

Refined Structures of Bobwhite Quail Lysozyme Uncomplexed and Complexed With the HyHEL-5 Fab Fragment

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ABSTRACT The HyHEL-5 antibody has more than a thousandfold lower affinity for bobwhite quail lysozyme (BWQL) than for hen egg-white lysozyme (HEL). Four sequence differences exist between BWQL and HEL, of which only one is involved in the interface with the Fab. The structure of bobwhite quail lysozyme has been determined in the uncomplexed state in two different crystal forms and in the complexed state with HyHEL-5, an anti-hen egg-white lysozyme Fab. Similar backbone conformations are observed in the three molecules of the two crystal forms of uncomplexed BWQL, although they show considerable variability in side-chain conformation. A relatively mobile segment in uncomplexed BWQL is observed to be part of the HyHEL-5 epitope. No major backbone conformational differences are observed in the lysozyme upon complex formation, but side-chain conformational differences are seen in surface residues that are involved in the interface with the antibody. The hydrogen bonding in the interface between BWQL and HyHEL-5 is similar to that in previously determined lysozyme-HyHEL-5 complexes. © 1996 Wiley-Liss, Inc.

Key words: avian lysozyme, anti-protein Fab, antibody-antigen complex

INTRODUCTION

The structures of several antibodies in complex with protein antigens have been determined, and some common features of antibody-protein interactions are beginning to emerge.^{1–21} Antiprotein antibodies have large combining sites, and do not show the pronounced grooves often observed in antipeptide or antihapten antibodies. The antibody-antigen combining surfaces show good shape complementarity with only a few water molecules buried in the interface.^{12,14,22,23} The specificity and binding affinity appear to be dominated by the interactions of a few residues,^{17,24} although 20 or more residues from

the antibody and protein may be buried in the interface.²⁵

In antibody-antigen complexes, neither antibody nor antigen shows major structural changes upon complex formation, although small conformational changes in both molecules have been observed. Changes in the antibody upon complex formation include side-chain rotations⁶ and displacements of V_H relative to V_L ,⁵ while changes in the protein antigen are mainly in regions with flexible surface loops.²³

One of the earliest protein-antibody complexes examined was that of hen egg-white lysozyme (HEL) complexed with the HyHEL-5 Fab fragment.^{2,21} The HyHEL-5 antibody was made by immunization with HEL, but binds to other avian lysozymes with varying affinities.²⁶ In particular, it binds to bobwhite quail lysozyme (BWQL) with a $K_D \sim 10^{-7}$ M, more than a thousandfold lower affinity than to HEL ($K_D \sim 10^{-11}$ M). BWQL has four sequence differences from HEL, of which only one, Arg 68_Y→Lys, is directly involved in the interface between the Fab and lysozyme. The other three differences, Thr 40_Y→Ser, Ile 55_Y→Val, and Ser 91_Y→Thr, are not exposed to bulk solvent or to the antigen-antibody interface. It was initially presumed that the Arg 68_Y→Lys (R68K) change was entirely responsible for the difference in affinity, and the structure of the

*The subscript L refers to Fab light chain residues, H to Fab heavy chain residues, and Y to lysozyme residues. The Fab residues are numbered according to Kabat numbering.²⁷

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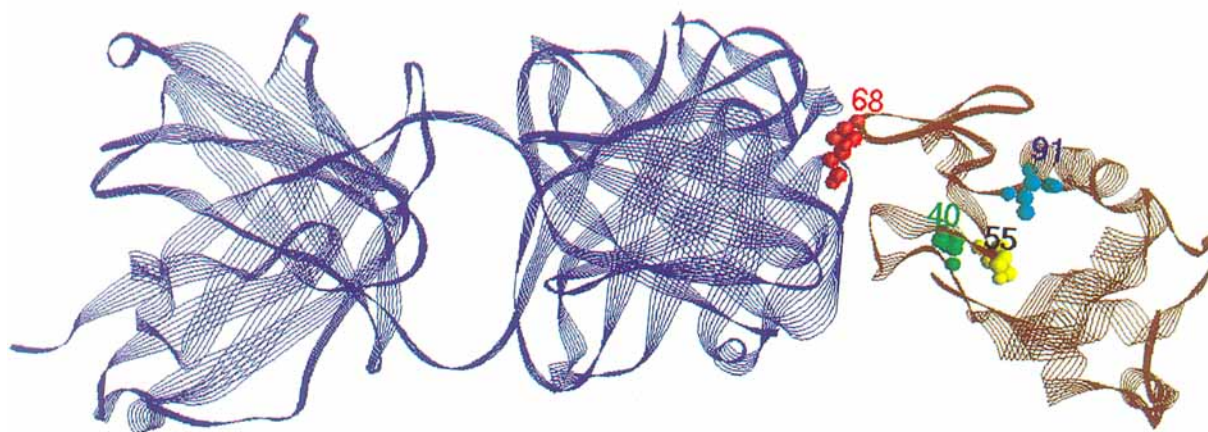


Fig. 1. RIBBONS²⁸ drawing of the bobwhite quail lysozyme-HyHEL-5 Fab complex. The Fab is in blue and the lysozyme in brown. Sequence differences between BWQL and HEL (residues 40, 55, 68, 91) are shown as spheres and are labeled.

HyHEL-5-R68K-HEL complex was examined for structural changes that might be responsible.¹⁷ However, binding studies have shown that the R68K lysozyme and BWQL have a small (2-fold) difference in binding affinity to the HyHEL-5 Fab (S. Smith-Gill, unpublished observations).

