Chapter 15
^{77}Se NMR Spectroscopy of Selenoproteins

Jun Liu and Sharon Rozovsky

Abstract One of the most essential contributions of selenium to biology is the specialized chemistry performed by selenoproteins. Elucidating the mechanisms by which selenoproteins govern the reactivity of their selenocysteine (Sec) requires exploring how the protein environment primes Sec interactions with substrates, prevents inactivation, and otherwise optimizes the use of this unique amino acid. ^{77}Se nuclear magnetic resonance (NMR) spectroscopy is a particularly powerful technique to study the chemical properties of selenocysteine, its conformational preferences and mobility, and the molecular interactions by which it is stabilized. Recent advances have simplified sample preparation and data analysis, extending the utilization of ^{77}Se in NMR studies of biological samples. These improvements include the development of efficient procedures for enriching proteins with the ^{77}Se isotope, the reports on NMR parameters of different selenoproteins that greatly expand the available basis for data analysis, and the progress in utilizing theoretical calculations for data interpretation. We discuss these areas of progress in ^{77}Se NMR of biological systems, and we consider the range of questions for which ^{77}Se NMR is most useful.

Keywords ^{77}Se NMR • Selenium NMR • Selenocysteine • Selenocystine • Selenopeptides • Selenoproteins • Selenoredox motifs

15.1 NMR Spectroscopy of Biological Macromolecules

NMR spectroscopy is a superb probe of the molecular environment and an unparalleled tool for understanding its electronic and chemical structure. It is routinely used to study the atomic structures, conformational mobility, and supramolecular organization of biological macromolecules. In addition, it can characterize their interactions with ligands, drugs and protein partners. Routine biological NMR relies on the nuclei ^{1}H, ^{13}C, ^{15}N, and ^{31}P, whose behavior in different environments is extensively documented. Theoretical calculations allow the NMR observables of these nuclei in

J. Liu • S. Rozovsky (✉)
Department of Chemistry and Biochemistry, University of Delaware,
Newark, DE 19716, USA
e-mail: rozovsky@udel.edu

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different chemical environments to be predicted, and their relation to the structural and functional properties of macromolecules to be elucidated [1].

Biological NMR utilizes a variety of other, less ubiquitous nuclei (for example, metals) that yield rich information about function. Likewise, ^{77}Se NMR is employed to study selenoproteins, since the chemistry centers on selenium's high reactivity and thus it is advantageous to directly probe it. Furthermore, ^{77}Se NMR is used as a spectroscopic surrogate of sulfur because the only NMR-sensitive isotope of sulfur, ^{33}S, is a low-sensitivity quadrupolar nucleus that cannot be utilized to study biological systems. This chapter focuses mostly on recent progress in the studies of selenoproteins because the fundamentals and historic perspectives of ^{77}Se NMR of biological systems have been summarized in a recent review [2].

15.2 Selenium's NMR Properties

Nuclear spin is an intrinsic property of the nucleus that must be larger than zero for the nucleus to interact with a magnetic field and be detected by NMR spectroscopy. Among selenium's six naturally occurring isotopes, only ^{77}Se, whose nuclear spin quantum number is ½, is NMR-active [3]. In general, the detection sensitivity is higher for spin ½ nuclei than for nuclei with larger spin numbers and their spectra are simpler to record and understand [4]. It is therefore fortuitous that ^{77}Se NMR is spin ½ and, like proton, carbon and nitrogen, can be readily detected with routine hardware and pulse sequences.

NMR is sensitive to the electronic structure because the local magnetic field near the observed nucleus reflects the interactions between the electron cloud and the external magnetic field [5, 6]. This results in the chemical shifts that report not only on covalent bonds, but also on non-covalent interactions, solvent structures, nearby charges, and other forces that influence the electron cloud [7]. Selenium has more electrons than carbon and nitrogen and those are held further away from the nucleus and occupy more orbitals. Thus, the external (spectrometer) magnetic field exerts a more extensive effect on its electron cloud. Therefore, while the chemical shift range for ^{13}C spectra in diamagnetic samples is ca. 300 ppm, the range for ^{77}Se spectra is over 3000 ppm (Fig. 15.1). This large range reflects ^{77}Se NMR's high sensitivity to the local environment, making it an excellent spectroscopic reporter on bonding, geometry, and electronic structure [2, 8–10]. However, this sensitivity comes at a price, as the large response of the electron cloud to the external magnetic field leads to broad resonances. These line widths, ranging from 50 to 500 Hz, depending on the intrinsic mobility of the specific site and the strength of the external magnetic field, decrease detection sensitivity and can potentially cause overlap of different resonances. Nevertheless, since the number of ^{77}Se sites in biological samples is typically low, this overlap is usually not a problem. Thus, the ^{77}Se spectrum is straightforward to detect with satisfactory sensitivity and is rich in information due to its pronounced chemical shift response, as exemplified in the following sections.

