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Conformations of Peptides Corresponding to Fatty Acylation Sites in Proteins

A CIRCULAR DICHROISM STUDY*

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Fatty acid acylation is a posttranslational modification found in membrane proteins that have hydrophobic sequences serving as transmembrane segments as well as those that do not have them. The fatty acids myristate and palmitate are linked through an amide bond to N-terminal glycine and SH of cysteine via a thioester bond, respectively. In order to elucidate whether or how fatty acid acylation would modulate peptide structure, especially in hydrophobic environment, we have carried out circular dichroism studies on synthetic peptides both hydrophobic and hydrophilic in nature, corresponding to fatty acylation sites and their fatty acyl derivatives. The hydrophilic peptides were ~12 residues in length as studies on proteins modified by site-directed mutagenesis indicated that a peptide segment of ~12 residues is sufficient to direct acylation as well as membrane association, especially when the fatty acid is myristic acid. The peptide corresponding to a transmembrane segment composed of 31 residues as well as its palmitoyl derivative was found to adopt α-helical structure. Acylation appeared to favor increased partitioning into micelles even in the case of a hydrophobic peptide. The hydrophilic peptides and their myristoyl or palmitoyl derivatives showed very little ordered structure in micelles. Our results suggest that the myristoyl and the palmitoyl moieties do not have the ability to "force" a hydrophilic peptide segment into a hydrophobic micellar environment. Thus, the mere presence of a fatty acid moiety may not be sufficient for membrane binding and recycling as is assumed especially in proteins in which no hydrophobic segment is present.

It is now becoming increasingly clear that small, linear peptide fragments of proteins, in aqueous solution, can exhibit defined conformational preferences for structures other than random-coil structure (1–8). Many structures like nascent helix (1, 2), reverse turns (4, 5), and ordered helix (6) have been characterized in short peptides. In fact, a 9-residue peptide corresponding to the internalization signal of low density lipoprotein receptor was found to adopt a reverse turn conformation in solution, correlating well with the proposal that internalization signals in coated pit receptors is a β-turn conformational motif (4, 5). This suggests that inference of structure drawn from studies on isolated short peptides could have relevance in biological processes. Apart from examining conformational preferences in water, the solvent trifluoroethanol (TFE)[1] has been used extensively in studying the conformation of peptides, especially while examining helix propensities (9). The solvent stabilizes the helical structure in peptides. In circular dichroism studies, this solvent is particularly useful in the case of peptides that exhibit limited solubility in water.

Fatty acid acylation is a posttranslational modification found in membrane proteins which have hydrophobic sequences serving as transmembrane segments as well as in proteins like Ras p21, which is relatively hydrophilic with almost all of its structure in the cytoplasm (10–12). The fatty acids, myristate and palmitate, are linked through an amide bond to N-terminal glycine and SH of cysteine via a thioester bond, respectively. In order to elucidate whether or how this fatty acid acylation would modulate peptide structure especially in hydrophobic environment, we have carried out circular dichroism studies on synthetic peptides corresponding to fatty acylation sites in proteins and their fatty acyl derivatives (Table I). We have synthesized peptides with myristic acid at the N-terminal glycine and with palmitic acid at the cysteine residue. The peptide sequences were chosen from fatty acylated proteins having hydrophobic membrane-spanning segments as well as hydrophilic sequences. Peptide 31R corresponds to the transmembrane segment of the vesicular stomatitis virus G protein (13), whereas peptides 12R, 14R, and 11R correspond to acylation sites in oncogene proteins. Peptide 12R corrresponds to the C-terminal end of the Ras protein (14), 14R corresponds to N-terminal region of the mouse mammary tumor virus (15), and 11R corresponds to the N-terminal portion of the oncogene product of HIV F/3′ orf, phosphorylated GTP-binding protein (16). Studies on proteins modified by site-directed mutagenesis (17, 18) as well as chimeric proteins (19) have indicated that peptide segments of ~12 residues are required to direct acylation as well as membrane association, especially when the fatty acid is myristic acid. Hence we have chosen peptides composed of 11–14 residues, except in the case of 31Pal where the transmembrane spanning region has been included, resulting in a peptide composed of 31 residues.

