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Soaking: the effect of osmotic shock on tetragonal lysozyme crystals

F. J. López-Jaramillo,* A. B. Moraleda, L. A. González-Ramírez, A. Carazo and J. M. García-Ruiz

Loboratorio de Estudios Cristalográficos, Instituto Andaluz de Ciencias de la Tierra, CSIC-UGRA, Facultad de Ciencias, Campus Fuentenueva, 18002 Granada, Spain

Correspondence e-mail: javier@lec.ugr.es

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Protein crystals crack when they are soaked in a solution with ionic strength sufficiently different from the environment in which they grew. It is demonstrated for the case of tetragonal lysozyme that the forces involved and the mechanisms that lead to the formation of cracks are different for hypertonic and hypotonic soaking. Tetragonal lysozyme crystals are very sensitive to hypotonic shocks and, after a certain waiting time, cracks always appear with a characteristic pattern perpendicular to the crystallographic *c* axis. Conversely, a hypertonic shock is better withstood: cracks do not display any deterministic pattern, are only visible at higher differences in ionic strength and after a certain time a phenomenon of crystal reconstruction occurs and the cracks vanish. At the lattice level, the unit-cell volume expands in hypotonic shock and shrinks under hypertonic conditions. However, the compression of the unit cell is anisotropic: the *c* axis is compressed to a minimum, beyond which it expands despite the unit-cell volume continuing to shrink. This behaviour is a direct consequence of the positive charge that the crystals bear and the existence of channels along the crystallographic *c* axis. Both features are responsible for the Gibbs–Donnan effect which limits the free exchange of ions and affects the movement of water inside the channels and bound to the protein.

1. Introduction

Unlike crystals of small molecules, macromolecular crystals have an open structure with an interconnected network of large channels filled with a solution in equilibrium with the mother solution from which the crystal grew (Drenth, 1994). Some of these solvent molecules are bound to the macromolecule and contribute to stabilize the crystal building, but others are actually part of the bulk solution. The latter can move freely along the interconnected network of channels and exchange with the medium surrounding the crystal.

This network of channels is responsible for the success of soaking (Stura & Gleichmann, 1999), a common operation in macromolecular crystallography to produce isomorphous derivatives, to bind or replace ligands from macromolecules in the crystal or to cryoprotect the crystal prior to data collection at low temperature. However, when the crystal is soaked in a solution of different ionic strength, a net flow of molecules between the crystal and the solution takes place, which may affect the stability of the crystalline building. In this paper, we study the effect of both hypertonic and hypotonic shocks on tetragonal lysozyme in order to understand the process underlying the formation of the cracks which are often observed during the soaking of crystals.

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2. Materials and methods

2.1. Crystal growth

Hen egg-white lysozyme was purchased from Sigma. Tetragonal crystals of lysozyme were grown at 293 $\pm$ 1 K by the batch method from a 0.025%(*w*/*v*) agarose medium containing 40 mg ml^{-1} lysozyme in 50 m*M* acetate buffer pH 4.5 and 3%(*w*/*v*) NaCl. Euhedric (well developed faces), well faceted and optically defect-free crystals were selected. They were rinsed with an equilibrated protein solution in 50 m*M* acetate buffer pH 4.5 with an NaCl concentration identical to the growth solution and only those specimens that remained optically perfect and defect-free underwent the cracking experiment.

2.2. Crystal cracking

The crystals were soaked in solutions with increasing (hypertonic shock) or decreasing (hypotonic shock) concentrations of NaCl buffered in 50 m*M* sodium acetate pH 4.5 until cracks became evident by microscopy. All buffers were at 293 K and the temperature remained constant during the experiment. The cracking behaviour was recorded by optical video-microscopy with a 14× magnification. The surface of the crystals was estimated from image analysis with the software *Image Tools* v1.27 from UTHSCA. The waiting time was measured with a precision of seconds and normalized to the surface area of the crystal. Each experiment was repeated ten times.

