Crystallographic Refinement of the Three-dimensional Structure of the FabD1.3-Lysozyme Complex at 2.5-Å Resolution*

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The three-dimensional crystal structure of the complex between the Fab from the monoclonal anti-lysozyme antibody D1.3 and the antigen, hen egg white lysozyme, has been refined by crystallographic techniques using x-ray intensity data to 2.5-Å resolution. The antibody contacts the antigen with residues from all its complementarity determining regions. Antigen residues 18–27 and 117–125 form a discontinuous antigenic determinant making hydrogen bonds and van der Waals interactions with the antibody. Water molecules at or near the antigen-antibody interface mediate some contacts between antigen and antibody. The fine specificity of antibody D1.3, which does not bind (Kd < 10^-12) avian lysozymes where Gln123 in the amino acid sequence is occupied by His, can be explained on the basis of the refined model.

The three-dimensional structure of a complex between the Fab of a mouse (BALB/c) monoclonal antibody (mAb), D1.3 (IgG1, k), and its specific antigen, hen egg white lysozyme (HEL), has been determined by x-ray diffraction at 2.8-Å resolution (Amit et al., 1986), followed by a preliminary refinement. The structural model thus obtained allowed the definition of the complete antigenic determinant as well as that of the combining site of the antibody. The antigenic determinant recognized by mAb D1.3 is discontinuous in nature, consisting of two stretches of the polypeptide chain of HEL centered around amino acid residues 20 and 120. At 2.8-Å resolution, no major conformational change was observed in the complexed antigen. The occurrence of conformational changes in the antibody could not be directly ascertained by a comparison with the free Fab since this had not been crystallized.

Fine specificity studies using a panel of anti-HEL antibodies (Harper et al., 1987) indicate that antibody D1.3 has a very low affinity for other avian lysozymes in which Gln123 is replaced by His. There are several possible structural explanations for the fine specificity of mAb D1.3, but selecting the correct one requires a model in which atomic contacts with the antigen can be precisely determined.

Since the structure of the FabD1.3-HEL complex was reported (Amit et al., 1986), FabD1.3 has been crystallized unliganded, and in a complex with the syngeneic, anti-idiotypic FabE225 (Boulot et al., 1987). In addition, the Fv segment of D1.3, consisting of the variable domains of the heavy and light polypeptide chains (VH and VL, respectively), has been cloned, expressed in Escherichia coli (Ward et al., 1989), and subsequently crystallized as the free Fv, and in a complex with the antigen, HEL (Boulot et al., 1990). The three-dimensional structures of these crystalline forms have been determined (Bhat et al., 1990) to 1.9-Å and 2.4-Å resolution, respectively. Comparison of the free and the antigen-bound Fv structures reveals a small relative displacement of the VH and VL domains. Establishing the possible occurrence of similar conformational differences between the free and the antigen-bound FabD1.3 is essential for a study of the antigen-binding reaction and for establishing a possible mechanism in the activation of effector functions by antibody molecules. This goal requires refined, high resolution structures of the free and antigen-bound FabD1.3. Thus, the crystallographic refinement of the atomic coordinates of the FabD1.3-HEL complex to 2.5-Å resolution presented here is an essential step for structure-function correlations in this experimental antigen-antibody system.

MATERIALS AND METHODS

Crystals of the FabD1.3-HEL complex were grown by macroseeding in hanging drops or capillaries, using 15 to 25% polyethylene glycol 8000, 0.1 M KPi, pH 6.0 (Mariuzza et al., 1980). Their space group is P21, with a = 90.0 Å, b = 143.5 Å, c = 49.3 Å, β = 120°, and one molecule of the complex per asymmetric unit. For this work, a new data set to 2.5-Å resolution was collected at the Biotechnology Resource, University of Virginia, Charlottesville, on the multiwire x-ray diffractometer (Sobottka et al., 1990), using two detectors placed at 87 cm from the crystal. CuKα radiation passing through a graphite monochromator was obtained from a rotating anode generator operated at 42 kV, 150 mA, with an effective x-ray focus size of 0.5 × 0.5 mm. Three crystals, measuring about 1.2 × 0.6 × 0.4 mm, were used. A total of 41,026 observations were scaled, reduced, and merged to give 20,475 independent reflections, 98% of the total expected between 5.5 and 2.5 Å (see Fig. 1). Intensity data between 5.5 and 2.8 Å, collected with one detector, gave a Rmerge = (Σ[Ii−<I>]/Σ[Ii]) of 0.077; data between 3.5 and 2.5 Å, collected with a second detector, were merged with the data collected with the first detector giving an overall Rmerge of 0.115. Reflections in the range 7.0 Å to 5.5 Å were those previously measured (Amit et al., 1986) using a four-circle diffractometer. In the final data set, 18,469 reflections had structure amplitudes |F| ≥ 2σ(F) and were retained for the crystallographic refinement reported here.

