Selenocysteine

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Site-Specific pK_a Determination of Selenocysteine Residues in Selenovasopressin by Using ^{77}Se NMR Spectroscopy**

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Selenium is predominantly present in biological systems in the form of the naturally occurring amino acid selenocysteine (Sec/U), which has been identified in all lineages of life.[1] It has been established as a biologically essential element for cellular redox balance, immunocompetence, male fertility, and prevention of cancer and chronic inflammation.[2] Sec is co-translationally inserted into proteins in response to UGA codons and, unlike the other 20 amino acids in the genetic code, Sec is synthesized universally on its own tRNA by using serine as an intermediate.[3] Some selenoproteins, such as glutathione peroxidase and thioredoxin reductase, have already been well characterized,[3] but the precise function of many of these proteins remains unknown, leading to a growing interest in the synthesis and study of selenoproteins. The persistence of Sec utilization in nature, including the maintenance of a complex and unorthodox biosynthetic machinery for Sec incorporation, suggests a selective advantage over non-Sec containing proteins. Indeed, it has been demonstrated that at least for some human selenoenzymes there is a 100- to 1000-fold decrease in catalytic activity when the Sec residues are replaced by Cys.[4]

Despite selenium and sulfur being neighboring chalcogens, Sec exhibits distinct chemical properties when compared to cysteine (Cys/C). These include higher nucleophilicity,[5] better leaving group character,[4a] higher susceptibility to nucleophilic attack,[6] and higher acidity.[5a,7] Similarly, diselenide, selenylsulfide, and disulfide bonds have distinct redox potentials, which allow preferential formation of diselenide over disulfide bonds.[8] Central to all these properties is the acidity of Sec reflected in the pK_a value of the selenol moiety. Currently only the pK_a value of the amino acid selenocysteine has been characterized and reported to be ca. 5.5.[5a,7,9] Experimental observation of Sec-containing peptides and proteins however suggest that the pK_a of Sec in proteins must be substantially lower than that found in free selenocysteine.[8b,10] While a significant amount of work has been carried out to establish the role of the different redox potentials of disulfide, selenylsulfide and diselenide bonds in peptides and proteins,[4a,8a,10a] the pK_a and thus a physiochemical basis for the reactivity of Sec in peptides and proteins remains elusive.

A particularly powerful method for directly probing the physical properties of selenium atoms in a peptide is nuclear magnetic resonance (NMR) spectroscopy.[11] The low natural abundance (7.6%) of the NMR-active isotope of selenium (^{77}Se) combined with the lack of synthetic access to adequate amounts of selenopeptides has previously hampered NMR-based investigations. However, recent studies that provide means of producing ^{77}Se-enriched Sec building blocks[12] as well as advances in synthetic incorporation of Sec into peptides and proteins by chemical synthesis and native chemical ligation has reinvigorated interest in studies of Sec-containing peptides and proteins using ^{77}Se NMR spectroscopy.[13]

Here we use ^{77}Se NMR spectroscopy to determine, for the first time, the pK_a values of two Sec residues incorporated into a bioactive peptide hormone and neurotransmitter (AVP, arginine vasopressin), which consists of a N-terminal cyclic 6-residue ring that is stabilized by an intramolecular diselenide bond (Figure 1).

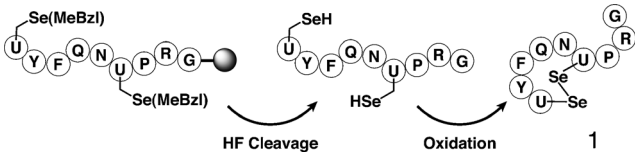

Figure 1. Chemical synthesis (Boc-SPPS), HF cleavage, and oxidation of selenovasopressin [C1U;C6U]-AVP.

