Crystallization and Structure Determination of an Autoimmune Antipoly(dT) Immunoglobulin Fab Fragment at 3.0 Å Resolution*

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HED10 is an autoimmune antibody (IgG) which shows considerable specificity for the single-stranded DNA poly(dT). Production of Fab fragments by papain digestion resulted in heterogeneity as judged by isoelectric focusing gels, which had a marked negative effect on crystallization. However, a single species of Fab with a pI of 7.6 could be isolated in good yield by DEAE-cellulose chromatography, and good crystals were produced by the hanging drop vapor diffusion method. The space group was P2₁, with cell dimensions, a = 64.2, b = 90.0, c = 42.3 Å, and β = 96.7°. These crystals diffract to about 2.2 Å resolution. The structure of Fab HED10 was solved by the molecular replacement method using the known structure of McPC603 and is refined to R = 27.2% at 3.0 Å resolution. Fab HED10 is more extended than McPC603 and has an elbow angle (between the variable and constant domains) of 162°, very similar to that observed in Fab KOL. The majority of the hypervariable regions are visible in the model.

Antibodies provide a family of proteins with very similar overall structure. The similarity of their architecture is accompanied by variations in their binding sites which result in their specificity for a large variety of molecules. Anti-DNA antibodies are the subject of intensive studies (for a review see Ref. 1). They provide the opportunity to investigate how proteins can recognize different DNA structures and/or sequences and the range of interactions that can provide the capability for such recognition. Crystallographic studies of these proteins and their complexes with DNA will provide an understanding of the protein-nucleic acid interactions that give rise to their specificity, whether it is for the configuration of the sugar-phosphate backbone or for particular bases. The properties of these proteins make them very interesting models for studying protein-nucleic acid interactions.

The determination of the structures of DNA-binding immunoglobulins will allow the comparison of their structures with those of proteins that have evolved to bind particular DNA structures or base sequences. This is particularly of interest in view of the observed similarities of three-dimensional structure and amino acid sequence among proteins which are involved in repression and activation of transcription (2-8).

Anti-DNA antibodies are also of interest because of their involvement in the disease systemic lupus erythematosus (9). The sera of patients with this autoimmune disorder (as well as animal models of the disease) frequently contain high titers of anti-DNA antibodies which have been implicated in the pathogenicity of systemic lupus erythematosus. It is thought that these antibodies form immune complexes which, for as yet unknown reasons, are not cleared from the system, resulting in tissue damage.

For these reasons HED10 is a particularly suitable antibody for structural studies. Not only was it derived from autoimmune mice (10) which develop a disease resembling human systemic lupus erythematosus but also it shows considerable specificity in its interactions with single-stranded DNA (11). Detailed binding studies revealed a strong preference for poly(dT) and poly(d(2BrU)) with the recognition of four consecutive bases. Its binding constant is strongly dependent upon ionic strength suggesting that two phosphates are involved in the interaction. Comparison of these two Fab fragments will be most instructive.

The structure of a number of Fab fragments (13-17), Bence-Jones proteins (18-20), and variable domain dimers, Fc (21-24) have been reported and are known at atomic resolution. In addition, the low resolution structure of an intact immunoglobulin molecule is also known (15, 25-27). Comparison of the structures of different Fab fragments (28) has revealed a common folding pattern for the variable and constant domains of the light (Vλ and Cλ) and heavy (VH and CH1) polypeptide chains and a similar association of Vλ-Vλ and Cλ-Cλ into the variable (V) and constant (C) domain pairs. There are only two polypeptide connections between the domain pairs, and there are relatively few interatomic contacts between them. For these reasons Fab molecules display considerable flexibility.

The high purity of the protein used for crystallization is very often an imperative in growing crystals suitable for x-ray diffraction experiments (29). We present here the methods used for the preparation of a homogenous HED10 Fab fragment, which was essential for the crystallization of the protein, and the determination of its structure at 3.0 Å resolution.