We have determined the structure of uncomplexed BWQL in two different crystal forms, as well as its complex with the HyHEL-5 Fab (Fig. 1). The monoclinic crystal form of uncomplexed BWQL has two molecules in the asymmetric unit, and, along with the hexagonal form, provides a basis for determining the structural changes that have taken place on complex formation. The complex structure has also been compared with the HyHEL-5-HEL complex and the HyHEL-5-R68K-HEL complex.

METHODS

Bobwhite Quail Lysozyme

A stock solution of BWQL was prepared by dissolving protein in distilled water at a concentration of 125.5 mg/ml. Seed crystals were grown by hanging drop vapor diffusion methods with a 10- μ l drop containing 5 mM sodium acetate, pH 4.0, 45 mg/ml BWQL, 0.625 mM formamide, and 6% (w/v) sodium nitrate, and a well containing 1 ml 10 mM sodium acetate, pH 4.0, 1.25 mM formamide, and 12% (w/v) sodium nitrate. After 3 to 7 days at room temperature (22°C), crystals grew as small to moderate plates. These crystals were transferred to 200- μ l solutions containing 20 mM sodium acetate, pH 4.0, 12.6 mg/ml protein, 5% (w/v) sodium nitrate, 1 mM formamide, and enlarged to $\sim 0.3 \times \sim 0.12 \times \sim 0.02$ mm. Crystals were mounted in thin-walled glass capillaries (Charles Supper Co., Natick, MA) for data collection on a FAST area detector system. The crystals diffracted to 1.9 Å and had symmetry con-

sistent with space group $P2_1$ with cell constants of $a = 28.0$ Å; $b = 62.6$ Å; $c = 60.7$ Å; $\beta = 91.6^\circ$. Data were processed with the program MADNES.²⁹ Statistics for the data are shown in Table I.

A second crystal form was grown from a 10 mg/ml protein solution, 164 mM cacodylate buffer at pH 6.0, 1 mM formamide, 45.5% sodium nitrate (added as solid salt without stirring). Crystals grew as hexagonal rods, and the biggest crystals were $\sim 0.15 \times 0.15 \times 1.3$ mm. These crystals diffracted to 2.0 Å, and the space group of this crystal form is $P6_122$, $a = b = 89.2$ Å; $c = 61.7$ Å. Data for this hexagonal form of BWQL were collected from a single crystal on a Siemens X-1000 area detector and processed with the program XGEN.³⁰ Statistics for the data are shown in Table I.

The monoclinic form of BWQL was determined by using the deposited monoclinic lysozyme coordinates (1LYM) from the Protein Data Bank (PDB),^{31,32} which were subsequently modified by superimposing the structure of tetragonal lysozyme (6LYZ) on each of the two monomers in the asymmetric unit as a starting model for refinement. The structure of hexagonal BWQL was determined by molecular replacement from entry 6LYZ using programs MERLOT³³ and BRUTE.³⁴ The structures were refined by using programs X-PLOR³⁵ and TNT.³⁶ Side chains with sequence differences were initially modeled as Ala, with the correct sequence built into difference density maps. The final refinement statistics are presented in Table II. The monoclinic BWQL form binds three nitrate ions, determined by the presence of well-defined trigonal planar density in the difference maps. Solvent atoms were added as long as their refined B factors remained below 60 Å². The maximum allowed B factor for protein atoms was 80 Å². An overall anisotropic B factor was

TABLE I. Data Collection Statistics

	HyHEL-5-BWQL complex	Monoclinic BWQL	Hexagonal BWQL
Resolution limit	2.6 Å	1.9 Å	2.0 Å
Detector	Siemens	FAST	Siemens
No. of observed reflections	30,801	43,399	49,907
No. of unique reflections	15,950	14,910	9,288
$\langle I/\sigma \rangle$	n/d	49.7	29.4
R-sym	0.074	0.0367	0.0565
Completeness	80.8%	85.0%	92.9%
Molecules/asymmetric unit	1	2	1

used in scaling F_{obs} to F_{calc} in both cases. The Ramachandran plots³⁷ for the monoclinic and hexagonal forms are shown in Figure 2A,B. Coordinates for the monoclinic (entry code 1DDK) and hexagonal crystal forms (entry code 1DKJ) are available from the Protein Data Bank.

HyHEL-5-BWQL Complex

Bobwhite quail lysozyme was purified from bobwhite quail eggs (Stevenson Game Bird Farm, P.O. Box 426, Riverside, TX 77367) according to the procedure described by Arnheim and colleagues.⁴⁰ Final purity of BWQL was assessed by using silver-stained SDS-PAGE. BWQL was stored at 4°C in the presence of 0.02–0.05% sodium azide. It was further verified that the purified protein bore the characteristic BWQL mutations as shown by electrospray ionization mass spectroscopy (performed by Gregory Rahn, Oneida Research Services, Whitesboro, NY 13492). The mass of HEL was found to be 14302.7 (the mass calculated based on the mRNA sequence is 14305.14) and that of BWQL to be 14261.33 (the mass calculated based on the mRNA sequence of HEL and the sequence of BWQL is 14263.10). Most importantly, the calculated and experimental mass difference between HEL and BWQL is 42.04 and 41.38, respectively, which are in close agreement, considering the experimental error in the mass determination. HyHEL-5 Fab was prepared as described by Chacko and coworkers.¹⁷

The protein solution for crystallization consisted of 1.5 µl each of 9.1 mg/ml Fab and 3.8 mg/ml bobwhite quail lysozyme. Crystals were grown by vapor diffusion against a well solution of 14% polyethylene glycol (PEG) 4000, 0.1 M imidazole pH 7.0, 0.007% spermine. The crystals were macroseeded to increase the crystal size. Data were collected from a single crystal of size $0.15 \times 0.15 \times 0.25$ mm at room temperature on a Siemens area detector. The data were processed using XDS.⁴¹ The crystals were isomorphous with the native HyHEL-5-HEL complex as well as the HyHEL-5-R68K-HEL crystals. The space group was $P2_1$ and the unit cell parameters a

$= 55.0$ Å, $b = 74.1$ Å, $c = 79.5$ Å, $\beta = 102.1^\circ$. Statistics for the data are presented in Table I.

