $N^{\eta 1}$
Gln^{53} $O^{\varepsilon 1}$	Asn^{93} $N^{\delta 2}$	Tyr^{33} O^{η}	Lys^{97} O
Gln^{53} $N^{\varepsilon 2}$	Asn^{93} $O^{\delta 1}$	Tyr^{50} O^{η}	Arg^{21} $N^{\eta 1}$
Ser^{91} O	Tyr^{20} O^{η}	Tyr^{50} O^{η}	Ser^{100} O
Asn^{92} O	Arg^{21} N	Tyr^{53} O	Asp^{101} $O^{\delta 1}$
Tyr^{96} O^{η}	Arg^{21} $N^{\eta 1}$	Tyr^{58} O^{η}	Gly^{102} N

From Padlan et al. (1989).

Sample preparation

All experiments were performed in 10 mM phosphate buffer at pH 8.0, unless noted otherwise. The ionic strength of the solution was adjusted to the stated value with NaCl and the value determined, using

$$I = 0.5\ \Sigma_i\ m_i z_i^2, \tag{1}$$

where m_i are the molarities and z_i the charges of each of the i species. The antibody and antigen were codialyzed overnight against the same buffer. The cosolute solutions were prepared gravimetrically and the pH adjusted after the cosolute was mixed in. The viscosities of cosolute solutions were determined with a Brookfield digital viscometer (model DV-II+); these agreed with published values (Wolf et al., 1988). The concentrations of the protein solutions were determined spectrophotometrically (Beckman DU-6500 spectrophotometer), using E_{280} values of 1.49, 1.43, and 2.64 for 1.0 mg/ml solutions of HyHEL-5, HyHEL-10, and HEL, respectively (Hibbits et al., 1994). Molarities of the protein solutions were calculated by using molecular weights of 14,305 for HEL and 150,000 for the two antibodies.

Kinetic measurements

A SPEX Fluorolog 212 fluorometer with Glan-Thompson polarizers was used to measure the change in anisotropy on antibody/antigen association or dissociation. Experiments were performed in L-format with a manual shutter after the excitation monochromator, to minimize photobleaching of the sample. The samples were excited at 490 nm with a 3.4-nm bandpass. A 520 nm cut-on filter (Omega Optical, Brattleboro, VT) was used on the emission side to maximize sensitivity. The kinetics of antibody/antigen association was studied, using a SFA-20 pneumatically driven, stopped-flow kinetics accessory (Hi-Tech, Salisbury, England) with a dead time estimated by the manufacturer to be less than 20 ms. The experiments were carried out under pseudo-first-order conditions by using a 10- or 20-fold excess of antibody binding sites as compared to the antigen concentration. The antibody and antigen solutions were taken into 1.0-ml syringes, and the two protein solutions were mixed at a volume ratio of 1:1. An external trigger interface (Instruments SA, Edison, NJ) was used for data acquisition with software supplied with the fluorometer. The temperature of the protein solutions was controlled to within ±0.8°C with circulating water baths (Haake Model A82). To increase the signal-to-noise ratio, kinetic traces of five different experiments for each polarizer position were averaged (Fig. 1). The initial readings corresponding to the dead time of the instrument (less than 1% of the whole) were not deleted before data analysis. The data files were converted into ASCII format and modified with Lotus for analysis by KINFIT (OLIS, Bogart, GA), using a successive integration algorithm. The best fit as judged by the residuals and the autocorrelation function, for all of the association kinetics data, was to the single-exponential equation

$$A_t = (A_f - A_b) \times \exp(-k_{obs}t) + A_b, \tag{2}$$

where A_t, A_f, and A_b are anisotropy of the sample at time t, free antigen and bound antigen respectively, and k_{obs} is the first-order rate constant. For each temperature, pH, and ionic strength, typically four different combinations of antibody and antigen concentrations were studied under pseudo-first-order conditions and the pseudo-first order rate constant (k_{obs}) determined for each combination. The second-order rate constant (k_{assn}) for association was determined by averaging the pseudo-first-order rate constants divided by the appropriate antibody concentration (linear regression gave similar results within experimental error).

The dissociation rate constant was determined by adding a 20-fold excess of unlabeled HEL (1.0 μM) to a solution of preformed antibody: fluorescein labeled HEL 1:2 complex (25 nM HyHEL-5 or HyHEL-10 and 50 nM fluorescein-labeled HEL). It was verified by using an 80-fold excess of unlabeled lysozyme that the dissociation followed first-order kinetics for both antibody/antigen complexes. For the dissociation of both HyHEL-5/HEL and HyHEL-10/HEL complexes, the anisotropy was measured at 30-min intervals for 390 min. In control experiments, it was separately

