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STEREOSPECIFIC AND STRUCTURE SPECIFIC RECOGNITION OF TWO- AND THREE-DIMENSIONALLY ORGANIZED SURFACES BY BIOLOGICAL MACROMOLECULES

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The interactions between proteins and crystal surfaces can range from totally non-specific physical adsorption of the macromolecule on the surface to the most highly specific complementarity. Among proteins, antibodies are the tool that nature evolved to recognize foreign invaders. Recognition occurs through complementarity of their binding sites with the surface of the invader.

Recognition in antibody-antigen interactions was studied using crystals and monolayers of cholesterol and its structural isomers as well as crystals of dinitrobenzene. Sequencing and modeling of specific antibodies show an impressive degree of complementarity between the antibodies and the organized surfaces they recognize. In the cholesterol system, one antibody binds to monolayers of cholesterol and entcholesterol with affinity in the range of 10^{-12} M and high stereoselectivity, but no enantioselectivity. We conclude that the low level of chirality exposed at the monolayer surface limits the chiral discrimination.

Keywords: antibodies; crystals; molecular recognition; monolayers; stereoselectivity; structural complementarity

Molecular recognition is a widely studied subject in chemistry. How does a molecule recognize another molecule leading to stereospecific interaction? This is a question that has been addressed ever since the basic rules of molecular structure have been unraveled. Later, questions concerning stereochemistry in the solid state have been also addressed, insofar as the crystalline order provided an additional variable to stereochemistry [1].

In the biological realm, the three dimensional arrangement of molecular moieties governs the selectivity and the reactivity of many enzymes, the

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recognition in protein-DNA interactions and a wide range of other processes [2]. By and large, molecular recognition between biological macromolecules and their substrates, or one with the other, is the molecular basis of life. The question of how biological macromolecules recognize solid surfaces has been however little considered, mainly because it was considered mostly, if not totally, non-specific. Indeed, hydrophobic and/or electrostatic interactions are overwhelming factors resulting in denaturation of proteins at their contact with solid surfaces in artificial environments. But has it necessarily to be so?

We have learnt during the last 15 years, especially from studies in the field of biomineralization, that interactions between proteins and crystal surfaces may be very specific, and may be exploited to control crystal formation in all its aspects, from polymorphism to nucleation, inhibition, growth and morphology [3].

Antibodies are the tools that vertebrates evolved to tackle all foreign invaders by virtue of molecular recognition. They thus appear to be ideal to study the rules of molecular recognition at interfaces with biological environments.

Crystals can be invaders and should be treated by the organism as any other invader, by producing antibodies specific to their surfaces. The question that we are asking is thus: are there antibodies capable of intrinsic 2-dimensional or three-dimensional recognition of repetitive organized surfaces? If the answer is yes, what are the rules of recognition?

Antibodies owe their identify and versatility at the same time to their sharing a common backbone structure for most part of their chains, and differing from each other in the sequence and conformation of six loops forming their binding sites [4].

Note that an antibody binding site has a size of about 1000 $Å^2$ [5,6], such that potentially it may interact with 5–15 molecular moieties emerging at a crystal surface, depending on the size and the organization of the molecules in the crystal. An antibody interacting with a crystal surface will thus feel not one molecule, but rather an arrangement of molecular moieties not dissimilar in nature from any macromolecular surface.

Crystals expose however different arrangements of molecules at different surfaces. Thus, at least in principle, different antibodies should recognize specially different surface.

During the last several years, we have been investigating antibody-crystal interactions in this perspective. We showed first that crystals can be antigenic, namely they can elicit the selection and amplification of antibodies that specifically recognize their exposed surfaces [7,8]. This may occur when the organism is challenged with endogenous crystals during the course of pathological crystal-associated diseases, such as gout, gall stone formation or possibly vascular calcification. Immune reaction can also be

stimulated when exogenous crystals are injected into the body tissues [9–11].

In order to study the nature of the interactions ensuing between antibodies and crystals, monoclonal antibodies, i.e., antibodies generated by one cell type and bearing all the same binding site, are needed. These were produced and isolated after stimulation of the murine immune system with crystals of 1,4-dinitrobenzene and cholesterol monobydrate, chosen as appropriate antigens [10,11].