The sensitivity of selenium nuclei to their electronic environment provides an ideal probe for titration experiments where reduced [C1U;C6U]-AVP (**1**) can be used to directly observe the protonation state of each of the two distinct Sec sites in this molecule. We used homonuclear NMR methods to assign the hydrogen resonances of **1**. These were then used in 2D heteronuclear NMR experiments, where the highly variable ^{77}Se signal, which changed from approximately −60 to −220 ppm upon deprotonation, could be correlated to the more stable sidechain β-methylene proton chemical shifts that only changed by 0.2 ppm during the titration (Figure 2). This provides a simple and rapid method for site-specific assignment of ^{77}Se atoms at varying pH. This 2D approach further benefits from the higher spin polarization of protons compared to ^{77}Se, allowing high quality spectra to be collected (insert in Figure 2). Further-

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more, the impressive chemical shift range of ^{77}Se allows NMR to be used to directly assess the oxidation and protonation state of selenium atoms. Based on our results and previous findings it can be summarized that positive chemical shifts, typically around 300 ppm, correspond to oxidized selenium atoms in diselenides. In [C1U;C6U]-AVP these were found to be 250 ppm and 359 ppm for the first and sixth residue, respectively (Figure S3 in the Supporting Information). We note that these shifts define the extremes of the ^{77}Se chemical shift scale of oxidized selenocysteines in peptides reported to date and are likely to be due to the many charged and aromatic moieties present in the intervening residues. Chemical shifts around −70 ppm correspond to protonated selenols, and those around −200 ppm correspond to selenolate atoms, all consistent with the progressive shielding of the ^{77}Se nucleus and consistent with previous ^{77}Se NMR studies on organic molecules.[11a] Remarkably, these values give a total chemical shift range of nearly 600 ppm for the same atom in different oxidation states. The large difference between the protonated and deprotonated forms is particularly important as it makes the method less sensitive to small spurious chemical shift variations (e.g., due to temperature, protein concentration etc.), thus allowing accurate and robust determination of pK_a values. The combination of these advantages has allowed us to very accurately measure site-specific pK_a values for multiple Sec residues in the same polypeptide by using ^{77}Se NMR spectroscopy.

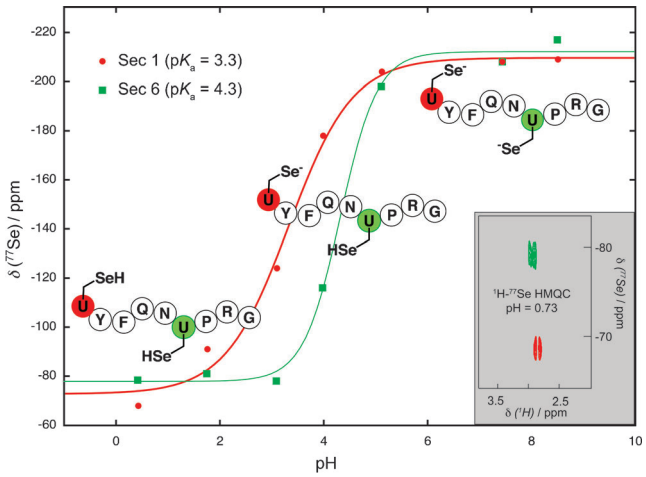

Figure 2. pH dependence of the ^{77}Se chemical shifts of selenols in reduced selenovasopressin. The insert is a ^{1}H-^{77}Se HMQC spectrum of reduced selenovasopressin at pH 0.73 showing correlations between the β protons and selenium atoms of the two selenocysteine residues through $^{3}J_{H\beta\text{-}Se}$ scalar couplings.

Interestingly, the two Sec residues in selenovasopressin were found to have significantly different pK_a values (Figure 2), with the Sec at positions 1 and 6 having pK_a values of 3.3 and 4.3, respectively. Both of these values are significantly lower than the values reported for free Sec (5.2–5.6) indicating that the complex local electronic environment in a peptide/protein strongly influences the protonation mechanism, as had been previously noted for active-site Cys residues in thiol-oxidoreductases.[14] Of the two pK_a values reported here, the value for the Sec at position 6 is likely to be more typical for selenoproteins as Sec residues have yet to be reported at the termini of naturally occurring peptides and proteins. The data reported here and that available for selenocystamine would, however, suggest that the pK_a value for Sec residues in peptides and proteins will be highly sequence dependent, opening up the intriguing possibility of predicting and driving chemical reactions such as alkylation or oxidation at a given site at a specific pH. To demonstrate this we alkylated **1** at a range of pH values and found that the N-terminal Sec residue was selectively alkylated at all pH values lower than 4 with regioselective alkylation at pH less than 2.5 (no alkylation was observed below pH 1.5). We observed progressive alkylation of both Sec residues at more basic pH values with both Sec residues completely alkylated at pH 8 within 20 min (Figure 3 and Figure S2). Thus, measurement of Sec pK_a values will not only improve our understanding of the chemistry of Sec residues in selenoproteins but it will also provide a means for introducing molecular tags or markers in a site-specific manner.

