Association and dissociation kinetics of bobwhite quail lysozyme with monoclonal antibody HyHEL-5

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The anti-hen egg lysozyme monoclonal antibody HyHEL-5 and its complexes with various species-variant and mutant lysozymes have been the subject of considerable experimental and theoretical investigation. The affinity of HyHEL-5 for bobwhite quail lysozyme (BWQL) is over 1000-fold lower than its affinity for the original antigen, hen egg lysozyme (HEL). This difference is believed to arise almost entirely from the replacement in BWQL of Arg68 by lysine. In this study, the association and dissociation kinetics of BWQL with HyHEL-5 were investigated under a variety of conditions and compared with previous results for HEL. HyHEL-5–BWQL association follows a bimolecular mechanism and the dissociation of the antibody–antigen complex is a first-order process. Changes in ionic strength (from 27 to 500 mM) and pH (from 6.0 to 10.0) produced about a 2-fold change in the association and dissociation rates. The effect of viscosity modifiers on the association reaction was also studied. The large difference in the HEL and BWQL affinities for HyHEL-5 is essentially due to differences in the dissociation rate constant.

Keywords: antibody/fluorescence polarization spectroscopy/lysozyme/stopped-flow kinetics

Introduction

The availability of crystal structures of antibody–protein antigen complexes has paved the way for a detailed thermodynamic and kinetic characterization of antibody–protein interactions. The most studied protein antigen is hen egg lysozyme (HEL). Many structures of HEL, avian species-variant and mutant lysozymes in complexes with antibodies have been determined in the past 10 years (reviewed by Davies and Cohen, 1996). The crystal structures of HyHEL-5 Fab in complexes with HEL (Sheriff et al., 1987; Cohen et al., 1996), BWQL (Chacko et al., 1996) and the R68K mutant of HEL (Chacko et al., 1995) have been solved. In both variant complexes, the Arg68→Lys mutation was observed to be accompanied by the insertion of a water molecule which mediates hydrogen bonding interactions between the shorter Lys68 side chain and residues on the HyHEL-5 Fab. The structures of BWQL free and complexed with HyHEL-5 showed no major backbone conformational changes in lysozyme upon complex formation, although side chain conformational differences were seen in surface residues involved in interaction(s) with the antibody.

Association of HEL and BWQL with HyHEL-5 is enthalpically driven and entropically unfavorable near physiological temperatures (Hibbits et al., 1994; Shick et al., 1997). The association constant for the interaction of HyHEL-5 with HEL is about 4×10¹⁰ M⁻¹ (Lavoie et al., 1990; Xavier et al., 1997). The association constant for the HyHEL-5–BWQL system is over three orders of magnitude lower, 1.5×10⁷ M⁻¹ by particle concentration fluorescence immunoassay (PCFIA) (Lavoie et al., 1990), 5×10⁶ M⁻¹ by fluorescence polarization spectroscopy (Xavier et al., 1997) and 8×10⁵ M⁻¹ by isothermal titration calorimetry (ITC) (Shick et al., 1997).

The most prominent structural and energetic feature of the HyHEL-5–HEL complex is the close contact between HEL arginines (Arg45 and Arg68) and 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) (‘H’ and ‘L’ represent antibody heavy and light chains, respectively, and ‘Y’ represents lysozyme). Calculations of pKₐ values of residues in the HyHEL-5–HEL complex and the proteins individually using a grid-based electrostatics model (McDonald et al., 1995) showed that the pKₐ values of several residues, including those involved in the Arg–Glu salt links, change significantly upon complex formation. Investigation of the role of electrostatics in the HyHEL-5–HEL system using a multigrid-based Newton method for solving the nonlinear Poisson–Boltzmann equation (Slagle et al., 1994) revealed that counterions neutralizing or reversing the charge of any of the residues involved in salt links in the HyHEL-5–HEL system always resulted in decreased binding affinities. In Brownian dynamics simulations of molecular recognition in the HyHEL-5–HEL system (Kozack and Subramaniam, 1993; Kozack et al., 1995) charged residues, particularly Glu-H35 and Glu-H50 of HyHEL-5, were predicted to exert a considerable influence on steering the two proteins into a favorable configuration for binding.

