X-ray quality crystals which diffract to high resolution (=1.9-2.1 Å) have been grown of an anti-peptide Fab and its complex with a 9-residue peptide antigen. Both crystals are monoclinic P2₁, with unit cell dimensions a = 90.3 Å, b = 82.9 Å, c = 73.4 Å, β = 122.5° for the native Fab and a = 63.9 Å, b = 73.0 Å, c = 49.1 Å, β = 120.6° for the complex. The peptide sequence corresponds to residues 100-108 of all influenza virus hemagglutinins (HA1) of the H3 subtype (1968-1987). The peptide antigen has been well characterized immunologically (Wilson, I. A., Nimam, H. L., Houghton, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767-778; Wilson, I. A., Bergmann, K. F., and Stura, E. A. (1986) in Vaccines '86 (Channock, R. M., Lerner, R. A., and Brown, F., eds) pp. 33-37, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), structurally, as a free peptide by NMR (Dyson, J. H., Cross, K. J., Houghton, R. A., Wilson, I. A., Wright, P. E., and Lerner, R. A. (1985) Nature 318, 480-483; Dyson, J. H., Lerner, R. A., and Wright, P. E. (1988) Annu. Rev. Biophys. Chem. 17, 305-324), as part of the intact antigen by x-ray crystallography (Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) Nature 289, 366-373) and by binding studies to the HA molecule (White, J. M., and Wilson, I. A. (1987) J. Cell Biol. 105, 2887-2896). Knowledge of the three-dimensional structure of the complex will elucidate the details of how anti-peptide antibodies recognize a small peptide antigen and provide insights into the recognition of the same sequence in the intact protein antigen. As both native Fab and the peptide-Fab complex have been crystallized, we can also determine in addition whether changes in the structure of the antibody accompany antigen binding. The nucleotide sequence of the mRNA coding region of the anti-peptide Fab has been determined to provide the amino acid sequence ultimately required for the high resolution three-dimensional structure determination.

Major advances in our understanding of the detailed interactions in antibody-antigen complexes have been made recently with the structure determinations of three Fab-protein complexes. The structure of two Fab-lysozyme complexes (Amit et al., 1985, 1986; Sheriff et al., 1987) and a neuraminidase-Fab complex (Colman et al., 1987a, 1987b) have shown the very close association of antibody and antigen. Although the structure of the native antigens are available for comparison with their structure in each of the solved complexes, in none of these cases could the structure of the free Fabs be determined as suitable native Fab crystals could not be obtained. Consequently, one cannot be certain of the extent to which antigen conformational changes accompany binding to these protein antigens.

Further advances in our understanding of antigenic determinants have arisen from the enormous proliferation of studies using synthetic peptides as immunogens (Arnon and Sela, 1969; Lerner, 1982, 1984; Berzofsky, 1985). Antibodies raised against such peptides have been shown to react not only with determinants previously identified by anti-protein antibodies but perhaps more importantly with epitopes not normally reactive when the intact protein is the immunogen (Green et al., 1982, Lerner, 1982, 1984; Berzofsky, 1985). These anti-peptide antibodies are currently being used as research tools for a multitude of purposes which include basic detection of a given antigen, recombinant protein purification, tissue specificity of viruses, serological testing, detection of biologically important conformational changes and vaccine development. Some pertinent examples of the latter include anti-peptide antibody neutralization of the foot-and-mouth disease (Francis et al., 1987), hepatitis-B virus (Thornton et al., 1987), polio virus (Chow et al., 1984), and malaria (Patarroyo et al., 1988).

In light of such studies, we wish to investigate in structural detail how an anti-peptide antibody can react with both a peptide immunogen and its cognate sequence in the parent antigen. It has been suggested for anti-peptide antibody recognition that flexibility of the peptide determinant in the intact antigen is important (Tainer et al., 1984, 1985). This correlation has also been made for some anti-protein antibodies (Westhof et al., 1984; Williams and Moore, 1985), although some controversy still exists in both systems as to what parameters correlate best with antigenicity (Moudallal et al., 1985; Thornton et al., 1986; Novotny et al., 1987; Geyssen et al., 1987).

