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Mapping spatial proximities of sulfhydryl groups in proteins using a fluorogenic cross-linker and mass spectrometry

Andrea Sinz*,[1] and Kuan Wang

Muscle Proteomics and Nanotechnology Section, Laboratory of Muscle Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

Chemical cross-linking of proteins in combination with mass spectrometric analysis of the reaction products has gained renewed interest as a method of obtaining distance constraints within a protein and determining a low-resolution three-dimensional structure. We present a method for identifying spatially close sulfhydryl groups in proteins employing chemical cross-linking with the fluorogenic, homobifunctional cross-linker dibromobimane, which cross-links thiol pairs within ~3–6 Å. The applicability of our strategy was demonstrated by cross-linking the sulfhydryl groups of Cys-18 and Cys-78 in γ-crystallin F, which are within a distance of 3.57 Å according to the X-ray structure. Intramolecularly cross-linked γ-crystallin was first separated from reaction side products by reversed-phase chromatography on a C-4 column. Subsequently, the fraction containing the reacted protein was enzymatically digested with trypsin, and the resulting peptide mixture was separated by a second reversed-phase chromatographic step on a C-18 column, in which the cross-linked peptides were tracked by their fluorescence. The cross-linking product between Cys-18 and Cys-78 in γ-crystallin F was identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. This strategy presents a rapid method for mapping sulfhydryl groups separated by a distance of ~3–6 Å within a protein.

Keywords: Chemical cross-linking; γ-Crystallin; Dibromobimane; Fluorescence; HPLC; MALDI-TOF mass spectrometry

Chemical cross-linking, a conceptually simple approach, has proven to be an efficient method for mapping distances within a protein and thus yielding low-resolution three-dimensional structural information on proteins [1,2]. The identification of a large number of cross-linking sites from complicated mixtures generated by chemical cross-linking, however, remains a daunting task. To overcome these difficulties, a method integrating chemical cross-linking and mass spectrometry and using the fluorogenic cross-linking reagent dibromobimane (DB)[2] has been developed. DB possesses two equivalent bromomethyl groups that are able to cross-link thiol pairs spanning ~6 Å [3,4]. DB is nonfluorescent in solution but becomes fluorescent when both of its alkylating groups have reacted, exhibiting an emission maximum at 477 nm when excited at 385 nm [5]. DB has been successfully applied to the study of protein and nucleic acid structure, association, and conformation. Examples include stabilization of a primary loop in the myosin

* Corresponding author. Fax: +49-341-9736115.
E-mail address: sinz@chemie.uni-leipzig.de (A. Sinz).
[1] Present address: Biotechnological-Biomedical Center, Faculty of Chemistry and Mineralogy, University of Leipzig, D-04103 Leipzig, Germany.

[2] *Abbreviations used:* DB, dibromobimane 4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; EDTA, ethylenediaminotetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; HCCA, α-cyano-4-hydroxy cinnamic acid; HPLC, high-performance liquid chromatography; MALDI-TOFMS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; TCEP-HCl, tris(2-carboxyethylphosphine) hydrochloride; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid.

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structure [4,6], determination of the spatial proximity of cysteine residues in ArsA ATPase [7], in lactose permease [8], and in cysteine-containing F-actin mutants [9], and identification of the residues comprising the drug-binding domain of P-gp [10]. DB has been used for cross-linking dimers of bovine seminal ribonuclease [11] and for mapping the interfaces between calmodulin and a fragment of the giant muscle protein nebulin [12]. DB can also be applied for cross-linking lysine residues if the ε amino groups are first thiolated (for example by 2-iminothiolane) [12].

Our current aim is to develop a rapid method to map pairwise those intrinsic sulfhydryl groups that are within a distance of ~3–6 Å of each other, especially for proteins that are not accessible to structural analysis by NMR spectroscopy or X-ray crystallography. These short-range distances would be essential to deduce rudimentary folding patterns of proteins.

Materials and methods

Chemicals

γ-crystallin from bovine eye lens was obtained from Sigma–Aldrich (St. Louis, MO) and used without further purification. Na-EDTA and Tes-Na of the highest available purity were also obtained from Sigma–Aldrich. TFA was obtained from Burdick & Jackson (Muskegon, MI). TCEP-HCl was obtained from Pierce Chemical (Rockford, IL). Trypsin (sequencing grade) was obtained from Promega (Madison, WI). DB was obtained from Calbiochem (La Jolla, CA) as "Thiolyte DB." Acetonitrile (HPLC-grade quality) was purchased from Mallinckrodt (St. Louis, MO). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA).

