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Use of Site-Directed Cysteine and Disulfide Chemistry to Probe Protein Structure and Dynamics: Applications to Soluble and Transmembrane Receptors of Bacterial Chemotaxis

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Abstract

Site-directed cysteine and disulfide chemistry is broadly useful in the analysis of protein structure and dynamics, and applications of this chemistry to the bacterial chemotaxis pathway have illustrated the kinds of information that can be generated. Notably, in many cases, cysteine and disulfide chemistry can be carried out in the native environment of the protein whether it be aqueous solution, a lipid bilayer, or a multiprotein complex. Moreover, the approach can tackle three types of problems crucial to a molecular understanding of a given protein: (1) it can map out 2° structure, 3° structure, and 4° structure; (2) it can analyze conformational changes and the structural basis of regulation by covalently trapping specific conformational or signaling states; and (3) it can uncover the spatial and temporal aspects of thermal fluctuations by detecting backbone and domain dynamics. The approach can provide structural information for many proteins inaccessible to high-resolution methods. Even when a high-resolution structure is available, the approach provides complementary information about regulatory mechanisms and thermal dynamics in the native environment. Finally, the approach can be applied to an entire protein, or to a specific domain or subdomain within the full-length protein, thereby facilitating a divide-and-conquer strategy in large systems or multiprotein complexes.

Rigorous application of the approach to a given protein, domain, or subdomain requires careful experimental design that adequately resolves the structural and dynamical information provided by the method. A full structural and dynamical analysis begins by scanning engineered cysteines throughout the region of interest. To determine 2° structure, the solvent exposure of each cysteine is determined by measuring its chemical reactivity, and the periodicity of exposure is analyzed. To probe 3° structure, 4° structure, and conformational regulation, pairs of cysteines are identified that rapidly form disulfide bonds and that retain function when induced to form a disulfide bond in the folded protein or complex. Finally, to map out thermal fluctuations in a protein of known structure, disulfide formation rates are measured between distal pairs of nonperturbing surface cysteines. This chapter details these methods and illustrates applications to two proteins from the bacterial chemotaxis pathway: the periplasmic galactose binding protein and the transmembrane aspartate receptor.

Introduction

The structures of many important proteins, as well as key aspects of protein regulation and dynamics, are not yet accessible to high-resolution methods. Site-directed cysteine and disulfide chemistry (Falke and Koshland, 1987; Falke *et al.*, 1986, 1988) provides an approach that can, in many cases, address these challenging questions by analyzing the structure and dynamics of a given protein in its native environment. The most prevalent application of site-directed cysteine and disulfide chemistry has been in structural studies of membrane protein systems and large multiprotein complexes (Akabas *et al.*, 1992; Bass and Falke, 1998, 1999; Chervitz and Falke, 1995, 1996; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1986, 1988; Hughson and Hazelbauer, 1996; Hughson *et al.*, 1997; Lee *et al.*, 1994; Miller *et al.*, 2006; Pakula and Simon, 1992; Sahin-Toth and Kaback, 1993; Todd *et al.*, 1989; van

Iwaarden *et al.*, 1991; Wu *et al.*, 1996). The structures of such large, insoluble, complicated systems are often the most difficult to analyze by high-resolution structural methods, making the structural constraints provided by the present chemical approach quite valuable. More broadly, the ability of site-directed cysteine and disulfide chemistry to analyze proteins in their native solution, membrane, or supermolecular environment enables rigorous investigation of the relationship between structural changes and regulation or function. Even in proteins of known high-resolution structure, site-directed cysteine and disulfide chemistry can provide important information about thermal fluctuations, yielding insights into the timescales and trajectories of long-range backbone and domain motions that are currently inaccessible to other methods (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995).

Site-Directed Cysteine and Disulfide Chemistry: History

Site-directed cysteine and disulfide chemistry was originally developed as a generalizable approach to protein structure and dynamics in the bacterial chemotaxis pathway (for example, Bass and Falke, 1998, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995; Chervitz and Falke, 1995, ¹⁹⁹⁶; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1986, ¹⁹⁸⁸; Hughson and Hazelbauer, 1996; Hughson *et al.*, 1997; Lee *et al.*, 1994; Miller *et al.*, 2006; Pakula and Simon, 1992), but the method is broadly applicable. As a result, novel extensions of the approach have been developed and implemented in a wide array of other systems (for example, Akabas *et al.*, 1992; Sahin-Toth and Kaback, 1993; Todd *et al.*, 1989; van Iwaarden *et al.*, 1991; Wu *et al.*, 1996). The present chapter focuses on the use of site-directed cysteine and disulfide chemistry to analyze 2° structure, 3° structure, 4° structure, the coupling between structure and regulation, and the nature of thermal backbone dynamics. To illustrate applications of these methods to specific proteins, published studies of components from the bacterial chemotaxis pathway are discussed. However, the central goal of this chapter is to facilitate the application of site-directed cysteine and disulfide methods to new protein systems. Thus, the chapter emphasizes the general design and implementation of these methods and key factors that should be considered when interpreting results.

The two components of the *Escherichia coli* and *Salmonella typhimurium* chemotaxis pathway that are used to illustrate the methods of the present chapter include a soluble binding protein and a transmembrane receptor (reviewed in Baker *et al.*, 2006; Falke *et al.*, 1987). The periplasmic galactose binding protein is a 36 kDa, monomeric soluble receptor that specifically binds the chemoattractants D-galactose and D-glucose in a cleft between its two domains connected by a flexible hinge (Vyas *et al.*, 1988). The transmembrane aspartate receptor is a 120 kDa homodimeric protein that assembles into larger oligomers (Ames *et al.*, 2002; Falke and Hazelbauer, 2001; Falke and Kim, 2000; Kim *et al.*, 1999; Milburn *et al.*, 1991; Studdert and Parkinson, 2004). This receptor binds the chemoattractant aspartate at a site on its periplasmic domain and sends a transmembrane signal through membrane-spanning helices to its cytoplasmic domain, where the signal regulates the bound histidine kinase CheA.

Site-directed cysteine and disulfide chemistry has revealed important structural, functional, and dynamical features in both these chemotaxis proteins. The approach was first used to analyze helix–helix packing in the periplasmic and transmembrane domains of the aspartate receptor and other chemoreceptors (Chervitz and Falke, 1995, 1996; Chervitz *et al.*, 1995; Falke and Koshland, 1987; Falke *et al.*, 1988; Hughson and Hazelbauer, 1996; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005), and a nonperturbing disulfide bond discovered in these studies was used to stabilize a dimeric construct of the isolated periplasmic domain for crystallization and X-ray structure determination (Falke and Koshland, 1987; Milburn *et al.*, 1991). The chemical studies were then extended to the cytoplasmic domain where they revealed two regions of helical secondary structure in the

HAMP subdomain and an extended 4-helix bundle forming the majority of the cytoplasmic domain (Bass and Falke, 1998, 1999; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Winston *et al.*, 2005). Overall, the 2°, 3°, and 4° structural constraints determined by site-directed cysteine and disulfide chemistry in the full-length, membrane-bound receptor have been confirmed by high-resolution X-ray structures of the isolated receptor periplasmic and cytoplasmic domains, and by an NMR structure of a distantly related HAMP subdomain (Hulko *et al.*, 2006). Together, these chemically defined constraints have greatly aided the development of the current working atomic model for the full-length receptor dimer (Kim *et al.*, 1999; Milburn *et al.*, 1991), although the current model of HAMP subdomain structure (Hulko *et al.*, 2006) has yet to be incorporated.

