Intramolecular signaling upon complexation

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ABSTRACT Crystal habits can be used as indicators of conformational changes in their constituent proteins. As in the conversion of unliganded hemoglobin to the oxygenated form, the addition of a small hapten to a suspension of platy crystals of an unliganded Fab (NC6.8) results in the immediate disintegration of the plates and their replacement with prisms of the ligand-protein complex. Examination of the native and liganded forms by X-ray crystallography reveals that the space groups and protein structures are different. During complexation there are ligand-induced conformational changes both in the antigen combining site (local alterations) and in more distal portions of the molecule (allosteric changes). There is an extension of the light chain (10 Å increase in length), a commensurate shortening of the heavy chain (by flexing), and a decrease in the "elbow bend" angle of 31° (184° to 153°). Relative to the variable domains, the constant domain pair moves mainly as a unit in such a way that the carboxyl end of the heavy chain is displaced by 19 Å. In an intact antibody this displacement may be relayed as a tug (by tensile forces) on the segment connecting the Fab to the Fc region, perhaps altering the orientations of the constituents responsible for such effector functions as complement activation.—Guddat, L. W., Shan, L., Fan, Z.-C., Andersen, K. N., Rosauer, R., Linthicum, D. S., Edmundson, A. B. Intramolecular signaling upon complexation. *FASEB J.* 9, 101-106 (1995)

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THREE POSSIBLE MECHANISMS FOR COMPLEMENT activation were proposed by Metzger (1) in 1978 and will be used as a basis for discussion in this article. *1)* In the aggregation model the presence of aggregated antigen-antibody complexes is sufficient to initiate complement lysis. *2)* The allosteric model suggests that antigen binding leads to distal conformational changes in the Fc to promote the binding of C1q, which is the first component in the complement cascade. *3)* In the distortive model the Fab arms are displaced from the Fc to expose an otherwise inaccessible docking site for C1q. All three models have some excellent structural features that do not seem to us to be mutually exclusive.

In some highly constrained antibodies, allosteric or distortive models requiring twist or waggle motions of the Fab arms are considered to be untenable. For example, a disulfide bond, genetically introduced to bridge residues H119 on the two Fab arms of an anti-dansyl antibody, markedly lowers the segmental flexibility but does not interfere with complement activation after ligand binding (2).

In contrast, we encountered a naturally occurring murine IgG2b (κ) antibody (NC6.8) in which the Fab exhibits a significant waggle motion during complex formation (3, 4). It has been possible to define these movements by comparisons of the crystal structures of the NC6.8 Fab in the presence and absence of a trisubstituted guanidine hapten with highly potent sweet taste properties. The NC6.8 comparative studies have provided us with new perspectives in interpreting earlier work on transmitted changes accompanying the binding of dinitrophenyl compounds to a human light chain dimer (5, 6).

ATTACHMENT SITE FOR C1q

The docking site for C1q includes three prominent residues on the surface of the CH2 domain of the Fc region of IgG antibodies: glutamic acid 318, lysine 320, and lysine 322 (7). These residues are located on the middle strand of the three-chain β-pleated sheet of the domain and are highlighted on the structure of the intact human (Mcg) IgG1 immunoglobulin shown in **Fig. 1** (8). Although human IgG1 antibodies with hinge deletions (e.g., Mcg, Dob, and Lec) do not fix complement (9, 10), the putative C1q attachment site in Mcg is indistinguishable from that in a human Fc fragment released from IgG molecules with normal hinge regions (11).

The deletion of the hinge per se may not be the only important factor. For example, a mouse-human chimeric IgG3 molecule constructed without a hinge region actually is more active than the wild-type IgG3 protein in complement-mediated cell lysis (12). A short-chain murine IgG2a antibody lacking a CH1 domain can be isolated in two forms, one with and one without the light chains linked by an interchain disulfide bond (13, 14). Only the type lacking the interchain disulfide binds C1q and activates complement (14). The interference by covalently immobilized CL domains can apparently be obviated by movements (possibly flexion) of the light chains in the absence of the disulfide bond.

