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Protein crystallization with a combination of hard and soft precipitants

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Much effort and progress have been made in understanding the nucleation and crystallization of globular proteins, and many techniques have been developed to crystallize proteins in the past decades. The advantages of the use of combined precipitants in protein crystallization have been much appreciated. Unfortunately, there is still no theory or empirical guide on how to combine so many precipitants and how to use combined precipitants, although many proteins have been crystallized successfully using combined precipitants. This report gives a proposal about how to use conventional precipitants to obtain protein crystals, based on a novel idea of hard and soft precipitant combinations.

1. Introduction

Although there are numerous physical-chemical approaches for supplying structural information, X-ray diffraction is still the major technique yielding experimentally, in useful mathematical terms, a detailed and precise description of protein structures, which serve as a basis for drug design and protein engineering (Drenth, 1994). However, this enormously powerful method is prevented from full applicability to particular proteins (*e.g.* enzymes or receptors or their complexes with the related drugs) by the fact that the proteins must first be crystallized. Not only must the crystals be grown, but the crystals must also be of high quality, suitable for X-ray diffraction analysis at high resolution (McPherson, 1990). Unfortunately, the properties of proteins in solution vary as a function of many environmental factors, including temperature, pH, ionic strength and some types of contaminating materials; their conformations change according to the environment, as proteins are structurally dynamic and microheterogeneous aggregating systems (Feigelson, 1988). Protein crystallization is often performed with a strictly empirical methodology because of poor understanding of the mechanism and forces by which the proteins are prompted to form and maintain crystals (Feigelson, 1988).

Generally, most attempts to obtain protein crystals rely upon a large array of trials in order to determine which combination of environmental factors (*e.g.* pH, precipitant type and concentration, temperature *etc.*) yield the required crystals. Once found, the crystallizing conditions are improved carefully through subsequent refinement in relatively narrow ranges. There are three approaches normally used in searching for initial conditions in the hanging-drop and sitting-drop vapour-diffusion methods. The first can be named the 'shotgun approach' or 'multiple factorial method' (Cudney *et al.*, 1994). This is a sparse-matrix screen utilizing a selected set from 30 to 60 or more different conditions which have proved successful

in other protein crystallizations. These contain a combination of the precipitants, buffers, effects and physical parameters most frequently reported in the literature. The shotgun approach allows one to perform efficient screening covering a broad range of the most popular and effective salts, polymers and organic solvents *versus* a wide range of pH values. Crystal Screen and Crystal Screen II are two examples of sparse-matrix formulations selected from the known crystallization conditions for biomacromolecules (Jancarik & Kim, 1991). The second approach, described as the 'netting approach' or 'grid-screen' method, works by screening a single precipitant against a range of pH values, and can be viewed as a two-dimensional grid with the sampling pH on one axis and precipitant concentration on the other (Weber, 1990, 1991; McPherson, 1990; Samudzi, 1992; Jancarik & Kim, 1991). This netting approach can cover a broad range of pH values and precipitant concentrations in order to provide the preliminary conditions or a narrow range for improving the crystallization conditions. In the third method, the incomplete factorial approach, the variables to be screened are randomly combined. The use of four sandwich boxes for screening the preliminary conditions described by McPherson (1992) is one example of this approach. Normally, these three methods should be utilized together in order to ensure a complete coverage of preliminary crystallization conditions.

However, it is not an easy task to perform protein crystallization in a short period of time, although some crystals of proteins have been grown successfully. It might be thought that as we have so many proteins available, some crystals must appear if we persevere. The question is now what we should do if all these empirical trials fail to crystallize the proteins in which we are interested. Do we have another promising option? A possible solution is to try again using an appropriate combination of hard and soft precipitants, chosen as described in this report.

2. Experimental procedures

2.1. Materials

Two proteins (NAD^+ glycohydrolase and acuthrombin-C) were isolated and purified from the venom of *Agkistrodon acutus*. 2-Methyl-2,4-pentanediol (MPD) was purchased from MERCK-Schuchardt (Germany). Polyethylene glycol (PEG) 4000 and PEG 8000 were purchased from Fluka (Switzerland). N-(morpholino)ethanesulfonic acid (MES) was produced by Sigma (USA). Other reagents and chemicals were of analytical grade from commercial sources.

