TOPICAL REVIEWS

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Use of Glycerol, Polyols and Other Protein Structure Stabilizing Agents in Protein Crystallization

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Abstract

A protein preparation to be used for crystallization should be homogeneous and should remain so throughout the course of a prolonged crystallization experiment. General methods for preparation of pure proteins and for prevention of their covalent modification (through proteolysis, sulfhydryl oxidation, *etc.*) during prolonged incubation are well known. Crystallographers are less aware of general methods for stabilization of proteins against non-covalent modifications (partial denaturation, heterogeneous aggregation) which can also introduce structural heterogeneity into a protein preparation. Related to this issue are methods to suppress protein conformational flexibility which can be a source of dynamic structural heterogeneity and which presents an entropic barrier to crystallization. However, for many years agents which stabilize protein structure have been described in the biochemical literature. Recently the most widely used of these structure-stabilizing agents, glycerol, was used to crystallize T7 RNA polymerase. The observation that this compound has general structure-stabilizing effects and that it was essential for crystallization of at least this one protein led to the suggestion that it might be generally useful in crystallizing flexible proteins and in inducing order in disordered segments of crystalline proteins. Subsequently, glycerol was used with good effect in the crystallization of a number of proteins. Other recent results suggest that soaking crystals in solutions containing glycerol can have 'structure-ordering' effects on the crystalline protein. These observations support the utility of glycerol in protein crystallization and suggest that the information in the biochemical literature on protein structure-stabilizing agents will find useful application in the field of protein crystal growth.

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Most macromolecular crystallography texts introduce the idea that it is critical that a protein preparation which is to be used for crystallization be 'pure' or 'homogeneous' (Blundell & Johnson, 1976; McPherson, 1982; McRee, 1993). It is usually stated explicitly that this means that the protein has been purified away from other cellular proteins. Other sources of structural heterogeneity may also be mentioned. These may include isotype heterogeneity, heterogeneity in the state of post-translational modification of the protein, the presence of proteolyzed species, and heterogeneity in the state of the protein's sulfhydryl groups. Established purification procedures, primarily chromatographic methods, are normally adequate for preparation of a protein sample for crystallization. It is also essential to maintain the homogeneity of a pure protein during what may be a prolonged crystallization experiment. General procedures, which have been borrowed from the protocols used by biochemists to stabilize proteins, exist for this purpose. Dithiothreitol (DTT) or β-mercaptoethanol may be added to maintain sulfhydryl groups in a reduced state. Metal chelators such as EGTA or EDTA may be added to sequester reactive metals which could bind the protein, as well as to inhibit the activity of any metal-dependent proteases which may be present in low amounts despite efforts to remove such enzymes during purification. Specific protease inhibitors may also be added.

Protease inhibitors, metal-ion chelators and β-mercaptoethanol are compounds which can be added to a crystallization mix to help maintain the structural homogeneity of a protein preparation against covalent modification. Another source of structural heterogeneity is non-covalent modification. There are multiple sources of non-covalent modification. Some proteins are known

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to be labile when purified away from their physiological environments (Shelanski, Gaskin & Cantor, 1973; Na & Timasheff, 1981; Pikula, Wrzosek & Famulski, 1991; Varga, Taylor & Martonosi, 1991). During the course of a long crystallization experiment such proteins may slowly accumulate partially or wholly denatured species which may aggregate. An initially homogenous protein preparation may, therefore, slowly accumulate aggregates of partially denatured protein and these aggregates may be deleterious to the growth of high-quality crystals. The dynamic conformational flexibility of the protein itself may be considered to be another source of non-covalent structural heterogeneity. In solution a protein molecule may exist as a number of interchanging conformers. These conformers may display the same overall fold, but may exhibit flexible surface-exposed loops, disordered termini, clefts and crevices within the protein which may open and close, have interdomain motions, *etc.* (Ansari *et al.*, 1985; Fraunfelder, 1985; Hong *et al.*, 1990; Kubo, 1966; Suzuki, 1975). When a protein is restricted in a crystal lattice, its conformational flexibility may be markedly restrained relative to its solution state. Zhu, Sage, Rigos, Morikis & Champion (1992), for example, showed that packing interactions in myoglobin crystals greatly restricted the conformational fluctuations of myoglobin as compared to its solution state. Since the number of protein conformers which can be accommodated in an amorphous precipitate is expected to be greater than what can be accommodated in a crystal, increasing conformational flexibility in a protein will increasingly favor amorphous precipitation over crystallization. We may, therefore, consider conformational flexibility to be an entropic barrier to crystallization (Sousa & Lafer, 1990; Sousa, Lafer & Wang, 1991). It is probably the case that we may expect conformational flexibility and denaturation to be more common barriers to the crystallization of larger multi-domain proteins which execute complex functions and which may bind multiple ligands or large macromolecules. Such barriers may be less common for smaller single-domain structurally robust proteins.

