Restricting the \( \psi \) Torsion Angle Has Stereoelectronic Consequences on a Scissile Bond: An Electronic Structure Analysis

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ABSTRACT: Protein motion is intimately linked to enzymatic catalysis, yet the stereoelectronic changes that accompany different conformational states of a substrate are poorly defined. Here we investigate the relationship between conformation and stereoelectronic effects of a scissile amide bond. Structural studies have revealed that the C-terminal glycine of ubiquitin and ubiquitin-like proteins adopts a syn \( (\psi \sim 0^\circ) \) or gauche \( (\psi \sim \pm 60^\circ) \) conformation upon interacting with deubiquitinasas/ubiquitin-like proteases. We used hybrid density functional theory and natural bond orbital analysis to understand how the stereoelectronic effects of the scissile bond change as a function of \( \varphi \) and \( \psi \) torsion angles. This led to the discovery that when \( \psi \) is between 30\(^\circ\) and –30\(^\circ\) the scissile bond becomes geometrically and electronically deformed. Geometric distortion occurs through pyramidalization of the carbonyl carbon and amide nitrogen. Electronic distortion is manifested by a decrease in the strength of the donor–acceptor interaction between the amide nitrogen and antibonding orbital \( (\pi^* ) \) of the carbonyl. Concomitant with the reduction in \( \pi_N \rightarrow \pi^* \) delocalization energy, the sp\(^2\) hybrid orbital of the carbonyl carbon becomes richer in p-character, suggesting the syn conformation causes the carbonyl carbon hybrid orbitals to adopt a geometry reminiscent of a tetrahedral-like intermediate. Our work reveals important insights into the role of substrate conformation in activating the reactive carbonyl of a scissile bond. These findings have implications for designing potent active site inhibitors based on the concept of transition state analogues.

Stereoelectronic effects dictate structure and reactivity in organic chemistry.\(^1\) The concept of stereoelectronic effects is rooted in the interactions between orbitals. According to frontier molecular orbital theory, chemical reactions require overlap between the highest occupied molecular orbitals (HOMOs) and the lowest unoccupied molecular orbitals (LUMOs) of the reactants. When orbitals are properly aligned, donor–acceptor interactions can occur, stabilizing conformations and transition states. Consider the case of chorismate mutase, an enzyme that catalyzes the key step in the shikimate pathway by converting chorismate to prephenate. The [3,3]-sigmatropic rearrangement of chorismate proceeds through a chairlike transition state in which orbitals are correctly aligned.\(^2,3\) Gaining access to the chair conformer, however, requires energy as other conformers are more populated in solution. Chorismate mutase facilitates this process by rapidly converting the nonproductive states to the chair conformation.\(^4\) This example illustrates that a structure resembling the transition state [also called a near attack conformer (NAC)] can be embedded within the Boltzmann distribution of ground state substrate conformations.\(^5\) The key is for an enzyme to perturb the distribution in favor of the NAC.

Our lab has been interested in examining whether the concept of NACs applies to the isopeptidase activity of deubiquitinases (DUBs) and ubiquitin-like (Ubl) proteases. DUBs and Ubl proteases catalyze the removal of ubiquitin (Ub) and Ubl proteins from target proteins by hydrolytically cleaving the isopeptide bond between the Ub/Ubl C-terminal glycine and the \( \varepsilon \)-amino group of a substrate lysine\(^6,7\) (Figure 1A). The general mechanism involves formation of a Michaelis complex with a Ub/Ubl–protein conjugate, nucleophilic attack of an active site thiolate on the C-terminal carbonyl carbon of Ub or a Ubl, generation of a thioester acyl–enzyme intermediate, and hydrolysis to liberate the free enzyme and Ub/Ubl (Figure 1B). Structural studies of Michaelis complexes have shown that the C-terminal glycine of Ub/Ubls (the P1 residue using the protease nomenclature) is confined to \( \psi \) angles that fluctuate between syn and gauche conformations \( (–60^\circ < \psi < 20^\circ) \)\(^8,13\) (Figure 1C and Table 1). According to quantum mechanics/molecular mechanics simulations, the syn conformation rapidly isomerizes back to the anti configuration in the absence of a protease.\(^14\) However, in the presence of an enzyme, the syn conformer is preferred because the vicinal NH groups of P1-Gly engage in a hydrogen bond network. The question is whether the syn configuration places the scissile carbonyl in a reactive conformation.

