SITE-SPECIFIC CHARACTERIZATION OF SELENOL-DISELENIDE EQUILIBRIA OF BIOMOLECULES

PhD thesis

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1. Introduction

The fundamental redox reactions of biochemical metabolism form reactive oxygen species (ROS) amid the natural respiration of every organism. The intracellular redox homeostasis is maintained mainly by oxidoreductase enzymes and coenzymes of the antioxidant system. These redox reactions, while diverse in character, are one- or two-electron transitions primarily taking the form of thiol-disulfide exchange.

Selenium-containing proteins, also called selenoproteins have an important role in maintaining the redox homeostasis, an inevitable precondition of life, which combats the agents of oxidative stress in living systems. The only amino acids that contain selenium are selenocysteine and (in case of forming diselenide bridges) selenocystine. Moreover, it is proved that the selenium-containing region is essential for the catalytic function.

It is a key feature of any thiol-disulfide, or the analogous selenol-diselenide redox system, that only the deprotonated thiolate or selenolate species are active in the redox process, i.e. only the anionic thiolate or selenolate can be oxidized directly into disulfides or diselenides. Since the deprotonated fraction of a selenol depends on the solution pH, the oxidation-reduction potential of selenol-containing biomolecules is also pH-dependent. Thus, a thorough characterization of the selenol-diselenide equilibria can be achieved by means of species-specific, so-called microscopic parameters.

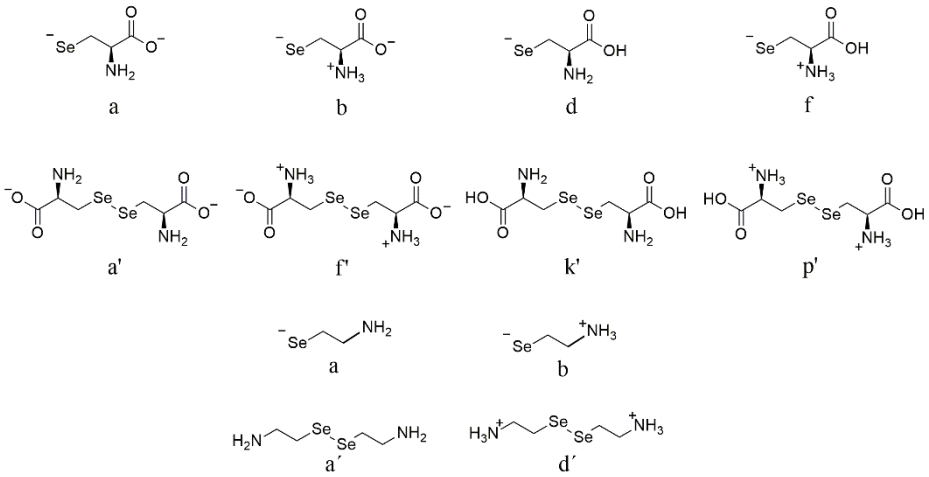

Figure 1: *Selenocysteine (top line) and selenocysteamine (third line) microspecies with deprotonated selenolate groups along with their diselenide microspecies which are formed by oxidation.*

The prevention and therapy of oxidative stress requires selective substances with known reducing power. The in-depth knowledge of the physico-chemical properties of these antioxidant compounds are particularly important in predicting the efficacy and safety of a potential therapeutic agent.

In the ^{77}Se NMR literature chemical shifts are given relative to various references with limited accuracy and consistency, however, precise chemical shifts are needed, especially for methods making further conclusions based on chemical shift data.

2. Objectives

The aim of the doctoral thesis is to characterize the co-dependent acid-base and redox properties of the selenol-diselenide equilibria in small molecules of biological importance. Moreover, our hypothesis was that there is a correlation between the microscopic acid-base, and redox parameters and also, the microscopic ^{1}H, ^{13}C and ^{77}Se NMR chemical shifts.

