Folding of β pep-4 β -sheet sandwich dimers and tetramers is influenced by aliphatic hydrophobic residues at the intersubunit interface

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For the designed peptide 33mer, β pep-4, formation of β -sheet structure [Ilyina, Roongta and Mayo (1997) *Biochemistry* **36**, 5245–5250] is thermodynamically linked to self-association. Dimers and tetramers are stabilized by interactions between hydrophobic residues lying on the hydrophobic faces of the amphipathic monomer subunits. The present study investigates the effects on folding and self-association of the substitution of two key hydrophobic residues (Ile²⁰ and Val²²) at the β -sheet sandwich interface of β pep-4. Single-site (I20L, I20V, I20A, V22L, V22I and V22A; where I20L corresponds to the substitution of Ile²⁰ with leucine etc.) and double-site (I20L/V22L and I20V/V22I) variants have been investigated. Like parent β pep-4, all variants can form dimers and tetramers. NOESY data indicate that the overall β -sheet fold and intersubunit β -strand alignments are the same in all variant tetramers. CD data for all variants indicate mostly β -sheet character in dimers and random coil character in monomers. Only for the V22I

variant is the β -sheet fold stabilized in the monomer state. Pulse-field gradient NMR-derived diffusion coefficients, measured as a function of peptide concentration, provide a means for deriving the distribution of monomer, dimer and tetramer states and, therefore, equilibrium association constants. Relative thermodynamic stabilities, which vary no more than approx. 0.5 kcal/mol (where 1 kcal $\equiv$ 4.184 kJ) from peptide to peptide, are I20V/V22I > I20V > I20L/V22L = β pep-4 (ΔG_D of 7.5 kcal/mol) = I20L = I20A > V22I > V22L > V22A for dimer formation and I20V > I20L/V22L > I20L > β pep-4 (ΔG_T of 6 kcal/mol) > V22I > I20V/V22I > V22L > I20A > V22A for tetramer formation. For the most part, dimer and/or tetramer stabilities are enhanced by the presence of valine and leucine and are attenuated by the presence of isoleucine and alanine.

Key words: CD, NMR, peptide, self-association, β -sheet conformation.

INTRODUCTION

β pep peptides are designed, water soluble, β -sheet-forming peptide 33mers [1]. While other β -sheet-forming peptides have been designed as monomers (e.g. β -hairpins [2–4]) or as β -sheet sandwich dimers (e.g. betabellins [5–7] and betadoublet [8]), β pep peptides self-associate primarily as tetramers at concentrations greater than a few mg/ml. β pep peptides, betabellins and betadoublets have a similar number of amino acid residues, i.e. 31–33, and are amphipathic, i.e. designed to self-associate through their hydrophobic faces. In these peptides, β -sheet formation is linked to the self-association process. β -Hairpins, on the other hand, are considerably shorter, less hydrophobic and are not amphipathic, by avoiding the sequential pattern of alternating hydrophobic and hydrophilic residues [6].

In most β -sheet-forming peptides, compact structure is lacking, and transient β -sheet conformation is characterized by a strong CD band at 217 nm and a more ‘random coil’ NMR spectrum [1]. β pep-4 is an exception and forms well-structured β -sheet sandwich tetramers that are composed of monomer subunits each having the same three-stranded anti-parallel β -sheet fold [9,10]. Two distinct dimer folds (D1 and D2), however, are observed, as depicted in Figure 1. In both types of dimer, the N-terminal strand from one monomer associates with that from the other in an anti-parallel fashion thereby extending the β -sheet to six-strands. The dimers differ at the monomer–monomer interface primarily by a two residue shift in the alignment of the

β -strands. In the compact tetramer state, dimers interact via the hydrophobic faces of their amphipathic structures. β pep-4 folding is consistent with known determinants of side-chain pairing and hydrophobic clustering in β -sheets [11–15].

The equilibrium scheme from unfolded monomer through molten globule-like dimer to well-folded tetramer [16] is shown below:

Monomer species are composed mostly of random coil structure with the possible presence of some partially folded β -sheet structure. Even though association of β pep-4 monomers into dimers could occur either via interaction of the hydrophobic faces of amphipathic β -sheet monomers or via continuation of the monomer anti-parallel β -sheet into a six-stranded structure like that found in well-structured β pep-4 tetramers [9,10] (see Figure 1), the former way is the more likely. If the latter were to occur, the hydrophobic surface of the amphipathic dimer would remain mostly exposed to solvent water, and this would be thermodynamically unfavourable, yielding very small populations of dimer, the opposite of which is observed [16]. Moreover, the temperature independence of the dimer equilibrium association constant, K_D , suggests hydrophobically-mediated dimerization, and the β pep-4 Val⁷ N-methyl variant, which cannot form six-stranded β -sheet type dimers, still dimerizes. Such β -sheet sandwich structures are not uncommon, e.g. betabellin [5–7] and betadoublet [8] peptides. Furthermore, β pep-4 dimers

Abbreviations used: HOHAHA, homonuclear Hartman–Hahn spectroscopy; NOE, nuclear Overhauser effect; PFG, pulsed-field gradient; ROESY, rotating-frame Overhauser enhancement spectroscopy.