2.3. Diffraction experiments

The osmotic shock was also analyzed by X-ray diffraction on an Enraf–Nonius rotating-anode generator operating at 40 kV and 90 mA. A single crystal was mounted in a 0.5 mm X-ray capillary glued to a magnetic base like those used in cryocrystallography. The crystal was aligned with its crystallographic *c* axis along the capillary in such a way that it did not slip during the data collection since the width of the crystal was of the same order (0.5 mm) as the internal diameter of the capillary. The soaking was carried out directly in the same X-ray capillary by exchanging the solution in contact with the crystal at the upper side of the capillary. After several replacements with fresh buffer of the desired ionic strength for 20 min, a short data set was collected. This procedure was repeated at 2.5, 3, 5, 7, 12, 16 and 20%(*w*/*v*) NaCl.

Typically, each data set consisted of six 1° oscillation images grouped into two wedges 90° apart. Data were collected at room temperature on a MAR 345 image-plate detector (MAR Research) placed at a distance of 100 mm. The exposure time was 4 min per frame and the wavelength was 1.5418 Å. Spots with $I/\sigma(I) > 20$ were autoindexed with *MOSFLM* 6.01 (Leslie, 1999) in the space group $P4_32_12$ to estimate a rough value of mosaicity, unit-cell parameters and crystal orientation. Post-refinement was carried out to obtain the final and more accurate parameters.

3. Results

Crystals were obtained by the batch method in a growth solution containing 0.025%(*w*/*v*) agarose. This low agarose concentration does not yield a gel but a non-Newtonian fluid that maintains the crystals suspended in the growth solution. This fact has two important consequences: (i) crystals can be manipulated as they are in a viscous solution and (ii) crystals show well developed faces because the growth is not limited by contact with other crystals or sedimentation to the bottom of the reactor.

Invariably, tetragonal lysozyme crystals crack if they are subjected to a large enough osmotic shock. Fig. 1 summarizes the results of the cracking experiment for tetragonal lysozyme crystals grown at 3%(*w*/*v*) NaCl. This pattern is shared by crystals grown at 6%(*w*/*v*) NaCl, with the only difference being that the events are shifted to higher values of NaCl concentration.

Under hypotonic shock ($\Delta C < 0$) cracking takes place when the gradient is larger than $|-0.5|$. Cracks always appear perpendicular to the crystallographic *c* axis (Fig. 2*a*) and display a characteristic pattern that is visible after a certain waiting time proportional to the inverse of the gradient and the surface area of the crystal.

Hypertonic shocks ($\Delta C > 0$) are better withstood by tetragonal lysozyme. The cracks do not show any deterministic pattern or follow any specific orientation (Fig. 2*b*) and they are observed only when the gradient is larger than $|+4|$, without any waiting time. Nevertheless, after a certain time, crystals seem to adapt to the new ionic strength conditions and they experience a visible phenomenon of reconstruction that leads to the disappearance of the cracks.

At the lattice level, insight into the differing behaviour can be obtained by diffracting the crystal *in situ* during the cracking experiment. However, one important source of lattice stress is manipulation, which is responsible for an increment in the mosaicity, a parameter that is expected to be affected by the osmotic shock. On the other hand, as depicted in Fig. 1, crystals dissolve below 1%(*w*/*v*) NaCl ($\Delta C = -2$) with a lag time that cannot be taken into account in the diffraction experiment. These two facts led us to (i) minimize the manipulation by soaking directly in the capillary in which the crystal was mounted and (ii) limit the diffraction experiment to a single hypotonic measurement at 2.5%(*w*/*v*) NaCl followed by hypertonic shock with measurements at 3, 5, 7, 12, 16 and 20%(*w*/*v*) NaCl.

