Protein engineering as a tool for crystallography

Stephen R Price and Kiyoshi Nagai

MRC Laboratory of Molecular Biology, Cambridge, UK

The generation of large quantities of protein by overexpression technology has enabled structural studies of many important molecules that are found in only minute quantities in the cell. An increasing number of structures of proteins overexpressed in non-native systems have been solved. Crystallographers now have an extremely powerful tool, namely protein engineering, for the generation of native and derivative crystals that diffract to high resolution. The mutation of residues or generation of compact domains through truncation has resulted in crystals with enhanced diffraction properties. Heavy atom derivative crystals isomorphous to the native protein may also be engineered either by introducing cysteines or by removing cysteines whose reaction with heavy-atom compounds results in poor crystals.

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Introduction

The first crystals grown of a newly studied protein are rarely of diffraction quality. If there is a secret to the preparation of crystals of biological macromolecules for high-resolution structural studies, it lies in the identification and exploitation of parameters critical to formation of the crystals. One parameter is ignored regularly — it has been used to improve the quality of native crystals and to facilitate the generation of derivative crystals — this parameter is the protein sequence itself, modification of which can be accomplished by protein engineering. The preparation of crystals represents an assay; one can change the crystallization conditions and monitor the effect on crystal quality. With this assay and a biochemical assay for functionality, mutations may be made in the protein that may improve the crystals whilst retaining the function. This is rather akin to attempting to crystallize the same protein from different native sources, an oft used approach in the days before protein engineering.

The two major obstacles to success in macromolecular crystallography are obtaining crystals that diffract well and the generation of their isomorphous heavy-atom derivatives. The use of overexpression systems not only allows us to obtain large quantities of proteins that normally exist in minute quantities in the cell, but also provides an opportunity to specifically modify protein molecules to overcome these obstacles. In this review, we deal neither with the overexpression of proteins nor with the initial crystallization trials, as these topics have been covered elsewhere [1,2]; instead, we focus on strategies either to obtain crystals with good diffraction properties or to improve existing crystals through protein engineering.

Use of protein engineering to improve crystals

Proteins often have a modular structure consisting of several distinct domains (independent folding units) that are tethered by flexible linkers. In the majority of cases, each domain may be treated as independent from each other for the purposes of a structural study. An understanding of the domain structure is often important in deciding upon the protein construct to crystallize. Clearly, a study of the actions of the protein as a whole requires a study of the protein as a whole; thus, although structures of a DNA-binding domain and an activation domain from a transcription factor help to explain DNA binding and activation, respectively, they may not always tell how DNA binding affects activation. Yet, information on the two separate functions is still of considerable interest.

Gross flexibility in a protein may be thought of as generating heterogeneity in structure. Removal of a flexible linker region between two domains in the study of each separately can aid in crystallization by minimizing any interfering effects resulting from this microheterogeneity. Protein engineering allows the production of these domains in isolation, without the need for proteolysis of the intact protein, which often results in heterogeneity itself. Identification of domain boundaries may be possible through comparisons of aligned protein sequences from different species. It is of vital importance, however, to have experimental evidence for the domain boundary from limited proteolysis of the wild-type protein using proteases of differing specificity. The precise cleavage site of each protease may be identified by amino-terminal sequencing and mass spectrometry of the cleaved fragments. Mass spectrometry also allows the identification of any microheterogeneity in the

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sample through partial processing of the amino-terminal methionine, post-translational modification or limited proteolysis, which can occur during purification of the sample. These types of microheterogeneity may be removed at the outset through, for example, engineering of an amino-terminal sequence that is processed fully [3,4••] or synthesis of a more compact domain resistant to proteases [5–7]. As a caveat to this section, it is worth mentioning the HIV-1 reverse transcriptase, where p66 and proteolysed p51 (which has lost the RNase H domain) form the active heterodimer. The equivalent parts in p66 and p51 assume substantially different tertiary structures [8], and any of the possible homodimers are inactive, which illustrates the need for a functional assay.