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Figure 1 Backbone fold for β pep-4 dimers 1 and 2

The backbone folds for the two types of dimer (1 and 2) of β pep-4 that form within the context of the tetramer [10] are shown. Amino acid substitutions have been made at residues Ile²⁰ and Val²² (boxed-in).

demonstrate spectroscopic characteristics of molten globules as demonstrated by significant amounts of β -sheet from CD experiments and the lack of compact structure from NMR analysis [16]. β pep-4 dimers associate to form well-folded β -sheet sandwich tetramers. One open structural question is whether dimers D1 and D2 associate to form a heterotetramer or whether they associate separately to form two types of homotetramers.

The present study investigates the effects on folding and self-association of the substitution of two key hydrophobic residues (Ile²⁰ and Val²², see Figure 1) positioned at the β -sheet sandwich interface of β pep-4 dimers and tetramers. Single-site (I20L, I20V, I20A, V22L, V22I and V22A; where I20L corresponds to the substitution of Ile²⁰ with leucine etc.) and double-site (I20L/V22L and I20V/V22I) variants are investigated. Pulsed-field gradient (PFG)-NMR self-diffusion measurements provide information on changes in the oligomer distribution as the β pep-4 variant peptide concentration is changed. This information allows derivation of fractional oligomer populations and equilibrium constants. NMR and CD data are used to assess conformational changes in the variants. The present study addresses the problem of designing peptide sequences that can form more stable β -sheet sandwich structures in a desired oligomer state.

EXPERIMENTAL

Peptide synthesis

Peptide 33mers, based on the amino acid sequence of β pep-4 (Figure 1), were synthesized on a Milligen Biosearch 9600 automated peptide synthesizer. The procedures used were based on Merrifield solid phase synthesis utilizing fluoren-9-yl-methoxycarbonyl chemistry [17]. After the sequences were obtained, the peptide support and side-chain protection groups were acid (trifluoroacetic acid and scavenger mixture) cleaved. Crude peptide was analysed for purity on a Hewlett-Packard 1090M analytical HPLC machine using a reverse-phase C₁₈ VyDac column. Peptides were approx. 90% pure. Further purification was performed using a preparative reverse-phase HPLC C₁₈ column using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. Peptides were then analysed for amino acid composition on a Beckman 6300 amino acid analyser by total hydrolysis of samples using 6 M HCl at 110 °C for 18–20 h. N-terminal sequencing confirmed peptide purity.

NMR measurements

For NMR measurements, freeze-dried peptide was dissolved either in ²H₂O or in H₂O/²H₂O (9:1, v/v). Protein concentration was normally in the range of 1–5 mM. pH was adjusted by adding microlitre quantities of NaOH or ³HCl to the peptide sample. NMR spectra were acquired on a Varian Inova Unity Plus 600 spectrometer.

Two-dimensional homonuclear magnetization transfer [homonuclear Hartman–Hahn spectroscopy (HOHAHA)] spectra, obtained by spin-locking with a MLEV-17 sequence [18] with a mixing time of 60 ms, were used to identify spin systems. NOESY and rotating-frame Overhauser enhancement spectroscopy (ROESY) experiments [19,20] were performed for conformational analysis. All two-dimensional NMR spectra were acquired in the time proportional phase incrementation ('TPPI') [21] or States [22,23] phase sensitive mode. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans, as well as during the mixing time in NOESY experiments. Two-dimensional NMR spectra were collected as 256–400 t1 experiments, each with 1024 or 2048 complex data points over a spectral width of 6 kHz in both dimensions with the carrier placed on the water resonance. For HOHAHA and NOESY spectra, normally 16 and 64 scans respectively were time-averaged per t1 experiment. Data were processed directly on the spectrometer or offline using FELIX (Molecular Simulations, Inc., San Diego, CA, U.S.A.) on a Silicon Graphics Institute workstation. Data sets were multiplied in both dimensions by a 30–60°-shifted sine-bell function and zero-filled to 2048 in the t1 dimension prior to Fourier transformation.

PFG-NMR self-diffusion measurements

PFG-NMR self-diffusion measurements were performed as described previously [16], using the PFG longitudinal eddy-current delay pulse sequence [24]. Measurements were performed in ²H₂O at temperatures of 5 and 40 °C. Peptide concentrations ranged from 0.05 to 20 mg/ml. Each diffusion constant, *D*, was determined from a series of 15 one-dimensional PFG spectra acquired using different gradient strength (*g*) values. Experimental decay curves were approximated as single exponentials. *D* values were derived from the decay behaviour of upfield methyl and methylene resonances (average of three resonances).

