ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Structural Biology 142 (2003) 88–97

Journal of Structural Biology

www.elsevier.com/locate/yjsbi

The protein as a variable in protein crystallization

Glenn E. Dale,* Christian Oefner, and Allan D'Arcy

Morphochem AG, WRO-1055/338, Schwarzwaldallee 215, CH-4058 Basel, Switzerland

Received 5 February 2003

Abstract

Strategies for growing protein crystals have for many years been essentially empirical, the protein, once purified to a certain homogeneity, being mixed with a selection of crystallization agents selected in a more or less trial-and-error fashion. Screening for the correct conditions has been made easier through automation and by the introduction of commercially available crystallization kits. Many parameters can be changed in these experiments, such as temperature, pH, and ionic strength, but perhaps the most important variable has been ignored, namely the protein. The crystallization properties of a protein vary greatly: some crystallize readily, whereas others have proven extremely difficult or even impossible to obtain in a crystalline state. The possibility of altering the intrinsic characteristics of a protein for crystallization has become a feasible strategy. Some historical perspectives and advances in this area will be reviewed.

Keywords: Crystallization; Protein modification; Mutagenesis; Light scattering; Truncation; Fusion proteins

1. Introduction

The importance of structural biology research has been highlighted in the past few years, not only as an integral part of drug discovery programs in the pharmaceutical industry but also through the efforts in academia and the many structural genomics programs that have been established throughout the world. The various genome projects have revealed numerous new genes believed to be involved in the disease process or essential for the diseased state. However, it is not the genes themselves that contribute to the disease but rather the proteins encoded by the genes. Now, in the postgenomic era, the focus has turned to the function of the various proteins and their role in disease manifestation. Many approaches have been used to shed light on this area, such as gene-knockout systems, overexpression models, and transgenic and yeast two-hybrid systems. However, since the function of a protein correlates with its 3D structure, ultimately, the structure of each gene product is required to fully understand its function. Therefore structure determination should be incorporated into the process of function assignment. Many advances have been made in the technology to support these initiatives; great progress has been made in bioinformatics, protein expression, purification, and methods for accelerating crystallization and X-ray data collection (Abola et al., 2000; Blundell et al., 2002; Boettner et al., 2002; Chayen and Saridakis, 2002; Dieckman et al., 2002; Karain et al., 2002; Leslie et al., 2002; Orengo et al., 1999; Peitsch, 2002; Schwede et al., 2000; Shih et al., 2002; Stevens, 2000a).

X-ray crystallography is still the most powerful technique for determining the three-dimensional structure of a protein. A prerequisite for such studies is the production of protein crystals of suitable quality, and this in many cases remains the rate-limiting step in the process. With the advent of commercially available sparse matrix screens it has become trivial to set up crystallization trials covering a huge range of conditions. Developments in liquid handling and miniaturization of the process have made it possible to set up thousands of different crystallization experiments per day (Krupka et al., 2002; Luft et al., 2001; Stevens, 2000b). Statistics arising from the various structure–genomics programs reveal variable success rates from the cloned protein to the structure determination (Service, 2002). For example,

* Corresponding author. Fax: +41-61-695-2122.
E-mail address: Glenn.dale@morphochem.ch (G.E. Dale).

1047-8477/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1047-8477(03)00041-8

figures taken from the Human Proteome Structural Genomics pilot project (Brookhaven National Laboratory, Einstein College of Medicine, New York, NY, USA: http://proteome.bnl.gov/progress.html) show that of the 120 proteins expressed, 19 (15.8%) yielded crystals suitable for structure determination. The program at the Berkeley Structural Genomics Center (Berkeley, CA, USA: http://www.strgen.org/status/progresstotals.html) obtained similar results, with 19.4% of the soluble expressed proteins providing diffraction-quality crystals. Other programs have had varying degrees of success ranging from identifying crystallization conditions for 31.2% of the expressed proteins (Lesley et al., 2002) to 3.1% of the proteins providing diffraction data (Joint Center for Structural Genomics: http://www.jcsg.org/scripts/prod/home.html). The majority of programs have obtained diffraction-quality crystals for approximately 10% of the expressed proteins (Claverie et al., 2002; Ding et al., 2002; Sulzenbacher et al., 2002). It is becoming increasingly clear that the effectiveness of a crystallization experiment is not directly proportional to the number of conditions tested; therefore, improvements are urgently required.

2. Screening and protein modification

The surprisingly low success rates in these initiatives could be due to the type of screening method or, more likely, due to the properties of the protein used. The use of dynamic light scattering has been described as a criterion for determining the crystallizability of a proteins (D'Arcy, 1994; Ferre-D'Amare and Burley, 1997; Zulauf and D'Arcy, 1992). In one study, it was estimated that 70% of proteins that show a monodisperse size distribution in a light-scattering experiment should give crystals using a standard sparse matrix screen using between 48 and 96 conditions (Zulauf and D'Arcy, 1992). In a structural genomics study DeTitta and coworkers used dynamic light-scattering as a selection criterion to choose proteins that were suitable for crystallization, but only 36% of those produced crystals using a screen with 1536 conditions (personal communication). This was possibly due to the use of paraffin oil in the microbatch experiment, as it does not allow for any significant evaporation of the drop and subsequent concentration of the protein during the crystallization trial. The effects of using different oils in microbatch experiments have recently been described, and experimental ways to improve the hit rate were proposed (D'Arcy et al., 2003).

Even when one considers the best case, in which approximately 70% of the soluble, monodisperse proteins give crystals in an initial screen, the remaining 30% of suitable proteins do not produce crystals or produce only crystals of poor quality. Longenecker and colleagues argue that the protein itself, rather than the precipitating agent, should be considered the most important variable in the screening process (Longenecker et al., 2001). In this light, as early as 1972 it was proposed that if crystals of a target protein cannot be grown, homologous proteins from another species should be considered (Campbell et al., 1972). Generally, these proteins differed by only a few amino acid residues, which are often found on the surface of the protein; nevertheless, the effect on crystallization could be dramatic. It has also been our experience that some proteins crystallize rather easily and under various conditions (e.g., porcine pancreatic elastase, *Staphylococcus aureus* DHNA, *S. aureus* DHFR), whereas other homologous proteins proved to be very difficult to crystallize (e.g., human neutrophil elastase, *Escherichia coli* DHNA, *Streptococcus pneumoniae* DHFR). It can be argued that those proteins that do not crystallize readily have surface properties that do not favor the formation of crystal contacts. It is still a very general practice to choose a protein from an alternative source for the crystallization but increasingly researchers are modifying the target protein to obtain usable crystals.

