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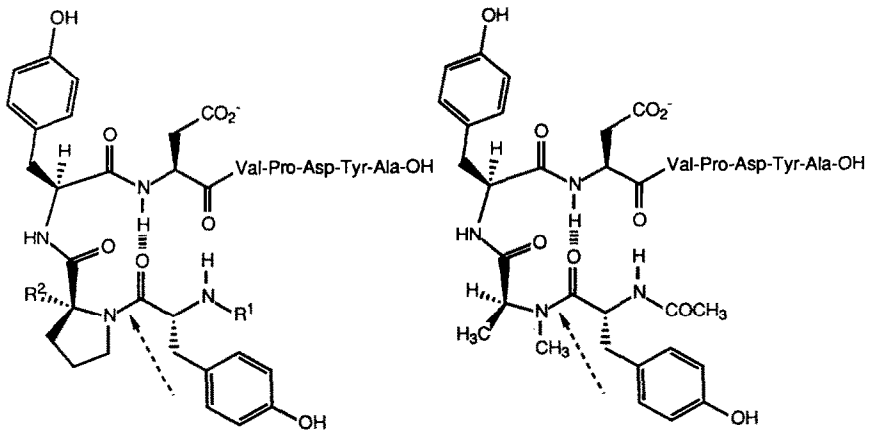
Probing the role of proline as a recognition element in peptide antigens

The role of the imino acid *S*-proline in controlling local conformation in oligopeptides is a topic of much pharmacological interest. Recent work on the design and construction of synthetic vaccines, and of antagonists to peptides such as bradykinin, has revealed its importance in controlling molecular structure responsible for observed biological activity. Additional impetus to research on this structural problem has been provided by the startling discovery that cyclophilin, a protein that binds the immunosuppressive drug cyclosporin A, can catalyse the slow isomerisation of X-Pro amide bonds in oligopeptides [1, 2]. We have attempted to probe the molecular mechanism by which proline exerts its conformational influence using a series of non-natural amino acid replacements in studies on the recognition of small peptides by monoclonal antibodies. Our interest in this topic arose from research aimed at detailing the molecular interactions responsible for the recognition of an immunogenic nonapeptide, H_2N-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-OH, **1**, (Fig. 1). This sequence occurs in the HA1 subunit of influenza virus hemagglutinin and has been shown to be highly immunogenic in raising antipeptide antibodies able to bind to the intact protein [3]. Two monoclonal antibodies (DB19/1 and DB19/25) were raised which tightly bound the nonapeptide **2**, which is related to **1** by acetylation of the N-terminal amino group. Significantly, it was determined, using standard amino acid replacement methods, that the recognition of **2** by the antibodies was dependent mainly upon the Tyr-Pro-Tyr-Asp segment of the peptide.* As NMR evidence had been reported indicating that, in aqueous solution, **1** exists in a highly populated conformation in which the residues Tyr-Pro-Tyr-Asp occupied a defined secondary structure (type II β-turn) [4], we decided to explore this segment of the peptide in more detail. In particular, the correlation between recognition and conformational constraints introduced into the oligopeptide by the *S*-proline residue in this region was investigated using a quantitative binding assay of the recognition of nonapeptide analogues **3** and **4** by DB19/1 and DB19/25. In these peptides, *S*-proline was replaced by the non-natural residues *S*-2-methylproline (2-MePro) and *S*-*N*-methylalanine (NMeAla) respectively. In both cases, the introduction of these analogue amino acids gave oligopeptides which were similar in their charge and steric properties with respect to **2**. However, we anticipated that the conformational dynamics of **3** and **4** would be altered significantly compared to that of **2** in the important tetrapeptide segment.

Molecular modelling was used to determine the extent to which the conformational minima of the peptide backbones of **2** and **3** were a subset of those available to **4**. Our results suggested that there was no strain energy penalty incurred upon folding **4** into a conformation about the NMeAla residue identical to that of *S*-proline in oligopeptide **2**. Thus, we envisaged that **2** and **4** could adopt similar backbone conformations upon binding to the antibody combining site. However, the dissociation constants for the complexes formed between **2**, **3** and **4** and each monoclonal antibody varied considerably. In the case of DB19/1 the variation in dissociation constant was over almost two orders of magnitude. NMR evidence was obtained which indicated that the conformational flexibility of peptides **2**, **3** and **4** was dissimilar in the key tetrapeptide segment. Indeed, amide chemical shift temperature dependence indicated that **3** was significantly less mobile than **2** in this region, presumably due to the additional steric constraints introduced by the methyl group at the C_α carbon of residue 2. Our current interpretation of these results is that the antibodies are extremely sensitive to the entropic component of the binding free energy change, arising from folding the oligopeptide into its bound form. These results are consistent with the view that the proline residue acts only to *restrict* the number of accessible peptide conformations and does not "lock" the molecule into a single conformer in which functional groups are held rigidly in spatial orientations necessary for optimal enthalpic interactions with the antibody.