2.2. The concept of hard and soft precipitants

In order to obtain crystals of a given protein, the protein solution must be transformed by some means as slowly as possible into a supersaturated state, so that its tendency to return to its equilibrium point will force soluble protein molecules into the solid state (crystalline or amorphous precipitant). Protein crystallization can be achieved in more than one way. A common event in crystallization methods is the increasing of the so-called effective concentration, usually by adding salts in order to immobilize the water molecules (salting out) and/or by adding organic solvents to diminish the repulsive forces between the protein molecules or to increase the attractive forces between them. The salts and organic solvents added to protein solutions during crystallization are often termed precipitants. Although precipitants have the common effect of increasing the effective concentration of the protein during the crystallization, there are specific interactions between particular precipitants and proteins. Therefore, a selection of the types of precipitants which should be considered and chosen is one of the most important or even the most critical factor in protein crystallization. Precipitants can be classified into three types for each particular protein, according to the strength of the interaction between the precipitant molecules and the protein molecules. Type I precipitants are called hard precipitants. They have strong interactions with protein molecules which can drive the protein molecules into the precipitated state (but rarely the crystal state) even at a low concentration. Type II precipitants are named soft precipitants and have weak interactions with protein molecules; they precipitate protein molecules only at higher concentrations. Other precipitants can be assigned as Type III precipitants. It is worth pointing out that this classification only has meaning for a particular protein. A particular precipitant may belong to different hard/soft classifications for different proteins.

Table 1
Some precipitants commonly used in protein crystallization.

Precipitant	Concentration usually used	Concentration as hard precipitant	Concentration as soft precipitant
MPD [%(*v*/*v*)]	15–60	<20	>50
PEG 4000 [%(*w*/*v*)]	10–30	<15	>30
PEG 8000 [%(*w*/*v*)]	10–30	<10	>30
$(NH_4)_2SO_4$ [%(*w*/*v*)]	50–80	<30	>80
Ethylene glycol [%(*v*/*v*)]	10–30	<10	>30
2-Propanol [%(*v*/*v*)]	5–30	<5	>30
Dioxane [%(*v*/*v*)]	10–35	<10	>35

2.3. Experimental determination of hard and soft precipitants

Determination of hard and soft precipitants can be made empirically from the precipitant concentrations generally used in crystal growth. In order to find out whether one precipitant should be considered to be a soft or a hard precipitant for a particular protein, experiments need to be designed in order to determine whether the precipitant precipitates the protein at lower or higher concentrations in the range normally used in protein crystallization. The classification is not unlimited and unconditional, as the normal concentration range of one precipitant being used in crystallization is undefined. Table 1 shows some precipitant examples in protein crystallization in our laboratory. For a given protein, two methods are often used to recognize whether a precipitant is hard or soft. One is from the screening of results of preliminary crystallization

Table 2
Initial screen for the crystallization conditions of NAD^+ glycohydrolase using combined hard and soft precipitants at pH 6.0, 6.5, 7.0, 7.5 and 8.0.

Hard precipitant	1.5 *M* $(NH_4)_2SO_4$	15% PEG 4000	15% PEG 8000
Soft precipitant (%)			
MPD	5, 10, 15	5, 10, 15	5, 10, 15
Ethylene glycol	5, 10, 15	5, 10, 15	5, 10, 15
2-Propanol	5, 10, 15	5, 10, 15	5, 10, 15
Dioxane	5, 10, 15	5, 10, 15	5, 10, 15

conditions by means of sparse-matrix screens, grid screens and/or incomplete factorial screens. Another is to determine the so-called 'precipitating point' – the concentration of the precipitant at which protein molecules begin to aggregate. It should be kept in mind that the hard and soft precipitant classification may depend on pH for some proteins if their stability in solution is very sensitive to pH.

2.4. Combination of hard and soft precipitants

Based on experience accumulated in practice, it seems that combined precipitants may posess advantages which an individual precipitant does not have in protein crystallization. Unfortunately, there is no theory or empirical guide to which kinds of precipitants should be chosen and how they should be combined. Here, we propose a method to solve the problem and to make crystallization easier. For a particular protein to be crystallized, its hard and soft precipitants should first be determined. One of its hard precipitants should then be combined with one of its soft precipitants, and in this combined precipitant the concentration of the hard precipitant should be about 10–20% higher than its precipitating point, while the concentration of the soft precipitant should be about 10–30% lower than its precipitating point. The presence of the soft precipitant seems to stabilize the protein in its hard precipitant environment and improve the protein's crystallization behaviour.