The discovery of engineered disulfide bonds that lock the receptor in its kinase-activating and -inactivating signaling states, termed “lock-on” and “lock-off” disulfides, respectively, were crucial to the development of current mechanistic models describing signal transmission through the transmembrane and cytoplasmic regions of the receptor (Bass and Falke, 1999; Chervitz and Falke, 1995, 1996; Starrett and Falke, 2005). These studies were carried in full-length, membrane-bound receptor in its working complex with CheA kinase. Independent work carried out in several laboratories has strongly supported the mechanistic conclusions of these site-directed cysteine and disulfide chemistry studies (Draheim *et al.*, 2005, 2006; Falke and Hazelbauer, 2001; Hughson and Hazelbauer, 1996; Lai *et al.*, 2006; Miller and Falke, 2004).

Finally, studies of backbone and domain motions in the known structures of the galactose binding protein and the aspartate receptor have shed light on the thermal backbone and domain motions that occur within these proteins in their native aqueous and membrane environments, respectively (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995). The findings reveal dramatic, long-range motions that occur on biologically meaningful timescales but are difficult to detect by other methods, suggesting that proteins may be significantly more dynamic than is generally believed.

Site-Directed Cysteine and Disulfide Chemistry: Applications and Limitations

The present chapter presents detailed procedures for three types of cysteine and disulfide chemistry: chemical reactivity scanning, disulfide mapping, and disulfide trapping. *Chemical reactivity scanning* measures the reactivities of adjacent residues with an aqueous probe, revealing local patterns of solvent exposure. The resulting patterns can identify 2° structure elements in an unknown structure or conformational changes in a known structure. *Disulfide mapping* measures disulfide bond formation rates, as well as the effects of disulfide formation on protein function, to map out contacts between pairs of positions in the 3° and 4° structure. The resulting information can provide proximity constraints that map out folding and packing in an unknown structure. In addition, the effects of engineered disulfides on function can resolve the structures of different signaling or activity states, and define the conformational changes that connect different states. *Disulfide trapping* measures disulfide formation rates between pairs of positions separated by different distances in a protein of known structure. The resulting data reveal the amplitudes, trajectories, and frequencies of long-range backbone and domain motions within the protein of interest.

When undertaking a study involving site-directed cysteine and disulfide chemistry, it is important to consider the caveats and limitations of this chemical approach. In particular, the method provides information about both structure and dynamics, and it is important to separate these two classes of information when designing and interpreting experiments (Bass and Falke, 1999; Careaga and Falke, 1992). For example, analysis of 2° structure via cysteine scanning and chemical reactivity analysis assumes that the element selected for study possesses distinct buried and exposed faces that will give rise to a periodic pattern of solvent exposure. While

this limitation is compatible with 2° elements on a protein surface, it prevents application of the method to completely buried elements in the protein interior. Many studies of 3° or 4° structure in various laboratories have attempted to detect packing contacts by measuring rates or extents of disulfide bond formation between pairs of engineered cysteines. Often, such rates are correlated with spatial proximities, but the fundamental determinants of disulfide formation rates are sulfhydryl–sulfhydryl collision frequencies and reactivities rather than simple proximities. Thus, it is important to design approaches that can resolve structural from dynamical contributions. In short, each of the caveats and limitations of site-directed cysteine and disulfide chemistry can be overcome by suitable experimental design and controls, as discussed below in the relevant sections.

Incorporation of an Affinity Tag and Creation of a Cysless Protein

Ideally, the protein selected for analysis by site-directed cysteine and disulfide chemistry should contain an affinity tag to facilitate rapid purification and should lack intrinsic cysteine residues. Many native proteins lack cysteine (Cys), while those possessing Cys residues can often be modified by a systematic approach to generate a fully functional cysteine-less (Cysless) derivative. The advantage of such a Cysless construct is that each Cys introduced at a selected surface position becomes a unique site for sulfhydryl and disulfide chemistry. In previous studies of bacterial chemotaxis components, the galactose binding protein and the transmembrane aspartate receptor required no affinity tag since both could be easily isolated to sufficient purity for sulfhydryl and disulfide chemistry; and, moreover, both of these proteins lack intrinsic Cys residues (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995; Chervitz and Falke, 1995, ¹⁹⁹⁶; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1988; Pakula and Simon, 1992; Winston *et al.*, 2005). By contrast, the histidine kinase CheA was more difficult to purify and thus was fused to an N-terminal 6-His affinity tag for ease of isolation (Miller *et al.*, 2006). In addition, native CheA possesses three intrinsic Cys residues that were substituted with an optimized combination of alanine and serine residues to generate a fully functional Cysless construct (Miller *et al.*, 2006). Chapter 1 in this volume (Bass *et al.*, 2007) outlines general strategies that can be employed to prepare a new protein for site-directed cysteine and disulfide chemistry, including the incorporation of a 6-His affinity tag, the creation of an optimized Cysless construct, and the importance of careful tests to exclude engineered proteins that exhibit perturbed structure, stability, or activity.

Choice of Positions for Cys Incorporation and Creation of a Mutant Library

One advantage of cysteine and disulfide chemistry is that it can be applied in a highly targeted fashion to a protein subdomain or domain if desired, or, at the other extreme, broadly applied to an entire protein. A typical study focuses on a specific region within the intact protein. Such adaptability facilitates divide-and-conquer strategies. To maximize the probability of a definitive conclusion within the target region, it is advisable to generate as many Cys substitutions as possible since the degree of certainty increases with the number of independent measurements. Moreover, whenever possible, experimental studies of the resulting Cys mutants are carried out on the intact protein in its native solution, membrane, or multiprotein complex environment.

Once a protein subdomain or domain has been chosen for analysis, single Cys substitutions are introduced at all positions within the selected region using high throughput methods to generate, express, and purify each Cys mutant. These methods are described in Chapter 1 of this volume (Bass *et al.*, 2007). Note that to avoid oxidation of the engineered Cys sulfhydryl groups during purification and storage, Cys mutant purification methods include reducing agent and minimize exposure to transition metals that catalyze oxidation, particularly Cu(II)

and Fe(III). During the final stages of purification, the concentration of reducing agent is decreased to a low level (typically, 10 μ M dithiothreitol, DTT) to protect the engineered sulfhydryls while minimizing interference with subsequent cysteine and disulfide reactions. In addition, a metal chelator is included (typically, 1 mM ethylene diamine tetraacetic acid, EDTA) for protection against transition metal-catalyzed oxidation, and mutants are flash-frozen in liquid nitrogen and stored at -80° to further minimize oxidative damage.

