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Enhanced stability of *cis* Pro-Pro peptide bond in Pro-Pro-Phe sequence motif

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Abstract **Identification of sequence motifs that favor *cis* peptide bonds in proteins is important for understanding and designing proteins containing turns mediated by cis peptide conformations. From ^{1}H NMR solution studies on short peptides, we show that the Pro-Pro peptide bond in Pro-Pro-Phe almost equally populates the *cis* and *trans* isomers, with the *cis* isomer stabilized by a CH$\cdots\pi$ interaction involving the terminal Pro and Phe. We also show that Phe is over-represented at sequence positions immediately following *cis* Pro-Pro motifs in known protein structures. Our results demonstrate that the Pro-Pro *cis* conformer in Pro-Pro-Phe sequence motifs is as important as the *trans* conformer, both in short peptides as well as in natively folded proteins.**

Keywords: *cis* Peptide; Proline; Sequence motif; CH–Pi interaction

1. Introduction

Cis peptide bonds, rarely observed in proteins [1–5] due to unfavorable steric interactions between $C^{\alpha}(i)$ and $C^{\alpha}(i+1)$ in the peptide unit Xaa(*i*)–Xaa(*i*+1), are known to exhibit a strong bias for Pro at the (*i*+1) position. The sequence bias arises from additional unfavorable $C^{\alpha}(i)$–$C^{\delta}(i+1)$ steric interactions, introduced by Pro in the *trans* conformers of Xaa-Pro units [6]. Nuclear magnetic resonance (NMR) studies of *cis–trans* equilibrium in short designed peptides [7–11] have shown that the Xaa-Pro motif exhibits the highest (23–38%) [7] *cis* content when Xaa is an aromatic residue, while the lowest *cis* content (6%) [7] is observed when Xaa is Pro. Yet, surprisingly, surveys of protein structures [1,7] indicate that along with the aromatic residues, Pro is also over-represented at the Xaa position in *cis* Xaa-Pro motifs. What is the origin of this discrepancy? In an earlier work [1] it was noted that in some proteins containing Pro-Pro *cis* bond, the first Pro residue was involved in a CH$\cdots\pi$ interaction [12–16] with Phe side-chain following the second Pro. However, there were other examples where no such interaction was present in Pro-Pro-Phe *cis* units (Fig. 1). The presence of a large number of many-body tertiary interactions in a folded protein restricts one to draw any clear conclusion about the role played by potential CH$\cdots\pi$ interactions in stabilizing the *cis* Pro-Pro conformer in Pro-Pro-Phe motifs. To overcome this problem, we synthesized two model peptides, PPF (Ac-Pro-Pro-Phe-NH_2) and PPA (Ac-Pro-Pro-Ala-NH_2). Since the peptides are devoid of all potential tertiary interactions similar to that present in a folded protein, the *cis*/*trans* equilibrium of the Pro-Pro unit in these peptides can only be influenced by local interactions. The purpose of this study is to determine if Pro-Pro *cis* conformation in PPF shows enhanced stability than in PPA and if it is accompanied by any CH$\cdots\pi$ interaction between Pro(1) and Phe(3).

2. Materials and methods

Peptides PPF and PPA were synthesized using standard solid phase Fmoc protocol (pentafluorophenyl ester [OPfp] activation) and purified by reverse phase HPLC (C18 column). The peptides were characterized by the presence of two consecutive Pro residues (total correlation spectroscopy [TOCSY] pattern and presence of $\alpha\alpha(i,i+1)$ or $\alpha\delta(i,i+1)$ nuclear Overhauser effect [NOE] crosspeaks) followed by a Phe residue (TOCSY pattern and presence of $\alpha N(i,i+1)$ NOE crosspeaks). ^{1}H NMR experiments were performed in dmso-d_6 and H_2O (Watergate solvent suppression) at 25 °C in a Bruker DRX 500 MHz spectrometer. After sequence-assignment the resonances were isomer-assigned from Pro-Pro NOE cross peaks as *cis* ($\alpha\alpha(i,i+1)$ crosspeaks) or *trans* ($\alpha\delta(i,i+1)$ crosspeaks). The Phe $^3J_{\alpha\beta}$ values were measured from double quantum filtered correlation spectroscopy (DQF-COSY) experiments.

