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ORIGINAL ARTICLE

Why do proteins use selenocysteine instead of cysteine?

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Abstract Selenocysteine is present in a variety of proteins and catalyzes the oxidation of thiols to disulfides and the reduction of disulfides to thiols. Here, we compare the kinetic and thermodynamic properties of cysteine with its selenium-containing analogon, selenocysteine. Reactions of simple selenols at pH 7 are up to four orders of magnitude faster than their sulfur analogs, depending on reaction type. In redox-related proteins, the use of selenium instead of sulfur can be used to tune electrode, or redox, potentials. Selenocysteine could also have a protective effect in proteins because its one-electron oxidized product, the selanyl radical, is not oxidizing enough to modify or destroy proteins, whereas the cysteine-thiyl radical can do this very rapidly.

Keywords Kinetics · Thermodynamics · Sulfur · Selenium · Radical damage · Redox

Introduction

Selenols are different from thiols. A selenol is three orders of magnitude more acidic [pK 5.3 (Huber and Criddle 1967)] than a thiol; selenols are more reducing than thiols (Besse et al. 1997) and selanyl radicals are less oxidizing than thiyl radicals and therefore more easily produced (Nauser et al. 2006). Selenocysteine (Sec, U) is less stable than cysteine (Cys) (Huber and Criddle 1967). Upon storage in acidic aqueous solution, we find a precipitate of red elemental selenium in the container while Cys is stable.

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The biosynthesis of Sec needs the selective use of selenium and has been described as "costly and inefficient" (Gromer et al. 2003). Therefore, one expects a clear advantage of Sec over Cys.

Mammals possess two well-known selenoproteins that carry out redox reactions. Glutathione peroxidase (GPx) oxidizes thiols to disulfides with an active site that contains a "single" Sec (Flohé et al. 1973), and thioredoxin reductases (TrxR) feature a selenosulfide bond (GCUG motif) in the active site that reduces disulfides to thiols.

Selenocysteine is known to catalytically oxidize thiols to disulfides with oxygen or hydrogen peroxide (Chaudière et al. 1992; Singh and Whitesides 1991). This is indeed a function of GPx: the reduction of H_2O_2 at the expense of GSH. The enzyme is less active if Sec is replaced by Cys (Rocher et al. 1992) which would suggest that selenoproteins have higher activity than sulfur proteins, a notion often found in literature. However, human peroxiredoxin 2 is a sulfur protein that has a similar rate of reaction with H_2O_2 as the selenoprotein GPx (Peskin et al. 2007). Thioredoxin reductases (TrxR) are essential for redox homeostasis in cells via reduction of disulfides: in mammals they feature a selenosulfide bond (GCUG motif) in the active site, but sulfur analogs of these proteins in other organisms often have very similar or even higher activities (Kanzok et al. 2001). In mutant TrxR of *Drosophila melanogaster*, it was found that the GCUG active site has approximately the same activity as the SCCS, wild-type active site (Gromer et al. 2003). Thus, native selenoproteins do not necessarily have higher activities than native sulfur proteins; furthermore, replacement of Cys by Sec in an enzyme active site does not increase its activity. Other factors than kinetics are likely to be relevant too.

Therefore, mutant studies may be misleading: aside from the mutation itself, one may need to change the active

site in ways that are poorly understood, because as redox catalyst, sulfur and selenium each requires a different environment. Therefore, it is conceivable that Sec and Cys will not function in an equally efficient way in the same protein structure. As an alternative and complementary strategy to mutant studies, we try to assess the chemical difference between selenols (Sec) and thiols (Cys) using simple model compounds. For the reaction of proteins with a single sulfur or selenium active site (GPx), we use selenocystamine and cystamine. These two compounds are more soluble and less likely to form aggregates than Sec and Cys in aqueous solution. For the study of redox reactions involving intramolecular sulfur–sulfur and sulfur–selenium bonds as in TrxR, we use DTT analogs, which do form such intramolecular bonds.

We report here on selenol and thiol chemistry that may lead to new hypotheses on the role of selenium in biochemistry.

Experimental

Stopped-flow experiments were carried out with an Applied Photophysics SX 17 MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK) at 25°C. The instrument was flushed with nitrogen; solutions were degassed with argon and transferred in air-tight syringes from Hamilton (Bonaduz, Switzerland) to minimize dioxygen concentration. Solutions were buffered with 5–10 mM bis–tris [bis (2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane]. The laser-flash photolysis and pulse radiolysis equipment have been described elsewhere (Nauser et al. 2008). All chemicals used were of the highest commercially available purity. Diselenothreitol (DSeT) and selenothiothreitol (SeTT) were synthesized and purified by D. Steinmann (unpublished). All solutions were freshly prepared.

