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# Rational Protein Crystallization by Mutational Surface Engineering

Ways & Means

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## Summary

**Protein crystallization constitutes a limiting step in structure determination by X-ray diffraction. Even if single crystals are available, inadequate physical quality may seriously limit the resolution of the available data and consequently the accuracy of the atomic model. Recent studies show that targeted mutagenesis of surface patches containing residues with large flexible side chains and their replacement with smaller amino acids lead to effective preparation of X-ray quality crystals of proteins otherwise recalcitrant to crystallization. Furthermore, this technique can also be used to obtain crystals of superior quality as compared to those grown for the wild-type protein, sometimes increasing the effective resolution by as much as 1 Å or more. Several recent examples of this new methodology suggest that the method has the potential to become a routine tool in protein crystallography.**

## Introduction

In spite of dramatic advances in macromolecular crystallography over the last three decades, preparation of well diffracting single crystals continues to be the time-limiting step. While structures can be often phased within minutes of rapid data collection experiments at synchrotron beamlines (Dauter, 2002), the effort involved in the preparation of X-ray quality crystals remains a substantial barrier to both high-throughput approaches and problem-oriented investigations. Current estimates based on information from the Structural Genomics Centers suggest that on average less than 30% of proteins expressed in soluble form in *E. coli* form crystals, of which only a portion yield X-ray quality specimens diffracting to 2.0 Å resolution or better (Dale et al., 2003).

As it is essentially impossible to predict crystallization conditions for any protein, the process relies on extensive screening of hundreds to thousands of conditions, including different precipitants, buffers, etc. Thus, development of high-throughput crystallization robots, able to prepare over 100,000 samples per day while miniaturizing the volumes, is often an integral part of the structural genomics efforts (Stevens, 2000). While these robots undoubtedly make an important impact on high-throughput research, they do not necessarily enhance the crystallization efficiency of new proteins because the success of protein crystallization is not directly correlated with the number of conditions tested (Segelke, 2001). Proteins that crystallize easily often do so with rather simple, commercially available screens. It has been estimated that ~80% of crystallizable proteins can be crystallized using only 50 different condition (Jancarik and Kim, 1991). In contrast, for proteins recalcitrant to crystallization, little is gained by extending the range of conditions. As the proverbial "low-hanging fruit," i.e., easily crystallized small- to medium-sized proteins, falls prey to high-throughput efforts, the "high-hanging fruit" presents an increasing challenge.

One of the ways to address the problem of efficient crystallization is homolog screening, pioneered over 50 years ago (Campbell et al., 1972; Kendrew et al., 1954). The concept is simple: if you cannot grow crystals of the protein you want, try growing crystals of a homologous protein with a similar or identical function. This approach, while often successful, has some drawbacks. It is not possible to predict, for example, if any of the homologs crystallize easier or at all. Also, each new gene must be subcloned with the purification procedures determined from scratch. Finally, for drug design purposes and other studies, a protein from a hyperthermophilic bacterium may be a relatively poor model for a human homolog, with perhaps no more than 15% sequence identity.

An alternative approach is to modify the target protein by recombinant methods. The first use of site-directed mutagenesis for crystal engineering was reported by Lawson et al. (1991), who reproduced crystal contacts from rat L ferritin in human ferritin H chain. A replacement of Lys86, found in the human sequence, with Glu, which occurs in rat, recreated a $Ca^{2+}$ binding bridge that mediates crystal contacts in the rat ortholog. Subsequently, McElroy et al. (1992) showed that mutagenesis of residues on the surface of thymidylate synthase resulted in dramatic changes in the protein's behavior in crystallization screens. Although this and subsequent reports clearly suggested an efficient experimental route, lack of specific protocols and guidelines as to how to alter the protein's surface appears to have precluded wider application of this strategy. Nonetheless, a number of proteins are known to have been crystallized in mutated form when wild-type crystals were unavailable, often by serendipity. For example, GroEL was crystallized using samples with two mutations accidentally introduced by PCR (Braig et al., 1994; Horwich, 2000).

