Preliminary Crystallographic Data and Primary Sequence for Anti-peptide Fab' B1312 and Its Complex with the C-helix Peptide from Myohemerythrin*

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Crystals of the Fab' fragment from the monoclonal anti-peptide antibody B1312 and of the Fab'-peptide antigen complex have been characterized. The monoclonal antibodies were raised against a synthetic homologue of the C-helix of myohemerythrin (residues 69–87 in myohemerythrin). The Fab'-peptide complex crystallizes in space group P622 with unit cell dimensions a = b = 142.5 Å, c = 101.5 Å, α = β = 90°, γ = 120°, and Z = 1. The native Fab' crystallizes in space group P212121 with unit cell dimensions a = 98.0 Å, b = 151.7 Å, c = 80.8 Å, α = β = γ = 90°, and Z = 2. Both crystal forms diffract to beyond 2.6 Å resolution. We also report the cDNA and predicted amino acid sequences for the variable regions of both the light and heavy chains of this anti-peptide antibody.

Recent advances in the production of anti-peptide antibodies have opened new avenues of investigation into the mechanisms of antibody-antigen specificity (Lerner, 1982). Synthetic peptides homologous to surface regions of proteins have been shown to elicit antibodies reactive with both the peptide antigen and the native protein. These anti-peptide antibodies have been found to be effective in protein purification (Field et al., 1988), as tools to help study protein folding, in serological testing and in detecting biologically important conformational changes (White and Wilson, 1987). Anti-peptide antibodies may also be useful in some instances as vaccines (Lerner, 1982). Progress has been made in this direction with anti-peptide antibodies reactive with foot-and-mouth disease virus, hepatitis-B virus, polio virus, and malaria (Francis et al., 1987; Thornton et al., 1987; Chow et al., 1984; Patarroyo et al., 1988).

Further insight into the structural aspects of antibody-antigen binding has been provided by the recent crystal structure solution of four antibody-protein complexes. The availability of these antibody-protein complexes is due largely to the advent of monoclonal antibody technology and the subsequent ability to select antibodies that bind to specific protein antigens. Three of these structures are of lysozyme-Fab complexes (D1.3, Amit et al., 1986; HyHEL-5, Sheriff et al., 1987; HyHEL-10, Davies et al., 1989) and one is of a neuraminidase-Fab complex (NC-41, Colman et al., 1987). In comparisons with the native antigen crystal structures, tertiary structural changes in the antigen upon binding Fab vary from minimal in the D1.3 Fab-lysozyme complex to more extensive in the NC-41-neuraminidase complex (see review by Davies et al., 1988). No structures have been reported for the identical free and complexed Fab although crystals have been obtained of two different monoclonal Fabs and their respective Fab-antigen complexes. (Stura et al., 1987a, 1987b; Schulze-Gahmen et al., 1988).

The monoclonal antibodies described in this study were raised against a peptide homologue of the C-helix of Mhr1 (residues 69–87) coupled to a protein carrier. All of the anti-peptide antibodies are from the fusion B13 (Fieser et al., 1987). By ELISA analysis of the omission- and replacement-series analogues, the antigenic determinants on the peptide homologue were mapped for each monoclonal antibody (Fieser et al., 1987). Antibodies C, I, and L recognize a site spanned by amino acids 69–73. Antibodies A and F recognize a site spanned by amino acids 79–84 (Fig. 1). Antibodies A and F bind peptide with equal or greater affinity than they bind Mhr. Interestingly, antibodies C, I, and L bind Mhr with greater affinity than they bind peptide.

Two-dimensional 1H NMR and circular dichroism (CD) studies have been used to examine the peptide structure in solution. The NMR experiments showed that the C-terminal region of the peptide in water forms a "nascent helix": the region quickly converts between different turn-like structures but does not form a stable helix (Dyson et al., 1988). The addition of trifluoroethanol to the peptide/water solution (60% trifluoroethanol) stabilizes the helix between residues 78–85. These results are confirmed by the CD experiments; these indicate no helix for the peptide/water solution, and 50% helix for the peptide/water/trifluoroethanol solution (Dyson et al., 1988).

