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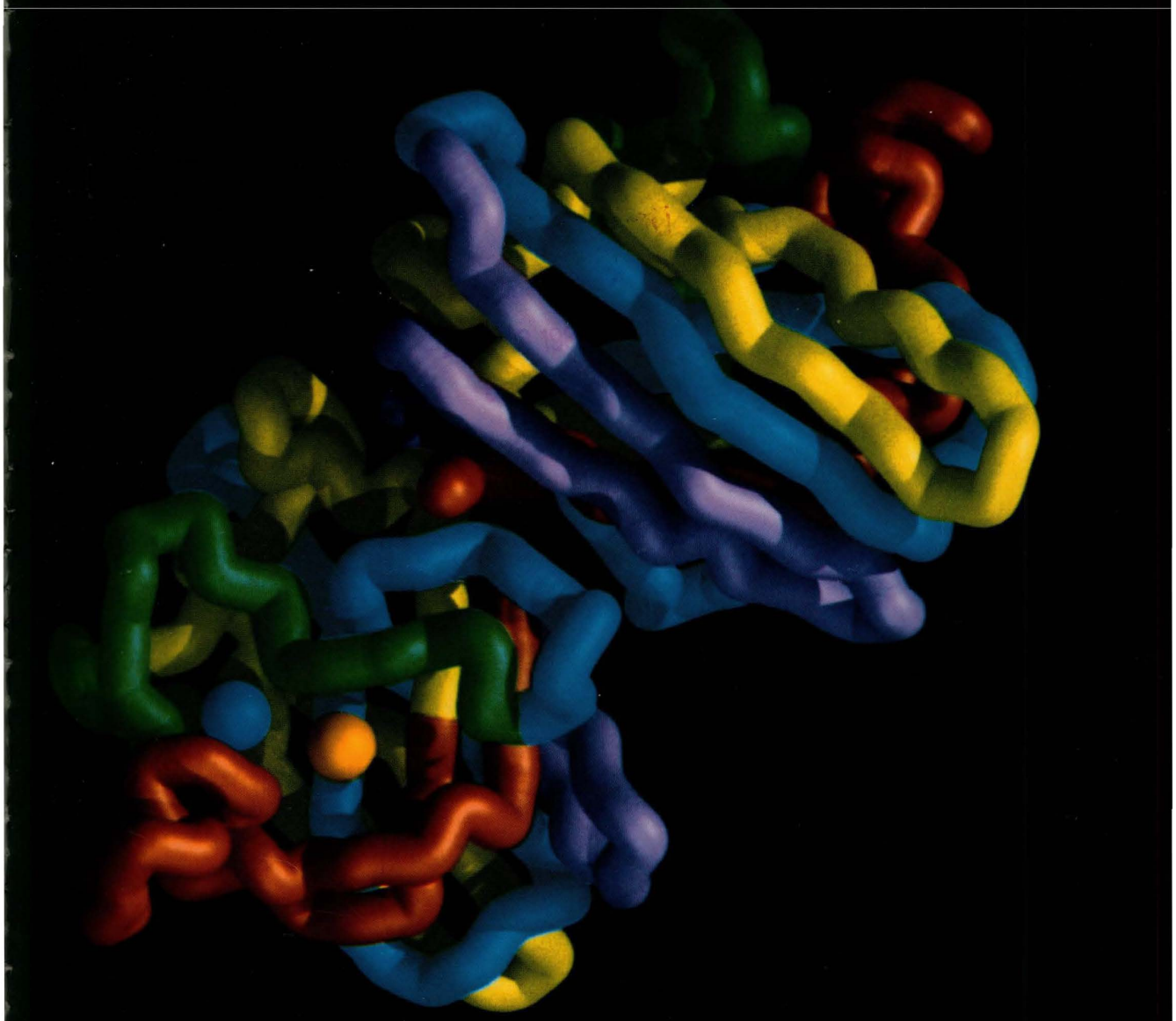

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Three-Dimensional Structure of a Fluorescein–Fab Complex Crystallized in 2-Methyl-2,4-pentanediol