EXPERIMENTAL PROCEDURES

Materials

Amino acids, dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, fatty acids, and ethanedithiol were purchased from Sigma, solvents were from Merck India, trifluoroacetic acid, thioanisole, and *m*-cresol were from Fluka. All Fmoc-protected amino acids were from Novabiochem, Nottingham, United Kingdom.

Methods

Peptide Synthesis—All peptides were synthesized by solid phase procedures. Peptide 31R (Table I) was synthesized using 2% hexane

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[1] The abbreviations used are: TFE, trifluoroethanol; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; OG, octyl glucoside.

TABLE I
Amino acid sequences of peptides corresponding to fatty acylation regions in proteins
Acm, acetamidomethyl; Pal, palmitoyl; Myr, myristoyl.

WKSSIASFFFIIGLIIGLFLVLRVGIKLC(Acm)IK	31R	Ref. 13
WKSSIASFFFIIGLIIGLFLVLRVGIKLC(Pal)IK	31Pal	Ref. 13
WKTPGC(Acm)VKIKKA	12R	Ref. 14
WKTPGC(Pal)VKIKKA	12Pal	Ref. 14
GLIWLLLLSLLEPS	14R	Ref. 15
Myr-GLIWLLLLSLLEPS	14Myr	Ref. 15
GGKWSKSSVVG	11R	Ref. 16
Myr-GGKWSKSSVVG	11Myr	Ref. 16

dioldiacrylate-cross-linked polystyrene resin (anchoring capacity of 2.32 meq/g of the resin) using *t*-butyloxycarbonyl chemistry by procedures described earlier (20). The attachment of the first amino acid to the resin was done by the cesium salt method of Gisin (21). The synthesis was carried out in a vessel described by Stewart and Young (22). Synthesis of peptides 12R, 14R, and 11R (Table I) were performed on a Pharmacia semi-automated synthesizer by Fmoc chemistry using Nova syn KA resin from Novabiochem. The first amino acid was attached to the resin by symmetric anhydride method (23). The acylation of the cysteine SH with palmitoyl chloride and of the N-terminal glycine with myristoyl chloride was carried out when peptides were attached to the resin, as described earlier, (24). After acylation, acidolytic cleavage of the peptides from the resin was done to obtain the acylated peptides.

The peptides with the exception of 31R and 31Pal were purified by fast performance liquid chromatography on a reverse phase pep RPC 5/5 (Pharmacia) column. Peptides 31R and 31Pal were purified by thorough washing with methanol and acetonitrile. Both the peptides exhibited limited solubility in these solvents and could be easily separated from the impurities which were soluble in methanol and acetonitrile The purity and composition of the peptides were determined by amino acid analysis on a LKB 4151 Alpha Plus amino acid analyzer, after hydrolysis *in vacuo* with trifluoroacetic acid, 6 N HCl (1:2). The purity of the nonacylated peptides was further confirmed by sequencing on 473A protein sequencer (Applied Biosystems). The presence of fatty acids was confirmed by analysis on a Hewlett-Packard 5840A gas-liquid chromatograph, after hydrolysis.

Circular Dichroism (CD) Measurements—Spectra were recorded on a Jobin-Yvon Dichrograph V spectropolarimeter in cells of 0.1-cm path length at 22 °C. The spectropolarimeter was calibrated with (±)-10-camphorsulfonic acid using θ_M at 290 nm = 7600 (25).

All peptide stock solutions were made in methanol (MeOH) and dimethyl sulfoxide (Me_2SO) and were quantitated by OD at 280 nm and quantitative amino acid analysis. Aliquots of required concentrations of peptide stock solution were transferred to test tubes and dried in vacuum. Spectroscopic grade TFE was added to make up the required concentration just before recording the spectra. For experiments in micelles, appropriate volumes and concentrations (above the critical micellar concentration of 8 mM sodium dodecyl sulfate (SDS) and 0.64% octyl glucoside (OG)) of detergent solution were added after drying the sample in a test tube, prior to recording of the spectra. The concentrations of SDS and OG for the experiments were 20 mM and 1% (w/v), respectively.

