Quantitative evaluation of the lengths of homobifunctional protein cross-linking reagents used as molecular rulers

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Abstract

Homobifunctional chemical cross-linking reagents are important tools for functional and structural characterization of proteins. Accurate measures of the lengths of these molecules currently are not available, despite their widespread use. Stochastic dynamics calculations now provide quantitative measures of the lengths, and length dispersions, of 32 widely used molecular rulers. Significant differences from published data have been found.

Keywords: Protein cross-linking; cross-linking reagents; molecular rulers; stochastic dynamics

Supplemental material: See www.proteinscience.org.

Bifunctional chemical cross-linking reagents and the intramolecular and intermolecular cross-linking of proteins and protein complexes have been instrumental in the structural and functional characterization of proteins. The importance of these investigations is reflected in the large and increasing arsenal of commercially available cross-linking reagents (Pierce Chemicals 1999). In the arena of structural studies, the cross-linking reagents are envisaged as molecular rulers that provide information on distances between the cross-linked amino acid residues that are pertinent to both the tertiary and quaternary arrangements of proteins (Fasold et al. 1971; Peters and Richards 1977). Such distance determinations are especially valuable in membrane proteins and other proteins that cannot be crystallized and frequently lead to important advances in mapping the protein topography (Swaney 1986; Kwaw et al. 2000). For crystallized proteins (Fasold et al. 1971) and lower resolution structures refined by computational methods (Holmes et al. 1990; Lorenz et al. 1993), the cross-linking results have provided valuable constraints for the modeling of such structures.

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The most reliable information is derived from zero-length reagents that induce a direct covalent link between cross-linked sites. Disulfide and carbodiimide cross-linking reactions are probably the most popular in this category (Kunkle et al. 1986). In contrast, most bifunctional reagents introduce a bridge between the cross-linked residues and contain flexible bonds; these do not provide a rigid yardstick of interresidue distances. Nevertheless, the distances between cross-linked sites frequently are represented by a single mean value provided by the reagent manufacturer (Sun and Kaback 1997; Nagy et al. 2000). One result of using such mean cross-linking span values is the assignment of incorrect distances to the linked pairs of residues. In studies designed to probe macromolecular flexibility by using homologous series of bifunctional reagents, the dynamic features of the protein may be misrepresented if the flexibility of the reagents is not accounted for (Kliche et al. 1999). Despite the many uses of these substances, an accurate description of the cross-linking span of the bifunctional reagents is not currently available. In this study, we have used stochastic molecular dynamics techniques to provide a more realistic quantification of the lengths of 32 popular homobifunctional reagents. The data provide more accurate information on the structure and dynamics of proteins probed with these reagents. The dynamics of both the protein and the cross-linking reagent will have an influence on the rates

of cross-linking. Our work deals with the most critical feature of how easy it is for the reagent to span various distances. Even then there will be some variation in the ability of various reagents to form cross-links with proteins containing diverse functional group orientation. These issues of orientation are not addressed in our considerations, and we only focus on the dynamic range of the reagents.

Computational methodology

Stochastic dynamics simulations are able to efficiently and completely sample the conformational space of a molecule without being limited to the local minimum in the region of the starting structure. The method simulates the effect of solvent on the conformation of small molecules by including the effects of random collisions with solvent and by introducing a frictional drag component (Leach 1996).

Each cross-linking reagent was fully minimized using the AMBER* force field (Mohamadi et al. 1990) as implemented in the `Macromodel` program. All simulations were performed at 298K. After a 50-psec equilibration period, each structure then was subjected to stochastic dynamics for 2.5 nsec using a generalized Born/solvent accessible (GB/SA) water solvation model (Still et al. 1990). The time step used was 1.5 femtosecond (fs). Every picosecond, a sample structure was taken, and the pertinent cross-linking span was measured. To ensure that there was adequate sampling, we plotted the ensemble of distances from each simulation as a function of time (Fig. 1B). The frequency of occurrence for each distance during the simulation also was plotted, providing distribution statistics (Fig. 1A). Statistics were collected at several time intervals to ensure that sufficient time was allowed to achieve equilibrium. The plots for oPDM are shown in Figure 1 as a representative example. Data of this type for all the cross-linking reagents studied here are given at www.proteinscience.org.

In several of the cases studied, the effect of attaching one end of the cross-linker to a protein was estimated by conjugating the reagent to a molecule of pentaiodobenzene thiolate. This heavy, but sterically unhindering, group increases the time taken to achieve equilibrium. With sufficient calculation times, however, the conjugated and unconjugated molecules obtained the same distribution statistics. Because the conformational changes in the reagents occur on the nanosecond time scale, and most cross-linking reactions occur on the order of seconds, attachment of a heavy group free of steric encumbrance should not change the effective length of these molecular rulers.

Results and Discussion

All of the cross-linkers in the study are reactive with either amines or sulfhydryl groups. They can be divided into five different series based on the type of reagent and their reactivity. The first two series are sulfhydryl-reactive molecules.