In addition to protein crystallography, heterogeneity in preparations of RNA for RNA crystallography or RNA–protein crystallography may be removed by engineering. The use of *cis*-acting hammerhead ribozymes to generate RNA with defined termini by *in vitro* transcription has been described [9]. Previous methodologies for the preparation of RNA for structural studies employed run-off transcription that utilized phage RNA polymerases. These methods limit the sequences that may be made, with the polymerase also generating heterogeneity at the 3′ terminus as it runs off. By utilizing two *cis*-acting ribozymes co-transcribed with the desired RNA sequence in between, the types of sequence possible are limited only by the specificity of the ribozymes (a considerable improvement on the limitations imposed by the polymerase), with 3′ heterogeneity removed because the 3′ terminus is generated by site-specific cleavage.

Engineering of crystal contacts

Crystal contacts are mediated by the interaction of surface residues and are dependent on the character of the interacting moities. When crystals are of poor quality, amino acid residues likely to be on the protein surface may be mutated to remove weak contacts or to introduce residues that may promote good surface interaction. A definitive selection of mutations is not possible in the absence of the three-dimensonal structure and knowledge of crystal contacts, but consideration of a few existing examples may be useful in designing such mutations. Brange *et al.* [10] made several mutants of insulin to prevent hexamer formation, which they had observed in their crystals. The replacement of small hydrophobic groups with large hydrophobic groups was not effective in destabilizing the protein contacts and larger side chains were readily accommodated by slight re-arrangements of packing. Electrostatic repulsion of charged side chains was much more effective in preventing hexamer formation. In the absence of detailed structural knowledge, it is dangerous to mutate a hydrophobic amino acid to a charged amino acid because a hydrophobic residue may be internal, and its replacement with a charged residue is likely to disrupt the core structure. Charged amino acids are likely to be solvent exposed and their mutation to uncharged hydrophilic groups or hydrophobic groups may alter crystal packing.

Some examples of how protein engineering has been used to improve crystals

GroEL

GroEL is a large protein assembly involved in the ATP-dependent folding of polypeptide chains. Seven identical subunits assemble into a torus with sevenfold rotational symmetry which, in turn, stacks back-to-back within the crystal [11••]. The original native crystals did not diffract well; however, a double point mutant carrying the (functionally null) Arg13→Gly (three-letter amino acid code) and Ala126→Val mutations produced crystals with considerably better diffraction qualities. These mutations were introduced by polymerase error during PCR and make the multimeric protein assembly less stable, although still functional. It is believed that the mutant crystals diffracted better than the native crystals because they had a lower affinity for peptides which co-purify with the native protein.

A complex of U1A spliceosomal protein and its cognate RNA hairpin

U1A protein is a component of the U1 small nuclear ribonucleoprotein particle involved in pre-mRNA splicing. In an attempt to crystallize a complex of U1A protein and its cognate RNA hairpin, RNAs with various stem lengths and overhanging nucleotides were initially added to the native protein fragment [4••]. Cubic crystals, similar in morphology to those obtained using the protein on its own, diffracting to only 7 Å resolution were obtained consistently. It was believed that crystal contacts within the protein–RNA crystal were the same as those within the crystals of the protein alone. If this assumption were true, then disruption of these contacts would likely result in a different crystal form, possibly with better diffraction qualities. Protein dimers interact within the protein crystal through a hydrophobic surface; a Gln39→Arg mutation was introduced into the protein in an attempt to disrupt this surface. During the PCR to introduce this mutation, an additional mutation, Tyr36→His, was also introduced in error. A combination of the double mutant protein and a 21-nucleotide RNA resulted in a hexagonal crystal form diffracting beyond 1.7 Å resolution. These two mutations form crucial crystal contacts, His36 is packed against a nucleotide base at the end of the stem and also interacts with the overhanging base [12]. Proteins containing either mutation singly did not produce crystals. Structural knowledge of the protein

Table 1. Examples of protein structure determinations that have been facilitated by protein engineering.

