Long-Range Changes in a Protein Antigen Due to Antigen–Antibody Interaction†

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ABSTRACT: Amide exchange kinetics were used to probe the conformation of hen egg-white lysozyme complexed with the anti-lysozyme monoclonal antibody HyHEL-5. Following the technique developed by Paterson et al. [(1990) *Science 249*, 755–759] we used two-dimensional NMR to measure amide exchange kinetics of the lysozyme amide protons in the lysozyme–antibody complex. A total of 15 amide protons showed altered exchange kinetics in the presence of the complex. Five of these 15 protons reside on residues that are found within the epitope as defined by X-ray crystallography. Five residues are located at the perimeter of the epitope. The remaining five residues are removed from the epitope. The perturbation of amide exchange rates at sites distant from the epitope indicates that the formation of antigen–antibody complexes can produce changes in the antigen at sites that are quite distant from the structural epitope.

A major goal of immunochemistry for the past few decades has been to define the molecular architecture of protein antigenic determinants (epitopes), the antibody combining site, and the interface formed upon interaction of antigen with antibody. In addition, significant efforts have been made to determine whether conformational changes are induced in either the antigen or antibody upon interaction that might lead to the activation of isotype-specific biological functions.

Toward this goal, recent X-ray crystallographic determination of the structures of antibodies in complex with several haptens (Satow et al., 1986; Herron et al., 1989; Brunger et al., 1991), peptides (Stanfield et al., 1990), carbohydrates (Cygler et al., 1991), and proteins (Amit et al., 1986; Sheriff et al., 1987; Padlan et al., 1989; Colman et al., 1987) has been reported. Although these structures have provided considerable and interesting results, the use of X-ray crystallography to study these interactions is in general limited to those antigen–antibody complexes for which appropriate crystals can be produced.

More recently, Paterson and her colleagues (Paterson et al., 1990) have described an alternative approach to mapping and defining antigenic sites on small proteins in solution. By determining the exchange rate of amide–hydrogens in the presence and absence of a monoclonal antibody (mAb),[1] they were able to define a region on the surface of horse heart cytochrome *c*, comprised of protected residues and their hydrogen bond acceptors, with a solvent-accessible surface area of approximately the same size and general shape of protein epitopes defined by X-ray diffraction methods. This region contained several residues that were determined, by mapping with variant antigens, to lie within the epitope defined by the mAb used (Cooper et al., 1987; Carbone & Paterson, 1985; Oertl et al., 1989; Collawan et al., 1988).

Although their study was of considerable significance, it suffered from the fact that the crystal structure of horse cytochrome *c* in complex with the mAb was not known and thus a direct comparison could not be made between the NMR and X-ray diffraction analyses. The study reported here was designed to overcome this deficiency by performing similar NMR analyses using an antigen (hen egg-white lysozyme; HEL) and a monoclonal antibody (HyHEL-5) for which the crystal structure of the complex has been determined to 2.8-Å resolution (Sheriff et al., 1987). The purpose of this study was 2-fold: to investigate the possibility that formation of the antigen–antibody complex can induce long-range conformational changes in the antigen and to critically test NMR methods for epitope mapping.

Our studies confirm those of Paterson et al. (1990) by demonstrating that amide–hydrogens of amino acids known to be in contact with or buried by antibody may be protected from exchange by interaction with antibody. As postulated by Patterson et al. (1990), we also show that residues outside the antigenic site can be significantly protected and that protection effects can be seen in residues quite distant from the epitope, suggesting long-range antibody-induced changes in the antigen.

MATERIALS AND METHODS

Antigen and Antibody. Hen egg-white lysozyme (HEL) was purchased from Sigma Chemical Co. (St. Louis, MO). The HyHEL-5 mAb was purified from ascites fluid by affinity chromatography on HEL conjugated to Affi-Gel-15 (Bio-Rad).

Hydrogen Exchange on Antibody-Bound HEL. This procedure was carried out essentially as described by Paterson et al. (1990). Affinity-purified HyHEL-5 mAb (400 mg) was conjugated to 65 mL (packed gel volume) of Affi-Gel-15 according to the manufacturer's instructions. This mAb–Affi-Gel matrix was poured into a 2.5-cm-diameter column with a sintered-glass filter and was thoroughly washed with phosphate-buffered saline (PBS), pH 7.4. Sixty milligrams of HEL in 30 mL of PBS, pH 7.4, containing 0.02% sodium azide, was slowly passed over the column which was then washed until the OD_{280} was less than 0.05. Hydrogen exchange was initiated by washing the column with 90 mL of 70 mM sodium phosphate buffer in D_2O, pD 7.25, containing 0.02% sodium azide. Approximately 30 min was required to

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[1] Abbreviations: HEL, hen egg-white lysozyme; HOHAHA, homonuclear Hartmann–Hahn; mAb, monoclonal antibody.

