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Protein crystals orientation in a magnetic field

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Abstract

Nucleation and crystal growth of hen egg-white lysozyme, bovine pancreatic trypsin inhibitor and porcine pancreatic α-amylase were carried out in the presence of a magnetic field of 1.25 T produced by small permanent magnets. Crystals were oriented in the magnetic field, except when heterogeneous nucleation occurred. The orientation of protein crystals in the presence of a magnetic field can be attributed to the anisotropic diamagnetic susceptibility of proteins resulting from the large anisotropy of the α-helices due to the axial alignment of the peptide bonds.

1. Introduction

According to Worcester (1978), many protein structures exhibit some diamagnetic anisotropy resulting from the contribution of the planar peptide bonds of the molecules and aromatic amino-acid residues. For α-helices, these bond planes are oriented parallel to the helix axis which is, therefore, of smallest diamagnetic susceptibility. This explains why proteins and synthetic polypeptides exhibit magnetic orientation with the α-helices parallel to the magnetic field. In the literature, most of the studies focus on the magnetic birefringence (Cotton–Mouton effect) of aromatic or aliphatic compounds (Lonsdale, 1939) or biological systems (Neugebauer *et al.*, 1977; Torbet *et al.*, 1981; Yamagishi, 1990). The diamagnetic anisotropy is characterized by observation of the formation of oriented fibrin, of small crystals or by the anisotropy of a scattering pattern. Only recently have protein crystals been nucleated and grown in the presence of a magnetic fields (Ataka *et al.* 1997; Sazaki *et al.* 1997; Wakayama *et al.* 1997). Moreover, crystal growth of benzophenone under high magnetic fields was reported recently (Katsuki *et al.* 1996).

The anisotropy between parallel and perpendicular diamagnetic susceptibilities, $\Delta\chi$ for an α-helix containing N peptides, was first calculated by Worcester (1978) from the data of Lonsdale (1939) and recalculated by Pauling (1979). The diamagnetic anisotropy will be dominated by the peptide backbone of the protein molecules when there are many parallel α-helices in the structure. On the other hand, if all aromatic rings of the molecules were nearly all oriented perpendicular to the α-helix axis, the diamagnetic anisotropy should be sharply reduced, although this is unlikely to occur in most proteins. From this it could be thought that protein structures containing many parallel α-helices should orient themselves parallel to the magnetic field. In fact this not the case for most of the proteins; there is a preferential direction corresponding to the resultant of the contribution of the α-helices present in the crystal.

In this communication we report preliminary results on the nucleation and growth of lysozyme, bovine pancreatic trypsin inhibitor (BPTI) and porcine pancreatic α-amylase in the presence of a magnetic field.

2. Materials and methods

2.1. *Proteins*

For several years we have been investigating the crystallization process of some proteins together with their behaviour in solution (diffusion coefficients, hydrodynamic and gyration radii, polydispersities *etc.*). The crystals under consideration are different polymorphs of porcine pancreatic α-amylase (Boistelle *et al.*, 1992; Veesler *et al.*, 1994), bovine pancreatic trypsin inhibitor (BPTI) (Lafont *et al.*, 1994, 1996; Veesler *et al.*, 1996) and different fish parvalbumins (Lafont, 1996). Since we have previously established their phase diagrams and the crystallization conditions, we decided to first carry out experiments with these proteins, together with chicken egg-white lysozyme which is easily available and also the protein most studied by crystal growers.

In the present work, since we only aimed at observing possible crystal orientation in a magnetic field, the solutions were prepared in such a way that nucleation occurred within 6–24 h. Supersaturation is defined here as the ratio $\beta = C/C_s$ where C is the actual concentration of the protein and C_s its saturation concentration, *i.e.* its solubility. Furthermore, the crystallization conditions were chosen so that the number of observed crystals was high enough to evidence the orientation effect, without being excessive and hinder this orientation. All experiments were carried out at 293 ± 1 K.

2.1.1. *Lysozyme*. Lysozyme is supplied as a lyophilized powder (chicken egg-white lysozyme reference No. L6876, Sigma Chemical Company) and used as received. We mixed two solutions to achieve supersaturation. Solution *A* contained the crystallization agent, NaCl, dissolved in a buffer of acetic acid (50 m*M*), pH = 4.5, adjusted by NaOH (1 *M*) and solution *B* contained the protein dissolved in the same buffer. After mixing solutions *A* and *B*, the final NaCl concentration was 0.7 *M* and the protein concentration 30 mg ml^{-1}, which corresponded to $\beta \simeq 10$ (Cacioppo & Pusey, 1991).