Table 1 Fraction of tetramer (f_T) at 40 °C

The error in f_T is estimated to be ± 0.03 (S.D.s) for any given value. In this respect, lower f_T values have a greater percentage error, since both the tetramer population and the NMR spectral signal-to-noise were lower. Errors can also arise from inaccuracies in determining the number of α H protons (estimated to be ± 1 proton) within the cut α H resonance envelope, as described in [16]. Calculated values for f_T using the minimization procedure are given in parentheses beside the experimentally determined values.

Peptide	Peptide concn (mg/ml)...	f_T					
		6	3	1.5	0.75	0.3	0.1
Parent (IIIV)		0.77 (0.87)	0.69 (0.75)	0.55 (0.71)	0.43 (0.59)	0.33 (0.4)	0.15 (0.19)
Variants							
LIV		0.72 (0.11)	0.66 (0.03)	0.59 (0.01)	0.49 (0.01)	0.36 (0.002)	0.18 (0.001)
VIV		0.63 (0.3)	0.54 (0.2)	0.47 (0.11)	0.4 (0.06)	0.32 (0.02)	0.1 (0.01)
AIV		0.22 (0.26)	0.16 (0.17)	0.13 (0.11)	0.11 (0.06)	0.05 (0.02)	0.01 (0.006)
III		0.81 (0.43)	0.75 (0.31)	0.63 (0.25)	0.5 (0.18)	0.32 (0.07)	0.13 (0.02)
III		0.65 (0.72)	0.6 (0.63)	0.54 (0.5)	0.44 (0.37)	0.25 (0.17)	0.1 (0.05)
IIA		0.06 (0.01)	— (0.004)	— (0.002)	— (0.0)	— (0.0)	— (0.0)
VII		0.56 (0.58)	0.51 (0.45)	0.45 (0.28)	0.39 (0.18)	0.32 (0.07)	0.15 (0.03)
LIL		0.65 (0.22)	0.58 (0.12)	0.53 (0.11)	0.45 (0.08)	0.36 (0.04)	0.19 (0.01)

Downfield-shifted α H resonances are only associated with well-folded tetramers and provided an experimentally-derived D value for the tetramer state. D values measured at upfield resonances vary considerably with changes in β pep-4 variant concentration and represent a weighted average over the equilibrium distribution of oligomer states, including tetramers. Slopes of decay curves are linear with changes in gradient strength.

Monomer, dimer and tetramer fractional populations derived from diffusion data

Using PFG diffusion data, fractional populations for monomer, dimer and tetramer states were derived essentially as described by Mayo and Ilyina [16], using the following equations:

$$D_{\text{OBS}} = f_M D_M + f_D D_D + f_T D_T \quad (1)$$

$$f_M + f_D + f_T = 1 \quad (2)$$

where f_M , f_D and f_T are the fractional populations for monomer, dimer and tetramer states respectively, and D_M , D_D and D_T are their respective diffusion coefficients. For parent β pep-4, D_M , D_D and D_T are known [16]. At 40 °C, for example, D_M is $30.4 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, D_D is $20.7 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and D_T is $15.6 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. For all variants, fractional populations for monomer, dimer and tetramer states were calculated by using eqn (1) and these diffusion coefficients for β pep-4 monomer, dimer and tetramer states. In this analysis, only dimer and tetramer oligomers were considered, since no evidence was found for trimer formation in β pep-4 [16]. To derive f_M , f_D and f_T , eqns (1) and (2) were used to fit the concentration dependence of D_{OBS} using a Monte Carlo procedure which minimized the function:

$$F = \sum [(D_{\text{OBS}} - D_{\text{CAL}})/D_{\text{CAL}}]^2 \quad (3)$$

Since f_M , f_D and f_T are functions of the dimer and tetramer equilibrium association constants, K_D and K_T , which are invariable with changes in peptide concentration, equations for K_D and K_T expressed in terms of f_M , f_D and f_T were used in the minimization of eqn (3). This procedure, therefore, allowed the derivation of f_M , f_D , f_T , K_D and K_T . In the present study $K_D = [\text{dimer}]/[\text{monomer}]^2$ and $K_T = [\text{tetramer}]/[\text{dimer}]^2$.

The best fits were obtained when D_{OBS} varied substantially over the peptide concentration range investigated. In some cases (parent peptide and variants I20A, V22I, V22A and I20V/V22I) the fractional population for tetramer, f_T , derived using this approach, compared favourably with f_T determined from the ratios of resonance areas for α H and aromatic resonances [16]. Calculated values for f_T using this minimization procedure are given in Table 1 in parentheses beside the experimentally determined values. For variants I20L, I20V, V22L and I20L/V22L, this approach worked less satisfactorily since calculated f_T was substantially less than experimental f_T . The reason for these poorer comparisons was not due to fitting errors, which were quite good, but rather to D_{OBS} being close to D_D and varying much less with changes in peptide concentration. In this case, f_D tended to result as the major contributor. This inaccuracy was overcome by fixing f_T at the experimentally determined value during the Monte Carlo minimization procedure. Errors in determining K_D and K_T ranged from 2 to 12%.