With these considerations in mind we may enumerate the requirements of 'structural homogeneity' of a protein sample for crystallization as follows. First we must obtain a preparation which is reasonably pure and homogeneous. We may then have to add inhibitors of proteolysis, oxidation and the actions of reactive metals to maintain the homogeneity of this preparation against covalent modification during crystallization. It may also be necessary to suppress the slow denaturation and aggregation of the protein and to restrict its conformational flexibility (make it less 'floppy'). Ideally, we would like to stabilize the protein in a predominant, compact conformer. It is the case that general procedures exist to obtain a pure protein preparation and to stabilize it against covalent modifications. However, crystallographers are less aware of general methods for stabilizing a protein against denaturation and denaturation-driven aggregation, and for suppressing its conformational flexibility. Extensive conformational flexibility may often be touted as a barrier to crystallization of certain proteins, but the solution to this problem has usually been seen in the definition of specific solution environments (pH, buffering agent, precipitant) which may stabilize a particular protein conformation, or the identification of ligands or effector molecules which may bind to a protein and thereby specifically stabilize one conformer. Unfortunately, the effectiveness of these defined solution environments, ligands, or effector molecules is usually limited to individual proteins. It is uncommonly the case that identification of these agents in specific cases is applicable to the crystallization of distinct proteins.

It would, therefore, be useful to identify agents which generally would help suppress conformational flexibility and stabilize proteins against denaturation. Biochemists have long been aware of such compounds. Polyols, in general, and glycerol in particular, have been used as structure-stabilizing agents for a number of years. (A partial list of the literature describing these effects would include: Ball, Hardt & Duddles, 1943; Boyer, 1945; Simpson & Kauzmann, 1953; Tanford, Buckley, De & Lively, 1962; Utter, Keeth & Scrutton, 1964; Gerlsma, 1968, 1970; Gerlsma & Sturr, 1972, 1974; Neucere & St Angelo, 1972; Frigon & Lee, 1972; Bradbury & Jakoby, 1972; Donovan, 1977; Lee & Timasheff, 1974, 1981; Back, Oakenfull & Smith, 1979; Arakawa & Timasheff, 1982*a*,*b*, 1985*a*,*b*; Gekko & Timasheff, 1981*a*,*b*; Timasheff & Arakawa, 1988.) One manifestation of the structure-stabilizing effects of these agents is their ability to increase the temperatures at which proteins denature. Glucose, sorbitol, sucrose, glycerol, and a number of other polyols have all been shown to increase protein thermal denaturation temperatures with increases of nearly 20 K reported with proteins in concentrated solutions of these compounds in some cases (Back, Oakenfull & Smith, 1979; Lee & Timasheff, 1981; Gekko & Timasheff, 1981*a*,*b*; Gerlsma & Sturr, 1972). Qualitative reports of the use of polyols to stabilize protein structure are also common. An extreme example is tubulin. This protein is very labile after purification and loses native structure within hours when placed in a 'typical' non-denaturing buffer. Upon addition of glycerol or sucrose, tubulin becomes much more stable and can be stored for weeks without measurable loss of activity (Shelanski, Gaskin & Cantor, 1973; Na & Timasheff, 1981). Glycerol similarly stabilizes membrane-bound Ca^{2+} ATPases (Pikula, Wrozek & Famulski, 1991; Varga, Taylor, Martonosi, 1991). We may also note that in commercial protein preparations glycerol is the most commonly used additive for stabilization of protein activity and structure.

While most of the work on protein structure-stabilizing agents has emphasized the effects of polyols, other classes of compounds are also known to have powerful structure-stabilizing effects. Methylated derivatives of glycine have been shown to increase protein thermal denaturation temperatures: 8.2 *M* sarcosine increases the thermal denaturation temperature of ribonuclease A by 22 K and its stabilization free energy by 30.1 kJ mol^{-1} at 338 K (Santoro, Liu, Khan, Hou & Bolen, 1992). Other classes of compounds which stabilize proteins against denaturation are also known (Yancey, Clark, Hand, Bowlus & Somero, 1982).