Results

Stopped flow

Cysteamine (0.1–50 mM) and 1 mM selenocystamine (diselenide) were mixed in the stopped flow and formation of selenosulfides and diselenides were monitored by absorption spectroscopy. From the kinetics traces, the rate constants for the equilibria 1 and 2 were derived (Table 1):

$$\mathrm{RSe^-} + \mathrm{RSSR} = \mathrm{RSeSR} + \mathrm{RSH} \quad (1)$$

$$\mathrm{RSe^-} + \mathrm{RSeSR} = \mathrm{RSeSeR} + \mathrm{RSH} \quad (2)$$

The measurements were carried out at pH 7. Given that pK_a (S–H) ≈ 8.4 (Huber and Criddle 1967), the thiols were mostly protonated. As thiolates are much more reactive nucleophiles than thiols, the reactivity observed at pH 7 is assigned to the deprotonated cysteamine (controls not shown). In contrast, the selenol group is fully deprotonated at physiological pH, with pK_a (Se-H) ≈ 5.3 (Huber and Criddle 1967). The exchange rate data in Table 1 show that at pH 7, $K_1 = 127$ and $K_2 = 200$. These equilibrium constants can be used to calculate the electrode potentials at pH 7 of $E^{\circ\prime}{}_4$ and $E^{\circ\prime}{}_5$ from a known $E^{\circ\prime}{}_3$:

$$\mathrm{RSSR} + 2\ \mathrm{e^-} + 2\ \mathrm{H^+} = 2\ \mathrm{RSH} \quad (3)$$

$$\mathrm{RSeSR} + 2\ \mathrm{e^-} + \mathrm{H^+} = \mathrm{RSe^-} + \mathrm{RSH} \quad (4)$$

$$\mathrm{RSeSeR} + 2\ \mathrm{e^-} = 2\ \mathrm{RSe^-} \quad (5)$$

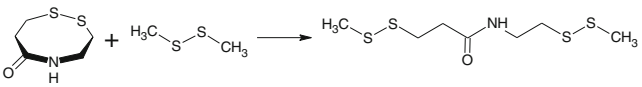
via the relation: $\mathrm{RTln}K = \Delta G^{\circ\prime} = -\mathrm{nF}\Delta E'$ and with $1 = 3 - 4$ and $2 = 4 - 5$ we calculate the difference between the electrode potentials of reactions 3, 4, and 5 at pH 7:

$$\Delta E'_1 = E^{\circ\prime}_3 - E^{\circ\prime}_4 = 63\mathrm{mV}(12\mathrm{kJ/mol}) \quad (6)$$

$$\Delta E'_2 = E^{\circ\prime}_4 - E'_5 = 68\mathrm{mV}(13\mathrm{kJ/mol}) \quad (7)$$

Calculations

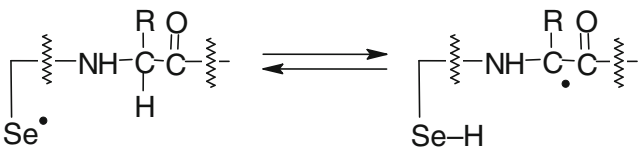
The ring strain energy, which is induced by oxidation of two vicinal Cys to an intramolecular disulfide bond, was estimated with the group equivalent reaction of the disulfide rearrangement (Bachrach 1990) of 5-aza-1,2-dithiacyclooctan-6-on (1,2,5-dithiazocan-6-on) with dimethyldisulfide to 3-(methyldisulfanyl)-*N*-[2-(methyldisulfanyl)ethyl] propanamide (reaction 8).



(8)

A reaction enthalpy of −18 kJ $\mathrm{mol^{-1}}$ was computed with Gaussian (Frisch et al. 2003) and a B3LYP/6-31+G** functional/basis set for optimization and frequency calculations. This result indicates that a ring strain of approximately 18 kJ $\mathrm{mol^{-1}}$ is to be expected in the oxidized peptide.

