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# Rapid Nitrogen Inversion Pathway in the *cis*/*trans* Isomerization of Selenoxo Peptide Bonds

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The peptide bonds of a protein backbone confer conformational constraints, of which the slow *cis*/*trans* isomerization defines an enzymatically targeted conformational interconversion of high biological significance.[1] The majority of experimental data for the spontaneous isomerization support the assumption that the barrier of *cis*/*trans* interconversion is rotational and not one controlled by nitrogen inversion in aqueous solution.[2] Moreover, the transition state of the enzymatically catalyzed isomerization is associated with motions resulting from peptide bond rotation.[3] Despite the findings described above, non-enzymatic pathways to enhance the conformational flexibility of the protein backbone by manipulating the electronic nature of the peptide bond are under debate.

Notably, the extreme p$K_a$ of the peptide bond [CO–NH–] might not completely prevent the transient formation of its imidate or iminol forms by electrostatic interactions in buried regions of a protein. This kind of behavior is related to the specific base-catalyzed dissociation of amide protons, which is the reason for the chemical exchange under basic conditions and is useful for probing protein folding and stability.[4] Peptide bond tautomerism is expected to participate in the unidirectional proton-transfer pathway in cytochrome c oxidase.[5] Nitrogen inversion is discussed as a potentially stabilizing step in the acylation pathway of serine proteases,[6] and the highly acidic carbonyl-amine form of the Asn side chain amide is discussed to play an essential role for amide group activation in the oligosaccharyltransferase-catalyzed Asn glycosylation.[7] By applying density function (DFT)-based simulations, the *cis*/*trans* isomerization is found to exhibit distinct pathways in the oxoamide and the iminol forms of polyglycine.[8] Although the characterization of peptide-bond-derived imidates is crucial to the understanding of proteinaceous amide reactivity and conformational interconversions, their short lifetime[9] and low abundance at moderate basic pH values[10] prevent a detailed characterization in aqueous solution. Chalcogen-substituted isosteres of the peptide bond may provide an alternative approach to the investigation of these properties, because they couple higher NH acidity with photocontrol of the *cis*/*trans* equilibrium. The thioxo substitution, due to its minimal perturbation of the peptide backbone,[11] is considered an ideal backbone modulation to serve as a hydrogen bonding probe,[12] reversible photoswitch[13] and potent fluorescence quencher.[14] The selenium-substituted analogues, selenoxo peptides, have also been recently synthesized and they feature a higher C–N rotational barrier, enhanced NH acidity and lower photoswitching energy than the thioxo peptides.[15] A further study of the oxo, thioxo and selenoxo peptide bonds has revealed that chalcogen atomic polarizability is the predominant factor determining several of their physicochemical properties.[16]

Spectrophotometric titration of Bz-VGAψ[CSe–NH]FA-$NH_2$ (Bz = benzoyl, ψ indicates a pseudo-peptide bond) yielded a p$K_a$ of 9.1 for the selenoxo amide proton dissociation (Figure 1). The ionic form featured a loss of UV absorbance at about 296 nm but an increase of absorbance at around 260 nm. The loss of the 296 nm band, which is attributed to π–π* transition of C=Se, clearly indicated that the double bond between carbon and selenium does not exist in the dissociated form. The new 260 nm band may result from the n–σ* transition of [C(Se$^-$)=N–], which is shifted to

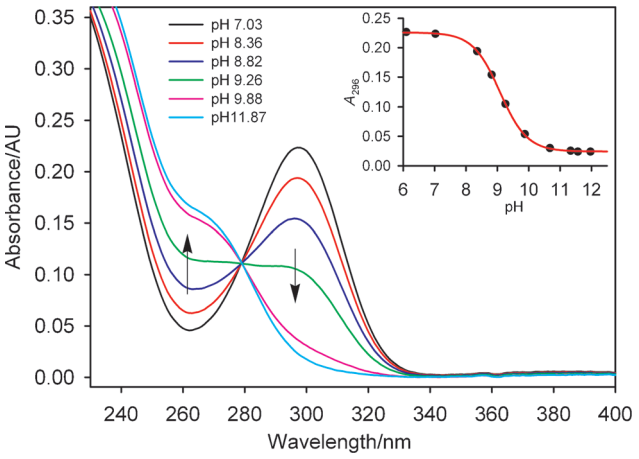


Figure 1. The pH-dependent UV spectra of Bz-VGAψ[CSe–NH]FA-$NH_2$ in aqueous solution at 20.0 °C. The absorbance at 296 nm was fitted to the Henderson–Hasselbalch equation, resulting in a p$K_a$ value of 9.1 for the selenoxo amide group.

