PROTEINS
Structure, Function, and Genetics
Editor-in-Chief
Cyrus Levinthal
Department of Biological Sciences • Columbia University • New York, New York
Executive Editor
George D. Rose
Department of Biological Chemistry • Hershey Medical Center, Pennsylvania State University • Hershey, Pennsylvania
Associate Editors
Thomas E. Creighton
Medical Research Council Laboratory of Molecular Biology, Cambridge, England

John Abelson
Division of Biology, California Institute of Technology, Pasadena, California

Robert L. Baldwin
Department of Biochemistry, Stanford University School of Medicine, Stanford, California

Herman J.C. Berendsen
Laboratory of Physical Chemistry, University of Groningen, Groningen, The Netherlands

David Botstein
Genentech Inc. • South San Francisco, California

Ralph Bradshaw
Department of Biological Chemistry, University of California, Los Angeles, California

Jane-Michel Claverie
Institut Pasteur, Unite d’Informatique Scientifique, Paris, France

David Eisenberg
Department of Molecular Biology, University of California at Santa Cruz, California

S. Walter Enghardter
Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Richard M. Fine
Department of Biological Sciences, Columbia University, New York, New York

Robert J. Fleterick
Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California

Lila M. Gierasch
Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas

Walter Gilbert
Biological Laboratories, Harvard University, Cambridge, Massachusetts

Nobuhiko Gó
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto, Japan

Jonathan Greer
Computer Assisted Molecular Design Group, Pharmaceutical Division, Abbott Laboratories, Abbott Park, Illinois

David Davies
Section of Molecular Structure, N.I.A.M.D.D., National Institutes of Health, Bethesda, Maryland

William DeGrado
Central Research and Development Department, E.I. du Pont de Nemours and Company, Wilmington, Delaware

Arnold T. Hager
The Agouron Institute, La Jolla, California

Jan Hermans
Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina

Barry Honig
Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York

Leroy Hood
Division of Biology, California Institute of Technology, Pasadena, California

Wayne L. Hubbell
Jules Stein Eye Institute, University of California School of Medicine, Los Angeles, California

Michael N.G. James
Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

Alwyn Jones
Department of Molecular Biology, Uppsala, Sweden

Arthur Karlin
Departments of Biochemistry and Neurology, College of Physicians and Surgeons, Columbia University, New York, New York

Martin Karplus
Department of Chemistry, Harvard University, Cambridge, Massachusetts

William Konigsberg
Departments of Biochemistry and Biophysics, Yale University School of Medicine, New Haven, Connecticut

Joseph Kraut
Department of Chemistry, University of California at San Diego, La Jolla, California

Robert Langridge
Computer Graphics Laboratory, Department of Pharmaceutical Chemistry, University of California, San Francisco, California

Eaton E. Lattman
Department of Biophysics, Johns Hopkins School of Medicine, Baltimore, Maryland

Dale L. Oxender
Center for Molecular Genetics, University of Michigan, Ann Arbor, Michigan

Alexander Rich
Department of Biophysics, Massachusetts Institute of Technology, Cambridge, Massachusetts

Editorial Board

Authorization to photocopy items for internal or personal use of specific clients, is granted by Alan R. Liss, Inc. for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of $0.50 per copy, plus $0.25 per page is paid directly to CCC, 27 Congress Street, Salem, MA 01970, 0887-3585/89 $0.50 + .25.


Subscription prices: Volumes 5&6, 1989, eight issues, $150.00. Special personal subscription rate for 1989: $75.00. For all 1989 subscriptions outside the US, please add $32.00 postage. Subscriptions at the personal rate must be made by personal check, credit card, bank draft or money order. All subscriptions outside North America will be sent by air. Payment must be made in US dollars drawn on a US bank. Change of address: Please send to publisher six weeks prior to move; enclose present mailing label with change of address. Claims for missing issues: Claims cannot be honored beyond four months after mailing date. Duplicate copies cannot be sent to replace issues not delivered because of failure to notify publisher of change of address. Cancellations: Subscriptions cancellations will not be accepted after the first issue has been mailed. Exclusive agent in Japan: Igaku Shoin Limited, Foreign Department, 1-28-36 Hongo, Bunkyo-ku, Tokyo 113, Japan. ¥ 5,400 for Volumes 5–6. (Air Cargo Service Only).

