Antibody organization on lipid films

Influence of pH and interchain disulphide reduction

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Two distinct lattice structures are observed for two-dimensional (2-D) antibody organization on phospholipid films. A low-order, small-unit-cell, square lattice is obtained at pH 7 and below for mouse IgE, mouse IgG_{2a} and IgG_{2b} and rabbit IgG. At pH 7.5 and above, the observed lattice structure switches to a large-unit-cell, hexagonal type for rabbit IgG and mouse IgE. Interchain disulphide reduction by exposure to 2 mM-dithiothreitol results in the formation of the compact 2-D lattice for all cases and for all pH conditions.

INTRODUCTION

Two-dimensional (2-D) self-organization of proteins bound to phospholipid films has been demonstrated for monoclonal mouse IgG₁ [1] and IgE [2] as well as for certain bacterial toxins [3,4], ribonucleotide reductase [5] and RNA polymerase [6]. The 2-D crystals so generated have been useful for obtaining high-resolution images of the proteins comprising the crystals [4,5,7]. The induction of ordered arrays on the lipid films may be a useful general approach to the study of proteins and other large macromolecules or macromolecular assemblies in physiological conditions.

In particular, a surface such as a phospholipid bilayer on which free 2-D mobility may be achieved [8] may allow different molecular conformations induced by different solution conditions to be readily visualized. If 2-D ordering can occur for the different induced conformations in physiologically relevant conditions, then the consequences of the conformational changes should be readily manifested in the lattice structure.

It is believed that 2-D organization can occur in a broad range of ionic conditions as long as a high density of the proteins is achieved at the membrane surface. In many instances, specific ligand binding fulfils this requirement, which may also be achieved by ionic interactions [6,9]. Thus, in general, a range of ionic conditions may be sampled in the growth of 2-D crystals, and the conditions for crystal growth appear to be less stringent than for 3-D crystal growth.

In the case of antibodies, previous studies have focused on several monoclonal antibodies which crystallized into well-ordered, large-unit-cell, hexagonal lattices. Antibody conformation could be clearly visualized and the antibodies for those structures were found to be in an open 'Y'-like conformation, with Fab arms at an angle of 120° to each other [10]. In extending these studies to other antibody classes, such as mouse IgG_{2a}, IgG_{2b} and rabbit IgG, as well as examining mouse IgE organization more thoroughly, a distinctly different 2-D organization was observed, consisting of a loosely ordered, very tightly packed lattice (unit cell 5.5 nm × 5.5 nm). This organization was strikingly pH-dependent for some antibodies, occurring readily at pH 7 and below, but switching to the large-unit-cell hexagonal ordering at higher pH for rabbit IgG and mouse IgE. Mouse IgG_{2a} and IgG_{2b}, however, failed to form any organized structure other than the compact lattice for all pH conditions.

One interpretation of these results is that the pH is an important driving force for the configurational switching between an open Y form and a closed, collapsed Y form of the antibody. It is also apparent that interchain disulphide reduction tends to drive rabbit IgG and mouse IgE to a closed configuration. The effect on mouse IgG_{2a} and IgG_{2b} was not observable, as these antibodies tend towards a closed configuration regardless of pH and salt concentrations, whereas for mouse IgG₁ the strong tendency to form hexagonal lattices at low as well as high pH is lost with the addition of dithiothreitol (DTT), but no ordering into a different lattice was observed. Previous electron microscopy studies [11,12] of antibody multimer formation induced by divalent haptens indicated that a variety of angles between the Fab arms are possible, and at pH 7.4 the Fab arms appear to be freely flexible and can sample freely Fab–Fab angles between 60° and 180°, the fully extended form. Only in the instance of dimer formation, in which the Fab arm angle is 0°, was there an indication of a small strain energy barrier. No systematic investigation of the effects of pH was undertaken, but the effects of mild reduction and alkylation were to increase dimer and trimer formation at the expense of higher multimers, which supports the interpretation of the observations in this study.