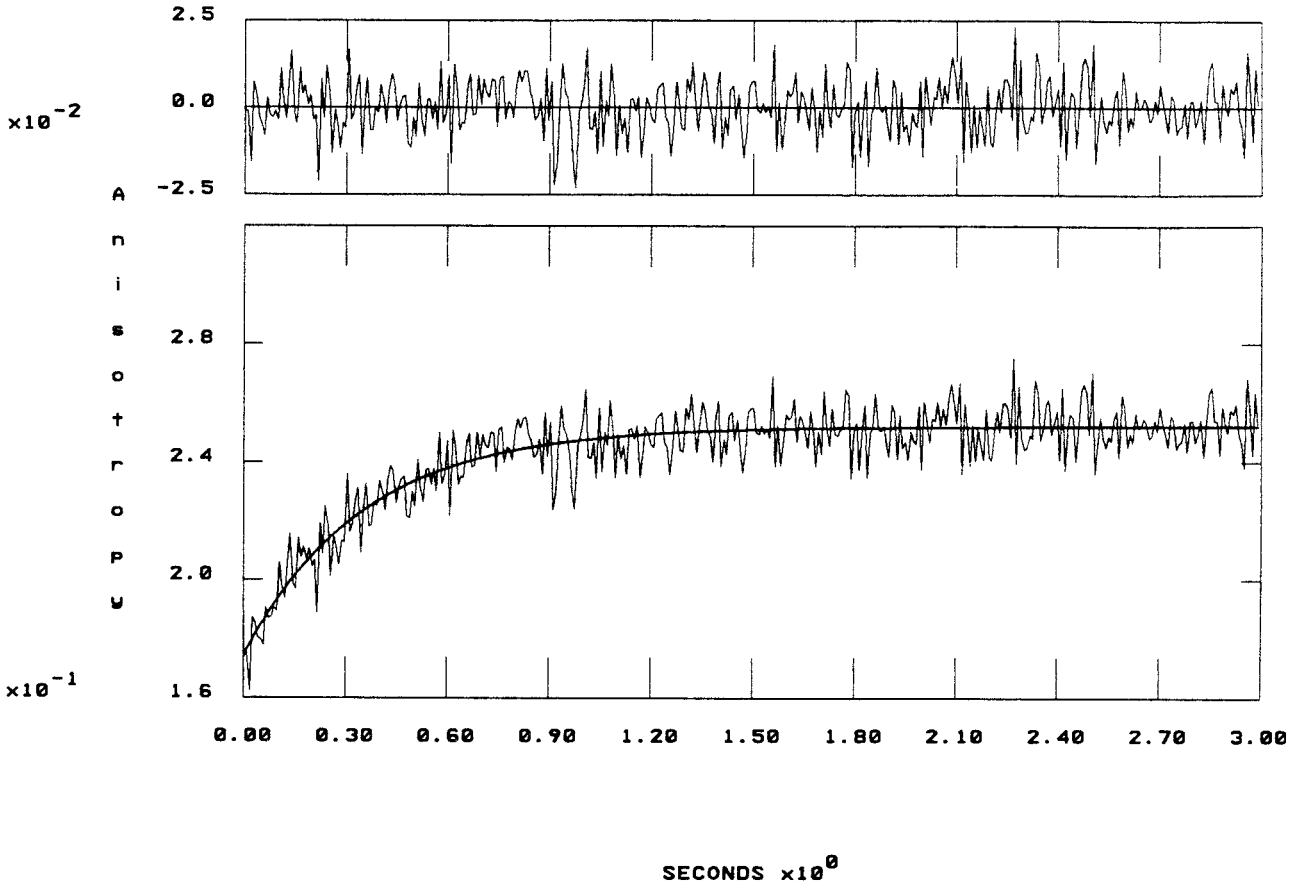

FIGURE 1 Kinetic fluorescence anisotropy trace for the association of 50 nM HEL with 250 nM HyHEL-5 (i.e., 500 nM binding sites) at 25°C, pH 8.0, and an ionic strength of 150 mM. The fit is to a first-order rate equation by successive integration fit using KINFIT. The upper panel shows the residuals.

determined that the anisotropy of the complex was constant to within ±0.002 for the duration of the experiment. All experiments were done in triplicate, and the data were fitted to Eq. 2.

Calculation of transition-state thermodynamic parameters

Activation parameters for the antibody/antigen interaction were obtained from the temperature dependence of k_{assn}, using the Eyring equation (Eyring and Eyring, 1963)

$$\frac{\Delta G^{\ddagger}}{T} = R \ln\left[\left(\frac{k_b T}{h}\right)\left(\frac{1}{k_{assn}}\right)\right], \quad (3)$$

where R is the gas constant, k_b is Boltzmann's constant, h is Planck's constant, and T is the absolute temperature, and

$$\frac{\Delta G^{\ddagger}}{T} = \frac{\Delta H^{\ddagger}}{T} - \Delta S^{\ddagger}. \quad (4)$$

A plot of $\Delta G^{\ddagger}/T$ versus $1/T$ gives the activation parameters. By analogy with equilibrium thermodynamics, the enthalpy of activation is related to the activation energy E_a, $E_a = \Delta H^{\ddagger} + RT$.

Alternatively, the activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ can be determined (Mummert and Voss, 1996) by using the activation energy (E_a) and preexponential factor (A) obtained from

$$\ln k_{assn} = -E_a/RT + \ln A. \quad (5)$$

The activation parameters obtained from Eq. 5 are within 10% of those determined with the Eyring formulation. The transmission coefficient determined from these parameters (Mummert and Voss, 1996) is close to unity for both antibody/antigen systems (at 25°C, 1.05 for HyHEL-5/HEL and 1.06 for HyHEL-10/HEL). Therefore the transition-state theory is appropriate for analysis of association in these systems.

Analysis of viscosity dependence of association rate constants

A simplified model introduced by von Smoluchowski (1917) can be used to determine the approximate magnitude of rate constants for diffusion-controlled interactions. It is assumed that the interacting molecules may be treated as spheres undergoing normal Brownian motion in a viscous fluid and that they interact when they come within a distance r_{AB} of each other. In the absence of any forces between the two molecules, it was shown that the second-order rate constant is

$$k_{assn} = 4000\pi r_{AB} N_A (D_A + D_B), \quad (6)$$

where N_A is Avogadro's number, D_A and D_B are the diffusion coefficients of the interacting species A and B in units of $m^2\ s^{-1}$, and r_{AB} is in meters. The Stokes-Einstein relationship for the diffusion coefficient of species i,

$$D_i = \frac{k_b T}{6\pi\eta r_i}, \quad (7)$$

where k_b is Boltzmann's constant, T is the absolute temperature, η is the solvent viscosity, and r_i is the radius, can be substituted in Eq. 6. Using $r_{AB} = r_A + r_B$, we obtain

$$k_{assn} = \frac{2000\, N_A k_b T}{3\eta}\left(2 + \frac{r_A}{r_B} + \frac{r_B}{r_A}\right). \quad (8)$$

Hence the association rate constant is predicted to be inversely proportional to the solvent viscosity. Kramers (1940) has shown that the relative association rate constant for a bimolecular interaction k^o_{assn}/k^v_{assn}, where k^o_{assn} is the rate constant in the absence and k^v_{assn} is the rate constant in the presence of a viscosity modifier, is linearly dependent on the relative viscosity (η/η_o) of the medium,

$$\frac{k^o_{assn}}{k^v_{assn}} = A + B\frac{\eta}{\eta_o}. \quad (9)$$

For the case of an ideal diffusion-limited interaction, $A = 0$ and $B = 1$.