Indexed by: BIOSIS Data Base • Cambridge Scientific Abstracts • Chemical Abstracts • Current Contents/Life Sciences-Science Citation Index (SCISEARCH) • Excerpta Medica • Index Medicus

Printed in the United States of America. Copyright © 1989 Alan R. Liss, Inc. The paper on which this journal is printed adheres to the requirements for library/archival stability.

PFIZER EX. 1586 Page 2
Three-Dimensional Structure of a Fluorescein–Fab Complex Crystallized in 2-Methyl-2,4-pentanediol

James N. Herron,1 Xiao-min He,1 Martha L. Mason,1 Edward W. Voss, Jr.,2 and Allen B. Edmundson1
1Department of Biology, University of Utah, Salt Lake City, Utah 84112 and 2Department of Microbiology, University of Illinois, Urbana-Champaign, Illinois 61801

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 antifluorescein antibody (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 antifluorescein 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: antifluorescein 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 IgG2a (κ) antibody that binds fluorescein with an association constant of $3.4 \times 10^{10}$ M$^{-1}$ in aqueous solution. This affinity decreases 300-fold in 47% (v/v) 2-methyl-2,4-pentanediol (MPD), the solvent used for co crystallization 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).$^17$–$^9$ 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-
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 e-amino group of lysine side chains. The 4-4-20 hybridoma was obtained by fusion of hyper-immune 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 cocrys­

tals of Fab molecules and protein antigens like lysozyme and influenza neuraminidase.16–19 Antibodies binding DNA have grooves as potential combining sites, and cocrys­

tals of Fabs and oligodeoxynucleotides are currently being subjected to X-ray analyses.20–23 The structure of an unliganded Fab (J539) with specificity for ga­

trene 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 protein25 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. Ligated 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 (∆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 diffrac­

tometer operated at 40 kV and 35 mA (CuKα, ra­

diation). 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 IgG2b, 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 meth­ods27–30 that the proteins were in the same orienta­

tions 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 refinement31,32 of the structure was initiated with the X-ray diffraction data4 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
of the light and heavy chains were altered to correspond to those of the 4-4-20 Fab. 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. Polypeptide backbones and amino acid side chains were fitted to 2Fo-Fe maps, in which Fo and Fe were observed and calculated structure factors. Fluorescein was located in a ΔF (Fo-Fe) 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 (Σ ||Fo||-|Fe|) 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 are illustrated in Figure 5. Amino acid sequences 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-
Fig. 1. Three-dimensional electron density (orange) corresponding to fluorescein (green) in the combining site of the 4-4-20 Fab. This electron density was obtained in a difference Fourier map after crystallographic refinement of the ligand-protein complex. A skeletal model of fluorescein was fitted to the electron-density by interactive computer graphics.

Fig. 2. Stereo diagram of α C tracings of the light (blue) and heavy (red) chains of the 4-4-20 Fab, with fluorescein (green) in the combining site. The "variable" domains are at the top and the "constant" domains are at the bottom. Disulfide bonds are represented by yellow bars.
Fluorescein Binding Site

Fluorescein fits into a slot lined by constituents of both the light and heavy chains (see Figs. 4 and 5), and participates in a network of interacting aromatic groups with the protein. There are 68 pairs of atoms of the ligand and protein separated by <6 Å. Of the potential interactions, 42 are associated with the xanthonyl moiety and 26 with the benzoyl group of fluorescein. This distribution is consistent with the assignment of the xanthonyl moiety as the "immunodominant" portion of the hapten.

Tryptophan L101 forms the bottom of the slot and tryptophan H33 and tyrosine L37 provide the sides. The width of the slot, as measured from the distance between the centroids of the rings of tryptophan H33 and tryptophan H52; another key residue in the region, is salt bridged to glutamic acid H59 (an alanine in BV04-01). Surprisingly, the second negative charge on fluorescein (the carboxylic acid) is not formally neutralized by a protein substituent. Instead, this group faces the solvent at an open end of the slot. Partial charge compensation is achieved by the formation of a hydrogen bond between the phenolic hydroxyl group of tyrosine L37 and one of the carboxylic acid oxygen atoms (the electron density in a 2F0 - Fc map is continuous between the two sets of atoms).