CD

CD spectra were measured on a JASCO JA-710 automatic recording spectropolarimeter coupled with a data processor. Curves were recorded digitally and fed through the data processor for signal averaging and baseline subtraction. Spectra were recorded from 278 to 313 K in the presence of 10 mM potassium phosphate, over a 185–250 nm range using a 0.5 mm pathlength thermally-jacketed quartz cuvette. Temperature was controlled using a NesLab (Portsmouth, NH, U.S.A.) water bath. Peptide concentration was varied from 0.013 to 0.4 mM. The scan speed was 20 nm/min. Spectra were signal-averaged eight times, and an equally signal-averaged solvent baseline was subtracted.

RESULTS

Since amino acid substitutions are limited to residues Ile²⁰ and Val²² within the tripeptide sequence I²⁰IV²² (see Figure 1), the abbreviations AIV, VIV, LIV, IIA, IIL, III, LIL and VII will be used for single-site variants I20A, I20V, I20L, V22A, V22L, V22I and for double-site variants I20L/V22L and I20V/V22I respectively.

Figure 2 NMR spectra for β pep-4 variant peptides

^1H NMR (600 MHz) spectra are shown for β pep-4 variant peptides in $^2\text{H}_2\text{O}$ (pH 5.8) at 10 mg/ml and 40 °C. Spectra were accumulated with 8192 data points over a 6 kHz sweep width and were processed with 1 Hz line broadening. Only the spectral region downfield from the H_2O resonance is shown.

Overall fold in tetramers is not affected by substitutions

Compact anti-parallel β -sheet folding of β pep-4 is thermodynamically linked to tetramer formation [16]. When dissociated, β pep-4 dimers and monomers are not compactly folded and NMR cannot be used to elucidate, for example, β -strand alignment. The β pep-4 tetramer state is identified in ^1H NMR spectra by the presence of downfield-shifted αH and NH resonances. In general, all β pep-4 variants fold similarly in the tetramer state. Figure 2 shows the aromatic and downfield αH resonance regions for β pep-4 variants at 10 mg/ml and 40 °C. All variants show the presence of downfield-shifted αH resonances. PFG-NMR self-diffusion measurements analysed using these αH resonances indicate that, as with β pep-4 [16], downfield-shifted αH resonances of the variants are also associated with the tetramer state. Relative to parent β pep-4 at the same concentration (see Figure 1 in Mayo and Ilyina [16]), only variants AIV and IIA show a decreased αH resonance area compared with that for the aromatic resonances, indicating a shift in tetramer stability that is addressed in the following section.

Figure 3 NOESY spectra for β pep-4 variants

The αH – αH cross-peak region from NOESY contour plots is shown for all variants of the β pep-4 peptide (10 mg/ml) in 20 mM potassium phosphate (pH 5.8) at 40 °C. Data were collected in $^2\text{H}_2\text{O}$ (99.99%). Hypercomplex free induction decays (256) containing 2048 data points each were collected, zero-filled to 2048 in t_1 and multiplied by a 60°-shifted sine-squared function in t_1 and t_2 prior to Fourier transformation. The NOESY mixing time was 0.2 s. Labelling of resonances is as discussed in the text. Boxed-in cross-peaks denote duplicate resonances, one from each dimer state.

To assess β -strand alignment and overall folding in tetramers of these variants, TOCSY and NOESY spectra were acquired, and resonance assignments were made by using the standard ^1H homonuclear approach, as well as by analogy to assigned spin systems in β pep-4. For all variants, Figure 3 displays the αH – αH region from NOESY spectra acquired in $^2\text{H}_2\text{O}$. Some αH – αH nuclear Overhauser effect (NOE) connectivities have been labelled. In all cases where αH resonance assignments could be made, αH – αH pairings indicate the same β -strand alignment as found in β pep-4. In this respect, the overall fold of the variants is essentially the same as that in β pep-4 tetramers [1,9,10]. Although some cross-peaks in NOESY spectra result from exchange between αH resonances associated with the two differently folded dimer states [9,10], these ROESY-identified

Figure 4 PFG-derived diffusion coefficients for β pep-4 and variants

PFG-NMR diffusion data are shown for β pep-4 and variants as a function of peptide concentration. Each diffusion coefficient was determined from a series of 15 one-dimensional PFG spectra acquired using different g values. Experimental decay curves were approximated as single exponentials. Experimental conditions were as follows: duration of the magnetic field gradient pulse (δ) = 4 ms; g = 1–45 G/cm; time between gradient pulses (Δ) = 34.2 ms; and the longitudinal eddy-current delay (T_e) = 100 μ s. Data points represent the average D values derived from the three different resonances used to measure them.

cross-peaks lie close to the diagonal and do not interfere with analysis of most actual NOEs.

Thermodynamic stability of dimers and tetramers

To assess the stability of dimer and tetramer states of the variants, ^1H NMR spectra and PFG-NMR self-diffusion measurements were made as a function of peptide concentration.

