Synchrotron Radiation Diffraction from Two-Dimensional Protein Crystals at the Air/Water Interface

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ABSTRACT Protein structure determination by classical x-ray crystallography requires three-dimensional crystals that are difficult to obtain for most proteins and especially for membrane proteins. An alternative is to grow two-dimensional (2D) crystals by adsorbing proteins to ligand-lipid monolayers at the surface of water. This confined geometry requires only small amounts of material and offers numerous advantages: self-assembly and ordering over micrometer scales is easier to obtain in two dimensions; although fully hydrated, the crystals are sufficiently rigid to be investigated by various techniques, such as electron crystallography or micromechanical measurements. Here we report structural studies, using grazing incidence synchrotron x-ray diffraction, of three different 2D protein crystals at the air-water interface, namely streptavidine, annexin V, and the transcription factor HupR. Using a set-up of high angular resolution, we observe narrow Bragg reflections showing long-range crystalline order in two dimensions. In the case of streptavidin the angular range of the observed diffraction corresponds to a resolution of 10 Å in plane and 14 Å normal to the plane. We show that this approach is complementary to electron crystallography but without the need for transfer of the monolayer onto a grid. Moreover, as the 2D crystals are accessible from the buffer solution, the formation and structure of protein complexes can be investigated in situ.

INTRODUCTION

Periodic ordering of proteins in two dimensions (2D) has proved to be a valuable alternative to conventional crystallography for determining molecular structures at high resolution by transmission electron microscopy. The most successful method for crystallizing soluble proteins in 2D relies on their specific anchoring to ligand-lipids inserted into a lipid monolayer at the air-water interface (Uzgiris and Kornberg, 1983; Kornberg and Darst, 1991; Brisson et al., 1994). Many soluble proteins expressed with polyhistidine tags for purification on nickel columns can also bind to new Ni-chelating lipids and form 2D crystals (Vénien-Bryan et al., 1997; Bischler et al., 1998). For electron microscopy, a delicate step of transfer (and possibly fast freezing) of these 2D crystals onto a solid support film is required, possibly leading to significant structural reorganization. In addition, the nonplanarity of the carbon film substrate can be another drawback for the determination of the full 3D structure, which requires precise tilting of the sample (Uzgiris and Kornberg, 1983).

Monochromatic synchrotron radiation under grazing incidence has been used for in situ diffraction studies of crystalline Langmuir films forming a 2D powder at the air-water interface (Als-Nielsen et al., 1994) and more recently of single layers of streptavidin (Haas et al., 1995) and of purple membranes (Verclas et al., 1999). But high-resolution structural determination of 2D protein crystals with large unit cell parameters requires the high angular resolution obtained from third-generation synchrotron beams to separate the numerous Bragg reflections that overlap at wide angles of diffraction. However, such investigations face additional difficulties: 1) the data collection procedure has to be adapted to the time scale of irradiation damage of the 2D crystals; 2) the in-plane spatial resolution, which is determined by the maximum angular range of measurable diffraction, may be limited by dynamic disorder, such as internal molecular motions and thermally excited capillary waves; 3) the in-plane elastic fluctuations, the role of which is known to be important in limiting the angular range of diffraction from 2D crystals, has been emphasized in recent experiments (Safinya and Shen, 1996; Zakri et al., 1997)

In this paper we discuss these different effects from diffraction studies of four protein-ligand systems known for producing 2D crystals:

Streptavidin crystallized under a biotinylated lipid monolayer, also investigated by Haas et al. (1995), who used synchrotron radiation, and by Scheuring et al. (1999), who used atomic force microscopy

Annexin V, which binds to negatively charged phospholipids in a Ca^{2+} buffer solution (Andree et al., 1992; Olofsson et al., 1994).