Tyrosine 103 extends toward the phenyl ring of fluorescein and tyrosine 102 contributes to the structural integrity of the binding site without actually being in contact with the hapten (see Fig. 4). There is a clear solvent channel for a lysine side chain to be connected to an isothiocyanate group in the para position of the phenyl ring, as would be expected for a hapten coupled to the KLH carrier protein in the immunogen. This solvent channel is shown in Figure 5.

Glutamine H50, which replaces an important arginine residue in the putative binding site of the BV04-01 Fab, is mostly buried in the presence of fluorescein in the 4-4-20 Fab. However, the polar end of the side chain (the carbonyl oxygen of the amide group) is within hydrogen bonding distance of the indole nitrogen of tryptophan H33. Arginine H52, another key residue in the BV04-01 binding region, is salt bridged to glutamic acid H59 (an alanine in BV04-01).
Fig. 4. Stereo diagram of the hapten binding site in the 4-4-20 Fab. Light (L) chain constituents are blue, heavy (H) chain components are red, and fluorescein is green. The xanthonyl (three ring) group of fluorescein is flanked by tyrosine L37 on the left and tryptophan H33 on the right, with tryptophan L101 forming the bottom of the slot. Enolic oxygen atoms on opposite corners of the xanthonyl group point toward histidine L31 (left) and arginine L39 (right). The phenyl carboxyl group of fluorescein (single ring) is located below tyrosines H103 (left) and H102 (right).

Fig. 5. Solvent-accessible surface dot representation of the hapten combining site, with a skeletal model of fluorescein superimposed. Note the free space above the para position of the phenylcarboxyl group of fluorescein. In the immunogen used to elicit the production of the 4-4-20 antibody, a lysine side chain on a carrier protein would be linked to an isothiocyanate group in the para position of the hapten. Lysine would presumably occupy the free space when the hapten entered the antibody combining site.

Crystal Packing Interactions in the 4-4-20 and BV04-01 Systems

Analyses of packing interactions revealed structural features that are conducive to the formation of 4-4-20 Fab crystals nearly isomorphous with those of the unliganded BV04-01 Fab. Light chain constituents of the 4-4-20 Fab were found to be involved in a large proportion (23 of the 26 examples in the survey) of major packing interactions. The light chain is also prominent in crystal packing of the unliganded BV04-01. The close similarities in amino acid sequences and conformations of the two light chains are apparently reflected in the packing of the parent Fabs, despite the differences in crystallizing media.

Sequestering of an MPD Molecule in the V\textsubscript{L}–V\textsubscript{H} Interface

In the interface of the variable domains below the fluorescein binding site (closest distance ~4.6 Å), there was a module of electron density that could not be assigned to either protein or hapten. This module had the size and shape expected for a molecule of MPD, and persisted in maps calculated after advanced stages of the crystallographic refinement. The cage electron density for the putative solvent molecule is shown with the surrounding protein constituents in Figure 7. MPD was omitted from the model used to calculate the phases for the difference Fourier map. The binding site for MPD was lined by both polar and apolar residues, such as threonine 99, serine 101, and tryptophans 47 and 108 of the heavy chain, and arginine 39, tyrosine 41, and tryptophan 101, and phenylalanine 103 of the light chain. Hydroxyl groups on threonine H99 and tyrosine L41 were located within hydrogen bonding distances of the 2- and 4-hydroxyl groups of MPD.
suited for a specialized high-affinity antibody for example, antibodies elicited to the liganded site of fluorescein. Space available for binding in the site is liganded, idiotypic state. These studies suggest the limited. While geometrically optimized, the Recent immunological studies support this view. For the to the ligand in the tryptophan residues are located in close juxtaposition the formation of such a tight complex was achieved by conformational adjustments in the protein. It is interesting to ask what factors contribute to a high-affinity site. There is a red shift in the absorption spectrum when fluorescein is bound to the antibody and other monoclonal anti­fluoresceyl antibodies. This shift has been attributed to hydrophobic effects involving tryptophan side chains. The model of the complex supports such a view, since two tryptophan residues are located in close juxtaposition to the ligand in the 4-4-20 combining site. However, strategically placed tryptophans are not sufficient to account for the high affinity of 4-4-20. For example, the 9-40 anti­fluoresceyl antibody has tryptophan residues in homologous positions in its amino acid sequence and a lower affinity for the ligand than 4-4-20.