Mayo and Ilyina [16] demonstrated that these data can be used to derive association equilibrium constants for dimer (K_D) and tetramer (K_T) formation, thereby quantifying aggregate stability. As with β pep-4, lowering the peptide concentration for any of these variants shifts the monomer $\leftrightarrow$ dimer $\leftrightarrow$ tetramer equilibrium away from tetramer as noted by a decrease in intensity of downfield-shifted αH resonances relative to the constant area under the aromatic resonance envelope (results not shown) [16]. The fraction of tetramer, f_T , in this equilibrium distribution was derived using the area under the downfield-shifted αH resonance envelope calibrated against the constant aromatic resonance area, as described by Mayo and Ilyina [16] and outlined in the Experimental section. Table 1 lists f_T values for parent and variant peptides.

Figure 4 plots PFG-NMR-derived diffusion coefficients versus peptide concentration. At 40 $^\circ\text{C}$, variant III behaves like the parent peptide (IIV) with D_{OBS} at high concentration indicating the presence of mostly tetramers. As the concentration is lowered, dissociation into dimers and monomers occurs. The two alanine-containing variants, IIA and AIV, form the least stable tetramers, IIA more so than AIV. All other variants, IIL, VIV, LIL, VII and LIV, follow nearly the same trend, apparently forming less stable tetramers and more stable dimers compared with the parent peptide, IIV. To quantify stability differences, fractional populations for monomer, dimer and tetramer states (f_M , f_D and f_T respectively) and equilibrium association constants, K_D and K_T , were derived as described in the Experimental section by basically fitting these diffusion data using eqns (1) and (2) and minimizing the error between D_{OBS} and D_{CAL} .

Best fits were obtained when D_{OBS} varied substantially over the peptide concentration range investigated. In these cases (parent peptide, IIV, and variants AIV, III, IIA and VII), the fractional population of tetramer, f_T , derived using this approach compared favourably with f_T determined experimentally from the ratios of resonance areas for αH and aromatic resonances (see Table 1) [16]. Calculated values for f_T resulting from the minimization procedure are given in Table 1 in parentheses beside the experimentally determined values. For variants LIV, VIV, IIL and LIL, this approach did not work satisfactorily since calculated f_T values were substantially less than experimental f_T values (see Table 1). The reason for these poorer comparisons was not due to fitting errors, which were quite good, but rather was the result of D_{OBS} being close to the value of D_D and varying relatively little with changes in peptide concentration. In this case, minimization tended to yield f_D as the major contributor. This false minimum was overcome by fixing f_T to the experimentally determined value during the Monte Carlo minimization procedure. Using either approach (free or fixed f_T),

Table 2 Equilibrium constants for dimer and tetramer formation at 40 $^\circ\text{C}$

In the present study $K_D = [\text{dimer}]/[\text{monomer}]^2$ and $K_T = [\text{tetramer}]/[\text{dimer}]^2$. Values of ΔG were calculated using the equation $\Delta G = -RT \times \ln K_i$ for the temperature 313 K. 1 kcal $\equiv$ 4.184 kJ.

Peptide	$K_D(\text{M}^{-1})$	ΔG_D (kcal/mol)	$K_T(\text{M}^{-1})$	ΔG_T (kcal/mol)
Parent (IIV)	1.7×10^5	7.5	1.6×10^4	6.0
Variants				
LIV	1.3×10^5	7.3	2.8×10^5	7.8
VIV	5.9×10^5	8.2	1.6×10^6	8.9
AIV	1.2×10^5	7.3	3.9×10^2	3.7
IIL	1.6×10^4	6.0	6.5×10^2	4.0
III	3.3×10^4	6.5	5.2×10^3	5.3
IIA	4.4×10^3	5.2	1.6×10^1	1.7
VII	5.1×10^5	9.6	1.9×10^3	4.7
LIL	2.3×10^5	7.7	6.8×10^5	8.3

Figure 5 CD spectra of β pep-4 variants

Far-UV CD spectra for β pep-4 variant peptides are shown as mean residue ellipticity versus wavelength (nm). Peptide concentration was varied from 0.013 to 0.4 mM as indicated in the Figure. The pH was adjusted to pH 6. Traces are shown for data collected at 40 °C. Other experimental variables are given in the Experimental section.

errors in determining K_D and K_T ranged from 2 to 12%. K_D , K_T and free energies for dimer and tetramer formation, ΔG_D and ΔG_T respectively, are given in Table 2. In general, K_T values for variants vary more than K_D values. This is manifest in ΔG values which range from 5.2 to 9.6 kcal/mol (where 1 kcal $\equiv$ 4.184 kJ) for dimer formation and from 1.7 to 8.9 kcal/mol for tetramer formation.

