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The Three Dimensional Structure of a Combining Region–Ligand Complex of Immunoglobulin NEW at 3.5-Å Resolution

(antibody-combining region/hypervariable positions/myeloma protein/vitamin K_1).

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ABSTRACT **IgG *New* binds ligands such as orceine, menadione, and uridine with a low affinity (K_0 about 1 × 10^3 liter/mol) and a γ-hydroxy derivative of vitamin K_1 with a higher affinity (K_0 = 1.7 × 10^5 liter/mol). Binding studies indicate that both the 2-methylnaphthoquinone rings and the phytyl tail of the vitamin K_1 hapten contribute to the total binding energy.**

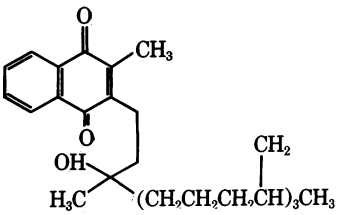
The binding of these ligands in the crystalline state has been investigated by difference Fourier maps of Fab′ *New*-ligand complexes at 6-Å resolution. A 3.5-Å resolution difference Fourier map obtained for the γ-hydroxy derivative of the vitamin K_1–Fab′ complex shows that this hapten is bound in a shallow groove or crevice between the light and the heavy chains, in close proximity to the polypeptide segments containing the hypervariable regions. At least 12 amino-acid residues from both the light and the heavy chains appear to be in close contact with the ligand. No major conformational changes were detected in the Fab′ fragment after ligand binding.

The antigen-binding region of an antibody is located within the V domain of the Fab′ fragment of the molecule. In an IgG immunoglobulin the V domain is composed of the N-terminal half of the light (L) chain and the N-terminal quarter of the heavy (H) chain. It has been proposed that the conformation and specificity of an antigen-binding site is determined by the segments of the L and H chains which show a high degree of variability in amino-acid sequence (1). Binding studies have suggested approximate dimensions of 34 × 12 × 7-Å for the combining region (2). X-ray crystallographic studies of the Fab′ fragment of human myeloma protein IgG *New* have shown that the hypervariable positions occur in the V domain at one end of the Fab′ fragment, exposed to the solvent, in an area of approximately 20 × 25 Å (3).

It has recently been demonstrated that IgG *New* binds, among other ligands, a γ-hydroxy derivative of vitamin K_1 (4). In this report we present the results of a 3.5-Å resolution crystallographic study of the binary complex of Feb′ *New* and the hydroxy derivative of vitamin K_1.

MATERIALS AND METHODS

Synthesis of Vitamin K_1OH. 3-(3′-hydroxy-3′,7′,11′,15′-tetra]-methylhexadecyl)-2-methyl-1,4-naphthoquinone (vit. K_1OH) was prepared by sulfuric-acid treatment of vitamin K_1 and purified on silica gel according to the method of Wagner *et al.* (5).



In agreement with values reported in the literature, in isooctane λmax = 244 nm ($E^{1\%}_{1\,cm}$ = 380), 249 nm, 264 nm, 273 nm ($E^{1\%}_{1\,cm}$ = 375), 325 nm. The mass spectrum shows a peak at m/e 468, which is probably the M^{-1} ion, and smaller peaks at m/e values corresponding to the M^{-2}, M^{-3} ions. A further set of peaks at, and close to, m/e 171 are probably due to fragment ions derived from the 2-methylnaphthoquinone rings. Radioiodination procedures to prepare [^{125}I]vit. K_1OH have been described (4).

Screening of IgG New for Ligand-binding Activity. The screening assay employed to test for ligand-binding activity is based on the observation that low affinity interactions between immunoglobulin-combining regions and haptenic ligands are relatively common (4). Although it is uncertain whether such low affinity binding is physiologically important, the displacement of a radioactive low affinity ligand can be used to indicate the binding of potential haptens with larger association constants.

