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Selenium and Sulfur in Exchange Reactions: A Comparative Study

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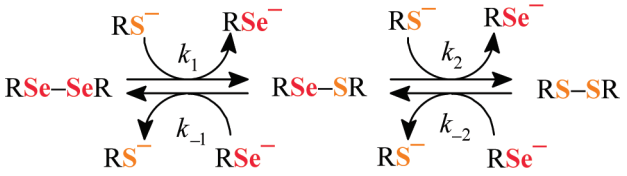

Cysteamine reduces selenocystamine to form hemiselenocystamine and then cystamine. The rate constants are $k_1 = 1.3 \times 10^5\ M^{-1}\ s^{-1}$; $k_{-1} = 2.6 \times 10^7\ M^{-1}\ s^{-1}$; $k_2 = 11\ M^{-1}\ s^{-1}$; and $k_{-2} = 1.4 \times 10^3\ M^{-1}\ s^{-1}$, respectively. Rate constants for reactions of cysteine/selenocystine are similar. Reaction rates of selenium as a nucleophile and as an electrophile are 2−3 and 4 orders of magnitude higher, respectively, than those of sulfur. Sulfides and selenides are comparable as leaving groups.

According to recent proteomics reports, selenium is present in at least 25 human protein families in the form of the amino acid selenocysteine (Sec)[1,2] Although the functions of only a few selenoproteins have been well characterized to date, many can be classified as redox enzymes in which Sec is part of the catalytic center.[3] Sec and Cys share many properties, as there are only minor differences between sulfur and selenium in terms of electronegativity, ionic radius, and available oxidation states.[4,5] An important difference, though, is that the pK_a of Sec is lower (5.3)[4] than that of Cys (8.3),[6] which makes Sec a considerably more potent nucleophile under neutral and acidic conditions.[4] Furthermore, selenium is softer than sulfur, with a polarizability volume of 3.8 Å (Se) compared to 2.9 Å (S).[5] The two elements have similar functions, and, for most selenoenzymes, there exist Cys-containing homologues.[7] During the catalytic cycles of selenoenzymes, formation and cleavage of selenylsulfides via exchange reactions are common.[8−11] However, in contrast to thiol-disulfide exchange reactions, only a few studies of analogous reactions involving selenium have been published.[12−17] It has been shown by NMR that the selenol-diselenide exchange reaction of selenocysteamine (SeCya) with selenocystamine ($SeCya_{ox}$) is 7 orders of magnitude faster at neutral pH than the analogous exchange reaction of cysteamine (Cya) with cystamine (Cya_{ox})[18] and that selenols catalyze the latter reaction.[19]

Recently, the authors of a computational study predicted that nucleophilic attack by thiols at selenium is both kinetically faster and thermodynamically more favorable than at sulfur.[20]

Herein, we compare sulfur and selenium as nucleophiles, electrophiles, and leaving groups in thiol-disulfide-like exchange reactions to better understand differences in reactivity between selenium and sulfur in biological systems. We use a published value for the electrode potential of the thiol/disulfide couple of dithiothreitol (DTT), $E^{o\prime}(DTT_{ox}/DTT)$,[21] as the linchpin to calculate critical equilibrium and rate constants.

We compare the kinetics of reduction of Sec_{ox} by Cys and of reduction of $SeCya_{ox}$ by Cya (Scheme 1), measured by stopped-flow spectrophotometry, from which we conclude that selenium promotes both nucleophilic and electrophilic exchange reactions, relative to sulfur.

The reduction of the diselenides by the thiols proceeds via a two-step process (Scheme 1) at pH 7 (Figure 1); the first phase of the reaction, the formation of the hemiselenocystine

(1) Abbreviations, systematic nomenclature (trivial names): DTT, dithiothreitol; DTT_{ox}, trans-1,2-dithiane-4,5-diol (oxidized dithiothreitol); Sec, selenocysteine; Sec_{ox}, selenocystine; Cys_{ox}, cystine; SeCya, selenocysteamine; $SeCya_{ox}$, selenocystamine; Cya, cysteamine; Cya_{ox}, cystamine; $hSeCys_{ox}$, hemiselenocystine; $hSeCya_{ox}$, hemiselenocystamine; GPx, glutathione peroxidases; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mops, 3-(*N*-morpholino)propanesulfonic acid.