Conformational analysis of dimer and monomer states

At lower peptide concentrations, tetramers dissociate, and as populations of dimers and monomers increase, CD and NMR provide information on the conformations of these lower aggregate states. This allows insight into how amino acid substitu-

tions in β pep-4 variants affect the β -sheet-forming potential of dimers and monomers. For this, CD and NMR spectra were acquired as a function of peptide concentration and spectral changes were noted. For CD measurements, variant peptide concentrations ranged from 0.05 mg/ml (0.013 mM) to 1.5 mg/ml (0.4 mM). At the lowest concentration, the population of tetramer at 40 °C is approx. 10%. At 5 °C, the tetramer population is less than approx. 5%. Based on trends occurring in CD traces as the peptide concentration is lowered (Figure 5), the relative amount of β -sheet in dimer and monomer states can be qualitatively deduced by observing the ratio of CD ellipticity at 217 nm to that at 205 nm (Table 3). The 217 nm CD band is characteristic of β -sheet conformation [25–27], whereas the CD band between 200 and 205 nm is characteristic of random structure distributions predicted for mostly unstructured peptides [27–29]. A 217/205 nm ratio of approx. 2 is expected for 90–100% β -sheet. At 1.5 mg/ml, where tetramer and dimer states dominate for most variants, this ratio was indeed approx. 2. Variants AIV and IIA represent the other extreme where dimer and monomer states dominate at 1.5 mg/ml and the ratio was 0.5 and 0.3 respectively. Since the monomer population has increased, this is consistent with the observation that variant monomers, like β pep-4 monomers [16], are usually predominantly in the random coil state. This view is reinforced by analysing CD spectra acquired at the lowest concentration and at the lower temperature of 5 °C (results not shown), where the monomer population is at least 80%. For all but one variant, the 217/205 nm ratio falls as the population of monomer increases. Variant III is the exception because even at 0.05 mg/ml, with monomer state dominating, the ratio remains near 2. This indicates that the variant III monomer state maintains a relatively stable β -sheet fold.

At intermediate concentrations, where the population of monomer is still minimal and the dimer population has increased substantially, the 217 nm CD band remains the more prominent. As with β pep-4, this observation suggests that dimers of these variants are composed mostly of β -sheet. This structure, however, is highly transient. This conclusion is based on analysis of TOCSY spectra acquired at low concentration (0.08 mM) to attenuate the tetramer population, which reveals that most dimer/monomer resonances fall close to random coil positions. This was the case with β pep-4 [16], and, like β pep-4, resonances from the tetramer state of variants are in slow chemical exchange (NMR chemical shift time scale) with resonances associated with dimer and monomer states. Since in these TOCSY spectra, many dimer/monomer spin systems are distinguishable from the more downfield-shifted tetramer ones, their chemical shifts can be compared with those given by the chemical shift index [30]. To

Table 3 Ratio of ellipticity at 217 nm to that at 205 nm for CD data acquired at 5 °C

Peptide	Peptide concn (mg/ml)...	Ratio				
		1.5	1.0	0.5	0.2	0.05
Parent (IIV)		2	2	1.8	1.2	1
Variants						
LIV		2	2	1.5	1.1	0.8
VIV		1.4	1.2	0.9	0.9	0.6
AIV		0.5	0.5	0.4	0.35	0.25
IIL		2	2	2	1.2	0.4
III		2	2	2	2	1.8
IIA		0.3	0.3	0.3	0.3	0.3
VII		2	2	1.5	1.0	0.7
LIL		2	2	1.8	1	0.8

Figure 6 TOCSY spectra for β pep-4 variants

TOCSY contour plots are shown for β pep-4 peptide variants in $^2\text{H}_2\text{O}$. The peptide concentration was 0.08 mM and the temperature was 40 °C. Hypercomplex free induction decays (256) containing 2048 data points each were collected and processed as discussed in the Experimental section. Data were zero-filled to 2048 in t1. The raw data were multiplied by a 30°-shifted sine-squared function in t1 and t2 prior to Fourier transformation. Labelling of resonances is as discussed in the text.

exemplify this, Figure 6 shows the serine $\alpha\text{H}/\beta\text{H}_2$ region for most of the β pep-4 variants. This region is perhaps the least overlapped, and the one in which the general assignment to serines can be made unambiguously. β pep-4, and each of its variants, has three serines, Ser¹, Ser⁸ and Ser³¹. Serine cross-peaks arising from the tetramer state are specifically labelled in Figure 6. αH resonances for Ser⁸ and Ser³¹ are downfield-shifted, consistent with their being located in strands one and three of the well-structured β -sheet, whereas the αH resonance for N-terminal residue Ser¹ falls near the random coil position for serine. The remaining distinguishable serine cross-peaks arise from dimer/monomer states (labelled Ser M/D) and also fall nearer the random coil position for the serine αH resonance. However, some chemical shift dispersion is noted with serine αH s resonating between 0.1 and 0.2 p.p.m. downfield from their random coil position. Similar comparisons can be made with other resonances (results not shown) as was done with β pep-4 [16].