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We sought to address this problem by investigating the geometric and stereoelectronic changes that occur in P1-Gly as a function of conformation. The primary stereoelectronic effect that influences the reactivity of an amide bond is the donor–acceptor interaction between the nitrogen lone pair and the adjacent carbonyl antibonding orbital (herein termed the $n_N \rightarrow \pi^*$ interaction). Unlike the chorismate example, the hypothesis is that activation of a scissile carbonyl requires a reduction in the level of orbital overlap by minimizing the $n_N \rightarrow \pi^*$ interaction. Consistent with this idea, model compounds of twisted amides bearing an orthogonal nitrogen lone pair and carbonyl $\pi^*$ orbital are $10^{-3}$–$10^{-4}$ times more susceptible to hydrolysis than planar amides. How proteases contort an imidic bond and reduce resonance stabilization in an actual peptide substrate is, however, unclear. A few reports suggest the key to activation is rotation around the $\omega$ torsion angle in the form of cis–trans isomerization.

Other studies argue that scissile bond distortion depends on $\psi$, as imide bond twisting and carbonyl pyramidalization occur over a range of $\psi$ angles (i.e., when $\psi$ is close to $\pm 30^\circ$, $\pm 90^\circ$, and $\pm 150^\circ$). Because the $\psi$ angle is confined for P1-Gly in Michaelis complexes of substrate-bound DUBs, we decided to focus on the relationship between the $n_N \rightarrow \pi^*$ interaction and $\psi$. Using hybrid density functional theory and natural bond orbital (NBO) analysis, we show that when P1-Gly of Ub/Ubl conjugates is forced to adopt a syn conformation ($-30^\circ \leq \psi \leq 30^\circ$) the carbonyl and amide nitrogen experience out-of-plane deformations and there is a corresponding decrease in the extent of $n_N \rightarrow \pi^*$ interaction. These geometric and electronic distortions are also accompanied by a reorganization of hybrid atomic orbitals. Our findings suggest that rotation around the $\psi$ torsion angle can activate the scissile bond for cleavage.

### Table 1. Torsion Angles ($\phi$, $\psi$, and $\omega$) of P1 and P2 Glycines in the Presence and Absence of DUBs and Ubl Proteases

<table>
<thead>
<tr>
<th>Protein Data Bank</th>
<th>Resolution (Å)</th>
<th>DUB/Ubl Protease</th>
<th>Ub/Ubl Conjugate</th>
<th>Scissile Bond</th>
<th>P1 $\phi$ (deg)</th>
<th>P1 $\psi$ (deg)</th>
<th>P2 $\phi$ (deg)</th>
<th>P2 $\psi$ (deg)</th>
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<tr>
<td>2IY0</td>
<td>2.8</td>
<td>SENP1</td>
<td>SUMO-1-RanGAP1</td>
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<td>$-163$</td>
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<td>$168$</td>
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<td>Ub-Nε-K11 peptide</td>
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<td>$-16$</td>
<td>$-153$</td>
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each conformer was calculated using the Boltzmann distribution equation and plotted as a function of $\psi$ and $\varphi$. The data are presented in Table S1.

**Out-of-Plane Deformations.** The out-of-plane deformation of the imidic C(=O)–N group in each conformer of tripeptide I can be described in terms of its internal orthogonal coordinates $\tau$, $\chi_C$, and $\chi_N$. The coordinate $\tau$ corresponds to the mean twisting angle around the C–N bond ranging from 0° (planar amide) to 90° (when the nitrogen lone pair is orthogonal to the carbonyl $\pi^*$ system), and $\chi_C$ and $\chi_N$ represent the out-of-plane bending angles of the C and N atoms, respectively (Figure 3). In a planar $sp^3$ hybridized system

