An approach to crystallizing proteins by metal-mediated synthetic symmetrization

Arthur Laganowsky,^{1,2†} Minglei Zhao,¹ Angela B. Soriaga,^{1,2}
Michael R. Sawaya,^{1,3} Duilio Cascio,¹ and Todd O. Yeates^{1,2*}

¹UCLA-DOE, Institute for Genomics and Proteomics, Los Angeles, California 90095-1570

²Department of Chemistry and Biochemistry, University of California, Los Angeles, California

³Howard Hughes Medical Institute-UCLA-DOE, Institute for Genomics and Proteomics, Los Angeles, California 90095-1570

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Abstract: Combining the concepts of synthetic symmetrization with the approach of engineering metal-binding sites, we have developed a new crystallization methodology termed metal-mediated synthetic symmetrization. In this method, pairs of histidine or cysteine mutations are introduced on the surface of target proteins, generating crystal lattice contacts or oligomeric assemblies upon coordination with metal. Metal-mediated synthetic symmetrization greatly expands the packing and oligomeric assembly possibilities of target proteins, thereby increasing the chances of growing diffraction-quality crystals. To demonstrate this method, we designed various T4 lysozyme (T4L) and maltose-binding protein (MBP) mutants and cocrystallized them with one of three metal ions: copper (Cu²⁺), nickel (Ni²⁺), or zinc (Zn²⁺). The approach resulted in 16 new crystal structures—eight for T4L and eight for MBP—displaying a variety of oligomeric assemblies and packing modes, representing in total 13 new and distinct crystal forms for these proteins. We discuss the potential utility of the method for crystallizing target proteins of unknown structure by engineering in pairs of histidine or cysteine residues. As an alternate strategy, we propose that the varied crystallization-prone forms of T4L or MBP engineered in this work could be used as crystallization chaperones, by fusing them genetically to target proteins of interest.

Keywords: protein crystallization; protein design; metal binding; symmetry

Introduction

A common bottleneck in protein X-ray crystallography is the ability to grow diffraction-quality crystals. Although high-throughput robotics now makes it possible to screen vast numbers of crystallization

conditions, some proteins remain recalcitrant to crystallization. Many approaches have been developed to improve the success rate for crystallization, for example, systematically truncating the target protein,^{1,2} methylating the lysine residues,^{3,4} removing post-translational modifications,^{5–7} screening homologues of the target protein for crystallization,^{8,9} fusing the target protein to a carrier protein,^{10–13} crystallizing racemic mixtures of the target protein,^{14–20} and cocrystallizing the target protein with antibodies or other binding proteins.^{21–23} A number of those methods have been reviewed.²⁴

Recently, rational mutagenesis of protein surface residues has been proposed to improve the crystallizability of target proteins, including methods referred to as “surface entropy reduction” (SER)²⁵ and “synthetic symmetrization” (or crystal lattice engineering).^{26–28} The SER method typically replaces

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Additional Supporting Information may be found in the online version of this article.

Arthur Laganowsky, Minglei Zhao, and Angela B. Soriaga contributed equally to this work.

[†]Laganowsky's present address is Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Oxford, UK.

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*Correspondence to: Todd O. Yeates, Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095-1569. E-mail: yeates@mbi.ucla.edu

solvent-exposed, flexible amino acids, such as Lys, Glu, and Gln, with less flexible residues, such as Ala. It has been successfully used for several protein targets,^{29–31} and a web-based server has been created to suggest suitable mutation sites in any given protein sequence.³⁰ Synthetic symmetrization involves introducing into the surface of a protein specific motifs likely to drive symmetric self-association. Motivation for the approach derives in part from the observation that protein oligomers tend to crystallize in space groups that support the point group symmetry of the oligomer.²⁶ In such cases, some of the contacts required to establish a crystal lattice are essentially built in to the oligomer, meaning that fewer fortuitous contacts are required.¹⁶ The general strategy is also amenable to variation; different modes of engineered oligomerization lead to distinctly different opportunities for crystallization. One method already demonstrated for synthetically dimerizing a protein is to introduce a single cysteine on the surface and then form an intermolecular disulfide bond. That method was applied first to crystallize bacteriophage T4 lysozyme (T4L) in several new crystal forms²⁶ and then to crystallize an enzyme of previously unknown structure.²⁷ In a parallel strategy (alternately coined crystal lattice engineering), a series of surface-exposed helix residues were mutated to leucine to promote dimerization by way of an intermolecular leucine zipper²⁸; a subsequent application of that approach produced crystals by way of heterotypic interactions between the engineered half-leucine zipper and a distinct surface region from another protein molecule, rather than by way of a symmetric self-association.³² Despite the successes demonstrated so far by the disulfide- and leucine-zipper-based approaches to synthetic symmetrization, they suffer from a limited type of symmetric association; both are designed to produce dimers.

It is known that metals play a very important role in oligomerization and crystallization of proteins.^{33–35} For example, in the high-resolution crystal structure of truncated alphaA crystallin,³⁶ a zinc-binding motif is formed by three chains, and zinc promotes protein oligomerization and crystallization. The compound tetrathiomolybdate, used in the treatment of copper metabolism disorders, reacts with the copper binding metallochaperone, Atx1, forming a stable complex.³⁷ The crystal structure of this complex reveals a trimer of Atx1 molecules mediated through the bound tetrathiomolybdate molecule. Tezcan and coworkers^{38–42} have studied metal-directed protein self-assembly on the model protein, cytochrome b₅₆₂, focusing primarily on the evolution of metal coordination in protein folds and complexes. Their work has shown that by introducing histidine mutations on the alpha helical surface of cytochrome b₅₆₂, the protein can oligomerize to form dimers to

tetramers, all of which are mediated through metal binding. Furthermore, the metals present within a protein crystal can provide the experimental phases needed for its structure determination.⁴³ Although the incorporation of selenomethionine into proteins has become a standard method for exploiting anomalous scattering information,⁴⁴ natural metal-binding sites containing copper, iron, nickel, cobalt, or zinc atoms have also been utilized for protein structure determination via anomalous dispersion (see Refs. 45–49 and references therein).

Here, we propose a new crystallization methodology, termed metal-mediated synthetic symmetrization, which combines the idea of synthetic symmetrization with strategies—following work by Tezcan *et al.*⁴²—for engineering metal-binding sites at protein interfaces. This method has the potential to produce a variety of new crystal forms by introducing new contacts between protein molecules in the lattice through metal binding interactions, thereby increasing the chances of obtaining diffraction-quality crystals. We demonstrate the applicability of metal-mediated symmetrization using two proteins commonly used as fusion partners for crystallization trials: T4L and maltose-binding protein (MBP). We discuss potential advantages of this method over current approaches and ways to use the method on proteins of unknown structure, through either direct mutations on the target protein or fusion of the target protein to metal-site mutants of T4L or MBP, which could serve as crystallization chaperones.