The effect of the osmotic shock on the unit-cell volume and the estimated mosaicity are summarized in Fig. 3. The volume of the unit cell (Fig. 3*a*) increased slightly without appreciable repercussion on the estimated mosaicity (Fig. 3*b*) when the crystal was subjected to hypotonic shock. The soaking back into a 3%(*w*/*v*) NaCl solution led to an increase of the estimated mosaicity to 150%, although neither external signs of stress nor an effect on the diffraction pattern were observed. On the other hand, despite the crystal being in hypertonic shock [$\Delta C = +0.5$ because it was equilibrated at 2.5%(*w*/*v*) NaCl] the unit cell expanded to the maximum value reached

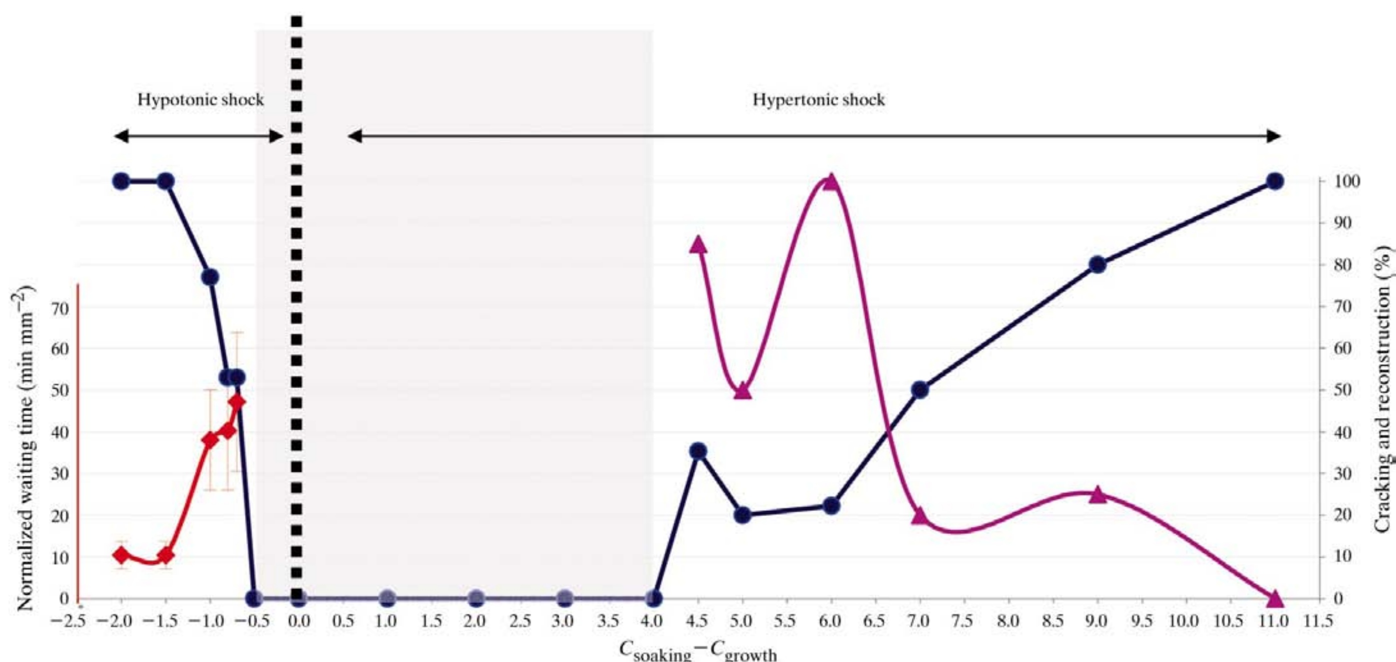

Figure 1
Behaviour of tetragonal lysozyme subjected to osmotic shock. For each soaking condition is plotted (i) the percentage of crystals that showed visible cracks (full circles), (ii) the percentage of cracked crystals that underwent the phenomenon of reconstruction (triangles), (iii) the waiting time (min) until the crack became visible in hypotonic shock normalized to the surface area of the crystal (mm^2) (squares). The shadowed area shows the osmotic shocks that do not provoke visible cracking and the dotted line marks the ionic strength under which the crystals grew.

