Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization in the presence of glycerol displaces water molecules in the structure of thaumatin

Christophe Charron,[a] Adel Kadri,[a] Marie-Claire Robert,[b] Richard Giegé[a] and Bernard Lorber[a]*

[a]Département 'Mécanismes et Macromolécules de la Synthèse Protéique et Cristallogenèse' UPR 9002, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 Rue René Descartes, F-67084 Strasbourg Cedex, France, and [b]Laboratoire de Minéralogie et Cristallographie, Universités Paris VI et VII, Associé au CNRS, Case 115, 4 Place Jussieu, F-75252 Paris Cedex 05, France

Correspondence e-mail: b.lorber@ibmc.u-strasbg.fr

Received 17 July 2002
Accepted 20 September 2002

PDB References: thaumatin crystallized with glycerol, 1lxz, r1lxzsf; thaumatin crystallized without glycerol, 1ly0, r1ly0sf.

The intensely sweet protein thaumatin has been crystallized at 293 K in the presence of sodium tartrate and 25%(*v*/*v*) glycerol for X-ray diffraction data collection at 100 K. A comparison of the three-dimensional structure model derived from a crystal grown in the presence of glycerol with that of a control deprived of this additive reveals only minor changes in the overall structure but a ∼20% reduction in the number of water molecules. X-ray topography analyses show that the overall quality of the crystals prepared in the presence of this cryoprotectant is enhanced.

1. Introduction

Pure glycerol (1,2,3-propanetriol; $C_3H_8O_3$) is a clear, viscous and dense glass-forming liquid having a sweet taste. It occurs naturally in fatty-acid esters present in animal and plant tissues. Since its discovery in olive oil by C. W. Scheele in 1779 (the name glycerin was coined by E. Chevreul in 1813), it has found a myriad of uses in all branches of industry. The structure of glycerol has been investigated by most physical methods. Neutron and X-ray diffraction studies indicate that in the liquid state the molecules are ordered in a network that is strongly hydrogen bonded, each monomer having 6–7 hydroxyl neighbours (Champeney *et al.*, 1986; Sarkar & Joarder, 1996).

Like most polyols and polysaccharides, glycerol stabilizes the native structure of proteins and the activity of enzymes in aqueous solution when it is the co-solvent (see, for example, Priev *et al.*, 1996; Farnum & Zukoski, 1999). As an osmolyte, it is strongly excluded from the protein domain and favours its hydration (Gekko & Timasheff, 1981; Arakawa & Timasheff, 1985). Its high viscosity strongly affects the thermodynamic and dynamic properties of proteins: it reduces the flexibility required for the conformational change of the polypeptide fold (Rosenberg *et al.*, 1989; Ansari *et al.*, 1992, Gonnelli & Strambini, 1993; Tsai *et al.*, 2000).

In protein crystallography, glycerol was one of the first substances used as a cryoprotective agent for X-ray diffraction data collection at sub-zero temperatures (Douzou *et al.*, 1975; Petsko, 1975). At 110 K, a 10–30%(*v*/*v*) concentration reduces the rate of ice nucleation and so favours the formation of a glass in which the protein crystal is stable (Rodgers, 1994). Glycerol and other structure-stabilizing compounds can increase the diffraction limit and the order in soft protein crystals (Sousa, 1995, and references therein). Despite the great interest in glycerol, no comparative study of a three-dimensional protein fold and of its hydration shell in the presence *versus* in the absence of glycerol has been reported.

Here, we report the effect of glycerol on monomeric thaumatin in crystals prepared in the presence and absence of this

© 2002 International Union of Crystallography
Printed in Denmark – all rights reserved

compound. Differences and similarities between both three-dimensional structures are described. Preliminary results from X-ray topography showing an improvement of the quality of the crystals containing glycerol are discussed.