Nylon screen discs were partially hydrolyzed with 3 N HCl and the resulting amino groups used as initiator foci for the polymerization of L-serine *N*-carboxyanhydride to polyserine strands (average length, 47 residues), at a density of one strand per 400 Å^2. IgG *New* was coupled to the nylon-polyserine whisker discs with glutaraldehyde, and the discs were incubated with each member of a bank of 20 radioactive compounds. Incubation of such haptens with nylon discs having ethanolamine-blocked polyserine chains served as controls. Binding of radioactive compounds is expressed as a ratio of counts bound to the discs containing IgG *New*, to the counts bound to control discs. Substantial ligand binding (in excess of 2.5 × control) was found with [^{14}C]folic acid, [^{3}H]uridine, [^{125}I]insulin, and with the peptide Asp-Glu-Gly-Pro-Tyr-Lys, (MSH-1, the N-terminal hexapeptide of MSH). The IgG *New*–[^{3}H]uridine complex (K_0 = 1 × 10^3 liter/mol at 20°)

Abbreviations: The nomenclature of immunoglobulins and their chains and fragments is as recommended in *Bull. W.H.O.* **30,** 447 (1964); vit. K_1OH, 3-(3′-hydroxy-3′,7′,11′,15′,tetramethyl hexadecyl) 2-methyl 1,4,naphthoquinone; Men, 2-methyl-1,4-naphthoquinone; MSH, β-melanocyte stimulating hormone; EDTA, disodium ethylenedinitrilotetraacetate.

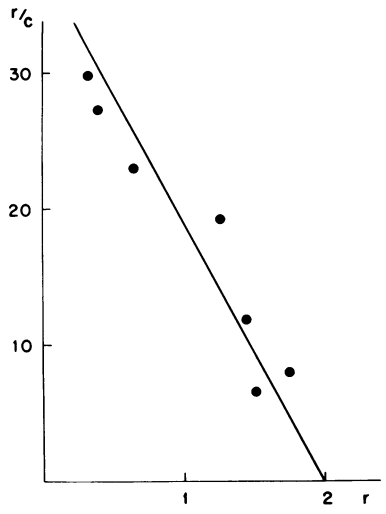


FIG. 1. Binding of the hapten [^{125}I]vit. K_1OH to the Fab fragment of IgG *New* as determined by equilibrium dialysis. *c*, free hapten concentration; *r*, no. of moles of hapten bound/7S monomer of IgG *New*.

was incubated with each of 24 nonradioactive amino acids and peptides, 17 carbohydrates and polysaccharides, 11 vitamins and coenzymes, 4 nucleic acids and nucleotides, 8 alkaloids, steroids, antibiotics, 13 aromatic dyes, and 14 aromatic and polycyclic compounds. Compounds of low water solubility (e.g., vit. K_1OH) were added as solutions in dioxane. Final dioxane concentration was less than 5%. In these competition experiments several weak interactions ($K_0 = 1.0 \times 10^3$ liter/mol) were found with compounds which included orceine, dichlorophenolindophenol, and coenzyme Q 50. These interactions were of the same order of magnitude as those of [^{3}H]uridine, [^{14}C]folic acid, and [^{125}I]MSH-1. IgG *New* was also found to bind Men ($K_0 = 1.0 \times 10^3$ liter/mole) and vit. K_1OH ($K_0 = 1.7 \times 10^5$ liter/mol). Binding constants were measured by equilibrium dialysis in 100-μl volume lucite cells at 20°.

Crystallographic Analysis. Complexes of Fab′ *New* with uridine, dichlorophenolindophenol, orceine, and Men were obtained by diffusion of the ligands into Fab′ crystals. Vit. K_1OH, which is barely soluble in water, was dissolved in 1.5 N phosphate (pH 6.9) containing 5% (v/v) dioxane. The final vit. K_1OH concentration was 1×10^{-5} M. Small intensity changes in the diffraction pattern of h0*l* precession photographs of Fab′ *New* were observed after diffusion of these compounds, indicating the possible formation of a crystalline ligand–protein complex. Dioxane (5%) alone did not produce any intensity changes. Three-dimensional difference Fourier syntheses were calculated using $|F|_{Fab'\ New+ligand} - |F|_{Fab'\ New}$ as coefficients and $\alpha_{Fab'\ New}$ as phases (3).