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Cholera toxin B-subunit (CTB) bound to monogangliosides (GM1) diluted in a lipid monolayer (Mosser and Brisson, 1991; Mosser et al., 1992)

HupR, a RNA transcription factor that shows the specific interaction between a polyhistidine extension engineered in the protein and a nickel atom chelated to a lipid molecule (C. Vénien-Bryan et al., 1997).

MATERIALS AND METHODS

Streptavidin (Boehringer, Ingelheim, Germany; concentration between 10 and 100 μg/ml) was bound to biotin-LC-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine diluted with dioleoylphosphatidylcholine (DOPC) at a ratio of 1:4. Two different buffer solutions were used, giving different results: The first was at pH 7 (Darst et al., 1991), and the second was not buffered (pH ~5.5) (Haas et al., 1995). Annexin V (Boehringer) was used at a concentration of 15 μg/ml. The lipid monolayers contained dioleoylphosphatidylserine and DOPC at a ratio of 1:4; the buffer is described by Pigault et al. (1994). CTB (Sigma) was used at a concentration of 15 μg/ml. GM1-lipids (Aldrich) were mixed with DOPC lipids at a molar ratio of 2:9. The buffer is described by Mosser and Brisson (1991). The histidine-tagged HupR was expressed as described by Toussaint et al. (1997) and was used at a concentration of 15 μg/ml. Nickel lipids, Ni-NTA-DOGA, were synthesized in C. Mioskowski's laboratory (Balavoine, 1998) and were diluted with DOPC at a ratio of 1:3. The buffer is described by Vénien-Bryan et al. (1997).

A monolayer of lipids and ligands at high lateral pressure (~50 mN/m) is deposited at the surface of the buffer solution, from a chloroform solution prepared with slight oversaturation (~115%) with respect to full coverage of the trough area. Protein is injected into the subphase, with a peristaltic pump and two capillaries, for input and output; circulation serves to stir the subphase.

The surface diffraction experiments were performed at the beamline ID10-Troika (Grübel et al., 1994) of the European Synchrotron Radiation Facility (ESRF), using a Si (111) monochromator and Ge (111) analyzer crystals, which permit a high angular resolution to be used, for separating the numerous Bragg reflections at a relatively low angle. The in-plane resolution of the scattering vector Q_{xy} was $\Delta Q_{xy} = 10^{-3}$ Å^{-1} (Zakri et al., 1997). Under grazing incidence geometry for surface structure analysis, the x-ray beam is totally reflected at the air-water interface, and only the evanescent wave propagating parallel to the interface over a depth of several tens of Å may be diffracted. The diffracted intensity is recorded versus the in-plane angle 2θ between the direct beam and the detector direction, which corresponds to the horizontal component of the scattering vector $Q_{xy} = (4\pi \sin \theta)/\lambda$. Because of the absence of periodicity along the vertical direction, crystalline monolayers produce Bragg rods elongated along the vertical direction (Als-Nielsen et al., 1994). The vertical component of the scattering vector is defined as $Q_z = (4\pi \sin \alpha)/\lambda$, where α is the vertical angle between the diffracted beam and the horizontal plane. A vertical position-sensitive detector was used to record the intensity profiles along the Bragg rods. After a first series of experiments using an x-ray beam with an energy of 9 keV ($\lambda = 1.407$ Å), the higher energy of 13 keV ($\lambda = 0.945$ Å) was preferred, to increase the lifetime of the 2D crystals. As shown in a previous paper, minimum deformation of the Bragg rods is achieved when the successive diffractions by the monolayer and the analyzer crystal are in nondispersive geometry (Zakri et al., 1997). The angular resolution was controlled using the narrow diffraction line of a dodecanol self-assembled monolayer at the surface of pure water. For the two different set-ups at 9 keV and 13 keV, the values found for $\Delta(2\theta)$ were 0.007° and 0.013°, respectively.

A special sample environment was used: helium is allowed to circulate through a transparent circular leak-proof box (400-mm diameter) with capton windows. Inside the box, two rectangular Teflon troughs (80 mm × 120 mm, depth 2 mm) were positioned on a precision translation stage. During the experiment, the troughs were regularly moved in such a way that the beam footprint (of width 0.1 mm) did not expose the same sample area for more than 30 s.