In this work, we studied the association and dissociation kinetics of the HyHEL-5–BWQL system by stopped-flow fluorescence polarization spectroscopy to examine the kinetic effects of the R68K mutation. The effects of temperature,
viscosity, ionic strength and pH on the association and dissociation rate constants were determined. The titration behaviors of individual amino acids of both the HyHEL-5–HEL and the HyHEL-5–BWQL complexes were predicted by continuum electrostatic methods (Gibas et al., 1997). The experimental results along with modeling insights suggest plausible explanations for the observed HyHEL-5 affinity difference between HEL and BWQL.

Materials and methods

Protein purification and labeling

HyHEL-5 was produced and purified as described by Xavier et al. (1997). Bobwhite quail eggs were obtained from Steven- son Game Bird Farm (Riverside, TX) and BWQL was purified according to the protocol of Arnheim et al. (1969), as modified by Shick et al. (1997).

BWQL was labeled with fluorescein isothiocyanate (Molecular Probes, Eugene, OR) using the protocol recommended by the manufacturer. Briefly, BWQL was labeled in sodium hydrogen carbonate buffer (pH 7.5) and dialyzed extensively to remove free dye. A protein fraction with a cut-on filter (Omega Optical, Brattleboro, VT) on the emission monochromator to minimize photobleaching and a 520 nm Thompson polarizers, a manual shutter after the excitation Experiments were performed in the L-format with Glan–

A SPEX Fluorolog 212 fluorimeter (Instruments SA, Edison, NJ) with an SFA-20 stopped-flow kinetics accessory (Hi-Tech, Salisbury, UK) was used to determine the combination of antibody and antigen concentrations, each divided by the appropriate antibody concentration. Linear regression of $k_{obs}$ versus antibody concentration gave similar results, within experimental error. The stopped-flow technique was used to study the dissociation kinetics of the HyHEL-5–BWQL system owing to the short lifetime of the complex. These experiments were performed by rapidly adding a 5- or 10-fold excess of unlabeled BWQL to a preformed antibody–labeled BWQL 1:2 complex (50 nM HyHEL-5 and 100 nM fluorescein-labeled BWQL). It was verified using up to 60-fold excess of unlabeled BWQL that the first-order kinetics of dissociation were independent of the competitor BWQL concentration. Experiments were carried out in triplicate at two different concentrations of unlabeled BWQL and the data were fitted to Equation 1.

Results

Temperature dependence of rate constants

The association rate constant for the HyHEL-5–BWQL system was measured in the temperature range 10–32°C (Table 1) and varies from $8.3 \times 10^6$ M$^{-1}$ s$^{-1}$ (10°C) to $2.6 \times 10^7$ M$^{-1}$ s$^{-1}$ (32°C). These values are within 2-fold of those for the HyHEL-5–HEL system in the same temperature range (Figure 1). The activation parameters for the HyHEL-5–BWQL system from the Eyring plot (Eyring and Eyring, 1963) was taken as the average of $k_{obs}$ determined for four different combinations of antibody and antigen concentrations, each divided by the appropriate antibody concentration. Linear regression of $k_{obs}$ versus antibody concentration gave similar results, within experimental error.

