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Conformational Analysis of Oxidized Peptide Fragments of the C-terminal Redox Center in Thioredoxin Reductases by NMR Spectroscopy†

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Abstract

Vicinal disulfide rings (VDRs) occur when a disulfide bond forms between adjacent cysteine residues in a protein and results in a rare eight-membered ring structure. This eight-membered ring has been found to exist in four major conformations in solution, divided between *cis* and *trans* conformers. Some selenoenzymes use a special type of VDR in which selenium replaces sulfur, generating a vicinal selenosulfide ring (VSeSR). Here we provide evidence that this substitution reduces ring strain, resulting in a strong preference for the *trans* conformation relative to *cis* in a VSeSR (*cis:trans* – 9:91). This was determined by using the “ γ -gauche effect” which makes use of both ¹H-NMR and two-dimensional (2D) NMR techniques for determining the amide bond conformational ratio. The presence of selenium in a VSeSR also lowers the dihedral strain energy (DSE) of the selenosulfide bond relative to the disulfide bond of VDRs. While *cis* amide geometry decreases strain on the amide bond, it increases strain on the scissile disulfide bond of the VDR found in thioredoxin reductase from *D. melanogaster* (DmTR). We hypothesize that the *cis* conformation of the VDR is the catalytically competent conformer for thiol/disulfide exchange. This hypothesis was investigated by computing the DSE of VDR and VSeSR conformers, the structure of which was determined by 2D NMR spectroscopy and energy minimization. The computed values of the VDR from DmTR are 16.5 kJ/mol DSE and 14.3 kJ/mol for the *C+* and *T-* conformers, respectively, supporting the hypothesis that the enzyme uses the *C+* conformer for thiol/disulfide exchange.

INTRODUCTION

High molecular weight (M_r) thioredoxin reductases (TRs) are head to tail homodimeric pyridine nucleotide disulfide oxidoreductases that are important enzymes for regulating the cellular redox state [1, 2]. The enzyme uses the coenzyme NADPH as a source of two

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electrons to reduce a tightly bound flavin (FAD) to form FADH₂, which in turn reduces an internal dithiol/disulfide cysteine (Cys) pair (N-terminal redox center). The N-terminal redox center then reduces a C-terminal redox center on the opposite subunit of the TR dimer. Ultimately, it is the reduced C-terminal redox center that passes these electrons to the protein substrate, Trx [3, 4]. TR has a similar mechanism and structure as glutathione reductase (GR), except that GR is missing the C-terminal redox center [5]. GR uses glutathione disulfide (GSSG) as a mobile acceptor of electrons, which then shuttles them to various targets in the cell. In TR, the C-terminal redox center functions as a covalently linked glutathione-like module [5], and exists as three different redox motifs. We have classified these motifs as types Ia, Ib, and II, respectively [6]. Each type has a different arrangement of redox active Cys and selenocysteine (Sec) residues. The sequence of amino acids in each type is as follows: type Ia uses a Cys-Sec dyad, type Ib uses a Cys-Cys dyad, and type II uses a Cys-Gly-Gly-Gly-Lys-Cys motif. Mammalian TRs (Sec-TRs) contain the rare Sec amino acid in a Cys-Sec dyad, but Sec appears not to be a requirement for catalysis since Cys-orthologs (Cys-TRs) of the mammalian enzyme catalyze the reduction of their cognate Trx with very high catalytic efficiency [7].

The catalytic cycle of type Ia and type Ib enzymes involves formation of either a vicinal disulfide ring (VDR) in the Cys-TR, or a vicinal selenosulfide ring (VSeSR) in the case of the Sec-TR (Figure 1). The VDR or VSeSR undergoes a thiol/disulfide exchange reaction with the N-terminal redox center during the catalytic cycle of the enzyme. The presence of selenium in a VSeSR accelerates the rate of the thiol/disulfide exchange reaction that occurs between the two redox centers due to a combination of chemical factors including: nucleophilicity, electrophilicity, and leaving group ability [8–10]. In addition to chemical differences between a VDR and VSeSR, there must also exist structural differences due to the size difference between selenium and sulfur, a theme that is a large part of this report.

The structure of VDRs has been the subject of previous investigation. Early work by Chandrasekaran and Balasubramanian [11, 12] predicted that the amide bond geometry of the VDR dipeptide L-cysteinyl-L-cysteine (H-[Cys-Cys]_{ox}-OH) should contain a nonplanar *cis* geometry with a dihedral angle (ω) of -12° . This was seemingly confirmed by X-ray crystallography to be -7° [13]. However, all VDRs found in protein structures contain *trans* amide geometry, although the value of ω commonly deviates $\sim 12^\circ$ from ideal 180° *trans* geometry [14]. Model studies of VDRs in peptides show that the amide bond exists in both *cis* and *trans* forms with interconversion between the two forms. The best work in this area has been done by Reitz and coworkers who showed by using NMR spectroscopy that the VDR dipeptide Ac-[Cys-Cys]_{ox}-NH₂ is quite flexible, with four conformations being observed in solution under physiological conditions [15]. They designated these conformers as *C*+, *C*−, *T*−, and *T*'−. The “*C*” and “*T*” descriptors denote *cis* and *trans* amide bond conformation respectively, while “+” and “−” indicate the helicity of the disulfide bond to be either $+90^\circ$ or -90° . This observation fits with later work that proposed VDRs might act as a type of conformational molecular redox switch [16]. This has been postulated for VDRs within the nicotinic acetylcholine receptor [17], human Rnase H1 [18], and ADAP [19]. Recent work however by Alewood, King, and coworkers showed that conformational

switching is not the purpose of the VDR from κ -hexatoxin-Hv1c, but rather it is involved in a “precisely configured hydrophobic interaction.” [20].