INTERACTION OF 1,4-DINITROBENZENE CRYSTALS WITH ANTIBODY 122B1

The 1,4-DNB structure is dominated by stacking interactions between the benzene rings along the *a* and *c* axes, with the stacks arranged in a herring-bone motif along the *b* axis. The crystals are delimited by faces {100}, {110}, {101} and {10$\bar{1}$}. The {10$\bar{1}$} flat faces expose the benzene rings edge-on to the faces, and display the herring-bone motif of the stacks. The other faces are rougher and display the various component motifs and orientations [10].

Among the selected antibodies, antibody 122B1 was found to interact specifically with the {10$\bar{1}$} faces [12]. The antibody variable region was subsequently sequenced, and the structure of its binding site was independently derived by molecular modeling, based on the structure of highly homologous antibodies, determined by X-ray diffraction. In contrast to the structure of other 'conventional' antibody binding sites, the derived model of antibody 122B1 is remarkably flat, and exposes many aromatic residues, in a predicted conformation edgeon to the interacting surface. Furthermore, the model can be docked on the structure of the recognized {10$\bar{1}$} face, such that six aromatic residues of the antibody appear to continue, each, one stacking motif of benzene molecules emerging at the crystal surface, without any apparent break in continuity (Fig. 1). A number of asparagine and serine residues in the binding their side-chain residues and the nitro-groups of the crystal molecules. The antibody thus appears to establish a very good geometrical fit to the crystal surface, reinforced by a net of interactions between appropriate amino acid side-chains and surface components [13].

INTERACTION OF CHOLESTEROL CRYSTALS WITH ANTIBODY 36A1

The structure of cholesterol monohydrate consists of bilayers of cholesterol molecules with intercalated water. The necessity to optimize the interactions between the rigid polycyclic ring systems generates an

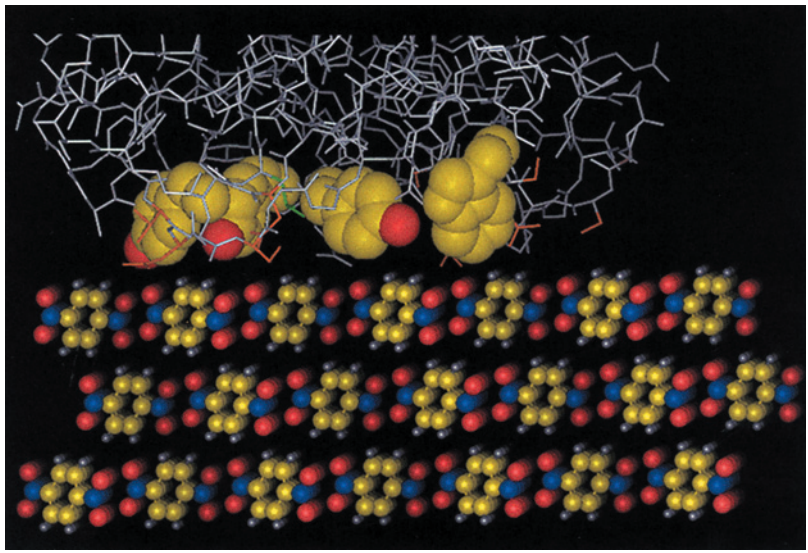

FIGURE 1 Docking model of antibody 122B1 (inline representation), on the $(10\bar{1})$ face of the 1,4-dinitrobenzene crystal (in ball and stick representation). The aromatic side chains of four amino acids in the antibody binding-site are rendered in space-filling representation, to highlight their striking match to the stacking motifs of the aromatic rings of dinitrobenzene emerging at the face. Two additional aromatic amino acids in the antibody binding-site are not visible. The antibody asparagine and serine residues that can bind to the nitro-groups of the crystal are marked in orange. White: antibody backbone and crystal hydrogen atoms. Yellow: dinitrobenzene and antibody aromatic rings. Red: oxygen atoms. Blue: nitrogen atoms.

unusual packing, of symmetry **P1**, with eight independent molecules per unit cell [14]. The crystals are delimited by large {001} faces parallel to the bilayers, which in water will expose the surface comprising the hydroxyl functional groups bound to water molecules. The {0*kl*} and {*h*0*l*} side faces of the crystals have mostly hydrophobic character. Some of the faces however, such as {100} and {011}, are parallel to molecular layers, and are thus flat and hydrophobic. Others, such as {010}, {101} and {301}, comprise molecular steps where one side of the step is hydrophilic, due to the exposed hydroxyls [11].