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longer wavelengths compared to the ionized selenol group in selenocysteine.[17] The negative charge should be localized at the Se due to its high atomic polarizability,[16] thus leading to the formation of a selenoimidate anion. Upon irradiation with 288 nm UV light, the *cis* isomer content increased, as characterized by a red-shift of the UV spectrum, and then recovered to the ground state *cis*/*trans* ratio after dark equilibration (Figure 2). Notably, only the selenoxo moiety but

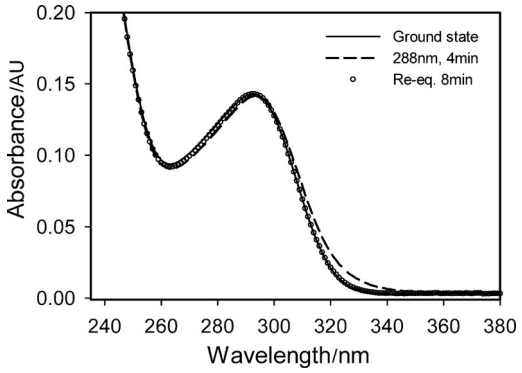



Figure 2. UV spectra characterizing the reversible photoisomerization of Bz-VGAψ[CSe–NH]FA-$NH_2$ (50 μM) in Gly-NaOH buffer (1 mM), pH 9.0, at 20.0 °C.

not any other ionized species contributed to the time-dependent UV absorbance at 320 nm. Typically, kinetic traces were recorded in the dark after the photostationary state was reached, which is characterized by a *cis* content of about 20% for the [CSe–NH] moiety. However, a lower value was observed at high pH under the same irradiation conditions.

In contrast to the relatively inert response of the *cis/trans* isomerization of oxoamides on pH variation,[18] the *cis* to *trans* isomerization rate of selenoxo peptides was dramatically accelerated at high pH (Figure 3). Intriguingly, time courses of the *cis* isomer decay varied depending on pH, following strict first-order kinetics under neutral conditions (Figure 3a), whereas sigmoidal kinetics was observed at alkaline pH (Figure 3b). Comparison of the shapes of the time courses obtained for Bz-VGAψ[CSe–NH]A-OMe and Bz-VGAψ[CSe–NH]AA-$NH_2$ (Figures S1a and S2 in the Supporting Information) did not reveal any dependence on peptide structure.

Next, it was established that the *cis* to *trans* isomerization of the selenoxo imidic bond [CSe–N<] in Bz-VGAψ[CSe–N]P-OMe was not accelerated at high pH values (Figure S3 in the Supporting Information). Therefore, we concluded that $OH^-$ ion attack on the selenocarbonyl carbon in the selenoxoamide state of the peptides was unsuitable for explaining the high isomerization rates found in Figure 3b. The deprotonation of the $C_\alpha$ atom attached to the C=Se bond could also be excluded, since this would lead to an epimerization that was not observed, as evidenced by the conservation of the HPLC profile and by NMR spectra (Figures S4 and S5 in the Supporting Information). Considering