Spectra were deconvoluted by convex constraint analysis as described by Perczel *et al.* (26). For all the spectra, 15 iterations were done.

RESULTS

The conformations of peptides were examined in TFE, a solvent known to promote helical conformation in peptides with helical propensity (2, 6), micelles of SDS which provide a simple hyrophilic/hydrophobic interface mimicking a membrane environment, octyl glucoside, a non-ionic detergent, and dioleoly phosphatidylcholine lipid vesicles.

Peptides with Palmitoyl Modification—The spectra of 31R and the palmitoylated peptide 31Pal in TFE and SDS are shown in Fig. 1. They are characterized by minima at ~207 and 222 nm with a crossover at ~204 nm. The θ_{207} and θ_{222} values and the appearance of the spectra argue for an α-helical conformation for the peptide. Results of convex analysis of the CD data shown in Fig. 1 are presented in Fig. 2. Only two pure components were obtained for the peptides. In TFE, both the peptides have a helical content ~66% with 34% unordered conformation. In SDS micelles, 31Pal shows increase in helical content as compared with 31R. The nonhelical component in Fig. 2, *b* and *d*, do not correspond to β-sheet or β-turn conformations. Using the description of Perczel *et al.* (26), we assign this component to additional chiral contribution. Palmitoylation appears to favor increased binding of 31Pal to SDS micelles as compared with 31R.

The spectra of 31R and 31Pal in OG micelles and results of spectral deconvolution are shown in Fig. 3. Peptide 31R shows a single minimum ~216 nm. A pure component with contribution of ~72% has very similar appearance to the experimentally obtained spectrum. The component contributing ~28% is not characteristic of helix, turn, or β-sheet conformations. The θ_M value of ~35,000 at the minimum argues against a β-sheet conformation that would result in $\theta_M = -18{,}000$. However, distorted helices (27) do exhibit a single minimum with $\theta_M < -18{,}000$. Hence it appears that the structure of 31R is that of a distorted helix in neutral micelles. The spectrum of 31Pal is typical of a helical structure that is also reflected in the "pure" component spectra.

The spectra of 12R and 12Pal in TFE and SDS are shown in Fig. 4. The spectrum of 12R in TFE shows a minimum at ~200 nm and a shoulder at ~225 nm. The θ_M values and the crossover that would be below 195 nm argue against the peptide existing in a predominantly ordered conformation. However, the spectra do not resemble those of "classical" random structure (28). The peptide has the sequence TPGC, which could form a β-turn (29, 30). In SDS, although the general appearance is similar to that in TFE, the θ_M value at the minimum is considerably more. The spectra of the acylated peptide 12Pal in TFE and SDS micelles are shown in Fig. 4, *c* and *d*. In TFE, the spectrum shows a minimum at ~215 nm with a crossover at ~197 nm. The spectra may be assigned to a β-turn conformation with residues TPGC participating in the turn. Acylation, here, seems to increase the proportion of peptide in a β-turn conformation. The spectrum in SDS is very much similar to that in TFE. Results of the convex analysis of CD curves shown in Fig. 4 are presented in Fig. 5. The spectra of 12R in TFE yield four pure components (Fig. 5*a*). *Curve I* is characteristic of unordered conformation and is present to an extent of ~ 41%. *Curves II* and *III* may be assigned to β-turn conformations. Their contributions to the structure are 33 and 11%, respectively. *Curve IV* cannot be unequivocally assigned to α-helix, β-sheet, or β-turn conformation. In SDS, Fig. 5*b* indicates a random component of ~44%. Although *curves II* and *III* cannot be unambiguously assigned, component IV may be assigned to β-turn conformation. Fig. 5*c* indicates a decrease in the extent of random conformation for 12Pal in TFE. The pure spectra indicate a β-turn contribution of 52% (*i.e. curves II* and *III*). Thus, palmitoylation at the middle of the peptide chain seems to favor β-turn formation. Surprisingly, acylation does not appear to favor β-turn conformation in the micellar environment of SDS as the spectrum (Fig. 4*d*) is very similar to 12R in SDS (Fig. 4*b*). The spectra of 12R and 12Pal in OG micelles were characteristic of peptides in unordered conformation. This sug-

