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NMR structural analysis of a peptide mimic of the bridging sheet of HIV-1 gp120 in methanol and water

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gp120 is a subunit of the Env (viral envelope protein) of HIV-1. The protein consists of inner and outer domains linked by a bridging sheet. Several gp120 residues that bind the neutralizing antibody 17b as well as the cellular co-receptor CCR5 (CC chemokine receptor 5), are located in the bridging sheet. Peptides that mimic the 17b-binding regions of gp120 would be useful potential immunogens for the generation of neutralizing antibodies against HIV-1. Towards this end, a 26-residue, four-stranded β-sheet peptide was designed on the basis of the structure of the bridging sheet, and its structure was characterized in methanol by NMR. In methanol, amide and α-proton resonances were well resolved and dispersed. A number of interstrand NOEs (nuclear Overhauser effects) were observed, providing good evidence for multiple turn β-hairpin structure. NOEs also provided good evidence for all Xxx–D-Pro bonds in the *trans* configuration and all three turns formed by a two residue D-Pro–Gly segment to be of type II′ turn. The structure conforms well to the designed four-stranded β-sheet structure. Approx. 20% of the peptide was estimated to adopt a folded conformation in water, as evidenced by CD spectroscopy. This was consistent with smaller, but still significant, downfield shifts of $C^{\alpha}H$ protons relative to random-coil values. A second peptide was designed with two disulphide bonds to further constrain the peptide backbone. While structured in methanol, this peptide, like the previous one, also exhibits only partial structure formation in water, as evidenced by CD spectroscopy.

Key words: CD4i, gp120, HIV, 17b, spectroscopy.

INTRODUCTION

A major lacuna in the search for an effective HIV vaccine is finding immunogens capable of eliciting neutralizing antibodies broadly cross-reactive against HIV-1. Most antibodies in HIV-1-infected individuals are directed against Env, the envelope surface glycoprotein of the virus. However, these antibodies are typically non-neutralizing [1,2]. Conformational flexibility masking of conserved epitopes and high mutability are important contributors to the lack of neutralizing response. The gp120 subunit of Env binds to the cellular receptor CD4 [3]. CD4 binding results in a conformational change that enables subsequent binding of gp120 to the co-receptors CCR5/CXCR4 (CC chemokine receptor 5/CX chemokine receptor 4). This conformational change results in the exposure of previously buried (cryptic) epitopes known as CD4i epitopes [4,5–7]. Recently, cross-linked complexes of gp120 with the first four extracellular domains of human CD4 (gp120:CD4) were used as immunogens in rhesus macaques (*Macaca mulatta*) [8]. The resulting antisera were shown to have broad neutralizing activity. A subsequent study [9] used single chains of gp120 fused to the first two extracellular domains of human CD4 (gp120:$CD4_{D12}$) as immunogens in guinea-pigs (*Cavia porcellus*). While the antisera did show broad neutralization, this was due to anti-CD4 antibodies. Although antibodies against CD4i epitopes were also produced, they did not contribute to neutralization. However, other studies have demonstrated that the sulphated antibodies 412d and E51, derived from HIV-1-infected individuals, were capable of enhanced neutralization of some primary isolates relative to the prototypical unsulphated antibodies 17b and 48d [10,11].

The structure of core gp120 [12] in a ternary complex with the first two extracellular domains of CD4 and the antibody 17b consists of inner and outer domains connected by a discontinuous bridging sheet. 17b binds to CD4i epitopes and the bridging sheet of gp120 forms a significant part of the CD4i epitope in 17b as well as other CD4i antibodies [10,11]. The bridging sheet was found to be absent in a recently determined structure of unliganded core gp120 [13] and thus appears to be formed only upon CD4 binding. Hence, in the present work, we attempted to design a peptide model of the bridging sheet. The motivation for the work is to design antigens that will immunofocus the antibody response to only the bridging sheet region. Such antigens will be useful tools with which to understand whether antibodies generated against CD4i epitopes can be broadly neutralizing. While there have been many successful attempts at designing β-hairpins [14–16], there have been only a few reports of the successful design of three-stranded β-sheets [17–20] and only one report of the *de novo* design of a peptide that forms a four-stranded β-sheet in aqueous solution [21]. By contrast, there have been several successful attempts to design α-helical peptides [22,23]. There have been extensive studies on the design of β-sheet and β-hairpin peptides based on the D-Pro–Gly-turn nucleating sequence. A key element in the design of β-hairpins is the nucleation of tight reverse turns of appropriate stereochemistry. The hairpins mostly accommodate type I′, type II′ and type I turns [24]. The torsion angles restraints of these turns

Abbreviations used: BS1PEP and BS2PEP, chemically synthesized bridging-sheet peptide mimics; CCR5, CC chemokine receptor 5; CXCR4, CX chemokine receptor 4; DTT, dithiothreitol; Env, HIV-1 viral envelope glycoprotein; GdmCl, guanidinium chloride; HSQC, heteronuclear single-quantum coherence; MALDI-MS, matrix-assisted laser-desorption–ionization MS; MBP, maltose-binding protein; MRE, mean residue ellipticity; Ni-NTA, Ni^{2+}-nitrilotriacetate; NOE, nuclear Overhauser effect; ROESY, rotating-frame Overhauser enhancement spectroscopy.

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[Ramachandran angles (Φ,ψ)] at the $i+1$ and $i+2$ residue for these turn types are: type I′ +55°, +40° and +80°, 10; type II′ +60°, −120°, and +90°, 0°; type I −65°, −30° and −90°, −10° (the accepted variation in angle being ±30° [25]) [26]. Both type I′ and type II′ turns require that Φ be positive for the $i+1$ residue. Positive Φ values are rarely adopted by L-amino acids. Such turns have a local right-handed twist that is compatible with the right-handed twist of strands in a β-sheet [27]. In designed synthetic peptides this is achieved by incorporating a D-proline residue in which the constraint of pyrrolidine ring formation restricts $\Phi_{D\text{-}Pro}$ to $\approx +60 \pm 20°$ [28]. L-Asparagine has also been used at the same position, on account of its high propensity to adopt positive Φ values in naturally occurring proteins. Comparative studies establish that D-Pro-Gly segments are superior to Asn-Gly segments in hairpin nucleation [29]. Early attempts towards the design of β-sheets had focused on the formation of tight turns of the right stereochemistry, but had faced the problem of limited solubility and aggregation [30]. In the present study we have used multiple D-Pro-Gly turn sequences placed appropriately within the gp120 bridging-sheet sequence to generate four-stranded β-sheet peptides that are 26 residues long and have analysed their solution conformations in methanol and water.