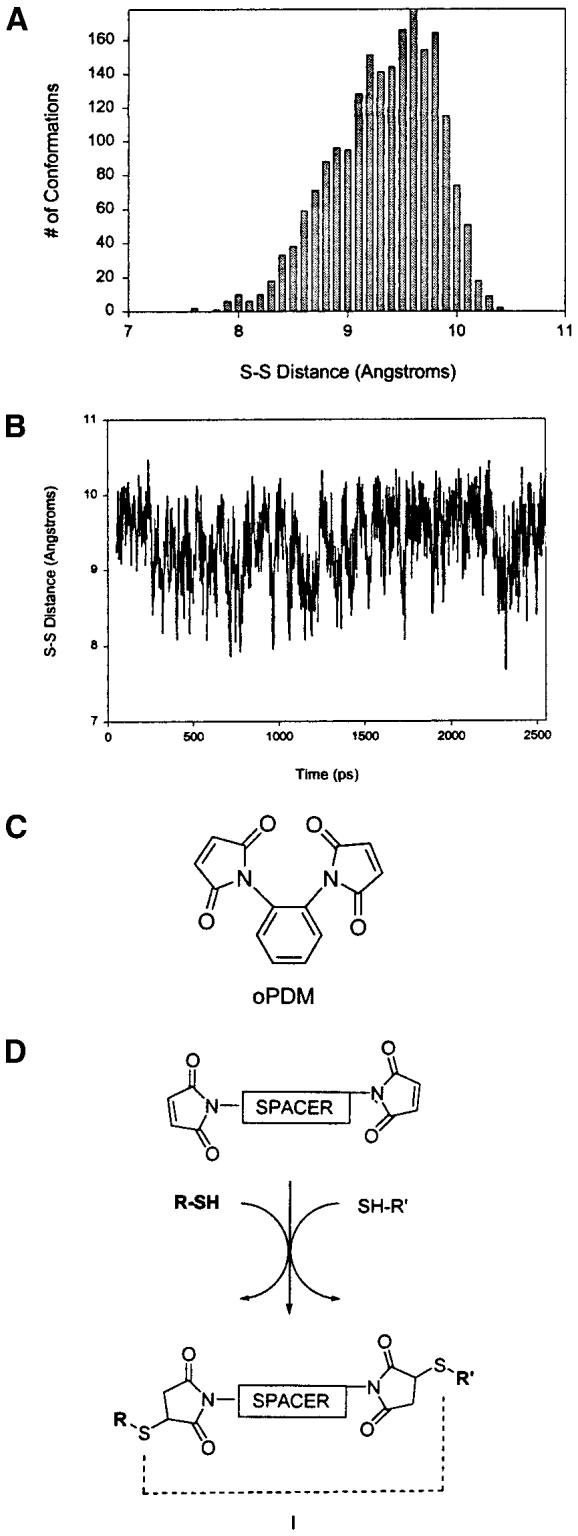

Fig. 1. *N,N′*-1,2-phenylenedimaleimide (oPDM). (*A*) Frequency vs. cross-linking span for oPDM; (*B*) cross-linking span vs. time for oPDM; (*C*) structure of oPDM; (*D*) reaction of oPDM and other bis-maleimide cross-linking reagents with sulfhydryl groups. l is the S-S distance and the cross-linking span of the reagent. (oPDM) *N,N′*-1,2-phenylenedimaleimide.

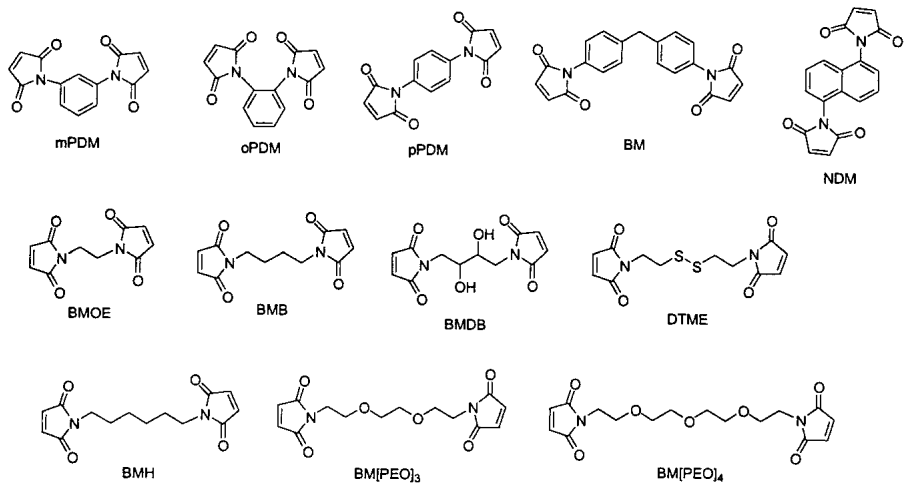

Fig. 2. The bis-maleimide cross-linking reagents. (mPDM) *N,N′*1,3-phenylenedimaleimide; (oPDM) *N,N′*-1,2-phenylenedimaleimide; (pPDM) *N,N′*-1,4-phenylenedimaleimide; (BM) *N,N′*-(methylene-4-1-phenylene)bismaleimide; (NDM) naphthalene-1,5-dimaleimide; (BMOE) bismaleimidoethane; (BMB) 1,4-bismaleimidobutane; (BMDB) 1,4-bis-maleimidyl-2,3-dihydroxybutane; (DTME) dithiobis-maleimdoethane; (BMH) 1,6-bismaleimidohexane; (BM[PEO]$_3$) 1,8-bismaleimidotriethyleneglycol; (BM[PEO]$_4$) 1,11-bis-maleimidotetraethyleneglycol.

This first group reacts by alkylation chemistry, whereas the second cross-links via disulfide exchange. The remaining categories represent amine-reactive chemicals. They can react with either the α-amines at the N terminus or the ε-amines of lysine residues to give either stable amide or amidine linkages.

The statistics for each reagent are compiled in Tables 1–5. Every distance (in angstroms) obtained from the simulation was used to compute the statistics. In each table, the average distance between S or N atoms attached to the linker molecule, the standard deviation of this value, the mode—or most probable distance, median distance, and the range of all distances obtained in a simulation are given and compared with the commonly cited distances. The cited distances for mPDM, oPDM, pPDM, BM, and NDM were obtained from force field energy minimizations as described by Nitao and Reisler (1998), whereas the distances for the C-6 through C-9 disulfides were determined by Faulstich (Kliche et al. 1999). Cross-linking spans for all the remaining molecules studied were obtained from the Pierce Chemicals *Double Agents Cross-Linking Reagents Selection Guide* published in 1999 (Pierce Chemicals 1999).