A representative list from pdb [17] (PISCES April 2005; 1608 chains; sequence identity <25%; $R \leqslant 2$ Å) [18] was surveyed for Pro-Pro motifs. The propensity of a residue Xaa immediately following a *cis* Pro-Pro motif is given by:

$$P_{\mathrm{Xaa}} = \frac{N_{\mathrm{cPPX}}/(N_{\mathrm{cPPX}} + N_{\mathrm{tPPX}})}{N_{\mathrm{cPP}}/(N_{\mathrm{cPP}} + N_{\mathrm{tPP}})} \quad (1)$$

where N_{cPPX}, N_{tPPX}, N_{cPP}, and N_{tPP} correspond to the total number *cis* Pro-Pro-Xaa units, *trans* Pro-Pro-Xaa units, *cis* Pro-Pro units and *trans* Pro-Pro units, respectively, in database. CH$\cdots\pi$ hydrogen bonds were identified using a fairly relaxed criteria: (i) distance between the center of Phe ring and $C^{\alpha}/C^{\delta} \leqslant 4.5$ Å, (ii) angle between the center of Phe ring, protons attached to C^{α}/C^{δ} atom and $C^{\alpha}/C^{\delta} > 110°$.

3. Results and discussion

3.1. Equilibrium population of cis conformers in PPA and PPF

The amide regions of the 1D ^{1}H NMR spectra of the peptides in dmso-d_6 are shown in Fig. 2a and b. In both peptides

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Abbreviations: NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; NOE, nuclear Overhauser effect; OPfp, pentafluorophenyl ester

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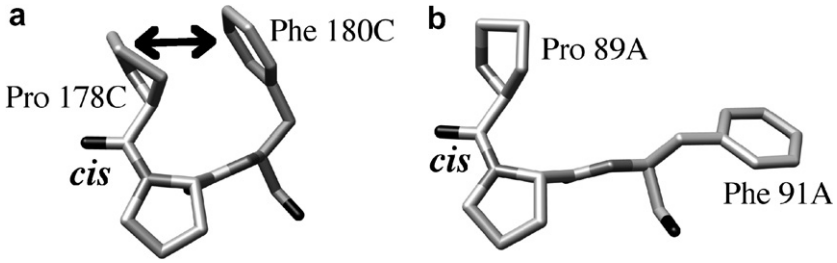

Fig. 1. Two examples of *cis* Pro-Pro peptide bond in Pro-Pro-Phe sequence motifs in proteins: (a) with Pro(*i*)-Phe(*i*+2) interaction (pdb code: 1nr0; $\chi 1 = -39.54$ (gauche+)) and (b) without Pro(*i*)-Phe(*i*+2) interaction (pdb code:1gsa; $\chi 1 = -174.64$ (*trans*)).

there are two Xaa-Pro bonds (Ac-Pro and Pro-Pro) each of which can be present as *cis* or *trans* giving rise to four possible conformers (cc, ct, tt, tc; see Fig. 3a). As can be seen in Fig. 2a and b, four amide peaks are observed for both peptides, PPF and PPA. The relative peak integrals, listed in Table 1, correlate with the relative populations of the four conformers. All four conformers of PPF were unambiguously assigned using NMR (TOCSY and nuclear Overhauser effect spectroscopy [NOESY]) experiments. For PPA, there was ambiguity in assigning the *trans*/*cis* state of the Ac-Pro bond. The combined populations of the *cis* Pro-Pro conformers (cc and tc) were: 42% (PPF) and 16% (PPA) in dmso-d_6 and 47% (PPF) and 17% (PPA) in H_2O. The results demonstrate that the Pro-Pro *cis* conformer experiences extra stability in PPF than in PPA. The origin of this extra stability, due to the presence of the Phe side-chain, was then examined.

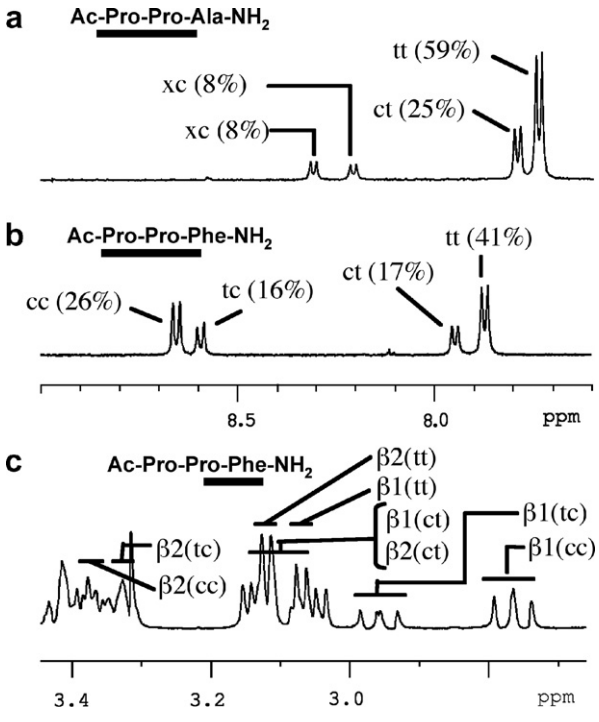

Fig. 2. 1D ^{1}H NMR spectra of: (a) Ala amide protons of PPA in dmso-d_6, (b) Phe amide protons of PPF in dmso-d_6, (c) Phe C^{β}–H protons of PPF in H_2O. Each amide peak is annotated as one of the four *cis*/*trans* isomers defined in Fig. 3a with the relative peak integral within parenthesis. For PPA the cc and tc isomers could not be unambiguously assigned and annotated as xc.

