THESIS

SOFTWARE TO DESIGN CROSSLINKS FOR PROTEIN CRYSTAL STABILIZATION

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ABSTRACT

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Programmable materials allow properties as specific locations in the material to be modified through reliable encoding. One class of such materials are protein crystals that allow changes to be made through genetic manipulation. Protein crystals are well-ordered and highly porous materials, but they are also easily dissolved, limiting their utility. Crosslinking techniques previously developed often have a deleterious effects on the crystal order. In this work, we introduce software to design specific crosslinks across protein crystal interfaces using disulfide and dityrosine crosslinks as well as a variety of small molecule crosslinkers used in protein conjugation. The software is a general tool for specific crosslinking that introduces a number of improvements on previous disulfide design software. Several of the disulfide and dityrosine designs were assembled in the lab and one of the disulfide crosslink designs was confirmed using X-ray diffraction.

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Motivation

What is a programmable material?

We define a programmable material as a macroscopic material whose properties at specific locations may be directly manipulated through reliable encoding. In addition, we may use such encoding to direct assembly of the material. Alternative definitions may focus on materials that change physical properties or shape based on some input. This work will focus on the first definition and, in particular, the ability to place atoms at specific locations within a material with precision approaching a few angstrom. Several approaches have been taken to reach this goal of programmed self-assembling materials. These approaches are predicated on the concept of self-assembly which describes the regular arrangement of atoms and molecules directed exclusively by interaction properties between the building blocks of the material. In metal organic frameworks, the designed interactions are those between metal ion centers and organic linkers (El-Kaderi et al. 2007). The substitution of longer linkers has resulted in MOFs with larger pores (Eddaoudi et al. 2002). Substitution of metal ions with different coordination geometry would be expected to rearrange the geometry of the assembly. Computational design of a MOF, in which selections from a panel of building blocks were fit to a specified topological arrangement, has been demonstrated as a way to reach specific absorbance properties (Gomez-Gualdron et al. 2014). Assembly of virus particles in a particular orientation has been demonstrated by designing the interactions between amino acid side chains in a specific location of the virus surface and the surface of a substrate (Chen et al. 2013; Huang et al. 2005; S.-W. Lee et al. 2002).

Another aspiration of this field is to define locations within the material for adding functional groups which may be used to attach other molecules. Examples of successful functionalization have included the attachment of dye molecules in an MOF (Hinterholzinger et al. 2012), and the capture of proteins (green fluorescent protein and lipase) on an MOF (Jung et al. 2011). In protein crystals, the natural biotin-binding

pocket of avidin has been used to capture dyes, enzymes, or nanoparticles labeled with biotin within co-crystallized cowpea mosaic virus-avidin (Liljeström, Mikkilä, and Kostiainen 2014).

Applications of nanoporous programmable material

Porous metal-organic frameworks have been proposed as novel materials for absorbing gases for storage (Rosi 2003) or separation (Li, Kuppler, and Zhou 2009), catalysis (J. Lee et al. 2009), and carriers for drug delivery (Horcajada et al. 2010). In one example of a highly porous MOF, the rectangular pores had apertures of ~9 × 12 Å (Li, Kuppler, and Zhou 2009). Perhaps the largest pore designed MOF has been assembled with pore apertures ranging up to 98Å which allowed green fluorescent protein (which measures about 34Å x 45Å) to pass into the pores (Deng et al. 2012).

Advantages of protein crystals over other approaches

In metal-organic frameworks designed to form large pores, the frameworks often form twinned interpenetrating structures with smaller pores instead (Furukawa et al. 2013). With some exceptions, MOFs lack hydrolytic and chemical stability (at least in natural gas storage applications) (Gomez-Gualdron et al. 2014). Ueno provided a useful review of recent demonstrations of the usefulness of proteins crystals as nanomaterials (Ueno 2013). While it may be possible to design a protein crystal with a bottom up approach (Lanci et al. 2012) as has been done with MOFs, we also have the opportunity to search the vast collection of crystal structures deposited in the Protein Data Bank for pore dimensions desired.

Protein crystals are highly-porous, well-ordered, three-dimensional materials with pore diameters commonly ranging up to five nanometers. This, combined with the availability of a wide range of tools for protein modification and conjugation, makes protein crystals an attractive target for development as novel materials for storage, separation, or templating. Two major hurdles to this development are that proteins are often difficult to crystallize, and that once crystals are obtained, they are generally very fragile and likely to dissolve following slight changes in solution conditions. This work addresses the latter of the two concerns through computational design of intermolecular crosslinks. Characterization of some designed crosslinks in protein crystals was completed.

We can use *E. coli* to produce 10-60 milligrams of protein per liter of culture using autoinduction. For higher production of proteins, filamentous fungal cell cultures have reached 150-900 mg per liter (Nyyssönen et al. 1993; Ward et al. 2004) and mammalian cell cultures have been used to obtain up to a gram per liter (Wurm 2004) with one example reaching 26 grams per liter (Jarvis 2008). Genetic modification of the protein's gene sequence through standard techniques allow easy modifications of the protein's amino acid sequence. Existing computational protein design tools can be applied to design problems in protein crystals. Well established methods for protein characterization can be used to study protein crystals; from X-ray crystallography, to high-performance liquid chromatography, to mass spectrometry.

If we scan the Protein Data Bank for crystal structures having specific attractive structures, we can also consider protein crystals as programmable materials in another sense: they are programmed to self-assemble.

Templating

Synthesis of nanoparticles often relies on high temperatures and harsh chemicals. These conditions may be avoided by templating nanoparticles with protein crystals. Protein crystals that have already been stabilized by glutaraldehyde crosslinking have already been used to template metal particles. Researchers have successfully accumulated ions of Pt^{2+} (Falkner et al. 2005), Au^{2+} (Wei et al. 2011; Takeda, Kondow, and Mafuné 2011; Slocik et al. 2004; Guli et al. 2010), Ag^{2+} (Guli et al. 2010), and Co^{2+}/Pt^{2+} (Abe et al. 2012) in order to form nanoparticles inside the pores of crosslinked protein crystal or by similar methods on virus particles. The metal particles can be closely aligned with the interior surfaces of the crystal pores (Falkner et al. 2005).

Templating of nanostructured metallic materials has also been demonstrated on the exterior of viral capsids. In this case, the ease of modifying the surface properties of the capsids through genetic methods allowed researchers to construct different template structures (Huang et al. 2005). Genetically modified phage particles have been designed to align perpendicularly to a zinc sulfide film in what was termed a liquid crystal due to the flexibility of orientations of the particles beyond the single attachment point of the phage display (S.-W. Lee et al. 2002). In a similarly modified bacteriophage, nanowires with potential application in dye-

sensitized solar cells were produced. Here, the loosely aligned particles were crosslinked together using glutaraldehyde to produce a hydrogel. To make the wires, gold nanoparticles were first captured on the phage surface at specific binding sites, and then the phage particles were coated with titanium dioxide (Chen et al. 2013).

