A pair of pyrene groups as a conformational probe for designed two α-helix polypeptides

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Peptides with two α-helix segments anchored on 2,2'-bipyridyl-4,4'-dicarboxylic acid and suberic acid have been designed and synthesized. L-1-Pyrenylalanine (Pya) was introduced near the centre of each α-helical segment as a CD and fluorescent probe to detect the arrangement of the two α-helix segments. The amphiphilic α-helical 14-peptide was designed with an amino acid sequence in which the hydrophobic amino acids, Leu and Pya, are deployed in the same manner as hydrophobic amino acids in coiled-coil proteins. The synthesis was carried out by a solid-phase synthesis on Kaiser's oxime resin and solution coupling with anchors. The probing behaviour of a pair of Pya residues was examined by CD and fluorescent measurements under various conditions, *e.g.* in aqueous and methanolic solutions, in the presence of guanidine hydrochloride (GuHCl) and under increasing temperature. The peptides showed a highly α-helical CD pattern in the far-UV region in aqueous solution. Furthermore, they showed strong split CD peaks with positive and negative maxima at longer and shorter wavelengths, respectively, at the pyrene absorption region (^1L_a and ^1B_b). These split CD signals decreased with the disruptions of the two α-helix structure by the addition of methanol, GuHCl and also with increasing temperature. On the other hand, the excimer emission of pyrene in fluorescence was very weak in aqueous solution, but it was transiently increased while the helix–helix interaction was relaxed to some extent with the addition of methanol. These facts indicate that the two pyrene groups are forced to be in close proximity and to be in a right-handed sense between the two α-helix structures in aqueous solution; whereas, the arrangement of the pyrene groups is loosened by the destruction of the 3D structure. Thus, the pyrene probe has been demonstrated to provide significant information about the arrangement of the two α-helix segments by the simple measurements of CD and fluorescence spectra.

A number of trials to design artificial proteins have been reported involving construction of 3D structures of polypeptides.[1-7] They commonly utilized the hydrophobic interaction between α-helices to form aggregation of segments with the amphiphilic α-helical motif.[8,9] Though the degree of secondary structure especially for an α-helix in a designed protein was characterized by the measurements of circular dichroism (CD) spectra in the far-UV region,[1-7] the orientation or arrangement of α-helical segments has not been precisely defined. The evaluation of the arrangement of segments hitherto requires the examination of the 3D structure by multi-dimensional NMR measurements[10] and X-ray crystallographic analysis.[11] These analyses, however, are not generally applicable for that purpose, because designed proteins are commonly in a stable but a molten globule-like state consisting of a mixture of low-energy conformations.[1a,10]

To examine the relationship between α-helical segments easily, we introduced a pair of L-1-pyrenylalanine (Pya) residues as CD and fluorescent probes into α-helix segments.[12,13] Though the pyrene group is rather bulky (pyrene is *ca.* 0.9 nm long, Trp is *ca.* 0.6 nm long), it has great advantages of strong fluorescence, excimer formation and an extended π-system to detect interchromophore interaction. Pyrene has been utilized as a probe for the evaluation of membrane fluidity and polymer conformation.[14-16] Detection of the pyrene excimer elucidated the phase-transition of membranes and the thermal transition of polymer conformations. Sisido *et al.* utilized Pya as a chromophoric amino acid in poly(amino acids) to design molecular electronic devices with a chromophoric array along an α-helix.[17] They applied the techniques of CD and fluorescence-detected CD to the evaluation of pyrene geometry as an amino acid side chain in the poly(Pya). Goedeweeck *et al.* also employed Pya as a tool for molecular dynamics study of dipeptides by intramolecular pyrene excimer formation.[18] Measurements of pyrene fluorescence and conformational calculations of diastereoisomers of Ac-DL-Pya-DL-Pya-OMe dipeptide elucidate the population of the extended and folded conformations of the dipeptides. Furthermore, Pya residues in hormonal,[19a] antimicrobial[19b] and amphiphilic α-helical[19c] peptides were utilized as a probe for the detection of the interaction between the peptides and phospholipid membranes. Thus, pyrene is a valuable tool for use in the study of peptide–macromolecular interaction.

We attempted to utilize the pyrene probe as an artificial amino acid, Pya, for the detection of the interaction between segments and conformational changes of polypeptides consisting of two α-helices (Fig. 1). That is, when two pyrene groups are in close proximity in the structure, the pyrene groups in the Pya residues would show the exciton interaction in CD and excimer emission in fluorescence to provide information about the arrangement of the α-helical segments in the 3D structures. In fact, the utility of Pya as a probe was demonstrated in detecting the orientation of the α-helical segments in a right-handed sense in a 2α-helix[12] and a 4α-helix bundle structures.[13] In the present study, we characterized the behaviour of a pair of pyrene groups in 2α-helix structures in various environments to confirm the probing method in the evaluation of the 3D structures of polypeptides. The peptides were designed to have

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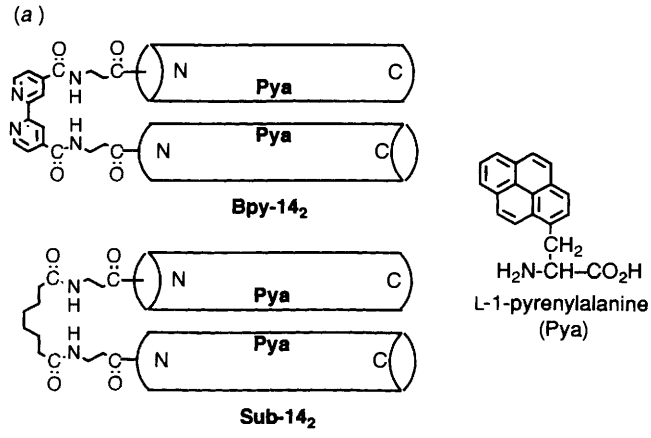

Fig. 1 Structure of the two α-helix peptides, Bpy-14_2 and Sub-14_2. (*a*) Illustration of Bpy-14_2 and Sub-14_2 and the structure of pyrenylalanine. (*b*) Amino acid sequence of the 14-peptide. (*c*) Helix wheel drawing of the two 14-peptides in coiled-coil form. (*d*) Illustration and structure of the antiparallel two α-helix peptide, Bpy-14_2(*anti*).

two amphiphilic α-helical 14-peptide segments containing Pya, which are anchored on a bipyridyl group (Fig. 1). CD and fluorescence measurements revealed that the two α-helix segments in the peptide, Bpy-14_2, are arranged in a right-handed sense in aqueous solution, although the 3D structure is disrupted in methanolic solution, in guanidine hydrochloride (GuHCl) solution and at higher temperatures. A 2α-helix peptide, Sub-14_2, with suberic acid as an anchoring group instead of bipyridine showed the pyrene behaviour similar to the original peptide. However, another 2α-helix peptide, Bpy-14_2(*anti*), with two α-helices in antiparallel orientation did not show any characteristic CD spectra for pyrene.