Chemical cross-linking of γ-crystallin

Reduction of γ-crystallin with TCEP-HCl. For this, 150 μl of a 133 μM solution of γ-crystallin was prepared in 5 mM Tes buffer containing 1 mM Na-EDTA, pH 7.6; 3 μl of a 1 M TCEP-HCl solution in water was added, giving a final TCEP-HCl concentration of 20 mM. The mixture was incubated at 50 °C for 30 min. After 30 min, reacted protein was separated from low-molecular-weight compounds by filtration with Microcon 10 filters (Amicon, Bedford, MA) and recovered in 100 μl 5 mM Tes-Na buffer, containing 1 mM Na-EDTA, pH 7.6. Na-EDTA was added to the buffer to complex heavy metal cations, which catalyze oxidation of sulfhydryl groups. To avoid oxidation of the protein, it was used immediately for reaction with DB.

Cross-linking reaction of γ-crystallin with DB. For this, 0.375 μl of a 10 mM stock solution of DB in acetonitrile was added to 25 μl of the solution of freshly reduced γ-crystallin, giving a final concentration of 150 μM DB. After 45 min reaction time at room temperature in the dark, the reaction was quenched by addition of dithiothreitol to 40 mM final concentration.

HPLC and enzymatic digestion. HPLC was performed on an HP1100 system (Agilent Technologies, Palo Alto, CA) equipped with UV photodiode array detector and fluorescence detector. The cross-linking reaction mixture was fractionated by reversed-phase HPLC using a C-4 column (Sephasil protein, 250 × 4.6 mm, 5 μm, Amersham Pharmacia Biotech, Piscataway, NJ) with a linear gradient from water + 0.1% TFA to acetonitrile + 0.1% TFA in 60 min at a flow rate of 800 μl/min. MALDI-TOFMS was performed to check the fractions for γ-crystallin. The HPLC fraction containing the cross-linked protein was dried in a vacuum concentrator (Speed-vac SC110, Savant Instruments, Holbrook, NY), redissolved in 50 mM NH_4HCO_3, pH 7.8, and digested with trypsin at 37 °C for 14 h using a substrate:enzyme ratio of 50:1 (w/w). After digestion, the tryptic peptide mixture was dried in a vacuum concentrator, redissolved in 80 μl water, and analyzed by MALDI-TOFMS. The peptide mixture was further fractionated by HPLC on a C-18 column (Jupiter, 250 × 2.0 mm, 5 μm, 300 Å; Phenomenex, Torrance, CA). For peptide elution, the following gradient was applied: 0–60 min, 98–62.5% A; 60–90 min, 62.5–37.5% A; 90–105 min, 37.5–2% A (at a flow rate of 200 μl/min, where A is water + 0.06% TFA and B is 80% acetonitrile/20% water + 0.052% TFA). Fractions were collected by fluorescence emission signals at $\lambda = 477$ nm after excitation at $\lambda = 385$ nm. The fractions were dried in a Speed-vac system, redissolved in 25 μl water, and stored at −20 °C before MALDI-TOFMS analysis.

MALDI-TOF mass spectrometry. All spectra were obtained on a Proflex III time of flight mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a nitrogen laser (337 nm). Spectra were obtained by summation of 50–70 laser shots. For protein analysis, the mass spectrometer was operated in linear mode. Sinapinic acid (Sigma) was used as matrix and used without further purification. A saturated solution of sinapinic acid in a mixture of 30% acetonitrile, 70% water (+0.1% TFA) was used. Samples were prepared by mixing 1 μl of sample solution with 4 μl of saturated matrix solution and spotting 1.3 μl onto a stainless steel target. The mixture was air dried before insertion into the mass spectrometer. Horse heart myoglobin ($[M+H]^+$ 16952), cytochrome *c* ($[M+H]^+$ 12361), and bovine serum albumin ($[M+H]^+$ 66431) were used as external calibrants. For peptide analysis, the instrument was operated in reflectron mode. α-Cyano-4-hydroxy cinnamic acid (HCCA) (Fluka, Milwaukee, WI) was used as matrix after recrystallization from ethanol. For peptide analysis, a saturated HCCA solution in a mixture of 30%

acetonitrile, 70% water (+0.1% TFA) was used and peptide samples were prepared in the same fashion as protein samples. Substance P ($[M+H]^+_{mono}$ 1347.74), bombesin ($[M+H]^+_{mono}$ 1691.82), and ACTH (18-39) ($[M+H]^+_{mono}$ 2465.20) were used for external calibration. Database searches were performed using the Swiss Prot Database (www.expasy.ch).