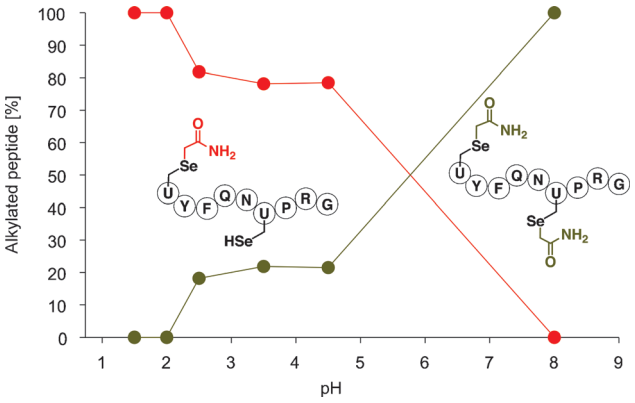

Figure 3. Reductive alkylation of selenovasopressin with TCEP and iodoacetamide. The red line shows the fraction of peptide alkylated only at U1 while the dark green line shows the fraction of peptide in which both Sec residues were alkylated. Selective alkylation of U1 was observed below pH 5, whilst at pH 8 complete alkylation of both Sec residues occurs.

The combination of the low pK_a values reported here and the known high reactivity of the selenolates might also provide an explanation as to why Sec incorporation does not follow the classic biochemical pathway of the other 20 natural amino acids. Nature elegantly deals with this reactive species by keeping the selenolate activated by hydrogen bonding to proximal functional groups which constitute catalytic triads, as observed in the crystal structure of the selenoenzymes glutathione peroxidase (hydrogen bonding with the imino group of a Trp residue and the amido group of a Gln residue)[15] and selenosubtilisin (hydrogen bonding with Asn and His).[16]

Finally, we note that the low natural abundance of ^{77}Se (7.58%) could in some cases lead to detection problems using this method. In such cases these limitations can be overcome by incorporation of ^{77}Se-enriched Sec residues into peptides

and proteins, thus requiring lower concentrations (10-fold) or shorter experiment time (100-fold).[13] The presented NMR approach in combination with isotope-enrichment methods can therefore be applied to a wide range of selenoproteins and other diverse systems containing multiple Sec sites. Possible difficulties in assignment of the reduced peptide can be resolved by the innovative variable labeling strategy recently suggested by Bulaj et al.,[17] making this approach a general and widely applicable one in enhancing our understanding of the fundamental properties of these essential biomolecules.

Experimental Section

Selenovasopressin (**1**, [C1U;C6U]-AVP, Figure 1) was synthesized by solid-phase peptide synthesis (SPPS) via Boc chemistry using the HBTU-mediated in situ neutralization protocol for chain assembly.[8b,18] 1D ^{1}H and 2D (COSY, TOCSY, NOESY) NMR spectra were recorded in H_2O and D_2O at 900 MHz for ^{1}H resonance assignment. ^{1}H chemical shifts assignments agreed with those found by Larive et al.[19] All ^{77}Se experiments were performed in D_2O using a 500 MHz Bruker AVANCE system equipped with a broadband probe. The chemical shifts of the two ^{77}Se sites were monitored using ^{1}H-^{77}Se HMQC spectra; the optimal value of $^1J_{(^{77}Se-^1H)}$ was determined to be 25 Hz. These 2D experiments required on average 10 h of data acquistion time at which point a 1D ^{1}H spectrum was re-measured to ensure the peptide was reduced. Chemical shifts were referenced to Sec, as previously described.[13] The pH-dependent ^{77}Se chemical shifts in the reduced state ranged from −60 ppm to −217 ppm. The pH dependence of oxidized **1** was also investigated by measuring the ^{77}Se chemical shift at pH 1 and 7, where a negligible (< 5 ppm) difference was found. Furthermore, the pH-dependent variation in the ^{1}H chemical shifts of the β-methylene protons of Sec residues in the reduced and oxidized state of **1** were found to be negligible (< 0.2 ppm).