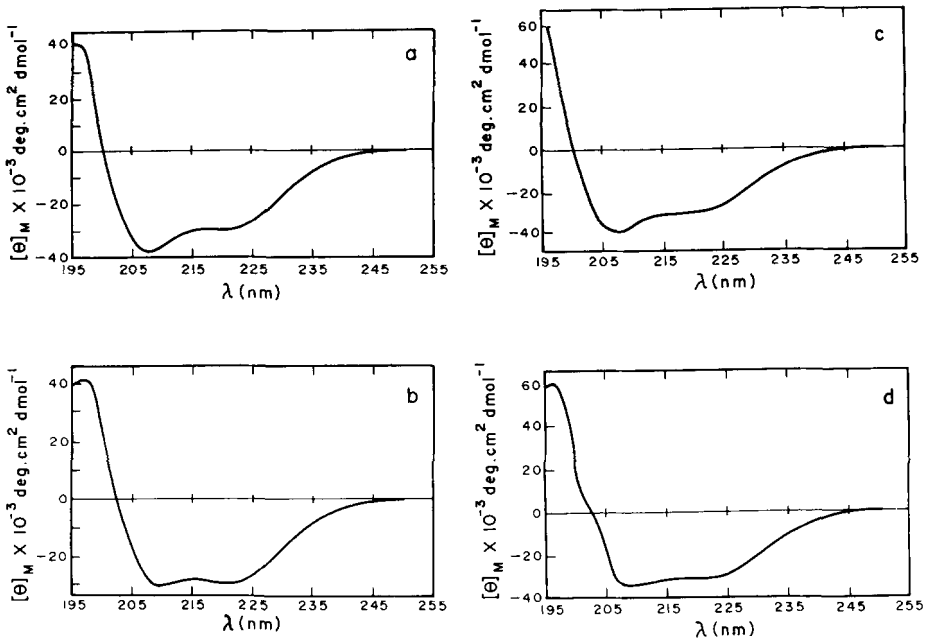

FIG. 1. **CD spectra of peptides 31R and 31Pal.** *a*, 31R in TFE; *b*, 31R in SDS micelles; *c*, 31Pal in TFE; *d*, 31Pal in SDS micelles. Peptide = 0.05 mM.