RESULTS AND DISCUSSION

For streptavidin we observed two different 2D structures from two different buffer solutions. Fig. 1 shows the grazing

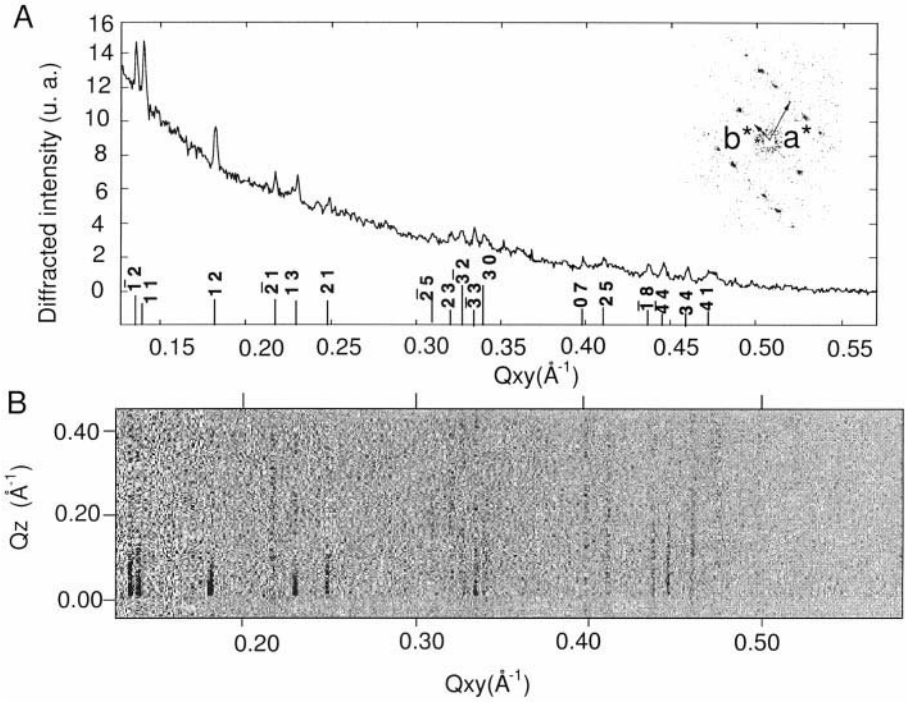

FIGURE 1 Surface x-ray diffraction pattern of streptavidin 2D crystals at the air-water interface. The spectrum was recorded at equilibrium, 10 h after sample preparation. (*A*) Diffracted intensity, integrated along the vertical direction (Q_z), as a function of the in-plane projection of the scattering vector Q_{xy}. Bragg peaks are indexed in the crystal unit cell: $a = 55.5$ Å, $b = 110.4$ Å, and $\gamma = 106.4°$. The inset shows the FT pattern of electron microscope pictures, on samples taken at the end of the experiment and negatively stained. (*B*) Reconstructed intensity map showing the vertical distribution of intensity obtained at each scattering angle. Bragg rods appear as vertical dark lines. The background due to diffuse scattering of x-rays by water, which is visible on *A*, has been subtracted to increase the contrast.