Results and discussion

Design and synthesis

The 14-peptide was designed to form an amphiphilic α-helical structure employing Ala, Leu and Lys residues. The probe, Pya residue, was introduced near the centre of the peptide (Fig. 1). The Pya and Leu residues were deployed on the α-helix segment in the same manner as hydrophobic amino acids in coiled-coil proteins[20] to define the packing mode between the two α-helix segments. Coiled-coil sequences in proteins consist of heptad repeats containing two characteristic hydrophobic positions, *i.e.* (-a-b-c-d-e-f-g-)$_n$ in which the a and d residues are hydrophobic ones such as Leu. The 14-peptide consists of two repeats of the sequence c-d-e-f-g-a-b where the a residue is Leu or Pya, d is Leu, f is hydrophilic Lys and the others are Ala residues. The C-terminal carboxylic acid was methylamidated to increase the stability of the α-helix structure. Though the 14-peptide is not long enough to form a coiled-coil structure, 14 residues in length would be sufficient to stabilize an α-helix structure (four turns in the α-helix at maximum) and to evaluate the helix–helix interaction.[1-3] To construct a parallel two α-helix structure, the two segments were anchored on 2,2′-bipyridyl-4,4′-dicarboxylic acid with a flexible spacer of β-Ala (Bpy-14_2, Fig. 1). Owing to its rigid and hydrophilic properties, the bipyridyl group seems to be a good candidate for the anchoring moiety. As a reference compound with a flexible anchoring group, a 2α-helix peptide with suberic acid (Sub-14_2) was also designed. Furthermore, another 2α-helix peptide with two α-helices in antiparallel orientation [Bpy-14_2(*anti*)] was designed employing different 14-peptides, Ac-14-peptide and H-14-peptide-$NHCH_3$, containing Pya. In the third 2α-peptide, two Pya residues are deployed at the sixth positions from the anchor facing each other.

The artificial amino acid, Pya, was quantitatively synthesized in pure form by the modified procedure of the reported method[18] with *Aspergillus* acylase. The peptide synthesis was carried out by the combined method of solid-phase synthesis on Kaiser's oxime resin[21] and solution synthesis. The peptide acid was synthesized by stepwise elongation of Boc-amino acids on the oxime resin and cleavage from the resin with *N*-hydroxypiperidine (HOPip) followed by reductive removal of the piperidyl ester. The 7-peptide methylamide was synthesized by the same method on the resin and the cleavage with methylamine. The 7-peptides were condensed in solution [dichloromethane (DCM)–trifluoroethanol (TFE) 3:1][22] to give the 14-peptides. Though the protected 7-peptides had poor solubility in dimethylformamide (DMF), the coupling solvent could dissolve the peptides to yield products. Consequently, the new strategy gave purer products in higher yield than the reported one employing fragment coupling by the solid-phase method on the oxime resin.[12] The H-14-peptide-$NHCH_3$ was condensed with the anchor, Bpy-(β-Ala)$_2$ or Sub-(β-Ala)$_2$, with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBt)[23] in dimethylsulfoxide (DMSO) to give protected Bpy-14_2 and Sub-14_2. To synthesize Bpy-14_2(*anti*), H-14-peptide-$NHCH_3$ and Ac-14-Peptide-OH were condensed with the anchor Bpy-(β-Ala)(Eda) (Eda, ethylenediamine) in a stepwise manner. The protecting groups in these peptides were removed with anhydrous HF and the obtained peptides were purified by gel-filtration and HPLC to a high purity (>95%). The bipyridyl peptide without Pya residues (Leu residues instead) was also synthesized by a similar method.

Spectral behaviour in aqueous solution

The CD spectrum of Bpy-14_2 was measured in a buffer solution and the result is shown in Fig. 2. The peptide showed a typical α-helical CD pattern with ellipticity at 222 nm of −22 000 deg cm^2 dmol^{-1} (75% α-helicity).[24] The monomeric 14-peptide-$NHCH_3$ was much less α-helical ($[\theta]_{222}$ = −5200 deg cm^2 dmol^{-1}). The stabilization of the α-helix structure was attributed

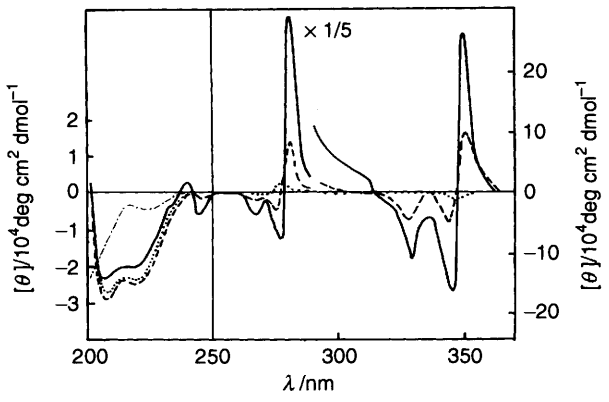

Fig. 2 CD spectra of Bpy-14$_2$ (———), Sub-14$_2$ (– – –), Bpy-14$_2$(*anti*) (•••••) and 14-peptide-NHCH$_3$ (– • – • –) in 2.0 × 10^{-2} mol dm^{-3} Tris·HCl, pH 7.4. [θ] at the amide region is the mean residual weight ellipticity and [θ] at the pyrene absorption region is the molar ellipticity for the peptide. Spectra at 250–290 nm are drawn to 1/5 scale. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}, 25 °C.

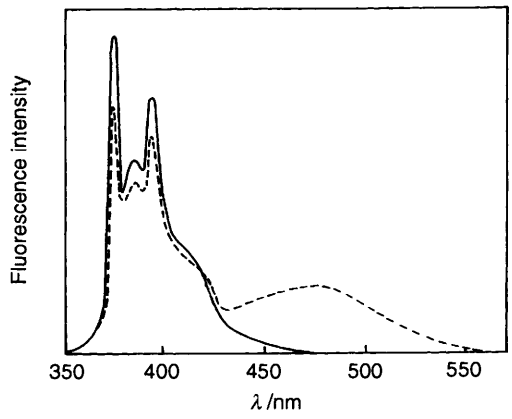

Fig. 3 Fluorescence spectra of Bpy-14$_2$ in 2.0 × 10^{-2} mol dm^{-3} Tris·HCl, pH 7.4 (——) and in 50% MeOH–Buffer (— — —). [Peptide] = 1.0 × 10^{-6} mol dm^{-3}. Excited at 342 nm at 25 °C.

