Self-organization of IgE immunoglobulins on phospholipid films

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Mouse monoclonal IgE anti-2,6-dinitrophenyl antibody (clone SPE-7) crystallizes in two dimensions when bound to a phospholipid monolayer or bilayer that has been combined with the hapten 2,6-dinitrophenyl groups. The two-dimensional lattice exhibits considerable local disorder, but long-range order is maintained over many unit cells of the lattice. The crystallization of IgE on the films is sensitive to IgE concentration in bulk solution. The optimum appears to be in the range 100–200 $\mu\text{g/ml}$. The crystallization tendency is diminished at pH lower than pH 6.5, and the crystals ‘melt out’ at a rather low temperature; no crystals were observed for 37 °C and above. The crystal growth rate is very much lower than was observed for monoclonal IgG₁ anti-2,6-dinitrophenyl antibody. Finally, the IgE lattice is very different from the two-dimensional lattice formed by IgG₁: the hexagonal lattice unit cell is smaller and has an open structure.

INTRODUCTION

Proteins are able to self-organize into two-dimensional arrays when bound to fluid phospholipid monolayers at high density and in an oriented fashion. The first example of this tendency was demonstrated for a monoclonal antibody [1], mouse IgG₁ anti-DNP, and a phospholipid monolayer that had been combined with the hapten DNP. Subsequently other proteins have been crystallized on lipid films, and include lectins [2], toxins [3] and an enzyme ribonucleotide reductase (H. Ribi & R. D. Kornberg, unpublished work). Such two-dimensional crystals are interesting from the structural point of view, as they enable the use of electron microscopy and imaging techniques [4] for the structural elucidation of the proteins composing the two-dimensional lattice. A well-known example of the application of such techniques is the analysis of two-dimensional crystals of bacteriorhodopsin to 0.7 nm (7 Å) resolution [5]. It is therefore a useful exercise to investigate the process of self-organization and to ask what limits the growth of crystals, what induces crystal nucleation and what the limits of resolution might be. In this way some general rules governing two-dimensional crystallization processes may be developed.

For IgG₁ the dynamics of crystal growth have been reported and the conditions for achieving large highly ordered crystals have been indicated [6]. Heretofore, no other classes of antibody have been successfully crystallized. It would be very useful to be able to induce other classes of immunoglobulins to crystallize, to enable a comparison of functional differences with structural differences as well as with differences in molecular interactions that lead to two-dimensional order.

In the present paper I report on the two-dimensional crystallization of IgE, a mouse monoclonal antibody against the hapten DNP. The conditions for crystal growth differ from those for IgG in several important respects. These differences are presumably due to differences in antibody structure and molecular interactions in the two-dimensional lattice. Finally, the IgE system is a convenient test system, as both the

monoclonal antibody and the modified phospholipid are commercially available. Therefore various aspects of the two-dimensional crystallization phenomena can be readily investigated with this system.

MATERIALS AND METHODS

Antibody

Mouse monoclonal IgE anti-DNP antibody (clone SPE-7) was purchased from ICN Immuno-Biologicals (Lisle, IL, U.S.A.). The antibody was further purified by h.p.l.c. (Waters 840 system) on a Bio-Rad TSK-250 gel-filtration column (600 mm $\times$ 7.5 mm) with 300 mM-NaCl/10 mM-sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min. The antibody was then concentrated and stored at 4 °C in 150 mM-NaCl/25 mM-Tris/HCl buffer, pH 7.5.

Phospholipids, monolayer formation and production of Langmuir–Blodgett films

N-(2,6-Dinitrophenyl)amino hexanoyl phosphatidylethanolamine was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). The lipid was diluted 10-fold in chloroform and spread on a water surface (4 cm $\times$ 4 cm $\times$ 0.5 cm Teflon trough) by adding 20 μl of the diluted lipid. The equilibrium surface pressure was measured to be about 350 $\mu\text{N/cm}$ (35 dyn/cm) on a torsion-wire film balance [7]. When a hydrophobic surface was passed through this monolayer (freshly carbon-shadowed electron-microscope grid) a phospholipid monolayer was transferred to this surface with the hydrocarbon tails of the lipid next to the solid surface. This monolayer was preserved in this orientation upon withdrawal through the interface and subsequent exposure to a drop of salt solution containing the antibody.

An alternative approach was to generate a bilayer by first making the solid-support film highly hydrophilic (by u.v. irradiation), and then passing the support grid out through the spread monolayer and then back in again. This approach led to highly reproducible deposition of phospholipid films [8], and most of the

Abbreviation used: DNP, 2,6-dinitrophenyl.

experiments reported here utilized this method of generating supported bilayers.

