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Ionic strength reducers: an efficient approach to protein purification and crystallization. Application to two Rop variants

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Detailed knowledge of the influence of various parameters on macromolecular solubility is essential for crystallization. The concept of so-called 'ionic strength reducers' provides insight into the changes in 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 purification and crystallization of proteins. Using two designed variants of the Rop protein as model systems, superior crystals have been obtained compared with conventional techniques. This procedure is particularly useful in cases where excessive nucleation leads to the growth of a large number of tiny crystals that are useless for crystallographic analysis.

1. Introduction

Preparation of diffraction-quality crystals remains the key problem in macromolecular structure determination. Crystal nucleation and growth occur in supersaturated solutions where the concentration of the macromolecule exceeds its equilibrium solubility value. Supersaturation depends on numerous parameters. Of these, the concentration and nature of salts and other specific components of the solution (precipitants) play an important role in the protein solubility. In previous studies (Papanikolau & Kokkinidis, 1997; Athanasiadis *et al.*, 1997; Andreeva *et al.*, 2000), we examined the effects of organic solvents and hydrophilic polymers (*e.g.* polyethylene glycol) on the solubility of macromolecules in aqueous electrolytic solutions. These components are called 'ionic strength reducers' and their effects on macromolecular solubility are treated in a generalized form of Green's equation (Riès-Kautt & Ducruix, 1992). It has been verified experimentally that for a given concentration of electrolyte, ionic strength reducers decrease macromolecular solubility under salting-in conditions and increase it under salting-out conditions. Addition of salt has the opposite effect.

In this report, we show that the properties of ionic strength reducers can be exploited in order to obtain superior results in the purification and crystallization of proteins compared with conventional techniques. The development of a simple crystallization strategy based on ionic strength reducers has consistently produced data-quality crystals of two designed variants of the *Escherichia coli* repressor of primer (Rop) protein, which are used as model systems. Rop is a homodimeric RNA-binding protein (Polisky, 1988) which forms a highly regular four-α-helical bundle (Banner *et al.*, 1987; Eberle *et al.*, 1991). Each monomer consists of two antiparallel α-helices joined by a short loop. The Rop mutants reported in this work are A31P, which is the replacement of loop residue Ala31 by Pro, and Δ(30D-34Q), which carries a deletion of five residues (Asp30–Gln34) in the loop region.

2. Experimental procedures

2.1. Purification

The oligonucleotide-directed mutagenesis of Rop has been described previously (Castagnoli *et al.*, 1989). For both mutants, bacteria (*E. coli* strain K38) were cultured in a 100 l fermenter using standard nutritients and conditions for *E. coli* growth. In a typical preparation, 50 g cell paste was thawed in 100 ml buffer containing 50 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 5%(*v*/*v*) glycerol, 1 m*M* DTT, 1 m*M* EDTA and subsequently incubated at 277 K for 1 h with 300 μg ml^{-1} lysozyme and 1 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF). The lysate was treated for 30 min with 10 m*M* $MgCl_2$ and 10 μg ml^{-1} DNAase and cell debris was removed by centrifugation (14 000*g* for 1 h). After this stage, different purification schemes were used for the two mutants.

For the purification of A31P the pH was adjusted to 8.5 and the supernatant was loaded onto a Q-Sepharose fast flow column (Pharmacia) equilibrated in 25 m*M* Tris buffer pH 8.5 (buffer *A*). The column was washed extensively (three bed volumes of buffer *A* followed by 20–30 bed volumes of 20 m*M* Tris buffer pH 7.5; buffer *B*). To elute the protein, a

linear gradient of 0–600 m*M* NaCl was applied. Protein-containing fractions were pooled and loaded onto an 80 ml hydroxylapatite column equilibrated in buffer *B*. The column was initially washed with buffer *B* and then with ten bed volumes of 10 m*M* phosphate buffer pH 6.8. A linear gradient of 10–300 m*M* phosphate was applied and protein-containing fractions were pooled and concentrated to 3 ml. The sample was loaded onto a S-100 Sephacryl (Pharmacia) gel-filtration column, from which the protein eluted as a dimer with a purity of at least 95% (as judged from SDS–polyacrylamide gels). After buffer exchange with 2 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 1 m*M* DDT, A31P was concentrated to 10–30 mg ml^{-1} (Bradford, 1976).