GLOBAL MOVEMENTS IN ANTIBODIES

With its shortened heavy chains and the interchain disulfide bond connecting the two light chains, the Mcg IgG is restricted in any global movements of the Fc region with respect to the Fab arms. In contrast, the murine Mab 231 antibody (subclass IgG2b) has hinge segments acting as long flexible tethers (15). The Mab 231 Fc is significantly offset from the vertical pseudodyad between the two Fab arms and is stabilized in this position by packing interactions with an adja-

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[2]Abbreviation: CD, circular dichroism.

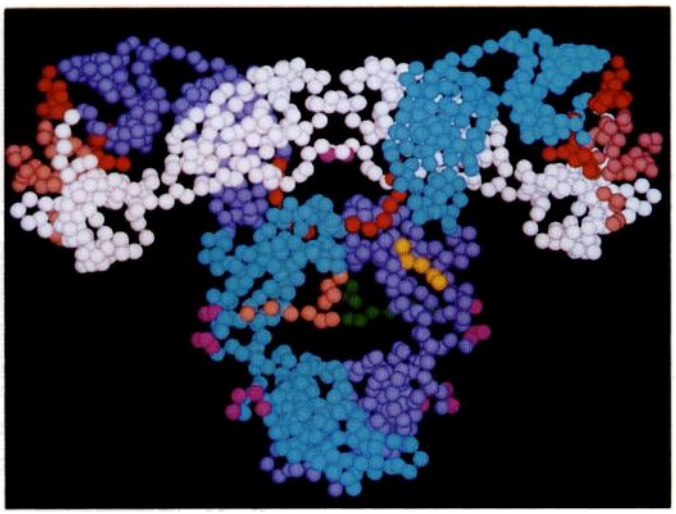

Figure 1. An α-carbon bead model of the Mcg IgG1 immunoglobulin. The Fab arms are located at the top of the T-shaped molecule and the Fc region is symmetrically oriented between and below the two Fabs. Light chains are represented by white spheres; the two heavy chains are colored purple and cyan; carbohydrates attached to the two CH2 domains are green and orange. Functionally important regions are highlighted in the following way: complementarity determining regions (CDRs) at the tips of the Fabs are colored red in the heavy chains and pink in the light chains; the interchain disulfide bond between the light chains is a magenta ball-and-stick model (partially hidden); the heptapeptide connector between the Fab and Fc units is in red; in CH2 the crucial region of the C1q attachment site is in yellow (the second equivalent site is located on the reverse side of the molecule); contact residues of the protein A docking sites are in magenta.

cent Fab in the crystal lattice. By extrapolation from this structure, we can begin to appreciate the range of motion possible with a loosely tethered Fc unit and its attendant sites for the mediation of biological functions. Without the benefit of such stringent packing interactions, two intact human immunoglobulins with normal hinge regions (Kol IgG1 and Zie IgG2) failed to display any significant contributions of their Fc regions in the X-ray analyses of their crystal structures; apparently the Fc regions adopted more than one conformation in the crystal lattices (16, 17).

CONFORMATIONAL CHANGES WITHIN THE FAB REGIONS

Although the inherent mobility of the Fc region is well established, there are few documented cases of transmitted signals generated during the antigen-binding events and transmitted from the Fab regions to the Fc unit (1). Direct 3-dimensional structural studies have not proved applicable to this question because so far there is no definitive crystallographic system, i.e., adequate quantities of crystalline high-affinity antibodies with normal hinge segments, discernable Fc regions, or well-characterized antigens that induce sizeable changes in distal structural elements upon complexation with the active site. Our laboratory therefore has concentrated on ligand-induced changes in the combining site and the adjacent CL and CH1 domains while continuing to search for such an elusive crystallographic system to bridge the activities of the Fab and Fc portions of an antibody.