2. Materials and methods

2.1. Chemicals and protein crystallization

All solutions were made up with ultrapure sterile water and were filtered through membranes with a 0.22 µm pore size (Ultrafree-MC; Cat. No. UFC30GV00, Millipore). DNase-, RNase- and protease-free glycerol (M_r = 92.09; d = 1.26) was a gift from Acros Organics (Geel, Belgium). *N*-[2-Acetamido]-2-iminodiacetic acid (ADA; Cat. No. A-9883), tartaric acid (Cat. No. T-5259) and lyophilized thaumatin I from *Thaumatoccus daniellii* (Cat. No. T-7638, Lot 108F00299) were purchased from Sigma. The protein (Swiss–Prot code P02883; 207 amino acids; M_r = 22 204) was dissolved in water and insoluble material was removed by centrifugation at 10 000 rev min^{-1}. After filtration, the concentration of soluble thaumatin was determined from the absorbance at 280 nm using an extinction coefficient of 1.25 for a 1 mg ml^{-1} solution (1 cm path length). Crystallization assays were set up at 293 K using the vapour-diffusion method in 20 µl hanging drops deposited on glass cover slips coated with a hydrophobic organosilane. The reservoir contained 1 ml of 0–25%(*v*/*v*) glycerol solution made with 0.73 *M* tartaric acid in 0.1 *M* ADA adjusted to pH 6.5 with NaOH. Drops were prepared by mixing 10 µl of protein solution at 35 mg ml^{-1} with 10 µl of reservoir solution.

2.2. X-ray diffraction data collection and structure refinement

Diffraction data were collected at LURE (Orsay, France) under cryogenic conditions on the wiggler beamline DW32 (λ = 0.9474 Å; beam diameter 300 µm) equipped with a MAR345 imaging-plate detector (MAR Research, Hamburg). A 700 µm long bipyramidal crystal was withdrawn from a crystallization drop and frozen at 100 K in the nitrogen stream. A control crystal of similar size (and volume) prepared without glycerol was flash-frozen after an ∼10 s dip in mother liquor containing 25%(*v*/*v*) glycerol. The crystal-to-detector distance was 175 mm and the exposure time was 120 s for 0.5° oscillations. Both data sets were processed in a similar manner using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996) (Table 1). The indexed intensities were converted to structure factors using *TRUNCATE* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) without any σ cutoff.

The structures were solved using a model derived from a crystal grown in the absence of glycerol and at room temperature (Ko *et al.*, 1994; PDB code 1thw). As the space groups of the crystals were identical, the first step of the structure determination was a rigid-body refinement. A total of 10% of each set of data were randomly selected for $R_{\rm free}$ calculations. A molecular-dynamics refinement using the simulated-annealing technique was performed using the *CNS* package (Brünger *et al.*, 1998) in the 30–1.36 Å range for the structure without glycerol and in the 30–1.25 Å range for that with glycerol. Electron-density maps were calculated to correct and rebuild the model wherever necessary using *O* (Jones *et al.*, 1991). At this stage, the electron-density maps revealed density corresponding to one tartrate molecule per asymmetric unit, as described previously (Ko *et al.*, 1994). The model was further refined and water molecules were defined using the *CNS* package (Brünger *et al.*, 1998). This was performed in several steps based on peaks of at least 3σ in the $F_o - F_c$ map and 1σ in the resulting $2F_o - F_c$ map. Cycles of positional and *B*-factor refinement and the location of water molecules were continued until no significant density (>3σ in $F_o - F_c$ maps) was left.

Table 1
Data-collection and refinement statistics for thaumatin crystals prepared in the absence or presence of 25%(*v*/*v*) glycerol.

Values in parentheses are for the highest resolution shell: 1.36–1.41 Å for the 0%(*v*/*v*) glycerol data and 1.25–1.29 Å for the 25%(*v*/*v*) glycerol data.