RESULTS

The association experiments were performed under pseudo-first-order conditions with excess antibody, as this was the unlabeled species. The second-order association rate constant (k_{assn}) can be determined by linear regression from a plot of the known antibody concentration versus the observed association rate constant, or by averaging the association rate constants obtained by dividing the k_{obs} by the appropriate antibody concentration. The two methods give association rate constants that agree within the experimental error of the stopped-flow method (in most cases within 15%). Figs. 2 and 3 show the determination of k_{assn} for the association of HyHEL-5 with HEL by the two methods at 25°C, pH 8.0, and an ionic strength of 150 mM.

From Fig. 3 the association rate constant for the interaction of HyHEL-5 with HEL is observed to be constant at $2.4 \pm 0.2 \times 10^7$ M^{-1} s^{-1} over a wide range of antibody concentrations, showing that the association process is bimolecular at 25°C, pH 8.0, and an ionic strength of 150 mM. For the same set of conditions, the association rate constant for the HyHEL-10/HEL system is $4.8 \pm 0.4 \times 10^6$ M^{-1} s^{-1}. The association of HyHEL-5 and HyHEL-10 with HEL followed bimolecular kinetics under all conditions tested (temperature, pH, ionic strength, and viscosity).

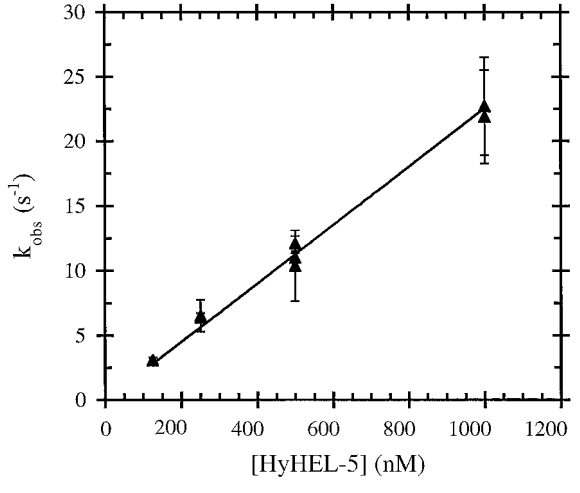

FIGURE 2 Plot of k_{obs} versus antibody concentration for the binding of HyHEL-5 with HEL at 25°C, pH 8.0, and an ionic strength of 150 mM, to obtain the association rate constant. Linear regression of the data gives an association rate constant of $2.3 \pm 0.2 \times 10^7$ M^{-1} s^{-1}.

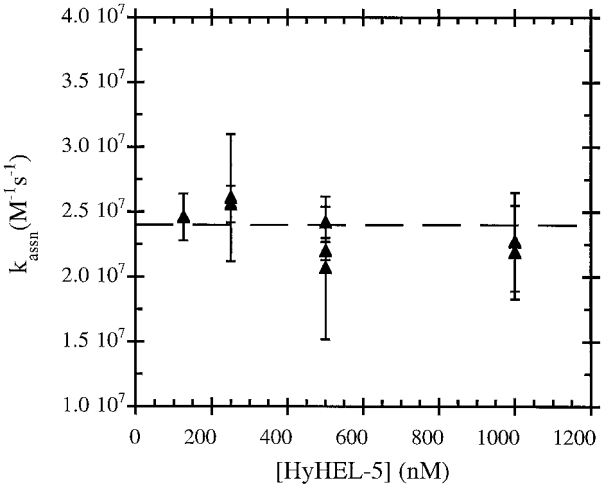

FIGURE 3 The association rate constants obtained for the binding of HyHEL-5 with HEL (at the conditions of Fig. 2) for different concentrations of the antibody and the antigen, giving an average k_{assn} of $2.4 \pm 0.2 \times 10^7$ M^{-1} s^{-1}.

Temperature dependence of association rate constants

The temperature dependence of the association of HyHEL-5 and HyHEL-10 with HEL was measured in the temperature range 10–40°C (Table 3). From the Eyring plot (Fig. 4), for the HyHEL-5/HEL system the thermodynamic interaction parameters are $\Delta H^\ddagger = 6.0$ kcal mol^{-1} and $\Delta S^\ddagger = -4.6$ cal mol^{-1} K^{-1}. Thus at 298 K there is an activation free energy barrier for the association reaction of $\Delta G^\ddagger = 7.4$ kcal mol^{-1}. Similarly for the HyHEL-10/HEL system (Fig. 4), the thermodynamic interaction parameters are $\Delta H^\ddagger = 9.4$ kcal mol^{-1}, $\Delta S^\ddagger = 3.5$ cal mol^{-1} K^{-1}, and an activation free energy barrier of $\Delta G^\ddagger = 8.3$ kcal mol^{-1} at 298 K. The activation free energy barrier for the association of HyHEL-5 with HEL is lower than that for HyHEL-10 by ~1

TABLE 3 Association rate constants for the HyHEL-5/HEL and the HyHEL-10/HEL systems at different temperatures