In studies on the catalytic mechanism of Sec-TR, we modeled a VSeSR as a *T*- conformer in the active site as the catalytically competent form to undergo thiol/disulfide exchange with the N-terminal redox center [21], even though the previous work discussed above allowed for other conformations. The basis of this prediction is that a VSeSR should be larger than a VDR due to longer S–Se and C–Se bonds in comparison to shorter S–S and C–S bonds. The larger ring size of a VSeSR should stabilize the *trans* form of the amide and result in fewer possible conformations. In the case of the Cys-TR from *D. melanogaster* (DmTR), we modeled the VDR as a *C*+ conformer due to a better fit in the active site of the enzyme and on the basis of structural superposition of the sulfur atom of the *C*+ conformer with the sulfur atoms of oxidized glutathione bound in the active site of glutathione reductase (GR). GR shares high structural homology with TR and catalyzes a near identical thiol/disulfide exchange reaction (Figure 2).

The questions we investigate in this report are: (i) how the substitution of selenium for sulfur affects the structure of a VDR using both one-dimensional and two-dimensional NMR spectroscopy, (ii) how the four different conformers described above influence the rate of the thiol/disulfide exchange reaction that occurs between the N- and C-terminal redox centers of types Ia and Ib TRs, and (iii) the structural similarity of the disulfide of GSSG with that of the *C*+ conformer found in type Ib TRs.

MATERIALS AND METHODS

Materials

Solvents for peptide synthesis were purchased from EMD Biosciences (San Diego, CA). Fmoc-amino acids were purchased from Synpep Corp (Dublin, CA). Resins for solid-phase synthesis were purchased from Applied Biosystems (Foster City, CA). All other chemicals were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). The HPLC system was from Shimadzu with a Symmetry® C₁₈–5 μ m column from Waters (4.6 $\times$ 150mm). A Voyager-DE™ PRO Workstation (Applied Biosystems) was used for mass spectral analysis of peptide samples.

Peptide Syntheses

Fmoc-selenocysteine(Mob)-OH was synthesized as had been reported previously [22, 23]. All peptides were synthesized with a Burrell Model 75 wrist-action shaker (Pittsburgh, PA). For each peptide synthesis the resin was first swelled in dichloromethane (DCM) for 30 min and washed six times with dimethylformamide (DMF). Deprotection of the N α -Fmoc protecting group was carried out using one 15-min agitation with a solution of 20% piperidine/80% DMF. Success of all deblocking and amino acid couplings were monitored qualitatively using a ninhydrin test [24]. Elongation of the peptides used standard Fmoc SPPS chemistry with 4 eq. (equivalents relative to peptide synthesis scale in mmoles) HBTU (0.05 M final concentration), 4 eq. HOBt, 4 eq. Fmoc-AA-OH, and 4 eq. TMP (collidine) in a 50% DCM/50% DMF solution with no preactivation time [25]. Peptides were synthesized

on a 0.3 mmole scale using Fmoc-Gly-PEG-PS-Resin (0.20 mmol/g loading), or Fmoc-Ser-PEG-PS-Resin (0.20 mmol/g loading). For deprotection of Sec(Mob) residues in peptide **7**, we used a well described procedure used previously by us that uses 2,2°-dithiobis(5-nitropyridine) (DTNP) for simultaneous deprotection and selenosulfide bond formation [26]. For the construction of peptides **1–4**, we used a modified procedure that also uses DTNP and this procedure has been previously described [27]. For the construction of peptides **5–6**, we used CLEAR-OXTM resin to form the vicinal disulfide bond. These peptides were cleaved from the resin with a cocktail consisting of 96:2:2 trifluoroacetic acid/triisopropylsilane/H₂O for 2 hr. The cleavage cocktails were dripped into cold, anhydrous diethyl ether to precipitate the peptide. The solution was then centrifuged to pellet the solid after which the solid was washed with cold, anhydrous diethyl ether and then pelleted by centrifugation (2x). The peptide was dissolved in a minimal amount of a water/acetonitrile mixture and then lyophilized. The product was then subjected to oxidation using CLEAR-OXTM resin. A 3-fold excess of resin (versus quantity of peptide) was swelled in DCM for 40 min, then washed with DCM, DMF, methanol, deionized water, and degassed 1:1 100 mM ammonium bicarbonate/acetonitrile 3 times each. Peptides **5** and **6** were then dissolved in the degassed ammonium bicarbonate/acetonitrile and added to the resin. After gentle agitation for 90 min, the peptide solution was filtered away from the resin and lyophilized. All oxidized peptides were purified by preparative reverse-phase HPLC and then lyophilized to obtain white crystals.

Conformational Dynamics and NMR

Proton and 2D NMR data were obtained with a Varian 500 spectrometer at 20 °C, unless otherwise noted. Chemical shifts for ¹H NMR and ¹³C NMR are reported in parts per million (ppm) relative to tetramethylsilane (δ = 0.00 ppm for ¹H NMR) or chloroform-*d* (δ = 77.0 ppm for ¹³C NMR) respectively. The resonances of all Cys (and/or Sec) residues were assigned based on TOCSY and COSY homonuclear correlations. ¹H-NMR intensity was used as the initial method to differentiate amino acid residues belonging to the four observed conformers. HMQC experiments then provided the ¹³C assignments for the β -carbons based on correlation with TOCSY and COSY proton resonance assignments. Long range HMQC, or HMBC, then confirmed the connectivity between the two Cys (and/or Sec) residues of each ring conformer. Homonuclear 2D-J experiments in H₂O as well as in D₂O provided the necessary coupling constants between the vicinal NH and α -proton, and between the α -proton and β -protons needed to provide the dihedral angles of ϕ and χ_1 . Using a Karplus curve for polar β -substituents (such as – SSR), four possible dihedral angles were realized for each homonuclear coupling constant obtained [28]. Heteronuclear coupling constants were then measured by quantifying the separation in the antiphase dimension of the HMBC proton peaks for each ¹³C resonance. This was used to determine if the ring carbonyl was synclinal or antiperiplanar to the β -proton for each Cys residue of the ring. By combining the homonuclear coupling constants between the α - and β -protons of each cysteine, with the heteronuclear coupling data, it was possible to identify the correct χ_1 dihedral angle from the four possibilities initially suggested by each homonuclear coupling constant and the Karplus curve [29]. The four conformers (*C*+, *C*–, *T*–, and *T'*–) depicted in Figure 4 were generated by combining the homonuclear and heteronuclear 3-J couplings as angle restrictions, with distance restrictions derived from ROESY experiments [30]. The *cis* or