The equilibrium of an intramolecular hydrogen abstraction at the $^{\alpha}$C–H by a selanyl radical is calculated as $K_{9a} \approx 10^{-11}$:

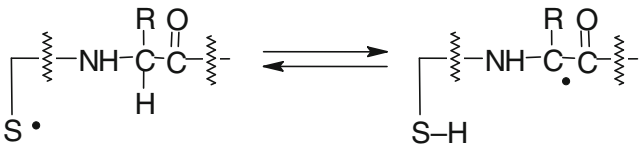


(9a)

Table 1 Reaction rates for exchange reactions, or two-electron transfers, with sulfur and selenium

Nucleophile	Electrophile	Reaction rate constant in $M^{-1}s^{-1}$		Reaction number
		(measured value, pH 7)	(calculated for pH 10)	
RS^-	R**S**-SR	3.6	90	(6)
RS^-	R**S**-SeR	11	275	(-1)
RS^-	R**Se**-SeR	1.3×10^5	3.3×10^6	(-2)
RSe^-	R**S**-SR	1400	1400	(1)
RSe^-	R**Se**-SR	2.6×10^7	2.6×10^7	(2)
RSe^-	R**Se**-SeR	1.7×10^7	1.7×10^7	(7)

In the electrophiles column, the central atom is printed in bold and the leaving group in gray
The rate constants k_3 and k_4 are taken from Pleasants et al. (Pleasants et al. 1989)



(9b)

The corresponding equilibria for thiols are approximately $K_{9b} \approx 0.1$ (Nauser et al. 2008), and as the difference of the bond dissociation energies (BDE) of S–H and Se–H are $\Delta BDE = BDE(RS\text{-}H) - BDE(RSe\text{-}H) \approx 55$ kJ mol^{-1} (Nauser et al. 2006), we can derive $K_{9a} = K_{9b} \times e^{-\Delta BDE/RT}$.

Pulse radiolysis

Radiolysis of dilute aqueous solutions produces mainly $HO^\bullet$ and $e^-_{aq}/H^\bullet$ radicals with known yields and has no direct effect on the solutes. Argon-saturated solutions of 0.1 mM DSeT and SeTT in their oxidized forms, Se–Se and Se–S, respectively, 0.1 M t-BuOH and 10 mM buffer were irradiated with doses of approximately 30 Gy (8 μM $HO^\bullet$, 8 μM e^-_{aq} and 1.7 μM $H^\bullet$). Under these conditions, the $HO^\bullet$ radicals formed are scavenged by *t*-BuOH and $e^-_{aq}/H^\bullet$ will react at a diffusion-controlled rate with DSeT/SeTT. After 2 μs (DSeT, blue squares in Fig. 1) or 50 μs (SeTT, red squares in Fig. 1), the absorptivity of the sample was measured. We measured the formation of the radical anions ($RSSR^{\bullet-}$, $RSeSR^{\bullet-}$ and $RSeSeR^{\bullet-}$). Establishment of the pK equilibrium of these species is dependent on the protonation rate constant, which is ca 10^{10} M^{-1} s^{-1}, but might take up to 1 ms at pH 7 as the H^+ concentration is only 10^{-7} M. Since we find a $pK_a > 7$ for SeTT, the protonation at pH values at or above the pK_a is slow in the microsecond timescale of the experiment and depends critically on the nature of the buffer and its concentration. As a control, we approached the equilibrium also with the "open" or reduced form: solutions of 0.1 mM SeTT (reduced form), 100 mM NaN_3 and 100 mM phosphate buffer were saturated with N_2O, irradiated with 30 Gy pulses and measured 2 μs after the pulse (red triangles in Fig. 1). Under these conditions, 19 μM $N_3^\bullet$ radicals are formed, which will rapidly oxidize the selenol to a selanyl radical. We find $pK_a(DSeT^{\bullet-}) = 2.4$ and $pK_a(SeTT^{\bullet-}) = 7.4$; the published value for DTT is $pK_a(DTT^{\bullet-}) = 5.2$ (Akhlag and von Sonntag 1987). These

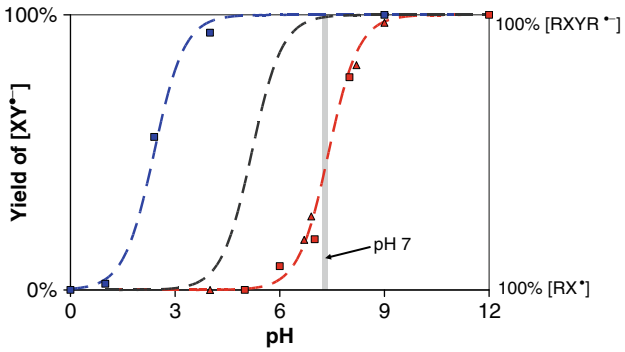


Fig. 1 pH dependence of the yields of $DSeT^{\bullet-}$ (*blue*), $DTT^{\bullet-}$ (*black*) and $SeTT^{\bullet-}$ (*red*). *Points* experimental measurements. Calculated curves are given for $DSeT^{\bullet-}$ ($pK_a = 2.4$), $DTT^{\bullet-}$ [$pK_a = 5.2$ (Akhlag and von Sonntag 1987)], and $SeTT^{\bullet-}$ ($pK_a = 7.4$). *Squares* one-electron reduction of DSeT/SeTT (oxidized disulfide/selenosulfide form) with $e^-_{aq}/H^\bullet$. *Triangles* one-electon oxidation of SeTT (reduced form) with $N_3^\bullet$ radicals

