ELSEVIER

Journal of Crystal Growth 210 (2000) 761–766

JOURNAL OF CRYSTAL GROWTH

www.elsevier.nl/locate/jcrysgro

Crystallization of the *E. coli* polyamine-induced protein: a novel procedure based on the concept of ionic strength reducers

Yannis Papanikolau[a,b], Renate Gessmann[b], Kyriacos Petratos[b], Kazuei Igarashi[c], Michael Kokkinidis[a,b,*]

[a]*Department of Biology, University of Crete, P.O. Box 2208, GR-71110 Heraklion, Crete, Greece*
[b]*Institute of Molecular Biology and Biotechnology (IMBB), P.O. Box 1527, GR-71110 Heraklion, Crete, Greece*
[c]*Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba 260, Japan*

Received 20 October 1999; accepted 23 November 1999
Communicated by N.E. Chayen

Abstract

Nucleation and growth of macromolecular crystals occur in supersaturated solutions the properties of which depend on numerous parameters that influence macromolecular solubility. Detailed knowledge of the effects of those parameters is essential for crystallization. The concept of the so-called "ionic strength reducers" provides insight into the changes of solubility induced by organic solvents and hydrophilic polymers in aqueous electrolytic solutions. A simple and efficient procedure is presented which exploits the properties of ionic strength reducers in the crystallization of proteins. Using this procedure in the crystallization of the *E.coli* polyamine-induced protein, superior crystals compared to conventional techniques have been obtained. The procedure combines microseeding with dialysis techniques and is applicable to other proteins, particularly in cases where conditions favoring both for nucleation and growth cannot be found, or in cases where excessive nucleation leads to the growth of a large number of very small crystals. © 2000 Elsevier Science B.V. All rights reserved.

PACS: 36.20; 87.15; 61.10

Keywords: Protein crystallization; Protein solubility; Ionic strength reducers; Microseeding; Microdialysis cells

1. Introduction

Protein crystallization is the first essential step in structural analysis by X-ray methods. Preparation of diffraction-quality crystals remains the key problem of macromolecular structure determination. Crystal nucleation and growth occur in supersaturated solutions where the concentration of the macromolecule exceeds its equilibrium solubility value. Supersaturation is achieved at high concentrations of the macromolecule and depends on numerous parameters, which influence its solubility. Among those parameters, the concentration and nature of specific components of the solution, commonly referred to as precipitants, play an

* Corresponding author. Department of Biology, University of Crete, P.O. Box 2208, GR-71409 Heraklion, Crete, Greece. Tel.: + 30-81-394455; fax: + 30-81-394351.
E-mail address: kokkinid@imbb.forth.gr (M. Kokkinidis)

0022-0248/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0022-0248(99)00817-9

important role. Better knowledge of the influence of those parameters on macromolecular solubility is thus essential for crystallization. In a recent study [1] we have examined the effects of organic solvents and hydrophilic polymers (e.g. polyethylene glycol) on the solubility of macromolecules in aqueous electrolytic solutions. These components of the solution are called "ionic strength reducers" [1] and their effects on macromolecular solubility are treated in a generalized form [1] of Green's equation [2]. From this generalized equation follows that for a given concentration of electrolyte, ionic strength reducers should decrease macromolecular solubility under salting-in conditions and increase it under salting-out conditions. Addition of salt should have opposite effects. These effects have been verified experimentally and they have been occasionally applied in chromatography and crystallization experiments [1].

In this report we present a simple and efficient crystallization strategy based on the concept of ionic strength reducers. Application of the new procedure has consistently produced data-quality crystals for the *E. coli* polyamine-induced (PI) protein, whereas conventional crystallization approaches produced poor results. The 517-residue PI protein derives its name from the fact that its synthesis is stimulated by polyamines [3]; it has been found to play an important role in cell growth [4]. The PI protein is an oligopeptide binding protein. It shares a high degree (84%) of amino acid sequence homology with the *S. typhimurium* OppA protein [5]. OppA is a periplasmic oligopeptide-binding protein belonging to a peptide transport system of Gram negative bacteria; it binds oligopeptides between two and five amino acids regardless of their sequence, with maximum specificity for tri- and tetrapeptides [6]. OppA from *S. typhimurium* has been subject of numerous investigations ranging from crystallographic to biochemical and thermodynamical studies [7–12]. The *E. coli* PI protein and the *S. typhimurium* OppA protein have the same number of residues and are both synthesized in a precursor form, which is processed by removal of the signal peptide to generate a mature protein of $M_r = 58$ kD.

