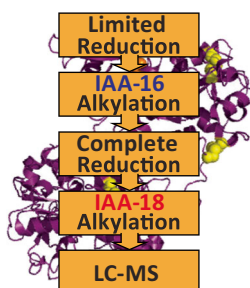


Identification of Reduction-Susceptible Disulfide Bonds in Transferrin by Differential Alkylation Using O¹⁶/O¹⁸ Labeled Iodoacetic Acid

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Abstract. Stabilization of native three-dimensional structure has been considered for decades to be the main function of disulfide bonds in proteins. More recently, it was becoming increasingly clear that in addition to this static role, disulfide bonds are also important for many other aspects of protein behavior, such as regulating protein function in a redox-sensitive fashion. Dynamic disulfide bonds can be taken advantage of as candidate anchor sites for site-specific modification (such as PEGylation of conjugation to a drug molecule), but are also frequently implicated in protein aggregation (through disulfide bond scrambling leading to formation of intermolecular covalent linkages). A common feature of all these labile disulfide bonds is their high susceptibility to reduction, as they need to be selectively regulated by either specific

local redox conditions *in vivo* or well-controlled experimental conditions *in vitro*. The ability to identify labile disulfide bonds in a cysteine-rich protein can be extremely beneficial for a variety of tasks ranging from understanding the mechanistic aspects of protein function to identification of troublesome “hot spots” in biopharmaceutical products. Herein, we describe a mass spectrometry (MS)-based method for reliable identification of labile disulfide bonds, which consists of limited reduction, differential alkylation with an O¹⁸-labeled reagent, and LC-MS/MS analysis. Application of this method to a cysteine-rich protein transferrin allows the majority of its native disulfide bonds to be measured for their reduction susceptibility, which appears to reflect both solvent accessibility and bond strain energy.

Keywords: Protein structure, Disulfide bonds, Disulfide reduction, Cysteine alkylation, Stable isotope, Isotope labeling

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Introduction

It is widely accepted that disulfide bonds play a significant role in maintaining and stabilizing the three-dimensional structure of proteins, primarily by decreasing the entropy of the unfolded state [1]. Those special covalent bonds are frequently found in extracellular proteins in which they can greatly promote the resistance of the proteins to proteolysis and denaturation in the harsh extracellular environment. More recently, it was realized that in addition to the structural role, some disulfide bonds also play an essential role in regulating molecular functions [2–4]. For example, catalytic disulfide

bonds represent a unique group of disulfide bonds that catalyze formation, reduction, and isomerization of other disulfide bonds in a protein substrate [5, 6]. Furthermore, some other disulfide bonds can regulate protein function allosterically by triggering a conformational change upon their reduction [7, 8]. Although most disulfide bonds are buried inside the protein core because of their hydrophobicity, a common feature of functional disulfide bonds is reduction susceptibility, as they need to be sensitive to the changes in redox conditions in order to switch between bonded and unbonded states.

In addition, reduction-susceptible disulfide bonds are frequently found to be responsible for the formation of protein aggregates via intermolecular disulfide scrambling. This raises a great concern regarding the safety of protein therapeutics due to correlation between the aggregation propensity and increased immunogenicity [9, 10]. For example, the dimerization of IgG2 molecule was reported to be a result of disulfide bond scrambling at the hinge region, where the disulfide bonds are

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extremely labile [11, 12]. Besides, the reduction-susceptible disulfide bonds are often hot spots for intramolecular disulfide bond scrambling, leading to the formation of non-native disulfide bonds [13].

Reduction-susceptible disulfide bonds have also been exploited to achieve site-specific modification of proteins. Several recent studies have described a novel disulfide-based protein modification approach in which the most susceptible disulfide bond in a protein is selectively reduced and followed by *bis*-alkylation to insert the modification reagent (e.g., activated PEG) [14–16]. Using mild reduction conditions, both the location and the number of modification can be controlled, making it an attractive alternative to traditional amine-based modification methods, in which almost all the solvent-accessible Lys residues will be modified to different extents [17]. In addition, as the reduced disulfide bond is re-bridged during *bis*-alkylation, the protein's tertiary structure is frequently maintained and the biological activity is mostly preserved [16]. Particularly, with the ever growing number of antibody-drug conjugates (ADCs), this precisely controlled modification approach is very attractive and is already enjoying notable popularity in this field [18].