A protein may be modified in a number of ways in order to improve the chances of obtaining crystals. Many proteins that have not crystallized in their native state could be readily crystallized as complexes. Complex formation and subsequent cocrystallization screens can be performed with cofactors, inhibitors, or even antibody fragments (Davis et al., 1990; Ostermeier et al., 1997; Prongay et al., 1990). The conformational changes induced upon such ligand binding may be favorable to the crystallization process by exposing new crystal contacts or by stabilizing the protein. In general, if substrate analogs, cofactors, or small-molecule inhibitors are available they should be included in the initial screening process. The production of antibody fragments for cocrystallization is nontrivial and in our experience (γ-interferon receptor/Fab complex and p75 TNF receptor/Fab complex) the diffraction quality of the crystals obtained was not satisfactory (unpublished results).

Proteins obtained from natural sources or eukaryotic expression systems may prove difficult to crystallize because of a high or heterogeneous carbohydrate content. Such proteins are often extremely soluble, and very high protein concentrations are needed to bring the protein to a state of supersaturation. Baker and coworkers first described the enzymatic removal of sugars to improve protein crystallization and which has subsequently been successfully used (Baker et al., 1994; Gruenunger-Leitch et al., 1996; Kostrewa et al., 1997, 1999; Oefner et al., 2000). In the case of neprilysin, large single crystals of the native glycosylated protein could be obtained; however, these crystals diffracted only to approximately 7 Å resolution, compared to 2.1 Å resolution for the deglycosylated protein (Dale et al., 2000).

A common phenomenon observed during a long crystallization experiment (>1 month) is that spontaneous proteolysis has occurred, producing a modified protein which crystallizes more readily than the native form (Holden et al., 1996; Leppanen et al., 1999; Sogabe et al., 2002). As early as 1982, McPherson suggested that if a molecule can undergo limited, controlled proteolysis, this form should be considered in the crystallization strategy (McPherson, 1982). When such proteolysis is observed it is advisable to analyze the content of crystals obtained to determine what kind of fragment has been generated in order to reproduce the modification in a controlled manner. This was our experience when trying to crystallize a complex of human tissue factor and Factor VIIa (Kirchhofer et al., 1995). Proteolysis had produced a fragment that gave crystals under one condition of a crystallization screen. Analysis on SDS–PAGE gels and mass spectrometry led us to the conclusion that subtilisin was most likely the proteolytic agent. A controlled digest was performed, the crystallization was reproduced, and the structure could be solved (Banner et al., 1996). More recently molecular biology has become a powerful tool to engineer proteins in order improve their crystallization properties. These modifications could be site-directed point mutations, N-terminal and C-terminal truncations, internal loop deletions, or the construction of fusion proteins to create molecules that form suitable protein crystals.

Within the cell, proteins have a natural potential to interact via hydrogen bonds and ionic and Van der Waals contacts and it is precisely these interactions which occur in the intermolecular packing within a protein crystal. It has been suggested that crystallization seems to depend predominantly on random protein–protein interactions, which have little in common with the physiological protein–protein recognition processes (Carugo and Argos, 1997; Janin and Rodier, 1995; Jelsch et al., 1998). However, it has also been observed that crystal contacts form in such a way as to avoid the inclusion of hydrophobic interactions (Dasgupta et al., 1997). For a protein recalcitrant to crystallization, mutation of lysine (the least favored residue in a crystal contact) to an arginine or glutamine (the most favored residues in crystal contacts) was a recommended strategy (Dasgupta et al., 1997).

3. Point mutations

There are a number of examples in which rational surface point mutations were used to engineer crystal contacts to generate a crystallizable protein (Table 1). One of the first examples was the engineering of human H-chain ferritin (Lawson et al., 1991). The horse spleen ferritin and rat liver L-chain ferritin gave large single crystals with $CdSO_4$ in vapor diffusion, but the addition of $CdSO_4$ to H-chain ferritin resulted in the instant formation of amorphous precipitate. Examination of the intermolecular crystal contact regions of the L-ferritins revealed that the molecules in the crystal are linked by double Cd^{2+} bridges. These are situated near the two-fold axis where pairs of aspartate and glutamine side chains from neighboring molecules form the ligands to the Cd^{2+} ions. The glutamine is not present in human H-chain ferritin but rather is replaced by lysine. The authors mutated Lys86 to Gln in the human H-chain ferritin, enabling the metal bridge contacts to form, resulting in crystals which diffract to 1.9 Å resolution (Figs. 1A and B).

In a seminal study with thymidylate synthase, Villafranca and co-workers wanted to determine the efficacy of altering a protein's intrinsic solvation properties to improve its crystallization (McElroy et al., 1992). Here the authors introduced 12 single-point mutations at 11 different positions on the surface of the protein. The mutations changed either the charge or the polarity of the wild-type amino acid. They concluded that there was no correlation between solubility trends and crystallizability; however, all mutations had an effect on crystallization and most mutations resulted in a more crystallizable protein. In a similar study with the 24-kDa fragment of the *E. coli* DNA gyrase B subunit, nine surface amino acids were mutated changing either the charge or the polarity of the wild-type amino acid. It was found that single amino acid changes on the surface of a protein could have a dramatic effect on the crystallization properties of the protein. Generally the mutations resulted in an improvement in the number of crystal-screen hits as well as in an improvement in crystal quality (D'Arcy et al., 1999). These results were in good agreement with the conclusions drawn from McElroy's study.

The human RhoGDI GTPase was used by Derewenda and colleagues as a model system to investigate the effect of Lys to Ala, Glu to Ala, and Glu to Asp mutagenesis on the protein's crystallization properties (Longenecker et al., 2001; Mateja et al., 2002). The authors hypothesized that surface residues with high conformational entropy, specifically lysines and glutamates, impede protein crystallization. Indeed, lysine is the least likely residue to be found in a crystal contact and similarly glutamate is very uncommon; however, both are found almost exclusively on the surface of a protein (Baud and Karlin, 1999; Dasgupta et al., 1997). All but one of the Glu to Ala (Asp) mutants produced crystals in the screen, whereas the wild-type protein failed to crystallize. All the Lys to Ala mutations resulted in proteins that crystallized. Four structures were solved and in three cases the crystal contacts of the new lattices were found precisely at the sites of the mutation. More recently, using aspartyl-tRNA synthetase (AspRS-1) from *Thermus thermophilus* as a model protein, Charron and

