Structural and computational investigations of the conformation of antigenic peptide fragments of human polymorphic epithelial mucin

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Human polymorphic epithelial mucins (PEM) are complex glycoproteins that are associated with breast and ovarian carcinomas. The PEM core protein consists of variable numbers of a tandem repeat sequence which contains a short antigenic hydrophilic region (Pro^1-Asp-Thr-Arg-Pro-Ala-Pro^7). High-field n.m.r. studies undertaken on antigenic 20- and 11-amino acid fragments of the PEM core protein in dimethyl sulphoxide have identified a type-I β-turn to be present in the region Pro^1-Asp-Thr-Arg^4. This region includes and overlaps the identified type-I (Asp^2-Thr-Arg^4) and type-II (Arg^4-Pro-Ala^6) epitopes of anti-PEM monoclonal antibodies. The studies indicate that the β-turn is stabilized by the presence of a salt-bridge interaction between Asp-2 and Arg-4. In order to probe the conformations accessible to the PEM peptides a computational study was undertaken independently on the peptide Pro-Asp-Thr-Arg-Pro using a modified Metropolis Monte Carlo algorithm. This study identified the n.m.r.-observed salt-bridge type-I β-turn as the major low-energy conformer. These results suggest that this structural motif may be involved in the immune recognition of PEM.

INTRODUCTION

Epithelial mucins are high-molecular-mass glycoproteins that are associated with human breast and ovarian carcinomas (Taylor-Papadimitriou & Gendler, 1988). Their apparent molecular mass is in excess of 300 kDa and they exhibit a genetic polymorphism demonstrable at both the DNA and protein level (Swallow et al., 1986; Gendler et al., 1988): these complex glycoproteins have been termed polymorphic epithelial mucin (PEM). cDNA clones coding for the core protein of the mucin have been obtained (Gendler et al., 1988) resulting in the identification of a large domain consisting of a highly conserved 20-amino acid tandem repeat sequence (Table 1).

A number of mouse monoclonal antibodies recognize determinants in the PEM molecule; most of these define epitopes contained within the 20-amino acid tandem repeat of the protein core. Secondary-structure predictions and hydropathicity calculations performed on this sequence have identified a hydrophilic domain extending over the first ten amino acids. It is within this domain that the determinants for the protein-core-specific antibodies are found (Price et al., 1990a).

Epitope-mapping experiments utilizing immobilized synthetic peptides for antibody-binding assays have identified two distinct epitope regions that are defined by these antibodies (Price et al., 1990a), each with their own fine specificity (Briggs et al., 1991). Type-I epitopes, which include the motif Asp^2-Thr-Arg^4, are defined by antibodies which have generally been raised against human milk fat globule membranes such as the antibodies HMFG-1 and HMFG-2 (Taylor-Papadimitriou et al., 1981). Type-II epitopes, which all contain Arg^4-Pro-Ala^6, are defined by antibodies typified by C595 (Price et al., 1990b) and NCRC-11 (Ellis et al., 1984), raised against either dissociated breast carcinoma cells or the purified mucin.

A knowledge of the structure of peptides in solution provides a model for investigating the recognition phenomena associated with the interaction of PEM with specific monoclonal antibodies. We have previously investigated the conformation of an 11-amino acid fragment of the tandem repeat, p(1–11) (Table 1), by n.m.r. spectroscopy (Tendler, 1990). This study revealed the presence of a type-I β-turn between residues Asp-2 and Arg-4, overlapping regions of the identified epitopes for both the type-I and type-II monoclonal antibodies.

This paper reports on our continuing structural studies on PEM-related peptides. The stabilization of the type-I β-turn in peptide p(1–11) and the structure of the full 20-amino acid peptide, p(1–20) (Table 1) have now been investigated by high-field n.m.r. spectroscopy. In addition, to probe the structure of the PEM epitope, a simulated annealing algorithm has been employed to search for low-energy minima in the PEM turn region of peptide p(1–5) (Table 1).

MATERIALS AND METHODS

Peptide chemistry

Peptide p(1–20), purified to > 98 % by reversed-phase h.p.l.c., was the kind gift of Celltech Ltd. (Slough, Bucks, U.K.). Peptides p(1–11) and p(7–20) were prepared and purified as previously described (Tendler, 1990; Price et al., 1990a). The peptides were analysed on an Applied Biosystems Inc. model 437A peptide sequencer and found to have the correct sequence.

Peptide p(1–20) with the guanidinyl group of Arg-4 blocked with p-hydroxyphenylglyoxal (HPG) was prepared according to the method of Yamasaki et al. (1980). Peptide p(1–20) (2 mg) was incubated with HPG (2 mg) for 16 h at 25 °C in 50 mM-sodium carbonate buffer at pH 10. Excess reagent was removed by passing the mixture down a Sephadex G-25 column into 50 mM-sodium carbonate buffer at pH 9. The extent of modification was determined to be > 85 % from the absorbance at 340 nm using the absorption coefficient 1.83×10^4 M$^{-1}\cdot$cm^{-1}