For all the experiments, 200 µl of the solutions mixture prepared as above were poured after filtration into cylindrical glass vessels, with a flat bottom, the internal diameter of which was 6 mm for a height of 15 mm.

2.1.2. *BPTI*. BPTI was supplied by Bayer A. G. (Wuppertal, Germany) as a lyophilized powder and used as received. The supersaturated solution was achieved by mixing two stock solutions *A* and *B*. BPTI was dissolved in solution *A*, a buffer of acetic acid (50 m*M*) adjusted to pH = 4.5 by NaOH (1 *M*). Solution *B* was prepared by dissolving either NaCl or $(NH_4)_2SO_4$ in the same buffer. After mixing *A* and *B*, the salt concentrations were 1.7 and 1.8 *M* in NaCl and $(NH_4)_2SO_4$

solutions, respectively, and the BPTI concentrations were 30 and 10 mg ml^{-1} in NaCl and $(NH_4)_2SO_4$ solutions, respectively. These concentrations corresponded to $\beta \simeq 1.6$ and $\beta \simeq 1.4$, respectively (Lafont *et al.*, 1994, 1997).

2.1.3. *α-Amylase*. This protein was supplied by Laboratoire de Biochimie, Biologie Moleculaire et Cellulaire (Marseille, France) in solution: 10 m*M* Tris–HCl at pH = 8, containing 6 m*M* NaCl, 1 m*M* $CaCl_2$ and 3 m*M* NaN_3. Unlike most proteins α-amylase crystallizes without any crystallization agent so that supersaturation may be achieved either by evaporation or, as in our case, by concentrating the sample solution through an Amicon membrane (Centricon-10). For nucleation experiments, we used protein solutions, the concentration of which was 50 mg ml^{-1} which corresponds to $\beta \simeq 10$ (Boistelle *et al.*, 1992). For the growth experiments of seed crystals the concentration was 40 mg ml^{-1} which correspond to $\beta \simeq 8$.

2.2. *Magnetic field*

In order to observe a possible orientation of the crystals, we first used the magnetic field of an electron paramagnetic resonance (EPR) spectrometer (Bruker SP 300E). The crystals were grown in the presence of a magnetic field of 1.2 T. After preparation of the solutions, the glass cells were immediately placed in the magnetic field. After a few hours, the cells were removed from the experimental set-up and the crystals observed by optical microscopy. The results we obtained were encouraging but there was also an important drawback in so far as we could not follow the crystallization process in a continuous way. For this reason we changed the set-up and used small permanent magnets, made of an Nd–Fe–B alloy (Goudsmit Company, France), which produced an intense magnetic field of 1.25 T. The dimension of the magnets were L = 25 mm for a width W = 9 mm and height H = 9 mm. Connected by the pole piece, we directly mounted them on an inverted microscope equipped with a video-camera system, so that the occurrence and growth of the crystals in the glass cell could be continuously observed and recorded. Fig. 1 is a top view of the system.

In these experiments the crystals rarely formed on the bottom of the glass cell. In fact, they occurred in the bulk of the solution and slightly decanted when their size increased. Besides, when the magnetic field induced crystal orientation, we easily observed that the crystallites were already oriented in the bulk, keeping this orientation when decanting, with only small fluctuation probably resulting from convection currents.

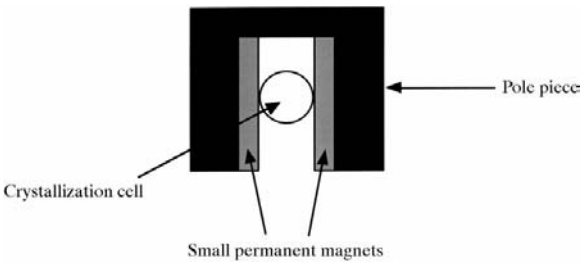

Fig. 1. Top view of the experimental device.

3. Results and discussion

3.1. *Lysozyme*

The tetragonal crystals of lysozyme nucleated in the magnetic field are perfectly aligned with their [001] axis parallel to the direction of the field (Fig. 2*a*). It is worth pointing out that in the absence of a magnetic field nucleation still occurred whereas crystals are randomly oriented. The [001] axis is of highest symmetry that means that each helix is repeated four times, then the projection of its axis on [001] is repeated four times whereas the projection on [010] and [100] is only repeated twice. For example, looking at the plane (100) (Fig. 2*b*), out of a total of 32 helices in a unit cell, 20 helices have an angle between [001] and their axis which is below 45° and only 12 have an angle between [010] and their axis which is below 45°. Thus, the resultant of the contribution of the helices present in a unit cell will be parallel to [001].