Most of the distributions obtained were basically gaussian in nature, although some were skewed slightly toward longer lengths. This is not surprising given that the lowest energy conformation of the alkyl bridged reagents is an extended conformation. There is only one fully extended conformation, however, and very little energy is required to rotate one or more bonds into gauche conformations. The number of populated conformations with eclipsed bonds is much lower than gauche conformations because of the relatively high energy of eclipsed conformations. Therefore, the mode of the distribution represents an ensemble of conformations all with the same cross-linking span. In a normal distribution, 97.7% of the data should fall within three standard deviations of the mean. In almost all of the cases studied, this is the case. Therefore, the average distance, coupled with the standard deviation, provides an accurate sense of the distribution data. For mPDM, for example, our recommended ruler length is 10.6 ± 0.5 Å.

The first series (Fig. 2) consists of maleimide-capped

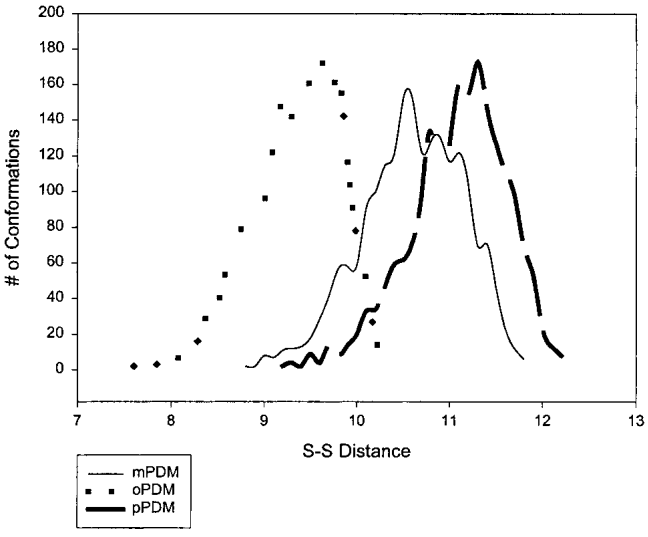

Fig. 3. Frequency plots showing overlap of mPDM, oPDM, and pPDM spans. (mPDM) *N,N′*1,3-phenylenedimaleimide; (oPDM) *N,N′*-1,2-phenylenedimaleimide; (pPDM) *N,N′*-1,4-phenylenedimaleimide.

Table 1. *Series 1: SH reactive Alkylation-based reagents*

Cross-linking reagent	Average S-S distance	Standard deviation	Mode	Median S-S distance	Other major modes	Range of S-S distances	Cited S-S distance[a]
mPDM	10.65	0.55	10.5	10.67	—	8.84–11.87	9.6–11.5
oPDM	9.39	0.47	9.6	9.44	—	7.67–10.47	5.2–7.8
pPDM	11.13	0.52	11.4	11.19	—	9.20–12.29	12.1–12.4
BM	14.53	1.51	15.0	14.80	—	9.40–17.34	14.9–15.4
NDM	12.31	0.47	12.3	12.35	—	10.33–13.52	12.4–12.9
BMOE	8.18	0.75	7.8	8.09	—	6.27–10.52	8.0
BMB	10.44	2.04	12.0	11.00	8.0	4.52–14.14	10.9
BMDB	11.99	0.52	12.0	12.06	—	9.48–13.18	10.2
BMH	10.16	2.41	11.5	10.55	—	3.47–15.64	16.1
DTME	12.43	1.60	13.6	12.61	—	6.68–16.12	13.3
$BM[PEO]_3$	8.83	1.81	9.0	8.83	—	3.51–14.26	14.7
$BM[PEO]_4$	9.51	2.34	9.2	9.39	—	3.51–16.56	17.8

(mPDM) *N*,*N*′1,3-phenylenedimaleimide; (oPDM) *N*,*N*′-1,2-phenylenedimaleimide; (pPDM) *N*,*N*′-1,4-phenylenedimaleimide; (BM) *N*,*N*′-(methylene-4-1-phenylene)bismaleimide; (NDM) naphthalene-1,5-dimaleimide; (BMOE) bismaleimidoethane; (BMB) 1,4-bismaleimidobutane; (BMDB) 1,4-bis-maleimidyl-2,3-dihydroxybutane; (BMH) 1,6-bismaleimidohexane; (DTME) dithio-bis-maleimdoethane; ($BM[PEO]_3$) 1,8-bis-maleimidotriethyleneglycol; ($BM[PEO]_4$) 1,11-bis-maleimidotetraethyleneglycol.

[a] For mPDM, oPDM, pPDM, BM, and NDM, see Nitao and Reisler (1998). All others, see Pierce Chemicals (1999).