The purification of Δ(30D-34Q) was based on the observation of typical ionic strength reducer effects, *i.e.* the protein becomes insoluble in the presence of 20%(*v*/*v*) ethanol and is solubilized again if 600 m*M* NaCl is added. The following scheme was developed: after buffer exchange with buffer *A*, supernatant was loaded onto a Q-Sepharose column. The column was washed extensively [initially with buffer *A* followed by buffer *B*, then with 20 m*M* Tris pH 8.5 containing 20%(*v*/*v*) ethanol and finally with 20 m*M* Tris pH 6.0 containing 20%(*v*/*v*) ethanol] and a linear gradient of 0.0–1.0 *M* NaCl in 20 m*M* bis-tris buffer pH 6.0 containing 20%(*v*/*v*) ethanol was applied. Fractions containing Δ(30D-34Q) eluted at approximately 600 m*M* NaCl. The purity of the protein in these fractions was sufficient for crystallization.

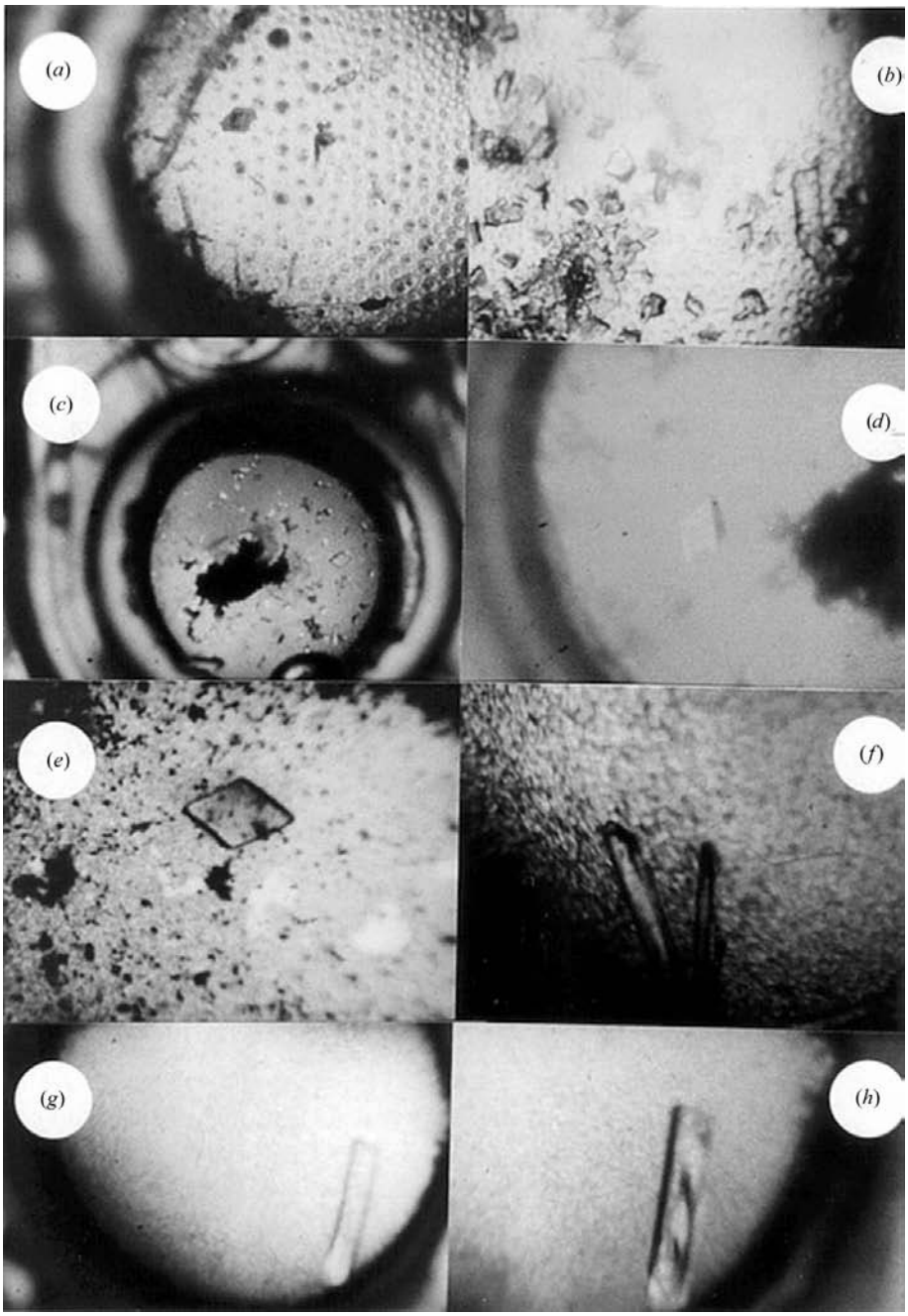

Figure 1
Crystallization of A31P. (*a*)–(*c*) Growth of a seed stock in microdialysis cells. (*d*), (*e*) Growth of lozenge-shaped crystals (space group $C222_1$) at pH 4.4 in cells to which seeds from the originally grown stock had been transferred. (*f*)–(*h*) Growth of rod-shaped crystals (space group *C*2) at pH 4.8 in cells to which seeds from the originally grown stock had been transferred.

2.2. Crystallization

Extensive trials to crystallize the proteins using conventional batch crystallization or hanging-drop vapour diffusion resulted in many tiny crystals, even though these techniques had been successfully employed previously in the crystallization of wild-type Rop and other Rop mutants (Kokkinidis *et al.*, 1993). A more controlled strategy was thus developed that utilizes ionic strength reducer effects and more specifically the observation that organic solvents, *e.g.* ethanol, methanol (ionic strength reducers), and salts, *e.g.