Among the isolated antibodies, antibody 36A1 selectively recognizes the {301} faces of the crystals [11]. These faces embody some of the characteristics of the {100} faces, with which they share the long hydrophobic stretches, while the step structure is identical to that of the {101} faces. Neither of the latter faces is however recognized by the antibody with an

affinity as high as that displayed on the {301} faces. The similarity in structure to the {100} faces, combined with the different affinity observed, induced us to suspect that antibody binding most probably occurs at steps.

When the antibody variable region was sequenced and its binding site structure modeled, we were delighted to find that it indeed assumes the shape of a step, with one hydrophobic and one hydrophilic side, decorated by 5 tyrosines arranged at distances matched to the distances between hydroxyls on the crystal step (18 Å) [13]. Because of the complexity and chirality of the structure, four distinct motifs of cholesterol molecules with different orientations emerge on these steps, two from the $+c$ side and two from the $-c$ side of the crystal. Upon docking onto the crystal surface, three out of the four motifs show a very remarkable geometrical and stereochemical match to the antibody binding site, two of them practically identical (Fig. 2)[#]. The fourth motif is not as well matched, because the angle formed by the molecular backbones on the step, 100°, does not match the angle of the antibody binding site, 85°. It is unfortunately impossible, because of technical reasons, to detect differences in antibody binding between the two opposite {301} faces. Were this possible, an additional demonstration of the subtle sensitivity of molecular recognition in the system might be provided. We do not know why antibody binding on the {101} faces is more limited than on the {301} faces, exposing the same step structure. We note that the size of each antibody molecule (IgM), spans approximately 200 Å in an extended conformation (Fig. 3, bottom). Each antibody molecule has ten binding sites, which multiple binding would amplify cooperatively the binding energy and thus the affinity of the antibody [4]. It is conceivable that the large distance between steps on the {301} faces better accommodates the large antibody molecules, allowing cooperative binding of one molecule at multiple sites on adjacent steps.

INTERACTION OF ANTIBODY 36A1 WITH CHOLESTEROL, EPI-CHOLESTEROL, AND ENT-CHOLESTEROL MONOLAYERS

Cholesterol may be deposited at the air water interface in the form of monolayers. Within compressed monolayers, the cholesterol molecules are organized in structured domains, 100 Å wide [15]. The cholesterol molecules, even in non-compressed monolayers, are presumably organized in

[#]In Figure 2 docking has been performed paying particular attention to the match between the antibody binding site and the two sides of the step exposed to it. The perfect topology of the (301) face implies, however, continuation of the step into another long hydrophobic stretch. Note that, as represented in Figure 2, this would clash with a part of the antibody not comprising the binding site, unless one further cholesterol molecule is removed.