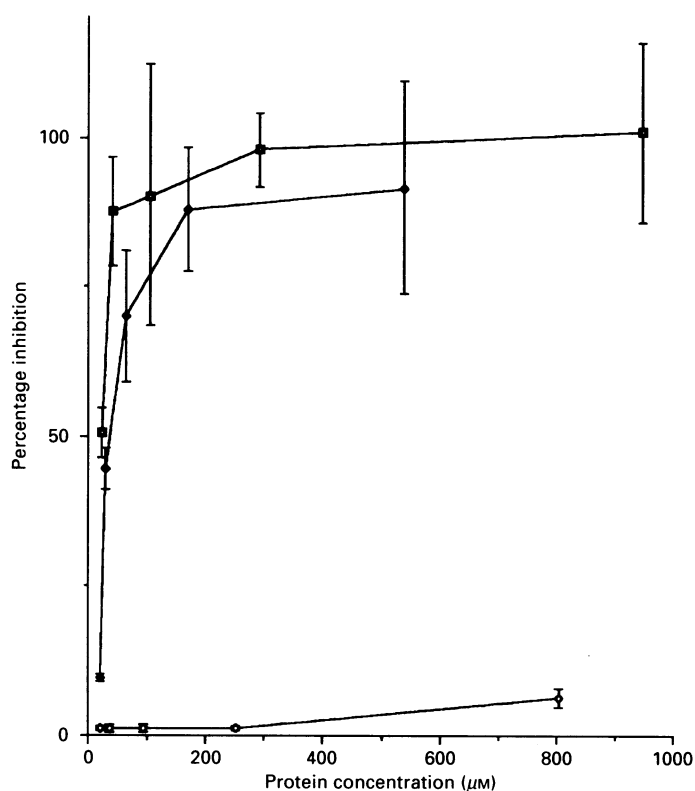**Table 1. Sequence of PEM-related peptides**

The one-letter notation for amino acids is used.

Peptide	Sequence			
p(1–20)	PDTR$\overset{5}{P}$	APGS$\overset{10}{T}$	APPA$\overset{15}{H}$	GVTS$\overset{20}{A}$
p(7–20)		PGST	APPAH	GVTSA
p(1–11)	PDTRP	APGST	A	
p(1–5)	PDTRP			

Fig. 1. Inhibition of PEM binding to the monoclonal antibody C595 by synthetic peptides in solution

The percentage inhibition of PEM binding to C595 by peptides at various concentrations was examined. ◆, p(1–20); □, p(1–11); ◇, p(7–20).

(Yamasaki et al., 1980). The modified peptide was dialysed into water and freeze-dried to give a 40 % yield.

Binding studies

The ability of peptides to block the binding of intact mucin to the anti-mucin monoclonal antibody C595 was measured by their competitive activity in a two-site immunoradiometric (sandwich) assay (Price et al., 1990b). The capture antibody, C595, [10 μl at 10 μg/ml in phosphate-buffered saline (PBS) at pH 7.3 containing 0.02% NaN$_3$], or PBS/NaN$_3$ alone, was adsorbed on to the walls of Terasaki Microtest plates for 18 h at 4 °C. The wells were aspirated and washed four times in casein buffer (0.1% casein in PBS), then incubated for 1 h at 25 °C in casein buffer to block non-specific binding. Portions (5 μl) of the peptide fragments at various concentrations (1 mg/ml–10 μg/ml) plus 5 μl of casein buffer were added to the wells, and the plates incubated for 2 h at 37 °C. The wells were aspirated and the plates washed four times with casein buffer. Portions (5 μl) of the

test peptide at equal concentrations to the previous incubation plus 5 μl of purified mucin [approx. 10 μg/ml as estimated from antigen–antibody titrations (Price et al., 1990b) in PBS plus 0.02% NaN$_3$] were added to the wells, and the plates incubated for a further 30 min at 4 °C. The plates were aspirated and washed four times with casein buffer. ^{125}I-labelled NCRC-11 antibody (as the radioactive tracer antibody to detect mucin captured on the solid phase) was added to the wells at 1.5 × 10^5 c.p.m./10 μl per well. After incubation for 1 h at 20 °C, the wells were aspirated, the plates washed six times with casein buffer, the wells were separated with a band saw and the radioactivity in each well was determined using an LKB γ-radiation counter.

N.m.r. spectroscopy

Samples for n.m.r. spectroscopy contained approx. 4 mM-peptide and 0.09 mM-tetramethylsilane in [^{2}H$_6$]dimethyl sulphoxide. ^{1}H n.m.r. experiments were undertaken at 400 MHz and 500 MHz on Bruker AM400 and AM500 n.m.r. spectrometers respectively. Over the range of 2–10 mM, no concentration-dependent change in chemical shift of the resonances of the peptide have been observed. Chemical-shift values (δ) (accurate to 0.01 p.p.m.) are reported relative to the tetramethylsilane resonance (δ 0.00 p.p.m.). The one-dimensional spectra were typically recorded at 303 K in 16 K data points and routinely zero-filled to 32 K data points to give a final resolution of 0.305 Hz/pt. The spectrum of the Arg-4 HPG-blocked peptide was recorded at 298 K. For the temperature studies, spectra were recorded between 293 and 318 K with 5 K increments. A 20 min period was allowed for temperature equilibration between each of the experiments. In the study of the amide resonances, the residual water resonance was suppressed by presaturation at all times except during the acquisition period. In the study of the very low field resonances no presaturation was used.