MATERIALS AND METHODS

Peptide design and purification

Recombinant bridging-sheet mimics that were based on a previous report [31] were constructed as fusions with MBP (maltose-binding protein). The two regions of gp120 that comprise the 17b binding epitope, corresponding to residues 419–435 and 119–205, were separately amplified by PCR using overhangs that had overlapping sequence with each other and one (βpep1) or two residue (βpep2) glycine linkers between the fragments. The PCR-generated fragments were mixed and subjected to overlap PCR. These were then cloned in the vector pMALp2 (as C-terminal fusions with MBP). To generate a construct with an additional helix, a part of the native helix of gp120 spanning the region 109–118 was used, along with the bridging-sheet region to generate the construct. The final construct, βpep3, comprised residues 109–209, 380–385 and 418–439 of gp120 linked sequentially to each other with single glycine linkers. This was cloned in the vector pMMHa [32] C-terminal to the hydrophobic TrpE leader sequence. DNA sequences of all the constructs were confirmed by DNA sequencing. Expression and purification of MBP fusions of βpep1 and βpep2 were performed using amylose affinity chromatography. The TrpE leader sequence has the property of directing fusion partners to the insoluble inclusion-body fraction and was purified by using published protocols for purifying TrpE fusion peptides [33]. Briefly, the TrpE fusion peptides were expressed in *Escherichia coli* and re-solubilized from inclusion bodies in 6 M GdmCl (guanidinium chloride). The TrpE fusion peptides were then purified using an Ni-NTA (Ni^{2+}-nitrilotriacetate) column under denaturing conditions. The purified protein was dialysed against 10 % (v/v) acetic acid and was digested then with CNBr in 1 % formic acid solution. The presence of a single methionine linker in between the TrpE leader sequence and the peptide assists in obtaining pure peptide after CNBr cleavage. The TrpE leader peptide was removed by dialysing the protein solution against PBS, pH 7.4, which resulted in the exclusive precipitation of TrpE peptide. Peptides containing D-Pro were commercially synthesized and purified further by reverse-phase HPLC on a 10 mm × 250 mm C18 column [Vydac; 5 μm particle size; 30 nm (300 Å) pore size]. Peptides were eluted with an acetonitrile/water gradient mobile phase containing 0.1 % trifluoroacetic acid. MALDI-MS (matrix-assisted laser-desorption–ionization MS) was used to confirm the peptide identities.

Far-UV CD spectroscopy

CD spectra were recorded on a Jasco (Eaton, MD, U.S.A.) J-715 C spectropolarimeter flushed with N_2. The spectra were recorded using a 0.1-cm-path-length cuvette with a scan rate of 10 nm/min and a time constant of 8 s. All the data respresent an average over a minimum of six scans and are presented in terms of MRE (mean residue ellipticity) as a function of wavelength [9]. Far-UV CD spectra were taken for all the peptides in water, methanol and water/methanol mixtures over the range of 250–200 nm. CD spectra were also recorded for the peptides at different pH values and in different osmolytes (betaine, sarcocine and trimethylamine *N*-oxide). The CD spectra for the fully unfolded peptides were recorded in 4 M GdmCl/5 mM phosphate buffer, pH 7.4, in the wavelength range 250–210 nm.

Calculation of population of folded peptides in aqueous solution

The calculation of the population of folded peptides was based on the assumption that the entire population of the peptide has a four-stranded β-sheet structure in 100 % methanol. This was assumed on the basis of the NMR data in methanol and from the magnitude of downfield shift of the $C^{\alpha}H$ chemical shifts in methanol (see below). The unfolded reference state was taken to be the CD spectra for the peptides in 4 M GdmCl. The population of folded molecules was calculated according to the following equation:

$$\text{Percentage of the population of the peptide in the folded state in aqueous solution} = \frac{([\theta]_{220,\text{Water}} - [\theta]_{220,\text{GdmCl}}) \times 100}{[\theta]_{220,\text{Methanol}} - [\theta]_{220,\text{GdmCl}}}$$

where $[\theta]_{220,\text{Water}}$ is the MRE of the peptide at 220 nm in water, $[\theta]_{220,\text{GdmCl}}$ is the MRE of the peptide at 220 nm in 4 M GdmCl and $[\theta]_{220,\text{Methanol}}$ is the MRE of the peptide at 220 nm in 100 % methanol. The 'folded state' refers to the four-stranded β-sheet structure found in 100 % methanol.

Re-oxidation of chemically synthesized bridging-sheet peptide mimic BS2PEP in aqueous solution

Purified oxidized BS2PEP was subjected to reduction by DTT (dithiothreitol). Peptide solution (1 mM in 2 mM Tris/HCl, pH 7.3) was boiled with 4 mM DTT for 10 min. To check for completeness of reduction, iodoacetamide was added to a small aliquot of the solution at a final concentration of 8 mM. The aliquot with iodoacetamide was incubated at room temperature in the dark for 30 min and the protein was desalted using a C18 ZipTip® pipette tip (Millipore), and complete reduction was confirmed by MALDI-MS. For reoxidation, the peptide reduced with DTT was dialysed extensively against water. The re-oxidation was confirmed by MALDI-MS.

Competition ELISAs of the peptides with 17b

These were perfomed in 96-well plates to which D7324 (a sheep antibody against the C-terminal 15 amino acids of gp120; 1 μg/ml; Cliniqua Corporation, Fallbrook, CA, U.S.A.) had been adsorbed after overnight incubation at 4 °C in capture buffer (100 mM $NaHCO_3$, pH 9.5). Plates were washed three times with PBS/0.5 % Tween-20 (PBST) and blocked with 200 μl of 5 % (w/v) non-fat milk in PBST. After a wash, 70 μl of native single-chain gp120–$CD4_{D12}$ (100 ng/ml) [9] was captured over the plate by incubating for 2 h. To this, 17b (5 μg/ml) preincubated with

different concentrations of the bridging-sheet mimics was added to the wells and incubated for 2 h at room temperature. Bound 17b was detected using a peroxidase-conjugated goat anti-human antibody (Sigma) at a dilution of 1:5000 and the chromogenic substrate *o*-phenylenediamine dihydrochloride.