The resulting library of single-Cys mutants is carefully tested for perturbations introduced by individual Cys substitutions. Often, the Cys substitution itself is relatively nonperturbing, since the Cys side chain is relatively small and sterically accommodating, while the sulfhydryl group can adapt to different electrostatic environments by varying between an apolar, protonated state and an anionic, deprotonated state (Careaga and Falke, 1992; Falke and Koshland, 1987; Falke *et al.*, 1986, 1988). Even when located at a buried position, Cys substitutions are often tolerated unless they replace a residue essential for folding stability. In a large library of Cys mutants, however, several mutants will typically be found to exhibit significant perturbations of stability or function. These perturbed mutants are best identified by appropriate stability and/or functional assays developed specifically for the target protein. Mutants found to be perturbed should then be eliminated from further studies.

Analysis of 2° Structure by Chemical Reactivity Scanning

Overview of Chemical Reactivity Scanning

In a typical protein, most secondary structure elements possess one face exposed to protein exterior, and another face exposed to the protein interior. When the solvent-exposed face of such an element is in contact with aqueous solution, its secondary structure can be elucidated by measuring the chemical reactivity of single Cys residues scanned throughout its length (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Winston *et al.*, 2005). Those positions exposed to aqueous solution will react rapidly with a bulky, charged, water-soluble alkylating reagent, while those positions exposed to the buried positions will react significantly more slowly. The periodicity of exposed and buried positions is characteristic of the type of secondary structure, ranging from 3.5 to 3.6 residues per cycle for α -helices to 2 residues per cycle for β -strands. To detect this periodicity, reaction conditions are chosen that enable initial rates of alkylation by the aqueous probe to be accurately measured in the region of interest. Several parameters of the reaction conditions can be adjusted as needed for the local environment to ensure that the initial rates can be determined on a convenient time scale. The method possesses three limitations that should be considered. First, the target secondary structure element must possess distinct aqueous and buried faces to ensure that the periodicity of chemical reactivity can be interpreted. Second, the method cannot easily be applied to transmembrane domains exposed to the hydrocarbon core of the bilayer, since alkylation reactions proceed too slowly to measure in such an environment. Third, the method requires sufficient quantities of protein that the alkylated product can be directly detected and quantitated. The present approach utilizes the sulfhydryl-specific aqueous probe 5-iodoacetamido-fluorescein (5-IAF) as the alkylating agent, which yields an easily quantitated fluorescent band on an SDS-PAGE gel for quantities of labeled protein exceeding 5 pmol.

The accuracy of chemical reactivity scanning in mapping out secondary structure elements has been tested and confirmed in the full-length, membrane-bound aspartate receptor (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Winston *et al.*, 2005). First, using the known high-resolution structure of the isolated periplasmic domain (Milburn *et al.*, 1991), positive control studies were carried out focusing on helix α 4 of the periplasmic domain in the intact receptor. Chemical reactivity scans of this region revealed a periodic pattern of reactivity that closely matched the oscillating pattern of solvent exposure observed for helix α 4 in the crystal structure, thereby directly establishing the validity of the

approach (Bass and Falke, 1998; Butler and Falke, 1998; Danielson *et al.*, 1997). Furthermore, the approach was applied to the unknown structure of the receptor cytoplasmic domain, again in the intact, membrane-bound receptor. Chemical reactivity scans revealed four local regions of α -helical periodicity, two of them separated by a region proposed to be a hairpin turn (Bass and Falke, 1998; Bass *et al.*, 1999; Danielson *et al.*, 1997; Winston *et al.*, 2005). The putative helical regions, with their distinct exposed and buried faces, were later confirmed to be amphiphilic and α -helical in crystal structures of the isolated cytoplasmic domain (Falke and Kim, 2000; Kim *et al.*, 1999; Park *et al.*, 2006). Similarly, this structure confirmed that the putative turn was indeed a hairpin. Another two regions of α -helical periodicity were observed in the cytoplasmic HAMP region, and one region lacking a defined secondary structure was detected (Butler and Falke, 1998). These three assignments were confirmed in a subsequent NMR structure of an isolated HAMP domain from a thermophilic bacterium (Hulko *et al.*, 2006), although the relevance of this structure to full-length, membrane-bound chemoreceptors has not yet been experimentally tested. Overall, however, the evidence to date indicates that the chemical reactivity scan accurately identifies water-exposed secondary structure elements on the surfaces of full-length proteins in their native environments. In principle, chemical reactivity scanning could also be employed to map out conformation changes via measurements of changing solvent exposure, but such changes have not yet been detected in the aspartate receptor, presumably because signal transduction triggers a subtle structural change too small to be easily detected by this method (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Falke and Hazelbauer, 2001; Winston *et al.*, 2005).

Chemical Reactivity Scanning: General Considerations

The following protocol for cysteine reactivity scanning was developed for the aqueous domains of the full-length, membrane-bound aspartate receptor (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Winston *et al.*, 2005) but is generalizable to other protein systems. For typical protein surface elements exposed to the bulk aqueous phase, the indicated 5-IAF reaction conditions yield a rate of alkylation easily measured by quenching the reaction at specific timepoints after probe addition, then quantitating the extent of cysteine labeling with fluorescein. However, if the protein surface targeted for study is highly charged, near a membrane surface, or poorly accessible to bulk solvent, the alkylation reaction could be too fast or slow to conveniently measure. In such a case, it is simple to adjust the reaction conditions to bring the alkylation time scale into the convenient range by increasing the pH (which increases the fraction of cysteine sulfhydryl in the reactive sulfanion state), temperature, and/or 5-IAF concentration to speed the reaction, or by decreasing one of these variables to slow the reaction.

Chemical Reactivity Scanning: Detailed Procedure

1. Each of the single-Cys engineered proteins is diluted to a final concentration of 5 μ M in labeling reaction buffer (10 mM NaH₂PO₄ pH to 6.5 with NaOH, 50 mM NaCl, 50 mM KCl, and 1 mM EDTA) on ice, yielding a total volume of 25 μ l per sample in a microfuge tube. Care should be taken that protein sulfhydryls are protected from oxidation during purification of the protein stock, and that the concentrations of residual reducing agents such as DTT are negligible (less than 25 μ M total sulfhydryl) relative to the 5-IAF concentration during the following alkylation reaction. Before proceeding with the 5-IAF labeling reaction, each sample is carefully mixed to ensure a homogenous solution and spun for 10 sec in a microfuge to bring the entire volume to the bottom of the tube.
2. The samples are moved to a 25° temperature bath, allowed to equilibrate for 1 min, then the alkylation reaction is initiated by adding 1.25 μ l of a 5-IAF stock (5 mM 5-

IAF in N,N'-dimethylformamide, DMF), yielding a final 5-IAF concentration of 250 μ M, with careful mixing before quickly returning to the 25° bath.

