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REVIEW

Conformational landscape of substituted prolines

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Abstract

The cyclic side chain of the amino acid proline confers unique conformational restraints on its backbone and side chain dihedral angles. This affects two equilibria—one at the backbone (*cis*/*trans*) and the other at the side chain (*endo*/*exo*). Substitutions on the proline ring impose additional steric and stereoelectronic effects that can further modulate both these equilibria, which in turn can also affect the backbone dihedral angle (ϕ, ψ) preferences. In this review, we have explored the conformational landscape of several termini capped mono-(2-, 3-, 4-, and 5-) substituted proline derivatives in the Cambridge Structural Database, correlating observed conformations with the nature of substituents and deciphering the underlying interactions for the observed structural biases. The impact of incorporating these derivatives within model peptides and proteins are also discussed for selected cases. Several of these substituents have been used to introduce bioorthogonal functionality and modulate structure-specific ligand recognition or used as spectroscopic probes. The incorporation of these diversely applicable functional groups, coupled with their ability to define an amino acid conformation via stereoelectronic effects, have a broad appeal among chemical biologists, molecular biophysicists, and medicinal chemists.

Keywords Proline · Substituted proline · Conformational restriction · Collagen · Exo-endo · Cis-trans

Introduction

The local backbone conformation of a polypeptide chain is determined by three dihedral angles (ϕ, ψ, and ω). While ω is almost always restricted to ~180° (*trans* conformation), the energetically allowed ϕ-ψ conformational space is given by the Ramachandran map (Sasisekharan and Ramachandran 1957; Ramachandran et al. 1963; Ramachandran and Sasisekharan 1968). The imino acid proline (Pro) contains a cyclic side chain fused with its backbone (Fig. 1). This imposes a severe constraint on the dihedral angle ϕ (~ − 65°) yielding a Pro-specific Ramachandran map with two major conformational basins—a right-handed helix (which includes both helix and turn) and a left-handed polyproline II helix (Fig. 2a). The ϕ-ψ map corresponding to residues preceding Pro also deviates significantly (MacArthur and Thornton 1991; Ho and Brasseur 2005) from the canonical Ramachandran map. The lack of an amide hydrogen atom precludes Pro from participating in backbone hydrogen bond which is why Pro rarely appears in the middle of an α-helix or in non-edge region of a β-sheet (Chou and Fasman 1974). The most favorable conformation of Pro is in tight turns (Venkatachalam 1968; Lewis et al. 1973; Richardson 1981; Wilmot and Thornton 1988). The cyclic side chain is responsible for two types of isomerization equilibria in Pro (Fig. 2b): (i) imide *cis*/*trans* isomerism that is associated with the main chain dihedral angle ω and (ii) *exo*/*endo* ring puckering that is associated with the side chain dihedral angles $\chi 1$ and $\chi 2$.

For the imide isomerization, both steric and stereo-electronic factors determine the relative stability of the *cis* and the *trans* conformers (Ramachandran and Mitra 1976; Newberry and Raines 2017). A non-Pro amino acid prefers the *trans* conformation since the corresponding *cis* conformation is associated with steric repulsion between its C^{α} atom and the C^{α} atom of the amino acid preceding it. For Pro, both *cis* and the *trans* conformations are associated with steric repulsions (C^{α}-C^{δ} in *trans* and C^{α}-C^{α} in *cis*) (Ramachandran and Mitra 1976). Therefore, for Xaa-Pro units, the *cis* conformation becomes experimentally detectable, although the *trans* conformation is still preferred

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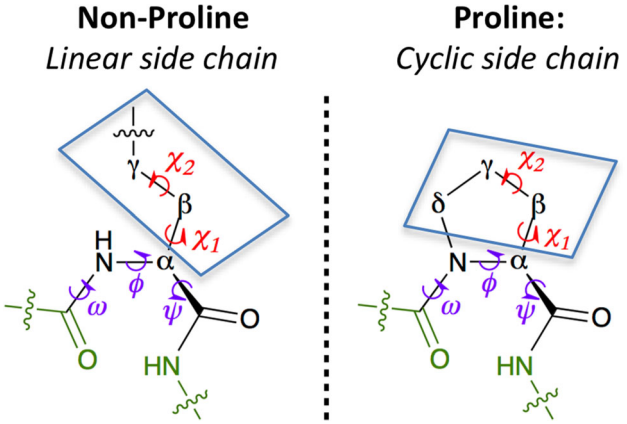

Fig. 1 A schematic depicting a generic non-proline α-amino acid (with linear side chain) and proline (with cyclic side chain). The amino acids are shown as part of a polypeptide chain where the main chain dihedral angles (ω, ϕ, and ψ) and the side chain dihedral angles (χ_1, χ_2, etc.) are shown in purple and red, respectively. Atoms of the preceding and following residues are shown in green, while the side chain atoms are boxed

due to the delocalization of the non-bonding electrons from the carbonyl oxygen (*n*) of the residue preceding Pro to the antibonding π* orbital of Pro carbonyl carbon (the $n \rightarrow \pi^*$ interaction; see Fig. 2b) (Bartlett et al. 2010; Zondlo 2010; Newberry and Raines 2017). The enhanced *cis*-content of Xaa-Pro can be further influenced by the presence of neighboring aromatic amino acids (Grathwohl and Wüthrich 1976; Hetzel and Wüthrich 1979; Yao et al. 1994; Wu and Raleigh 1998; Pal and Chakrabarti 1999; Meng et al. 2006; Thomas et al. 2006; Zondlo 2013). For example, in a series of designed peptides containing -Xaa-Pro-, the *cis*-content was found to be 37% when Xaa was Trp compared with 7% when Xaa was Ala (Wu and Raleigh 1998; Reimer et al. 1998). Similarly, analysis of Xaa-Pro in folded proteins showed a significantly higher *cis*-content of Xaa-Pro when Xaa was aromatic (12.4% versus 5% for Xaa = Trp and Ala, respectively). Non-contiguous C–H/ π interactions from aromatic groups can also significantly alter the *cis*-content of Xaa-Pro (Kemmink and Creighton 1993, 1995; Nardi et al. 2000; Dasgupta et al. 2007; Ganguly et al. 2012, 2013).

Substitutions on the Pro ring can affect both the *cis*/*trans* and the *endo*/*exo* equilibria. A classic example is the effect of 4*R*-hydroxyproline (Hyp) (Fischer 1902), a naturally occurring imino acid (Plimmer 1912) derived from the post-translational modification (PTM) of Pro by the enzyme prolyl 4-hydroxylase (Gorres and Raines 2010). Hyp is mostly found in collagen, the most abundant protein in humans. Collagen contains three polypeptide chains consisting of (Xaa-Yaa-Gly)$_n$ repeats ($n \sim 300$) (Fietzek and Kühn 1975) that winds around each other to form a right-handed triple helix providing significant tensile strength (Sasisekharan and Ramachandran 1957). While the Xaa is mostly Pro, either a Pro or a Hyp often occupies the position Yaa. While