MATERIALS AND METHODS

The preparation of the HED10 IgG from the hybridoma cell line was as described by Lee et al. (10). The immunoglobulin isotype was

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determined by solid-phase radioimmunoassays with the aid of isotype-specific anti-mouse IgGs (Zymed Laboratories). The HED10 Fab fragment was produced by incubating IgG with papain (Sigma), typically for 4 h at 37 °C in 0.25 M NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM 2-mercaptoethanol (11). The papain was inactivated with iodoacetamide, and the digest was dialyzed versus 10 mM Tris-HCl, pH 8.0, before being loaded on isoelectric focusing (IEF) gels for analysis, or applied to a DEAE-Sepacel column equilibrated with the same Tris buffer for purification. The Fc portion was retained by the DEAE-Sepacel and eluted late in a 0–0.5 M NaCl gradient. The Fab fragments eluted in the flow through or early in the gradient.

The Fab fragment was crystallized by the vapor diffusion technique (30). The protein, 7.5 mg/ml in 5% polyethylene glycol (PEG) 8000 (Sigma), 0.05 M NaCl and 25 mM MOPS, pH 6.8–7.0, was equilibrated through the vapor phase with 10% PEG 8000 and 50 mM MOPS buffer, pH 7.0. Before data collection the crystals were equilibrated against 15% PEG 8000, 0.1 M NaCl, and 50 mM MOPS, pH 7.0.

Isoelectric focusing in polyacrylamide gels was carried out according to the Pharmacia product manual (31).

Data Collection—The intensities were collected on a SYNTEx P2, diffractometer equipped with a long detector arm (crystal to detector distance of 55 cm). Since the crystals were sensitive to radiation and decayed relatively quickly in the x-ray beam, the data were collected by scanning only the tops of the peaks (32, 33). Each reflection was scanned in seven steps over a narrow ω-range (0.10–0.15°) and checked to ensure that the top of the peak was scanned. Five consecutive steps within the top of the peak were used to derive the net intensity. For each reflection the background was measured for 2 s on either side of the peak with a 0.9° offset in the ω angle. Ten reflections, distributed over the reciprocal space and covering the 2θ range investigated with each crystal, were measured every 100 reflections and used later for proper scaling and correction for decay. Approximately 100 reflections were measured per hour. Data collection continued until the intensities of the control reflections declined on average to 50% of their original intensities.

To estimate the background for each reflection Fletterick et al. (33) made the assumption that the background is a slowly varying function independent of χ and that the dependence of 2θ and φ could be separated. They measured the background at the end of data collection for one value of χ as a function of 2θ and for one value of 2θ as a function of φ, and the background at any position was calculated based on these observations.

Application of this procedure to our data sometimes led to large negative intensities which showed a systematic pattern, indicating that for some φ angles the background was overestimated. This is due to the oversimplified description of the angular dependence of the background. To obtain a better estimate of the background around each reflection, the (6,2θ) plane was divided into a grid with δ(φ) = 5° and δ(2θ) determined empirically from the 2θ dependence of the background. An average background was calculated for each grid point from the actual short (4 s/reflection) background measurements of reflections with φ and 2θ within ± (δ(φ),δ(2θ)) from that grid point. Measurements differing by more than 3σ from the mean were discarded and the mean value recalculated. For each reflection the average background from the nearest grid point was taken as an estimate. Most of those were based on a total measurement time between 50 and 100 s. With the background estimated in this way only a few reflections had negative intensities below the −3σ(1) level. The absorption correction was estimated according to the method of North et al. (34), and the anisotropic decay correction was derived from the reflections after Fletterick et al. (33).

Data to 3Å resolution were collected from two crystals (Table I). The symmetry equivalent reflections from each crystal were averaged (Rmerge = 0.04 and 0.08, respectively) and then merged together (Rmerge = 0.10). Data for two heavy atom derivatives, K3Hgl and HgCl2, were collected and processed in a similar way. Some statistical information is given in Table I.