Reduction-susceptible disulfide bonds represent a unique group of disulfide bonds that are extremely active both *in vivo* and *in vitro*. Thus, identification of these disulfide bonds in a protein is highly desirable. Generally, it is safe to assume that reduction-susceptible disulfide bonds are frequently located at the surface of a protein so that they are accessible to reducing agents. Using the crystal structure of a protein, the surface accessibility of each disulfide bond can be easily measured and its relative susceptibility can be estimated. More recently, a computational approach was developed for identification of solvent-accessible disulfide bonds using published structural information [19]. Despite the ease of using such models, it is important to emphasize that the static image generated by X-ray crystallography cannot fully represent the dynamic properties of a protein in solution. In addition to solvent accessibility, other factors also contribute to the reduction susceptibility of disulfide bonds. For example, thermally unstable disulfide bonds that possess high dihedral strain energy frequently show greater susceptibility than others [3, 20]. Furthermore, the presence of positively charged residues can also promote the susceptibility of its nearby disulfide bonds by providing an electrostatically favorable environment with increased local concentration of thiolated anion [21]. Mechanical force was recently introduced as another factor to affect the susceptibility of disulfide bonds, although those studies were currently limited to engineered disulfide bonds [22, 23].

The existence of multiple factors complicates the identification of reduction-susceptible disulfide bonds. X-ray crystallography has proven to be effective in identifying solvent-accessible disulfide bonds and even estimate the dihedral strain energy (DSE) using high-resolution structure data [3]. However, prediction of disulfide bond susceptibility based on crystal structure is not always reliable. For proteins with no crystal structure available, the challenge to identify susceptible

disulfide bonds is further amplified. Fortunately, the rapid development of mass spectrometry (MS)-based techniques provides a powerful tool to tackle this challenging task. Particularly, applying a differential alkylation strategy with stable isotope-labeled alkylation reagents, MS-based methods have been developed to investigate the cysteine oxidation status [24]. More recently, a similar approach was applied to rank the susceptibility of disulfide bonds in human IgG1 antibodies [25]. In this study, we propose a MS-based method consisting of limited reduction, differential alkylation, and LC-MS/MS analysis for the identification of reduction-susceptible disulfide bonds. The O¹⁶- and O¹⁸-labeled iodoacetic acid was used for differential alkylation, and the latter can be easily prepared *in-house* using iodoacetic acid and O¹⁸-enriched water [26].

Human serum transferrin (Tf), an 80 kDa iron-binding protein with 19 disulfide bonds, is used as the model to demonstrate the feasibility of the new method. The capability of Tf to deliver iron into cells or across the physiological barriers via transferrin receptor (TfR)-mediated endocytosis/transcytosis has been widely explored to achieve targeted drug delivery [27, 28]. Indeed, Tf has been a part of a number of biopharmaceutical products that are currently under development [29]. Identification of reduction-susceptible disulfide bonds in Tf could greatly benefit the on-going efforts to develop Tf-based therapeutics from different aspects. First, with the ever growing number of Tf-drug conjugates [30–33], the susceptible disulfide bond in Tf provides a potential drug conjugation site that can be precisely controlled. Furthermore, from the quality control perspective, identification of reduction-susceptible disulfide bonds could also reveal the potential scrambling sites so that they can be closely monitored. Finally, the possible functional role of disulfide bonds in Tf or in Tf-based iron-delivery pathway has rarely been explored. Identification of reduction-susceptible disulfide bonds could certainly facilitate these on-going efforts.

Experimental

Non-glycosylated human serum Tf was provided by Professor Anne B. Mason (University of Vermont College of Medicine, Burlington, VT); H₂O¹⁸ (97% purity), iodoacetic acid (IAA), dithiothreitol (DTT), and proteomic-grade trypsin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used in this work were of analytical grade or higher. O¹⁸-labeled iodoacetic acid (IAA-18) solution was prepared using a previously reported protocol [26].

