Protein Structures

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Direct Visualization of Disulfide Bonds through Diselenide Proxies Using ^{77}Se NMR Spectroscopy**

*Mehdi Mobli, Aline Dantas de Araújo, Lynette K. Lambert, Gregory K. Pierens, Monique J. Windley, Graham M. Nicholson, Paul F. Alewood, and Glenn F. King**

Disulfide bridges are a common posttranslational modification in proteins that result from the formation of a covalent bond between the thiol groups of two cysteine residues. Disulfide bridges are important for directing and stabilizing the three-dimensional (3D) structure of proteins[1] and they are often functionally critical.[2,3] Incorrect pairing of disulfide bonds typically leads to a nonnative 3D fold accompanied by a loss of protein function. The importance of this structural motif is highlighted by the fact that 24% of the circa 53000 protein structures deposited in the Protein Data Bank (PDB) contain at least one disulfide bond.

Disulfide bonds are highly prevalent in three important classes of secreted proteins: peptide hormones, growth factors, and peptide toxins excreted in animal venoms.[4] NMR spectroscopy is an ideal technique for determining the 3D structure of these small proteins; of the 4285 structures available in the PDB for proteins smaller than 8 kDa, 71% were determined using NMR methods. However, this raises a conundrum—NMR spectroscopy is a poor method for establishing disulfide-bond connectivities owing to the absence of a sulfur isotope with favorable NMR properties. In some cases, dipolar interactions between the β-methylene protons of covalently linked cysteine residues can be used to infer disulfide bridges,[5] but these connectivities are often ambiguous when multiple disulfide bridges are present.[6,7] Thus, disulfide bonds often constitute a "blind spot" in NMR structural analyses.

One possible solution to this "disulfide blind spot" would be to replace the NMR-inactive ^{32}S nucleus with a nucleus of similar chemical, but more favorable magnetic, properties. Such a replacement occurs naturally, albeit infrequently. Selenocysteine (Sec), often referred to as the 21st amino acid, is a genetically encoded amino acid residue that is identical to cysteine except for the replacement of sulfur with selenium.[8,9] The human selenoproteome comprises 25 selenoproteins.[10] Sec residues can be introduced into proteins by chemical synthesis[11–14] or recombinant expression,[15,16] and replacement of disulfide bonds with diselenide bonds has been demonstrated previously not to alter protein structure or function.[13,14,17]

With a natural abundance of 7.6%, ^{77}Se is an NMR-active $s = 1/2$ nucleus that provides a route for direct determination of disulfide bridge connectivities using NMR scalar couplings. We demonstrate this approach using a 37-residue spider toxin (κ-ACTX-Hv1c) that contains four disulfide bonds, including a rare and functionally critical vicinal disulfide bridge between the adjacent cysteine residues Cys13 and Cys14.[2] Since recombinant expression would lead to replacement of all Cys residues with Sec, for the sake of simplicity we used solid-phase peptide synthesis to produce a toxin in which only Cys13 and Cys14 were replaced with Sec residues (see the Supporting Information).

We determined the 3D solution structure of the [Sec13,Sec14] toxin using homonuclear NMR methods (see the Supporting Information) and found it to be equivalent to that of the native toxin; the two structures can be superimposed over the well-ordered region (residues 1–34) with a backbone root-mean-square deviation (rmsd) of 0.91 Å (Figure 1a). κ-ACTX-Hv1c is a high-affinity blocker of calcium-activated potassium (K_{Ca}) channels in insects, and the vicinal disulfide bond is a key component of the channel binding site.[2,18,19] Figure 1b shows that the [Sec13,Sec14] variant is equipotent with the native toxin in blocking K_{Ca} currents in cockroach neurons (see the Supporting Information). Thus, replacing the functionally critical vicinal disulfide bridge in κ-ACTX-Hv1c with a vicinal diselenide bond does not affect either the folding or function of the protein.

Figure 2 shows that the diselenide connectivities in the [Sec13,Sec14] toxin (which serve as a proxy for the disulfide connectivities) can be unequivocally determined from scalar couplings present in a 2D ^{1}H–^{77}Se heteronuclear multiple bond correlation (HMBC) experiment. The two intense cross-peaks in Figure 2b result from large intraresidue two-bond couplings (ca. 35 Hz) between each pair of Sec methylene protons and the ^{77}Se nucleus of the same residue. The two less intense but clearly visible cross-peaks arise from interresidue three-bond couplings across the diselenide bond between methylene protons and ^{77}Se. This latter coupling, although small (ca. 2 Hz), allows unequivocal assignment of the diselenide bond connectivity.

[*] Dr. M. Mobli, Dr. A. D. de Araújo, Prof. P. F. Alewood, Prof. G. F. King
Institute for Molecular Bioscience, The University of Queensland
St. Lucia QLD 4072 (Australia)
Fax: (+61) 7-3346-2101
E-mail: glenn.king@imb.uq.edu.au
Homepage: http://www.imb.uq.edu.au
Dr. L. K. Lambert, Dr. G. K. Pierens
Centre for Magnetic Resonance, The University of Queensland
St. Lucia QLD 4072 (Australia)
M. J. Windley, Prof. G. M. Nicholson
Department of Medical and Molecular Biosciences
University of Technology, Sydney (Australia)