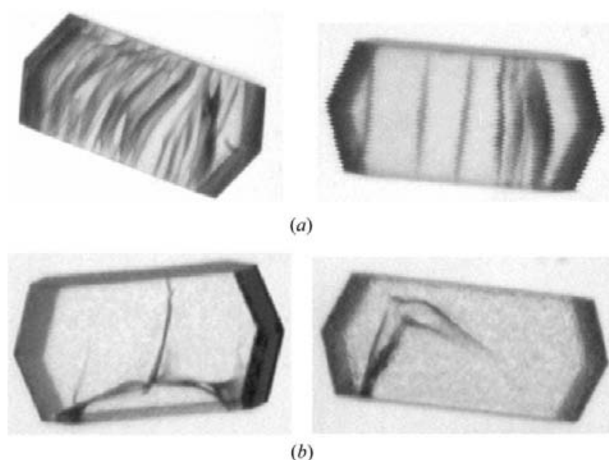

Figure 2
Under hypotonic conditions (*a*) cracks are always perpendicular to the crystallographic *c* axis (*a*). Under hypertonic conditions (*b*) cracks do not display any characteristic feature.

during the experiment. The next soakings at 5 and 7%(*w*/*v*) NaCl provoked shrinkage of the unit cell and re-established the mosaicity to the normal values without any visible sign of cracking. This trend continued up to 12%(*w*/*v*) NaCl, although some cracks were visible at the beginning of the soaking until the crystal adapted to the new ionic strength. At 16%(*w*/*v*) NaCl (ΔC = +13), as expected from the previous result shown in Fig. 1, the phenomenon of reconstruction did not take place and the persistent cracks were responsible for the failure to autoindex the diffraction pattern.

Surprisingly, when the crystal was soaked at 20%(*w*/*v*) NaCl, some of the cracks produced at 16%(*w*/*v*) NaCl vanished and the diffraction pattern was successfully indexed, showing that the unit-cell volume shrank. However, at the lattice level the reconstruction was not so successful since the estimated mosaicity increased up to 130% of the initial value. To fully explore the adaptability of tetragonal lysozyme crystals to drastic osmotic shock, the crystal was kept at 20%(*w*/*v*) NaCl for 48 h. This treatment provoked severe cracking, poor diffraction patterns and autoindexation failure.

Analysis of the unit-cell axes reveals important details of the mechanism behind the cracking process in tetragonal lysozme crystals. Results are depicted in Fig. 4. Under hypotonic conditions the regions of the lattice more affected by the osmotic shock are those that lie on the plane formed by the *a* and *b* axes, and they are responsible for the increase in the volume of the unit cell (Fig. 3*a*). Conversely, the net effect of hypertonic shock is a reduction in the unit-cell volume but, as for hypotonic shock, the crystallographic axes follow different pathways. Although the shrinkage of the *a* and *b* axes fits a straight line very well with a regression coefficient close to 0.99, the *c* axis shows a complex behavior: it shrinks very sharply to reach a minimum at 7%(*w*/*v*) NaCl, beyond which it increases with the ionic strength.

4. Discussion

Although cracking is produced by both hypertonic and hypotonic shocks, the forces involved and the mechanisms that lead to the crack formation are different. These differences

can be rationalized on the basis of a Gibbs–Donnan effect, a well known phenomenon that takes place when two solutions with different concentrations are separated by a selectively permeable membrane which does not allow the free diffusion of some of the species in solution. This provokes an imbalance in the distribution of particles on both sides of the membrane, which leads to a charge difference across the membrane (Levine, 2001). Two features of the tetragonal lysozyme crystals lead to a Gibbs–Donnan effect: (i) the wide channel along the crystallographic *c* axis, a consequence of the arrangement of the molecules in the unit cell (Fig. 5), that allows the free diffusion of solutes and (ii) the positive net charge at pH 4.6 that limits the free diffusion of ions and is responsible for the 12 mV of experimentally calculated ζ potential (Holmes *et al.*, 1997).