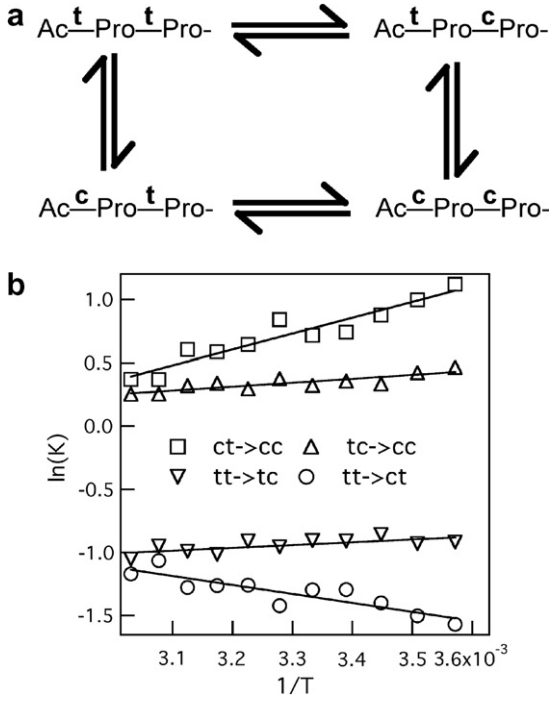

Fig. 3. (a) A schematic representation of multiple equilibria for the peptides PPA and PPF. (b) Temperature dependence of the four microscopic equilibrium constants (shown in Fig. 3a).

Table 1
Relative populations of *cis* and *trans* conformers in PPF and PPA[a]

	PPF		PPA	
	H_2O	dmso-d_6	H_2O	dmso-d_6
cc#	29	26	8	8
tc#	18	16	9	8
ct	9	17	37	25
tt	44	41	46	59

[a]Relative populations were estimated from integrals of NMR amide peak signals. # For PPA the *cis*/*trans* states of the Ac-Pro peptide bond could not be confirmed by NMR when the Pro-Pro bond was *cis*, so cc and tc should be read as xc, where x indicates unassigned *cis*/*trans* states.

3.2. Interacting Pro(1) and Phe(3) side chains in Pro-(cis)-Pro-Phe

Closely interacting Pro(1)-Phe(3) side-chains, as found in some proteins (Fig. 1a), may give rise to the observed stability of Pro-Pro *cis* conformers in PPF. A consequence of such close proximity is the ring current effect on C^{α}–H protons of Pro(1). Indeed a systematic upfield shift was observed in the chemical

shifts of Pro(1) C^{α}–H protons for the two *cis* isomers (cc: 2.90 and tc: 3.32 ppm) when compared to the *trans* isomers (ct: 4.52 and tt: 4.34 ppm) or the Pro(2) C^{α}–H chemical shifts of all four isomers (cc: 4.02, tc: 4.05, ct: 4.15 and tt: 4.10 ppm) of PPF in H_2O. In dmso-d_6, one of the two *cis* isomers (cc: 3.39 ppm) exhibited an upfield shift. The upfield shifts therefore points towards a closely interacting Pro and Phe side-chain, exclusively in *cis* isomers. The absence of upfield shifted Pro(1) C^{α}–H proton in one *cis* isomer (tc in dmso) may not necessarily mean an absence of Pro(1)-Phe(3) interaction since ring current shift depends not only on the proximity of a proton and the Phe ring but also on their mutual orientations.

Further evidence of closely interacting Pro(1) and Phe(3) side chains in the *cis* conformers of PPF came from the observed $^3J_{\alpha\beta}$ coupling constants of Phe(3). For both the *cis* isomers, the $^3J_{\alpha\beta}$ values were a combination of two numbers, one large and the other small (<5 Hz; ~12/13 Hz) indicating restricted rotation of the Phe ring [19] in both DMSO-d_6 and H_2O (Fig. 2c). On the other hand, for the *trans* conformers the $^3J_{\alpha\beta}$ values were roughly equal (~6–7 Hz), indicating considerable rotation about the $\chi 1$ angle [19]. The observation of restricted rotation of Phe side-chain in a three-residue peptide is remarkable. The combined observation of restricted rotation of the Phe side chain and the upfield shift of Pro(1) C^{α}–H chemical shifts clearly demonstrate that there is a strong Pro(1)-Phe(3) side chain interaction in the *cis* (and not *trans*) conformers of peptide PPF.