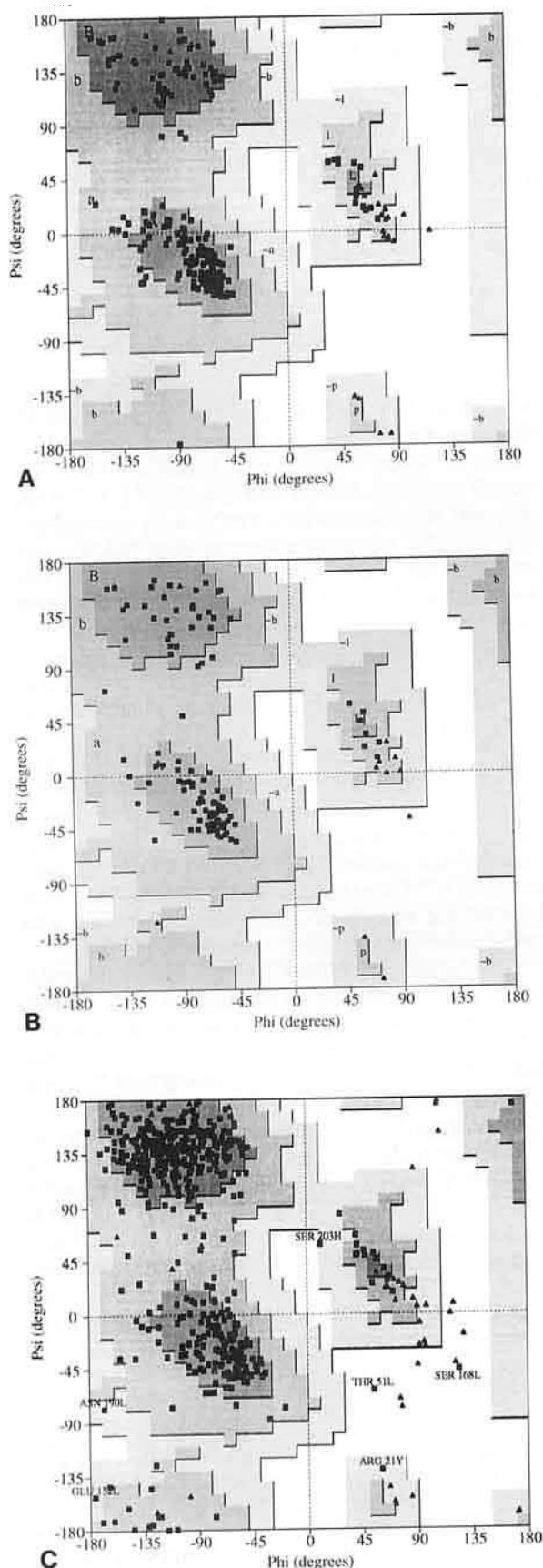
The R value between the HyHEL-5-HEL observed structure factors and the HyHEL-5-BWQL observed structure factors was 25.4 for unweighted F 's, and 19.1 when the statistics were weighted by $1/\sigma^2$. A preliminary difference map between the two was examined around the sites where the sequences differ, and the observations are listed in Table III.

The structure was refined by energy minimization in XPLOR³⁵ using the parameters of Engh and Huber⁴² with electrostatic charges turned off. The initial model used was that of the HyHEL-5-HEL complex.²¹ Residues that differed in the HEL and BWQL sequences were deleted in their entirety from the structure before refinement. The deleted residues were built back into the model from the difference Fourier and the $2||F_o| - |F_c||$ maps using the program O.^{43,44} The structure was further refined with alternating rounds of positional refinement and model building until the R value was 23.2%.

The program MAPMAN⁴⁴ was used to pick significant peaks in the $||F_o| - |F_c||$ map ($10 - 2.5$ Å) that might be attributed to water molecules. All peaks at a height greater than 3.0σ were marked as water molecules. The data were further refined through positional and overall B factor refinement. The $||F_o| - |F_c||$ and $2||F_o| - |F_c||$ maps were then displayed, and water molecules were preserved if they had $2||F_o| - |F_c||$ density greater than 1σ , made hydrogen bonds (<3.4 Å) to a suitable protein atom, and had B factors less than 50 Å^2 . Table II shows the final statistics for the refined structure (PDB entry code 1BQL).⁴⁵ The Ramachandran plot for the structure is shown in Figure 2C. Surfaces were calculated using program MS⁴⁶ with a probe radius of 1.7 Å.

RESULTS AND DISCUSSION

Comparison of bobwhite quail lysozyme with the refined structure of tetragonal hen egg-white lysozyme (PDB entry 1LSE)⁴⁷ reveals only limited structural changes (see Fig. 3). Three of the four sequence differences (T40S, I55V, S91T) correspond



to side chains that are buried within the molecule. These conservative mutations yield a decrease in side-chain volume of only $27 \text{ \AA}^3$ (one methylene group). The changes in sequence do not cause any backbone rearrangement around those locations: for example, the root-mean-square (RMS) deviation of 125 backbone atoms within $10 \text{ \AA}$ of I55V C α is $0.33 \text{ \AA}$ for the hexagonal form and 0.29 and $0.4 \text{ \AA}$ for the two molecules of the monoclinic form. Similar results are observed around T40S and S91T. The remaining difference, R68K, is in a solvent-exposed surface position, and is partially disordered in at least some of the BWQL structures. The only significant conformational change observed is a rotation of the side chain of Phe 3 about the χ_1 dihedral by about 20° . The side chain of Phe 3 moves toward the C β s of T40S and I55V (which are $4\text{--}5 \text{ \AA}$ away) as the local atomic volume is decreased in BWQL relative to HEL. This change is consistently observed in all three uncomplexed BWQL molecules when compared with tetragonal HEL, triclinic HEL (PDB entry 2LZT),⁴⁸ and monoclinic HEL (PDB entry 1LYS).⁴⁹