3. At timepoints of 2.5, 5, and 10 min, aliquots of 5 μ l are removed and quenched by addition to a microfuge tube containing 1 μ l of quench solution (500 mM stock of β -mercaptoethanol in water) with careful mixing.
4. Immediately after the 10 min aliquot is quenched, another 5 μ l aliquot is removed and transferred to a microfuge tube containing 1 μ l of denaturation solution (4% SDS in water) with careful mixing. This endpoint sample is heated to 95° for 2 min to ensure complete unfolding and reaction of all free Cys residues with 5-IAF. Then, the endpoint sample is removed from the heat and 1 μ l quench solution is added with careful mixing.
5. The four quenched aliquots are each pelleted in a microfuge to deposit the sample at the bottom of the tube. Then, they are carefully mixed with an equal volume of 2 $\times$ Laemmli nonreducing sample buffer and heated to 95° for 2 min. Longer heating times and higher temperatures are avoided to minimize heat-triggered membrane protein aggregation.
6. The samples are loaded and run side-by-side on a nonreducing Laemmli SDS-PAGE gel. To ensure that equal quantities of protein are compared, 10 μ l of each timepoint sample and 12 μ l of the endpoint sample are loaded. The gel is formulated so that the target protein exhibits a relative migration (R_f) of approximately 0.5, thereby maximizing resolution from other proteins and from free fluor at the dye front. Before staining with Coomassie or another protein stain, which typically quenches fluorescence, the fluorescent bands are visualized by placing the gel on a standard ultraviolet (UV) light box and imaged by digital photography, ensuring that none of the bands are saturated. Imaging software is used to quantitate the relative fluorescence of the bands. Following quantitation of fluorescence, the gel can be stained with Coomassie and used to determine the purities and relative concentrations of the different protein samples by digital photography quantitation of the resulting Coomassie absorbance.
7. To determine the extent of 5-IAF labeling at each timepoint, the fluorescence intensity of each sample for a given timepoint is divided by the intensity of the endpoint sample. If the Coomassie analysis reveals significant discrepancies in the amount of protein in different lanes, the Coomassie intensities can be used to normalize the fluorescence intensities of each lane to the same protein level prior to division by the endpoint fluorescence intensity.
8. Once the initial 5-IAF reaction time courses have been determined for a subset of positions, it may be necessary to adjust the reaction rate. It is useful to optimize the reaction rate so that virtually all the positions examined yield between 5 and 75% labeling at either the 2.5 or 5 min timepoint. This optimization ensures that each position is labeled to a sufficient extent to be quantitated, while the reaction has not proceeded to completion and thus remains in or near its linear range. To decrease or increase the speed of the reaction, one can decrease or increase, respectively, either the pH or temperature of the reaction.
9. After optimization, the labeling reaction is carried out in triplicate for each position to enable comparison of means and standard deviations for different Cys positions. Multiple positions can be tested in a set of simultaneous reactions, with the exact number depending on the timepoint chosen for sampling the reaction. In each set of reactions, two control reactions are carried out for a selected, well-characterized Cys position that serves as an internal standard when comparing different sets. For each

reaction in a set, a given single-Cys protein is diluted to 5 μM final concentration, as in step 1, except that the total volume is now halved to 12.5 μl and the newly optimized reaction buffer is utilized. After mixing, spinning, and equilibration to the optimized temperature, 5-IAF is added to a final concentration of 250 μM as in step 2, and the reaction is allowed to proceed. At the optimized timepoint, two 5 μl aliquots are removed from each reaction. One is quenched as in step 3, the other is denatured and reacted to completion before quenching as in step 4. Then, the quenched samples are analyzed by SDS-PAGE and digital imaging as described in steps 5 through 7, yielding a quantitative extent of 5-IAF labeling for each reaction. By running multiple sets of reactions consecutively, as many as 20 engineered Cys positions can be analyzed per day. Care should be taken that the control Cys position included in all sets yields similar extents of 5-IAF labeling in different sets run on the same or different days.

Chemical Reactivity Scanning: Interpretation of Results

To analyze the chemical reactivity data and identify 2° structure elements, the mean chemical reactivities and standard deviations of consecutive engineered Cys positions are plotted as a function of residue number. Secondary structure elements with distinct water-exposed and buried faces are recognized via characteristic periodicities imposed by their oscillating solvent exposures. β -strands, for example, exhibit a periodicity of 2 residues in their pattern of chemical reactivities, since consecutive positions alternately face aqueous solution and the protein interior and thereby yield high and low relative reactivities, respectively. By contrast, α -helices exhibit a significantly longer periodicity of 3.5 (coiled-coil helix) or 3.6 (standard helix) residues and are thus easily distinguished from strands. Regions that lack a defined periodicity may represent linkers between structural elements. Published studies of the transmembrane aspartate receptor discussed previously further illustrate the use of chemical reactivity data to identify 2° structure elements, turns, and linkers (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Winston *et al.*, 2005) in the full-length receptor embedded in its native membrane.

Disulfide Mapping of Spatial Proximity and Conformational Changes

Overview of Disulfide Mapping

In principle, disulfide bond formation can map out contacts between pairs of positions in the folded protein structure. For example, disulfide mapping approaches first shed light on the helix-helix packing within the periplasmic, transmembrane, and cytoplasmic domains of the full-length, membrane-bound aspartate receptor (Bass and Falke, 1996; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1988; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005). The well-defined distance and angular constraints of the disulfide bond ensure that each Cys pair that forms a disulfide bond lies in close proximity and also satisfies strict angular constraints during the disulfide formation reaction (Careaga and Falke, 1992). Thus, the ability of two Cys positions to rapidly form a disulfide bond has been widely assumed to be a signature of proximity within the equilibrium structure. While this is often the case, it is important to note that disulfide bonds are sometimes formed between Cys residues that are distal in the equilibrium structure but able to collide during structural fluctuations away from this structure (Bass *et al.*, 1999; Butler and Falke, 1996; Careaga and Falke, 1992). (Such thermal fluctuations can be analyzed by the disulfide trapping method described later.) Moreover, the environmental dependence of Cys chemical reactivities can also modulate disulfide formation rates; for example, a highly reactive pair could form a disulfide more rapidly than a more proximal pair with a higher collision rate. (Such unusual reactivities can generally be detected by comparison of 5-IAF reactivities

measured for Cys residues on the same face of a structural element, as described in the preceding section on chemical reactivity measurements.) To rigorously detect proximities in the active equilibrium structure, it is useful to measure both (1) the rate of disulfide formation and (2) the effect of disulfide formation on protein activity. The information provided by the latter functional analysis of engineered disulfide bonds can also, in many cases, elucidate conformational changes among different signaling or functional states.