The starting atomic coordinates were those obtained at 2.8-Å resolution (Amit et al., 1986). The amino acid sequences of VH and VL have been determined (Verhoeyen et al., 1988; Ward et al., 1989); those of the constant domains of the heavy and the light chains, Cγ1 and Cλ, respectively, were taken from Kabat et al. (1987). Refinement proceeded by iterative use of the program PROLSQ (Hendrickson and Konnert, 1980; Hendrickson, 1985) followed by model building.
Crystal Structure of FabD1.3-Lysozyme Complex

FIG. 1. Crystallographic R-factor and percentage of theoretically possible reflections as a function of resolution. The line marked 0.35 Å is taken from a Luzzati plot (Luzzati, 1952) and represents a statistical error of 0.35 Å in the atomic positions of the model.

with the program FRODO (Jones, 1978; Pflugrath et al., 1984) on an Evans and Sutherland PS390 graphics system. Difference Fourier (2Fo − Fc), (Fo − Fc) and OMIT-maps (Bhat and Cohen, 1984) were used to correct the model. Individual atomic temperature factors were introduced. At the end of three cycles of rebuilding and refinement, the R-factor (R = Σ(|Fo| − |Fc|)/Σ|Fo|) was 28%, with a root mean square deviation in bond lengths of 0.030 Å. The program X-PLOR (Brünger et al., 1987) was then used to perform one cycle of refinement, giving an R-factor of 23.7% with a root mean square deviation in bond lengths of 0.024 Å. The resulting electron density map gave better indications for manual improvements to the model.

After these corrections were applied, a second X-PLOR cycle gave an R-factor of 20.5% with a root mean square deviation of 0.017 Å from ideal bond lengths. Thirty water molecules for which there were clear electron densities and acceptable hydrogen bonds were subsequently included in the model, and their coordinates and temperature factors were refined with X-PLOR. Three cycles of X-PLOR conjugate gradient minimization positional (without simulated annealing) and individual B-factor refinement were performed giving a final R-factor of 18.4% (19.5% for all reflections) with a root mean square deviation of 0.013 Å and 3.3° from ideal bond lengths and angles. The root mean square deviation of the angle χi from its nearest ideal value is 16.3° (Bhat et al., 1979; Hendrickson and Konnert, 1980). An estimate of the intrinsic accuracy of the atomic positions (Luzzati, 1952) indicated a mean error of 0.35 Å (see Fig. 1). A list of the atomic coordinates has been sent for deposit at the Brookhaven Data Bank.

The calculation of interatomic contacts between atoms of the antigen and the antibody was performed with the same maximum contacting distances used by Sheriff et al. (1987) and Tulip et al. (1989) (see Table I).

Enzyme-linked immunosorbent assays to test direct binding of monoclonal antibodies to hen and turkey egg white lysozymes were performed as described (Harper et al., 1987).