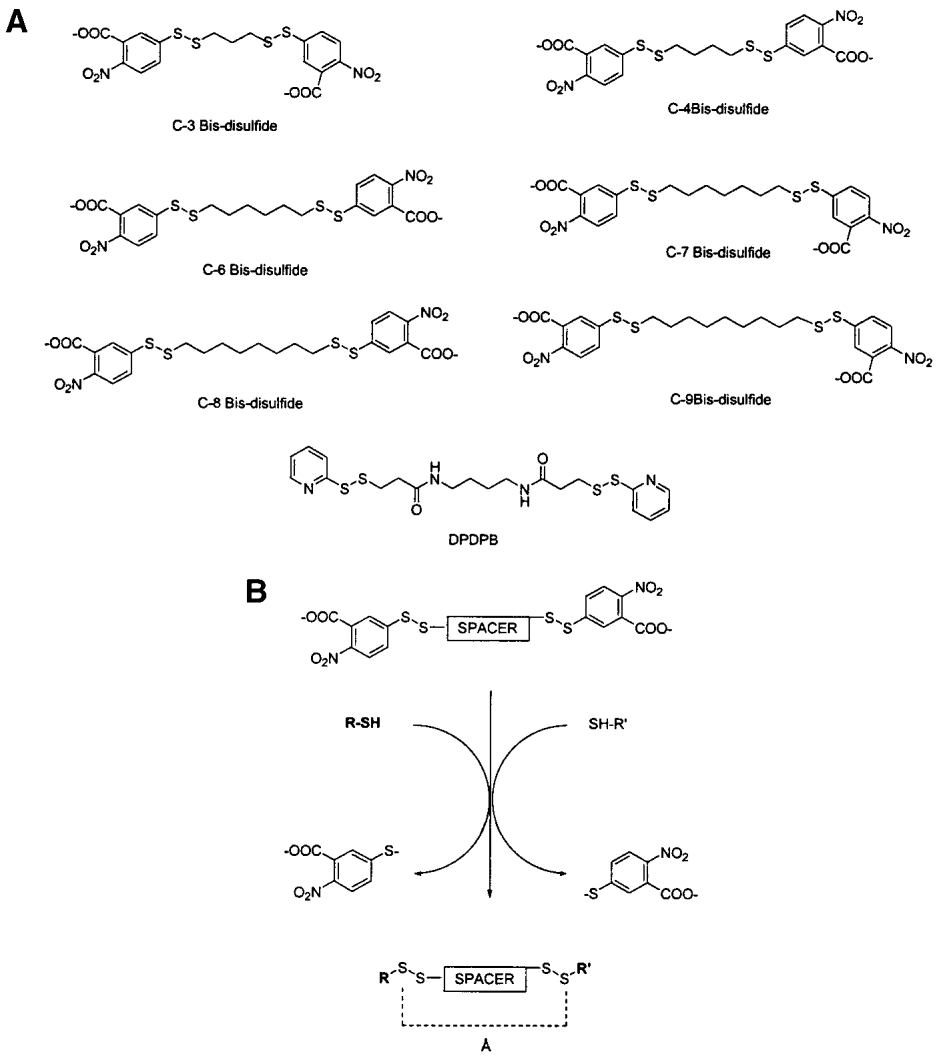

Fig. 4. Disulfide exchange cross-linking reagents. (*A*) Structures of the arene-disulfide exchange reagents. (*B*) Reaction of bis-disulfide cross-linking reagents with sulfhydryl groups. Å is the cross-linking span of the reagent.

Table 2. *Series 2: Sh-reactive disulfide exchange reagents*

Cross-linking reagent	Average S-S distance	Standard deviation	Mode	Median S-S distance	Other major modes	Range of S-S distances	Cited S-S distance
C-3 disulfide	6.87	1.35	7.8	7.3	4.3, 5.9	3.90–8.90	8.6
C-4 disulfide	8.07	1.42	9.7	8.2	8.2	4.10–10.30	9.9
C-6 disulfide	10.37	1.24	10.4	10.50	12.3	5.95–12.70	12.5
C-7 disulfide	11.61	1.40	12.5	12.04	—	6.89–14.10	13.8
C-8 disulfide	11.50	2.09	13.8	11.75	—	6.08–14.71	15.1
C-9 disulfide	12.24	2.02	12.2	12.37	—	6.73–16.16	16.4
DPBDP	14.65	1.44	15.7	14.88	—	9.29–18.20	19.9

The activated disulfides are the C-3, C-4, and C-6 through C-9 disulfides and 1,4-di-(3′-[2-pyridyldithio]-propionamido) butane (DPDPB).
[a] See reference Pierce Chemicals (1999).

reagents that cross-link by addition of the sulfhydryl group of a cysteine residue to the double bond in the maleimide (Fig. 1D). In this series, the resultant sulfur to sulfur distance was monitored in the derivatives formed by the addition of methanethiol to the double bond of each maleimide. This group includes *N*,*N*′1,3-phenylenedimaleimide (mPDM; for some representative examples, see Chang and Flaks 1972; Moroney et al. 1982; Nadeau et al. 1997; Nitao and Reisler 1998), *N*,*N*′-1,2-phenylenedimaleimide (oPDM; for some representative examples, see Chang and Flaks 1972; Oda and Funatsu 1979; Wells et al. 1980; Masuho et al. 1982; Moroney et al. 1982; Nadeau et al. 1997; Nitao and Reisler 1998; Yu et al. 1998; Wang and Kaback 1999), *N*,*N*′-1,4-phenylenedimaleimide (pPDM; for some representative examples, see Ohara et al. 1982; Valenzuela et al. 1984; Chaussepied et al. 1988; King et al. 1991; Hester-