* NaCl, ammonium sulfate, have opposite effects on the solubility of both Rop mutants. At low ionic strength, organic solvents reduce the solubility of A31P and Δ(30D-34Q), but this effect can be reversed by an increase in salt concentration. The new crystallization procedure consists of a microseeding and a macroseeding step (Stura & Wilson, 1992), both performed in microdialysis cells (Ducruix & Giegé, 1992), 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.

For the crystallization of A31P, an initial stock of crystals was grown by conventional batch crystallization from a protein solution containing 50 m*M* citrate buffer pH 4.4–4.8 and 20% ethanol. Two crystal morphologies were obtained: rod-shaped crystals at pH 4.8 and lozenge-shaped crystals at pH 4.4. These crystals provided seeds for the subsequent microseeding step. In this step (Figs. 1*a*–*c*), microscopic seeds obtained by crushing a crystal were transferred to a microdialysis cell containing approximately 50 µl protein solution with 450 m*M* NaCl, 37% ethanol, 100 m*M* citrate buffer pH 4.4 or 4.8, 1 m*M* DTT and 1 m*M* EDTA. Seeds from lozenge-

shaped and rod-shaped crystals were transferred to protein solutions of pH 4.4 and 4.8, respectively. The cell was sealed in a Petri dish containing a 3 ml reservoir of 200 m*M* NaCl, 37% ethanol, 100 m*M* citrate buffer pH 4.4 or 4.8, 1 m*M* DTT and 1 m*M* EDTA. The seeds grew into small lozenge- or rod-shaped crystals. After reaching a size of approximately 0.1 mm, the best of them were thoroughly washed with reservoir buffer and transferred to microdialysis cells (macroseeding) containing approximately 50 µl of protein solution with 450 m*M* NaCl, 37% ethanol, 100 m*M* citrate buffer pH 4.4 (for lozenge-shaped crystals) or 4.8 (for rod-shaped crystals), 1 m*M* DTT and 1 m*M* EDTA. The cells were sealed in small Petri dishes containing 3 ml reservoirs of 250 m*M* NaCl, 37% ethanol, 100 m*M* citrate buffer pH 4.4 or 4.8, 1 m*M* DTT and 1 m*M* EDTA. Under those conditions, the macroseeds grew, usually reaching 0.8–1.0 mm along the longest dimensions (Figs. 1*d*–1*h*).