Large backbone differences between the three molecules of uncomplexed BWQL and tetragonal HEL (see Fig. 3) are observed near the C terminus of the chain, and at 25Y–26Y where the peptide plane of tetragonal HEL is flipped relative to BWQL, triclinic HEL, and monoclinic HEL. The remaining regions of large deviation between BWQL and HEL backbones occur in the loops around residues 45 and 70, which are part of the HyHEL-5 epitope. These loops, along with the loop around Gly 102_Y have been identified as very flexible in free and complexed lysozyme structures^{21,49,50} and have relatively high main-chain B factors in the current BWQL structures. The largest discernible differences between BWQL and HEL are comparable to the differences between different copies of BWQL in the monoclinic and hexagonal crystal forms (RMS differences of $0.6\text{--}0.7 \text{ \AA}$). The four nitrate ions observed in the monoclinic form are located near Arg 122_Y in molecule 1, and Arg 122_Y, Trp 63_Y, and Ser 81_Y in molecule 2.

The bobwhite quail lysozyme does not undergo any major backbone conformational changes upon complex formation with the HyHEL-5 antibody. Figure 4 shows the comparison of BWQL in the complex with the three molecules of uncomplexed BWQL. Apart from the mobile regions at the N and C termini of the lysozyme chain, large backbone differ-

Fig. 2. Ramachandran plot of ϕ - ψ angles for (A) monoclinic BWQL, molecules 1 and 2, (B) hexagonal BWQL, and (C) HyHEL-5-BWQL complex. The regions are shaded according to PROCHECK³⁹ with the most favored regions being darkest and the disallowed regions lightest. Glycine residues are shown as triangles. Residues in generously allowed and disallowed regions are labeled. No appreciable difference in the quality of the plot for molecules 1 and 2 of monoclinic BWQL was observed.

TABLE II. Refinement Statistics

	HyHEL-5- BWQL complex	Monoclinic BWQL	Hexagonal BWQL
Resolution limits	8–2.6	6–1.9	6–2.0
Sigma cutoff on F^o s	2.0	1.0	1.0
No. of reflections	14,290	14,283	8,539
Overall anisotropic ΔB factors		B11 = -4.5 B22 = 4.0 B33 = 0.6 B13 = 1.6 (all others 0.0)	B11 = -1.9 B22 = -1.9 B33 = 3.9 B12 = 0.8 (all others 0.0)
	n/d		
R value	19.1%	15.7%	17.7%
R -free ³⁸	29.1%	n/d	n/d
No. of protein atoms	4240	1996	998
No. of water molecules	81	154	62
No. of hetero atoms	—	16	—
RMSD bond lengths	0.009	0.012	0.012
RMSD bond angles	1.9	1.6	1.6
RMSD dihedrals	27	25	24
RMSD impropers	1.7	2.1	2.1
RMSD B for bonded atoms	4.5	2.5	2.4

TABLE III. Initial ($F_{\text{HyHEL-5-HEL}} - F_{\text{HyHEL-5-BWQL}}$) Map Features at Mutation Sites

Residue	HEL	BWQL	Map Feature at This Position
40	Thr	Ser	2.2 σ negative density peak near one arm of Thr
55	Ile	Val	No features above 2.0 σ
68	Arg	Lys	5.0 σ negative density peak on the terminal nitrogens of the Arg
91	Ser	Thr	4.5 σ positive density peak near C β of Ser

ences are seen around residues 45_Y–48_Y, 71_Y–72_Y, and 102_Y, which are flexible loops in the uncomplexed BWQL and HEL structures. The four positions in which BWQL and HEL differ were examined in the three molecules of uncomplexed BWQL and the three HyHEL-5-lysozyme complexes (i.e., HyHEL-5-BWQL, HyHEL-5-HEL, and HyHEL-5-R68K-HEL,⁵¹ and are discussed below.

Residue 40_Y is a Thr in the HEL and a Ser in BWQL, whose side chain points away from the interface in all the uncomplexed and complexed structures. The Ser in BWQL essentially follows the conformation of the O γ 1 of the Thr in HEL. Residue 55_Y, a Val in BWQL, has a similar conformation in the three molecules of uncomplexed BWQL. This residue is modeled as a different rotamer in the HyHEL-5-BWQL complex, but at the resolution of the complex structure, it could possibly be modeled in either rotamer. Residue 91_Y has a similar conformation in all the structures, with the O γ 1 in Thr of the HEL complexes following the O γ of the Ser in the uncomplexed BWQL and the HyHEL-5-BWQL complex.

Residue 68_Y is particularly interesting, since it has been shown to be an important determinant of

specificity and binding affinity along with Arg 45_Y.^{52,53} In the HyHEL-5-HEL complex, Arg 45_Y and 68_Y form a ridge on the lysozyme that spatially complements a cleft in the Fab, and most of the interactions between the Fab and lysozyme are made by these two arginines. Residue 68_Y is a Lys in BWQL, and is the only sequence difference between HEL and BWQL that is involved in the antibody-antigen interface. Figure 5 shows the different conformations adopted by residues 45_Y and 68_Y in the two crystal forms of BWQL and the three HyHEL-5-lysozyme complexes. It can be seen that the side chains are very mobile in the uncomplexed BWQL molecules, but have quite similar conformations in the Fab-lysozyme complexes. In the complex with BWQL, Lys 68_Y does not appear to be disordered as was observed in the HyHEL-5-R68K-HEL complex, but has a single conformation whose terminal nitrogen lies approximately between the two disordered nitrogen positions in the R68K-HEL complex. However, as in that complex, a new water molecule is bound in place of the terminal nitrogens of the equivalent arginine in HEL providing a water-mediated hydrogen bond between the Lys 68_Y and Glu 50_H.