* Brennand DM *et al.*, manuscript submitted for publication.



4

1: $R^1 = H$, $R^2 = H$

2: $R^1 = COCH_3$, $R^2 = H$

3: $R^1 = COCH_3$, $R^2 = CH_3$

Fig. 1. Structures of nonapeptides **1–4**. Arrowed bond indicates that about which *cis-trans* isomers are observed for peptides **1**, **2** and **4**.

Methods

Murine monoclonal antibodies with high affinities for nonapeptides **1** and **2** were obtained following standard protocols. All oligopeptides were synthesised using continuous flow solid phase synthesis, in which amino acids were used as their N-protected FMOC* derivatives and were coupled as symmetrical anhydrides. After removal from the resin, the peptides were purified using LH-20 Sephadex eluting with 1% aqueous acetic acid, followed by reverse phase HPLC upon a C_{18} column using gradient elution over 30 min (10% MeCN/0.2% TFA/H_2O to 90% MeCN/0.2% TFA/H_2O). Purity was assessed using analytical reverse phase, and anion exchange, HPLC. Tritiated peptides **2**, **3** and **4** were prepared by stirring the relevant nonapeptide (10 mg) with [^{3}H]acetic anhydride (100 mCi), in dilute sodium hydroxide solution, at room temperature for 3 hr. After removal of solvent and excess reagents from the reaction solution, exchangeable tritium was removed by repeated lyophilisation of the peptide from distilled water. The acetylated materials were also purified by chromatography on LH-20 Sephadex (eluting with 0.5% ammonium acetate in pH 7 buffer) followed by reverse phase HPLC using identical conditions to those outlined above. After purification, labelled peptides were dissolved in distilled water (in the range 0.1 to 0.3 mg/mL), and their concentrations determined using UV absorption spectroscopy. Extinction coefficients at 277 nm at 293° K had been measured for standard concentrations of unlabelled material. The specific activity of each of the labelled peptides **2**, **3** and **4** was then computed using liquid scintillation counting of these solutions.

Dissociation constants for the interaction of **2**, **3** and **4** with the two monoclonals (DB19/1 and DB19/25) were determined using equilibrium ultrafiltration measurements. In general, the antibody was mixed with the tritiated peptide as a solution in 5 mL phosphate-buffered saline and incubated at 291° K for 16 hr. Two aliquots (1 mL) were then filtered separately through an Amicon Centrifree micropartition membrane by centrifugation at 1900 *g* for 5 min. The concentration of the unbound peptide could then be measured by liquid scintillation counting of an aliquot of the filtrate. Experiments were performed using a series of peptide concentrations, and the dissociation constants were determined from a Scatchard plot. The extent of non-specific binding of the peptides was estimated by repetition of the experiments in the presence of an irrelevant antibody (RJD2A10).

Full assignments of the proton NMR spectra for compounds **2**, **3** and **4** were obtained using a combination of COSY and TOCSY experiments [5]. Information on the solution phase conformational preferences of the acetylated peptides was then collected (a) by determining the temperature dependence of the chemical shift of the amide NH resonances [6], and (b) from measurements of interproton distances using rotating frame NOE experiments [7]. Molecular modelling of the residues comprising the key tetrapeptide region, in both **3** and **4**, was carried out by generating large numbers of initial molecular geometries (2904 and 3661 respectively) by systematic variation of rotatable bonds [8]. Starting structures in which pairs of atoms were closer than 1.5Å were eliminated from the subsequent calculations. All structures were energy minimised using the AMBER potential energy functions and parameters [9] as implemented in the MacroModel† software package. For the calculations the tetrapeptides were modelled as their *N*-acetylated *N'*-methylamide derivatives, and the microscopic dielectric constant ε was set to 2.25. This minimised the introduction of conformational artifacts due to strong charge–charge interactions in the gas-phase modelling. All conformations within 20 kJ/mol of the global energy minimum were retained for analysis, duplicate structures being removed by an automatic superimposition procedure.