$\chi_C$ and $\chi_N$ should be 0°. The geometrical meaning of $\tau$, $\chi_C$, and $\chi_N$ can be understood by defining the four torsion angles $\omega_1 = O$–C–N–C, $\omega_2 = C\alpha$–C–N–H, $\omega_3 = C\alpha$–C–N–C, and $\omega_4 = O$–C–N–H. Using these angles $\tau = (\omega_1 + \omega_2)/2$, $\chi_C = \omega_1 - \omega_1 + \pi$ (mod 2$\pi$), and $\chi_N = \omega_2 - \omega_2 + \pi$ (mod 2$\pi$). As a second measure of out-of-plane deformations, we calculated the sum of the bond angles around the P1 carbonyl group of tripeptide I as a function of $\varphi$, $\psi$ angles. For a perfectly planar system the sum is 360° and it should decrease as the carbonyl deviates from planarity. The coordinates for each conformer are presented in Table S2.

**Changes in Resonance Stabilization.** NBO analysis was performed using NBO 6.0.34 interfaced into Gaussian 09. NBO analysis transforms the nonorthogonal atomic orbitals from the HF wave function into natural atomic orbitals (NAOs), natural hybrid orbitals (NHOs), and natural bond orbitals (NBOs). NBO transformations yield filled orbitals with high occupancies, which allow for the analysis of donor–acceptor interactions through second-order perturbation theory. The energy values from the second-order perturbation method provide a reasonable qualitative description of the magnitude of resonance stabilization through the $n\pi \rightarrow \pi^*$ interactions.35,36 NBO deletion analysis was also performed using the B3LYP/6-311+G(2d,p)-optimized structures. It is important to point out that the NBO method overestimates charge-transfer energies compared to the results of other localized wave function methods.37 Nevertheless, the trends obtained from NBO calculations are the same as those produced by these other methods. More importantly, the overestimation of charge-transfer energies will be the same for each conformer in this study. NBO second-order perturbation data are presented in Table S3.

**Hybrid Orbital Distortions.** NBO transformations lead to NHOs, which represent optimal fits to electronic occupancies in terms of known angular properties of atomic orbitals. NHOs can therefore be used to measure percent p character and percent s character.38 The hybridization data for each conformer are presented in Table S4.

## RESULTS

### The P1 Residue Is Conformationally Restricted in the Presence of DUBs and Ubl Proteases.

Structures of catalytically inactive forms of cysteine-dependent DUBs and Ubl proteases bound to native substrates provide important insights into the geometrical changes that occur upon forming a Michaelis complex. To date, eight structures have been reported at resolutions between 1.9 and 3.2 Å (Table 1).40–43 In all cases, the DUB or Ubl protease has been rendered inactive through a C-to-S or C-to-A substitution. Conformational analysis of the scissile bond reveals the $\psi$ torsion angle of P1-Gly decreases upon formation of a Michaelis complex (Table 1; $\psi$ moves from $\pm160°$ in the absence of a protease to between $-60°$ and $20°$ in the presence of a protease). When $\psi \approx 0°$, the vicinal NH groups of P1-Gly are essentially eclipsing one another in a syn configuration (Figure 1C). For comparison, we also analyzed the conformation at the P2 site. These data demonstrate that upon engagement of a protease, conformational changes are largely limited to the P1 position, as the geometry at P2 remains relatively invariant (average P2 glycine $\psi = 154°$).

What restricts the conformation of P1-Gly is a backbone carbonyl of the protease. The cysteine protease DUBs and SENPs are characterized by the presence of a Cys-His-Asp/Asn catalytic triad.39 According to available structures, the catalytic Cys is invariably positioned at the N-terminus of an $\alpha$-helix, while the His and Asp/Asn residues are located within $\beta$-strands or loop regions. In all of the Michaelis complexes, there is a backbone carbonyl next to the catalytic His that is pointing toward the two NH groups of P1-Gly (Figure 1C). This carbonyl forms bifurcated hydrogen bonds, as measurements reveal hydrogen bonding criteria are satisfied in each structure; the donor–acceptor distances are 2.86 ± 0.16 and 3.06 ± 0.19 Å, the H···O distances 1.90 ± 0.19 and 2.19 ± 0.14 Å, and N–H···O bond angles are 160.0 ± 9.9° and 145.9 ± 15.0°.40 Because the strength of bifurcated hydrogen bonds is estimated to be $\sim2$–3 kcal mol$^{-1}$,41 formation of such an interaction likely compensates for the allylic strain imposed by the syn configuration. We propose bifurcated hydrogen bonds are conserved in each Michaelis complex, as superposition aligns the active sites of all cysteine-dependent DUBs and Ubl proteases.