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ABSTRACT **The crystal structure of a fluorescein–Fab (4-4-20) complex was determined at 2.7 Å resolution by molecular replacement methods. The starting model was the refined 2.7 Å structure of unliganded Fab from an autoantibody (BV04-01) with specificity for single-stranded DNA. In the 4-4-20 complex fluorescein fits tightly into a relatively deep slot formed by a network of tryptophan and tyrosine side chains. The planar xanthonyl ring of the hapten is accommodated at the bottom of the slot while the phenylcarboxyl group interfaces with solvent. Tyrosine 37 (light chain) and tryptophan 33 (heavy chain) flank the xanthonyl group and tryptophan 101 (light chain) provides the floor of the combining site. Tyrosine 103 (heavy chain) is situated near the phenyl ring of the hapten and tyrosine 102 (heavy chain) forms part of the boundary of the slot. Histidine 31 and arginine 39 of the light chain are located in positions adjacent to the two enolic groups at opposite ends of the xanthonyl ring, and thus account for neutralization of one of two negative charges in the haptenic dianion. Formation of an enol-arginine ion pair in a region of low dielectric constant may account for an incremental increase in affinity of 2–3 orders of magnitude in the 4-4-20 molecule relative to other members of an idiotypic family of monoclonal antifluorescyl antibodies. The phenyl carboxyl group of fluorescein appears to be hydrogen bonded to the phenolic hydroxyl group of tyrosine 37 of the light chain. A molecule of 2-methyl-2,4-pentanediol (MPD), trapped in the interface of the variable domains just below the fluorescein binding site, may be partly responsible for the decrease in affinity for the hapten in MPD.**

Key words: antifluorescyl monoclonal antibody, high-affinity binding site, effects of MPD on hapten binding

INTRODUCTION

The antifluorescein system is well suited for studying the molecular basis of antigenic specificity because it offers both a wide range of binding affinities (10^5–10^{10} M^{-1}), and a variety of experimental techniques for correlating antigen binding affinities, kinetics, and thermodynamics.[1–9] Furthermore, it has been possible to develop a family of idiotypically cross-reactive antibodies in which individual monoclonals vary in affinity over a 1000-fold range.[9] Amino acid sequences recently determined for eight of these antibodies in one of our laboratories (E.W.V.) show that at least six were derived from the same germline variable genes. Thus, the antifluorescein idiotype family can be used to further the understanding of both idiotypy and affinity maturation. In this report, we describe the three-dimensional structure of a complex of dianionic fluorescein with the antigen-binding fragment from the antibody (4-4-20) with the highest affinity in this idiotype family.

The 4-4-20 monoclonal is an IgG_{2a} (κ) antibody that binds fluorescein with an association constant of 3.4×10^{10} M^{-1} in aqueous solution. This affinity decreases 300-fold in 47% (v/v) 2-methyl-2,4-pentanediol (MPD), the solvent used for cocrystallization of the 4-4-20 Fab with fluorescein hapten.[5] The antibody is a highly specialized molecule that does not cross-react with rhodamine compounds.[1,6] In the formation of a complex with the 4-4-20 antibody, fluorescein satisfied criteria for a site-filling ligand, with the xanthonyl ring behaving as the "immunodominant" moiety.[6] Despite its relatively large size and distinctive chemical features, fluorescein induces a diverse immune response when injected as a conjugate with keyhole limpet hemocyanin (KLH).[1,7–9] Interpretation of binding studies have sometimes been ambiguous with heterogeneous populations of antibodies. It therefore seemed appropriate to determine the mode of binding in a single molecular species, particularly one with high affinity for fluorescein.

This crystal system affords a rare opportunity to consider both the structural features responsible for high-affinity binding and the effects of solvent in lowering that affinity. The complex will also be ex-

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amined to assess the influence of the carrier protein (KLH) on the location and orientation of the hapten in the combining site. In the preparation of the immunogen, molecules of the isothiocyanate derivative of fluorescein amine (isomer I) were coupled to KLH, with the principal reactions presumed to involve the ϵ-amino group of lysine side chains. The 4-4-20 hybridoma was obtained by fusion of hyperimmune splenocytes stimulated with the fluorescein–KLH conjugate.