NMR spectroscopy

NMR samples were prepared by dissolving the peptide in 100 % [*methyl*-^{2}H]methanol ('$C^2H_3O^1H$'), after which spectra were recorded. To improve resolution in the Cα region, spectra were also recorded in 100 % [^{2}H]methanol ('$C^2H_3O^2H$'). For studies in water, the NMR samples were prepared by dissolving peptide in $^1H_2O/^2H_2O$ (9:1, v/v). To improve resolution in the Cα region, spectra were also recorded in 100 % ^2H_2O. The concentration of peptides used was approx. 2 mM in all cases. For ^{1}H–^{15}N HSQC (heteronuclear single-quantum coherence) spectra as a function of methanol concentration, equimolar solutions of peptide in water and methanol were mixed in different amounts. The spectra were recorded at natural abundance of ^{15}N. One- and two-dimensional ^{1}H-NMR spectra were recorded on a 500 MHz Bruker spectrometer. For methanol-containing solutions, the residual methyl resonance in [*methyl*-^{2}H]methanol was used as an internal standard for chemical-shift referencing [34]. In all experiments involving two-dimensional NMR, spectra were measured in the phase-sensitive mode using time-proportional phase incrementation. TOCSY [35] and ROESY (rotating-frame Overhauser enhancement spectroscopy) [36] spectra were measured with standard pulse sequences. Usually, spectra were recorded with 2K points in the directly detected (F2) dimension and 450 points in the F1 dimension. A mixing time of 100–150 ms was used for TOCSY and 200–250 ms for ROESY experiments. Solvent suppression was achieved using presaturation and watergate sequences with standard pulse sequences available in the Bruker library. Data were processed on a Silicon Graphics Indy workstation using XWINNMR software and on a Pentium IV PC with MestRec3.7 (www.mestrec.com). Typically, a shifted (π/2) sine-squared window function was applied in both the dimensions. Data in F1 were zero-filled to obtain a final matrix of 2K $\times$ 2K data set.

RESULTS

Peptide design

The bridging sheet in intact gp120 is composed of four antiparallel discontinuous strands (strands 20, 21, 2 and 3) comprising regions 423–434 and 120–201 of gp120. In gp120, strands 20 and 21 are joined by a short hexapeptide of sequence MWQKVG, whereas strands 2 and 3 are connected by the long variable V1/V2 loop that is about 60–70 residues in length. Several different strategies were employed to design peptide analogues of the bridging sheet. The initial designs were based on a previous report claiming that a bridging-sheet peptide derived directly from the gp120 of the NLDH10 isolate of HIV-1 had the ability to bind conformational mAbs like IgGb12 (a CD4 binding site antibody) and 17b (a CD4i binding antibody) [31]. The peptide reported contained the 421–437 region of gp120 joined to the 118–207 region with a glycine linker in between. The construct contained the V1/V2 loop and was expressed as an MBP fusion. In the present study we expressed bridging-sheet peptides similar to the ones reported, but derived from JRFL gp120. JRFL is a CCR5 tropic primary isolate of HIV-1 that is relatively difficult to neutralize. We have previously used JRFL gp120 derivatives as immunogens in guinea-pigs [9]. In these constructs, the 419–435 region of gp120 was linked to the 119–205 region using one or two glycine residues respectively. The region 419–421 makes important contacts with 17b and hence was included in our design. Since residues 435–437 and 118 do not contribute to 17b binding, these were omitted from our design. From geometric criteria of turn formation it was also found that the best linkage retaining the native structure was achievable by linking residues 435 and 119. Peptides were expressed as fusions with MBP. The yields of the purified peptides were low (about 100 μg/litre of culture), and they were inactive when assayed for 17b binding. Another construct (βpep3) that contained an additional helix to pack behind the β-sheet was also generated and expressed. This peptide was expressed as a fusion with TrpE leader sequence and then purified by Ni-NTA affinity chromatography. The TrpE leader was removed from the peptide by CNBr cleavage at the single methionine residue present between the TrpE leader and βpep3. The yield of the purified peptide was about 150 μg/litre of culture. MALDI-MS showed that the peptide did not undergo any chemical modification upon CNBr treatment and had the desired mass. This peptide also did not exhibit 17b binding. The CD spectrum of this peptide showed that it is unstructured in water and is largely helical in methanol (results not shown). Hence further studies with these peptides were not pursued. Since our attempts to express and purify these peptides in bacteria resulted in low yields of inactive peptides, a different approach using chemically synthesized peptides was attempted.

The first of these peptides (henceforth termed 'BS1PEP') contains three D-Pro-Gly segments. The peptides were designed based on the bridging-sheet sequence of JRFL gp120 (Figure 1A). Only one face of the bridging sheet interacts with 17b, and the residues on this face of the sheet were left untouched. The V1/V2 loop is not present in the core gp120 structure and is not required for 17b binding. The V1/V2 loop is thought to shield CD4i epitopes [31] and was omitted from the designed peptides. The connectivities of the strands were altered to connect the adjacent strands (Figure 1B). The turns were formed from sequences of D-Pro-Gly. D-Pro-Gly is known to be a turn nucleator [37], whereas the flanking residues, namely lysine and serine, were added to ensure that all 17b binding residues remained on the same face of the sheet. Lysine and serine were chosen owing to their propensity to occur at such positions in naturally occuring proteins (as analysed from protein structures available in the PDB database [25]). The residues of gp120 interacting with 17b were extracted from the co-ordinates of the complex as described previously [38] using a program kindly provided by Dr C. Ramakrishnan (Molecular Biophyscis Unit, Indian Institute of Science, Bangalore-12, India). The residues of gp120 that show more than 10 % decrease in their accessible surface area upon complex formation are taken to be interacting residues. Although the residues on the 17b binding face were retained, the other face of the sheet was modified to introduce residues that are β-branched and have high β-sheet propensity. Hydrophobic residues were generally not used, in order to avoid aggregation problems. The residues of the sheet that do not interact with 17b, but interact with the rest of gp120 and are hydrophobic, were also determined and were mutated to polar residues to prevent aggregation of the peptide. We ensured the edge strands contained at least one positively charged lysine residue to prevent edge–edge aggregation. The sequence of the first peptide (BS1PEP) is shown in Figure 1(C). A second peptide (BS2PEP) was also designed with two interstrand disulphide groups introduced in peptide BS1PEP to further constrain the peptide to a β-hairpin conformation (Figure 1C). The disulphide groups were positioned at the non-hydrogen-bonded pairs of the β-sheet, as it was observed that most disulphide groups bridging adjacent antiparallel strands of a β-sheet in naturally occurring proteins are between the