The working solution of Tf (9.5 μM) was prepared in 20 μL of 50 mM potassium phosphate buffer at pH 7.4. The limited reduction was initiated by the addition of DTT to a concentration of 10 mM, and the sample was incubated at 37°C for 5 min. Subsequently, the reduction was quenched by a 25-fold dilution using the alkylation solution consisted of 10 mM of O¹⁶-labeled iodoacetic acid (IAA-16) and 6 M of guanidine hydrochloride at pH 8.0. The alkylation of reduced cysteine

residues was achieved by incubating the solution at 37°C for 30 min in the dark. Subsequently, the excess of IAA-16 was removed by three times buffer exchange using the denaturing buffer (50 mM of potassium phosphate, 6 M of guanidine hydrochloride, pH 8.0), using a Vivaspin sample concentrator with 10 kDa MWCO membrane. The complete reduction was achieved by the addition of DTT to a concentration of 10 mM and incubation at 37°C for 30 min. Finally, all the reduced cysteine residues were alkylated with 30 mM of IAA-18 at 37°C for 30 min in the dark, and the tryptic digestion was performed at an enzyme/substrate ratio of 1:40 at 37°C overnight. The entire workflow is presented in Figure 1.

The tryptic digests of Tf were analyzed by nanoLC-MS/MS using an LC Packings Ultimate (Dionex/Thermo Fisher Scientific, Sunnyvale, CA, USA) nano-HPLC system coupled with a QStar-XL (AB SCIEX, Toronto, Canada) hybrid quadrupole/TOF MS system. A previously reported instrument set-up and method were applied for the nanoLC-MS/MS analysis [34].

Results and Discussion

The IAA-18 solution (500 mM) can be readily prepared by dissolving 80.0 mg of iodoacetic acid (IAA) in 495 μ L of O¹⁸-enriched water (ca. 97% H₂O¹⁸) and incubating in the presence of 1% trifluoroacetic acid at 50°C for 1 d [26]. At low pH, the two carboxylic oxygen atoms in IAA quickly exchange with O¹⁸ atoms in O¹⁸-enriched water, leading to a mass increase of 4 Da. Compared with C¹³-labeled alkylation reagents that are commercially available and more frequently used [25, 35], IAA-18 provides larger mass shift (4 Da) between differentially alkylated peptides, which is beneficial for simple quantitation. Analysis of a Cys-containing peptide from IAA-18 alkylated Tf showed that only less than 1% of peptides did not incorporate any O¹⁸ atom (black trace in Figure 2a). Although the completeness of the labeling is limited by the purity of the H₂O¹⁸, the ratio of fully labeled products (+4 Da) to partially labeled products (+2 Da) is always constant among all the Cys-containing peptides (data not shown), as the label is introduced during the alkylation step [26]. Based on the isotopic distribution of the Cys-containing peptide, the reduction level of the disulfide bond under native conditions can be estimated using the following equation:

