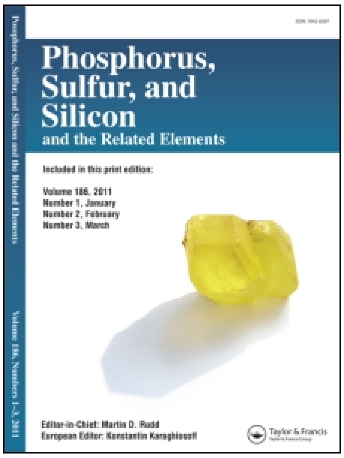

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MAGNETIC RESONANCE STUDIES OF SELENIUM CONTAINING COMPOUNDS

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MAGNETIC RESONANCE STUDIES OF SELENIUM CONTAINING COMPOUNDS

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Abstract The utility of selenium-77 nuclear magnetic resonance in studying the properties of a range of selenium-containing compounds from small organoselenium molecules to macromolecules which contain selenium bound covalently is discussed. Relaxation times, chemical shifts and spin-spin coupling constants are all useful indicators of structure, function and bonding in these systems. The origin of selenium-77 chemical shifts in organoselenium compounds as a function of the polarizability of the selenium electron cloud and the well-known γ-effect are explored. Finally, the use of selenium-77 NMR spectroscopy in investigating biochemical systems is illustrated. For example, we show that selenium-containing inhibitors and substrates reflect the mechanism of their binding to enzymes as a result of the sensitivity of the selenium-77 chemical shift.

INTRODUCTION

Our entry into selenium chemistry and selenium-77 nuclear magnetic resonance studies was a result of a research program being conducted under the auspices of the Environmental Protection Agency on NMR investigations of the heavy metals cadmium and mercury.[1,2] The toxicity of these metals clearly involves their binding by sulfur in biological systems and we initially considered performing ^{33}S NMR experiments on model systems. However, ^{33}S NMR spectroscopy is relatively difficult experimentally due to the quadrupolar nature of the nucleus ($I = 3/2$) and the low natural abundance (0.76%).[3] Biochemical NMR studies of sulfur would present almost insurmountable problems. However, from our readings, it became clear that selenium and sulfur in many respects are very similar chemically [4,5] and that NMR studies of the selenium-77 nucleus might be very useful. Selenium-77 is a spin 1/2 nucleus with a natural abundance of 7.58% and although its relative receptivity with respect to the hydrogen nucleus is only 5.26×10^{-4}, it is 2.98 times more sensitive than carbon-13.[3] It is the only isotope of the six naturally occurring selenium isotopes which possesses a nuclear spin. In addition selenium-75, which decays by beta emission, has been used extensively as a radio tracer in biochemical studies of selenium compounds. Additionally, selenium-77 has an extremely large chemical shift range (~3000 ppm) and shows a dramatic sensitivity in chemical shifts to subtle electronic changes.[6] For example, if dimethyl selenide is taken as the chemical shift reference at O ppm, the replacement of a methyl group by a hydrogen atom to give methyl selenol, CH_3SeH, shields the selenium resonance by 160 ppm! If we replace the methyl groups in $(CH_3)_2Se$ by ethyl groups, the selenium resonance is deshielded by 220 ppm. An interest in understanding, at least in a qualitative sense, the origin of ^{77}Se chemical shifts in organoselenium molecules has led us to a detailed study of selenium-77 substituent-caused chemical shifts in selenols, selenides and diselenides.[7] By examining solvent effects on ^{77}Se chemical shifts of dialky selenides and dialkyl diselenides in halocarbon solvents of varying molecular polarizabilities, we have extrapolated intermolecular polarizability to an intramolecular polarizability concept whereby dispersion forces within a molecule exerted by neighboring alkyl groups influence selenium shielding. This effect, along with the well-known "γ effect" offers a clear understanding of a large number of ^{77}Se chemical shifts in organoselenium compounds. Although based on more limited data, it also appears that this intramolecular polarizability concept also explains the even larger substituent caused chemical shifts for the ^{125}Te nucleus.[7]