MATERIALS AND METHODS

Antibodies and protein labels

Rabbit anti-2,4-dinitrophenyl (DNP) was purchased from Gateway Immunosera as an affinity-purified IgG fraction. The antibody was not further purified. It ran as a single peak in gel-filtration analysis and gave two principal bands corresponding to the heavy and light chains in SDS/PAGE electrophoresis. Rabbit anti-fluorescein was purchased from Reagents, Inc. (Windham, ME, U.S.A.) as an IgG fraction and was not further purified. Whole rabbit anti-DNP serum was obtained from ICN. Goat anti-(rabbit IgG) serum and Protein A were obtained from ICN. Anti-(Protein A) serum was purchased from Sigma. DNP-BSA and DNP-haemocyanin were obtained from Calbiochem. Mouse monoclonal IgE (anti-DNP) was purchased from ICN, and mouse monoclonal IgG_{2a} and IgG_{2b} (anti-fluorescein) were provided by Dr. J. Owicki (Molecular Devices, Palo Alto, CA, U.S.A.) and Dr. A. Cantor (Stanford School of Medicine, Stanford, CA, U.S.A.).

Fig. 1. Electron micrographs of antibody arrays

(a) Electron micrograph of rabbit antibody arrays on a bilayer of DNP-PE/PC, after a 15 h incubation at room temperature. The electron microscope grid was floated on antibody solution (50 mM-NaCl/10 mM-Tris, pH 8.5). The grid was then washed with a drop of water and stained with 1% uranyl acetate. A hexagonal array is seen in the centre and linear chain stacking is seen in the lower left and upper left of figure. Bar = 50 nm. Inset: Image diffraction pattern from hexagonal array. (b) Electron micrograph of rabbit antibody array on a bilayer of DNP-PE/PC (1:3) after a 30 min incubation at room temperature. An electron microscope grid with the phospholipid bilayer was floated on 200 μ g of antibody/ml in 10 mM-phosphate buffer (pH 6.5)/150 mM-NaCl. The grid was then washed with a drop of water and stained with 1% uranyl acetate. Bar = 50 nm. Inset: Image diffraction pattern from array shown.

Phospholipids

The phospholipids *N*-dinitrophenylaminophosphatidylethanolamine (DNP-PE), *N*-dinitrophenylaminohexanoylphosphatidylethanolamine (DNP-Hex-PE), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and fluorescein-PE (F-PE) were purchased from Avanti Polar Lipids. Fluorescein-glycine-PE (FGPE) was a gift from Dr. J. Owicki and Dr. A. Cantor.

Preparation of supported bilayers

The spreading and sequential transfer of phospholipid monolayers from a water surface to carbon-shadowed electron microscope grids has been described in detail previously [13]. At an equilibrium surface pressure of 0.3–0.4 mN/cm, the monolayers readily transfer to the hydrophilic carbon surface as the grids are dipped through the interface. After a withdrawal through the interface with a spread monolayer, a single monolayer is picked up with polar heads abutting the carbon surface [14]. A second immersion through the interface results in the pick-up of the second half of the bilayer. The grids were withdrawn again into air and allowed to dry before further use. The last withdrawal was also through a spread monolayer of haptinated lipid or through PE to prevent surface tension forces from disrupting the bilayer.

2-D crystal growth and electron microscopy

The grids with the attached bilayers were floated on 20 μ l drops (in microtitre tissue culture plates) of buffer containing the antibody of interest. Depending on the pH desired, the buffers used in these experiments were 10 mM-phosphate, 25 mM-Tris, 20 mM-Bistris, or 20 mM-Hepes in 150 mM-NaCl. Salt concentrations were varied in some experiments as indicated below. The antibody concentration varied from 50 to 500 μ g/ml. After incubating the grids on the antibody solution for the desired

time, the grids were washed with a drop of water and stained for a short time with 1% uranyl acetate. Electron microscopy was done with a Hitachi 600. Electron micrographs were examined with an area-measuring program to score for crystal content. Image analysis was done on a Zeiss image analyser.