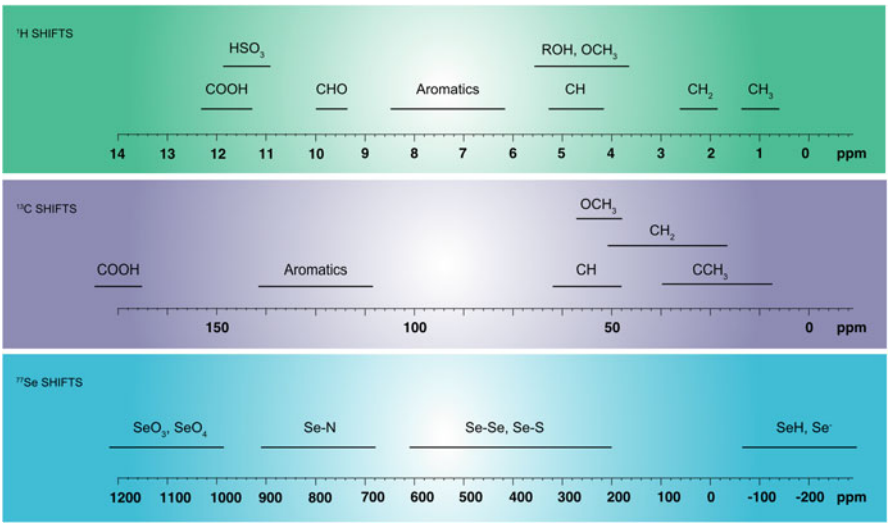

Fig. 15.1 Characteristic chemical shifts for biologically relevant ^{1}H, ^{13}C and ^{77}Se chemical groups. ^{77}Se chemical shifts cover a range of over 3000 ppm. The figure was adapted from references [4, 8, 9]

^{77}Se NMR is abundantly used in studies of materials, small molecules and selenopeptides. Extensive surveys describing ^{77}Se NMR of small compounds and materials can be found in comprehensive reviews [8–13]. However, only a handful of publications have utilized ^{77}Se NMR for studies of biological systems [14–17] and ^{77}Se is far from being routinely exploited. This can be attributed to the following major bottlenecks: a lack of protocols to enrich proteins with the NMR-active ^{77}Se isotope, and the challenge of interpreting NMR data in the absence of a significant body of research. Recent advances in sample preparation and data analysis [11–13, 18, 19] discussed in the following sections, however, render ^{77}Se NMR broadly applicable for biological systems.

15.3 ^{77}Se Isotopic Enrichment of Proteins for NMR Studies

Since not all nuclei are NMR active or their sensitivity is low, biological samples typically need isotopic enrichment with NMR-active nuclei. Similar to ^{13}C and ^{15}N, whose NMR-active isotopes make-up only a small percentage of the total isotopes in the natural abundance sample, ^{77}Se natural abundance is only 7.63 %, and, hence, isotopic enrichment is required. For ^{13}C and ^{15}N enrichment, recombinant proteins are usually prepared using ^{13}C-glucose and ^{15}N-ammonium chloride as the sole carbon and nitrogen sources in *Escherichia coli* growth media. *E. coli* then synthesizes the amino acids from these basic building blocks. A similar approach can be undertaken for ^{77}Se enrichment, but high concentrations of selenocompounds are toxic to