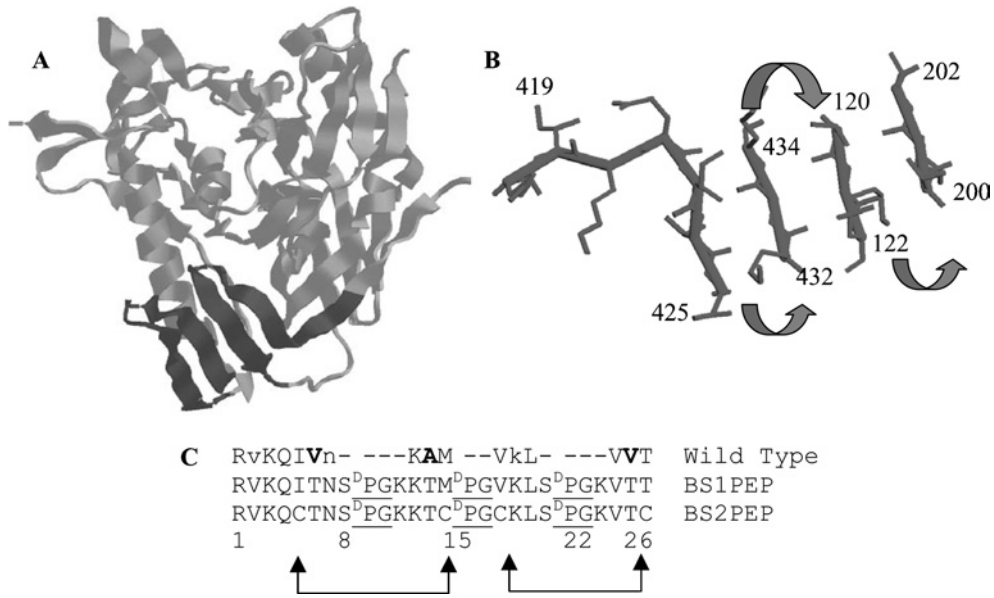

Figure 1 Bridging-sheet region of gp120 and peptide design

(**A**) The crystal structure of core gp120 [12] (PDB code: 1g9m) is shown with the bridging-sheet region coloured black. (**B**) The connectivity of the designed peptide is shown with corresponding numbering from intact gp120. The four strands were connected with D-Pro-Gly (^{D}PG) turn nucleating residues. (**C**) Sequences of wild-type bridging-sheet residues derived from gp120, BS1PEP and BS2PEP as obtained after reconnecting the strands of gp120 and introduction of appropriate residue substitutions. The residues in bold in wild-type could potentially cause aggregation of the peptide and hence were replaced by polar residues. The residues were chosen so that they had a high propensity for β-sheet formation and could solubilize the peptide. Excess positive charges were introduced at the edge strands to disfavour edge–edge aggregation. The residues that were important for binding to 17b were left unchanged and are represented in upper-case in the top (wild-type) sequence. Putative turn regions are underlined. The sequence of peptide BS2PEP has four residues of BS1PEP substituted with cystine to aid the formation of two interstrand disulphide bonds. The desired disulphide connectivity is shown schematically along with the sequence of BS2PEP.

non-hydrogen bonded pairs (K. Chakraborty and R.Varadarajan, unpublished work). To facilitate the formation of correct disulphide groups in BS2PEP, the peptide was air-oxidized in methanol. Methanol was chosen as the solvent for oxidation as BS1PEP was shown to have the desired four-stranded β-sheet structure in methanol (see below). The purity of all the peptides was greater than 95 % as obtained from MS and ^{1}H-NMR. Monoisotopic masses obtained from MALDI were 2751.2 Da for BS1PEP (expected 2751.6 Da), 2714 Da for oxidized BS2PEP (expected 2714.4 Da) and 2718.1 Da for reduced BS2PEP (expected 2718.4 Da). The oxidized form of BS2PEP (henceforth referred to as BS2PEP unless otherwise stated) clearly had the two disulphide groups formed, and there were no cross-linked dimers or other oligomers present. In an activity assay by competition ELISA, both the peptides were unable to compete out 17b binding to gp120–$CD4_{D12}$.

CD spectroscopy

Figure 2 shows the CD spectra of BS1PEP (Figure 2A) and BS2PEP (Figure 2B) in different concentrations of methanol in water. The CD spectra of the peptides in methanol do not conform to the classical spectra for a β-sheet, which should have a single negative band at 217 nm [39]. In methanol, BS1PEP shows a strong negative band at 220 nm, with a shoulder at 210 nm. However, NMR data (see below) suggest that the peptide does adopt the desired β-sheet conformation. BS2PEP in methanol shows a strong negative band at 210 nm. No α-helix was present in either peptide, as the NMR data do not show significant intensity for sequential N_iH-$N_{i+1}H$ NOEs (nuclear Overhauser effects), which are generally characteristic of helical structure. The presence of three D-Pro-Gly segments in the sequence also makes the formation of an α-helix unlikely. The CD spectra of the peptides in aqueous solution show that they are not well folded. However, the absence of positive ellipticity at 215 nm and the low amplitude of negative signal at 200 nm indicates that a fraction of the peptide retains significant secondary structure in aqueous solution [40]. To check whether any residual structure is present in water, spectra for the peptides were also recorded in 4 M GdmCl. The spectra show that, in both peptides, there is an isosbestic point in water, methanol and water/methanol mixtures. This suggests that there are two species in solution for both peptides. Assuming that the peptides form close to 100 % β-sheet in 100 % methanol (details are given below), approx. 20 % of the population of BS1PEP and 17 % of the population of BS2PEP was calculated to be structured in water. CD spectra of the peptides in different concentrations of methanol suggest that the transition of the peptides from random-coil to folded structure with increasing concentration of methanol is gradual and non-co-operative. The differences in shapes between the CD spectra for the two peptides are in part due to the spectral contribution of the two disulphide groups present in BS2PEP. The reduced form of BS2PEP shows a CD spectrum similar to that of BS1PEP in water, methanol and water/methanol mixtures (Figure 2C). Approx. 20 % of the population of the reduced BS2PEP also appeared to be structured in water.

NMR spectroscopy

BS1PEP yields well-resolved 500 MHz ^{1}H-NMR spectra at 300 K in [*methyl*-^{2}H]methanol. The sharp resonances are consistent with a major population of monomeric structure in methanol. In contrast, the 500 MHz ^{1}H-NMR spectra of BS2PEP was not well dispersed at 300 K, but was well dispersed at 313K, so further studies with BS2PEP were done at 313K unless otherwise mentioned. Linewidths for both peptides were not concentration-dependent over the range of 0.5–4 mM, indicating the absence of dimers and other higher oligomers in solution. The monomeric state of the peptides was also confirmed by gel-filtration