We have crystallized the Fab' fragment of the anti-peptide monoclonal antibody B1312 in its native form and in a complex with the synthetic peptide corresponding to the C-helix of Mhr. Preliminary data collection results indicate that both crystal forms are excellent candidates for successful three-dimensional structure determination. The cDNA and the predicted amino acid sequences for the variable regions of the light and heavy chains of B1312 Fab' are also reported. This

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1 The abbreviations used are: Mhr, myohemerythrin; ELISA, enzyme-linked immunoadsorbent assay; HPLC, high performance liquid chromatography; MPD, methylpentanediol.
amino acid sequence is necessary for the high resolution structure determination of the Fab' and Fab'-peptide complex. When completed, these two crystal structures will provide a comparison of the structure of a free Fab' and its complex with antigen. Parallel studies are being carried out in this laboratory on crystals of Fab'. Future studies will then allow a comparison of the secondary structure of the peptide when bound to different Fabs at two separate epitopes.

EXPERIMENTAL PROCEDURES

Immunological Materials and Methods

Monoclonal Antibody Production—Protein conjugates were prepared of keyhole limpet hemocyanin linked to a peptide homologue corresponding to residues 69–87 of Mhr. The spleen cells of immunized mice (strain A/J) were fused to cells of the P3X63-Ag8.653 murine myeloma line by the method of Galfre and Milstein (1981) and Fieser et al. (1987).

IgG Purification and Fab' Production—IgGs were purified from ascites fluid by ammonium sulfate precipitation. Ammonium sulfate was first added to ascites fluid to a final concentration of 20%. After centrifugation the resulting supernatant was brought to a final concentration of 50% saturated ammonium sulfate. The resulting precipitate was resuspended in phosphate-buffered saline and dialyzed overnight against 10 mM Tris-HCl, pH 8.0. The crude IgG was then purified by anion exchange chromatography on a Synchropak Q300 column (Pharmacia). The IgG purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (Fab')' fragments were produced by digesting the IgG with 50 μg/ml pepsin, pH 3.5. After a 1-h digest, the reaction was halted by raising the pH to 7.0. Fab' fragments were produced by reducing the (Fab)2 with 10 mM cysteine for 2 h at 25 °C. The reduction was stopped by the addition of 30 mM iodoacetamide in the absence of light at 4 °C for 4 h. The Fab' fragments were then dialyzed and concentrated against 10 mM Tris-HCl, pH 8.0. The Fab' fragments were purified by size-exclusion chromatography on a Sephacryl Sf-300 column (Pharmacia). The concentrated fractions were then further purified by anion exchange chromatography on a monoQ column (Pharmacia) and analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fragments appear as a single band on silver stained polyacrylamide gels. The Fab' fragments bind both peptide and Mhr with similar affinity to that of the intact IgG (∕M 5 5.40 × 10^10 m for Mhr to IgG).

Affinity Measurements

Relative affinities of peptide and Mhr for the antibody B1312 were determined by inhibition radioimmunoassay where the solid phase was Mhr and the peptide or Mhr are in solution along with radiolabeled monoclonal antibody (Fieser et al., 1987).

The peptide homologue of the Mhr C-helix was synthesized in the laboratory of Dr. R. A. Houghten by standard techniques (Houghten, 1985). The peptides were then purified by HPLC on a Vydac C18 reverse-phase column (Dyson et al., 1988).

Nucleotide Sequencing

Total hybridoma RNA was isolated (Chomczynski and Sacchi, 1987) and poly(A)′ mRNA prepared by selection with oligo(dT) cellulose (Maniatis et al., 1982). cDNA was synthesized (Promega cDNA synthesis system) from 1 μg of poly(A)′ mRNA and ligated into a gem 4 arms (Promega). Heavy and light chain variable region-specific DNAs were selectively cloned by the polymerase chain reaction (Saiki et al., 1988) using T7 primer and the respective immunoglobulin primers (C H = 5′-AGATCCAGGGCC4TGATA-3′; C K = TGGATGTCGCAAATG (C K from Pharmacia)). Heavy and light chain polymerase chain reaction products were purified by 2% agarose gel electrophoresis, phosphorylated with T4 polynucleotide kinase (Boehringer Mannheim), and ligated into dephosphorylated pGEM 7zf plasmid DNA (Promega). Plasmid primer SP6 (Promega) and C H or C K primers were used to sequence minipreps of pGEM 7zf.

