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α-Helical conformation in the C-terminal anchoring domains of *E. coli* penicillin-binding proteins 4, 5 and 6

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Abstract

The *E. coli* low molecular mass penicillin-binding proteins (PBP's) are penicillin sensitive, enzymes involved in the terminal stages of peptidoglycan biosynthesesis. These PBP's are believed to anchor to the periplasmic face of the inner membrane via C-terminal amphiphilic α-helices but to date the only support for this hypothesis has been obtained from theoretical analysis. In this paper, the conformational behaviour of synthetic peptides corresponding to these C-terminal anchoring domains was studied as a function of solvent, pH, sodium dodecyl sulphate micelles and phospholipid (DOPC, DOPG) vesicles using circular dichroism (CD) spectroscopy. The CD data showed that in 2,2,2-trifluoroethanol or sodium dodecylsulphate, all three peptides have the capacity to form an α-helical conformation but in aqueous solution or in the presence of phospholipid vesicles only those peptides corresponding to the PBP5 and PBP6 C-termini were observed to do so. A pH dependent loss of α-helical conformation in the peptide corresponding to the PBP5 C-terminus was found to correlate with the susceptibility of PBP5 to membrane extraction. This correlation would agree with the hypothesis that an α-helical conformation is required for membrane interaction of the PBP5 C-terminal region. © 1997 Elsevier Science B.V.

Keywords: Penicillin-binding protein; Amphiphilic α-helix; Membrane anchor; Circular dichroism

1. Introduction

The *E. coli* low molecular mass penicillin-binding proteins (PBP's) comprise PBP4, PBP5 and PBP6 and are penicillin sensitive enzymes which are believed to play a regulatory role in the terminal stages of peptidoglycan biosynthesis. These proteins possess DD-carboxypeptidase activity and additionally PBP4 has DD-endopeptidase activity [1]. The low molecular mass PBP's are targeted to the inner membrane via cleavable N-terminal signal sequences [2–4] and upon translocation become associated with the periplasmic face of the inner membrane. Generally, membrane associated proteins may be classified as either integral or non integral. Integral membrane proteins are anchored via single or multiple hydrophobic transmembrane segments [5] whilst non-integral membrane proteins are essentially water soluble but can weakly associate with the membrane

Abbreviations: PBP, penicillin-binding protein; P4, P5 and P6, peptides corresponding to the C-termini of PBP4, PBP5 and PBP6 (Table 1); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; SUV's, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol; CD, circular dichroism; SDS, sodium dodecylsulphate; PG, phosphatidylglycerol

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either directly through non-covalent interactions or more strongly via covalent modification [6,7]. However, it appears that the *E. coli* low molecular mass PBP's associate with the bilayer via a novel mechanism, a C-terminal amphiphilic α-helical anchoring domain [1].

Deletion analysis has indicated that the C-terminal 18 residues of PBP5 [3,8] and the C-terminal 19 residues of PBP6 [9] are essential for efficient membrane interaction and fusion of the C-terminal domain of PBP5 to the periplasmic protein, β-lactamase, resulted in a hybrid protein which was able to bind to the membrane [10]. When the amino acid residues of these C-terminal domains are represented as two dimensional axial projections [11] the resulting distribution of residues demonstrate the potential to form amphiphilic α-helices (Fig. 1). In the case of PBP5 the significance of the α-helix was further emphasised by the fact that incorporation of a proline residue, with its ability to disrupt or distort an α-helical conformation, destabilised the anchoring of PBP5 to the inner membrane [14]. A theoretical analysis of the primary sequences of the C-terminal regions of PBP5 and PBP6 has predicted that these domains would show an α-helix forming potential and surface