The stopped-flow technique was used to study the dissociation kinetics of the HyHEL-5–BWQL system owing to the short lifetime of the complex. These experiments were performed by rapidly adding a 5- or 10-fold excess of unlabeled BWQL to a preformed antibody–labeled BWQL 1:2 complex (50 nM HyHEL-5 and 100 nM fluorescein-labeled BWQL). It was verified using up to 60-fold excess of unlabeled BWQL that the first-order kinetics of dissociation were independent of the competitor BWQL concentration. Experiments were carried out in triplicate at two different concentrations of unlabeled BWQL and the data were fitted to Equation 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$k_{assn}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{diss}$ (s$^{-1}$)</th>
<th>$K_a = {k_{assn}/k_{diss}}$(M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.3 ± 0.7 × 10$^6$</td>
<td>0.10 ± 0.01</td>
<td>8.3 × 10$^7$</td>
</tr>
<tr>
<td>17</td>
<td>1.1 ± 0.1 × 10$^7$</td>
<td>0.31 ± 0.02</td>
<td>3.5 × 10$^7$</td>
</tr>
<tr>
<td>25</td>
<td>1.8 ± 0.3 × 10$^7$</td>
<td>0.96 ± 0.06</td>
<td>1.9 × 10$^7$</td>
</tr>
<tr>
<td>32</td>
<td>2.6 ± 0.6 × 10$^7$</td>
<td>2.7 ± 0.3</td>
<td>9.6 × 10$^6$</td>
</tr>
<tr>
<td>Relative viscosity$^b$</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sucrose:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (0% w/w)</td>
<td>1.8 ± 0.3 × 10$^7$</td>
<td>0.96 ± 0.06</td>
<td>1.9 × 10$^7$</td>
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<tr>
<td>Glycerol$^b$:</td>
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</tr>
<tr>
<td>1.00 (0% w/w)</td>
<td>1.8 ± 0.3 × 10$^7$</td>
<td>0.96 ± 0.06</td>
<td>1.9 × 10$^7$</td>
</tr>
<tr>
<td>Ionic strength (mM)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>27</td>
<td>2.3 ± 0.3 × 10$^7$</td>
<td>1.2 ± 0.05</td>
<td>1.9 × 10$^7$</td>
</tr>
<tr>
<td>50</td>
<td>2.0 ± 0.3 × 10$^7$</td>
<td>1.12 ± 0.07</td>
<td>1.8 × 10$^7$</td>
</tr>
<tr>
<td>150</td>
<td>1.8 ± 0.3 × 10$^7$</td>
<td>0.96 ± 0.06</td>
<td>1.9 × 10$^7$</td>
</tr>
<tr>
<td>500</td>
<td>1.2 ± 0.1 × 10$^7$</td>
<td>0.75 ± 0.09</td>
<td>1.6 × 10$^7$</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>9.0 ± 2.2 × 10$^6$</td>
<td>1.4 ± 0.2</td>
<td>6.4 × 10$^6$</td>
</tr>
<tr>
<td>7.0</td>
<td>1.3 ± 0.2 × 10$^7$</td>
<td>0.93 ± 0.06</td>
<td>1.4 × 10$^7$</td>
</tr>
<tr>
<td>8.0</td>
<td>1.8 ± 0.3 × 10$^7$</td>
<td>0.96 ± 0.06</td>
<td>1.9 × 10$^7$</td>
</tr>
<tr>
<td>10.0</td>
<td>1.6 ± 0.2 × 10$^7$</td>
<td>1.4 ± 0.1</td>
<td>1.1 × 10$^7$</td>
</tr>
</tbody>
</table>

aAll experiments were carried out in 10 mM sodium phosphate, with the basis of comparison being 25°C, pH 8.0 and an ionic strength of 150 mM.

bThe viscosities were determined using a Brookfield digital viscometer (Model DV-II+®) and agreed with published values (Wolf et al., 1988).

cFrom Xavier et al. (1997).
(Figure 1) are $\Delta H^\ddagger = 8.5$ kcal/mol, $\Delta S^\ddagger = 3.2$ cal/mol.K and $\Delta G^\ddagger (298 \text{ K}) = 7.5$ kcal/mol. The corresponding values for the HyHEL-5–HEL system are $\Delta H^\ddagger = 6.0$ kcal/mol, $\Delta S^\ddagger = -4.6$ cal/mol.K and $\Delta G^\ddagger (298 \text{ K}) = 7.4$ kcal/mol (Xavier and Willson, 1998). Hence both HEL and BWQL have similar rates and activation free energy barriers for interacting with HyHEL-5, but interestingly $\Delta S^\ddagger$ has opposite signs for the two systems.

The effect of temperature on the dissociation rate constant for the HyHEL-5–BWQL complex is also tabulated in Table I. The dissociation rate constant shows an Arrhenius dependence with Eyring parameters $\Delta H^\ddagger = 24.9$ kcal/mol, $\Delta S^\ddagger = 25.2$ cal/mol.K and $\Delta G^\ddagger (298 \text{ K}) = 17.4$ kcal/mol. At 25°C, pH 8.0 and an ionic strength of 150 mM, the dissociation rate constant for the HyHEL-5–HEL complex is $2.2 \times 10^{-4}$ s$^{-1}$, whereas for the same conditions the dissociation rate constant for the HyHEL-5–BWQL complex is 0.96 s$^{-1}$. The Arg68→Lys mutation, therefore, has only a slight effect on the association reaction but produces a reduction of four orders of magnitude in the half-life of the antibody–antigen complex.

Both association and dissociation rate constants increase with increasing temperature and the $k_{\text{diss}}$ shows a larger increase (27-fold vs 3-fold) than $k_{\text{assn}}$, leading to a lower thermodynamic stability of the HyHEL-5–BWQL complex at higher temperature.