The first objective was to determine the macroscopic and microscopic parameters to quantify the acid-base properties of selenium-containing molecules.

The second objective was to determine the species-specific redox equilibrium constants of the selenol-diselenide equilibria against dithiothreitol and consequently the species-specific standard redox potentials. For the latter, the standard redox potential of deprotonated dithiothreitol was determined relative to glutathione, for which the microscopic redox parameters have already been described.

In order to obtain precise chemical shift data our final aim was to introduce an internal chemical shift reference for aqueous ^{77}Se NMR measurements.

3. Methods

For the determination of the macroscopic protonation constants ^{1}H NMR pH titrations were used. The site-specific protonation constants were determined on the basis of macroconstants, the occasionally symmetrical structure of some molecules and data from auxiliary compounds, using the deductive method. To obtain species-specific ^{1}H, ^{13}C and ^{77}Se NMR chemical shift data NMR spectra were recorded on the plateaus of the titration curves.

The species-specific redox equilibrium constants and standard potential values were obtained by recording and evaluating quantitative ^{1}H NMR spectra of samples in which the redox equilibria had been achieved between the diselenides and dithiothreitol.

For the evaluation of ^{1}H NMR-pH titration nonlinear curve fitting was used by OriginPro 8.0 (OriginLab Corp., Northampton, MA, USA) sofware. The regression analysis was carried out on the platform of OriginPro as well.

The integration and quantitative evaluation of the NMR spectra was done with ACD/NMR Processor Academic Edition v12.01 (Advanced Chemistry Development, Toronto, ON, Canada) software.

The potential ^{77}Se NMR chemical shift reference compounds were searched, investigating six different experimental parameters (pH, temperature, ionic strength, concentration, D_2O-content, time elapsed after sample preparation).

4. Results

The macroscopic protonation constants of the four investigated molecules (selenocysteine, selenocystine, selenocysteamine and selenocystamine) have been determined by ^{1}H NMR pH titration method. The macroconstants of selenocystine dimethyl ester have also been determined for further calculations (Table 1).

Table 1: *The protonation macroconstants of the selenol-diselenide redox pairs and selenocystine dimethyl ester*

	Seleno-cysteine	Seleno-cystine	Dimethyl-ester*	Seleno-cysteamine	Seleno-cystamine
logK_1	10.18 ± 0.02	9.19 ± 0.02	6.74 ± 0.12	10.87 ± 0.03	9.62 ± 0.04
logK_2	5.52 ± 0.03	8.22 ± 0.02	5.82 ± 0.32	6.26 ± 0.02	8.48 ± 0.12
logK_3	2.01 ± 0.04	2.25 ± 0.05	-		
logK_4	-	1.58 ± 0.07	-		

* dimethyl ester derivative of selenocystine

The relationships between the macroscopic (K) and microscopic (k) protonation constants exemplified for selenocysteine are as follows:

$$\beta_1 = K_1 = k^{O} + k^{Se} + k^{N} \quad (1)$$

$$\beta_2 = K_1 \cdot K_2 = k^{O} \cdot k_{O}^{Se} + k^{N} \cdot k_{N}^{O} + k^{N} \cdot k_{N}^{Se} = \ldots \quad (2)$$

$$\beta_3 = K_1 \cdot K_2 \cdot K_3 = k^{O} \cdot k_{O}^{Se} \cdot k_{SeO}^{N} = k^{N} \cdot k_{N}^{O} \cdot k_{NO}^{Se} = \ldots \quad (3)$$

where O, Se and N denote the carboxylate, selenolate and amino groups, respectively, and as an example k_{O}^{Se} microconstant describes the

protonating selenolate group (Se) when the carboxylate group (O) is protonated.