To understand how locking P1-Gly into a syn conformation could affect the energetics of the system, we constructed a Ramachandran surface of tripeptide I, a model for the isopeptide linkage (Figure 2). This was achieved by performing density functional theory (DFT) calculations at the B3LYP/6-311+G(2d,p) level. The $\varphi$ torsion angle was varied in 30° increments, and $\psi$ was rotated in 10° increments, with subsequent energy minimization. The fractional population of each conformer was then plotted as a function of $\varphi$ and $\psi$ (Figure 4A and Table S1). The resulting contour map is in accord with the canonical clustering of glycine conformations at $\varphi = 180°$ and $\psi = 0°$.42,43 Placing the $\varphi\psi$ coordinates from each of the crystal structures on the Ramachandran plot indicates the syn and gauche conformations are in allowed space, with energies that are $\sim1$–3 kcal mol$^{-1}$ higher than those of the anti configurations observed in the absence of a protease (Figure 4B).
Figure 4. C-Terminal glycine of Ub/Ubls that adopts a syn conformation when bound to DUBs and Ubl proteases. (A) Ramachandran plot for P1-Gly of tripeptide I. Fractional populations were calculated using the Boltzmann distribution equation. Red corresponds to heavily populated conformers, while blue represents high-energy species. (B) Coordinates for P1-Gly of protease-bound and unbound Ub/Ubl conjugates are mapped on the Ramachandran plot for glycine. The allowed conformational space is colored blue, and the forbidden conformations are colored gray.

The Scissile Bond Is Distorted in Michaelis Complexes. With the identification of the syn conformer within the Boltzmann distribution of ground state conformations, we sought to determine whether the scissile bond is distorted in this configuration. Out-of-plane deformations of an amide bond can be described by the sum of bond angles as well as angles $\chi^0$, $\chi^0 \phi$, and $\tau$ (Table S2).

We calculated the sum using three bond angles: $\alpha = C - C', O$, $N - C' - O$, and $\alpha = C - C', N$. Any deviation from trigonal planar geometry should lead to a sum of $<360^\circ$. As evidenced by what ostensibly look like horizontal stripes in $\phi, \psi$ space, there are small (<0.3°) but significant (>2σ) deviations in trigonal planar geometry (Figure 5A). This indicates that the geometry of the carbonyl is dependent on the $\psi$ torsion angle. The most significant distortions ($\Sigma \sim 5\sigma$) occur when glycine has $\phi, \psi$ coordinates of (60°,60°) and (140°,25°). On the left side of the map ($\phi < 0^\circ$), there is a smaller departure from trigonal planar geometry. However, there are points within this region where the sum is approximately 4σ, e.g., when the $\phi, \psi$ coordinates are ($-140^\circ,-25^\circ$) and ($-60^\circ,-60^\circ$).

A plot of $\chi_C$ as a function of $\phi$ and $\psi$ reflects what we observe for the sum of the bond angles (Figure 5B). The largest $\chi_C$ values are 6° at (140°,25°) and −5° at ($-140^\circ,-25^\circ$), with a mean of 0.5°. Comparing these values to those obtained from crystal structures of serine proteases bound to proteinaceous inhibitors ($\chi_C$ between −10° and −20°) indicates a smaller degree of pyramidalization. Nevertheless, $\chi_C$ values at the extremes strongly deviate from the mean, and it is at the extremes where the greatest overlap exists with the syn configuration of P1-Gly.