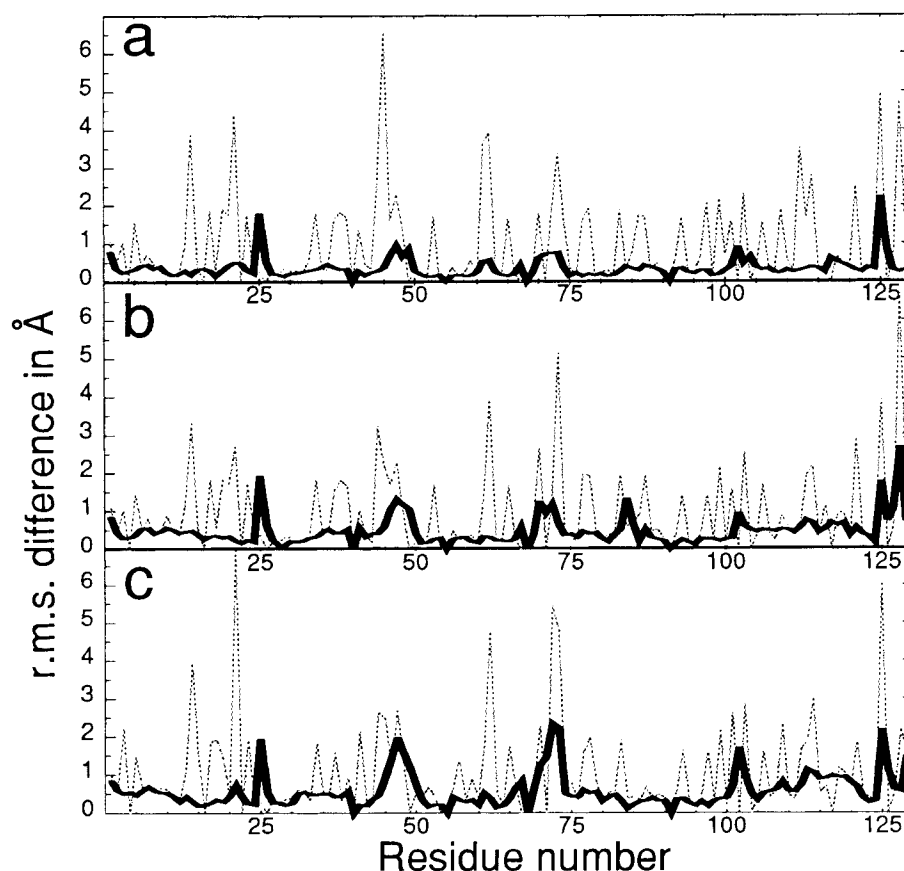


Fig. 3. Comparison of the tetragonal HEL⁴⁷ (PDB entry 1LSE) with uncomplexed bobwhite quail lysozyme (a) monoclinic form, molecule 1; (b) monoclinic form, molecule 2; and (c) hexagonal form. The RMS difference between backbone atoms (bold lines) and side-chain atoms (dotted lines) is shown for each residue. C α , N, C, and O atoms were included in the backbone calculations. Residues 40, 55, 68, and 91 appear to have zero RMS differences due to the sequence differences between BWQL and HEL in those positions.

Water Molecules in the Complex

Of the 81 bound water molecules in the refined model for the HyHEL-5-BWQL complex, two water molecules (Wat 62, Wat 70) make bridging contacts between the Fab (heavy chain) and lysozyme. Wat 62 is bound in the position of the guanidinium group of the equivalent Arg 68_Y in HEL, and is also observed in the HyHEL-5-R68K-HEL complex. It is fully buried in the center of the interface, and forms hydrogen bonds to Glu 50_H, Asn 58_H of the Fab as well as to Thr 51_Y and Lys 68_Y of BWQL. The second "bridging" water, Wat 70, makes hydrogen bonds to the O γ of Ser 56_H and O γ 1 of Thr 43_Y, and is fully buried near the edge of the interface.

Four waters (Wat 29, Wat 32, Wat 45, and Wat 66) make "bridging" hydrogen bonds between the light and heavy chains of the Fab. Two of these (Wat 32, Wat 45) are buried near the Fab-BWQL interface but make hydrogen bonds only to Fab residues. A third, Wat 29, mediates a hydrogen bond between backbone oxygens of Pro 125_H of the CH1 domain and Ala 119_L of the CL domain, and is exposed to

bulk solvent. Wat 66, which is conserved in all three HyHEL-5-lysozyme complex structures, is peripheral to the interface and hydrogen bonds to the backbone oxygens of Arg 93_L and Tyr 59_H.

One additional highly conserved water molecule (Wat 55) is the only one that is seen in the other two complexes as well as in triclinic HEL (PDB reference 2LZT)⁵⁵ and tetragonal HEL (PDB reference 1LSE),⁴⁷ and all three molecules of uncomplexed BWQL. It is internal to the lysozyme, buried just below the lysozyme epitope near residue 68_Y. This water is hydrogen-bonded to the N ϵ of Arg 68_Y in HEL, but is unable to make the same bond in BWQL where the residue is a Lys. In spite of its location near the Arg/Lys 68_Y position, this water is unlikely to play a direct part in stabilizing the complex, as it makes the same hydrogen bonds in uncomplexed and complexed BWQL. Analysis of the refined structure of HyHEL-5:HEL²¹ also indicates that this water is not involved in the formation of the complex with HEL.