RESULTS
Protein Purification and Crystallization—An examination of the microheterogeneity of Fab fragments that were used for crystallization experiments was initially undertaken be-

\[ \text{IEF} \text{, isoelectric focusing; PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid.} \]

\[ \text{TABLE I} \]

| Statistical information on HED10 native and derivative data sets |
|---------------------------------------------------------------|
| Native | K3Hgl | HgCl2 |
| No. of crystals | 2 | 1 | 1 |
| Resolution | 3.0 Å | 6.0 Å | 5.0 Å |
| No. of measured reflections | 10,994 | 1,385 | 4,961 |
| No. of independent reflections | 9,695 | 1,279 | 2,193 |
| No. of reflections >2σ(f) | 7,504 | 1,141 | 2,096 |
| \( R_{\text{merge}} \) | 0.04 (0.08) | 0.035 | 0.024 |

**FIG. 1. Papain digestion of HED10 IgG.** The polyacrylamide isoelectric focusing gel was run with a pH gradient of 4–9 (acidic at the bottom). The approximate pI of the different Fab bands are indicated. Lane a is a dissolved crystal. The papain digestions in lanes b–f were carried out as described under “Materials and Methods”. The weight ratios of papain to IgG were: b, 1:50; c, 1:100; d, 1:250; e, 1:500; and f, 1:1000. cause one preparation yielded crystals while subsequent preparations did not. Analysis of the different preparations of Fab by IEF indicated that they were, in fact, a mixture of several species with pI values between 7.0 and 8.5. The preparation that crystallized contained the same pI species as the preparations that did not crystallize but the distributions differed. Analysis of the products of the papain digestion of IgG HED10 by IEF revealed that the conditions of the digestion affected the distribution of pI species produced. By altering the IgG/papain ratio, it was found that the distribution of the different species could be altered (Fig. 1). Digestions at a constant ratio of IgG/papain for different periods of time were not as effective in altering the distribution of products (data not shown). Presumably the papain became inhibited, although this was not verified. In the case of HED10, the major initial product of papain digestion is clearly subjected to further proteolysis to yield a species with a pI of 7.6. At still higher levels of papain further degradation occurs to smaller molecular weight material. Although the distribution of the pI species could be changed by altering the conditions of the papain digestion, this was not in itself sufficient for obtaining high quality crystals for x-ray diffraction measurements. IEF analysis of single crystals (see Fig. 1) revealed that they contained predominantly

1 The abbreviations used are: IEF, isoelectric focusing; PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid.
the pl 7.6 species and very little, if any, of the others. This information, combined with the analysis of the products of the papain digestion and their dependence on the conditions of the digestion, allowed the adaptation of the digestion to provide for maximal production of the pl species that occurred in the crystals. Although this species could have been purified by preparative IEF, it was found that it could be purified from the other Fab fragments by DEAE-cellulose chromatography (Fig. 2). This allowed a simpler purification with better overall yield.

The HED10 pl 7.6 Fab fragment was crystallized as described above. The protein crystallizes in the monoclinic space group P21, with cell dimensions a = 64.2, b = 90.0, c = 42.3Å, and β = 96.7°. One Fab fragment of 50,000 daltons per asymmetric unit would result in a Vₐ of 2.43 Å³/dalton, which is in the range generally observed for protein crystals (35). The x-ray diffraction pattern of these crystals extends to about 2.2 Å resolution. The purity of the protein dramatically prevents the nucleation of crystal growth. In some preparations containing a distribution of species such as those described above. The protein crystallizes in the monoclinic space group P2₁ with cell dimensions

A

Fig. 2. Purification of pl 7.6 Fab fragment. A, elution profile from a DEAE-Sephacel column (1.5 x 15 cm). The gradient was from 0 to 0.5 M NaCl in a total volume of 500 ml. The fractions were 2.5 ml. B, Polyacrylamide isoelectric focusing gel of selected fractions from the DEAE-Sephacel chromatography. Lane a is the papain digest used; lanes b-h are fractions 10, 25, 27, 29, 30, 31, and 32, respectively.