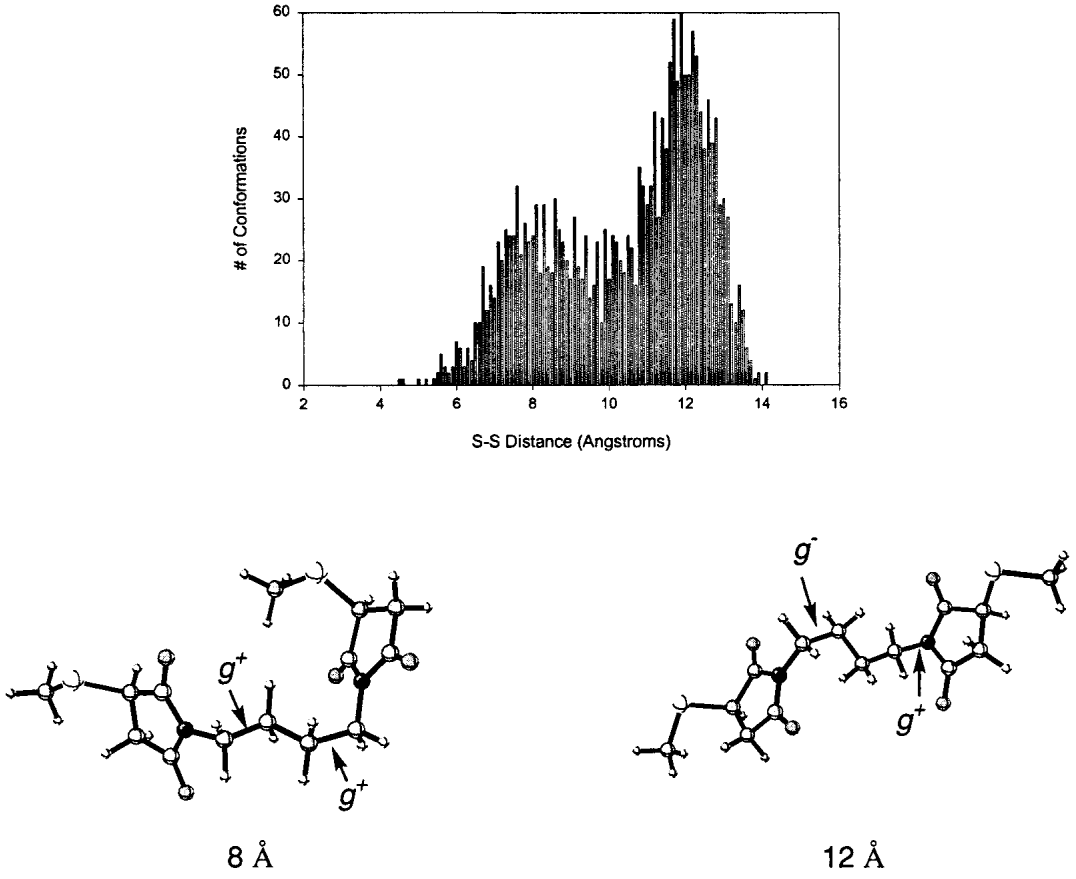

Fig. 5. Frequency plot for 1,4-bismaleimidobutane (BMB). The structures shown represent conformations of BMB with cross-linking spans corresponding to the two modes. Both conformations have two gauche NCCC dihedral angles.

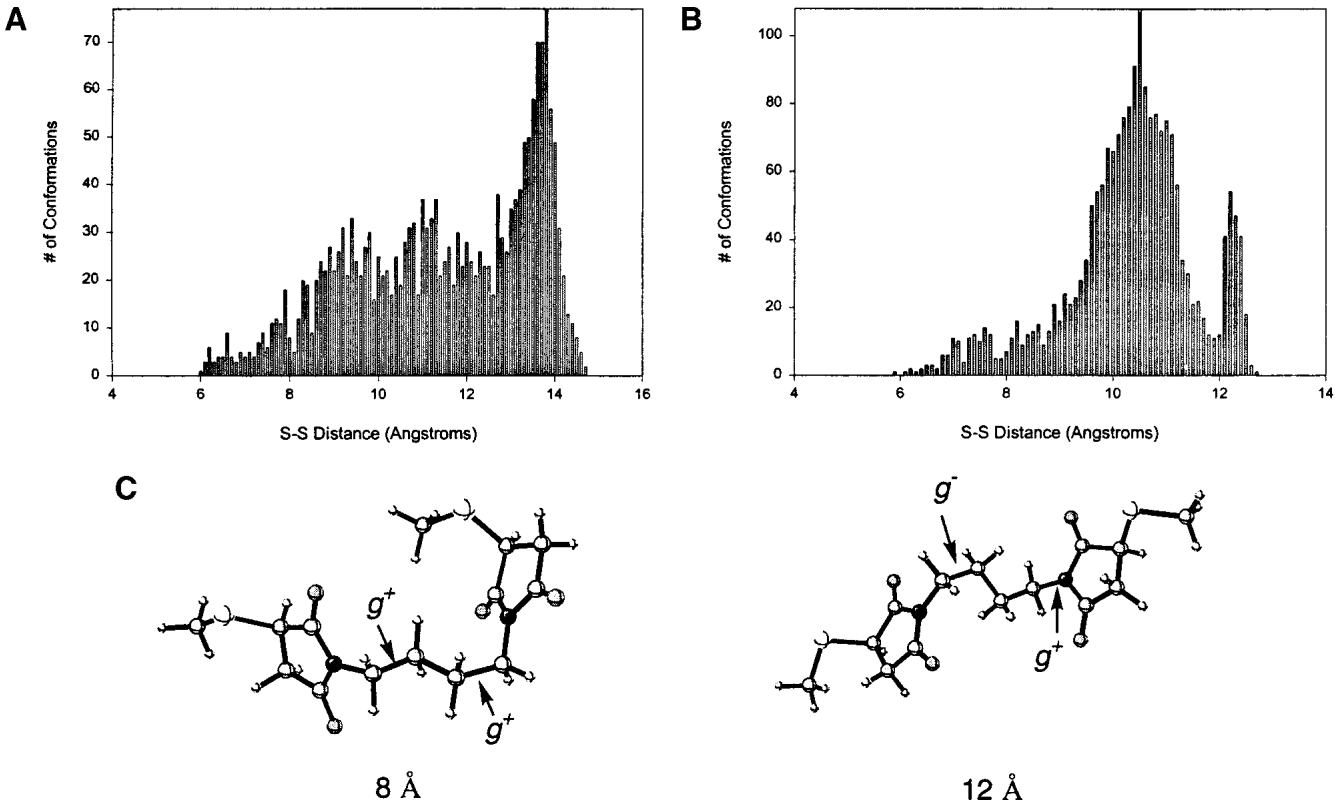

Fig. 6. C-6 and C-8 disulfides. (*A*) Frequency plot for C-8 arene disulfide; (*B*) frequency plot for C-6 arene disulfide; (*C*) two major conformations of the C-6 disulfide reagent and the corresponding S-S distances.

kamp et al. 1993; Bubis et al. 1995; Nadeau et al. 1997; Polosukhina and Highsmith 1997; Nitao and Reisler 1998; Wang and Kaback 1999), *N*,*N*′-(methylene-4-1-phenylene)bismaleimide(BM; for some representative examples, see Coggins 1996; Nitao and Reisler 1998), naphthalene-1,5-dimaleimide (NDM; for some representative examples, see Wells et al. 1980; Miller et al. 1982; Moroney et al. 1982; Perkins et al. 1984; Nitao and Reisler 1998), bismaleimidoethane (BMOE; e.g., see Cheronis et al. 1992; Tanaka et al. 1996), 1,4-bismaleimidobutane (BMB; e.g., see Cheronis et al. 1992; Schwarzer et al. 1997; Franke et al. 1999), 1,4-bis-maleimidyl-2,3-dihydroxybutane (BMDB; e.g., see Bauer and Hagen 1997; Schwarzer et al. 1997), 1,6-bismaleimidohexane (BMH; e.g., see Goldberg et al. 1991; King et al. 1991; Ishiguro et al. 1992; Greve and McClelland 1994; Hansen and Barklis 1995; McDermott et al. 1996; Stults 1997; Margolin et al. 1998; Franke and Pingoud 1999; Wang and Kaback 1999; Archambault 2000; Rappsilber et al. 2000), dithio-bis-maleimdoethane (DTME); e.g., see Moroney et al. 1982; Kato et al. 1986), 1,8-bismaleimidotriethyleneglycol (BM[PEO]$_3$; e.g., see Alexander and Speranza 1989; Kossmehl et al. 1995), and 1,11-bis-maleimidotetraethyleneglycol (BM[PEO]$_4$).