Temperature (± 0.8°C)	k_{assn} (M^{-1} s^{-1}) (HyHEL-5/HEL)	k_{assn} (M^{-1} s^{-1}) (HyHEL-10/HEL)
10	$1.3 \pm 0.1 \times 10^7$	$1.7 \pm 0.1 \times 10^6$
17	$1.7 \pm 0.1 \times 10^7$	$3.0 \pm 0.4 \times 10^6$
25	$2.4 \pm 0.2 \times 10^7$	$4.8 \pm 0.4 \times 10^6$
32	$2.9 \pm 0.1 \times 10^7$	$6.8 \pm 0.2 \times 10^6$
40	$3.9 \pm 0.2 \times 10^7$	$9.5 \pm 0.6 \times 10^6$

Sodium phosphate buffer (10 mM), pH 8.0, was supplemented with sufficient NaCl (123 mM) to adjust the ionic strength to 150 mM.

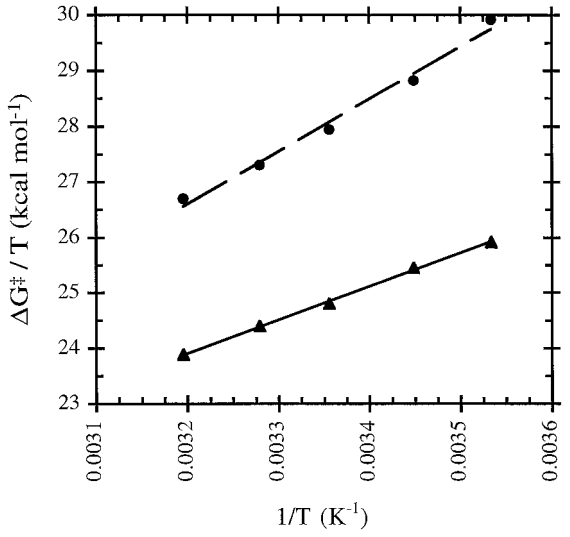

FIGURE 4 Eyring plot showing the temperature dependence of k_{assn} for the association of HyHEL-5 (▲) and HyHEL-10 (●) with HEL. All experiments in 10 mM sodium phosphate buffer, pH 8.0, supplemented with sufficient NaCl (123 mM) to adjust the ionic strength to 150 mM.

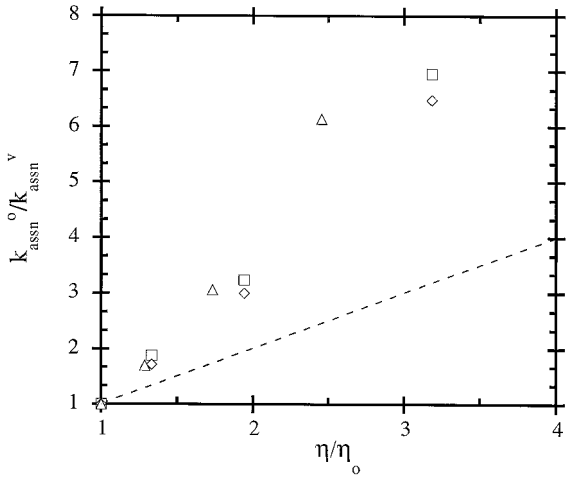

FIGURE 5 The effect of viscosity on the association rate constant for the binding of HyHEL-5 (sucrose (□) and glycerol (△)) and HyHEL-10 (sucrose (◇)) with HEL at 25°C, pH 8.0, and an ionic strength of 150 mM. The line represents the case of an ideal diffusion-controlled interaction.

kcal mol^{-1}. The difference in the sign of $\Delta S^{\ddagger}$ for the association of the two antibodies with HEL suggests that there could be a difference in the type of intermediate or energy barrier for association.

Viscosity dependence of association rate constants

Sucrose and glycerol were used to probe the viscosity dependence of k_{assn} for the interaction of HyHEL-5 and HyHEL-10 with HEL. Two cosolutes were used to control for effects other than viscosity. The k_{assn} of both antibodies binding HEL is decreased in the presence of the cosolute(s) (Fig. 5). For the HyHEL-5/HEL system, the rate constant is slightly more sensitive to solvent viscosity when glycerol is used at higher concentrations, possibly indicating a small chemical effect. For the association of HyHEL-5 with bobwhite quail lysozyme (BWQL), sucrose appeared to exhibit specific interactions at high concentrations (Xavier and Willson, unpublished results). The decrease in k_{assn} in the presence of both sucrose and glycerol shows a greater than reciprocal change with increasing viscosity, suggesting that the association is primarily diffusion-limited. The slope and the intercept values for Kramers theory are 2.73 and −1.82 (HyHEL-5/HEL system in sucrose), 3.57 and −2.81 (HyHEL-5/HEL system in glycerol), and 2.53 and −1.66 (HyHEL-10/HEL system in sucrose). In addition to chemical or nonspecific effects of the cosolutes, it is probable that the larger than expected decrease in the association constant could be due to the osmotic pressure exerted by the cosolute solutions. Osmotic pressure effects have been observed for the interaction of HyHEL-5 with BWQL; on an increase of the concentration of glycerol to 30% (w/w; relative viscosity 2.45), the equilibrium association constant decreases by about twofold (Xavier et al., 1997).

Ionic strength dependence of association rate constants

The HyHEL-5/HEL complex has two buried salt links, whereas the HyHEL-10/HEL has a single solvent-exposed salt link (Davies and Cohen, 1996). Brownian dynamics computational results for the HyHEL-5/HEL system suggests an increase of 10-fold in the rate constant on going from high to low ionic strength (Kozack et al., 1995). Thus the salt links, in the case of the HyHEL-5/HEL system, may be important in "steering" the two proteins during the association process (Kozack et al., 1995) and thus could affect the orientational requirements for association.