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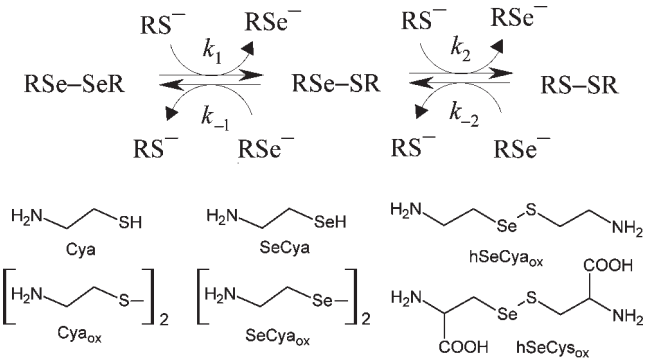
SCHEME 1



(hSeCys$_{ox}$)/hemiselenocystamine (hSeCya$_{ox}$), is complete in ca. 60 ms, while the latter phase continues for ca. 20 s (Figure 1, inset) in our experiments. Rate constants for the reactions can be derived from the rates of absorbance change, and from the absorbance of the selenolate produced after 60 ms and 20 s, the equilibrium constants K_1 and K_2, respectively, were calculated.

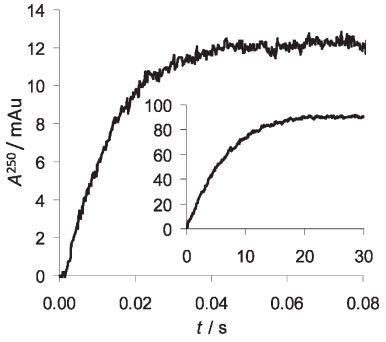


FIGURE 1. Formation of Sec, monitored at 250 nm, upon rapid mixing of Cys and Sec$_{ox}$. At low concentrations of Cys (2×10^{-5} M) and Sec$_{ox}$ (5×10^{-5} M), the first equilibrium, Cys + Sec$_{ox}$ = hSeCys$_{ox}$ + Sec, is attained within 60 ms. Inset: At 10 mM Cys and 5×10^{-5} Sec$_{ox}$, the second equilibrium, hSeCys$_{ox}$ + Cys = Cys$_{ox}$ + Sec, is reached within 20 s.

To determine the equilibrium constants for the reactions in Scheme 1, we needed to know the molar absorptivities of Sec and SeCya. The selenolates are the only species that absorb significantly at 250 nm at pH 7, and absorbance changes caused by other species were neglected. The extinction coefficients of the selenolates were measured after anaerobic reduction of the diselenides with sodium borohydride in the stopped-flow spectrophotometer. We performed the reaction at pH 10 to prevent evolution of H_2 during the spectroscopic measurements. We multiplied the molar absorptivity obtained at pH 10 ($\varepsilon_{250} = 6450 \pm 50$ M^{-1} cm^{-1} for both Sec and SeCya) by the ratio of selenolates present at neutral pH (96−99%)[22] to calculate the ε_{250} at pH 7 (6300 ± 200 M^{-1} cm^{-1}).

To determine the two-electron electrode potential $E^{o\prime}$(SeCya$_{ox}$/2SeCya), we mixed SeCya$_{ox}$ with DTT at pH 7 in a stopped-flow spectrophotometer. Initial concentrations were typically 0.1 mM DTT, 5 mM oxidized dithiothreitol

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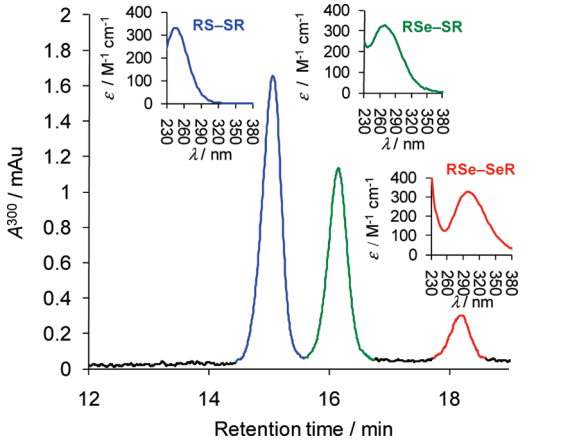