Table 1
Examples of point mutations used to crystallize proteins

Structure (source)	Mutations	Comments	Reference
Human H-chain ferritin	Lys86 to Gln	Engineered intermolecular crystal contact via metal bridge	Lawson et al. (1991)
Human thymidylate synthase	12 point mutations	Investigated the correlation of intrinsic solubility with crystallizability	McElroy et al. (1992)
E. coli ROP protein	4 point mutations; 1 insertion	Attempted to correlate a protein's stability with the crystallization properties	Kokkinidis et al. (1993)
E. coli glutathione reductase	Double point mutation	Attempted to engineer contacts and observed a shortening of the crystallization time	Mittl and Schulz (1994)
Catalytic domain of HIV integrase (50–212)	Phe185 to Lys	Constructed 29 mutants changing hydrophobic to Lys; one mutant gave a soluble, crystallizable protein	Dyda et al. (1994)
E.coli GroEL	2 point mutations	Mutations introduced by PCR led to crystallizable protein	Braig et al. (1994)
Human cytomegalovirus protease catalytic domain	4 point mutations	Constructed a point mutation to decrease autoproteolysis followed bv 3 point mutants for crystallizability	Chen et al. (1996)
Human leptin	Trp100 to Glu	Single mutation produed a protein with improved solubility and propensity to crystallize	Zhang et al. (1997)
E. coli OmpA	3 point mutations	Two serendipitous mutations followed by a third rational led to a crystallizable protein	Pautsch et al. (1999)
24-kDa fragment of DNA gyrase from *E. coli*	9 point mutations	Surface mutants to investigate influence on crystallizability; all mutations had an effect	D'Arcy et al. (1999)
Human RhoGDl GTPase	Lys to Ala; Glu to Ala or Asp	Effect of surface mutants on crystallizability; all mutations except one crystallized	Longenecker et al. (2001), Mateja et al. (2002)
T. termophilus aspartyl-tRNA synthase	7 point mutations	Disrupted and introduced crystal contacts; increase in contacts correlated with crystallizability	Charron et al. (2002)

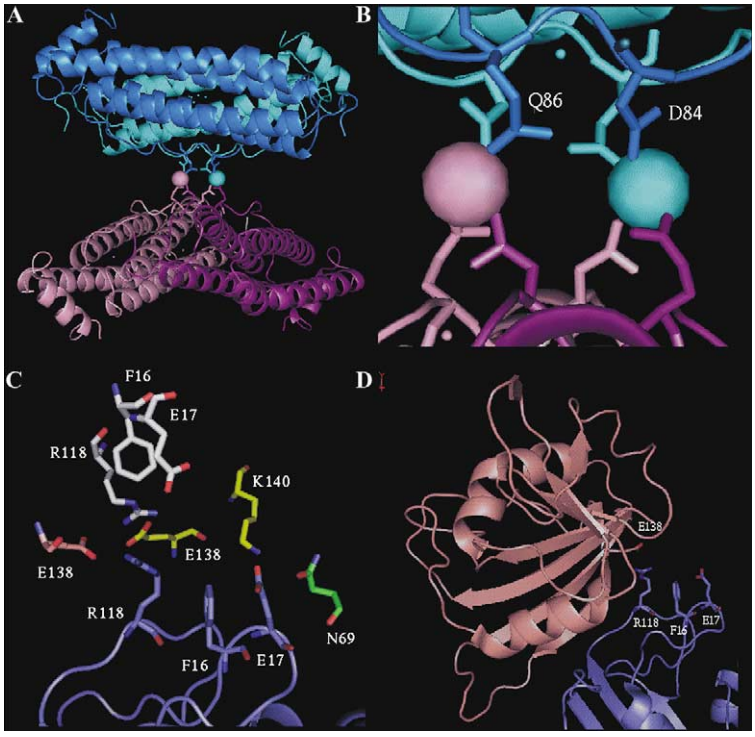

Fig. 1. (A and B) Tetrameric arrangement of human ferritin near the calcium binding site involving D84 and Q86 and their symmetry equivalents. (C) Crystal contacts as observed in the hexagonal crystal form of the wild-type chromosomal *S. aureus* DHFR (1DFR). All symmetry-related molecules are indicated with different colors. (D) The crystal contacts observed in the tetragonal crystal form of the mutant plasmid-encoded trimethoprim-resistant S1 DHFR from *S. aureus*. All figures were generated using PyMOL (DeLano (2001)).

co-workers analyzed the crystal contacts in the orthorhombic space group and constructed seven variant proteins with mutations at contact positions (Charron et al., 2002). The variants were chosen to have an altered charge distribution at their surface, modified local surface hydrophobicity, or perturbation of the H-bond pattern involved in crystal contacts. The authors found a correlation between disruption/addition of intermolecular interactions in the crystal packing and the crystallizability of AspRS-1 mutations: the disruption of contacts hinders crystallization and the addition of contacts favors it.

Several other examples of the crystallization of mutant proteins, in which the wild-type protein failed to crystallize, have been reported. The structural characterization of the human cytomegalovirus protease catalytic domain was possible only with directed mutagenesis. Initially a mutant was made to eliminate autoproteolysis and was followed by four single-point mutations on surface loops to optimize the crystallization properties (Chen et al., 1996). During the work with central core domain HIV integrase (50-512) the authors speculated that a fortuitous replacement of solvent-accessible hydrophobic residues might lead to a protein that was more soluble and less aggregated. They used a strategy of replacing hydrophobic residues by lysine to alter the solubility of the protein, and eventually the F185K mutation showed a dramatic increase in solubility and led to crystallization (Dyda et al., 1994; Jenkins et al., 1995). In a similar manner, residues proposed to be solvent exposed (based on homology models) were mutated in the plasmid-encoded trimethoprim-resistant S1 DHFR from *S. aureus.* A double mutant showed a dramatic increase in solubility and the protein could be purified and crystallized; however, only poorly diffracting crystals could be obtained (Dale et al., 1994). An analysis of the crystal contacts observed in the hexagonal crystal form of the wild-type *S. aureus* chromosomal DHFR diffracting to 2.5 Å revealed the presence of three key residues, E16, F17, and R118, forming a complex set of interactions (Fig. 1C). The corresponding residues in the S1 DHFR were mutated to the wild-type sequence with the goal of reproducing the interface. Although better diffracting crystals could be obtained (1.9 Å resolution), a different crystal contact has been created in the tetragonal space group (Fig. 1D; Dale et al., manuscript in preparation). This study underscores the difficulty in rationalizing and predicting crystal contacts. A point mutation alters only a small fraction of the protein surface area and the influence of the remaining differences is difficult to predict. In the case of histidine ammonia lyase from *P. putida,* the tetrameric enzyme gave rise to undefined aggregates in the absence of thiols, and suitable crystals could not be obtained (Schwede et al., 1999). The solvent accessibility along the chain was predicted from the amino acid sequence and the only cysteine that was predicted to be solvent exposed was mutated to alanine. This abolished all undefined aggregations and readily yielded crystals diffracting isotropically to 1.8 Å resolution. However, not all mutations that resulted in crystalline protein were rational. In a number of cases serendipity played a role in the ability of the protein to crystallize. In the case of the chaperone GroEL the wild-type protein gave crystals that diffracted poorly. A double mutation that was inadvertently introduced by PCR during the cloning step, however, produced crystals with considerably better diffraction qualities (Braig et al., 1994). Another example is the bacterial outer membrane protein OmpA. Attempts to crystallize the protein had failed, the authors had turned to mass production of the protein in inclusion bodies, and the purified protein had produced very thin crystals (Pautsch et al., 1999). Using electrospray mass spectroscopy it was observed that the protein had a lower MW than expected and resequencing of the clone revealed the presence of two mutations in the external region of the protein. The authors then added a third mutation in the external region (Lys107 to Tyr), creating a protein that crystallized reproducibly and diffracted beyond 2 Å resolution.