LIGAND-INDUCED ALLOSTERIC CHANGES IN A LIGHT CHAIN DIMER

The light chain shown in Fig. 1 has an amino acid sequence identical to that of the urinary Bence-Jones protein from the same patient (Mcg). When complexed with bis(dinitrophenyl) lysine, the Mcg Bence-Jones dimer crystallizes predominantly as bundles of needles rather than the prismatic trigonal forms produced by the unliganded (native) protein (5, 6, 18). These needles are unsuitable for X-ray analyses. However, ligand-induced changes can be monitored by circular dichroism (CD)[2] spectroscopy after harvesting and solubilizing the needles. Extensive alterations in the aromatic CD spectra (relative to the native protein) were interpreted as evidence for significant environmental shifts of coupled tyrosine and tryptophan residues that are located at the distal ends of the CL domains (positions 189 and 195). These spectral changes do not appear to be attributable to ligand-induced protein aggregation, as evidenced by the behavior of the complexes on sizing columns of Sephadex G-100.

The indirect evidence for the transmission of ligand-induced effects is reinforced by the reverse experiments. After needles are dissolved and dialyzed to remove the ligand, the interchain disulfide bond linking the light chains is reduced and allowed to reoxidize slowly (6). The resulting protein gives a normal CD spectrum and crystallizes like the original native form. Presumably, the release of constraints permits the components of the dimer to revert to their starting conformations. This conclusion has been verified by X-ray analyses of the restructured crystals.

Recently we found a system in which the transmission of signals could be directly demonstrated in a ligand-protein complex with Mcg. After diffusion of a neurohormone hexapeptide into a preexisting crystal (native form) of the dimer, there were distinct conformational adjustments in the contact residues of the active site, in polypeptide segments immediately adjacent to the active site, and in the switch region between the V and C domains (L. Hanson and A. Edmundson, unpublished results). Cocrystallization trials with this peptide led solely to the production of needles, as predicted.

LIGAND-INDUCED CONFORMATIONAL CHANGES IN A MURINE FAB

General properties of the antibody and its hapten

The NC6.8 Fab also shows local and transmitted conformational changes on binding of a small ligand, N-(*p*-cyanophenyl)-N'-(diphenylmethyl)-N",-(carboxymethyl)guanidine (4). Nine murine monoclonal antibodies raised against this highly immunogenic compound were produced in relatively large quantities by hybridoma technology (3). The affinities of these antibodies for the ligand varied over three orders of magnitude from 10^6 to 10^9 M^{-1}.

The trisubstituted guanidinium derivative has proved to be an almost ideal hapten. It has a cyanophenyl component, a positive and a negative charge (zwitterion) for electrostatic interactions, and two aromatic (diphenyl) groups for hydrophobic interactions. This hapten is one of the most potent biological agents known (200,000 times sweeter than sucrose) and its interactions with antibodies may act as a paradigm for the activity of sweet-tasting compounds. As expected, the highest potencies are associated with ligands optimized for spatial and electrostatic complementarity with the putative receptors (19). A special role is reserved for the

p-cyanophenyl ring, which is believed to interact directly in a π-π system in a sweet taste receptor (20).

When the guanidine ligand was added to crystals (thin plates) of the native (unliganded) NC6.8 Fab the crystals immediately started to dissolve, and new crystals with different shapes (prisms) began to form next to them (see **Fig. 2**). The crystals of the complex were more robust and easier to manipulate. Formation of this complex led to structural alterations that could not be accommodated within the crystal lattice of the unliganded protein. X-ray analyses of the native and liganded forms indicated that the volume occupied by the protein increased by 11% (2.52 to 2.78 Å^3/Da) and the space group changed from monoclinic C2 to orthorhombic $P2_12_12$ (4).

Binding of hapten by an induced fit mechanism

Depending on the choice of the probe radius (1.4–1.7 Å), estimates of the total area of the hapten range from 663 to 730 Å^2 (21). About 81–84% of this area is buried when the ligand is bound to NC6.8. Only 218–260 Å^2 of the protein surface is buried because the protein side chains acting as contact residues for the hapten tend to be partially exposed to solvent even after complex formation.