trans peptide geometry (ω , Figure 1) was then set as 0° or 180° based on the HMQC γ -effect (Figure 5). Vicinal α,β - and geminal β,β -NOE integrations were used as internal standards for the *cis* conformers to determine the distance between α -protons of the ring, while the NOE integrations between the *N*-terminal methyl group and its adjacent amide proton were used as internal standards to determine distances in the *trans* conformers. These close contacts are depicted in Figure 8A [31–33]. Sulfur helicity (indicated as + or – for each conformer) was not specified as a geometric restriction. Rather, MOPAC AM1 and MINDO/3 minimization of the free energy of formation provided the peptidyl side chain dihedrals and sulfur helicity. The 0° or 180° ω -restrictions were then removed and additional energy minimization was allowed for fine adjustments of ω , as well as the dihedral angle between the amide carbonyl oxygen and amide proton of the Cys-Cys or Cys-Sec dyad. These experiments resulted in the proposed conformations, depicted in Figure 4. All spectra collected in this investigation are given in the Supporting Information.

RESULTS AND DISCUSSION

Synthesis of VDR peptides

As mentioned in the Introduction, type Ia and type Ib TRs contain either oxidized Cys-Sec or Cys-Cys dyads as part of their respective C-terminal redox centers. Type Ib redox centers have small residues flanking each side of the Cys-Cys dyad (either Gly, Thr, or Ser). To study the effect that different flanking residues have on the conformation of the dyad of Cys-TRs, VDR constructs **1–4** were synthesized. Targets **5** and **6** were chosen to determine how the stereochemistry of Cys impacts VDR conformer population. Last, VSeSR **7** was selected to mimic the C-terminal redox center of Sec-TR (see Table 1 for the structures of these peptides). The synthesis of **1** has been previously been reported by us, and others [15, 34]. The construction of targets **2–4** and **7** was accomplished as described in the Methods using protocols previously described by us [26, 27]. Construction of targets **5–6** was more challenging and we employed ClearOX™ resin in order to make the vicinal disulfide bond [35]. ClearOX™ resin was developed to promote intramolecular disulfide bond formation, while disfavoring both intermolecular disulfide bond formation and oligomerization. This resin makes use of immobilized DTNB that acts as the oxidant on cross-linked ethoxylate acrylate resin. After oxidation with ClearOX™ resin, the peptide solution was lyophilized and then analyzed by HPLC. Analytical HPLC showed that peptides **5–6** were ~90% pure (data not shown). The peptides were purified by preparative HPLC as described in the methods and then lyophilized before being subjected to spectroscopic analysis.

Complexity of VDR conformation revealed by one-dimensional ^1H -NMR

Figure 3A shows the ^1H -NMR spectra of VDR **3**. The complexity of the spectrum is evident upon casual inspection. The multiple resonances shown in the spectrum are due to the presence of the four conformers described in the Introduction and depicted in Figure 4. The ^1H -NMR spectrum of the VSeSR (Figure 3B) is less complex because there are fewer conformers present. This was expected due to the larger ring size conferred by the substitution of selenium for sulfur, as this substitution results in longer S–Se and C–Se bonds compared to S–S and C–S bonds. The ^1H -NMR spectrum of VDR **5**, containing a Cys

residue with inverted C α -H stereochemistry, is the least complex because it exists as a single conformer as explained in the legends to Figures 3 and 4.

Structure determination of ring structures

All constructs were subjected to: (i) ROESY experiments for determination of *cis* amide resonances due to the strong Cys2- α Cys3- α through space proton coupling in the *cis* conformer, (ii) TOCSY and COSY experiments for determining skeletal structure of the ring, (iii) 2Dj-homonuclear, HMQC-, and HMBC-heteronuclear coupling experiments for elucidation of dihedral angles, and (iv) MOPAC AM1 and MINDO/3 energy minimizations for determination of disulfide or selenosulfide helicity. The *cis* conformers both showed strong ROESY through space interactions between the Cys α -protons (Figure 4, ROESY interactions shown in blue). The *trans* conformers displayed different ROESY contacts with the *T*- conformation having a close through space relationship between the NH of Cys2 and the NH of Cys3 and the *T'*- conformation having one between the NH of Cys3 and the β -proton of Cys2. Based on the dihedral constraints and energy minimization experiments, models of VDRs 3–5, and VSeSR 7 were constructed as PDB files and these models were used to calculate the dihedral angles used in the analyses presented in later sections.

The use of the γ -gauche effect to determine conformeric ratios of VDRs and VSeSRs

The complexity of the ^1H -NMR spectra of VDRs 1–4 is due to the presence of multiple *cis* and *trans* conformers. During the course of our investigation to determine the relative populations of each conformer, we discovered that a HMQC γ -gauche effect could be used to distinguish *cis* and *trans* conformers [36]. The γ -gauche effect arises from steric clashes at the α -stereocenters that make up the VDR or VSeSR system and produces a shift in the ^{13}C resonance of both allylic carbons as a result of steric crowding and increased electron density at these sites for a *cis* double bond (Figure 5) [37, 38]. This enhanced electron density, when compared to a *trans* olefin, results in an upfield shift in the ^{13}C -NMR for the carbons adjacent to the site of unsaturation [39]. This rule can be extended to VDRs and VSeSRs since the amide bond has partial carbon-nitrogen double bond character [40]. Thus using the γ -gauche effect one would predict that the α -carbons of both Cys residues would be shifted upfield for the *cis* conformers relative to the *trans* conformers, and indeed that is the case. Each *trans* conformer can be easily identified by the unique combination of a downfield α -carbon with an upfield α -proton. In the *T*- conformer the Cys2 α -proton is shifted upfield as a result of being within the diamagnetic anisotropic shielding cone provided by the adjacent carbonyl. In a similar fashion the *T'*- conformer has an upfield α -proton for Cys3 as a result of the diamagnetic anisotropic shielding cone provided by the adjacent amide bond due to its partial carbon nitrogen double bond character. Interestingly the γ -gauche effect was also observed with the β -carbons of the Cys residues as well, and the HMQC heteronuclear C-H correlation experiment shown in Figure 6 illustrates the ease by which the γ -gauche effect can be used to differentiate *cis* from *trans* conformations in VDRs and VSeSRs. Subsequent ^1H -NMR integration provides the conformer ratios detailed in Table 1.