For the crystallization of Δ(30D-34Q), an initial crystal stock was obtained by conventional crystallization techniques. In the first step, microscopic seeds were added to a microdialysis cell containing approximately 50 µl protein solution with 900 m*M* NaCl, 45% methanol, 50 m*M* bis-tris buffer pH 6.2, 1 m*M* DTT and 1 m*M* EDTA. The cell was sealed in a Petri dish containing a 3 ml reservoir of 750 m*M* NaCl, 45% methanol, 50 m*M* bis-tris buffer pH 6.2, 1 m*M* DTT and 1 m*M* EDTA. The seeds grew into small crystals with dimensions of approximately 0.1 mm (Figs. 2*a* and 2*b*). The best-formed small crystals were thoroughly washed with reservoir buffer and in the subsequent macroseeding step they were transferred to microdialysis cells containing approximately 50 µl protein solution with 900 m*M* NaCl, 45% methanol, 50 m*M* bis-tris buffer pH 6.2, 1 m*M* DTT and 1 m*M* EDTA. The NaCl concentration in the protein solution was reduced by sealing each cell in a small Petri dish containing 3 ml of 800 m*M* NaCl, 45% methanol, 50 m*M* bis-tris buffer pH 6.2, 1 m*M* DTT and 1 m*M* EDTA. Under those conditions, the small crystals grew for several days and lozenge-shaped crystals up to a size of 0.8 mm along the longest dimension were obtained (Figs. 2*c* and 2*d*).