2.5. Crystal growth

Two new proteins (NAD^+ glycohydrolase and acuthrombin-C) purified from the venom of *A. acutus* were crystallized using combined precipitants. From the screening results of the preliminary crystallization conditions it was found that $(NH_4)_2SO_4$, PEG 4000 and PEG 8000 were hard precipitants and MPD, dixoane, ethylene glycol and 2-propanol were soft precipitants for NAD^+ glycohydrolase. For acuthrombin-C, PEG 4000, PEG 8000 and 2-propanol were hard precipitants and ethylene glycol was a soft precipitant. By adding precipitant (in 1 µl aliquots) to 20 µl of the protein solutions (40 mg ml^{-1}) until the proteins began to precipitate, the precipitating points of those precipitants were also determined. For NAD^+ glycohydrolase, the precipitating points of $(NH_4)_2SO_4$, PEG 4000, PEG 8000, MPD, ethylene glycol, dixoane and 2-propanol were 1.2 *M*, 10%(*w*/*v*), 8%(*w*/*v*), 55%(*v*/*v*), 30%(*v*/*v*), 35%(*v*/*v*) and 25%(*v*/*v*), respectively. For acuthrombin-C, the precipitating points of PEG 4000, PEG 8000, 2-propanol and ethylene glycol were 10%(*w*/*v*), 8%(*w*/*v*), 8%(*v*/*v*) and 25%(*v*/*v*), respectively.

In the case of NAD^+ glycohydrolase, the three hard precipitants [$(NH_4)_2SO_4$, PEG 4000 and PEG 8000] and the four soft precipitants (MPD, dioxane, ethylene glycol and 2-propanol) were combined to generate a 3 × 4 matrix. A single concentration of each of the hard