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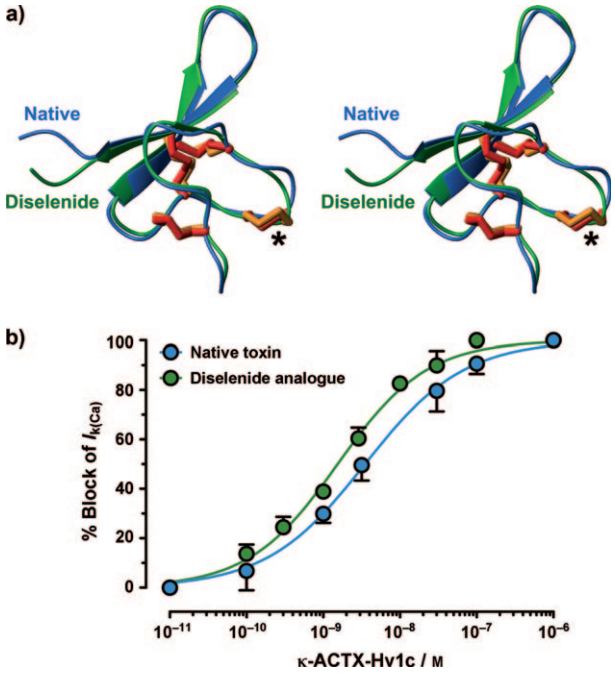

Figure 1. Structure and function of a [Sec13,Sec14] analogue of the spider toxin κ-ACTX-Hv1c. a) Stereoview of the 3D structure of the [Sec13,Sec14] analogue determined in the current study (green backbone with orange disulfide/diselenide bonds) superimposed on the previously determined structure of the native toxin (blue backbone with red disulfide bonds; PDB file 1L0). The lowest-energy structure in each ensemble was used for the overlay. The vicinal disulfide/diselenide bond is highlighted with an asterisk. b) Concentration-dependence of the blocking of K_{Ca} currents in cockroach dorsal unpaired median (DUM) neurons by native κ-ACTX-Hv1c (green circle) and the [Sec13,Sec14] analogue (blue circle). The calculated IC_{50} values were 3.5 ± 1.2 nM and 1.5 ± 0.3 nM, respectively. See the Supporting Information for experimental details.

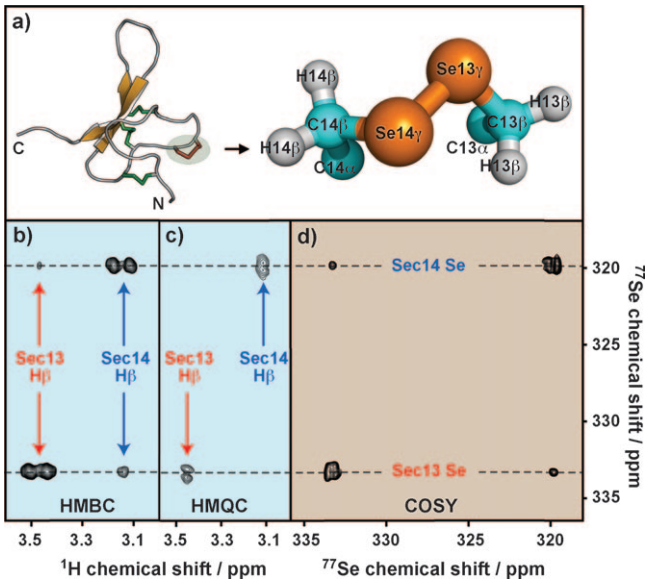

Figure 2. Direct determination of disulfide-bond connectivities using ^{77}Se NMR spectroscopy. a) 3D structure of the [Sec13,Sec14] analogue of κ-ACTX-Hv1c.[2] The three disulfide bonds and single diselenide bond are shown as green and red tubes, respectively. A magnified version of the vicinal diselenide bridge is shown on the right with the NMR-active nuclei labeled. b) ^{1}H–^{77}Se HMBC, c) ^{1}H–^{77}Se HMQC, and d) ^{77}Se–^{77}Se COSY spectra of the [Sec13, Sec14] analogue acquired at 500 MHz. The spectrum shown in (b) was acquired using a natural-abundance sample, whereas the spectra shown in (c) and (d) were acquired using a sample containing ^{77}Se-enriched Sec residues. See the Supporting Information for experimental details.

Additional experiments applicable to larger proteins become possible if the cysteine residues are replaced with Sec enriched with ^{77}Se. This involves incorporation of ^{77}Se-enriched metallic selenium into the appropriate selenocysteine framework for subsequent use in peptide synthesis or recombinant expression. We synthesized a version of κ-ACTX-Hv1c in which Cys13 and Cys14 were replaced with Sec residues enriched with 98% ^{77}Se (see the Supporting Information). This allowed direct observation of diselenide bonds through one-bond ^{77}Se–^{77}Se scalar couplings in a two-dimensional ^{77}Se–^{77}Se COSY experiment (Figure 2d). The connected Sec residues were then assigned by a simple 2D heteronuclear multiple quantum coherence (HMQC) experiment that allowed each ^{77}Se nucleus to be correlated with the β-methylene protons of the same Sec residue (Figure 2c). This NMR approach is the first method to allow direct observation of disulfide bridges in native proteins in solution.

Although the methodology described here is ideally suited to synthetic peptides, it is equally applicable to larger proteins produced using recombinant methods.[20] Sec incorporation can be achieved by growing cysteine auxotrophs of *E. coli*[15,16] or yeast[21] in media enriched with Sec. We anticipate that this new approach will stimulate the study of disulfide bridges in much the same way that the study of hydrogen bonds in proteins has been stimulated by the development of methods for their direct observation using NMR spectroscopy.[22,23]

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