incidence diffraction pattern of 2D crystals of streptavidin at pH 5.5 (the same as in Haas et al., 1995). Several diffraction peaks (integrated along Q_z) are clearly visible, superimposed over a background due to diffuse scattering from the water subphase. Indeed the peaks closest to the origin correspond to diffraction angles 2θ not greater than 1.3°. In this experiment ~20 Bragg reflections were detected and observed over several subsequent measurements. The width of the Bragg peaks was that of the instrumental resolution (0.013°). In direct space, the best in-plane resolution achieved was ~10 Å. After each experiment, 2D crystals were transferred onto electron microscopy grids, and the electron micrographs were computer analyzed, using standard Fourier techniques (Kornberg and Darst, 1991). The x-ray Bragg peaks could thus be indexed, using unit cell parameters close to those calculated by electron image analysis. As shown in Fig. 1 *A*, the agreement is very good, with no peak deviating by more than 0.005° from its calculated position. For each diffraction peak, the intensity distribution along Q_z was recorded using a vertical position-sensitive detector (Fig. 1 *B*). Each peak in Fig. 1 *A* corresponds to an extended Bragg rod, as expected for a 2D crystal (Figs. 1 *B* and 2). For each (*h, k*) Bragg reflection the intensity distribution along Q_z gives the structure factor $F(h, k, Q_z)$, which contains the structural information along *c*, the crystallographic axis normal to the monolayer (Als-Nielsen et al., 1994). Some Bragg rods show intensity up to the edge of the detector at $Q_z = 0.45$ Å^{-1}, which corresponds to a resolution of 14 Å along *c*.

Fig. 3 shows similar maps of diffracted intensity by 2D crystals of HupR, before (Fig. 3 *A*) and after (Fig. 3 *B*) injection in the subphase of 0.5% glutaraldehyde, a well-known protein cross-linker (Ku et al., 1993). Under the same conditions of measurements, one observes that the positions of the two lowest order peaks are not affected by the glutaraldehyde cross-linking, but their intensity increases significantly, and two additional peaks appear at higher Q_{xy}. As for streptavidin, the linewidths of the diffraction peaks are essentially determined by the instrumental resolution. This result confirms that the size of the crystalline patches is larger than several micrometers.

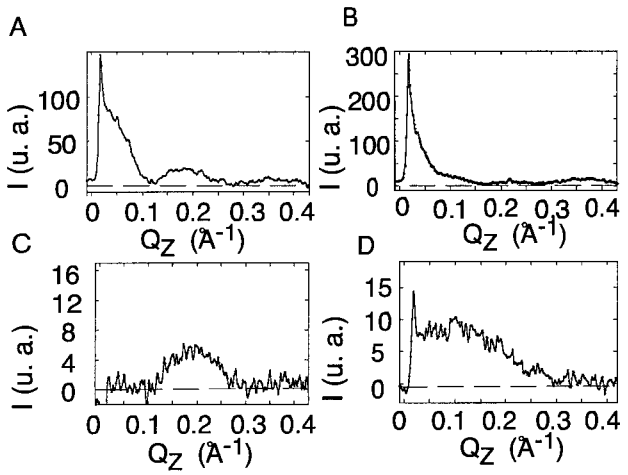

FIGURE 2 Intensity profiles recorded along the Bragg rods for streptavidin 2D protein crystals (after background subtraction). In *A* the first minimum at $Q_z = 0.12$ Å corresponds to a crystal layer thickness on the order of 50 Å.