Analysis of cross-linking products

Signals showing exclusively in fluorescent HPLC fractions of the tryptic peptide mixture were subjected to analysis by the in-house software NIH-XL [12].

Results

The goal of our studies was to develop a method for rapid isolation and identification of spatially close sulfhydryl groups within a protein by applying the bifunctional, fluorogenic cross-linking reagent DB in combination with MALDI-TOF mass spectrometric analysis of the cross-linking products. To demonstrate feasibility we first applied an in-house C program (G.W. Offer, unpublished results) to search for high-resolution proteins possessing at least one cysteine pair, in which the two sulfur atoms are within a distance of 3–6 Å from each other (Protein Data Bank; available at: http://www.rcsb.org/pdb/). Based on its easy availability, we chose γ-crystallin F to optimize experimental protocols. The sulfhydryl groups of Cys-18 and Cys-78 of γ-crystallin F are within a distance of 3.57 Å. Since both are located on the surface of the protein, they are easily accessible to the cross-linking reagent. The commercially available bovine lens γ-crystallin is a mixture of several γ-crystallins, being closely related in sequence [13].

Cross-linking reaction of γ-crystallin with DB

γ-crystallin F was reduced with TCEP before conducting the cross-linking experiments to ensure that the sulfhydryl groups were in the reduced form. After the cross-linking reaction with DB, the modified protein was separated from reaction side products by reversed-phase HPLC using a C-4 column (Fig. 1). Fluorescence detection showed several peaks with high intensities at