Despite the detailed structural characterization of the *S. typhimurium* OppA protein and the expected structural homologies between *S. typhimurium* OppA and the *E. coli* PI protein, determination of the crystal structure of the latter should reveal additional structural information which is important for the understanding of those periplasmic oligopeptide-binding proteins. Because the sequences of the two proteins exhibit some variation in the substrate binding regions and in domain linking segments, comparison of the structural details of their interactions with oligopeptides, of the hydration of their peptide-binding pockets (hydration is functionally important), and of their domain movements, should be particularly informative. In addition, there are indications that some features of the *S. typhimurium* OppA crystal structure (e.g. the angle of opening between domains in the unliganded form [10]) are influenced by crystal packing. Determination of the structure of the *E. coli* PI protein in a different crystalline environment could thus reveal deviations from the *S. typhimurium* OppA structure. It is noteworthy, that important crystal contacts in the crystallographic studies of the liganded form of the *S. typhimurium* OppA protein are mediated by uranium ions from the crystallization buffer [7–11].

2. Experimental procedure

2.1. Purification of the protein

Bacteria (*E. coli* strain K38) were cultured in a 30 l fermenter using standard nutrients and conditions for *E. coli* growth. Approximately 100–140 g cells were harvested in each fermentation and stored at 200 K. For a typical purification, 50–60 g cell paste was thawed in 100 ml buffer containing 50 mM Tris HCl pH 7.5, 200 mM NaCl, 5% (v/v) glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 μg/ml phenylmethyl sulfonyl fluoride (PMSF) and subsequently incubated at 277 K for 1 h with 300 μg/ml lysozyme. The lysate was treated for 30 min with 10 mM $MgCl_2$ and 10 μg/ml DNAase. After removing the precipitate by centrifugation and 4-fold dilution with 67 mM Hepes buffer pH 6.5, the extract was loaded onto an 80 ml S-fast flow Sepharose column (Pharmacia)

equilibrated in 50 mM Hepes buffer pH 6.5. The column was first washed with the equilibration buffer (5 bed volumes) and then with 50 mM Hepes pH 7.8 (30–40 bed volumes). The protein was obtained by step elution with 50 mM Hepes pH 7.8 containing 200 mM NaCl. Protein containing fractions were pooled and the buffer was changed by diafiltration to 20 mM Tris HCl pH 8.5. The sample was then loaded onto a 60 ml Q-fast flow Sepharose column (Pharmacia) equilibrated with 20 mM Tris buffer pH 8.7. After wash with the equilibration buffer and an extensive wash with 20 mM Tris buffer, pH 8.2 (10 bed volumes), a 500 ml linear gradient (0–400 mM) in NaCl was applied and the protein eluted at 150 mM NaCl. Protein containg fractions were pooled, concentrated to a volume of approximately 2.5 ml and loaded onto a 500 ml S-200 Sephacryl (Pharmacia) gel filtration column equilibrated with 20 mM Tris HCl, pH 8.2 containing 150 mM NaCl. The PI protein elutes as a monomer with a purity of approximately 95% as estimated from a SDS/polyacrylamide gel. After buffer change to 2 mM Tris HCl, pH 7.5, 5 mM NaCl, 1mM EDTA, 1 mM DTT, the protein was concentrated to 10–20 mg/ml using Amicon Centricon ten microconcentrators. A total of 5 mg pure PI protein was the yield from 50 to 60 g cell paste. All purification steps were carried out at 277 K. After the first crystallization of the purified protein, N-terminal sequencing was used to unambiguously identify the crystallized sample as the PI protein. This sample was also used in every new purification as a marker for the identification of the protein in SDS/polyacrylamide gels.