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completely wash the column; thus the zero time point actually corresponds to 30 min of exchange. For the zero point, loading and exchanging were done at 4 °C using cold solutions. For the 14-, 48-, and 96-h time points the column was allowed to stand at room temperature (23 °C) for various times to permit exchange between protein hydrogen and the deuterium in the exchange buffer. The control and the antibody-bound samples for each time point were prepared at the same time, and thus they have identical thermal histories.

At the appropriate times, the column was moved into the cold room (4 °C), and the bound HEL was eluted using cold 0.2 M deuterated acetic acid in D_2O, containing 0.02% sodium azide, pD 2.5. The protein-containing fractions were pooled, and the pD was adjusted to 3.2 with NaOH in D_2O. This solution was concentrated by centrifugation in Centriprep-10 and Centricon-10 (Amicon Corp., Danvers, MA) ultrafiltration tubes that had been prewashed with the elution buffer.

Nonbound control samples were prepared by passing the protein solution over a Sephadex G-50 column preequilibrated with the sodium phosphate/D_2O buffer, pD 7.25. The protein-containing fractions were pooled and allowed to exchange for the various times. At the appropriate interval, these control samples were concentrated and transferred into the deuterated acetic acid/D_2O elution buffer by passage over a Sephadex G-50 column preequilibrated with the pD 2.5 elution buffer. The pD of this final protein solution was adjusted to 3.2, and the protein was concentrated as described above.

To assess the degree of nonspecific interaction between HEL and the column matrix, an anti-staphylococcal nuclease antibody was coupled to Affi-Gel-10 as described above. Both the anti-HEL and the anti-nuclease antibodies are of the class IgG1 and thus would only show sequence differences in the hypervariable region of the immunoglobulin. Direct binding of HEL to the anti-nuclease affinity resin was determined by measuring the amount of unbound HEL as a function of total HEL concentrations in the range of 8–70 μM. Measurements of amide exchange in the presence of the anti-nuclease column were done by dissolving HEL in D_2O buffer and mixing this solution of HEL with the anti-nuclease resin. Under the conditions used for the exchange experiment approximately 25% of the HEL is bound to the resin. After 48 h of exchange the HEL was washed off the anti-nuclease resin, deuterated acetic acid was added to 0.2 M, and the pD was adjusted to 3.8. The protein was concentrated as described above.

NMR Experiments. A HOHAHA (Homonuclear Hartmann–Hahn) experiment (Bax & Davis, 1985) was used to obtain cross peaks between amide and H_α protons. This type of experiment was used because of the increased sensitivity over a COSY (correlation spectroscopy) experiment and the presence of amide–β cross peaks in the HOHAHA spectrum which aided in the assignment of resonances. Experiments were performed on a General Electric Omega spectrometer operating at 500.13 MHz. The sample concentration was 3.0–5.0 mM, and all experiments were performed at 25 °C. A z-filtered pulse sequence was used (Rance, 1987) with the isotropic mixing effected by a 60-ms MLEV17 mixing scheme (Bax & Davis, 1985). The 90° pulse length was 27 μs for the isotropic mixing, and a 90° pulse width of 20 μs was used for the other pulses. The spectral width in both dimensions was 7220 Hz. This was sampled with 2048 complex points in t_2 and 256 complex points in t_1. Quadrature in the t_1 dimension was obtained by shifting the phase of the first pulse by 90°. Each t_1 slice was the sum of 64 scans giving a total data acquisition time of 9 h. The time domain data were processed using FELIX (Hare Research Inc., Woodinville, WA). A cosine-squared function was applied in both dimensions to force the free induction decay to zero at the last data point. The final digital resolution was 3.5 Hz/point in t_2 and 7 Hz/point in t_1.

The intensities of the cross peaks were determined by measuring the amplitude of a t_1 slice through the highest point of a cross peak. Although the apodization function used will introduce some degree of non-Lorentzian character into the line shape, the quantification of cross-peak intensities by measuring peak amplitudes is valid provided the line shape is the same from experiment to experiment. We observed no differences in the line shape of cross peaks in any of the experiments. In order to correct for differences in the protein concentration between samples, the measured intensities were normalized using the cross peak associated with the H5 and H7 protons of Trp108. The relative error associated with each measurement was estimated by taking the ratio between the noise (peak to peak) and the intensity of the cross peak observed in the control spectrum for $t = 0$. Depending on the amplitude of the cross peak, the error ranged from 5% to 15%.