The disulfide mapping approach described herein can be applied to most proteins, except those that contain large numbers of intrinsic Cys residues that would complicate the analysis. Useful disulfide mapping strategies are illustrated by published and ongoing studies of the aspartate receptor and other bacterial chemoreceptors (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005). Disulfide mapping studies first elucidated the packing of the four membrane-spanning helices in the transmembrane region of the receptors (Pakula and Simon, 1992), and the resulting packing model has been strongly supported by subsequent chemical, spectroscopic, and modeling studies in several chemoreceptors (Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Kim *et al.*, 1999; Lee *et al.*, 1994; Ottemann *et al.*, 1999), although a high-resolution structure is not yet available for the transmembrane region. Other studies used disulfide mapping to determine the helix-helix packing geometry within the cytoplasmic domain of the full-length, membrane-bound receptor, yielding an extended 4-helix bundle model for this domain (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Danielson *et al.*, 1997; Hughson *et al.*, 1997; Winston *et al.*, 2005), which has been confirmed by high-resolution X-ray crystal structures of cytoplasmic domain fragments (Kim *et al.*, 1999; Park *et al.*, 2006). Together, these and other studies of bacterial chemoreceptors have demonstrated the accuracy of disulfide mapping in the determination of various packing interactions within full-length proteins in their native environment, including both transmembrane and aqueous domains.

Disulfide Mapping: General Considerations

The following disulfide mapping protocol was developed for the aspartate receptor bacterial chemotaxis (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005), but is generalizable to other membrane and soluble proteins. For proteins containing intrinsic Cys residues, the first step is generally to remove them by substitution with Ala or Ser, especially in the domain targeted for disulfide mapping, using the procedure described in Chapter 1 of this volume (Bass *et al.*, 2007). Intrinsic Cys residues buried in other domains will generally not interfere and need not be removed.

One key to successful implementation of the disulfide mapping approach is the choice of suitable Cys pairs for proximity testing. The pairs chosen are defined by the nature of the target protein system. The simplest application of disulfide mapping is the analysis of a symmetric packing interface between two identical subunits, since the introduction of a single Cys in the primary structure of the subunit generates a pair of symmetric Cys residues at the interface, as illustrated in early disulfide mapping studies of the aspartate receptor (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005). In this special case, a simple, model-independent scanning approach is sufficient to map out contacts between the two subunits. To carry out such a dimer analysis, single Cys residues are introduced at consecutive positions in the region being analyzed, using the site-directed mutagenesis procedures described in Chapter 1 of this volume (Bass *et al.*, 2007). Each single-Cys mutant is expressed and purified, yielding a homodimer possessing two Cys residues at symmetric positions. The resulting library of Cys mutants is tested for perturbations

due to individual Cys substitutions using stability and/or activity assays developed specifically for the target protein, and perturbed mutants are discarded. The surviving pool of active, stable mutants is subjected to the disulfide mapping procedure.

More generally, however, a model-independent scanning analysis of 3° or 4° structure within a protein or complex is not practical, since all possible combinations between different pair of positions would need to be tested for proximity, yielding a problem of enormous combinatorial complexity. Instead, a model-dependent approach is much more efficient in such applications, utilizing disulfide mapping to test specific predictions of the model by examining carefully chosen pairs of engineered Cys residues for proximity. To choose appropriate pairs of positions for proximity testing, the model structure is examined to find candidate adjacent positions. If an atomic model is available, each selected pair should possess a β -carbon to β -carbon distance in the range 4 to 8 Å (Careaga and Falke, 1992a,b). Depending on the level of confidence regarding the model, it is often advisable to test other registers of adjacent structural elements by examining additional pairs that would be closer in an alternate register. Finally, it is important to include negative controls, including pairs of Cys positions predicted by the model to be too distant for disulfide bond formation, with a β -carbon to β -carbon distance exceeding 12 Å (although this distance should be larger if the protein is unusually dynamic) (Careaga and Falke, 1992). Before disulfide mapping studies are carried out, the effects of individual Cys pairs on protein stability and/or function are determined by appropriate assays, and perturbed proteins are discarded.

Two different types of chemistry can be used to carry out disulfide mapping reactions, depending on the specific protein system. For transmembrane domains, in which pairs of Cys residues are buried deep in the hydrocarbon core of a phospholipid bilayer, the use of molecular iodine (I_2) as the oxidation agent often yields the most efficient disulfide production (Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Pakula and Simon, 1992). In this chemistry, the apolar I_2 molecule is added to initiate the reaction, whereupon it partitions selectively into the apolar membrane core and efficiently oxidizes pairs of Cys residues to form disulfide bonds. For aqueous proteins or domains, the use of ambient molecular oxygen (O_2) as the oxidation agent and Cu(II) (1,10-phenanthroline)₃ as a redox catalyst is generally preferred (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1988; Hughson *et al.*, 1997; Lee *et al.*, 1994; Winston *et al.*, 2005). Here, the catalyst is added to initiate the reaction, whereupon it catalyzes the production of diffusible reactive intermediates that can penetrate protein interiors and drive disulfide bond formation (Careaga and Falke, 1992). Since the catalyst is charged it does not partition well into membranes, and although it can catalyze disulfide formation near the aqueous surface of a bilayer, it is generally not effective deeper in the membrane.

In both the I_2 and O_2 /Cu(II) (1,10-phenanthroline)₃ chemistries, the detailed reaction mechanism appears to proceed through a series of free radical and anionic intermediates, generating a disulfide bond as one product and I^- or HO^- anions as the other product, respectively (Careaga and Falke, 1992). Additional sulfhydryl oxidation reactions that produce sulfinic and sulfenic acids compete with disulfide formation; thus, only the most proximal Cys pairs with the highest disulfide formation rates yield final extent s of disulfide product approaching 100% (Careaga and Falke, 1992). Since the goal is to compare the initial rates of the disulfide formation reactions between different Cys pairs, it may be necessary to adjust the rate of the oxidation reaction to yield a time course that is more convenient for initial rate measurements. One advantage of the O_2 /Cu(II) (1,10-phenanthroline)₃ chemistry is the ease with which it can be tuned to generate different rates in a variety of protein systems and environments. Disulfide mapping using O_2 /Cu(II) (1,10-phenanthroline)₃ chemistry has even

been successfully applied in living cells (Hughson *et al.*, 1997), although this application is beyond the scope of the current chapter.

The final step in a full disulfide mapping analysis is carried out after oxidation reactions have revealed a set of Cys pairs that rapidly form disulfide bonds, and thus potentially identify proximities in the protein structure. To rigorously identify Cys pairs that are adjacent in the native, protein fold, rather than formed by rapid fluctuations away from the equilibrium structure, the effects of these disulfides on protein activity are tested. Cys pairs that rapidly form disulfide bonds retaining protein activity provide strong evidence for proximity between specific positions in the equilibrium structure. Moreover, analysis of functional effects can reveal mechanistically important disulfide bonds that covalently, but reversibly, trap the protein in specific signaling or other functional states, such as the function-retaining, lock-on, and lock-off disulfide bonds identified in disulfide mapping studies the aspartate receptor (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Winston *et al.*, 2005). Such disulfides place strong structural constraints on the mechanisms of conformational changes, including transitions between different functional states such as on–off switching.