VDR 1 displayed four conformers as had been previously shown, two being *cis* and two *trans* [15]. We used the γ -gauche effect discussed above in combination with ^1H -NMR

integration to calculate a total *trans:cis* ratio of 59:41 for VDR **1**. Removal of the amino acetyl-substituent results in a significant increase in the *cis* amide population with a ratio of *trans* to *cis* conformers in VDR **2** being 23:77. This is a result of the missing carbonyl oxygen of the acetyl or amino acid at the *N*-terminus of the ring. The oxygen of this carbonyl forms a seven-membered hydrogen-bond with the proton of the amide group within the VDR and helps to stabilize the *T*– conformation. The *T'*– conformation does not possess this hydrogen-bonding interaction (Figure 4), thus the missing carbonyl has no impact on its formation, as opposed to the *T*– VDR [15]. As a result the *T*– conformer was not observed for VDR **2**.

VDR constructs **3** and **4** mimic the redox active C-terminal tail of Cys-TR enzymes from *C. elegans* and *D. melanogaster*, respectively. VDR **3** was very similar to **1**, with a *trans:cis* ratio of 61:39, while compound **4** displayed a slightly inverted *trans:cis* ratio of 48:52. Each VDR (**1–4**) also showed a preference for disulfide helicity within either *trans* or *cis* conformation. For both *trans* amide conformers, only negative disulfide helicity was observed, with the *T*– conformation being preferred. Both positive and negative helicity was observed in the case of the *cis* amide conformers. The *C*+ conformer predominated over the *C*– conformer in each VDR (Table 1).

VDRs and VSeSRs have different conformational ratios

In contrast to the results for the VDRs above, the amide bond conformation for VSeSR **7** was observed to be overwhelmingly in the *trans* conformation, with a total *trans:cis* ratio of 91:9. In general, the same HMQC pattern of H-C correlations observed for the *trans* and *cis* conformers of VDR **3** (Figure 6A) were also observed for VSeSR **7** (Figure 6B). Thus the *trans* conformers were easily recognized by an upfield α -proton correlated to a downfield α -carbon within the VSeSR. Similar to **3**, Cys2 of the *T*– conformer possesses a shielded α -proton correlated with a deshielded α -carbon while the *T'*– conformer possesses a shielded α -proton correlated with a deshielded α -carbon on Sec3. VSeSR **7** also showed an upfield shift for the β -carbons on the selenocysteine residue for both *cis* and *trans* conformations. This was expected due to their closer position to selenium relative to sulfur. This difference aside, there were conformational similarities. For VDRs **1–4** and VSeSR **7**, the major *trans* conformer is *T*– and the major *cis* conformer is *C*+. The VSeSR *trans* conformations observed both had negative disulfide helicity as was also found in the analogous VDR.

As the data shows in Table 1, substitution of selenium for sulfur results in a ring system in which the amide bond is found mainly as one of two *trans* conformers, with the *cis* conformer in low abundance. These data support our hypothesis that the longer S–Se and C–Se bonds within the VSeSR lowers the amide bond strain due to a slightly larger ring size, which can accommodate *trans* geometry within a medium sized ring. An amide-containing ring must contain at least nine atoms for the ring to be nearly all in the *trans* conformation. A ring size of eight atoms is the transition point between *cis* and *trans* conformers since they are nearly isoenergetic [41]. The interplay between overall ring strain and pendant amino acid steric clashing results in an equilibrium population of *cis* and *trans* conformers with the equilibrium position determined by the lowest energy conformation. For both VDRs and VSeSRs pendant amino acid steric clashes are particular noteworthy in the *cis* conformation.

VSeSRs are more flexible due to the presence of selenium

The larger ring size of the VSeSR also imparts much greater flexibility compared to the VDR as illustrated by the comparison of ROSEY spectra of VDR **4** with VSeSR **7** collected at various temperatures as shown in Figure 7. The existence of multiple VDR conformers implies a barrier to isomerization at room temperature with slow interconversion between conformers. The data presented in Figure 7 for VDR **4** (also observed for **3**) suggests that the *cis* conformers, *C*⁺ and *C*[−], coalesce at 40 °C. After coalescence to a single resonance, interconversion between the *cis* form and the two *trans* conformers becomes more pronounced. This model is shown in Figure 8. *Trans-trans* exchange peaks appear to be residual magnetization that is transferred from *T*[−] to *cis* to *T*[′][−] and back again, since the *cis-trans* exchange peaks integrate to approximately eight times the magnitude of the *trans-trans* exchange peaks. Furthermore the most intense exchange peaks were observed at the Cys2 β-protons followed by the Cys2 α-protons, which suggests that this is the pivot point for conformer interconversion.