The lifetime (inverse of the rate constant) of the amide protons was determined by fitting the intensities to an exponential function using a nonlinear least-squares program (Johnson & Frasier, 1985). The parameters of the fit were the amplitude and the lifetime. For those amide protons which showed intermediate to long lifetimes the amplitudes obtained by fitting the intensity data from complexed or noncomplexed lysozyme were within the estimated error of the intensity measurement. In situations where the amide proton had a short lifetime when not complexed with the antibody (see Figure 2, Asn46), we added an additional zero time point to the intensity data. The value of this point was the intensity of the corresponding peak in the complexed lysozyme. For the case of exchange in the presence of the anti-nuclease column we measured the signal intensities at 48 h and used this intensity to estimate the lifetime. An exchange experiment with free HEL was performed at the same time.

We express the degree of protection of the amide as a protection factor, f, which is the ratio of the proton–amide lifetime in the complex to that for free lysozyme. Protection factors calculated in this manner can be directly compared to those presented by Paterson et al. (1990).

RESULTS

The HOHAHA spectra for lysozyme exchanged in the presence and absence of antibody are shown in Figure 1. Assignments of the amide–α-proton cross peaks were obtained by comparing the chemical shifts of the amide and H_α protons to those reported by Redfield and Dobson (1988). In crowded regions of the spectra the chemical shifts of the β-protons and the reported amide–proton lifetimes (Pedersen, 1991) were used to confirm the assignments. The chemical shifts of those peaks with significant intensity are given in Table I. Most of the cross peaks which we detected correspond with those defined by Redfield and Dobson (1988) to arise from amide–protons with slow to intermediate exchange rates. We could not detect cross peaks from three slowly exchanging residues: Leu75, Cys80, and Ala95. This may be a consequence of the isotropic mixing time used in the HOHAHA experiment. We were able to detect a number of peaks which were shown to have fast exchange kinetics by Redfield and Dobson. However, these peaks disappeared within 14 h of exchange, and it is not possible to quantify the exchange rate of these peaks with the exchange times used in this study.

Intensities were measured for all NH–C_αH cross peaks in both the control and the bound sample. Selected examples

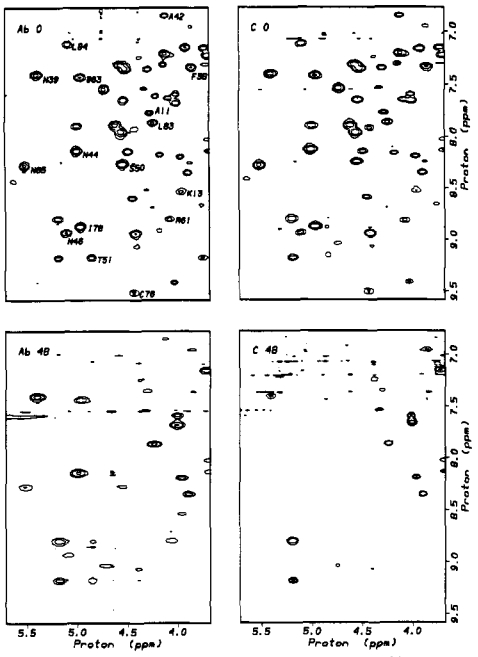

FIGURE 1: NMR spectra of lysozyme under different exchange conditions. Two-dimensional HOHAHA spectra of the amide–α-proton cross-peak region are shown. The f_1 axis is the ordinate, and the f_2 axis is the abscissa. The two left panels show the spectra of lysozyme exchanged in the antibody–antigen complex after 0 h (Ab 0, top left) and 48 h (Ab 48, bottom left). The corresponding controls are shown in the right panels.

of the amide intensity decay curves are shown in Figure 2. These intensities were fit to exponential decays to give the mean lifetimes of the amide–protons shown in Table I. The protection factors, f, are also given in Table I. These were found to be greater than or equal to 1 for all amide–protons with the exception of Leu84, which showed enhanced exchange in the antibody–lysozyme complex. Ratios that are greater than 1.8 were considered significant on the basis of the standard error of the nonlinear regression. For example, compare Asn27 (f = 1.1) to Ala11 (f = 1.9).