TCEP (tris(2-carboxyethyl)phosphine) reduction studies were conducted to determine the optimal conditions for fast and complete reduction of **1**. Chemical shift changes in the less crowded ^{1}H aromatic region were monitored to assess the oxidation state of **1**. Full reduction was achieved after 5 min at pH 3–4 using an excess of 10-fold TCEP. pK_a titration of both Sec residues (at positions 1 and 6) was carried out under an inert atmosphere in susceptibility-matched Shigemi microcells. For each titration point, **1** was reduced with a 10-fold excess of TCEP at pH 3–4 for 10 min followed by pH adjustment using the appropriate buffer. All pH values measured in D_2O solution (pD) were converted to pH values using the relationship pD = pH + 0.44.[20] The converted pH values were 0.43, 1.76, 3.10, 3.99, 5.12, 7.46, and 8.07. The samples were purged for 10 min with nitrogen before being transferred into the Shigemi microcell and sealed with parafilm. The concentrations were kept constant at 1 mM for **1** and 10 mM for TCEP.

The chemical shifts were fitted to Equation (1),

$$\delta_i = \frac{A-B}{1+e^{\left(\frac{D-pH_i}{C}\right)}} + B \qquad (1)$$

where δ_i is the chemical shift at pH_i, A and B are the start and end points of the titration, and C and D are the slope and midpoint of the curve. The fitting was performed using the least squares based fit function in *gnuplot* (http://www.gnuplot.info). The pK_a of Sec sites 1 and 6 were found to be 3.3 ± 0.1 and 4.3 ± 0.1, respectively. Alkylation was performed by first reducing **1** as above followed by addition of appropriate buffer and 1 mM iodoacetamide. The reaction mixture was subjected to MS analysis after 30 min (see the Supporting Information for details).