FIGURE 2. Reversed-phase HPLC analysis of Cya$_{ox}$ (RS−SR), SeCya$_{ox}$ (RSe−SeR), and hSeCya$_{ox}$ (RSe−SR) obtained from reaction of 10 mM Cya and 2 mM SeCya with O_2; Uptisphere 5 μm HDOC18 (250 mm ×10 mm) column; H_2O/CH_3CN mobile phase with 0.1% TFA; 30 °C; detection at 300 nm; molar absorptivities for quatification of products obtained from UV−vis spectra (insets).

(DTT$_{ox}$), and 0.05 mM SeCya$_{ox}$ before mixing. $E^{o\prime}$(SeCya$_{ox}$/2SeCya) = −368 ± 4 mV, from K_{eq} ([DTT$_{ox}$][SeCya]^2)/([DTT][SeCya$_{ox}$]) = 0.04 ± 0.01 M and the published value for $E^{o\prime}$(DTT$_{ox}$/DTT) = −327 mV.[21] Singh et al.[19] used a similar approach with K_{eq} ([DTT$_{ox}$][SeCya]^2)/([DTT]-[SeCya$_{ox}$]) = 0.14 M to obtain a value of −352 mV.

Upon rapid mixing of Cya and SeCya$_{ox}$, we find K_1 = (5 ± 1) × 10^{-3}, and we calculated $\Delta E_1^{o\prime}$ = −69 ± 3 mV. From $E^{o\prime}$(SeCya$_{ox}$/2SeCya) = −368 ± 4 mV and $\Delta E_1^{o\prime}$ = −69 ± 3 mV, we obtain $E^{o\prime}$(hSeCya$_{ox}$/Cya,SeCya) = −299 ± 5 mV. We used this $E^{o\prime}$(hSeCya$_{ox}$/Cya,SeCya) and subtracted $E^{o\prime}$(Cya$_{ox}$/2Cya)[16] to calculate $\Delta E_2^{o\prime}$ = −63 ± 5 mV, from which we calculated K_2 = (8 ± 4) × 10^{-3}. From the direct observation of equilibrium 2 upon mixing Cya with SeCya$_{ox}$, the determination of K_2 and, thus, of ΔE_2° is difficult because, during the 20 − 30 s that elapse before equilibrium is reached, oxidation of the selenide by O_2 could corrupt the absorbance measurement at the end of the experiment. Further, the presence of even very small amounts of Cya$_{ox}$ in the Cya shifts the equilibrium and leads to incorrect results, and we indeed obtained irreproducible values for ΔE_2° from measurements of K_2 upon mixing Cya with SeCya$_{ox}$.

From the values of $\Delta E_1^{o\prime}$ and $\Delta E_2^{o\prime}$, we can predict the equilibrium distribution of diselenides, selenylsulfides and disulfides in solution mixtures, e.g.:

$$\text{Cya}_{ox} + \text{SeCya}_{ox} = 2\text{hSeCya}_{ox} \qquad (3)$$

We mixed 10 mM Cya with 2 mM SeCya, oxidized them in air at pH 7, and analyzed the products by HPLC (Figure 2) to measure the equilibrium concentrations of Cya$_{ox}$, SeCya$_{ox}$, and hSeCya$_{ox}$; from K_3 = 0.30 ± 0.09, we calculate $\Delta E_3^{o\prime}$ = −8 ± 2 mV. This value agrees well with the $K_3 = K_1/K_2$ measured by stopped-flow spectrophotometry ($\Delta E_3^{o\prime}$ = ($\Delta E_1^{o\prime} - \Delta E_2^{o\prime}$) = −6 ± 6 mV).