Quenching of fluorescence is characteristic of anti­fluoresceyl antibodies. The maximum quenching constant for the 4-4-20 molecule is greater than the $Q_{\text{max}}$ values of some antibodies (e.g., 20-4-4), but slightly smaller than those for other antibodies (e.g., 20-20-3) with lower affinities for fluorescein. Moreover, $Q_{\text{max}}$ for 4-4-20 actually increases in MPD while the ligand affinity decreases. These results indicate that there is not a strong correlation between fluorescence quenching and high affinity. Interactions resulting in quenching appear to be very diverse. Tryptophan has been invoked as a possible participant in such interactions and in one case (20-20-3) histidine was suggested to be involved in the protonation of the enolic group of bound fluorescein. The 4-4-20 active site contains both tryptophan and histidine residues in positions that should be favorable for fluorescence quenching.

Model-based hypotheses seem to be especially useful in explaining deuterium fluorescence enhancement and iodine quenching of bound ligand in anti­fluoresceyl antibodies. The liganded 4-4-20 molecule shows about 188% fluorescence enhancement in deuterium oxide (relative to water). Such experiments afford a measure of the degree of hydrogen bonding between ligand and protein. The enhancement for 4-4-20 was 10 times greater than values for four other anti­fluoresceyl monoclonal antibodies and hydrogen bonding was therefore considered to be very important for ligand binding.

The structure of the complex is consistent with this proposal and can be used to identify the most prominent hydrogen bonds (enol with histidine L31 and phenyl carboxyl group with tyrosine L37). Only 2.5% quenching of bound fluorescein was noted when the liganded 4-4-20 IgG was titrated with potassium iodide. This observation indicated that fluorescein bound in the 4-4-20 active site was essentially inaccessible to iodide. Quenching was higher (8.4–15.5%) in four other antibodies with lower affinities.

**DISCUSSION**

A simple, yet restricted combining site seems well suited for a specialized high-affinity antibody for fluorescein. Space available for binding in the site is limited. While geometrically optimized, the aromatic, electrostatic, and hydrogen bonding interactions are relatively few in number. We suspect that the formation of such a tight complex was accomplished by conformational adjustments in the protein.

Recent immunological studies support this view. For example, antibodies elicited to the liganded site of the 4-4-20 antibody were not reactive with the nonliganded, idiotypic state. These studies suggest the generation of new epitopes on ligand binding as a consequence of induced conformational changes.

Is it interesting to ask what factors contribute to a high-affinity site. There is a red shift in the absorption spectrum when fluorescein is bound to the 4-4-20 and other monoclonal anti­fluoresceyl antibodies. This shift has been attributed to hydrophobic effects involving tryptophan side chains. The model of the complex suggests such a view, since two tryptophan residues are located in close juxtaposition to the ligand in the 4-4-20 combining site. However, strategically placed tryptophans are not sufficient to account for the high affinity of 4-4-20. For example, the 9-40 anti­fluoresceyl antibody has tryptophan residues in homologous positions in its amino acid sequence and a lower affinity for the ligand than 4-4-20.

Quenching of fluorescence is characteristic of anti­fluoresceyl antibodies. The maximum quenching constant for the 4-4-20 molecule is greater than the $Q_{\text{max}}$ values of some antibodies (e.g., 20-4-4), but slightly smaller than those for other antibodies (e.g., 20-20-3) with lower affinities for fluorescein. Moreover, $Q_{\text{max}}$ for 4-4-20 actually increases in MPD while the ligand affinity decreases. These results indicate that there is not a strong correlation between fluorescence quenching and high affinity. Interactions resulting in quenching appear to be very diverse. Tryptophan has been invoked as a possible participant in such interactions and in one case (20-20-3) histidine was suggested to be involved in the protonation of the enolic group of bound fluorescein. The 4-4-20 active site contains both tryptophan and histidine residues in positions that should be favorable for fluorescence quenching.

Model-based hypotheses seem to be especially useful in explaining deuterium fluorescence enhancement and iodine quenching of bound ligand in anti­fluoresceyl antibodies. The liganded 4-4-20 molecule shows about 188% fluorescence enhancement in deuterium oxide (relative to water). Such experiments afford a measure of the degree of hydrogen bonding between ligand and protein. The enhancement for 4-4-20 was 10 times greater than values for four other anti­fluoresceyl monoclonal antibodies and hydrogen bonding was therefore considered to be very important for ligand binding.