In another example of nano-structured templating using proteins, researchers templated polyacrylamide within lysozyme pores. Crystals were crosslinked with glutaraldehyde and soaked in acrylamide before initiating polymerization (Cohen-Hadar et al. 2006).

Pore functionalization

Protein crystal pore surfaces are easily modified both with DNA encoded amino acid sequence changes and post-expression or post-crystallization chemical modifications. For example, residues exposed to the pores of a protein crystal can be mutated to cysteine, enabling attachment of other molecules including proteins via maleimide-activated crosslinkers (Hermanson 2013). Ueno and coworkers demonstrated this by attaching maleimide derivatives of fluorescein, eosin, and Tris(bipyridine)ruthenium(II) ($[Ru(bpy)3]^{2+}$) to the pore surfaces of myoglobin crystals (Koshiyama et al. 2010).

Background

Porous Protein Crystals as Programmable Materials

Protein crystals are highly ordered three-dimensional arrays of molecules in which the positions of atoms within the crystal may be determined within a few angstrom using X-ray diffraction. Protein crystals are typically highly porous and frequently have solvent content ranging from 27% to 65% (Matthews 1968) with an average of 51% (Chruszcz et al. 2008). Pore diameters typically range from 2 to 10 nm (Vilenchik et al. 1998). This combination of atomic level order and high porosity provide an intriguing opportunity to template other materials complementary to the crystal pore shape. Their high void volume makes protein crystals as attractive targets for development of novel storage materials and the range of pore sizes makes them potential targets for designing novel separation media.

There are few other materials with this combination of porosity, pore size, and internal order. Protein crystals may be considered programmable in that the amino acid sequence may be reliably modified by standard DNA manipulation methods. Substitution of one amino acid for another may be used to change the physical properties at the site of the substitution. Such modifications may include introducing attachment sites at specific locations in the crystal, changing the properties of the crystal at specific locations, or altering the pore structure of the native protein crystal.

Challenges of using protein crystals

However, the applicability of protein crystals to the problems of nanoscale programmable materials is hindered by several challenges. First and foremost, protein crystals are generally minimally stable, and slight changes in the solution conditions (including changes in precipitant concentration, protein concentration, temperature, or pH) may result in the crystal dissolving. Once a protein dissolves, recrystallization will depend on the ability to change the solution conditions back to those favorable to crystallization without losing the protein.

A major challenge of working with any protein in solution stems from their minimal stability against unfolding. With relatively mild variations from native physiological conditions, the folded structure of a protein may easily denature into an unstructured or semi-structured polymer. While refolding a denatured protein may be possible, hydrophobic regions of unfolded protein are prone to binding one another, resulting ultimately in a disordered aggregate. This aggregation is often irreversible. Protein crystals have some advantages compared to proteins in solution in terms of susceptibility to unfolding. For example, dry lipase crystals demonstrated improved shelf stability (activity after storage at room temperature) compared with non-crystalline aggregate (Shenoy et al. 2001).

In addition, protein crystals can be cracked or broken by slight mechanical forces. For example, protein crystals may be chipped or cracked simply by a clumsy attempt to loop a crystal. A rapid change in the solution conditions may also result in a cracked crystal due to differences in the osmotic pressure exerted on different parts of the crystal.

Overcoming Fragility with Glutaraldehyde Crosslinking

Glutaraldehyde crosslinking is commonly used to overcome the challenge of protein crystal instability. This method was introduced in 1964 by Quiocho and Richards (Quiocho and Richards 1964) as a way to prevent crystals from dissolving in cryoprotectant solutions used in X-ray crystallography and it continues to be utilized and improved. Glutaraldehyde is generally thought to mainly react with primary amines in lysine side chains. Wine et al. confirmed this crosslink by using X-ray crystallography to identify glutaraldehyde crosslinks between lysine nitrogen atoms found in hen egg while lysozyme (HEWL) crystals (Wine et al. 2007). Glutaraldehyde crosslinking can result in long term crystal stability in water (St. Clair and Navia 1992). Cohen-Hadar et al. showed that the pore surfaces and diffraction of lysozyme crystals were mostly preserved during glutaraldehyde crosslinking. Acrylamide polymerization within the pores demonstrated the viability of using crosslinked crystals for templating (Cohen-Hadar et al. 2006). Crosslinking enzyme crystals with glutaraldehyde has been promoted as way to use these fragile molecules in challenging industrial processes and enable recovery (Margolin and Navia 2001).

Crosslinked Enzyme Crystals

Beyond templating other materials, crosslinking may be applied to enzyme crystals to produce stable and easily recovered solid particles for catalysis. Enzyme crystals can retain all or most of the activity of enzymes in solution while offering the attractive property of easy physical separation of these particles from the reaction mixture. Crosslinking or immobilization of enzymes by crosslinking them to a solid surface provide mechanical stability which is essential for the use of enzymes in practical industrial process conditions. In enzyme crystals, the concentration of glutaraldehyde in the crosslinking solution has been positively correlated with activity retention over time (St. Clair and Navia 1992). Crosslinked enzyme crystals of each of the following enzymes have been demonstrated: lipase (Lalonde et al. 1995), alcohol dehydrogenase (K. M. Lee et al. 1986), thermolysin for asparatame production (St. Clair and Navia 1992), thermolysin for peptide synthesis (Persichetti et al. 1995), chloroperoxidase (Ayala et al. 2002), aldolase (Sobolov et al. 1994), subtilisin (Noritomi et al. 2007), glucoamylase (Abraham et al. 2004), and laccase (Shenoy et al. 2001). Better still, crosslinked enzyme crystals have been shown in many instances to preserve activity in conditions that would denature the monomeric enzyme (e.g., 50%THF (St. Clair and Navia 1992), 30% DMSO (St. Clair and Navia 1992), DMF (Persichetti et al. 1995), acetone (Persichetti et al. 1995), elevated temperature (Ayala et al. 2002; Abraham et al. 2004), acetonitrile (Sobolov et al. 1994), dioxane (Sobolov et al. 1994), and mixed organic media (Noritomi et al. 2007)). The results of one study also suggested that the impeller speeds necessary to keep a stirred tank homogenous would cause little breakage of glutaraldehyde crosslinked crystals (T. S. Lee, Turner, and Lye 2002).