We recently proposed a protocol for modification of the surface of the target protein, involving local and limited mutational exchange of large hydrophilic residues for alanines, as a rational strategy aimed at preparation of X-ray quality crystals. The procedure leads to local reduction of conformational entropy and generates contact-forming, conformationally homogeneous surface patches. The method shows considerable potential, and to date allowed for the crystallization of several novel proteins. It has also been used to generate new crystal forms diffracting to much higher resolution than the wild-type protein crystals. This paper is intended to

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briefly review the novel strategy and selected examples of its successful application.

## Results

### The Concept of Rational Surface Engineering for Crystallization

In very general terms, the free energy change that drives crystallization can be expressed as a sum of three components:

$$\Delta G = \Delta H - T(\Delta S_{protein} + \Delta S_{solvent}).$$

Given that the enthalpy values of intermolecular interactions in the crystal lattice are typically small, crystallization is very sensitive to entropy changes involving both the solvent and the protein. Incorporation of protein molecules into the lattice carries a negative entropy term, and this is an inescapable thermodynamic cost. This unfavorable term may be overcome by positive entropy from the release of water molecules bound to the protein as it is incorporated into the lattice. There is some evidence that the latter effects may at least in some cases exert a determining driving force in crystallization (Vekilov, 2003; Vekilov et al., 2002), even though in absolute terms this may amount to the release of no more than a few water molecules.

What is less appreciated is that the formation of crystal contacts involving ordering of surface side chains involves further loss of entropy. Thus, a protein with a surface populated with larger hydrophilic residues may in effect have an "entropy shield" preventing formation of intermolecular contacts required for crystallization. It should therefore be possible to overcome this barrier by selective replacement of bulky surface residues with small amino acids, e.g., Ala, with little or no conformational entropy.

Among the candidate exposed polar amino acids, lysines and glutamates play a particular role. With very few exceptions, they are located on the surface (Baud and Karlin, 1999), they have a large solvent exposed surface, and their side chains are characterized by high conformational entropy of $\sim$2 kcal/mol under normal conditions (Avbelj and Fele, 1998). Both lysines and glutamates are typically disfavored at interfaces in protein-protein complexes (Conte et al., 1999), most probably because the entropic cost of their incorporation into an ordered interface is prohibitive. We hypothesized that Lys and Glu residues can constitute a good target for surface modification and consequent crystal engineering.

### The Test Case—Human RhoGDI

In order to test the above hypothesis, we first studied the crystallization properties of a number of mutants of the human protein RhoGDI—the Rho-specific guanine nucleotide exchange inhibitor. This relatively small protein (202 residues) contains a disordered N-terminal domain and an immunoglobulin-like C-terminal domain comprising 130 amino acids. The human RhoGDI has a high combined Lys/Glu content of nearly 20% but is not an exception in this regard among many cytosolic regulatory proteins. Even without the disordered N-terminal fragment, RhoGDI is very difficult to crystallize, and the known crystals of the wild-type domain diffract to 2.5 Å with three molecules in the asymmetric unit (Keep et al., 1997).

A number of mutants of RhoGDI with between one and four Lys and/or Glu residues replaced with alanines were screened for crystallization properties (Longenecker et al., 2001a; Mateja et al., 2002). The multiple mutants involved residues sufficiently close together in sequence to allow for the use of a single primer in the QuikChange mutagenesis protocol. The results were very encouraging. Virtually every mutation significantly altered the crystallization properties of RhoGDI (Figure 1). Single-site mutants showed significant differences in the kinetics of crystallization as compared to the wild-type protein, albeit without changing the crystal symmetry. In contrast, multiple mutations yielded a number of new crystal forms. The crystal structures of selected mutants revealed that the new crystal forms contain crystals contacts formed directly by the mutated epitopes, as predicted by the hypothesis. Moreover, some of the crystals exhibited diffraction properties superior to the wild-type crystals, including the double-mutant E154A/E155A, which yielded data to 1.25 Å resolution (Mateja et al., 2002).