Hapten linkage to phospholipids

In order to assess the dependence of antibody crystallization on the details of the hapten linkage to the phospholipid, several different linkages were used for each of the two haptens studied. Fluorescein was linked directly to the amino group of PE in one case and, in the other, it was linked to this group through a triglycine peptide [15]. For DNP, a direct linkage to PE was used as well as linkage through a six-carbon spacer.

RESULTS AND DISCUSSION

Rabbit anti-DNP IgG antibodies form several types of 2-D arrays, as shown in Fig. 1. At high pH (7.5–8.5), hexagonal lattices formed as well as linear stacks (Fig. 1a). These types of antibody lattices have been well described for mouse monoclonal IgG₁ [1,10,16]. The linear stacks were spaced 15 nm apart and image enhancement views show what appear to be a 1-D stacking of antibodies against each other in linear chains. The hexagonal lattice is a more complex packing motif, with six antibody molecules per unit cell (31.5 nm $\times$ 31.5 nm). Detailed analysis of such a lattice structure revealed an open Y antibody conformation, i.e. with an angle of 120° between Fab–Fab arms [10]. Such an open conformation would also account for the spacing of the linear chains (Fig. 1a).

As the pH was lowered below pH 7.5 a very different ordering occurred for the rabbit IgG. A very compact square lattice was observed (5.5 nm $\times$ 5.5 nm). Figs. 1(b) and 2 show the lattice

Fig. 2. Electron micrograph of the antibody array after image enhancement by a low pass filtering procedure on a Zeiss image analyser

Inset: Fourier filtered image of lattice; bar = 5 nm.

structure obtained after incubation for 30 min on a bilayer of DNP-PE/PC (1:4). The antibody was at 200 $\mu\text{g}/\text{ml}$ in 10 mM-phosphate (pH 6.5)/150 mM-NaCl. The lattice structure filled the available surface area on the electron microscope grid. An identical lattice structure was formed for a bilayer of FGPE/PC (1:5) and rabbit anti-fluorescein IgG. The use of whole rabbit antiserum to DNP resulted in the growth of 2-D arrays but at a slower rate and with limited surface coverage (10–20 % of grid surface) after a 10 h incubation.

Mouse monoclonal IgE was also organized into the compact square lattice form below pH 6.5 and at pH 7.5 with 2 mM-DTT present, but otherwise, at higher pH and without DTT, the organization proceeded into open hexagonal lattices as previously described [2]. By contrast, the only organization observed for mouse IgG_{2a} and IgG_{2b} was the square lattice form, regardless of pH and the presence or absence of DTT. Mouse IgG₁ was not studied in these experiments, but in prior investigations [16], mouse IgG₁ retained a tendency to form hexagonal lattice structures even at low pH (with much lowered crystal growth rates). A drastic loss in hexagonal and linear crystallization incidence was noted in the presence of DTT at a concentration of greater than 1 mM. There was no optical diffraction from regions of apparent disorder; hence there was no other organization that could be detected. Thus, for this antibody, there is a strong tendency to form the open lattice structure; this tendency is decreased with lowered pH and with the addition of DTT, but it is not replaced by another organized structure as happens for mouse IgE and for rabbit IgG.

The rabbit IgG organization on the phospholipid proceeds very rapidly, as indicated in Fig. 3. In 150 mM-NaCl and at room temperature, the most rapid organization occurred for a 2 % (molar) haptinated lipid concentration in the bilayer. In that case, the time to organize into arrays over one-half of the surface area was only about 8 min. This time increased to about 30 min for hapten concentrations greater than 20 % and also at the other extreme of very low hapten concentrations, i.e. 1 % or less.

Although the organization is fast, it does not produce a well-ordered lattice. A variety of procedures were undertaken to attempt to increase the order. These included annealing a preformed lattice by raising the temperature (up to maximum of 50 °C) then cooling; varying incubation temperature and salinity; including or excluding trace divalent ions; varying haptinated

Fig. 3. Rabbit anti-DNP organization of DNP-PE/PC lipid bilayers for various antibody concentrations and hapten-lipid fractions in the lipid bilayer

○, 2 % DNP-PE, 500 μg of antibody/ml, pH 6.5; □, 5 % DNP-PE, 500 $\mu\text{g}/\text{ml}$, pH 6.5; △, 20 % DNP-PE, 100 $\mu\text{g}/\text{ml}$, pH 6.8; ●, 30 % DNP-PE, 100 $\mu\text{g}/\text{ml}$, pH 6.8.

lipid concentration in the bilayer and antibody concentrations in solution; and slowing the rate of antibody binding through diffusion-limited transport. All attempts to increase the order failed. Very pure monoclonal antibodies also did not yield better order for the square lattice.