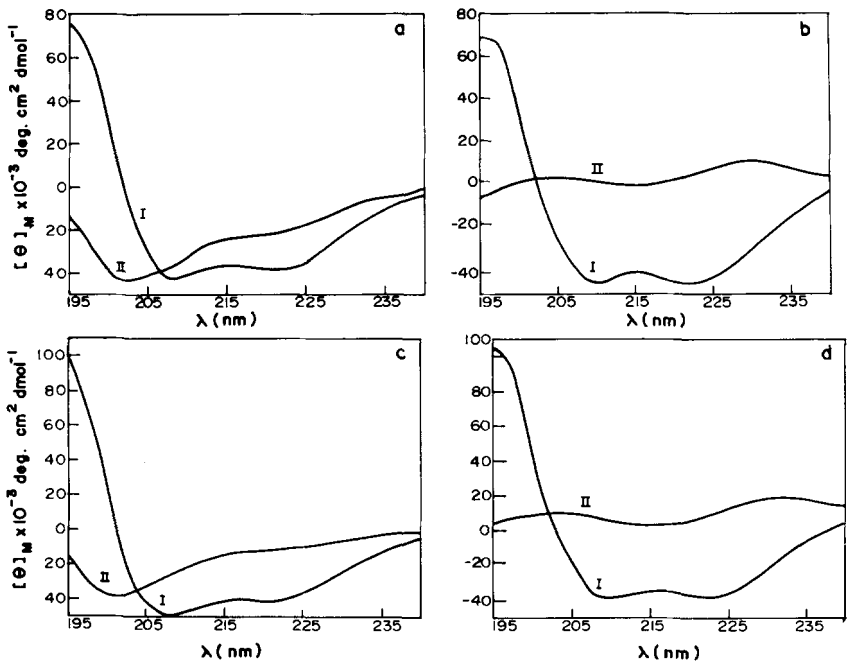

FIG. 2. **Pure components obtained after convex constraint analysis of the CD spectra of peptides 31R and 31Pal.** *a*, 31R in TFE: *curve I*, 74%; *curve II*, 32%. *b*, 31R in SDS: *curve I*, 66%; *curve II*, 33%. *c*, 31Pal in TFE: *curve I*, 66%; *curve II*, 34%. *d*, 31Pal in SDS; *curve I*, 85%; *curve II*, 15%.

gests that although acylation would result in increased hydrophobicity of the peptide and consequently more favorable partitioning into micelles, it does not happen. Hence, the mere presence of the fatty acid may not be sufficient for association with membranes especially when the peptide chain in the vicinity of the acylation site is hydrophilic.

Peptides with Myristoyl Modification—The spectra of 14R in TFE and SDS are shown in Fig. 6, *a* and *b*. The spectra show two negative bands with a crossover at ~200 nm, which is characteristic of predominantly helical conformation. The spectra suggest that the peptide is considerably more ordered in SDS as compared with TFE. The spectra of the N-terminally myristoylated peptide 14Myr are shown in Fig. 6, *c* and *d*. The TFE spectrum is very similar to that of the nonacylated peptide. However, the SDS spectrum, though having two negative bands, has lower θ_M values compared with the nonacylated peptide. Convex constraint analysis of spectra in Fig. 6 is shown in Fig. 7. The results presented in Fig. 7, *a* and *b*,

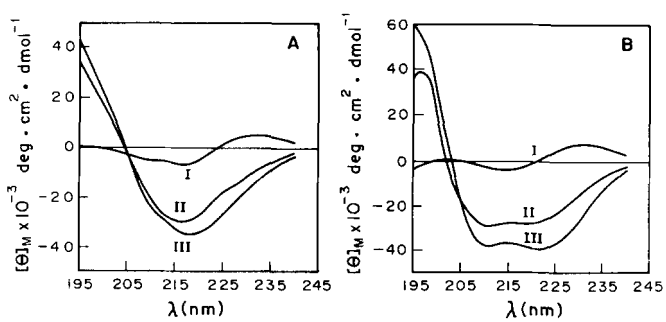

FIG. 3. **CD spectra of peptides 31R and 31Pal in octyl glucoside micelles and the pure components obtained after convex constraint analysis of the CD spectra.** *A*, 31R: *curve I*, 28%; *curve II*, experimental spectra; *curve III*, 72%. *B*, 31Pal: *curve I*, 25%; *curve II*, experimental spectra; *curve III*, 75%. Peptide = 0.05 mM.