### Crystallization of Novel Proteins by Surface Engineering

The promising results obtained using the model system of RhoGDI paved the way for the application of the technique to novel proteins, otherwise resistant to crystallization or yielding relatively low-quality crystals. Some of the examples of studies that were brought to fruition by surface engineering include the following:

*1. The RGSL Domain from PDZRhoGEF* (Garrard et al., 2001; Longenecker et al., 2001b). The RGSL domains target the proteins that contain them to the $\alpha$ subunits of trimeric G proteins. The respective domain from the guanine nucleotide exchange factor PDZRhoGEF would not crystallize in the wild-type form. Five mutants were prepared: E90A/K91A, E123A/E126A, E131A/E134A, E171A/K172A, and K183A/E185A/E186A. All were expressed in *E. coli*, purified, and screened for crystallization using the commercial Hampton Research Crystal Screens I and II. Out of the five, the triple-mutant K183A/E185A/E186A yielded high-quality single crystals straight from the screen. The structure was readily solved by MAD using SeMet-labeled protein, and revealed an intimate crystal contact formed by two symmetry-related mutated loops (Figure 2).

*2. The* Yersinia pestis *LcrV Antigen* (Derewenda et al., 2004). LcrV (V antigen) is a multifunctional virulence factor in *Yersinia pestis*, the causative agent of plague. The protein regulates the translocation of cytotoxic effector proteins into the cytosol of mammalian cells via a type III secretion system and is an active and passive mediator of resistance to disease. Several groups have unsuccessfully tried to crystallize wild-type LcrV. Five multiple mutants (K40A/D41A/K42A, K54A/D55A/E57A, K72A/K73A, E155A/E156A/E159A, and K214A/E217A/K218A) were made for the N-terminally truncated ($\Delta$1–

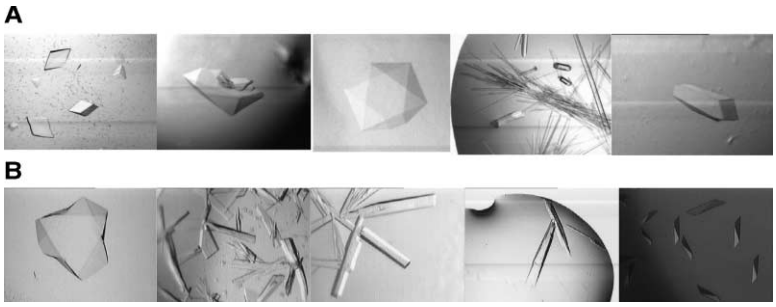


Figure 1. A Selection of Crystals

Obtained using (A) K → A mutants and (B) E → A mutants of human RhoGDI. For details see Longenecker et al. (2001a) and Mateja et al. (2002).

Δ23) LcrV protein, which has a wild-type activity. At the same time, to enhance sample homogeneity, the single cysteine in LcrV (Cys274) was replaced with a serine residue. Four mutants gave "hits" in screens. One, K40A/D41A/K42A, gave crystals that after optimization of conditions diffracted to 2.2 Å resolution. These crystals were used to solve the structure of LcrV by the MAD method. The refined model of the LcrV structure reveals that the epitope with the triple-surface mutation (K41A/D42A/K43A) mediates a crystal contact with the neighboring