Challenges of Glutaraldehyde Crosslinking

Many of the difficulties using glutaraldehyde to crosslink crystals arise from its potential to form a variety of different crosslinks. Though lysine-to-lysine crosslinks are expected to be the primary target of glutaraldehyde crosslinking, reactions with thiols, phenols, and imidazoles are also possible (Walt and Agayn 1994; Migneault et al. 2004; Habeeb and Hiramoto 1968; Wine et al. 2007). In one study, lysine residues as well as serine and arginine residues were modified by glutaraldehyde crosslinking (Ayala et al. 2002). If an

enzyme is the target of crosslinking, a lysine may be active in catalysis (e.g., aldolase), and glutaraldehyde may not be used as it could destroy the enzyme's activity.

In addition to reacting with several amino acid side chains, glutaraldehyde may polymerize through an aldol condensation reaction in basic solutions or when there is a high concentration of monomer (Wine et al. 2007). There have been conflicting reports about whether glutaraldehyde polymerization only occurs at high pH. Korn et al. (1972) studied the various forms of glutaraldehyde in aqueous solution which they found to include free, monomeric glutaraldehyde, the cyclic hemiacetal of a hydrate form, and oligomers of glutaraldehyde. They concluded that glutaraldehyde polymerizes at all pHs, but polymerizes in a different way in alkaline conditions (Korn, Feairheller, and Filachoine 1972). The presence of polymers improves stabilization of crystals by forming a variety of crosslinks reaching different distances, but may reduce diffusion rates (Walt and Agayn 1994). The presence of oligomers of different lengths that may react with several side chains could lead to the formation of many diverse linkages in a cob-web of disordered crosslinks in protein crystal pores. Such a construction would occlude the pores and disrupt the ordered nature of the crystal. Indeed, glutaraldehyde is associated with a decrease in crystal resolution (from 1.3Å to 1.6Å (Wine et al. 2007)and from 1.7Å to 2.0Å (Cohen-Hadar et al. 2006)). In addition it's been reported that glutaraldehyde crosslinking in lysozyme crystals resulted in a small expansion (1.8%) in the a-dimension of the crystal unit cell and a small contraction in the c-dimension (1.4%) (Guli et al. 2010).

Lusty (1999) pointed out that, with glutaraldehyde crosslinking, it is "often difficult to establish the required intermolecular cross-links without loss of diffraction quality" and suggested reducing the glutaraldehyde concentration in solution by using vapor-diffusion as a solution. This work also suggests that non-specific crosslinking plays a role in diffraction loss and observes that stabilization by glutaraldehyde crosslinking is limited by the number and positions of the lysines in the protein (Lusty 1999). In one example of diffraction loss, the diffraction resolution was checked at each stage of the previously mentioned polyacrylamide templating. Crosslinking lysozyme crystals by glutaraldehyde slightly decreased diffraction resolution from 1.7Å to 2.0 Å. Soaking in the acrylamide reduced resolution to 2.6 Å for crosslinked crystals, while un-crosslinked crystals were severely damaged by this step of the process. Polymerization of acrylamide

within the crystal introduced rigidity without impacting resolution which was measured at 2.5 Å (Cohen-Hadar et al. 2006).

Glutaraldehyde crosslinked enzyme crystals generally perform poorly when the substrate is large or when the reaction is fast and diffusion limited (Govardhan 1999). Simply put, volumetric activity must suffer if enzymes in the crystal interior are not working at capacity. The observed specific activity has in fact been lower for some crosslinked enzyme crystals compared to the same enzyme in solution (Roy and Abraham 2006). Crosslinked crystals may also restrict conformational changes necessary for activity and crosslinking may modify residues essential to activity (Govardhan 1999). On the other hand, crosslinking that doesn't restrict diffusion or restrict a conformation change necessary for catalysis may allow the enzyme to achieve higher activity at a higher temperature than could be withstood by the enzyme in solution (Abraham et al. 2004). Fortunately, transport concerns can be addressed very easily by simply growing smaller crystals though a trade-off must be considered in that smaller crystals will be more challenging to filter from the reaction mixture. The critical thickness for a crosslinked enzyme crystal, above which the reaction rate is diffusion limited, depends on the reaction kinetics, enzyme concentration, and diffusion constant of the substrate in the crystal. This critical thickness may be as little as the thickness of the crystal unit cell for fast reactions, and on the order of several microns for relatively slower reactions (Margolin and Navia 2001).

Although glutaraldehyde crosslinking does often lead to stable crystals, its reactivity with multiple side chains and potential for glutaraldehyde polymerization may cause widespread modification of the crystal, loss of diffraction, and possibly lead to the partial blocking of pores. Glutaraldehyde crosslinking may depend on the pH of the solution, temperature, the presence of primary amines in the solution, and the age of the glutaraldehyde and, in our own work, glutaraldehyde crosslinking has been difficult to reliably reproduce. For these reasons, we would like to develop crosslinking methods that more reproducible and offer greater control.

Alternative crosslinking methods

Clearly, some of the control over atomic placement in protein crystals is lost if they are crosslinked by glutaraldehyde. As such, the ability to label them a programmed material is lost. This is observed both in

the difficulty controlling the behavior of glutaraldehyde as well as in the often observed effect of decreasing the resolution of X-ray diffraction. To fulfill our vision of a stable, highly-ordered material, improved methods of crosslinking need to be developed. Several options are available to address the crosslinking problem with more specificity. Two natural amino acids lend themselves to direct crosslinking with the minimum modification necessary: a pair of cysteine residues may readily crosslink in the oxidizing environment of the atmosphere, and a pair of tyrosine residues may be oxidized by a variety of slightly more aggressive means to form dityrosine. Many natural materials rely on these crosslinks for strength. Structural proteins relying on natural crosslinks are of special note in this context. In addition, several small molecules may be used to crosslink proteins in a more specific manner than glutaraldehyde. These may have greater reactive specificity and may not have the potential for polymerizing. Crosslinking by disulfides, dityrosines, or some of these small molecule crosslinkers may stabilize protein crystals without surrendering the specificity needed to retain their properties as programmable materials.

Natural crosslinked materials

Many naturally-occurring proteins rely on crosslinking to provide mechanical strength and resilience. Keratins rely on disulfide crosslinks between either the coiled coils of alpha-helices (alpha-karatins) or between beta-sheets (beta-keratins). These include keratins found in silk, the hair, hooves, horns and claws of mammals, and the scales, shells, and claws of reptiles, and the feathers, beaks, and claws of birds. Less well known are the dityrosine crosslinks found in a variety of natural materials. More expansive review of these materials is provided below.