Results

Rationale and design of mutations

On the basis of previous studies regarding natural or engineered metal-binding sites, we rationalized that mutations of solvent-exposed residue pairs i and $i + 4$ of a helix^{50–53} or i and $i + 3$ of a helix-loop³⁷ to a pair of either histidine or cysteine residues would induce homooligomeric assemblies upon incubation with metal. Specifically, we predicted the metal site within these assemblies to take on a tetrahedral or octahedral coordination, in which the mutated histidines or cysteines among the molecules participate in the metal coordination. Such coordination geometries are commonly found in proteins^{33,54} and have been engineered previously.^{37–42} On the basis of the crystal structures of T4L and MBP in each protein, we chose three pairs of solvent-accessible residues that are located close to the ends of helices (Fig. 1). The introduction of metal-binding sites near the ends of helices, compared with sites in the middle of helices, was expected to cause fewer steric clashes and allow greater coordination possibilities among molecules. In both T4L and MBP, the mutations were chosen to be distant from the C-terminus, to avoid potential interference with a fusion protein

Figure 1. Surface residues of T4 lysozyme (T4L) and maltose-binding protein (MBP) selected for mutations. (a) T4L is represented as a cartoon (blue). Three pairs of residues are labeled (red, yellow and green), each corresponding to one double-residue mutant: D61/K65 to histidines, R125/E128 to cysteines, R76/R80 to either histidines or cysteines in two different constructs. A quadruple histidine mutant for D61/K65/R76/R80 was also made. (b) MBP is represented as a cartoon (orange). Three pairs of residues are labeled (green, blue and purple), each corresponding to one double histidine mutant. All selected pairs are three or four residues apart and are close to the ends of helices. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

that might be attached. The determination of which residues are on the surface-exposed side of a helix was straightforward in our test experiments with T4L and MBP, whereas this represents a challenge when the structure is unknown. A recent application of disulfide-based synthetic symmetrization²⁷ illustrates the use of sequence alignments and bioinformatics tools to make reasonable choices for engineering residues on the sides of helices.

T4L and MBP mutants—most containing two mutations and one containing four mutations (summarized in Table I)—were expressed, purified, and subjected to cocrystallization in the presence of either copper (Cu^{2+}), nickel (Ni^{2+}), or zinc (Zn^{2+}). The ratio of metal to protein was ~ 1.5 to 1. In some cases, the addition of metal induced slight precipitation of the protein-metal solution that could be reversed by the addition of EDTA. However, no additional purification step, besides filtration through a $0.22\ \mu\text{m}$ membrane, was performed once the metal was added (see Methods for details). Crystallization was carried out using standard commercial crystallization screens (typically only three to four 96-well experiments). For all of the mutant constructs, crystals formed in a variety of conditions within 1 week, and in some cases within 2 h, of setting up crystal screens. All structures were solved from crystals mounted from preliminary screens without any further optimization (Supporting Information Table 3).

T4L histidine mutants

For T4L, we created two double-histidine mutants, T4L^{76H/80H} and T4L^{61H/65H}, and one quadruple-histidine mutant, T4L^{61H/65H/76H/80H}, chosen to emulate the engineering design by Tezcan *et al.*³³ in which four histidine mutations are introduced as two pairs

of proximal residues in a long helix. Crystallization of these mutants with the various metals resulted in eight crystal structures (Table I), which represent six distinct crystal forms; in some mutants, nickel and copper produced similar metal-binding sites and crystal packing arrangements. The crystal forms obtained were all different from those observed before for T4L.⁵⁵ The molecular structures and crystal packing diagrams are shown in Figures 2 and 3, and crystallographic statistics are shown in Supporting Information Table 1.

Different combinations of mutations and metals led to various oligomeric forms of T4L. The protein can form either dimers [Fig. 2(a–c,e)] or trimers [Fig. 2(d)] through metal coordination, illustrating a greater range of oligomerization possibilities compared with other methods of symmetrization. Moreover, in one case, a metal-mediated dimer further associated into a hexameric ring within the crystal asymmetric unit [Fig. 2(e)]. With respect to metal site formation, the crystallization results were likewise variable and unpredictable. The same mutant sometimes crystallized in different forms in the presence of different metals [Fig. 2(a,e)] or even in different forms with the same metal [Fig. 2(b,d)]. Unexpectedly, in some structures one of the mutated histidines is not in coordination with the metal [Fig. 2(b–d)]. Instead, either natural (wild type) surface residues or solvent molecules complete the coordination (Table I). However, in all of the cases where this occurs, the neighboring free histidine is in a π stacking arrangement with the coordinating histidine [Fig. 2(b–d)], indicating it may be necessary for the coordination. Finally, not all the T4L molecules in the asymmetric unit are coordinated by the metals. In two cases, a noncoordinated molecule is present

Table 1. Characteristics of Metal Interactions for Various T4L and MBP Mutants and Their Crystals

	Residues mutated	Metal	Space group	Residues involved in metal coordination ^c	Type of oligomerization ^a	Geometry of metal–ligand interactions	Corresponding Figure
T4L ^{76H/80H}	R76, R80	Cu	P4 ₁ 3SB9	H76, H80	Local/2	Octahedral	2a
T4L4H ^{61H/65H/76H/80H}	D61, K65, R76, R80	Zn	P2 ₁ 2 ₁ 2 ₁ 3SBA	H76, H80	Local/3	Dist. ^b Octahedral	2e
		Cu	P2 ₁ 2 ₁ 2 ₁ 3SB6	H61, H65, D72, ^c H76	Local/2	Dist. Square planar	2b
		Cu	H3 3SB7	H61	Crystallographic/3	Dist. Tetrahedral	2d
		Ni	P2 ₁ 2 ₁ 2 ₁	H76, H80	Local/2	Dist. Octahedral	Not shown
T4L ^{61H/65H}	D61, K65	Cu	P2 ₁ 2 ₁ 2 ₁ 3SB8	H65, Q69, G-1	Local/2	Dist. Square planar	2c
T4L ^{125C/128C}	R125, E128	Zn	H32 3SB5	C125, C128	Crystallographic/3	Tetrahedral	6a
T4L ^{76C/80C}	R76, R80	None ^d	P422 3SBB	C76, C80	Crystallographic/222	N/A	6b
MBP ^{216H/220H}	A216, K220	Cu ^e	P2 ₁ 3SES	H216, E222, H40	Local/2	Dist. Tetrahedral	4a
		Ni ^e	P2 ₁ 3SET	H216, E222, H40	Local/2	Dist. Tetrahedral	Not shown
		Ni ^e	P2 ₁ 2 ₁ 2 ₁ 3SEX	H216, E222, H40	Local/2	Dist. Tetrahedral	Not shown
		Zn	C2 3SEW	H216, E222, E39	Crystallographic/2 ₁	Dist. Tetrahedral	4b
MBP ^{26H/30H}	K26, K30	Zn	C2 3SEY	H216, H220, E222, E39, D56	Crystallographic/2 ₁	Dist. Tetrahedral	Not shown
		Zn	P2 ₁ 3SEU	H216, H220, E222, E310	Crystallographic/2 ₁	Dist. Tetrahedral	4c
		Zn	P2 ₁ 3SER	H26, H30, D165	Local/Screw	Dist. Tetrahedral	4d
		Zn	P2 ₁ 2 ₁ 2 ₁ 3SEV	H310, H314, E289, E292	Local/3	Dist. Tetrahedral	4e
MBP ^{310H/314H}	E310, K314	Zn					