Some of the most important recent results in the area of the influence of solvent composition on protein conformational flexibility have come from spectroscopic studies. Using EPR, Bizzarri & Cannistraro (1992, 1993) showed that addition of glycerol or sucrose to solutions of myoglobin caused a dramatic narrowing of the conformational substrate distribution of this protein. In another example, Cioni & Strambini (1994) used tryptophan phosphorescence decay to measure protein flexibility in response to changes in pressure and the addition of 70%(*v*/*v*) glycerol. Their results revealed that addition of glycerol favored the assumption of more compact and rigid protein conformations. These observations represent some of the first direct evidence that these agents can not only stabilize protein structure, but can also suppress protein conformational flexibility, making proteins less 'floppy'. This implies that these agents can help overcome the entropic barrier to crystallization posed by excessive conformational flexibility.

The biochemical literature, therefore, presents the crystallographer with a number of compounds with demonstrated general stabilizing and flexibility suppressing effects on protein structure. Glycerol appears to be the most widely used of these compounds for protein stabilization. The outlined arguments suggest that these compounds might be useful in crystallizing proteins where lability or extensive conformational flexibility pose barriers to crystallization. Over the past few years this idea has received a good deal of experimental confirmation.

T7 RNA polymerase (RNAP) was crystallized in the presence of 25–30% glycerol and is a case where the effect of glycerol on crystallization was characterized and discussed at length (Sousa & Lafer, 1990; Sousa, Lafer & Wang, 1991). T7 RNAP is a moderately labile protein as demonstrated by its tendency to denature and aggregate during incubation in low ionic strength buffers, exceptional protease sensitivity and a thermal denaturation profile which reveals partial denaturation at temperatures as low as 303–308 K (Davanloo, Rosenberg, Dunn & Studier, 1984; Sousa, Lafer & Wang, 1991; Osumi-Davis *et al.*, 1994). T7 RNAP is a large protein (99 kDa) with multiple subdomains, at least one of which is demonstrably flexible, and it undergoes conformational transitions during transcription (Sousa, Chung, Rose & Wang, 1993; Sousa, Rose & Wang, 1995; Bonner, Lafer & Sousa, 1994; Sousa, Patra & Lafer, 1992). These results suggest that T7 RNAP is a conformationally flexible protein with poorer than average structural stability. It was found that both glycerol and sucrose stabilized this protein against denaturation and aggregation. It was also found that crystallization of T7 RNAP was absolutely dependent on the presence of at least 15%(*v*/*v*) glycerol in the crystallization solution, consistent with the idea that glycerol would be useful in the crystallization of labile or conformationally flexible proteins. Based on the specific observations with T7 RNAP crystallization and the observations that glycerol and polyols are general protein structure-stabilizing agents, it was suggested that these agents would be found to be generally useful in protein crystallization (Sousa, Lafer & Wang, 1991; Sousa & Lafer, 1990).

Subsequently, glycerol was demonstrated to be essential in the crystallization of a chimeric T7/T3 RNAP (Sousa, Chung, McAllister, Wang & Lafer, 1990; Sousa, Chung, Wang & Lafer, 1992). While this chimeric polymerase crystallized in a different space group from T7 RNAP, its structural similarity to T7 RNAP meant that its crystallization provided only weak support for the suggestion that glycerol would be found to be generally useful in protein crystallization. Stronger support for this came from the observations of Pechik, Nachman, Ingham & Gilliland (1993) who reported that, in the absence of glycerol, only precipitate or microcrystals could be obtained from a solution of a 40 kDa fibronectin fragment with ammonium sulfate used as a precipitant. Adding glycerol [15–25%(*v*/*v*)] increased the size and quality of the crystals which could be grown, and the best crystals grew at the highest glycerol concentration. Very recently, the use of glycerol in protein crystallization has become increasingly common with the effects of glycerol usually reported as essential for crystallization (*i.e.* only an amorphous precipitate could be obtained without glycerol) or as suppressing the growth of microcrystals to allow the more controlled growth of large, high-quality crystals (Umland *et al.*, 1994; Kohlstaedt, Wang, Friedman, Rice & Steitz, 1992; Zhang, Van Etten, Lawrence & Stauffacher, 1994; Hu *et al.*, 1994; Anderson, Prince, Yu, McEntee & Goodman, 1994; Reinisch, Chen, Verdine & Lipscomb, 1994; Rodseth *et al.*, 1994). It has also been reported that glycerol can be useful in the growth of two-dimensional membrane protein crystals (Varga, Taylor & Martonosi, 1991; Pikula, Wrzosek & Famulski, 1991). It is noteworthy that the trend to use glycerol in crystallization is quite new. In fact, in reporting their use of glycerol to grow crystals of the DNA endonuclease III, Kuo, McRee, Cunningham & Trainor (1992) remark that their results ran counter to an analysis of the crystallization database (Gilliland, 1988) and popular wisdom which held that glycerol