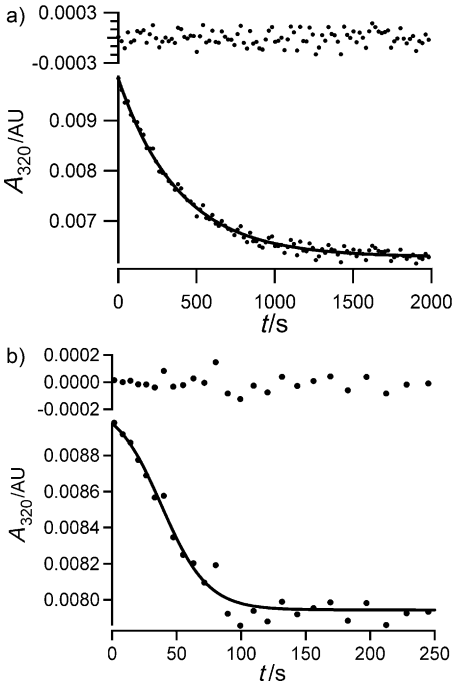



Figure 3. Time course of the *cis* decay after UV irradiation (288 nm, 4 min) observed for Bz-VGAψ[CSe–NH]FA-$NH_2$ (4.5 μM) at: a) pH 7.1 (1 mM phosphate buffer), and b) pH 9.9 (1 mM Gly-NaOH buffer) at 20.0 °C. The dark reaction was monitored at 320 nm. From the time course at pH 7.1, a first-order rate constant of $2.58\times10^{-3}$ $s^{-1}$ was calculated. The time course at pH 9.9 was fitted by Equation (1), resulting in the following parameters: $A_{00}=0.001$ AU, $b=42.3$, $\Delta A=0.0011$ AU, $k_{app}=0.053$ $s^{-1}$. The solid lines represent least-square fits. The plots of the residuals are given above each graph.

the rate versus pH profile, the highest interconversion rate could be observed in a pH region corresponding to the complete formation of the selenoimidate anion (Figure 4). Given the structural features of this species, which must make rotational movements more energy demanding, nitrogen inversion represents an alternative pathway for the rapid *cis* isomer decay. Notably, the *E*/*Z* interconversion of the *N*-arylformimidate anion has been discussed to take

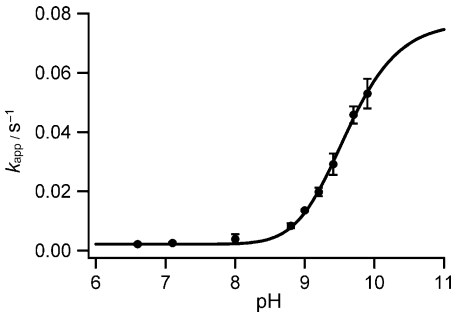
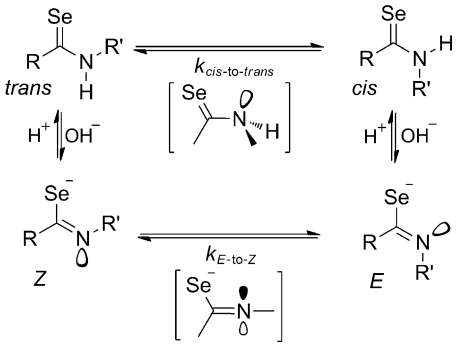



Figure 4. The pH-dependent *cis* to *trans* isomerization of Bz-VGAψ-[CSe–NH]FA-$NH_2$ (4.5 μM) at 20.0 °C. Data for pH < 8 were measured in phosphate buffer (1 mM), and data for pH ≥ 8 were measured in Gly-NaOH buffer (1 mM). The pH–$k_{app}$ plot was fitted by Equation (2) with the experimental restrictions $k_{cis\text{-}to\text{-}trans}=0.0022$ $s^{-1}$ and $pK^{trans}=9.1$, resulting in the parameters $k^{obs}_{E\text{-}to\text{-}Z}=0.077$ $s^{-1}$ and $pK^{cis}=9.3$.




Scheme 1. The *cis*/*trans* isomerization, nitrogen inversion and protonation/deprotonation of selenoxo peptides after leaving the photostationary state for the dark reaction. Putative transition states are shown in brackets.[22]

place via a nitrogen inversion pathway in an organic solvent.[19] Since the proton transfer steps are fairly fast and not rate-limiting for the slow conformational interconversions in aqueous solution, the *trans* and *cis* conformers could rapidly equilibrate with the *Z* and *E* selenoimidate anions, respectively, in the photostationary state (Scheme 1). In addition, the sigmoidal decay curves at high pH values (Figure 3b, and Figures S1 and S2) and the linear concentration dependence of isomerization rates (Figure 5) indicate the