The bis-maleimide reagents oPDM, mPDM, pPDM, and BM are perhaps among the most frequently used molecular rulers of intersulfide distances in proteins. They owe their popularity to their high reactivity and specificity and the perceived lack of length overlap among them (Perkins et al. 1984; Nitao and Reisler 1998; Kwaw et al. 2000). Our results (Table 1) show an overlap in the range of S-S distances that can be bridged by these reagents, particularly mPDM and pPDM (Fig. 3). The range of distances in the tables is derived from all distances with a contribution of one of more conformers to the distribution. The values obtained for mPDM are quite close to those cited from literature whereas the width of the distribution found here for pPDM is very

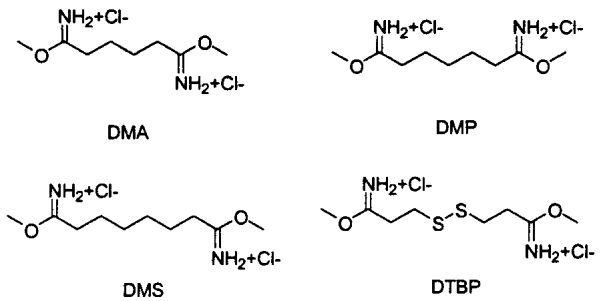

Fig. 7. The bis-imidoester cross-linking reagents. (DMA) dimethyl adipimidate; (DMP) dimethyl pimelimidiate; (DMS) dimethyl suberimidate; (DTBP) dimethyl 3,3′-dithiobis-propionimidate.

Table 3. *Series 3: NH_3 reactive imidoester class of cross-linking reagents*

Cross-linking reagent	Average N-N distance	Standard deviation	Mode	Median N-N distance	Other major modes	Range of N-N distances	Cited N-N distance[a]
DMA	7.40	0.58	7.4	7.47	—	4.03–8.99	8.6
DMP	8.36	0.90	8.5	8.47	—	4.46–10.29	9.2
DMS	9.39	1.03	9.5	9.59	—	6.82–11.60	11.0
DTBP	8.41	1.05	8.3	8.54	—	3.93–11.07	11.9

(DMA) dimethyl adipimidate; (DMP) dimethyl pimelimidiate; (DMS) dimethyl suberimidate; (DTBP) dimethyl 3,3′-dithiobis-propionimidate.
[a] See reference Pierce Chemicals (1999).

different from cited, and the cited oPDM values (5.2–7.8 Å) are entirely different from those found here (7.67–10.47 Å). Although in some cases the overlap regions may represent only a small fraction of the overall conformations of the reagents, the very existence of such overlap must be taken into account in the analysis of protein cross-linking kinetics by these reagents. The various rates of cross-linking may be as much influenced by protein dynamics as by that of the reagent making it more difficult to assess the conformational fluctuations of the protein.

$BM[PEO]_3$ and $BM[PEO]_4$ prefer gauche conformations in solution and, as such, provide two of the most striking examples of large differences between the cited sulfur–sulfur distance and the most common distance from dynamics. In triethylene glycol, the distance cited for the spacer arm length is 14.7 Å, whereas we obtain a distance of 8.8 Å. An analysis of this conformation, obtained from the dynamics simulation, shows that the CO bonds adopt gauche conformations, thus bringing the two reactive ends of the molecule closer together. This gauche preference is well known in such molecules (Eliel 1965) and makes an even larger difference in the tetraethylene glycol case. Our calculations show that the most populated distance is ~9.5 Å. Literature values commonly cited are almost twice this length (17.8 Å).

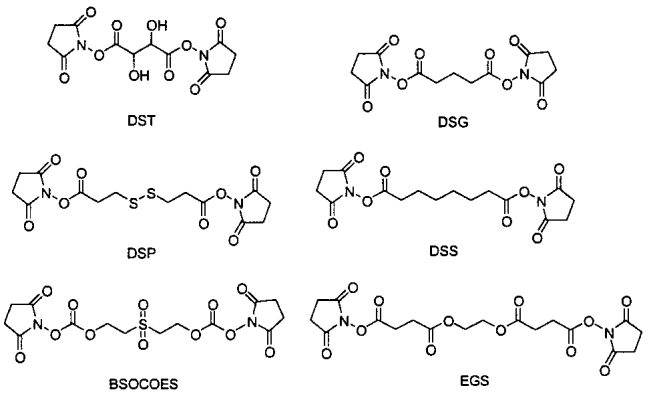

Fig. 8. The bis-*N*-hydroxysuccinimide ester cross-linking reagents. (DST) disuccimimidyl tartarate; (DSG) disuccinimidyl glutarate; (DSP) dithiobis-(succinimidylpropionate); (DSS) disuccinimidyl suberate; (BSOCOES) bis(2-[succinimidooxycarbonyloxy]ethyl)sulfone; (EGS) ethyleneglycol bis-(succinimidylsuccinate).

The second series (Fig. 4A) consists of sulfhydryl-reactive reagents capped by aryl disulfides. These activated disulfides are the C-3, C-4, and C-6 through C-9 disulfides (Kliche et al. 1999), and 1,4-di-(3′-[2-pyridyldithio]-propionamido) butane (DPDPB) (e.g., see Russell-Jones et al. 1994; Zecherle et al. 1992; Lai 1997; Middleton et al. 1997; Wolff et al. 1998, 1999). The distance between the outermost sulfurs was monitored, because these reagents cross-link through disulfide exchange reactions (Fig. 4B). In addition, the cross-linker can be cleaved through reducing reagents like dithiothreitol (DTT; Kumar et al. 1991; Hovinen et al. 1993) or other disulfide reductants.