For molecules having two basic sites and symmetrical structure the protonation microconstants can be determined directly from the macroconstant values. For example, the microscopic protonation constants of a symmetrical compound with two amino groups (N and N′) are:

$$\log k^{\mathrm{N}} = \log k^{\mathrm{N}'} = \log K_1 - \log 2 \tag{4}$$

$$\log k_{\mathrm{N}}^{\mathrm{N}'} = \log k_{\mathrm{N}'}^{\mathrm{N}} = \log K_2 + \log 2 \tag{5}$$

For polyprotic molecules the pair-interactivity parameter shows to what extent the protonation of a basic side reduces the basicity of the other basic site. The pair-interactivity parameter between the carboxylate and amino group of selenocysteine is defined as shown below:

$$\begin{aligned} \log \Delta E_{\mathrm{N/O}} &= \log k^{\mathrm{O}} - \log k_{\mathrm{N}}^{\mathrm{O}} = \log k_{\mathrm{Se}}^{\mathrm{O}} - \log k_{\mathrm{NSe}}^{\mathrm{O}} = \\ &= \log k^{\mathrm{N}} - \log k_{\mathrm{O}}^{\mathrm{N}} = \log k_{\mathrm{Se}}^{\mathrm{N}} - \log k_{\mathrm{SeO}}^{\mathrm{N}} \end{aligned} \tag{6}$$

Using the deductive method, the pair-interactivity parameters can be imported from molecules having analogous moieties.

Based on the above-mentioned relationships all 34 microscopic protonation constants of the four investigated molecules could be determined (Table 2).

Table 2: *The microscopic protonation constants of the investigated molecules. The constants describing the major protonation pathways are in bold.*

Selenocysteine		Selenocystine	
$\mathbf{\log k^{N}}$	**10.18 ± 0.02**	$\mathbf{\log k^{N} = \log k^{N'}}$	**8.89 ± 0.02**
$\log k^{Se}$	6.81 ± 0.04	$\log k^{O} = \log k^{O'}$	4.40 ± 0.13
$\log k^{O}$	5.02 ± 0.05	$\mathbf{\log k_{N}^{N'} = \log k_{N'}^{N}}$	**8.52 ± 0.04**
$\mathbf{\log k_{N}^{Se}}$	**5.52 ± 0.03**	$\log k_{N'}^{O} = \log k_{N}^{O'}$	3.84 ± 0.05
$\log k_{N}^{O}$	3.13 ± 0.05	$\log k_{N}^{O} = \log k_{N'}^{O'}$	2.51 ± 0.13
$\log k_{Se}^{N}$	8.89 ± 0.02	$\log k_{O'}^{N} = \log k_{O}^{N'}$	8.33 ± 0.12
$\log k_{Se}^{O}$	3.90 ± 0.04	$\log k_{O}^{N} = \log k_{O'}^{N'}$	7.00 ± 0.02
$\log k_{O}^{N}$	8.29 ± 0.02	$\log k_{O'}^{O} = \log k_{O}^{O'}$	4.33 ± 0.14
$\log k_{O}^{Se}$	5.69 ± 0.05	$\mathbf{\log k_{NN'}^{O} = \log k_{NN'}^{O'}}$	**1.95 ± 0.05**
$\mathbf{\log k_{NSe}^{O}}$	**2.01 ± 0.04**	$\log k_{N'O}^{N} = \log k_{NO'}^{N'}$	6.63 ± 0.04
$\log k_{NO}^{Se}$	4.40 ± 0.06	$\log k_{NO'}^{O} = \log k_{N'O}^{O'}$	2.44 ± 0.14
$\log k_{SeO}^{N}$	7.00 ± 0.04	$\log k_{N'O'}^{N} = \log k_{NO}^{N'}$	7.96 ± 0.12
Selenocysteamine		$\log k_{N'O'}^{O} = \log k_{NO}^{O'}$	3.77 ± 0.07
$\mathbf{\log k^{N}}$	**10.87 ± 0.03**	$\log k_{OO'}^{N} = \log k_{OO'}^{N'}$	6.44 ± 0.12
$\log k^{Se}$	7.55 ± 0.03	$\mathbf{\log k_{NN'O}^{O'} = \log k_{NN'O'}^{O}}$	**1.88 ± 0.07**
$\mathbf{\log k_{N}^{Se}}$	**6.26 ± 0.02**	$\log k_{NOO'}^{N'} = \log k_{N'OO'}^{N}$	6.07 ± 0.12
$\log k_{Se}^{N}$	9.58 ± 0.04	Selenocystamine	
		$\mathbf{\log k^{N} = \log k^{N'}}$	**9.32 ± 0.04**
		$\mathbf{\log k_{N}^{N'} = \log k_{N'}^{N}}$	**8.78 ± 0.12**