$$\text{Reduction (\%)} = \frac{I_1}{I_5 \cdot (1 + \alpha)} \quad (1)$$

where α ($\alpha=0.14$ in this work, and is only affected by the purity

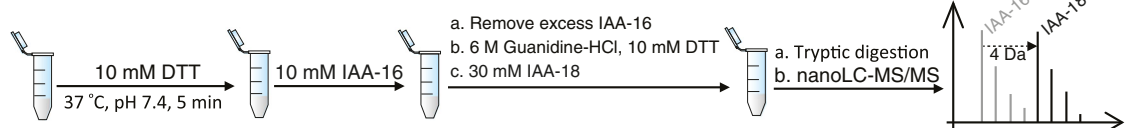


Figure 1. A schematic presentation of the experimental procedure

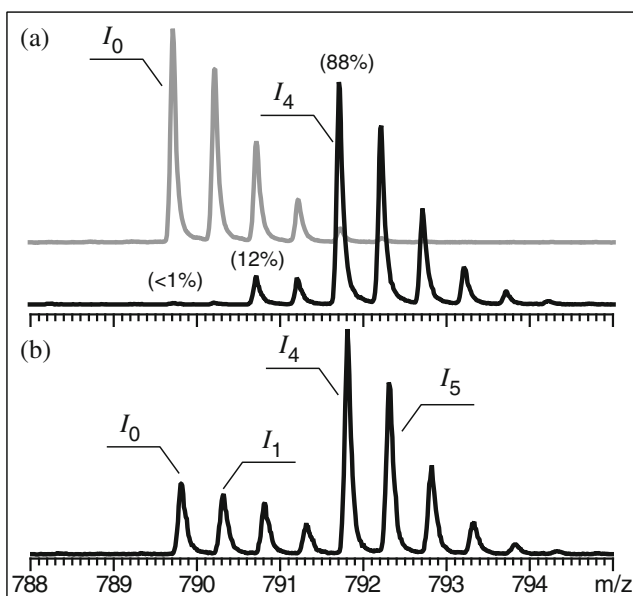


Figure 2. Isotopic distribution of a doubly charged peptide ion FDEEFSEGC*APGSK alkylated by either IAA-16 (grey trace) or IAA-18 (black trace) (a), and isotopic distribution of this peptide differentially alkylated by IAA-16 and IAA-18 after 5 min of reduction by 10 mM DTT under native conditions (b)

of H₂O¹⁸) is the fraction of partially labeled peptides [26]; and I₁ and I₅ represent the observed relative intensities of the second isotopic peaks for the O¹⁶-labeled peptides and fully O¹⁸-labeled peptides, respectively (Figure 2b).

It is assumed that by reduction of a disulfide bond with DTT, the two participating cysteine residues are converted to free sulfhydryls simultaneously and are subsequently alkylated by IAA-16. Thus, the extent of a disulfide bond reduction could be calculated from the isotopic distribution of either one of the two differentially alkylated cysteine residues. This is confirmed by analyzing the O¹⁸ content for six pairs of cysteine residues, which form disulfide bonds in Tf (see Figure 3). The ability to base the measurements of the extent of a disulfide reduction on the O¹⁸ content of a single participating cysteine residue is very useful vis-a-vis improving the coverage of disulfide bonds, as it is not uncommon for some cysteine-containing tryptic peptides to elude detection by LC-MS because of poor recovery or inadequate size. In addition, the comparable reduction levels from the two pairing cysteine residues also demonstrated that no significant scrambling event occurred during the limited reduction step.

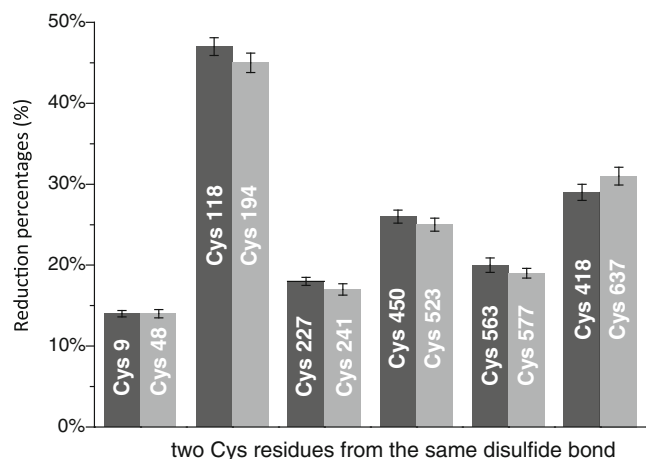


Figure 3. Comparison of the reduction levels of several disulfide bonds calculated using both participating cysteine residues in a pair (shown in black and grey). The error bars represent standard deviation from three replicate measurements

Although it is relatively straightforward to calculate the extent of reduction of a disulfide bond when proteolytic peptides do not contain more than one cysteine residue, this task becomes significantly more challenging when multiple cysteine residues are present in a single peptide. An example is shown in Figure 4, where isotopic distribution of peptide ion EGTC*PEAPTDEC*KPVK is very convoluted because of the presence of two differentially alkylated cysteine residues. The reduction levels of both disulfide bonds could be readily calculated using other peptides containing matching cysteine residues (SAGWNIPIGLLYC*DLPEPR and APNHAVVTRKDKEAC*VHK). However, should either of these two peptides fail to provide detectable ionic signal, the convoluted isotopic distribution of the peptide EGTC*PEAPTDEC*KPVK would become essential for the

analysis of the extent of reduction of the disulfide bonds. For example, if peptide APNHAVVTRKDKEAC*VHK cannot be detected by LC-MS because of poor recovery, the reduction level of the second disulfide bond can still be calculated using the equations described in Figure 4. The derivation of the equations is described in [Supplementary Material](#).