4. Truncations and deletions

Proteins often have a modular structure consisting of several distinct domains that are tethered by flexible linkers. Because of the problems of protein solubility, size, or complexity it is often advantageous to work with truncated proteins, domains of proteins, or individual subunits of multimeric proteins rather than the native protein. Gross flexibility in a protein may be thought of as generating heterogeneity in structure. One general aspect of protein crystallization that is universally recognized is that rigid, stable proteins are much more likely to crystallize than proteins that are internally flexible or have dynamic surfaces. Thus, deleting or removing the flexible regions at the termini or within a protein may help in the crystallization by minimizing any interfering effects from the microheterogeneity. N-terminal and C-terminal deletions and truncations have become standard protocol in recombinant protein expression. However, insertions and deletions within the protein have received little attention, in part because of the expectation that they will severely compromise the stability of the folded conformation (Shortle and Sondek, 1995). In this view, it was quite surprising when the genetic analysis of a number of proteins revealed that insertions and deletions were frequently well tolerated, causing relatively small effects on biological function (Ay et al., 1998; Betton et al., 1997; Nagi and Regan, 1997; Nugent et al., 1996; Thompson and Eisenberg, 1999; Zhou et al., 1996).

Indeed there are several examples in which deletions have been employed successfully to improve the crystal properties of the protein (Table 2). Using the three-dimensional structure of an active, disulfide cross-linked dimer of the ligand-binding domain of the *S. typhimurium* aspartate receptor, Yeh and co-workers removed the regions which were disordered in the initial electron-density map (Milburn et al., 1991; Yeh et al., 1996). They deleted the residues from the N- and C-terminus of the ligand-binding domain, improving the resolution from 3 to 1.85 Å. In a similar study with the 24-kDa fragment of DNA gyrase from *S. aureus,* crystallization trials with the purified wild-type enzyme were only partially successful. It was possible to grow crystals diffracting to 3 Å resolution only in the presence of the ligand cyclothialidine (Dale et al., 1999). Analysis of the X-ray structure of the SaGyrB (1-234)–cyclothialidine binary complex as well as comparison to the *E. coli* p24-novobiocin complex revealed two regions for which there was no electron density. In the *E. coli* complex electron density was lacking for the N-terminal 13 amino acids and the loop region comprising residues 99–118 was also disordered (Kostrewa et al., unpublished results; Lewis et al., 1996). These regions correspond to residues 1–23 and 109–127 of the *S. aureus* enzyme (Brockbank and Barth, 1993). These regions were systematically removed by deletion mutagenesis and in the initial crystallization trials only the mutants with the deletion of loop region corresponding to residues 105–127 crystallized readily and diffracted to approximately 2 Å resolution. In the case of the ligand binding domain of the ionotropic glutamate receptor crystallization was feasible only after a process of limited proteolysis followed by deletion mutagenesis. The combined approach culminated with an active, stable protein that gave crystals which diffracted beyond 1.5 Å resolution (Armstrong et al., 1998; Chen et al., 1998).

5. Fusion proteins

Recombinant techniques in which genes or gene fragments are spliced together to form gene fusions have become a standard tool for biochemistry and biotechnology, in particular to produce a soluble protein and to facilitate the purification from a crude extract (Ford et al., 1991; Sheibani, 1999; Uhlen and Moks, 1990). However, there are very few examples of fusion proteins being crystallized (Table 2). Perhaps the most reasonable explanation is the inherent flexibility of a fusion protein, mainly due to the addition of linker segments between the gene of interest and the fusion partner. The linker segments code for protease cleavage sites and provide DNA restriction sites to simplify cloning. The principle of using a crystallization tag of known tertiary structure is appealing since the molecular replacement method using the known structure of the fusion partner can, in principle, be applied more easily than for crystals

Table 2
Examples of truncations, deletions, and fusion proteins for crystallization

Structure (source)	Mutations	Comments	Reference
S. typhimurium aspartate receptor	N- and C-terminal deletions	Regions of protein for which no electron density was observed were deleted from the terminii	Yeh et al. (1996)
Ionotropic glutamate receptor iGluR2	Multiple deletions and truncations	Limited proteolysis, deletion mutagenesis, and multiple sequence alignment identified the minimal functional ligand binding domain	Chen et al. (1998)
24-kDa fragment of DNA gyrase from *S. aureus*	Residues 1–22 and 105–126 were deleted	Residues for which no electron density was observed in the initial map were systematically deleted	Dale et al. (1999)
B. macerans 1,3-1,4-β-glucanase/ 1,4-β-xylanase of *B. licheniformis* fusion	Insertion into loop	Insertion of entire xylanase domain into a surface loop of glucanase	Ay et al. (1998)
Bovine poly(A) polymerase	C-terminal deletion of 226 residues	WT protein failed to crystallize; mutant formed high-quality crystals	Martin et al. (2000)
DNA-binding domain of DNA replication-related element-binding factor	GST fusion	Fusion to GST with thrombin cleavage site	Kuge et al. (1997)
Human T-cell leukemia virus type I transmembrane protein gp21	MBP fusion	Gp21 (335–445) was fused to MBP via a 3-Ala linker to stabilize the helix	Center et al. (1998)
D. discoideum dynamin GTPase domain	Myosin II fusion	The dynamin A GTPase domain (2–316) was fused to the C-terminus of the *D. discoideum* myosin II catalytic domain	Niemann et al. (2001)
SarR from *S. aureus*	MBP fusion	Fused to MBP via a truncated linker for reduced flexibility	Liu et al. (2001)

of the target protein alone. Fusion proteins, or crystallization tags of known tertiary structure, have been used for the crystallization of short peptides and their structures could be subsequently determined (Dale et al., unpublished results; Donahue et al., 1994; Lim et al., 1994).