The possibility that the altered exchange kinetics are due to nonspecific binding between HEL and the column was investigated by measuring the intensity of the proton resonance lines after 48 h of exchange in the presence of the anti-nuclease affinity column. The signal intensities of the amide–protons of residues Lys13 and Leu83 were sufficiently large to quantify from these spectra. The protection factors for these residues were found to be 1.8 and 1.4, respectively. Signals could not be detected for Ala11, Phe38, Trp63, and Cys76. The absence of signals from these residues places an upper limit on the lifetime to approximately 10–15 h. This estimate of the lifetime in the presence of the anti-nuclease column matrix results in an *upper* limit of the protection factors for residues Ala11, Phe38, Trp63, and Cys76 of 1.1, 2.0, 1.2, and 1.5, respectively. Under the conditions of the experiment (e.g., 25% of the HEL bound to the anti-nuclease column) a protection factor of 1.33 would be observed if the binding of HEL to the anti-nuclease column matrix significantly reduced the exchange rate of the amide–protons. A comparison of the expected versus the observed protection factors indicates that nonspecific binding of HEL to the affinity matrix can result in a decrease of the amide exchange rates of a number of residues. To assess the effect of the nonspecific binding of HEL to the resin during the measurements of exchange rates, it is necessary to determine the amount of HEL nonspecifically bound to the HyHEL-5 affinity matrix. The apparent affinity constant of nonspecific binding of HEL to the anti-nuclease column was found to be 10^4 M^{-1}, and the apparent number of sites was found to be 20 μM. The binding affinity of HEL to HyHEL-5 is 4×10^{10} M^{-1}, with an error of less than 5%. This binding constant was determined by two different sequential saturation affinity assays with essentially identical results. Both were modifications of the method described by Friquet et al. (1985) and are described in detail elsewhere (Kam-Morgan et al., 1985; Lavoie et al., 1991). Using the affinity constant for HEL binding to the anti-nuclease resin and to the HyHEL-5 resin, it can be calculated that greater than 99.9% of the HEL is bound to HyHEL-5 and less than 0.1% of the HEL is nonspecifically bound to the matrix. Given that the amount of nonspecific binding is very low in the presence of HyHEL-5, the protection factors obtained in this study arise from specific interactions between HyHEL-5 and its antigen.

The structure of the complex between lysozyme and HyHEL-5 has been determined by X-ray crystallography (Sheriff et al., 1987). On the basis of this structure the residues in lysozyme can be divided into four classes (see Table II): residues that (1) contact the immunoglobulin (contact residues), (2) are not in contact but are buried at the interface between the two proteins (buried residues), (3) either are hydrogen bond donor/acceptors or are adjacent to residues from the above two classes (boundary residues), and (4) are distant from the epitope (remote residues). We can quantify amide exchange for 3 of the 14 contact residues. Of these, 2 (Asn44 and Asn46) were significantly protected in the complex, while the third, Leu84, showed enhanced amide exchange. Of the 9 residues buried at the interface, we can quantify amide exchange for 2 residues (Thr51 and Arg61); both showed significant protection in the complex. We could measure significant protection factors for 5 boundary residues (Asn39, Ala42, Ser50, Asn65, and Leu83). On the basis of the measurements of amide exchange kinetics by Pedersen et al. (1991), it may be possible to obtain information on 2 additional contact residues (Gln41, Asp48) and 1 buried residue (Ser85) by employing a lower pH during the exchange period. However, this would leave 7 contact residues and 4 buried residues whose amide exchange rates are sufficiently fast that the amide protons could not be detected with the NMR methods used in these experiments, regardless of the degree of protection in the antibody–antigen complex. The remaining 5 residues that have significant protection factors are not found near the epitope. One of these, Trp63, has a protection factor which is as large as that found for Arg61, a buried residue. The protection factors for the remaining remote residues (Ala11, Lys13, Phe38, Cys76) are small but significant.

DISCUSSION

One of the points we wish to address is whether amide exchange measurements can be used for epitope mapping. The locations, in the three-dimensional structure of lysozyme, of all residues with altered amide exchange kinetics are shown in Figure 3. As indicated in this figure, the locations of residues with significant protection factors do not accurately reflect the epitope as defined by crystallography (the structural epitope). This discrepancy results from two factors. First, the amide exchange rate of a large number of contact and buried residues is too fast to measure with the NMR experiment used in this study. It is apparent that the accurate mapping of epitopes by this method in some systems will require the measurement of protection factors of those residues that exchange rapidly with solvent. Second, changes in amide

Table I: Properties of Amide–H of Hen Egg-White Lysozyme in the Presence and Absence of Antibody