The approach presented above would fail if none of the peptides containing a single cysteine residue (SAGWNIPIGLLYC*DLPEPR or APNHAVVTRKDKEAC*VHK) were available for analysis. In this situation, deconvolution of the isotopic distribution of C*STSSLLEAC*TFR to determine the O¹⁸ content of each alkylated thiol group would require the use of tandem mass spectrometry. As shown in Figure 5c, the O¹⁸ content of the cysteine residue in the C-terminal part of the peptide (and hence the reduction level of the corresponding disulfide bond) could be calculated based on the isotopic distribution of CID-generated γ -fragments, which incorporate that residue, but not the other cysteine (e.g., γ_7^+). The O¹⁸ content of the cysteine residue in the N-terminal half of this peptide can be subsequently determined using the strategy described above. It is worth noting that in order to achieve reliable quantitation based on the tandem mass spectra, it is critically important to ensure that both the efficiency of mass isolation and fragmentation for the two differentially alkylated peptide ions be identical. Although the fragmentation efficiency of the two peptide ions is unlikely to be sensitive to the presence of O¹⁸ isotopes [36], avoiding a bias during the mass isolation step requires that a relatively broad mass selection window be used. A control experiment where very low collision energy (5 eV) was used confirmed the absence of the mass-bias during the isolation step of ions representing differentially alkylated peptides (see Figure 5b).

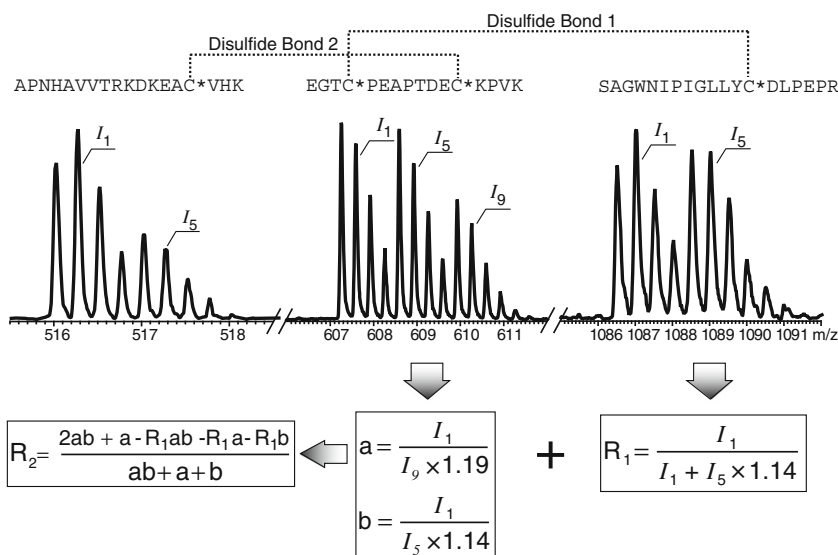


Figure 4. Isotopic distributions of three tryptic peptides that form two adjacent disulfide bonds. The equations describe the calculation of reduction level of one disulfide bond using isotopic distributions of two peptides; R_1 and R_2 represent the reduction levels of disulfide bond 1 and disulfide bond 2, respectively