Aspects of rabbit antibody binding and ordering are summarized in Table 1. Arrays could form even from unpurified whole rabbit serum, but heat denaturation and premixing with DNP-BSA or anti-rabbit IgG were procedures that would preclude array formation or significant antibody binding. Finally, after a standard incubation of anti-DNP IgG in which more than 75 % of the lipid is covered with arrays, a pH 4 wash removed all structures and left a blank grid surface as observed in the electron microscope.

Different hapten linkages were utilized. For DNP, both direct and hexacarbon linking to PE resulted in antibody binding and array formation, with no noticeable differences in order quality or rates of crystallization. The same result was obtained for the hapten fluorescein when a direct and triglycine linkage to PE was utilized.

There was no upper limit on the DNP-PE concentration in the lipid bilayer. However, the fastest ordering occurred for low hapten concentrations (2 %) in either PC or PE. For fluorescein there was an upper limit to the hapten concentration. No arrays were observed for 100 % F-PE and very few for 75 % F-PE. High fractional array coverage occurred only at 20–30 % F-PE or FGPE.

The organization of antibodies into square lattice arrays was dependent on pH (Fig. 4) at 10 mM-phosphate and 150 mM-NaCl and with rabbit anti-DNP antibody. Above pH 7 the tendency to form the compact arrays was lost, but this was reversible on return to a lower pH. At pH 8.1 the addition of 2 mM-DTT restored the tendency to form the square lattice arrays (Fig. 4). A similar tendency was observed for mouse monoclonal IgE. At the higher pH conditions IgE formed extensive hexagonal lattices, and rabbit IgG formed both hexagonal lattices and linear stacks, but at a low frequency (e.g. less than 10 % of lipid area). Mouse monoclonal IgG_{2a} and IgG_{2b} formed only the square lattice arrays, regardless of pH.

At pH 7.5–8.0, a decrease in the salt concentration promotes loss of the square lattice organization, and an increase of salt, restores the organization. Hence charge shielding is an important effect at the higher pH conditions. It may be that such ionic

Table 1. Rabbit IgG organization on phospholipid bilayers

Incubation with the test antibody was at room temperature for 3 h in 10 mM-phosphate and 150 mM-NaCl, pH 6.8, for each experiment. The antibody concentration was in the range of 200–500 µg/ml.

Hapten	Antibody (rabbit unless noted)	Ordering (% of lipid surface with arrays)
DNP	IgG fraction	75–100
Fluorescein	IgG fraction	75–100
DNP	Whole serum	10–20
DNP (heat-treated)	IgG	0
DNP (premixed)	IgG + goat anti-(rabbit IgG)	0
DNP (premixed)	IgG + DNP-BSA	0
DNP (post-crystallization)	pH 4 wash	No antibody remains
DNP (premixed)	Goat IgG + rabbit IgG	0

* Against DNP.

Fig. 4. Effect of pH on rabbit antibody crystallization into square lattice arrays

Comparisons were for identical conditions other than the pH of the phosphate buffers used and the presence of 2 mM-DTT in some of the incubations at high pH.

interactions promote the antibody conformation changes that lead to different lattice types observed as a function of pH.

In the case of mouse IgG_{2a} and IgG_{2b}, there does not appear to be any conformational opening or closing of the Fab arms as a function of pH, as only one lattice type was observed. For these antibodies there was no observable effect of varying the salt concentration during the antibody binding and incubation step.