^a For each mutant, the metal may be involved in either crystallographic or local contacts. Highest symmetry elements mediated by metal coordination are listed in international Hermann–Mauguin notation when possible.
^b Dist. = Distorted
^c Residues in italics are natural amino acids that participate in metal-coordination.
^d Assembly is mediated by disulfide bonds.
^e Same structure in the asymmetric unit.

Figure 2. Crystal structures of metal-mediated symmetrization of T4 Lysozyme (T4L) histidine mutants. For each panel, a close-up view of the metal binding site is shown as an inset with coordinating residues labeled. Copper and zinc atoms are shown as bronze and grey spheres, respectively. Coordination to metal atoms is shown as dashed yellow lines. (a) The mutated histidine residues of T4L^{76H/80H} form a twofold NCS (noncrystallographic symmetry) dimer mediated through the bound copper atom. T4L^{76H/80H} has similar packing and interactions when cocrystallized with nickel (data not shown). Two coordinating water molecules are shown in red. (b) The quadruple histidine mutant, T4L^{61H/65H/76H/80H}, forms a dimer through two copper atoms. Three mutated histidine residues, H61/H65/H76, and a natural aspartate, D72, complete each copper binding site. (c) The double histidine mutant, T4L^{61H/65H}, forms a dimer (purple and green) through two copper atoms. This dimer is accompanied by a non-copper bound molecule (blue) packed within one asymmetric unit. Each copper site is formed by one mutated histidine residue, H65, a natural glutamine, Q69, and completed by N-terminal glycine residues derived from the tobacco etch virus (TEV) protease cleavage site. (d) The four histidine mutant, T4L^{61H/65H/76H/80H}, also forms a trimer. The copper binding site is formed by the mutated histidine, H61, located on a threefold crystallographic axis. Three other protein molecules in the asymmetric unit, not bound to copper ions, are also shown (light yellow, light orange and light red). (e) The double histidine mutant, T4L^{76H/80H}, also forms a hexameric ring structure mediated by three zinc atoms. The zinc binding sites are formed by two mutated histidine residues, H76/H80, from two neighboring molecules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Fig. 2(c,d)]. The variations in metal site geometry and protein oligomerization enable the numerous new crystal forms observed here for T4L (Table I and Fig. 3).

MBP histidine mutants

To demonstrate that this methodology applies not only to T4L but also to a second protein, MBP, we prepared three double-histidine mutants of MBP – MBP^{216H/220H}, MBP^{26H/30H}, and MBP^{310H/314H} – and crystallized them in the presence of metals (Table I). The molecular structures and crystal packing diagrams of these mutants are shown in Figures 4 and 5, and crystallographic statistics are shown in Supporting Information Table 2. Similar to T4L histidine mutants, MBP mutants can either dimerize

[Fig. 4(a)] or trimerize [Fig. 4(e)] upon addition of metal ions. In one case, a dinuclear zinc site is present [Fig. 4(c)]. Notably for the MBP mutants, metal coordination can be involved in lattice contacts and drive the formation of metal-mediated polymers [Figs. 4(b–d) and 5]. Depending on the crystallization conditions and type of metal, a single mutant construct MBP^{216H/220H} can pack into three different forms [Fig. 4(a–c)]. Interestingly, in all the structures, natural surface residues, usually Glu and Asp, participate in the coordination, and all molecules in the asymmetric unit are coordinated by the metals. The wild-type MBP construct used as the starting point in this study crystallized exclusively in space group P6₁ in previous work. The structures of all the MBP mutants shown here are distinct

Figure 3. Crystal packing of T4L histidine mutants. (a–e) The crystal packings of five T4L histidine mutants are shown. The annotation below each image indicates the construct, the cocrystallizing metal and the space group. The metal binding sites are highlighted by red dots. Different chains in the asymmetric unit are colored differently. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at the lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

from the P6₁ crystal form obtained with wild-type MBP and, therefore, represent new packing modes driven by metal coordination.

T4L cysteine mutants

Considering that cysteine residues are also commonly found in coordination with metals—zinc finger proteins being a particularly well-studied

case^{54,56}—we explored whether cysteine mutations could also be used for metal-mediated symmetrization. We generated two double-cysteine mutants of T4L: T4L^{76C/80C} and T4L^{125C/128C} (Fig. 6 and Supporting Information Table 1) following the design of an *i* and *i* + 3 mutation at a helix-loop. The T4L^{125C/128C} mutant was inspired by the crystal structure of copper-binding Atx1 in complex with

Figure 4. Crystal structures of metal-mediated symmetrization of maltose-binding protein (MBP) histidine mutants. The insets show close-up views of the metal binding sites. Metal atoms are colored the same as described in Figure 1. Water and chloride ions are shown as blue and green spheres, respectively. (a–c) A double histidine mutant, MBP^{216H/220H}, forms a variety of metal mediated interactions. (a) A copper mediated dimer is formed through the mutated histidine, H216, the natural histidine, H40, and glutamate, E222, residues. (b) A polymeric assembly is created through crystal lattice contacts mediated by zinc atoms. The zinc binding site is formed by the mutated histidine, H216, and the natural glutamate residues, E39/E222. (c) Crystal lattice contacts are produced through zinc binding. Two zinc atoms are bound by the two mutated histidines, H216/H220, the two natural glutamates, E222/E310, and also by acetate ions (orange). The MBP^{216H/220H} mutant protein displays the versatility of metal binding through use of different metals. (d) Crystal lattice contacts are mediated through zinc atoms for the double histidine mutant, MBP^{26H/30H}. The two mutated histidines, H26/H30, and the natural aspartate, D165, form the zinc binding site. (e) The double histidine mutant, MBP^{310H/314H}, forms a trimer assembled through three zinc atoms. Two natural glutamates, E292/E289, and the mutated histidines, H310/H314, complete each zinc binding site. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tetrathiomolybdate and copper, in which natural Cys15 and Cys18 of Atx1 participate in a complex metal binding network.³⁷ Both mutants were purified in the presence of 1 mM dithiothreitol (DTT) to prevent cysteine oxidation, and crystallization was carried out in the presence of zinc. Interestingly, T4L^{125C/128C} crystallized as a trimer mediated by a novel multinuclear zinc sulfur cluster, which to our knowledge has not been reported previously [Fig. 6(a) and Supporting Information Fig. 1]. Four zinc atoms form a tetrahedron with six sulfur atoms—two from each protein—coordinating from six edges, forming an adamantane-like cage. The zinc sulfur cluster is reminiscent of the copper (Cu¹⁺) sulfur cluster stabilized by Atx-1 and tetrathiomolybdate in anaerobic conditions.³⁷ The second double cysteine mutant, T4L^{76C/80C}, crystallized in more than 60% of the crystallization conditions from an initial screen, and some crystals diffracted to ~ 1.5 Å without any further optimization. The structure of this mutant revealed a D₂ tetramer covalently linked by four di-