Glycerol concentration [%(*v*/*v*)]	0	25
Temperature of crystallization (K)	293	293
Temperature of data collection (K)	100	100
Space group	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 57.9$, $c = 150.0$	$a = b = 57.8$, $c = 150.0$
Resolution† (Å)	1.36	1.25
$R_{\rm sym}$ (%)	4.9 (15.1)	4.6 (14.7)
Completeness (%)	99.9 (99.9)	96.8 (96.9)
Average $I/\sigma(I)$ (overall)	18.0	21.0
Multiplicity (overall)	7.3	6.7
Resolution used in refinement (Å)	30.0–1.36	30.0–1.25
$R_{\rm factor}$ (%)	19.5	17.8
$R_{\rm free}$‡ (%)	20.4	19.5
R.m.s. deviations from ideal		
Bond lengths (Å)	0.005	0.011
Bond angles (°)	1.31	1.58
No. of protein atoms	1552	1552
No. of water molecules	323	262
No. of tartrate molecules	1	1
Average *B* factor (Å^2)		
Protein atoms	10.69	10.16
Water molecules	21.57	19.81

† Resolution corresponds to the reflections in the last shell having an intensity/background ratio > 3 and an $R_{\rm sym}$ < 20%. ‡ 10% of the reflections were used for computing $R_{\rm free}$.

The stereochemical acceptability of the structure was verified using the program *PROCHECK* (Laskowski *et al.*, 1993). The refinement parameters are given in Table 1. The hydrogen bonds were determined in both structures using the program *HBPLUS* (McDonald & Thornton, 1994).

2.3. X-ray topography analysis

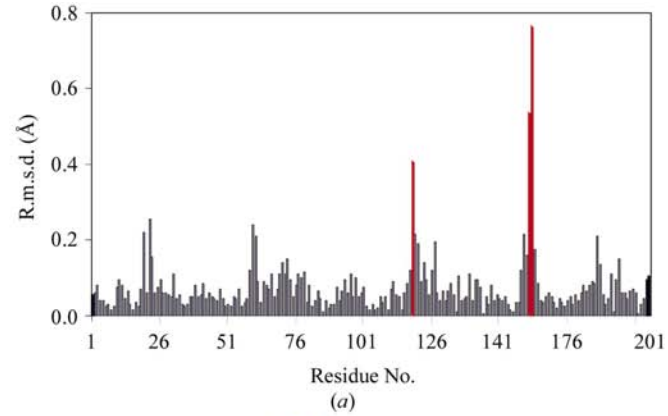
Rocking curves and X-ray topography images were recorded to compare the crystalline quality of crystals grown with and without addition of glycerol. Details of the experimental setting at beamline D25 of LURE (Fourme *et al.*, 1995) and of the analytical procedure (Lorber, Sauter, Ng *et al.*, 1999) have been described previously. The degree of perfec-

tion of thaumatin crystals was estimated by measuring the full-width at half-maximum (FWHM) of individual (00*l*) and (*hk*0) Bragg reflection profiles. The FWHM $w_{(00l)}$ and $w_{(hk0)}$ measure the global misorientation of the blocks forming the crystals in directions that are respectively normal and parallel to the *c* axis of the bipyramid (Lorber, Sauter, Ng *et al.*, 1999; Robert *et al.*, 2001). X-ray topography images were recorded on film when the intensity of the diffraction was sufficient. The contrasted regions of the topographs reveal defects.