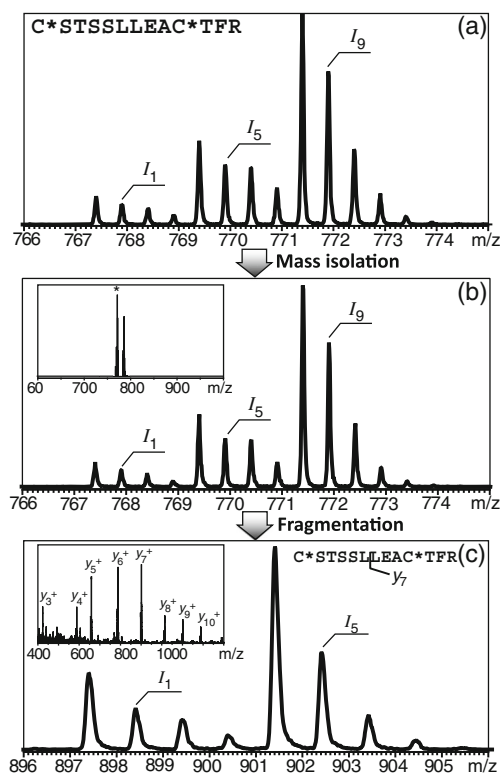


Figure 5. Measurement of O¹⁸ content for two cysteine residues in peptide C*STSSLLEAC*TFR using tandem MS. (a) The isotopic distribution of differentially alkylated peptide C*STSSLLEAC*TFR. (b) Mass isolation of the whole isotopic cluster to the first quadrupole using ‘low resolution mass selection’ setting and low collision energy. (c) The isotopic distribution of fragment ion y_7^+ from differentially alkylated peptide C*STSSLLEAC*TFR

Using the above described approaches, reduction susceptibility of 15 disulfide bonds in Tf have been measured and are shown in Table 1 (the remaining four disulfide bonds could not

be detected when trypsin was used to digest Tf; if a complete coverage of all disulfide bonds is desired, other proteolytic enzymes would have to be used in parallel to produce complementary sets of fragment peptides). The 15 disulfide bonds whose reduction susceptibility has been characterized exhibit a wide range of lability, showing reduction levels from 8% to 60% after 5 min of reduction by 10 mM DTT under native conditions. In order to correlate the susceptibility with its contributing factors, those disulfide bonds were also assessed for the solvent-accessible surface areas (SASA), the secondary structural features as well as the dihedral strain energies (DSE) [37]. The recently solved crystal structure of holo-human Tf (PDB ID 8v83) was used for these analyses [38]. As shown in Table 1, the solvent accessibility of a disulfide bond plays an important role in determining its susceptibility to reduction. Disulfide bonds in which at least one participating cysteine residue is completely sequestered from the solvent in the protein interior (such as Cys9-Cys48, Cys19-Cys39, Cys227-241, Cys355-368, Cys450-Cys523, Cys484-Cys498, and Cys563-577) showed higher resistance to reduction under native conditions (less than 20% reduced in 5 min). All other disulfide bonds (for which the crystal structure shows at least some solvent accessibility) were found to be more susceptible to reduction with DTT. In addition to solvent exposure, the secondary structure in the immediate environment of the cysteine residues also exerts significant influence of the reduction susceptibility of the corresponding disulfide bond. For example, disulfide bonds connecting α -helices or β -strands (Cys9-Cys48, Cys19-Cys39, and Cys355-Cys368) were found to be more resistant to reduction compared with disulfide bonds, which connect flexible loop regions (Cys137-Cys331 and Cys339-Cys596). In fact, disulfide bonds located in the flexible regions are more susceptible to reduction, even if their solvent accessibility is relatively low according to the crystal structure (we note that SASA calculations for residues located within highly dynamic regions should be taken with a certain degree

Table 1. Structural Analysis of Disulfide Bonds in Human Tf

Cys-1	Cys-1 secondary structure	Cys-1 SASA ^a (Å ²)	Cys-2	Cys-2 secondary structure	Cys-2 SASA (Å ²)	DSE ^b (KJ/mol)	Reduction level ^c
9	Extended strand	0	48	Alpha helix	0	13.0	11.2±1.3% ^d
19	Alpha helix	0	39	Strand	38	11.8	8.6±1.1%
118	Strand	0	194	Alpha helix	0	16.3	35.8±0.6%
227	Loops or irregular	2	241	Bend	1	14.6	11.6±0.7%
137	3 ₁₀ Helix	47	331	Loops or irregular	44	15.7	43.8%±1.2%
339	Loops or irregular	46	596	Alpha helix	3.5	39.5	60.7%±5.0%
345	Strand	1	377	Alpha helix	0	22.7	28.6±1.7%
355	Alpha helix	0	368	Extended strand	19	8.38	15.5±1.6%
402	Hydrogen bonded turn	16	674	Alpha helix	26	5.64	21.0±1.5%
418	3-Helix (3 ₁₀ helix)	11	637	Extended strand	7.2	14.7	26.1±0.7%
450	Strand	0	523	Alpha helix	0	16.1	17.0±1.8%
474	Bend	46	665	3 ₁₀ helix	33	13.4	24.8±0.9%
484	Strand	0	498	Loops or irregular	6	8.52	18.3±0.5%
563	Loops or irregular	6	577	Bend	3	21.0	15.1±1.1%
615	Loops or irregular	53	620	Loops or irregular	12	14.3	32.3±3.8%