	assignments[a]		amide–H lifetime[b]						
			mAb bound			free			
residue	N–H	H_α	low	mean	high	low	mean	high	f[c]
Phe3	8.91	4.18		0			0		
Gly4	8.52	4.31		0			0		
Leu8	8.67	3.76	13	26	50	14	20	27	1.3
Ala9	8.43	3.58		>500			>500		
Ala10	8.19	3.98		>500			>500		
Ala11	7.77	4.28	24	26	29	12	14	16	1.9
Met12	9.14	3.44		>500			>500		
Lys13	8.53	3.97	54	94	168	27	36	46	2.6
His15	7.32	4.56		0			0		
Leu17	7.15	3.92		0			0		
Tyr23	7.65	4.53		0			0		
Asn27	8.19	4.18	9	10	11	8.6	8.8	9	1.1
Trp28	7.15	3.73		>500			>500		
Val29	7.57	3.42		>500			>500		
Cys30	8.03	2.48		>500			>500		
Ala31	8.14	3.70		>500			>500		
Ala32	7.60	4.01		>500			>500		
Lys33	7.96	2.54		>500			>500		
Phe34	7.35	4.30	62	92	134	42	55	70	1.7
Glu35	8.60	4.44		0			0		
Ser36	7.88	4.58		0			0		
Asn37	8.14	4.49		0			0		
Phe38	7.34	3.86	13	17	20	7.4	7.5	7.6	2.3
Asn39	7.41	5.40	164	252	1300	30	42	57	6.0
Thr40	9.42	4.03		0			0		
Gln41	7.92	4.43		0			0		
Ala42	6.85	4.12	58	74	93	6.2	6.4	6.5	11
Asn44	8.14	5.00	149	244	426	5.4	5.5	5.6	44
Asn46	8.93	5.11	39	52	67	0	0.8	32	65
Ser50	8.27	4.54	17	22	29	1.4	5.8	11	3.8
Thr51	9.17	4.86	40	56	76	8.0	8.1	8.2	6.9
Asp52	8.80	5.19		>500			>500		
Tyr53	9.04	4.74		>500			>500		
Gly54	9.06	4.39		>500			>500		
Leu56	8.94	4.42		0			0		
Gln57	7.98	3.35		>500			>500		
Ile58	7.68	4.01		>500			>500		
Asn59	8.43	5.63		0			0		
Ser60	9.17	5.19		>500			>500		
Arg61	8.80	4.08	112	232	704	14	19	26	12
Trp63	7.44	4.97	74	174	740	12	13	15	13
Cys64	7.60	5.83		>500			>500		
Asn65	8.28	5.52	72	76	81	10	11	12	6.9
Cys76	9.52	4.44	17	31	52	10	11	12	2.8
Ile78	8.87	4.97	4.4	7.3	12	4.8	4.9	5.0	1.5
Ala82	7.62	4.09		0					>1
Leu83	7.86	4.24		>500		63	79	98	>6
Leu84	7.12	5.09	0.1	0.5	2.2	7.5	7.6	7.7	0.06
Val92	8.41	3.09		>500			>500		
Asn93	8.69	4.25		0			0		
Cys94	7.90	4.99		0			0		
Lys96	8.02	3.70		>500			>500		
Lys97	7.21	4.12	14	17	22	14	18	23	0.94
Ile98	7.98	2.76		>500			>500		
Val99	8.35	3.90		>500			>500		
Trp108	7.88	4.62		0			0		
Trp111	7.22	3.70		0			0		
Arg112	8.29	3.36		0			0		
Asn113	7.95	4.56		0			0		
Cys115	7.35	4.53		0			0		
Trp123	7.63	4.10		0			0		
Ile124	7.54	4.73		0			0		
Arg125	7.33	4.14		0			0		

[a] Chemical shifts of the amide and H protons. [b] Mean lifetime, in hours, of the amide–proton ± one standard error (low and high). 0, proton was observed but the exchange rate was sufficiently rapid that it could not be measured under the conditions of this experiment. >500, proton was observed but its exchange rate was sufficiently slow that it could not be measured under the conditions of this experiment. [c] f, ratio of mean lifetimes for bound and free.

exchange are also found to occur in boundary and remote residues. It is not possible to use protection factors to distinguish boundary residues from those which are located in the antigen–antibody interface. For example, protection factors for the boundary residues Leu83 and Ala42 exceed that found for the buried residue Thr51. Because of these factors, measurement of amide exchange rates may not be broadly applicable for mapping epitopes in antigen–antibody

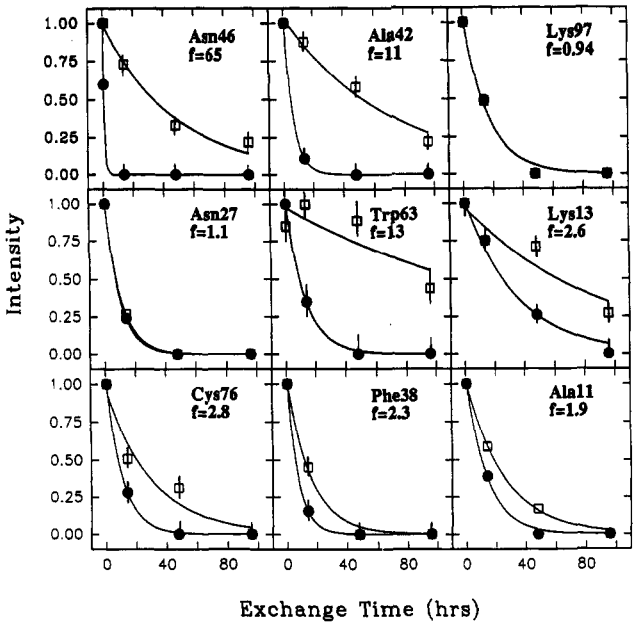

FIGURE 2: Exchange kinetics of selected amide protons. The intensity of the NH–H_α proton cross peaks are shown as a function of the exchange time. The normalized intensity is shown on the abscissa, and the exchange time is indicated on the ordinate. The residue is shown in the upper right corner of each frame. Measured intensities in the absence of antibody are indicated by closed circles. Intensities in the presence of antibody are indicated by open squares. The error bars associated with each point represent the estimated error in intensity measurement for each particular residue. Error bars that are smaller than the symbols are not shown.

interactions or for mapping contact regions in protein–protein interactions.