Effect of α-carbon stereochemistry on VDR ring geometry

Incorporation of D-Cys into the VDR (VDRs **5** and **6** and abbreviated as *unnat*-VDR in Table 1) has a large impact on ring geometry. In both cases only one conformer was observed, and in both cases the amide of the ring was in a *trans* conformation. The single conformation observed in **5** and **6** was not affected by temperature (5–80°C), or by pH of the solution. The inversion of configuration at either Cys residue allows for an extended non-eclipsing conformation with the only difference being the helicity about the disulfide bond. The *trans* conformations observed for these *unnat*-VDRs are not analogous, but is similar, to what was observed previously with compounds **1–4**. However, since compounds **5** and **6** are enantiomeric it is impossible to specifically discern which disulfide/amide orientation belongs to which *unnat*-VDR since they display the same NMR spectra. These *trans* conformers differ from those observed for VDRs **1–4** because the α-protons has an anti-periplanar relationship to at least one β-proton for both Cys residues. Thus we designate these conformers as *trans antiperiplanar* or *TA*. One of the *trans unnat*-VDRs shares the same amide orientation (amide carbonyl down) relative to the ring as the VDR *T*[−] conformer in which both Cys residues have L-stereochemistry (a “natural” VDR), while the other has an amide orientation the same as the natural VDR *T*[′][−] conformer (amide carbonyl up). The *unnat*-VDR in the *T*[−] amide conformation has negative disulfide helicity, while the *unnat*-VDR in the *T*[′][−] amide conformation possesses positive helicity. These conformers are therefore designated as either *TA*[−] or *TA*[′]⁺ and are mirror images of each other. A similar analysis to the above was done for a VDR containing penicillamines with inverted stereochemistry about the α-carbon [42]. Our analysis is supportive of this prior analysis, but is distinct because of two important factors: (i) the use of a more biological example (Cys instead of penicillamine), and (ii) our conformational analyses includes the description of the antiperiplanar arrangement that exists between the α-protons.

An explanation for the different conformers based on energetics

The parent compound of the VDRs constructed in this paper is 1,2-dithia-5-azacyclooctan-6-one (the parent VDR shown in the left panel of Figure 1). It has been shown by NMR to

exist as a single *trans* conformer [34, 41]. In this ring, there are no alkyl groups on the α -carbons that must be placed in such a way as to avoid steric strain. All of the hydrogen atoms are in a conformation that avoids eclipsing interactions, which results in the ring being in a rigid *trans* conformation. VDRs with pendant groups, such as the ones reported on here, contend with multiple types of competing steric clashes resulting in four different conformations that are relatively close in energy, as calculations by Hudaky have shown [14]. Below we quantify useful types of strain energy parameters in each of the different conformers of a VDR and VSeSR to provide useful insight on how enzymes catalyzing thiol/disulfide exchange reactions might use different conformations of a VDR to their catalytic advantage.

Calculation of amide strain energy

In both *cis* and *trans* conformers of VDRs, the amide lone pair is slightly out of phase with the π system of the carbonyl. The result is a strained amide bond. The amount of strain in the amide bond is given by $E = A \sin^2 \omega$ and has a local minimum at 0° and a global minimum at 180° . This amide strain energy was tabulated for each different major *cis* and *trans* conformer of VDRs **3–5**, and VSeSR **7** and is given in Table 2. The results demonstrate that strain on a *trans* amide bond is alleviated by adopting a *cis* conformation (compare values for *T*– and *C*+ in Table 2) [14]. However in order to adopt this *cis* conformation, the main peptide chain at the α -carbons on either side of the ring must be placed in a pseudoequatorial position to avoid eclipsing interactions at the α – and β –carbon of the ring, resulting in steric strain as a result of these unfavorable close contacts. This interplay of competing steric clashes and ring strain results in the four different conformations of a VDR.

The amide strain energy for the *T*– conformer of VSeSR **7** stands out in the data. The amide strain energy for **7** is ~ 10 kJ/mol lower than VDR **3** and **4**. This is because ω is closer to 180° in VSeSR **7** than in the VDRs. The presence of the selenium atom in the ring results in a larger ring size and a larger value of ω , which in turn lowers the amide strain energy. This lower amide strain energy allows VSeSR **7** to be predominantly in the *trans* conformation. It should be remembered that amide strain energy is but one factor that determines ring geometry and this parameter does not take into account the other types of steric clashes as we have discussed above.

Calculation of dihedral strain energy

Another useful energetic term for calculating strain energy in a VDR or VSeSR is “dihedral strain energy” (DSE). The DSE is a parameter that is used to quantify the amount of strain in a *disulfide bond* [43–45]. The angles that define a disulfide bond are χ_1 , χ_2 , χ_3 , χ_1' , and χ_2' , where χ_1 is the angle about the $C\alpha$ – $C\beta$ bond, χ_2 is the angle about the $C\beta$ – $S\gamma$ bond, and χ_3 is the angle about the two $S\gamma$ atoms (Figure 1). The prime designation denotes the dihedral angles on one side of the ring from the other side. These five angles allowed for the generation of 3D models of the *C*+ and *T*– conformers of VDR **3**, **4**, and **5** as well as VSeSR **7**. From this data we calculated the DSE for each conformer and the results are given in Table 2. The DSE offers valuable insights into the conformational preferences of each compound. The data shows that VSeSR **7** has a significantly lower DSE than VDRs **3** and **4**.

and can help explain why **7** prefers the *T*- form, even though the amide strain energy is lower in the *C*+ form of **7**. The presence of selenium in **7** lowers the DSE because it causes the ring to be larger, and more flexible, resulting in an overall lowering of ring strain. A similar case to **7** is *unnat*-VDR **5**, containing an inverted C- α stereocenter, which has a strained amide bond due to the significant deviation of ω from 180°, but has a low DSE. When one of the α -stereocenters is D and the other L, ring strain, as reflected by DSE, is minimized due to the *anti*-arrangement of these stereocenters, allowing for equatorial placement of both bulky peptidyl side chains. The *trans* conformer dominates even though the amide bond is strained because the overall strain in the ring is lower due to the arrangement of pendant groups.

Our results in relation to the mechanism TR – a hypothesis

The mechanisms of GR and TR share a common thiol/disulfide exchange reaction as shown in Figure 2. In the case of GR, the thiolate from the N-terminal redox center attacks the disulfide bond of oxidized glutathione (GSSG), releasing one molecule of reduced glutathione (GSH) and forming a mixed disulfide bond intermediate. One way that GR could accelerate the rate of this thiol/disulfide exchange reaction is by placing strain on the disulfide bond when GSSG is bound to the enzyme. This would make the disulfide bond more electrophilic and more reactive. In the analogous thiol/disulfide exchange reaction in TR, a VDR or VSeSR takes the place of GSSG. We have previously noted that the sulfur atoms of a VDR will superpose with the sulfur atoms of GSSG, as it is found bound in the active-site of GR, when the amide of the VDR adopts a *cis* geometry [21]. Superposition of the sulfur atoms is not possible when the VDR amide bond has *trans* geometry (Figure 9). This bound structure of GSSG is on a pathway to undergo a thiol/disulfide exchange reaction and is therefore in a configuration that will lead to the transition state. It is intriguing that sulfur-atom superposition only occurs with *cis* geometry; this leads to the hypothesis that a VDR with *cis* amide geometry can become strained and is the preferred form to undergo a thiol/disulfide exchange reaction such as occurs between the N-terminal redox center and the VDR of the C-terminal redox center in Cys-TRs.