A model of the hapten in the combining site is depicted in **Fig. 3.** Details of the hapten-antibody interactions are given in ref 4 and in the Fig. 3 legend. This ligand is bound by end-on insertion into a narrow fissure, with the cyanophenyl group in the lead and the diphenyl rings partially protruding into bulk solvent at the entrance to the site. The cyanide group is firmly wedged between the light and heavy chain backbone segments at glycine L91 and serine H97 (compare Fig. 3 and Fig. 4). We speculate that this penetration sets off the series of events leading to the distal conformational changes discussed below. Preliminary kinetic analyses support this hypothesis. There appears to be a two-step "on" reaction, with the usual diffusion-dependent interaction followed by a second stage of probable conformational adjustments (D. S. Linthicum et al., unpublished results).

Tryptophan H33 is probably the dominant residue involved in the quenching of fluorescence when the ligand is bound (22). Its orientation in the native protein is changed to make room for the entering ligand and it then packs tightly against the rigid guanido group, as well as against the carbon atoms of the acetic acid moiety.

Tyrosine H96 is a key residue in the restructuring of CDR3 during the formation of the complex. In the native protein CDR3 forms a γ-turn, with a hydrogen bond between the backbone amide group of tyrosine H96 and the carbonyl oxygen of serine H98. This hydrogen bond is broken in the complex because the backbone at tyrosine H96 moves 2.1 Å toward the ligand. The CDR3 loop expands at the top and a new hydrogen bond is established between the carbonyl oxygen of tyrosine H96 and a protonated guanido nitrogen of the hapten. The side chain of tyrosine H96 swings upward 4.5 Å to interact with the diphenyl rings and thereby locks them into the binding site. Concomitantly, the backbone at serine H97 moves toward the ligand and the carbonyl oxygen stacks with the cyanophenyl ring (23, 24). The tyrosine L96 phenolic group moves to stack with this ring on the opposite side. On the basis of these conformational adjustments, the mechanism of binding can be classified as another example of induced fit (25–30).