The arene disulfide reagents of series 2 also show striking differences between the cited values and those obtained from this work (Table 2). The implications are significant. For example, Faulstich and coworkers (Kliche et al. 1999) tried to determine the distance between SH1 and SH2 in rabbit skeletal myosin subfragment 1 by monitoring the cross-linking kinetics by using a series of these arene disulfide reagents. The reagent with the fastest kinetics was believed to be the one with the optimal fit. From these kinetic experiments, the authors concluded that the distance between SH1 and SH2 must be >15 Å. The dynamic nature of the cross-linking reagents, however, was not taken into account, nor was the fact that the most frequently observed conformations had distances much shorter than those cited. In addition, there is substantial overlap in the lengths that are easily obtainable in this series of reagents, adding another layer of difficulty. The observed kinetics probably result from both the conformational fluctuations of the cross-linking reagent and the dynamic processes of the protein. When highly flexible cross-links are used, concrete conclusions about protein structures are difficult.

The distributions of BMB, C-6 disulfide, C-7 disulfide, and C-8 disulfide appear to be a composite of two overlapping Gaussian functions. In all cases, one of the modes is much higher than the other, and this mode is what is reported in the tabulated data. All other statistics take into account the full distribution of data. The existence of the second mode can be understood by examining the confor-

Table 4. *Series 4: NH_3 reactive N-hydroxysuccinimide esters*

Cross-linking reagent	Average N-N distance	Standard deviation	Mode	Median N-N distance	Other major modes	Range of N-N distances	Cited N-N distance[a]
DSG	6.22	0.68	6.4	6.35	—	3.12–7.49	7.7
DSP	8.04	1.08	8.1	8.11	—	4.63–10.65	12.0
DSS	8.88	1.20	9.2	9.16	—	5.58–11.42	11.4
DST	4.06	0.62	3.6	4.03	—	2.45–5.84	6.4
EGS	9.11	2.31	9.8	9.40	—	3.23–14.79	16.1
BSOCOES	10.62	0.97	10.7	10.72	11.6	6.81–12.43	13.0

(DSG) disuccinimidyl glutarate; (DSP) dithiobis(succinimidylpropionate); (DSS) disuccinimidyl suberate; (DST) disuccimimidyl tartarate; (EGS) ethyleneglycol bis-(succinimidylsuccinate); (BSOCOES) bis(2-[succinimidooxycarbonyloxy]ethyl)sulfone.
[a] See reference Pierce Chemicals (1999).

mations of the reagent that compose the lesser mode. For example, in BMB (Fig. 5) the mode at 12 Å represents the case in which there are two gauche N-C-C-C conformations, one g^+ and the other g^-. The minor mode at 8 Å, however, is the case in which both of these are g^+, and the terminal groups are closer together. Some of the sparsely populated distances between two modes represent cases in which there may be one gauche and one or two anticonformations. Similar situations exist for the C-7 and C-8 arene disulfide (Fig. 6A) molecules, except that there is a relatively narrow distribution overlapping with a very broad one. This is not surprising, because as the molecule becomes more flexible, there are many more possible conformations that can be populated without a large energy penalty. The mode in both of these cases represents conformations that have two gauche carbon–carbon bonds.

The C-6 arene bis-disulfide (Figure 6B) shows some of the features of the longer arene disulfides but has a notable narrow distribution with a mode at 12.2 Å. The mode with the longer distance represents a molecule in which both of the C-S-S-C dihedral angles are ~90°. The most common length, on the other hand, results from one of the C-C bonds being in a gauche conformation in addition to the two C-S-S-C dihedral angles being ~90°. An example of one of these conformations is shown in Figure 6C.

Series 3, shown in Figure 7 and Table 3, represents the imidoester class of cross-linking reagents. This class of reagents shows considerable selectivity for primary amines and has very little cross-reactivity with other possible nucleophiles in the protein. The imidoester reacts with an amine to form an amidine linkage that carries a positive charge at physiological pH (Kiehm and Ji 1977; Wilbur 1992). The N(amidine) to N(amidine) distance—resulting from displacement of methoxide by methyl amine—was used to determine the cross-linking span. The reagents studied were dimethyl adipimidate (DMA; e.g., see Hartman and Wold 1967; Niehaus and Wold 1970; Wang and Kassell 1974; Yu and Carter 1976; Pennathur-Das et al. 1982; Wasylewska et al. 1987; Garlick et al. 1992; Erarslan and Ertan 1995; Rappsilber et al. 2000), dimethyl pimelimidiate (DMP; e.g., see Cohlberg et al. 1972; Davies and Kaplan 1972; Hitchcock 1975; Sinha and Brew 1981; Koga 1987; Bar-Peled and Raikhel 1996), dimethyl suberimidate (DMS; e.g., see Shoshan-Barmatz et al. 1995; Gotte et al. 1997; Vanhoutte and Malaisse 1997; Watty et al. 1997, 1998), and dimethyl 3,3′-dithiobis-propionimidate (DTBP; e.g., see Lloyd and Weitzman 1987; Stros and Kolibalova 1987; Shivdasani and Thomas 1988; Cornell 1989; Dubey et al. 1989; Erarslan and Ertan 1995).