sulfide bonds [Fig. 6(b)]. The tetramer is constituted by two dimers that have similar structures to the copper-bound T4L^{76H/80H} [Fig. 2(a)]. The disulfide bonds are all homotypic, with the two Cys₇₆-Cys₇₆ bonds lying on one dyad axis of symmetry and the two Cys₈₀-Cys₈₀ bonds lying on one of the other dyad axes. Surprisingly, no metal ions were observed in the electron density, although zinc ions were added to the crystallization drops. For both double cysteine mutants of T4L, the crystal packing modes are novel (Fig. 7), although in the second case, there was no mediation through zinc.

Discussion

In this study, we demonstrate a crystallization method that combines the concept of synthetic symmetrization with the approach of engineering metal binding sites. We generated several different double or quadruple histidine mutants of proteins T4L and MBP, which were chosen in part for their prior use as fusion proteins. On incubating these constructs

Figure 5. Crystal packing of MBP histidine mutants. (a–e) Crystal packings of five MBP histidine mutants are shown. The annotation below each image indicates the construct, the cocrystallizing metal and the space group. The metal binding sites are highlighted by red dots. Different chains in the asymmetric unit are colored differently in panels a, d, and e. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with various metals (Cu²⁺, Ni²⁺, and Zn²⁺), we obtained 14 new crystal structures, representing 11 new and distinct crystal forms, showing various symmetries and packing modes mediated by the added metal ions (Table I). Moreover, we crystallized two different double cysteine mutants of T4L with added zinc ions, and obtained two further distinct crystal forms exhibiting more complex packing arrangements, in one case based on direct disulfide bonding rather than metal-mediated interactions.

We obtained crystals with new symmetry and packing from all the mutation pairs we tested. Some mutants crystallized with great ease and variety. For example, MBP^{216H/220H} and T4L^{61H/65H/76H/80H} could be crystallized in many different forms, depending on the crystallization condition and the type of metal. Crystals having the same unit cell dimensions and symmetry as the wild-type protein (P₃₂21 for T4L and P6₁ for MBP) were also sometimes obtained, but at a relatively low rate (less

Figure 6. Crystal structures of metal-mediated symmetrization of T4 Lysozyme (T4L) double cysteine mutants. (a) The double cysteine mutant, T4L^{125C/128C}, forms a trimeric complex assembled through a zinc cluster. Three pairs of cysteines from neighboring molecules coordinate four zinc atoms in an adamantane-like structure stabilized by four chloride ions (shown in the inset). (b) A covalent tetramer is formed by the double cysteine mutant, T4L^{76C/80C}, through disulfide bonds. Shown are four molecules related by D₂ (or 222) symmetry. Four disulfide bonds between the mutated cysteines, C76 and C80, covalently link the four protein molecules into a ring. The disulfide bonds are highlighted in the inset. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than 1/3 of the time). Analysis of the crystallization conditions for all mutants (Supporting Information Table 3) shows that conditions that facilitated metal-mediated oligomeric assembly tended to either have high concentrations of salt or polyethylene glycol of various molecular weights.

The metal ions were incorporated into the crystal lattice in different ways in different crystals. In a number of structures, the metal fell on a crystallographic axis (Table I), suggesting that the metal-induced homooligomeric assembly helped drive lattice formation. This is especially apparent in the polymeric assemblies of MBP^{216H/220H} and MBP^{26H/30H} in the presence of zinc (Fig. 5), in which metal coordination generates helical arrangements of subunits compatible with crystal symmetry. Both types of situation support the notion that symmetrized oligomers may

gain an advantage over asymmetric monomers in crystal formation. We note that in all the crystal structures obtained, despite the variety of protein oligomeric states induced, that generally the individual metal ions added were coordinated by two subunits. We also observed that in slightly more than half of the structures, only one of the two introduced histidines participated in the coordination. In those cases, natural surface residues, usually Glu or Asp, or solvent molecules were involved in metal binding instead. This suggests the possibility in future work of combining Glu and Asp with single or double His mutations for metal-mediated synthetic symmetrization.

The oligomeric protein arrangements observed by metal-mediated symmetrization were highly variable. It is likely that even greater diversity could have been realized through additional

investigations. For several constructs, there were too many favorable leads from initial crystallization trials to permit a complete characterization of all the possible crystal forms. Furthermore, other experimental variables that might have produced additional crystal forms were not investigated. The relative concentration of metal ions was held fixed in our experiments, although this variable might be expected to influence the oligomeric modes of metal-mediated protein association.

Polymorphic assembly behavior was also observed under different crystallization conditions for some individual combinations of protein constructs and metals. This was evident in the multiple structures of T4L^{61H/65H/76H/80H} with copper, for example, in which slight variations in the crystallization conditions (Supporting Information Table 3) led to outcomes in which the geometry of the metal coordination by T4L molecules varied from square planar to tetrahedral [Table I and Fig. 2(b,d)]. In such cases, alternate metal-driven protein arrangements may be favored by slightly different solution conditions, or by different crystal packing arrangements. The likelihood that multiple distinct arrangements coexist in solution in some cases raises the possibility that heterogeneity could hinder crystallization. However, the multitude of our successful crystallization results, including multiple distinct forms from some individual combinations of protein construct and metal ion, argues otherwise. The reversibility of metal coordination probably has a positive effect in this regard, making it possible for favorable crystal packing arrangements to drive otherwise heterogeneous mixtures into specific, well-ordered crystal forms. Furthermore, the coordination geometries of the metal sites were often slightly distorted (Table I), suggesting that flexibility in metal coordination could also help enable the formation of well-ordered crystal packing arrangements.