The k_{assn} values for the binding of the two antibodies were determined at different ionic strengths to probe the importance of the computationally observed "electrostatic steering" in the HyHEL-5/HEL system (Fig. 6). Comparison of the ionic strength dependence of the rate constants for three antibody/protein antigen systems (Table 4) shows that k_{assn} increases by two- or threefold on going from high ionic strength (i.e., "nonsteering") to low ionic strength conditions. This change in the association rate constant could be explained in terms of the interacting proteins being oppositely charged at pH 8.0. HEL is strongly positively charged at pH 8.0 (Righetti et al., 1981), and mouse immunoglobulins (IgGs) usually have a pI between 6.0 and 7.5 (Hamilton et al., 1987; Raman et al., 1992). At pH 8.0 the net charges of the Fv fragments of HyHEL-5 and HyHEL-10 calculated from the amino acid sequence are 0 and −5, and those obtained by electrostatic calculations are −1 and −3, respectively (S. M. McDonald, personal communication).

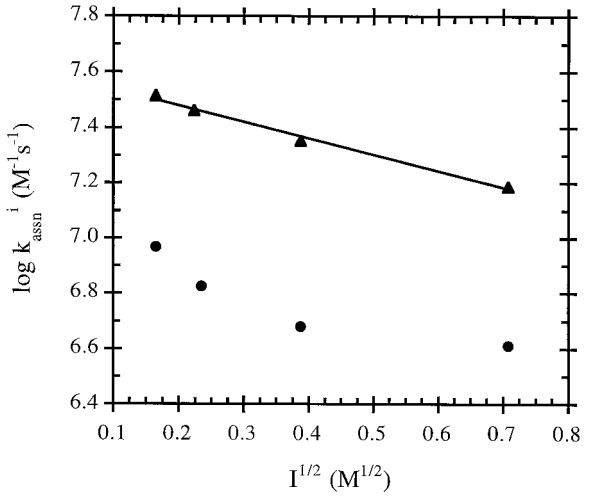

FIGURE 6 Plot showing the ionic strength dependence of k_{assn} for the association of HyHEL-5 (▲) and HyHEL-10 (●) with HEL at 25°C and pH 8.0. The data for HyHEL-5/HEL association have been fitted to $\log k^{i}_{assn} = \log k^{o}_{assn} + z_{Ag}z_{Ab}I^{1/2}$, where k^{i}_{assn} is the association rate constant at an ionic strength I, $k_{assn}{}^{o}$ is the association rate constant at zero ionic strength (by extrapolation), and z_{Ag} and z_{Ab} are charges on the antigen and antibody, respectively (Alberty and Hammes, 1958; Hammes, 1978), although the equation is strictly applicable only at low ionic strength.

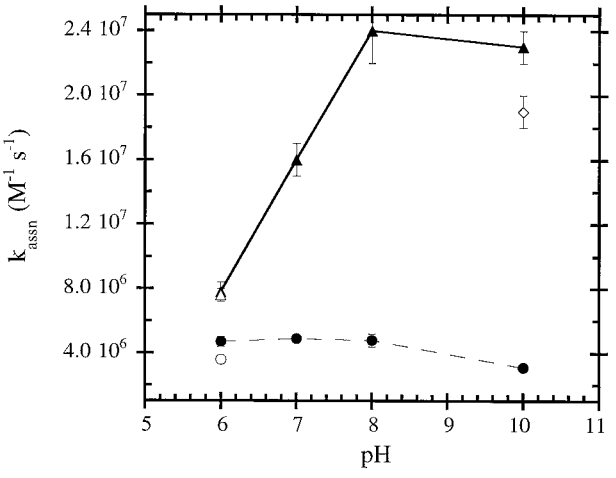

FIGURE 7 Plot showing the pH dependence of k_{assn} for the association of HyHEL-5 (▲, △, ◇) and HyHEL-10 (●, ○) with HEL at 25°C and 150 mM ionic strength. The experiments were performed in 10 mM sodium phosphate (▲, ●), 10 mM MES (▲, ●), and 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (◇), respectively.

pH dependence of association rate constants

Electrostatic calculations on the HyHEL-5/HEL system showed that several residues, especially those involved in the salt links, had pK_a values that changed significantly on formation of the antibody/antigen complex (McDonald et al., 1995). In contrast, isothermal titration calorimetry studies using buffers with different enthalpies of ionization for the association of HEL with HyHEL-5 (Hibbits et al., 1994) and HyHEL-10 (Shick and Willson, unpublished results) did not show significant proton linkage effects. The effect of pH on the association rate constant was determined in the pH range 6.0–10.0, to investigate the effect of net charge on the rate constant for the two antibodies interacting with HEL. The k_{assn} for the interaction of HyHEL-5 with HEL is significantly more pH sensitive and declines at pH near the estimated pI (Fig. 7).