Two-dimensional phase-sensitive correlation (COSY) experiments were acquired at 400 MHz into 512 × 2 K files. The files were zero-filled to 1 K in F1 and analysed according to the method described by Wüthrich (1986). Homonuclear Hartmann-Hahn (HOHAHA) experiments (Braunschweiler & Ernst, 1983) were recorded at 400 MHz and 500 MHz using an MLEV-17 pulse sequence. The spectra were acquired in the phase-sensitive mode using time proportional phase incrementation (TPPI). In the experiments at 500 MHz, 440 × 2 K files were acquired, and at 400 MHz 512 × 2 K files were acquired. The 400 MHz experiment was recorded with a mixing time of 120 ms, and the 500 MHz experiments were recorded with mixing times of 50 ms, 80 ms and 120 ms respectively. The rotating Overhauser spectroscopy (ROESY) experiment (Bax & Davis, 1985) was acquired at 500 MHz. The spectrum was recorded in the phase-sensitive mode with continuous wave-spin locking and TPPI; 512 × 2 K files were collected. The spin locking time used for this experiment was 200 ms.

Computational chemistry

The model peptide (acetyl)-Pro-Asp-Thr-Arg-Pro-(N-methyl-amide) was graphically constructed in a fully extended conformation for the simulated annealing calculations. The aspartate and arginine residues were modelled with ionic sidechains. Empirical partial atomic charges were added by CHARGE2 (Abraham & Smith, 1988; Abraham et al., 1991) and all molecular mechanics and molecular dynamics calculations were performed using the COSMIC(90) force field with a dielectric constant of one (Morley et al., 1991; Vinter et al., 1987).

A novel simulated annealing algorithm was employed to search conformational space and hunt for low-energy minima. Based on the Metropolis Monte Carlo method (Metropolis et al.,

Table 2. Chemical shifts (p.p.m.) of the assigned amino acid residues for peptide p(1–20) recorded at 303 K

Residue	NH	αH	βH	Others
Pro[1]	8.43	4.18	2.25, 1.86	γH 1.86
				δH 3.23, 3.18
Asp[2]	8.85	4.71	2.79, 2.55	
Thr[3]	7.70	4.19	3.96	γH 1.03
				OH 4.86
Arg[4]	7.93	4.51	1.70, 1.53	γH 1.53
				δH 3.10
				NH 7.13, 7.52
Pro[5]	–	4.33	2.00, 1.75	γH 1.83
				δH 3.65, 3.50
Ala[6]	8.07	4.48	1.17	
	9.17 (min)			
Pro[7]	–	4.30	1.98, 1.77	γH 1.83
				δH 3.63
Gly[8]	8.16	3.75, 3.70		
Ser[9]	7.77	4.31	3.59, 3.51	
Thr[10]	7.72	4.18	4.03	γH 1.02
				OH 4.82
Ala[11]	7.84	4.50	1.17	
Pro[12]	–	4.54	2.11, 1.73	γH 1.89
				δH 3.67, 3.44
Pro[13]*	–	4.46	2.14, 1.73	γH 1.73
				δH 3.40
Ala[14]	8.04	4.15	1.17	
His[15]	7.94	4.46	3.05, 2.95	C2H 6.58
				C4H 7.14
Gly[16]	8.18	3.89, 3.73		
Val[17]	8.03	4.28	2.20	γH 0.86
Thr[18]	7.83	4.33	3.98	γH 1.04
Ser[19]	7.78	4.38	3.63, 3.56	OH 5.05
Ala[20]	8.06	4.19	1.25	

* Assignments determined at 298 K.

1953), it uses short bursts of molecular dynamics, with kinetic energy concentrated into bond rotation, to generate trial configurations of the system. Over a sufficient number of trials the available energy states are sampled according to their Boltzmann probability. These instantaneous conformations can either be minimized at regular intervals or alternatively the system can be slowly cooled to trap the molecule in low-energy minima.

The procedure utilized can be summarized as follows:

1. Calculate initial energy (E_{old}) of starting co-ordinates.

2. Randomly select a fraction of the total number of rotatable bonds.

3. Assign atomic velocities to set the selected bonds spinning in either a clockwise or anti-clockwise direction. This was done in practice by temporarily rotating each bond by $\pm 5°$ and setting the velocities proportional to the atomic displacements.

4. Renormalize velocities to current temperature.

5. Run a brief dynamics trajectory for a specified number of iterations to generate new atomic co-ordinates. Integration is by the 'leapfrog' algorithm (Verlet, 1967) using a 1 fs time step.

6. Calculate energy of trial configuration (E_{new}) and compare with E_{old}. The trial is accepted if either $E_{new} - E_{old} < 0$ or $\exp(-(E_{new} - E_{old})/RT) > \text{RAN}[0, 1]$ where RAN[0, 1] is a random number between 0 and 1. Update E_{old} (energy of last accepted step).

7. If trial was accepted, then continue with current dynamics trajectory (loop back to step 4); otherwise begin new trajectory from the last accepted configuration (loop back to step 2).