The structure of the complex is consistent with this proposal and can be used to identify the most prominent hydrogen bonds (enol with histidine L31 and phenyl carboxyl group with tyrosine L37). Only 2.5% quenching of bound fluorescein was noted when the liganded 4-4-20 IgG was titrated with potassium iodide. This observation indicated that fluorescein bound in the 4-4-20 active site was essentially inaccessible to iodide. Quenching was higher (8.4–15.5%) in four other antibodies with lower affinities.

**DISCUSSION**

A simple, yet restricted combining site seems well suited for a specialized high-affinity antibody for fluorescein. Space available for binding in the site is limited. While geometrically optimized, the aromatic, electrostatic, and hydrogen bonding interactions are relatively few in number. We suspect that the formation of such a tight complex was accompanied by conformational adjustments in the protein.

Recent immunological studies support this view. For example, antibodies elicited to the liganded site of the 4-4-20 antibody were not reactive with the nonliganded, idiotypic state. These studies suggest the generation of new epitopes on ligand binding as a consequence of induced conformational changes.

It is interesting to ask what factors contribute to a high-affinity site. There is a red shift in the absorption spectrum when fluorescein is bound to the 4-4-20 and other monoclonal anti­fluoresceyl antibodies. This shift has been attributed to hydrophobic effects involving tryptophan side chains. The model of the complex supports such a view, since two tryptophan residues are located in close juxtaposition to the ligand in the 4-4-20 combining site. However, strategically placed tryptophans are not sufficient to account for the high affinity of 4-4-20. For example, the 9-40 anti­fluoresceyl antibody has tryptophan residues in homologous positions in its amino acid sequence and a lower affinity for the ligand than 4-4-20.

Quenching of fluorescence is characteristic of anti­fluoresceyl antibodies. The maximum quenching constant for the 4-4-20 molecule is greater than the $Q_{\text{max}}$ values of some antibodies (e.g., 20-4-4), but slightly smaller than those for other antibodies (e.g., 20-20-3) with lower affinities for fluorescein. Moreover, $Q_{\text{max}}$ for 4-4-20 actually increases in MPD while the ligand affinity decreases. These results indicate that there is not a strong correlation between fluorescence quenching and high affinity. Interactions resulting in quenching appear to be very diverse. Tryptophan has been invoked as a possible participant in such interactions and in one case (20-20-3) histidine was suggested to be involved in the protonation of the enolic group of bound fluorescein. The 4-4-20 active site contains both tryptophan and histidine residues in positions that should be favorable for fluorescence quenching.

Model-based hypotheses seem to be especially useful in explaining deuterium fluorescence enhancement and iodine quenching of bound ligand in anti­fluoresceyl antibodies. The liganded 4-4-20 molecule shows about 188% fluorescence enhancement in deuterium oxide (relative to water). Such experiments afford a measure of the degree of hydrogen bonding between ligand and protein. The enhancement for 4-4-20 was 10 times greater than values for four other anti­fluoresceyl monoclonal antibodies and hydrogen bonding was therefore considered to be very important for ligand binding.

The structure of the complex is consistent with this proposal and can be used to identify the most prominent hydrogen bonds (enol with histidine L31 and phenyl carboxyl group with tyrosine L37). Only 2.5% quenching of bound fluorescein was noted when the liganded 4-4-20 IgG was titrated with potassium iodide. This observation indicated that fluorescein bound in the 4-4-20 active site was essentially inaccessible to iodide. Quenching was higher (8.4–15.5%) in four other antibodies with lower affinities.
Fig. 7. Stereo diagram of the cage electron density (lavender) corresponding to a molecule of MPD in its binding site just below that of fluorescein in the V domain interface. Key residues of the light (blue) and heavy chains (red) are labeled with three-letter abbreviations. The hydroxyl groups of threonine H99 and tyrosine L41 may form hydrogen bonds with the 2-and 4-hydroxyl groups of MPD.

for fluorescein. Figures 4 and 5 show a very tight fit of the ligand in the site, and the presence of tyrosine 102 and 103 further shields the bound ligand from additional reactants. These tyrosine residues are not present in the sequence of the 9-40 antibody, in which bound fluorescein is more accessible to iodide.