Downfield-shifted αH resonances indicate the presence of transient β -sheet conformation in dimer/monomer states of these variants. Since in the fully-folded tetramer state, the two serines within the β -sheet are approx. 1 p.p.m. downfield-shifted from their random coil position, a shift change of 0.1–0.2 p.p.m.

should correspond to approx. 10–20% β -sheet formation. This estimate is smaller than that derived using CD. However, it is not unusual for percentage estimates derived from CD and NMR to vary in this way. For variant III, for example, β -sheet content in the monomer state is estimated to be approx. 100% by CD (see above), whereas, by using NMR chemical shift changes, it is estimated to be no greater than approx. 20%. One reason for this discrepancy is that CD is sensitive to ϕ and ψ angles and β -strand formation, whereas NMR chemical shifts are sensitive to β -strand proximity, i.e. β -sheet formation. Regardless of this, results can be compared in a relative sense.

Since high resolution structures of these dimers cannot be elucidated, it is not known if the β -strands and their alignments in the β -sheet are the same in the dimer as those in the well-folded tetramer [10]. Some insight into this can be derived from αH chemical shifts of variant dimer resonances. In most variant dimers, it can be determined that both valines, two serines, the methionine residue, one arginine, at least two lysines and most of the isoleucine and leucine residues appear to be involved in β -sheet structure. This distribution is similar to that in β -strands in a monomer subunit of the tetramer. Moreover, the unique alanine residue, Ala¹⁶, which is positioned within a loop between β -strands one and two in a monomer subunit of the tetramer, exhibits a random coil αH chemical shift in the dimer, consistent with it not being part of a β -strand in the dimer. That loop sequence also contains two lysine residues, and, in the dimer state, approx. two lysine residues do show random coil chemical shifts. While this is by no means proof that the transient β -sheet fold in the dimer is the same as in the tetramer, it is suggestive.

DISCUSSION

Overall, β pep-4 variants fold in the same way as the parent peptide [9,10,16]. In this respect, none of the single-site or double-site amino acid substitutions significantly disturbs the overall fold relative to parent β pep-4. On the other hand, the fold stability in these variants is quite dependent on the particular substitution. Two general observations can be made: (1) isoleucine and alanine destabilize dimer and/or tetramer formation, and (2) valine and leucine stabilize dimer and/or tetramer formation. For promotion of β -sheet sandwich fold stability in dimers and tetramers, the overall ranking of these aliphatic hydrophobic residues is: valine > leucine > isoleucine > alanine. This scale is different from the β -sheet propensity scales of Minor and Kim [31,32] (isoleucine > valine > leucine > alanine) and of Chou and Fasman [33,34] (valine > isoleucine > leucine > alanine). One reason for these differences rests in how these scales were generated. Minor and Kim developed their scale by making amino acid substitutions at the helix-sheet interface of α/β -structured B1 domain from Protein G. In this regard, residue packings were between the hydrophobic surfaces of the amphipathic helix and sheet, which are expected to be different from those between two β -sheet domains as is the case with β pep peptides. Chou and Fasman used a statistical approach with a number of protein structures, and their results are biased by the particular database used. It may be that, for β -sheet sandwich folds, the ranking found in the present study is more appropriate. Context dictates which residues can be used and are optimal [31], and this, of course, varies from protein to protein. For example, with helix coiled-coils, even β -sheet-promoting isoleucine can be used at the helix-helix interface to yield a very stable structure [35].

Considering the β pep-4 folding scheme, shown in the Introduction section, the monomer state of most of these variants, like that of the parent peptide, is primarily random coil with

some highly transient β -sheet conformation. However, the presence of two juxtapositioned isoleucine residues in variant III produces a relatively more stable β -sheet fold in the monomer state. This is demonstrated by the persistence of a strong CD band at 217 nm at low peptide concentration where the monomer state is dominant. Surprisingly or not, this is consistent with isoleucine being at the top of β -sheet-forming propensity scales [32,33]. In the absence of a structure, one can only speculate that the two isoleucine residues interact with each other and with neighbouring cross-strand hydrophobic residues (Met⁹, Leu¹¹, Ile³⁰ and Leu³²) to stabilize the β -sheet in the monomer state. Isoleucine appears to be a good residue for designing peptides to fold as monomeric β -sheets. With variant III, increased fold stability in the monomer state may act to shift the monomer $\leftrightarrow$ dimer equilibrium away from the dimer state. Although conformational entropy must be overcome on making the transition from a random-coil monomer to a collapsed dimer, the variant III monomer, relative to the other variants, has a more stable β -sheet conformation, which is probably rearranged on association and formation of the dimer state. Alternatively, the positioning of the two isoleucine residues at the monomer–monomer hydrophobic interface may destabilize the dimer state, thereby shifting the equilibrium to the monomer state.