In this study, we investigate the interaction of a peptide, corresponding to a sequence of the influenza virus hemagglutinin (residues HA1 100-108), with an anti-peptide antibody.
The original influenza peptide study was initiated by Green et al. (1982), who investigated the reactivity of 25 different anti-peptide (HA1) rabbit antisera with intact hemagglutinin or influenza virus. An analysis of the location of the reactive peptides based on the x-ray structure of the A/Ann/68 influenza virus HA (Wilson et al., 1981), showed that the entire surface of the HA molecule could be considered antigenic (Green et al., 1982). A panel of 21 mouse monoclonal antibodies was then raised against one of these peptides (HA1 75-110) by Niman et al. (1983), and their fine specificity was determined (Wilson et al., 1984, 1986; Wilson, 1985; Houghten, 1985; Houghten et al., 1986). The major immunodominant site lies in the trimer interface in the intact HA (Wilson et al., 1984) consistent with the fact that the antibodies react in solution at neutral pH only with monomeric HA1 "tops" (residues 27-328 (Kc ~10 M⁻¹)) (Wilson, 1985; Wilson et al., 1986).

Recently, these antibodies have provided evidence for a pH-induced conformational change in the HA trimeric structure as might well occur in the biologically important HA-mediated membrane fusion event between the virus and the host cell endosomal membrane (White and Wilson, 1987). In addition, the free peptide (HA1 98-106) has been shown to have a surprisingly high percentage of type 1 β-turns in water (Dyson et al., 1989). This has led to the proposal of a possible role for the folding of peptide in solution in producing protein-reactive anti-peptide antibodies (Dyson et al., 1988).

In this paper, we report the crystallization of the Fab fragment of one of our panel of anti-peptide antibodies (17/9) with and without bound peptide. Preliminary crystallographic data and binding studies indicate promise for the successful high resolution structure determination of both the native Fab and the peptide-Fab complex, aided by the deduced amino acid sequence data presented here.

**EXPERIMENTAL PROCEDURES**

**Antibody**—The monoclonal antibody 17/9 (IgG2α) is a subclone of monoclonal antibody H17D05, which was raised against a 36-amino acid residue peptide corresponding to influenza virus hemagglutinin HA1 (residues 75-110), coupled to keyhole limpet hemocyanin as described by Niman et al., 1983.

**Papain Cleavage of Monoclonal Antibody**—Mercuriapain (Sigma) was incubated in PBS, containing 10 mM cysteine and 2 mM EDTA, at a concentration of 1 mg/ml for 15 min at 37 °C. The active enzyme was added to a 100-fold molar excess of 1M NaCl, pH 8.4 (0.1 ml) in 1 ml of buffer (15X-5 cm) equilibrated in PBS and the protein concentration determined by measuring the absorbance at 280 nm (Arnon, 1970). The papain was added to the ammonium sulfate precipitated IgG, which had been dialyzed against PBS at pH 7, in a 1:50 (w/w) ratio (enzyme-to-antibody). The cleavage reaction was allowed to proceed for 90 min at 37 °C and was then stopped by the addition of iodoacetamide to a final concentration of 20 mM.

**Purification of the Fab Fragment**—The crude papain digest was fractionated by size-exclusion chromatography on an AcA 44 column (LKB, Bromma, Sweden) (100 × 2.5 cm) with 0.1 M sodium acetate buffer, pH 5.1, at a flow rate of 0.5 ml/min. The tracial eluate was analyzed both by absorbance measurements at 280 nm and by non-reducing sodium dodecyl sulfate-gel electrophoresis. In nonreducing gels, the Fab fragment appears as a band at 43 kDa whereas the Fc fragment shows up as a 20-22 kDa band. The Fab fragment containing fractions were combined, concentrated, and dialyzed against 10 mM Tris/acetate buffer, pH 7.8, which was the starting buffer for the following anion exchange chromatography.