E. coli necessitating the development of labeling protocols with suitable yield and affordable building blocks.

We have developed a ^{77}Se isotopic labeling method for replacing sulfur with ^{77}Se by heterologous expression in *E. coli* [20]. This technique relies on commercial elemental ^{77}Se instead of the expensive and hazardous synthesis of selenocompounds, thus providing a facile and cost-effective route for the insertion of the NMR-active isotope ^{77}Se into proteins. In essence, the method replaces all sulfur sources in the media with selenium using ^{77}Se-selenite. The incorporation is random, leading to a substitution of all sulfur sites and generating ^{77}Se-substituted proteins that contain multiple Sec and selenomethionine (Sem) residues. By fine-tuning the ratio of sulfur to selenium in the growth media, it is possible to control how much sulfur in proteins is replaced with selenium. In other words, for proteins that originally had disulfide or selenylsulfide (Se-S) bonds, it is possible to poise the sample for dominance of either diselenide (Se-Se) or selenylsulfide bonds, as desired.

A second method for ^{77}Se isotopic labeling by heterologous expression in bacteria relies on harnessing *E. coli* genetic incorporation machinery to overexpress selenoproteins. In this procedure, the ancillary proteins that assist Sec synthesis are overexpressed to meet the high level demand for Sec incorporation [21]. A sulfur-rich growth medium is supplemented with low concentrations of ^{77}Se-selenite as the sole source of selenium. *E. coli* then synthesizes and incorporates ^{77}Sec into the target protein in a site specific fashion [13, 22]. While this method allows for the presence of multiple, unaltered cysteines (Cys), it can only label one Sec residue in the protein. In addition, it is realistically restricted to the subset of proteins with a C-terminal located Sec (for additional discussion see reference [2]). This procedure has been employed to generate a series of non-native selenoproteins with representative selenoredox motifs for ^{77}Se NMR investigation [13].

Heterologous expression is not the only path for ^{77}Se enrichment of proteins. Initially, investigations of biological systems by ^{77}Se NMR relied on reacting proteins with ^{77}Se-labeled compounds [15–17]. Another early incorporation method was purification of erythrocyte glutathione peroxidase from lambs fed with ^{77}Se-selenite in their diets [14], but this route was limited as only a few selenoproteins can be readily purified from natural sources. Hence, it became increasingly popular to employ peptide synthesis using ^{77}Sec [12]. ^{77}Sec and chemically protected forms that are compatible with peptide synthesis are not commercially available, but procedures for their synthesis have been published [23]. However, this approach is constrained by the size and properties of the peptide.

An alternative method to prepare selenoproteins for biophysical studies is native chemical ligation (NCL), which relies on an amide-forming reaction to generate peptides and proteins from their respective fragments [24]. This approach has long been employed by selenium biochemists [25], but because it relies on solid-phase peptide synthesis, its scope is limited by the properties of the selenopeptide. Indeed, NCL has only been reported for one native selenoprotein so far, thioredoxin reductase 1 [26], and has never been employed for enrichment with ^{77}Se. Recently we have devised a novel expressed protein ligation procedure that is compatible with the production of

^{77}Se-labeled proteins [J Liu, Q Chen, S Rozovsky, manuscript submitted]. Instead of utilizing peptide synthesis, we produce selenoprotein fragments for ligation in *E. coli*, alleviating the size restriction. This flexible approach allows for the preparation of selenoproteins with Sec at any location in the protein. It is also compatible with labeling of more than one Sec residue, tolerating the presence of Cys residues, and isotopic labeling with additional nuclei such as ^{13}C and ^{15}N.

15.4 Identification of Chemical Species by ^{77}Se NMR

With the realization that selenium is covalently incorporated in proteins came an interest in recording the identity and diversity of chemical species that partake in biochemical reactions. Hilvert and coworkers reported the chemical shifts of a selenosubtilisin that was prepared by reacting the catalytic serine of the bacterial protease subtilisin with ^{77}Se enriched hydrogen selenide [17]. They charted the chemical shifts of selenosubtilisin selenolate (R-Se$^-$), seleninic acid (R-O_2H), and selenylsulfide forms (R-Se-S-R′) under native conditions. This work was groundbreaking in demonstrating that it is possible to collect NMR spectra with sufficient detection sensitivity for this 27.4 kDa protein at what is now considered low magnetic fields of 200 and 300 MHz. Another seminal study was performed by Gettins and coworkers on erythrocyte glutathione peroxidase and other non-native selenoproteins [14, 15]. Even though these were conducted under denaturing conditions, they demonstrated the potential of biological ^{77}Se NMR.