As in the HyHEL-5-HEL complex, the shape com-

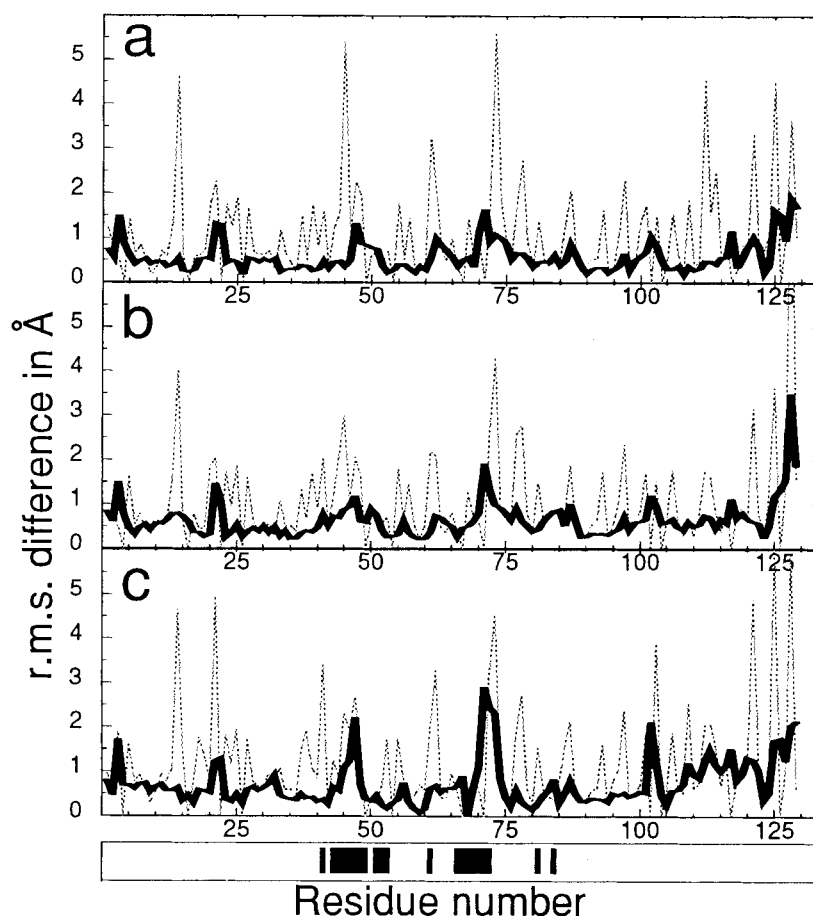


Fig. 4. RMS differences per residue between BWQL in complex and uncomplexed BWQL in the (a) monoclinic form, molecule 1; (b) monoclinic form, molecule 2; and (c) hexagonal form for backbone atoms (bold lines) and side-chain atoms (dotted lines). The RMS backbone difference from BWQL in complex was 0.6 Å and 0.7 Å, respectively, for the two molecules of the monoclinic form, and 0.4 Å for the hexagonal form. Bars below the plots indicate residues that are partially or fully buried in complex formation.

plementarity between antigen and antibody is good, as evidenced by the fact that there are only two water molecules buried in the interface. Apart from the cavities in which we have modeled waters 62 and 70, no additional cavities are observed in the interface that are large enough to hold a water molecule. The resolution of this structure precludes location of further water molecules on the edge of the interface which may mediate antibody-antigen interaction. An extensive cavity between the V_L and V_H domains is observed of approximately the same volume as that in the HyHEL-5-HEL complex,²¹ and three of the four waters in this cavity match those seen in that complex.

Comparison With Other HyHEL-5-Lysozyme Complexes

Three segments of BWQL make up the epitope with HyHEL-5, as in the HyHEL-5-HEL complex.^{2,21} The Fab residues involved in complex for-

mation are the same, with the addition of Gly 53_H, which contributes less than 3 Å² to the interface in the HyHEL-5-BWQL complex. The BWQL surface area in the interface of this complex is 740 Å², which, given the coordinate uncertainty in both structures, compares well to the 750 Å² in the HyHEL-5-HEL complex. Figure 6 shows the results of the coordinate comparison between the complexes of HyHEL-5 with BWQL, HEL and the R68K-HEL. The RMS differences between the complexes are similar (0.3 Å for the HyHEL-5-HEL complex and 0.4 Å for the HyHEL-5-R68K-HEL complex), but more residues have large backbone and side-chain differences from the HyHEL-5-R68K-HEL complex. Of the three complexes, the high-resolution data was the weakest for the HyHEL-5-R68K-HEL complex, and several surface residues in that structure had poor electron density.

In the comparison with the native HyHEL-5-HEL complex, the four residues with backbone dif-