Runs were performed using 200 000 Monte Carlo trials at a constant temperature of 900 K. At each trial, half of the rotatable bonds were selected for rotation. The Metropolis test was applied

at 1 fs intervals. It was found necessary to restrain peptide bonds to the trans-configuration by means of an extra harmonic torsional potential with a force constant of 25 kcal/mol per rad². No other restraints were used in these calculations.

The co-ordinates were archived every 1000 trials and fully energy minimized by 540 cycles of a conjugate gradients minimizer to give 200 conformations. Structures within 29.3 kJ/mol (7 kcal/mol) of the lowest energy conformer found were extracted for further analysis. Duplicate structures (those with a heavy-atom root-mean-square difference of less than 0.005 nm) were removed. Each run, including minimizations, took approximately 22 h central-processor-unit time on an Apollo DN10000 (5.6 million floating-point operations/s).

RESULTS

Binding studies

The inhibition of binding of urinary mucin to the C595 antibody by peptides p(1–11), p(7–20) and p(1–20) is presented graphically in Fig. 1. Peptides p(1–11) and p(1–20) showed significant inhibition of antigen binding compared with peptide p(7–20), which failed to affect this binding. These results confirm that peptides that contain the previously identified epitope for the antibody C595 Arg[4]-Pro-Ala-Pro can inhibit the binding of the anti-mucin monoclonal antibody to the intact urinary mucin.

N.m.r. spectroscopy

The assignments of the proton resonances of peptide p(1–20) (Table 2) were made on the basis of chemical shift and connectivity in one-dimensional and two-dimensional COSY and HOHAHA experiments using the methods previously described
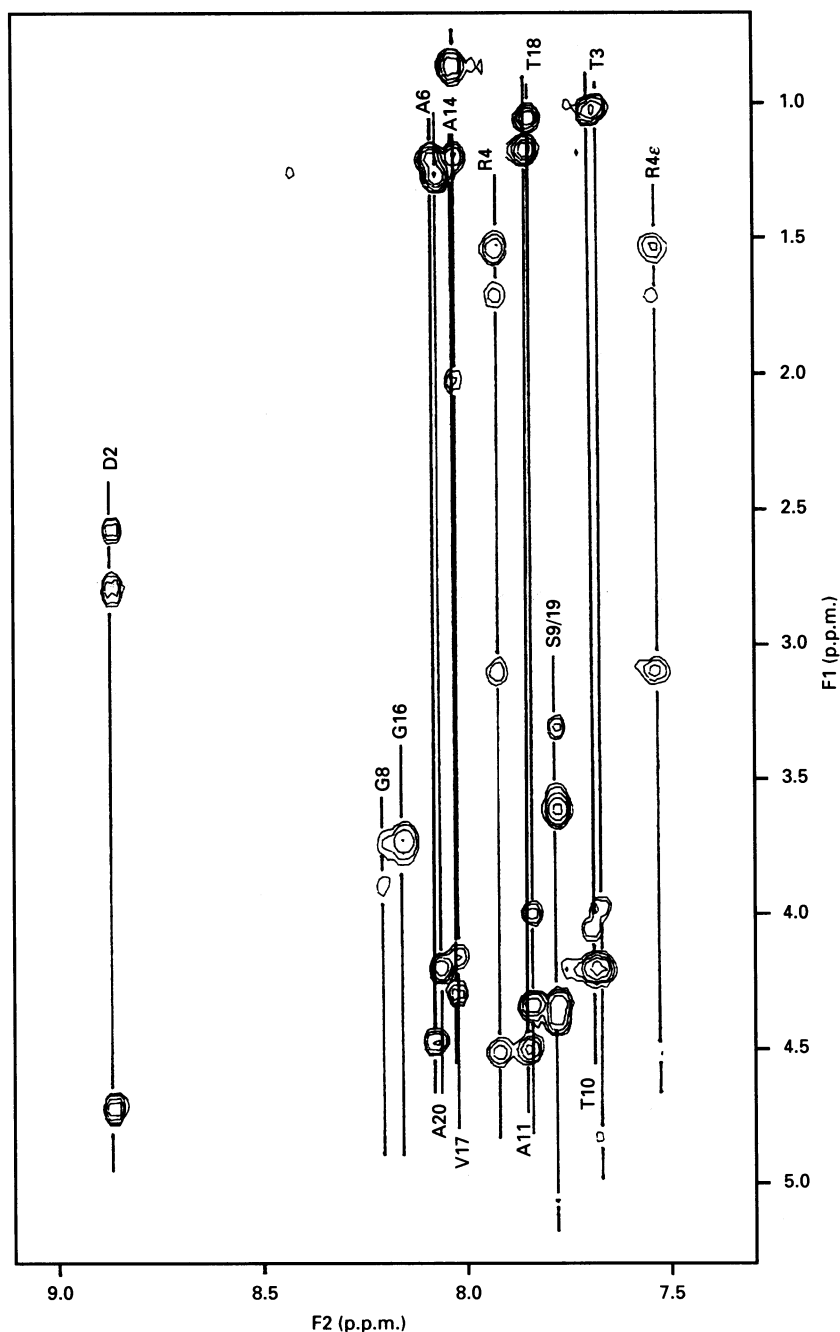
Fig. 2. Amide to aliphatic region of the ¹H HOHAHA spectrum of peptide p(1–20)