Structure (source)	Mutations*	Comments*	References
RNA-binding domain of U1A protein (*Homo sapiens*)	Successful mutants included Ser29→Cys, Gln39→Cys, Gln54→Cys, Glu61→Cys, Ser71→Cys and Gln85→Cys. Unsuccessful mutants included Thr6→Cys, Thr11→Cys, Ser63→Cys and Asp79→Cys	The Gln85→Cys mutant without heavy atoms produced crystals larger than the wild type and was used as the 'native' dataset. All mutants were pre-reacted with heavy-atom compounds, with the exception of Gln85→Cys	[6,26]
RNA-binding domain of U1A protein complexed with a stem-loop of RNA (*H. sapiens*)	Gln39→Arg, Tyr36→His and others. For a discussion, see [4••]	Two surface mutations, Gln39→Arg and Tyr36→Cys proved critical for crystallization. In addition, many mutations were made in order to generate a completely processed amino terminus	[4••,12]
Ribosomal protein L6 (*Bacillus stearothermophilus*)	Val124→Cys. (No natural cysteines are found in L6)	This protein, purified from the native source, was crystallized in 1983, but no derivatives could be made. Overexpression allowed the engineering of the Val124→Cys mutation. Val124 was chosen because *E. coli* L6 protein has a cysteine at this position. A selenomethionine derivative is also used.	[17]
Ribosomal protein L9 (*Bacillus stearothermophilus*)	Successful mutants included Asn27→Cys, Glu100→Cys, Leu35→Met and Leu124→Met. Unsuccessful mutants were Asn20→Cys, Gln33→Cys, Thr40→Cys lys45→Cys and Gln105→Cys	Crystals of L9 protein purified from *B. stearothermophilus* were first grown in 1979, but the structure proved insoluble. Protein engineering enabled solution of the structure. Of all the mutations, Asn27→Cys and Glu100→Cys gave the best crystals, although crystals of the former mutants were in a different space group. Leu35→Met and Leu124→Met were introduced in order to label with selenomethionine; their presence proved invaluable in chain tracing	[18]
Cutinase (*Fusarium solani*)	Ser4→Cys, Ser92→Cys, Ser120→Cys and Ser129→Cys	Each serine is mutated to cysteine in turn. Out of the 14 possible mutants, four crystallized isomorphously to the native enzyme, leading to useful derivatives. Interestingly, one of the mutations was to catalytically active serine	[19]
Tenascin, fibronectin type III domain (*H. sapiens*)	Not applicable	A selenomethionine derivative was used Data were collected at four wavelengths around the selenium absorption edge	[31]
Ubiquitin-conjugating enzyme E2 (*Arabidopsis thaliana*)	Cys114→Ser	Reaction of the two cysteines in the wild type with heavy-atom compounds resulted in derivatives non-isomorphous to the native crystals. This Cys114→Ser mutation solved the problem; reaction of the remaining cysteines with heavy-atom compounds resulted in three useful derivatives	[23]
Interferon-γ (*H. sapiens*)	Five carboxy-terminal residues deleted by protein engineering	The carboxyl terminus was targeted because it was known to be protease sensitive. A compact domain was generated by the mutations	[5,40]

*Amino acids are given in the three-letter code.

component was used to design one of the mutations, but in the absence of the structure of the cubic form, it is not possible to tell whether the hydrophobic surface is used to pack the complex in the cubic crystals of the native protein.

HIV integrase

HIV integrase is an enzyme involved in the integration of viral DNA into the host genome. The intact protein, which comprises 288 amino acid residues, is required for 3′ processing and DNA strand transfer activities, but a fragment consisting of residues 50–212 is capable of carrying out the integration reaction. Crystals of the native protein diffract poorly and Dyda *et al.* [13••] have undertaken a systematic approach to mutating hydrophobic residues. A single amino acid substitution of Phe185→Lys results in a protein with improved solubility and native activity. The structure has been solved to 2.5 Å resolution. The mutated protein

assembles as dimers in solution, whereas the native protein forms more complex aggregates. The amino group of the mutated lysine residue is hydrogen bonded to the main-chain carbonyl group of Ala105 of the adjacent molecule within the dimer. It remains to be seen whether engineering of this sort will become commonplace for the generation of better native crystals with a view towards the solution of other structural problems in biology (see also [14]).