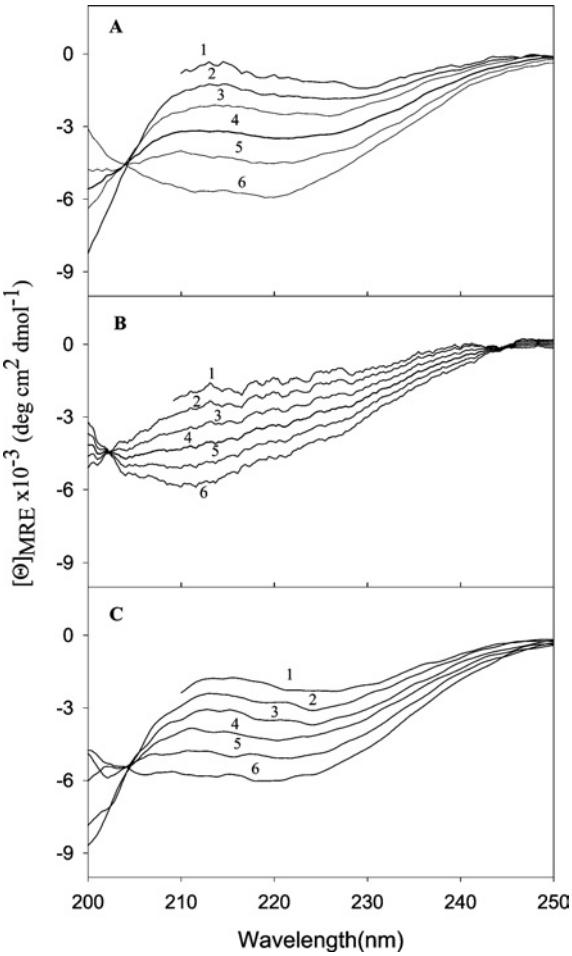

Figure 2 Far UV CD spectra of peptides BS1PEP and BS2PEP

Spectra were obtained with 30 μM peptide in 5 mM Hepes/5 mM glycine/5mM citrate, pH 7.4, and various concentrations of methanol. The peptides BS1PEP (**A**), BS2PEP (**B**) and BS2PEP in reduced form (**C**) show a gradual increase in secondary-structural content with increasing concentration of methanol [4 M GdmCl (trace 1), 0 % methanol (trace 2), 25 % (v/v) methanol (trace 3), 50 % methanol (trace 4), 75 % methanol (trace 5) and 100 % methanol (trace 6)]. The presence of isosbestic points in all cases suggest that there is a two-state folding transition with increasing methanol concentration. In the presence of 4 M GdmCl, data points below 210 nm were 'noisy' because of sample absorbance and were therefore deleted.

chromatography (Figure 3). Complete sequence-specific assignments of resonances was achieved using a combination of TOCSY experiments to identify the spin systems and ROESY experiments to identify the near-neighbour connectivities [41]. The information in Supplementary Table 1 (http://www.BiochemJ.org/bj/390/bj3900573add.htm) summarizes the chemical shifts of all the assigned protons in BS1PEP and BS2PEP. Large values of coupling constants ($^3J_{C\alpha H\text{-}NH} \geqslant 8.0$ Hz) are observed for several resonances which could be measured accurately from a resolution enhanced one-dimensional spectrum. These values are consistent with dihedral angles (φ) being in the extended sheet region of the Ramachandran map [42].

The C$^\alpha$H chemical shifts are known to be downfield-shifted with respect to random-coil values in the case of a β-sheet and upfield-shifted when the amino acids are in an α-helix [43,44]. The plot of the difference in chemical shifts of C$^\alpha$H for the peptides from random-coil values as shown in Figure 4 corroborate well with the desired β-sheet structure of the peptides. The random-coil C$^\alpha$H chemical shifts for the various amino acids in methanol were taken from [45]. These values were quite similar (difference less

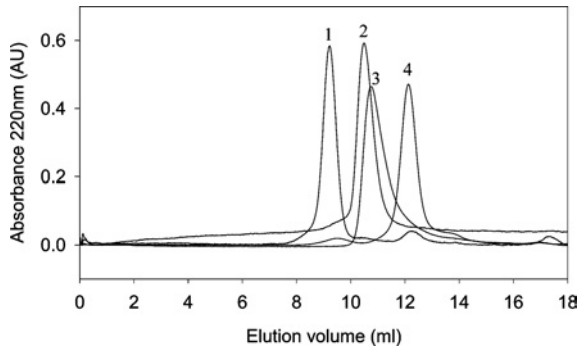

Figure 3 Gel-filtration chromatography to determine peptide oligomerization state using a Superdex peptide column

A 100 μg portion of each of the peptide was used for loading. Insulin (peak 1, $\approx$5.7 kDa) and a 1.6 kDa (peak 4) control peptide were run to calibrate the column. BS1PEP (peak 2) and BS2PEP (peak 3) were eluted in between the two peaks, indicating that both peptides were monomeric (expected monomeric mass of $\approx$2.7 kDa and dimeric mass of $\approx$5.4 kDa). Abbreviation used: AU, absorbance units.

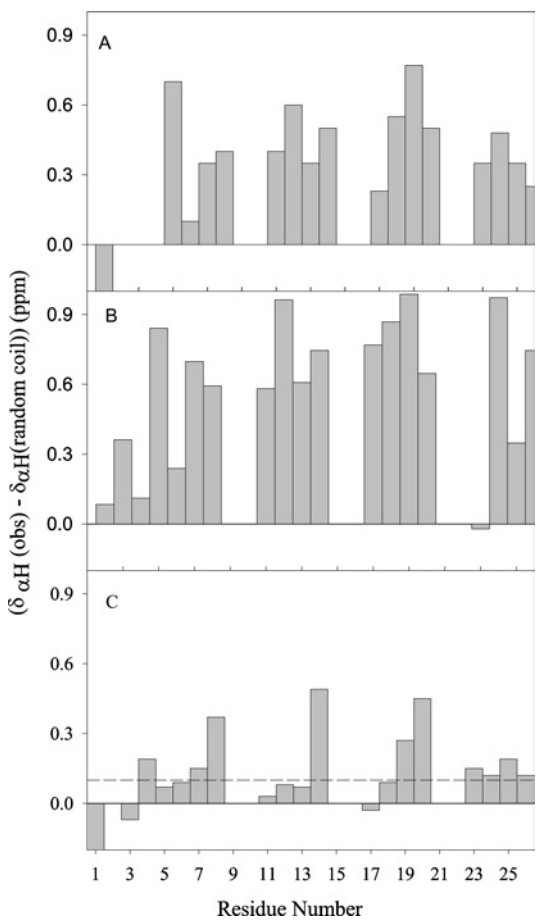

Figure 4 Change in chemical shift of C$^\alpha$H protons from random-coil values for the described peptides

C$^\alpha$H protons of residues designed to be in the extended strand region of the peptides exhibited significant downfield shifts in the case of both BS1PEP (**A**) and BS2PEP (**B**) in methanol. Smaller but significant downfield shifts were observed (obs) for BS1PEP in aqueous solution (**C**). The horizontal broken line indicates the cut-off value for significant downfield shifts of 0.1 p.p.m. ('ppm') (see the text for details). The random-coil chemical-shift values of C$^\alpha$H for the various residues in methanol and water were obtained from [45] and [44] respectively. Blank spaces indicate the positions of D-Pro-Gly turn sequences.