Dityrosine crosslinks in nature

DiMarco and Giulivi provide a good review of the natural occurrences of dityrosine in structural proteins and oxidatively damaged proteins, as well as the analytical methods for detecting dityrosine (DiMarco and Giulivi 2007). Dityrosine crosslinks have been identified in a wide variety of structural proteins such as resilin found in insect cuticles including the rubbery tendon that attaches a dragonfly's wing to its body (Andersen 1964), elastin (LaBella et al. 1967), fibroin (Raven, Earland, and Little 1971), cuticle collagen

(Sakura and Fujimoto 1984), caddisfly silk fibers (Wang et al. 2014), and keratin (Raven, Earland, and Little 1971). Dityrosine has also been implicated in a variety of diseases including the of β-amyloid plaques implicated in Alzheimer's disease (Al-Hilaly et al. 2013) and the protein aggregates in cataracts. Oxidative stress may lead to dityrosine crosslinking resulting in protein aggregation and inactivation.

The presence of dityrosine in a wide array of strong, elastic proteins fibers suggests these crosslinks play a vital role in the physical properties of these proteins. Elvin et al. (2005) demonstrated that dityrosine crosslinks contributed to the impressive physical properties seen in spider silks and insect cuticles. From a gene coding for the resilin in fruit fly wing tendons, these researchers synthesized a highly elastic material with higher resilience than both low- and high-resilience rubbers (Elvin et al. 2005). Similarly, the β-amyloid peptide plaques associated with Alzheimer's disease have been approximated in vitro by producing dityrosine crosslinked fibers of the implicated peptide sequence (Kok et al. 2013).

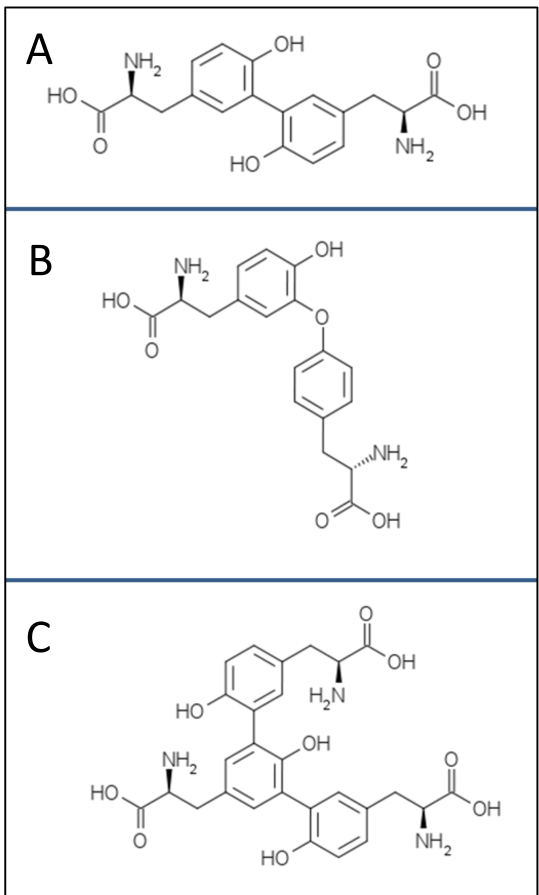

Figure 1: Chemical structure of three forms of tyrosine conjugation observed in natural materials: A) dityrosine, B) isodityrosine, and C) trityrosine.

The dityrosine bond between epsilon carbons of tyrosine has been more frequently reported, but isodityrosine crosslink has also been identified in cell wall calli of potato plants (Fry 1982) and trityrosine has been identified in resilin (Andersen 1964).

Engineered disulfide crosslinks

Researchers have attempted to mimic nature in stabilizing proteins by introducing disulfide bonds. Site directed mutagenesis can reliably be used to change the amino acid sequence of a protein in order to introduce a pair of cysteine residues. The question of which positions

in the protein backbone are capable of forming disulfide bonds following mutation presents an immediate challenge.

Many methods of disulfide design have been implemented by researchers over time. The simplest, though perhaps more painstaking, approach has been to manually pick residue pairs located within an appropriate alpha-carbon (Cα) distance cutoff. This approach has been used to design disulfide crosslinks between enzymes to effect substrate channeling where the proximity of the enzymes benefits the overall conversion rate (Hirakawa, Kakitani, and Nagamune 2013). Not everyone will have the skill and patience required to successfully design disulfide crosslinks by eye, and several computational approaches have been developed. Computational design of disulfides facilitates screening the entire protein for potential disulfide crosslinks and the ranking of the designs for feasibility.

An early computational approach built cysteine sidechains into the existing backbone to find residue pairs that could potentially provide a good disulfide bond distance. Clashing atom positions between the sulfur atoms in the disulfide and neighboring atoms were avoided, though the publication does not make clear how this if this was a manual check or part of the software. In this case, the purpose was to stabilize an enzyme (subtilisin) against denaturation (Wells and Powers 1986). Several researchers have automated similar processes with computer programs to scan proteins for appropriate site for disulfide crosslink introduction. The statistical preferences of disulfide geometries in protein structures are generally relied on to provide a target for finding optimal sites for introducing disulfide bonds. Several surveys of disulfide geometries have been undertaken to understand distribution of geometry of disulfide bonds in protein structures in the PDB (Petersen, Jonson, and Petersen 1999; Sowdhamini et al. 1989). DSDBase is a recent example that is available online which provides a database of disulfide bond geometries found in the Protein DataBank, including Cα–Cα, Cβ–Cβ, and S-S dihedral angles as well as the number of residues in the loop closed by the disulfide bond (Vinayagam 2004).

Proteus (Pabo and Suchanek 1986)

Pabo and Suchanek (1986) were the first to publish a computational program (called Proteus) for designing disulfide bonds in proteins. Their program scanned residue pairs for those meeting the appropriate

distance requirements and attempted to build the disulfide using a local coordinate system for each candidate pair. Proteus was written in LISP and FORTRAN and had approximately 500 functions. This program took 15 minutes of CPU time to screen a 184 residues in a dimer. The researchers completed a manual check for clashing atom positions as well as an energy refinement of the surrounding residues. Sauer et al. (1986) used Proteus to design intermolecular disulfide crosslinks between the monomers in a lambda repressor dimer. In experiments, the designed disulfide bond provided the dimer with resistance to denaturation by urea. Interestingly, the researchers also tested what they expected to be an unfavorable disulfide, mutating TYR85 to cysteine rather than TYR88. In a reducing environment, the Y85C mutant denatured at the same concentration urea as the wild type. In an oxidizing environment, on the other hand, the Y85C mutant denatured at a lower urea concentration, suggesting that the unfavorable disulfide may have had a negative effect on the folding stability of the protein. Studies of the thermal denaturation of each design followed a similar pattern to what was observed in the chemical denaturation experiments (Sauer et al. 1986).