**Viscosity dependence of association rate constants**

In the presence of increasing concentrations of sucrose and glycerol, the association rate constant for the HyHEL-5–BWQL system shows the same dependence on viscosity as the HyHEL-5–HEL system (Figure 2). While a given increase in viscosity produces the same decrease in association rate constant whether produced by addition of sucrose or glycerol, the dependence on viscosity is not the simple inverse relationship expected for a strictly diffusion-controlled reaction. In addition, these solutes differ in their influence on dissociation rate constant, possibly owing to interactions of sucrose with HEL at high concentrations (K.A.Xavier and R.C.Willson, unpublished results).

**Ionic strength dependence of rate constants**

The association rate constants of HyHEL-5 with HEL and BWQL were determined at different ionic strengths to probe the importance of the computationally observed 'electrostatic steering' in this antibody–antigen system. On increasing the ionic strength from 27 to 500 mM, the rate constant decreased by about 2-fold for both HyHEL-5–HEL and HyHEL-5–BWQL association (Table I and Figure 3). The dissociation rate constant for the HyHEL-5–BWQL changed by less than 2-fold in the same ionic strength range (Table I). The changes in the association and dissociation rate constants over the ionic strength range offset each other, resulting in an equilibrium association constant that changed little with ionic strength.

**pH dependence of rate constants**

Isothermal titration calorimetric experiments with the HyHEL-5–BWQL system in buffers with different enthalpies of ionization suggested the possibility of a small proton-linkage effect (Shick et al., 1997), whereas no such effect was observed for the HyHEL-5–HEL system (Hibbits et al., 1994). The effect of pH on the association and dissociation rate constants for the interaction of HyHEL-5 with BWQL was determined (Table I) to investigate the effect of net charge on the rate constants. In the pH range 6.0–10.0 both HEL and BWQL are positively charged (Righetti et al., 1981), while mouse IgGs usually have pI between 6.0 and 7.5 (Hamilton et al., 1987; Raman et al., 1992). On changing the pH from 6.0 to 8.0, $k_{\text{assn}}$ increases by 2-fold for HyHEL-5–BWQL
association, whereas it increases 3-fold for HyHEL-5–HEL association (Figure 4). In the pH range 6.0–10.0, the dissociation rate constant for the HyHEL-5–BWQL system changes by less than 2-fold, with the change occurring in the pH region near the estimated $pI$ of the antibody.

Equilibrium association constants

The equilibrium association constant ($K_a$) for the HyHEL-5–BWQL system calculated from the kinetic data at 25°C and pH 8.0 is $1.9 \times 10^7$ M$^{-1}$ (Table I). This is in reasonable agreement with the $K_a$ values that were obtained by PCFIA ($1.5 \times 10^7$ M$^{-1}$) (Lavie et al., 1990), fluorescence polarization spectroscopy ($5 \times 10^6$ M$^{-1}$) (Xavier et al., 1997) and isothermal titration calorimetry ($8 \times 10^6$ M$^{-1}$) (Shick et al., 1997), considering the differences in assay conditions.

Discussion

Most differences in the structures of the HyHEL-5–BWQL complex (Chacko et al., 1996) and the HyHEL-5–HEL complex (Sheriff et al., 1987; Cohen et al., 1996) involve the salt link interactions and hydrogen bonding between the two molecules. These structures suggest that the contacts between BWQL and the antibody are not as close as those with HEL and that the interface may be more accessible to water molecules (although the BWQL complex structure is not yet at a sufficient resolution to resolve many bound water molecules). Osmotic stress experiments indicate that at least 6–12 water molecules are taken up in the association of HyHEL-5 with BWQL (Xavier et al., 1997). In both the HyHEL-5–R68K-HEL and HyHEL-5–BWQL complexes a water molecule has been observed bound in place of the terminal nitrogens of HEL Arg68 (Chacko et al., 1995, 1996). There are also fewer hydrogen bonds between HyHEL-5 and BWQL than between HyHEL-5 and HEL (see Table 4 in Chacko et al., 1996). In particular, the hydrogen bonds to Lys68 of BWQL are fewer in number and are mediated by water molecules.