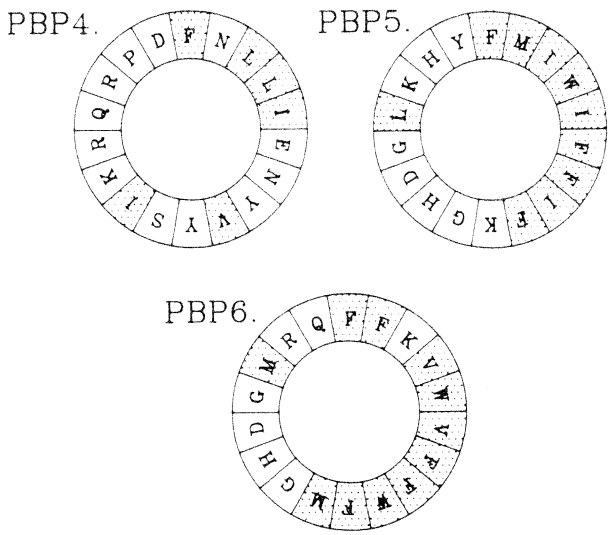

Fig. 1. Two dimensional axial projections of the C-terminal residues of the low molecular weight PBP's. When the C-terminal regions of PBP4 [12], PBP5 and PBP6 [13] are plotted as two dimensional axial projections [11] the general segregation of the hydrophobic (shaded) and the hydrophilic residues demonstrate the potential of these regions to form amphiphilic α-helical structures.

Table 1
The primary structures of the peptides P4, P5 and P6

Protein	C-terminal residues
PBP4	RRIPLVRFESRLYKDIYQNN-COOH
PBP5	GNFFGKIIDYIKLMFHHWFG-COOH
PBP6	GGFFGRVWDFVMMKFHQWFGSWFS-COOH

The peptides P4, P5 and P6 possess the primary structures of the C-terminal domains of PBP4, PBP5 and PBP6, respectively [12,13].

activity comparable to that of the polypeptide melittin, a toxin known to be active at the membrane interface [15]. The susceptibility of PBP5 and PBP6 to perturbants, particularly chaotropic agents such as the thiocyanate ion and urea, has suggested that the membrane association of these proteins is predominantly hydrophobic. Together, these data led to the generally accepted model for the membrane anchoring of PBP5 and PBP6 in which C-terminal amphiphilic α-helices lie at the membrane interface with their hydrophobic arcs interacting with the bilayer core [16,17].

The C-terminal 18 amino acid residues of PBP4 may be represented in the form of a two dimensional axial projection in a similar manner to those of PBP5 and PBP6 and this region exhibits the potential to form a weakly amphiphilic α-helix (Fig. 1). A theoretical analysis of the primary sequence of the PBP4 C-terminal region has predicted that this region has the potential to form either an α-helix or β-sheet structure with almost equal levels of amphiphilicity [18]. If the region was to adopt an α-helical conformation, the helix would be only weakly surface active compared to PBP5 and PBP6 [15]. The membrane interaction of PBP4 is highly susceptible to ionic perturbants, particularly sodium chloride. This implies that PBP4-membrane anchoring is predominantly electrostatic in nature which is in contrast to the mainly hydrophobic membrane binding of the other low molecular mass PBP's. Taken with the fact that overproduction of PBP4 results in only 5% of the protein being membrane bound compared to 100% in the cases of PBP5 and PBP6, this may indicate that PBP4-membrane interaction proceeds via a different mechanism to that of the other low molecular mass PBP's and may not involve the C-terminal domain of the protein (Harris and Phoenix, unpublished data).

As yet, the ability of the C-terminal regions of PBP4, PBP5 and PBP6 to form α-helices is untested. Here, circular dichroism (CD) is used to determine if synthetic peptides (Table 1), which possess primary structures corresponding to those of the C-terminal anchoring domains of the low molecular mass PBP's, are able to form an α-helical conformation; as a function of pH, solvent and membrane mimetic environments. These peptides correspond to regions identified by theoretical analysis as forming potential anchor domains [19].

2. Materials and methods

2.1. Materials

Synthetic peptides P4, P5 and P6, possessing primary structures which correspond to the C-terminal domains of PBP4, PBP5 and PBP6 (Table 1) respectively were purchased from the Department of Biochemistry, University of Liverpool. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Sigma. All solvents were of spectroscopic grade.