Another potential advantage of metal-mediated synthetic symmetrization is the phasing power introduced by metals. The metal sites are well defined in most of our structures (Supporting Information Table I and II), and anomalous signals were observed for most of them, despite that the data were not collected using an X-ray wavelength close to the absorption edge for the metals introduced (Supporting Information Fig. 1). For the T4L^{125C/128C} mutant, we confirmed that experimental phases could be obtained using the anomalous scattering from the zinc atoms (Supporting Information Fig. 1). By using synchrotron radiation tuned to the optimal wavelength, we anticipate that it will be generally possible to obtain diffraction phases from crystals grown by the metal-mediated symmetrization approach.

On the basis of our findings, we propose that one could utilize the concept of metal-mediated synthetic symmetrization to crystallize more difficult

Figure 7. Crystal packing of T4L cysteine mutants (a and b). Crystal packings of two T4L cysteine mutants are shown. The annotation below each image indicates the construct, the cocrystallizing metal and the space group. The metal binding sites are highlighted by red dots. Note that the packing of T4L^{76C/80C} is mediated by disulfide bonds instead of metals. Different chains in the asymmetric unit are colored differently in panel a. Molecules related by crystallographic symmetry are shown in the same color. The packing arrangements are shown projected along one of the unit cell edges as denoted by a coordinate system at lower left corner of each image. A projection of the unit cell is also shown by a black quadrilateral in each image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

protein targets that have eluded crystallization using traditional methodologies. Figure 8 summarizes the “rational mutagenesis of surface residues” methods that have been developed to facilitate protein crystallization. In contrast to previously proposed methods, our approach allows the formation of

Figure 8. A scheme summarizing approaches for crystallizing protein targets by rational mutagenesis of surface residues. A protein (red box) can be mutated according to the idea of “surface entropy reduction”³⁰ (orange box), which typically involves replacing long flexible amino acid side chains by alanine. Alternatively, or in combination, surface residues can be modified in a way that specifically promotes symmetric oligomerization. This general idea is referred to as “synthetic symmetrization”²⁶, a closely related idea has been called “crystal lattice engineering.”²⁸ Single cysteine mutations have been used successfully for dimerization^{26,27} (green box). A method based on inserting multiple leucine residues in a surface helix has been used successfully by others (blue box).^{28,32} In this work we propose metal-mediated synthetic symmetrization (purple boxes), which involves introducing either double histidine mutations (lower right box) or double cysteine mutations (lower left box), followed by the addition of metal ions. The metal-mediated approach leads to a rich variety of oligomeric arrangements and crystal packing opportunities. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oligomers in diverse arrangements and symmetries, giving rise to greatly expanded opportunities to grow diffraction-quality crystals. The flexibility of our approach also allows it to be potentially combined with methods such as SER in which high-entropy surface residues can be mutated.

We propose two variations by which the approach could be used to promote the crystallization of new target proteins: (i) crystallizing fusion constructs in which a target protein is fused to the various engineered forms of T4L or MBP described here, or (ii) directly crystallizing a target protein after mutating pairs of surface residues to histidine or cysteine. The first approach is based on the observation that both T4L and MBP have been successfully used to crystallize otherwise difficult proteins, including membrane proteins and amyloid proteins.^{10,57–61} The mutation pairs characterized in this study could be readily used to increase the chance of getting crystals. The crystal packing arrangements of such fusion proteins would depend on surface properties of the target protein and would therefore likely be different from the structures presented here. In the second, direct approach, positions for making mutation pairs in a target protein

would be chosen based on predictions of secondary structure and surface exposure, or based on a homology model, when available. Double histidine or cysteine mutations are preferably introduced close to the ends of a helix (Fig. 1). For long helices, two histidine pairs can be introduced at both ends. The spacing between the two histidines are preferably i and $i + 4$, and for cysteines either i and $i + 3$ or i and $i + 4$. Theoretically, histidine mutations should have a broader application, because they will not interfere with native cysteines. Although additional purification of oligomeric species after metal addition was not necessary for success in our studies, this step might be useful in confirming metal-mediated assembly and optimizing the chances of success with more difficult protein targets.

Considering that it is easy to introduce double mutations, and all the crystals in this study were solved directly from robotic screens without any optimization, one could apply the approach relatively easily to generate and test several varied constructs for a given protein of interest. Such an approach could prove valuable for crystallizing asymmetric proteins or protein complexes that have eluded traditional crystallization methodologies.

Methods

T4L plasmid construction

Cysteine-less T4L (a kind gift from Mark Fleissner and Wayne Hubbell at UCLA), residues 1–162, was PCR amplified with Platinum Taq Polymerase (Invitrogen, Carlsbad, CA). The N-terminal primers contained a six base pair overhang, NdeI restriction site, TEV protease cleavage site, and a short linker of residues GP to aid TEV protease cleavage. The C-terminal primer contained a stop codon, XhoI restriction site, and a three base pair overhang. The PCR product was agarose gel purified and extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR product and pET28b (Novagen, Gibbstown, NJ) were digested with NdeI and XhoI according to manufacturer protocol (New England Biolabs, Ipswich, MA). Digested pET28b and T4L products were gel purified and extracted (as described above). DNA concentrations were determined using BioPhotometer UV/VIS Photometer (Eppendorf, Westbury, NY). The vector pET28 and T4L were ligated using the Quick Ligation Kit (New England Biolabs, Ipswich, MA) according to manufacturer protocol, and transformed into *E. coli* cell line TOP10 (Invitrogen, Carlsbad, CA). A colony was grown overnight, and the pET28-TEV-T4L plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA).

T4L and MBP mutants

All mutations in the DNA sequence were performed on the pET28-TEV-T4L or the pMal-a1 (a kind gift from

Prof. Cynthia Wolberger at Johns Hopkins University) plasmids using a Site-Directed Mutagenesis kit (Quick-Change II XL, Agilent, Santa Clara, CA) with site-directed primers designed using manufacturers Quick-Change Primer Design Program available online (Agilent, Santa Clara, CA), according to the manufacturer's protocol. The final constructs were sequenced prior to transformation into *E. coli* expression cell line BL21 (DE3) gold cells (Novagen, Gibbstown, NJ).

Protein expression

A single colony was inoculated into LB Miller Broth (Fisher BioReagents, Fisher Scientific, Pittsburgh, PA) supplemented with 30 $\mu\text{g/mL}$ Kanamycin (Fisher Scientific, Pittsburgh, PA; LB_{Kan}) or 100 $\mu\text{g/mL}$ Ampicillin (Fisher Scientific, Pittsburgh, PA; LB_{Amp}) for T4L and MBP mutants, respectively. One liter of LB_{Kan} or LB_{Amp} in a 2-L shaker flask was inoculated with 7 mL of overnight culture and grown at 37°C until the culture reached an $\text{OD}_{600} = 0.6\text{--}0.8$. For T4L mutants, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and grown for 1.5 h at 37°C. For MBP mutants, IPTG was added to a final concentration of 1 mM, and grown for 4 h at 37°C. Cells were harvested by centrifugation at 5,000g for 10 min at 4°C. The cell pellet was frozen and stored at -80°C prior to purification.