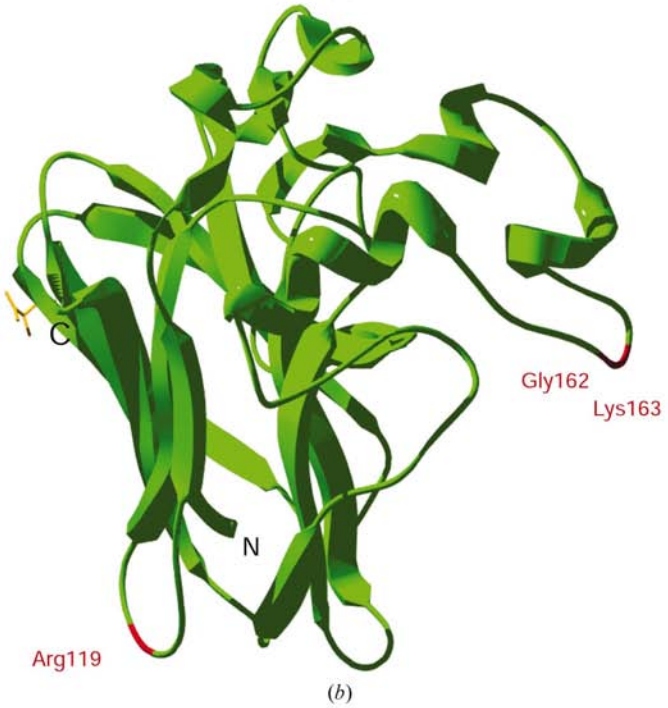
3. Results

Thaumatin crystallizes as tetragonal bipyramids in the presence of sodium tartrate as the crystallizing agent either with or without 25%(*v*/*v*) glycerol. All crystals belong to space group $P4_12_12$ (Table 1). Their unit-cell parameters are similar to those of previously reported crystals (Ko *et al.*, 1994). X-ray diffraction data were collected at a synchrotron beamline. The resolution range was 30–1.36 Å and 30–1.25 Å for crystals grown without and with 25%(*v*/*v*) glycerol, respectively. Overall, the data set of the crystal that was prepared without glycerol had an R_{sym} of 4.9% on intensities ($R_{sym} = \sum|I - \langle I\rangle|/\sum I$) and was 99.9% complete; that of the crystal grown with 25%(*v*/*v*) glycerol had an R_{sym} of 4.6 and was 96.9% complete (no strategy program was used to optimize data collection) (Table 1).

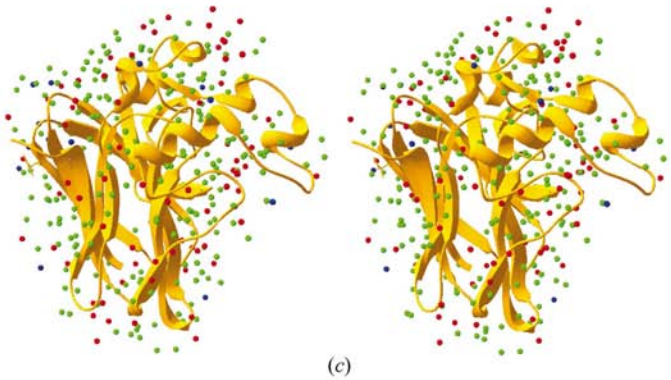
The structure without glycerol was refined to a final *R* factor of 19.5% and an R_{free} of 20.4%. The asymmetric unit contains one molecule of thaumatin (207 residues), one molecule of tartrate and 323 molecules of water. On the other hand, the structure of thaumatin with glycerol was refined to a final *R* factor of 17.8% and an R_{free} of 19.5%; using the same computational steps, only 262 water molecules could be defined. The average value of the *B* factor of the protein atoms is similar in both structures (10.69 and 10.16 Å^2 in the folds without and with glycerol, respectively). Similarly, water molecules in both structures have close average *B* factors (B = 21.57 and 19.81 Å^2 in the folds without and with glycerol, respectively). The superimposition of the coordinates of the C^α atoms and of the main-chain atoms in the structures derived from the crystal grown in the presence of glycerol (PDB code 1lxz) onto those in the structure derived from a crystal prepared in the absence of glycerol (PDB code 1ly0) gives small r.m.s.d. values (0.107 and 0.151 Å, respectively; see Table 1). In Fig. 1(*a*), C^α deviations that are >0.3 Å are coloured red. The three amino-acid residues Arg119, Gly162 and Lys163 which have an r.m.s.d. $\geq$ 0.4 Å are located in two distant loops at the surface of the protein (Fig. 1*b*). In the crystal packing, Gly162 and Lys163 are proximal to Arg119 belonging to another thaumatin molecule and also to Asp60 of a third molecule. All these residues point into a solvent channel.