2.2. Preparation of phospholipid vesicles

Small unilamellar vesicles (SUV's) of DOPC and DOPG (phospholipid content 4.5 mM) were prepared according to Keller et al. [20]. The lipid–chloroform solutions were dried with nitrogen gas and hydrated with aqueous buffer pH 7.4 comprising 50 mM NaCl, 5 mM piperazine-*N*,N′-bis(2-ethane-sulphonic acid) (PIPES) and 1 mM ethylenediaminetetraacetic acid (EDTA). The resulting cloudy suspensions were ultra-sonicated at 4°C with a Soniprep 150 (amplitude 10 μm) until clear suspensions resulted (ca. 30 cycles of 30 s) which were then centrifuged (15 min, 3000 × g, 4°C).

2.3. Preparation of peptide solutions

Peptide solutions (0.1 mM) were prepared in either water, 5% (v/v) 2,2,2-trifluoroethanol (TFE) or 25 mM sodium dodecylsulphate (SDS). The peptides were also solubilised in SUV's of DOPC and DOPG to give a molar ratio of peptide to lipid of 1:50 [20].

2.4. Determination of CD spectra

CD spectra were recorded using a nitrogen flushed JASCO J720 Spectropolarimeter, employing a 4 s time constant, a 10 nm min^{-1} scan speed, a spectral bandwidth of 1 nm and a 0.02 cm cell pathlength. Spectra obtained from peptides in the presence of DOPC and DOPG vesicles were reported as $\Delta A = A_L - A_R$, since problems were encountered with the determination of peptide concentration. In all other cases, spectra were reported in terms of $\Delta\epsilon = \epsilon_L - \epsilon_R$ (M^{-1} cm^{-1}) based upon a mean molecular weight per amino acid residue in accordance with impending IUPAC–IUB recommendations. The estimation of secondary structure from CD data was analysed with GRAMS/386 using a principle component regression (PCR) method (Drake, unpublished data).

3. Results and discussion

Fig. 2 indicates that in aqueous solution P4 has an irregular structure and that P6, which has very low water solubility, possesses a spectrum characteristic of β-strand aggregation. However, P5 shows an estimated 29% of α-helical conformation in its structure. Fig. 4 shows that in a supramicellular concentration of SDS P5 shows a remarkably high α-helical content of 62% compared to 40% and 20% α-helical content for P6 and P4, respectively. In contrast, it can be seen from Fig. 3 that in the presence of TFE the structure of P6 shows 32% α-helical content whereas those of P5 and P4 show 28% and 25% respectively. These results show that these peptides possess the ability to adopt α-helical conformations depending upon their environment. This implies that the C-termini of PBP4, PBP5 and PBP6 have the ability to form amphiphilic α-helices although the level of helicity is dependent on the environment.

In vesicles of DOPG (Fig. 5) and DOPC (Fig. 6) P5 exhibited spectra characteristic of α-helical conformation. P6 showed a predominantly soluble α-helical conformation in the presence of DOPG vesicles (Fig. 5) but an irregular structure with DOPC vesicles (Fig. 6). With both vesicle types, P4 adopted a largely irregular structure (Figs. 5 and 6). Phospho-

lipid vesicles mimic membrane environments more appropriately than either TFE or SDS micelles. The fact that under membrane mimetic conditions P4 did not adopt an α-helical conformation may be interpreted to support the theoretical prediction that the PBP4 C-terminal region may not form an α-helix or would be only weakly surface active [15]. In contrast, P5 not only readily adopted an α-helical conformation in the presence of membrane mimetic vesicles but showed a strong tendency to do so in all cases examined (Figs. 2–7). This further supports the generally accepted idea that PBP5 associates with the membrane via an amphiphilic C-terminal α-helix [10,16] and that this membrane association has no requirement for the presence of anionic phospholipids