However, amide exchange provides additional information about antigen–antibody interaction beyond mapping the epitope. One of the factors which affects amide exchange in protein antigen–antibody complexes is the exclusion of solvent from the interfacial region. Paterson et al. (1990) have considered the effect of binding affinity on the protection factor. In studies with cytochrome *c* it was found that several amide protons have protection factors close to that predicted from the binding affinity. This was interpreted by Paterson et al. to indicate that these amides did not exchange in the antigen–antibody complex. In the complex of HEL and HyHEL-5 a maximum protection factor of 1200 could be observed, based on a binding constant of 4×10^{10}. In this study we find that the contact and buried residues Asn44, Asn46, Thr51, and Arg61 have large protection factors but these are all much less than those predicted from the binding constant. This suggests that significant amide exchange occurs in the antigen–antibody complex. Davies et al. (1990) show that only two water molecules remain in the interface between HyHEL-5 and HEL. Therefore, on the basis of crystallographic data, the interface is protected from solvent. However, on the basis of the measured protection factors presented here, we suggest that the interfacial region of the antibody–antigen complex must be solvent accessible to some degree. Solvent penetration may be facilitated by transient opening of the interfacial region without dissociation of the complex. This event may be either dampened in the crystal due to protein–protein contacts or sufficiently infrequent to affect the time-averaged scattering of X-rays.

Most of the observed protection factors for the buried and contact residues were greater than 1. The sole exception was displayed by the contact residue Leu84. Several factors could account for the increased exchange rate of this amide proton

Table II: Lack of Correlation between Protection and H-Bond Distances

residue	acceptor[a]	exchange rate[b]	*f*[c]	D_{free}[d]	D_{bound}[d]
contact					
Gln41	Asn39 s	0			
Thr43	nil				
Asn44	Asp52 m	5	44	1.9	2.4
Arg45	nil				
Asn46	Ser50 m	1	65	1.9	1.9
Thr47	nil				
Asp48	nil	0			
Gly49	Asn46 m				
Tyr53	Ile58 m	>500			
Gly67	Asn65 m				
Arg68	Asp66 s				
Thr69	nil				
Pro70					
Leu84	Ser81 m	7.6	0.06	2.0	2.0
buried					
Thr51	Ser60 s	8	6.9	2.0	1.9
Asp52	Asn44 m	>500			
Arg61	Asn59 m	19	12	2.1	2.0
Asp66	nil				
Gly71	nil				
Ser72	nil				
Pro79					
Ser81	nil				
Ser85	nil				
boundary					
Asn39	Ser36 m	42	6	2.5	2.2
Ala42	Asn39 m	6.4	11	2.3	2.3
Ser50	Asp48 s	5.8	3.8	2.0	2.7
Ile58	Tyr53 m	>500			
Asn59	nil	0			
Asn65	Ile78 m	11	6.9	2.0	2.4
Ala82	Pro79 m	0			
Leu83	Cys80 m	79	>6	2.1	2.5
remote					
Ala11	Glu7 m	14	1.9	2.1	2.7
Lys13	Ala9 m	36	2.6	2.0	2.3
Phe38	Ala32 m	7.5	2.3	2.5	2.3
Trp63	Asn59 m	13	13	2.3	2.9
Cys76	Trp63 m	11	2.8	1.9	1.9

[a] Hydrogen bond oxygen acceptor of amide–H: s, side chain; m, main chain. [b] Mean lifetime of amide–proton of free (unbound) lysozyme. [c] *f*, ratio of mean lifetime of amide–protons in antibody-bound (B) and free (F) forms. [d] Hydrogen bond distances in free (D_{free}) and antibody-bound (D_{bound}) forms. Measured as hydrogen to oxygen distances using Sybyl (Tripos Associates, Inc., St. Louis, MO) and coordinate sets derived from Sheriff et al. (1987) and Ramanandham et al. (1990) for the bound and free forms, respectively.

in the antibody–antigen complex. These factors include changes in hydrogen bonding of the Leu84 amide proton, changes in the electrostatic environment of Leu84 which could change the pK_a of the amide proton, changes in the structure of bound water in the region of Leu84, and an increase in protein flexibility in this region of the complex. Electrostatic effects can be excluded because there are no additional charged residues from the antibody near Leu84 that may alter its pK_a. Increased flexibility in the region of Leu84 also appears unlikely on the basis of discussion of the amide exchange kinetics of boundary and remote residues below. However, a careful analysis of the crystal structure of bound and free lysozyme shows that the hydroxyl group of Ser85 in HEL is altered in the complex. This may have an effect on the exchange kinetics of Leu84 by changing the organization of the bound water in this region of the protein.