However, these early studies were, for the most part, restricted to proteins that were prepared by chemical synthesis. Thus, it remained unclear if protein samples containing multiple Sec and Sem residues would yield significant signals at moderate to high magnetic field strengths, and to what extent the broad signals would lead to signal overlap. Our group reported the first NMR detection of a ^{77}Se-substituted protein that contained numerous Sec and Sem residues. These multiple selenium resonances were detected at moderate and high magnetic fields of 14.1 and 19.97 T (^{1}H frequency of 600 MHz and 850 MHz, respectively). Most importantly they were highly sensitive to changes in the chemical environment [11]. We studied augmenter of liver regeneration (ALR), a sulfhydryl oxidase in the mitochondrial intermembrane space. ALR employs a CXXC motif, in conjunction with a flavin cofactor, to catalyze the formation of disulfide bonds (Fig. 15.2). In its secreted form, it is a 32 kDa homodimer that employs six Cys residues in each monomer to form interfacial and internal disulfide bonds. Thus ALR contains both structural and catalytic Cys. The sulfur sites of all six Cys and two Met residues were substituted to selenium, using a defined medium with a prefixed ratio of selenium/sulfur. By controlling this ratio, it was possible to poise the proportion of diselenide and selenylsulfide bonds in the selenium-substituted ALR. To better understand the ramifications of substituting sulfur to selenium, we determined the thermal stability and activity of selenium-substituted ALR and the X-ray crystal structure of ALR enriched with 90 % selenium [20, 27] to a 1.5 Å resolution (PDB entry 3U5S). This high

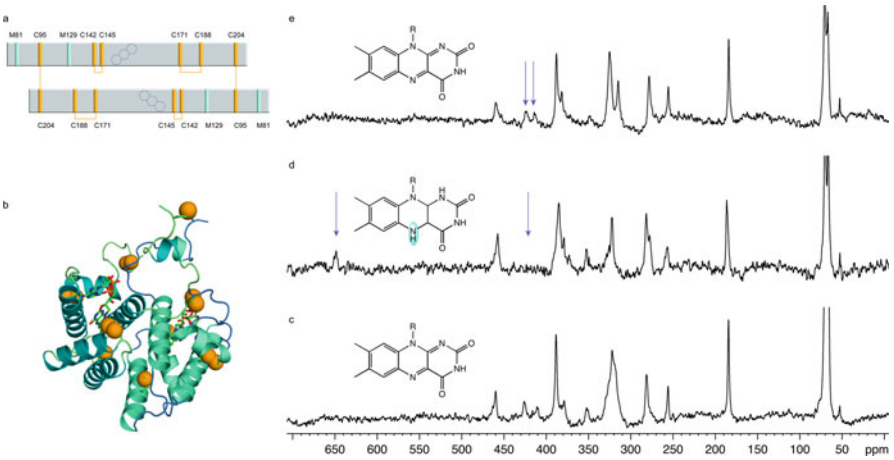

Fig. 15.2 ^{77}Se NMR investigations of the sulfhydryl oxidase ALR. (**a**) ALR is a 32 kDa homodimer enzyme with 12 Cys (*yellow*) and four Met (*green*) residues. Each active site contains a flavin cofactor (*purple*), and two catalytic Cys residues (C142–C145). Selenium-substituted ALR maintains its activity. (**b**) The selenium in different Sec (*orange spheres*) are shown in the crystal structure of selenium-rich ALR (PDB entry 3U5S). (**c**–**e**) ^{77}Se NMR characterization of the flavin reduction in ALR. For ^{77}Se NMR, the sulfur was replaced with selenium to create a protein rich in selenylsulfide bonds. All NMR spectra were acquired at 20 °C and 14.1 T (^{1}H frequency of 600 MHz). Sem resonances between 0 and 150 ppm are truncated for clarity. (**c**) Proton-decoupled ^{77}Se spectrum of ALR with 50 % selenium enrichment. The *inset* shows oxidized FAD. (**d**) Spectrum of ALR with reduced FAD under anaerobic conditions. *Arrows* indicate missing resonances at 412 and 426 ppm and a new resonance at 651 ppm. The *inset* shows reduced FAD. (**e**) Spectrum of ALR when FAD was reoxidized following removal of the reducing agent. The two resonances missing in (**d**) reappeared, suggesting that they arise from the catalytic residues in the immediate vicinity of the FAD. The *inset* shows oxidized FAD. Adapted from reference [20]

resolution allowed us to contrast the bond length and dihedral angles between the sulfur-based and selenium-rich proteins.