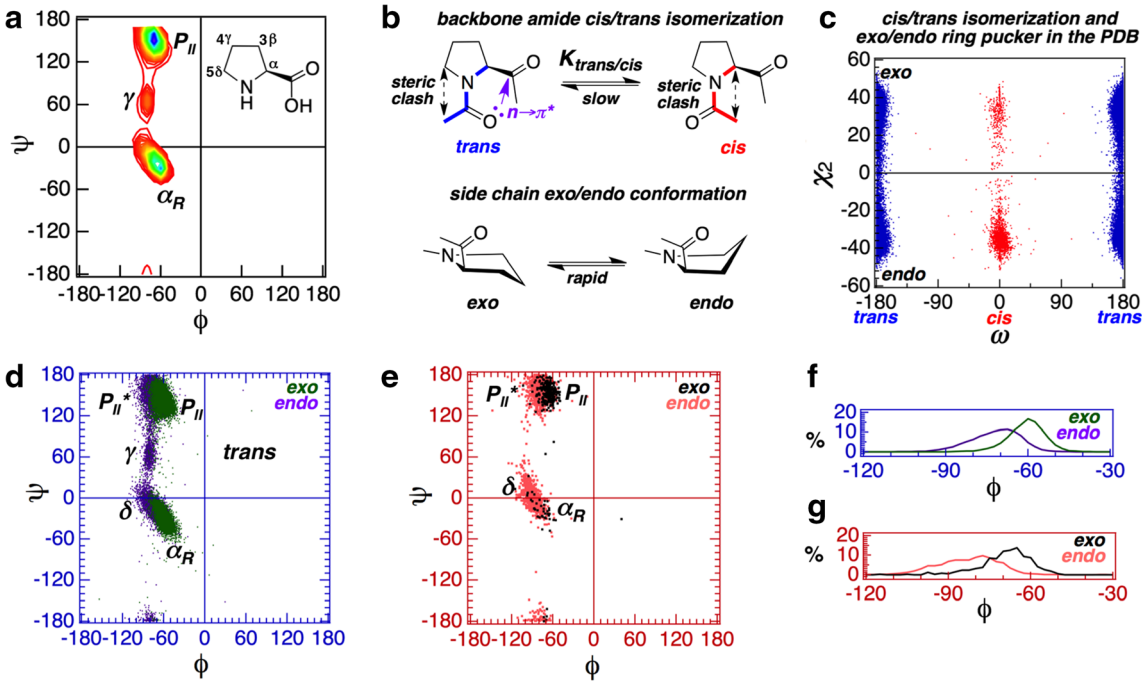

Fig. 2 **a** Observed distribution of ϕ-ψ dihedral angles for Pro in PDB. Atom numbering of Pro side chain is shown in the inset. **b** Two distinct isomerization equilibria in Pro: *cis*/*trans* and *exo*/*endo*. Both steric and stereoelectronic effects operative in the *cis* and the *trans* states are shown. **c** Observed distribution of ω-χ2 angles for Pro in PDB. The angle χ2 reflects *endo* ($\chi 2 < 0$)/*exo* ($\chi 2 > 0$) ring puckering, while the angle ω reflects *cis* ($\omega \sim 0$)/*trans* ($\omega \sim \pm 180$) imide conformation. **d** Observed distributions of ϕ-ψ dihedral angles for *trans* Pro (*exo* and *endo*) in PDB. **e** Observed distributions of ϕ-ψ dihedral angles for *cis* Pro (*exo* and *endo*) in PDB. **f** Distribution of ϕ dihedral angles for *trans-exo* and *trans-endo* conformers (from panel **d**). **g** Distribution of ϕ dihedral angles for *cis-exo* and *cis-endo* conformers (from panel **e**)

hydroxylation of Pro is known to increase the stability of collagen (Berg and Prockop 1973), the frequency of Hyp (at Yaa position) directly contributes to thermal stability of collagen. Originally it was hypothesized that water molecule–mediated hydrogen bonding between the hydroxyl group of Hyp and the carbonyl oxygen from the adjacent chains contributed to the enhanced stability of the collagen triple helix structure (Hospital et al. 1979). However, using 4*R*-fluroproline (Flp), a synthetic Pro analog, Raines and coworkers disproved this hypothesis by generating a hyperstable collagen mimic with Pro-Flp-Gly repeats (Holmgren et al. 1999). The hyperstability was observed despite fluorine being a weaker hydrogen bond acceptor than the hydroxyl oxygen in Hyp. Raines et. al. and other groups firmly established that a stereoelectronic effect, via the hydroxyl group in Hyp (reviewed in detail later), pre-organizes the Yaa position to an *exo* ring pucker and *trans* amide conformation thereby imparting stabilization (Holmgren et al. 1999; Bretscher et al. 2001; Jenkins and Raines 2002; DeRider et al. 2002; Hodges and Raines 2003; Shoulders et al. 2006, 2009; Shoulders and Raines 2009a, b). This method of investigation, i.e., to use substituted proline residues, was later extended to other systems as well.

Because of the ease of synthesis from commercially cheap starting materials (mainly Hyp), the use of 4-substituted Pro analogs in the field of chemical and structural biology has increased significantly in the past few decades. Such modifications are achievable by making Fmoc/Boc protected 4-substituted Pro analogs using Hyp in solution phase and incorporating them into peptides via standard solution-phase or solid-phase peptide synthesis protocols or even more enticingly, via the alluring "proline editing" (Thomas et al. 2005; Pandey et al. 2013) method on a pre-synthesized sequence on resin. Coupled with the increasing interest in ^{19}F NMR (Dorai 2015; Dahanayake et al. 2018) and a promising future of ^{19}F MRI (Dahanayake et al. 2018; Rose-Sperling et al. 2019) probes, 4-fluoroprolines, or fluorine-containing proline analogs in general, have justifiably dominated research in chemical biology. Recently, the impact of conformationally defined 4-fluoroprolines and other fluorine containing proline analogs on peptide structure and their application as ^{19}F NMR probes were comprehensively reviewed (Verhoork et al. 2018). Other 4-substituted proline analogs (mainly fluoro- and methylprolines) that are related to collagen stabilization/destabilization were also reviewed (Shoulders and Raines 2009b; Kubyshkin 2019). A suite of modifications and substitutions on prolines other than fluoroproline or merely 4-subtituted prolines are available. Each of these substitutions is unique in their ability to control the conformational landscape (ω, ϕ, ψ, $\chi1$, and $\chi2$) that is accessible to the proline scaffold. This review gives an overview of the conformational preference for the substituted prolines in the minimal amino acid context (singly or doubly capped Pro) as well as within a short peptide or protein context, where available.

Conformational preference of unsubstituted proline

Before discussing the conformational landscape of substituted prolines, here, we briefly summarize the key conformational features associated with unsubstituted proline. As mentioned in the previous section, the origin of specific conformational features of Pro lies in its cyclic side chain. The cyclic side chain not only restricts the back bone angle ϕ to approximately $-65°$ but also, uniquely, makes two side chain conformation states available to Pro: *endo* and *exo*. In addition, the cyclic side chain also allows Pro to access the *cis* amide (imide in case of Pro) isomer in addition to the canonical *trans* isomer. The occurrence of the *endo* and the *exo* side chain conformations is correlated with the backbone *cis* and *trans* conformations. This is evident from Fig. 2c which shows that the *endo* conformation strongly favors the *cis* isomer but the *exo* conformation has no such preference. Similarly, Fig. 2d, where the ϕ-ψ distribution of *trans* Pro (in proteins) are shown, shows that the *endo* and the *exo* conformations prefer slightly different ϕ-ψ distributions. The γ-basin, occupied by a small fraction of *endo-trans* Pro is empty for the corresponding *exo-trans* Pro. Similarly, as shown in Fig. 2e, the *endo* and the *exo* conformations prefer slightly different ϕ-ψ distribution for *cis* Pro (in proteins) as well. Specifically, compared with the *exo* conformations, the *endo* conformations in both *trans* (Fig. 2f) and *cis* (Fig. 2g) Pro prefer a broader distribution of ϕ with the mean shifted towards lower values (*trans-endo* $-70.75° \pm 9.21°$; *trans-exo* $-59.43° \pm 7.09°$; *cis-endo* $-81.62° \pm 11.37°$; *cis-exo* $-67.43° \pm 11.31°$). In summary, the side chain ring puckering of Pro can affect its backbone conformational preference. Therefore, any perturbation on the ring, like substitution of ring hydrogen atoms, can affect the conformational landscape of Pro. In the following sections, we have surveyed the conformational landscape of α(2)-, β-(3), γ(4)-, and δ(5)-substituted prolines using structures from the Cambridge Structural Database (CSD).