3.2. *BPTI*

When BPTI crystallizes from NaCl solutions, it belongs to a hexagonal space group, the experimental morphology being made of a bipyramid with 12 faces (isocelohedron) elongated along the [001] direction, sometimes truncated at low supersaturation by {0001} faces (Lafont *et al.*, 1996). In the magnetic

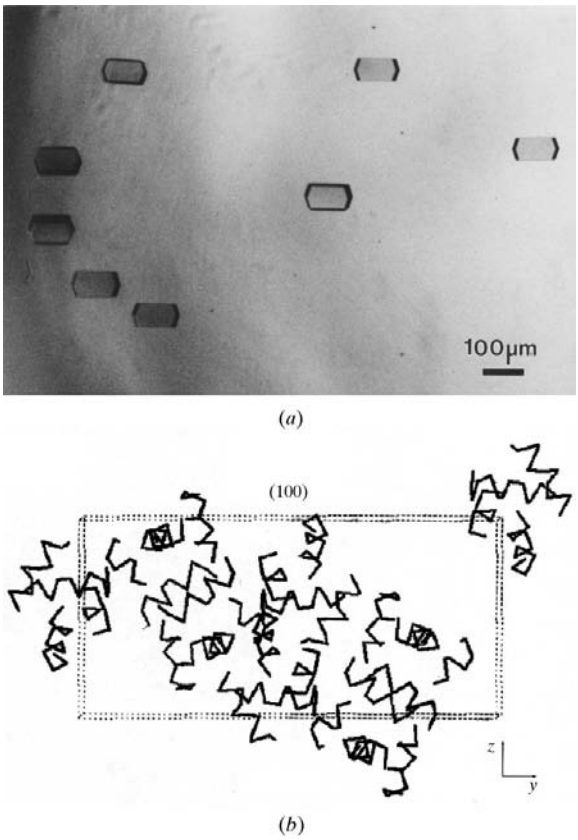

Fig. 2. (*a*) Orientation of tetragonal crystals of lysozyme nucleated in a magnetic field of 1.25 T. The direction of the magnetic field was parallel to the horizon. (*b*) Projection of the unit cell of tetragonal lysozyme in (100), only the α-helices are represented using the graphic tool *TURBO-FRODO* Viewer X11 implemented on an HP 9000 C160.

field all crystals are aligned with their [001] axis parallel to the field.

If grown from $(NH)_4SO_4$ solutions, BPTI crystals may form either in this hexagonal system or in a monoclinic system. In the case of the hexagonal crystal the habit is the same as in NaCl solutions but the crystal faces are significantly rounded, probably because of surface roughness. In the latter case the crystals are rod like with a pseudo-hexagonal cross section. In the magnetic field the hexagonal crystals have the same orientation as in the NaCl solutions (Fig. 3*a*). On the other hand, the monoclinic crystals have their elongation axis perpendicular to the magnetic field (Fig. 3*b*). It is noteworthy that twinned hexagonal crystals are deposited on the bottom of the glass cell with an orientation corresponding to the bisecting line of the angle formed by the two crystalline elements (Fig. 3*a*)

3.3. *α-Amylase*

When the crystals of α-amylase were nucleated from clear solution they were always observed growing at the bottom of the cell with no orientation of the crystals for either orthorhombic polymorphs *A* or *B* (Boistelle *et al.*, 1992). This is in contrast to the growth experiments of seed crystals of polymorph *A* for which crystals are found oriented with their [001] axis perpendicular to the direction of the magnetic field (Fig. 4).