(*a*)



(*b*)



(*c*)

Figure 1
Comparison of the thaumatin structures in the absence and in the presence of 25%(*v*/*v*) glycerol. (*a*) Histogram of the r.m.s. deviation as a function of C^α atom position along the polypeptide chains. Deviations between the atomic coordinates of C^α atoms in the structure model derived from crystals prepared with and without glycerol that are >0.3 Å are shown in red. (*b*) Residues with a r.m.s.d. > 0.3 Å (in red) in the ribbon representation of the thaumatin fold. One tartrate ion is drawn in yellow on the left-hand side near the C-terminus. (*c*) Stereoview displaying the positions of the water molecules around the thaumatin fold. The color code is green for common water sites, red for water sites found only in the structure deprived of glycerol and blue for sites that are specific to the structure with glycerol.

In the structure model computed from data collected from the crystal prepared without glycerol, the hydration shell encompasses 323 water molecules. There are only 262 such molecules in the model built with the data collected from a crystal prepared with glycerol. To compare the locations of the water sites, the two structures were superimposed using a least-squares procedure as described above. We considered the centre of a first site to be enclosed in the van der Waals envelope of another site if they are closer than 1.4 Å. According to this criterion, 247 water sites (drawn in green in Fig. 1*c*) are equivalent in both structures. Interestingly, half of the 76 water molecules that are specific to the fold obtained in the absence of glycerol are not directly in contact with the protein, but are only hydrogen bonded to other water molecules. The other half interacts either with residues of the polypeptide backbone or with side chains of acidic, basic or neutral residues. Of the 15 water molecules that are specific to the structure with glycerol, six interact strictly with water, one with tartrate and the others with various atoms of the protein. On the other hand, 80% of water molecules that are common to both structures do interact with protein atoms. Hence, the additional water sites that are found in the absence of glycerol are preferentially located in the second hydration shell and do not interact directly with the protein. Moreover, these additional water molecules have a higher B factor (average B = 28.65 Å^2) than those that are common to both structures (average B = 19.24 Å^2).

Previous X-ray topography studies of thaumatin crystals can be summarized as follows. (i) Gel-grown crystals have narrower Bragg reflection profiles than solution-grown crystals (the profiles of the latter are sometimes composed of several peaks arising from the existence of several domains; Fourme *et al.*, 1995; Vidal *et al.*, 1999; Lorber, Sauter, Ng *et al.*, 1999). (ii) Crystals prepared in agarose gel under microgravity are of superior quality (Lorber, Sauter, Robert *et al.*, 1999). (iii) Except when the crystals are prepared by the batch technique (Kadri *et al.*, 2002), all from a given experiment generally have $w_{(00l)}$ and $w_{(hk0)}$ values that are distributed over a relatively narrow range (plus or minus about 6 arcsec around the mean). Here, a preliminary study of the crystalline quality (*i.e.* the degree of perfection) performed on three thaumatin control crystals and on three crystals grown with glycerol has given the following results. For the control crystals, the $w_{(00l)}$ values were respectively not measurable (multidomain), 13 and 13″, and the corresponding $w_{(hk0)}$ values were 35, 35 and 22″. For the crystals grown with 15%(*v*/*v*) glycerol, $w_{(00l)}$ values were 9.5, 6.5 and 11″, and the $w_{(hk0)}$ values were 23′, 29 and 42″, respectively. While there is no striking difference in $w_{(hk0)}$, the values of $w_{(00l)}$ measured for crystals grown with glycerol are the lowest values that have ever been obtained on thaumatin crystals. A FWHM as low as 6.5″ is well below all values that have been measured on more than 200 thaumatin crystals prepared under various conditions either on earth or under microgravity.