The association of both BWQL and HEL with HyHEL-5 is primarily diffusion limited, as suggested by the effect of viscosity modifiers on the association rate constants and by the association rate constants being within an order of magnitude of the calculated diffusion limit for molecules of this size ($4–7 \times 10^7$ M$^{-1}$ s$^{-1}$) using the modified Smoluchowski equation (von Hippel and Berg, 1989; Xavier and Willson, 1998). The decrease in the association rate is greater than that expected for simple diffusion control, suggesting that in addition to translation at large separation, there may be other limiting processes. The factors responsible could include orientational (Berg, 1985; Berg and von Hippel, 1985), hydrodynamic (Berg and von Hippel, 1985), varying effectiveness of diffusive entrapment (Northrup and Erickson, 1992) or electrostatic changes or titration. The activation energetics for the two systems, however, are significantly different. The activation entropy, enthalpy and free energy are all likely to include contributions from the solvent since the kinetics suggest diffusion-influenced reaction (for instance, from the temperature dependence of the solvent viscosity). The association kinetics of the two systems also show similar dependences on ionic strength and pH. Comparison with the previously reported ionic strength dependence of the association rate constants for monoclonal antibodies against small globular proteins (Raman et al., 1992; Xavier and Willson, 1998), suggests that the observed increase in $k_{assn}$ of about 2-fold on decreasing the ionic strength from 500 mM (no 'steering') to 27 mM could be simply due to decreased screening of the opposite net charges of the two proteins at the given pH. The $k_{assn}$ shows a 2-fold reduction with decreasing pH, with much of the reduction occurring in the pH region near the estimated $pI$ of the antibody. The half-life of the HyHEL-5–BWQL complex shows a more even value with changing pH. These 2-fold or smaller changes in the association and dissociation rate constants can be compared with the four orders of magnitude change in the dissociation rate constant for the two complexes. Our data show that the major kinetic effect of the Arg68→Lys mutation is on the half-life of the antibody–antigen complex.

The energetic effects of the Arg68→Lys substitution are illustrated by comparison of isothermal titration calorimetric results for HyHEL-5 association with BWQL and CQL (Shick et al., 1997). Like BWQL, CQL bears the three ‘quail’ mutations T40S, I55V and S91T, but has arginine at position 68. At 25°C the $\Delta G^\circ$ (BWQL→CQL) of 5.1 kcal/mol arises approximately one-quarter from change in association enthalpy ($\Delta H^\circ = 1.3$ kcal/mol) and the remainder from a change in entropy [$\Delta(\Delta S^\circ) = -3.8$ kcal/mol]. There is a loss of at least one hydrogen bond between HyHEL-5–HEL and HyHEL-5–BWQL complexes (Chacko et al., 1996), which may account partially for the observed loss of enthalpic driving force. An additional water molecule is bound in place of the terminal nitrogens of HEL Arg68 in the HyHEL-5–BWQL complex, which may be responsible for some of the observed unfavorable entropy of interaction. An estimate of the unfavorable entropic contribution associated with the binding of a single water molecule in the complexes that have Lys in place of Arg is ~3 kcal/mol (Fersht, 1985).

The HyHEL-5–HEL and HyHEL-5–BWQL systems show similar patterns of $pK_a$ shifts and protonation behavior upon association by continuum electrostatic methods (Gibas et al., 1997). A significant shift in $pK_a$ values was seen for several residues of the HyHEL-5 antibody and a few residues of HEL upon complexation (McDonald et al., 1995) and similar shifts were observed for the HyHEL-5–BWQL system (Gibas et al., 1997). Residues with large shifts included all four residues involved in the salt links (Glu-H35, Glu-H50, Arg-Y45 and Lys-Y68) and other residues in the interface between the two molecules (Tyr-L33, Tyr-H101, Tyr-Y53 and Asp-Y66). Glu-H35 and particularly Glu-H50 show a tendency to deprotonate on complex formation in both systems at low ionic strength. At physiological ionic strength (150 mM) there is little tendency for deprotonation. Nearly all the residues that tended to lose
or gain a proton upon complexation are involved in salt links or hydrogen bonding interactions between the antibody and the antigen. The largest pKₐ shifts and net loss of a proton were seen for Glu-H50, which is involved in salt link interactions with Arg-Y45 and Arg/Lys-Y68. The protonation calculations suggest that the antibody interacts with the two antigens similarly but the thermodynamic driving force is lower for the BWQL system owing to the interface contacts being less intimate.

Based on structural information from crystallography, electrostatic calculations and kinetics of both HEL and BWQL interacting with HyHEL-5, the lower affinity of the HyHEL-5–BWQL system is the result of the lower half-life of the complex, which is probably due to poorer packing of the antibody–antigen interface compared with the HyHEL-5–HEL system.

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References


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Kinetics of anti-lysozyme antibody