values are influenced both by the pK_a of the non-radical thiol or selenol and the equilibrium constant K_{10} (for the case, where a reaction between thiolate and thiyl radical occurs),

$$RS^{\bullet} + RS^{-} = RSSR^{\bullet -} \quad (10)$$

Laser-flash photolysis

Ar-saturated aqueous solutions of 1 mM selenocystine, 3–17 mM monohydrogen-ascorbate and 10 mM phosphate buffer (pH 7.4) were irradiated with 355 nm light pulses. The photolysis caused homolysis of the Se–Se bond (Nauser et al. 2006) and the selanyl radicals so generated reacted with monohydrogen-ascorbate to form ascorbyl radicals, which were detected at 360 nm. A rate constant of $k = (8 \pm 2) \times 10^7$ M^{-1} s^{-1} was determined.

Discussion

Tuning of equilibria and kinetics of two-electron transfers

Nucleophilic reactions of cystamine at pH 7 are approximately two to three orders of magnitude slower than those of selenocystamine, as can be seen by comparison of reactions (6) and (1) or (−2) and (7) in Table 1. Similar values for reactions with protein mutants have been reported by Metanis et al. (Metanis et al. 2006). In comparison, the leaving group (sulfur vs. selenium) has a negligible influence on the rate constant [see reactions (6) and (−1) or (2) and (7)], again in agreement with Metanis' study. Importantly, if the central atom in the electrophile is changed from sulfur to selenium, reactions (1) and (2) or (−1) and (−2) are four orders of magnitude faster. Improved reactivity as nucleophile or electrophile may be why selenium is used in reactions of GPx. However, we must not forget that cysteamine may react slower because of the thiol group being protonated. In specialized proteins, where active site thiols are fully deprotonated, the advantage of selenium over sulfur may be limited (Peskin et al. 2007).

Minor changes in protein folding have the potential to strongly influence kinetics and thermodynamics. Intramolecular bond formation is subject to geometrical constraints. Certain geometries favor bond formation, while others do not. The "ring strain" effected by the S–S, Se–S or Se–Se bond formation can have an important influence on equilibria. For instance, a small strain of 10 kJ mol^{-1} changes the redox potential by 59 mV and the equilibrium constant by a factor of 50 and that may result in a change of reaction rate up to a factor of 50. Thus, chemical reactions can be extremely sensitive to small structural changes. From the equilibria in Table 1 and the two-electron electrode potential of DTT at pH 7 $E^{\circ\prime}$(DTT) = −332 mV (Cleland 1964), we calculate $E^{\circ\prime}$(SeTT) = −394 mV and $E^{\circ\prime}$(DSeT) = −464 mV. The electrode potential of RSeSR ($E^{\circ\prime}{}_4$) lies exactly in the middle of those of RSSR ($E^{\circ\prime}{}_3$) and RSeSeR ($E^{\circ\prime}{}_5$). In principle, selenosulfide or diselenide may be used to tune stabilities and equilibria in biological redox chains. In TrxR for example, the reduction of the active site takes place via reduction of FAD and a disulfide bond close to the FAD, followed by a long range two-electron transfer to the active site. The disulfide bond close to the FAD is located in a rigid area of the protein and probably has little strain. The active site selenosulfide on the mobile C-terminal end of the protein has a ring strain of approximately 18 kJ mol^{-1}, which results in a change of the two-electron electrode potential by +93 mV. One reason for the use of Sec in the GCUG active site might be the compensation of the ring strain of +93 mV with a lower electrode potential $E^{\circ\prime}{}_4$ for selenium—sulfur compared to $E^{\circ\prime}{}_3$ for sulfur—sulfur ($\Delta E_1{}' = -63$ mV). In this case, the electrode potential of the active site is closer to that of the disulfide close to the FAD and of the substrate thioredoxin (Trx). Under the assumption that the potential of the wild-type TrxR and Trx are similar, a mutant with an SCCS sequence in the TrxR active site would have a 63 mV higher potential than Trx. The equilibrium constant of a two-electron process with such a potential difference of 63 mV is $K = [\text{TrxR}]/[\text{Trx}] \approx 125$. In vivo, we would not expect both TrxR and Trx to be fully reduced. The major reductant (99%) then will be TrxR not Trx, and direct reduction of disulfides (GSSG) by TrxR, bypassing Trx, is expected. From the point of view of thermodynamics, the GCUG active site is a more powerful reductant than SCCS. It may react with more substrates and it allows the matching Trx to be a stronger reductant too.