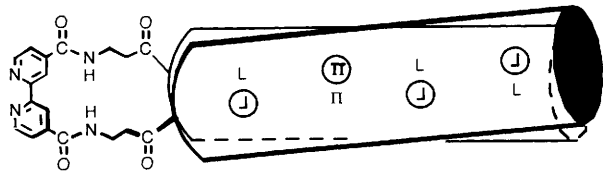

Fig. 4 Proposed arrangement of two α-helices in Bpy-14$_2$ in a coiled-coil-like form. The circled and reversed Π and L denote Pya and Leu residues on the opposite side of the front helix and other residues are of the rear helix. Two helical rods may be arranged with a twisting angle of *ca.* 18°.[20c] Two helices of peptides in coiled-coil form are also arranged in this sense and wrap around each other to form a left-handed supercoil.[26]

to the interaction between the hydrophobic face of the amphiphilic α-helical segments.[1-3] Another notable CD result was that at the pyrene absorption region especially at 345 (^1L_a band of pyrene) and 278 nm (^1B_b band), strong CD peaks were induced which were split into a positive peak at longer wavelengths and a negative peak at lower wavelengths (Fig. 2). This result indicates that the two pyrene groups are fixed in close proximity in the two α-helix structure and, furthermore, they are arranged in a right-handed sense according to the exciton chirality method.[25] Sisido *et al.* reported on poly(Pya) that the polypeptide showed induced CD peaks of pyrenes in which only one at 240 nm (^1B_a band) was split with positive ellipticity at shorter wavelengths.[17] This fact indicated that the screw sense of the transition moment belonging to the two successive pyrenyl groups on the α-helical rod was in a left-handed arrangement. In contrast to their results, the pyrene rings between the α-helical segments displayed split CD signals in a right-handed sense both in the ^1B_b and ^1L_a bands. Because the ellipticities of the CD signals are much larger than those in poly(Pya) and Ac-Pya-OMe,[17] the side chain pyrenes in Bpy-14$_2$ are more rigidly fixed in the structure than those in the polyamino acids or the amino acid derivatives.

On the other hand, Sub-14$_2$, which has a more flexible anchor, showed an α-helical CD ([θ]$_{222}$ = −25 000 deg cm^2 dmol^{-1}, 85% α-helicity) and split CD signals in the pyrene region similar to those of Bpy-14$_2$. The intensity in the CD splitting of Bpy-14$_2$ was greater than that of Sub-14$_2$. This fact indicates that the two pyrene groups in Bpy-14$_2$ are more strongly fixed in the 2α structure probably due to the rigid anchor. If the intensity of the splitting could indicate the extent of packing of the side chains on the α-helices, the side chains in Bpy-14$_2$ would be better packed than those in Sub-14$_2$. However, α-helicity of Sub-14$_2$ is slightly larger than that of Bpy-14$_2$. Because suberic acid is so much more flexible than the bipyridyl group that the peptide segments increase their motion, the two segments in Sub-14$_2$ may more easily interact with each other than those in Bpy-14$_2$ resulting in higher α-helicity. It seems that α-helicity is not correlated to the extent of packing of the α-helices. The parent peptide containing Leu instead of Pya also showed similar α-helicity ([θ]$_{222}$ = −20 500 deg cm^2 dmol^{-1}), therefore, the effect of Pya residues on the stability of the α-helix is not significant.

In the fluorescence spectra in the buffer (Fig. 3), both peptides showed the monomeric fluorescence of pyrene without excimer emission. The excimer emission could not be observed at a peptide concentration ranging from 1 to 40 × 10^{-6} mol dm^{-3}, indicating that the peptide is not in aggregated form. The fluorescence data suggest that the two pyrenes are fixed in close proximity, but owing to their geometry the excimer is not formed. The simple 14-peptide also showed only monomer emission.

These results in CD and fluorescence spectra suggest that the two α-helical segments containing Pya are arranged in a right-handed sense (Fig. 4). This arrangement is consistent with the orientation of the two α-helical segments in the coiled-coil form.[20] It is noteworthy that the bipyridyl peptide with the 14-peptides in antiparallel relationship [Bpy-14$_2$(*anti*)] did not show either any characteristic CD or a split CD in aqueous solution, though the ellipticity at 222 nm was −25 500 deg cm^2 dmol^{-1} (Fig. 2). This would be due to the fact that the two pyrene groups on the antiparallel α-helices are oriented in the opposite direction, because the side chains are directed to the N-terminal of each α-helix.[26] This fact strongly suggests that the two α-helical segments in the coiled-coil-like form are responsible for the split CD signals of the pyrene probe.‡[,2]

Spectral behaviour in methanolic solution

The CD and fluorescence spectra of both Bpy-14$_2$ and Sub-14$_2$

‡ Furthermore, the two α-helix peptide on the bipyridyl group with another 14-peptide (Ala-Leu-Glu-Gln-Lys-Pya-Ala-Ala-Leu-Glu-Gln-Lys-Leu-Ala-NHCH$_3$) did not show the split CD at neutral pH, but it appeared under basic conditions (pH > 10) (unpublished data, Mihara and Nishino). In this peptide, the foregoing amino acid prior to Pya is not Ala with a small side chain, but Lys, and pyrene side chains are placed under restraint conditions by the salt bridging of Lys and Glu between the two segments at neutral pH which is not adopted in Bpy-14$_2$. Under basic conditions, the salt bridge is cancelled, and then two pyrene rings are fixed at the positions to give the split CD. These data support the assumption that two pyrene rings are oriented around the preceding Ala side chains on the helix rods of Bpy-14$_2$ in a right-handed sense.

were measured in various MeOH contents (Figs. 5 and 6) to examine the helix–helix interaction under different hydrophilic conditions. With increasing MeOH content, the split CD signals were decreased and disappeared at 30–35% MeOH, although the α-helicities were not changed (Fig. 6). On the other hand, the excimer emission of pyrene in the fluorescence was increased and reached a maximum at 60% MeOH; then it began to decrease gradually to 100% MeOH (Fig. 6). These results indicate that the two pyrene groups fixed between the two α-helical segments are gradually untied and become mobile with the addition of MeOH [Fig. 6(*e*)]. The folded two α-helix structure is also unfastened with the addition of MeOH, resulting in the disappearance of CD splittings and an increase in the chance of excimer formation up to 60% MeOH. In MeOH, the two pyrene groups are completely apart from each other resulting in a significant decrease in the excimer. The α-helicity was maintained because of the stabilization of the α-helix by MeOH.