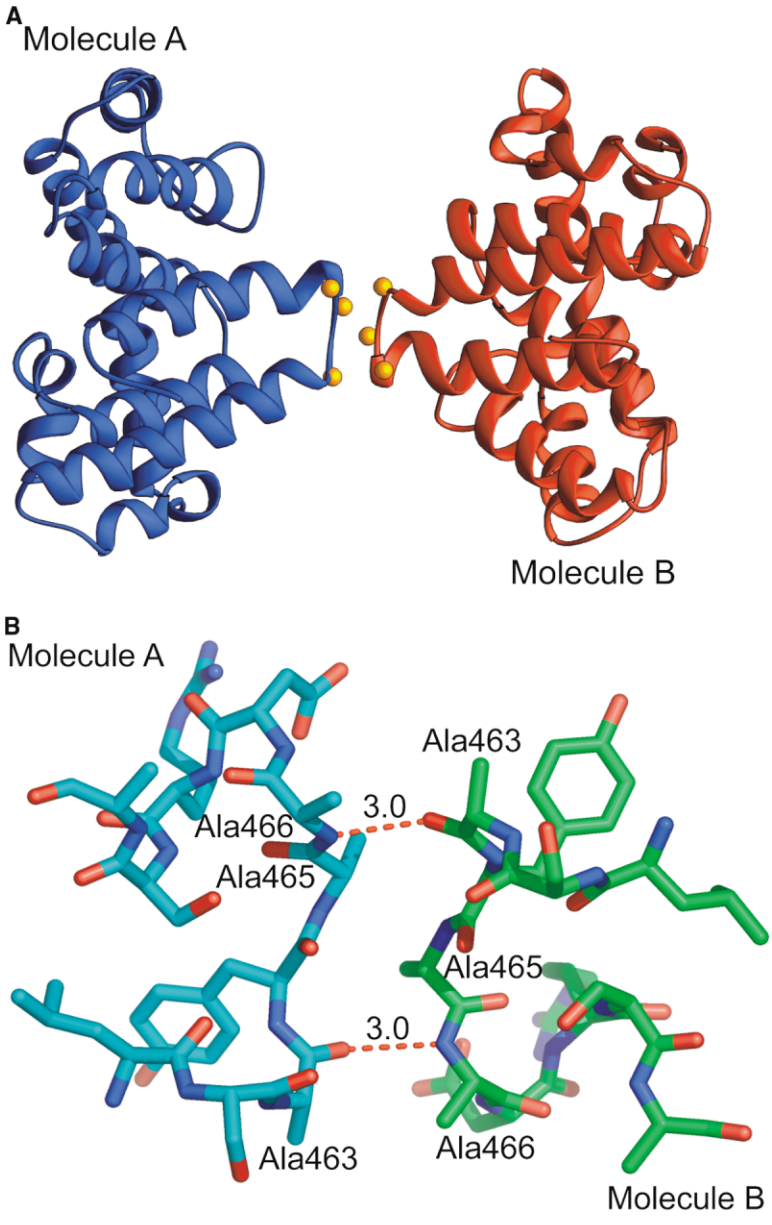


Figure 2. The RGSL Domain of PDZRhoGEF

(A) Arrangement of two molecules related by a 2-fold axis in the $P6_122$ unit cell, showing the three mutated sites.

(B) Close-up of the symmetric crystal contact.