Various approaches have been used to design fusion proteins as crystallization tags. Many attempts to clone into the multiple cloning site of a commercial vector have met with limited success. More recently, researchers have modified the vector to reduce the flexibility and the size of the linker, with limited success, however (Center et al., 1998; Liu et al., 2001). Prive and co-workers proposed inserting a carrier molecule into the loops of proteins as the tag (Prive and Kaback, 1996; Prive et al., 1994). Perhaps the first example of fusion tags used for the crystallization of a protein was the DNA-binding domain of DNA replication-element related-binding factor (DREF) fused to glutathione *S*-transferase (GST) (Kuge et al., 1997). GST and the DNA binding domain of DREF were linked by the sequence Asp-Leu-Val-Pro-Arg-Gly-Ser, and since the structure of this linker is unknown the general applicability of the GST tag for carrier-protein-driven crystallization is difficult to predict. Additionally, the maltose binding protein (MBP) has been applied successfully as a crystallization tag. Fusions to MBP were used to determine the structure of the gp21 ectodomain segment (lacking a fusion peptide and the 20 C-terminal residues) of the human T cell leukemia virus type I transmembrane protein as well as the *Staphylococcus* accessory regulator (SAR) from *S. aureus* (Center et al., 1998; Liu et al., 2001). Crystals were not obtained for gp21 when connected to MBP via a flexible linker but were obtained when the MBP C-terminal α-helix was connected to the gp21 N-terminal α-helical sequence via three alanine residues. The N-terminal α-helix of gp21 is roughly perpendicular to the C-terminal helix of MBP via the linker sequence. In the case of SAR, the protein was fused to the C-terminus of MBP using a truncated linker in order to reduce flexibility. The SAR dimer is located at the top of two individual MBP molecules, connected by two loops with residues from both SAR and modified MBP.

6. Perspectives

There has been remarkable progress in the method development in protein crystallography in recent years, and the challenge to determine the three-dimensional structures of all the proteins in the human genome has been taken up by the many structural genomics initiatives. With the advances that have been made for improved data collection at synchrotron sources and software developments for structure determination the speed at which structures can be solved has been dramatically accelerated. Although advances have also been seen in the area of automation and miniaturization of crystallization experiments, the production of high-quality protein crystals remains an area where many problems need to be solved. This is highlighted by the relatively low ratio of proteins cloned and expressed to those producing suitable crystals in the structural genomics initiatives. We have learned important lessons from these studies. The size of the crystallization screen used is not necessarily proportional to the number of hits that can be expected, but rather the quality of the proteins used should be carefully controlled. Many investigators have found that dynamic light scattering has proved to be a simple and reliable tool for this purpose. DeTitta and co-workers have confirmed this in their structural genomics study in which proteins were first analyzed using dynamic light scattering; their success rate is significantly higher than that in most of the other studies (Service, 2002).

However, even in cases where the protein is both soluble and suitable for crystallization there are still many examples which it proves difficult or impossible to obtain crystals. There are considerable data to support the proposal of Longenecker et al. (2001), who have correctly observed that "the protein itself rather then the precipitating agent should be considered as the most important variable in the screening process." Many ways to modify a protein's crystallization properties have been described over the years: complex formation, enzymatic deglycosylation, and limited proteolysis are some examples. By far the most exciting development has been in the application of mutagenesis to alter a protein's crystallization properties.

One conclusion that can be drawn from the various studies is that mutations can have a dramatic effect on the crystallization behavior of a protein and that an improvement in crystal quality can generally be obtained. Perhaps most important is that the various studies have demonstrated that only a limited number of mutations are required to achieve an improvement in yield or quality of crystals. However, although there are numerous examples of mutagenesis as a tool to enhance crystallization it has not been used widely. This is probably due, in part, to the lack of established protocols with regard to the type of mutation that is required. In addition, defining a rational strategy was by no means a trivial task.

In our opinion, the use of fusion proteins will probably be minimal, as it appears that the choice of linker sequence and of fusion partner for each protein will be critical to the success of the approach. Although there have been successful examples, our experience with fusion proteins has been somewhat sobering. We have attempted fusing a number of proteins to GST, MBP, and thioredoxin with both flexible and stabilized linkers

without success. Moreover, we have tried the carrier protein approach, inserting cytochrome b_{562} into loops of flexible regions of both the 24-kDa fragment of DNA gyrase from *S. aureus* and the *S. aureus* S1 DHFR without success (Dale et al., unpublished results). On a similar note, the use of deletion mutants will be most appropriate in the initial phases of producing a soluble protein amenable to crystallization. Employing deletions or insertions at a later stage for improving crystallization or crystal quality will probably be limited to examples in which an initial electron density map is available. In this case, we recommend considering the secondary structure linker fragments from known protein structures retaining the geometry and distance constraints while incorporating residues favorable for crystal contact formation.

In the cases in which a protein fails to crystallize or only crystals of poor quality are obtained we feel that rational, surface mutagenesis should be regarded as a suitable tool in the preparation of X-ray-quality protein crystals. For proteins for which the structure of a homologue is available, a valid approach is to mutate residues predicted to be on the surface of the protein to those residues of the homologue that are favorable for crystal contact formation. It is important to note that not all mutations will result in crystal contacts, although the alteration of the surface properties may lead to suitable crystals. In the case in which no structural information is available, we recommend mutating lysine and glutamate residues (the least favored residues in crystal contacts) with an approximately 90% chance of being surface exposed to arginine or glutamine residues (the most favored residues found in crystal contacts) (Baud and Karlin, 1999; Dasgupta et al., 1997).

In conclusion, the amount of knowledge generated by the various studies cited in this review should serve to define strategies for protein characterization, crystallization screening, and protein modification using rational mutagenesis, which will allow us to establish reliable protocols to accelerate the path from gene to structure. The underlying lesson from this study, which cannot be overemphasized, is that the protein itself remains the most important variable in the crystallization process.

Acknowledgments

The authors thank Alice Douangamath and Aengus MacSweeney for critically reading the manuscript.

References

Abola, E., Kuhn, P., Earnest, T., Stevens, R.C., 2000. Automation of X-ray crystallography. Nat. Struct. Biol. 7 (Suppl.), 973–977.

Armstrong, N., Sun, Y., Chen, G.Q., Gouaux, E., 1998. Structure of a glutamate-receptor ligand-binding core in complex with kainate. Nature 395, 913–917.

Ay, J., Gotz, F., Borriss, R., Heinemann, U., 1998. Structure and function of the Bacillus hybrid enzyme GluXyn-1: native-like jellyroll fold preserved after insertion of autonomous globular domain. Proc. Natl. Acad. Sci. USA 95, 6613–6618.

Baker, H.M., Day, C.L., Norris, G.E., Baker, E.N., 1994. Enzymatic deglycosylation as a tool for crystallization of mammalian binding proteins. Acta Crystallogr. D 50, 380–384.

Banner, D.W., D'Arcy, A., Chene, C., Winkler, F.K., Guha, A., Konigsberg, W.H., Nemerson, Y., Kirchhofer, D., 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature 380, 41–46.