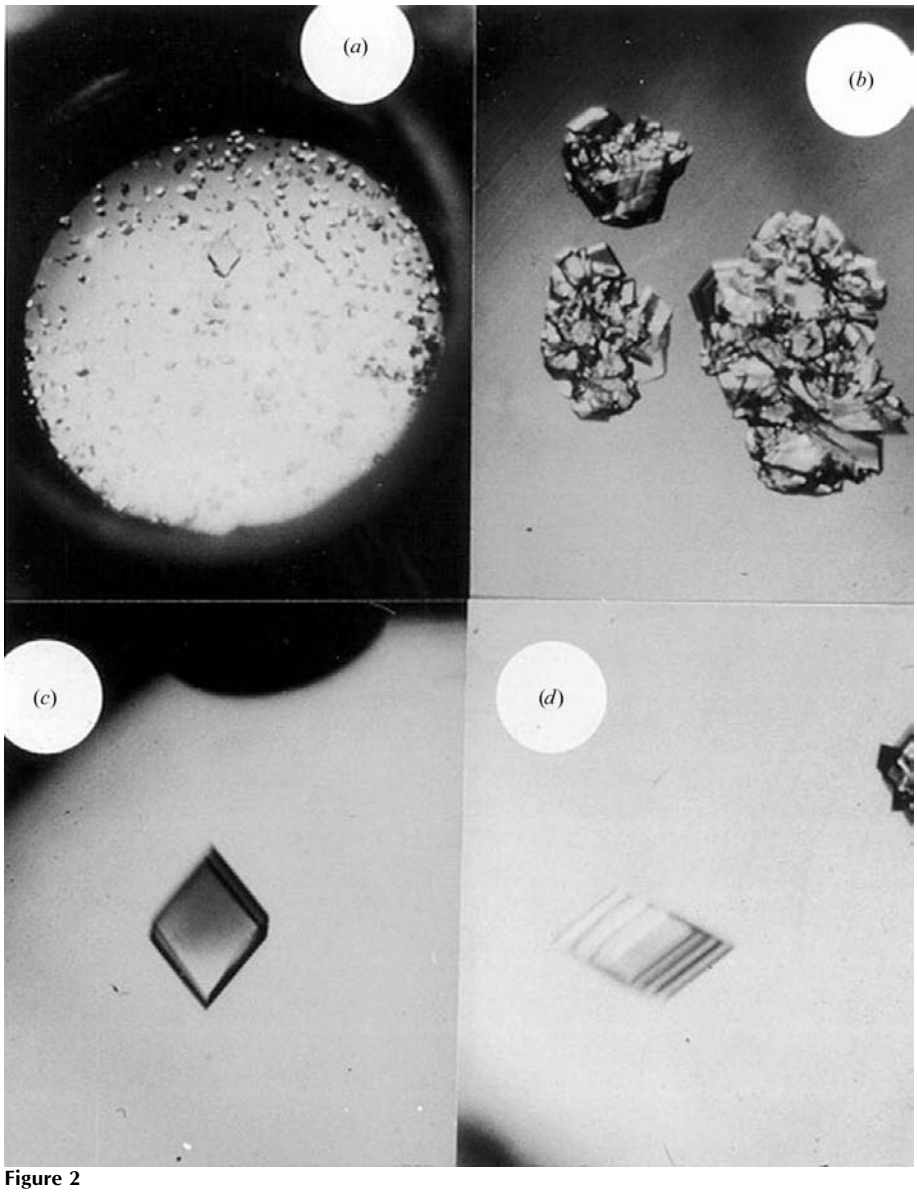

Figure 2
Crystallization of Δ(30D-34Q). (*a*) Growth of a seed stock in a microdialysis cell. (*b*) A cluster of crystals grown by conventional techniques. (*c*) and (*d*) Growth of data-quality crystals in cells to which seeds from the originally grown stock had been transferred. The longest dimension of the lozenge-shaped crystals shown in (*c*) and (*d*) is 0.6 mm.

2.3. X-ray analysis

Crystals of both mutants were characterized by the collection of X-ray diffraction data at room temperature. Data from the Δ(30D-34Q) crystals were measured using a MAR Research imaging-plate detector mounted on a Rigaku RU-3HR rotating-anode X-ray generator (Ni-filtered Cu $K\alpha$ radiation focused with a MAR Research double nickel-coated mirror system) operating at 50 kV and 100 mA with a 300 µm point focus. The rotation method (Arndt & Wonacott, 1977) was used. Oscillation frames of width 1.0° were recorded. Intensities were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). A31P crystals were measured on an automated Enraf–Nonius CAD-4 diffractometer operating at 40 kV and 26 mA (Cu $K\alpha$ radiation) in the $\omega/2\theta$ scan mode. Diffractometer data were processed using the *SDP* package (Frenz, 1985) and programs written by us. The data were corrected for absorption using an empirical absorption curve obtained from the variation of strong Bragg reflections as the crystal was rotated about the normal to the corresponding planes (North *et al.*, 1968).

3. Results and discussion

After purification, both proteins were stable for several weeks. Application of a crystallization procedure based on ionic strength reducers resulted in crystals suitable for X-ray analysis unlike conventional methods. Statistics from the data collection are presented in Table 1. Δ(30D-34Q) crystallizes in the monoclinic space group *C*2, with

Table 1
Data-collection statistics and Matthews coefficients for Δ(30D-34Q) and A31P.

	Δ(30D-34Q)	A31P	A31P
Space group	Monoclinic	Orthorhombic	Monoclinic
Resolution limit (Å)	2.02	1.8	1.9
Completeness (%)	95.3	100	97
No. unique reflections	7628	5103	8828
No. reflections	50953	N/A	32241
$\langle I/\sigma(I)\rangle$	5.1	6.2	5.9
$R_{\rm merge}$ (%)	6.6	N/A	7.2
$V_{\rm M}$ † (Å^3 Da^{-1})	2.25‡	1.86§	1.99‡
Solvent content (%)	45	34	38

† Matthews (1968). ‡ Assuming two molecules in the asymmetric unit. § Assuming one molecule in the asymmetric unit.

unit-cell parameters a = 54.48, b = 42.57, c = 51.72 Å, β = 104.70°.

A31P crystallizes in two crystal forms. The lozenge-shaped crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 30.4, b = 42.1, c = 81.4 Å. The rod-shaped crystals of A31P belong to the monoclinic space group $C2$ and have unit-cell parameters a = 94.4, b = 24.1, c = 64.4 Å, β = 130.6°.

In conclusion, the studies described in this work provide encouraging evidence regarding the applicability of the concept of ionic strength reducers in the development of efficient protocols for the purification and crystallization of proteins. Application of this concept in ion-exchange chromatography provides in a single chromatographic step Δ(30D-34Q) protein of sufficient purity and quantity for crystallization experiments. More importantly, for both Rop mutants a crystallization protocol based on the use of ionic strength reducers produced superior results compared with conventional techniques. The introduction of the crystallization procedure described in this work, which combines controlled changes in protein solubility (using the effects of ionic strength reducers) with seeding techniques, was essential for the crystallization of both proteins. This simple and efficient crystallization approach may be applied to other proteins, particularly in cases where conditions which are favourable both for nucleation and growth cannot be found or in cases where excessive nucleation leads to the growth of a large number of tiny crystals that are useless for crystallographic analysis. Our observations should warrant further investigation of the ionic strength reducer concept as potentially generally applicable in macromolecular crystallization.

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