In the 3000 ppm range of ^{77}Se chemical shifts, there appear to be relatively well-defined ranges for selenols, selenides and diselenides and, in general, it appears that selenium halides and oxyhalides have very deshielded resonances and the selenide anion Se^{-2}, and the hydrogen selenide ion, HSe^{-}, are the most shielded resonances.[6] Selenocarbonyl compounds (selones) are anomalous in that their ^{77}Se chemical shifts span almost the entire range. For example, COSe has a ^{77}Se chemical shift of -447 ppm [8] while the most deshielded resonance for this class of compounds occurs at 2162 ppm for $(t\text{-}Bu)_2CSe$.[8] It is also interesting to note that simple electronegativity arguments break down here if one examines the chemical shifts for $Se=C=O$ (-447 ppm), $Se=C=S$ (102 ppm) and $Se=C=Se$ (331 ppm).[8] Clearly the ^{77}Se chemical shifts are exactly opposite to what would be predicted based on the relative electronegativities of oxygen, sulfur and selenium.

Another example of the sensitivity of ^{77}Se chemical shifts to extremely small electronic changes was demonstrated in a study in our laboratories of the ^{77}Se chemical shifts in H_2Se, HDSe and D_2Se.[9] These three materials in an NMR tube exhibited well-separated selenium resonances of over 7 ppm! This is by far the largest secondary isotope shift observed in high resolution NMR spectroscopy for any spin isotope and corresponds to almost the entire normal chemical shift range in ^{1}H NMR.

When we entered the field of selenium NMR spectroscopy, a number of standards had been utilized as chemical shift references including neat dimethyl selenide, [10] neat seleninyl chloride ($SeOCl_2$), [11] aqueous selenonic acid, [12] selenophene [13-16] and 4,4'-dimethyldiphenyl diselenide. [17] Because of the sensitivity of selenium chemical shifts mentioned above, we felt that it was important that a single standard be selected and that the ^{77}Se chemical shift of that compound be well characterized with respect to solvent, concentration and temperature effects. A 60% (v/v) solution of dimethyl selenide in $CDCl_3$ was chosen because of the commercial availability of $(CH_3)_2Se$, its relative inexpensiveness, its relatively low toxicity and the fact that a good signal-to noise ratio can be obtained in a single pulse on a 60% solution.

The ^{77}Se chemical shift was found to vary by approximately 9 ppm from neat $(CH_3)_2Se$ to a 1.25% solution in $CDCl_3$ while its temperature dependence varied by approximately 2.5 ppm over a 100 degree range (223-323 K). The largest variation in chemical shifts occurred in different solvents with the extremes being 7.13 ppm for C_6H_{12} and -14.33 for DMSO.[18] The need clearly exists for conditions to be carefully controlled and stated when reporting ^{77}Se chemical shifts.

The more we became involved in selenium chemistry and selenium NMR spectroscopy, the more interested we became in studying the feasibility of examining selenium in biological systems by NMR spectroscopy. Selenium biochemistry has been of interest to a relatively large number of researchers and a great deal of progress has been made in the last decade in the identification and characterization of selenium containing biological macromolecules.[19] The realization that low concentrations of selenium in these large systems could pose significant experimental difficulties involved us almost immediately in relaxation time studies. [20,21] To take full advantage of the substantial gains in signal-to-noise ratios obtained by Fourier transform NMR methods, a knowledge of the inherent spin-lattice relation time, T_1, of the selenium nucleus is desirable since it influences the time duration of the experiments via the recycle time between pulses.

Relaxation time studies for a relatively large number of organoselenium molecules basically demonstrated several important points. First, there was a total lack of a Se-{^{1}H} Nuclear Overhauser Effect enhancement which eliminates the dipole-dipole (DD) mechanism as an effective mode of relaxation for selenium. This was even true for selenols where a hydrogen is directly bonded to a selenium atom and is due to the relatively long Se-H bond distances and the inverse r^6 dependence on this distance in dipole-dipole relaxation. Second, Arrhenius plots of the temperature dependence of the observed relaxation rates of selenium in relatively small molecules exhibited a straight line with negative slope indicating that the spin-rotation (SR) mechanism was the dominant mode of relaxation for the selenium nucleus. Naturally, this result was of great concern to us since, if this mechanism were to be the dominant relaxation mechanism in macromolecules which tumble relatively slowly in solution, NMR experiments on selenoproteins and selenoenzymes would be prohibitively long. However, relaxation time experiments on larger molecules as a function of temperature demonstrated that as spin rotation became less and less efficient, the chemical shift anisotropy (CSA) mechanism becomes a dominant mode of relaxation for selenium-77. Thus we were encouraged that we would be able to observe selenium resonance in large molecules in a reasonable amount of spectrometer time.