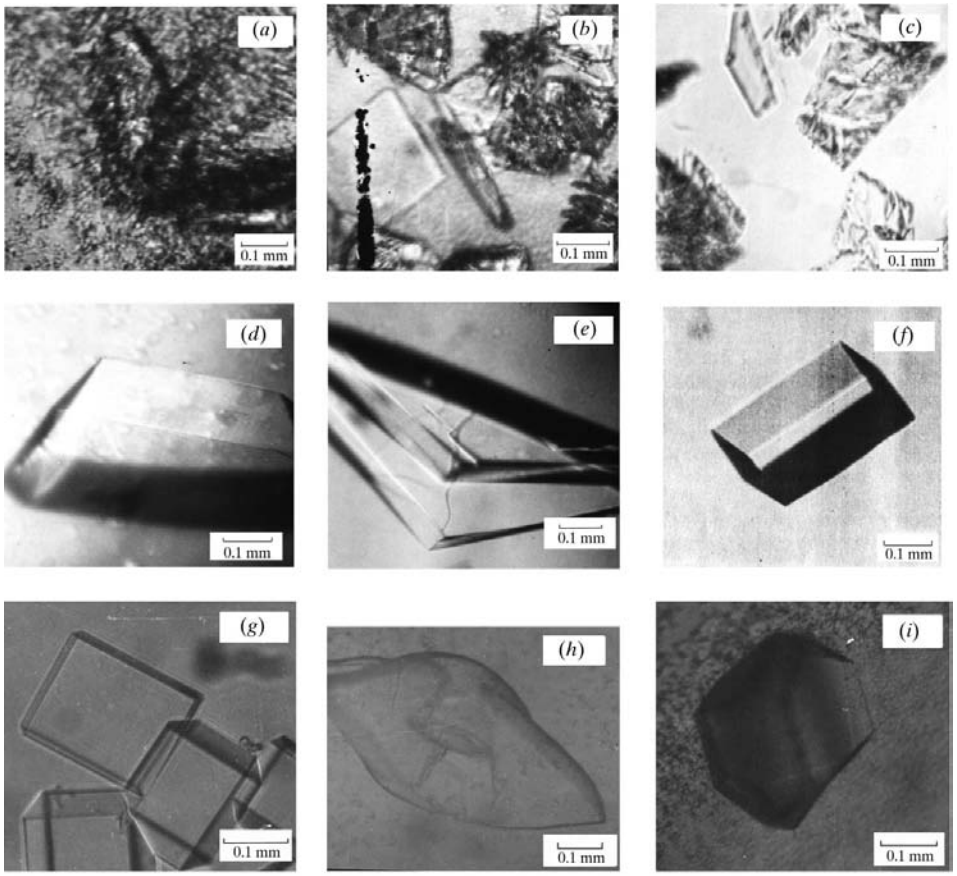

Figure 1
Crystals of NAD^+ glycohydrolase. (*a*) Form *A* crystal, (*b*) form *B* crystal, (*c*) form *C* crystal, (*d*) form *D* crystal, (*e*) form *E* crystal, (*f*) form *F* crystal, (*g*) form *G* crystal, (*h*) form *H* crystal, (*i*) crystal of NAD^+ glycohydrolase produced by the hanging-drop vapour-diffusion method using MPD as precipitant. 2 µl of protein solution (40 mg ml^{-1} in distilled water) was mixed with an equal volume of reservoir solution [55%(*v*/*v*) MPD, 0.04 *M* Tris–HCl pH 7.4] and equilibrated against 0.5 ml of the same reservoir solution. Crystals were obtained one month later.

Table 3
Composition of the reservoirs used in the crystallization of NAD^+ glycohydrolase.

	$(NH_4)_2SO_4$	PEG 4000	PEG 8000
MPD	Form *A* 1.6 *M* $(NH_4)_2SO_4$, 15% MPD 0.2 *M* MES/NaOH pH 6.5	Form *B* 15% PEG 4000, 10% MPD, 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.0	Form *C* 15% PEG 8000, 10% MPD 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.0
Ethylene glycol	No crystals	Form *D* 15% PEG 4000, 10% ethylene glycol, 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.0	Form *E* 15% PEG 8000, 10% ethylene glycol, 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.0
2-Propanol	No crystals	Form *F* 12% PEG 4000, 10% 2-propanol, 0.2 *M* Na_2HPO4/NaH_2PO_4 pH 7.4	Form *G* 10% PEG 8000, 10% 2-propanol, 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.4
Dioxane	Form *H* 1.6 *M* $(NH_4)_2SO_4$, 10% dioxane 0.2 *M* MES/NaOH pH 6.5	No crystals	No crystals

Table 4
Initial screen for the crystallization conditions of acuthrombin-C using combined hard and soft precipitants at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

	Hard precipitant		
	15% PEG 4000	10% PEG 8000	10% 2-propanol
Soft precipitant (%)			
Ethylene glycol	5, 10, 15	5, 10, 15	5, 10, 15

precipitants [1.5 *M* $(NH_4)_2SO_4$, 15%(*w*/*v*) PEG 4000 and 15%(*w*/*v*) PEG 8000] and three concentrations of each of the soft precipitants [5, 10 and 15%(*v*/*v*)] were selected to be used in initial crystallization trials at pH 6.0, 6.5, 7.0, 7.5 and 8.0 (Table 2). The crystals were grown by the conventional hanging-drop vapour-diffusion method using tissue-culture plates and siliconized glass cover slips (McPherson, 1982) at room temperature (about 273 K). In all screens, 2 µl of the protein solution (40 mg ml^{-1} in distilled water) was mixed with an equal volume of reservoir solution and equilibrated against 0.5 ml of the same reservoir solution. Eight crystal forms appeared one or two weeks later. Using the narrower grids, improved quality crystals were obtained (Table 3; Fig. 1).

For acuthrombin-C, a single concentration of each of its hard precipitants [15%(*w*/*v*) PEG 4000, 10%(*w*/*v*) PEG 8000 and 10%(*v*/*v*) 2-propanol] and three concentrations of each of the soft precipitant [5, 10 and 15%(*v*/*v*)] were combined and used in initial crystallization trials at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (Table 4). The crystals were also grown by the hanging-drop vapour-diffusion method using tissue-culture plates and siliconized glass cover slips (McPherson, 1982) at room temperature (about 273 K). In all screens, 2 µl of the protein solution (20 mg ml^{-1} in 0.2 *M* NaCl) was mixed with an equal volume of reservoir solution and equilibrated against 0.5 ml of the same reservoir solution. Three crystal forms were obtained one week later (Table 5; Fig. 2).

3. Results

Eight forms of NAD^+ glycohydrolase crystals and three forms of acuthrombin-C crystals were obtained in the initial crystallization trials using combinations of hard and soft precipitants. As controls, no crystals of NAD^+ glycohydrolase were obtained by the grid-screen method using a single precipitant [$(NH_4)_2SO_4$, PEG 4000, PEG 8000, ethylene glycol, dioxane or 2-propanol]. Using MPD as precipitant, small crystals of NAD^+ glycohydrolase (with maximum dimensions of 0.2 × 0.2 × 0.3 mm) appeared (Fig. 1). Needle-like crystals of acuthrombin-C were also obtained by the grid-screen method using PEG 4000 or PEG 8000 as precipitant (Fig. 2), but no crystals were observed using 2-propanol or ethylene glycol as precipitant.