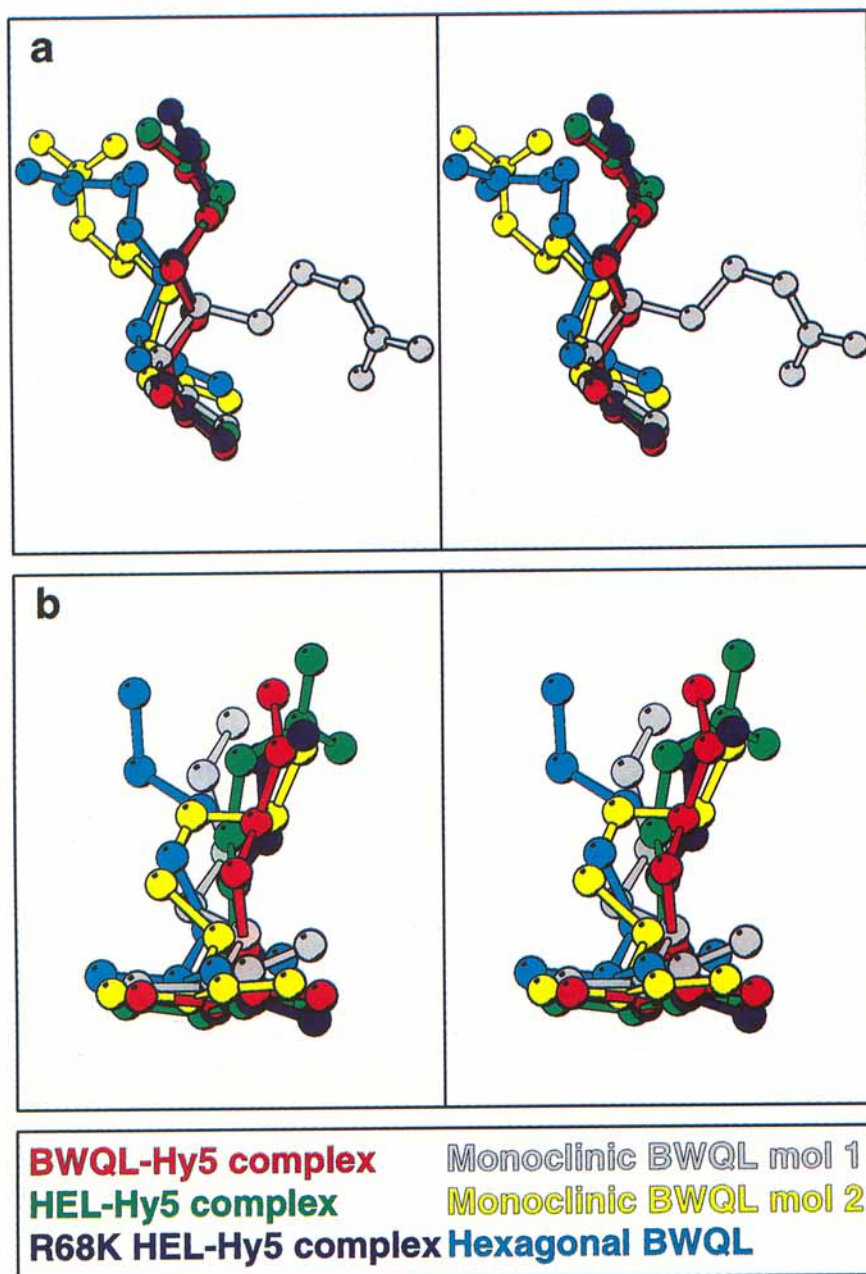


Fig. 5. Conformations adopted by residues (a) 45_Y and (b) 68_Y in the structures described. Residue 45_Y is an Arg in all the structures. Residue 68_Y is an Arg in the HyHEL-5-HEL complex, and a Lys in all the other structures. In the HyHEL-5-R68K-HEL complex, the Lys has dual conformation. Figure drawn with MOLSCRIPT.⁵⁴

ferences greater than three standard deviations above the RMS difference of 0.3 Å were Glu 132_H, Thr 70_H, Gly 22_Y, and Leu 129_Y. All four residues are surface residues on the far side of the molecules from the interface region. Side-chain residues showing differences greater than three standard deviations above the RMS difference (0.6 Å) are Cys 6_Y and Leu 129_Y. Both residues are surface residues, and the latter is the terminal residue of the lyso-

zyme chain. Although the Cys 6_Y has moved relative to the HEL-Fab complex, the disulfide bond between that residue and Cys 127_Y is preserved with almost no change in the bond distance, due to a small compensating change in position of the S_Y of Cys 127_Y.

Similar results are observed with the comparison to HyHEL-5-R68K-HEL complex. Only 12 residues have backbone RMS differences greater than three

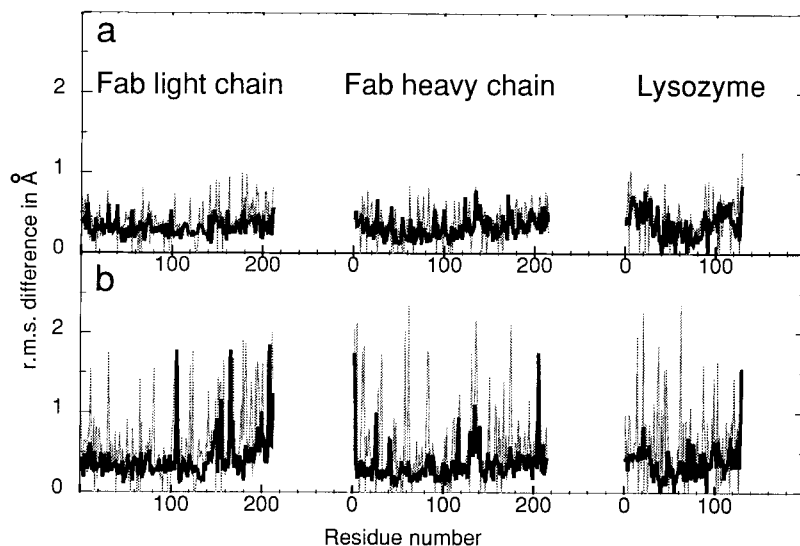


Fig. 6. Comparison between HyHEL-5-BWQL complex and related complexes. The RMS difference between backbone atoms (bold lines) and side-chain atoms (dotted lines) is shown for each residue. (a): Comparison with HyHEL-5-HEL complex. RMS backbone difference was 0.3 Å. (b): Comparison with R68K HyHEL-5-R68K-HEL complex. RMS backbone difference was 0.4 Å.

standard deviations above the RMS difference of 0.4 Å. Of these, six residues are at the N or C terminus of a polypeptide chain and are poorly ordered. Two residues in the light chain, Arg 106_L and Asp 165_L, are surface residues with poor density in the complex with R68K-HEL. Another residue, Glu 122_H, is a mobile surface residue with a high B factor in all the complexes. The remaining residues are in the Fab light chain, away from the antigen interface. Side-chain residues with large conformational differences are all surface residues with poor density and high B factors in at least one of the two complexes.