'was commonly believed to both hinder crystallization and reduce crystalline order' and was therefore 'an uncommon additive in crystallizations'. These reports demonstrate that glycerol was rarely, if ever, used in crystallization just a few years ago but has recently become a common additive in crystallization solutions. They support the earlier suggestion that glycerol would be found to be generally useful in protein crystallization.

More tentative support has been obtained for the suggestion that transferring crystals of proteins which contain disordered regions into solutions of glycerol or other polyols might enhance the order and structure of those regions (Sousa, Lafer & Wang, 1991). Rould, Perona & Steitz (1991) showed that soaking co-crystals of aminoacyl tRNA synthetase–tRNA in 20% glycerol followed by cooling to 265 K increased their diffraction limits and the order of disordered regions in these crystals. Crystals of reverse transcriptase soaked in glycerol solutions and cooled also exhibited greater stability, lifetimes and improved diffraction (Manni, 1994). The primary rationale for the use of glycerol in these cases was as a cryoprotectant. Most of the first cases of data collection from cooled crystals reported the use of ethylene glycol or organic solvents as cryoprotectants (Petsko, 1975; Hope *et al.*, 1989; Hansen, Bartels, Wittmann & Yonath, 1989; von Boehlen *et al.*, 1991). Since then glycerol has become increasingly favored as a cryoprotectant (Coleman *et al.*, 1994; Reinisch, Chen, Verdine & Lipscomb, 1994; Rould, Perona & Steitz, 1991; Manni, 1994). It is sometimes reported that the glycerol soaking and cooling processes increase the diffraction limits or order of crystals. Because these studies almost always report both solvent modifications and temperature reductions, it is impossible to identify which step is having an effect. It would be useful if investigators would report the effects on diffraction of soaking in cryoprotectant alone so as to make it possible to distinguish between the effects of solvent modification and the effects of temperature variation.

Thermodynamically, the mechanism of the structure-stabilizing effects of glycerol and polyols may be understood to be a consequence of their preferential exclusion from interaction with protein surfaces which prefer to interact with water (Donovan, 1977; Arakawa & Timasheff, 1982*a*,*b*, 1985*a*,*b*; Gekko & Timasheff, 1981*a*,*b*). This preferential hydration also explains the protein precipitating effects of agents such as salts, PEG and MPD. Some precipitating agents can also have structure-stabilizing effects, and both their precipitating and structure-stabilizing effects may be understood to be consequences of the preference protein surfaces have for interaction with water *versus* these agents. Oversimplifying, we may say that addition of these agents to a protein solution essentially reduces the availability of water for protein solvation. As a consequence their presence favors changes in the system which reduce the amount of water solvable protein surface area. These changes may involve the burying of protein surface accompanying the association of two protein molecules (intermolecular precipitation) as well as changes that stabilize the protein's structure and suppress its conformational flexibility (stabilization of compact conformers; condensation of flexible, exposed loops; closing up of clefts and crevices; *i.e.* what might be described as intramolecular precipitation). Compounds such as urea, thiocyanate and guanidinium are powerful protein solubilizing and structure destabilizing agents. The effects of these agents, directly opposed to those of protein precipitating/structure-stabilizing compounds, are reflected in their protein interaction properties: proteins preferentially bind these solubilizing and structure destabilizing agents (Bradbury & Jakoby, 1972; Arakawa & Timasheff, 1982*a*,*b*).