3.3. Energetic of cis–trans equilibrium

As shown in Fig. 3a, there are four microscopic equilibria between distinct *trans*/*cis* species in peptides PPF and PPA. The corresponding equilibrium constants can be estimated from the ratio of amide peak integrals corresponding to the four species (cc, ct, tc and tt) for respective peptides, as shown in Table 1. Of these, the tt → tc equilibrium (equilibrium constant K_{tc}) is relevant to folded protein structures and we will focus on this. For peptide PPF, K_{tc} and the corresponding free energies ΔG_{tc} ($-RT\ln K_{tc}$; $T = 298$ K) are 18/44 and 0.53 kcal/mol in H_2O, and 16/41 and 0.56 kcal/mol in dmso. The *cis*/*trans* states of the Ac-Pro bond in peptide PPA for the two minor conformers (*cis* Pro-Pro unit) could not be assigned unambiguously leading to two possible values of K_{tc} (8/46 or 9/46) and ΔG_{tc} (1.03 or 0.97 kcal/mol) in H_2O and two possible values of K_{tc} (both values 8/59) and ΔG_{tc} (both values 1.18 kcal/mol) in dmso. In terms of free energies, the *cis* forms of both peptides are unfavorable compared to the corresponding *trans* forms. However, the *cis* form is less unfavorable than the *trans* from in peptide PPF than in peptide PPA, both in H_2O (ΔG_{tc} (PPA) − ΔG_{tc} (PPF) = 0.41–0.47 kcal/mol), and in dmso (ΔG_{tc} (PPA) − ΔG_{tc} (PPF) = 0.62 kcal/mol). This extra stability of the *cis* isomer in PPF, about 0.4–0.6 kcal/mol, arises solely due to the presence of Phe instead of Ala side-chain in PPF.

The entropic and enthalpic components of the free energy difference were estimated from the temperature dependence of K_{tc} (derived from NMR spectra) as shown in Fig. 3b. A van't Hoff analysis yielded $\Delta H = -0.45$ kcal/mol and $\Delta S = -3.34$ cal/mol/deg for PPF, and $\Delta H = 0.62$ kcal/mol and $\Delta S = -1.59$ cal/mol/deg for PPA in H_2O. For both peptides, the *cis* conformer is entropically disfavored (1.00 kcal/mol for PPF and 0.47 kcal/mol for PPA at 298 K). However, the trend in enthalpic stability of the *cis* form is opposite in PPF and in PPA. The *cis* form is enthalpically favored in PPF and disfavored in PPA. The net effect of Ala to Phe substitution is therefore an enthalpic stabilization of the *cis* form by −1.07 (−0.45 to 0.62) kcal/mol and a corresponding entropic destabilization by 0.53 (1.0 − 0.47) kcal/mol (at 298 K).

A model where the *cis* isomer in PPF is stabilized by CH···π interaction between Pro(1) and Phe(3) predicts that the presence of the Phe ring in the *cis* form will make it entropically disfavored (locking of Phe rings) and enthalpically favored by an energy corresponding to that arising from a CH···π hydrogen bond. The experimentally observed entropic and the enthalpic components for the *cis* form of PPF, after subtracting appropriate energy and entropy components of PPA, are consistent with this model. The *cis* form of PPF is indeed associated with an additional unfavorable entropic component (0.53 kcal/mol) and is favored by an enthalpic component (−1.07 kcal/mol), compatible with the reported CH···π hydrogen bond energy of −0.88 kcal/mol [20].

3.4. Occurrence of Pro-(cis)-Pro-Phe motifs in known protein structures

NMR results showed that the Pro-Pro peptide bond is almost equally distributed between the *cis* and the *trans* conformational states in PPF. Without any tertiary structure, PPF is representative of the unfolded state of a protein. What is the fate of the Pro-Pro peptide bond in Pro-Pro-Phe motifs in a folded protein? To address this question we computed propensities of residues to be present at the (i+2) position of *cis* Pro-Pro motifs in proteins and the associated z-score (Table 2). The highest propensity is shown by Phe, followed by Tyr and His, all capable of exhibiting CH···π interaction. It should be pointed out that due to sparse data, Trp, Met and Cys have been left out of the current analysis. Out of a total 28 Pro-Pro-Phe motifs,