Thus, when the crystal is soaked in a hypotonic solution, the osmotic pressure inside the crystal is higher than that in the buffer and it leads to a massive entrance of water at the same time that some ions, mainly cations, leave the crystal to restore isotonicity (Fig. 6). This entrance of water takes place through the channels and creates an overpressure along the *c* axis that is transmitted to the lattices laying on the plane formed by the *a* and *b* axes. This idea is experimentally supported by the fact that the length of the *c* axis remains invariant since the overpressure can be relieved in that direction without displacing protein molecules. This is in full agreement with the results reported by Weik *et al.* (2001), who found that the volume expansion in trigonal *Tc*AChE crystals is anisotropic; the cell axis along the solvent channels shows half of the relative expansion of the other cell axes. The existence of the channels also explains the influence of the surface on the

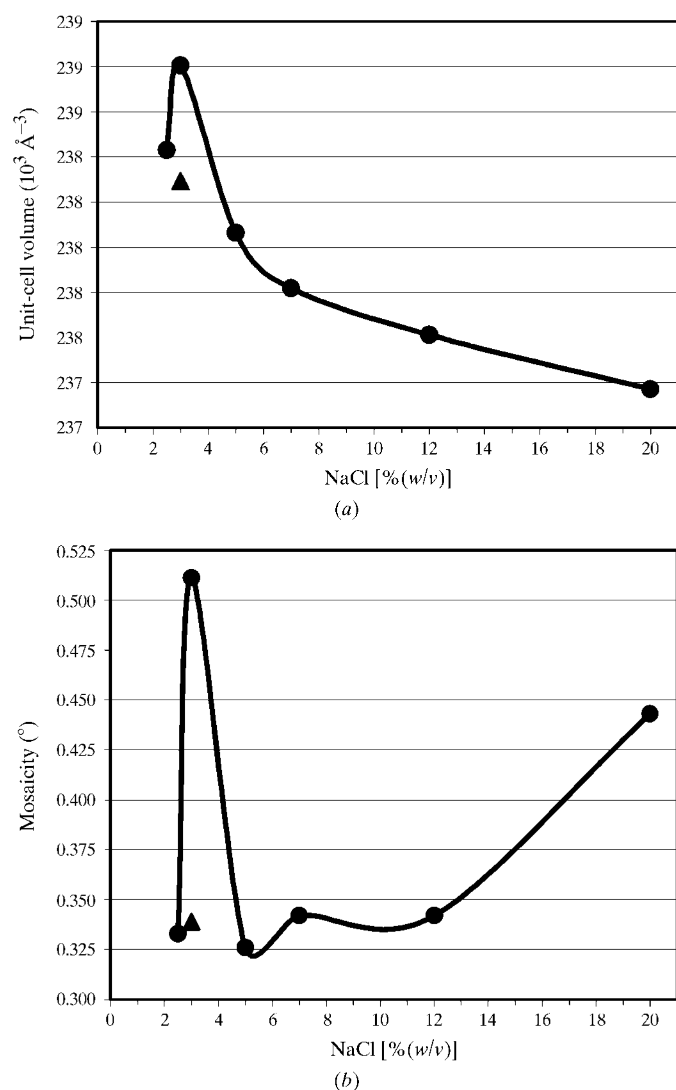

Figure 3
Effect of the osmotic shock on the unit-cell volume (*a*) and mosaicity (*b*). The values of both parameters before starting the soaking process are shown as a triangle; for the mosaicity the triangle value 0.3° is in fact the beam divergence (approx 0.3°), as it is now well known that in general normally prepared tetragonal lysozyme crystals have a near-zero mosaicity (*i.e.* < 0.01°). The crystals subjected to adverse osmotic shock show increased mosaicity [all values plotted need the beam angular smearing factor (∼0.3°) deconvoluting out].