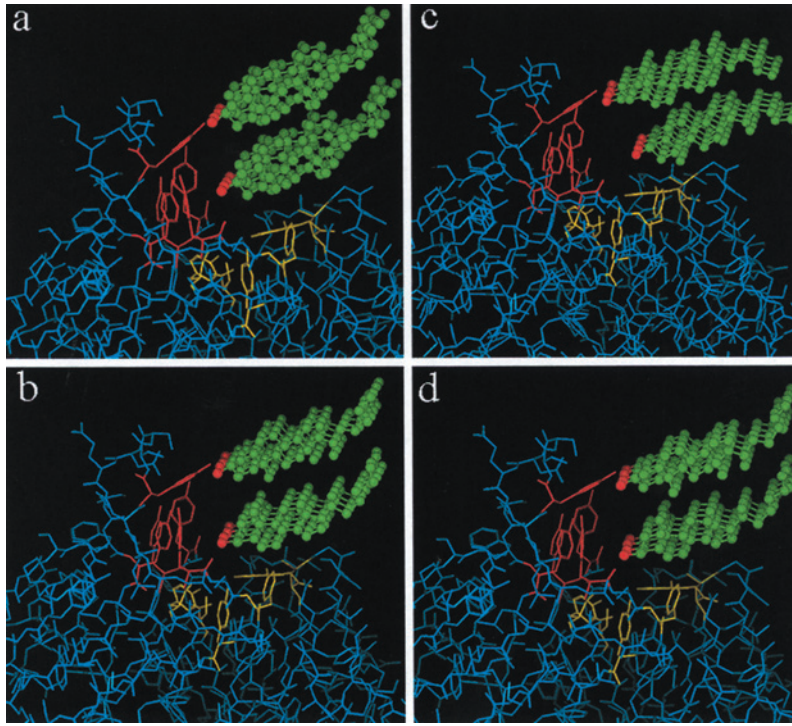

FIGURE 2 (a), (b), (c): Docking models of antibody 36A1 (in line representation) on the four distinct motifs of cholesterol molecules (in ball and stick representation) emerging with different orientations on the steps at the (301) face of the cholesterol monohydrate crystal. Two of the motifs, practically identical, are represented in (a). Three out of the four motifs, shown in (a) and (b), shows a very remarkable geometrical and stereochemical match to the antibody binding-site. The fourth, shown in (c), does not match well the antibody binding-site. (d): Docking model of antibody 36A1 on the steps of the (301) face of ent-cholesterol. For convenience, only one motif of ent-cholesterol molecules is shown, which corresponds to the one shown in (b) for cholesterol. Clearly, the chirality of the molecule is not easily distinguished on the crystal step arrangement. Cyan: antibody backbone. Red: hydroxyl oxygen atoms of the cholesterol molecules and tyrosines in the antibody binding-site. Green: cholesterol backbone. Yellow: hydrophobic residues in the antibody binding-site.

domains of structure not dissimilar from that of a (001) layer in the crystal of cholesterol monohydrate, with the hydroxyl moieties contacting the water, and the hydrophobic part in air (Fig. 3, top). The structure is bound to be more relaxed, with greater molecular mobility both within the domains and at their boundaries. This is an interesting structure to study,

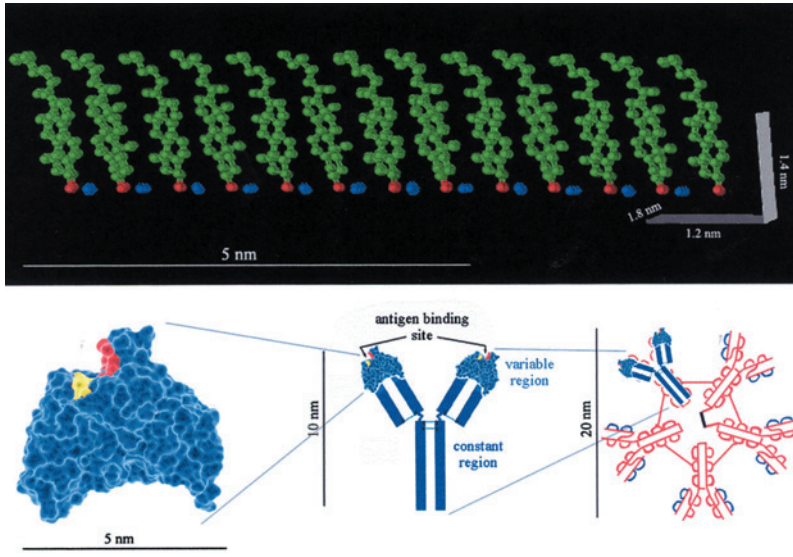

FIGURE 3 *Top*: Packing arrangement of a (001) layer in the crystal of cholesterol monohydrate. The layer has been drawn in analogy to the arrangement of an ideal crystalline domain of a cholesterol monolayer, with the hydroxyl moieties contacting the water, and the hydrophobic part in air. At the boundary of the domain, a step (in gray) has been drawn in scale, with the geometry of the binding site of antibody 36A1. Green: Cholesterol backbone. Red: hydroxyl oxygen atoms of cholesterol molecules. Blue: water molecules from the cholesterol monohydrate structure. *Bottom*: Schematic representation showing the typical size-scale of an antibody variable region (from the model of antibody 36A1) (left), of an IgG molecules (center), and of an IgM molecule (right).

insofar as it is intermediate between the crystal structure and the possible arrangement of cholesterol in some biological systems, in particular in cell membranes.