Fig. 2(*a*) displays one of the best X-ray topographs recorded so far; it is from a thaumatin crystal that was prepared in agarose gel under microgravity on board the space shuttle and was characterized by a $w_{(00l)}$ value of only 10″ (Lorber, Sauter, Robert *et al.*, 1999). Although all facets of the tetragonal bipyramid are well developed, the strong contrast in its central part reveals a main defect arising from strain in the core. The latter arises from a misorientation between pyramidal growth sectors growing in the opposing $+c$ and $-c$ directions that originates at the level of the seed. The intensity diffracted by two crystals containing glycerol was sufficient to record topography images (no image was recorded for control crystals because we were essentially searching for crystals that were better than the best ones ever obtained under any conditions). The topograph of the crystal with the lowest $w_{(00l)}$ value of 6.5″ is shown in Fig. 2(*b*). Like most crystals prepared in solution, the habit of the crystal is not complete; its bottom part is missing because it was in contact with the solid surface of the crystallization well. This crystal displays the same features as that in Fig. 2(*a*), but the lesser contrast in the central part confirms that it has less defects.

4. Discussion and conclusion

A wide array of proteins have been crystallized in the presence of glycerol and more than 200 are found in the Protein Data Bank whose structure contains one or several ordered glycerol molecules. In contrast, no glycerol molecules are visible in the electron-density map of thaumatin. Although this additive does not significantly alter the protein fold (Fig. 1*b*), the major effect occurs at the level of its hydration (or solvation) layer.

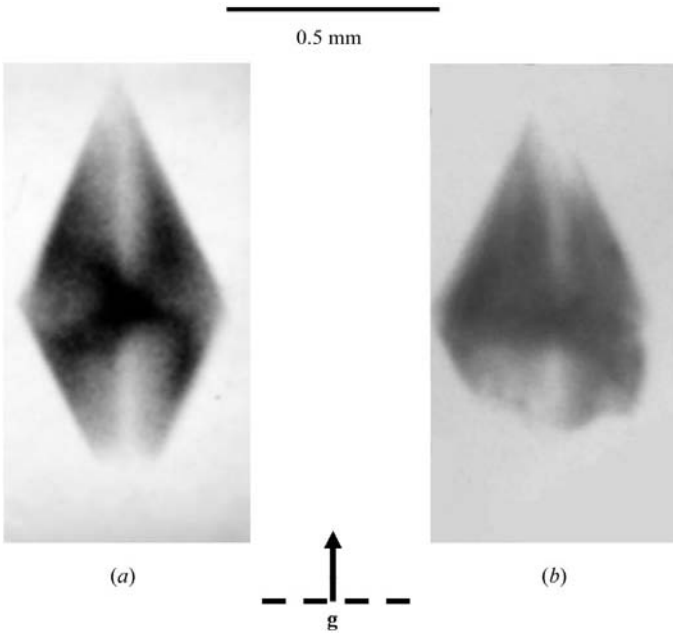


Figure 2
X-ray topographs of thaumatin crystals prepared in the absence or presence of 15%(*v*/*v*) glycerol. The topographs were taken at the angular position corresponding to the maximum intensity of the (00*l*) reflection profiles. The dotted line is a trace of the reflector planes and **g** is the diffraction vector. (*a*) One of the best crystals that was grown so far in agarose gel under microgravity (FWHM = 10″); (*b*) a crystal grown on earth in solution under similar conditions but with 15%(*v*/*v*) glycerol (FWHM = 6.5″).

Indeed, there are about 25% less ordered water molecules in the presence of 25%(*v*/*v*) glycerol (Table 1). This is consistent with the observation that this triol displaces selected protein-bound water molecules. Using several other small-sized solutes (such as sucrose and ethylene or polyethylene glycol) that are commonly employed as co-solvents to increase the osmotic pressure, it has been elegantly demonstrated that a few such water molecules play a critical role in catalytic activity or in allosteric regulation (see *e.g.* Kornblatt & Hui Bon Hoa, 1990; Di Primo *et al.*, 1992; Colombo *et al.*, 1992). These water molecules are involved in extensive networks of hydrogen bonds and are an integral part of the protein structure (see, for example, Gregoriou *et al.*, 1998).