Electron-microscope grids

Silver electron-microscope grids were coated with nitrocellulose in a standard way and then shadowed with carbon in a low vacuum. Freshly prepared grids were sufficiently hydrophobic that the monolayer-transfer procedure could be utilized in depositing the phospholipid film, and this was the procedure of the early experiments. For stored grids, and as a matter of good lipid coverage and good lipid film stability on the carbon, the bilayer-transfer approach was used and became the standard method of lipid transfer.

The coated grids were floated on 20 μ l drops of antibody solution for various salt concentrations, pH values and temperatures in a 25 mM-Tris buffer. The micro-titre plates in which the drops were placed were kept in a humid atmosphere throughout the incubation period.

Electron microscopy

After the incubation period, the grids were lifted off and washed with either one drop of water or one drop

of 1% uranyl acetate solution. Then a drop of uranyl acetate was held on the grid for 30 s. The drop was drained off and the grid was allowed to dry. The electron microscopy was done on a Hitachi model 600 instrument.

Area measurements

The degree of ordering was monitored by obtaining low-magnification images (17000) and analysing these images with an area-integration program (Digital Paintbrush on an Apple II+ computer).

Image analysis

Electron micrographs were image-analysed on a Zeiss Image Processor system.

RESULTS AND DISCUSSION

Fig. 1 shows an IgE crystal grown in a 12 h incubation at 23 °C in 150 mM-NaCl/25 mM-Tris/HCl buffer, pH 7.4, and 100 μ g/ml antibody concentration. The crystal growth is extensive and appears to proceed from many nucleation centres. The ordering in this case approaches nearly 100% of the lipid surface area. It can be observed

Fig. 1. Electron micrograph of IgE crystals after a 12 h incubation of a phospholipid film with IgE at 100 μ g/ml at 23 °C at pH 7.4 in the presence of 150 mM-NaCl

Inset: digital Fourier transform of the micrograph image.

Fig. 2. Filtered image of the electron micrograph of Fig. 1 obtained by using the transform spots shown in the inset

in the inset that the optical diffraction from such micrographs gives sharp diffraction spots but that the intensity of spots rapidly declines with order number. Typically this is indicative of local disorder in the lattice with long-range order preserved. A filtered image of the lattice is shown in Fig. 2. This relatively low-resolution image shows several interesting features: (a) the lattice is an open hexagonal structure with none of the intricate Fc contacts that are present in the IgG₁ hexagonal arrays [6–9]; (b) the vertices of the lattice are fuzzy, presumably owing to the local disorder and the consequent lack of higher-order reflexions in the Fourier transforms of the electron micrographs; (c) there is no evidence in any of the electron-microscope images of another crystal phase such as the linear stacking phase [6] readily observed for IgG₁.

These observations suggest that the Fc portion of the antibody molecule in this case does not allow the same highly ordered molecular contacts that arise in IgG₁ crystal growth. In this case there is not enough room in the unit cell to form the sixfold Fc contacts that are found for IgG₁ because of the larger Fc of the IgE molecule. This difference in the molecules also must be the reason for the lack of linear strands for IgE. Although the present data cannot distinguish between

various packing models in the IgE lattice, the molecular packing is clearly different from that found for the IgG lattice. To demonstrate this, lattice decoration with DNP-(bovine serum albumin) was carried out. It was found that DNP-(bovine serum albumin) does indeed bind to the IgE lattice after it is formed, but at alternating vertices of the lattice (results not shown). Thus one model would be that the Fab portion of the molecule is oriented vertically with respect to the film; one vertex of the lattice would be formed from a cluster of such Fab domains and the other vertex would arise from Fc contacts. This working model also suggests that the IgE must be very stiff in order to pack in this way. Indeed, if the temperature is raised, the IgE lattice 'melts out' readily, as shown in Fig. 3. This is again in contrast with the behaviour found for IgG₁, which readily crystallizes as the temperature is raised even to 50 °C. The decrease in ordering for temperatures below 20 °C is a question of dynamics. For example, the fractional ordering for 12 °C can be in excess of 50% if the incubation time is increased from about 12 h to 48 h. Furthermore, in the presence of 0.5–1 mM-dithiothreitol, a condition that promotes flexibility in the molecule by virtue of reduction of the disulphide bonds at the hinge of the molecule [10], no ordering was observed.