Results and Discussion

The dissociation constants K_d for the interaction of

* Abbreviations: FMOC, fluorenylmethyloxycarbonyl; TFA, trifluoroacetic acid; MeCN, acetonitrile; COSY, correlated spectroscopy; TOCSY, total coherence transfer spectroscopy; and NOE, nuclear Overhauser effect.

† Available from Professor W. C. Still, Department of Chemistry, Columbia University, New York.

Table 1. Equilibrium dissociation constants for the complexes formed between the nonapeptides **2**, **3** and **4**, and monoclonal antibodies DB19/1 and DB19/25

	Antibody			
	DB19/1		DB19/25	
Peptide	K_d (M) at 293°K	ΔG_{293} (kJ/mol)	K_d (M) at 293°K	ΔG_{293} (kJ/mol)
2	$(1.8 \pm 0.2) \times 10^{-7}$	−37.8	$(1.8 \pm 0.2) \times 10^{-8}$	−43.4
3	$(3.6 \pm 0.5) \times 10^{-9}$	−47.4	$(5.7 \pm 0.4) \times 10^{-9}$	−46.2
4	$(4.2 \pm 0.3) \times 10^{-6}$	−30.2	$(6.0 \pm 0.5) \times 10^{-7}$	−34.9

peptides **2**, **3** and **4** with the two monoclonal antibodies DB19/1 and DB19/25 are given in Table 1, together with the free energies of binding computed from the expression $\Delta G = RT\log_e(K_d)$.

Introduction of the methyl group at the α-carbon of proline-2 improved the binding of **3**, relative to that of reference peptide **2**, by 9.6 kJ/mol to monoclonal DB19/1. Nonapeptide **3** was also recognised by DB19/25, although the binding free energy was only lowered by 2.8 kJ/mol. Hence there was no apparent difficulty in accommodating the steric bulk of the additional methyl group within the combining site of either antibody. The free energy change upon transferring a methyl substituent from water into a hydrophobic environment has been estimated at 3–5 kJ/mol [10]. The increased binding of **3** with DB19/25 may only have reflected changes in the solvent entropy as a result of burying the methyl group within the protein. This explanation seemed unable to account for the total difference in free energy observed for the interaction of **3** with DB19/1 relative to **2**. Even more striking was the reduced affinity for nonapeptide **4** shown by both antibodies. Although removing a methylene group and replacing it with two hydrogen atoms was expected to increase the binding free energy of **4** relative to **2** by 3–4 kJ/mol, as a consequence of the "hydrophobic" effect, the observed changes were 8.5 and 7.6 kJ/mol with DB19/25 and DB19/1 respectively. Since a crystallographic study had suggested that antibodies interact with protein antigens using a "handshake" mechanism [11], the structural properties of both partners were likely to be modified during the recognition event. This was also consistent with ideas on the thermodynamics of flexible peptide hormones interacting with cellular receptors [12]. Since we anticipated that **4** could be folded into a conformation identical with that of **2** about the proline residue, the loss in recognition was surprising. Hence, a molecular modelling study of the key tetrapeptide region of **4** was undertaken to ensure that there were no enthalpic barriers to its adoption of conformations similar to those accessible to **2** and **3**.

Given the computational requirements needed to locate all energy minima for molecules with greater than 8 rotatable bonds, the conformational search was carried out for the tetrapeptides AcNH-Tyr-2-MePro-Tyr-Asp-NMe (**5**) and AcNH-Tyr-NMeAla-Tyr-Asp-NMe (**6**) as models for the nonapeptides **3** and **4** respectively. The backbone torsion angles (ϕ,ψ) were selected as measures of the molecular conformational preferences, since the enthalpic barriers to sidechain rotation were small relative to those

Table 2. Conformational properties of low energy structures computed for tetrapeptide model peptides **5** and **6**