The crystallization medium containing a high concentration of glycerol has some peculiar properties compared with the medium deprived of glycerol. For instance, a 25%(*v*/*v*) aqueous glycerol solution has an osmotic pressure of 10 MPa, equivalent to 100 times atmospheric pressure. This may seem a high value, but thaumatin in aqueous solution (deprived of glycerol) is in fact not denatured when it is subjected to a 25-fold higher hydrostatic pressure (Kadri *et al.*, 2002). At atmospheric pressure and in the presence of glycerol, the rate of equilibration of protein-solution droplets by vapour diffusion is significantly reduced; we observed that the first thaumatin crystal appeared after 30 d in the presence of 25%(*v*/*v*) glycerol and after 18 d in 15%(*v*/*v*) glycerol, compared with after 3 d in its absence. In addition, the viscosity of the crystallizing solution [η/η_0 = 2.06 for an aqueous 25%(*v*/*v*) solution at 293 K] may attenuate convection sufficiently to favour matter transport by diffusion. Under such conditions, the crystal-growth rate may be limited by the kinetics of molecular incorporation and this may contribute to enhancing the order within the lattice. The preliminary results of our analysis of the crystalline quality are encouraging. More systematic investigations are necessary to assess the improvement of the degree of perfection of the crystal nucleus when nucleation occurs in the presence of glycerol.

In the experiments reported here, the solubility of the protein was 2.5 and 6 times greater in the presence of 15 and 25%(*v*/*v*) glycerol, respectively [mean *S* (with standard deviation) in mg ml^{-1} of four independent experiments was 5.6 (0.9) and 14 (1)] than that in its absence [where *S* = 2.2 (0.2) mg ml^{-1}]. The preferential hydration of proteins in glycerol–water mixtures (Gekko & Timasheff, 1981) and the weak interactions controlling protein stability and association (Timasheff, 1993) have been documented. These phenomena suggest that protein–protein interactions in solution (and possibly in the crystal) might be affected. Experimentally, it has been shown that glycerol changes the interaction and the solubility of bovine pancreatic trypsin inhibitor by increasing the intermolecular repulsion (this leads to an increase in the second virial coefficient; Farnum & Zukoski, 1999). This effect may be much stronger in the particular case of enzymes binding polysaccharides. For instance, glycerol binds at the catalytic site of glycogen phosphorylase and competes with the substrate glucose-1-P and its analogues, thus preventing the formation of complexes (Tsitsanou *et al.*, 1999). For this reason, the conditions for cryoprotection had to be changed for this protein.

For protein crystallization and crystallography, glycerol is an attractive additive because it permits the vitrification of aqueous solutions and the collection of X-ray diffraction data under cryogenic conditions. Despite the practical aspects of this cryoprotectant, experimenters should be aware that it can bind in an ordered way to the protein when it is added as a stabilizing agent during purification and/or crystallization. A very short soaking time (<30 s after crystallization has ceased) may be sufficient to observe it in the electron-density map (see, for example, Adler *et al.*, 2000). A comparison of structures containing glycerol shows that one or several molecules may associate more or less specifically to surface residues. Glycerol also diffuses into clefts or into deep pockets. Once it has been in contact with the protein prior to crystallization, it may stick to it and hinder its assembly by occupying potential crystal contacts. For instance, cytoplasmic yeast aspartyl-tRNA synthetase could never be crystallized when it was purified in the presence of glycerol (Sauter *et al.*, 1999).

We acknowledge financial support from CNES and greatly appreciate the beam time that was allocated to this project by LURE. We are grateful to R. Fourme, T. Prangé and B. Capelle for their invaluable help on the beamlines. CC was the recipient of a CNES fellowship. AK thanks Dr G. Jenner for support and Professor M. Damak and the Ministère de l'Enseignement Supérieur de Tunisie for a fellowship.

References

Adler, M., Davey, D. D., Philipps, G. B., Kin, S.-H., Jancarik, J., Rumennik, G., Light, D. R. & Whitlow, M. (2000). *Biochemistry*, **39**, 12534–12542.

Ansari, A., Jones, C. M., Henry, E. R., Hofrichter, J. & Eaton, W. A. (1992). *Science*, **256**, 1796–1798.