At this point we felt that it was very important that we demonstrate the feasibility of observing selenium in large biomolecules. Although one of our ultimate goals was to observe selenium in naturally occurring selenoproteins, we felt that, in view of the many experimental difficulties which would have to be overcome with these natural systems, a more promising initial strategy would be to introduce selenium into large biomolecules via a selenium-containing chemical modification reagent. To this end, we synthesized and characterized 6,6'-diselenobis-(3-nitrobenzoic acid) (DSNB)[22] which is a selenium analog of the well known Ellman's reagent, [23] 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB). The new selenium reagent, DSNB, was shown to behave in a very similar manner to DTNB, reacting specifically and quantitatively with free sulfhydryl groups to form a selenenyl sulfide and the intensely yellow-colored dianion, 3-nitro-6-selenobenzoate. The molar absorption coefficient of the chromophoric dianion is 10,000 at 432 nm in dilute aqueous solution. Sulfhydryl content as determined by DSNB in reduced and denatured ribonuclease, reduced and denatured lysozyme, native papain and native and denatured thymidylate synthase compared very favorably with DTNB determinations. An added benefit was that, unlike DTNB which undergoes alkaline decomposition at pH values greater than 9, DSNB is stable to hydrolysis even in 0.05 M NaOH.

During the course of this project with DSNB, an examination of the literature revealed that there were no reports of ^{77}Se NMR studies of selenenyl sulfides. Since our modification reagent would produce this linkage in the biological systems which we wished to utilize for our feasibility study, it was necessary to synthesize a series of model selenenyl sulfides and characterize their ^{77}Se chemical shift range. Accordingly we synthesized and studied 23 selenenyl sulfides which were of the general form of a nitrophenyl moiety bound to selenium and an alkyl or aryl group bound to sulfur, *i.e.* R-Se-S-R'. [24] Even though the compounds were relatively similar, the total chemical shift range was over 320 ppm and the study achieved its goal of establishing a chemical shift range for these types of selenenyl sulfides from 416 to 738 ppm with respect to 60% $(CH_3)_2Se$ in $CDCl_3$.

The systems chosen for our feasibility study of observing NMR signals of ^{77}Se covalently attached to proteins were denatured and reduced ribonuclease-A and denatured and reduced lysozyme. [25] Both of these enzymes are small biomacromolecules (ribonuclease, 124 amino acids; lysozyme, 129 amino acids) which, in their native state, possess four disulfide bonds. Thus, in their reduced state, eight sulfhydryls are available to react with DSNB to produce eight selenium-sulfur covalent bonds. To our satisfaction the NMR feasibility study was definitely positive. A 1.6 mM ribonuclease sample and a 0.86 mM lysozyme sample, both of which had been denatured and reduced and had reacted with DSNB, both produced five ^{77}Se resonances in the chemical shift range for selenenyl sulfides. These experiments were long-term FT spectral accumulations and required isotopically enriched ^{77}Se, but it was clear that ^{77}Se NMR spectroscopy in high molecular weight systems was definitely possible. The source of five resonances in each spectrum was probably due to a combination of rather subtle changes in the electronic environment of the eight different selenium atoms and to the persistance of some secondary structure in the denatured protein.