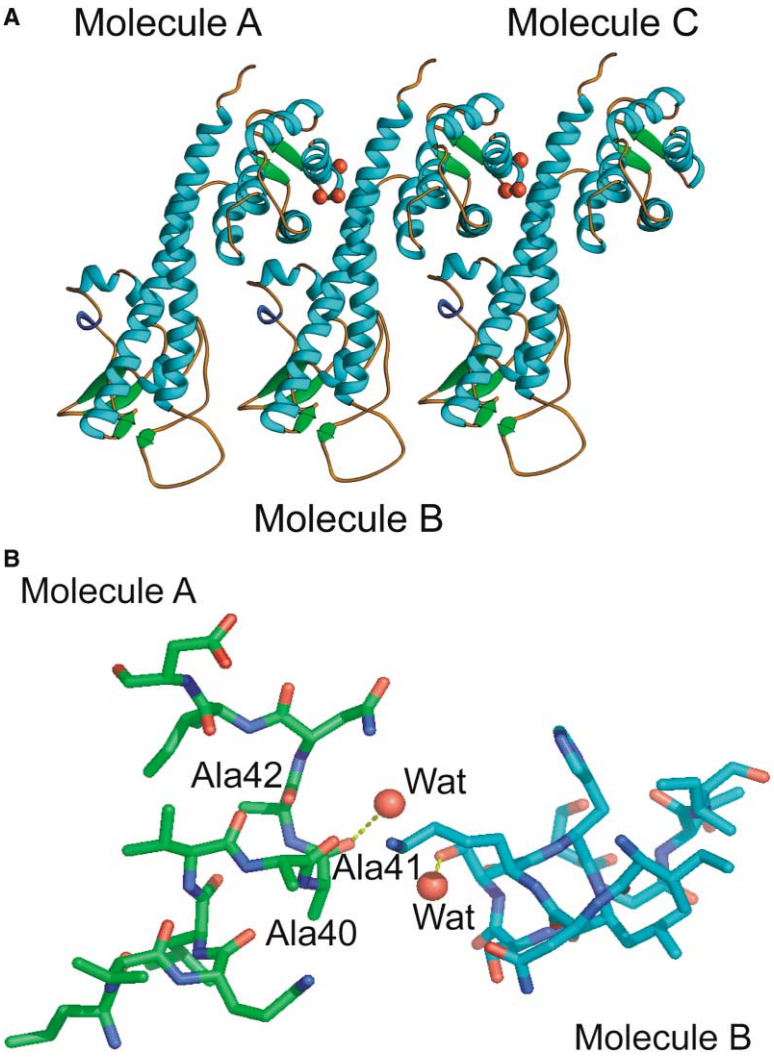


Figure 3. The Lcrv Antigen

(A) Packing of molecules in the P1 lattice.

(B) Details of the crystal contact involving the mutated patch.

molecule. These three residues constitute the last turn of helix $\alpha$-1, which forms an exposed knob that fits between two adjacent molecule in the crystal lattice (Figure 3). The absence of the long side chains that occupy these positions in the wild-type structure allowed the protein molecules to approach one another without excessive loss of conformational entropy.

*3. The Product of YkoF Gene from* B. subtilis (Y. Devedjiev et al., submitted). This protein, one of the targets of the Midwest Structural Genomics Center, failed to yield crystals in the wild-type form. Two double mutants were prepared, K33A/K34A and K112A/E114A, and the former gave high-quality crystals diffracting to 1.6 Å resolution. The structure was solved readily by MAD. The refined structure revealed a tertiary fold containing an internal repeat of an $(\alpha/\beta/\alpha)_2$ motif and a tightly associated homodimer in the asymmetric unit. The key crystal contact occurs between the homodimers and is mediated by the equivalent mutated surface patches from each of the two molecules. The exposed backbone carbonyls create an ideal $Ca^{2+}$ binding site between the molecules (Figure 4), and indeed $Ca^{2+}$ ions are required for these crystals to grow.

*4. The CUE:Ubiquitin Complex* (Prag et al., 2003). The Hurley group at NIH used the surface modification strategy to obtain crystals of the complex of the CUE domain of Vps9p with ubiquitin. The CUE domains are found in proteins participating in trafficking and ubiquitination pathways. A single double mutant, K435A/K436A, was generated, and its complex with ubiquitin yielded high-quality crystals diffracting to 1.7 Å resolution, with two CUE and two ubiquitin molecules in the asymmetric unit. As in the other examples, the mutated epitopes are involved in the crystal contacts, although no direct H bonds are seen. Instead, it appears that the lysines found in the wild-type protein would have caused steric problems, and the proximity of other positively charged residues would have made this contact unfavorable.

*5. The Tyrosine Kinase Domain of the Insulin-like Growth Factor-1 Receptor* (Munshi et al., 2003). The IGF-1R is a potential target for anticancer drug therapy (Baserga, 1996). However, the crystal structure of the unphosphorylated apo form of the kinase domain was solved at 2.7 Å resolution, too low for proper drug optimization. The Merck investigators prepared three mutants, K1025A/K1026A, E1067A/E1069A, and K1237A/E1238A/