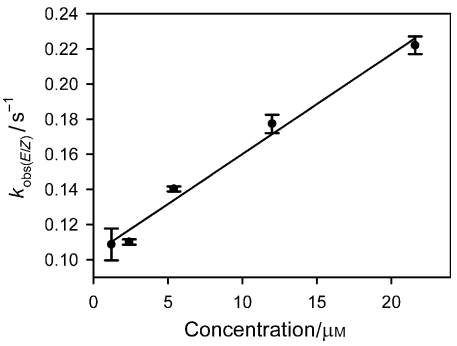



Figure 5. Concentration dependence of $k^{obs}_{E\text{-to-}Z}$ obtained for Bz-VGAψ-[CSe–NH]A-OMe in Gly-NaOH buffer (1 mM, pH 9.7) at 20.0 °C. The $k^{obs}_{E\text{-to-}Z}$ values were derived from Equation (2), by applying $k_{cis\text{-to-}trans}$ = 0.003 s$^{-1}$, p$K^{trans}$ = 9.8 and p$K^{cis}$ = 9.7 (Figure S1b). The linear fit resulted in an intercept corresponding to $k^{uncat}_{E\text{-to-}Z}$ = 0.10 s$^{-1}$.

possibility of an autocatalytic term in the *cis* isomer decay, which most likely is due to the transient presence of the *Z* selenoimidate anion for nucleophilic attack on the imine carbon atom of the *E* conformer, thus adding to the rate of apparent *cis* isomer decay (Scheme 1). The resulting tetrahedral adduct has a disrupted amide resonance and will thus exhibit a very low energy barrier to isomerization. This type of catalysis is consistent with data indicating a very high nucleophilicity of anionic selenium compounds.[20]

In the experiment described in Figure 4, the intermolecular term of *cis* isomer decay was held small. Furthermore, for secondary amide peptide bonds the *cis* content in the dark is negligible,[21] and the *E* content should also be very low at equilibrium due to a strong lone pair repulsion in the *E* configuration. Hence, we assumed in the analyses that $k_{cis\text{-to-}trans} \gg k_{trans\text{-to-}cis}$ and $k_{E\text{-to-}Z} \gg k_{Z\text{-to-}E}$, thus allowing us to ignore the reverse reactions in Scheme 1.

Solving the rate equations for the *cis* isomer decay (see the Supporting Information) based on the mechanism depicted in Scheme 1 results in Equations (1) and (2):

$$A_{320} = \frac{k_{app}}{b + \left(\frac{k_{app}}{\Delta A} - b\right) e^{k_{app} t}} + A_{00} \quad (1)$$

$$k_{app} = \frac{\frac{10^{(pH - pK^{cis})}}{1 + 10^{(pK^{trans} - pH)}} k^{obs}_{E-to-Z} + k_{cis-to-trans}}{1 + 10^{(pH - pK^{cis})}} \quad (2)$$

where $\Delta A$ is the amplitude of the absorbance increase after irradiation, $A_{00}$ is the absorbance at infinite time, $b$ is a constant at a given pH (see the Supporting Information), $k_{app}$ is the apparent rate constant for *cis* to *trans* relaxation, $k_{cis\text{-to-}trans}$ is the rate constant of *cis* to *trans* isomerization via the C–N rotational pathway, $k^{obs}_{E\text{-to-}Z}$ is the observed rate constant of *E* to *Z* conversion in the selenoimidate anion, and $t$ is the reaction time.

The sigmoidal character of the time course depicted in Figure 3b is perfectly described by Equation (1). Based on Equation (2), the apparent rate constant for the *cis* to *trans* decay of the peptide at a given pH value contains a rotational and an inversion term for the selenoxo and the selenoimidate anion, respectively. Consequently, the pH–$k_{app}$ plot (Figure 4) could be fitted by Equation (2), resulting in an observed rate constant of 0.077 s$^{-1}$ for *E* to *Z* conversion of the selenoimidate anion, which represents a 35-fold rate increase as compared to the C–N rotational pathway that applies for the neutral selenoxo peptide. The solvent deuterium kinetic isotope effect (SKIE), defined as $k^{obs}_{E\text{-to-}Z}$ ($H_2O$)/$k^{obs}_{E\text{-to-}Z}$ ($D_2O$) = 1.13 ± 0.04, was measured with Bz-VGAψ-[CSe–NH]FA-$NH_2$ (4.5 μM) by using the same ratio between the ionized and unionized species (pH 9.2/pD 9.85, Figure S6 in the Supporting Information). This SKIE value indicates that solvent reorganization occurs in the transition state of nitrogen inversion to a much higher extent than in the case of the rotational pathway, for which the value of $k_{cis\text{-to-}trans}$ ($H_2O$)/$k_{cis\text{-to-}trans}$ ($D_2O$) = 1.004 ± 0.008 is consistent with the SKIE obtained for *cis*/*trans* isomerization via the rotational pathway in oxoamide bonds.[18a,23]