To investigate this hypothesis, we used the published crystallographic coordinates of GSSG bound to the active site of GR to calculate the DSE presented in the disulfide bond [46] and the result is listed in Table 2. The DSE of the disulfide bond of bound GSSG is 19.5 kJ/mol. This is a significantly high number based on the study by Hogg and coworkers [44]. In the data set examined by Hogg, it was found that disulfides that were part of catalytic active sites in enzymes were strained and had a mean DSE of 20.8 kJ/mol. As a point of comparison, it was found that structural disulfides had a mean DSE of ~10 kJ/mol. When the bound GSSG is attacked by the active site thiolate, a mixed disulfide complex is formed (Figure 2A). This mixed disulfide between glutathione and GR then undergoes a second thiol/disulfide exchange reaction to produce oxidized enzyme and a second molecule of GSH. A structure of the mixed disulfide complex also exists [46] and we used these coordinates to calculate the DSE of this mixed disulfide. The computed DSE for this mixed disulfide complex is 19.75 kJ/mol (Table 2). Both of these calculated results support the use of DSE as a parameter to evaluate the reactivity of a disulfide bond.

VDR constructs **3** and **4** mimic the redox active C-terminal tail of Cys-TR enzymes from *C. elegans* and *D. melanogaster*, respectively. The data in Table 2 shows that the DSE is higher in VDR **4** compared to VDR **3**. VDR **4** has pendant Ser residues, while VDR **3** has pendant Gly residues. In the case of DmTR it has been proposed that the function of the C-terminal Ser residue is to increase the nucleophilicity of the adjacent Cys residue to help compensate for the absence of a nucleophilic selenium atom [47]. The data presented here suggests that an additional function of the pendant Ser residues in this VDR is to increase the DSE, accelerating the thiol/disulfide exchange reaction that occurs between the VDR and the N-terminal redox center of the enzyme (Figure 2B). In DmTR, mutation of the C-terminal Ser residue to Gly decreases the catalytic constant for the reduction of its target substrate [47]. This may be because this mutation relieves some of the strain on the ring and slows the rate of thiol/disulfide exchange between the two redox centers. We note that the TR from *C. elegans* (CeTR) uses pendant Gly residues in its VDR and this contradicts our hypothesis. This contradiction might be reconciled if the active site architecture of CeTR is smaller and this crowding provides additional transition state strain on the ring. The smaller Gly residues would be needed to fit in the smaller space.

With respect to selenium-containing VSeSR **7**, a recent study by Brandt and coworkers should be discussed in comparison to our results [48]. The results of DFT calculations with VSeSR **7** showed that the *cis* conformer was more stable by 20.92 kJ/mol relative to the *trans* conformer [48]. This contrasts with our experimental observations that the *trans* conformer of **7** predominates in solution as determined by NMR spectroscopy. It also contrasts with our computed data of DSE for bound GSSG in the active-site of GR, where the *s-cis*-like conformation of bound GSSG was found to be strained. However, the work of Brandt and coworkers provides some important insight into the mechanism of Sec-TR. If both type Ia and type Ib TRs use the same mechanism, then both enzymes would want to maximize the rate of thiol/disulfide exchange that occurs between the N- and C-terminal redox centers. This might be achieved if both enzymes strain their respective -S-S- or -S-Se- bonds of the C-terminal redox center by converting them to “an energy-rich form” [48]. The results of our work here suggest that the *s-cis* conformation of **4** and **7** would help to accelerate the rate of thiol/disulfide exchange.

CONCLUSIONS

The data presented here has shown how substitution of selenium for sulfur in a VDR affects the structure and conformational preferences of the ring. A major conclusion is that the use of selenium in a VSeSR results in a ring with mostly *trans* amide geometry, while VDRs exist in multiple conformations in solution due to interplay between overall ring strain and pendant amino acid clashing. Substitution of selenium for sulfur in these types of rings lowers both the amide strain energy and the DSE due to a larger ring size. The *C+* conformation of a VDR results in strain on the disulfide bond, depending on the identity of pendant groups, but at the same time relieves strain on the amide bond. The strain placed on the disulfide bond may be used to accelerate the rate of thiol/disulfide exchange reactions. This idea is strengthened by the finding that the sulfur atoms of glutathione disulfide bound in the active site of GR can only be superposed with the sulfur atoms of a VDR as found in the *C+* conformation, and not the *T-* conformation. The *C+* conformation of a VDR has

higher DSE when the pendant amino acids are serine, as is the case for DmTR. The relatively high DSE in VDR **4** could accelerate the thiol/disulfide exchange reaction that occurs between N- and C-terminal redox centers of the enzyme. While the data presented here supports this hypothesis, it can only be verified with a crystal structure of DmTR in a conformation that shows the molecular details of this thiol/disulfide exchange reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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conformers displayed two downfield ^{13}C signals for each Cys α -carbon (relative to *cis* conformers), which were coupled with one downfield and one upfield ^1H signal for the Cys α -protons.

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Figure 1.

Structure of the parent VDR, 1,2-dithia-5-azacyclooctan-6-one (*left panel*) and a general structure of a VDR (X=S) resulting from disulfide-bond formation between vicinal cysteines (*right panel*). In the VDR shown in the right panel, angles about the ring system are indicated using standard peptide nomenclature (ψ , ω , ϕ , and χ). The left portion of the ring, or Cys2, ring dihedrals are labeled as χ while the right portion of the ring, or Cys3, dihedrals are labeled as χ' . Both cysteine residues share ω (amide bond dihedral) and χ_3 (disulfide bond dihedral). The general structure of a VSeSR (X=Se) resulting from selenosulfide-bond formation between vicinal cysteine and selenocysteine residues possesses the same ring nomenclature described above. Selenium in the form of selenocysteine replaces Cys3 in VSeSR 7 studied here.

