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Probing dipeptide *trans*/*cis* stereochemistry using pH control of thiopeptide analogues, and application to the PepT1 transporter

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The stereochemistry of thiodipeptides of proline [*e.g.* Ala-Ψ[CS-N]-Pro] can be controlled using pH, allowing the *trans*-preference for substrates of the peptide transporter PepT1 to be confirmed.

The stereochemistry of the peptide bond is generally accepted as being *trans*, but there are now many examples that indicate that this is not always the case. In particular, the secondary amino acid proline has a much smaller thermodynamic preference for the *trans*-stereochemistry; several examples of *cis*-prolyl peptides or proteins are now documented, and *cis–trans* isomerisation can be used as a 'switch', which can be induced by isomerase enzymes.[1–4]

We have been studying the peptide transporter PepT1, which is an essential eukaryotic membrane protein that actively transports di- and tri-peptides across the wall of the small intestine.[5–7] The preferred stereochemistry of the peptide bond has been probed by us using molecular mechanics based modeling.[8] One idea that arose from this work was that the *cis* stereochemistry might explain the high affinity of dipeptides for PepT1 as, in their zwitterionic form, there is little difference in the energy of the two isomers due to an intramolecular salt bridge (see Fig. 1).

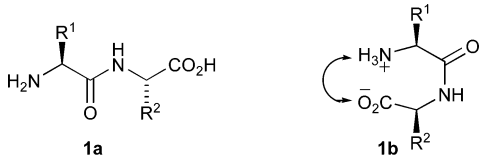

Fig. 1 Dipeptides in the *trans* (**1a**) and *cis* (**1b**) conformations.

Elegant work by Brandsch *et al.* had employed configurationally more stable thiopeptides to probe this question,[9] utilizing thiodipeptides of Ala-Pro, but we have developed and extended this work in two ways: a) We have used pH to control the *trans* : *cis* ratio. b) We have used other thiodipeptides to confirm the *trans*-stereochemical preference of PepT1.

Thioamides undergo slower rotation of the C–N bond than the corresponding amides (typical $t_{1/2}$ measured in hours instead of milliseconds at RT);[10] this provides a convenient handle to analyze peptide bond stereochemistry using such analogues. It is well known that the amides of secondary amines show the presence of both *trans* and *cis* isomers by NMR, and we wondered if the ionization of thiodipeptides might be used to control and temporarily lock the *trans* : *cis* ratio. Accordingly, we prepared the known thiodipeptide Ala-Ψ[CS-N]-Pro,[9] and incubated it for 48 h at RT at various pH values. Analysis by ^{1}H-NMR readily allowed us to determine the *trans* : *cis* ratio, with the methyl doublet of the alanyl residue being a clear marker (Fig. 2).

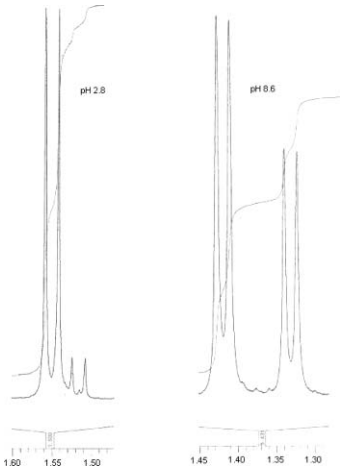

Fig. 2 Methyl doublets of alanine in the ^{1}H spectra NMR (D_2O, 400 MHz) of Ala-Ψ[CS-N]-Pro at pH 2 (*trans* : *cis* = 9 : 1) and pH 7 (*trans* : *cis* = 3 : 2).

We demonstrated that pH control of stereochemistry was achievable, with a dramatic reduction in the *trans* content at pH ≤ 3.5 (Fig. 3); the stereochemical assignment was confirmed by NOESY experiments (see Fig. 4).

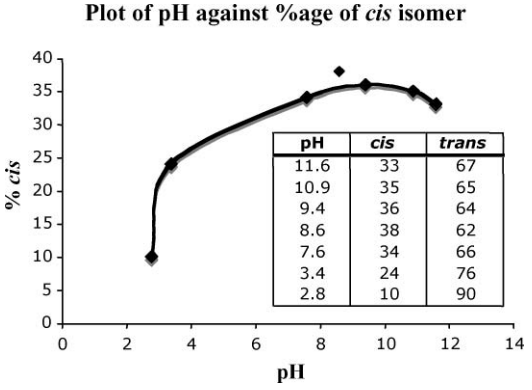

Fig. 3 Variation of *trans* : *cis* ratio with pH for Ala-Ψ[CS-N]-Pro.