A number of residues outside the structural epitope show significant protection factors. Large protection factors are observed for a number of boundary residues, some of which are hydrogen bond acceptors for residues contained in the contact/buried region. Regardless of the interaction of

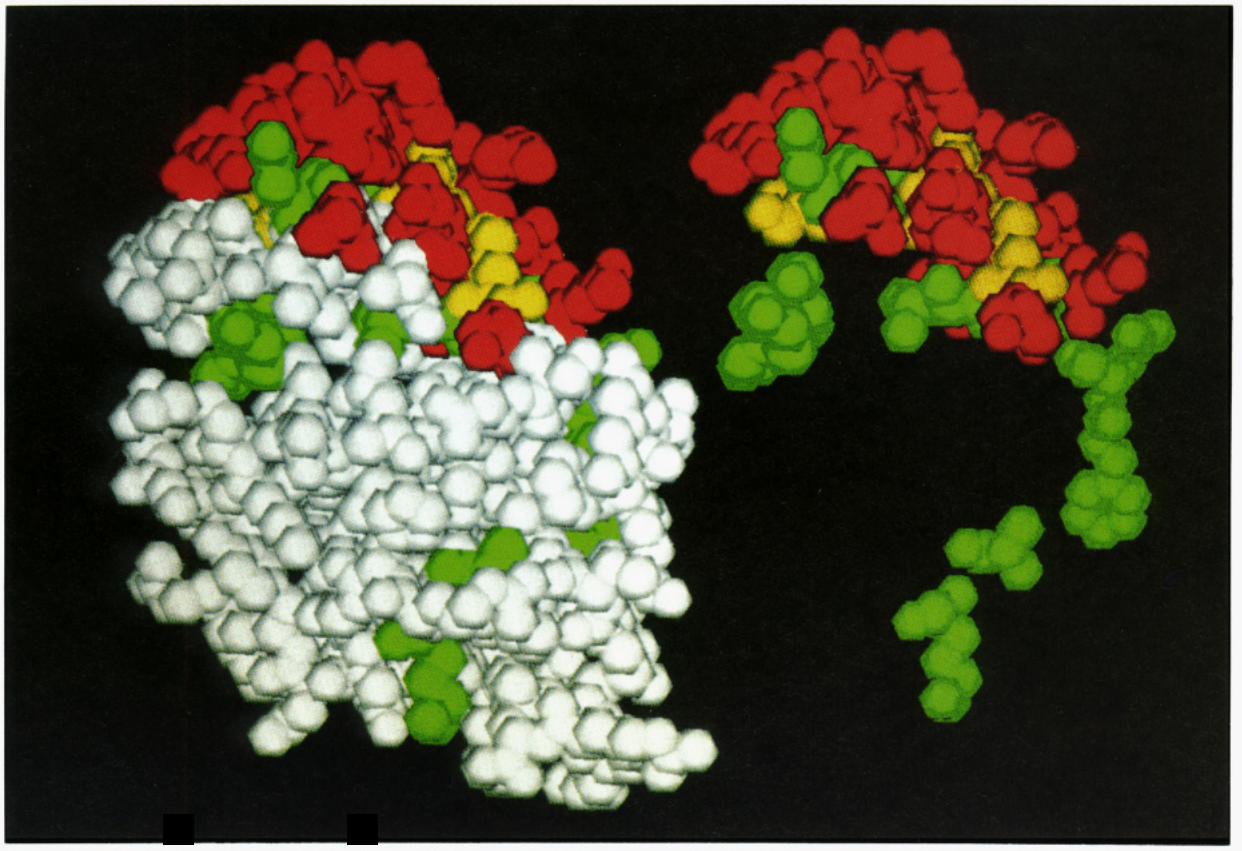

FIGURE 3: Model of lysozyme. The left structure is a space-filling model of lysozyme. The right structure shows those boundary and remote residues with altered exchange kinetics plus all contact and buried residues. Red residues in these models are contact or buried residues with exchange kinetics that are either too fast or too slow to be used for protection measurements. Yellow residues are contact or buried residues with altered exchange kinetics. Green residues are boundary or remote residues with altered exchange kinetics.

boundary residues with the contact/buried residues, it is clear the changes in the amide exchange rates can be observed in regions of the protein which are just outside the epitope. Conformational changes in the antigen due to antibody binding could affect the amide exchange of the boundary and remote residues. The crystallographic studies on the HyHEL-5 antigen complex detected a number of changes in the structure of the antigen due to antibody binding (Sheriff, 1987). The most significant changes were a movement of Pro70 (a contact residue) and a ring-flip of the indole ring of Trp63 (a remote residue). With the exception of the structural changes mentioned above (Ser85, Pro70, Trp63), there are no other apparent changes in the structure of main-chain atoms of HEL that occur as a result of antibody bonding. Thus, the changes in amide exchange rates of the boundary residues cannot be rationalized in terms of changes in hydrogen bond lengths (see Table II) or other structural differences between the two forms of lysozyme.