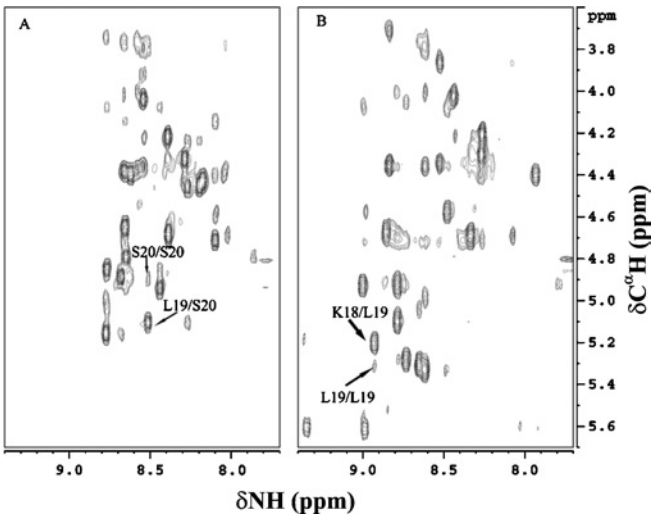

Figure 5 C$^{\alpha}$H-NH region of ROESY spectra for BS1PEP (A) and BS2PEP (B) in [*methyl*-^{2}H]methanol illustrating the observed $d_{\alpha N}$ (C$^{\alpha}$H$_i$ NH$_{i+1}$ and C$^{\alpha}$H$_i$ NH$_i$) interactions

Representative C$^{\alpha}$H$_i$ NH$_{i+1}$ and C$^{\alpha}$H$_i$ NH$_i$ cross-peaks are indicated to highlight the fact that the latter ones are weaker than the former, indicative of an extended strand structure.

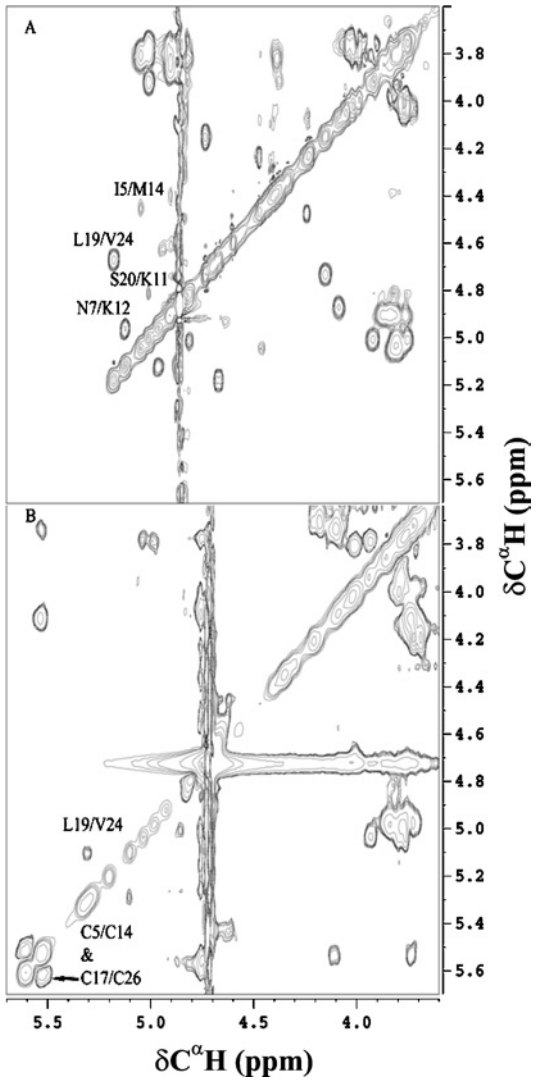

Figure 6 C$^{\alpha}$H-C$^{\alpha}$H region of the ROESY spectra for BS1PEP (A) and BS2PEP (B) in [^{2}H]methanol illustrating the $d_{\alpha\alpha}$ connectivities

Representative connectivities are shown as cross-peaks in the spectra that are labelled with the corresponding residue numbers.

than 0.1 p.p.m.) from previously published values of random-coil C$^{\alpha}$H chemical shifts in water [43,44]. The magnitudes of the downfield shifts as well as the $^{3}J_{C\alpha H\text{-}NH}$ coupling constants were significantly larger for BS2PEP than for BS1PEP. This indicates that the disulphide bonds have contributed positively to structure formation.

Figure 5 and Figure 6 show the ROESY spectra which illustrate the observed $d_{\alpha N}$ (C$^{\alpha}$H$_i$ NH$_{i+1}$ and C$^{\alpha}$H$_i$ NH$_i$) and $d_{\alpha\alpha}$ (C$^{\alpha}$H$_i$ C$^{\alpha}$H$_j$) connectivities respectively. In the case of a β-strand, intense $d_{\alpha N}$ cross-peaks are expected, whereas the intraresidue C$^{\alpha}$H$_i$ NH$_i$ cross-peaks should be weak [39]. Figure 5(A) for BS1PEP and Figure 5(B) for BS2PEP shows that this is indeed the case. Interstrand NOE peaks between C$^{\alpha}$H of residues that are distant in sequence are a clear indication of proper registry of strand segments. The expected registry of the designed antiparallel β-sheets are shown in Figure 7, along with the observed inter-strand NOEs. All the NOEs observed are consistent with the designed structure, and there were no NOEs consistent with any other strand registry. The observation of four distinct $d_{\alpha\alpha}$ connectivities between I5 (using the one-letter amino acid notation) and M14, N7 and K12, K11and S20 and L19 and V24 in case of BS1PEP (Figure 6A) further suggests that the peptide assumes the desired fold as shown in Figure 7(A). The $d_{\alpha\alpha}$ NOEs between residues T13 and K18 and between V17 and T26 were not observed. This was because these residue pairs had similar C$^{\alpha}$H chemical shifts and hence the cross-peaks overlapped with diagonal peaks. $d_{\alpha\alpha}$ connectivities between T13 and K18 and L19 and V24 were observed in case of BS2PEP (Figure 6B). Many of the expected connectivities were not observed due to the close proximity of the chemical shifts of the C$^{\alpha}$H protons in the respective pairs. In addition to $d_{\alpha\alpha}$, all d_{NN} connectivites characteristic of the three turn regions of the peptide were observed for both the peptides (Figure 8). Like the the $d_{\alpha\alpha}$ ones, the d_{NN} connectivities between residues that are far apart in sequence and characteristic of the inter-strand amino acid pairs in registry with respect to hydrogen bonding also provide evidence of the desired fold. Several such NOEs were observed in the ROESY spectra of BS1PEP (S8 and K11, M14 and V17, S20 and K23) (Figure 8A). Other d_{NN} connectivities (T6 and T13, K12 and L19, K18 and T25) could not be observed, owing to the accidental proximity of the NH$_i$ chemical shifts for the partners of each pair. In the ROESY spectra of BS2PEP (Figure 8B), several long-range d_{NN} connectivities (T6 and T13, S8 and K11, G16 and C17, K18 and T25, S20 and K23) were also observed. These, together with the $d_{\alpha\alpha}$ connectivities, downfield shifts for C$^{\alpha}$H and high coupling constant for C$^{\alpha}$H-NH, provide strong evidence for the desired strand alignment and structure (Figure 7) of both peptides in methanol. The data also justify the assumption that close to 100% of the peptide molecules form a folded four-stranded antiparallel β-sheet in 100% methanol. Long-range side-chain connectivities were not observed, most probably because of the dynamic behaviour of the side chains. Extensive structure calculations were not done, as the number of NMR restraints obtained from these experiments were not sufficient for structure calculation.