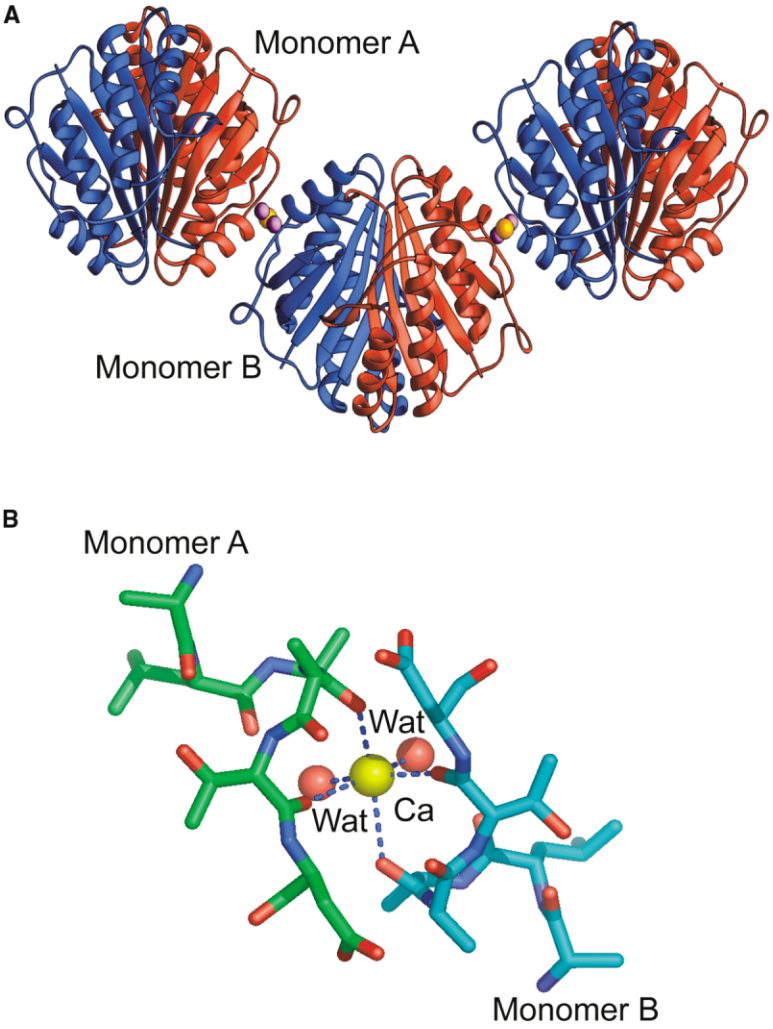


Figure 4. YkoF Crystal Structure

(A) Packing of molecules along the $2_1$ axis mediated by the Ca binding site.

(B) Details of the Ca-mediated crystal contact.

E1239A. Of these, the second double mutant yielded crystals diffracting to 1.5 Å resolution. Interestingly, the 1067/1069 sites occur at the N terminus of the kinase insert region, which in the wild-type crystals is disordered. In contrast, in the mutant crystals, the fragment is completely ordered. Comparison of the two structures shows that, in the wild-type lattice, E1067 and E1069 would have been in proximity of E1253 and E1254, creating an unstable negatively charged constellation. Thus, the reason for the crystallization of this mutant is similar to that behind the CUE:ubiquitin complex.

## Discussion

Rational protein surface engineering offers an effective crystallization strategy through engineering of patches conducive to crystal-contact formation. Among the examples of successful application of this protocol, we note a clear causal relationship between the type of the mutations and the intermolecular contacts in the resulting crystals. The technique is readily applicable to any soluble protein, using the QuikChange mutagenesis kit with primers introducing two or more mutations of the K → A and/or E→A type, in sites located in relative proximity to the amino acid sequence (Wang and Malcolm, 1999). While the prospects appear very promising, these results also raise important questions:

### 1. How Do We Choose Sites for Mutagenesis?

As already pointed out, lysines and glutamates are found predominantly on the protein's surface, with only 6% and 12%, respectively, buried (Baud and Karlin, 1999). If two or more of these residues occur in close proximity as a cluster in the sequence, the surface location is a virtual certainty. But which cluster to choose? Statistically, both residues occur preferentially in $\alpha$ helices and turns, much less frequently in $\beta$ structures. For lysines, the distribution among $\alpha$, $\beta$, and coil structures is 47%, 16%, and 37%, while for glutamates 38%, 17%, and 45% (Baud and Karlin, 1999). Consequently, the K/E clusters also show a tendency to occur with high probability in either helices or surface loops. Those in helices may be less useful for engineering purposes because their main chain carbonyl and amide groups are engaged in intrahelical H bonds and are not available for crystal contact interactions. Based on the examples of successful applications, tight turns offer the best opportunities for crystal contact engineering. If structures of

homologs of the target protein are available, they may be used to facilitate the design of the mutations. Alternatively, secondary structure predictions can be used to locate tight turns of up to six residues, containing Lys, Glu, or both (Chou, 2000).