T4L protein purification

The cell pellet was thawed and resuspended in buffer A [50 mM sodium phosphate, 0.3M sodium chloride, and 20 mM imidazole (pH = 8.0)] supplemented with Halt Protease Inhibitor Cocktail (Peirce, Thermo Fisher Scientific, Rockford, IL) and 1 mM DTT for cysteine containing mutants at 25 mL per 1 L of culture volume. The resuspended was culture sonicated and centrifuged at 12,000g for 25 min at 4°C. The clarified lysate was filtered through a 0.45- μm syringe filtration device (HPF Millex-HV, catalog no. SLHVM25NS, Millipore, Billerica, MA) before loading onto a 5-mL HisTrap-HP column (GE Healthcare, Piscataway, NJ). The HisTrap-HP column was washed with five column volumes of buffer A and protein eluted with linear gradient to 100% in four column volumes of buffer B [50 mM sodium phosphate, 0.3M sodium chloride, and 500 mM imidazole, (pH = 8.0)]. For cysteine containing mutants, buffers A and B were supplemented with 5 mM beta-mercaptoethanol (BME). Protein eluted around 40–60% buffer B and peak fractions were pooled. TEV protease, produced and purified as described,³⁶ was added at a volume of 1/100th the pooled volume, and ethylenediaminetetraacetic acid (EDTA) was added to final concentration of 1 mM. After 20–30 min, pooled protein was transferred to a Slide-A-Lyzer 10,000 MWCO dialysis cassette (Pierce, Thermo Fisher Scientific, Rockford, IL), and dialyzed against buffer C (20 mM TRIS pH 8.0, 200 mM sodium chloride, 20

mM imidazole, 1 mM DTT) at room temperature overnight. The dialyzed protein fraction was transferred to a 50 mL conical falcon tube (Fisher Scientific, Pittsburgh, PA). After 2 days of TEV protease cleavage, cut protein was passed over a 5 mL HisTrap HP column pre-equilibrated in buffer A and the flow-through containing His-tag removed T4L was collected and concentrated prior to loading onto a Superdex Prep Grade 75 gel filtration column equilibrated in GF buffer [100 mM sodium chloride, 1 mM DTT, and 20 mM TRIS (pH = 8.0)]. Peak fractions were pooled and concentrated. Protein concentration was determined by UV absorbance at 280 nm with extinction coefficient of $24750\text{ M}^{-1}\text{ cm}^{-1}$ of protein.

MBP protein purification

Cells were resuspended in a lysis buffer containing 100 mM Tris-HCl (pH = 8.0), 100 mM NaCl, and 1 mM EDTA. Phenylmethylsulfonyl fluoride (PMSF) was added to the cell resuspension to a final concentration of 1 mM. Clarified cell lysate, as described above, was loaded onto a self-packed amylose column (150 mL column volume, resin from New England Biolabs, Ipswich, MA). The column was first washed with Buffer A [20 mM Tris-HCl (pH = 8.0) and 100 mM NaCl] for one column volume and then eluted with Buffer B [20 mM Tris-HCl (pH = 8.0), 100 mM NaCl, and 10 mM maltose]. Peak fractions were pooled and concentrated using an Amicon Ultra-15 concentrator (30 kDa MW cutoff; Millipore, Billerica, MA) prior to loading onto a Superdex S-200 column (GE Healthcare, Piscataway, NJ) equilibrated in SEC buffer [20 mM Tris-HCl (pH = 8.0), 100 mM NaCl, and 5 mM maltose]. Peak fractions were pooled and concentrated. Protein concentration was determined by absorbance at 280 nm with the calculated extinction coefficient of $67800\text{ M}^{-1}\text{ cm}^{-1}$.

Protein crystallization

Concentrated T4L and MBP protein mutants were diluted to $\sim 1\text{ mM}$ and supplemented with 1.25–1.5 mM of metal: copper sulfate, nickel sulfate, or zinc acetate. Protein solutions containing metals were filtered through a 0.22 μm Ultrafree-MC centrifugal filter device (Amicon, Bedford, MA) prior to crystallization experiments in hanging drop plates. Crystallization experiments were carried out at the UCLA crystallization core facility (<http://www.doe-mbi.ucla.edu/facilities/crystallization>) and stored at 20°C. The crystallization conditions are provided in Supporting Information Table 3.

Structure determination

All data were collected at 100 K at Advanced Photon Source (Chicago, IL) beam lines 24-ID-C and 24-ID-E, and in-house on a Rigaku Raxis-IV++ imaging plate detector using Cu K α radiation from a Rigaku FRe+ rotating anode generator with confocal optics

(Supporting Information Table 1). Single crystals were cryoprotected with glycerol and mounted with CrystalCap HT Cryoloops (Hampton Research, Aliso Viejo, CA). Crystals were flash-cooled in liquid nitrogen prior to data collection. All data were processed using DENZO/SCALEPACK⁶² or XDS/XSCALE.⁶³ Initial phases of T4L and MBP mutants were calculated by molecular replacement using structures with PDB codes 3LZM and 1ANF, respectively, as search models using PHASER.⁶⁴ Model building was done using COOT.⁶⁵ All model refinement was done using REFMAC,⁶⁶ PHENIX,⁶⁷ and BUSTER.⁶⁸