The consecutive equilibrium constants K_1 and K_2 for the reduction of Sec$_{ox}$ by Cys are equivalent, $K_1 = K_2 = (4 \pm 1) \times 10^{-3}$, which corresponds to electrode potential differences of $\Delta E_1^{o\prime} = \Delta E_2^{o\prime} = -72 \pm 4$ mV. From this difference and

TABLE 1. Rate Constants for Exchange Reactions of Selenols and Thiols with Disulfides, Selenylsulfides, Diselenides and Hydrogen Peroxide at pH 7

reaction				rate constant (M^{-1} s^{-1})	
Nu[a]	El[b]	LG[c]		Cya/SeCya$_{ox}$	Cys/Sec$_{ox}$
RSH	RSe−SeR	RSe$^-$	k_1	$(1.3 \pm 0.2) \times 10^5$	$(1.0 \pm 0.2) \times 10^5$
RSe$^-$	RSe−SR	RS$^-$	k_{-1}	$(2.6 \pm 0.2) \times 10^7$	$(2.6 \pm 0.2) \times 10^7$
RSH	RS−SeR	RRSe$^-$	k_2	11 ± 3	7 ± 2
RSe$^-$	RS−SR	RS$^-$	k_{-2}	$(1.4 \pm 0.3) \times 10^3$	$(1.8 \pm 0.4) \times 10^3$
RSH	RS−SR	RS$^-$	k_4	3.6 ± 0.1[d]	
RSe$^-$	RSe−SeR	RSe$^-$	k_5	$(1.7 \pm 0.2) \times 10^7$[e]	
RSH	HO−OH	HO$^-$	k_6	1.1[f]; 2.9[g]	1.0[c]; 2.9[g]
RSe$^-$	HO−OH	HO$^-$	k_7	9.7×10^2[h]	

[a]Nucleophile, RSH stands for the equilibrium mixture RSH and RS$^-$. [b]Electrophile. [c]Leaving group. [d]pD = 7.43.[18] [e]pD < 7.6.[18] [f]pH = 7.4, 20 °C.[24] [g]pH 7.4, 37 °C.[25] [h]pH = 6.8, 20 °C.[26]

$E^{\circ\prime}$(Sec$_{ox}$/2Sec) = -383 ± 8 mV,[23] we calculate $E^{\circ\prime}$(hSeCys$_{ox}$/Cys,Sec) = -311 ± 9 mV. Solutions containing Cys are less sensitive to oxidation, and we were able to measure K_2 directly via the absorbance changes in kinetics traces attributed to equilibrium 2, from which we calculate $E^{\circ\prime}$(Cys$_{ox}$/2Cys) − $E^{\circ\prime}$(Sec$_{ox}$/2Sec) = −145 mV, in excellent agreement with the sum $\Delta E_1^{\circ\prime} + \Delta E_2^{\circ\prime} = -144 \pm 9$ mV. We were also able to determine K_{eq}([DTT][Sec$_{ox}$])/([DTT$_{ox}$][Sec]^2) = 90 M^{-1} by stopped-flow spectrophotometry, which fits with the electrode potential of $E^{\circ\prime}$(Sec$_{ox}$/2Sec) = -383 ± 8 mV.[23] We can derive mechanistic information regarding the relative effectiveness of selenium versus sulfur as an electrophile, nucleophile, or leaving group by comparing the reactions and corresponding rate constants in Table 1.

First, we compare reactions of each nucleophile with a given electrophile and leaving group (e.g., reactions 1 vs 5 or −2 vs 4): the rate constants for nucleophilic reactions of SeCya are 2−3 orders of magnitude higher than those of Cya, as reported by Singh and Kats.[14] Similar enhancement is noted for nucleophilic attack on H_2O_2 (reactions 6 vs 7), reactions that are catalyzed by Sec- or Cys-containing glutathione peroxidases (GPx) in vivo. The thiolate, not the thiol, is the reactive nucleophile, and, thus, the difference in reactivity reduces to 1−2 orders of magnitude in a basic environment where both selenols and thiols are deprotonated.

Next, we compare reactions of each electrophile with a given nucleophile (reactions 1 vs 2 and −1 vs −2): the reactions of selenium as an electrophile are 4 orders of magnitude faster than those of sulfur. Similarly, nucleophilic attack of cyanide on $PhSeSO_2Ar$ is 5 orders of magnitude faster than on $PhSSO_2Ar$.[27]

Finally, we note that rate constants for selenolate and thiolate as the leaving group at neutral pH are comparable (reactions 5 vs −1 and 2 vs 4).