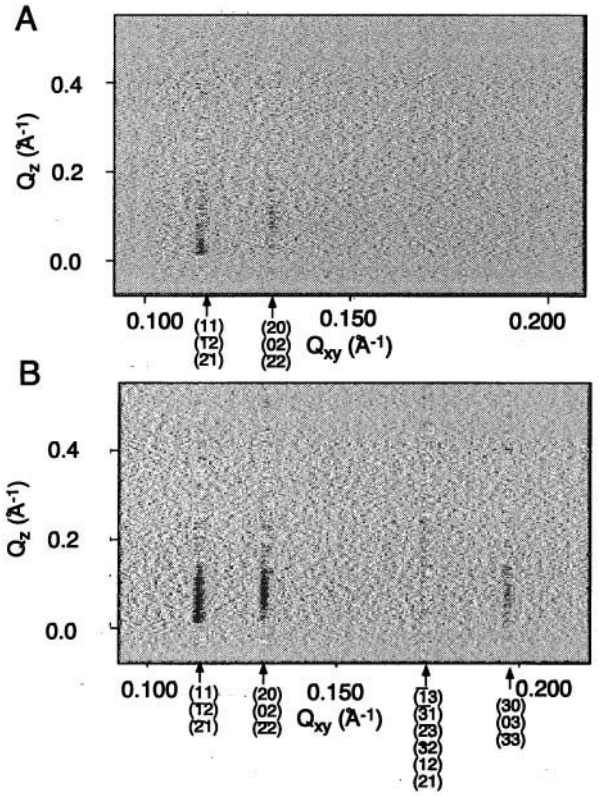

FIGURE 3 Reconstructed intensity map for HupR 2D crystals bound to nickel lipids. (*A*) After 10 h of incubation, just before the glutaraldehyde injection. (*B*) Same monolayer, but 1 h after injection of 0.5% glutaraldehyde. Bragg peaks are indexed in the 2D unit cell: $a = b = 111$ Å and $\gamma = 120°$.

The 2D crystals were found to be highly sensitive to radiation damage: the intensity of the Bragg reflections faded out exponentially with a time constant of less than 1 min, under an incident beam flux on the order of 6×10^{10} photons/s spread over an area of ~5 mm^2. For the same incident flux, the lifetime increased by a factor of 2 when x-rays of energy 13 keV instead of 9 keV were used. Taking into account the grazing incidence geometry, the fading dose can be evaluated to be on the order of 1 MGy (MJ/kg), which is comparable to that evaluated from electron microscopy results (Unwin and Henderson, 1975; Henderson, 1995; Stark et al., 1996). It is worth noticing the positive effect of glutaraldehyde that increased the lifetime of the crystals by a factor of 3. Nevertheless, for collection of the diffraction data, it was necessary to continuously move the trough across the x-ray beam.

Table 1 gives a summary of the crystallographic results for the four systems that have been investigated. The lattice parameters are very close to those determined by electron microscopy, but no diffraction was obtained with CTB, although eight samples were investigated. This might be due to a high sensitivity to radiation damage. Another possibility is that CTB crystals observed by electron microscopy appear during transfer of the monolayer from the water surface to the carbon layer supported by the electron microscope grid, although it is unlikely that large 2D crystals can appear during such transfer, which is a rather fast process. For the three other systems, at least two Bragg peaks were reproducibly observed for each sample. It has to be noticed that the two buffers used for streptavidin studies produced two different crystallographic orderings. The first is a square cell of area 6700 Å^2; the other one is a more compact cell (area 5800 Å^2) and is closer to hexagonal symmetry. We are currently investigating the relevant parameters for obtaining one or the other structure, as it appears that pH might not be the only important one. Measurements of the shear elastic constant μ (described by Vénien-Bryan et al., 1998) give a macroscopic determination of the rigidity of the crystalline layers, which shows a correlation with the Q-range of observable diffraction Q_{max}. The higher the macroscopic shear modulus, the larger is Q_{max}. The effect of glutaraldehyde cross-linking clearly confirms this correlation. Moreover, no diffraction is observed for CTB monolayers, which have a low shear modulus.

The next question to address is whether elastic fluctuations of 2D crystals introduce an intrinsic limitation of the resolution. The theoretical limit of spatial resolution $2\pi/Q_{cutoff}$ can be estimated from the measured value of the shear elastic constant μ: $Q_{cutoff} = \sqrt{4\pi\mu/kT}$, where T is the absolute temperature and k is the Boltzmann constant (Zakri et al., 1997). Below Q_{cutoff} the peaks are expected to broaden. Table 1 shows that the observed Bragg reflections are below this theoretical limit, although it is almost reached in some cases, such as streptavidin at pH 7. Nevertheless, the real value of Q_{cutoff} is probably underestimated because the value of μ measured on a 2D powder is likely to be lower than that of the constitutive crystals, because of the softness of the grain boundaries (Vénien-Bryan et al., 1998). The above evaluation is also consistent with the absence of broadening of the observed diffraction peaks with an increase in the scattering angle. We therefore conclude that the limitation imposed by the elastic fluctuations is not directly responsible for the limited range of observations of Bragg peaks. Instead the limitation comes most likely from the contribution of the local disorder to the Debye-Waller factor. In complex molecules like proteins, internal groups may experience independent motions. The experiments studying the effect of glutaraldehyde indicate that this disorder is dynamic: if it were a frozen disorder (as in a glass), the addition of protein cross-linker would not have revealed new Bragg peaks. The experiments presented here rather suggest that the protein cross-linker reduces the local motions, which are thermally excited. Notice that the links added to the 2D crystal by the cross-linker also stiffen the layer at large spatial scales, as detected in the macroscopic rigidity measurement (see Table 1).