For 90 % ^{77}Se-substituted ALR, it was possible to collect overnight data on a high magnetic field of 19.97 T (^{1}H frequency of 850 MHz) with adequate signal to noise ratio [2]. For a 50 % ^{77}Se-substituted ALR at the moderate magnetic field of 14.1 T (^{1}H frequency of 600 MHz), the acquisition time was close to 2 days. Therefore, it is possible to acquire informative ^{77}Se NMR spectra using conventional acquisition times. In addition to acquiring ^{77}Se spectra with resolved resonances and a wide range of chemical shifts, we further demonstrated that these resonances are responsive to the reduction of ALR's flavin adenine dinucleotide (FAD) cofactor. The FAD was specifically reduced with sodium dithionite in an anaerobic NMR tube, changing the environment next to the CXXC active-site motif. Figure 15.2 displays the spectra of ALR before, during, and following reduction of the FAD. Several resonances shifted upon reduction of FAD, and two of these (between 400 and 450 ppm) disappeared, but are restored when the reductant is removed. These two resonances were tentatively assigned to be the catalytic residues next to the FAD. A new resonance appeared downfield at 651 ppm in the presence of sodium dithionite, and can be

attributed to a reversible modification on a cysteine. Accordingly, this resonance disappeared when the reducing agent was removed. Hence, the Sec is acting as a sensitive probe of the chemical transformations in the active site and the FAD state.

15.5 The Connectivity of Diselenide Bonds

The connectivity of a diselenide bond (i.e., the identity of the residues that form the diselenide bond) can be traced using NMR experiments that examine the properties of the selenium bonds. Such approaches rely on measuring the scalar coupling, a property of the chemical bond. Scalar coupling (also called J coupling and indirect spin-spin coupling) arises from the influence of electrons in the chemical bond on the tendency of the atoms' spins to align [4]. This coupling can only be measured if atoms are bonded together or through a small number of chemical bonds. The sign and magnitude of scalar coupling depends on the molecular geometry and thus can be used to extract information on which atoms participate in a given bond, and what are the bond and torsion angles form. This approach was first demonstrated for the 37-mer insecticidal neurotoxin k-ACTX-Hv1c [12]. The vicinal disulfide bond of this spider toxin was converted to a diselenide bond using solid-phase peptide synthesis. Diselenide connectivity was measured in the k-ACTX-Hv1c by both ^{1}H–^{77}Se Heteronuclear Multiple Bond Correlation (HMBC) and ^{77}Se-^{77}Se Correlation Spectroscopy (COSY) [12, 28]. Since then, additional seleno-toxins have been characterized in a similar fashion [29].

In selenoproteins, the selenylsulfide bonds are significantly more important for the catalytic cycle than the diselenide bonds [30, 31]. For these selenylsulfide bonds, it is only possible to correlate the selenium and hydrogen atoms so HMBC is the experiment of choice. We have shown that it is possible to record HMBC for the selenylsulfide bond in selenoproteins, specifically in a 12 kDa protein containing a Gly-Cys-Sec-Gly redox motif, see Figure 4 of reference [2].

15.6 Measurements of Sec pK_a in Selenoproteins and Selenopeptides

NMR spectroscopy is useful for measuring the pK_a of residues including that of Sec. The chemical shift difference between protonated selenols and the deprotonated selenolate is in excess of 100 ppm, allowing easy differentiation between the two. This chemical shift difference can be plotted against pH, and analysis of the titration curve yields pK_a values. Hilvert and coworkers established that in selenosubtilisin, the pK_a of Sec was lower than 4.0, but they could not extend these studies to lower pH due to the instability of the native fold of the protein [17]. However, selenopeptides lend themselves to investigations at acidic pH and thus can provide information on the contributions of nearby residues. The pK_as of two Sec residues in the 9-mer

peptide selenovasopressin (UYFQNUPRG) were reported as 3.3 and 4.3 at positions 1 and 6, respectively, and these pK_a values were used for selective alkylation [18].