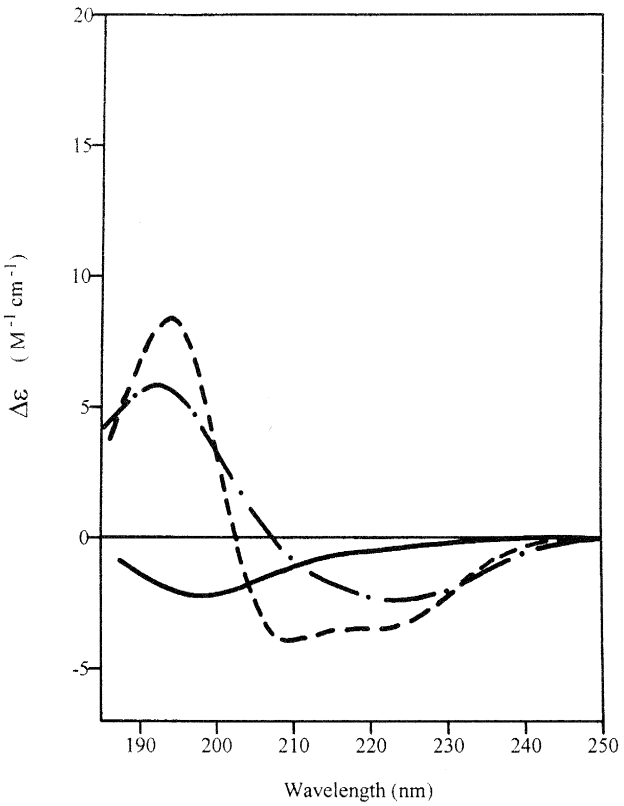

Fig. 2. The CD spectra of P4, P5 and P6 in aqueous solution. CD was used to determine the structures of P4 (solid line), P5 dashed line) and P6 (dash-dotted line) in aqueous solution, at their intrinsic pH. For 0.1 mM P5, this pH was measured as pH 4.1 and the peptide readily adopted an α-helical conformation which constituted 29% of the P5 structure. By contrast, P6 was sparingly soluble, exhibiting a β-strand structure typical of aggregation whereas 0.1 mM P4 was found to possess an intrinsic pH of 4.4 and demonstrated an irregular structure.

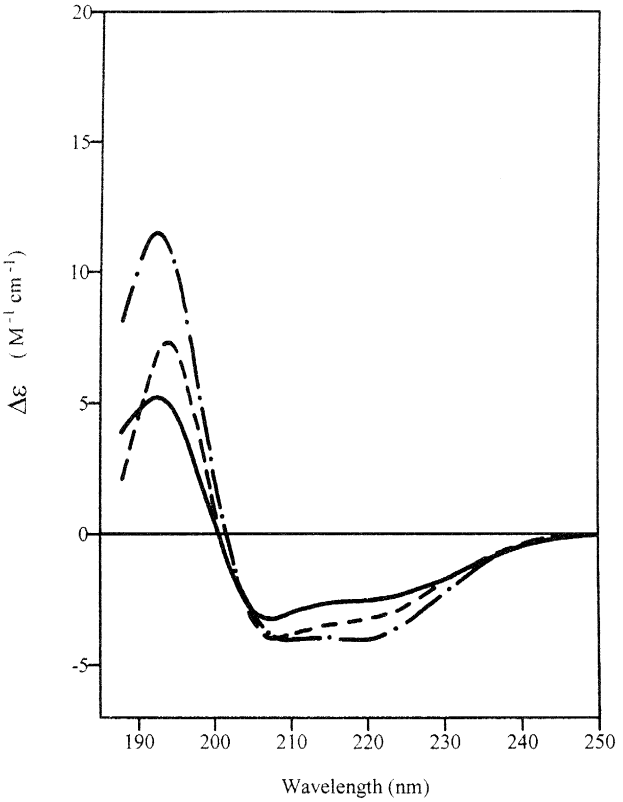

Fig. 3. The CD spectra of P4, P5 and P6 in aqueous TFE. CD was used to determine the structures of P4 (solid line), P5 (dashed line) and P6 (dash-dotted line) in 5% (v/v) TFE. At a concentration of 0.1 mM, the intrinsic pH of these peptide solutions were determined as pH 5.7, 4.1 and 4.9 and with estimated α-helix contribution to the peptide structures of 25%, 28% and 32%, respectively.