Baud, F., Karlin, S., 1999. Measures of residue density in protein structures. Proc. Natl. Acad. Sci. USA 96, 12494–12499.

Betton, J.M., Jacob, J.P., Hofnung, M., Broome-Smith, J.K., 1997. Creating a bifunctional protein by insertion of beta-lactamase into the maltodextrin-binding protein. Nat. Biotechnol. 15, 1276–1279.

Blundell, T.L., Jhoti, H., Abell, C., 2002. High-throughput crystallography for lead discovery in drug design. Nat. Rev. Drug Discovery 1, 45–54.

Boettner, M., Prinz, B., Holz, C., Stahl, U., Lang, C., 2002. High-throughput screening for expression of heterologous proteins in the yeast Pichia pastoris. J. Biotechnol. 99, 51–62.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimiak, A., Horwich, A.L., Sigler, P.B., 1994. The crystal structure of the bacterial chaperonin GroEL at 2.8 A. Nature 371, 578–586.

Brockbank, S.M., Barth, P.T., 1993. Cloning, sequencing, and expression of the DNA gyrase genes from Staphylococcus aureus. J. Bacteriol. 175, 3269–3277.

Campbell, J.W., Duee, E., Hodgson, G., Mercer, W.D., Stammers, D.K., Wendell, P.L., Muirhead, H., Watson, H.C., 1972. X-ray diffraction studies on enzymes in the glycolytic pathway. Cold Spring Harbor Symp. Quant. Biol. 36, 165–170.

Carugo, O., Argos, P., 1997. Protein–protein crystal-packing contacts. Protein Sci. 6, 2261–2263.

Center, R.J., Kobe, B., Wilson, K.A., The, T., Howlett, G.J., Kemp, B.E., Poumbourios, P., 1998. Crystallization of a trimeric human T cell leukemia virus type 1 gp21 ectodomain fragment as a chimera with maltose-binding protein. Protein Sci. 7, 1612–1619.

Charron, C., Kern, D., Giege, R., 2002. Crystal contacts engineering of aspartyl-tRNA synthetase from Thermus thermophilus: effects on crystallizability. Acta Crystallogr. D Biol. Crystallogr. 58, 1729–1733.

Chayen, N.E., Saridakis, E., 2002. Protein crystallization for genomics: towards high-throughput optimization techniques. Acta Crystallogr. D Biol. Crystallogr. 58, 921–927.

Chen, G.Q., Sun, Y., Jin, R., Gouaux, E., 1998. Probing the ligand binding domain of the GluR2 receptor by proteolysis and deletion mutagenesis defines domain boundaries and yields a crystallizable construct. Protein Sci. 12, 2623–2630.

Chen, P., Tsuge, H., Almassy, R.J., Gribskov, C.L., Katoh, S., Vanderpool, D.L., Margosiak, S.A., Pinko, C., Matthews, D.A., Kan, C.C., 1996. Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad. Cell 86, 835–843.

Claverie, J.M., Monchois, V., Audic, S., Poirot, O., Abergel, C., 2002. In search of new anti-bacterial target genes: a comparative/structural genomics approach. Comb. Chem. High Throughput Screen. 5, 511–522.

Dale, G.E., Broger, C., Langen, H., D'Arcy, A., Stuber, D., 1994. Improving protein solubility through rationally designed amino acid replacements: solubilization of the trimethoprim-resistant type S1 dihydrofolate reductase. Protein Eng. 7, 933–939.

Dale, G.E., D'Arcy, B., Yuvaniyama, C., Wipf, B., Oefner, C., D'Arcy, A., 2000. Purification and crystallization of the extracellular domain of human neutral endopeptidase (neprilysin) expressed in Pichia pastoris. Acta Crystallogr. D 56, 894–897.

Dale, G.E., Kostrewa, D., Gsell, B., Stieger, M., D'Arcy, A., 1999. Crystal engineering: deletion mutagenesis of the24 kDa fragment of the DNA gyrase B subunit from Staphylococcus aureus. Acta Crystallogr. D 55, 1626–1629.

D'Arcy, A., 1994. Crystallising proteins—A rational approach. Acta Crystallogr. D 50, 469–471.

D'Arcy, A., Mac Sweeney, A., Haber, A., 2003. The advantages of using a modified microbatch method for rapid screening of protein crystallization conditions. Acta Crystallogr. D, in press.

D'Arcy, A., Stihle, M., Kostrewa, D.A., Dale, G., 1999. Crystal engineering: a case study using the24 kDa fragment of the DNA gyrase B subunit from *Escherichia coli*. Acta Crystallogr. D 55, 1623–1625.

Dasgupta, S., Iyer, G.H., Bryant, S.H., Lawrence, C.E., Bell, J.A., 1997. Nature of contacts between protein molecules in crystal lattices and between subunits of protein oligomers. Proteins 28, 494–514.

Davis, S.J., Brady, R.L., Barclay, A.N., Harlos, K., Dodson, G.G., Williams, A.F., 1990. Crystallization of a soluble form of the rat T-cell surface glycoprotein CD4 complexed with Fab from the W3/25 monoclonal antibody. J. Mol. Biol. 213, 7–10.

DeLano, W.L., 2001. The PyMOL User's Manual. DeLano Scientific, San Carlos, CA.

Dieckman, L., Gu, M., Stols, L., Donnelly, M.I., Collart, F.R., 2002. High throughput methods for gene cloning and expression. Protein Expr. Purif. 25, 1–7.

Ding, H.T., Ren, H., Chen, Q., Fang, G., Li, L.F., Li, R., Wang, Z., Jia, X.Y., Liang, Y.H., Hu, M.H., Li, Y., Luo, J.C., Gu, X.C., Su, X.D., Luo, M., Lu, S.Y., 2002. Parallel cloning, expression, purification and crystallization of human proteins for structural genomics. Acta Crystallogr. D Biol. Crystallogr. 58, 2102–2108.

Donahue, J.P, Patel, H., Anderson, W.F., Hawiger, J., 1994. Three-dimensional structure of the platelet integrin recognition segment of the fibrinogen gamma chain obtained by carrier protein-driven crystallization. Proc. Natl. Acad. Sci. USA 91, 12178–12182.

Dyda, F., Hickman, A.B., Jenkins, T.M., Engelman, A., Craigie, R., Davies, D.R., 1994. Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. Science 266, 1981–1986.

Ferre-D'Amare, A., Burley, S., 1997. Dynamic light scattering in evaluating crystallisability of macromolecules. Methods Enzymol. 276, 157–166.

Ford, C.F., Suominen, I., Glatz, C.E., 1991. Fusion tails for the recovery and purification of recombinant proteins. Protein Expr. Purif. 2, 95–107.