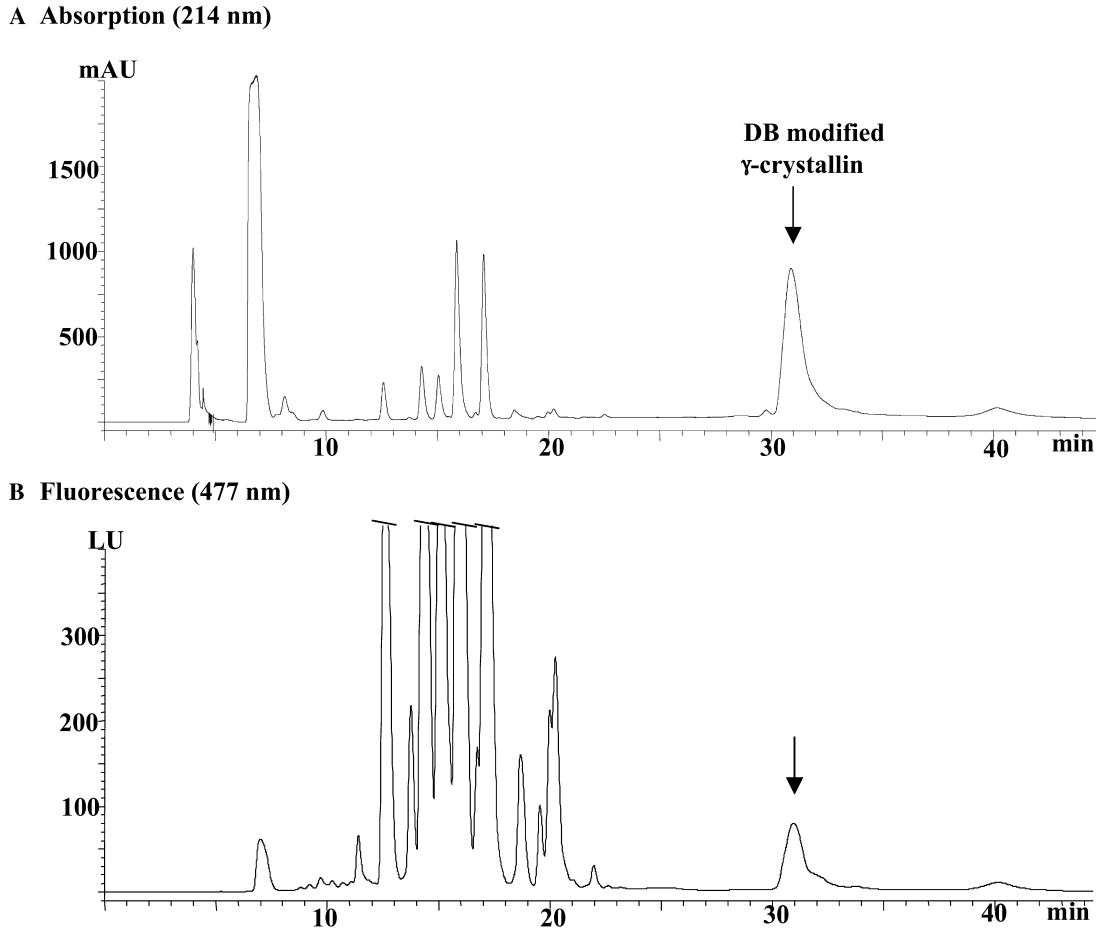

Fig. 1. Reversed-phase HPLC (C-4) of the reaction mixture of γ-crystallin with DB. (A) UV detection 214 nm. (B) Fluorescence detection. Excitation wavelength, 385 nm; emission wavelength, 477 nm. Au: absorption units, Lu: fluorescence units.