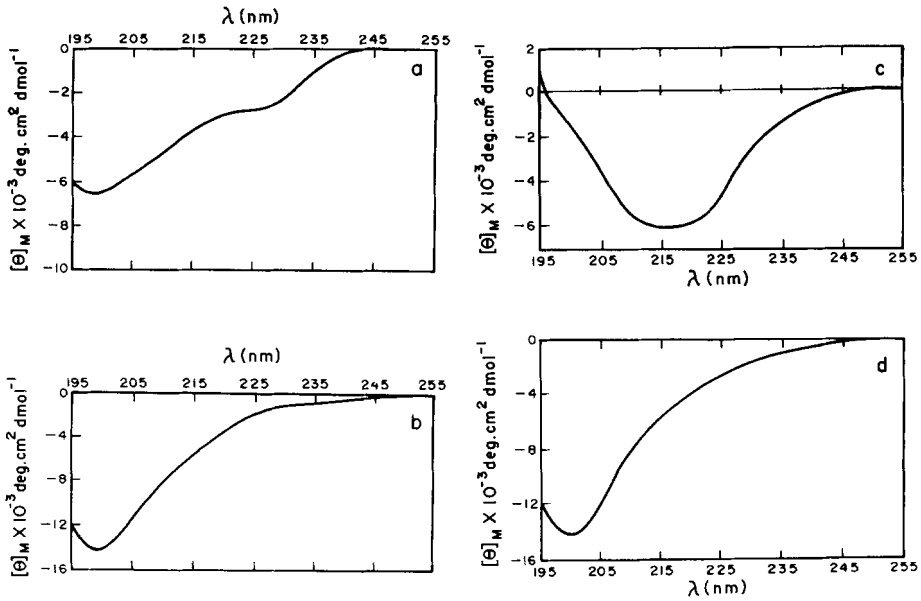

FIG. 4. **CD spectra of peptides 12R and 12Pal.** *a*, 12R in TFE; *b*, 12R in SDS micelles; *c*, 12Pal in TFE; *d*, 12Pal in SDS micelles. Peptide = 0.1 mM.

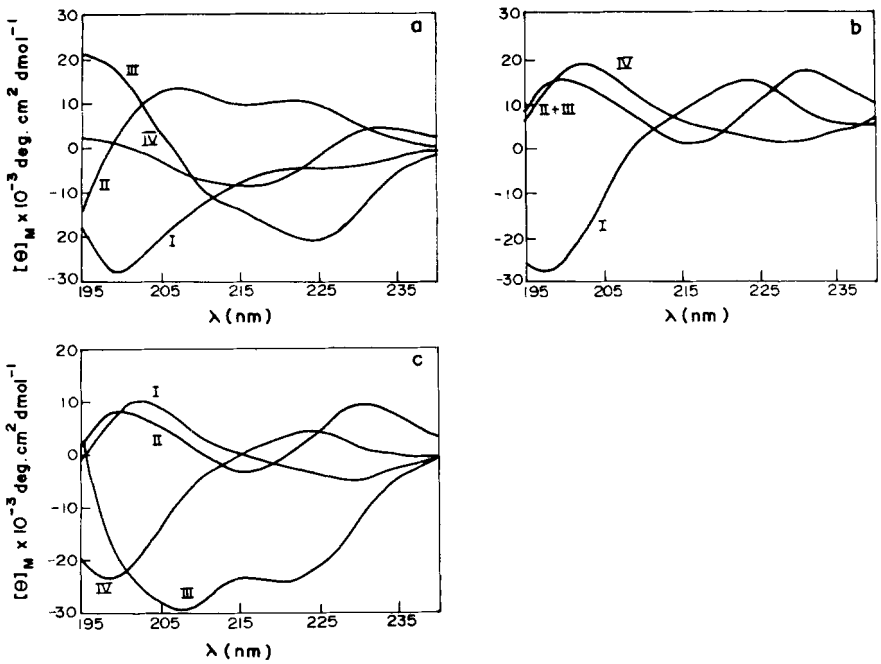

FIG. 5. **Pure components obtained after convex constraint analysis of the CD spectra of peptides 12R and 12Pal.** *a*, 12R in TFE: *curve I*, 41%; *curve II*, 33%; *curve III*, 11%; *curve IV*, 10%. *b*, 12R in SDS: *curve I*, 44%; *curves II + III*, 39%; *curve IV*, 18%. *c*, 12Pal in TFE; *curve I*, 36%; *curve II*, 20%; *curve III*, 16%; *curve IV*, 21%.