Disulfide Mapping: Detailed Procedure

1. Each di-Cys protein of interest is diluted to a final concentration of 2 μM in disulfide reaction buffer (buffered with either 20 mM NaH_2PO_4 , pH to 7.0 with NaOH or 20 mM Tris base, pH to 7.5 with HCl, and also containing 10% (v/v) glycerol, 100 mM NaCl, and 5 mM EDTA), yielding a total volume of 22.5 μl per sample in a microfuge tube. Published studies of the aspartate receptor and the galactose binding protein have successfully utilized the phosphate buffer at pH 7.0, while current studies of CheA use Tris buffer at pH 7.5 to increase the basicity and thus the disulfide formation rate (Gloor and Falke, unpublished). Ideally, both buffer systems should be tested on a subset of mutants to see whether one yields more convenient disulfide formation time courses or better data. In all cases, care should be taken that protein sulfhydryls are protected from oxidation during purification of the protein stock, and that the concentrations of residual reducing agents, such as DTT, are negligible (less than 5 μM total sulfhydryl) during the following disulfide reaction. Note that protein concentrations higher than low micromolar may lead to undesired intermolecular collisions and disulfide formation, particularly for small soluble proteins that diffuse rapidly. Before proceeding with the disulfide reaction, each sample is carefully mixed to ensure a homogenous solution and spun for 10 sec in a microfuge to bring the entire volume to the bottom of the tube.
2. The samples are moved to a 25° temperature bath, allowed to equilibrate for 1 min, then the disulfide reaction is initiated by adding 2.5 μl of the appropriate initiator stock with careful mixing before quickly returning to the 25° bath. The initiator stock varies with the type of oxidation chemistry chosen for the target system. For structural studies of aqueous proteins or domains, including regions near a membrane–water interface, the $\text{O}_2/\text{Cu(II)}$ (1,10-phenanthroline)₃ chemistry is preferred because its disulfide formation rate can be easily adjusted as needed for different systems. In this case, ambient O_2 (approx. 200 μM) within the sample is the oxidant and the initiator stock is 5 mM catalyst Cu(II) (1,10-phenanthroline)₃, created by addition of metal ion (100 μl of 0.5 M CuSO_4 in water) and chelator (175 μl of 1 M 1,10-phenanthroline in absolute EtOH) to water (9.725 ml). Dilution of the initiator stock into the sample yields a final catalyst concentration of 0.5 mM Cu(II) (1,10-phenanthroline)₃. For studies of transmembrane domains, I_2 chemistry is preferred due to its greater efficiency of disulfide formation in the apolar membrane environment. In this case,

the oxidant is I_2 and the initiator stock is 10 mM I_2 in EtOH, created by dissolving iodine crystals in absolute EtOH.

3. At each selected timepoint, 6 μ l of the reaction is removed and quenched by addition to a microfuge tube containing 6 μ l of quench solution (either 4 $\times$ nonreducing Laemmli sample buffer for membrane samples to ensure complete solubilization, or 2 $\times$ non-reducing Laemmli sample buffer for nonmembrane samples, and, in both cases, also containing 10 mM EDTA and 40 mM N-ethyl maleimide to quench the redox chemistry). Immediately after careful mixing, the tube is sealed and incubated at 95° for 2 min to ensure complete quenching. Typically, timepoints of 10, 30, and 300 sec are sufficient to contain the linear initial phase of the reaction timecourse.
4. For each time course, a zero point is generated by diluting the same di-Cys protein to a final concentration of 2 μ M in disulfide reaction buffer, yielding a total volume of 9 μ l in a microfuge tube. To this sample is added 1 μ l of water or EtOH, depending on whether the $O_2/Cu(II)$ (1,10-phenanthroline)₃ or I_2 chemistry is utilized, respectively, then 6 μ l is removed and quenched as described in step 3. The purpose of this zero point is to determine the level of spontaneous disulfide formation that occurred prior to addition of initiator.
5. The zero and timepoint samples are pelleted in a microfuge to deposit each sample at the bottom of the tube. Then, these samples (10 μ l each) are loaded side-by-side on a nonreducing Laemmli SDS-PAGE gel formulated so that the unreacted protein band exhibits maximal resolution from the disulfide-containing product band. The gel is stained with a linear dye such as Coomassie, and the stained bands are imaged by digital photography. Finally, the bands in each sample corresponding to the unreacted protein and the disulfide-containing product protein are quantitated by integration of their dye absorbances.

Note that the migration of the disulfide-containing product band can differ dramatically depending on the nature and location of the disulfide bond. If the disulfide bond is formed between two different polypeptide chains, then the disulfide-containing product band will migrate more slowly than the unreacted protein band, typically in the size range expected for the covalent complex (Falke *et al.*, 1988). If, however, the disulfide bond is formed via an intramolecular reaction between two Cys residues in the same polypeptide chain, then the disulfide-containing product band will migrate more rapidly than the unreacted band (Falke *et al.*, 1988). Moreover, subtle differences in migration are typically seen between different disulfide products of the same class (inter- or intra-chain) when the location of the disulfide bond is changed in the primary structure (Falke *et al.*, 1988). These complex effects on gel migration arise from the covalent crosslink imposed by the disulfide bond, which alters the average conformation and dynamics of the denatured polypeptide chain while it migrates through the gel matrix. If one is uncertain whether a given band is a true disulfide-containing product or a contaminant, disulfide-containing bands can be identified by rerunning the gel samples under reducing conditions.

6. To determine the extent of disulfide bond formation (E) at each timepoint, the integrated absorbances of the unreacted protein (U) and the disulfide-containing product (D) are used to calculate the ratio $E = D/(D + U)$. This ratio represents the fraction of total protein converted to disulfide-containing product.
7. Once the initial disulfide formation time courses have been determined for a range of positions, it may be necessary to adjust the reaction rate. It is useful to optimize the reaction rate so that virtually all the positions examined yield between 5 and 75% disulfide formation at a single timepoint, typically either 10 or 30 sec. This optimization ensures that as many Cys pairs as possible yield sufficient disulfide bond

formation to be quantitated, while at the same time the reaction has not proceeded to completion and thus remains in or near its linear range. To decrease or increase the speed of the reaction, one can decrease or increase, respectively, the pH or temperature of the reaction. Alternatively, reducing the glycerol concentration will generally increase the reaction rate, since this protein stabilizer reduces thermal fluctuations, and therefore collision rates and disulfide formation rates (Butler and Falke, 1996). For the $\text{O}_2/\text{Cu(II)}$ (1,10-phenanthroline) $_3$ chemistry, the concentration of the redox catalyst can also be increased to speed the reaction or decreased to slow the reaction, respectively. Notably, increasing the catalyst to a concentration in excess of EDTA greatly speeds the reaction because the latter chelator no longer buffers the Cu(II) (see step 9). For the I_2 chemistry, the reaction rate is more difficult to adjust, but, in some cases, is moderately sensitive to the final I_2 concentration.