2.2. Crystallization

Crystallization trials were initially performed using conventional hanging-drop vapor diffusion [13] with tissue-culture plates and siliconized glass cover slips [14]. Crystals appeared in drops containing equal volumes of protein concentrate and a reservoir solution of 20% w/v polyethylene glycol (PEG) 6000 in the pH range 5.0–7.0. The crystals usually exhibited poorly defined morphologies and showed high mosaicity. Furthermore, it has not been easy to reproduce the results of those crystallization experiments. A general observation was that in the case of the PI protein it is not simple to find with conventional techniques optimum conditions for both crystal nucleation and growth. For better crystals, a more controlled crystallization procedure based on the concept of ionic strength reducers [1] was developed. The basis of the crystallization strategy is the observation that PEG 6000 and salts have opposite effects on the solubility of the PI protein: at low ionic strength, PEG 6000 reduces the solubility of the protein, but this effect can be reversed by an increase in salt concentration. This typical ionic strength reducer behavior gives the ability to fine-tune protein solubility by carefully balancing PEG and salt concentrations and provides the basis for the growth of data-quality crystals.

The new crystallization procedure combines dialysis methods with seeding techniques; changes in the balance of ammonium sulfate and PEG 6000 (which acts as ionic strength reducer) concentrations are used to change protein solubility. Each crystallization experiment consists of a microseeding [15] step performed in a microdialysis cell [13] where protein solubility can be conveniently manipulated by equilibrating the protein solution across the dialysis membrane against a reservoir containing suitable concentrations of salts and ionic strength reducers. Prior to microseeding, a stock of microscopic seed was obtained by crushing crystals grown either by conventional crystallization techniques (see above) or in microdialysis cells where a protein solution containing 120 mM ammonium sulfate, 17% PEG 6000, 20 mM Na citrate buffer pH 6.4 was equilibrated against a reservoir containing 50 mM ammonium sulfate, 17% PEG 6000, 20 mM Na citrate buffer pH 6.4 (the quality of those crystals was generally not sufficient for crystallographic analysis).

In the microseeding step, some microscopic seeds were transferred to a 50 μl protein solution in a microdialysis cell containing 120 mM ammonium sulfate, 17% PEG 6000, 20 mM Na citrate buffer pH 6.4. Subsequent reduction of the ammonium sulfate concentration in the protein solution across the dialysis membrane (this was achieved by immersing the cell into a 3 ml reservoir containing the same PEG 6000 concentration as the protein