SSBOND(Hazes and Dijkstra 1988)

Written in FORTRAN 77, SSBOND accepted structural data input in Protein Data Bank format, generated potential Cβ and Sγ positions for each residue based on the backbone atom coordinates for that residue, calculated an energy score of the bond conformation, and optimized the geometry based on the scores. Candidates were filtered by Cβ-Cβ distance and Cβ1-Sγ2 distance before the energy minimization took place. The bond energies used by SSBOND were taken from the GROMOS molecular dynamics and energy refinement program package and the minimization followed a least squares regression algorithm. The program was validated by comparing attempts to recapitulate disulfides in known structures. The authors acknowledged that the program ignored potential clashes between the designed disulfide and neighboring side atoms, and did not account for packing defects due to cysteine mutations. For a protein with 274 residues, the program took only ~5 minutes of CPU time.

MODIP (Sowdhamini et al. 1989) /MODIP2 (Dani, Ramakrishnan, and Varadarajan 2003)

MODIP (Modelling Of Disulfides In Proteins) was designed in a manner more similar to Proteus than to SSBOND, adding a grading system for ranking the disulfide geometries designed. Grades were simply assigned based on whether the design fit bond length and dihedral angle cutoffs that were based on the distribution of these values measured from structures deposited Protein Data Bank. The software was validated by attempting to recapitulate natural disulfide bonds hidden from the program.

In 2003, MODIP was revised (MODIP2) to include a steric clash check between the introduced disulfide and nearby residues. The ideal geometry in the program was also updated based on an updated Protein Data Bank. The 6017 structures in the Data Bank were narrowed to 172 by eliminating those lacking disulfide bonds, those with resolution worse than 2.0Å, and those with sequence homology more than 40%. The authors observed that many previously designed disulfides destabilized the proteins they were intended to stabilize and hypothesized that this was due to steric hindrance of the disulfide. The previous grading system was updated with a penalty assigned for any non-zero van der Waals clash between the sulfur atoms of the introduced cysteines and neighboring atoms. A simple energy function of the form $A * exp^{-\mu r} - B * r^{-6}$ (where A and B depend on the atom interacting with the sulfur and r is the distance of the interaction) was used to penalize clashes.

Disulfide by Design ™ (Dombkowski 2003) / Disulfide by Design 2.0 ™ (Craig and Dombkowski 2013)

Also in 2003, Dombkowski described another program to design disulfide crosslinks, Disulfide by Design ™. Similar to its predecessors, the program scanned residues for backbone geometries that could provide adequate disulfide bond geometries. In this case, the geometry was optimized based on an energy function fit to a statistical survey of disulfide geometries by Petersen et al. (1999). Disulfide by Design ™ identified 706 of 710 existing disulfide bonds when "hidden" from the program (Dombkowski 2003). Anthony et al. (2002) used Disulfide by Design ™ to design a disulfide bond in *E. coli* RNA polymerase to facilitate the study of conformational changes during sigma[70] binding (Anthony, Dombkowski, and Burgess

2002). Craig and Dombkowski later updated Disulfide by Design and made it available on the internet (Disulfide by Design 2.0, 2013), allowing structures to be imported directly from the PDB. The updated program provided a disulfide bond energy score as well as the B-factor of the residues to be mutated to cysteine on the basis of other studies that found a correlation between this value and whether or not the disulfide was stabilizing (Craig and Dombkowski 2013).

Molecular Dynamics

Sanchez-Romero et al. (2013) successfully incorporated molecular dynamics (MD) simulation in the design of a disulfide-stabilized phytase. The authors identified flexible regions of the protein using MD simulations at elevated temperature and analysis of the isotropic root mean square deviation of Cα atoms. Residues within these regions were chosen for mutation based on the backbone geometry (no further details were provided). Six designs were constructed: three with a single disulfide, two with a pair of disulfides, and one with three disulfide crosslinks. All six of their designed variants achieved their maximum activities at temperatures higher than found for the wild-type protein by up to 10°C. Thermal denaturation of the disulfide-enhanced designs occurred at 1.3-12.1°C higher than in the wild-type protein with the single-disulfide designs at the lower end and the triple-disulfide design with the largest improvement (Sanchez-Romero et al. 2013)

Disulfides in the context of crystals

An early successful introduction of disulfide crosslinks at protein-protein interfaces was reported by Yang et al. (2000). In this case, the crystallographic interface was used to model cysteine mutants that would create disulfide crosslinks. The mutation sites were apparently manually designed as no mention is made of software used for this purpose. Polymers of T4 lysozyme were then made both from lysozyme in solution and crystallized lysozyme mutants by exposing the protein to oxygen. The yield of polymers was much higher from oxidized crystals than oxidized monomers in solution. Interestingly, these polymers were exploited as a means of studying monomer unfolding by mechanical stress through scanning force microscopy (SFM) (Yang et al. 2000).

Srinivasan et al. (2002) promoted disulfide crosslinked protein crystals as a way to form protein fibers which they called crystine. The disulfide modeling program MODIP was used to predict sites in crystallographic interfaces that would support disulfide crosslinking across the interface. Only one out of three designs produced was successfully crystallized. The authors noted that even though the crosslinking was only one-dimensional, the crystals were difficult to dissolve. Dissolution of these crystals resulted in bundled fibers with diameters up to 7nm in which several crosslinked chains are held together by non-covalent interactions. In contrast to the random three dimensional network produced by glutaraldehyde crosslinking, disulfide crosslink preserved the order of a protein crystal (Srinivasan et al. 2002).

Crystallization of a coiled coil domain from a human protein BAP29 resulted in intermolecular disulfide bonds that produced one-dimensional protein polymers as in crystine. These crystals were shown to contain hexagonal pores with dimensions 7.5 nm x 9.0 nm (Quistgaard 2014). Finally, Heinz and Matthews reported that designed intermolecular disulfide crosslinks in T4 lysozyme resulted in more rapid crystallization (Heinz and Matthews 1994).

Improved disulfide engineering

The software developed in this work improves on the methods previously used. First, the geometry constraints have been updated based on more recent review of the disulfide geometries found in protein crystal structures found in the Protein Data Bank (see Supporting information). Second, the software may optionally allow the protein backbone to sample alternative conformations around the design site, potentially finding better optimized disulfide geometries. This reflects the natural backbone flexibility of proteins at room temperature and allows a broader sampling of space to assist the software in finding optimized disulfide bond geometries. Third, side chains located near the designed disulfide are allowed to move out of the way of the designed disulfide in order to avoid clashes. Combinatorial optimization of the design site allows the software to consider disulfides that would clash with the known structure, accepting only designs that can relieve such clashes by finding better positions for the clashing side chains. Lastly, this software is designed as a more general purpose crosslink design tool that includes the capability to design dityrosine crosslinks and a number of small molecule crosslinkers. The addition of further crosslinking molecules is also an automated

process. Ultimately, the software should scale, providing diverse candidate options to crosslink native and/or mutated protein-protein interactions.