The 17/9 Fab was purified by ion exchange chromatography on DEAE-cellulose (DE52, Whatman, Springfield Mill, Maidstone, Kent, United Kingdom). A salt gradient from 0-0.25 M sodium acetate in 10 mM Tris/acetate, pH 7.8, was used for developing a 20-ml column with a flow rate of 1.5 ml/min. The total gradient volume was 200 ml. Under these conditions the Fab fragment elutes as a major peak with a shoulder very early in the gradient. Contaminating Fc fragments are eluted at much higher salt concentrations. The Fab fragment, eluting in the major front peak, was concentrated and dialyzed against 0.1 M sodium acetate buffer, pH 5.5, 0.02% sodium azide, for crystallization.

The purification procedures were monitored by analytical scale size exclusion HPLC as well as sodium dodecyl sulfate-gel electrophoresis. Final analysis of the purified Fab by sodium dodecyl sulfate-gel electrophoresis and isoelectric focusing gels revealed only one band. The Fab binding activity for peptide remained unchanged from that of the intact IgG as judged from peptide binding studies in ELISA.

**ELISA**—The relative affinity of the 17/9 IgG for various overlapping peptides was determined by inhibition-ELISA analysis (Table 1). For this purpose peptide 1 (Table 1), dissolved in 0.17 M borate, pH 9.0, 30-39% (w/v) PEG 600, twice crystallization was inhibited. Aliquots of 50 μl of 1% bovine serum albumin in PBS, was added in 5-fold molar excess of the Fab solution (10 mg/ml) to the plate. The plates were washed with deionized water and blocked with 150 μl of 1% bovine serum albumin in PBS/well for 1 h at 37 °C. The plates were again washed with deionized water before the addition of 50 μl serial 2-fold dilutions of antibody in 1% bovine serum albumin in PBS/well. The antibody dilutions were incubated for 2 h at 37 °C. After washing with deionized water the bound antibody was detected by adding 50 μl of peroxidase conjugated goat anti-mouse IgG (Organon Technika, Capp Division, Westchester, PA) at a 1:500 dilution in 1% bovine serum albumin in PBS for 1 h at 37 °C. The conjugated antibody was removed, the plate was washed with deionized water. The amount of conjugated antibody bound/well was quantitated spectrophotometrically after 100 μl of a solution containing 4 mg of 2,2'-azino-di-[3-ethyl-benzthiazoline sultonate] (Boehringer Mannheim) and 3 μl of 30% hydrogen peroxide in 10 ml of 0.05 M phosphate buffer, pH 4, was added to each well. The developing color was quantitated after 15 min by absorbance measurements at 415 nm.

To measure the relative affinity of the antibody for various peptides, 50 μl of a solution containing both the antibody, at a concentration corresponding to 50% binding in the ELISA assay, and different inhibitor peptides, at a range of concentrations, were added to wells which had been coated with peptide 1. Otherwise the assay procedure remained the same.

**Nucleotide Sequencing**—The amino acid sequence of the antibody Fab was determined by sequencing the mRNA from the hybridoma. The mRNA was isolated using standard methods (Cheng et al., 1979). DNA was sequenced using the dideoxy chain termination methodology (Sanger et al., 1980), using oligodeoxynucleotide primers end-labeled with 32P and reverse transcriptase (Hamlyn et al., 1978; Gellibert, 1987). In areas of ambiguity, cDNA was synthesized from mRNA using specific primers followed by sequencing using specific primers end-labeled with 32P. The HA polymerase Klenow fragment, and Sequenase (USB, Cleveland, OH). The problems of the endogenous SP2/0-Ag14 light chain, which is essentially identical in sequence to the mouse light chain PKAPPA (11244), (Rabbit et al., 1980), were overcome by the synthesis of two oligonucleotides complementary to sequences 5G-25 and 17101, which resolved any only one ambiguity. These two primers were used in combination, labeled and unlabeled, in order to increase the specificity of the sequencing, by specific competition for these homologous sites. Compressions in the sequencing were resolved using 7 M urea and 40% formamide gels. The oligodeoxynucleotide primers were synthesized on an Applied Biosystems Synthesizer and used without further purification.