Conformation of the peptides in water

The 500 MHz ^{1}H NMR spectra of BS1PEP in water was reasonably well dispersed in the amide region of the spectra. Complete

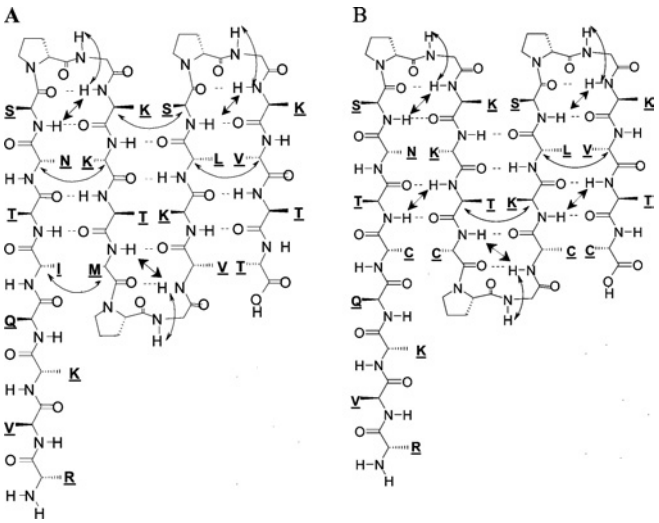

Figure 7 Designed strand alignment of the peptides along with the observed long-range NOEs

The NOEs are shown as double-headed arrows in the case of BS1PEP (**A**) and BS2PEP (**B**), with the straight double-headed arrows representing NH-NH NOEs and the curved double-headed arrows representing $C^{\alpha}H$-$C^{\alpha}H$ NOEs.

assignments of the different spin systems of BS1PEP were made with the aid of TOCSY and ROESY experiments. The 500 MHz ^{1}H-NMR spectra of BS2PEP in water was poorly dispersed in the amide region at all temperatures studied. Hence, for this peptide, no sequence specific assignments could be made and no further structural studies were pursued.

The $C^{\alpha}H$ chemical shifts for the residues of BS1PEP expected to be in the extended conformation are also slightly downfield-shifted with respect to the random-coil values. Values of the downfield shift greater than 0.1 p.p.m. are generally considered significant [44]. A recent report [46] indicated that chemical shifts in aqueous solution are influenced by solvent accessibility as well as by secondary structure, and that highly solvent accessible residues can have chemical shifts similar to random-coil values, despite being in regions of secondary structure. The turn regions are well formed in water, as the long-range d_{NN} connectivities for all the three-turn region were present in the ROESY spectra. Other inter-strand $d_{\alpha N}$ and $d_{\alpha\alpha}$ connectivities were not observed. This is indicative of disruption of the structure established in [*methyl*-^{2}H]methanol.

The ^{1}H–^{15}N HSQC spectra, at natural abundance of ^{15}N, of the peptide BS1PEP in four different concentrations of methanol in water were recorded to observe the structural transition from methanol to water (Figure 9). Sequence-specific assignments of all the cross-peaks were possible. The change in conformation of the peptide with increasing percentage of water was clearly evident from the shift of the cross-peaks. At each concentration of methanol, only a single peak was observed for each amide, indicating that the two conformations detected by CD are rapidly interconverting on the NMR timescale. The HSQC spectra of BS2PEP could not be taken in different concentrations of methanol because the NH region of the spectra was not well dispersed, even in 25 % water.

NMR and CD spectroscopy of BS2PEP reoxidized in aqueous solution

BS2PEP that had been oxidized and structurally characterized in methanol was reduced and reoxidized in aqueous solution. The re-

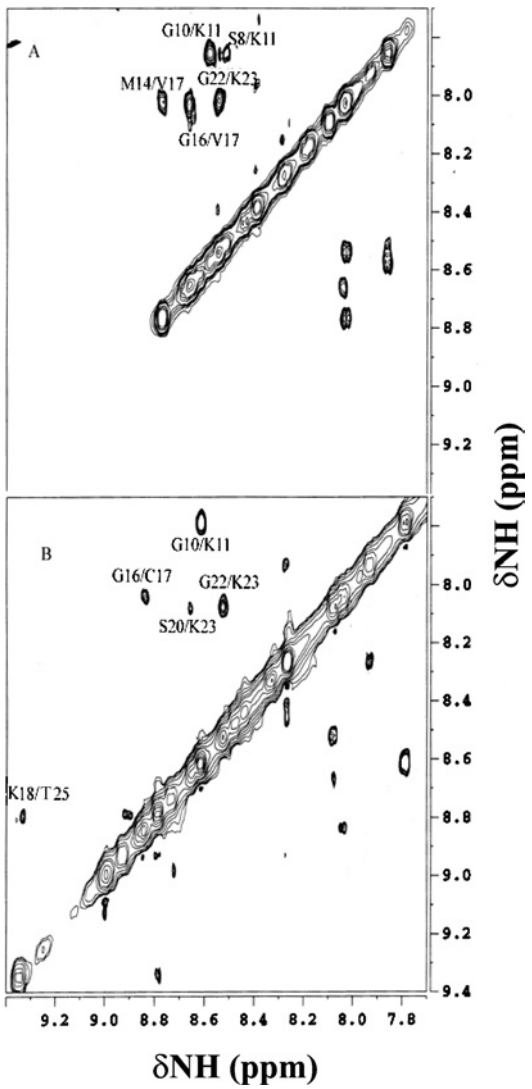

Figure 8 NH-NH region of the ROESY spectra for BS1PEP (A) and BS2PEP (B) in [*methyl*-^{2}H]methanol

The representative d_{NN} connectivities are shown as cross-peaks in the spectra that are labelled with the corresponding residue numbers.

oxidized peptide was dissolved in [*methyl*-^{2}H]methanol and the 500 MHz ^{1}H-NMR spectrum was recorded. The spectrum was identical with that acquired in [*methyl*-^{2}H]methanol of BS2PEP that had been oxidized in methanol (Figure 10). The CD spectra of the reoxidized BS2PEP in both methanol and water were identical with that of the original BS2PEP that had been originally oxidized in methanol. The possibility of incomplete reduction was ruled out by monitoring reduction by MALDI-MS after carboxymethylation of free cystine residues with iodoacetamide. The mass obtained for the carboxymethylated BS2PEP was 2947.2 Da, whereas the expected mass of BS2PEP modified with four carboxymethyl groups on cystine residues was 2947.4 Da. This proved that the four cystine residues were in the reduced state after treatment with DTT. The fact that, in spite of there being three possible combinations of the two disulphide bonds, the right disulphide bonds are formed in aqueous solution indicates that the peptide has residual structure in water that aids in the correct disulphide pairing.