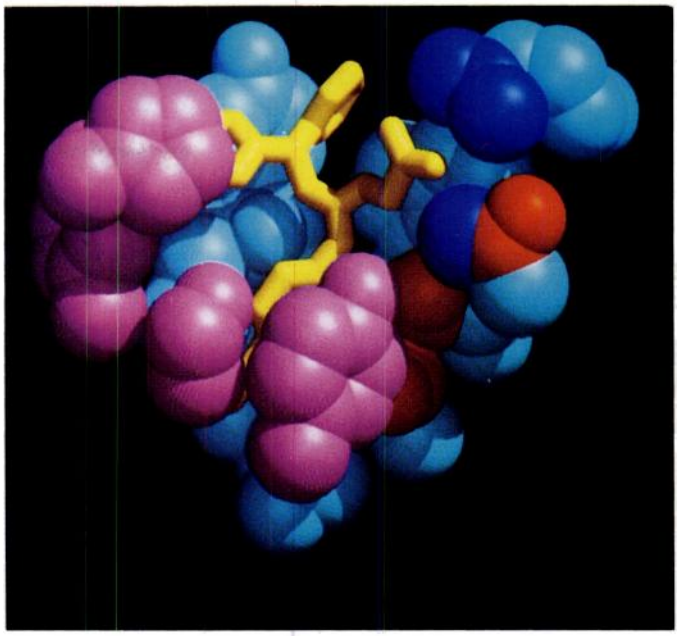

Figure 3. Corey-Pauling-Koltun (CPK) model of key active site constituents of the NC6.8 Fab, with a stick model of the trisubstituted guanidine hapten superimposed. The following color code is used: light chain components in magenta, heavy chain residues in cyan, guanido group of arginine H56 (top right) in dark blue, amide group of asparagine H58 (below arginine H56) in red and dark blue, carboxyl group of glutamic acid H50 (below asparagine H58) in red, and the hapten in yellow. Note that the cyanophenyl group of the hapten (lower left) is wedged between the light and heavy chains and is stacked against tyrosine L96 (lower right). One of the diphenyl rings (upper part of hapten) is located between histidine L27d (upper left) and tyrosine H96 (top center). H96 is the tyrosine side chain that moves 4.5 Å on complexation to help seal the hapten in place. Arginine H56 and asparagine H58 neutralize the acetic acid side chain of the hapten (upper right), and glutamic acid H50 forms an ion pair with the guanido group (planar Y-shaped group in the middle of the ligand). Tryptophan H33 is just visible behind the guanido and acetic acid moieties. This drawing was made with the program MIDAS (35).

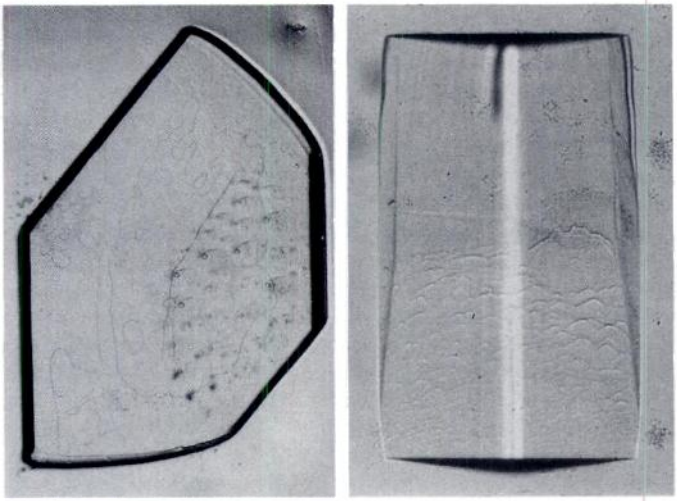

Figure 2. Crystal forms of the native NC6.8 Fab (left) and its complex with N-(*p*-cyanophenyl)-N'-(diphenylmethyl)-N",-(carboxymethyl)guanidine. The differences in the two crystal habits of NC6.8 reflect the conformational changes that have occurred in complex formation with its hapten.

Allosteric conformational changes on complexation

When the structures of the native and ligand proteins were simultaneously displayed on the graphics screen (see **Fig. 4**), one of the first features to be recognized was the extension of the length of the light chain by 10 Å in the complex. This extension was accompanied by an increase of 20° (77° to 97°) in the bend angle between the VL and CL domains. The heavy chain was commensurately shortened by a flexion of 22° (97° to 75°) around the switch region between the VH and CH1 domains. It is interesting to note that the structural arrangements in the unliganded NC6.8 are reversed relative to those in the Mcg IgG1, the Mcg Bence-Jones dimer, and many other Fabs where the light chains are extended and the heavy chains are flexed. On complexation of NC6.8, the light and heavy chains revert in unison to their more familiar structural patterns.

The reciprocal changes in the light and heavy chains are reflected in striking changes in the elbow bend angle between the pseudodyads. On ligand binding this angle decreases 31° (184° to 153°), by far the largest change recorded for complex formation among current Fab structures (31). Note also that the elbow bend of the native protein exceeds 180°, a relatively unusual but not unique feature of NC6.8.

Several lines of evidence indicate that the changes responsible for the contraction of the elbow bend angle are mediated around the flexible switch regions and involve mainly rigid body movements of pairs of domains. To measure these shifts, VL-VH and CL-CH1 pairs in the native Fab are first aligned as units on their counterparts in the complex. Individual domains are enclosed in a cylindrical envelope and then moved into more exact coincidence with the corresponding domains in the complex. These additional movements are recorded in terms of translations and three angles of rotation: a "barrel roll" of the cylinder about its long axis (x-axis vector), a tilt (cant) in the y-direction, and a swivel in the z-direction. The extra movements required for superposition are very small in all domains. For example, the translation of VL is only 0.05 Å and the roll, cant, and swivel angles are −1.84°, 0.12°, and 0.85°, respectively.