Once having demonstrated that resonances could be observed from ^{77}Se nuclei covalently attached to proteins, we chose to extend our biochemical studies to determine whether this technique would be useful in characterizing the interaction of selenium-containing substrates or inhibitors with enzymes. We selected chymotrypsin as a model enzyme for these studies because, as a readily available and inexpensive digestive enzyme, its structural, functional, and mechanistic properties have been widely reported. Chymotrypsin is a protein of MW 24,300 which exhibits specificity for catalyzing the hydrolysis of peptide bonds whose carbonyl groups are donated by aromatic amino acid residues in protein and peptide substrates. This enzyme, which also promotes the hydrolysis of synthetic substrates containing ester and amide linkages, is a member of a group of enzymes known as serine proteases, whose mechanism of action features the transient acylation of the nucleophilic side chain hydroxyl group of the active site serine residue. To begin our studies, we designed and synthesized sodium (phenylselenyl) acetate, ($PhSeCH_2COO^-Na^+$) [25], whose methylene congener, 3-phenylpropanoate[26], is a known inhibitor ($K_I = 25$ mM) of chymotrypsin. The former compound was readily prepared by the following route: sodium phenylselenolate, resulting from the sodium borohydride reduction of diphenyldiselenide, was reacted with iodoacetic acid, and the mixture was treated with 6N NaOH in methylene chloride to yield the sodium salt of (phenylselenyl) acetate. Following purification by recrystallization and standard characterization, sodium (phenylselenyl) acetate was evaluated as an inhibitor of chymotrypsin and evidenced a respectable K_I value of 20 mM. We were now in a position to pose the following question: Is the selenium-77 chemical shift sensitivity sufficient to reflect the binding of a selenoinhibitor to chymotrypsin? In addressing this question we designed a ^{77}Se NMR experiment to investigate the titration of chymotrypsin (4.49 mM in 0.1 M K_2HPO_4 buffer, pH 6.8) with (phenylselenyl) acetate containing ^{77}Se at natural abundance (7.58%). The experiment was performed on a Bruker WP-200 instrument utilizing a 15-mm broad-band probe, and chemical shifts were internally referenced to 30 mM trimethylselenonium iodide, which exhibited a single resonance whose line width was found to be unaffected by the presence of enzyme or inhibitor. Since increasing ratios of chymotrypsin to inhibitor produced a broadening (from 12 Hz for free inhibitor up to as much as 54 Hz for enzyme: inhibitor mixtures) and shielding of the selenium resonance of the inhibitor, we deduced that these data were consistent with the presence of a reversible enzyme-inhibitor complex with the inhibitor in rapid exchange between the enzyme-bound and free states. Since such a system does not permit the direct measurement of the selenium chemical shift of the enzyme: inhibitor complex, we employed a two state model and equations developed by Raftery and coworkers [27] to calculate K_I from the NMR data and to estimate, Δ, the chemical shift of the enzyme: inhibitor complex from the NMR measurements. The reader is referred to a paper by Mullen *et al.* [25] and to Dr. Mullen's Ph.D. thesis [28] for a comprehensive discussion of these calculations. The K_I for (phenylselenyl) acetate calculated from the NMR data was 19.7 mM which is in excellent agreement with the value of K_I (20 mM) determined in kinetic studies. The value of Δ was calculated to be -39.8 ppm to higher shielding! To appreciate the significance of this enormous change in chemical shift resulting from the binding of the inhibitor to the enzyme, one must realize that this value of Δ is an order of magnitude larger than those for inhibitors whose binding to chymotrypsin was studied by proton, carbon-13, and fluorine-19 NMR. We postulate that this large change in chemical shift reflects a combination of possible factors such as polar field and polarizability effects, changes in the γ-effect resulting from restricted conformation of the inhibitor in the active site of the enzyme, and possibly the generation of a chemically different intermediate(s) of the inhibitor as a result of binding. Whatever the explanation, it is clear that Se-77 NMR is capable of detecting enzyme: inhibitor interactions.

Quite naturally, the success of the latter investigations led us to pose a second question regarding the utility of Se-77 NMR spectroscopy in studying enzymatic systems. That is, could Se-77 NMR be employed to characterize the formation of a covalent acyl-enzyme intermediate resulting from the reaction of chymotrypsin with a selenium-containing substrate?

To answer this question we evaluated the p-nitrophenyl ester of (phenylselenyl) acetate as a potential substrate for chymotrypsin [29]. The chymotrypsin catalyzed hydrolysis of the substrate yielded Michaelis-Menten type kinetics with an apparent $K_M = 5.16 \times 10^{-6}$ M. By analogy to the action of this enzyme on related ester substrates, a minimal catalytic mechanism would involve three steps: (1) formation of a noncovalent enzyme: substrate complex, (2) acylation of the side chain of serine-195 to form a covalent acyl-enzyme intermediate, and (3) deacylation of the latter intermediate to form the product carboxylate and regeneration of active enzyme. The next experimental step was to determine if the substrate functioned as an active site titrant at relatively low pH values (pH 4-5) where the deacylation process is slow but the acylation step is rapid. Thus, incubation of a four fold excess of p-nitrophenyl (phenylselenyl) acetate with chymotrypsin at pH 5.0 and 3°C resulted in a burst release of an amount of p-nitrophenol which was within 5% of the active site concentration as determined with the active site titrant, cinnamoyl imidazole. This result together with the fact that a very slow turnover rate of substrate (3.9×10^{-9} Ms^{-1}) occurs after the burst, provided strong evidence that the substrate was reacting with the enzyme to form a stabilized acyl-enzyme species under these conditions.