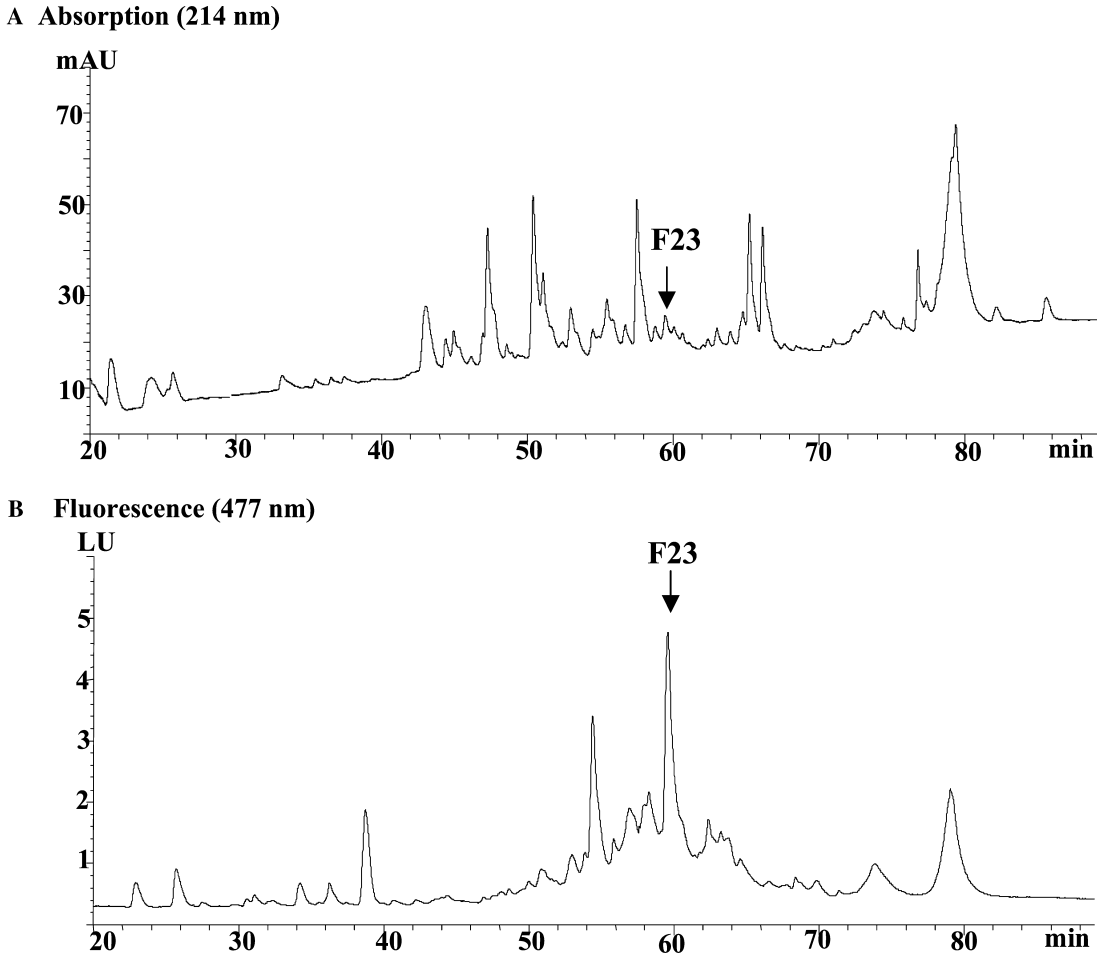

Fig. 2. Reversed-phase HPLC (C-18) of the tryptic peptide mixture of DB-modified γ-crystallin. (A) UV detection 214 nm. (B) Fluorescence detection. Excitation wavelength, 385 nm; emission wavelength, 477 nm.

retention times between ~12 and ~22 min. These peaks were assigned by LC/MS to contain low-molecular-weight DB reaction products. The peak with a retention time of 31 min, corresponding to DB-modified γ-crystallin, was digested with trypsin. The tryptic peptide mixture was analyzed by MALDI-TOFMS, showing unmodified peptides for the γ-crystallins A, B, D, E, and F as the most prominent signals (data not shown). The tryptic peptide mixture was separated by C-18 HPLC, tracking DB-positive fractions by their fluorescence at 477 nm (Fig. 2). The fluorescence chromatogram shows only a few peaks with high intensity, indicating that DB had reacted at only a limited number of sites. From a comparison with the absorption chromatogram at 214 nm it is clear that fluorescent peptides were minor components in the digest. This demonstrates the attribute of the fluorogenic cross-linker in identifying cross-linked peptides. Fractions were collected according to the fluorescence at 477 nm and the most abundant peaks eluting at retention times of 38.7 min (fraction 12), 54.4 min (fraction 21), 59.6 min (fraction 23), and 79.0 min (fraction 33) were subjected to MALDI-TOFMS analysis.

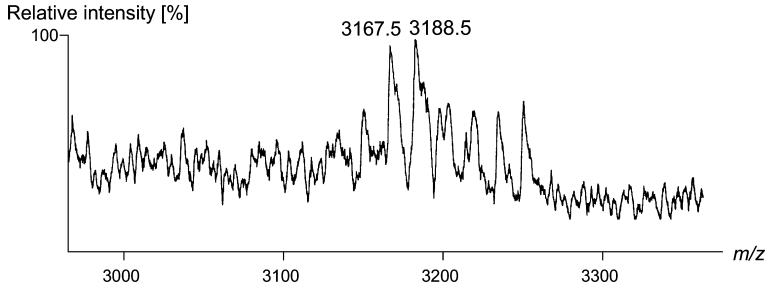

Fig. 3. Enlarged region of the MALDI-TOF mass spectrum of fraction 23 (F23) from C-18 HPLC (Fig.2).

Analysis of cross-linking products. Signals appearing exclusively in fluorescent HPLC fractions were used for analysis by NIH-XL. This software calculates theoretical molecular weights of cross-linked peptide pairs originating from the digestion of a given protein with a specified enzyme and compares the results to experimentally obtained values from mass spectrometric analysis. Some signals were assigned to the polyacrylamide gel matrix, to peptides from non-cross-linked protein, and to autolytic peptides of trypsin. In fraction 23 of the C-18 HPLC separation (Fig. 2), a signal at *m/z* 3167.5 was detected, which was found to match with a cross-linking product of γ-crystallin F, consisting of amino acids 77–79 and 10–31 cross-linked with DB (calculated molecular weight 3167.5) (Fig. 3). Also visible is a signal at *m/z* 3188.5 corresponding to a peptide comprising amino acids 10–36 (Fig. 3). Interestingly, in three other highly fluorescent fractions no cross-linked peptides were detected. The fluorescence of these fractions appeared to have originated from peptides that have been modified by DB hydrolyzed at one reactive site.

Discussion

The agreement of chemical cross-linking with the crystallographic spacing of the two sulfhydryls of γ-crystallin F is encouraging. DB thus appears to be a promising reagent for a rapid method to map pairwise those sulfhydryl groups that are within a distance of ~3–6 Å of each other. These short-range distances would be essential to deduce rudimentary folding patterns of proteins.