The present work adds to previous crystallographic studies of complexes in which small molecules were diffused into crystals of human and murine immunoglobulin fragments with binding sites shaped like cavities or shallow depressions.[10–15] These studies have helped define the molecular basis of low- and medium-affinity interactions and have provided the background to broaden our understanding of antigenic specificity. Block-end types of interactions over large external surfaces have been studied in cocrystals of Fab molecules and protein antigens like lysozyme and influenza neuraminidase.[16–19] Antibodies binding DNA have grooves as potential combining sites, and cocrystals of Fabs and oligodeoxynucleotides are currently being subjected to X-ray analyses.[20–23] The structure of an unliganded Fab (J539) with specificity for galactan in carbohydrates has also been determined.[24] A model for the binding of the ligand was proposed on the basis of solution studies and the structure of the combining site.

Recently, a single-chain antigen-binding protein[25] was constructed to simulate the Fv fragment of the 4-4-20 antibody (an Fv fragment consists of the "variable" domains of the heavy and light chains). The three-dimensional structure of the 4-4-20 Fab should prove very useful in assessing the properties and applications of the single chain protein.

MATERIALS AND METHODS

Preparation of Fluorescein–Fab Complex

Procedures for the isolation and purification of the 4-4-20 monoclonal antibody and fluorescein–Fab complex were described in a previous article.[5] In outline the Fab fragments were prepared by hydrolysis of fluorescein–IgG complexes with papain. Liganded Fabs were purified by chromatofocusing on 15 ml Pharmacia PBE 94 columns, with a linear pH gradient of 9 to 6 formed with Pharmacia Polybuffer 96. Because of potential destructive effects of dye-sensitized photooxidation by free fluorescein, it was necessary to protect the liganded protein from light at all stages of the procedures. Columns, tubes, and vessels used in affinity chromatography, chromatofocusing, dialysis, enzymatic hydrolysis, and crystallization trials were all covered with aluminum foil. All manipulations and transfers were carried out in reduced light. Under such precautions the liganded Fab was eluted from the chromatofocusing column as a single component with a pI of 7.2.

After dialysis against 50 mM sodium phosphate, pH 7.2, the solution of the fluorescein–Fab complex was concentrated to 25 mg/ml by ultrafiltration (the acceptable range for crystallization was 10–30 mg/ml). The complex was crystallized by a batch method in an environment with strict light and temperature (12–14°C) control. Graded aliquots of MPD were added to 40 μl samples of the liganded protein in flat-bottomed glass vials. Crystals appeared in 2 days with final MPD concentrations of 38–60% (v/v), the optimum being ~47%. Bladed crystals suitable for X-ray analysis grew to dimensions of 0.6 × 0.35 × 0.3 mm (l × w × d) in 1–2 months. Green fluorescence, attributable to the dissociation of fluorescein from the complex in MPD, was observed in each crystallization tube.

Collection of X-Ray Diffraction Data

The fluorescein–Fab complex crystallized in the triclinic space group $P1$, with a = 58.3, b = 43.9, and c = 42.5 Å; α = 82.1, β = 87.3, and γ = 84.6°.[5] Crystals disintegrated at temperatures >22°C, but were mechanically stable even in the X-ray beam when the ambient temperature was maintained at 12–14°C. Temperature instability of the complex in MPD had also been noted in solution.[5] For example, irreversible increases in the standard free-energy changes ($\Delta G°$) in the liganded IgG and Fab molecules occurred in 40% MPD at relatively low temperatures (transition temperature of 30°C).

A single crystal was used to collect X-ray diffraction data to 2.7 Å resolution with a Nicolet P21 diffractometer operated at 40 kV and 35 mA (CuK_α radiation). The data set included 11,116 unique reflections, of which 9120 (82.0%) were observed at intensity levels >1.5 standard deviations (based on counting statistics).

Determination of the Three-Dimensional Structure of the Liganded Fab

The fluorescein–Fab complex crystallized in the same space group ($P1$) as the unliganded Fab of the BV04-01 IgG_{2b} autoantibody, with specificity for single-stranded DNA.[5,26] Unit cell dimensions for the two crystals were nearly identical and one of us (X-M.H.) found by molecular replacement methods[27–30] that the proteins were in the same orientations in these unit cells. With the refined 2.7 Å structure of the BV04-01 Fab as starting model, the orientation of the 4-4-20 Fab was determined more accurately with rotation function programs.