α-substituted proline

A variety of α-substituted Pro (Fig. 3a) have been investigated by several research groups, mostly using X-ray crystallography. Here we survey representative Pro α-substitutions as summarized in Table S1. Substitutions at the C^{α} position favors the *trans* over the *cis* imide conformation due to steric clash between the α-substituent and the side chain of the preceding residue (Torbeev et al. 2012) (see Fig. 3b). Solution ^{1}H NMR data on N-acetylated αMep and α-trifluoromethylproline

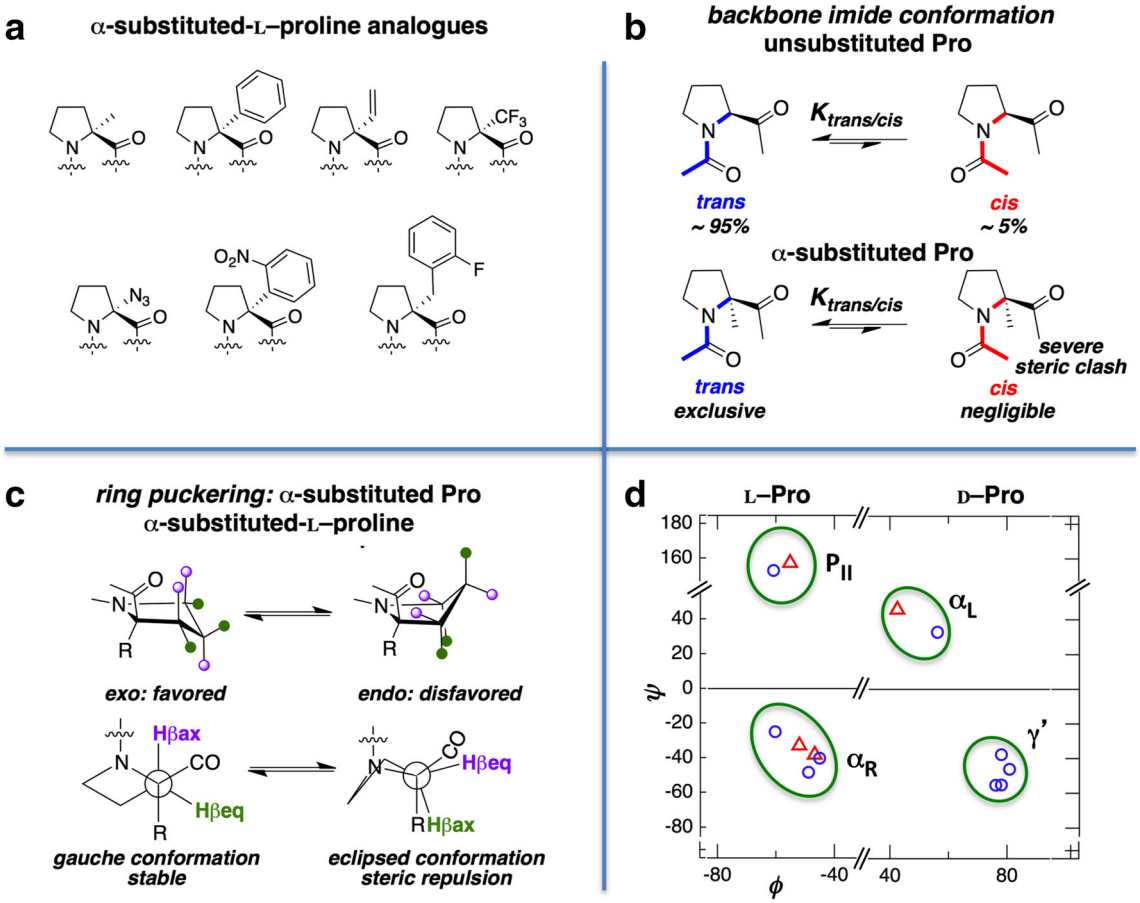

Fig. 3 **a** Representative α-substituted L-Pro analogs discussed in this article (see Table S1 for structural details). **b** A schematic showing the effect (steric clash in the *cis* conformation) of α-substitution on *cis*/*trans* equilibrium. **c** A schematic showing the effect (steric clash in the *endo* puckered state) of α-substitution on *endo*/*exo* equilibrium. **d** Ramachandran plot for α-substituted L-Pro and D-Pro analogs (circle *trans*; triangle *cis*)

expectedly show near negligible *cis* amide population in D_2O (Kubyshkin et al. 2018). In addition, α-substitution can also influence Pro ring puckering. The *exo* ring pucker is favored for L-Pro while the *endo* ring pucker is favored for D-Pro. This is because the alternate puckering states (*exo* for D-Pro and *endo* for L-Pro) are in all-eclipsed conformations and therefore sterically disfavored (Fig. 3c). Substitutions at the C^{α} position are also expected to impose restrictions on the accessible ϕ-ψ phase space in a manner analogous to that of α-amino-*iso*-butyric acid (Aib). Crystallographic (Flippen-Anderson et al. 1983; Torbeev et al. 2012; Mykhailiuk et al. 2017) and spectroscopic data, coupled with theoretical calculations, indeed show that the back bone preferences of α-methylproline (αMep) are restricted to the helical region of the Ramachandran plot (De Poli et al. 2009).

The geometric restriction of the αMep was exploited to build several structural scaffolds in conjunction with other amino acids. Toniolo and co-workers have designed several scaffolds with β-II, β-II', β-III, and β-III' conformation using a combination of αMep, Aib, and Ala in homochiral and heterochiral peptides (Moretto et al. 2008). In a suite of systematic studies, they showed that although αMep largely prefers the compact 3_{10}- and α-helical conformations (Toniolo 1989; Crisma and Toniolo 2015; Drouillat et al. 2018), it also has the tendency to explore the semi-extended P_{II} conformation (Kang and Park 2014). Interestingly, compared with unsubstituted proline, αMep showed a remarkable tendency to promote a β-turn conformation when present at the $i+1$ position (De Poli et al. 2009).

Similar to αMep, α-vinylproline exhibited the α_R conformation (Reuter et al. 2015). However, incorporation of a phenyl group at the C^{α} position results in conformations associated with slightly lower ϕ and higher ψ values (Fig. 3d). Except for Boc-α-phenylproline, which also crystallized in a *cis* conformer (Tamazyan et al. 2004), α-phenyl-D-proline (Tamazyan et al. 2002, 2008) and α-(4-chlorophenyl)-D-proline (Polindara-García and Miranda 2012) appear in the γ'-basin (Fig. 2d) with an *endo* ring pucker. The nitrophenyl substitution on L-proline however, exemplified as α-(4-nitrophenyl)

proline, expectedly showed α_R conformation with a *trans* amide bond (Foschi et al. 2010). Finally, two more independent studies on azido- and benzylic-substituted Pro, α-azido-L-proline and α-(2-fluorobenzyl)-L-proline, yielded P_{II} conformation (Lynch et al. 1995; Rajalakshmi et al. 2013). It is to be noted that for α-substituted proline, only αMep has been studied systematically. The conformational data for the other proline analogs were generated from non-systematic independent studies, often with results from synthesis that generated racemic mixtures of these "monopeptides." In many cases, the absolute stereochemistries from the resulting "monopeptides" were not determined via X-ray crystallography.

Among the α-substituted proline analogs, αMep is the most popular choice for biochemical and structural biology applications. This is expected as among all the α-substituted prolines, αMep has the least effect on the hydrophobicity of the unsubstituted proline, promotes *trans* amide conformation, and offers restricted ϕ-ψ space. Similar to the polymeric chain of proline, poly-αMep chains strongly favor the left-handed polyproline conformation (P_{II}) in solution (Kang and Park 2014). αMep has been incorporated into several polypeptide chains and proteins of biological significance including the hormone bradykinin (positions 3 and 7) (Welsh et al. 1992), the Asn-Pro-Asn-Ala (NPNA) repeat motif of CS Surface protein in *P. falciparium* (Nanzer et al. 1997), mimotope of apolipoprotein H (Sem et al. 1998), and the antimicrobial peptide buforin 2 (BF2) (Kobayashi et al. 2004). These substitutions provided valuable mechanistic insights about the structure-function relationships. In bradykinin, substitution of either Pro3 or Pro7 with αMep induces disorder-to-order transition nucleating a type II β-turn (Juvvadi et al. 1992; Welsh et al. 1992; Pierson et al. 2013). Similar results were obtained for Pro to αMep substitution in the NPNA repeat motifs (Bisang et al. 1995). The Pro to αMep substitution for the mimotope of apolipoprotein H resulted in the antigenic peptide to adopt a distorted type I β-turn and improved its binding to anti-cardiolipin antibodies. The same substitution obliterated the *cis-trans* isomerism in the antimicrobial peptide BF2 but had no effect on its translocation via lipid membranes implying that the *cis-trans* isomerization is not mechanistically involved in BF2's translocation (Kobayashi et al. 2004). Substitution of Pro32 by αMep in β2-microglobulin, a 99-residue human protein implicated in dialysis-related amyloidsis, increases the tendency of the protein to oligomerize (Azinas et al. 2011). Using the Pro32αMep mutation, it was shown that Pro32 is constrained to the *trans* amide conformation initiating a misfolding that is directly responsible for the amyloid nucleation, providing a window of opportunity to study and target dialysis-related amyloidosis in the early stages (Torbeev et al. 2015).