[21]. PBP6 possesses similar anchoring characteristics to PBP5 [17] and therefore, it might have been expected that the peptide P6 would have an α-helix forming capacity comparable to that of P5 which indeed was observed in TFE, supramicellular SDS and in the presence of DOPG vesicles (Figs. 3–5). However, unlike P5, in the presence of DOPC vesicles, P6 showed no α-helical content (Fig. 6) which indicates that there are differences between the two systems and indeed, despite its potential amphiphilicity, a higher level of hydrophobicity is associated with the structure of P6 [18]. This hydrophobicity is probably responsible for the low water solubility of P6 and the aggregation of the peptide in the presence of DOPC vesicles.

In aqueous solution, the conformation of P5 was found to be pH dependent (Fig. 7) whereas both P6

and P4 showed no pH dependence under the conditions tested. At pH 4.1, P5 shows a CD spectrum rich in α-helix conformation. With increasing pH, the α-helix content of P5 rises until at pH 6.2 a maximum is observed. Thereafter the α-helical contribution falls until at pH 8.3 and 10.3 peptide aggregation–precipitation accompanied by a large loss of α-helical structure is observed. At pH 11.6 the peptide regains solubility and the predominant conformation is α-helical. This model would be consistent with the proposal of Phoenix and Pratt [16], who suggested that upon translocation a pH related stabilisation of the PBP5 C-terminal region leads to α-helix formation. These latter findings correlate well with the pH dependent loss of α-helical content in P5 and together these data suggest that the strength of PBP5-membrane binding could be related to the α-helical content of the proteins C-terminal region, with high α-helicity corresponding to strong PBP5-membrane interaction.

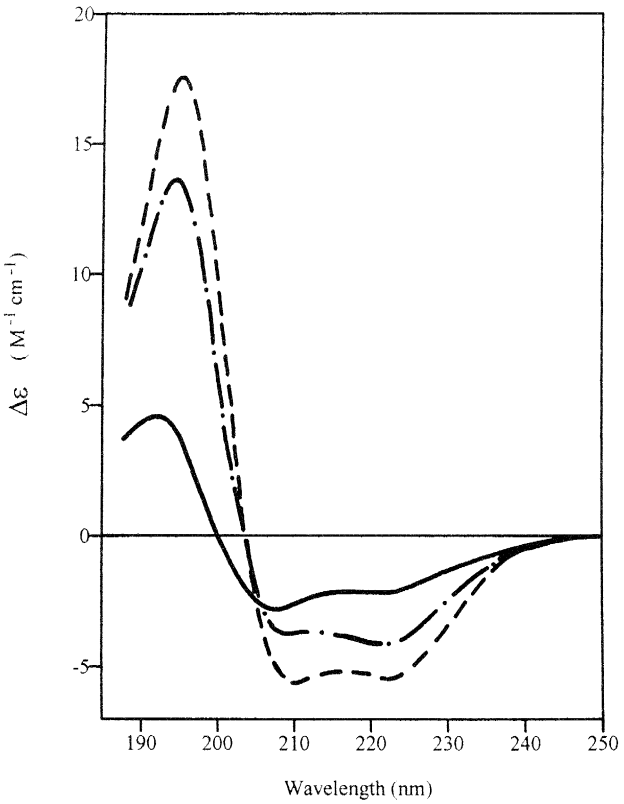

Fig. 4. The CD spectra of P4, P5 and P6 in the presence of SDS. CD was used to determine the structures of P4 (solid line), P5 dashed line) and P6 (dash-dotted line) in the presence of 25 mM SDS. All of the peptides were soluble and were able to adopt α-helical conformation. For each peptide at a concentration of 0.1 mM, the estimated α-helical contribution to their respective structures was 20%, 62% and 40%.