**Crystallization Procedures**—The native 17/9 Fab and the Fab-peptide complex were crystallized using the hanging drop vapor-diffusion method (for a general review of crystallization methods, see Jancarik and Petsko, 1988). For crystallization, 0.1 M sodium phosphate, pH 6.2-6.5, 0.1 M NaCl, 5-30% (w/v) polyethylene glycol 6000, and reservoir buffer, 0.2 M sodium acetate/maleate, pH 6.5-6.5, 0.1 M NaCl, 30-39% (w/v) PEG 600, were mixed in the drop and equilibrated against 1 ml of the reservoir buffer. For the cocryltrallization experiments, peptide 4 (HA1 100-108, Table 1) was added in 5-fold molar excess of the Fab solution (10 mg/ml in 0.1 M sodium acetate buffer, pH 5.5). If the peptide concentration was raised to 10-15-fold molar excess, crystallization was inhibited. Aliquots of the Fab-peptide solution (2 μl) were combined with reservoir solution, 7-12% (w/v) PEG 600, 0.02% sodium azide, at a ratio of 3:1 or 4:3 (v/v) and equilibrated against 1 ml of the reservoir buffer as before. All crystallizations were then allowed to equilibrate at 22.4 °C in a constant temperature incubator.

Attempts to diffuse peptide 4 (Table 1) into native crystals pro-

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2 K. Bowdish and J. Hicks, unpublished observations.
TABLE I
Relative affinities for overlapping antigenic peptides for monoclonal antibody 17/9, raised against peptide 75-110 (X31 numbering) of influenza virus X-47 HAI

| Peptide residues in influenza virus X-47 hemagglutinin | Peptide concentration for 50% inhibition in ELISA |
|------------------------------------------------------|--------------------------------------------------|
| 1° 88-110                                            | 10^{-4}                                          |
| 2 98-110                                             | 3 \times 10^{-7}                                |
| 3 98-106                                             | 3.5 \times 10^{-6}                              |
| 4 100-108                                            | 3 \times 10^{-7}                                |
| 5 98-104                                             | No inhibition                                   |

*The corresponding peptide sequences are:
1. V-E-R-S-K-A-F-S-N-C-Y-P-Y-D-V-P-D-Y-A-S-L-R-S
2. Y-P-Y-D-V-P-D-Y-A-S-L-R-S
3. Y-P-Y-D-V-P-D-Y-A
4. Y-D-V-P-D-Y-A-S-L
5. Y-P-Y-D-V-P-D

reduced deep cracks in the crystals if the initial concentration of the peptide solution was high (>10 \mu M). However, equilibration of native crystals with 1 \mu M peptide in 0.2 M imidazole/malate, pH 6.0, 0.1 M NaCl, 50% (w/v) PEG 600 followed by a gradual increase in the peptide concentration leaves most crystals optically intact. Typically the peptide concentration can be raised stepwise from 1 \mu M to 10 mM with a 10-fold increase each day.