4-substituted prolines

Consequences of substituting the hydrogen atoms at γ-carbon

Substitution at the γ-carbon (position 4) of Pro yields two diastereoisomers, 4*S* and 4*R*. As shown in Fig. 4a, each of these can be present either in the *endo* or the *exo* ring puckered state. The relative stabilities of the *endo*/*exo* conformations depend on the nature of the substitution (X) and the particular diastereoisomeric state. When X is bulky, sterics become the key determinant and 4*R*-*endo* is favored over 4*R*-*exo* while 4*S*-*exo* is favored over 4*S*-*endo*. The origin of this can be understood from the orientation of X in the pyrrolidine ring. The relative orientation of the imide nitrogen atom and X is *gauche* in 4*R*-*exo* and *anti* in 4*R*-*endo*. Since *gauche* is more sterically crowded than *anti*, steric factors would favor the 4*R*-*endo* (*anti*) conformation. The steric bias against 4*R*-*exo* is further augmented by the fact that X is *axial* in 4*R*-*exo* but *equatorial* in 4*R*-*endo*. Similarly, 4*S*-*exo* is sterically favored over 4*S*-*endo* due to the fact that the relative orientation of the imide nitrogen atom and X is *anti* (and X is *equatorial*) in 4*S*-*endo*. The situation is reversed if the substituent X is electron-withdrawing in nature.

When X is electron-withdrawing, the C–X and the C–N bonds will show a preference for the *gauche* conformation. The origin of this "*gauche* effect" (O'Hagan et al. 2000; Mooney et al. 2002) is hyperconjugative interaction. As shown in Fig. 4b, the electron-withdrawing group X will render the antibonding orbital associated with the C–X bond (σ^*_{C-X}) highly electron deficient. The *gauche* conformation will be favored over the *anti* conformation because only in the *gauche* conformation can the electron deficient σ^*_{C-X} orbital participate in hyperconjugative interaction with bonding orbitals associated with the Cδ–H or the Cβ–H bonds ($\sigma_{C\delta-H}$ or $\sigma_{C\beta-H}$) (Fig. 4b). In summary, this leads to a strong preference for the *exo* pucker of the pyrrolidine ring with an electron-withdrawing group as a 4*R* substituent or an *endo* pucker of the pyrrolidine ring with an electron-withdrawing group as a 4*S* substituent. It should be noted that the *exo* ring pucker is associated with compact secondary structures, e.g., α_R, PP_{II} while the *endo* favors extended conformation (Dunbrack and Karplus 1994). The *endo* ring pucker is also strongly associated with the *cis* imide conformation. Pyrroline ring pucker correlates with the Pro main chain conformation, and therefore, the chemical nature and the stereotopic position of the 4-substituent can be directly exploited in molecular designs.

Crystal structures of terminally blocked 4-substituted proline

A number of crystal structures have been solved for 4-substituted Pro (see Table S2 for details). The ϕ-ψ angle

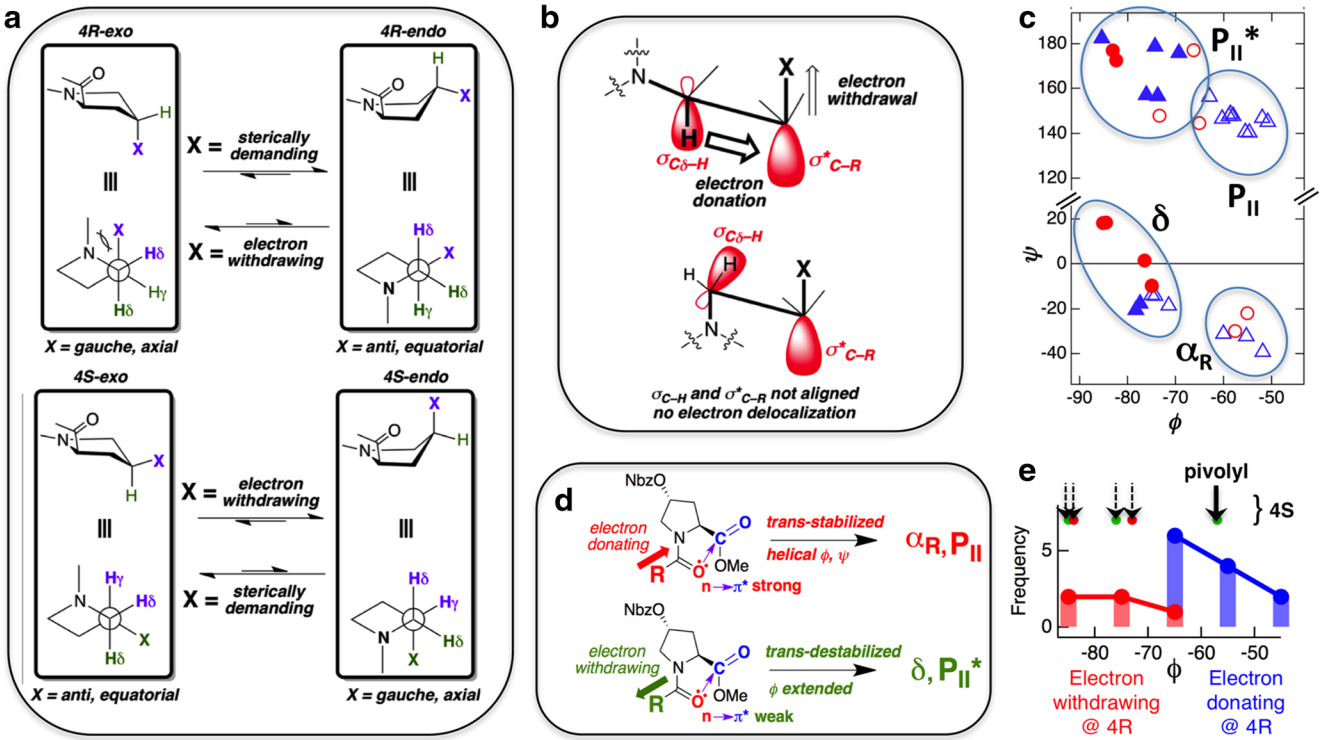

Fig. 4 **a** Schematic showing the effects of a sterically demanding or an electron-withdrawing substituent at position 4 of Pro. **b** A schematic depicting the gauche effect—a stereoelectronic effect that stabilizes the *gauche* conformer when an electron-withdrawing group is present at position 4 of Pro. **c** Ramachandran plot for γ-substituted L-Pro analogs (triangle *trans*; circle *cis*; filled symbols represent *endo* while open symbols represent *exo*). More details about the structures used are given in Table S2. **d** A schematic depicting the effect of electron-withdrawing versus electron-donating substitutions at the N-cap position of 4-nitrobenzoatehydroxyproline on backbone dihedral angles, mediated by $n \rightarrow \pi^*$ interactions. The nitrobenzoate group (NBz) is an electron-withdrawing substituent and hence it favors the *exo* ring pucker when substituted at the 4*R* position and *endo* ring pucker when substituted at the 4*S* position. **e** Distribution of dihedral angle ϕ in 4*R*-substituted proline, separated in two groups: one with electron-donating (blue) and the other with electron-withdrawing (red) N-cap. The corresponding 4*S* variants are also shown (arrows on the top) where all except the pivolyl N-cap show extended ϕ

distribution of these structures is shown in Fig. 4c. In general, they are distributed into four clusters (α_R, δ, P_{II}, and P_{II}*) in the Ramachandran map without much difference in trends between *trans* and *cis* conformations. However, *endo* ring puckered structures (filled symbols in Fig. 4c) show a clear tendency to appear at lower ϕ and higher ψ values. The most common 4-substituted Pro is the naturally occurring Hyp. With the electron-withdrawing hydroxyl group occupying the 4*R* position, Hyp prefers the *exo* ring pucker and adopts either the α_R (Clegg et al. 2003; Lübben et al. 2014) or the P_{II} conformation (Panasik et al. 1994), exhibiting the *trans* imide bond. In contrast, 4*S*-hydroxyproline (hyp), which does not occur in nature, prefers the *endo* ring pucker with a *cis* amide bond and appears mostly in the δ-basin (Shoulders et al. 2010). Consistent with hydroxyproline, other electron-withdrawing groups at the 4*R* position (e.g., methoxy-, aryloxy-, fluro-, nitrobenzoate, and azido-) exhibit an *exo* ring pucker and are associated with either the α_R or the P_{II} conformation (Panasik et al. 1994; Sonntag et al. 2006; Kotch et al. 2008; Ghosh et al. 2014; Pandey et al. 2014; Forbes et al. 2016). When present at the 4*S* position, the electron-withdrawing groups favors the *endo* ring pucker and the δ-/P_{II}* basin in the Ramachandran plot (Webb and Eigenbrot 1991; Anderson et al. 1996; Shoulders et al. 2010; Siebler et al. 2015; Szcześniak et al. 2015; Forbes et al. 2016).