In enzymes, e.g., GPx, the microenvironment of the active site plays a critical role and has to be taken into account when comparing Sec- and Cys-containing variants. A Cys residue in an active site must be deprotonated under physiological conditions to act as a nucleophile,[28,29] and thus, only the smaller difference in nucleophilicity of ca. 1−2 orders of magnitude is expected for Sec versus Cys in enzymes with similar functions. In GPx, nucleophilic attack of the thiolate/selenolate on the peroxide substrate is the rate-limiting step: indeed, for the Sec-containing pig heart enzyme GPx-4, the rate constant for the attack at phosphatidylcholine hydroperoxide is 1×10^7 M^{-1} s^{-1}, compared to 1.3×10^6 M^{-1} s^{-1} [30,31] for the corresponding Cys-containing enzyme of *Drosophila melanogaster*. Similarly, for the Sec-containing bovine cGPx, the rate constant for attack at H_2O_2 is 1 order of magnitude higher than that of the Cys-containing human peroxiredoxin 2.[32,33] Although comparisons of enzymes from different organisms or that have different functions may have limited validity, these literature values agree reasonably well with our findings of a 10-fold rate enhancement when selenium acts as a nucleophile.

Additionally, leaving group protonation by proximate amino acid residues may influence the rate of reaction. It is argued that, in a hydrophobic active-site environment, deprotonated Cys is a worse leaving group; in an environment where protonation of the leaving group is assisted, differences in reactivity between Sec and Cys may be small.[34]

Thus, from Table 1 it is clear that the rate enhancement of selenium over sulfur as an electrophile (ca. 10^4) is 2 orders of magnitude greater than that of the nucleophilic enhancement; selenium as a leaving group is approximately equivalent to sulfur.

The thermodynamics as well as the kinetics of an enzyme reaction are altered as a function of whether selenium replaces sulfur in an active site. The electrode potentials of selenylsulfides are ca. 70 mV lower than the potentials of corresponding disulfides according to our study and that of Metanis et al.;[15] thus, the selenylsulfides are considerably more difficult to reduce.[9] This difference in electrode potentials may be used advantageously to tune enzyme equilibria in different compartments.

Experimental Section

All chemicals were of highest commercially available quality and were, aside from Cya, used as received. Millipore Milli-Q water (18.2 MΩ resistance) was used in all experiments.

Because small amounts of disulfide impurities can strongly influence the redox equilibria under study, thiols were analyzed before experiments for the presence of disulfides by HPLC. Cya contained 1.5% Cya$_{ox}$ and was, therefore, reduced with $NaBH_4$ (0.01−0.1 M) and trace amounts of Sec$_{ox}$ ($< 10^{-5}$ M) as a catalyst. DTT contained 0.5% DTT$_{ox}$, which was taken into account in the equilibrium calculations, and Cys contained only negligible levels of impurities.

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Stopped-flow experiments were carried out with an Applied Photophysics SX 17 MV stopped flow spectrophotometer at 25 °C. The instrument was flushed with nitrogen; solutions were degassed with argon and transferred in airtight syringes (Hellma GmbH, Müllheim, Germany) to minimize dioxygen contamination. O_2 concentrations during experiments are estimated to be below 2 μM. Solutions were buffered with 5−10 mM bis-tris, mops, hepes, or phosphate buffer. Kinetics traces were analyzed with singular value decomposition and nonlinear regression modeling by means of the Levenberg−Marquard method.[35]

HPLC analysis was performed with a Hewlett-Packard (Agilent) HPLC, with a HP-1050 autosampler and quaternary pump, a HP-1100 diode-array detector and Agilent-1100 degasser. The stationary phase consisted of a C-18 250 mm × 10 mm column (Uptisphere 5 μm HDOC18) from Interchim Inc., operated at 30 °C. The mobile phase was composed of mixtures of H_2O/CH_3CN containing 0.1% trifluoroacetic acid. Detection was at 300 nm; extinction coefficients used to quatify products were obtained by UV−vis spectroscopy.

UV−vis spectroscopy was carried out with a SPECORD 200 or SPECORD 250 spectrophotometer from Analytik Jena in 0.1−2 cm quartz cells from Hellma GmbH.

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