Arakawa, T. & Timasheff, S. N. (1985). *Biophys. J.* **47**, 411–414.

Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.

Champeney, D. C., Joarder, R. N. & Dore, J. C. (1986). *Mol. Phys.* **58**, 337–347.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Colombo, M. F., Rau, D. C. & Parsegian, V. A. (1992). *Science*, **256**, 655–659.

Di Primo, C., Sligar, S. G., Hui Bon Hoa, G. & Douzou, P. (1992). *FEBS Lett.* **312**, 252–254.

Douzou, P., Hui Bon Hoa, G. & Petsko, G. A. (1975). *J. Mol. Biol.* **96**, 367–380.

Farnum, M. & Zukoski, C. (1999). *Biophys. J.* **76**, 2716–2726.

Fourme, R., Ducruix, A., Riès-Kautt, M. & Capelle, B. (1995). *J. Synchrotron Rad.* **2**, 136–142.

Gekko, K. & Timasheff, S. N. (1981). *Biochemistry*, **20**, 4667–4676.

Gonnelli, M. & Strambini, G. B. (1993). *Biophys. J.* **65**, 131–137.

Gregoriou, M., Noble, M. E. M., Watson, K. A., Garman, E. F., Krulle, T. M., Delafuente, C., Fleet, G. W. J., Oikonomakos, N. G. & Johnson, L. N. (1998). *Protein Sci.* **7**, 915–927.

Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.

Kadri, A., Lorber, B., Jenner, G. & Giegé, R. (2002). *J. Cryst. Growth*, **245**, 109–120.
Ko, T.-P., Day, J., Greenwood, A. & McPherson, A. (1994). *Acta Cryst.* D**50**, 813–825.
Kornblatt, J. A. & Hui Bon Hoa, G. (1990). *Biochemistry*, **29**, 9370–9376.
Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
Lorber, B., Sauter, C., Ng, J. D., Zhu, D. W., Giegé, R., Vidal, O., Robert, M. C. & Capelle, B. (1999). *J. Cryst. Growth*, **204**, 357–368.
Lorber, B., Sauter, C., Robert, M. C., Capelle, B. & Giegé, R. (1999). *Acta Cryst.* D**55**, 1491–1494.
McDonald, I. K. & Thornton, J. M. (1994). *J. Mol. Biol.* **238**, 777–793.
Otwinowski, Z. & Minor, W. (1996). *Methods Enzymol.* **276**, 307–326.
Petsko, G. A. (1975). *J. Mol. Biol.* **96**, 381–392.
Priev, A., Almagor, A., Yedgar, S. & Gavish, B. (1996). *Biochemistry*, **35**, 2061–2066.
Robert, M. C., Capelle, B., Lorber, B. & Giegé, R. (2001). *J. Cryst. Growth*, **232**, 489–497.
Rodgers, D. W. (1994). *Structure*, **2**, 1135–1140.
Rosenberg, A., Ng, K. & Punyicski, M. (1989). *J. Mol. Liq.* **42**, 31–43.
Sarkar, S. & Joarder, R. N. (1996). *Phys. Lett. A*, **222**, 195–208.
Sauter, C., Lorber, B., Kern, D., Cavarelli, J., Moras, D. & Giegé, R. (1999). *Acta Cryst.* D**55**, 149–156.
Sousa, R. (1995). *Acta Cryst.* D**51**, 271–277.
Timasheff, S. (1993). *Annu. Rev. Biophys. Biomol. Struct.* **22**, 67–97.
Tsai, A. M., Neumann, D. A. & Bell, L. N. (2000). *Biophys. J.* **79**, 2728–2732.
Tsitsanou, K. E., Oikaonomakos, N. G., Zographos, S. E., Skamnaki, V. T., Gregoriou, M., Watson, K. A., Johnson, L. N. & Fleet, G. W. J. (1999). *Protein Sci.* **8**, 741–749.
Vidal, O., Robert, M. C., Arnoux, B. & Capelle, B. (1999). *J. Cryst. Growth*, **196**, 559–571.
Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.