The non-electron-withdrawing methyl, vinyl, or phenyl groups, when present as a 4*R* substituent, exhibit the *endo* ring pucker appearing in the P_{II}*/δ region of the Ramachandran plot, while the same groups when present as 4*S* substituents exhibit the *exo* ring pucker and α_R/P_{II} conformation (Flippen-Anderson et al. 1983; Tamaki et al. 2001; Valle and Goodman 2002; Jones et al. 2011; Hack et al. 2013). Interestingly, despite chlorine being more electronegative than carbon, and therefore qualifying as an electron-withdrawing group, chloroproline behaves differently than fluoroprolines. The 4*R*-chloroproline (Clp) exhibits an *endo* ring pucker in the δ-basin, as opposed to Flp (or Hyp) which appears in the α_R/P_{II}-basin with *exo* ring pucker. The 4*S*-chloroproline (clp) exhibits the *exo* ring pucker in P_{II}-basin, similar to 4*S*-alkylprolines and in sharp contrast to flp or hyp. This is possibly due to the large size of the chlorine atom and the steric necessity in this case outcompete the electronic effects that would have resulted from its electronegativity alone (Shoulders et al. 2008; Park et al. 2012). Interestingly, for

the 4*R*-azidoproline (Azp) crystal, both *cis* and *trans* isomers were observed in the same asymmetric unit (Sonntag et al. 2006). The *cis* and the *trans* isomers are associated with the *endo* and the *exo* ring pucker, respectively, as expected. The *cis-endo* crystal exhibits the P_{II}* conformation, while the *trans-exo* crystal exhibits the P_{II} conformation.

Tuning structure-function of peptides and proteins using 4-substituted proline

Selective tuning of the main chain conformation via stereospecific substitution at the γ-carbon of the proline residue has been justifiably exploited to alter/modify protein conformation and function. With Hyp being a cheap commercial source, chemists have conveniently designed several synthetic routes to make enantiopure 4-substituted proline derivatives in high yields using classical solution-phase synthesis and "editing" proline residues on the solid-phase within peptides (Thomas et al. 2005; Pandey et al. 2013). The free 4-substituted amino acids were genetically incorporated within proteins either via the use of appropriate auxotrophs or via unnatural amino acid incorporation approaches based on clever use of the amber stop codon/tRNA suppressors (Kim et al. 2004; Chalker and Davis 2010; Wals and Ovaa 2014; Kato 2015; Smolskaya and Andreev 2019). The incorporation of these 4-substituted proline residues provides valuable insights into protein structure and function, thereby subsequently leading to the design of important compounds for therapeutics including conformationally biased biological probes, inhibitors, and peptidomimetic drugs (Tressler and Zondlo 2014). A few selected examples of the use of 4-substituted prolines are discussed below.

In the individual strand of the collagen repeats [Xaa-Yaa-Gly]$_n$, modified proline residues at either the Xaa or the Yaa position can significantly affect the stability of the triple helix assembly. The 4-substituted prolines with preference for the *exo* ring pucker, e.g., Hyp, Flp, Mop (4*R*-methoxyproline), and mep (4*S*-methylproline), increase the stability of the model collagen triple helix. Incorporation of 4-substituted proline residues with a preference for *endo* ring pucker leads to destabilization of the collagen assembly (Holmgren et al. 1999; Hodges and Raines 2003; Kotch et al. 2008). Other than providing mechanistic insights into the stability of the native collagen triple helix, 4-substituted prolines can be used in design and development of non-native collagen with tunable mechanical properties (Kubyshkin 2019). A detailed discussion of the mechanism collagen stability explored via 4-substituted proline has been reviewed elsewhere (Jenkins and Raines 2002; Shoulders and Raines 2009b).

Rational design of structures via the incorporation of 4-substituted proline residues in place of proline has also been investigated in proteins and model peptides other than collagen. The consensus pentaresidue repeat sequence in elastin, VPGVG, undergoes a reversible temperature-dependent assembly that is driven by its intrinsic hydrophobicity (Urry et al. 1997). During this assembly process, the proline-glycine motif within these pentaresidue repeats forms type II β-turn structures and is critical to the phase separation event (Urry et al. 1985). Three repeats containing the cyclic version of the VPGVG sequence show that the proline residue exhibits an *exo* ring pucker, and the main chain dihedral angles ($\phi,\psi = -53°, +140°$) are compatible with the $(i+1)$th position of a β-turn (Cook et al. 1980). Replacement of the proline residues with Flp, that favor the *exo* ring pucker, selected the type II β-turn conformation and induced elastin transition at a lower temperature (favorable mechanism). In contrast, flp induced multiple conformational states, disfavored the type II β-turn conformation, and increased the transition temperature for the elastin assembly (unfavorable mechanism) (Kim et al. 2005). In the Trp-cage miniprotein, the incorporation of the strongly *exo* ring pucker promoting 4*R*-(4-nitrobenzoate)-hydroxyproline and 4*R*-(4-trifluromethylbenzoate)-hydroxyproline at the Pro12 position resulted in a dramatic increase of the stability of the protein, where both Flp and Hyp only modestly increased the protein stability (Naduthambi and Zondlo 2006). This finding advocates for the use of 4-substituted derivatives other than the commonly used fluoroprolines or hydroxyprolines to modulate protein structure and function.

The global replacement of 32 proline residues with Flp in KlenTaq DNA polymerase did not result in any loss of function for the protein despite causing a slight loss in the thermostablity of the protein (Holzberger and Marx 2010; Holzberger et al. 2012). Similarly, replacement of 10 proline residues with flp in EGFP resulted in faster refolding of the protein due to better accommodation of the *endo* ring puckered substituted proline in the engineered variant (Steiner et al. 2008; Moroder and Budisa 2010). Interestingly, attempts to express the KlenTaq DNA polymerase with flp and EGFP with Flp were unsuccessful probably because the misfolding of these protein variants rendered them insoluble (Holzberger and Marx 2010). All three proline residues in ubiquitin—Pro19, Pro37, and Pro38—were replaced with Flp leading to a greater stability of the engineered protein (Crespo and Rubini 2011). In the WW domain of Pin1, Pro37 exhibits an *endo* ring pucker (Mortenson et al. 2018). The Pro37 is crucial to the structural stability in the hydrophobic core of the Pin1 WW domain; a P37A mutation resulted in an unstable protein (Jäger et al. 2009). The mutation of Pro37 with a 4-substituted proline residue provided valuable insights into the role of designing the preference of the proline ring pucker and exploiting the effects of hydrophobicity on the stability of the WW domain and subsequently its ligand binding ability. While the *exo* pucker promoting mutations, P37Flp, P37Hyp and P37Mop, were destabilizing, the *endo* pucker promoting P37flp mutation had a stabilizing effect on the structure of the

Pin1 WW domain. Interestingly, although the *endo* pucker-favoring P37hyp and P37mop variants exhibited greater stability for the WW domain than their corresponding *exo* ring-promoting P37Hyp and P37Mop mutations, the P37hyp and P37mop mutants were less stable than the wild type Pin1 WW domain due to solvation and steric restraints. Nonetheless, the overall stability of the Pin1 WW domain positively correlated with its ability to bind the specific phospho-peptide (Huang and Horng 2015), advocating the role of 4-substituted proline derivatives to provide important molecular insights into the structure-activity relationship of protein-protein interactions (Tang et al. 2014). However, it should be pointed out that the thermodynamic consequences of using a 4-substituted proline residue with a preorganized ring puckered state may become complex if other non-covalent interactions including solvation play a dominant role. (Kantharaju et al. 2009; Rubini et al. 2013).