TABLE 1 Summary of x-rays and shear rigidity results on different protein monolayers at the water surface

Proteins	Symmetry group (2D)	Lattice parameters			No. of molecules per unit cell	Resolution $2\pi/Q_{max}$ (Å)	Shear rigidity μ (mN/m)		Expected resolution limit $2\pi/Q_{cutoff}$ (Å)
		a (Å)	b (Å)	γ (°)					
Cholera toxin B	No diffraction observed							5 ± 1	16
Annexin V	p6	184	184	120	3	35		3 ± 1	21
HupR native crystals	p6	111	111	120	3	50	(10 ± 2)*	70 ± 5	4,5
HupR after injection of glutaraldehyde	p6	111	111	120	3	30	$(30 \pm 5)^{\dagger}$		
Streptavidin pH 7.4	c2mm	82	82	90	2	13		12 ± 3	10
Streptavidin pH 5.5 native crystals	p2	55.5	110.4	106.4	2	13		90 ± 10	4
Streptavidin pH 5.5 after injection of glutaraldehyde	p2	55.5	110.4	106.4	2	10		200 ± 20	2,5

The lattice parameters are those determined by x-ray grazing incidence diffraction. The resolution column $2\pi/Q_{max}$ corresponds to the spatial period of the highest observable reflection. The column μ is the measurement of the shear rigidity recorded during separate experiments. This elastic constant corresponds to the resistance to flow and is characteristic of the existence of a 2D solid. The last column, $2\pi/Q_{cutoff} = 2\pi/\sqrt{4\pi\mu/kT}$, represents the expected resolution limit imposed by elastic fluctuations and calculated from the previous column.

*Taken just before the addition of glutaraldehyde; equilibrium was not reached.

†Taken after glutaraldehyde injection; equilibrium was not reached.

CONCLUSION

The results of grazing incidence x-ray diffraction from three protein-ligand systems exhibit narrow Bragg peaks, which demonstrate long-range 2D crystalline order at the air/water interface, but for CTB no diffraction has been observed, in contrast to previous electron microscopy results (after transfer on a carbon-coated grid). The diffraction data are not yet sufficient for structural determination at atomic resolution because of the limited Q-range of the detectable intensities. To overcome the limited lifetime of crystals under irradiation ($\sim$1 min), it was necessary to use a translation stage to move the sample across the beam. The analysis of the shear rigidity of the 2D crystals shows that long-range elastic fluctuations are not able to increase the linewidth of observable diffraction peaks or to reduce the maximum Q-range. Our conclusion is that the resolution on the molecular level is essentially limited by dynamic disorder, which can be reduced by stiffening the crystals with protein cross-linkers.

Grazing incidence x-ray diffraction on water can now be thought as a complement to electron microscopy. The results presented here also open the possibility of achieving medium-resolution structural determination from protein monolayers at solid-water interfaces, where the molecular dynamic disorder should be reduced. The approach of this paper provides a new strategy not only for molecules that cannot be ordered regularly in 3D, such as membrane proteins, but also for in situ crystallographic studies of conformational changes and of the formation of protein complexes at interfaces.

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