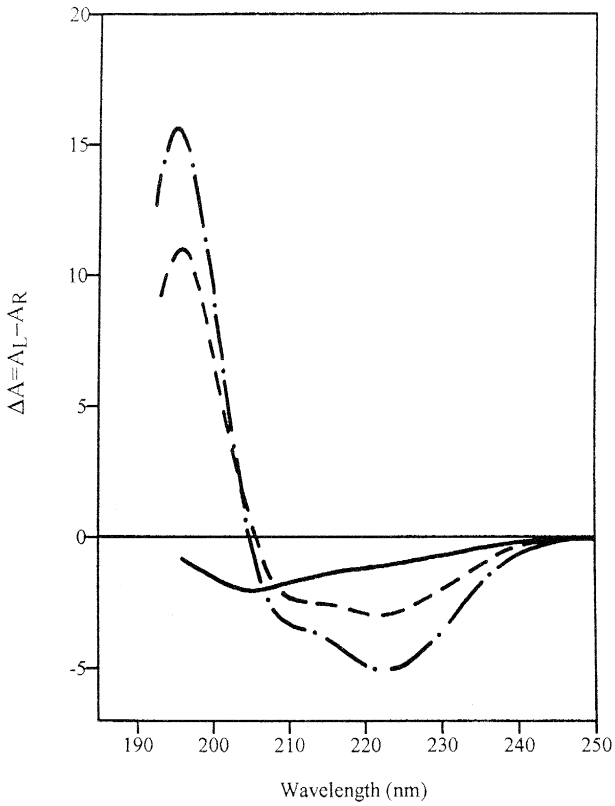

Fig. 5. The CD spectra of P4, P5 and P6 in the presence of DOPG vesicles. CD was used to determine the structures of P4 (solid line), P5 dashed line) and P6 (dash-dotted line) in the presence of SUV's of DOPG (phospholipid content 4.5 mM). P6 was not soluble under these conditions. P5 displayed a spectrum characteristic of α-helical conformation whereas that of P4 indicated a largely irregular structure.

In conclusion, for the first time, it has been shown directly that the amino acid residues comprising the C-terminal domains of PBP4, PBP5 and PBP6 have the capacity to adopt α-helical conformations. Despite this, in the presence of phospholipid vesicles, those residues corresponding to the PBP4 C-terminal domain formed no detectable α-helix. This is consistent with other data suggesting that this domain would only be weakly surface active and may not have a

role in the PBP4 anchoring mechanism. In contrast, a peptide corresponding to the C-terminal domain of PBP5 demonstrated a strong tendency towards α-helical formation in the presence of phospholipid vesicles and the pH dependent loss of α-helix in this peptide showed a correlation with the pH dependent susceptibility of PBP5 to membrane extraction [16]. This data is consistent with the hypothesis that an amphiphilic α-helical domain at the C-terminus of the protein is necessary for membrane interaction. A peptide corresponding to the C-terminal domain of PBP6 exhibited a tendency towards α-helical formation in the presence of SDS, TFE and vesicles formed from DOPG but not in the presence of DOPC. This would again be consistent with the hypothesis that the PBP6 C-terminus can form an α-helical anchor but suggests there may be differences between P5 and P6 which require further investigation.