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References

- Dong A, Xu X, Edwards AM, Chang C, Chruszcz M, Cuff M, Cymborowski M, Di Leo R, Egorova O, Evdokimova E, Filippova E, Gu J, Guthrie J, Ignatchenko A, Joachimiak A, Klostermann N, Kim Y, Korniyenko Y, Minor W, Que Q, Savchenko A, Skarina T, Tan K, Yakunin A, Yee A, Yim V, Zhang R, Zheng H, Akutsu M, Arrowsmith C, Avvakumov GV, Bochkarev A, Dahlgren LG, Dhe-Paganon S, Dimov S, Dombrovski L, Finerty P, Jr, Flodin S, Flores A, Graslund S, Hammarstrom M, Herman MD, Hong BS, Hui R, Johansson I, Liu Y, Nilsson M, Nedyalkova L, Nordlund P, Nyman T, Min J, Ouyang H, Park HW, Qi C, Rabeh W, Shen L, Shen Y, Sukumard D, Tempel W, Tong Y, Tresagues L, Vedadi M, Walker JR, Weigelt J, Welin M, Wu H, Xiao T, Zeng H, Zhu H (2007) In situ proteolysis for protein crystallization and structure determination. *Nat Methods* 4:1019–1021.
- Pantazatos D, Kim JS, Klock HE, Stevens RC, Wilson IA, Lesley SA, Woods VL, Jr (2004) Rapid refinement of crystallographic protein construct definition employing enhanced hydrogen/deuterium exchange MS. *Proc Natl Acad Sci USA* 101:751–756.
- Rypniewski WR, Holden HM, Rayment I (1993) Structural consequences of reductive methylation of lysine residues in hen egg white lysozyme: an X-ray analysis at 1.8-Å resolution. *Biochemistry* 32:9851–9858.
- Walter TS, Meier C, Assenberg R, Au KF, Ren J, Verma A, Nettleship JE, Owens RJ, Stuart DI, Grimes JM (2006) Lysine methylation as a routine rescue strategy for protein crystallization. *Structure* 14:1617–1622.
- Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettleship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, Dwek RA, Jones EY, Owens RJ, Davis SJ (2007) Glycoprotein structural genomics: solving the glycosylation problem. *Structure* 15:267–273.
- Gordon K, Redelinghuys P, Schwager SL, Ehlers MR, Papageorgiou AC, Natesh R, Acharya KR, Sturrock ED (2003) Deglycosylation, processing and crystallization of human testis angiotensin-converting enzyme. *Biochem J* 371:437–442.
- Lee X, Ahmed FR, Hiram T, Huber CP, Rose DR, To R, Hasnain S, Tam A, Mort JS (1990) Crystallization of recombinant rat cathepsin B. *J Biol Chem* 265: 5950–5951.
- Jaroszewski L, Slabinski L, Wooley J, Deacon AM, Lesley SA, Wilson IA, Godzik A (2008) Genome pool strategy for structural coverage of protein families. *Structure* 16: 1659–1667.
- Chang G, Spencer RH, Lee AT, Barclay MT, Rees DC (1998) Structure of the MscL homolog from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel. *Science* 282:2220–2226.
- Prive GG, Verner GE, Weitzman C, Zen KH, Eisenberg D, Kaback HR (1994) Fusion proteins as tools for crystallization: the lactose permease from *Escherichia coli*. *Acta Crystallogr D Biol Crystallogr* 50:375–379.
- Smyth DR, Mrozkiewicz MK, McGrath WJ, Listwan P, Kobe B (2003) Crystal structures of fusion proteins with large-affinity tags. *Protein Sci* 12:1313–1322.
- Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318:1266–1273.
- Nauli S, Farr S, Lee YJ, Kim HY, Faham S, Bowie JU (2007) Polymer-driven crystallization. *Protein Sci* 16: 2542–2551.
- Patterson WR, Anderson DH, DeGrado WF, Cascio D, Eisenberg D (1999) Centrosymmetric bilayers in the 0.75 Å resolution structure of a designed alpha-helical peptide, D, L-Alpha-1. *Protein Sci* 8:1410–1422.
- Pentelute BL, Gates ZP, Tereshko V, Dashnau JL, Vanderkooi JM, Kossiakoff AA, Kent SB (2008) X-ray structure of snow flea antifreeze protein determined by racemic crystallization of synthetic protein enantiomers. *J Am Chem Soc* 130:9695–9701.
- Wukovitz SW, Yeates TO (1995) Why protein crystals favour some space-groups over others. *Nat Struct Biol* 2:1062–1067.
- Zawadzke LE, Berg JM (1993) The structure of a centrosymmetric protein crystal. *Proteins* 16:301–305.
- Hung LW, Kohmura M, Ariyoshi Y, Kim SH (1999) Structural differences in D and L-monellin in the crystals of racemic mixture. *J Mol Biol* 285:311–321.
- Banigan JR, Mandal K, Sawaya MR, Thammavongsa V, Hendrickx AP, Schneewind O, Yeates TO, Kent SB (2010) Determination of the X-ray structure of the snake venom protein omwarpin by total chemical synthesis and racemic protein crystallography. *Protein Sci* 19:1840–1849.
- Matthews BW (2009) Racemic crystallography—easy crystals and easy structures: what's not to like? *Protein Sci* 18:1135–1138.
- Koide S (2009) Engineering of recombinant crystallization chaperones. *Curr Opin Struct Biol* 19:449–457.
- Kovari LC, Momany C, Rossmann MG (1995) The use of antibody fragments for crystallization and structure determinations. *Structure* 3:1291–1293.
- Sennhauser G, Grutter MG (2008) Chaperone-assisted crystallography with DARPins. *Structure* 16:1443–1453.
- Derewenda ZS (2010) Application of protein engineering to enhance crystallizability and improve crystal properties. *Acta Crystallogr D Biol Crystallogr* 66: 604–615.
- Longenecker KL, Garrard SM, Sheffield PJ, Derewenda ZS (2001) Protein crystallization by rational mutagenesis of surface residues: Lys to Ala mutations promote crystallization of RhoGDI. *Acta Crystallogr D Biol Crystallogr* 57:679–688.