Gruenunger-Leitch, F., D'Arcy, A., D'Arcy, B., Chene, C., 1996. Deglycosylation of proteins for crystallization using recombinant fusion protein glycosidases. Protein Sci. 12, 2617–2622.

Holden, H.M., Wesenberg, G., Raynes, D.A., Hartshorne, D.J., Guerriero, V., Rayment, I., 1996. Molecular structure of a proteolytic fragment of TLP20. Acta Crystallogr. D 52, 1153–1160.

Janin, J., Rodier, F., 1995. Protein–protein interaction at crystal contacts. Proteins 23, 580–587.

Jelsch, C., Longhi, S., Cambillau, C., 1998. Packing forces in nine crystal forms of cutinase. Proteins 31, 320–333.

Jenkins, T.M., Hickman, A.B., Dyda, F., Ghirlando, R., Davies, D.R., Craigie, R., 1995. Catalytic domain of human immunodeficiency virus type1 integrase: identification of a soluble mutant by systematic replacement of hydrophobic residues. Proc. Natl. Acad. Sci. USA 92, 6057–6061.

Karain, W.I., Bourenkov, G.P., Blume, H., Bartunik, H.D., 2002. Automated mounting, centering and screening of crystals for high-throughput protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 58, 1519–1522.

Kirchhofer, D., Guha, A., Nemerson, Y., Konigsberg, W.H., Vilbois, F., Chene, C., Banner, D.W., D'Arcy, A., 1995. Activation of blood coagulation factor VIIa with cleaved tissue factor extracellular domain and crystallization of the active complex. Proteins 22, 419–425.

Kokkinidis, M., Vlassi, M., Papanikolaou, Y., Kotsifaki, D., Kingswell, A., Tsernoglou, D., Hinz, H.J., 1993. Correlation between protein stability and crystal properties of designed ROP variants. Proteins 16, 214–216.

Kostrewa, D., Grüninger-Leitch, F., D'Arcy, A., Broger, C., Mitchell, D., van Loon, A.P.G.M., 1997. Crystal structure of phytase from Aspergillus ficuum at 2.5 Å resolution. Nat. Struct. Biol. 4, 185–190.

Kostrewa, D., Wyss, M., D'Arcy, A., van Loon, A.P., 1999. Crystal structure of Aspergillus niger pH 2.5 acid phosphatase at 2. 4 Å resolution. J. Mol. Biol. 288, 965–974.

Krupka, H.I., Rupp, B., Segelke, B.W., Lekin, T.P., Wright, D., Wu, H.C., Todd, P., Azarani, A., 2002. The high-speed Hydra-Plus-One system for automated high-throughput protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 58, 1523–1526.

Kuge, M., Fujii, Y., Shimizu, T., Hirose, F., Matsukage, A., Hakoshima, T., 1997. Use of a fusion protein to obtain crystals suitable for X-ray analysis: crystallization of a GST-fused protein containing the DNA-binding domain of DNA replication-related element-binding factor, DREF. Protein Sci. 6, 1783–1786.

Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Smith, J.M., Livingstone, J.C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., 1991. Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. Nature 349, 541–544.

Leppanen, V.M., Parast, C.V., Wong, K.K., Kozarich, J.W., Goldman, A., 1999. Purification and crystallization of a proteolytic fragment of *Escherichia coli* pyruvate formate-lyase. Acta Crystallogr. D Biol. Crystallogr. 55, 531–533.

Lesley, S.A., Kuhn, P., Godzik, A., Deacon, A.M., Mathews, I., Kreusch, A., Spraggon, G., Klock, H.E., McMullan, D., Shin, T., Vincent, J., Robb, A., Brinen, L.S., Miller, M.D., McPhillips, T.M., Miller, M.A., Scheibe, D., Canaves, J.M., Guda, C., Jaroszewski, L., Selby, T.L., Elsliger, M.A., Wooley, J., Taylor, S.S., Hodgson, K.O., Wilson, I.A., Schultz, P.G., Stevens, R.C., 2002. Structural genomics of the Thermotoga maritima proteome implemented in a high-throughput structure determination pipeline. Proc. Natl. Acad. Sci. USA 99, 11664–11669.

Leslie, A.G., Powell, H.R., Winter, G., Svensson, O., Spruce, D., McSweeney, S., Love, D., Kinder, S., Duke, E., Nave, C., 2002. Automation of the collection and processing of X-ray diffraction data—A generic approach. Acta Crystallogr. D Biol. Crystallogr. 58, 1924–1928.

Lewis, R.J., Singh, O.M., Smith, C.V., Skarzynski, T., Maxwell, A., Wonacott, A.J., Wigley, D.B., 1996. The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. EMBO J. 15, 1412–1420.

Lim, K., Ho, J.X., Keeling, K., Gilliland, G.L., Ji, X., Ruker, F., Carter, D.C., 1994. Three-dimensional structure of Schistosoma japonicum glutathione *S*-transferase fused with a six-amino acid conserved neutralizing epitope of gp41 from HIV. Protein Sci. 3, 2233–2244.

Liu, Y., Manna, A., Li, R., Martin, W.E., Murphy, R.C., Cheung, A.L., Zhang, G., 2001. Crystal structure of the SarR protein from Staphylococcus aureus. Proc. Natl. Acad. Sci. USA 98, 6877–6882.

Longenecker, K.L., Garrard, S.M., Sheffield, P.J., Derewenda, Z.S., 2001. Protein crystallization by rational mutagenesis of surface residues: Lys to Ala mutations promote crystallization of RhoGDI. Acta Crystallogr. D 57, 679–688.

Luft, J.R., Wolfley, J., Jurisica, I., Glasgow, J., Fortier, S., DeTitta, G.T., 2001. Macromolecular crystallization in a high throughput laboratory—The search phase. J. Cryst. Growth 232, 591–595.

Martin, G., Keller, W., Doublie, S., 2000. Crystal structure of mammalian poly(A) polymerase in complex with an analog of ATP. EMBO J. 19, 4193–4203.

Mateja, A., Devedjiev, Y., Krowarsch, D., Longenecker, K., Dauter, Z., Otlewski, J., Derewenda, Z.S., 2002. The impact of GluAla and GluAsp mutations on the crystallization properties of RhoGDI: the structure of RhoGDI at 1.3 Å resolution. Acta Crystallogr. D 58, 1983–1991.

McElroy, H.E., Sisson, G.W., Schoettlin, W.E., Aust, R.M., Villafranca, J.E., 1992. J. Cryst. Growth 122, 265–272.

McPherson, A., 1982. Preparation and Analysis of Protein Crystals. Wiley, New York.