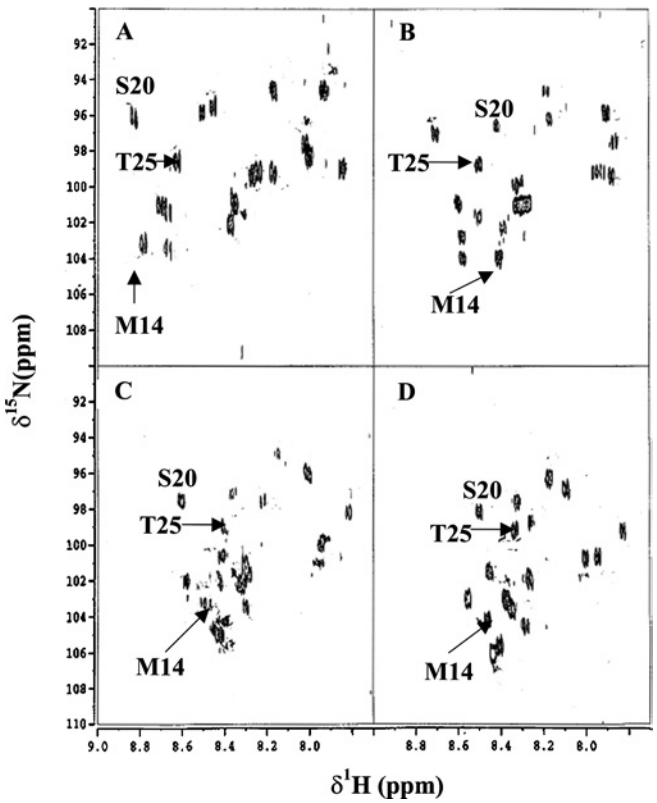

Figure 9 HSQC spectra for BS1PEP as a function of methanol concentration

The HSQC spectra for BS1PEP were recorded in 100% (v/v) methanol (**A**), 67% methanol (**B**), 33% methanol (**C**) and 100% water (**D**). Representative cross-peaks corresponding to amide protons are labelled to highlight the alteration in chemical shifts on decreasing methanol concentration. All the amide protons exhibited a single cross-peak at all methanol concentrations, indicating that the conformational exchange between the folded and the disordered form was fast compared with the NMR timescale.

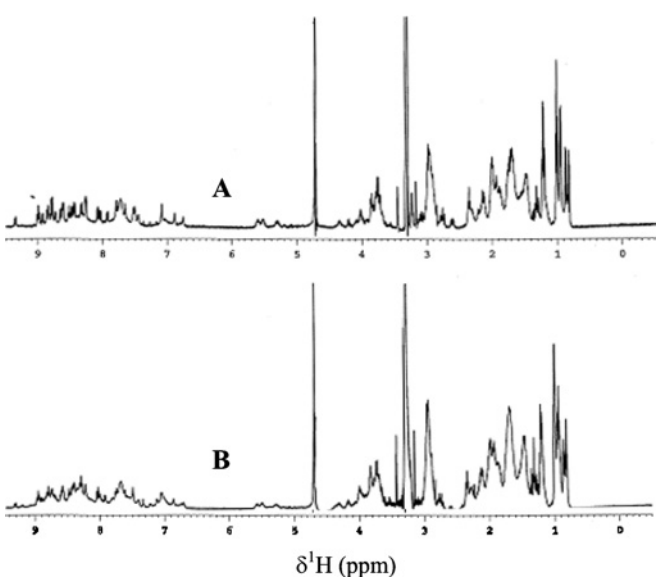

Figure 10 ^{1}H-NMR spectra for BS2PEP and re-oxidized BS2PEP recorded in 100% (v/v) [*methyl*-^{2}H]methanol

The spectra for BS2PEP oxidized in methanol (**A**) and BS2PEP reduced and oxidized in aqueous solution (**B**) are identical, indicating that both have the same structure and hence the same disulphide connectivity.

DISCUSSION

In contrast with a previous report [31], we were unable to obtain 17b binding with MBP fusions of bridging-sheet mimics. In the previous report, no data to support 17b binding was presented. The low yields and poor solubilities of these constructs prevented detailed characterization in our hands. The present work indicates that the designed peptides, BS1PEP and BS2PEP, have structures that are similar to the expected ones, albeit in methanol. As with previous studies that have shown that peptides with D-Pro-Gly sequences are good turn nucleators, we found that the four-stranded β-sheet was well formed in methanol. The peptides BS1PEP and BS2PEP fold predominantly into the expected four-stranded β-sheet conformation in methanol. The NOE data are consistent with a monomeric four-stranded β-sheet structure in which all three D-Pro-Gly β-turns are formed, resulting in appropriate strand registry. Solvation of the edge strands and positioning of charged residues at the edge strands prevent the formation of oligomeric sheet structure. The turn regions of the peptides are well formed in water, but evidence for proper inter-strand registry in aqueous solution was lacking. CD spectroscopy studies show that about 20% of the peptides are folded in a β-sheet structure in aqueous solution. Values of $C^{\alpha}H$ chemical shifts for BS1PEP and the correctly formed disulphide bonds for BS2PEP are additional evidence that suggest that some fraction of the peptides are structured in aqueous solution. The present study also shows that inter-strand cross-linking of strand 1 with 2 as well as strand 3 with 4 with disulphide bonds did not enhance the extent of secondary structure in aqueous solution, although some enhancement was observed in methanol (Figures 4A and 4B). These studies show that the presence of turn nucleating sequences and inter-strand cross-linking via disulphide bonds is insufficient to induce β-sheet formation in aqueous solution. This is likely due to the lack of a hydrophobic core in the designed structure. Future attempts will be made to introduce additional secondary-structure elements that will pack against the face of the β-sheet that is not involved in 17b binding. The residues that interact with 17b are on one face of the β-sheet and hence only residues of the other face are amenable to further design. Previous successful designs of three stranded sheets [18–20], as well as the single reported design of a four-stranded β-sheet [21], were not constrained by functional requirements. Besides the use of D-Pro-Gly turn sequences, the earlier studies also incorporated hydrophobic interactions, inter-strand salt bridges and β-branched residues. These branched residues display a preference for extended conformations which minimize steric repulsion of the β substituent with the main chain. In the present case there were several constraints on the designed sequence. All residues involved in 17b binding were kept unchanged. Aromatic–aromatic interactions as well as other hydrophobinc interactions were also avoided for two reasons. First, these would enhance the aggregation propensity of the peptide. Secondly, it has been shown that hydrophobic interactions between adjacent strands lead also to twisting of the strands [47]. In the present case we required a flat β-sheet. This is the first study that has attempted to synthesize a peptide that mimics the bridging sheet of gp120. Though only a small population of the peptide is structured in water, it exhibits the desired structure in methanol, suggesting that the present peptide is a useful platform for further designs of a second generation of peptides that better mimic the bridging sheet of gp120 in aqueous solutions.

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