In order to achieve maximum sensitivity in the Se-77 NMR experiments we decided to utilize substrate which had been enriched in the selenium-77 isotope. The enriched substrate was synthesized by the following procedure: the product resulting from the reaction of bromobenzene and magnesium was reacted with ^{77}Se metal to form the corresponding selenomagnesium bromide Grignard reagent, which was treated with iodoacetate to yield the selenium-77 enriched (phenylselenyl) acetic acid, followed by dicyclohexylcarbodiimide coupling with p-nitrophenol to yield the desired ester. Samples for ^{77}Se NMR spectroscopy were prepared by incubating chymotrypsin (2.6 mM) with an equivalent amount of substrate in 0.1 M acetate buffer at pH 5.0; two aliquots of the latter solution were removed and carefully adjusted to pH 3.8 and 2.0, respectively. Selenium-77 NMR spectra of these samples revealed one minor peak and one major peak. No resonance was observed at 350 ppm, the chemical shift of p-nitrophenyl (phenylselenyl) acetate. The major resonance was found at 275.1 ppm for pH 5.0, at 276 ppm for pH 3.8, and was split into two resonances, 276.3 and 277.3 at pH 2.0. These resonances were assigned to (phenylselenyl) acetyl chymotrypsin, the acyl-enzyme intermediate, on the basis of the following evidence. (1) The size of the 276 ppm resonance decreased with increasing temperature (277→292K) with the concomitant increase in the size of resonance assigned to the product, (phenylselenyl) acetate, thus illustrating the transitory nature of the acyl-enzyme intermediate. (2) Such acyl-enzyme intermediates of chymotrypsin exhibit the capability of acyl transfer to alternate acceptors. Incubation of the (phenylselenyl) acetyl chymotrypsin with a 1M solution of hydrazine sulfate followed by adjustment of the pH to 7.85 led to a spectrum which showed that the resonance at 276 ppm was replaced by two resonances at 332.3 ppm and 302.2 ppm which were assigned to protonated (phenylselenyl) acetyl hydrazide and (phenylselenyl) acetate, respectively. (3) Denaturation of the proposed acyl-enzyme intermediate sample resulted in the complete replacement of the resonance at 276 ppm by a sharp peak at 335 ppm, a value which is very similar to that observed independently for methyl (phenylselenyl) acetate (333 ppm). (4) We also noted that the resonance for the denatured (phenylselenyl) acetyl chymotrypsin remained at 335 ppm on increasing the pH to 9.16.

These experimental observations are all in accord with the assignment of the resonance at 275 ppm to the acyl-enzyme species. We were interested to find that the chemical shift of the acyl-enzyme intermediate (275 ppm) was much closer to that for phenylselenoacetaldehyde (257 ppm) than to the chemical shifts of the denatured acyl anzyme (335 ppm), the model methyl ester of (phenylselenyl) acetate (333 ppm), or the actual substrate (350 ppm). Compared to the resonance of the methyl ester, the environment of the enzymic active site produces a 58 ppm perturbation in chemical shift of the selenium nucleus in the covalently bound acyl group. Such an alteration in chemical shift could be attributed to a resonance-perturbed ester linkage and/or steric compression of nonbonding orbitals at the selenium nucleus. Whatever their origin, the chemical shift differences between the various species certainly are large and the relaxation mechanisms are sufficient to permit ready observation of NMR signals.

These studies have established the feasibility of selenium-77 NMR spectroscopy in the investigation of the interaction between enzymes and their substrates, products, inhibitors, etc. We are very interested in extending these studies to other enzymes and selenoligands, selenocoenzymes, and selenium-containing chemical modification agents and affinity reagents.

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