Dissociation kinetics of the HyHEL-5/HEL and the HyHEL-10/HEL complexes

The dissociation of the antibody/antigen complexes was studied by the addition of excess unlabeled lysozyme (20-fold, except where noted) to preformed antibody/fluoresceinated lysozyme 1:2 complexes (Fig. 8). In control experiments, when buffer was added instead of unlabeled lysozyme (change in total volume of less than 0.5%), the anisotropy was stable to within ±0.002 for 400 min. The dissociation rate constant (k_{diss}) for the HyHEL-5/HEL system is $2.20 \pm 0.02 \times 10^{-4}$ s^{-1} at 25°C, pH 8.0, and an ionic strength of 150 mM. A k_{diss} of 2.13×10^{-4} s^{-1} was obtained under the same conditions in an experiment using an 80-fold excess of unlabeled lysozyme, indicating that the dissociation process is first order. In experiments to determine k_{diss} for the HyHEL-10/HEL system, data could be obtained only for the first 390 min, because after the first 6.5 h the change in anisotropy between measurements was less than the error involved in the measurement. These results were analyzed assuming that the anisotropy of

TABLE 4 Ionic strength dependence of antibody/protein antigen association rate constants*

Ionic strength (mM)	2B5/cytochrome *c* (experiment)[#]	HyHEL-5/HEL (experiment)	HyHEL-5/HEL (computation)[§]	HyHEL-10/HEL (experiment)
5	1×10^6	—	8.6×10^6	—
27	—	$3.3 \pm 0.2 \times 10^7$	—	$9.3 \pm 0.4 \times 10^6$
50	6×10^5	$2.9 \pm 0.4 \times 10^7$	2.4×10^6	$6.7 \pm 0.4 \times 10^6$
150	4×10^5	$2.4 \pm 0.2 \times 10^7$	1.7×10^6	$4.8 \pm 0.4 \times 10^6$
≥500	$3 \times 10^{5\P}$	$1.5 \pm 0.1 \times 10^{7\|\|}$	$8.0 \times 10^{5\P}$	$4.1 \pm 0.2 \times 10^{6\|\|}$

*Association rate constants are in units of M^{-1} s^{-1}.
[#]Raman et al. (1992).
[§]Kozack et al. (1995).
[¶]Determined at an ionic strength of 1.0 M.
[||]Determined at an ionic strength of 500 mM.

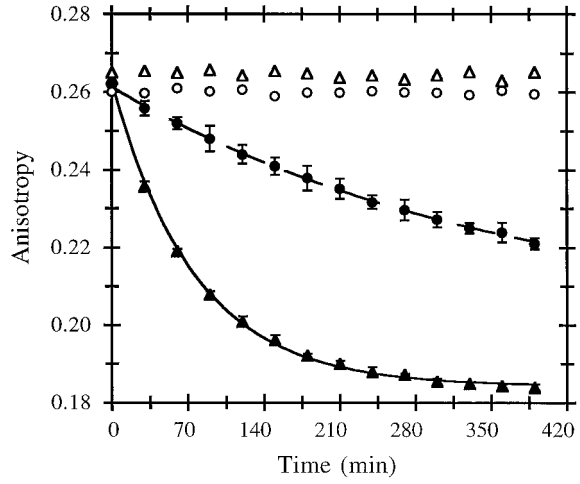

FIGURE 8 Change in anisotropy with time of preformed HyHEL-5/HEL (▲) and HyHEL-10/HEL (●) complexes on the addition of 20-fold excess of unlabeled HEL. Unfilled symbols are the anisotropy of corresponding antibody/antigen complexes to which buffer was added in place of unlabeled lysozyme. These experiments were performed at 25°C in 10 mM sodium phosphate, pH 8.0, with sufficient NaCl added to adjust the ionic strength to 150 mM; the data were fitted to Eq. 2.

"fully" dissociated HyHEL-10/HEL complex was similar to that obtained experimentally for the HyHEL-5/HEL complex. The dissociation rate constant for the HyHEL-10/HEL system is $\sim 3.2 \pm 0.2 \times 10^{-5}$ s^{-1} at 25°C, pH 8.0, and an ionic strength of 150 mM. The k_{diss} from a control experiment with an 80-fold excess of nonlabeled lysozyme was $\sim 3.7 \times 10^{-5}$ s^{-1}; thus the dissociation process appears to be first order (the difference between the two cases is probably due to the inaccuracy in the value used for the anisotropy of the dissociated HyHEL-10/HEL complex). Thus the half-life of the HyHEL-10/HEL complex is about sevenfold longer than that of the HyHEL-5/HEL complex.

For both HyHEL-5/HEL and HyHEL-10/HEL systems, the association is second order and the dissociation is first order; hence it is possible to estimate the equilibrium dissociation constant from the kinetic results (Table 5). The equilibrium dissociation constants obtained from the kinetic data are in reasonable agreement with literature values determined by particle concentration fluorescence immunoassay (PCFIA), considering differences in the assay conditions (Lavoie et al., 1990, 1992; using the methodology developed by Friguet et al. (1985) for measuring solution affinity constants using a solid-phase assay). Thus a model with a single "encounter complex" appears to be adequate to account for the experimentally determined association and dissociation rate constants for the interaction of HEL with both HyHEL-5 and HyHEL-10. The affinities of these two secondary response antibodies are close to the range suggested as an affinity ceiling for antibodies produced in normal immune responses (Foote and Eisen, 1995).

TABLE 5 Rate and equilibrium constants for the HyHEL-5/HEL and the HyHEL-10/HEL systems*

System	k_{assn}[#] (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (pM) (calculated)	K_d (pM) (literature)
HyHEL-5/HEL	$1.5–3.3 \times 10^7$	2.2×10^{-4}	6.7–14.7	~25[§]
HyHEL-10/HEL	$4.1–9.3 \times 10^6$	$\sim 3.2 \times 10^{-5}$	3.4–7.8	~22[¶]

*Sodium phosphate buffer was used at pH 8.0 and 25°C.
[#]Range of k_{assn} values is given for different ionic strengths.
[§]Determined by PCFIA (Lavoie et al., 1990).
[¶]Determined by PCFIA (Lavoie et al., 1992).