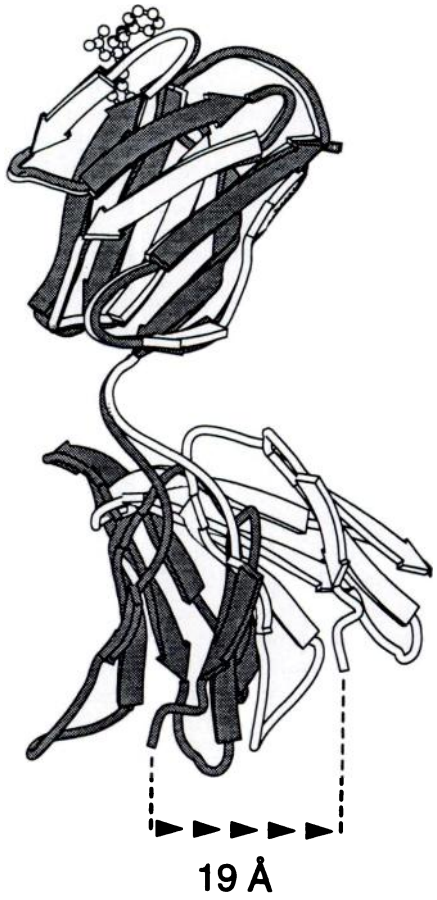

Figure 5. Diagrammatic representation of the shifts of the CH1 domain during complexation. Models of the native and liganded forms are constructed as in Fig. 4. The V domains of the two forms are superimposed with the program INSIGHT II (37) and the models are displayed in a view accentuating the 19 Å displacement of the carboxyl-terminal segment of the heavy chain (see text).

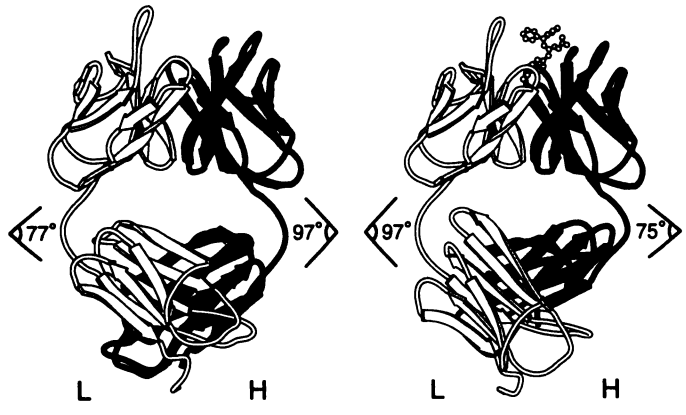

Figure 4. Schematic drawings of the crystal structures of the native (left) and liganded (right) forms of the NC6.8 Fab, as prepared with the program MOLSCRIPT (36). Directional arrows representing strands of β-pleated sheet are superimposed on tubular sections of polypeptide backbone: white for the light chains and gray for the heavy chains. The trisubstituted guanidine hapten is presented as a ball-and-stick model in its position between the variable domains at the top of the Fab on the right. Note that the overall length of the light chain is extended (by 10 Å) and the heavy chain is shortened proportionately.

In the next test the crossover angles within each pair of domains were found to remain virtually the same after complexation. VL crosses VH at an angle of 92° in the native protein and 91° in the complex. For the CL-CH1 dimer, the corresponding values are 109° in the unliganded form and 108° in the liganded molecule. Together, these measurements indicate that the rigid body shifts of individual domains are very limited within the V and C pairs. Moreover, the secondary structures of comparable domains are virtually indistinguishable in the native and liganded forms. This observation underlines our general impression that the most stable portions of the domain structures (i.e., the β-pleated sheets) are not appreciably affected by complex formation.