8. After optimization, the disulfide formation reaction is carried out in triplicate for each Cys pair at the chosen timepoint to enable comparison of means and standard deviations. Multiple positions can be tested in a set of simultaneous reactions, with the exact number depending on the timepoint chosen for sampling the reaction and the preference of the investigator. In each set of reactions, two control reactions are carried out for a selected, well-characterized Cys pair that serves as an internal standard when comparing different sets. For each reaction in a set, a given di-Cys protein is diluted to 2 μM final concentration, as in step 1, except that the total volume is now 9 μl and the newly optimized reaction buffer is utilized. After mixing, spinning, and equilibration to the optimized temperature, 1 μl optimized initiator stock is added as in step 2, and the reaction is allowed to proceed. At the chosen timepoint, a 6 μl aliquot is removed and quenched as in step 3. Then, the quenched samples are analyzed by SDS-PAGE and digital imaging as described in steps 5 and 6, yielding a quantitative extent of disulfide bond formation for each reaction. By running multiple reactions consecutively, a set as large as 20 engineered Cys pairs can be analyzed in triplicate per day. Care should be taken that the control Cys pair included in all sets yields similar extents of disulfide formation in different sets run on the same or different days.
9. After the rapidly forming disulfide bonds have been identified, the effects of these disulfides on activity are tested. First, a stronger oxidation reaction is used to drive disulfide formation to completion by adjusting the pH, temperature, glycerol concentration, or the oxidation components. When the $\text{O}_2/\text{Cu(II)}$ (1,10-phenanthroline) $_3$ chemistry is used, rapid reactions can often be conveniently driven to completion by decreasing the EDTA concentration of the disulfide reaction buffer (step 1, from 5.0 mM down to 0.8 mM), while increasing the final concentration of Cu(II) (1,10-phenanthroline) $_3$ in the reaction (step 2, from 0.5 mM up to 1.0 mM). These changes yield an excess of redox catalyst relative to EDTA (which, in the standard reaction, buffers most of the Cu(II) and slows the reaction rate for convenient measurement), thereby dramatically increasing the reaction rate. After the reaction is completed, EDTA is added to a final concentration of 2.0 mM to slow further oxidation, or to 10 mM if the activity assay does not require free metal ions such as Mg^{2+} or Ca^{2+} which should be added in excess of EDTA. Finally, the protein activity of this oxidized sample is measured in comparison to an equivalent reduced sample generated by treatment with 20 mM DTT rather than oxidation components for the same length of time.

Disulfide Mapping: Interpretation of Results

Typically, Cys residues that are in close proximity within the native equilibrium structure will exhibit the most rapid rates of disulfide formation, and their reactions will proceed to form

nearly 100% disulfide product when driven to completion (Careaga and Falke, 1992). Such rapid disulfide reactions are fast enough to overwhelm the competing reactions that oxidize Cys sulfhydryls to oxyacids incapable of forming disulfide bonds, while slower disulfide reactions exhibit lower final extents of disulfide product even when the reaction is driven to completion, because competing oxidation reactions generate nondisulfide products (Careaga and Falke, 1992). Disulfide bond formation can occur only if, during the transition state of the reaction, the two Cys pairs can approach each other with a maximum distance of 4.6 Å between their α -carbons, and with angular constraints satisfied as well (Careaga and Falke, 1992). However, it has been observed that Cys residues much farther apart in the equilibrium structure can also collide and form a disulfide bond due to protein thermal fluctuations that produce long-range backbone motions. For example, even in the extremely stable galactose binding protein saturated with its ligand, collisions yielding disulfide formation are detected between Cys residues whose α -carbons are separated by 19.8 Å in the crystal structure (Careaga and Falke, 1992). In this study, proximal Cys pairs yielded higher disulfide formation rates than did distal pairs, but the detection of disulfide formation alone was not sufficient to indicate proximity.

To rigorously identify Cys pairs that are nearby in the equilibrium structure, two general approaches are useful. First, by measuring the disulfide formation rates of a large number of Cys pairs under identical conditions, a pattern of rates will often emerge that can be correlated with structural elements to identify multiple proximities between two contact faces. To detect such spatial patterns, it is helpful to classify the disulfide formation rates into three groups—rapid, intermediate, and slow—then to search for structural models in which the distances between the corresponding Cys pairs are short, intermediate, and long. The success of this approach typically depends on the observation of multiple Cys pairs that exhibit high disulfide formation rates and are correlated in space at the same packing interfaces (Bass and Falke, 1999). Second, by measuring the functional effects of disulfide bonds on protein activity, a subset of rapidly formed disulfides that retain normal function, or that trap the protein in a specific native signaling or functional state with a measurable activity, may be identified (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Winston *et al.*, 2005). Such functional disulfide bonds provide the strongest evidence that the disulfide-crosslinked positions are indeed adjacent in the native, active structure. Moreover, functional disulfide bonds can provide important constraints that are useful in elucidating the molecular mechanisms and conformational changes of proteins that switch among different functional states.

Both the spatial pattern analysis and functional analysis of disulfide bond reactions have been successfully employed in the full-length, membrane-bound aspartate receptor to identify contact surfaces in the periplasmic, transmembrane, and cytoplasmic domains (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Falke and Koshland, 1987; Falke *et al.*, 1988; Hughson *et al.*, 1997; Lee *et al.*, 1994; Pakula and Simon, 1992; Winston *et al.*, 2005). Moreover, functional analysis of disulfide bonds has played an important role in defining the current mechanistic models for conformational transmembrane and adaptation signals in the receptor (Bass and Falke, 1998; Bass *et al.*, 1999; Butler and Falke, 1998; Chervitz and Falke, 1995; Chervitz *et al.*, 1995; Danielson *et al.*, 1997; Falke and Hazelbauer, 2001; Winston *et al.*, 2005). Together, these studies have identified 22 functional disulfide bonds within the receptor that can be divided into three categories: (1) function-retaining disulfides that retain normal transmembrane signaling and kinase regulation, (2) lock-on disulfides that trap the receptor in its native on-state, and (3) lock-off disulfides that trap the receptor in its native off-state (where the on- and off-states are characterized by multiple, asymmetric activity assays). The information provided by these functional disulfides has directly confirmed contacts between specific pairs of positions in the native 3° and 4° structure of the active receptor in its complex

with the kinase CheA. In addition, careful analysis of the functional effects has revealed regions of the receptor that are static during signal transduction, as well as regions that undergo conformational transitions. Furthermore, comparison of the locations of the lock-on and lock-off disulfides in the receptor structure has revealed the piston-type displacement of a specific membrane-spanning helix that is responsible for transmembrane signaling (Chervitz and Falke, 1995, 1996; Chervitz *et al.*, 1995). In short, the disulfide mapping method yields not only 3° and 4° contacts, but also can define conformational transitions by covalently trapping different functional states identified by their signature activities.

Disulfide Trapping of Thermal Backbone and Domain Motions

Overview of Disulfide Trapping

Proteins are highly dynamic structures, but often it is difficult to detect the long-range backbone motions that occur in their native solution and membrane environments. Disulfide trapping uses the formation of a disulfide bond during a collision between two Cys residues to detect and covalently trap the thermal backbone fluctuation that generated the collision (Careaga and Falke, 1992; Falke and Koshland, 1987). Since these collisions are stored and accumulate with time, even rare backbone fluctuations difficult to detect by other methods can be analyzed. Moreover, by examining the disulfide formation patterns and rates of multiple Cys pairs, information can be obtained about the trajectories and frequencies of the observed backbone fluctuations. The approach is most powerful when applied to a protein of known high-resolution structure, thereby facilitating interpretation of the spatial implications of the results, particularly with regard to amplitude and trajectory measurements.