Use of protein engineering in the generation of isomorphous heavy-atom derivatives

Conventionally, heavy-atom derivatives are obtained by soaking crystals in a solution of a heavy-atom compound. Heavy-atom compounds covalently react with amino acid side chains (e.g. cysteine, methionine and histidine), form ionic bonds with charged groups, or become trapped in a binding pocket (for a review, see [15]). The properties of heavy-atom compounds and examples of their use can be found in Blundell and Johnson [16]. Searching for derivatives involves soaking different heavy-atom compounds into native crystals, with preliminary data collection and data processing. This process can be time-consuming and sometimes requires many precious crystals. Serious problems arise when reactive amino acids are located near crystal contacts. In these cases, attachment of heavy-atom compounds to these amino acids often causes cracking of crystals or results in non-isomorphous crystals. Sometimes proteins do not contain binding sites for heavy-atom compounds, thus making it difficult to prepare heavy-atom derivatives. Protein engineering has been used to introduce cysteine residues into the protein, ideally on the surface where they are accessible to solvent and unlikely to disrupt the core structure of the protein [6,12,17–19]. When no cysteines are present in the wild-type protein, this technique represents a significant improvement on previous methods, which involved labelling less reactive groups. If soaking results in the cracking of crystals, some of the reactive residues can be mutated to unreactive amino acids [20–23].

The use of protein mutagenesis for the preparation of heavy-atom derivatives was first achieved using T4 lysozyme [24] and applied to solve the structure of γδ resolvase [25]. Serine is similar to cysteine in size and a Ser→Cys mutation is unlikely to cause a structural change in the protein or to affect crystal packing. Although cysteine is structurally similar to serine, the heavy-atom bound form is not; thus, there is no reason *a priori* to favour a Ser→Cys mutation over all others. In addition, serine can be buried, so it may be better to choose residues more likely to be on the surface of the protein (e.g. a charged amino acid or glutamine), although at present, trial and error is probably the best strategy for success. By introducing a cysteine at what one hopes is a surface site, one runs the risk of creating unnatural disulphide linked protein dimers. This may be avoided either by reacting the cysteine mutant with the derivatizing compound before setting up crystallizations or through crystallizing in the presence of a reducing agent such as dithiothreitol. Pre-reaction guarantees 100% occupancy of the heavy-atom sites, whereas soaking makes use of crystal packing forces that more likely, but not necessarily, constrain the crystal to a structure that is isomorphous to the native protein (for a more complete discussion, see [26]). Table 1 shows examples of protein structures that have been facilitated in solution by protein engineering.

Use of selenomethionine-containing proteins as heavy-atom derivatives

Unlike the mutagenesis method discussed above, the use of selenomethionine does not require further modification of the protein sequence [17,27–37]. Incorporating selenomethionine into proteins through overexpression in bacteria grown with seleno-L-methionine as the only source of methionine is, in principle, likely to yield derivative crystals isomorphous to the native. The chemistry of selenium is similar to that of sulphur (they are both in the same group of the periodic table). Furthermore, methionine is not likely to be involved in critical crystal contacts. The advantages of this method are that incorporation of the heavy atom should be close to complete (thus, simplifying considerably the solution of the patterson map), and that the identity and number of sites is known unambiguously, with each site acting as a landmark when tracing the electron density. Until recently, selenomethionine-labelled proteins had to be overexpressed in a strain of *Escherichia coli* auxotrophic for methionine biosynthesis. Yet, Bottomley *et al.* [38] have reported the almost complete incorporation of selenomethionine into vascular cellular adhesion molecule type 1 by utilizing a non-auxotrophic strain grown in the presence of seleno-L-methionine as the sole source of methionine. Methionines have also been incorporated into a protein in order to label with selenomethionine [39]. In addition, the number of structures solved using only selenomethionine-derived phases is increasing steadily [31,34,36]. (As a precautionary note, readers should be aware that all heavy-atom containing compounds and selenomethionine are toxic and should be treated with great care.)

Conclusions

Protein engineering may be used to help in the improvement of crystals and in the generation of heavy-atom derivatives. When used to complement existing methods, its application can only increase the likelihood of success in macromolecular crystallization.

Indeed, in some cases, the use of protein engineering has been critical to the successful completion of the project.

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SR Price and K Nagai, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.