Peptide Synthesis

The amino acid sequence of the C-helix peptide from myohemerythrin. The peptide corresponds to residues 69–87 in Mhr. Two determinants have been mapped onto the peptide; residues 69–73 and 79–84 (Fieser et al., 1987). Residues 78–85 have been shown to exist as a nascent helix in solution by two-dimensional NMR (Dyson et al., 1988). The determinant of the anti-peptide antibody B1312 corresponds to residues 69–73, GVVPH.

FIG. 1. The amino acid sequence of the C-helix peptide from myohemerythrin. The peptide corresponds to residues 69–87 in Mhr. Two determinants have been mapped onto the peptide; residues 69–73 and 79–84 (Fieser et al., 1987). Residues 78–85 have been shown to exist as a nascent helix in solution by two-dimensional NMR (Dyson et al., 1988). The determinant of the anti-peptide antibody B1312 corresponds to residues 69–73, GVVPH.

FIG. 2. Photomicrograph of crystals of the B1312 Fab'-peptide complex. The crystals grow as hexagonal discs with dimensions of up to 0.8 mm in diameter × 0.2 mm in thickness from a mixed solution of 1.85 M monobasic sodium phosphate and 1.85 M dibasic potassium phosphate, pH 5.75. The photomicrograph was taken at × 60 magnification between crossed polarisers.

FIG. 3. Photomicrograph of crystals of the native Fab'. The crystals grow as rectangular rods with dimensions of up to 1.0 × 0.2 × 0.2 mm from 1.1 M sodium citrate, 1% MPD, pH 6.0. The photomicrograph was taken at × 60 magnification between crossed polarisers.
containing inserts. Two to three heavy and light chain sequences were obtained using Sequenase (United States Biochemicals).

Crystalization Procedures

Crystal conditions were screened and crystals grown using the microvapor diffusion method and other standard crystallization techniques (McPherson, 1982). All crystals were grown by a modified sitting drop method (Stura et al., 1989). Initial Fab'-peptide complex crystallization experiments revealed a precipitate with consistent oval morphology at 1.5 M sodium citrate, pH 6.0. These precipitates were then used to seed other sitting drops at lower salt concentrations. Crystals with hexagonal morphology grew from these and successive seed transfers and optimization of the conditions yielded Fab'-peptide complex crystals of excellent quality (Fig. 2). Macroseeding was carried out with a modification of the technique used by Thaller et al. (1989). All seeds used were of good morphology, ranging from 0.01 to 0.1 mm, and were used when fresh, since older crystals often displayed various surface defects. Optimization of the crystallization conditions involved the replacement of citrate by 1.85 M monobasic sodium phosphate and 1.85 M dibasic potassium phosphate mixed to pH 5.75. The protein solution in the initial sitting drop contained 8 mg/ml Fab' and 0.5 mg/ml 19-mer peptide (EVVPHKMKHDFLE-KGGL). Protein drops were 14 μl in size with a ratio of protein to buffer of 1:3.1.

Native crystals were obtained only when MPD was used as an additive (1–5%) with sodium citrate as the precipitant at pH 5–7 (Fig. 3). Optimal conditions were determined to be 1.1 M sodium citrate, 1% MPD, pH 6.0. Protein (16 mg/ml) and precipitant were mixed in a 2:1 ratio with a typical drop size of 7.5 μl. Crystals appeared after 4 days. No crystals have yet been obtained in the absence of MPD. The production of limited nuclei by small quantities of additives such as MPD has been observed previously in our laboratory.

Crystal Analysis

To verify the presence of peptide in the Fab'-peptide complex crystals, the crystals were analyzed by reverse-phase HPLC. The crystals were washed twice with well solution for approximately 10 min each wash and then dissolved in 0.1% trifluoroacetic acid. This solution was then applied to a Vydac C18 reverse-phase column. The elution buffer consisted of 40 mM acid.

Data Collection

Data were collected using the Siemens-Nicolet-Xentronics area detector mounted on an Elliott GX-18 rotating anode x-ray generator operating at 40 kV, 55 mA with 100 μm focus and Franks focusing mirrors (Harrison, 1968). Data were reduced using the Xenogen package of programs (Howard et al., 1987) modified to run on the Sun workstation by Dan Bloch, Michael Siani, and Patricia O'Reilly in our laboratory.