RESULTS AND DISCUSSION

Most of the carbonyl groups of the main chains of the FabD1.3-HEL complex can be oriented with confidence in the electron density maps. The major corrections applied to the preceding 2.8 Å resolution model were in the Cγ1 and Cε domains. The polypeptide chains can be traced throughout, except for Cε1 residues 130 to 136, forming part of an external loop poised to solvent, for which there is no clear electron density. In addition, several other loops near the terminal cystine of the Cγ1 and Cε chains are based on irregular density, namely residues Cγ1 154 to 158, Cε 199 to 203, and Cε1 187 to 194. For the latter, a cis-Pro109 seems to fit better in the electron density than a trans, but this interpretation is not definitive. By contrast, the region around the interchain disulfide, which is difficult to trace in many Fab structures, is stabilized by contacts between neighboring molecules and could be easily interpreted in the maps. In the variable domains, a major correction was made in the orientation of VH Leu26, which was exposed to solvent in the 2.8 Å model and is now buried. This change in VH affects the adjacent residue Thr30 in that it is not in contact with HEL as in the initial model (see below). Also, the Vl Pro99 previously built as trans has now been corrected to the cis form; this modification does not lead to significant changes in the antigen-antibody contacts previously reported. These two changes in the complementarity determining regions (CDRs) bring them into conformity with the canonical structures derived from other immunoglobulins and with their predicted conformation (Chothia et al., 1986).

Within the limits of the current resolution, the V regions and, in particular, the antigen-antibody interface are well defined throughout without obvious ambiguities in the positioning of the main chain or the side chains. The Ramachandran plots (Ramachandran and Sasiskharan, 1968) (Fig. 2) indicate that the angular distribution conforms well with those seen in highly refined high resolution structures. Thus, the extension of the resolution from 2.8 Å to 2.5 Å and the refinement reported here result in an improvement in the R-factor (18.4% with a root mean square deviation from ideal bond lengths of 0.013 Å, compared to 28% and 0.03 Å) and in the estimated error of the atomic coordinates (0.35 Å compared to 0.6 Å).

As described before (Amit et al., 1986), the angle made by the pseudo-2-fold axes relating VH to VL and Cγ1 to Cε is of about 176°, giving an elongated Fab structure with few intra-

FIG. 2. Ramachandran plots of the model at 2.5 Å resolution. ○, Gly; x, other residues. a, FAB D1.3 (432 residues); b, lysozyme in the Fab-HEL complex (129 residues).
chain, interdomain contacts. In the crystal lattice, the longer axis of the Fab-HEL complex is roughly parallel to the b axis of the unit cell. Contacts between neighboring molecules of the complex along that axis occur between the C-terminal part of the Fab residues near the N and C terminus of HEL (Arg\textsuperscript{2}, Cys\textsuperscript{6}, Gly\textsuperscript{126}, Cys\textsuperscript{137}, and Arg\textsuperscript{286}).

Antigen-Antibody Contacts—Contacts between atoms of antigen and antibody are listed in Table I. The list is based on maximum van der Waals radii and hydrogen bond distances for the atoms involved in the contacts as explained in Table I. These distances are the same as used by Sheriff et al. (1987) and Tulip et al. (1989) so that the epitopes defined in different crystallographic studies can be consistently compared. Direct contacts with the antigen are made by VL residues Tyr\textsuperscript{22}, Tyr\textsuperscript{26}, Thr\textsuperscript{29}, Phe\textsuperscript{31}, Trp\textsuperscript{32}, and Ser\textsuperscript{33}, and by VH residues Gly\textsuperscript{31}, Trp\textsuperscript{52}, Gly\textsuperscript{55}, Asp\textsuperscript{54}, Asp\textsuperscript{56}, Tyr\textsuperscript{57}, and Arg\textsuperscript{58}. Some of these and, in addition, VH Arg\textsuperscript{58}, contact antigen residues through water molecules 803, 805, 806, 807, 810, 815, 816, and 822 (water molecules are numbered 801 to 830; Fig. 3b). Since at 2.5-Å resolution not all water molecules may be unequivocally located, there could be additional interactions mediated by them. A comparison with antigen contacts made by FvD1.3 (Bhat et al., 1990) shows substantial agreement although with some differences which can mostly be explained by the estimated error (about 0.35 Å) in the atomic coordinates. Other differences with Amit et al. (1986) are due to the crystallographic refinement of atomic coordinates reported here and to the distances used in defining contacting atoms.