**2. Is Entropy Really the Underlying Factor?**

There is no question that the removal of a charged, polar amino acid, and its replacement with Ala, has diverse consequences beyond the creation of a low-entropy surface patch. However, we note that, in many examples given above, the results of mutagenesis are very consistent with the notion that entropic phenomena play a major role. Once a bulky side chain is removed, the main chain carbonyls and amides become available to mediate crystal contacts without excessive entropy loss, and indeed the crystal contacts occur precisely at the mutated sites. This would not necessarily be the case if electrostatic effects were dominant. However, both steric and electrostatic effects are bound to have an impact, as can be inferred from the structure of the CUE:ubiquitin complex (vide supra).

**3. Can the Protein's Function Be Inadvertently Affected by the Mutations?**

If one seeks structural insights into the protein's known function, then obviously any mutants under investigation should be assessed using a functional assay. A blind application of the mutational approach to, for example, class I aminoacyl-tRNA synthetases, which contain a functional motif KMSKS, would certainly lead to the enzyme's impairment. In our studies of the RGSL domain, all mutants were assayed for their ability to bind $G\alpha_{13}$ (Longenecker et al., 2001b). In most cases, adequate data on functional residues exist to assist with the design of the appropriate mutational strategy. On the other hand, when the structure is sought as the first step in protein's characterization, i.e., in structural genomics projects, the availability of an atomic model of what might even be an inactive mutant is still extremely valuable. As with all scientific experiments, the limitations of the approach should always be born in mind and taken carefully into consideration.

**4. Do the Mutations Reduce Protein's Solubility?**

As might be expected, the K → A and E → A mutations almost invariably lower the protein's solubility. This is not inconsistent with the theoretical premises and not deleterious to the experiment, unless solubility is critically compromised. There is no question that the method is applicable primarily to well soluble proteins, where a loss of two or three polar residues is not a problem. For less soluble proteins, alternate strategies may also work (see below).

**5. Are K → A and E → A Mutations the Only Choice?**

We conducted two additional studies using other types of mutations. It has been suggested before that mutating K to R might constitute a valid strategy for rational crystallization (Dasgupta et al., 1997) . We investigated this possibility in RhoGDI, and we found that K → R mutations yield less dramatic results, but nonetheless out of nine mutants we obtained one crystal form, easily grown from ammonium sulfate, which diffracted to 1.6 Å resolution (Czepas et al., 2004). The refined structure shows how the two arginines introduced on the surface in place of lysines mediate a key contact by sequestering sulfate ions. In another study, we crystallized the N-terminal domain of the human protein doublecortin by replacing two lysines with aspartates (M.H. Kim et al., submitted). One should note, however, that the principle of replacing large residues disfavored at protein-protein interfaces with smaller ones with lower conformational entropy is generally upheld. The advantage of alternative mutations is that they do not impact on a protein's solubility as much as the alanine mutants.

**6. Is There a Danger of Destabilizing the Protein by Removal of Charged Surface Residues?**

Experimental studies showed that typically E → A mutants are less stable than the wild-type protein (Mateja et al., 2002). However, there appears to be no correlation between stability of the mutant and crystal quality: the E154A/E155A double mutant of RhoGDI, significantly less stable than the wild-type protein, yields superb crystals. Crystal quality is the derivative of the crystal lattice formed by crystal contacts and does not appear to be correlated with intrinsic thermodynamic stability of the protein.

**7. Is the Structure of the Protein Affected by the Mutations?**

Comparative analyses of the several structures of K → A and E → A mutants of RhoGDI reveal no differences in the backbone conformation of magnitude exceeding expectations based on the participation of these structural elements in crystal packing epitopes. We believe that this result, as well as numerous other studies of protein mutants, strongly suggests that the danger of altering the local secondary structure by limited mutagenesis is minimal.

The examples of successful structure determinations, based on crystals obtained using the rational surface engineering protocol, suggest that the method bears significant promise. As more examples emerge, it will be possible to refine the strategy to make it even more powerful and effective.

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