Thiol Exchange

In order to control the oxidation state of cysteine residues, several chemicals for reducing and oxidizing the thiol groups are available. β-mercaptoethanol (BME) is somewhat volatile and can therefore be used to reduce cysteine residues without direct addition and dilution of the sample. This may be useful for reducing protein crystals that may dissolve on dilution of protein and/or salt in the liquid they are stored in. BME has a fairly short lifetime in aqueous solution and requires fresh addition daily to maintain reducing conditions. Dithiothreitol (DTT) is less volatile than BME and slightly more stable, lasting 3-7 days in solution. The lack of volatility necessitates direct addition of DTT to the drop to be reduced. The stability of DTT decreases at pH values away from 7. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) is more stable than BME or DTT, lasting 2-3 weeks in solution. It is not volatile. TCEP solutions have a pH of ~2, so precautions should be taken to not modify the pH of solutions when TCEP is added. The structures of the reducing and oxidizing forms of these species and others are shown below in Table 1.

Figure 2: Thiol exchange illustrated with dithiolthreitol.

Although disulfide crosslinks may be easily formed by exposure to air, it may be useful to accelerate formation. Several chemical means of doing so are available. These have primarily been used to facilitate disulfide exchange in order to assist in protein folding.

Table 1: Structures of chemicals used for thiol exchange (to oxidize or reduce).

Chemical	Form used to reduce disulfides	Form used to oxidize disulfides

β-mercaptoethanol		
Dithiolthreitol		
TCEP-HCl		
Glutathione		
Cysteine		

Selenocysteine	HOOC, NH_2, O, NH, SeH, NH, COOH, O	HO, O, N, O, N, Se, Se, O, OH, NH_2, OH, O, NH_2, O, N, O, N, O, HO
Hydrogen peroxide		H, H, O–O

Engineered dityrosine crosslinks

Although less widely known than disulfide crosslinking due to being much less reactive in physiological conditions, dityrosine crosslinking has been used to study capsid dynamics during infection (Horowitz, Finn, and Asokan 2012), to study protein-protein interactions (David A. Fancy and Kodadek 1999), and to immobilize proteins on a surface (Endrizzi et al. 2006). With its implicated role in disease, dityrosine has also been proposed as a fluorescent probe for diagnosing oxidative stress (Malencik and Anderson 2003). Notably, UV light can accelerate dityrosine production, presumably due to radical formation. For example, pharmaceutical proteins including insulin could include dityrosine formation leading to dimerization and inactivation (Correia et al. 2012).

Researchers have induced dityrosine formation in proteins to mimic resilient materials found in nature, as a means to study transient protein-protein interactions, and to study dityrosine's role in disease. A variety of techniques have been used to oxidize pairs of tyrosine amino acids to dityrosine, including metal-catalyzed oxidation, photooxidation, and enzymatic oxidation.

Protein oxidation

Oxidation of proteins has been of great concern both in understanding mechanisms of age-related diseases and in protecting pharmaceutical proteins from degradation before use. Oxidation of proteins may

proceed by a variety of mechanisms and may be initiated by chemical oxidants or by light exposure. Kerwin and Remmele Jr. (2007) provide a useful review of the mechanisms of photo oxidation of proteins. These mechanisms start with the excitation of an electron in the protein backbone or in the side chains of tryptophan, tyrosine, phenylalanine, or cysteine. This can lead to formation of a triplet state, reaction with oxygen to form a peroxy radical, rejection of an aqueous electron, or relaxation to the ground state. Which of these occurs is influenced by the pH, temperature, polarity, nearby side chains, and protein structure around the excited electron. (Kerwin and Remmele 2007). UV radiation has also been used to generate hydroxyl radicals from hydrogen peroxide to study the solvent accessible surface areas of proteins. Side chains accessible to the radicals are modified and can be detected mass spectrometry.

Peroxymonosulfate triple salt (Oxone®)

The formulation Oxone® consists of potassium peroxymonosulfate, potassium bisulfate, and potassium sulfate. Kennedy and Stock (1960) studied the use of potassium peroxymonosulfate to oxidize organic substances. The triple salt mixture can be stored with little loss of active oxygen and is less hazardous than peroxymonosulfuric acid on its own. The presence of the bisulfate ion results in strongly acidic solutions (authors observed pH 2). Peroxymonosulfate ion may, in theory, split into a hydroxyl radical and sulfate ion-radical. Based on this, the primary difference between the use of peroxymonosulfate and ammonium persulfate in aqueous solution appears to be the generation of a hydroxyl radical in addition to the sulfate ion-radical generated by both (Kennedy and Stock 1960). Ball and Edwards (1956) found that the degradation rate of Caro's acid (later identified as peroxymonosulfuric acid) increases with pH (over the range 6 to 8) and that cobalt ion at 10^{-8} M increases degradation rate (Ball and Edwards 1956).

The manufacturer's specification sheet for Oxone® (DuPont ™) also indicates poor stability above pH 6.0. At 32°C and pH 9.0, 50% active oxygen decomposition is reached after about an hour. Nickel, cobalt, and manganese are strong catalysts for the decomposition of Oxone® in solution. Anipsitakis et al. (2008) found that either cobalt or UV-C light (254nm) are effective activating peroxymonosulfate for radical mediated destruction of common pool water contaminants. Bicarbonate ions were effective quenchers of the

reaction, suggesting a possible method for stopping protein oxidative crosslinking reactions (Anipsitakis, Tufano, and Dionysiou 2008).

Targeted Crosslinking by Metal-Catalyzed Oxidation

In many cases, oxidative crosslinking has been targeted to a specific site on the protein containing a metal-binding site and a tyrosine. This is an attractive method for dityrosine crosslinking because it is potentially less likely to result in oxidative modification of the protein crystal than other methods. This is important in keeping with our goal to produce a well ordered material. In metal catalyzed oxidations of tyrosine to dityrosine, a peroxide oxidizes Ni(II) to Ni(III), producing an oxygen radical species and a negatively charged oxygen species. Ammonium persulfate (APS), magnesium monoperoxyphthalate (MMPP), and potassium monoperoxysulfate have been used as the peroxide. The Ni(III) ion can be stabilized when complexed with the nitrogen atoms of an oligopeptide (Bossu and Margerum 1976). Two examples of the peptides used to complex nickel for oxidative crosslinking are glycine-glycine-hisitidine (GGH) (Endrizzi et al. 2006) or the hexahistidine tag commonly used in immobilized metal affinity chromatography purification (D A Fancy and Kodadek 1998; D A Fancy et al. 1996). Ni(III) attacks a tyrosine residue forming a tyrosyl radical which may then attack a second nearby tyrosine forming dityrosine. Crosslinking mediated by an attached hexahistidine tag has been observed to only occur near the tag (D A Fancy et al. 1996).