RESULTS

Fig. 1 shows that myeloma IgG *New* binds [^{125}I]vit. K_1OH ($K_0 = 1.7 \times 10^5$ liter/mol) at a molar ratio (vit. K_1OH: IgG *New*) of 2:1 which is consistent with the value observed for a hapten–antibody (IgG) system. Men, which corresponds to the quinone portion of vit. K_1OH without the phytyl chain, binds to IgG *New* with a lower affinity constant, $K_0 = 1 \times 10^3$ liter/mol.

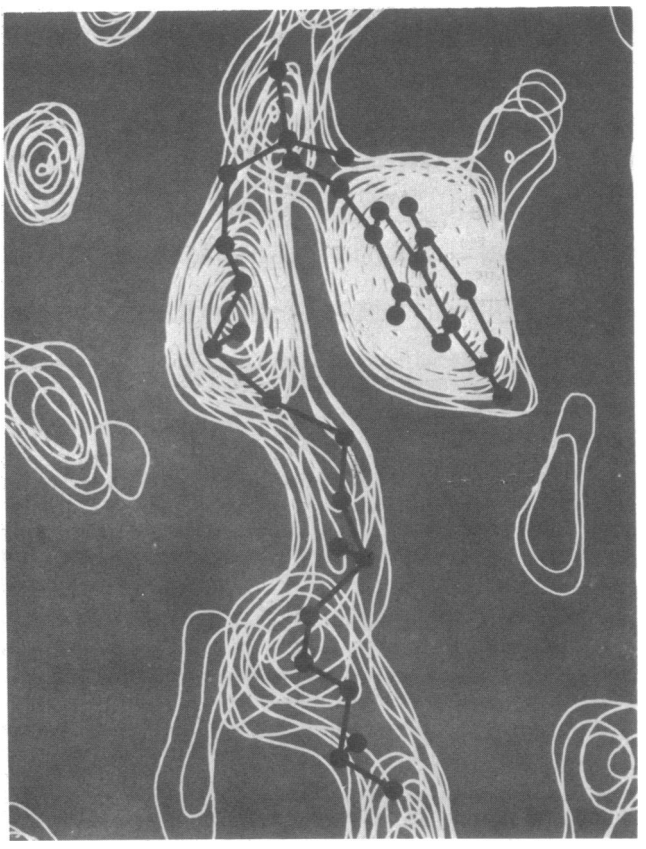


FIG. 2. Model of vit. K_1OH superimposed on the major peak of the 3.5-Å resolution difference Fourier map of the Fab′ *New*–vit. K_1OH complex.

Difference Fourier maps at 6-Å resolution were calculated for each of the crystalline complexes obtained by diffusion of uridine, dichlorophenolindophenol, orceine, Men, and vit. K_1OH into crystals of Fab′ *New*. These maps showed several peaks of positive electron density, the highest of which was located close to the region of hypervariable positions at one end of Fab′ *New*. Since this peak is very close to one of the positions occupied by heavy atoms ($x = 0.42$–0.45, $y = 0.50$, $z = 0.02$, ref. 6) in several isomorphous derivatives, it could be interpreted that the apparent ligand peak was partly or totally produced by errors resulting from the application of the isomorphous replacement method in the determination of x-ray phases. In order to test this possibility, difference Fourier maps with coefficients $|F|_{Fab'} - |F|_{Fab}$, $|F|_{IFab'} - |F|_{Fab'}$, $|F|_{Fab'+EDTA} - |F|_{Fab'}$ (where $|F|_{IFab'}$ and $|F|_{Fab'+EDTA}$ are the structure factors of an iodinated and an EDTA-treated derivative of Fab′, respectively) and native Fab′ phases were calculated as controls. The control maps did not show any peaks of electron density at the heavy atom (and ligand) binding sites. In view of these results, a difference Fourier map with coefficients $|F|_{Fab'\ vit.\ K_1OH} - |F|_{Fab'}$, including 6000 reflexions, was calculated at a resolution of 3.5 Å. The peak of electron density at the site described above was the dominant feature of the 3.5-Å difference Fourier map; other peaks that were observed at 6-Å resolution had a density barely above background in the 3.5-Å map. Furthermore, the shape of the dominant electron density peak is consistent with the distinctive molecular geometry of vit. K_1OH (see Fig. 2).