The 400 MHz ¹H HOHAHA spectrum of peptide p(1–20) dissolved in [²H₆]dimethyl sulphoxide was recorded at 303 K. The capital letters are the amino acids given in the one-letter notation, and the number refers to the position of the residue in the sequence.

by Wüthrich (1986). The aliphatic to amide region of a HOHAHA experiment is displayed in Fig. 2. As with peptide p(1–11) (Tendler, 1990), few through-space connections were observed in the nuclear Overhauser effect spectroscopy (NOESY) experiment due to the tumbling rate of the peptide (results not shown). The rotating Overhauser effect spectroscopy (ROESY) experiment was therefore utilized to investigate the through-space connectivity of the peptide. The amide region of this experiment is displayed in Fig. 3. A summary of the short-range dipolar interactions is given in Table 3.

In order to investigate whether elements of secondary structure are present in peptide p(1–20), the temperature-dependences of the chemical shift of the amide proton resonances have been

investigated; the results are presented in Table 4. If the amide proton is freely accessible to the solvent, a temperature coefficient of approx. 0.006 p.p.m. upfield/degree of temperature increase would be expected. In the case of an amide proton that is hydrogen-bonded, this exchange phenomenon will be hindered, resulting in a shift of the resonance at a rate of approx. 0.003 p.p.m./K. The Asp-2 amide proton has the lowest rate of movement at 0.0026 p.p.m./K. This result combined with the low-field nature of this resonance suggests that the Asp-2 amide proton may be involved in a hydrogen bond. The results similarly suggest that the amide protons of Arg-4, Ser-9, His-15 and Ser-19 with rates of movement below 0.0048 p.p.m./K are not directly accessible to the solvent.
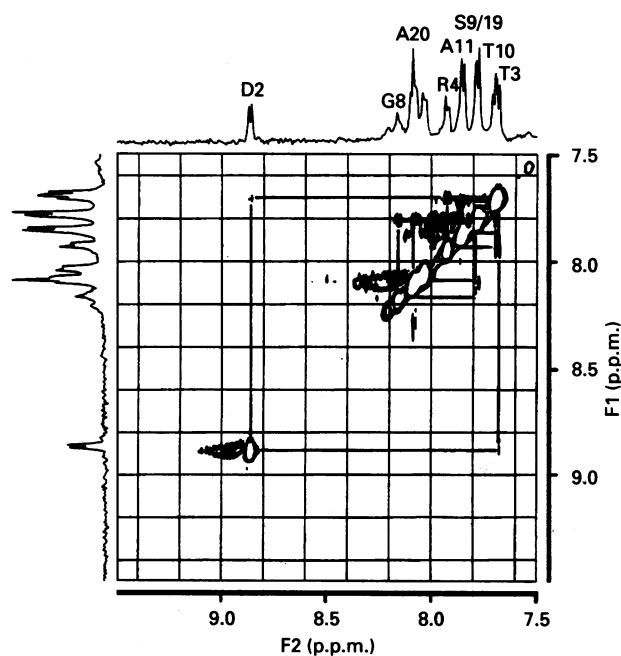
Fig. 3. Amide-to-amide region of the ^{1}H ROESY spectrum of peptide p(1–20)

The 500 MHz ^{1}H ROESY spectrum of peptide p(1–20) dissolved in [^{2}H$_6$]dimethyl sulphoxide was recorded at 303 K. Projections of the amide region of the one-dimensional spectrum are shown along both axes. The capital letters are as defined in Fig. 2.

Table 3. Summary of dipolar connections observed in peptide p(1–20)

The lines below the sequence represent the sequential $C\alpha H_i$–NH_{i+1} ($d_{\alpha N+1}$), NH_i–NH_{i+1} (d_{NN+1}) and $C\beta H_i$–NH_{i+1} ($d_{\beta N+1}$) nuclear Overhauser effects observed. The one-letter notation for amino acids is used.

Peptide	Sequence
p(1–20)	P D T R $\overset{5}{P}$ A P G S $\overset{10}{T}$ A P P A $\overset{15}{H}$ G V T S $\overset{20}{A}$
$d_{\alpha N+1}$*	
d_{NN+1}	
$d_{\beta N+1}$*	

* In the case of proline residues, connections are observed to the δ-protons.