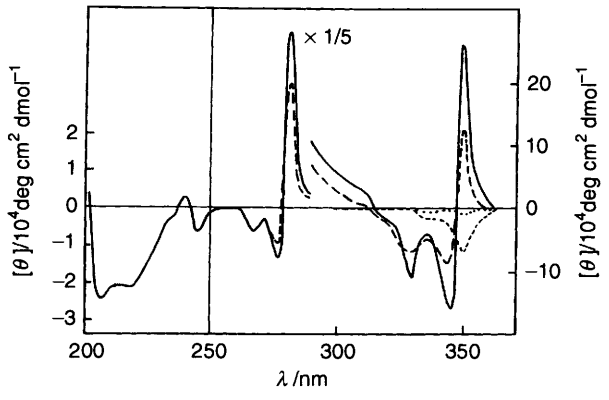

Fig. 5 CD spectra of Bpy-14$_2$ in 2.0 × 10^{-2} mol dm^{-3} Tris·HCl, pH 7.4 (——), 25% MeOH (— — —), 50% MeOH (– – – –) and MeOH (· · · ·). [θ] at the amide region is the mean residual weight ellipticity, and [θ] at the pyrene absorption region is the molar ellipticity for the peptide. Spectra at 250–290 nm are drawn to 1/5 scale. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}, 25 °C.

In comparing Bpy-14$_2$ with Sub-14$_2$, the split CD of Sub-14$_2$ disappeared at a lower MeOH content than did that of Bpy-14$_2$ (Fig. 6). This fact is consistent with the result that the side chains in Bpy-14$_2$ are better packed than those in Sub-14$_2$. It is noteworthy that at *ca.* 30% MeOH content in both peptides, where the split CD almost disappeared, the increase in the excimer emission was interrupted. This suggests the existence of a metastable state of the 3D structure such as a molten globule.[10] In such a metastable state, the secondary structure and the 2α-helix structure are maintained (α-helicity is not changed and excimer formation indicates that the two segments are still in close proximity), whereas the side chains are not fixed and are free to move (split CD disappeared) [Fig. 6(*e*)].

Effects of guanidine hydrochloride and temperature

The pyrene probe was further characterized by the behaviour under the disruption of the secondary structure by the addition of guanidine hydrochloride (GuHCl) (Figs. 7 and 8). The α-helix structures were stable to 4 mol dm^{-3} GuHCl and then became almost random structures at 7.5 mol dm^{-3}. The GuHCl concentrations at the half denatured structure ($C_{0.5}$) were 6.7 and 7.0 mol dm^{-3} for Bpy-14$_2$ and Sub-14$_2$, respectively. The α-helix structure is very stable toward GuHCl denaturation compared with native proteins such as other *de novo* designed proteins.[1,3] On the other hand, the free energies of folding in the absence of the denaturant ΔG_{H_2O} were −4.7 and −4.8, respectively, for Bpy-14$_2$ and Sub-14$_2$. The slight difference in these values between the two peptides is coincident with that in the α-helicities between the peptides. These values, however, are smaller than those of the designed proteins α2 (−12.8) and α4 (−22.5), but equivalent to that of helichrome (−4.4) and α-lactalbumin (−4.2).[1,3] It is important to note that the intensity of the split CD ([θ]$_{350}$) was decreased in parallel to the curve of [θ]$_{222}$ both in the cases of Bpy-14$_2$ and Sub-14$_2$. These facts indicate that under the conditions of GuHCl at more than 4 mol dm^{-3}, the secondary structure is gradually disrupted and the orientation of the pyrene side chains is cancelled in a parallel incident. When the secondary structure is significantly