In contrast, when the VL-VH pairs are superimposed, the following additional movements are necessary to restore the CL-CH1 module of the complex to its original position in the native protein: a translation of 10.9 Å and rotations of −6.0° (roll), 35.3° (cant), and −5.0° (swivel). The large change in the elbow bend angle can thus be described in terms of a composite motion, of which a sizeable translation and a tilt of the C pair are the largest components.

The anchoring effect of the interchain disulfide bond between the last residue in the light chain and residue 128 of the heavy chain is one of the main reasons why the C-pair moves as a unit. As in murine IgG2a antibodies (32), the interchain half-cystine of the NC6.8 IgG2b heavy chain is lo-

cated only 15 residues from the amino end of the CH1 domain sequence (and is thus encoded by the CH1 gene). In the 3-dimensional structure, this residue is located on a loop at the distal end of the CH1 domain immediately opposite the COOH-terminal half-cystine of the light chain. Because of this arrangement, the last segment (β-pleated sheet strand 3−3) of the CH1 domain is constrained only by hydrogen bonds with an adjacent β-strand (3−2), and not by the interchain disulfide bond that anchors it to the light chain in other subclasses. Leucine H223, which is the last residue encoded by the CH1 gene, is also the last residue held closely to the body of the domain by such hydrogen bonding.

The 3−3 β-strand in CH1 and the first hinge component (glutamic acid H224) are well defined in the electron density maps of both the liganded and native forms of the NC6.8 Fab. The shift in the position of the carboxyl end can therefore be measured quite accurately after complexation. We found that the α-carbon atom of glutamic acid H224 is displaced 19 Å as a consequence of ligand binding in an active site more than 70 Å away (see **Fig. 5**).

These observations provide a mechanical model for the transmission of allosteric changes from the antigen combining site to the distal end of the Fab regions. The large displacement of the CH1 domain should result in a significant tug on the tether connecting the Fab with the Fc regions. We can only speculate that the tensile effects will be further transmitted to the sites involved in complement binding and lysis.

Are the allosteric changes due to crystal packing forces?

In discussions of intramolecular signaling, it is important to consider whether crystal packing forces are responsible for the observed structural changes, as opposed to allosteric effects induced by ligand binding. It is especially important in the hapten-Fab complexes of NC6.8, because the aggregation of IgG antibodies and the attachment of C1q are generally associated with the binding of larger and often polyvalent antigens (1).

We chose to approach this problem by examining the complexes of NC6.8 with two other cross-reacting ligands: *1)* a 2-dimethyl-4-methyl-bicyclo[2.2.1] heptanyl derivative of guanidine, and *2)* N-cyclooctyl-guanidine acetic acid. Both compounds lack the cyanophenyl moiety and bind to NC6.8 with affinity constants two orders of magnitude lower than that of the immunogenic hapten. The two compounds cocrystallize with NC6.8 in forms similar to the plates of the native protein rather than the prisms of the cyanophenyl adduct (see Fig. 2). Moreover, the space group (monoclinic C2) and the unit cell dimensions are identical to those of the native protein.

X-ray analyses of the complexes reveal that each compound is bound in a more superficial subsite than the cyanophenyl hapten and the wedging effect of the latter is missing. Although the crystallographic refinement of each structure is still in progress, it is clear that the mobile H96 tyrosine (CDR3) is in the down position (the "native" conformation). In addition, there are only minimal disturbances of the starting orientations of the remainder of CDR3, the switch regions and the C domain dimers. Of the three ligands, therefore, only the cyanophenyl hapten triggers the observed distal changes in the NC6.8 protein structure.

These findings reinforce the impression of two types of crystal habits in the NC6.8 series, one associated with little or no change in the structure of the protein and one signifying local and transmitted alterations accompanying ligand binding. We concluded that the crystal packing interactions are not responsible for the apparent allosteric effects. This conclusion is consistent with the results of other studies. For example, the structures of bovine pancreatic trypsin inhibitor incorporated into crystals are believed to be selected from the population of conformational variants already present in solution, rather than being dictated by crystal packing forces (33).