Figure 2.

(A) Thiol/disulfide exchange reaction between GR and bound GSSG. The catalytic residues are: Cys_{CT} (charge-transfer cysteine), Cys_{IC} (interchange-cysteine), and HisH⁺ (general acid/base catalyst). Cys_{CT} is involved in charge-transfer complexation with FAD, Cys_{IC} is the active site nucleophile that initiates the attack onto the disulfide bond of the substrate, and His helps to deprotonate the nucleophile and the resultant HisH⁺ transfers a proton to the leaving group. (B) DmTR catalyzes an identical thiol/disulfide exchange reaction except that the "substrate" is the vicinal disulfide bond tethered to the opposite subunit. The use of *cis* amide geometry relieves the amide bond strain, while simultaneously putting strain on the scissile bond as shown by the high DSE in the C+ conformer of 4.

Figure 3.

^1H -NMR of VDR **3** (A), VSeSR **7** (B), and *unnat*-VDR **5** (C) taken at 20 °C. It is important to note the decrease in complexity in the ^1H -NMR spectra. As is apparent by visual inspection, VDR **3** has the most complex spectrum, VSeSR **7** has a spectrum that is significantly less complex and *unnat*-VDR **5** has the least complex spectrum. This is due to the number of conformers present in each ring system. VDR **3** has four dominant conformations (C^+ , C^- , T^- , T'^-), two *cis* and two *trans* (39:61 *cis/trans*) with respect to the amide double bond character in the ring. This contrasts with VSeSR **7**, which has three conformations (C^+ , T^- , T'^-), but now the *trans* conformers are preferred (9:91 *cis/trans*). The *unnat*-VDR **5** peptide only has one *trans* conformation due to the change in α -stereochemistry, which results in a staggered array about the VDR ring system. Due to the enantiomeric relationship between *unnat*-VDR **5** and *unnat*-VDR **6**, their respective ^1H -NMR spectra are identical. They do possess different disulfide helicities as a result of their enantiomeric relationship and are designated as TA^+ or TA^- as described in the legend to

Figure 4.

Figure 4.

Ring conformations for VDRs and *unnat*-VDRs (X = S or Se). Close through-space ROESY interactions are shown in blue. As described in the text, VDRs are comprised of four conformations (C^+ , C^- , T^- , T'^-), while VSeSRs are observed to exist in one of three conformations (C^+ , T^- , T'^-). The two *trans* conformations are labeled as T^- and T'^- . These conformations differ in the ring being either in an extended chair-chair conformation (T'^-) or in a twist-chair conformation (T'^-). The two *cis* conformers (C^+ and C^-) both exist in a boat-chair conformation and only differ by the helicity of the disulfide bond (positive or negative). The VDRs containing one D -Cys residue (*unnat*-VDR) only possessed one *trans* conformation. This conformation was different from the ring systems containing only L -Cys due to the inverted α -carbon stereochemistry. While the *unnat*-VDRs possess a *trans* amide-bond geometry there is a distinctive antiperiplanar arrangement of the α -protons of each cysteine. As a result these are labeled as TA^- and TA^+ . Due to the enantiomeric nature of unnatural VDRs **5** and **6** it is impossible to distinguish the helicity between the two. Unnatural VDR **5** is represented in the TA^- conformation while VDR **6** is represented in the TA^+ conformation.

Figure 5.

Comparison of amide bond geometry with olefin geometry and the γ -gauche effect. Enhanced electron density at the α -carbon of a *cis* olefin or *cis* amide results in an upfield shift in these two ^{13}C resonances. The relative lack of electron density at the α -carbon of a *trans* olefin or *trans* amide results (in comparison to the *cis* forms) in a downfield shift in these two ^{13}C resonances.

Figure 6.

The γ -gauche effect allows for efficient determination of *cis* and *trans* amide conformations within the ring. As a result of close contacts about the *cis* form of the VDR or VSeSR, these *cis* conformers have enhanced electron density at the α -carbons of the Cys and/or Sec residues that results in a diagnostic upfield shift in the ^{13}C -NMR for the carbons adjacent to the unsaturation. This γ -effect was observed in the HMQC spectra for VDRs such as Ac-Gly-[Cys-Cys]_{ox}-Gly-OH (**3**) shown in *panel A* (boxed in blue). The *trans* conformations also show a distinctive H-C correlation of a downfield proton with a downfield carbon, along with a H-C correlation of an upfield proton with a downfield carbon (boxed in red). This is a result of close contacts between the α -proton and the ring carbonyl in the *T*-conformation or between the α -proton and the amide bond in the *T'*-conformation. The distinctive pairing observed in the *trans* conformers for VDR **3** was also observed in the HMQC spectrum of Ac-Gly-[Cys-Sec]_{ox}-Gly-OH (**7**), as shown in *panel B* (boxed in red). The γ -gauche effect is not as clear in the case of VSeSR **7** (boxed in blue), when compared to VDR **3**. However the same general HMQC pattern of H-C correlations that is observed for the *cis* and *trans* conformers of VDR **3** (*panel A*) were also observed for VSeSR **7** (*panel B*). ROESY experiments were also conducted for verification of the conformation.

Figure 7.