For the characterization of the redox properties, only the conditional, pH-dependent redox equilibrium constants could be directly determined. The concentration values were obtained from quantitative ^{1}H NMR spectra.

$$K_C = \frac{[RSeH]^2 \cdot [DTT_{ox.}]}{[DTT] \cdot [RSeSeR]} \tag{7}$$

Taking into consideration that the acid-base and redox equilibria are coexisting and codependent, further calculations were needed to obtain the species-specific, pH-independent redox equilibrium constant can be defined. The above mentioned microscopic constant specified for the selenocysteine microspecies *b* which is produced from selenocystine microspecies *f′* by reduction is the following:

$$k^b = \frac{[b]^2 \cdot [DTT_{ox.}]}{[DTT^{2-}] \cdot [f']} = \frac{[CysSeH]^2 \cdot \chi_b^2 \cdot [DTT_{ox.}]}{[DTT] \cdot \chi_{DTT^{2-}} \cdot [CysSeSeCys] \cdot \chi_{f'}} = \\ = K_C \cdot \frac{\chi_b^2}{\chi_{DTT^{2-}} \cdot \chi_{f'}} \tag{8}$$

where χ_b and $\chi_{DTT^{2-}}$ are the respective mole fractions of microspecies *b* and the deprotonated dithiothreitol at a given pH, which can be calculated in the knowledge of the microscopic protonation constants. Using the obtained species-specific redox equilibrium constants and the standard potential of the other reactant (i.e. dithiothreitol) the microscopic standard redox potential of the investigated selenol-diselenid redox systems can be calculated (Table 3).

$$E^\circ_b = E^\circ_{DTT_{ox.}/DTT^{2-}} + \frac{R \cdot T}{z \cdot F} \cdot \ln \frac{[b]^2 \cdot [DTT_{ox.}]}{[f'] \cdot [DTT^{2-}]} = \\ = E^\circ_{DTT_{ox.}/DTT^{2-}} + \frac{R \cdot T}{z \cdot F} \cdot \ln k^b \tag{9}$$

where E° is the standard redox potential, R is the universal gas constant, T is the absolute temperature, z is the number of electrons transferred in the redox half reaction, F is the Faraday constant.

Table 3: *Species-specific standard redox potential of the two selenol-diselenide redox systems*

Selenocystine/ Selenocysteine		Selenocystamine/ Selenocysteamine	
species	E° (V)	species	E° (V)
a	-0.482 ± 0.01	a	-0.513 ± 0.006
b	-0.395 ± 0.01	b	-0.405 ± 0.006
d	-0.444 ± 0.01		
f	-0.323 ± 0.01		

In the evaluation process we have found correlation between the determined microscopic parameters (Figure 2 and 3).