From this and previous work [12], it is clear that, while the fluorogenic property of DB can be usefully exploited as a marker for cross-linked peptides, its fluorescence is not restricted to products with two sulfhydryls. Indeed, fluorescence is generated irrespective of whether DB reacts with two SH groups of a protein or whether it modifies only one SH group and is hydrolyzed at the other reactive site. This necessitates a careful mass spectrometric analysis of all fluorescent peaks. However, since hydrolysis of DB seems to be slower than the cross-linking reaction, effective cross-linking was achieved in up to 60 min without overwhelming hydrolysis of DB [12]. This side reaction thus represents a minor drawback. We demonstrated that separation of intramolecularly cross-linked protein from other reaction products in a relatively simple cross-linking reaction mixture can easily be performed by HPLC instead of using SDS–PAGE, thus avoiding problems caused by SDS for subsequent mass spectrometric analysis [14].

Chemical cross-linking in general reveals intermediate or transient conformations of a protein and thus offers a more complete picture of the structural dynamics of the protein in solution than the static X-ray structure. For γ-crystallin F, cross-linking between Cys-18 and Cys-78 is consistent with the inter-SH distance calculated from crystallographic data (Fig. 4). In contrast, for thiolated calmodulin, DB cross-linking of two distant domains in the crystal structure clearly indicates the flexible nature of the interdomain helix [12].

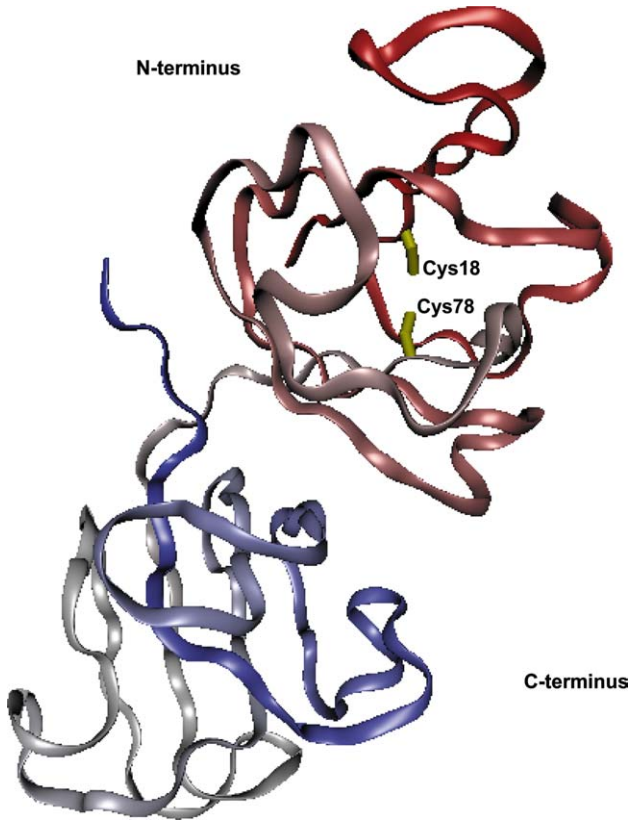

Fig. 4. X-ray structure of γ-crystallin F (pdb entry 1A45), in which the cross-linked Cys-18 and Cys-78 are indicated.

As is the case with the interpretation of any cross-linking data, the absence of cross-linking does not necessarily indicate the absence of proximal reactive groups in the protein. Factors such as orientation and accessibility of reactive groups also affect the outcome. We have applied the DB cross-linking to tropomyosin and failed to obtain appreciable cross-linking of the proximal SH pairs in the tropomyosin homodimers (A. Sinz and K. Wang, unpublished data). The rigidity of DB may require favorable orientations of the SH pair before effective cross-linking is achieved. If this is the case, then DB might be explored as a conformational probe for the relative orientation of the SH groups.

In summary, we demonstrated that by integrating fluorogenic cross-linking with DB, enrichment by HPLC, and identification of cross-linked peptides by fluorescence and MALDI-TOF mass spectrometry, valuable information about inter-SH distances can be defined within a protein. The strategy is also likely to be of general and broad application in the understanding of architecture of cellular organelles and protein assemblies,

since intrinsic proximal sulfhydryl groups that can be easily oxidized to disulfides are abundant, e.g., in cytoskeletal proteins or red blood cell membranes [15]. These pairwise distances would be important structural markers in understanding the molecular interfaces between different proteins in the complexes.

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