After the formation of square arrays, either of anti-DNP or anti-fluorescein IgG, further binding studies were performed in order to assess the orientation of the antibodies in the lattice. Grids with the formed antibody arrays were exposed for various lengths of time to buffers containing 500 µg of DNP-BSA or DNP-haemocyanin/ml. No binding of the label proteins was observed with electron microscopy and no changes could be seen in the image diffraction patterns. On the other hand, if the preformed lattice arrays were incubated subsequently with a Protein A solution (250 µg/ml) all lattice structure was lost, and no image diffraction spots were observed. We conclude that the antigen-binding sites of the bound antibody are unavailable after the square lattice forms, but that the Fc region of the antibodies is exposed to the solution and can easily bind to Protein A.

The hexagonal and linear chain organization has been well described previously. The square lattice organization is one with considerable local disorder in the arrays (sharp first-order diffraction spots; no higher order diffraction). The cause of the

variation in the mean lattice position of this type of organization is unknown. The local disorder could not be alleviated by ionic conditions (salt concentration, different buffers, pH variation within the permissive range), hapten surface density and hapten linkage differences, or by temperature. The limitations of the lattice dimensions demand a 'closed' form of the antibody, with Fab arms close together (e.g. with one antibody per unit cell and Fab binding sites anchored at each vertex of the lattice, the diagonal distance spanning the unit cell is 7.8 nm and this implies a 60° angle between Fab arms). Perhaps this conformation is more variable than the open Y conformation found in the hexagonal arrays. For example, there is an enhanced segmental flexibility in the presence of DTT [17–19], which is precisely the condition promoting square lattice organization and the closed Y conformation in my experiments. Square lattice arrays of similar quality have been obtained for mouse monoclonal IgG_{2a}, IgG_{2b} and IgE. Hence it is unlikely that heterogeneity in itself is the principal reason for the local disorder found in the square lattices.

If antibody conformation is the dominant factor in the array formation, then we might expect that inter-heavy-chain disulphide reduction might perturb the conformation at a given pH or that binding to the antibody Fc domain might also perturb the Fab configuration. Fig. 4 shows that the addition of 2 mM-DTT at pH 8.1 restored a square lattice organization (to about an 80% fractional occupancy). Apparently the release of the hinge disulphide link between the heavy chains (which is known to occur at mild reduction conditions such as used here [20]) allows the antibody to relax back into a closed Y configuration. This interpretation is supported by the observation that, in multimer formation through a divalent hapten, mild reduction followed by alkylation resulted in an increase of dimers and trimers at the expense of the higher multimers [12].

Significantly, rabbit IgG F(ab)₂ fragments derived from pepsin digestion followed by gel filtration purification give only square lattice formation even at a non-permissive pH value of 8.1 (results not shown). Thus it would appear that the critical residue or residues responsible for the array formation differences as a function of pH (and therefore of whether open or closed antibody conformations are assumed) lie on the CH₂ domain beyond the pepsin cleavage point. Also, as noted above, the inter-heavy-chain disulphide bridge is also involved in these antibody conformational shifts, as mild reducing conditions restored square lattice formation under conditions of high pH and at 150 mM-NaCl.

It will be of interest to investigate whether these observed changes in 2-D lattice structure can be correlated with hydro-

dynamic diffusion changes in solution. Polarization spectroscopy [21] in the nanosecond range has yielded much data on segmented flexibility of different antibody classes [19] and even on chimaeric antibodies [22]. Configurational changes such as those inferred from the present studies may be reflected in hydrodynamic diffusion changes as a function of pH and interchain disulphide reduction. If so, such global configurational changes may contribute to the effect observed with polarization spectroscopy.

We would also expect that, in antibody multimer formation studies [12], the multimer distribution would be shifted to low multimers by lowering the pH below 7, and that mouse IgG_{2a} and IgG_{2b} would form predominantly low multimers regardless of pH. In this respect, the behaviour of mouse IgG₁ would be most interesting, as the 2-D crystallization technique used here cannot explicitly indicate a conformational change as a function of pH or through a mild reduction treatment. Finally, the rapid organizational tendency of antibodies bound to phospholipid membranes demonstrated here may be an important factor in 2-D diffusion measurements on such systems [23].

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