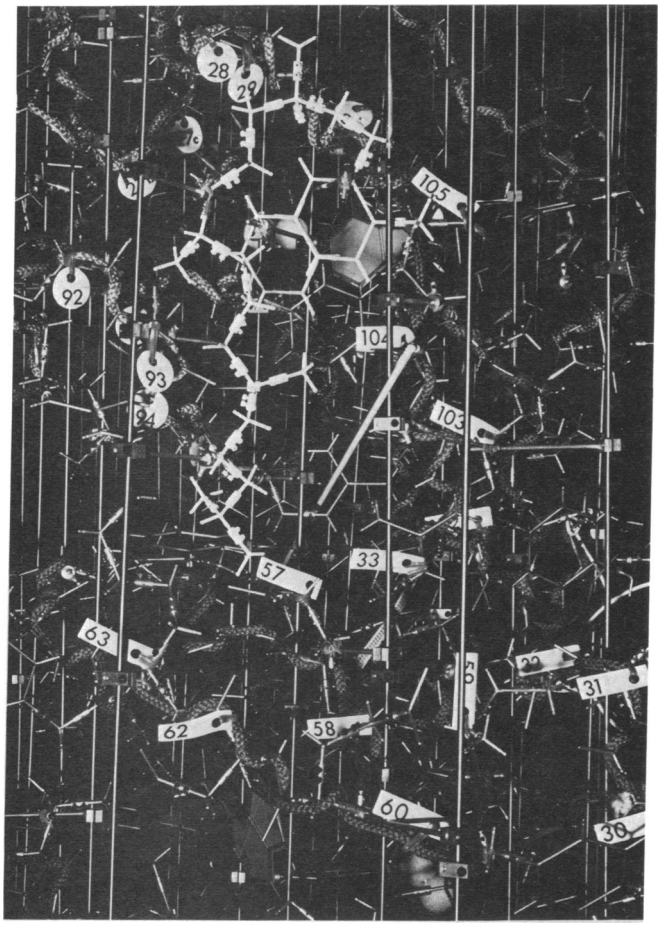


FIG 3. View of the vit. K_1OH–Fab' *New* model showing the spatial relationship of vit. K_1OH (in white) and the hypervariable positions of the L chain (numbered in round tags) and the H chain (rectangular tags). The Fab' *New* model was built from a 2.8-Å resolution Fourier map (3).