(a) Peptide **5**	AcNH-Tyr-2-MePro-Tyr-Asp-NMe				
Relative energy (kJ/mol)	ϕ_{i+1}* (deg)	ψ_{i+1}* (deg)	ϕ_{i+2}* (deg)	ψ_{i+2}* (deg)	β-Turn type†
0.0 (**A**)	−54.7	−23.5	−86.3	−8.7	I
0.6	−55.3	−27.1	−91.5	−39.5	III
4.3	−71.9	45.9	−161.7	−51.2	
7.1	−71.0	57.2	−78.5	64.7	
(b) Peptide **6**	AcNH-Tyr-NMeAla-Tyr-Asp-NMe				
Relative energy (kJ/mol)	ϕ_{i+1}* (deg)	ψ_{i+1}* (deg)	ϕ_{i+2}* (deg)	ψ_{i+2}* (deg)	β-Turn type†
0.0 (**A**)	57.7	−119.9	−82.4	−44.7	II′
0.2 (**B**)	−57.5	−39.1	−60.0	−47.6	I
4.3	59.1	−126.3	−66.9	−48.5	II′
6.2	−125.3	62.3	59.6	38.5	
8.8	59.0	−122.3	−81.9	−43.8	II′

* The torsion angles in the table are defined such that residues i + 1 and i + 2 correspond to the amino acids in positions 2 and 3 relative to the N-terminus of the oligopeptide, i.e. in **3** residue i + 1 is *S*-α-methylproline, while in **4** residue i + 1 is *S*-*N*-methylalanine.

† Although there have been various classifications of β-turn types, the proposed types correspond to those described by Wilmot and Thornton [14].

determined during deformation of the ϕ,ψ angles. Generation of initial conformations was performed using the systematic search algorithm of Still and Lipton [8]. Subsequent energy minimisation using both block-diagonal Newton–Raphson and conjugate gradient methods [13] gave a set of structures for both **5** and **6** within 20 kJ/mol of the lowest energy conformation (Table 2).

For the peptide containing the *S*-2-methylproline residue, a single well-defined conformation **5A** was found to be the global energy minimum. The backbone torsion angles in the MePro-Tyr segment corresponded to those defining a type I β-turn. However, for tetrapeptide **6** in which *S*-*N*-methylalanine had replaced *S*-proline, the global minimum conformation **6A** was located on the potential surface at a point only 0.2 kJ/mol below that of the structure of next lowest energy. Significantly, the peptide backbone of **6B** superimposed identically onto that determined for the global minimum structure **5A** about the critical region. Since in the other low energy structure **6A** the ϕ-angle of the *S*-*N*-methylalanine residue was found to be +57.5°, the peptide backbone could not be identical to any oligopeptide containing *S*-proline, since the ϕ-angle of this imino acid must have a negative value due to geometric constraints imposed by its location within the five-membered ring. From these studies it was clear that the enthalpic penalty (0.2 kJ/mol) to folding **4** into a shape identical with that of **2** or **3** in the peptide/antibody complex was insufficient to account for the observed differences in the binding constants of the peptides. In addition, evidence was also obtained which suggested that the proline replacements had not introduced any major perturbations into the electrostatic properties of the nonapeptide analogues, relative to those of **2**.

NMR studies of the oligopeptides **2**, **3** and **4** were also undertaken to investigate the conformational features of these molecules in solution. These included the measurement of NH chemical shift dependencies and the determination of relative interproton distances using rotating frame NOE experiments. Since the relevance of solution phase conformations of the peptides to that of their complexed forms had yet to be established, we focused upon determining the relative flexibilities of the three analogues. The most immediate manifestation of the ability of the peptides to explore conformational space was the ratio of *cis*- and *trans*-isomers about the amide bond between the first two residues in the peptide (arrowed in Fig. 1). The ^{1}H NMR spectrum of **3** showed only seven well-defined NH signals, indicating that it existed almost exclusively as the *trans*-isomer (>95%), whereas those of **2** and **4** contained two sets of NH resonances showing that these peptides existed in both forms (*ca.* 1:1). When the chemical shifts of the NH signals in the *trans*-form (since this was the only conformer which could adopt a type I, or II, β-turn) were measured as a function of temperature, we found that the temperature coefficients of the NH protons of residues Tyr-3 and Asp-4 were low for peptide **3** (Table 3); indeed, the Asp-4 NH resonance showed no alteration in chemical shift over the temperature range examined. This was consistent with its participation in a strong intramolecular hydrogen bond. This effect has been ascribed to shielding of the proton involved in the intramolecular interaction from the solvent [6].