The residue Pro62 present in the villin headpiece protein exhibits an *exo* ring pucker, and its Hγ atom is involved in a C–H···π interaction with the Trp64 (Bhattacharyya and Chakrabarti 2003). Surprisingly, all *exo* pucker promoting 4-substituted proline derivatives (Hyp, Flp, and Mop) destabilized the villin headpiece protein and so did all *endo* pucker promoting substituents (hyp and mop) (Zheng et al. 2010). Substitution of the Hγ at Pro62 possibly weakens the proline-aromatic C–H···π interaction, which is crucial in maintaining the local structure. Only flp (which promotes the *endo* ring pucker) at position 62 marginally increased the stability of the villin headpiece protein, which is attributed to its hydrophobic effect (Hsu et al. 2015). The HPK1-derived proline rich sequence (PPPLPPKPKF) that recognizes and binds to the SH3 domain of the murine cortactin ($SH3_{m-cort}$) via formation of the left-handed polyproline structure (Feng et al. 1994; Rubini et al. 2010) did not show a gain in function when *exo* ring pucker promoting, and P_{II} stabilizing 4-substituted prolines were incorporated in place of the proline residues (Borgogno and Ruzza 2013).

4-substituted proline in polyproline helices

Proline peptides can exist in two conformations: (i) the left-handed polyproline helix (PP_{II}) with all *trans* peptide bonds and (ii) the right-handed polyproline helix (PP_I) with all *cis* peptide bonds (Cowan et al. 1955; Kurtz et al. 1956; Traub and Shmueli 1963). While the PP_{II} conformation is common in protein structures and is stabilized in aqueous environment, PP_I helix is rarely encountered in proteins and can form only in organic solvents. The 4*R*-substituted prolines with electron-withdrawing substituents promote the *exo* ring pucker and increase the population of $n \rightarrow \pi^*$ interaction-driven *trans* peptide bonds. The *exo* ring pucker is also associated with a more compact ϕ distribution for the proline residue ($\sim -60°$ to $-70°$) and is therefore expected to contribute favorably to the stability of a PP_{II} helix. In contrast, the PP_I helix should be preferred by *cis* peptide bond promoting *endo* ring pucker-favored electron-withdrawing substituents at the 4*S* position of the proline. Expectedly, Flp, Hyp, Mop, and 4*R*-azidoproline (Azp) were found to stabilize the PP_{II} conformation compared with unsubstituted proline in homo-polymeric peptides (Horng and Raines 2006). Interestingly, $(Flp)_{10}$ and $(Hyp)_{10}$ peptides are resistant to form PP_I structures even after long incubation in organic solvents while $(Pro)_{10}$ showed significant PP_I conformation under identical conditions (Chiang et al. 2009). The peptides with 4*S*-substituents (e.g., $(flp)_{10}$, $(hyp)_{10}$, $(azp)_9$) showed more stable PP_I structure in organic solvents and less stable PP_{II} structure in aqueous solution compared with their all proline analog (Chiang et al. 2009).

Apart from affecting the stability of the PP_{II} and PP_I helices, incorporation of 4-substituted proline residues in polyproline peptides modulate the transitional barrier of the PP_I/PP_{II} interconversion. While incorporation of a single Flp residue registers an increase in the transition barrier of $PP_{II} \rightarrow PP_I$ conversion (compared with Hyp, Mop, and Pro), incorporation of a flp residue registers the largest decrease in the $PP_I \rightarrow PP_{II}$ transition energy (compared with hyp, mop, and Pro) (Chiang et al. 2009). Therefore, both the chemical nature and the stereochemistry of the substituent modulate the thermodynamic and kinetic properties of the polyproline oligomers. Encouragingly, homo and hetero functionalization of the 4-azidoproline via Huisgen's 1,3-dipolar cycloaddition (popularly referred as "click chemistry") in polyproline peptides showed that the PP_{II} helical fold remains intact following the reaction. This has the potential for developing proline-based structural scaffolds with their use in bioorthogonal applications and medicinal chemistry (Kümin et al. 2007; Siebler et al. 2015). Potential applications for utilization of 4-substitued prolines with defined impact of peptide backbone conformation have been demonstrated. Cell-penetrating peptides with 4-substituted prolines were successfully designed where the structure of the peptide was stabilized in the PP_{II} conformation (Fillon et al. 2005). With the incorporation of both the 4*R* and 4*S* perfluoro-*tert*-butyl ethers of hydroxyproline in peptides, the potential for developing conformationally restrained and highly sensitive ^{19}F NMR (9 equivalent ^{19}F nuclei) probes is getting realized (Tressler and Zondlo 2014; Verhoork et al. 2018). Replacement of the central proline residue with 4S-azidoproline in the peptide catalyst ^{D}Pro-Pro-Glu, which catalyzes C–C bond formation, resulted in an increase of the enantiopurity of the product (Schnitzer and Wennemers 2018).

Tuning $n \rightarrow \pi^*$ interaction with 4-substituted proline

The main chain conformation of a proline residue can be further controlled via differential N-capping groups that can

directly modulate the $n \rightarrow \pi^{*}$··interaction (Fig. 4d). This was achieved via the utilization of "monopeptides"—4-substituted nitrobenzoate esters of 4-hydroxyproline. The 4*R*-(4-nitrobenzoate)-hydroxyproline (Hnb) is known to prefer the *exo* ring pucker, while the 4*S*-(4-nitrobenzoate)-hydroxyproline (hnb) prefers the *endo* ring pucker, consistent with the *gauche* effect (Pandey et al. 2014). Electron-donating groups at the N-terminal of the *exo* pucker-promoting Hnb, e.g., pivaloyl, *iso*-butyryl, propionyl, and acetyl, strengthened the $n \rightarrow \pi^{*}$ interaction (Fig. 4d), thereby leading to a compact helical backbone (PP_{II} or α_R) (Fig. 4e) and even stabilized α-helical conformations without any hydrogen bond (Wenzell et al. 2019). In contrast, electron-withdrawing groups at the N-terminal of Hnb (e.g., fluoroacetyl, trifluoroacetyl) weakened $n \rightarrow \pi^{*}$ interactions, thereby promoting extended conformations (Fig. 4e).

In contrast to Hnb, hnb is associated with the *endo* ring pucker and promotes *cis* imide conformation, the latter incompatible with $n \rightarrow \pi^{*}$ interaction. Therefore, in general, hnb promotes extended conformations (δ, P_{II}*), independent of the identity of its N-cap. The only exception to this is the pivaloyl N-cap group, which, due to its unusually strong electron-donating property, shows evidence of an $n \rightarrow \pi^{*}$ interaction and occupies the P_{II} conformational basin (Costantini et al. 2019) (Fig. 4e).

3-substituted prolines

As shown in Fig. 5 a and b, a number of 3-substituted L-Pro (as well as D-Pro) analogs have been studied, both at 3*R* and 3*S* positions (see Table S3 for details). Substitutions at the 3*R* and 3*S* positions have effects on the *exo*/*endo* puckering equilibrium analogous to that of 4-substituted prolines. Thus, an electron-withdrawing substituent at 3*R* favors the *exo* ring pucker while the same group at 3*S* favors the *endo* ring pucker, consistent with the "*gauche*" effect (Fig. 4b). Similarly, a bulky alkyl or aryl substituent at 3*R* favors the *endo* ring pucker while the same group at 3*S* favors the *exo* ring pucker, due to differential steric repulsion effects associated with the axial/equatorial positions (Fig. 5c–d).