Table IV shows the possible hydrogen bonds between the Fab and the lysozyme in the complex. The hydrogen-bonding pattern is very similar to that in the previously determined complexes. As in the HyHEL-5-R68K complex, Lys 68_Y makes water-mediated hydrogen bonds to the Fab that replicate some of the hydrogen bonds made by the equivalent Arg in HEL. However, these water-mediated bonds are fewer in number than in the R68K complex where we see a dual conformation for the lysine, and no direct hydrogen bonds are made by residue 68_Y to the Fab. The resolution of these structures do not permit hypotheses about the small difference in binding affinity between R68K-HEL and BWQL for the HyHEL-5 Fab.

Analysis of the hydrogen-bonding pattern in the interface shows that fewer hydrogen bonds are made in this complex as compared to the HyHEL-5-HEL complex. Calculations of the energies of the water-mediated bonds versus protein-protein hydrogen bonds are beyond the scope of this paper. Never-

theless, HyHEL-5 Fab makes one or two fewer hydrogen bonds to BWQL than to HEL, which must contribute to the decrease in affinity. Titration calorimetry has shown that the association of HEL with HyHEL-5 is enthalpically driven, while the entropic contribution is unfavorable.⁵⁷ Similar results were obtained for the association of BWQL with HyHEL-5; the over three orders of magnitude reduction in affinity is due to approximately equal unfavorable changes in ΔH and ΔS compared to the HyHEL-5-HEL association (K.A. Shick et al., unpublished results). The hydrogen bonds observed in the antibody-antigen interface will make significant contributions to the association enthalpy.

CONCLUSIONS

Before any of the crystallographic studies on HyHEL-5-lysozyme complexes, epitope mapping using avian species variants had identified the HyHEL-5 antibody epitope on hen egg-white lysozyme (HEL). Bobwhite quail lysozyme (BWQL) binds a thousand-fold less tightly to HyHEL-5 than does HEL. This focused attention on residue 68 in the lysozyme, as the Arg 68_Y-Lys is the only sequence difference between BWQL and HEL that is involved in the antibody-antigen interface. Three other sequence differences lie away from the interface region. To examine the structural changes that are associated with this change in affinity, we had earlier determined the structure of the HyHEL-5-R68K-HEL complex, and have now determined the structures of BWQL in two uncomplexed crystal forms, also complexed with the HyHEL-5 Fab.

TABLE IV. A. Hydrogen Bonds Between Fab and Lysozyme in Complex Structure

Fab atom	BWQL atom	Distance (Å)
Trp 91 _L O	Arg 45 _Y Nη2	3.1
Trp 91 _L (center of ring) ⁵⁶	Arg 45 _Y Nη2	3.6
Gly 92 _L O	Arg 45 _Y Nε	3.1
Gly 92 _L O	Arg 45 _Y Nη2	2.8
Arg 93 _L N	Asn 46 _Y O	3.3
Arg 93 _L Nε	Arg 45 _Y O	2.8
Arg 93 _L Nη2	Asn 44 _Y Oδ1	2.9
Arg 93 _L Nη2	Arg 45 _Y O	3.1
Arg 93 _L O	Arg 45 _Y Nε	3.1
Trp 33 _H Nε1	Tyr 53 _Y Oη	3.1
Glu 50 _H Oε2	Arg 45 _Y Nη1	2.9
Ser 56 _H Oγ	Gln 41 _Y O	2.6
Tyr 101 _H N	Gly 67 _Y O	2.8

B. Bonds Mediated by Water Molecules

Fab	Water	Lysozyme
Glu 50 _H Oε2	62	Lys 68 _Y Nζ
Asn 59 _H Nδ2		Thr 51 _Y Oγ1
Ser 56 _H Oγ	70	Thr 43 _Y Oγ1

Although four sequence differences exist between BWQL and HEL, only small differences are observed between the unbound BWQL and HEL molecules. Three of the four sequence differences are buried within the lysozyme molecule, and cause only a small decrease in side-chain volume, while the fourth, R68K, is a mobile surface-exposed side chain with conformational variability in the three BWQL molecules. The structural differences between BWQL and HEL are of the same order of magnitude as those between the different molecules of the uncomplexed BWQL. The most mobile part of the molecule is the HyHEL-5 epitope.

Changes in the lysozyme upon complex formation are localized to the side chains of residues in the epitope region. Three of the residues with sequence differences between HEL and BWQL have essentially similar structures in the uncomplexed BWQL molecules and the HyHEL-5-BWQL complex. The fourth, R68K, the only one involved in the HyHEL-5 epitope, shows a large decrease in mobility upon complex formation. The hydrogen bonding patterns in the Fab-lysozyme interface show that there is a net decrease in hydrogen bonding when Arg 68 is replaced by a lysine, as was observed for the HyHEL-5-R68K-HEL complex. In contrast to that complex, no dual conformation was observed for the lysine in the current structure. Two water molecules are fully buried in the interface, and make bridging hydrogen bonds to both the Fab and the lysozyme. Although some studies have observed a small difference in the affinity of R68K-HEL and BWQL for the HyHEL-5 Fab, the resolution of this complex does

not permit speculations about the structural causes for this phenomenon.

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