Further information about the structure of peptides in solution may be obtained from through-space connections observed between protons in the ROESY experiment. This information directly provides distance restraints that may be imposed on the protons involved. The pattern of connectivity between the amide protons of Asp[2]-Thr[3]-Arg[4] is consistent with the presence of a type-I β-turn being present in peptide p(1–20) (Wüthrich et al., 1984). This turn overlaps the epitopes for the type-I and type-II monoclonal antibodies. These findings are in agreement with the data obtained for the p(1–11) fragment (Tendler, 1990). The pattern of connectivity between Gly[8]-Ser[9] and Thr[10]-Ala[11] may also indicate structure in this region: secondary-structure calculations have previously predicted a turn in the region Gly[8]-Ser[9]-Thr[10] (Price et al., 1990a). The observation of through-space connections between the CβH protons of Ala-6 and the CδH protons of Pro-7 and between the CβH protons of Ala-11 and the CδH protons of Pro-12 provide further structural restraints

Table 4. Rate of upfield movement of the amide proton resonances with temperature

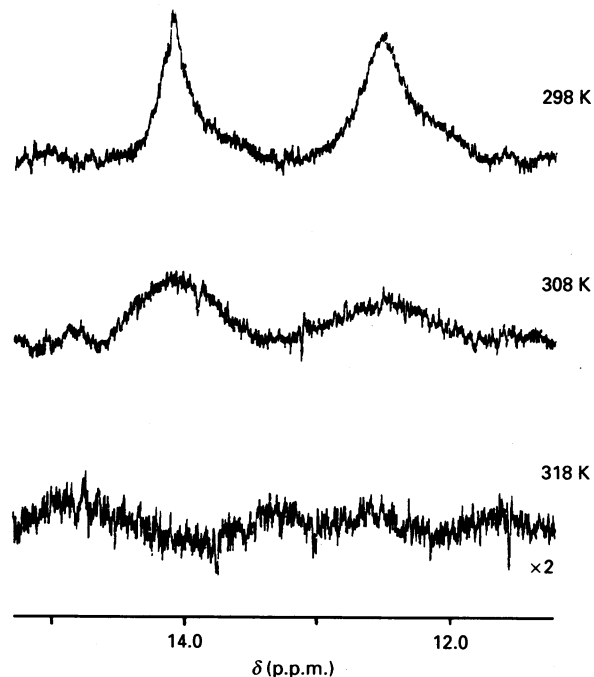| Residue	Rate of movement (p.p.m./K)
Asp[2]	0.0026
Thr[3]	0.0050
Arg[4]	0.0036
Ala[6]	0.0060
Gly[8]	0.0052
Ser[9]	0.0046
Thr[10]	0.0050
Ala[11]	0.0062
Ala[14]	0.0062
His[15]	0.0032
Gly[16]	0.0050
Val[17]	0.0060
Thr[18]	0.0060
Ser[19]	0.0042
Ala[20]	0.0062

Fig. 4. Temperature-dependence of the salt-bridge proton resonances of peptide p(1–20) dissolved in [^{2}H$_6$]dimethyl sulphoxide

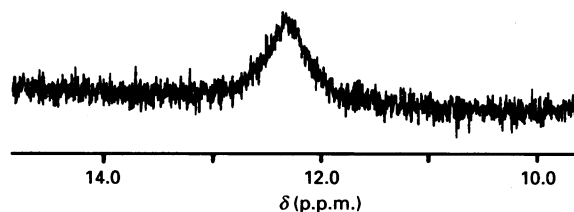Fig. 5. The salt-bridge proton resonance of peptide p(1–11) dissolved in [^{2}H$_6$]dimethyl sulphoxide

for peptide p(1–20); however, these constraints are not sufficient to define these regions of the peptide. The presence of the proposed secondary structure is supported by the large number

Table 5. Lineshape analysis of the temperature-dependence of exchange of the salt-bridge proton with the solvent

Peptide	Chemical shift (313 K) (p.p.m.)	Exchange rate (313 K) (s⁻¹)	Activation energy (kJ/mol)
p(1–11)	12.46	154	55.4
p(1–20)	12.17	137	60.5
	13.94	161	61.5

of βH_i–amide$_{i+1}$ proton through-space connections that are observed (Table 3).

In order to probe the stabilization of the type-I β-turn identified in the region of Asp²-Thr³-Arg⁴, two very-low-field resonances present in the spectrum of peptide p(1–20) at 12.42 and 14.03 p.p.m. (Fig. 4) have been investigated. The assignment of these resonances has been undertaken by preparing peptide p(1–20) where Arg-4 has been blocked with HPG. In the HPG-treated peptide, the low-field resonances and the broad guanidino-NH₂ resonances of Arg-4 are not observed at the same chemical shift (results not shown). In addition, the amide proton of Asp-2 ceases to resonate at the low field position. In the low-field region of the spectrum of the p(1–11) peptide, a single resonance at 12.46 p.p.m., as shown in Fig. 5, was observed.

The peptide p(1–20) low-field signals have temperature-dependence characteristic of the resonances, being dominated by the natural linewidth at low temperatures and exchange contributions at elevated temperatures (Bevan *et al.*, 1985). Line-shape analysis of this temperature-dependence of exchange has been undertaken according to the method of Bevan *et al.* (1985) in order to determine the dynamics of the exchange process and the corresponding energies of activation. The exchange rates and activation energies (Table 5) of each of the low-field resonances in peptide p(1–20) and the single low-field resonance in peptide p(1–11) are consistent with the presence of a proton involved in an ionic-type interaction. On the basis of these observations and the results with the HPG-blocked peptide p(1–20), it is postulated that this proton is involved in a salt-bridge-type interaction between the carboxylate side chain of Asp-2 and the guanidinium group of Arg-4. The fact that two resonances are observed for the proton in peptide p(1–20) suggest that the salt-bridge can exist in two conformations, each with similar populations and dynamics. In the case of peptide p(1–11), only one conformation of the salt-bridge is observed.

