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Attempts to rationalize protein crystallization using relative crystallizability

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Abstract

Protein crystal growth (PCG) remains the bottleneck of crystallography despite many decades of study. The nucleation zone in the two-dimensional-phase diagram has been used to evaluate the relative crystallizability of proteins, which is expressed as a percentage over the phase area delineated by experimental protein and precipitating agent concentration ranges. For protein-salts which are subject to a direct temperature effect on solubility, as represented by Egg Lysozyme, a decrease in temperature augments the nucleation zone percentage whereas for those with retrograde solubility as a function of temperature, for example fructose-1,6-bisphosphatase in the presence and absence of AMP, an increase in temperature can significantly enhance the relative crystallizability. These results have been confirmed by the number of "hits" using PEGs as precipitating agents in Sparse Matrix Screen experiments for different proteins and are in excellent agreement with the relative crystallizability. The relationship between solubility dependence, relative crystallizability and crystallization success, has been evidenced. Such crystallizability can become a guide to identify efficient crystallization regions, providing a rational approach to PCG and structural biology.

Keywords: Protein crystallization; Relative crystallizability; Nucleation curve; "Hits"; Two-dimensional-phase diagram; Temperature-dependent solubility; Sparse Matrix Screen

1. Introduction

Protein crystal growth (PCG)[3] has provided the basis for crystallography with which most structural entities currently found in the PDB bank have been determined (Berman et al., 2000; Lattman, 1994). This trial and error process has consumed intense scientific effort (Giege and Ducruix, 1999; McPherson, 1998), but a rational approach to PCG is needed for the efficient development of structural biology. It has been indicated that much remains unclear in this domain however, and that the protein crystallization research community will be quite active for many years to come (Wiencek, 1999).

In this study, we attempt to find a physico-chemical parameter that can be measured experimentally that can provide a rationale to efficiently find new crystallization conditions. Among the possible variable crystallization parameters temperature has been studied first due to the ease with which it may be modified. Temperature effects on crystal quantity, size, and quality have been widely studied (Christopher et al., 1998; www.hamtonresearch.com/support/xtslexp.html; Giege and Mikol, 1989; Han and Lin, 2000; Judge et al., 1999; Lorber and Giege, 1992; McPherson, 1990; Rosenberger et al., 1993), but here we focus on the search for first crystals using temperature as a probe. As a result of continuous work carried out during recent years, we have proposed the term "relative crystallizability" of proteins as a new measurable parameter and have carried out its quantitative evaluation for changing the PCG limiting step. We established phase diagrams (protein

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[3] *Abbreviations used:* PCG, protein crystal growth; PEG, polyethylene glycol; SmFru-1,6-P2ase, snake muscle fructose-1,6-bisphosphatase.

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concentration versus precipitating agent concentrations), at different temperatures for several protein forms. The nucleation zone where spontaneous nucleation occurs has been used as a reference for crystallizability, or "relative crystallizability" and is defined as the proportion of this zone area over the total phase area within the experimental protein and precipitating agent concentration range in the diagram. This initial idea of crystallizability has been verified by the number of 'hits', where crystals are obtained under conditions with the same precipitating agent in Sparse Matrix Screen experiments, showing the success rate of crystallization.

2. Materials and methods

The native form of snake muscle fructose-1,6-bisphosphatase (SmFru-1,6-P2ase) was prepared as described (Xu et al., 1982). The enzyme was stored at 277 K in 10 mM Tris–HCl buffer adjusted to pH 7.5 containing 20% glycerol, 0.2 mM EDTA, and 0.4 mM DTT. AMP was added to the enzyme sample to a final concentration of 1 mM just before crystallization. The other proteins were purchased from Sigma, their names and sources are listed in Table 1.

2.1. Crystallization at different temperatures

All crystallizations were carried out by vapor diffusion in hanging drops, which were initiated by mixing one volume of protein with one volume of reservoir solution. Crystal-solution phase diagrams were designed to plot the initial protein concentration versus the precipitating agent concentration under each crystallization condition. The multiwells were placed in incubators at different temperatures (288, 295, 300, and 303 K).

Crystallization of SmFru-1,6-P2ase was previously described (Zhu et al., 1999). Lysozyme, catalase, elastase, ribonuclease A, trypsin, bovine IgG, and concanavalin crystallizations are as described (Christopher et al., 1998), and ribonuclease S crystallization is given in (www.hamton-research.com/support/xtslexp.html). These enzyme forms are included in Table 1.

2.2. Two-dimensional phase diagrams and solubility measurement

Crystals were observed on video images taken with an optical microscope (LEICA MZ APO) equipped with a color CCD camera (Sony). In the phase diagram plotting protein concentration versus precipitating agent concentration, nucleation, and precipitation curves were determined by visual examination at 50-fold magnification. After an equilibration period of 50 days, the residual protein concentration in equilibrium with crystals was titrated using a dye-binding assay (Bradford, 1976; Zhu et al., 2001) to determine the solubility curve. Such equilibrium can reach the 95% level after approximately 10 days. The precipitation zone is denoted by the region above the precipitation curve while the nucleation zone, where spontaneous nucleation occurs, lies between the precipitation and nucleation curves. The metastable zone is situated between the nucleation and solubility curves where crystals grow, while the area below the solubility curve is the under-saturated zone (e.g., Fig. 1).

Lysozyme phase diagrams were determined after crystallization at pH 7.5 in Hepes buffer, in the presence of 3–32% (w/v) PEG 3350, 0.2 M ammonium sulphate, and 1–60 mg/ml protein. SmFru-1,6-P2ase phase diagrams in the presence or absence of AMP were determined by crystallization at pH 8.5 (Tris–HCl), in the presence of 0.39 M $MgCl_2$ and PEG 3350 from 18.5 to 30%, while the protein concentration varied from 1 to 30 mg/ml.

The nucleation zone area was calculated using Excel by determining the area between the precipitation curve and the nucleation curve in the experimental ranges of protein and precipitating agent concentrations mentioned above, as shown in the two-dimensional phase diagrams.

2.3. Evaluation of pH modification which may result from temperature variation

The pH of the crystallization solutions at different temperatures was monitored carefully. In the crystallization solution of Lysozyme, the pH variation was less than ±0.1

Table 1
Success rate of protein crystallization, by experimental "hits," in Sparse Matrix Screening[a]

Protein	Success rate at different temperature				"Hits"-temperature correlation[b]
	Temperature				
	288 K	295 K (%)	300 K (%)	303 K (%)	
Lysozyme (from chicken egg white)	45.8%	33.3	29.2		Decremental
Catalase (from bovine liver)	58.3%	54.2	50		Decremental
Ribonuclease A (from Type III bovine pancreas)	16.7	16.7	8.3		No dependence[b]
Ribonuclease S (from bovine pancreas)	29.2	25	16		Decremental
Trypsin (from bovine pancreas)	16.7	12.5	8.3		Decremental
SmFru-1,6-P2ase (from snake muscle, in the presence of AMP)	4.2%	12.5		25	Incremental
SmFru-1,6-P2ase (from snake muscle, in absence of AMP)	8.3%	12.5	16.7	21	Incremental
Elastase (from porcine pancreas)	0%	0	12.5		Incremental
Concanavlin A (from canavalia ensiformis)	4.2%	8.3	16.7		Incremental
Bovine IgG (from serum)	4.2%	0	0		No dependence[b]

[a] Crystals were obtained at different temperatures, using the 24 conditions with PEG as precipitating agent in Sparse Matrix Screen.
[b] No dependence, no demonstrated temperature-dependent solubility.