^aSASA: solvent accessible surface area of Cys residues

^bDSE: dihedral strain energy

^cThe reduction level of each disulfide bond was calculated after 5 min of reduction by 10 mM of DTT under native conditions

^dThe values were determined by three replicate experiments

of skepticism, as the crystal structure of such regions does not reflect the entire volume of the conformational space available to proteins in solution and, instead, shows significant bias towards configurations that are favored by the packing forces during the crystallization process).

Lastly, the geometry of a disulfide bond determines its potential energy, known as dihedral strain energy (DSE), which also influences the reduction susceptibility. Using the five successive χ angles, the strain energy of a disulfide bond can be estimated by the following empirical formula [39]:

$$\begin{aligned} \text{DSE (kJ} \cdot \text{mol}^{-1}) &= 8.37(1 + \cos 3\chi_1) + 8.37(1 + \cos 3\chi_1') \\ &+ 4.18(1 + \cos 3\chi_2) + 4.18(1 + \cos 3\chi_2') \\ &+ 14.64(1 + \cos 2\chi_3) + 2.51(1 + \cos 3\chi_3) \end{aligned} \quad (2)$$

where χ_1 and χ_1' are the dihedral angles of the two C $_{\alpha}$ -C $_{\beta}$ bonds, χ_2 and χ_2' are the dihedral angles of the two C $_{\beta}$ -S $_{\gamma}$ bonds, and χ_3 is the dihedral angle of the S $_{\gamma}$ -S $_{\gamma}$ bond. Based on the crystal structure, the dihedral strain energies of the 15 disulfide bonds were calculated (see Table 1). This analysis revealed two disulfide bonds, Cys345-Cys377 and Cys339-Cys596 (Figure 6), carried exceptionally high strain energy of 22.7 KJ/mol and 39.5 KJ/mol, respectively, compared with a mean value of 14.8 KJ/mol found in a recent study of 6874 unique disulfide bonds [3]. Although completely buried inside, disulfide bond Cys345-Cys377 in the C-lobe of Tf exhibited unusually high susceptibility (29%), particularly when compared with the analogous disulfide bond Cys9-Cys48 (11%) in the homologous N-lobe. Despite the similar solvent accessibility and secondary structure, disulfide bond Cys345-Cys377

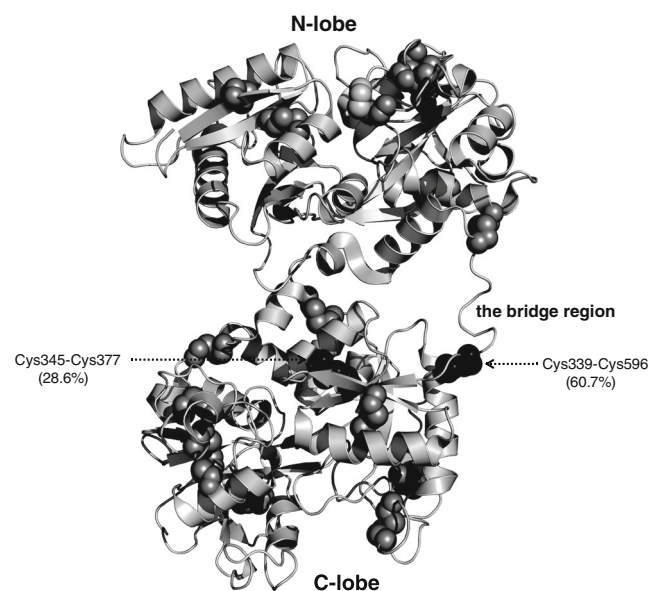


Figure 6. Crystal structure of the diferric form of human Tf (PDB 3V83) showing all disulfide bonds in spheres. Disulfide bonds Cys339-Cys596 and Cys345-Cys377 are shown as black spheres

was nearly three times more susceptible than disulfide bond Cys9-Cys48. This discrepancy is likely attributed to the difference in dihedral strain energy of the two disulfide bonds (Cys9-Cys48: 13.0 KJ/mol and Cys345-Cys377: 22.7 KJ/mol).