DISCUSSION

Determination of the association and dissociation rate constants for the interaction of HyHEL-5 and HyHEL-10 with HEL can help clarify the mechanism(s) and energetics of these protein-protein interactions. The association of both HyHEL-5 and HyHEL-10 with lysozyme follows a simple bimolecular rate expression. The other kinetically characterized monoclonal antibody/protein antigen systems, D1.3 binding HEL (Foote and Winter, 1992) and anti-cytochrome *c* antibodies 2B5 and 5F8 binding cytochrome *c* (Raman et al., 1992), also show bimolecular association. The dissociation of HyHEL-5/HEL and HyHEL-10/HEL complexes follows first-order kinetics. The equilibrium association constants calculated from k_{assn} and k_{diss} for HyHEL-5/HEL and HyHEL-10/HEL systems are in reasonable agreement with the values obtained by equilibrium measurements (Lavoie et al., 1990, 1992). In contrast, large differences in kinetics-derived and directly determined equilibrium association constants for monoclonal antibodies raised against the C-terminal fragment (F_2) of *E. coli* tryptophan synthase β_2 subunit, binding polypeptide fragments of F_2 obtained by chemical cleavage, have been suggested to be due to conformational adaptation (Friguet et al., 1989). Similar conformational adaptation has also been observed in the cross-reactions of monoclonal antibodies raised against different coiled-coil peptides (Leder et al., 1995) and for the cross-reaction of turkey egg lysozyme with anti-HEL antibody D1.3 (Braden et al., 1996).

The association of both antibodies with lysozyme follows Arrhenius kinetics, and $\Delta H^{\ddagger}$ is 6.0 kcal mol^{-1} and 9.4 kcal mol^{-1} for HyHEL-5/HEL and HyHEL-10/HEL systems, respectively. The $\Delta H^{\ddagger}$ for the association of the D1.3 Fv fragment and D1.3 mutants is in the range 5.4–9.2 kcal mol^{-1} (Ito et al., 1995). The anti-cytochrome *c* antibodies, 2B5 and 5F8, have $\Delta H^{\ddagger}$ of 5.5 kcal mol^{-1} and 3.8 kcal mol^{-1}, respectively. At 298 K, $T\Delta S^{\ddagger}$ for HyHEL-5/HEL association is an unfavorable -1.4 kcal mol^{-1}; in contrast, the $T\Delta S^{\ddagger}$ for HyHEL-10/HEL association is a favorable 1.0 kcal mol^{-1}. For D1.3 mutants binding HEL, the $T\Delta S^{\ddagger}$ is in the range -3.3–0.6 kcal mol^{-1} (Ito et al., 1995), whereas 2B5 and 5F8 bind cytochrome *c* with significantly unfavorable activation entropies of -3.8 kcal mol^{-1} and -5.2 kcal mol^{-1}, respectively. The significant difference in the values of $\Delta G^{\ddagger}$ for these antibody/antigen systems (although HyHEL-5, HyHEL-10, 2B5, and 5F8 all appear, based on the viscosity dependence of k_{assn}, to be substantially diffusion-

limited) makes a mechanistic interpretation of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ difficult. In the formation of the encounter complex, significant solvent rearrangement and loss of translational and rotational entropy of both proteins is expected. The $T\Delta S^{\ddagger}$ values observed for the various antibody/antigen systems suggest that the net contribution of these processes to association is dependent on the particular system, whereas $\Delta H^{\ddagger}$ always contributes unfavorably to formation of the encounter complex. Rearrangement of solvent molecules could result in a positive contribution to $\Delta H^{\ddagger}$ and a negative contribution to $\Delta S^{\ddagger}$ (Privalov and Gill, 1988). For the association of D1.3 with HEL, solvent rearrangement at the interface has been observed (Braden et al., 1995b). Osmotic pressure experiments also indicate the importance of water molecules in D1.3/HEL (Goldbaum et al., 1996) and HyHEL-5/BWQL (Xavier et al., 1997) association. In addition to diffusional constraints, chemical and energetic barriers may also be important in the association of both HyHEL-5 and HyHEL-10 with HEL.

The diffusion coefficients for lysozyme and IgG are 11.2×10^{-7} cm^2 s^{-1} and 4.0×10^{-7} cm^2 s^{-1}, and the radii of gyration are 15.2 Å and 70 Å, respectively (Tyn and Gusek, 1990). Using the fractional area buried when lysozyme interacts with HyHEL-5 or HyHEL-10 (Davies and Cohen, 1996) to calculate the unitless interaction parameter κ (determined by the fractions of the surfaces of the antigen and antibody that interact), the modified Smoluchowski equation (von Hippel and Berg, 1989),

$$k_{assn} = 4000\pi r_{AB}\, \kappa f N_A (D_A + D_B), \qquad (10)$$

where f is a unitless factor (here assumed to be 1.0) to account for electrostatic attraction or repulsion between the two interacting molecules, gives a diffusion-limited association rate constant of $\sim 4\text{–}7 \times 10^7$ M^{-1} s^{-1}. The k_{assn} for the binding of HyHEL-5 with HEL is only about twofold lower than this calculated diffusion limit, and hence the HyHEL-5/HEL binding appears to be largely diffusion-controlled. The k_{assn} values for the binding of antibody and antigen in the HyHEL-10/HEL, D1.3/HEL (Foote and Winter, 1992), 2B5/cytochrome *c*, and 5F8/cytochrome *c* (Raman et al., 1992) systems are about an order of magnitude lower than the calculated "diffusion limit"; the values of k_{assn} are 4.8, 1.4, 0.65, and 1.5 ($\times 10^6$ M^{-1} s^{-1}), respectively. (HEL and cytochrome *c* are of similar size and hence would have similar diffusion coefficients.) However, the binding of antibodies HyHEL-5, HyHEL-10, 2B5, and 5F8 with their respective antigens is substantially diffusion-limited on the basis of experiments using viscosity modifiers. The factors responsible for this difference could be orientational (Berg and von Hippel, 1985; Berg, 1985), hydrodynamic (Berg and von Hippel, 1985), varying effectiveness of diffusive entrapment (Northrup and Erickson, 1992), electrostatic, etc. The association rate constants for these antibody/antigen systems are in the range of values observed for protein-protein association (Fersht, 1985).