A total of five residues that are remote from the structural epitope (Ala11, Lys13, Phe38, Trp63, Cys76) show reduced amide exchange rates in the antibody–antigen complex. The protection factor of Trp63 observed in this study can be rationalized by assuming that the altered orientation of the indole ring in the complex between HEL and HyHEL-5 protects the Trp63 amide proton from exchange. The orientation of the indole ring of Trp63 in the complex could arise in several ways. A trivial explanation is that the orientation of the indole ring is the same in solution as in the antigen–antibody complex and that the indole ring assumes the other orientation in crystals of lysozyme. This explanation can be discounted by NMR studies on free lysozyme. A NOESY experiment (States et al., 1982) shows that both the HE1 and HZ3 protons of Trp63 are within 4 Å of the side chain of Ile98. This indicates that the predominant solution conformation is similar to that found in the crystal structure of unbound lysozyme. However, we observe that the resonance line for the amide proton of Trp62 is split with intensity ratios of approximately 1:10 (data not shown). The position of the indole ring of Trp63 would have an effect on the chemical shift of the amide proton of Trp62. On the basis of this evidence it appears that the conformation of Trp63 is heterogeneous in solution and that either HyHEL-5 binds preferentially to the minor component of the population or the equilibrium between the two orientations is shifted by formation of the antigen–antibody complex. Heterogeneity in the conformation of Trp side chains has been observed in other systems. For example, ^{13}C NMR studies of dihydrofolate reductase (London et al., 1979) show evidence of two conformational states of a Trp residue in the native protein. Interestingly, this heterogeneity is removed when ligand is bound.

Four additional remote residues (Cys76, Phe38, Lys13, Ala11) show altered exchange kinetics as a result of formation of the complex between antigen and antibody. The altered exchange kinetics of these remote residues cannot be explained by solvent exclusion because these residues are not part of the interface. In addition, there are no significant differences in the structure of lysozyme and the lysozyme–antibody complex in the area of these residues. It is interesting to note that the protection factors for a number of residues that are outside the antigenic site are very similar (Ala11, $f = 1.9$; Lys13, $f = 2.6$; Phe34, $f = 1.7$; Phe38, $f = 2.3$) although the lifetimes in the unbound state range from 7.5 h (Phe38) to 55 h (Phe34). This indicates that the region of the protein containing these residues is responding to the formation of the antibody–antigen

complex in a coordinated manner. A similar situation has been observed in allosteric effects on the exchange rate of amide protons in hemoglobin (Englander et al., 1992).

The decrease in amide exchange at both the boundary and remote sites may represent a reduction in fluctuations (local unfolding) of the protein as a consequence of the antibody binding. The effect of antibody binding on the exchange rate of amide–protons appears to be propagated from the epitope region to Ala11 via the connection of Asn39 to Phe38, followed by an interaction of the phenolic ring of Phe38 with the helix containing Ala11 and Lys13. Clearly, the effects are largest for the boundary residues, but a significant change in exchange kinetics is observed for a number of resides that are quite distant from the epitope. The effects of protein fluctuations on amide exchange in lysozyme have recently been addressed by Pedersen et al. (1991). In this case, the amide exchange properties of crystalline lysozyme were compared to those of lysozyme in solution. It was found that the rate of amide exchange was markedly reduced in the crystalline state. The altered exchange rates could not be correlated with a decrease in solvent exposure of the amide residues due to protein–protein contacts within the crystal. The conclusion arrived at by Pedersen et al. is that the reduction of amide exchange rates in the crystal is due to an overall reduction of protein fluctuations in the crystal.

A decrease in the local unfolding properties of the protein corresponds to a reduction in the number of accessible energy states of the protein. The thermodynamic consequence of this is a reduction in the heat capacity of the system. Reduction in the heat capacity of proteins due to ligand binding has been observed in a large number of systems (Biltonen & Langerman, 1979), and the formation of antigen–antibody complexes may be another example of this phenomenon.

In conclusion, as shown in the present case, amide exchange may be useful in identifing the general location of epitopes on protein antigens. However, the difficulty associated with the measurement of certain exchange rates and the substantial number of boundary and remote residues with altered amide exchange rates indicate that this method may not be a general method for mapping epitopes on protein antigens. However, the protection factors found for residues distant from the epitope indicate that long-range changes can occur in protein antigens due to the formation of protein–antibody complexes. In this case these changes may manifest themselves as changes in the dynamics of the antigen molecule.

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