Structure Solution—The structures of three immunoglobulin Fab fragments, NEW (36), McPC603 (14), and KOL (15) have been determined at atomic resolution and are well-refined. The comparison of their structures (28) shows that the conformation of each of the domains of the heavy and light chains are similar among the three proteins. The largest differences are within the hypervariable loops. The V and C domain pairs of the Fab molecule are connected by short polypeptide links, one within the heavy chain and one within the light chain, providing substantial flexibility in the so-called "switch" or "elbow" region. The elbow angle, defined as the angle between the pseudo 2-fold axes of symmetry of the Vi-Vh and Ci-CHl domain pairs, varies between 132° (McPC603, (14)) and 166° (KOL, (15)). This variation is even greater when the light chain dimers Mcg (113°, (18)) and Loc (97°, (19)) are considered. The three good models of an Fab molecule were available, molecular replacement seemed to be the method of choice for the structure determination. Although the amino acid sequence of HED10 is at present only partially known, the homology to McPC603 is expected to be higher than that of KOL or NEW. McPC603 was obtained from the same species (mouse) as HED10 and its light chain was also a κ-type. Since only one gene codes for the Ck(κ) region in mouse (37), the sequence of that part of both proteins had to be nearly identical. The heavy chain of McPC603 belongs to class α while that of HED10 belongs to class γ2A and the similarities of their amino acid sequences are less pronounced (38).

Determination of Orientation—A series of rotation functions were calculated, employing the fast rotation function algorithm (39) with the McPC603 Fab fragment as a model. Parameters such as the resolution shell, the integration radii, and the number of reflections were varied. Two peaks, corresponding approximately to a 30° difference in the orientation of the model, appeared consistently in all calculations. Since a difference in the elbow angle between McPC603 and HED10 was expected, these results were interpreted as an indication of a different spatial arrangement of V and C domain pairs in HED10 as compared to McPC603. According to that assumption one peak corresponded to the correct orientation of the V domain pair (and the incorrect orientation of C domain) while the other indicated the orientation of the C domain pair. To test this hypothesis, and to determine which peak was related to which pair, a series of rotation functions were calculated using only the V or the C domain pair as a model. In each case only one dominant peak was observed and it was at one of the two positions obtained previously (Table II). A detailed account of these calculations and comparison with the results obtained by using other Fab structures as starting models will be presented elsewhere.²

Determination of the Position of the Molecule within the Unit Cell—In the polar space group P2₁, the dimensionality of the problem of finding a proper position within the asymmetric unit of the correctly oriented molecule is decreased from three to two. Only translations along the x- and z-axes have to be determined while translation along the y-axis may be arbitrarily set to zero. To determine the x- and z-translations, the model was first rotated into the orientation indicated by the rotation function peak and then moved in small steps (1Å or less) within the part of the x,z-plane corresponding to one permissible origin. The correlation coefficient

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between the observed and the calculated structure factors was computed for each position of the model and the maximum located (40). Only data within the resolution shell 6–4 Å, containing 2581 reflections (> 2σ(I)), were included in these calculations. Such searches were performed independently for V and C domain pairs. The highest correlation coefficient for each pair corresponded to a very similar translation (see Table II). To determine the relative translation along the y-axis between the two pairs, they were positioned according to their respective peaks in the x,z-plane. The correlation coefficient was then calculated as a function of the translation of the C domain pair along the y-axis. The maximum correlation coefficient was \( r = 0.45 \). At this stage the Fab fragment was divided into four rigid domains, V\(_L\), C\(_L\), V\(_H\), C\(_H\), and the orientation and position of each of them was adjusted stepwise (within a narrow angular and translational range) to maximize the correlation coefficient. The final results are shown in Table II. As an independent test of the correctness of this model, phases determined by it were used to calculate difference maps for the two available derivatives. The highest peaks in these maps corresponded to the positions of heavy atoms derived independently from the analysis of the difference Patterson function. The K\(_2\)HgI\(_4\) derivative was also used independently to determine the translations of correctly oriented domains.