2D ROESY of peptide Ac-Ser-[Cys-Cys]_{ox}-Ser-OH (**4**) (*top panels*) and peptide Ac-Gly-[Cys-Sec]_{ox}-Gly-OH (**7**) (*bottom panels*). Spectra were taken either at 5 °C (*left panels*) or 40 °C (*right panels*). 2D ROESY experiments with magnetic relaxation resulted exclusively in positive intensity exchange peaks (ignoring the few dispersive TOCSY bleed through peaks), which allowed for tracking of each ring proton in each ring conformation. The temperature at which ROESY exchange peaks first appear allow for the observation of interconversion between *cis-cis*, *cis-trans*, and *trans-trans* conformers. For Ac-Ser-[Cys-Cys]_{ox}-Ser-OH (**4**), *cis-cis* exchange peaks were observed at 5 °C (*top left panel*), long before the ¹H-NMR spectra showed broadened resonances for the *cis* VDR conformers present in **4**. *Cis-trans* exchange peaks were not observed until 40 °C (*top right panel*), and *trans-trans* exchange peaks were only observed at 80 °C (data not shown). Studies of Ac-Gly-[Cys-Sec]_{ox}-Gly-OH (**7**) show that selenium imparts a significant degree of flexibility to the 8-membered VSeSR ring. This was anticipated based on longer bond-lengths due to the larger size of selenium. This is evidenced by the ¹H-NMR spectrum, which shows that the C⁺ and C[−] conformers resonances coalesce at 20 °C for VSeSR **7** (data not shown), whereas for VDR **4** this did not occur until 40 °C. Comparison of the ROESY spectra of VDR **4** and VSeSR **7** shown here further highlights the flexibility imparted to the ring by substitution of selenium for sulfur. The ROESY spectrum of VSeSR **7** at 5 °C (*bottom left*) shows that *cis-cis* exchange peaks and *cis-trans* exchange peaks were observed in contrast to VDR **4** at the same temperature (*top left*). At 20 °C the two *cis* forms of VSeSR **7** coalesce to a single *cis* conformer. For the sake of similar comparison to VDR **4** we show this coalescing occurring at 40 °C (*bottom right panel*). No *trans-trans* exchange peaks are observed for VSeSR **7** because it exists as a single *trans* conformer at this temperature. The observed coalescence of C⁺ and C[−] conformers at lower temperature in the VSeSR in comparison to the VDR, highlights the increased flexibility observed in VSeSR systems relative to VDR systems.

Figure 8.

Conformational interconversion between VDR **3** conformations (*panel A*). C+ and C- conformational interconversion occurs first at low temperatures. As the temperature increases the C+ and C- conformers merge and become the intermediary pivot point for conversion into T- and T'- conformations. Unfavorable close contacts are shown in red along with corresponding distances. Favorable hydrogen-bonding interactions present in the T- conformation are shown in blue. *Unnat*-VDRs **5** (*panel B*) and **6** (*panel C*) only possess one conformation. The extended chair-chair conformation that is observed is devoid of steric clashes, when compared to the natural VDRs. The stereochemical swap at the α -carbon now places both of the bulky peptidyl side chains in extended equatorial positions. The natural VDRs have at least one of these large peptidyl side-chains in either a pseudoequatorial position, or axial position, which results in the conformational ring dynamics that is observed in the VDRs studied here.

Figure 9.

Superposition of the disulfide bond of VDR **4** in the *C*+ conformation with the disulfide bond of GSSG bound in the active site of GR (*left panel*). Superposition of the disulfide bond of VDR **4** in the *T*- conformation with the disulfide bond of GSSG bound in the active site of GR (*right panel*). As can be clearly seen, overlap of the sulfur atoms of two structures only occurs with the *C*+ conformer.

Table 1Conformeric ratios of peptide VDRs determined by NMR spectroscopy^a.

<p>VDR $R_1 = \text{Ac}; R_2 = \text{NH}_2$ (1) $R_1 = \text{H}; R_2 = \text{Gly-OH}$ (2) $R_1 = \text{Ac-Gly}; R_2 = \text{Gly-OH}$ (3) $R_1 = \text{Ac-Ser}; R_2 = \text{Ser-OH}$ (4)</p>	<p>unnat-VDR $(S,R)\text{-3}$ (5) $(R,S)\text{-3}$ (6)</p>	<p>VSeSR $R_1 = \text{Ac-Gly}; R_2 = \text{Gly-OH}$ (7)</p>
VDR ($C+$; $C-$; $T-$; $T'-$)	unnat-VDR ($TA-$ or $TA'+$)	VSeSR ($C+$; $C-$; $T-$; $T'-$)
1 (36; 5; 42; 17)	5 (100)	7 (9; n/o; 70; 21)
2 (67; 10; n/o; 23)	6 (100)	
3 (33; 6; 47; 14)		
4 (47; 5; 40; 8)		

^aFor all peptides homonuclear 3J and heteronuclear 3J angle restrictions that were determined from 2Dj and HMBC experiments, followed by energy minimization to elucidate ring geometry, were in agreement with previous experimental data and theoretical computations [15].

Table 2

Calculated strain energy parameters of peptide VDRs.

Oxidized Peptide ^a	Geometry	ω (degrees)	Amide strain energy ^b (kJ/mol)	DSE ^c (kJ/mol)
Ac-SCCS-OH (4)	C+	-2.0	0.15	16.5
Ac-GCCG-OH (3)	C+	4.3	0.71	10.4
Ac-GCUG-OH (7)	C+	3.8	0.55	14.1
Ac-SCCS-OH (4)	T-	-152.9	26.1	14.3
Ac-GCCG-OH (3)	T-	-151.0	29.5	13.2
Ac-GCUG-OH (7)	T-	-158.8	16.4	9.1
Ac-GC _L C _D G-OH (5)	TA	-151.6	28.4	8.6
GSSG	—	—	—	19.5
Mixed disulfide	—	—	—	19.75

^aPeptide sequences are abbreviated using standard one-letter codes for the amino acids. Note that U is the one-letter abbreviations for selenocysteine.

^bThe amide strain energy is calculated from $E = A \sin^2 \omega$ where A is 125.52 kJ/mol.

^cDSE is equal to $8.37(1 + \cos 3\chi_1) + 8.37(1 + \cos 3\chi_1') + 4.18(1 + \cos 3\chi_2) + 4.18(1 + \cos 3\chi_2') + 14.64(1 + \cos 2\chi_3) + 2.51(1 + \cos 3\chi_3)$ as reported in [43].