These observations are consistent with the proposed lattice packing model, but of course do not prove it. Attempts to increase the local order were not successful. Lower incubation temperatures for longer times did not improve the order, nor did the use of fixing agents before staining. A different clone of IgE was also used (a gift from D. Baird), with no improvement. There remained the possibility that either the condition of the antibody or the molecular heterogeneity in the samples was limiting the crystallization results. The purchased material was seldom satisfactory as received. The tendency to crystallize varied from lot to lot. There was significant IgG contamination in the samples, as well as low- M_r contaminants. In order to achieve reliable and reproducible crystallization behaviour it was necessary to re-purify the IgE with h.p.l.c. gel filtration. After the purification, IgE crystallization could be induced readily, as described below, but there was no change in the quality of the ordering. Hence it may be that the observed local disorder is inherent in this system for physiological conditions and ambient temperatures.

Figs. 4 and 5 show the salt- and pH-dependence of IgE ordering. There is no significant salt effect until about 0.27 M-NaCl, whereupon there is a rapid decline in the fractional area that is ordered. Likewise there is no pH

Fig. 3. IgE crystallization on phospholipid films as a function of incubation temperature

Fig. 4. IgE crystallization: dependence on pH in the presence of 150 mM-NaCl

Fig. 5. IgE crystallization: dependence on salt concentration at pH 7.4

effect above pH 7, but below there is a decrease in ordering and very few crystals were observed for pH 5.9. Evidently the molecular contacts of the two-dimensional lattice are enhanced by ionizable groups, which can be titrated or shielded by salt ions. Again, the IgG₁ results are in contrast with this: no shielding effects were observed, and high pH retarded formation of the hexagonal lattice. Further differences from IgG₁ crystal growth are in the rate of crystal growth and in the dependence of crystal growth on antibody concentration. For typical incubation conditions (100 μ g of IgE/ml, 23 °C) no ordered domains were observed for incubations of less than 5 h, 50% of the area was ordered after about 9 h, and greater than 80% after 12 h. Thus the crystal growth is very slow by IgG₁ standards (50% ordering into hexagonal domains after only 1 h) [6]. Secondly, the IgE-concentration-dependence for ordering was found to be rather strong: between the concentrations of 100 and 200 μ g/ml crystal growth proceeded well, as described above, but little ordering (less than 5% of available area) was observed for concentrations below 50 μ g/ml or above 300 μ g/ml. It is believed that a sort of a jamming limit mechanism retards crystal growth at the high-concentration end. The decline in ordering at the low-concentration end may be due to low affinity, particularly if the IgE is binding to the phospholipid in a univalent fashion. In other words, in the low-concentration limit, local clustering of IgE may not occur frequently enough along the surface to drive crystal growth.

With this argument in mind, it would be instructive to investigate the dependence of crystal growth on hapten concentration. In particular, the jamming mechanism should be alleviated with less surface hapten, and, if our inference about the low-concentration decline in ordering is correct, crystal growth should be retarded dramatically below a certain hapten density. A systematic investigation of this question has not been done. Several hapten-dilution experiments were performed, however. *N*-(2,6-Dinitrophenyl)amino hexanoyl phosphatidyl-

ethanolamine was diluted with phosphatidylcholine down to 20% of total spread lipid, and IgG₁ crystal growth was observed to occur readily. In the case of IgE and canonical incubation conditions (100 μ g/ml, 23 °C, 15 h), a reasonable degree of ordering was observed (40% of the surface area) for 50% *N*-(2,6-dinitrophenyl)amino hexanoyl phosphatidylethanolamine lipid content. However, further hapten dilutions were not done, nor was the concomitant IgE-concentration-dependence investigated. One difficulty in this type of investigation is that the hapten density on the phospholipid film is probably quite different from that in bulk organic solvent, owing to the possibility of sequestering the DNP group in the hydrocarbon region of the film [11], a phenomenon previously observed in fluorescence studies of phospholipid monolayers. Hence it may be desirable to use a linker different from the present hexanoic acid in attaching the DNP group to phosphatidylethanolamine.

In summary, IgE forms two-dimensional crystals with considerable local disorder, which is retained under all conditions investigated. The molecular crystal interactions are quite weak, and the crystal is easily disrupted ('melted') at temperatures above 30 °C. The ordering of IgE appears to be rather sensitive to concentration of antibody in the bulk incubation solution. The IgE-DNP system is readily accessible, and questions relating to two-dimensional protein ordering or to IgE interactions can be readily investigated. In particular, the question of what can be done to remove local disorder remains an important bottleneck to further structural analysis.

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