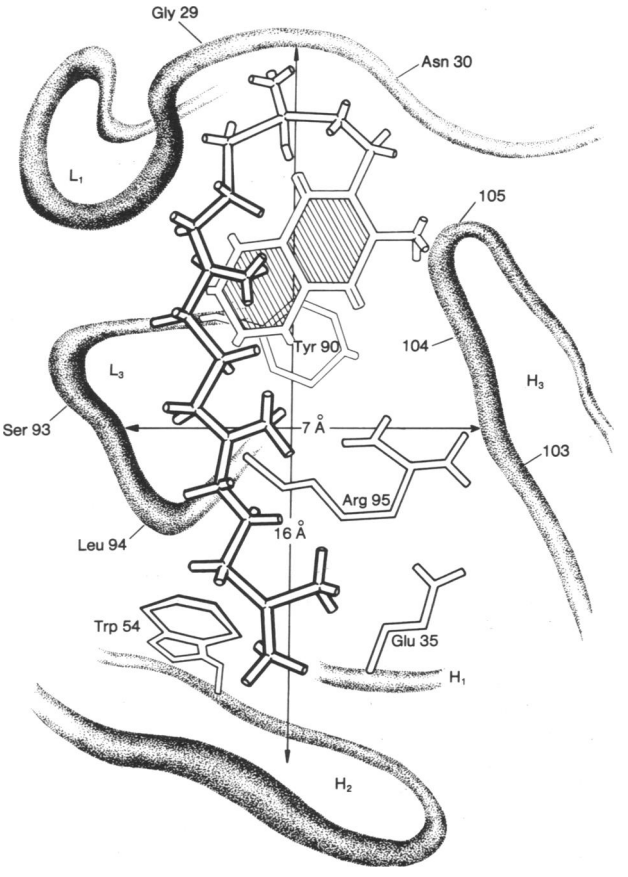


FIG. 4. Schematic drawing of vit. K_1OH bound to the combining region of IgG *New* (after a drawing by Irving Geis). L_1 and L_3 indicate the approximate location of the first and third hypervariable regions of the L chain. H_1, H_2, and H_3 designate hypervariable regions of the H chain. L-chain residues Tyr 90 and Arg 95 are at the bottom of the shallow groove or crevice between the H and L chains. Trp 54 (an invariable residue in human H-chain sequences) and Glu 35 (tentative assignment, not based on actual sequence data) are in close contact with the end of the phytyl chain of vit. K_1OH. See *text* for a description of other contacts between vit. K_1OH and L- and H-chain residues.

Sections of the difference map were placed over the 2.8-Å Fourier map of Fab' *New* obtained before (3). Using an optical comparator (7), a molecular model of vit. K_1OH was brought to correspondence with the difference Fourier map and the Kendrew skeletal model of Fab' *New* (Fig. 3). In this model (Fig. 3), the quinone moiety of vit. K_1OH appears tightly bound at the top of a shallow groove measuring 15-Å × 6-Å and 6-Å deep in the "active" region of the Fab', bordered by both the H and L chains (3). Vit. K_1OH makes close contacts with the polypeptide chain backbone and with the side chains of tyrosine 90 in the L chain (L 3, Fig. 4) and residue 104 in the H chain (H 3, Fig. 4) (L and H-chain residues are designated by the numbers given in ref 3).

DISCUSSION

Potentially, x-ray crystallographic studies of antigen–antibody complexes should be of value in answering many important biological questions. Such studies may help determine the structural basis for the high specificity and avidity displayed by antibodies in the immune response, the role of the hypervariable regions of amino-acid sequence in the conformation of the active site, the spatial extent of the antigen-binding region and the presence and number of subsites. In addition, such studies could give evidence of possible conformational changes that may be necessary for complement binding and for the biological "signal" that may originate in a cell-surface immunoglobulin receptor following antigen binding.

The ligand–immunoglobulin complex presented here can be used as a preliminary model to help define some of the structural parameters involved in an antibody–antigen reaction. From the results obtained in this work the following conclusions can be reached.

(*i*) The site of vit. K_1OH binding is located on the relatively flat end of Fab', next to the hypervariable residues of the L and H chains in a region which was defined before as the "active site" of the Fab' *New* molecule (3). Inside this region the Men moiety of vit. K_1OH is wedged in the upper part of a shallow groove which is surrounded by hypervariable residues. This groove or crevice occurs between the H and L chains and has the approximate dimensions 16 × 7 Å with a depth of 6 Å (3). Other low affinity ligands that were studied at 6-Å resolution (i.e., Men, orceine, and uridine), bind in the same upper part of the interchain crevice or groove. Crystallographic studies of the phosphorylcholine-binding Fab' fragment from

murine myeloma protein McPC 603 at 4.5-Å resolution have also located the hapten-binding site in a cleft between the V_L and V_H homology regions (8).