Computational chemistry

The simulated annealing algorithm identified three conformers

that were located within 29.3 kJ/mol of the lowest energy minimum found. The structures of these conformers are shown in Fig. 6, and energies, backbone dihedral angles and selected interatomic distances are listed in Table 6. The lowest-energy structure is in a type-I β-turn about the residues Pro¹-Asp-Thr-Arg, as indicated by the pro¹ O–Arg⁴ amide hydrogen bond and the aspartate and threonine dihedral angles, which are within $\pm 30°$ of the idealized type-I β-turn angles. Conformers 2 and 3 have energies 22.2 and 29.3 kJ/mol respectively above conformer 1 and would not therefore be significantly populated at room temperature. In conformer 2 the hydrogen bond stabilizing the β-turn has been broken and the backbone dihedral angles do not fit into any of the known turn categories. In the third conformation, the β-turn is again present. A strong ionic interaction is present in all three structures between the carboxylate group of aspartate and the guanidinium group of arginine, with the contact distance closest in conformation 2.

A full list of the hydrogen bonds exhibited by the three conformations is presented in Table 7. As expected, the two oxygens of the aspartate carboxylate group are involved in the majority of the hydrogen bonds formed, although the pattern of bonding varies between the three conformers with bonds being observed with the amide protons of aspartate and threonine in conformer 1, threonine and arginine in conformer 2, and threonine in conformer 3. The threonine hydroxy group forms hydrogen bonds to one or both of the carboxylate oxygens in all three structures.
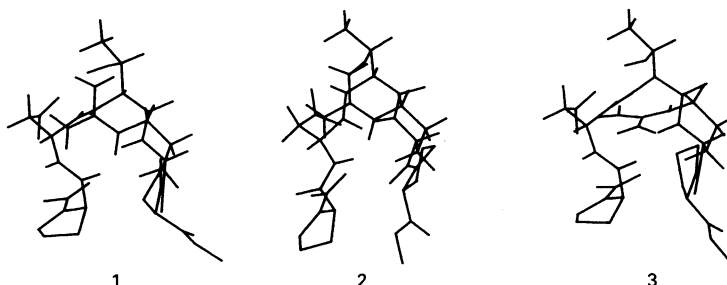
Table 6. Low-energy conformers of p(1–5) identified by simulated annealing

Conformer...	1	2	3	Ideal type-I β-turn
Energy*	−906.4	−884.2	−877.1	
Torsions†				
Asp² phi	−43.7	−160.4	−42.8	−60.0
Asp² psi	−53.6	−105.1	−62.1	−30.0
Thr³ phi	−118.4	−54.0	−104.8	−90.0
Thr³ psi	18.5	−18.0	13.4	0.0
Distances‡				
Pro¹ Cα–Arg⁴ Cα	0.418	0.492	0.438	
Pro¹ O–Arg⁴ NH (amide)	0.197	0.490	0.192	
Asp² γC–Arg⁴ ζC	0.330	0.291	0.344	

* COSMIC(90) force field energy (kJ/mol).
† Backbone dihedral angles (degrees).
‡ Distances between specified atoms (nm).

1　　　　**2**　　　　**3**

Fig. 6. Low-energy conformers 1–3 of peptide p(1–5) identified by simulated annealing

The backbone and side chains of the peptides (as defined in Table 6) are shown.

Table 7. Hydrogen bonds observed in low-energy structures

The criteria for selection use a cut-off of 0.25 nm for the H..X distance and 120° for the Y-H..X angle.

Acceptor	Donor	Conformer 1		Conformer 2		Conformer 3	
		Distance (nm)	Angle (°)	Distance (nm)	Angle (°)	Distance (nm)	Angle (°)
Pro O	Arg NH	1.97	145			0.192	150
Asp Oδ1/2	Asp NH	0.190	121*				
	Thr NH	0.193	128†	0.209	151†	0.182	143†
	Arg NH			0.183	161*		
	Thr OH	0.180	148†	0.185	157†	0.181	152†
				0.230	140*		
	Arg NH₂‡					0.187	147*
	Arg NH₂§					0.187	149*

* Hydrogen-bonded to Asp Oδ2 oxygen.
† Hydrogen-bonded to Asp Oδ1 oxygen.
‡ Hydrogen-bonded to Arg guanidinyl NH₂ H1.
§ Hydrogen-bonded to Arg guanidinyl NH₂ H2.

DISCUSSION

The antibody-binding studies presented in this paper show that in an aqueous environment the PEM-related peptides and PEM protein compete for the same binding site on the monoclonal antibody C595. The role of the solvent, however, is unclear in these binding experiments: fluorescent binding studies in our laboratories have indicated that the binding sites of both type-I (HMFG-2) and type-II (C595) anti-PEM monoclonal antibodies contain many hydrophobic tryptophan residues (M. J. Scanlon, M. R. Price and S. J. B. Tendler, unpublished results). Hence, the antigenic peptides when docking into the binding site of the antibody enter a hydrophobic environment, which may in effect resemble that found in dimethyl sulphoxide.