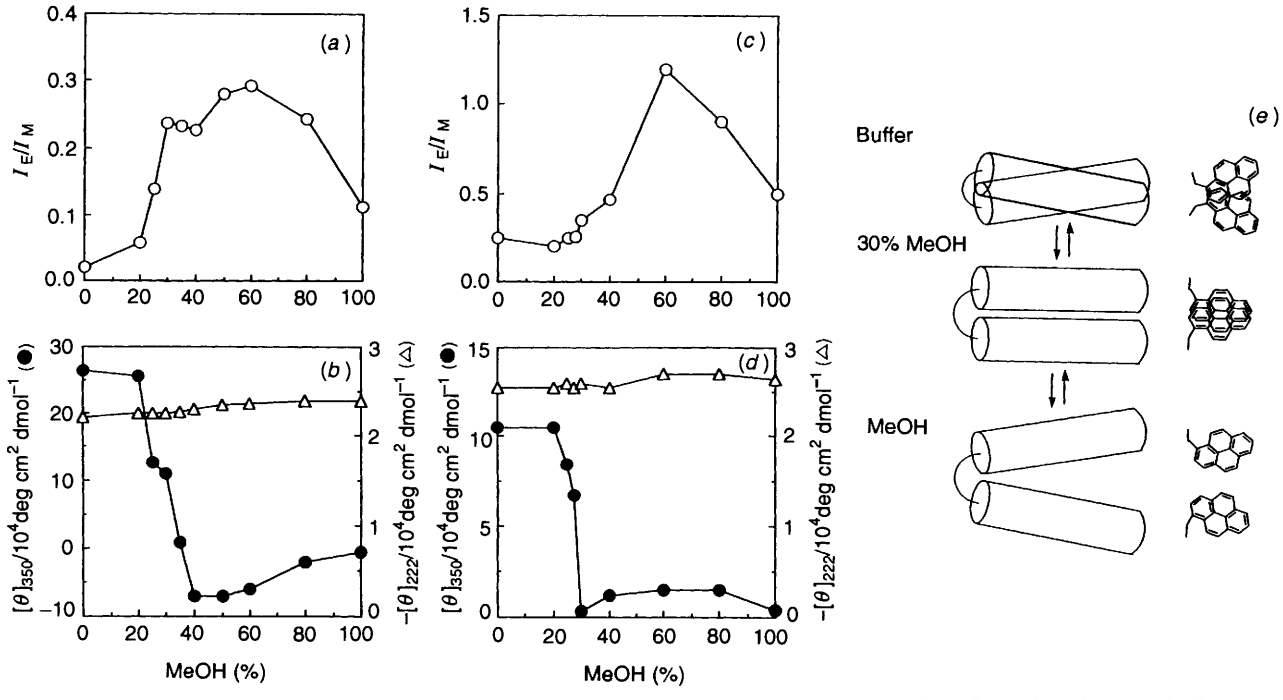

Fig. 6 Dependences of fluorescence and CD spectra on MeOH content. (*a*) and (*c*) Dependences of excimer formation of pyrenes for Bpy-14$_2$ and Sub-14$_2$, respectively. I_E/I_M denotes the ratio of fluorescence intensities at 480 nm to 395 nm. [Peptide] = 1.0 × 10^{-6} mol dm^{-3}. Excited at 342 nm at 25 °C. (*b*) and (*d*) Dependences of molar and mean residual weight ellipticities at 350 and 222 nm, respectively. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}, 25 °C. (*e*) Illustration of the conformation of the two α-helix peptide and interaction of two pyrenes under various conditions.

denatured at a GuHCl concentration of more than 6 mol dm^{-3}, compulsive orientation of the pyrene groups is lost, resulting in the disappearance of the split CD. Thus, the pyrene probe behaves in exact dependence on the peptide conformation.

The temperature dependences of the conformation and the pyrene probe were also examined from 3 to 70 °C (Figs. 7 and 9). Though the α-helix structure ($[\theta]_{222}$) was gradually decreased with the increase in temperature, the split CD signal ($[\theta]_{350}$) was decreased almost linearly with the increase in temperature; the half value was about 40 °C. The temperature dependence of the pyrene CD indicates that the secondary structure is not so disrupted by a temperature increase, although the side chains around Pya become mobile. On the contrary, at 3 °C the intensities of the splittings increased along with the increased α-helicities compared with those at 25 °C, indicating a more rigid orientation of Pya side chains in the structure at lower temperature.

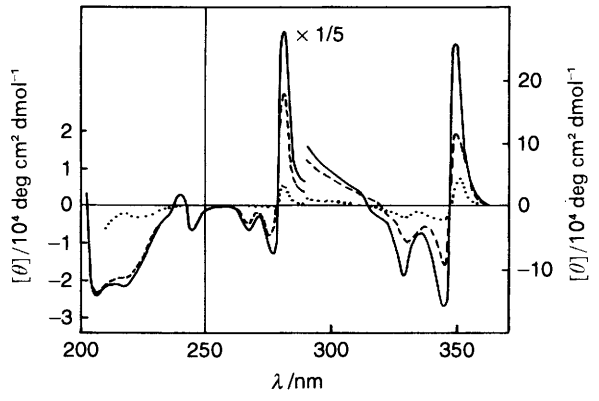

Fig. 7 CD spectra of Bpy-14_2 in 2.0 × 10^{-2} mol dm^{-3} Tris·HCl, pH 7.4 at 25 °C (——), at 60 °C (— — —) and in the presence of 7.5 mol dm^{-3} GuHCl at 25 °C (· · · ·). [θ] at the amide region is the mean residual weight ellipticity and [θ] at the pyrene absorption region is the molar ellipticity for the peptide. Spectra at 250–290 nm are drawn to 1/5 scale. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}.

Conclusions

It has been demonstrated that a pair of pyrene groups in a designed 2α-helix polypeptide probe the arrangement of the α-helices and the conformational changes very well under various conditions, though the 3D structure and the orientation of the pyrene groups should be precisely defined by NMR spectroscopy or X-ray crystallographic analysis. The sense in the arrangement of the two α-helix segments coincided with that in coiled-coil proteins. The probe represented the separation of the two α-helices by the addition of MeOH and the disruption of the conformation and of the side-chain packing by the addition of GuHCl and temperature increase. The pyrene probe in the two peptides behaved almost similarly under various conditions, though the anchor structures are different. The probe could be utilized for easy detection of the segment-arrangement in *de novo* designed artificial proteins. Furthermore, the resulting information on the pyrene arrangements will be applicable for designing the artifical proteins equipped with arranged functional chromophores in the defined 3D structure of polypeptides.

Experimental

Materials and methods

All chemicals and solvents were of reagent or HPLC grade. Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical Co. (Hiroshima, Japan). *p*-Nitrobenzophenone oxime resin was prepared according to the reported method.[21d,e] Solid-phase peptide synthesis was carried out manually in a glass vessel. Fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS-DX-300

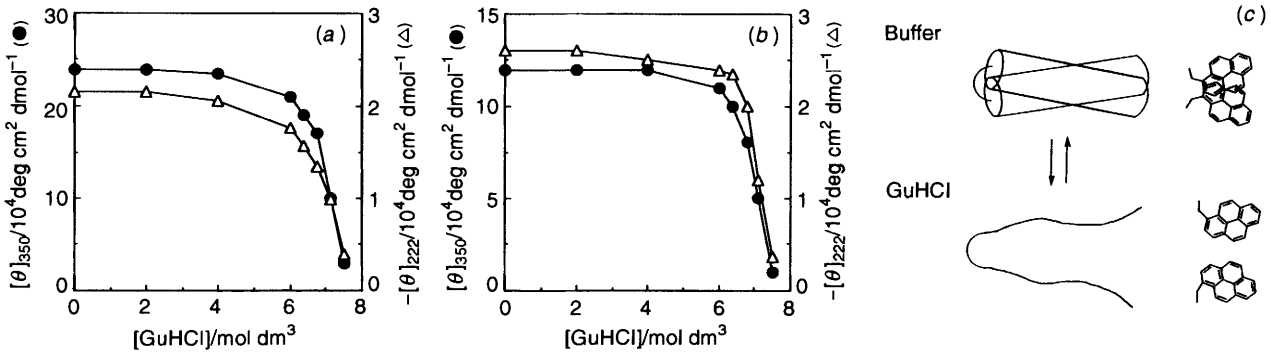

Fig. 8 Dependences of molar and mean residual weight ellipticities at 350 and 222 nm, respectively, on GuHCl concentration [(*a*) and (*b*) for Bpy-14_2 and Sub-14_2, respectively]. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}, 25 °C. (*c*) Illustration of the conformation of the two α-helix peptide and interaction of two pyrenes under the two conditions.