Model Building and Partial Refinement—One of the major concerns in the process of refining a protein at medium resolution on the basis of molecular replacement results is the bias introduced by the starting model (41). To minimize this effect a rather conservative approach was used in building the first model of the HED10 Fab molecule. Since the amino acid sequence of the HED10 Fab fragment is at present only partially known (about 23 NH\(_2\)-terminal residues of each chain plus the C\(_L\) domain), the amino acid sequence of McPC603 was used. The C\(_L\) backbones of three Fab fragments, McPC603, KOL, and NEW were visually compared on a graphic system. Residues forming part of the chain that adopted different conformations in these molecules, were removed from the starting model. This included, of course, the hypervariable loops and the switch region. A total of about 2400 atoms out of 3399 of McPC603 were then used to phase the reflections. An electron density map was calculated using the coefficients \( 2F_o - DF_c \) (42), which has the property of minimizing the model bias. The map was displayed on the MMS-X graphics system with the help of the program M3 (43). The parts of the chain that were not used for the phasing of this map and were clearly recognizable, were then added to the model. In the phase set where no sequence information was available, the side chains were altered according to the shape of the electron density. This model contained 2822 atoms. The R-factor calculated at this point was 38.0%. Eight cycles of restrained least squares refinement (44) with relatively rigid restraints reduced the R-factor to 32.7%. The model was then refined on the graphics system to a new electron density map calculated with \( 2F_o - DF_c \) coefficients and phases calculated from the improved model. It was possible at this point to follow the polypeptide chain in the switch region, as well as parts of the hypervariable regions. The next eight cycles of refinement further reduced the R-factor to 27.2%. The model will be refined further when the resolution of the diffraction data is extended and the amino acid sequence is determined.

### DISCUSSION

Analysis of Papain Digestion for Producing Fab Fragments—Papain digestion of the HED10 IgG was carried out with the aim of reducing the molecular weight and the conformational heterogeneity due to the flexibility of the intact IgG. This increases the probability of obtaining suitable crystals. One side effect is that the papain digestion introduces chemical heterogeneity due, presumably, to variability in the position or number of proteolytic cleavages. Our results show that, in the case of HED10, it is important to purify the Fab fragments produced to obtain crystals suitable for x-ray diffraction analysis. IEF provides a convenient and sensitive method for analyzing the products of the papain treatment and optimizing the conditions that are used for the digestion. In this case we were fortunate to obtain the initial crystals which allowed us to determine what pl species was incorporated in the crystal and accordingly adapt our procedures to purify that particular species. In most cases it will be useful to carry out crystallization trials on each of the purified pl species in order to obtain the most satisfactory crystals (29) whether or not the initial mixture of Fab fragments crystallize. When there are problems with the reproducibility of crystallization, or the size or the quality of crystals, careful purification to yield a chemically and conformationally pure sample is often a good first step towards alleviating the problem.

Crystal Structure of HED10—The structure of the HED10 Fab fragment has been solved at 3 Å resolution by the molecular replacement method. The conformation of Fab HED10

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**Table II**

| Domain | \( \alpha \) | \( \beta \) | \( \gamma \) | Height | Correlation coefficient |
|--------|---------------|---------------|----------------|--------|------------------------|
| \( V_L \) - \( V_H \) | 110 | 102 | 259 | 5.5 | 4.7 |
| \( C_L \) - \( C_H \) | 82 | 100 | 255 | 6.1 | 4.5 |

### Details of molecular replacement solution rotation search

| Domain | \( \Delta x \) | \( \Delta y \) | \( \Delta z \) | Correlation coefficient |
|--------|---------------|---------------|----------------|------------------------|
| \( V_L \) - \( V_H \) | 26.5 Å | 0.0 Å | 16.0 Å | 0.260 |
| \( C_L \) - \( C_H \) | 29.5 Å | 0.5 Å | 17.0 Å | 0.195 |

*Numbers given are the ratio of peak height to the rms value of the maps.*

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2 M. Cygler, A. Boodhoo, J. S. Lee, and W. F. Anderson, unpublished observations.
FIG. 3. Stereoscopic view of HED10 Fab molecule. Part of a molecule related by $Z_1$ symmetry is also shown to illustrate the intermolecular contacts along the $y$-axis. The dashed lines show possible position of the complementarity determining region 1 that was not included in the current model. The S-S bridges are indicated by solid lines joining the $C_\alpha$ atoms of the appropriate cysteine residues.