In examining the effect of the mono-substitution at the 3-position of Pro, care should be taken with the nomenclature. Several substituents alter the stereochemical nomenclature,

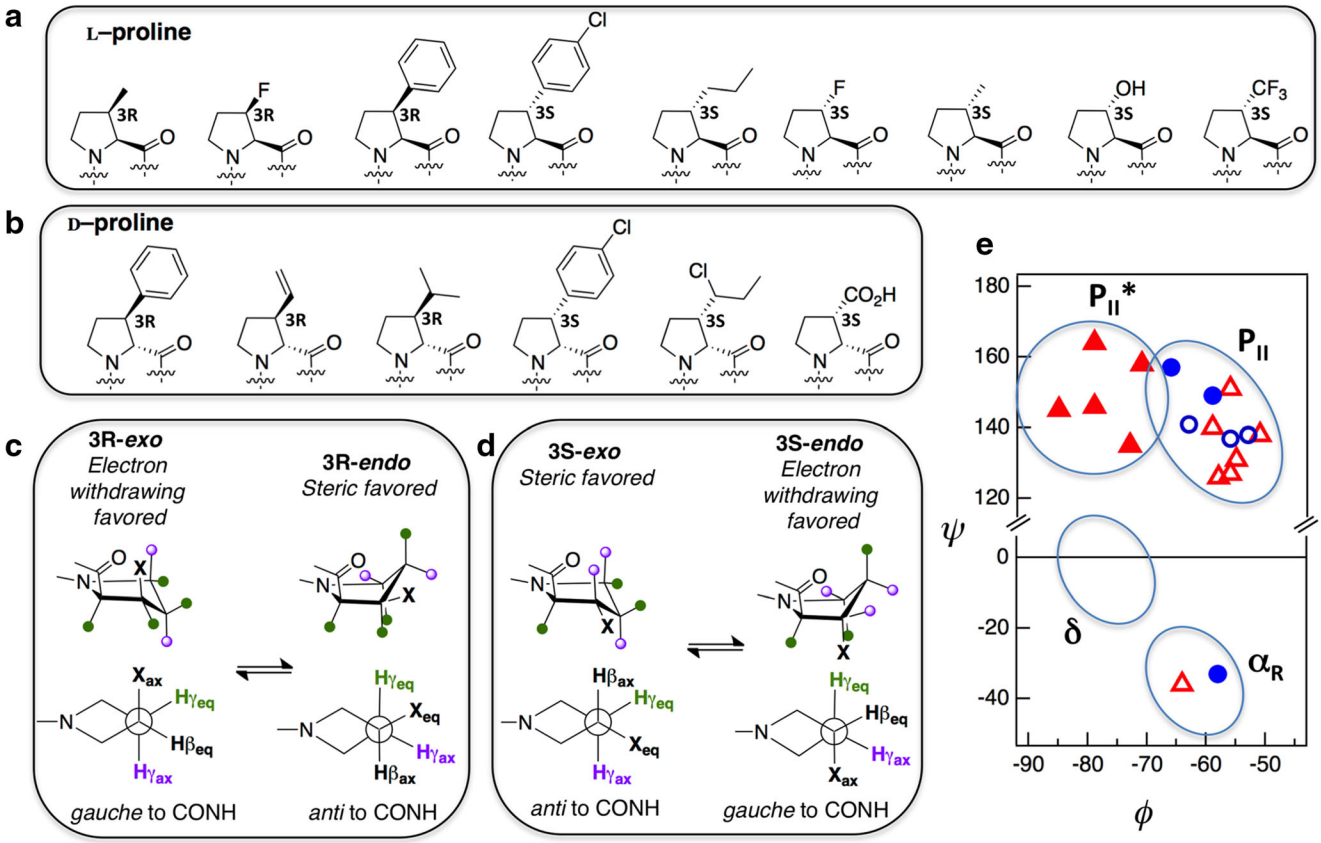

Fig. 5 **a** Representative 3-substituted L-Pro analogs discussed in this article (see Table S3 for structural details). The substituents are labeled according to the stereochemistry of the prochiral hydrogen atom. **b** Representative 3-substituted D-Pro analogs discussed in this article (see Table S3 for structural details). **c** Schematic showing the differential effect of 3*R* substitution (bulky steric group versus electron-withdrawing group). **d** Schematic showing the differential effect of 3*S* substitution (bulky steric group versus electron-withdrawing group). **e** Ramachandran plot for 3-substituted L-Pro analogs (triangle *trans*; circle *cis*; open symbols *exo*; filled symbols *endo*)

both at positions 3 and 2, depending on the priority of the substituent group according to the Cahn-Ingold-Prelog nomenclature for the determination of the absolute stereochemistry. Thus, the replacement of the pro-3*R* hydrogen atom with a methyl group results in a (2*S*,3*R*)-methylproline, while a phenyl group substitution at the same position would result in a (2*S*,3*S*)-phenylproline. Substitution of any of the two β-hydrogen atoms by a fluorine atom results in a change of nomenclature to 2*R* for L-proline, instead of 2*S*.

The *endo* ring pucker is exhibited by (2*S*,3*R*)-methylproline and (2*S*,3*S*)-phenylproline (in both cases, the substituents occupy the *pro*-3*R* position) (Flippen-Anderson et al. 1983; Flamant-Robin et al. 2002), while (2*S*,3*S*)-methylproline, (2*S*,3*S*)-(*n*-propyl)-proline, (2*S*,3*R*)-phenylproline, and (2*S*,3*R*)-(4-chlorophenyl)-proline exhibit the *exo* ring pucker (all substituents occupy the *pro*-3*S* position) (Flippen-Anderson et al. 1983; Zhong and Carlson 2006; Tran et al. 2008; Reuter et al. 2011; Huy et al. 2011; Fatás et al. 2011). This is consistent with the favorable placement of the sterically demanding group in the pseudo-equatorial position; in contrast, the electron-withdrawing substituents (–OH, and –F) occupy the pseudo-axial position (Fig. 4 c and d). This is also consistent with the "*gauche* effect," via which the *pro*-3*S* substitutions on β-carbon ((2*R*,3*S*)-fluoroproline and (2*S*,3*S*)-hydroxyproline) exhibit the *endo* ring pucker (Jenkins et al. 2003; Kim et al. 2006; Davies et al. 2018) and the *pro*-3*R* substitutions on β-carbon ((2*R*,3*R*)-fluoroproline) exhibits the *exo* ring pucker (Kim et al. 2006). Such 3-substituted fluoroproline derivatives were used as molecular probes to study prolyl *cis*-trans isomerization via ^{19}F NMR (Kim et al. 2006; Thomas et al. 2009). Since fluorine and hydrogen atoms are nearly isosteric and the trifluoromethyl group is only modestly electron-withdrawing (comparable to either hydroxyl and fluorine), (2*S*,3*S*)-trifluoromethylproline exhibits multiple conformations that access both the *exo* and the *endo* ring pucker conformations, with multiple backbone conformations within the same crystal structure (Tolmachova et al. 2018).

The ϕ-ψ distribution for 3-substituted L-Pro analogs is shown in Fig. 5e. Compared with the ϕ-ψ distribution for 4-substituted L-Pro analogs (Fig. 4c), there are two main differences. The δ-basin is empty for 3-substituted Pro (this could be due to less number of total 3-substituted structures). The other difference is that for 3-substituted Pro, all *cis-endo* conformations show compact ϕ ($> -70°$), while all *trans-endo* conformations show extended ϕ ($< -70°$). Among the 3-substituted alkyl and aryl prolines, β-phenylprolines were used as β-turn scaffolds (Fatás et al. 2011). With a strong electron–donating and $n \rightarrow \pi^*$ interaction-promoting pivaloyl group at the N-terminus of the proline analog, the β-phenylprolines were locked in a P_{II} conformation that is compatible with $i + 1$ position of a type II turn. Coupled with a ^{D}Phe residue at its C-terminal, β-phenylprolines, via aromatic-aromatic interactions, stabilize and promote turn conformations with *trans* amide bonds. In a peptide template, MT-II (Ac-Nle-c[Asp-His-^{D}Phe-Arg-Trp-Lys]-NH_2]) replacement of the His residue with β-phenylproline analogs, where the phenyl ring is substituted by H, -Cl, -Br, -CF_3, and -OMe, showed a suite of conformations. The conformations with a compact ϕ ($> -70°$) had a slightly lower IC_{50} values for melanocortin receptors, while the extended ϕ ($< -70°$) had much lower IC_{50}. All peptides with β-pheylproline analogs had a much lower IC_{50} value than with the peptide containing unsubstituted proline residue, advocating the role of 3-substituted proline residues and their conformational importance in medicinal chemistry (Cai et al. 2004).