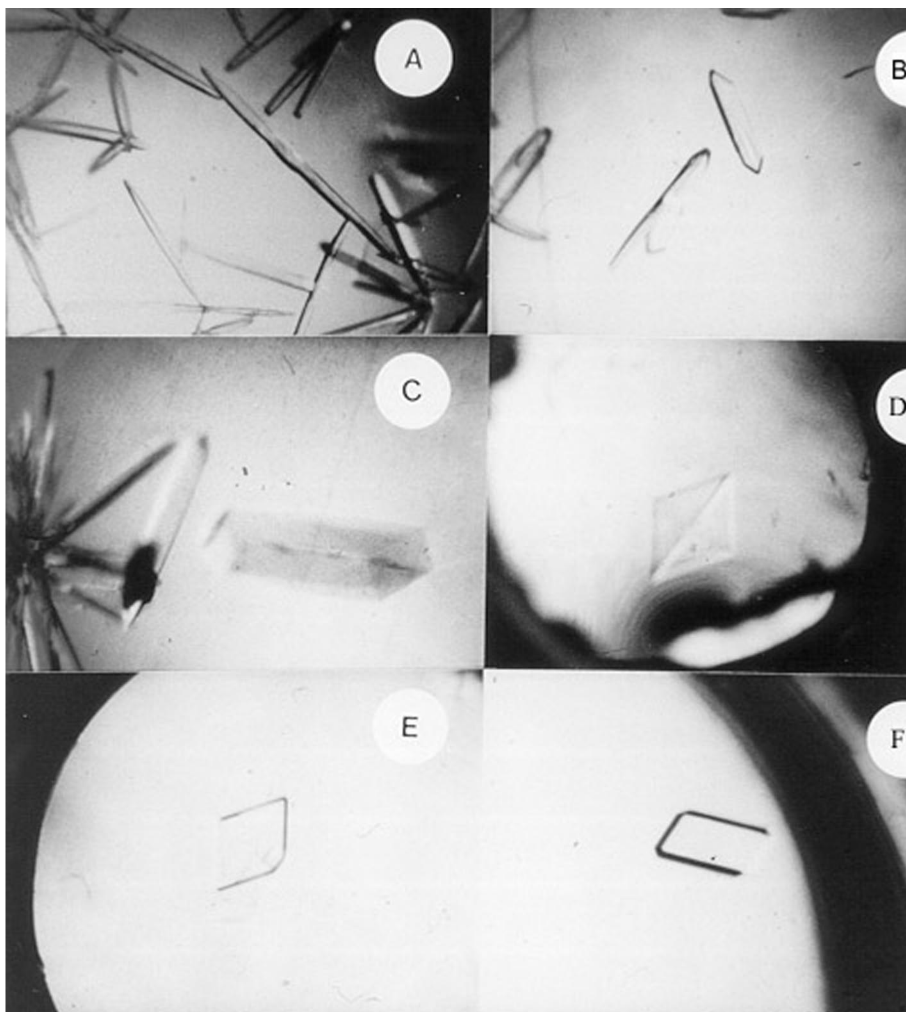

Fig. 1. Crystallization of the *E.coli* PI protein. (A)–(C) Crystals (not suitable for crystallographic work) grown by conventional techniques. (D)–(F) Data-quality crystals (space group $P2_12_12_1$) grown with the procedure described in this work.

solution but lower ammonium sulfate concentration, i.e. 80 mM ammonium sulfate, 17% PEG 6000, 20 mM Na citrate buffer pH 6.4) led to a gradual decrease of protein solubility and to favorable growth conditions for the microscopic seeds. The growth of crystal seeds extended over a period of several weeks. The crystals obtained were parallelepiped- or lozenge-shaped, up to a size of 0.8– 1.5 mm along the longest edge (Fig. 1). All crystallizations were conducted at 270 K.

2.3. X-ray analysis

Data collection work was performed at 100 K using a cryoprotected crystal (the stabilizing solution contained 55% PEG 400 as cryoprotectant agent) which was mounted in a stream of nitrogen gas using a Hampton Research cryoloop. A second crystal was mounted in a glass capillary and diffraction data were collected at room temperature. Diffraction data were collected at the EMBL/DESY

synchrotron radiation beamline X11 ($\lambda = 0.927$ Å) using a MAR 345 imaging plate detector. The rotation method [16] was used. Oscillation frames of width 1.0° were recorded. Intensities were integrated with DENZO and scaled with SCALEPACK [17].

3. Results and discussion

The crystals have the symmetry of spacegroup $P2_12_12_1$. Unit cell parameters and diffraction statistics are summarized in Table 1. Assuming one molecule per asymmetric unit, the Matthews coefficients [18] V_M vary from 1.9 (cryoprotected crystal) to 2.75 Å^3 Da^{-1} (crystal measured at room temperature). These correspond to a solvent content of 35 and 55%, respectively. The set of native diffraction data collected from the cryoprotected crystal extends to the present resolution limit of 2.5 Å, while the second dataset measured at room temperature extends to 2.7 Å; this data set, however, is incomplete because the crystal suffered radiation damage. Molecular-replacement calculations for the determination of the crystal structure are under way.

Table 1
Diffraction statistics of the cryoprotected crystal (A) and the crystal measured at room temperature (B)

Crystal	A	B
Collection temperature (K)	100	290
a (Å)	53.2	59.7
b (Å)	72.0	83.7
c (Å)	116.4	128.4
Resolution (Å)	2.5	2.7
Unique reflections	15903	15991
Completeness (%)	99	80
I/sigma	9.9	10.1
R_{sym} (%)	9.9	11.2