Molecules interacting after coming together via diffusion may experience many collisions before separation, and may undergo different relative orientations during these collisions. Usually diffusion control is indicated by linear viscosity dependence of the normalized association rate constant. It is often assumed that such a result implies rate limitation at the stage of approach of the two molecules from their initial wide separation. It appears that linear viscosity dependence also applies to more complicated binding interactions with orientational constraints and short-range interactions (Berg and von Hippel, 1985). The association rate constant for both HyHEL-5 and HyHEL-10 binding with lysozyme decreases nonlinearly with increasing viscosity. The decrease in the association rate is greater than that expected for simple diffusion control (Eq. 9), suggesting that in addition to translation at large separation, there may be another limiting process. The experimentally determined association rate constant is often considered as the sum of two individual "resistances" of diffusion and intrinsic reaction in series (Lauffenberger and Linderman, 1993). However, this model does not fit our data well with positive constants. Although association is clearly limited by a viscosity-responsive process, the nonunitary dependence on viscosity, the relatively high enthalpies of activation, and the opposite signs of the entropies of activation suggest the action of other effects in the overall process of association. The greater than theoretically expected decrease in the association rate constant could be due to osmotic pressure effects of the cosolute on the binding process (Xavier et al., 1997). It is unlikely that the cosolutes, glycerol and sucrose, induce any significant conformational changes in the antigen or the antibody; however, there was evidence for a small chemical effect with sucrose for the HyHEL-5/BWQL system (Xavier and Willson, unpublished results). Furthermore, the similar association rates of HyHEL-5 with HEL at similar viscosities in the presence of glycerol and sucrose suggest that the cosolutes act primarily as viscosity modifiers.

The association rate for the interaction of HyHEL-5 with HEL is about an order of magnitude greater than the association rate constants for other monoclonal antibodies interacting with protein antigens of similar size (Table 4). Kozack et al. (1995) have suggested that the charge complementarity between the binding sites of the antibody and antigen in the case of the HyHEL-5/HEL system may be important in "steering" the two proteins during the association process, thereby overcoming some of the orientational constraints on protein-protein association. The association rate for the HyHEL-5/HEL interaction increases by about twofold as the ionic strength is decreased from 500 mM ("no-steering" condition, due to effective shielding of the charges) to 27 mM; this is similar to the increase in association rate constant observed for HyHEL-10, 2B5, and 5F8 binding their respective antigens. This increase in association rate constants with decreasing ionic strength could be due to the augmentation of the complementary electrostatic fields. In a study of electrostatic potentials of antibody/antigen complexes at physiological salt concentrations (equivalent to 150 mM NaCl), the electrostatic fields did not

extend far into the solvent (~4 Å; Novotny and Sharp, 1992). It was also suggested that the electrostatic interactions between proteins essentially oppose complex formation because of the energetic cost of desolvation, and that the charge complementarity of the two protein surfaces primarily defines specificity (Novotny and Sharp, 1992). The increased rate constants for the binding of 2B5 and 5F8 with cytochrome *c* with decreasing ionic strength, qualitatively similar to that observed with the anti-lysozyme antibodies, is probably due to the opposite net charges on the two proteins at the experimental pH of 7.0 (Raman et al., 1992). Studies are presently under way to determine the effects on the association kinetics of mutating the two glutamic acids in the HyHEL-5 binding site.

To further understand the effect of electrostatic fields of the two proteins on the association process, the association rate constants for the interaction of the two antibodies with lysozyme were studied as a function of pH, in the range 6.0–10.0. It was observed that HyHEL-5 association with HEL is pH-sensitive and that of HyHEL-10 with HEL is only slightly affected by pH. The pI of the two antibodies is expected to be in the pH range 6.0–10.0; hence an explanation based only on the net charge of the proteins cannot explain the observed HyHEL-10/HEL results. A significant difference between the two antibodies with respect to their interaction with HEL is that HyHEL-5 forms two salt links with HEL (Cohen et al., 1996), whereas HyHEL-10 forms a solvent-exposed weak salt link between Asp-H32 and Lys^{97} of HEL (Padlan et al., 1989). Mutation of Asp-H32 to Ala, Asn, or Glu showed that this salt link was not energetically significant (Tsumoto et al., 1996). Based on the results of electrostatic calculations made by McDonald et al. (1995), the arginines and glutamic acids at the interface may contribute to the observed pH dependence of HyHEL-5/HEL association. It is likely that pK_a changes may occur after formation of the "encounter complex," which could then improve the probability of complex formation. Experiments with mutants of both antibodies and HEL are under way to further address these issues.

The dissociation of HyHEL-5/HEL and HyHEL-10/HEL complexes follows first-order kinetics with half-lives at 25°C of ~3,150 s and ~21,660 s, respectively. The functionally significant residues in the HyHEL-5/HEL interface are the HEL arginines 45 and 68 and the antibody glutamic acids H35 and H50 (Novotny et al., 1989; Lavoie et al., 1990), whereas the binding site of HyHEL-10 contains a disproportionate number of aromatic side chains that interact with the antigen (Padlan et al., 1989). The interaction energetics of human growth hormone with a panel of monoclonal antibodies (Jin et al., 1992; Jin and Wells, 1994) and its receptor (Clackson and Wells, 1995; reviewed in Wells, 1996) is dominated by a few residues. If the interaction of the two high-affinity secondary immune response antibodies, HyHEL-5 and HyHEL-10, with HEL is significantly affected by a few residues, as has been suggested by the studies to date, then the two antibodies achieve similar affinities for HEL by using different strategies, as evidenced by the difference in their association and dissociation rate constants, and the type of residues involved in the protein-protein interactions.

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