These possibilities motivated us to test the recognition of the isolated antibodies, specific to cholesterol monohydrate, for monolayers of cholesterol at the air-water interface.

Will the antibody be able to recognize these relaxed arrangements, and if yes, can it function as a reporter of supramolecular structure? At the boundaries of the monolayers domains, motifs of cholesterol molecules should be exposed forming structures similar, if not identical, to those of the steps binding antibody 36A1 on the {301} faces (Fig. 3, top).

Antibody 36A1 was indeed shown to be able to bind to such monolayers at the air water interface, when injected in the subphase in concentrations

as low as 10^{-12} M and in the presence of a tenfold excess of a different non-specific antibody [16,17].

Under the same conditions, antibody 36A1 does not bind at may appreciable extent to monolayers of epicholesterol, the epimer of cholestrol where the hydroxyl group is bound at position 3α (axial), rather than 3β (equatorial) [18]. Binding is not observed even when the antibody is present in the subphase at concentrations three orders of magnitude higher than for the cholesterol [17]. Antibody 36A1 does, however, bind to monolayers of ent-Cholesterol, the enantimer of cholesterol, in a manner indistinguishable from that of cholesterol [17].

The absence of binding to epicholesterol monolayers is explained in terms of the more acute angel imposed to the step by the hydroxyl position. The type of bad matching that may be expected due to different angles imposed on the step, relative to the antibody binding site, is exemplified in Figure 2c for one of the cholesterol motifs exposed on the ($\bar{3}0\bar{1}$) face. The absence of binding to epicholesterol monolayers also shown that the degree of recognition manifested on the crystal is preserved, notwithstanding the higher mobility of the single molecules and the more relaxed structure. The high affinity, together with the lack of enantiospecificity manifested in the binding to the ent-cholesterol monolayers, testimony that chirality is not expressed in the molecular arrangement of the cholestrol enantiomers at the air-water interface. Similar lack of enantiospecificity might be expected on crystals of ent-cholestrol as well, judging from the docking of the antibody to the {301} step in this structure (Fig. 2).

We are presently studying a case in which enantioselective as well as stereoselective recognition is manifested by an antibody on a crystal surface antigen. This was observed in an antibody raised and selected after stimulation of the murine immune system with crystals of the tripeptide Leu-Leu-Tyr, an immunostimulant present in cow milk casein. In contrast to cholesterol, the chirality of the single tripeptide molecules is clearly expressed, in the latter case, at the crystal surface.

LABELING OF CHOLESTEROL DOMAINS IN CELL MEMBRANES WITH ANTIBODY 58A1

Cholesterol is carried in the blood stream by the so-called LDL and HDL (low and high density lipid) particles, respectively. LDL particles, composed of cholesterol esters, unesterified cholesterol, phospholipids and proteins, are internalized by cells such as macrophages into lysosomes.

Inside the lysosome, the cholesterol esters are hydrolyzed to cholesterol, which is then trafficked to the membrane. From there, it can be expelled in

part from the cell, in part is trafficked back and store in the cell interior, and in part remains in the cell membrane, which becomes overloaded with cholesterol relative to its normal composition.

Kruth *et al*. showed that antibody 58B1, another antibody specifically recognizing cholesterol monohydrate crystals, is able to label the membrane of macrophages, which have been overloaded with cholesterol. They also showed that the labeling is sensitive to all the agents that influence cholesterol trafficking, in the expected manner [19]. It was concluded that overloading of cholesterol into the membrane causes its aggregation into domains of organized cholesterol, which are recognized by the antibodies.

As different antibodies were observed to label different cell cholesterol pools, it follows that they can be possibly used to learn about the structure of these organized domains. Different organized domains may furthermore be formed under different conditions in healthy or diseased cell membranes, such as in the so-called cholesterol rafts, which have recently been the focus of much attention [20,21].

The antibodies raised against, and recognizing specifically the surfaces of cholesterol monohydrate crystals, offer a rare chance to probe the structure of these organized domains, which is so far unknown.

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