FIG. 4. Crystal packing showing molecules in the $x,z$-plane. Possible position of complementarity determining region 1 is shown by dashed lines.

that is observed in the crystal exhibits some differences from McPC603, which was used as a model for the rotation/translation searches. The most significant difference is in the magnitude of the elbow angle. The HED10 molecule is much more extended than McPC603 with an elbow angle of 162°, compared to 132° for the latter. In this respect HED10 resembles Fab KOL (15) which has an elbow angle of 166° and is the most extended of the Fab molecules and light chain dimers that have been investigated to date by x-ray crystallography. There are also differences between HED10 and McPC603 at the level of the association of the light and heavy chains into the V and C domain pairs. These differences are immediately visible from the somewhat different values of the final rotation angles and translation parameters (Table II) for the $V_L$ and $V_H$, and $C_L$ and $C_H$ domains. The transformation that superimposes the $V_L$ onto the $V_H$ domain in HED10 is composed of a rotation by 175.6° and a translation of 0.4 Å along the rotation axis (calculated with the program HOMOLOGY (45) based on positions of $C_\alpha$ atoms only). Corresponding values for McPC603 are: 173.0° and 0.2 Å. Similarly, for the superposition of $C_L$ on $C_H$ the values are 168.8°, 1.6 Å and 170.7°, 2.7 Å for HED10 and McPC603, respectively. Values for HED10 are based on coordinates refined to $R = 27.2\%$ and may change slightly upon further refinement. The $C_\alpha$ atom representation of the HED10 Fab molecule is shown in Fig. 3.

In the recently determined structure of the light chain dimer Loc a somewhat different association of the two $V_L$ domains was observed than is found in other immunoglobulin fragments (19). While the transformation relating the $V_L$ and $V_H$ domains usually has a very small translational component along the pseudo 2-fold axis (less than 0.5 Å) in Loc this has a value of 3.5 Å. The relative translation of the two $V_L$ domains in the direction perpendicular to the axis of the $\beta$-barrel results in the closing of the central cavity in the antigen-binding region. Rather than forming a binding pocket or cavity this creates a site that protrudes from the protein surface. They speculate that such a different mode of association may, if present in other immunoglobulins, contribute at yet another level to the diversity of the immune response system. It is, however, equally possible that the association mode observed in Loc is a result of having two identical domains forced to form a complex. Up to now, the mode of association within the V domains that is observed in Fab fragments does not show such differences.

The electron density corresponding to the first complementarity determining regions of both the heavy and light chains is low and difficult to interpret at this stage and is not represented in the current model. At this moment, we attribute the weak density to the relatively large errors in our current phases rather than to disorder or large thermal motion of that segment of the polypeptide chain. The density in the
other hypervariable regions and the switch region was stronger, and it was possible to trace the chain in these locations.

**Packing of Molecules in the Crystal**—The molecules in the crystal form layers parallel to the x,z-plane. Two contacting layers are related by the 2-fold screw symmetry. Molecules in one layer interact in a head-to-tail fashion, with the V domains of one molecule packed against the C domain of the neighboring one, forming infinite lines in the [110] direction. This mode of packing, where the antigen-binding region of a Fab molecule packs tightly against symmetry-related molecules, is often observed in crystals of Fab fragments (28). The detailed nature of these interactions in HED10 crystals is not yet known since the electron density in the antigen-binding region is only partly interpreted. The contacts between symmetry related molecules, is often observed in crystals of Fab fragments (28). The overall architecture would appear to be reported. The antigen-binding site rather than in the structure itself.

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