Brown et al. (1995) used GGH-Ni(II) complex in the presence of MMPP to catalyze protein crosslinking between self-associating proteins. These oxidative crosslinking reactions were found to require the presence of the peptide, nickel, and oxidant. The metal-peptide complex and oxidant needed to be present in excess of the proteins. Oxone ® was also effective in crosslinking, but hydrogen peroxide and sodium ascorbate in combination were not. Replacing nickel with copper in the peptide complex resulted in no crosslinking. Generally, reactions were carried out at 10 μM protein (gp32), 100 μM GGH-Ni(II), and 100 μM MMPP for 5 min in 50mM phosphate, pH7.0 and 150mM NaCl (Brown et al. 1998).

Horowitz et al. (2012) used oxidative crosslinking to study conformational changes in viral protein subunits during cell infection. Similarly to Brown et al., the researchers utilized the GGH-Ni(II) complex and MMPP to effect crosslinking between subunits. Oddly, protein precipitation was observed when the oxidizing

reagents were premixed but not when added sequentially. Mutation of tyrosine to phenylalanine was used to determine the residues responsible for crosslinking: the Y704F mutation completely prevented crosslinking. Crosslinking was carried out in the following conditions: 10mM Ni/GGH & 10mM MMPP for 20 hours at 22°C, quenched by 150mM β-mercaptoethanol (Horowitz, Finn, and Asokan 2012).

Gill et al. (1997) demonstrated site specific crosslinking relying nickel binding sites. These researchers pointed out that nickel in solution exhibits little oxidative chemistry unless bound to a peptide or protein. RNase (400μM) and 50μM nickel acetate were incubated for 30 minutes at room temperature in 10mM sodium borate, pH10. 800μM potassium monopersulfate was allowed to oxidize the protein for 30 minutes, quenched by SDS-PAGE loading buffer. The optimal yield was found at pH 10. The presence of 4mM EDTA completely inhibited crosslinking. Cobalt catalyzed reactions had similar efficiency, while other transition metals (iron, manganese, palladium, copper, magnesium, zinc) were less effective or not at all effective. Other oxidants, MMPP and meta-chloroperoxybenzoic acid (MCPBA), were also effective in crosslinking. Free tyrosine at a two-fold excess to protein was an effective inhibitor of the reaction. Though generation of hydroxyl radical by the oxidized metal-peptide complex is possible, the presence of radical scavengers, mannitol or methanol, only partially inhibited crosslinking. This suggested that the reactive intermediates were either highly localized or unaffected by hydrogen atom donors. Modifications other than crosslinking were not prevalent. Amino acid analysis indicated "few, if any" modifications to the monomer bands in denatured gels of the oxidation products. Although protein fragmentation can result from oxidation, the authors noted that no fragmentation was observed in this work. Inhibition of crosslinking by free tyrosine, tryptophan, and, to a much lesser extent, histidine suggested that amino acids of these types in the protein were implicated in dimerization (Gill et al. 1997).

If specificity of oxidation is a concern, the work of Bridgewater et al. (2006) provides some guidance. This work optimized the specificity of metal-catalyzed oxidation to determine the metal binding sites in proteins through mass spectrometry. 10mM ascorbate and 1mM persulfate provided the best specificity of modification. The highest yield of modified protein was found at 10mM ascorbate, in the presence air and persulfate catalyzed by nickel (63%) or copper (65%) (Bridgewater, Lim, and Vachet 2006).

Hexahistidine tag as oxidative crosslinking site

Stayner et al. (2005) proposed using the hexahistidine (H6) tag often used to purify recombinant proteins as the metal-binding site to catalyze covalently crosslinked monomers. Several positions were tested for the substitution of tyrosine residues relative to the H6-tags to optimize the yield of crosslinks. In this case, nickel ions not only catalyzed crosslinking, but also mediated dimerization between the H6-tags. Again, here it was observed that the "metal complex prevents the reactive free radicals from diffusing away and thereby localizes the cross-linking reaction." Little or no crosslinking was observed in designs with the tyrosine on the N-terminus of the N-terminal H6-tag, while crosslinking did occur with the tyrosine between the tag and the rest of the protein. The authors suggested that this may be due to the effect of the local environment of the tyrosyl radical's lifetime (Stayner et al. 2005). In one case, a tyrosine was placed near an N-terminal GGH tag in self-associating ecotin. Interestingly, the researchers observed a novel tyrosine-glycine crosslink rather than the expected dityrosine (Person et al. 2001).

Alternative methods for dityrosine formation

This review has focused on metal-catalyzed oxidation for dityrosine formation. Other methods exist, the common element being the generation of radicals which may be generated by photosensitizers and light, radiation, or enzymatically. Kodadek et al. (2005) provide a useful review of protein oxidation in service of crosslinking proteins together in order to study protein-protein interactions. At the time, $Ru(bpy)^{2+}{}_3$, ammonium persulfate, and light systems showed some promising results showing their utility even in complex mixtures of proteins existing in crude cellular extracts. (Kodadek, Durouxrichard, and Bonnafous 2005). In addition to crosslinking with metal-catalyzed oxidation, Gill et al. (1997) crosslinked using 0.08 uM horseradish peroxidase and 800μM H_2O_2 in place of the nickel and monopersulfate (Gill et al. 1997). Minamihata also generated dityrosine enzymatically using a peroxidase in the presence of H_2O_2 (Minamihata, Goto, and Kamiya 2011). Dityrosine has been reported as a product after exposure to X-ray radiation (Boguta and Dancewicz 1981), exposure to ultraviolet light (Correia et al. 2012), and exposure to visible light in the presence of $Ru(bpy)_3^{2+}$ and ammonium persulfate (D A Fancy and Kodadek 1998; Kodadek, Durouxrichard, and Bonnafous 2005). The observation that persulfate may be photolysed in water with

308nm light to two sulfate anion radicals (Gau et al. 2010) suggests yet another method of dityrosine formation.

Dityrosine fluorescence

Many methods of detecting dityrosine have been used. One attractive feature of dityrosine is its fluorescence which allows detection of the crosslink by spectroscopy. Dityrosine exhibits fluorescence with an excitation spectra of around 315 nm in basic conditions, shifting to around 276 nm in acid conditions, and a emission spectra around 410 nm (Sakura and Fujimoto 1984). Peak fluorescence intensity of free dityrosine at pH 2.8 was observed to be less than half the intensity at pH 9.9 (Malencik and Anderson 2003). Kungl et al. correlated decreasing quantum yield of dityrosine fluorescence with increasing proton concentration and proposed that the local environment of buried dityrosine contributes to nonlinear quenching of fluorescence (Kungl et al. 1994). Huggins et al. observed that radiolytic oxidation resulted in the formation of other uncharacterized fluorescent products while metal-catalyzed oxidation resulted in proteins where 50% (in the case of lysozyme) or 100% (in RNase) of fluorescence was attributable to dityrosine. While the fluorescence of tryptophan was lost after oxidation, proteins that had been oxidized were, nonetheless, 4-10 times more fluorescent, likely due to the introduced dityrosine fluorescence (Huggins et al. 1993).