Another important observation concerns the highly reduction susceptible disulfide bond Cys339-Cys596, which exhibited the highest extent of reduction (>60%) following a 5 min exposure to DTT (due to a combination of high solvent accessibility for participating cysteine residues and unusually high strain energy of 39.5 KJ/mol). This disulfide bond is located in the inter-lobe bridge region and is unique to serum transferrin (but is not found in other members of transferrin family, such as lactoferrin and ovotransferrin). However, unlike the inter-lobe bridge region in lactoferrin (which adopts a helical conformation), this region in Tf is unstructured; the presence of a disulfide bond in this region is predicted to constrain the relative movements of the N-lobe and C-lobe [40], and the resulting tension may be responsible for the high strain energy of this disulfide bond. Unlike lactoferrin and ovotransferrin, serum Tf not only sequesters and stores iron (protecting it from forming insoluble hydroxide, and also limiting its availability to various pathogens), but also delivers it to cells through the process of receptor-mediated endocytosis. Both lobes of Tf participate in binding to TfR at the cell surface, and the protein is believed to remain bound to TfR throughout the entire endocytotic cycle. However, Tf must undergo a relatively large-scale conformational transition while inside the endosome in order to release iron from a metal binding cleft in each lobe (from the so-called “closed” state to the “open” state), and it is not inconceivable to hypothesize that the reduction-prone disulfide bond reinforcing the connection between the two lobes may play a role in this transition. Endosomal uptake and activation of several proteins are known to be assisted by disulfide reduction [41], although the efficiency and even the occurrence of these processes appears to be dependent on a specific protein [42]. The presence of the inter-lobe disulfide Cys339-Cys596 at neutral pH might be critical for facilitating Tf/TfR association at the cell surface by “fixing” the protein conformation in a state that is recognized by the receptor with highest affinity. At the same time, the anomalous reduction susceptibility of this disulfide might be critical for the ability of Tf to undergo conformational transitions followed by cleavage of this bond inside the endosome, thereby affording the protein more conformational freedom despite the fact that it remains bound to the receptor. This would certainly facilitate transition from the closed to open conformations in each lobe of Tf by reducing the entropic penalty (it is important to note that the protein does exhibit anomalous conformational heterogeneity at endosomal pH despite being complexed to the receptor), which in fact prevents meaningful interpretation of electron density maps even in low-resolution cryo-EM experiments [43].

Conclusions

A new LC-MS/MS-based method has been developed and demonstrated for identification of reduction-susceptible disulfide bonds in proteins using a differential alkylation strategy. This study was carried out under near native conditions, so that it could be more widely applied to other proteins compared with cyanylation-based approach [4]. In addition, the differential alkylation strategy in our approach can provide quantitative assessment of the susceptibility of site-specific disulfide bonds, which cannot be achieved by intact mass-based measurement [44]. Based on the isotopic distributions of cysteine-containing peptides as well as the fragment ions, 15 disulfide bonds in Tf can be measured for their reduction susceptibility. We also discussed various factors that contribute to disulfide bond reduction susceptibility, including solvent accessibility, secondary structure, and dihedral strain energy. Although it remains to be seen whether and/or how the disulfide bonds play a role in Tf endocytosis, the identification of reduction-susceptible disulfide bonds in Tf certainly supports this possibility and reveals the most likely disulfide bonds that might possess a functional role. In addition, a recent study using FRET imaging technique have observed the disulfide bond reduction during the folate receptor-mediated endocytosis and the co-localization with Tf in endosomes [45], suggesting the presence of a reductive capacity during the Tf endocytosis. Despite the debates on the overall redox potential of endosomes [42], evidence exists that disulfide reduction is an important factor in endocytotic uptake of several proteins. In addition to its likely functional importance, the anomalous reduction susceptibility of disulfide bond Cys339–Cys596 could also be explored as a potential derivatization site to achieve site-specific conjugation of drug molecules. Finally, the ability to measure Tf disulfide bonds for their reduction susceptibility will be very useful for the design of robust and efficient analytical protocols to monitor disulfide bond scrambling in stability and quality control studies of transferrin-based therapeutics.

Acknowledgments

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