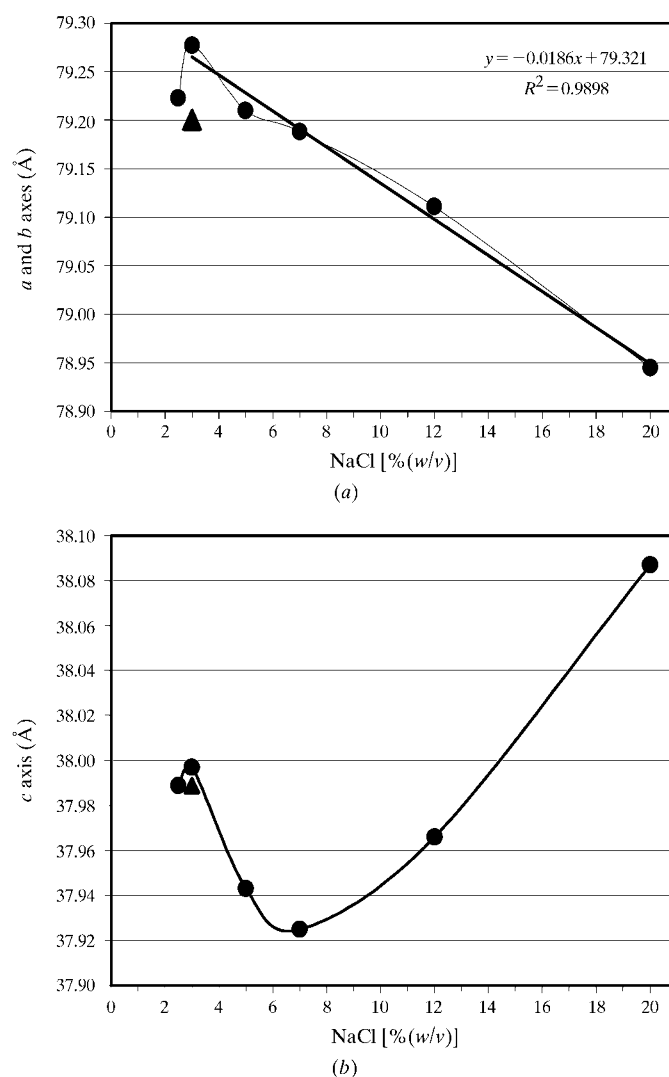

Figure 4
Effect of osmotic shock on the crystallographic *a* and *b* axes (*a*) and *c* axis (*b*). The lengths of the axes before starting the soaking process are shown as triangular dots.

waiting time, since the larger the surface, the more channels are available.

The return of the crystal in hypotonic shock to the original isotonic solution is a source of stress that increases the mosaicity by up to 150% of the starting value, despite the absence of visible signs of cracking. This unexpected value of the mosaicity may be a transition state in the pathway that the crystal undergoes to reach isotonicity with the medium. This is consistent with the existence of a measurable lag time before the cracking that is longer as the osmotic shock is softer.

Under hypertonic shock, the cracking process is completely different (Fig. 6). The osmotic pressure inside the crystal is lower and it tends to capture ions from the solution, but owing to the high charge of the crystal, cations will seldom be incorporated into the unit cell. On the other hand, the incorporation of anions is also difficult because the lattice has to accommodate the negative charge that they bear. The crystal will exchange water molecules and undergo a dehydration process that leads to shrinkage in the unit-cell parameters.

This exchange of water accompanied by shrinkage of the unit cell is consistent with the studies by Kundrot & Richards, 1988) and by Kodandapani *et al.* (1990) on the effect of high pressure and dehydration on the solvent content of tetragonal lysozyme. The former authors found that only about 63% of the water molecules in the structure of tetragonal lysozyme pressurized at 100 MPa had equivalents in the native form. The latter reported a similar result after comparing the structure of both native and low-humidity forms. Similar results have been published for the orthorhombic form (Sukumar *et al.*, 1999).