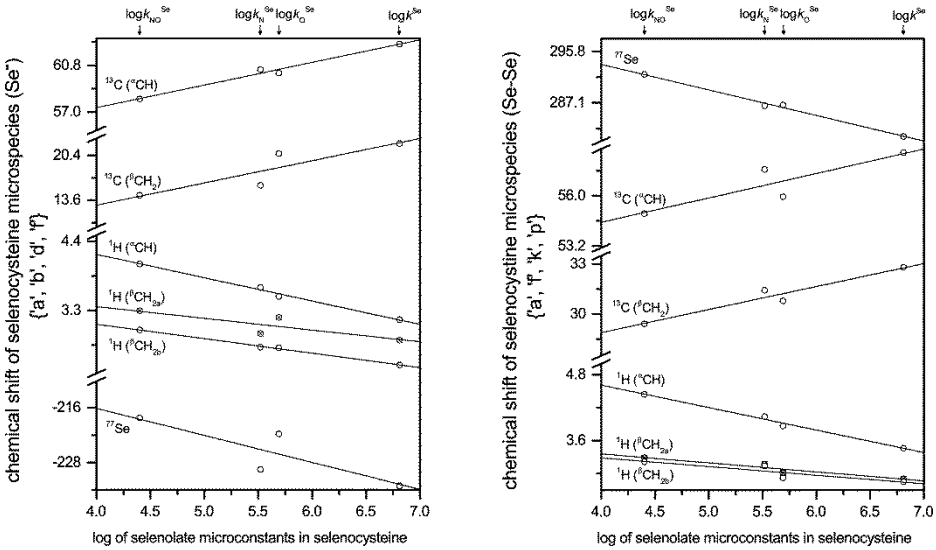

Figure 2: *The correlation between the basicity of the selenolate groups and the species-specific chemical shifts.*

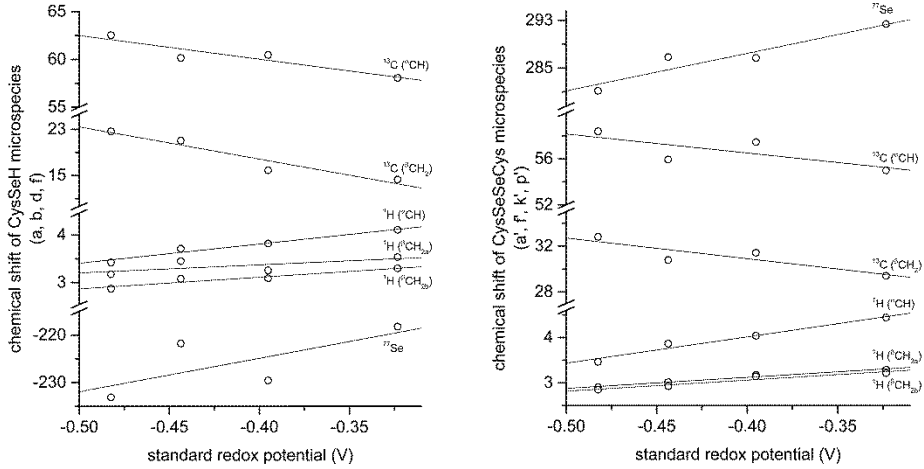

Figure 3: *The correlation between the species-specific standard redox potentials and the species-specific chemical shifts.*

Comparing the parameters describing the selenol-diselenide and the thiol-disulfide pairs, it can be observed that the log*k* vs *E*° curves are parallel, which means that the changes of basicity influence the redox properties to the same extent. However, the selenium atom ensures higher reducing power than sulfur.

To find a robust, reliable ^{77}Se NMR chemical shift reference compound, ^{77}Se NMR chemical shift of the selenate ions and ^{1}H NMR chemical shifts of DSS were recorded as a function of six different experimental parameters. The resulting data are also given in the percentage of the spectral window to provide straightforward comparability (Table 4).

Table 4: *The chemical shift of selenate ions (^{77}Se) and DSS (^{1}H) investigated respective to six different experimental parameters. To get comparable data we also give the result in the percentage of the spectral window of the given NMR nucleus. The pH effect and stability were analyzed at 0,15 mol/dm^3 (above) and 1 mol/dm^3 (below) ionic strength.*