3-hydroxyproline and collagen stability

Although rare, *trans*-3-hydroxyproline (2*S*,3*S*)-hydroxyproline (3-Hyp), like Hyp, is also found in nature, produced via proline PTM. The formation of 3-Hyp is not due to the promiscuity of prolyl-4-hydroxylase, as was initially hypothesized. Rather, proline PTM by prolyl 3-hydroxylase (P3H) specific to Pro-4Hyp-Gly sequences incorporates the 3-Hyp residues (Hudson and Eyre 2013). Interestingly, proline residues that are substrates of P3H are strongly conserved (Hudson et al. 2014).

Only one to two residues of 3-Hyp are found in type I and type II collagen, while this number marginally increases to 3–6 residues in types V and XI collagen. Type IV collagens have the highest 3-Hyp content (10% of all the hydroxyproline variants) (Weis et al. 2010; Eyre et al. 2011; Hudson et al. 2012). Surprisingly, the presence of 3-Hyp destabilizes the collagen triple helix assembly. Although the triple helices from $(3Hyp\text{-}4Hyp\text{-}Gly)_n$ sequences are more stable than the unmodified $(Pro\text{-}Pro\text{-}Gly)_n$ sequences, the triple helix assembly is slightly unstable compared with $(Pro\text{-}4Hyp\text{-}Gly)_n$ sequences. The extent of destabilization of the triple helix assembly is much less if 3-Hyp is in position Xaa of the $(Xaa\text{-}Yaa\text{-}Gly)_n$ sequence rather than when at position Yaa. The position Yaa strongly prefers an *exo* ring pucker, and because of the "*gauche* effect," the *endo* ring pucker is preferred for 3-Hyp. This is the reason behind the observed decrease in the stability of $(Xaa\text{-}3Hyp\text{-}Gly)_n$ collagen peptides (Jenkins et al. 2003).

5-substituted proline

Substitution of Pro at the 5 (or δ) position is unique since this is the only ring position where substitutions are devoid of any amide "*gauche* effect," as applicable for 3- or 4-substituted Pro. In other words, for 5-substituted Pro, none of the electron donating C–H bonds are aligned with the σ* of the C^{δ}–X bond. Therefore, only steric repulsions guide the pyrrolidine ring pucker (Fig. 6a). In unsubstituted Pro, the *pro*-5*R*

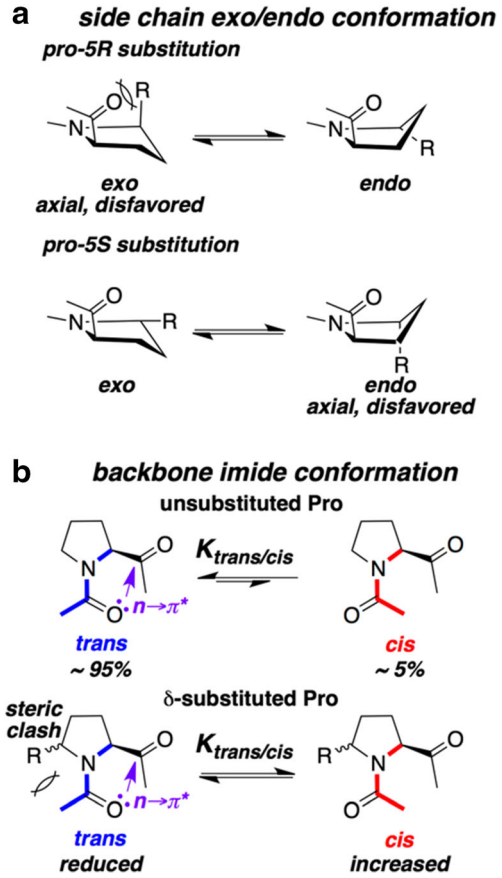

Fig. 6 **a** A schematic showing how steric clash in 5-substituted Pro increases the *endo*-content for 5*R* substitution while the same steric effect increases the *exo*-content for 5*S* substitution. **b** A schematic showing how steric clash in 5-substituted Pro increases the *cis*-content

hydrogen atom is pseudo-*equatorial* in the *endo* ring pucker conformation and pseudo-*axial* in the *exo* ring pucker conformation. Therefore, substituents at *pro*-5*R* position favor the *endo* ring pucker in the pyrrolidine ring. Similarly, *pro*-5*S* substituents prefer the *exo* ring pucker. The crystal structures of the "monopeptides" that were explored (Table S4) from the Cambridge Structural Database are consistent with this hypothesis (Masumi et al. 1982; Flippen-Anderson et al. 1983; Lynch et al. 1995; Gilchrist et al. 1997; Arndt et al. 1997; Soave et al. 1997; Mulzer et al. 2000; Hussaini and Moloney 2003; Matsumura et al. 2006; Moloney et al. 2006; Onomura et al. 2008; Reuter et al. 2011; Rodríguez et al. 2015; Beatty et al. 2016; Salih et al. 2016; Kubyshkin and Budisa 2017).

In addition, substitutions at the C^{δ} position also enhance the population of the *cis* imide conformation (Fig. 6b). This is due to the increased steric repulsion for the *trans* isomer, making it more disfavored compared with the unsubstituted proline. The $K_{trans/cis}$ value for Xaa-Pro unit in model peptides is around 19 (95% of the prolyl amides in *trans* conformation). Incorporation of methyl, trifluoromethyl, or *tert*-butyl groups at position 5 results in a monotonic decrease of $K_{trans/cis}$ (4.6, 2.8, and 0.82, respectively) with the degree of substitution (Lummis et al. 2005; Kubyshkin et al. 2018). In other words, *tert*-butyl substitution at the C^{δ}-position produces almost equal populations of *cis* and *trans* isomers. The *trans* → *cis* isomerization of the conserved hinge proline residue in the M2-M3 loops of the cation-selective nicotinic aetylcholine and 5-HT_3 receptors opens the pore of the neurotransmitter ion-gated channel. The use of 5-substituted proline residues, which increases the population of the *cis* imide bond, provides valuable mechanistic insights for these receptors (Lummis et al. 2005).

Summary and future outlook

The backbone ϕ angle of Pro is severely constrained due to the cyclic nature of its side chain. In addition, only Pro, among all amino acids, can access the *cis* backbone isomer. The unique conformational restrictions of Pro can be modulated further by suitable substitutions at various ring positions. Two such variants, 4-Hyp and 3-Hyp, are found to occur naturally in the protein collagen where they alter collagen stabilities. The mechanism behind this is a special stereoelectronic effect. In addition to the natural variants, a large number of Pro substitutions (at all the four positions: α-, 3-, 4-, and 5-) have been studied over the past two decades. For α- and 5-substitutions, steric factors play the dominant role in determining the conformational fate of the molecule. On the other hand, for 3- and 4-substituted Pro, the final conformational bias is determined by an interplay of steric interactions and the stereoelectronic *gauche* effect. Depending on the nature of substitution and the resulting dominant isomer (*cis*/*trans* and *exo*/*endo*), 3-,4- and 5-substituted Pro can assume either a compact (appearing in the α_R- or the P_{II}-basin) or a more extended (appearing in the δ- or the P_{II}*-basin) conformation. In general, the *endo* pucker favors an extended conformation while the *exo* pucker favors a more compact conformation. α-substituted Pro, on the other hand, is always restricted to the compact conformation (appearing in the α_R- or the P_{II}-basin). In all cases, the *trans*-*exo* conformation is associated with a stronger $n \rightarrow \pi^*$ interaction compared with the *trans*-*endo* conformation. Substituted Pro has a wide range of applications. Substituted Pro has been used in studies where a specific conformation (of a naturally occurring Pro) was designed to be stabilized (or destabilized). They have been used as ^{19}F NMR probes, and seleno-proline derivatives hold promise as a ^{77}Se NMR probe. They can be useful in medicinal chemistry due to the stereochemical control they offer in controlling distance between attached functional groups. The stereochemical control added with capacity to work as a sensitive ^{19}F NMR probe makes them attractive to probe intrinsically disordered proteins. Although we have discussed only singly substituted prolines, multi-substituted proline offers added steric and stereoelectronic controls.

Authors' contribution HKG performed the literature search. HKG and GB analyzed the data and wrote the review.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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