An intriguing possibility offered by these experiments is that a similar approach can be employed to record the redox potentials of selenopeptide and selenoproteins. However, this application is still awaiting demonstration.

15.7 Conformational Preferences and Dynamics of Sec

In addition to its sensitivity to the chemical environment, the strength of NMR lies in its ability to probe conformational dynamics on all time scales relevant for function of proteins (10^{-9}–10^{2} s). The ability of selenium to report on the conformational preferences and mobility of Sec at different sites was examined with a series of proteins whose local Sec environment was systematically changed. Specifically, we have recorded the NMR spectra of proteins into which we had placed Sec in different redox motifs (i.e., the patterns of amino acids next to Sec) [13]. In these selenoproteins, the reactive Sec formed a selenylsulfide bond with a neighboring Cys. The reactivity of Sec is influenced by the size of the ring formed in the oxidized state and the identity of the residues forming this redox motif. Using solution-state ^{77}Se NMR spectroscopy, we probed the conformations of these redox motifs in proteins, and the flexibility of Sec in both the reduced and oxidized states. As the size of the ring formed in the oxidized state increased, the NMR spectra display evidence for increased mobility at the selenium site, and also the presence of additional ring conformations. At the reduced state, the width of resonances suggests that serine in the redox motif can form a hydrogen bond to Sec.

A unique aspect of this study [13] is that theoretical calculations of the magnetic shielding were used to understand the conformational preferences of the oxidized selenylsulfide-containing rings. Not only do they illustrate the range of chemical shifts expected in Sec in similar protein environments, but they also demonstrate how conformation and thus weak non-bonding molecular interactions can be inferred from ^{77}Se NMR. This study is the first to combine experimental measurements and Density Functional Theory (DFT) calculations to investigate the origin of selenium NMR parameters in biological systems. These calculations provide a comprehensive interpretation of chemical shifts of selenium and insight into their origin. Overall this work deepens our understanding of the chemical reactivity of selenoproteins.

15.8 ^{77}Se NMR Sensitivity to the Local Environment

As discussed, the chemical shift contains information on how the electrons in the molecules respond to the external magnetic field, and thus report on the electronic structure, the chemical bond, and the protein environment [32]. This shift is not merely a single number, but has orientation-dependent values (which are usually

summarized in a tensor) that reflect how the molecule is positioned in the external magnetic field [6]. In solution-state NMR, the rapid molecular tumbling averages this orientation dependence of the chemical shifts. In contrast, in solid-state NMR (SSNMR), the arrested motion of the molecules allows the individual elements of the chemical shift tensor to be recorded. SSNMR samples can be large proteins, protein complexes, protein crystals, and other molecular states that are incompatible with solution-state NMR [33]. However, despite SSNMR's high information content and extensive flexibility with sample conditions, SSNMR has not yet been applied to biological systems with ^{77}Se.

In preparation for applying ^{77}Se SSNMR to macromolecules, we recently reported the selenium magnetic shielding tensor of the model compound L-selenocystine [19]. This study is notable, because DFT calculations of the selenium magnetic shielding tensor were utilized to explain the reported L-selenocystine chemical shift tensor (Fig. 15.3). We found that even though the crystal structure of L-selenocystine is unknown, the Se-Se torsional angle can be deduced using calculations of the magnetic shielding (i.e., chemical shift). Thus, this study demonstrates the ability to extract structural information from the chemical shift tensor for selenium-containing molecules with unknown molecular geometry. More importantly, it presents a systematic investigation of the fit of the experimental data to the calculations when various structural effects, such as protonation state, protein environment, substituent, or conformation, are varied. Consequently, this work provides fundamental information for future studies of Sec and Sem in different protein environments.