The methylnaphthoquinone ring of vit. K_1OH makes close contact with the phenolic ring of L-chain tyrosine 90, located at the bottom of the crevice, with the backbone and side chain of H-chain residue 104, and with the backbone of L-chain residues 29 and 30. Starting from the 2-methyl-1,4 naphthoquinone moiety the phytyl tail extends upwards and loops around (Fig. 4) making close contacts with L-chain residues 29 and 30 (Gly, Asn respectively, ref. 3) and proceeds downward making close contact with the backbone of L-chain residues 93, 94, and H-chain residue 104, and towards its end with the side groups of H-chain residues 54 (a constant Trp residue in human H-chain sequences), 57, and 63. The vit. K_1OH contact site is composed of at least 10–12 amino acids from both the H and L chains. This number is a minimum estimate and will be revised, together with the nature of the contacts, when the H-chain sequence becomes available (Nakashima, Konigsberg, Richards, Chen, and Poljak, in preparation) and when a higher resolution Fourier map of the vit. K_1OH–Fab′ *New* complex is calculated. Furthermore, binding of other ligands may involve different contacts distributed over a larger area of the binding region.

(*ii*) Men, which corresponds to the quinone moiety of vitamin K_1, binds to IgG *New* with a much lower affinity constant than that of vit. K_1OH. Since the quinone rings of both haptens are bound at the same crystallographic site (although they may differ in orientation) it is reasonable to conclude that substantial portions of the total binding energy are derived from both the interactions of the quinone rings and those of the phytyl chain with the protein. Thus, the model presented here gives a structural basis for the difference in affinity constants observed with Men and vit. K_1OH.

(*iii*) It has been postulated that conformational changes may take place after antigen binding and that these changes are necessary for complement fixation and for a signal from the immunoglobulin receptor which activates the antibody-producing cell. Inspection of the vit. K_1OH difference Fourier map indicates that binding of this ligand does not introduce major structural changes in the conformation of the crystalline Fab′ *New* fragment. However, the present 3.5-Å analysis does not exclude smaller changes. A similar result was reported in the 4.5-Å resolution study of the phosphorylcholine–McPC603 complex (8).

It is reasonable to ask what physiological function would be fulfilled by an antibody directed against vitamin K_1 and whether the Fab′ *New*–vit. K_1OH crystalline complex represents an "unphysiological" artifact. It is known that when vitamin K_1 is coupled to a macromolecular carrier, antibodies directed against vitamin K_1 may be elicited. In the early stage of an immune response, such antibodies have an average intrinsic binding constant of 5×10^4 liter/mol to 1×10^7 liter/mol, and thus could include species having a K_0 in the range of 1 to 2×10^5 liter/mol as observed for the vit. K_1OH–IgG *New* complex. Moreover, anti-idiotypic antibodies against IgG *New* combine with some of the immunoglobulin species (resolved by isoelectric focusing) from the serum of a rabbit immunized with a vitamin K_1–bovine gamma globulin complex (Varga, Rosenstein, and Richards, in preparation). Naphthoquinone derivatives, such as 2-hydroxy-3-methyl-1,4 naphthoquinone in the form of an acetone–soluble lipid associated precursor have been extracted from *Mycobacterium tuberculosis* (9) and *Corynebacterium diphtheria* (10). Also, the the custom of forming skin and hair protein complexes with 2-hydroxynaphthoquinone (henna) has continued from the Egyptian empire and Old Testament times (11) to the present day. There are, therefore, vitamin K_1-like potential antigens present in the human environment which could explain the presence of cell clones and immunoglobulin secretions which recognize these haptens.

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