Monomers of all variants associate to form β -sheet sandwich dimers that exhibit characteristics of molten globules [36,37], i.e. considerable secondary conformation (β -strand in this case) with little, if any, fixed tertiary structure. Substitution of Ile²⁰ in parent peptide IIV with either leucine (LIV) or alanine (AIV) has essentially no effect on dimer stability. The same can be said for the double leucine variant LIL. However, substitution of Ile²⁰ with valine (VIV) increases dimer stability by 3–4-fold. On the other hand, substitution of Val²² with any other aliphatic hydrophobic residue, especially alanine, reduces dimer stability. In variant III, reduced dimer stability due to substitution at position Val²² can be compensated for by switching the positions of Ile²⁰ and Val²² as in the double-site variant VII. This greatly increases dimer stability by 25-fold compared with the parent peptide. Although β pep dimers are molten globule-like and as such lack compact structure, having two valine residues or the valine–isoleucine combination at these positions apparently optimizes hydrophobic side-chain interactions at the monomer–monomer interface, thereby promoting increased dimer stability. This structural flexibility is also the reason why alanine in variant AIV does not decrease dimer stability. However, an alanine at position 22 (IIA) greatly decreases dimer stability. This comparison alone suggests that these molten globule-like collapsed states require some, possibly specific, residue contacts within the core and that these must be maintained for stability.

Molten globule-like β -sheet sandwich dimers then associate to form compactly folded β -sheet sandwich tetramers. For this to occur, the β -strands from opposing subunits in the dimers must associate in an anti-parallel fashion. When the β -strand conformations are optimal, tetramer formation is promoted. Tetramer stability is a different story because it is folded compactly. In this regard, side-chain packing, and not just transient side-chain interactions, as most likely occur in the dimer state, is crucial to stability. As expected from ‘cavity-creating’ mutation studies on lysozyme [38] and staphylococcal nuclease [39], substitution of Ile²⁰ or Val²² with alanine decreases tetramer stability. Moreover, placing a bulkier leucine or isoleucine residue at position 22 also reduces tetramer stability, albeit less so for substitution with isoleucine (III). On the other hand, substitution of Ile²⁰ with leucine (LIV), and more so with valine (VIV), greatly increases tetramer stability. Switching the positions of Ile²⁰ and Val²² in double variant VII, unlike its neutral effect on dimer stability,

decreases tetramer stability, whereas placing leucines in both positions (LIL) results in a 40-fold increase in stability. Variants LIV, LIL and VIV foster tetramer formation, whereas all other substitutions are detrimental. In fact, positioning two valines either side of Ile²⁰ (VIV) is optimal. Inclusion of a single isoleucine residue reduces tetramer stability, suggesting that isoleucine does not pack optimally within the tetramer hydrophobic core. This conclusion was based on comparison, for example, of variants VIV and LIV with III, and of IIV with III. In these cases, substitution with an isoleucine residue makes ΔG_T more positive by approx. 1–3 kcal/mol. Energetically, tetramer stability (ΔG_T) is decreased or increased from approx. 0.7 to 4 kcal/mol depending on the particular substitution. This range is consistent with that found, for example, in hydrophobic-substituted mutants of staphylococcal nuclease [39].

In addition, strand register in anti-parallel β -sheets is known to determine stability [11,12]. Wouters and Curmi [12] found that the most significant pair correlations were between cystines due to disulphide bond formation and between charged or polar residues with electrostatic compatibility. These authors also noted that, for non-hydrogen-bonded β - and γ -branched residue pairs, a correlation exists between the directionality in side-chain packing and the handedness of the twist of the β -sheet. In β pep-4 variants, these are the types of residues (isoleucine and valine) that are being substituted. However, since there are no high-resolution structures for β pep-4 variants, it is impossible to compare structures for an in-depth analysis. Nevertheless, changes in the twist of the β -sheet can affect fold stability and this may contribute to the observed differences in stability among variants.

Overall, β pep dimers are more stable than tetramers and both are more stable at higher temperature than at lower temperature. Lowering the temperature from 40 to 5 °C destabilizes dimer and tetramer states in most cases only up to approx. 4-fold. This general trend is consistent with the hydrophobic effect [40], and suggests that β -sheet sandwich folding is primarily entropy-driven. In general, β pep dimers are more thermodynamically stable than tetramers (Table 2). Exclusion of hydrophobic residues from polar solvent is the primary force that drives overall β pep-4 folding, and while fixed tertiary structure may contribute to a better defined and, therefore, a more stable subunit interface, the hydrophobic surface area of contact between molten globule-like dimer subunits is considerably greater than that between these dimer types in the tetramer. At the dimer–dimer (tetramer) interface, both hydrophobic contacts and hydrogen bonding between anti-parallel β -strands contribute to ΔG_T . A hydrophobic contact surface area, therefore, presumably dictates the larger free energy of monomer association regardless of internal flexibility in molten globule-like dimers. In fact, while considerable conformational entropy probably remains in molten globule-like dimers, it is relatively reduced in tetramers (D1, D2). A reduction in conformational entropy (internal flexibility) contributes to a more positive ΔG .

Conclusions

Single- and double-site hydrophobic core substituted variants of β pep-4 all form similar β -sheet sandwich folds compared with the parent peptide. Fold stability in these variants, however, is dependent on the particular substitution. In general, isoleucine and alanine residues destabilize dimer and/or tetramer formation, whereas valine and leucine residues stabilize dimer and/or tetramer formation. These results will aid in the design of β -sheet-forming peptides and in improving the hydrophobic core-mediated stability of peptides and proteins.

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