Possible mechanism for the large domain shifts

The mechanism by which the cyanophenyl moiety induces the domain shifts in NC6.8 still has facets we do not understand. One general hypothesis centers around the subtle loosening of the interdomain contacts to allow the light chain to extend and the heavy chain to flex. When these contacts are tabulated, both the VL-VH and the CL-CH1 pairing interactions are found to be reduced by about 20% in the formation of the complex. The distribution of these changes is interesting. In the V domains the alterations are mainly correlated with the direct effects of the ligand on the protein structure. Along the articulating surfaces of the C domains, the principal adjustments are seen in the polypeptide strands immediately following the switch regions where the chain movements are most accentuated.

At the location of the deepest penetration of the hapten into the active site, the α-carbon atoms of glycine L91 and serine H98 are displaced the same distance (1.69 Å and 1.73 Å) by the wedging action of the cyanophenyl group. In the accompanying expansion of the CDR3 loop of the heavy chain, the α-carbon positions are shifted 2.0 Å (Gly H95), 1.9 Å (Tyr H96), 1.7 Å (Ser H97), 1.1 Å (Ser H98), 0.81 Å (Met H100k), and 0.72 Å (Asp H101). The heavy-chain backbone segments of the native and liganded forms come back into register (α-carbon displacements of forms 0.2–0.4 Å) at the next residue (Tyr H102). After residue 108, however, the heavy chain of the complex again diverges to participate in the large interdomain movements.

In the light chain the cyanophenyl group recruits tyrosine L96, which in the native protein is stacked with tryptophan H47 (ring-to-ring distance of 3.5 Å) and is also hydrogen bonded with the carboxyl group of glutamic acid H50. This shift of tyrosine L96 from H47 to the cyanophenyl ring is one of the key events and perhaps the driving force for other conformational adjustments. Its effects are transmitted up and down the chain, resulting in the severance of a total of 23 interatomic contacts between Pro L95 and Trp H47, between Tyr L96 and Trp H47, and between Phe L98 and Leu H45. As in the heavy chains, the large backbone movements begin four to five residues (Leu L104) before the end of the VL domain and extend three to four residues (Ala L112) into the CL domain.

A related murine antibody, NC10.14 (34), binds the same hapten as NC6.8 but has a light chain of a different subclass (λ vs. κ) and a γ2b heavy chain from a different VH family (Q52 vs. J558). Although the affinities of the two antibodies (10^9 M^{-1}) are comparable, the sequences of the CDRs lining the active sites are radically different. Preliminary results indicate that the elbow bend angle of the hapten-10.14 complex (168°) is halfway between those of the native (184°) and liganded forms (153°) of NC6.8. Moreover, the ligand does not penetrate into the active site as far as it does in NC6.8, and the binding interactions are less intricate. We await a comparison of the structures of the native and liganded forms to see if the domain shifts in NC6.8 are diminished or absent in NC10.14.

CONCLUDING REMARKS

In combination, the NC6.8 and its hapten complex provide an excellent system for the study of intramolecular signaling by X-ray crystallographic techniques. This system is currently being treated as a hapten-restricted allosteric model, because it is not implemented when cross-reactive ligands lack the critical cyanophenyl moiety.

The ligand-protein complexes of the NC6.8 Fab and the Mcg light chain dimer display common features in their ligand binding and signaling systems. *1)* In both proteins the ligands responsible for the induction of distal alterations first penetrate deeply into the active sites and cause local conformational changes. *2)* Each system is characterized by two types of crystals that act as indicators of the native and altered conformations of the constituent proteins.

Allosteric signals of the types occurring in the Fab regions of NC6.8 are structurally possible in almost all classes of antibodies. Whether they occur or not seems to be highly dependent on the chemical and physical properties of the ligand, at least in the NC6.8 and Mcg series. FJ

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