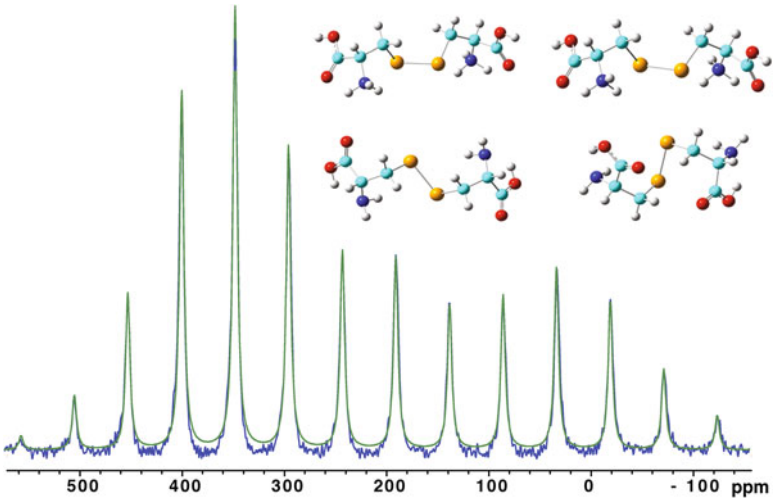

Fig. 15.3 ^{77}Se solid-state NMR spectra of L-selenocystine. *Blue*: ^{77}Se-^{1}H cross polarization magic angle spinning spectra of L-selenocystine recorded at 11.75 T, a temperature of 261 K, and a spinning rate of 5 kHz. *Green*: best fit simulated spinning chemical shift anisotropy sideband patterns for L-selenocystine at a spinning rate of 5 kHz. *Inset* shows models of representative selenocystine structures used for DFT calculations. Adapted from reference [19]

15.9 Data Interpretation

Due to the paucity of biological ^{77}Se studies, there is no comprehensive knowledge base to help determine the influence of the local environment (i.e., neighboring residues, hydrogen bonding, disulfide/diselenide/selenylsulfide interchange and their dynamics, local pH environment, etc.) on selenium chemical shifts in macromolecules. Consequently, the bottleneck for biological ^{77}Se NMR is neither sample preparation nor data acquisition, but rather data interpretation.

Fortunately, quantum calculations of chemical shifts in proteins are transforming NMR data interpretation, and thus can aid data interpretation in ways such as assignments of individual resonances and charting their molecular environment. These calculations have become an invaluable tool for biological NMR and they are now practically indispensable for the assignment of proteins' chemical shifts [34], the determination of their structure, characterization of minor structural conformers, and studies of ligand-protein interactions [1, 35, 36]. However, the magnetic shielding of ^{77}Se is somewhat more difficult to calculate than that of lighter nuclei such as ^{13}C and ^{15}N, since for higher atomic number nuclei, it is necessary to incorporate a more extensive description of electron correlation [37]. Thus, in the case of ^{77}Se, when the crystal structure is taken into account, the theoretical and calculated values differ typically by 5–15 % [38]. For biological systems it is more difficult to predict and understand chemical shifts. However, good agreement was found between calculations of isotropic chemical shifts for selenoproteins and selenol amino acids [39–41] and the experimental data reported by Odom, Gettins, and Hilvert [14–17] (see Sect. 15.4).

We have worked to build the knowledge base and to advance data interpretation by recording ^{77}Se chemical shifts through systematic surveys of ^{77}Se NMR parameters in biological systems [13]. In this work, theoretical calculations of the isotropic chemical shifts have been used to understand the conformational preferences of the oxidized selenylsulfide-containing rings. This was the first study to infer nonbonding interactions of Sec and its conformations (see Sect. 15.7). We also employed DFT calculations to understand ^{77}Se chemical shifts of the model compound L-selenocystine (see Sect. 15.8). This has advanced our understanding of how different variables in DFT calculations influence prediction of ^{77}Se chemical shifts of Sec and Sem residues measured in proteins.

15.10 Concluding Remarks

^{77}Se NMR of biological systems has become broadly accessible due to the development of high-yield and low-cost methods for ^{77}Se-isotopic enrichment, as well as the reports on the detection of Sec and Sem residues in various proteins. In addition, the quantum mechanical calculations of ^{77}Se magnetic shielding continue to improve in accuracy and ease, allowing for their routine implementation in studies of

biological systems. The rise in ^{77}Se NMR studies of different classes of biological macromolecules is leading to improved data interpretation and better integration of biological ^{77}Se NMR into routine use.

New directions include the use of ^{77}Se as a local probe of conformational changes. Selenium is an excellent probe for molecular motion since its magnetic shielding interaction can be used to detect dynamics that occur on the microsecond time scale, a range relevant to many biological processes. Other particularly exciting directions are investigations of native selenoproteins by both solution and solid-state NMR to understand general thematic questions pertaining to selenoproteins.

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