Crystallographic refinement[31,32] of the structure was initiated with the X-ray diffraction data[5] for the 4-4-20 Fab and the atomic model of the BV04-01 Fab.[22,23] After 30 cycles of refinement with 2.7–6.0 Å data (8304 reflections), the amino acid sequences

TABLE I. Refinement Data

	Actual rms deviation	Target value
Average ΔF*	46.6	
R-factor	0.215	
$< B >$ in Å^2†	15.5	
No. of reflections, $I > 1.5\ \sigma\ (I)$‡	8304	
Root mean square (rms) deviations		
From ideal distance (Å)		
Bond distance	0.028	0.03
Angle distance	0.055	0.04
Planar 1-4 distance	0.032	0.03
Hydrogen bond distance§	0.132	0.05
rms deviation from planarity (Å)	0.014	0.025
rms deviation from ideal chirality (Å^3)	0.210	0.150
rms deviation from permitted contact distances (Å)		
Single torsion contacts	0.280	0.500
Multiple torsion contacts	0.400	0.500
Possible hydrogen bond	0.409	0.500
rms deviation from ideal torsion angles (°)		
For prespecified ϕ, ψ angles	30.8	15.0
For planar group (0 or 180)	6.3	3.0
For staggered group (± 60 or 180)	26.9	15.0
For orthonormal group (± 90)	28.1	15.0

*The weight for the structure factor refinement was obtained from the equation $\omega = (1/\sigma)^2$, where $\sigma = 40 - 278 \times [\sin(\theta)/\lambda - 1/6]$.
†Average temperature factor.
‡Resolution limits of 2.7–6.0 Å.
§Explicit hydrogen bonds for β-pleated sheets.

of the light and heavy chains were altered to correspond to those of the 4-4-20 Fab.[34] The model of the 4-4-20 Fab was improved through alternating cycles of refinement and interactive model building on an Evans and Sutherland PS300 graphics system with the FRODO program.[35,36] Polypeptide backbones and amino acid side chains were fitted to $2F_o - F_c$ maps, in which F_o and F_c were observed and calculated structure factors. Fluorescein was located in a ΔF $(F_o - F_c)$ map, for which the phase angles were calculated from the refined atomic model of the protein (atomic coordinates for fluorescein were omitted). The ligand–protein complex was subjected to additional cycles of refinement until the R factor $(\Sigma \| F_o | - | F_c \| / \Sigma | F_o |)$ began to plateau at its current value of 0.180 (before idealization of bond lengths and angles; 0.215 after).

RESULTS

Description of the Three-Dimensional Structure of the Fluorescein–Fab Complex

The results of the crystallographic refinement of the complex are presented in Table I. This structure could be refined very quickly because of the great similarities with the structure of the unliganded BV04-01 Fab.

Figure 1 contains the three-dimensional "cage" electron density to which a skeletal model of fluorescein was fitted by interactive computer graphics. The torsion angle measured between the xanthonyl and benzoyl rings was 73° in the bound hapten.

An αC skeletal model of the 4-4-20 Fab is shown as a stereo pair in Figure 2, with a model of fluorescein codisplayed in the binding site. Tracings of the αC chains of the 4-4-20 and BV04-01 Fabs are superimposed in Figure 3. Details of the structures of the ligand and combining site are presented in Figure 4 (skeletal models). Solvent-accessible surfaces[37] are illustrated in Figure 5. Amino acid sequences[34] for the hypervariable regions are listed in Figure 6.

Polypeptide chains could be traced unambiguously in both the light and heavy chains of the 4-4-20 complex. Significantly, the third hypervariable loop, which was difficult to follow in the heavy chain of the BV04-01 Fab, was well defined in the fluorescein–Fab complex. In the presence of ligand, constituents of this loop were found to have small temperature factors (B values), characteristic of regions with low mobility.

Comparison of the Structures of the 4-4-20 and BV04-01 Fabs

The 4-4-20 Fab is an extended molecule in which the pseudotwofold axes between the pairs of variable and constant domains are nearly colinear (i.e., the measured "elbow bend" angle between the two pseudodiads is 171°). Except for the third hypervariable loops, in which the 4-4-20 heavy chain is shorter than the BV04-01 sequence by three residues, the αC tracings are remarkably similar in the two proteins. This similarity is readily understand-

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