indicate predominantly helical conformation for 14R with increase in helical content in SDS as compared with TFE. Component III in Fig. 6*a*, though not representative of a typical α-helical spectrum, could conceivably result from a distorted helical spectrum. The deconvoluted spectra of 14Myr in TFE indicates considerable conformational heterogeneity. β-Turn components (III and IV) appear to be substantial components in addition to α-helical conformation. Fig. 7*d* indicates that there is no increase in helical content in SDS but increased contribution from turn and even possibly β-sheet conformation. Although a visual comparison of Fig. 6, *b* and *d*, would indicate a decrease in ordered structure on myristoylation, deconvolution indicates that there is actually a *decrease* in percentage random conformation in micelles. While in 14R, only α-helical conformation is discernible in SDS, 14Myr, in SDS shows conformational heterogeneity with contribution from α-helix, β-turn, and β-sheet. The spectra of 14R and 14Myr in OG micelles were very similar to those in SDS micelles. Thus myristoylation at the N terminus clearly modulates conformation of the peptide in TFE and micelles.

The spectra of 11R in TFE and SDS are shown in Fig. 8, *a* and *b*. It is evident that only a fraction of the peptide exists in ordered conformation. The small fraction of ordered conformation could conceivably be a β-turn structure. The spectra of the myristoylated peptide 11Myr shown in Fig. 8, *c* and *d*. are very similar to those of the nonacylated peptide. Results of convex constraint analysis of the CD spectra in Fig. 8 are shown in Fig. 9. The pure components and weights indicate predominantly unordered conformation (~52%). The curves indicate that the residual ordered conformation consists of β-turns. Myristoylation does not appear to be sufficient for partitioning of 11Myr into micelles. CD spectra indicated that peptides 11R and 11Myr did not partition into OG micelles.

Peptides 31R, 31Pal, 14R, and 14Myr yielded spectra in micelles as shown in Figs. 1 and 6 only when the concentration of the detergent was above the critical micellar concentration. Below critical micellar concentration precipitation was observed. Satisfactory CD spectra could not be obtained in lipid

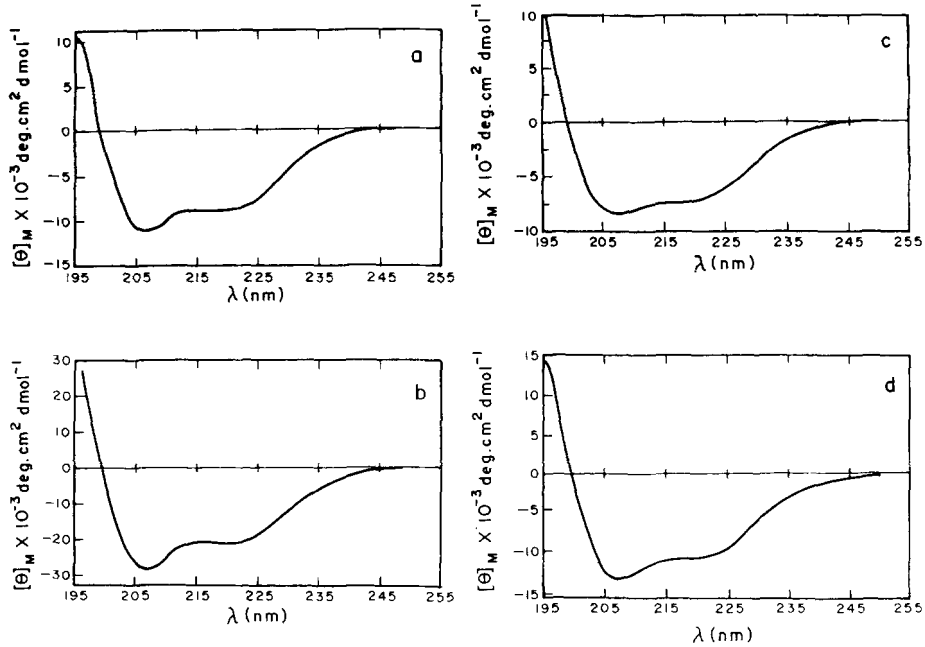

FIG. 6. **CD spectra of peptides 14R and 14Myr.** *a*, 14R in TFE; *b*, 14R in SDS micelles; *c*, 14Myr in TFE; *d*, 14Myr in SDS micelles. Peptide = 0.1 mM.