RESULTS AND DISCUSSION

Peptide Binding—In order to determine the minimum-sized peptide for the crystallization experiments, we determined the relative affinities of the 17/9 IgG to a set of overlapping peptides, from the major antigenic site (HAI residues 98-110) of the immunizing 36-amino acid peptide (Wilson et al., 1984) (Table I). The binding studies defined the minimum antigenic determinant to be part of peptide 4 (residues 100-108), which is in agreement with affinity measurements for other monoclonal antibodies against the same 36-mer peptide (Wilson, 1985; Wilson et al., 1986). The precise delineation of the site to residues 101-106 was determined by exhaustive peptide substitution and deletion studies by Houghten (1985; 1986). Consistent with the often deleterious effect of free amino and carboxyl-terminal peptide residues on binding by an antibody, peptide 4 (residues 100-108) was shown to be the minimum-sized peptide for high affinity binding (Table I, Wilson et al., 1986). The concentration of this peptide necessary for 50% inhibition in an ELISA assay when competed with the standard 23-mer (peptide 1) as absorbed antigen, is 3 \times 10^{-7} M. Affinity measurements in the presence of ammonium sulfate or PEG gave results similar to those in PBS buffer. In addition, the peptide binding results were quantitatively the same in both solution immune precipitation assays, and ELISA (Wilson, 1985; Wilson et al., 1986).

Native Fab 17/9 Crystals—The native Fab 17/9 crystallizes in the monoclinic space group P21, with unit cell dimensions of a = 90.3 Å, b = 82.9 Å, c = 73.4 Å, and \( \beta = 122.5^\circ \). The crystals typically grow to dimensions of 0.6 x 0.2 x 0.2 mm in 2-4 days (Fig. 1). An assumption of two Fab molecules in the asymmetric unit results in a packing density, \( V_m \), of 2.28 Å³/dalton or a solvent content of approximately 46% (Matthews, 1968). The hk0 zone showing the systematic absences due to the 2\( \bar{1} \) screw axis along the b axis is shown in Fig. 2. A striking feature of this zone is that at low resolution (6 Å) the reflections \( h + k = 2n \) are much more strongly than those where \( h + k = 2n + 1 \). A plot of the intensity of the \( h + k \) even versus \( h + k \) odd reflections shows this trend is significant to around 4.5 Å resolution. These pseudoeextinctions suggest that the second molecule in the asymmetric unit is centered in the a b projection with respect to the first molecule. Precession photos of the h01 zone and the 0k1 zone also show pseudoextinctions at low resolution such that \( h = 2n \) and \( k = 2n \), respectively. These special conditions indicate that the spacegroup can be considered as C121 at low resolution where the two molecules in the P21 asymmetric unit are located at \( (x, y, z) \) and \( (x + \frac{1}{2}, y + \frac{1}{2}, z) \).

The crystals are exceptionally well-ordered diffracting to an anti-peptide Fab 17/9 crystal. These high quality prismatic crystals were grown from PEG 600 imidazole-malate buffer, pH 5.6-6.5 as described under "Experimental Procedures." These crystals diffract to at least 1.9 Å resolution. The crystal shown is 0.6 x 0.2 x 0.2 mm and the photomicrograph was taken at \( \times 60 \) magnification between crossed polarisers.

\( ^3K. F. \) Bergmann and I. A. Wilson, unpublished data.
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better than 1.9 Å resolution as determined from oscillation photographs taken at the Stanford synchrotron x-ray laboratory (Fig. 3). X-ray crystallographic data to 2.0 Å resolution have been collected on a Nicolet-Xentronics area detector and reduced to give an unweighted absolute R-factor on intensities of 8.6%. We are currently attempting to solve this structure by molecular replacement, using the five available Fab coordinate sets in the Brookhaven data bank, as has been successfully reported for four Fab structures (Cygler et al., 1987, 1988; Sheriff et al., 1987; Vitali et al., 1987; Prasad et al., 1988). However, several of the peptide antigens have been iodinated or mercurated and can be used as heavy atom derivatives if this should prove necessary.

FIG. 5. 1° oscillation photograph of a Fab-peptide complex crystal taken in a general orientation at the Stanford synchrotron radiation laboratory. The exposure time was 274 s at 47 mA, 3 GeV, using a 0.2-mm collimator, with a crystal-to-film distance of 100 mm at 1.08 Å wavelength. The dimensions of the crystal were 0.5 × 0.04 × 0.01 mm. The reflections at the edge of the film are at 2.1 Å resolution.