Milburn, M.V., Prive, G.G., Milligan, D.L., Scott, W.G., Yeh, J., Jancarik, J., Koshland Jr., D.E., Kim, S.H., 1991. Three-dimensional structures of the ligand-binding domain of the bacterial aspartate receptor with and without a ligand. Science 254, 1342–1347.

Mittl, P.R., Schulz, G.E., 1994. Structure of glutathione reductase from *Escherichia coli* at 1.86 Å resolution: comparison with the enzyme from human erythrocytes. Protein Sci. 3, 799–809.

Nagi, A.D., Regan, L., 1997. An inverse correlation between loop length and stability in a four-helix-bundle protein. Fold Des. 2, 67–75.

Niemann, H.H., Knetsch, M.L., Scherer, A., Manstein, D.J., Kull, F.J., 2001. Crystal structure of a dynamin GTPase domain in both nucleotide-free and GDP-bound forms. EMBO J. 20, 5813–5821.

Nugent, P.G., Albert, A., Orprayoon, P., Wilsher, J., Pitts, J.E., Blundell, T.L., Dhanaraj, V., 1996. Protein engineering loops in aspartic proteinases: site-directed mutagenesis, biochemical characterization and X-ray analysis of chymosin with a replaced loop from rhizopuspepsin. Protein Eng. 9, 885–893.

Oefner, C., D'Arcy, A., Hennig, M., Winkler, F.K., Dale, G.E., 2000. Structure of human neutral endopeptidase (Neprilysin) complexed with phosphoramidon. J. Mol. Biol. 296, 341–349.

Orengo, C.A., Todd, A.E., Thornton, J.M., 1999. From protein structure to function. Curr. Opin. Struct. Biol. 9, 374–382.

Ostermeier, C., Harrenga, A., Ermler, U., Michel, H., 1997. Structure at 2.7 Å resolution of the Paracoccus denitrificans two-subunit cytochrome c oxidase complexed with an antibody FV fragment. Proc. Natl. Acad. Sci. USA 94, 10547–10553.

Pautsch, A., Vogt, J., Model, K., Siebold, C., Schulz, G.E., 1999. Strategy for membrane protein crystallization exemplified with OmpA and OmpX. Proteins 1, 167–172.

Peitsch, M.C., 2002. About the use of protein models. Bioinformatics 7, 934–938.

Prive, G.G., Kaback, H.R., 1996. Engineering the lac permease for purification and crystallization. J. Bioenerg. Biomembr. 28, 29–34.

Prive, G.G., Verner, G.E., Weitzman, C., Zen, K.H., Eisenberg, D., Kaback, H.R., 1994. Fusion proteins as tools for crystallisation: the lactose permease from *Escherichia coli*. Acta Crystallogr. D 50, 375–379.

Prongay, A.J., Smith, T.J., Rossmann, M.G., Ehrlich, L.S., Carter, C.A., McClure, J., 1990. Preparation and crystallization of a human immunodeficiency virus p24–Fab complex. Protein Eng. 7, 933–939.

Schwede, T., Diemand, A., Guex, N., Peitsch, M.C., 2000. Protein structure computing in the genomic era. Res. Microbiol. 151, 107–112.

Schwede, T.F., Badeker, M., Langer, M., Retey, J., Schulz, G.E., 1999. Homogenization and crystallization of histidine ammonia-lyase by exchange of a surface cysteine residue. Protein Eng. 2, 151–153.

Service, R.F., 2002. Structural genomics. Tapping DNA for structures produces a trickle. Science 298, 948–950.

Sheibani, N., 1999. Prokaryotic gene fusion expression systems and their use in structural and functional studies of proteins. Prep. Biochem. Biotechnol. 29, 77–90.

Shih, Y.P., Kung, W.M, Chen, J.C., Yeh, C.H., Wang, A.H., Wang, T.F., 2002. High-throughput screening of soluble recombinant proteins. Protein Sci. 11, 1714–1719.

Shortle, D., Sondek, J., 1995. The emerging role of insertions and deletions in protein engineering. Curr. Opin. Biotechnol. 4, 387–393.

Sogabe, S., Masubuchi, M., Sakata, K., Fukami, T.A., Morikami, K., Shiratori, Y., Ebiike, H., Kawasaki, K., Aoki, Y., Shimma, N., D'Arcy, A., Winkler, F.K., Banner, D.W., Ohtsuka, T., 2002. Crystal structures of Candida albicans N-myristoyltransferase with two distinct inhibitors. Chem. Biol. 10, 1119–1128.

Stevens, R.C., 2000a. High-throughput protein crystallization. Curr. Opin. Struct. Biol. 5, 558–563.

Stevens, R.C., 2000b. Design of high-throughput methods of protein production for structural biology. Struct. Fold. Des. 8, R177–185.

Sulzenbacher, G., Gruez, A., Roig-Zamboni, V., Spinelli, S., Valencia, C., Pagot, F., Vincentelli, R., Bignon, C., Salomoni, A., Grisel, S., Maurin, D., Huyghe, C., Johansson, K., Grassick, A., Roussel, A., Bourne, Y., Perrier, S., Miallau, L., Cantau, P., Blanc, E., Genevois, M., Grossi, A., Zenatti, A., Campanacci, V., Cambillau, C., 2002. A medium-throughput crystallization approach. Acta Crystallogr. D Biol. Crystallogr. 58, 2109–21015.

Thompson, M.J., Eisenberg, D., 1999. Transproteomic evidence of a loop-deletion mechanism for enhancing protein thermostability. J. Mol. Biol. 290, 595–604.

Uhlen, M., Moks, T., 1990. Gene fusions for purpose of expression: an introduction. Methods Enzymol. 185, 129–143.

Yeh, J.I., Biemann, H.P., Prive, G.G., Pandit, J., Koshland Jr., D.E., Kim, S.H., 1996. High-resolution structures of the ligand binding domain of the wild-type bacterial aspartate receptor. J. Mol. Biol. 262, 186–201.

Zhang, F., Basinski, M.B., Beals, J.M., Briggs, S.L., Churgay, L.M., Clawson, D.K., DiMarchi, R.D., Furman, T.C., Hale, J.E., Hsiung, H.M., Schoner, B.E., Smith, D.P., Zhang, X.Y., Wery, J.P., Schevitz, R.W., 1997. Crystal structure of the obese protein leptin-E100. Nature 387, 206–209.

Zhou, H.X., Hoess, R.H., DeGrado, W.F., 1996. In vitro evolution of thermodynamically stable turns. Nat. Struct. Biol. 3, 446–451.

Zulauf, M., D'Arcy, A., 1992. Light scattering of proteins as a criterion for crystallization. J. Cryst. Growth 122, 102–106.

Further reading

Stevens, R.C., Yokoyama, S., Wilson, I.A., 2000. Global efforts in structural genomics. Science 294, 89–92.