From the concentration dependence (Figure 5), the *E* to *Z* inversion rate without the autocatalytical term ($k^{uncat}_{E\text{-to-}Z}$) could be extrapolated. Therefore, measuring the temperature dependence of $k^{uncat}_{E\text{-to-}Z}$ allowed the determination of the activation parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the nitrogen inversion pathway in comparison to those found for C–N rotation. Based on the linear Eyring plot (Figure S7 in the Supporting Information), the activation energy $\Delta G^{\ddagger}$ for *E* to *Z* inversion in Bz-VGAψ[CSe–NH]A-OMe was thus determined to be 18.4 kcal mol$^{-1}$ (Table S1 in the Supporting Information), which is 2.3 kcal mol$^{-1}$ lower than the C–N ro-

tation barrier of 20.7 kcal mol$^{-1}$ measured at pH 6.5. The large negative entropy of the *E* to *Z* inversion indicates marked solvent participation and reorganization.

For the sake of comparison, the pH-dependence of *cis* to *trans* decay in the thioxo peptide Bz-VGAψ[CS–NH]FA-$NH_2$ was also evaluated. The p$K_a$ of the thioxo group was determined spectrophotometrically at 266 nm to be equal to 11.0 (Figure S8 in the Supporting Information). Surprisingly, the presence of thioimidate anion showed that the *cis* isomer decay is even slower than that of the unionized thioxoamide and the time courses did not reveal autocatalysis (Figure 6 and Figure S8b). It is therefore reasonable to

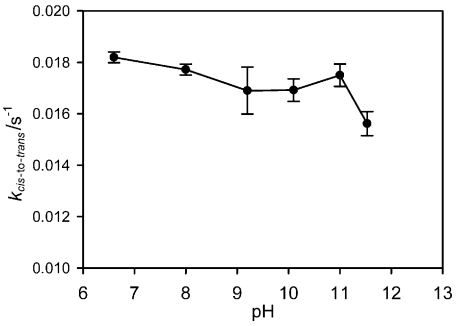



Figure 6. The pH-dependence of the *cis* to *trans* decay measured for Bz-VGAψ[CS–NH]FA-$NH_2$ at 20.0 °C. Excess *cis* isomer was achieved by irradiation with 260 nm UV light for 4 min. The *cis* decay was subsequently monitored at 290 nm. Data for pH < 8 and pH > 10.8 were measured in phosphate buffer (1 mM); data for 8 ≤ pH < 10.8 were measured in Gly-NaOH buffer (1 mM).

assume a nitrogen inversion rate that is similar or slightly slower than the rate of the rotational pathway. The *E* to *Z* inversion barrier of the *N*-benzylformimidate anion in [$D_6$]DMSO has been determined as 23.3 kcal mol$^{-1}$,[24] and in case of the *N*-benzylthioformimidate anion in MeOD as 19.6 kcal mol$^{-1}$.[25] Both values are above the inversion barrier of the selenoimidate anion in aqueous solution of 18.6 kcal mol$^{-1}$.

Even though the polarity of the solvents used is quite different, a linear decreasing trend is clearly observed in the $\Delta G^{\ddagger}_{E\text{-to-}Z}$ versus chalcogen polarizability profile (Figure S9 in the Supporting Information), which is in agreement with previous findings that the chalcogen atomic polarizability dominates many electronic properties of [CX–NR–] (X = O, S, Se; R = H, alkyl).[16] Certainly, it is clear that the introduction of photoresponsive chalcogens in a polypeptide backbone opens up a convenient way to manipulate and to characterize backbone dynamics of a polypeptide chain in a site-specific manner. The selenoxo peptide bond, due to its low p$K_a$ and rapid *E*/*Z* inversion, is of special interest, since the formation of the selenoimidate anion results in an enhancement of peptide bond *cis*/*trans* isomerization dynamics via a reaction pathway previously undetected in aqueous solution.

At this point, our data do not support a similar isomerization rate enhancement for the oxoimidate species potentially formed in the interior of proteins. However, as it became obvious from the intramolecular catalysis of peptidyl prolyl isomerization, peptide bond properties might change considerably owing to amino acid sequence specific interactions.[23,26]

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**Keywords:** chalcogens · isomerization · nitrogen inversion · selenoimidate anion · selenoxo peptides

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