Twelve lysozyme residues make contact with the antibody combining site. They are Asp\textsuperscript{86}, Asn\textsuperscript{89}, Gly\textsuperscript{125}, Ser\textsuperscript{136}, Asp\textsuperscript{127}, Gly\textsuperscript{128}, Thr\textsuperscript{130}, Asp\textsuperscript{131}, Val\textsuperscript{132}, Gln\textsuperscript{134}, Ile\textsuperscript{135}, and Arg\textsuperscript{136} (Table I). The list of contacting residues is in agreement with that of Bhat et al. (1990), except for Tyr\textsuperscript{127} and Lys\textsuperscript{128}. In the Fv structure, Tyr\textsuperscript{127} makes a single van der Waals contact with VL Asp\textsuperscript{135}, and Lys\textsuperscript{128} makes a hydrogen bond (3.2 Å) with O of FvD1.3 Tyr\textsuperscript{127}. In the FabD1.3-HEL complex, the corresponding hydrogen-bonded atoms occur at a distance of 4.0 Å. The HEL residues in contact with the antibody contribute to a discontinuous epitope. Additional contacts are made through water molecules, as discussed below.

A least squares fit of the Ca atoms of FabD1.3-bound HEL with those of free HEL in its tetragonal form at 1.6-Å resolution (Blake et al., 1965) gives a root mean square difference of 2.9 Å.

### Table I

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*Contacts between the HEL Gly\textsuperscript{121} N-2 and the aromatic ring of the side chain of VL Tyr\textsuperscript{26} can be taken (Levitt and Perutz, 1988) as a hydrogen bond interaction.*
of 0.48 Å. The largest difference, 1.1 Å, is observed for the Ca of Leu19. This C-terminal residue is very mobile as reflected by a large temperature factor. Thus, as noted before (Amit et al., 1986), no major change of conformation can be observed in the bound HEL. Changes in the orientation of its amino acid side chains relative to free lysozyme (Blake et al., 1965) can be observed in areas that are in contact with the antibody as well as in other areas which are not. However, a change in the conformation of Asn19 (a rotation of about 100° around a Ca-Cβ axis) can be explained by the formation of the complex with the antibody. In the crystal structure of native HEL, the N62 of Asn19 makes a hydrogen bond with the O61 of Asp18. This conformation of Asn19 could not be kept in the complex with D1.3 since it would lead to disallowed van der Waals contacts with Vλ Tyr50.

Using the program MS (Connolly, 1983), the sum of the areas of the solvent-buried surfaces of HEL and D1.3 after complex formation was found to be 1290 Å². For this calculation, the radius of the probe was taken to be 1.4 Å.

The pattern of hydrogen bonds (Fig. 3a) reveals the complexity and complementarity of the antigen-antibody interaction. Through these bonds, antibody main and side chains contact the epitope main and side chains. For example, main chain atoms of Vλ CDR3 (O of Phe51, N of Ser50) are hydrogen-bonded to atoms of a side chain (Nε2 and Oɛ1 of Gln51) of the antigen. In addition, the close approach of antigen and antibody allows main chain atoms of Vβ CDR2 to make close hydrogen bonds with main chain atoms of the antigenic determinant. Water molecules buried in the antigen-antibody interface contribute to the hydrogen bonding pattern between antigen and antibody (see below).

Water Molecules—Peaks in difference electron density maps consistently appearing at the later stages of crystallographic refinement have been interpreted as indicating the presence of solvent molecules. Since the current study does not have the resolution required for an unequivocal assignment, only those showing good electron density, low temperature factors, and close proximity to hydrogen bond donors or acceptors were retained. As a consequence, only 30 well ordered, buried, or partially buried water molecules, most of which have also been observed in the FvD1.3-HEL complex (Bhat et al., 1990), were added to the model (Fig. 4). Some, such as 805, 809, and 822 (Fig. 3b), are in contact with both the antigen and the antibody and are buried in their interface. Water molecules 815 and 816, which also make a bridge between antigen and antibody (Fig. 3b), are largely exposed to the bulk solvent. Other possible water molecules such as 806, 809, and 810 fill a cavity in the VH-VL interface opening onto the antigen-antibody contacting area. However, an alternative explanation for some of the electron density peaks assigned to these water molecules is that they correspond to one of two partially occupied positions of Vλ Tyr50.