The division of these compounds into two groups – those which preferentially interact with proteins to solubilize and destabilize them, and those which are preferentially repelled from proteins and favor protein precipitation, structure-stabilization, and flexibility suppressed – is useful, but represents an oversimplification. Some of these agents (glycerol and other polyols) are efficient at stabilizing structure but are not particularly effective protein precipitants. If addition of glycerol to a crystallization mix encouraged precipitation then it might be expected that a lower concentration of precipitant would be required to effect precipitation in the presence of glycerol than in its absence. However, none of the crystallization reports cited above report the use of lower concentrations of precipitant upon addition of increasing concentrations of glycerol to the crystallization mix. In the crystallization of T7 RNAP addition of glycerol to the crystallization mix increased the solubility of the protein leading to an increase in the concentration of precipitant (ammonium phosphate or sulfate) required to cause precipitation. The observations of Pechik, Nachman, Ingham & Gilliland (1993); Anderson, Prince, Yu, McEntee & Goodman (1994); and Zhang, Van Etten, Lawrence & Stauffacher (1994) also suggest increased protein solubility, or at least decreased precipitation and crystal nucleation, with increasing glycerol concentrations at constant precipitant concentrations.

These observations suggest that glycerol can act as a genuine cosolvent in these systems. This would be consistent with reports that glycerol can penetrate a protein's hydration layer when present at concentrations of 10–40% and may interact directly with proteins when present at higher concentrations (Stauff & Metrotra, 1961). However, this would appear opposed to the idea that preferential repulsion from a protein's surface is responsible for the structure-stabilizing effects of glycerol. Reconciliation of these two ideas requires an appreciation of the complex balance of forces operating and the mosaic character of a protein's surface. Glycerol

is strongly repelled from the hydrophobic regions of a protein but is not repelled from polar regions (Gekko & Timasheff, 1981*a*,*b*; Timasheff & Arakawa, 1989). The balance between repulsion from hydrophobic groups, attraction to polar groups, and attraction to solvating water molecules leads to a net preferential hydration of proteins (reduced concentration of glycerol in the immediate domain of the protein relative to bulk solvent) but not to complete exclusion of glycerol from the vicinity of the protein. Thermodynamically we may explain the structure-stabilizing effects of glycerol in terms of this preferential hydration, but a fuller description of the effects of a solvent component on protein structure-stabilization and precipitation requires a consideration of the chemical nature of the solvent component. Hydrophobic interactions may play a dominant role in the intramolecular forces that stabilize native structure and favor the assumption of more rigid, compact conformers. Hydrophobic interactions may be less important in intermolecular precipitation which is driven by the association of solvent-exposed, largely polar, surfaces. The degree to which a solvent component, relative to water, is attracted or repelled from hydrophobic *versus* polar regions may, therefore, cause it to differentially favor structure-stabilization or protein precipitation. To a first approximation, division of solvent compounds into 'preferentially excluded, structure-stabilizing, protein precipitating' and 'preferentially interacting, structure destabilizing, protein solubilizing' is useful and for some compounds may be fully accurate. For example compounds which show strong, overall repulsion from proteins both in their native and denatured states (*i.e.* strong 'salting-out' salts; Timasheff & Arakawa, 1989) can be expected to be both structure-stabilizing and precipitating since they simply favor processes which minimize the solvent-exposed protein surface. However, it appears that we may distinguish compounds which display more selective affinities for different types of protein surface. Thus, glycerol, which shows strong repulsion from hydrophobic regions but little or no repulsion from polar regions, favors structure-stabilization over precipitation. On the other hand, common protein precipitating agents such as MPD and PEG which may stabilize proteins at low temperatures are net protein structure destabilizers at higher temperatures, lowering their thermal denaturation temperatures, because these non-polar solvents preferentially bind to hydrophobic regions that become exposed upon protein denaturation (Hammes & Schimmel, 1967; Arakawa & Timasheff, 1985*a*,*b*; Pittz & Bello, 1971).

For the crystal grower seeking control over the solubility, structural stability and conformational flexibility of his protein the most important consideration may be that the studies carried out by the protein chemists identify agents which differentially effect these parameters. It is important to highlight this point since some commonly used precipitants for crystallization also provide structure-stabilizing effects. A barrier to crystallization may persist, however, if the concentration of precipitant which causes phase separation is less than that required to sufficiently stabilize the protein to favor crystallization. Glycerol, and perhaps some related compounds, can allow this barrier to be overcome because they provide structure-stabilization and conformational flexibility suppression while minimally encouraging precipitation. The critical issue is to be able to decouple structure-stabilization and flexibility suppression from precipitation so as to allow independent control of these parameters to favor crystallization.