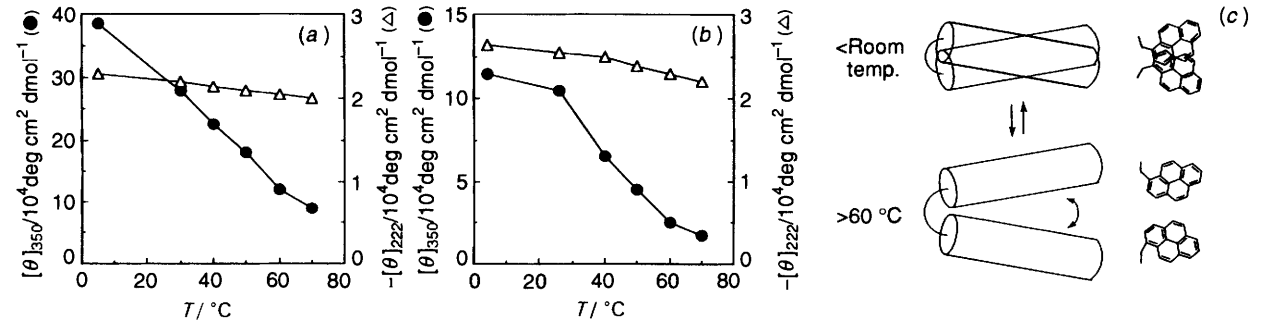

Fig. 9 Dependences of molar and mean residual weight ellipticities at 350 and 222 nm, respectively, on temperature [(*a*) and (*b*) for Bpy-14_2 and Sub-14_2, respectively]. [Peptide] = 3.0 × 10^{-5} mol dm^{-3}, 25 °C. (*c*) Illustration of the conformation of the two α-helix peptide and interaction of two pyrenes under the two conditions.

mass spectrometer equipped with JEOL JMA-3100 mass data analysis system by using dithiothreitol as a matrix and xenon for bombardment. Amino acid analyses were carried out on a JEOL JLC-300 system with ninhydrin detection after hydrolysis in 6 mol dm^{-3} HCl at 110 °C for 24 h in a sealed tube. HPLC was carried out on a MS-GEL C18 column (Asahi Glass, Tokyo) (4.6 × 150 mm or 10 × 250 mm) by employing a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4200 UV–VIS detector and a Hitachi D-2500 chromato-integrator. Solvent systems for HPLC are shown in the text. Thin-layer chromatography (TLC) was performed on Wakogel B-5 with indicated solvent systems.

***tert*-Butyloxycarbonyl-L-1-pyrenylalanine (Boc-Pya).** Several methods[17,18] have been reported for the preparation of L-Pya including asymmetric synthesis.[19a] For the simple and large scale (*ca.* 30 g) preparation of L-Pya, we developed the following procedure with optical resolution of Ac-DL-Pya using *Aspergillus* acylase. DL-Pya was prepared according to the reported method.[18,27]

Ac-DL-Pya.—To the solution of DL-Pya (87 g, 300 mmol) in 1 mol dm^{-3} NaOH (600 cm^3), acetic anhydride (45 cm^3, 450 mmol) was added in three parts over 1.5 h. The product was precipitated during the addition of acetic anhydride. The reaction was followed by TLC and, if necessary, 2 mol dm^{-3} NaOH (150 cm^3) and acetic anhydride (30 cm^3) were added. After 2 h, the pH was adjusted to 3 with 5 mol dm^{-3} HCl. The precipitates were filtered and washed with water. The product was dissolved in hot MeOH (1000 cm^3) and the insoluble materials were filtered off. The solution was evaporated and the residues were solidified with diethyl ether and light petroleum to give brown powder: 96 g (96%); TLC, R_f 0.66 ($CHCl_3$–MeOH–AcOH 50:10:2).

L-*Pya.*—Ac-DL-Pya (96 g, 290 mmol) was suspended in water (1000 cm^3) and the pH was adjusted to 7 with 2 mol dm^{-3} NaOH (65 cm^3) to dissolve. To the dark solution was added $CoCl_2 \cdot 6H_2O$ (250 mg) dissolved in water (5 cm^3) and *Aspergillus genus* acylase (15 g) (Tokyo Kasei) dissolved in water (100 cm^3), whose insoluble sand-like materials were filtered off. The solution was allowed to stand at 38 °C for 2 days. The precipitated L-Pya was collected and washed with water. Since the crude product contained small amounts of Ac-D(L)-Pya, the product was washed with MeOH several times to give purer L-Pya: 31 g (74%); TLC, R_f 0.32 ($CHCl_3$–MeOH–AcOH 50:10:2).

Boc-Pya.—To the suspension of L-Pya (29 g, 100 mmol) in water (120 cm^3) and dioxane (120 cm^3) were added Et_3N (21 cm^3, 150 mmol) and di-*tert*-butyl dicarbonate (26 g, 120 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 3 h and at room temperature overnight. After removal of dioxane, citric acid was added to give a solution at pH 3. The resulting product was extracted with ethyl acetate and washed with water, and then dried ($MgSO_4$). After evaporation, the residues were crystallized with diethyl ether and light petroleum. The off-white product was purified by silica-gel (Merck 60) chromatography ($CHCl_3$–MeOH–AcOH 90:10:2) to give a pure compound: 36 g (92%); TLC, R_f 0.94 ($CHCl_3$–MeOH–AcOH 50:10:2); $[\alpha]_D^{25}$ −73.3° (*c* 0.30 in MeOH) and −82.0° (*c* 0.30 in DMF);[19a] λ_{max}(MeOH)/nm 342 (ε/dm^3 mol^{-1} cm^{-1} 38 200), 327 (26 700), 313 (11 100), 277 (40 300), 266 (22 500), 256 (10 600), 243 (59 600) and 235 (37 700).