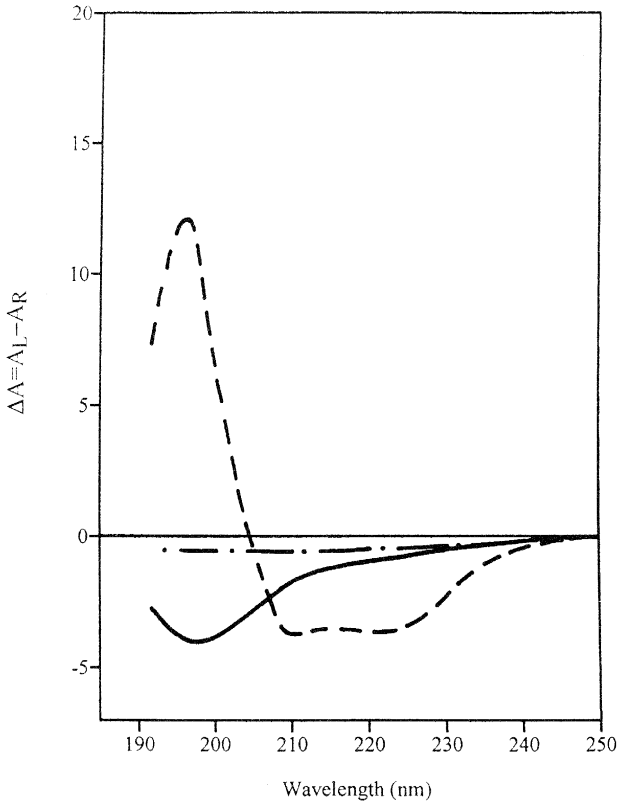

Fig. 6. The CD spectra of P4, P5 and P6 in the presence of DOPC vesicles. CD was used to determine the structures P4 (solid line), P5 (dashed line) and P6 (dash–dotted line) in the presence of SUV's of DOPC (phospholipid content 4.5 mM). P5 exhibited a spectrum indicating the presence of α-helical conformation whereas those of P4 and P6 did not.

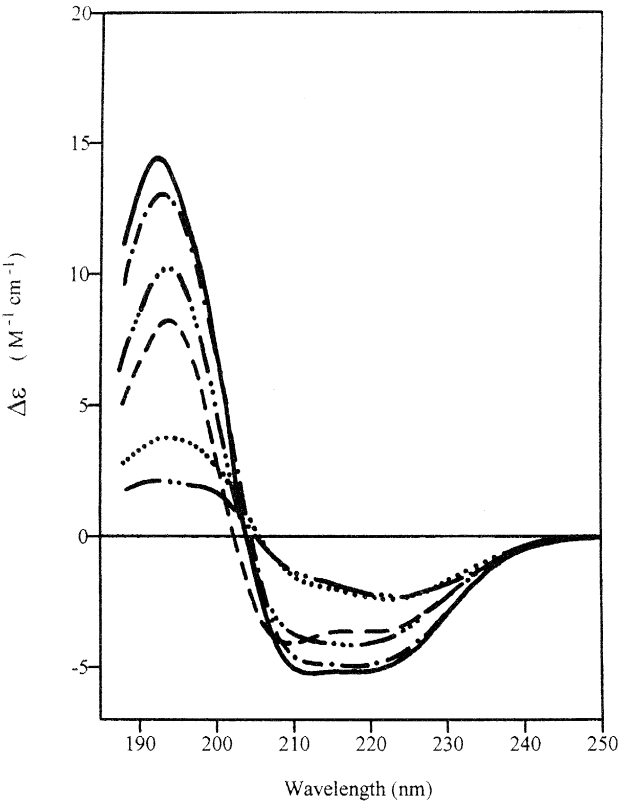

Fig. 7. The CD spectra of aqueous P5 as a function of pH. CD was used to determine the structure of 0.1 mM P5 at pH 4.1 (dashed line), pH 6.2 (solid line), pH 7 (dash–dot line), pH 8.3 (dash–dot–dot line), pH 10.3 (dotted line) and pH 11.6 (dash–dot–dot–dot line). At pH 4.1, the intrinsic pH of the peptide, α-helical structure was indicated. As pH was increased, significant β-strand contributions to the P5 structure were indicated by the red-shifting of the spectra cross-over points (at ca. 205 nm). At pH 6.2, a maximum in the α-helix content occurred which then decreased with increasing pH until at pH 8.3 and 10.3, the major contribution to P5 structure was from β-strand conformations and precipitation of the peptide occurred. At pH 11.6, P5 regained solubility and the α-helical contribution to structure predominated.

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