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[1] a) G. V. Kryukov, S. Castellano, S. V. Novoselov, A. V. Lobanov, O. Zehtab, R. Guigo, V. N. Gladyshev, *Science* **2003**, *300*, 1439; b) D. Behne, H. Pfiefer, D. Rothlein, A. Kyriakopoulos in *Proceedings of the 10th International Symposium on Trace Elements in Man and Animals*, Plenum, New York, **2000**, p. 29.
[2] a) M. P. Rayman, *Lancet* **2000**, *356*, 233; b) J. Chen, M. J. Berry, *J. Neurochem.* **2003**, *86*, 1; c) L. Schomburg, U. Schweizer, J. Koehrle, *Cell. Mol. Life Sci.* **2004**, *61*, 1988.
[3] L. V. Papp, J. Lu, A. Holmgren, K. K. Khanna, *Antioxid. Redox Signaling* **2007**, *9*, 775.
[4] a) L. Zhong, E. S. Arner, A. Holmgren, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5854; b) S. R. Lee, S. Bar-Noy, J. Kwon, R. L. Levine, T. C. Stadtman, S. G. Rhee, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2521; c) S. Gromer, L. Johansson, H. Bauer, L. D. Arscott, S. Rauch, D. P. Ballou, C. H. Williams, Jr., R. H. Schirmer, E. S. J. Arner, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12618; d) M. J. Axley, A. Bock, T. C. Stadtman, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 8450.
[5] a) R. E. Huber, R. S. Criddle, *Arch. Biochem. Biophys.* **1967**, *122*, 164; b) R. G. Pearson, H. R. Sobel, J. Songstad, *J. Am. Chem. Soc.* **1968**, *90*, 319; c) R. J. Hondal, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2001**, *123*, 5140.
[6] a) J. Beld, K. J. Woycechowsky, D. Hilvert, *Biochemistry* **2008**, *47*, 6985; b) T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, N. Fujii, *Chem. Pharm. Bull.* **1993**, *41*, 502.
[7] a) B. Nygard, *Ark. Kemi* **1967**, *27*, 341; b) A. P. Arnold, K. S. Tan, D. L. Rabenstein, *Inorg. Chem.* **1986**, *25*, 2433.
[8] a) D. Besse, F. Siedler, T. Diercks, H. Kessler, L. Moroder, *Angew. Chem.* **1997**, *109*, 915; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 883; b) M. Muttenthaler, S. T. Nevin, A. A. Grishin, S. T. Ngo, P. T. Choy, N. L. Daly, S.-H. Hu, C. J. Armishaw, C.-I. A. Wang, R. J. Lewis, J. L. Martin, P. G. Noakes, D. J. Craik, D. J. Adams, P. F. Alewood, *J. Am. Chem. Soc.* **2010**, *132*, 3514; c) A. Walewska, M.-M. Zhang, J. Skalicky Jack, D. Yoshikami, M. Olivera Baldomero, G. Bulaj, *Angew. Chem.* **2009**, *121*, 2255; *Angew. Chem. Int. Ed.* **2009**, *48*, 2221; d) S. Pegoraro, S. Fiori, J. Cramer, S. Rudolph-Böhner, L. Moroder, *Protein Sci.* **1999**, *8*, 1605.
[9] K.-S. Tan, A. P. Arnold, D. L. Rabenstein, *Can. J. Chem.* **1988**, *66*, 54.
[10] a) N. Metanis, E. Keinan, P. E. Dawson, *J. Am. Chem. Soc.* **2006**, *128*, 16684; b) K. L. House, A. R. Garber, R. B. Dunlap, J. D. Odom, D. Hilvert, *Biochemistry* **1993**, *32*, 3468.
[11] a) H. Duddeck, *Prog. Nucl. Magn. Reson. Spectrosc.* **1995**, *27*, 1; b) J. O. Boles, W. H. Tolleson, J. C. Schmidt, R. B. Dunlap, J. D. Odom, *J. Biol. Chem.* **1992**, *267*, 22217.
[12] a) E. M. Stocking, J. N. Schwarz, H. Senn, M. Salzmann, L. A. Silks, *J. Chem. Soc. Perkin Trans. 1* **1997**, 2443; b) A. H. G. Siebum, W. S. Woo, J. Raap, J. Lugtenburg, *Eur. J. Org. Chem.* **2004**, 2905.
[13] M. Mobli, A. D. de Araujo, L. K. Lambert, G. K. Pierens, M. J. Windley, G. M. Nicholson, P. F. Alewood, G. F. King, *Angew. Chem.* **2009**, *121*, 9476; *Angew. Chem. Int. Ed.* **2009**, *48*, 9312.
[14] a) C. Cabrele, S. Fiori, S. Pegoraro, L. Moroder, *Chem. Biol.* **2002**, *9*, 731; b) E. Mössner, M. Huber-Wunderlich, R. Glocks-

huber, *Protein Sci.* **1998**, *7*, 1233; c) M. Huber-Wunderlich, R. Glockshuber, *Folding Des.* **1998**, *3*, 161; d) T. Kortemme, T. E. Creighton, *J. Mol. Biol.* **1995**, *253*, 799.
[15] a) O. Epp, R. Ladenstein, A. Wendel, *Eur. J. Biochem. FEBS* **1983**, *133*, 51; b) G.-m. Luo, X.-j. Ren, J.-q. Liu, Y. Mu, J.-c. Shen, *Curr. Med. Chem.* **2003**, *10*, 1151.
[16] R. Syed, Z. P. Wu, J. M. Hogle, D. Hilvert, *Biochemistry* **1993**, *32*, 6157.
[17] A. Walewska, J. J. Skalicky, D. R. Davis, M. M. Zhang, E. Lopez-Vera, M. Watkins, T. S. Han, D. Yoshikami, B. M. Olivera, G. Bulaj, *J. Am. Chem. Soc.* **2008**, *130*, 14280.
[18] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180.
[19] C. Larive, D. L. Rabenstein, *Magn. Reson. Chem.* **1991**, *29*, 409.
[20] K. Mikkelsen, S. O. Nielsen, *J. Phys. Chem.* **1960**, *64*, 632.