FIG. 6. 7° precession photograph of a Fab-peptide complex crystal. The space group is P2₁ with unit cell dimensions a = 63.9 Å, b = 73.0 Å, c = 49.1 Å, and β = 120.6°. The 0k1 zone is shown with h° vertical and k° horizontal. The maximum resolution shown is 6.3 Å. The systematic screw-axis extinctions can be seen on the b axis. The exposure time was 49 h at 40 mA × 40 kV on a GX-20 Elliott rotating anode on a crystal of dimensions 0.6 × 0.02 × 0.01 mm, at a crystal-to-film distance of 100 mm.

17/9 Fab-Peptide Complex Crystals—The crystals grown from the Fab-peptide solutions are also in the space group P2₁, but with unit cell dimensions of a = 63.9 Å, b = 73.0 Å, c = 49.1 Å, and β = 120.6°. It should be noted that both the Fab-peptide complex and the native molecule crystallize in
unit cells having one edge close to the 72.4 Å dimension common to λ light chain dimer crystals (Schiffer et al., 1985). For these crystals the constant domains form an “infinite” β-sheet pattern resulting in the 72.4 Å repeat. Conceivably, similar interactions could be occurring with both our two Fab crystal forms. The Fab-peptide complex crystals grow as long thin rods with dimensions of 1.0 × 0.02 × 0.01 mm within 1–2 weeks (Fig. 4). One Fab molecule in the asymmetric unit results in a packing density, V_m of 0.19 Å^3/Å, or a solvent content of 38%. The crystals diffract to at least 2.1 Å resolution as determined at the Stanford synchrotron radiation laboratory (SSRL) (Fig. 5). A 7° precession photo of the 0k1 zone is shown in Fig. 6. These results suggest that a high resolution crystallographic analysis of these cocryrstals will be possible which will provide an independent solution of the Fab-peptide complex. Attempts to obtain larger crystals are in progress.

In order to confirm the presence of peptide in the cocystals, various control experiments were performed. Crystalization experiments with either Fab or peptide alone under the crystallization conditions, characteristic for the assumed Fab-peptide complexes gave no crystals. Reverse-phase HPLC analysis of complex crystals, which were washed twice with the well solution and then dissolved in 0.1% trifluoroacetic acid, showed an approximate 1:1 (mol/mol) ratio of peptide to Fab. It could of course be argued that the peptide is simply trapped in the solvent channels and not specifically bound in the antibody-binding site. However, the relatively high affinity of the peptide for the antigen even in high salt or PEG suggests that specific Fab-peptide complexes predominate in the crystall.

**Analysis of Gene and Protein Sequence**—The cDNA sequences determined for the light and heavy chains are shown in Table II. The VH sequence demonstrated that this was part of the VH 7183 gene family, one of the two VH region families proximal to the J_H D region (Alt et al., 1987). Those sequences found to show the highest similarity for the VH region were that of an anti-dinitrophenyl antibody (Riley et al., 1986) and VH 7183 gene member, (Yancopoulos et al., 1984) an anti-CEA antibody (Cabilly et al., 1984), an anti-SRBC antibody (Ollo et al., 1984) and an anti-β-(1,6)galactan (Hartmann and Rudikoff, 1984) VH gene. The analysis of the VH region demonstrated only two nucleotide sequences with significant similarity. The anti-lysozyme loop (gloop) antibody (Darsley and Rees, 1985) and the anti-dinitrophenyl antibody (Riley et al., 1986) showed 87 and 69% similarity, respectively. Comparison of the amino acid sequence of Fab 17/9 with that of the Fab fragments of the two Fab region families was under the crys-

| Protein sequence numbers are consistent with Kabat numbering  |  |  |
|---|---|---|
| Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M., Gottleman, K. S. (1987) *Sequences of Proteins of Immunological Interest*, 4th ed., Public Health Service, National Institutes of Health, Washington, D. C. |  |  |
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