Synthesis of anchors

4,4′-Di(β-alaninocarbonyl)-2,2′-bipyridyl [Bpy(β-Ala)$_2$] and 3,3′-(hexamethylenedicarbonyldiamino)dipropionic acid [Sub(β-Ala)$_2$]. 2,2′-Bipyridyl-4,4′-dicarboxylic acid (1.0 g, 4.0 mmol) was refluxed with thionyl chloride (5 cm^3) for 3 h to give 2,2′-bipyridyl-4,4′-dicarboxyl chloride (100%). The obtained acid chloride and β-Ala-OMe·HCl (1.7 g, 12 mmol) were dissolved in DCM (30 cm^3) and then Et_3N (2.8 cm^3, 20 mmol) was added to the solution at 0 °C. The mixture was stirred at 0 °C for 2 h and at room temperature overnight. After addition of $CHCl_3$, the organic phase was washed with 4% $NaHCO_3$, 10% citric acid and water, and then dried ($MgSO_4$). After evaporation, the residues were solidified with methanol–diethyl ether: 960 mg, (58%); TLC, R_f 0.65 ($CHCl_3$–MeOH 5:1); FABMS *m/z* 415 [(M + H)$^+$]. The obtained 2,2′-bipyridyl-4,4′-dicarboxyl di-β-alanine methyl ester [Bpy(β-Ala-OMe)$_2$] (410 mg, 1.0 mmol) was saponificated with 1 mol dm^{-3} NaOH (3 cm^3) in MeOH and DCM (1:1, 10 cm^3) at room temperature for 1 h. After evaporation, the pH was adjusted to 3 with 1 mol dm^{-3} HCl. The resulting precipitates were collected and washed with a small amount of cold water to give Bpy(β-Ala)$_2$: 310 mg (80%); TLC, R_f 0.13 ($CHCl_3$–MeOH–AcOH 50:10:2); FABMS *m/z* 387 [(M + H)$^+$]. Sub(β-Ala)$_2$ was synthesized by the same method described above: FABMS *m/z* 317 [(M + H)$^+$].

4-β-Alaninocarbonyl-4′-(2-aminoethylamino)-2,2′-bipyridyl [Bpy(β-Ala)(Eda)]. 2,2′-Bipyridyl-4,4′-dicarboxylic acid chloride described above (500 mg, 2.0 mmol) was treated with a mixture of β-Ala-OMe·HCl (420 mg, 3.0 mmol) and mono-Boc-ethylenediamine hydrochloride (Boc-Eda·HCl) (600 mg, 3.0 mmol) in the presence of Et_3N (1.4 cm^3, 10 mmol) in DCM (10 cm^3) at 0 °C for 2 h and at room temperature overnight to give a mixture (400 mg) of three compounds, Bpy(β-Ala-OMe)$_2$, Bpy(Eda-Boc)$_2$ and Bpy(β-Ala-OMe)(Eda-Boc). The mixture was treated with 1 mol dm^{-3} NaOH (1.5 cm^3) in MeOH and DCM (1:1, 5 cm^3) at room temperature for 1 h. After addition of water, the aqueous phase was washed with $CHCl_3$. The pH of the aqueous phase was adjusted to 3, and the resulting precipitates were collected and washed with a small amount of cold water to give a mixture (200 mg) of Bpy(β-Ala-OH)$_2$ and Bpy(β-Ala-OH)(Eda-Boc). The mixture was treated with trifluoroacetic acid (TFA) (2 cm^3) at 0 °C for 30 min. After evaporation, the residues were solidified with diethyl ether. The Boc-removed mixture was dissolved in water and its pH was adjusted to 4. The solution was subjected to a column of Amberlite IR-45 (a weak anion exchange resin, AcO^- form, 10 × 200 mm). The desired compound Bpy(β-Ala)(Eda) was passed through the column. The fractions including the compound were pooled and evaporated, and then the residues were solidified with MeOH to give Bpy(β-Ala)(Eda): 80 mg, (11% total yield); TLC, R_f 0.10 (butan-1-ol–AcOH–pyridine–water 4:1:1:2); FABMS *m/z* 358 [(M + H)$^+$]. For condensation with peptides, Bpy(β-Ala)(Eda) was converted to Bpy(β-Ala)(Eda-Boc) with di-*tert*-butyl dicarbonate.

Peptide syntheses

Protected 7-peptides. Boc-Ala-X-Ala-Lys(ClZ)-Ala-Y-Ala-oxime resins (X, Y = Leu or Pya) (ClZ, 2-chlorobenzyloxycarbonyl) were synthesized manually by stepwise elongation of Boc-amino acid on a *p*-nitrobenzophenone oxime resin. The following procedure was repeated from Boc-Ala-oxime resin (2.0 g, 1.0 mmol Ala):[21d] (*i*) washing twice with DCM, (*ii*) washing with 25% TFA–DCM, (*iii*) deprotection with 25% TFA–DCM (30 min), (*iv*) washing twice with DCM, (*v*) washing with propan-2-ol, (*vi*) washing three times with DCM, (*vii*) washing with DMF, (*viii*) coupling Boc-AA (Boc-Ala, Boc-Leu·H_2O, Boc-Lys(ClZ) and Boc-Pya) (3.0 equiv.), BOP (3.0 equiv.), HOBt·H_2O (3.0 equiv.) and diprop-2-ylethylamine (DIEA) (5.0 equiv.) in DMF (30 min), (*ix*) washing three times with DMF, (*x*) washing twice with DCM. Solvents were 15 cm^3 g^{-1} resin. Coupling efficiency was checked by the Kaiser test.[28] To synthesize Ac-peptide resin, the Boc-deprotected peptide resin was treated with acetic anhydride (5.0 equiv.) and DIEA (2.0 equiv.) in DCM for 30 min.

To obtain the protected 7-peptide-OH, the peptide resin was shaken with HOPip (5.0 equiv.) in DMF for 2 days. The peptide

solution was filtered and washed twice with DCM–TFE (3:1). The solvent was evaporated and the residues (Peptide-OPip) were dissolved in the same solvent and acetic acid (1:1). To this solution sodium dithionite ($Na_2S_2O_4$) (5.0 equiv.) was added and the solution was stirred for 1 h. The solvent was evaporated and the residues were solidified with water to give protected 7-peptide-OH. Boc-7-peptide-OPip was obtained without reductive removal of Pip ester. To obtain Boc-7-peptide-$NHCH_3$, the peptide resin was shaken with $NHCH_3$·HCl (5.0 equiv.) in the presence of AcOH (5.0 equiv.) and DIEA (5.0 equiv.) in DMF for 2 days. The peptide solution was filtered and washed twice with DCM–TFE (3:1). The solvent was evaporated and the residues were solidifed with 10% citric acid and collected and washed with water.

All protected peptides were washed with hot MeOH to be purified to over 92% purity (yield 80–90%). The purity was checked on RP-HPLC (MS-GEL C4 column, 4.6 × 150 mm, with a linear gradient of 30–100% acetonitrile–0.1% TFA over 30 min). Peptides were identified by the molecular ion peak $(M + H)^+$ or $(M + Na)^+$ on FABMS; Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Pya-Ala-OH, *m/z* 1106 $[(M + Na)^+]$; Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-$NHCH_3$, *m/z* 939 $[(M + H)^+]$; Ac-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-OH, *m/z* 890 $[(M + Na)^+]$; Boc-Ala-Pya-Ala-Lys(ClZ)-Ala-Leu-Ala-OPip, *m/z* 1167 $[(M + H)^+]$.