Although no visible cracking is observed in a wide range of hypertonic solutions, at the lattice level the cell reflects the effect of small osmotic pressures and reacts with compression of the unit cell along the three crystallographic axes. Beyond 7%(*w*/*v*) NaCl ($\Delta C > 4$), when cracking becomes visible, the pathway that leads to unit-cell shrinkage changes and the length of the crystallographic *c* axis increases with the ionic strength. The crystal cracks without any preferential direction because the dehydration process removes the water molecules that interact weakly with the protein. This is consistent with the fact that 93% of the water molecules with three or more interactions with the protein in the low-humidity form have equivalents in the native form. This ratio decreases to 68 and

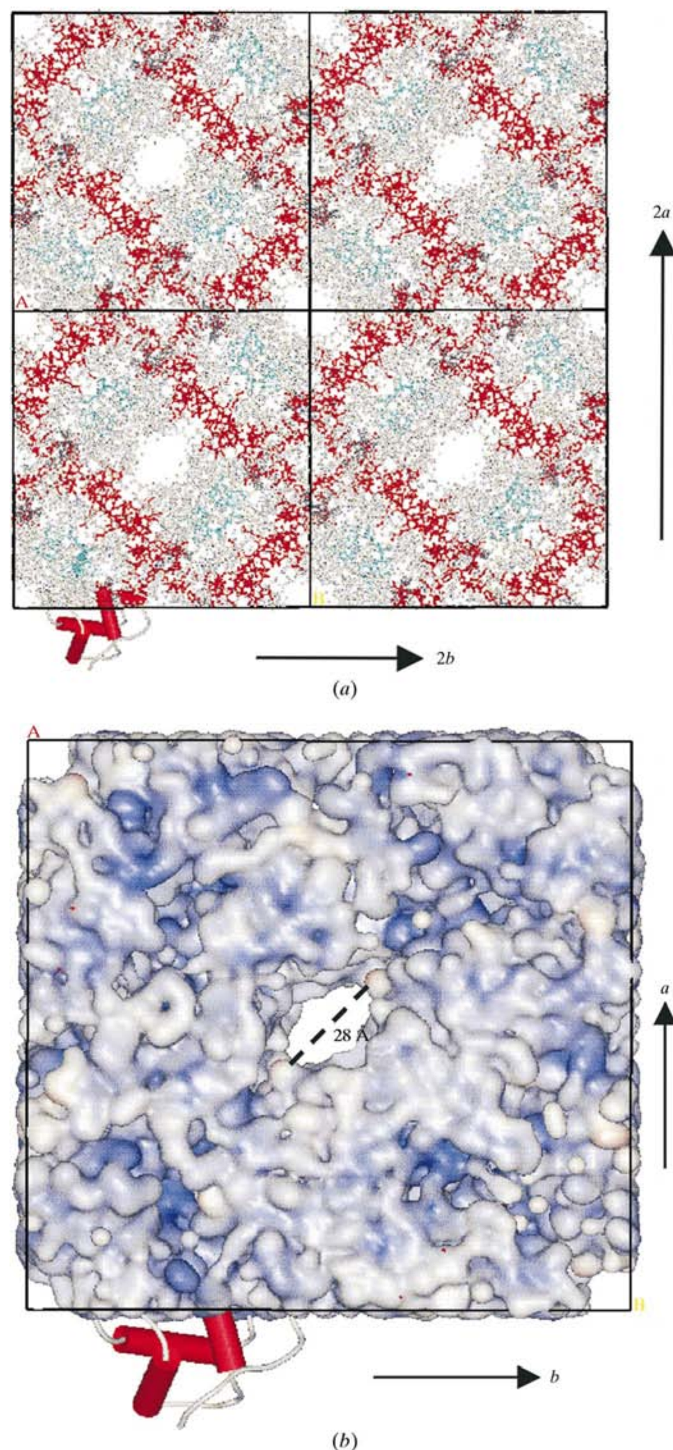

Figure 5
Packing diagram for tetragonal lysozyme viewed down the crystallographic *c* axis (*a*). The channels have a size of 12 × 28 Å and the surface around them has a positive electrostatic positive potential (*b*) responsible for the Gibbs–Donnan effect. The images were computed with *WebLab ViewerLite* v3.4 from Molecular Simulations Inc.