† In memory of Ian Collier (1960–2002).

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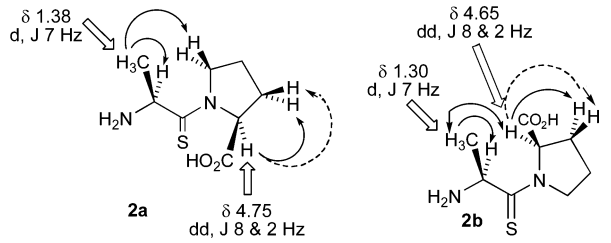


Fig. 4 Selected NOE data from the alanyl-Me and prolyl-α-H for Ala-Ψ[CS-N]-Pro in D_2O at pH 7 for the *trans* (**2a**) and *cis* (**2b**) conformers.

Table 1 Inhibition of uptake of [^{3}H]-D-Phe-L-Gln into PepT1 expressing oocytes by Ala-Ψ[CS-N]-Pro[11]

Pre-incubation	Estimated *trans* : *cis*	Expt. K_i
pH 7	67 : 33	0.56 mM (s.e. = 0.07)
pH 3	90 : 10	0.40 mM (s.e. = 0.08)

Knowing that the thiopeptides are configurationally stable over time-scales up to a few hours ($t_{\frac{1}{2}}$ *ca* 12 h), we compared the K_i values for Ala-Ψ[CS-N]-Pro containing 33% or 10% of the *cis*-isomer, and these results are shown in Table 1.

If the *trans*-isomer binds much more strongly than the *cis*-isomer, we would expect the apparent K_i to be about 1.3 times higher for thiodipeptide pre-incubated at pH 7, as was indeed observed. This both agrees with the results of Brandsch *et al.* (and hence verifying that *trans*/*cis* control using pH can be used to probe biological activity), and confirms the preferred *trans* stereochemistry for Ala-Ψ[CS-N]-Pro.

Nevertheless, proline is uniquely a secondary amine amongst DNA encoded amino acids, and it might have been possible for the carboxylic acid of this residue to have been located appropriately for binding of the *trans* amide, whereas all other dipeptides might have preferred the *cis* stereochemistry (Fig. 5).

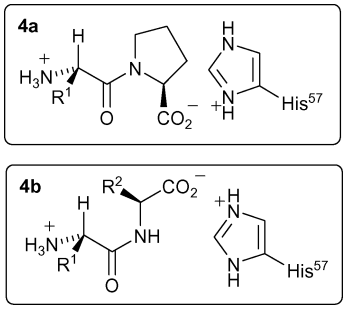
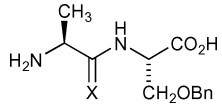


Fig. 5 Possible interactions of dipeptides with His[57]; **4a** shows how a Xxx-Pro dipeptide might form a salt bridge *via* the *trans*-peptide conformer, whereas **4b** shows how dipeptides with other residues at position 2 might also form a salt bridge *via* the *cis*-peptide conformer.

We therefore prepared the simple thiodipeptide **5b**, which was almost entirely in its *trans* stereochemistry (confirmed by NMR analysis), but with a high kinetic barrier to rotation. This thiodipeptide showed binding to PepT1 that was similar to its dipeptide analogues **5a**, and a large range of other thiodipeptides demonstrated similar binding. As thiopeptides are configurationally stable during transport, this result crucially indicates that the *trans* stereochemistry is preferred at the first amide bond.



5a X = O K_i = 0.23 M (s.e. 0.05)

5b X = S K_i = 0.29 M (s.e. 0.01)

In summary, we have developed a method for controlling the *trans* : *cis* ratio in prolyl dipeptides using pH, and demonstrated that this can be used to analyze stereochemical preferences in the binding to proteins. Moreover, we have unambiguously determined that the *trans*-peptide bond is essential for high affinity binding to PepT1.

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