A fourth class (Fig. 8; Table 4) represents the amine-reactive *N*-hydroxysuccinimide esters. The esters react with amine functionality on the protein to form stable amide bonds and release two molecules of *N*-hydroxysuccinimide (NHS). The esters tend to react with the α-amines at the N terminus and the ε-amines of lysine residues. It is also possible for these reagents to react with sulfhydryl and hydroxyl groups, but this does not lead to stable adduct formation. The cross-linking spans of disuccinimidyl glutarate (DSG; e.g., see Eisman and Schnaare 1996; Horiguchi et al. 1997; Pan et al. 1998; Holwerda 1999; Xu et al. 1999; Korsgren et al. 2000), dithiobis(succinimidylpropionate) (DSP; e.g., see Walleczek et al. 1989; Meunier et al. 1991; Poruchynsky and Atkinson 1991; Jansson et al. 1996; Stout and Kirley 1996; Tanaka et al. 1996; Carl et al. 1998), disuccinimidyl suberate (DSS; e.g., see Chain and Malkin 1991; Pliszka 1993; Persson and Ezban 1994; Loester et al. 1995; Stout and Kirley 1996; Carl et al. 1998; Leonard et al. 1998), disuccimimidyl tartarate (DST; e.g., see Bragg and Hou 1986; Farries et al. 1988; Mita et al. 1989; Miyazaki et al. 1990; Plouet and Moukadiri 1990; Amiranoff and Lori-

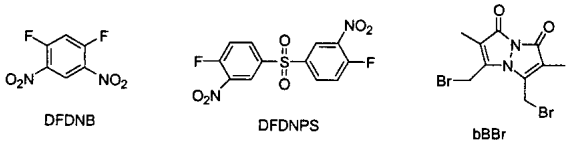

Fig. 9. The small rigid cross-linking reagents. (DFDNB) 1,5-difluro-2,4-dinitrobenzene; (DFDNPS) 4,4′-difluoro3,3′-dinitrodiphenylsulfone; (bBBr) dibromobimane.

Table 5. *Series 5 miscellaneous rigid cross-linking reagents*

Cross-linking reagent	Average distance	Standard deviation	Mode	Median distance	Other major modes	Range of distances	Cited distance[a]
DFDNB	4.91	0.07	4.9	4.91	—	4.73–5.11	3.0
DFDNPS	9.74	0.33	9.7	9.75	—	8.28–10.64	3.0
bBBr	4.88	0.57	5.0	4.99	3.6	3.17–6.61	

(DFDNB) 1,5-difluro-2,4-dinitrobenzene; (DFDNPS) 4,4′-difluoro3,3′-dinitrodiphenylsulfone; (bBBr) dibromobimane.
[a] See reference Pierce Chemicals (1999).

net-Laburthe 1991; Chen et al. 1992; Maman et al. 1994; Horiguchi et al. 1997), ethyleneglycol bis-(succinimidylsuccinate) (EGS; e.g., see Huey and Hugli 1985; Bladon et al. 1989; Caplow and Shanks 1990; Geisler et al. 1992), and bis(2-[succinimidooxycarbonyloxy]ethyl)sulfone (BSOCOES; e.g., see Smith et al. 1986; Svoboda et al. 1988a, 1988b; Schoffelmeer et al. 1989; Fujioka et al. 1990) were studied. In this series, methyl amine was again added to both ends, and the N(amide) to N(amide) bond distance was monitored.

The cited distances for all of the molecules studied in this series are outside the range of distance found through our calculations. Minimization of the fully extended conformations of these molecules with AMBER and measurement of the maximum achievable distances reveal that the longer distances cited could be obtained only by introducing considerable angle strain into the linker.

The final group (Fig. 9; Table 5) consists of three short and nearly rigid cross-linking reagents: 1,5-difluro-2,4-dinitrobenzene (DFDNB; e.g., see Mayeux et al. 1991; Herzig et al. 1995; Krupenko et al. 1995; Shoshan-Barmatz et al. 1995; Herzig et al. 1996), 4,4′-difluoro3,3′-dinitrodiphenylsulfone (DFDNPS; e.g., see Givol 1969; Modesto and Pesce 1971; Hsia et al. 1984), and dibromobimane (bBBr; e.g., see Kim and Raines 1995; Bhattacharjee and Rosen 1996; Wu et al. 1996; Loo and Clarke 1997, 1999; Konno et al. 2000). The first two are amine reactive through nucleophilic aromatic substitution to give aryl amines. Reactions with other nucleophiles (such as sulfhydryls, imidazoles, and phenolates) are possible, but these are reversible. In these two cases, the resultant aryl amine nitrogen to aryl amine nitrogen was measured for each conformation. The various cross-linking spans result mainly from molecular vibrations because the reagents are basically rigid. Of greatest interest is that both reagents have cross-linking spans significantly greater than the 3 Å cited in the literature. Nevertheless, DFDNB and bBBr are the reagents of choice among the large group of thiol cross-linking reagents (Tables 1 and 2) for bridging cysteine residues over relatively short distances (~5 Å).

Dibromobimane reacts mainly with thiol groups in an S_N2 manner to yield thioether linkages. As before, the cross-linking span was determined from the sulfur to sulfur distance resulting from nucleophilic displacement of the bromides by methyl thiolate. The various conformers in this case result from rotations about the resultant sulfur–carbon bonds.

Conclusions

The most striking result of this work is that the distances cited for many commonly used protein cross-linkers are highly improbable. In other cases, our calculations show that the linker can achieve a broader range of end-to-end distances than normally has been recognized in the literature.

In most cases, the cross-linking distances cited previously in the literature were obtained by measuring the distances between the two reactive groups in a fully extended conformation (Pierce Chemical Company, pers. comm.)—even in polyethylene glycol molecules $BM[PEO]_3$ and $BM[PEO]_4$ that are known to prefer gauche conformations; these cited values assume that there is only one significantly populated conformation of the molecule in solution. In fact, even in mostly rigid molecules there are several different conformations (with differing cross-linking spans) that are easily accessible in solution. In only two of the 32 cases studied was the average distance obtained from the dynamics simulation within 0.5 Å of the cited distance. In all other cases, the most populated states had lengths that were considerably different from the fully extended conformation. In addition, the literature value was only minimally populated.

The distances obtained from these dynamics simulations give more realistic lengths for these molecular rulers. We recommend the use of the statistical average plus or minus the standard deviation as a new measure of these lengths. The new distances should be a useful tool for studies of protein structure and function and should provide guidelines for the appropriate choice of cross-linking reagents.

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