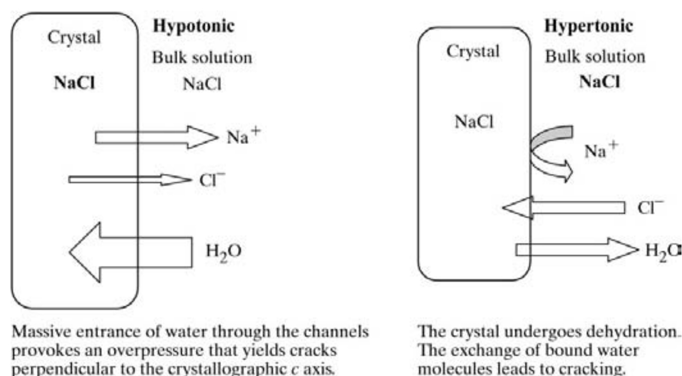

Figure 6
Diagrammatic sketch showing the proposed exchange of solutes between the surrounding solution and a tetragonal lysozyme crystal subjected to an osmotic shock. The movement of ions is limited by the Gibbs–Donnan effect and the exchange of water is the main factor responsible for the response to the osmotic shock.

53% for waters with two and one interactions, respectively (Kodandapani *et al.*, 1990).

This different behaviour of the crystallographic *c* axis explains the observations reported by Kundrot & Richards (1987), who noticed that crystals grown at 4.85%(*w*/*v*) NaCl cracked when they were pressurized beyond 40 MPa, but that when those same crystals were incubated at 8.1%(*w*/*v*) NaCl they did not crack even at 100 MPa. It is now clear that at 4.8%(*w*/*v*) NaCl the crystals were in the region where pressure leads to shrinkage along the three axes. The soaking at 8.1%(*w*/*v*) NaCl moves the crystal to a different compression regime that withstands hydrostatic pressures of 100 MPa. This second regime probably implies the exchange of water more tightly bound to the protein and explains the formation of cracks beyond 7%(*w*/*v*) NaCl. Subsequent rearrangements are responsible for the phenomenon of reconstruction, but beyond a certain ionic strength very tightly bound water molecules are removed and the cracking becomes irreversible.

5. Conclusions

In tetragonal lysozyme crystals, both channels along the *c* axis and charge lead to a Gibbs–Donnan effect that limits the exchange of ions under osmotic shock. The crystal building experiences a gradient of pressure created by the osmosis-driven exchange of mainly water molecules that leads to the cracking of the crystal.

Under hypotonic shock, the cracking behaviour is deterministic and irreversible, the consequence of a massive entrance of water through the channels along the *c* axis that creates an overpressure transmitted to the lattice on the plane formed by the *a* and *b* axes. Hence, the surface of the crystal (*i.e.* number of channels) influences the waiting time and determines that cracks always appear perpendicular to the *c* axis.

Under hypertonic shock the crystal undergoes a dehydration process without any preferential direction. Cracks appear when water molecules tightly bound to the protein are exchanged and subsequent rearrangements are responsible for the phenomenon of reconstruction, but beyond a certain ionic strength very tightly bound water molecules are removed and the cracking becomes irreversible. The dehydration process leads to shrinkage in the unit cell that is anisotropic: the *c* axis is compressed to a minimum beyond which it expands despite the unit-cell volume continuing to shrink. This change in the compression regime coincides with the soaking conditions to avoid cracking reported in experiments at high hydrostatic pressure.

The results reported here for tetragonal lysozyme may be shared by other macromolecules, since both charge and channels that lead to a Gibbs–Donnan effect are common features of protein crystals. As a practical application, it is clear from our results that soaking should be always carried out in mild hypertonic conditions and that if cracking takes place it is worth waiting for the reconstruction of the crystal building.

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