26. Banatao DR, Cascio D, Crowley CS, Fleissner MR, Tienson HL, Yeates TO (2006) An approach to crystallizing proteins by synthetic symmetrization. *Proc Natl Acad Sci USA* 103:16230–16235.
27. Forse GJ, Ram N, Banatao DR, Cascio D, Sawaya MR, Klock HE, Lesley SA, Yeates TO (2011) Synthetic symmetrization in the crystallization and structure determination of CclA from *Thermotoga maritima*. *Protein Sci* 20:168–178.
28. Yamada H, Tamada T, Kosaka M, Miyata K, Fujiki S, Tano M, Moriya M, Yamanishi M, Honjo E, Tada H, Ino T, Yamaguchi H, Futami J, Seno M, Nomoto T, Hirata T, Yoshimura M, Kuroki R (2007) 'Crystal lattice engineering,' an approach to engineer protein crystal contacts by creating intermolecular symmetry: crystallization and structure determination of a mutant human RNase 1 with a hydrophobic interface of leucines. *Protein Sci* 16:1389–1397.
29. Cieslik M, Derewenda ZS (2009) The role of entropy and polarity in intermolecular contacts in protein crystals. *Acta Crystallogr D Biol Crystallogr* 65:500–509.
30. Goldschmidt L, Cooper DR, Derewenda ZS, Eisenberg D (2007) Toward rational protein crystallization: a Web server for the design of crystallizable protein variants. *Protein Sci* 16:1569–1576.
31. Price WN, II, Chen Y, Handelman SK, Neely H, Manor P, Karlin R, Nair R, Liu J, Baran M, Everett J, Tong SN, Forouhar F, Swaminathan SS, Acton T, Xiao R, Luft JR, Lauricella A, DeTitta GT, Rost B, Montelione GT, Hunt JF (2009) Understanding the physical properties that control protein crystallization by analysis of large-scale experimental data. *Nat Biotechnol* 27:51–57.
32. Guo M, Shapiro R, Schimmel P, Yang XL (2010) Introduction of a leucine half-zipper engenders multiple high-quality crystals of a recalcitrant tRNA synthetase. *Acta Crystallogr D Biol Crystallogr* 66:243–250.
33. Harding MM (2001) Geometry of metal-ligand interactions in proteins. *Acta Crystallogr D Biol Crystallogr* 57:401–411.
34. Harding MM (2004) The architecture of metal coordination groups in proteins. *Acta Crystallogr D Biol Crystallogr* 60:849–859.
35. Tamames JA, Ramos MJ (2010) Metals in proteins: cluster analysis studies. *J Mol Model* 17:429–442.
36. Laganowsky A, Benesch JL, Landau M, Ding L, Sawaya MR, Cascio D, Huang Q, Robinson CV, Horwitz J, Eisenberg D (2010) Crystal structures of truncated alphaA and alphaB crystallins reveal structural mechanisms of polydispersity important for eye lens function. *Protein Sci* 19:1031–1043.
37. Alvarez HM, Xue Y, Robinson CD, Canalizo-Hernandez MA, Marvin RG, Kelly RA, Mondragon A, Penner-Hahn JE, O'Halloran TV (2010) Tetrathiomolybdate inhibits copper trafficking proteins through metal cluster formation. *Science* 327:331–334.
38. Brodin JD, Medina-Morales A, Ni T, Salgado EN, Ambroggio XI, Tezcan FA (2010) Evolution of metal selectivity in templated protein interfaces. *J Am Chem Soc* 132:8610–8617.
39. Ni TW, Tezcan FA (2010) Structural characterization of a microperoxidase inside a metal-directed protein cage. *Angew Chem Int Ed Engl* 49:7014–7018.
40. Radford RJ, Nguyen PC, Tezcan FA (2010) Modular and versatile hybrid coordination motifs on alpha-helical protein surfaces. *Inorg Chem* 49:7106–7115.
41. Salgado EN, Ambroggio XI, Brodin JD, Lewis RA, Kuhlman B, Tezcan FA (2010) Metal templated design of protein interfaces. *Proc Natl Acad Sci USA* 107:1827–1832.
42. Salgado EN, Radford RJ, Tezcan FA (2010) Metal-directed protein self-assembly. *Acc Chem Res* 43:661–672.
43. Guss JM, Merritt EA, Phizackerley RP, Hedman B, Murata M, Hodgson KO, Freeman HC (1988) Phase determination by multiple-wavelength x-ray diffraction: crystal structure of a basic "blue" copper protein from cucumbers. *Science* 241:806–811.
44. Hendrickson WA, Horton JR, LeMaster DM (1990) Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *EMBO J* 9:1665–1672.
45. Guncar G, Wang CI, Forwood JK, Teh T, Catanzariti AM, Ellis JG, Dodds PN, Kobe B (2007) The use of Co²⁺ for crystallization and structure determination, using a conventional monochromatic X-ray source, of flax rust avirulence protein. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 63:209–213.
46. Hendrickson WA (1991) Determination of macromolecular structures from anomalous diffraction of synchrotron radiation. *Science* 254:51–58.
47. Hendrickson WA (1999) Maturation of MAD phasing for the determination of macromolecular structures. *J Synchrotron Radiat* 6:845–851.
48. Kang SO, Wuerges J, Lee JW, Yim YI, Yim HS, Carugo KD (2004) Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. *Proc Natl Acad Sci USA* 101:8569–8574.
49. Borths EL, Locher KP, Lee AT, Rees DC (2002) The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. *Proc Natl Acad Sci USA* 99:16642–16647.
50. Regan L (1993) The design of metal-binding sites in proteins. *Annu Rev Biophys Biomol Struct* 22:257–287.
51. Ghadiri MR, Choi C (1990) Secondary structure nucleation in peptides—transition-metal ion stabilized alpha-helices. *J Am Chem Soc* 112:1630–1632.
52. Handel TM, Williams SA, DeGrado WF (1993) Metal ion-dependent modulation of the dynamics of a designed protein. *Science* 261:879–885.
53. Krantz BA, Sosnick TR (2001) Engineered metal binding sites map the heterogeneous folding landscape of a coiled coil. *Nat Struct Biol* 8:1042–1047.
54. Dokmanic I, Sikic M, Tomic S (2008) Metals in proteins: correlation between the metal-ion type, coordination number and the amino-acid residues involved in the coordination. *Acta Crystallogr D Biol Crystallogr* 64:257–263.
55. Baase WA, Liu L, Tronrud DE, Matthews BW (2010) Lessons from the lysozyme of phage T4. *Protein Sci* 19:631–641.
56. Michel SL, Berg JM (2002) Building a metal binding domain, one half at a time. *Chem Biol* 9:667–668.
57. Moon AF, Mueller GA, Zhong X, Pedersen LC (2010) A synergistic approach to protein crystallization: combination of a fixed-arm carrier with surface entropy reduction. *Protein Sci* 19:901–913.
58. Zhao M, Cascio D, Sawaya MR, Eisenberg D (2011) Structures of segments of alpha-synuclein fused to maltose-binding protein suggest intermediate states during amyloid formation. *Protein Sci* 20:996–1004.
59. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258–1265.
60. Wiltzius JJ, Sievers SA, Sawaya MR, Eisenberg D (2009) Atomic structures of IAPP (amylin) fusions

- suggest a mechanism for fibrillation and the role of insulin in the process. *Protein Sci* 18:1521–1530.
61. Kobe B, Center RJ, Kemp BE, Pombourios P (1999) Crystal structure of human T cell leukemia virus type 1 gp21 ectodomain crystallized as a maltose-binding protein chimera reveals structural evolution of retroviral transmembrane proteins. *P Natl Acad Sci USA* 96:4319–4324.
  62. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
  63. Kabsch W (2010) XDS. *Acta Crystallogr D Biol Crystallogr* 66:125–132.
  64. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser crystallographic software. *J Appl Crystallogr* 40:658–674.
  65. Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132.
  66. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53:240–255.
  67. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC (2002) PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 58:1948–1954.
  68. Blanc E, Roversi P, Vornrhein C, Flensburg C, Lea SM, Bricogne G (2004) Refinement of severely incomplete structures with maximum likelihood in BUSTER-TNT. *Acta Crystallogr D Biol Crystallogr* 60:2210–2221.