The large crystals obtained were characterized at room temperature (298 K) using a MAR Research imaging plate (diameter 300 mm) mounted on a MAR Research X-ray generator with a graphite monochromator and a sealed copper-target tube. The working tube voltage and current were 40 kV and 50 mA, respectively. The crystal-to-imaging

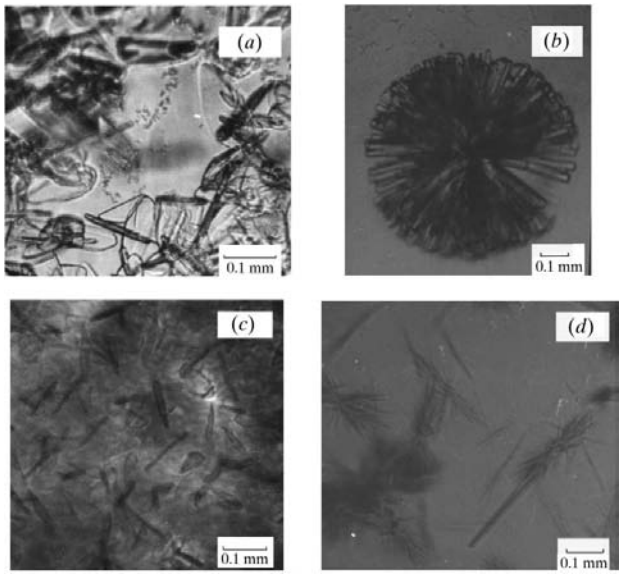

Figure 2
Crystals of acuthrombin-C. (*a*) Form *A* crystal, (*b*) form *B* crystal, (*c*) form *C* crystal, (*d*) needle-like crystals of acuthrombin-C produced by the hanging-drop vapour-diffusion method using PEG 4000 as precipitant. 2 µl of protein solution (20 mg ml^{-1} in 0.2 *M* NaCl) was mixed with an equal volume of reservoir solution [12%(*w*/*v*) PEG 4000, 0.04 *M* MES–NaOH pH 6.5] and equilibrated against 0.5 ml of the same reservoir solution. Crystals were obtained one week later.

Table 5
Crystallization conditions of acuthrombin-C using combined hard precipitants and soft precipitant (ethylene glycol).

PEG 4000	PEG 8000	2-Propanol
Form *A* 12% PEG 4000, 10% ethylene glycol, 0.2 *M* Mes/NaOH pH 6.0	Form *B* 10% PEG 8000, 10% ethylene glycol, 0.2 *M* Mes/NaOH pH 6.6	Form *C* 10% 2-propanol, 10% ethylene glycol, 0.2 *M* Na_2HPO_4/NaH_2PO_4 pH 7.0

plate distance was 120 mm. A 1° oscillation angle and 600 s exposure time were set for each imaging frame. The NAD^+ glycohydrolase crystals were found to have a low X-ray diffraction limit. Other crystals were too small to be characterized by X-ray diffraction. All eight forms of the NAD^+ glycohydrolase and three forms of the acuthrombin-C crystals were proved to be protein crystals by SDS–PAGE after collecting the crystals from the crystallizing drops.

4. Discussion

The advantages of using combined precipitants are being increasingly appreciated. The precipitant solutions required can be obtained from conventional commercial sources such as 'Crystal Screen' and 'Crystal Screen II', in which there are many kinds of combined precipitants. However, it is not easy to produce the effective precipitant combination by oneself using a rational procedure. This proposal regarding the recognition of hard and soft precipitants for a given protein may provide a possible guide to which kinds of precipitants should be chosen and how to combine them in order to crystallize a particular protein of particular interest. Based on the experiences at our laboratory, the possibility of successfully obtaining protein crystals was dramatically higher (67% for NAD^+ glycohydrolase). Unfortunately, the crystals grown by this method were not of good quality. The possible reasons for this may be complicated and the crystals obtained may be improved by further trials. Clearly, it is worthwhile to try this method if other methods have been tried and no crystals have appeared. In addition, if one wants to obtain different forms of crystals for a particular protein, this method may be very useful. The most important stage in this method is the determination of the hard and soft precipitants for the protein to be crystallized. The classification of precipitants often changes for different proteins. As a general phenomenon, it is notable that soft precipitants are often organic solvents.

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