RESULTS AND DISCUSSION

Antibody-Peptide Complex—Space groups and unit cell parameters were determined by precession photography. The Fab'-peptide complex crystals are hexagonal with space group P6_322 and unit cell parameters a = b = 142.5 Å, c = 101.5 Å, α = β = γ = 90°, and one molecule per asymmetric unit (Fig. 4). The crystals have a V_m value of 2.9 Å³/dalton (Matthews, 1968) with the protein occupying 43% of the volume of the crystal assuming a molecular mass of 52,000 daltons for the complex and a protein partial specific volume of 0.74 cm³/g. The crystals grow as hexagonal discs with a diameter of up to 0.8 mm and thickness of about 0.2 mm (Fig. 2). These crystals diffract to at least 2.6 Å resolution. Data complete to 2.8 Å resolution have been collected and processed from a single crystal. The R factor on intensities is 14.2% for data between infinity and 2.59 Å resolution.

Several results appear to substantiate the presence of peptide in these crystals. For example, attempts to crystallize the Fab' without added peptide under conditions identical to those required for growth of Fab'-peptide crystals were unsuccessful. Furthermore, analysis of extensively washed crystals by reverse-phase HPLC showed an approximate 1:1 molar ratio of Fab' to peptide.

Native Antibody—The native Fab' crystals are orthorhombic in space group P2_12_1, with cell constants of a = 98.0 Å, b = 151.7 Å, c = 80.8 Å, α = β = γ = 90° and two molecules per asymmetric unit (Fig. 5). The crystals have a V_m value of 3.0 Å³/dalton, and the protein occupies 41% of the volume of the crystal assuming a molecular mass of 50,000 daltons for the Fab' monomer. The crystals grow as rectangular rods with dimensions of up to 1.0 × 0.2 × 0.2 mm (Fig. 3) and diffract to at least 2.6 Å resolution. Data have been collected to 2.77 Å resolution and have an R factor on intensities of 12.9%.

Analysis of Gene and Protein Sequence—The cDNA nucleotide sequence and predicted amino acid sequence for the variable regions of the light and heavy chains of B1312 are shown in Table I. The B1312 heavy chain shows greater than 90% nucleotide sequence identity to several antibodies: 93.2%...
with the anti-dinitrophenyl antibody, a member of the VH
SerCysAlaAlaSerGlyPheThrPheSerArgCysAla~etSerTrpValArgGlnThr
AspValLeuMetThrGlnValProLeuSerLeuProValSerGlyGlyAspLeuValLysPr~lyGlySerLe~ys~u
GAn;TTTTGATGACCCAAACTCCACTCTCCCTGCCTGT~GTCTTGGAGAT~GCCTCC
LeuGlnMetSerSerLeuArgSerGluAspThrAlaIle~rTyrCysThrArgTyrSer
CTACAGAn;AGCAGTCTGAGGTCTGAGGACACGGCCATTTATTACTGTACAAGATACTCT
GluValGlnLeuValGluSerGlyGlyAspLeuValLysPr~lyGlySerLe~ys~u
GAGGTGCAGCTGGTGGAGTCTGGGGGAGACTTAGn;~GCCTGGA~TCCCTG~CTC
ProThrPheGlyGlyGlyThrLysLeuGluIleLysArgAla
SerGlyValProAspArgPheSerGlySerGlySerGlyTysIle
TyrLeuGlnLysPrffilyGlnSerProLysLeuLeuIle~rLysValSerAs~gPhe
SerArgValGluAlaGluAspLeuGlyValTyrTyrCysPheGlnGlySerHisValPro
ProAspThrValLysGlyArgPheIleIleSerArgAspAsnAlaArgAsnThrLeuSer
60 70
7183 family  (Riley
ProAspThrValLysGlyArgPheIleIleSerArgAspAsnAlaArgAsnThrLeuSer
et al.,
1987a, 1987b). The greatest homology for
greatest homology with  the anti-lysozyme loop antibody  (Darsley  and
1984). In a  comparison of the
4 Rotation func-
4 To
date, results of the molecular replacement analyses are very
promising for the solution of both the Fab’ and the Fab’-
peptide complex crystal structures.

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Structural Studies of an Anti-peptide Fab' and Fab'-Peptide Complex

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