It is now generally accepted that conformationally unrestricted peptides exist in a number of interconverting structures in solution: the n.m.r. time-scale is such that only an averaged weighted conformation is observed (Dyson et al., 1990). It is clear from this study that peptide p(1–20) exists in a number of conformations: two conformations are directly observed for the salt-bridge proton, and the amide proton of Ala-6 resonates at two distinct positions. Although a number of through-space connections are observed in the ROESY experiment, there is no guarantee that these connections are present in the same conformers. However, the pattern of connections between Asp²-Thr³-Arg⁴ strongly suggests that a type-I β-turn is present in this region (Wüthrich et al., 1984); a similar element of structure has been identified in peptide p(1–11) (Tendler, 1990). This structural motif explains the relatively low rate of exchange of the Arg-4 amide proton, although it provides little explanation for the rate of exchange of the Asp-2 amide proton. In addition, further elements of secondary structure have been identified in the region of Gly⁸-Ser⁹ and Thr¹⁰-Ala¹¹. The observation of a through-space connection in the region of Ser¹⁹-Ala²⁰ combined with the low rate of exchange of the Ser-19 amide proton may suggest that additional structure is present in this region.

The observation of the two very-low-field resonances at 12.42 and 14.03 p.p.m. and their subsequent assignment to a salt-bridge proton between Asp-2 and Arg-4 has allowed us to postulate that the type I β-turn is stabilized by this ionic interaction. In peptide p(1–20), the observation of the two resonance positions for this proton suggests that two conformations of the salt-bridge are present, with lineshape analysis identifying that these are equally populated with similar dynamic

properties. In the case of peptide p(1–11), the salt-bridge proton resonates at only one very-low-field position; however, lineshape analysis reveals that this resonance has similar dynamic properties to those observed for the two sites in peptide p(1–20). This suggests that in peptide p(1–11) a similar salt-bridge is present in a single conformation.

In order to investigate the conformation of the region Pro¹-Asp-Thr-Arg-Pro⁵, a novel simulated annealing algorithm has been employed. The results of this study have identified that three low-energy conformations exist, with conformation 1 being > 99 % populated at room temperature. In this conformation the backbone of the peptide forms a type-I β-turn about the residues Asp²-Thr-Arg that is produced by a hydrogen bond between the carbonyl of Pro-1 and the amide proton of the Arg-4. Further stabilization of this turn is provided by a salt-bridge interaction between the carboxylate of the aspartate and the guanidinium group of the arginine, with additional hydrogen bonds being observed between the carboxylate and the amide proton of the aspartate and the hydroxy group of the threonine. The structure of this conformer fits extremely well with the solution n.m.r. data observed with peptide p(1–20), with the identified type-I -β-turn between Asp²-Thr-Arg and the ionic interaction between the aspartate and the arginine residues. The particularly low-field nature and low exchange rate of Asp-2 may be explained by the modelled hydrogen bond between the Asp-2 carboxylate and its amide proton.

Epitope mapping and replacement-net experiments (Price et al., 1990a; Briggs et al., 1991) utilizing tethered synthetic peptides have allowed us previously to identify that the epitope for the PEM type-I monoclonal antibodies includes the region Asp²-Thr-Arg⁴ and that the epitope for the type-II monoclonal antibodies is Arg⁴-Pro-Ala⁶ (Price et al., 1990a). For the antibody to recognize the peptide, presentation of the epitope is required. We and others have previously identified that structural motifs such as β-turns may be involved in the antibody recognition of antigens (Williamson et al., 1986; Tendler, 1990; Dyson et al., 1990). These regions of structure, however, will be masked by the amino acid side chains that will be in a set orientation. The identified folding in dimethyl sulphoxide of the salt-bridge-locked Asp-Thr-Arg region of the peptide produces a very compact near-globular unit, as shown in the CPK representation of conformer 1 in Fig. 7. This region of structure directly overlaps the identified epitopes for both the type-I and type-II anti-PEM monoclonal antibodies.
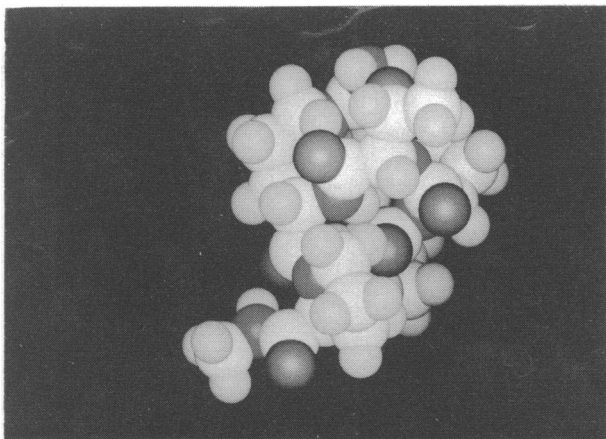
Fig. 7. CPK (Corey–Pauling–Koltun) model of conformer 1 displaying the globular nature of the salt-bridge-locked type-I β-turn

These results suggest that the salt-bridge-locked type-I β-turn may be present on the surface of the intact PEM and that this structural motif has a role in the immune recognition of PEM by type-I and -II anti-PEM monoclonal antibodies.

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