The interactions of tyrosine L37 with fluorescein appear to be important in orienting the ligand in the high-affinity site. The stacking of the phenyl group of tyrosine with the xanthonyl ring was further stabilized by a hydrogen bond between the phenolic hydroxyl group and the phenylcarboxyl moiety of fluorescein. The dual interaction of fluorescein with tyrosine also played a major role in fixing the torsion angle between the xanthonyl and phenyl rings, which would have greater freedom to rotate in solution.

Studies of the effects of pH on lifetimes of dissociation indicated that ionizable groups (pK > 8.0) in the 4-4-20 active site contribute to the binding of fluorescein. Lifetime maxima at pH 6.5–7.0 suggested that the xanthonyl moiety is negatively charged when bound to the 4-4-20 (the pK of the enolic group is 6.744). In the 3-D structure of the complex the two enolic groups of fluorescein are bracketed by the ionizable side chains of histidine L31 and arginine L39. At the pH used for crystallization the histidine side chain would be expected to be in the uncharged form. The formation of an ion pair with arginine probably makes a very significant contribution to the binding energy, particularly in a region of low dielectric constant. In the 9-40 antibody this arginine is replaced by histidine, a substitution that could partially account for the decrease in affinity relative to 4-4-20.

Rhodamine 110 and rhodamine B, structural analogs of fluorescein with amino (110) and diethylamino (B) groups substituted for the enolic oxygen atoms, do not bind to 4-4-20. Acylamino groups...
would have formal positive charges at relatively low pH values, (<5), at which there would be electrostatic repulsion by like charges on the histidine L31 and arginine L39 side chains. At pH 8 the only charge on a rhodamine molecule would reside on the phenylcarboxyl group. A monoanionic ligand would be incompatible with the pH profile of binding, which indicates a strong dependence on the presence of a diamin. In the case of rhodamine B, there is an additional steric factor to consider. For example, tetramethyl rhodamine formed a tight complex with the Mgc Bence-Jones dimer, but the tetraethyl derivative (rhodamine B) could not be accommodated in the binding site. Exposure to rhodamine B led to destruction of a crystal of the Mgc dimer in 5 hours.13

The crystal structure of the fluorescein-Mgc complex provides further insight into the minimal requirements for low-affinity binding of the ligand. As in 4-4-20, the ligand was accommodated in a network of aromatic residues. The xanthonyl ring of fluorescein was more deeply immersed in the cavity than the benzoyl moiety and participated in more interactions (14 vs 6) with the protein. A tyrosine side chain was wedged between the xanthonyl and benzoyl groups and largely dictated the torsion angle of 57° between these two rings. At the pH (6.2) of the crystals fluorescein was a monoanion and the charged group (phenylcarboxyl moiety) was oriented toward bulk solvent at the entrance of the binding cavity. In summary, the low-affinity site was more voluminous and there were fewer ligand–protein interactions. Aromatic rings did not stack, and potential negative charges were not balanced by basic side chains on the protein.

Thermodynamic studies indicated that the temperature stabilities of both the intact 4-4-20 antibody and Fab were significantly decreased in the presence of 40% (v/v) MPD.5 These studies also suggested that the decrease in affinity for fluorescein in MPD was mainly attributable to conformational changes in the protein. Future comparisons of the present structure with that of the same complex crystallized in polyethylene glycol6 will hopefully provide greater understanding of the general effects of these solvents.

Since MPD was added to the crystallization mixture after the formation of the hapten–Fab complex, we speculate that fluorescein would be dissociated from the protein prior to the admission of such a sizable solvent molecule to a deeper portion of the variable domain interface. Irrespective of the mode of entry of MPD, the local perturbations would be expected to have significant effects on the affinity constant of a hapten bound in a neighboring site.

ACKNOWLEDGMENTS

This article is dedicated to our late colleague, Martha Mason, who prepared the Fabs and crystals used in this work. We thank Kathryn Ely for advice and encouragement, Brad Nelson and Barbara Staker for photography and art work, Judy Baker for preparing the manuscript for publication, and Lynne Herron for aid in designing the graphics pictures. This work was supported by Grant CA 19616, awarded by the National Cancer Institute, Department of Health and Human Services (to A.B.E.), Grant AI 22898 (to J.N.H.), Grant AI 20860 (to E.W.V.), and N.I.H. Division of Research Resources Biomedical Research Grant RR07092 (to A.B.E.).

REFERENCES