Protected 14-peptides. *Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Pya-Ala-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-NHCH₃*.—Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-$NHCH_3$ (470 mg, 0.5 mmol) was treated with TFA (5 cm^3) at 0 °C for 30 min. After evaporation the residues were solidified with diethyl ether. The obtained H-7-peptide-$NHCH_3$·TFA (100%) and Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Pya-Ala-OH (540 mg, 0.5 mmol) were condensed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (190 mg, 1.0 mmol) in the presence of HOBt·H_2O (150 mg, 1.0 mmol) and Et_3N (0.10 cm^3, 0.75 mmol) in DCM–TFE (3:1)[22] (20 cm^3) at 0 °C for 2 days. The solvent was evaporated and the residues were solidified with water to give the protected Boc-14-peptide-$NHCH_3$. The crude 14-peptide was purified by washing with DMF: 740 mg (78%); FABMS *m/z* 1903 $[(M + H)^+]$.

Ac-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-Ala-Pya-Ala-Lys-(ClZ)-Ala-Leu-Ala-OH.—Ac-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-OH (430 mg, 0.5 mmol) and H-Ala-Pya-Ala-Lys(ClZ)-Ala-Leu-Ala-OPip·TFA obtained from Boc-7-peptide-OPip (550 mg, 0.5 mmol) were condensed in the same manner. The coupling solution was diluted with the same volume of AcOH and then $Na_2S_2O_4$ (5.0 equiv.) was added. The solution was stirred at room temperature for 1 h to give Ac-14-peptide-OH: 650 mg (71%); FABMS *m/z* 1854 $[(M + Na)^+]$.

2α-Helix peptides. *Bpy-14₂ and Sub-14₂*.—After removal of the Boc group of Boc-Ala-Leu-Ala-Lys(ClZ)-Ala-Pya-Ala-Ala-Leu-Ala-Lys-(ClZ)-Ala-Leu-Ala-$NHCH_3$ (290 mg, 0.15 mmol) with TFA at 0 °C for 30 min, the resulting H-14-peptide-$NHCH_3$·TFA was coupled with Bpy(β-Ala)$_2$ (20 mg, 0.05 mmol) with BOP (130 mg, 0.3 mmol) and HOBt·H_2O (46 mg, 0.3 mmol) with Et_3N (0.09 cm^3, 0.6 mmol) in DMSO (5 cm^3) at room temperature for 3 days. To the reaction mixture water and MeOH was added to give a crude protected peptide (300 mg). The protecting groups were removed with anhydrous HF in the presence of anisole at 0 °C for 60 min. The two-segment 28-peptide was purified by gel-filtration with Sephadex G-50 (10% acetic acid). The 28-peptide was eluted before the 14-peptide and the single-chained Bpy-14-peptide. The peptide Bpy-14_2 was further purified with RP-HPLC (MS-GEL C4, 10 × 250 mm, with a linear gradient of 60–80% acetonitrile–0.1% TFA over 20 min) to give the product with a single peak on analytical HPLC (MS-GEL C4, 4.6 × 150 mm with a linear gradient of 60–80% acetonitrile–0.1% TFA over 30 min): 48 mg (29%) based on the anchor; amino acid analysis $Ala_{16.0}$ (16), $Leu_{6.27}$ (6), β-$Ala_{1.80}$ (2), $Lys_{4.11}$ (4); λ_{max}(MeOH)/nm 342 (ε/dm^3 mol^{-1} cm^{-1} 69 600), 326 (46 600), 312 (25 400), 299 (20 000), 276 (86 600), 242 (149 000) and 233 (123 000). Sub-14_2 (52 mg, 33% yield) was synthesized by the same procedure using Sub(β-Ala)$_2$ (0.05 mmol): amino acid analysis $Ala_{16.0}$ (16), $Leu_{6.35}$ (6), β-$Ala_{1.77}$ (2), $Lys_{4.20}$ (4). The molecular weight of both peptides was estimated to be 4000 by gel-filtration on a Sephadex G-50 column (10% AcOH, 2.0 × 85 cm).

Bpy-14₂(anti).—Bpy(β-Ala)(Eda-Boc) (21 mg, 0.045 mmol) was coupled with H-Ala-Leu-Ala-Lys(ClZ)-Ala-Pya-Ala-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-$NHCH_3$·TFA (60 mg, 0.03 mmol) with BOP (45 mg, 0.1 mmol), HOBt·H_2O (15 mg, 0.1 mmol) and Et_3N (0.03 cm^3, 0.2 mmol) in DMSO (5 cm^3) at room temperature for 2 days. Water and MeOH was added to the reaction mixture to give the single-chain peptide, Bpy(β-Ala-14-peptide-$NHCH_3$)(Eda-Boc) (80 mg). After removal of the Boc group with TFA at 0 °C for 30 min, the obtained Bpy(β-Ala-14-peptide-$NHCH_3$)(Eda·TFA) was coupled with Ac-Ala-Leu-Ala-Lys(ClZ)-Ala-Leu-Ala-Ala-Pya-Ala-Lys(ClZ)-Ala-Leu-Ala-OH (80 mg, 0.045 mmol) with BOP (45 mg, 0.1 mmol), HOBt·H_2O (15 mg, 0.1 mmol) and Et_3N (0.03 cm^3, 0.2 mmol) in DMSO (5 cm^3) at 0 °C for 3 days. The protected Bpy-14_2(*anti*) was solidified by the addition of water and MeOH; 150 mg. The protecting groups were removed with anhydrous HF in the presence of anisole at 0 °C for 60 min and the crude peptide was purified in the same manner as described above: 17 mg (18%); amino acid analysis $Ala_{16.0}$ (16), $Leu_{6.08}$ (6), β-$Ala_{0.85}$ (1), $Lys_{4.23}$ (4).

CD measurements

CD spectra were recorded on a JASCO 500A spectropolarimeter equipped with Taiyo thermo supplier EZ-100 using a quartz cell with 1 mm pathlength. Peptides were dissolved in 2.0×10^{-2} mol dm^{-3} Tris·HCl buffer (pH 7.4), methanol–buffer or in the presence of GuHCl in peptide concentration of 3.0×10^{-5} mol dm^{-3}.

Fluorescence measurements

Fluorescence spectra were recorded on a Hitachi 650-10S fluorescence spectrophotometer. Peptides were dissolved in 2.0×10^{-2} mol dm^{-3} Tris·HCl buffer (pH 7.4) or methanol–buffer in peptide concentration of 1.0×10^{-6} mol dm^{-3}.

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