Preferential interaction provides an accepted thermodynamic explanation for the observed effects of different solvent compounds on protein stability. The molecular mechanisms which cause different compounds to interact differentially with proteins are less clear and a number of explanations have been advanced each of which may make varying contributions in specific cases. Steric effects are likely to play a role in many cases. Since water is an exceptionally small molecule, most cosolvents will be less able to penetrate crevices in the protein structure thus creating zones in the protein from which the cosolvent is excluded relative to water (Gekko & Timasheff, 1981*a*). Water also has an exceptionally high hydrogen-bonding capacity so an increase in the concentration of a cosolvent leads to a decrease in the solvent hydrogen-bond rupturing density, which in turn can stabilize protein structure by favoring protein intramolecular hydrogen bonding over protein–solvent hydrogen bonding (Utter, Keech & Scrutton, 1964; Gerlsma, 1968). The latter mechanism may be considered a special case of the general principle that cosolvents will be excluded from protein surfaces whenever water can form more favorable chemical interactions. Another example would be the repulsion of non-polar cosolvents from native proteins (Timasheff & Arakawa, 1988; Pittz & Timasheff, 1978).

Other proposed mechanisms are dependent not on the nature of the interaction between the cosolvent and protein but on the interaction between the cosolvent and water. A large number of the structure-stabilizing agents have a cohesive effect on water structure, manifested in an increase in surface tension upon their dissolution in aqueous solutions (Sinanoglu & Abdulnur, 1964). Presumably, the increased energy of cavity formation in such a solution stabilizes the native state, and encourages the stabilization of compact conformers and precipitation as mechanisms which reduce net cavity size. Glycerol is unusual in not causing an increase in surface tension, but its enhancement of water structure is manifested instead as an increase in the hydrophobic effect. Glycerol has been shown to form an 'ideal' solution in water: when it enters into the water lattice it strengthens water structure (Scatchard, Hamer & Wood, 1938). Its effects on water

structure may be thought of as opposed to the effects of denaturants like urea or guanidinium which disrupt water structure. This enhancement of water structure increases the hydrophobic effect. Other polyols have also been shown to enhance water structure and increase the hydrophobic effect though, unlike the case for glycerol, these effects are associated with an increase in surface tension (McDuffie, Quinn & Libovitz, 1962; Sinanoglu & Abdulnur, 1965; Stern & O'Connor, 1972; Tait, Sugget, Franks, Ablett & Quickenden, 1972; Franks, Ravenhill & Reid, 1972; Tanford, 1973; Oakenfull & Fenwick, 1979). A quantitative measure of the degree to which these compounds can increase the hydrophobic effect is provided by Back, Oakenfull & Smith (1979), who showed that 2 *M* sucrose or 6 *M* glycerol increased the free energy of the hydrophobically driven interaction of decyltrimethylammonium carboxylates from $-1.5\,\text{kJ mol}^{-1}$ (in water alone) to -2.3 or $-2.6\,\text{kJ mol}^{-1}$, respectively.

Steric effects, unfavorable chemical interactions between the cosolvent and the protein (relative to interactions between water and the protein), and effects on water structure leading to increased surface tension and/or hydrophobicity may all contribute to the structure-stabilizing and flexibility suppressing effects of structure-stabilizing agents, but whatever the mechanisms the immediately relevant experimental observations for the crystallographer are that these compounds help in crystallizing proteins. Certainly there is a good deal of evidence for this, at least for glycerol. There is also suggestive evidence that glycerol soaking may improve order in crystalline proteins. Since the original suggestion that glycerol might be generally useful for protein crystallization was based on its successful application in the single case of T7 RNAP and on its membership in a group of compounds with general protein structure-stabilizing effects, we may reasonably expect that some of these other compounds might also be generally useful in protein crystallization. It is possible that crystallization of some proteins will work better with some of these compounds than with others. Moore, Gulbis, Dodson, Demple & Moody (1994) recently reported the use of 300 m*M* glucose or maltose in the crystallization of a DNA methyltransferase. Cocktails of these agents may have effects superior to their use singly. The use of structure-stabilizing agents like glycerol should be a part of the methodological repertoire of the protein crystal grower. Familiarity with the biochemical literature on protein structure-stabilizing agents would be useful in the application and further extension of this methodology in crystal growth.

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