In mutant studies of the TrxR of *Drosophila melanogaster*, the activity of the wild-type SCCS active site is similar to that of the mutant GCUG active site. In contrast, a protein with the mutant active site sequence GCCG is almost inactive (Gromer et al. 2003). The ionization and solubility of Sec are different from Cys, which may influence the folding of TrxR. Because Sec is fully deprotonated at physiological pH, we speculate that Sec in the GCUG C-terminus is used to (1) increase its hydrophilicity and its accessibility to Trx and/or (2) improve its affinity to that protein. We speculate that the two serines in the active site SCCS serve a similar purpose. It should be noted that because TrxR and Trx are matched pairs, TrxR may perform poorly with Trx of a different species (Kanzok et al. 2001). The matched pair concept may also explain why there are also wild-type TrxR with the active site sequence GCCG (Lacey and Hondal 2006): presumably, the Trx active site compensates for the poor hydrophilicity

and the different electrode potential of the GCCG active site.

Does Sec protect in accidental one-electron transfers?

Thiyl radicals may rapidly and efficiently degrade peptides (Nauser et al. 2008). We therefore also considered radical reactions in the assessment of advantages or disadvantages of Sec in proteins. In contrast to the thiyl radicals, there are no known deleterious reactions of disulfide radical anions with peptides. Hydrogen abstraction from the protein backbone (reaction 6a) by selanyl radicals can practically be excluded, because $K_{9a} \approx 10^{-11}$. So, even if k_{-9a} would be extremely large, say 10^{10} s^{-1}, the abstraction rate would be only $k_{9a} = 0.1$ s^{-1}, too slow to be relevant because monohydrogen-ascorbate, at a concentration of 0.1 mM, would repair selanyl radicals five orders of magnitude faster.

For a prediction of possibly deleterious reaction pathways involving thiyl radical species, it is crucial to know their speciation: if there is a thiol in proximity, equilibrium 10 may be shifted far to the right. The resulting disulfide radical anion would react in a diffusion-controlled way with oxygen to form disulfide and superoxide, which in turn would be detoxified by superoxide dismutase (Winterbourn 1993). If equilibrium 10 lies on the left, intramolecular abstraction of hydrogen from the peptide backbone would occur (reaction 9b). Subsequent reaction with oxygen to peroxyl radicals and the destruction of the peptide are highly probable (Nauser et al. 2004; Nauser et al. 2008). For DTT and its analogs SeTT and DSeT, the distribution profiles of the corresponding radical species are given in Fig. 1 and can be summarized by pK-values for the radical anions. At pH 7, both DTT and DSeT radicals are present as radical anions, but SeTT would produce significant amounts of selanyl radical and thiol. Based on this information alone, no distinct protective effect can be assigned to the use of Sec because neither disulfide radical anions nor selanyl radicals are considered harmful. Until now, we did not take into account that DTT and its analogs are six-membered rings with virtually no strain. However, an eight membered ring in a peptide such as SCCS would have a strain of approximately 18 kJ mol^{-1}. This disfavors the formation of the radical anion because the equilibrium constant K_{10} and that of its (Se, S) and (Se, Se) analogs are now lower by three orders of magnitude. Then equilibrium 10 would not be shifted much to the right, if at all, and significant yields of thiyl or selanyl radicals could be expected. If one-electron transfers occur in TrxR, one might expect in the SCCS active site formation of deleterious thiyl radicals, as compared to innocuous selanyl radicals in the GCUG active site. Therefore, Sec could be protective.

Conclusion

In several types of reaction, Sec reacts faster than Cys. However, kinetic properties are probably not the only reason for the use of Sec in vivo. Selenosulfides may be used in electron transfer reactions to compensate ring strain and, compared to Cys, Sec might cause a protein to fold differently, or the substrate to bind differently. Moreover, in sites which are exposed to one-electron oxidation, for example during oxidative stress, Sec would be protective.

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