Potential problems with protein crystal oxidation

Although many of the journal articles reviewed here suggest that oxidative crosslinking forming dityrosine can be achieved without other modifications, generating radicals to oxidize protein crystals does present some risk of unintended changes.

Oxidation for Footprinting and Solvent Exposed Surfaces

Several oxidative methods have been used for footprinting studies in which protein surfaces are modified except where the surface is buried in an interface with another protein or within the interior of the same protein. These methods include hydroxyl radical-generating methods, sulfate radical-generating methods, and synchrotron X-ray radiation methods (Maleknia, Brenowitz, and Chance 1999). These methods are predicated on the basis that the less accessible buried side chains are less likely to be modified than those

on the surfaces exposed to solvent. Within 10ms of exposure to synchrotron X-ray radiation, oxidative modification of proteins can be detected. High energy photons rapidly ionize water molecules which react with water to produce hydroxyl radicals. In the presence of air, superoxide anions and hydroperoxyl radicals form. In work by Maleknia et al. (1999), mass spectrometry was used to observe synchrotron radiation of several peptides causing hydroxylation of phenylalanine, methionine, tyrosine, and cysteine, and oxidation of proline, methionine, cysteine, and modification of tryptophan, histidine, and leucine. For the peptides studied the relative reactivity of amino acids was as follows: Cys, Met ≫ Phe, Tyr > Trp > Pro > His, Leu. The extent of oxidation was highly influenced by the solvent accessibility. Reactions with the hydroxyl radical are favored by stabilization of the resulting radical (e.g., near double bonds, aromatic rings, and heteroatoms) (Maleknia, Brenowitz, and Chance 1999). Stayner et al. (2005) found some evidence of fragmentation during oxidation for dityrosine formation with the cleaved site being near the tyrosine designed to crosslink (Stayner et al. 2005).

Hydoxyl radicals are used in chemical footprinting to study protein-protein interactions. This is possible because the hydroxyl radical cannot access sidechains buried within the interface, leaving them unmodified in contrast to those exposed to solvent. Sulfate radicals have also been proposed as footprinting agents because they better approximate the water-accessible surface due to the similar size. Sulfate radicals are stronger oxidants than hydroxyl radicals, with a reduction potential of 2430mV versus 1900mV for hydroxyl radicals.

In one such study, LC/MS/MS allowed fraction of residues modified comparison. In a procedure called fast photochemical oxidation of proteins (FPOP), 308 nm light was used to generate sulfate radicals from persulfate in order to nonspecifically label solvent exposed side chains. Over half of the 20 common amino acid are reliably modified by this method. For our purposes however, it is worth noting that the percentage of side chains modified by this method was generally less than 8%. Comparing only those residues fully exposed to solvent, the order of sulfate radical reactivity to each residue was found to be as follows: M > Y = W > F = E = H > S > P > D = T > K = Q > L = V = I. "[Compared with hydroxyl radicals,] Persulfate FPOP is more reactive with His and Tyr and equally reactive to Met, Trp, Glu, and Ser." The

authors found a correlation between solvent accessible surface area (SASA) and extent of modification. This suggests that SASA may be an additional consideration to be used in the design of oxidative crosslinks relying on radical chemistry (Gau et al. 2010).

Small molecule crosslinkers

A wide variety of cross-linkers have been developed for use in protein-protein interaction studies. These crosslinkers are generally more specific in their reactivity than glutaraldehyde. These crosslinkers may have different active groups and may be homobifunctional, heterobifunctional, or may have more than two active groups for crosslinking. They also have a variety of linker lengths so that the user may choose a crosslinker tailored to a specific interface of interest. For the purposes of stabilizing known crystal interfaces, a variety of linkers may be tested for how well they fit into each interface.

Some examples of reactive groups include heterobifunctional carboxyl-to-amine linkers, homobifunctional amine-reactive linkers (NHS esters and imidoesters), homobifunctional sulfhydryl-reactive (maleimides), aldehyde-reactive groups, promiscuous photoinitiated linkers (aryl azide, diazirine groups on one end, NHS ester on the other end reactive with amines), and hydroxyl-reactive linkers (isocyanate). The wide field of available linkers can be narrowed down by selecting the reactive groups to target and the distance between reactive groups to be crosslinked.

Crosslinking with selective reactivity can provide information about the proximity of atoms within a protein or across a protein-protein interface. Transient interactions between proteins can be identified by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) combined with software (many are available) to disentangle the mass spectra observed. Following trypsin digest the fragments include a mixture of unconjugated peptide, conjugated, but un-crosslinked peptides, and crosslinked peptides. Depending on the number of reactive groups present in the side chains of each peptide, there may be a number side chains each with a different modification (Mädler et al. 2009). Such methods could also be useful for identifying crosslinks within protein crystals that have been designed by our software.

Project overview

In this work, general software for designing specific crosslinks between protein molecules was developed for the purpose of stabilizing protein crystals. Several of the designs were then constructed and characterized. The following sections (Methods, Results and Discussion) were redacted due to insufficient embargo/access restrictions offered via ProQuest publication agreement.

Conclusions

The practical use of protein crystals as porous materials necessitates the development of new methods for stabilizing them against dissolving. In many cases, such as in templating, it will be advantageous to preserve the sub-nanometer scale order of the atoms in the crystal. The most widely used current methods of protein crystal stabilization, glutaraldehyde crosslinking, often degrades this order because it can oligomerize or react with several amino acid side chains leading to a variety of crosslinks or non-crosslinking additions. We developed new general purpose software for the design of intermolecular crosslinks in protein crystals that are expected to preserve their as-grown order. The software may be used to identify sites in the protein crystal that may be crosslinked by natural amino acid crosslinking by tyrosines or cysteines. Such designs may be easily constructed using site-directed mutation to substitute amino acids programmed in the wild-type gene sequence followed by oxidation. In addition, the software may also be used to design specific intermolecular crosslinks using small molecule crosslinkers that are widely used in protein conjugation. The software developed in this work improves on previous crosslink design software by allowing backbone flexibility, optimizing nearby sidechains around designed crosslinks if possible, and by expanding the types of crosslinks that can be designed. The design tools may also be used to identify which small molecule crosslinkers may be used bind two monomers in a crystal with or without making mutations to the given amino acid sequence. Lastly, the software provides tools to enable further expansion by users in the small molecule crosslinkers available for design.

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