From the $\chi_C$ map, we can also extract information about the direction of pyramidalization. This is important as it relates to which prochiral face of an amide bond a nucleophile will add. A negative $\chi_C$ value predisposes the carbonyl carbon to si face addition. Conversely, a positive $\chi_C$ value means the carbonyl carbon is prone to re face attack. Focusing in on regions of the map occupied by the Michaelis complexes (Table 1 and Figure 4B), we find that in the lower left quadrant ($-170^\circ < \phi < 170^\circ$) and ($-140^\circ < \psi < 170^\circ$), regions of the map are predominantly blue, indicating that glycine is more likely to adopt a syn conformation when bound to DUBs and Ubl proteases.

Figure 5. Conformational dependence of scissile bond distortion. (A) Contour map showing deviations from trigonal planar geometry as a function of $\phi$ and $\psi$ torsion angles. The sum of bond angles $\alpha = C - C', O$, $N - C' - O$, and $\alpha = C - C', N$ was calculated for each geometry-optimized structure of tripeptide I. The scale from blue to red represents the deviation from the idealized 360° geometry. The standard deviation (σ) for these changes is 0.05 (determined using $\phi = 180^\circ$, ±150°, ±120°, and ±60°). Distortions considered significant are >2σ (shown from green to red). (B) Contour map of $\chi_C$ as a function of $\phi$ and $\psi$ torsion angles. Regions in blue (negative) and red (positive) represent the greatest deviations from planar ($\chi_C = 0$) geometry. The mean $\chi_C$ value is 0.5° (determined using $\phi = 180^\circ$, ±150°, ±120°, and ±60°). (C) Contour map of $\chi_N$ as a function of $\phi$ and $\psi$ torsion angles. Regions in blue (negative) and red (positive) represent the greatest deviations from planar ($\chi_N = 0$) geometry. The mean $\chi_N$ value is −3.4 (determined using $\phi = 180^\circ$, ±150°, ±120°, and ±60°). (D) Contour map of $\tau$ as a function of $\phi$ and $\psi$ torsion angles. Regions in blue (negative) and red (positive) represent the greatest twisting motion. The mean $\tau$ value is −0.4.
Nitrogen pyramidalization is accompanied by an sp2 rehybridization. During this transition, there is greater mixing of the nitrogen s and p atomic orbitals, which decreases the energy of the nitrogen lone pair (nN). Because the magnitude of the donor–acceptor nN → π* interaction is inversely proportional to the difference in energy between nN and the carbonyl π* system, lowering the energy of the lone pair will lead to a decrease in the strength of the interaction. Nitrogen pyramidalization should therefore coincide with a decrease in the nN → π* interaction. To test this, we turned to NBO analysis as it transforms a quantum mechanical wave function into orbitals corresponding to Lewis structures with localized bonds and lone pairs.45

A delocalization energy landscape for P1-Gly of tripeptide I shows qked changes in the magnitude of the nN → π* interaction. When ψ and ψ are 180°, the lone pair of the amide nitrogen is maximally delocalized into the π* of the carbonyl (Figure 6A). The absolute value of resonance stabilization is overestimated by second-order perturbation measurements37 (~80 kcal mol⁻¹) because it does not include the increase in the number of steric repulsions resulting from geometric changes associated with resonance delocalization. The empirical resonance energy corresponds approximately to the free energy of activation for rotation around the imidic C–N bond (reported to be 11–13 kcal mol⁻¹ for glycine),46 because resonance is completely lost in the transition state of this isomerization. Therefore, it is important to consider relative changes in NBO-derived stabilization energies, not the absolute energy values. Further inspection of the surface reveals two distinct regions where there is a significant decrease in the nN → π* interaction: one is on the left side when −180° < ψ < −100°, and the other is on the right side when 40° < ψ < 180° (Figure 6A). In both cases, the magnitude of the nN → π* interaction diminishes at ψ angles between 30° and −30°. A decrease in resonance stabilization is not observed for the adjacent P2 glycine (Table S3). As ψ diverges from 180°, the strong nN → π* interaction is replaced with a much weaker interaction between the nitrogen lone pair and the σ* orbital of the C=O bond.