In conclusion, the studies described in this work provide encouraging evidence about the applicability of the concept of ionic strength reducers in the development of efficient protocols for the crystallization of proteins: application of this concept in the growth of PI protein crystals using salting-in has produced superior results compared to conventional techniques. Furthermore, the use of uranium ions, which were essential for the crystallization of the *S. typhimurium* OppA protein, was not necessary with the new procedure. Generally, crystallization using salting-in is difficult, because ionic strengths are so low that buffering becomes problematic and concentration gradients across a dialysis membrane must be extremely small. For the PI protein a solubility in the order of 10–20 mg/ml can be maintained (in the absence of PEG) at ionic strengths corresponding to less than 5 mM ammonium sulfate or NaCl. This low value of ionic strength makes conventional salting-in experiments extremely difficult to control. The most important effect of the ionic strength reducer in the crystallization of the PI protein was thus to bring the protein solubility curve into a range which can be conveniently exploited for crystal growth at ammonium sulfate concentrations of around 100 mM. Under an ionic strength in this range, a useful buffer concentration can be maintained and ammonium sulfate gradients across a dialysis membrane can be easily controlled. The specific crystallization strategy described in this work, which combines controlled changes of protein solubility (exploiting the effects of ionic strength reducers) with seeding techniques, was essential for the production of data-quality crystals. This simple and efficient crystallization approach may be applied to other proteins, for example in cases where conditions that are favorable both for crystal nucleation and growth cannot be found, or in cases where excessive nucleation leads to the growth of a large number of tiny crystals which are useless for crystallographic analysis. Our observations should warrant further investigation of the ionic strength reducer concept as potentially generally applicable in macromolecular crystallization.

The ongoing structural analysis of the *E. coli* PI protein will provide further insight into the structural basis of the properties of periplasmic oligopeptide binding proteins.

Acknowledgements

The authors are indebted to Dr. P. Tucker for advice and assistance. We would like to acknowledge support by the EU BIOTECHNOLOGY

grant BIO4-CT98-0086 and provision of beam time at EMBL/DESY, Hamburg.

References

[1] Y. Papanikolau, M. Kokkinidis, Prot. Eng. 10 (1997) 847.
[2] M. Ries-Kautt, A. Ducruix, in: A. Ducruix, A.R. Giegé (Eds.), Crystallization of Nucleic Acids and Proteins, IRL Press, New York, 1992, pp. 195–218.
[3] K. Igarashi, T. Saisho, M. Yuguchi, K. Kashiwagi, J. Biol. Chem. 272 (1997) 4058.
[4] K. Mitsui, K. Igarashi, T. Kakegawa, S. Hirose, Biochemistry 23 (1984) 2679.
[5] K. Kashiwagi, Y. Yamaguchi, Y. Sakai, H. Kobayashi, K. Igarashi, J. Biol. Chem. 265 (1990) 8387.
[6] C.A. Guyer, D.G. Morgan, J.V. Staros, J. Bacteriol. 168 (1986) 775.
[7] J.R.H. Tame, G.N. Murshudov, E.J. Dodson, T.K. Neil, G.G. Dodson, C.F. Higgins, A.J. Wilkinson, Science 264 (1994) 1578.
[8] J.R.H. Tame, E.J. Dodson, G.N. Murshudov, C.F. Higgins, A.J. Wilkinson, Structure 3 (1995) 1395.
[9] J.R.H. Tame, S.H. Sleigh, A.J. Wilkinson, J.E. Ladbury, Struct. Biol. 3 (1996) 998.
[10] S.H. Sleigh, J.R.H. Tame, E.J. Dodson, A.J. Wilkinson, Biochemistry 36 (1997) 9747.
[11] S.H. Sleigh, P.R. Seavers, A.J. Wilkinson, J.E. Ladbury, J.R.H. Tame, J. Mol. Biol. 291 (1999) 393.
[12] T.G. Davies, R.E. Hubbard, J.R. Tame, Protein Sci. 8 (1999) 1432.
[13] A. Ducruix, R. Giegé, in: A. Ducruix, A.R. Giegé (Eds.), Crystallization of Nucleic Acids and Proteins, IRL Press, New York, 1992, pp. 73–98.
[14] A. McPherson, in: Crystallization of Biological Macromolecules, Cold Spring Harbor, Cold Spring Harbor Laboratory Press, New York, 1999, pp. 188–191.
[15] E.A. Stura, I.A. Wilson, in: A. Ducruix, A.R. Giegé (Eds.), Crystallization of Nucleic Acids and Proteins, IRL Press, New York, 1992, pp. 99–126.
[16] U.W. Arndt, A.J. Wonacott, in: U.W. Arndt, A.J. Wonacott (Eds.), The Rotation Method in Crystallography, North-Holland, Amsterdam, 1977, pp. 5–18.
[17] Z. Otwinowski, W. Minor, Meth. Enzymol. 276 (1996) 307.
[18] B.W. Matthews, J. Mol. Biol. 33 (1968) 491.