Significantly, all of the NH resonances in **4** possessed temperature coefficients in the range expected for the case of a linear peptide adopting a "random-coil" conformation, strongly supporting the absence of any well-defined intramolecular hydrogen bonding. This is qualitative evidence for the hypothesis that this compound was exploring many allowed conformations in solution, and that therefore it has higher flexibility than **3**. Further data have been obtained from a preliminary analysis of the rotating frame NOE spectra for **3** and **4**. In that of **3**, there was an NOE effect between the NH protons of residues Asp-4 and Try-3 which was significantly larger than any of the other $NH_i -> NH_{i+1}$ NOEs observed in this molecule.* These data therefore supported the population of a β-turn structure about the initial four residues of **3**. Similar experiments indicated that the conformational behaviour of **2** was intermediate between that observed for nonapeptides **3** and **4**.

In the light of the NMR measurements, together with the computational studies upon the sterically allowed peptide conformations, we propose that the recognition of the flexible nonapeptide is controlled to a significant extent by its conformational entropy. This energy penalty must be proportional to the number of accessible solution phase conformations of the oligopeptide undergoing binding within the antibody combining site. The flexibility of an oligopeptide in solution, *in addition* to the amino acid sequence, must therefore be considered as a key factor in its recognition by complementary protein receptors. We note that this information is not easily obtained from the X-ray crystallographic analysis of a peptide/receptor complex. Experiments are underway to confirm our hypothesis, involving the measurement of the solvation energies of **2** and its analogues, and the evaluation of the van't Hoff isotherms for complex formation between peptides **2**, **3** and **4** and the monoclonals. Results will be reported in due course.

Conclusion

We have presented experimental evidence that the recognition of flexible, linear oligopeptides by monoclonal antibodies is sensitive to the entropy penality incurred when the peptide becomes constrained to a well-defined region

* Compare the measurements of Dyson *et al.* [4].

Table 3. Temperature coefficients of selected NH resonances in peptides **2** (*trans* conformer)*, **3** (*trans* conformer)* and **4** in 90:10 H_2O/D_2O solution at *ca.* pH 6

	Temperature dependencies ($\Delta\delta$ ppb/°K)		
	Oligopeptide		
Residue NH†	**2**	**3**	**4**
Tyr-3	−7.1	−3.3	−6.9
Asp-4	**−4.6**	**0.0**	**−5.0**
Val-5	−7.4	−7.1	−8.0
Asp-7	−8.3	−8.7	−8.2

* These nonapeptides existed as a mixture of *cis* and *trans* isomers about the amide bond linking the first two residues in the molecule. All measurements of NH chemical shift δ were made at 500 MHz.

† All resonances for each isomer were unambiguously determined using 2D NMR methods.

of its conformation space upon binding. The possible implications of this observation for the development of synthetic vaccines will be outlined elsewhere.* This aspect of recognition is often difficult to probe using natural amino acid replacements due to the difficulty of maintaining the steric and electrostatic features of the natural substrate for the receptor. In utilising the non-natural analogues of *S*-proline, the overall charge of the peptide analogues was maintained as were the number of hydrogen bonding sites available for interaction with the receptor. However, as judged by NMR methods, these non-natural amino acids modified the conformational mobilities of analogues. Their use as replacements for *S*-proline in other biologically important substrates, such as bradykinin, may allow some determination of the relative importance of dynamic properties in systems involving the interaction of flexible proline-containing peptides with cellular receptors.

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Zinc fingers and molecular recognition. Structure and nucleic acid binding studies of an HIV zinc finger-like domain

Retroviruses encode for synthesis of a *gag* polyprotein which binds to viral RNA and anchors it to the cell wall for budding [1–3]. All retroviral *gag* proteins [and their nucleic acid binding protein (NABP*) proteolysis products] contain conserved amino acid sequences of the type C-X_2-C-X_4-H-X_4-C (X = variable amino acid residue) [4–6], which have been implicated in zinc binding [7, 8]. Although NABPs in the mature virus lack zinc [8, 9], probably due to the presence of internal disulfides [10], a growing body of evidence provides support for the physiological relevance of the zinc binding at the *gag*-stage of the retroviral life cycle [7, 8, 11–13]. We have determined the structure of an

* Abbreviations: NABP, nucleic acid binding protein; RMSD, route mean square deviation; 2D NOESY, two-dimensional nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser spectroscopy; and DG, distance geometry.