No buried water molecules were found inside any of the domains of FabD1.3 except for 802, H-bonded to Vλ Nε1 Trp35 (3.0 Å), O Thr31 (2.8 Å), and O Ser48 (3.1 Å), and for 803, H-bonded to VΗ O Tyr25 (3.2 Å), O Ile81 (2.9 Å), Nε Lys35 (3.1 Å).
partridge, turkey, California quail, pheasant, and guinea hen are found in the interface between CHI and CL and between VH and VL. Water molecules fill the gaps as discussed above for 805, 807, 811, 818, 822, and 826. Water molecules 811, 818, 822, and 826 are in a pocket between the antigen and the antibody, close to VH Gly 121.

Antigen Variability and Antibody Specificity—The reactivity patterns of monoclonal anti-lysozyme antibodies were studied using a panel of eight different avian lysozymes (Harper et al., 1987). Antibody D1.3 reacts with high affinity only with HEL and bobwhite quail lysozyme which have a Gln residue at position 121. In the avian egg white lysozymes from partridge, turkey, California quail, pheasant, and guinea hen that were used to study the fine specificity of mAb D1.3, position 121 is replaced by His. As can be seen in Table I and Fig. 5, Gln 121 makes a large number of close contacts with the antibody. It penetrates a hydrophobic pocket where it is surrounded by the aromatic side chains of VH Tyr 32, VL Tyr 92, and VH Tyr 101. The atoms of its side chain make a large number of van der Waals contacts with atoms of VH, VL, and VH Tyr 101. In addition, its side chain amide nitrogen and hydroxyl make three buried H bonds with the antibody. The hydrophobic cavity penetrated by Gln 121, lined by the aromatic side chains of the antibody, is closed toward the solvent side by Arg 125 and Asp 119 of HEL, which are linked to each other by a salt bridge.

Among the possible explanations for the very low affinity of D1.3 for the heterologous lysozymes are: 1) His 121 may be too bulky for the tight pocket occupied by Gln in the FabD1.3-HEL complex; 2) His 121 could be charged and consequently unstable in the hydrophobic pocket described above; and 3) at least one hydrogen bond would be lost when His 121 is substituted for Gln 121.

The second explanation has been ruled out by performing direct binding enzyme-linked immunosorbent assays. These indicate no binding of D1.3 to plates coated with turkey egg white lysozyme (TEL) at pH values from 4.5 to 9.0, a range that goes from a predominantly charged to an uncharged form of the imidazole side chain of His. As a control, the recognition of HEL by mAb D1.3 and that of HEL and TEL by mAb D122.13 (Harper et al., 1987) are not affected in that pH range. In addition, the loss of H bonds resulting from the replacement Gln by His at position 121 could contribute to the much lower affinity of D1.3 for the variant lysozymes. It is interesting to note in this context that VH D1.3 can bind HEL by itself with an affinity constant only one order of magnitude smaller than that of the whole mAb D1.3 or its Fv (Ward et al., 1989). In the VH-HEL interaction, some of the hydrogen bonds made by the side chain of Gln 121 with VL residues (see Table I and Fig. 5) may not be formed.

The explanation invoking steric hindrance would be fully compatible with the refined structure reported here. A histidine residue could not fit into the hydrophobic cavity formed by VH Tyr 32, Trp 92, and VH Tyr 101 and further enclosed by lysozyme residues Asp 119 and Arg 125. The 2 latter residues are linked to each other by a salt bridge which would in all likelihood prevent a displacement of the side chain 121 toward the solvent. In this tightly packed cavity, the histidine side chain would make a number of disallowed contacts with antibody atoms. Thus, a simple interpretation of the lack of recognition by antibody D1.3 of heterologous antigens in which position 121 is occupied by His could be steric hindrance. We cannot, however, preclude the possibility of small structural adjustments around the pocket for Gln 121 to accommodate the larger amino acid side chain. Should this be the case, the loss of one hydrogen bond and the hydrogen-aromatic interaction would lead to a less favorable free energy gain in comparison to the complex formed with HEL. Whatever the true mechanism may be, this structure provides a basis for explaining the frequently observed escape of viral and microbial antigens from antibody neutralization through the occurrence of point mutations affecting their antigenic determinants.

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