To reinforce the validity of these computational results, we also performed deletion analysis. Computational deletion of donor–acceptor interactions is based on quasi-variational theory instead of perturbation theory and provides a second measure of the nN → π* interaction.38 The results of deletion analyses are in good agreement with those from second-order perturbation theory (Figure 6B). For instance, at ψ = 0°, quasi-variational theory estimates a 74% decrease in the extent of the nN → π* interaction relative to the median, and perturbation theory yields an 80% decrease. Our NBO results are therefore consistent with the prediction that nitrogen pyramidalization and loss of resonance stabilization overlap in several regions within the conformational landscape of glycine. More importantly, these results highlight the relationship between ψ and electronic changes to the scissile carbonyl.
The syn Conformation Distorts the Carbonyl Orbitals in the Scissile Bond. Next, we sought to investigate whether changes in electronic delocalization and carbonyl geometry are accompanied by a rehybridization of the carbonyl orbitals. Using the natural hybrid orbitals from NBO analyses, we measured the p character in interhybrid orbitals that comprise the σ framework of the carbonyl.38 For an ideal sp²-hybridized carbonyl carbon, the p character should be approximately 66%. Indeed, this is the case for most of the rotamer states of I when ψ is maintained at 150° (Figure 7A). However, when ψ closes in on 0°, there is a sharp increase in p character, reaching ~83% when the vicinal NH groups are fully eclipsed. These results suggest the carbonyl rehybridizes from sp² to sp³ as the glycine is forced into a syn configuration.

To understand how the carbonyl could undergo rehybridization yet retain an overall geometry resembling a trigonal carbon, we examined the localized NBOs of the σ and π systems. Comparing the NBOs of the anti and syn conformers reveals that in the latter the σ bond no longer lies along the C=O internuclear axis (Figure 7B). Instead, there is a tilt in the hybrid orbitals giving rise to a bonding orbital that is above the axis. The π system is almost the mirror image. Unlike a canonical π bond, the p orbitals are not at a 90° angle, as the axis of the carbon p orbital is again tilted by ~30° with respect to the C=O internuclear axis. This results in a rather unique bonding arrangement, with strong overlap between the lobes of the π orbitals that are tilted toward one another and relatively weak overlap with the lobes that are leaning away from each other. The antibonding π* NBO shows a similar disposition of orbitals. Together, these results suggest that although the C=O bond remains intact in the syn configuration the orbitals resemble a sp³-like geometry. This can have significant implications for reactivity, as the carbonyl does not have to undergo a complete rehybridization during attack by an active site thiolate.

**DISCUSSION**

In this study, we used a quantum mechanical approach to show that the conformational restriction imposed by DUBs has significant electronic and geometric consequences on the scissile bond. Structures of Michaelis-like complexes composed of cysteine-dependent DUBs/Ubl proteases and native substrates reveal a conserved bifurcated hydrogen bonding network between a backbone carbonyl of the enzyme and the vicinal NH groups of the C-terminal Ub/Ubl glycine residue. This forces glycine into a syn configuration where the vicinal NH groups are nearly eclipsing one another. Using structure correlation analysis, a method pioneered by Bürgi and Dunitz,47 we show that the conformations of glycine in crystal structures of Michaelis complexes correspond to distorted geometries. The carbonyl carbon and the amide nitrogen experience out-of-plane deformations in the syn conformation. Consequently, the canonical amide resonance stabilization is attenuated, and the amount of p character in the carbonyl carbon hybrid orbitals is increased. Our work therefore establishes that P1-Gly of DUB substrates is geometrically and electronically distorted to the extent that the scissile carbonyl resembles a putative tetrahedral transition state for acyl−enzyme formation.