Parameter	Range	$\Delta\delta^{77}_{SeO_4^{2-}}$ Se	relative uncertainty	$\Delta\delta^{1}_{DSS}$ H	relative uncertainty
T (K)	293–328	1.49	0.053%	0.29	0.63%
c (mol/dm^3)	0.0025–0.5	0.46	0.016%	0.10	0.22%
I (mol/dm^3)	0.15–1	0.59	0.021%	0.08	0.17%
pH	1.36–11.68	2.78	0.099%	0.03	0.07%
	0.66–13.09	4.97	0.178%	0.14	0.30%
V/V% D_2O	5–100%	0.35	0.013%	0.02	0.04%
t (days)	127	0.05	0.002%	0.02	0.04%
	133	0.08	0.003%	0.01	0.02%
Reliable pH ranges					
pH	3.40–11.68	0.03	0.001%	0.04	0.09%
	3.55–13.09	0.11	0.004%	0.11	0.24%

Based on the results selenate is applicable in wide pH range as the first introduced internal chemical shift reference for aqueous ^{77}Se NMR measurements. The exact chemical shift referred to dimethyl selenide is 1048.65 ppm, and respective to the TMS ^{1}H signal is 1046.40 ppm.

5. Conclusions

- The acid-base properties of selenocysteine, the 21st amino acid and its derivatives were characterized at the macroscopic and microscopic level.
- The species-specific ^{77}Se, ^{1}H and ^{13}C NMR chemical shifts were determined for most NMR active nuclei in the microspecies of the four molecules studied.
- The pH-independent, species-specific standard redox potential of deprotonated dithiothreitol was determined by using quantitative ^{1}H NMR and a calculation method developed for this purpose.
- Quantitative ^{1}H NMR spectroscopy was used to determine the pH-dependent, apparent redox equilibrium constant of the two selenol/diselenide systems against dithiothreitol, and subsequently the pH-independent, species-specific redox equilibrium constants and standard redox potentials.
- Correlation was found between the microscopic protonation constants and the standard redox potentials describing a given selenolate group.
- Another correlation was found between the microscopic redox potentials characterizing the selenolate-diselenide transformations and the species-specific chemical shifts either for the selenol or the diselenide form. The correlation gives an earlier nonexisting chance to estimate the site-specific redox potentials of macromolecules containing several selenium atoms. This correlation also allows the

behavioural prediction of these compounds in the body, and in the longer term to treat diseases caused by oxidative stress.

- The enhanced reducing properties of selenol diselenide pairs was interpreted and compared to the previously characterized thiol disulfide systems.
- Selenate was introduced as a robust internal chemical shift reference for aqueous ^{77}Se NMR measurements.

6. Publications

6.1. Publications pertaining primarily to the doctoral thesis

1. **Pálla T**, Mirzahosseini A, Noszál B. (2020) The species-specific acid-base and multinuclear magnetic resonance properties of selenocysteamine, selenocysteine, and their homodiselenides. Chem Phys Lett, 741: 137076.

2. **Pálla T**, Mirzahosseini A, Noszál B. (2020) Species-Specific, pH-Independent, Standard Redox Potential of Selenocysteine and Selenocysteamine. Antioxidants, 9: 465.

6.2. Publications pertaining to related subjects

1. Mirzahosseini A, **Pálla T**, Orgován G, Tóth G, Noszál B. (2018) Dopamine: Acid-base properties and membrane penetration capacity. J Pharm Biomed Anal, 158: 346-350.

2. **Pálla T**, Tóth G, Kraszni M, Mirzahosseini A, Noszál B. (2018) Population, basicity and partition of short-lived conformers. Characterization of baclofen and pregabalin, the biaxial, doubly rotating drug molecules. Eur J Pharm Sci, 123: 327-334.

3. Mirzahosseini A, **Pálla T**, Orgován G, Tóth G, Noszál B. (2019) Characterization of the species-specific acid-base equilibria of adrenaline and noradrenaline. J Pharm Biomed Anal, 170: 215-219.

4. **Pálla T**, Fogarasi E, Noszál B, Tóth G. (2019) Characterization of the Site-Specific Acid-Base Equilibria of 3-Nitrotyrosine. Chem Biodivers, 16: e1900358.