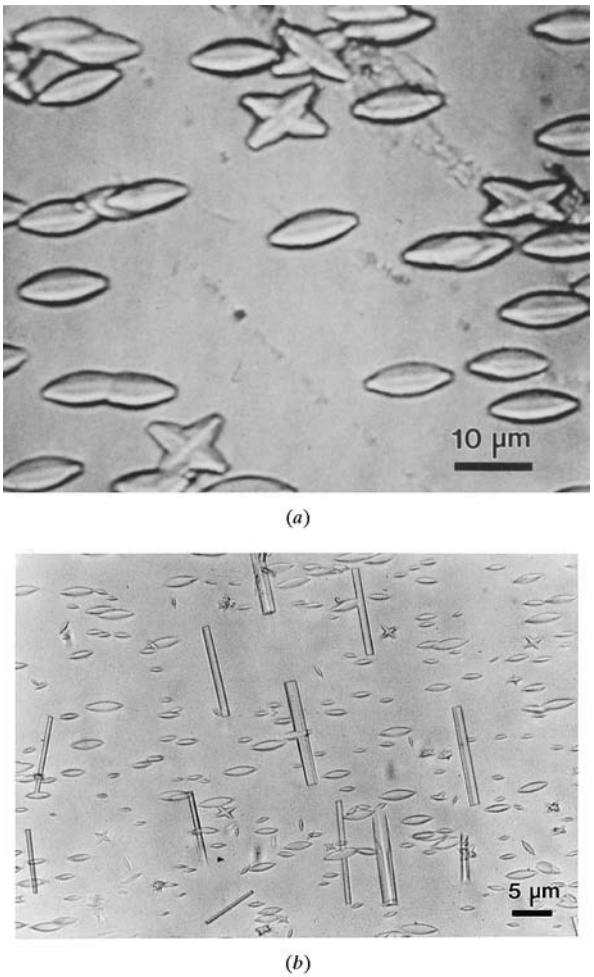

Fig. 3. Orientation of BPTI crystals nucleated in a magnetic field of 1.25 T. (*a*) Hexagonal modification. (*b*) Hexagonal and monoclinic or orthorhombic modification. The direction of the magnetic field was parallel to the horizon.

In the case of nucleation experiments, the driving force of the heterogeneous nucleation is related to the adhesion energy of the solute molecules of α-amylase onto the substrate (glass cell). This energy is probably greater than the one resulting from the magnetic field, and for this reason the crystals of α-amylase are not oriented. In contrast to this case, when crystals are seeded within the solution in the presence of the magnetic field the force resulting from the interaction between the nucleus and the substrate is cancelled and crystals of α-amylase are aligned along the direction of the magnetic field. By contrast, lysozyme and BPTI crystals which have been nucleated into the volume (homogeneous nucleation) are found oriented.

Generally speaking, orientation will occur if the energy difference between aligned and non-aligned units, ΔE, is greater than the thermal energy kT. ΔE is proportional to $|\Delta\chi B^2|$, were B is the magnetic field (T). This energy was estimated for a molecule of lysozyme under a field of 10 T to be about 10^{-2} J mol^{-1} (Sazaki *et al.*, 1997). The authors have calculated the diamagnetic anisotropy of lysozyme molecule under 10 T from the atomic coordinates of a lysozyme molecule and from the difference in the magnetic susceptibilities for an α-helix and a β-sheet structures. Consequently, under a field of 1.25 T ΔE is about 10^{-4} J mol^{-1}, this value being much smaller than the thermal energy at 293 K, 2435 J mol^{-1}. Individual molecules are probably not oriented in solution. However, if we consider small crystals containing n molecules ΔE will be comparable to the thermal energy for a value of n equal to about 10^7. In that case crystals will be oriented.

In this communication we reported preliminary results which clearly showed the orientation of crystals of different proteins nucleated and grown in the presence of the magnetic field. These experiments have shown that crystals are oriented in the case of homogeneous nucleation, for lysozyme and BPTI, and crystals are stuck at the bottom of the cell and not oriented in the case of heterogeneous nucleation for α-amylase. These experiments can be easily reproduced in the

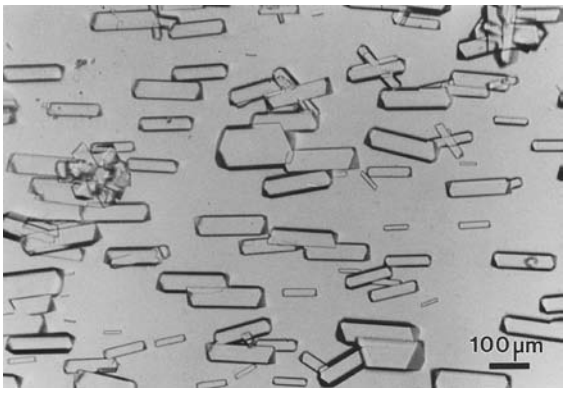

Fig. 4. Orientation of polymorph *A* of α-amylase in a magnetic field of 1.25 T. The direction of the magnetic field was parallel to the horizon and the [001] axis.

laboratory and require no special apparatus, the magnetic field being produced by small permanent magnets. In the future we will study the effect of the magnetic field on the nucleation rate, the crystal habit and crystal growth rates. The influence of the magnetic field on the crystal quality will also be studied.

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