Our results shed light on how proteases twist the imidic bond and weaken the nN → π* interaction. Conformational analysis of tripeptide I shows a correlation between the nN → π* interaction and peptide planarity. Via examination of the allowed region of the Ramachandran plot for glycine in I, it is evident that the magnitude of the nN → π* interaction is at its lowest when ψ is between 30° and −30°. Similarly, there is significant deviation from planarity (as measured by both the degree of carbonyl and nitrogen pyramidalization) when ψ is between 40° and −40°. These results are entirely consistent with the model that holds that ψ modulates both electronic (nN → π* interaction) and geometric (ψC) parameters.23 From a stereoelectronic point of view, the importance of ψ could be a result of steric interactions. As ψ moves closer to 0°, the four-electron closed-shell repulsion between the filled orbitals of the vicinal NH groups could force the carbonyl and nitrogen to pyramidalize,55 which in turn would lead to a reduction in the nN → π* interaction. Thus, when ψ is restricted to small angles, DUBs, and likely many other cysteine proteases, promote the loss of resonance stabilization and render the carbonyl more reactive.

We also observe a direct correlation between the loss of resonance stabilization and an increase in the percent p character of the carbonyl sp³ orbital. This increase manifests from a distortion of both the σ and π bonds. With the former, there is a loss of bonding along the internuclear C=O axis, whereas with the latter, a plane of symmetry is lost between the orbitals on both faces of the C=O bond. The net effect is a bonding pattern reminiscent of an sp³-like carbon that is induced by simply rotating around the ψ torsion angle. This partial rehybridization of the scissile carbonyl could also contribute to the free energy of activation associated with bond cleavage. A fundamental step in the cleavage of Ub/Ubl−protein conjugates by cysteine-dependent DUBs/Ubl proteases is nucleophilic addition by an active site thiolate into the π* orbital of the substrate carbonyl. If the carbonyl is forced to adopt an sp³-like geometry prior to thiolate addition, then less reorganizational energy is required for the formation of a tetrahedral species.

The conformational plasticity of glycine is likely critical to achieving a configuration conducive to nucleophilic attack. The steric constraints of amino acid side chains prevent non-glycyl residues from occupying φ,ψ space with positive φ values.42,49 Without a side chain, glycine can then populate an amount of φ,ψ space much larger that of non-glycyl residues. In fact,
nearly the whole right side ($\varphi > 0^\circ$) of the Ramachandran plot for glycine is allowed. We propose it is the right side of the Ramachandran plot that has the greatest functional consequences. Structure correlation analysis reveals that the scissile bond has coordinates of $(+\tau,+\chi_N,\chi_C)$ or $(-\tau,-\chi_N,+\chi_C)$, depending on whether glycine occupies the left or right side of the Ramachandran plot, respectively. When $\chi_C$ is negative, the carbonyl of glycine is predisposed to $si$ face addition (Figure 8). However, because $\tau$ and $\chi_N$ are positive when $\chi_C$ is negative, the lone pair on nitrogen would be pointing in the opposite direction from the imidazolium species. Computational studies of cysteine proteases have shown that the transfer of a proton from the active site histidine is concerted with nucleophilic attack by the cysteine thiolate ion. When this is taken into account, an alternative scheme would involve $si$ face addition to the carbonyl when it is pyramidalized upward $(+\chi_C)$. This would place the nitrogen lone pair in the vicinity of the imidazolium ring and allow for protonation to occur simultaneously with nucleophilic attack. We favor this model for the following reasons. First, the degree of carbonyl pyramidalization is quite small and is probably less important in activating the carbonyl than nitrogen pyramidalization. Second, the largest window in which geometric and electronic distortions occur is on the right side of the Ramachandran plot.

Our findings have led to a molecular model for how DUBs/Ub proteases activate their substrates for cleavage. We propose these enzymes twist P1-Gly into a $syn$ configuration, destabilizing and distorting the scissile carbonyl. Flexibility in both the Ub/Ubl core as well as the C-terminus is likely to play a role in promoting an environment that perturbs the scissile carbonyl and ultimately drives catalysis. Our results have implications for not only our fundamental understanding of DUBs and Ubl proteases but also the design of transition state analogues that could bind with high affinity to the active sites of these enzymes. By mimicking a tetrahedral intermediate with a vicinal NH group in an eclipsing conformation, future studies will aim to develop molecules that act as tight-binding inhibitors.

Figure 8. Schematic of $si$ face addition of the active site thiolate to the carbonyl of P1-Gly.


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