Table 2
Occurrence of *cis* and *trans* Pro-Pro peptide bonds in Pro-Pro-Xaa motifs in proteins

Xaa	E_{cPPX}[a]	N_{cPPX}[b]	N_{tPPX}[c]	P_{Xaa}[d]	z[e]
Phe	2.13	10	18	4.68	5.39
Tyr	1.83	6	18	3.28	3.09
His	1.30	3	14	2.31	1.50
Cys	0.53	1	6	1.87	0.64
Ala	4.12	7	47	1.70	1.42
Arg	2.52	3	30	1.19	0.31
Ile	1.91	2	23	1.05	0.07
Asn	1.98	2	24	1.01	0.01
Leu	4.12	4	50	0.97	−0.06
Thr	3.20	3	39	0.94	−0.11
Lys	2.97	2	37	0.67	−0.57
Gly	4.96	3	62	0.61	−0.88
Val	3.66	2	46	0.55	−0.87
Asp	2.06	1	26	0.49	−0.74
Gln	2.13	1	27	0.47	−0.78
Ser	3.89	1	50	0.26	−1.47
Glu	4.96	1	64	0.20	−1.78
Met	0.61	0	8	0.0	−0.78
Trp	0.46	0	6	0.0	−0.68
Pro	2.67	0	35	0.0	−1.64
Total		52	630		

Number of (a) expected *cis* Pro-Pro-Xaa peptide bonds, (b) observed *cis* Pro-Pro-Xaa peptide bonds, and, (c) observed *trans* Pro-Pro-Xaa peptide bonds. (d) Propensity (Eq. (1)), of *cis* Pro-Pro-Xaa peptide bond. (e) z-value: $(N_{cPPX}–E_{cPPX})/\mathrm{SQRT}\{E_{cPPX}(N - E_{cPPX})/N\}$ where N is the total number of Pro-Pro (*cis* and *trans*) in the data base. $|z| \geqslant 1.96$ signifies 95% confidence level.

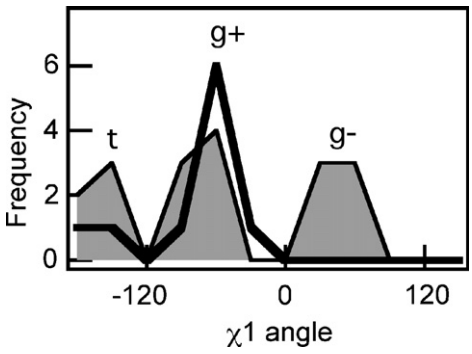

Fig. 4. χ1 angle distribution of Phe residues following *cis* (thick line) and *trans* (shaded) Pro-Pro motifs in proteins.

ten were found to be present in the *cis* and 18 in the *trans* conformation. The side chain angle χ1 of Phe residues also showed very different distributions when present in the *cis* and the *trans* Pro-Pro-Phe motifs (Fig. 4). As was observed for PPF, the *trans* conformers showed no preference among the three canonical side chain isomers (t, g+, g−) while the *cis* isomer was found to almost exclusively (8/10) populate the g+ state. As a rule we found a Pro-Pro VIb β-turn [21,22], followed by Phe ($\chi1 = g+$ and $\phi \sim -94° \pm 24°$ always showed the Pro(i)-Phe(i+2) CH$\cdots\pi$ interaction (4/10 cases). No Pro(i)-Phe(i+2) CH$\cdots\pi$ interaction was present in any of the *trans* conformers.

4. Summary and perspectives

We have demonstrated that the Pro-Pro peptide bond in the sequence motif Pro-Pro-Phe is predisposed to be present in the *cis* as well as the *trans* conformation with comparable likelihood. When present in a short peptide, devoid of any tertiary interaction, a CH$\cdots\pi$ interaction, between the first Pro residue and the Phe side-chain is the origin for the stability of the *cis* state. In proteins the Pro-Pro unit in Pro-Pro-Phe motifs also exhibited a high propensity to be present in the *cis* state, with or without the CH$\cdots\pi$ interaction. Incorporation of the sequence motif Pro-Pro-Phe may be useful in designing peptides, for example, peptides that contain a *cis* bond (type VI β-turn) [11,23] or where a Pro-Pro motif is used as a nucleating template for peptides [24]. The higher probability of having a *cis* peptide bond, which is not compatible with polyproline II conformation [25], may also be a reason why aromatic residues has a very low frequency of occurrence in polyproline II helices. In addition, our results point to new subtleties in the mechanism of protein folding, especially how the *cis–trans* isomerization [26] of Pro-Pro peptide bond can be modulated by the type of the residue following it.

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