The initial disulfide trapping studies were carried out in the transmembrane aspartate receptor and the aqueous galactose binding protein (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995; Falke and Koshland, 1987; Falke *et al.*, 1988). Like chemical reactivity scanning and disulfide mapping, the disulfide trapping approach can be applied to full-length proteins in their native environment. Dramatic backbone motions were detected in both proteins, including relative sliding motions of adjacent α -helices with amplitudes as large as 15 Å, and hinge-twisting motions yielding rotations of adjacent domains up to 36°. The estimated frequencies of the detected collisions ranged from 10 to 104 per sec. Since the engineered Cys pairs used to trap these motions were introduced at surface sites and had little or no detectable effect on protein stability or activity, the motions appear to be intrinsic features of the native proteins in their native membrane or aqueous environments, respectively.

Disulfide Trapping: General Considerations

The following disulfide trapping procedures were developed for the aspartate receptor and galactose binding protein of bacterial chemotaxis (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995; Falke *et al.*, 1988; Gloor and Falke, unpublished), but are generalizable to other membrane and soluble proteins. As noted for other types of cysteine and disulfide chemistry, the first step in applying disulfide trapping to a new protein system is to use methods described in Chapter 1 of this volume (Bass *et al.*, 2007) to remove intrinsic Cys residues by substitution with Ala or Ser, especially in the domain targeted for disulfide trapping. Intrinsic Cys residues buried in other domains will generally not interfere and need not be removed.

Successful implementation of the disulfide trapping approach requires careful selection of suitable pairs of positions for Cys substitution and subsequent collisional analysis. Since the most powerful application of disulfide trapping is to analyze backbone and domain motions in a protein of known high-resolution structure (Bass and Falke, 1999; Butler and Falke, 1996;

Careaga and Falke, 1992; Careaga *et al.*, 1995), the focus here is on such systems. Pairs are chosen at surface positions in the known structure to minimize perturbations of structure and dynamics and to maximize accessibility to the oxidation chemistry. In general, conserved surface residues are avoided because their replacement would perturb activity or stability. To map out the trajectory for a relative motion between two different structural elements, one Cys pair is created with the two Cys residues at adjacent positions, one on each element. Then, other Cys pairs are created keeping one Cys residue, the probe residue, at a fixed position while the other residue is moved to a range of other positions. This strategy enables analysis of the range of positions contacted by the probe residue on an adjacent structural element. After the resulting di-Cys library is created, expressed, and purified, the effects of individual Cys pairs on protein stability and/or activity are measured by appropriate assays, and perturbed proteins are discarded while the unperturbed proteins are subjected to disulfide trapping analysis.

The remaining steps in the disulfide trapping procedure are similar to those outlined for disulfide mapping. In principle, the same two types of chemistry can be used to carry out disulfide trapping reactions: I₂ oxidation for analysis of collisions within transmembrane domains and O₂/Cu(II) (1,10-phenanthroline)₃ oxidation for analysis of collisions within aqueous domains and proteins. To date, only the latter chemistry has been tested in disulfide trapping studies, and the procedure has been applied only to soluble proteins and to the aqueous domains of membrane-imbedded proteins (Bass and Falke, 1999; Butler and Falke, 1996; Careaga and Falke, 1992; Careaga *et al.*, 1995; Gloor and Falke, unpublished). The following procedure summarizes how the individual steps of the previously mentioned disulfide mapping procedure are modified for disulfide trapping using the O₂/Cu(II) (1,10-phenanthroline)₃ chemistry.

Disulfide Trapping: Detailed Procedure

1. Each selected di-Cys protein of interest is diluted to a final concentration of 2 μ M in disulfide reaction buffer, yielding a total volume of 22.5 μ l per sample prepared in a microfuge tube, as indicated in step 1 of the previously mentioned disulfide mapping procedure.
2. The Cu(II) (1,10-phenanthroline)₃ initiator stock is added as indicated in step 2 of disulfide mapping.
3. At each selected timepoint, typically, 10, 30, and 300 sec, an aliquot of the reaction is removed and quenched as in step 3 of disulfide mapping.
4. For each time course, a zero point is generated as in step 4 of disulfide mapping.
5. The zero and timepoint samples are run side-by-side on a gel. The resulting stained bands are imaged and integrated as in step 5 of disulfide mapping. The bands corresponding to the unreacted protein and disulfide-containing product are identified by their relative migration on the gel, where an intra-chain disulfide-containing product will typically migrate slightly faster than the unreacted protein. By contrast, an inter-chain disulfide-containing product will migrate slower than the unreacted protein, at about the same R_f expected for the MW of the covalent product (step 5).
6. The extent of disulfide bond formation (E) at each timepoint is calculated as described in step 6 of disulfide mapping.
7. If necessary, the reaction rate is adjusted for convenient measurement of disulfide reaction time courses, as described in step 7 of disulfide mapping.
8. After optimization, the disulfide formation time course is measured, at least in duplicate, for each Cys pair. Multiple positions can be tested in a set of simultaneous time courses, with the exact number depending on the timepoints chosen. In each set

of time courses, one control time course is carried out for a selected, well-characterized Cys pair that serves as an internal standard when comparing different sets.

9. Each time course is best-fit via nonlinear least squares analysis to the following equation for a two-component reaction scheme that includes both disulfide formation and a competing oxidation reaction:

$$F_{ss}(t) = \frac{k_{ss}}{k_{ss} + k_2} \left[1 - e^{-(k_{ss} + k_2)t} \right] \quad (1)$$

where $F_{ss}(t)$ is the fraction of the di-Cys protein converted to disulfide-containing product, k_{ss} is the rate constant for the disulfide formation reaction, and k_2 is the rate constant for the competing reaction. This analysis separates out the desired rate constant for the disulfide formation reaction (k_{ss}) and enables direct comparison of this rate constant for different Cys pairs reacted under the same oxidation conditions.

Disulfide Trapping: Interpretation of Results

Analysis of disulfide formation time courses for an array of Cys pairs differing in the distance between the probe and test positions determines both the amplitude and kinetics of the observed thermal backbone fluctuations. Each Cys pair that yields measurable disulfide bond formation detects a thermal collision produced by the observed fluctuation. These colliding Cys pairs are mapped on the surface of the known protein structure, revealing the spatial pattern of the detected collisions and defining both the amplitude and the trajectory of the fluctuation. If desired, the frequency of each collision can be estimated by determining the fraction of Cys pair collisions successfully converted to a disulfide, as described in detail elsewhere. Overall, the disulfide trapping method provides the most powerful approach currently available for analyzing the amplitudes, trajectories, and frequencies of rare, long-range backbone and domain motions present within many proteins in their native environment. Systematic application of the approach to a diverse array of proteins from different structural and functional families could significantly further the molecular understanding of protein thermal dynamics.

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