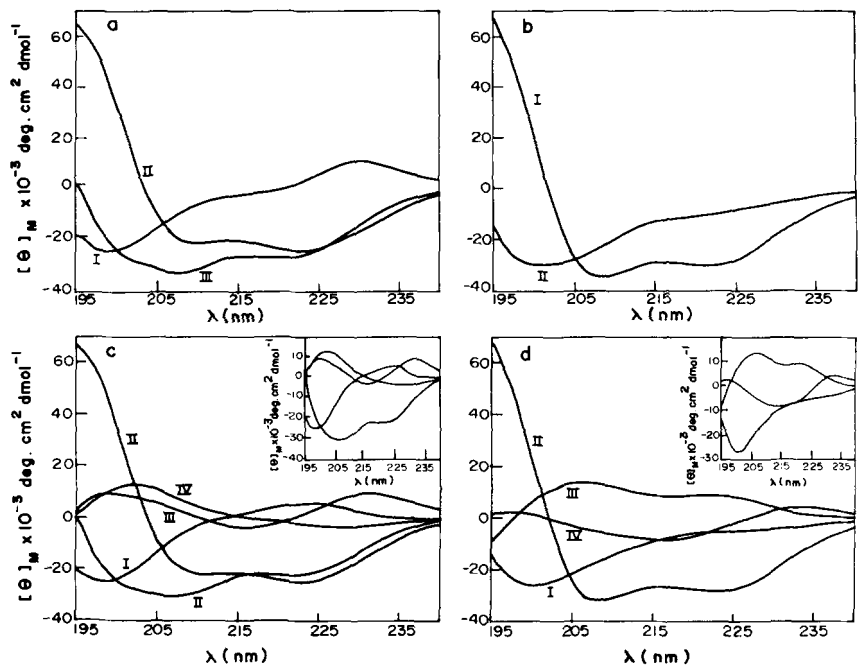

FIG. 7. **Pure components obtained after convex constraint analysis of the CD spectra of peptides 14R and 14Myr.** *a*, 14R in TFE: *curve I*, 46%; *curve II*, 28%; *curve III*, 5%. *b*, 14R in SDS: *curve I*, 48%; *curve II*, 44%. *c*, 14Myr in TFE; *curve I*, 39%; *curve II*, 24%; *curve III*, 13%; *curve IV*, 15%; *curve V*, 9%. *d*, 14Myr in SDS; *curve I*, 33%; *curve II*, 68%; *curve III*, 19%; *curve IV*, 16%. *Inset* in *c* and *d* correspond to components having θ_M extrema between −30 and +10.

vesicles composed of dioleoyl phosphatidylcholine due to intense scattering. The CD spectra were independent of the peptide concentrations in the range of 0.025 mM to 0.2 mM, indicating the absence of aggregation in this concentration range.

DISCUSSION

The conformation of peptides corresponding to acylation sites in proteins have been examined by circular dichroism in TFE a solvent of low dielectric constant, stabilizing α-helical structures and micelles of SDS and OG. Micelles of SDS offer a simple hydrophilic- hydrophobic interface and have been used extensively as a mimic of membrane active peptides. The spectra were deconvoluted into their pure components by the method of Perczel *et al.* (26). This has aided in revealing structural features not easily discernible by visual inspection of the spectra. Peptide 31R adopts an α-helical structure in a medium of low dielectric constant as would be expected of a peptide corresponding to a transmembrane region. While 14R tends to adopt helical conformation, 12R and 11R are largely unordered with a fraction of peptide possibly occurring in distorted helical or β-turn conformation. The crystal structure of the Ras protein shows an α-helix structure in the C-terminal region (31). Although detailed structures of the parent proteins of 11R and 14R are not available, the homologous proteins show preference for helical or β-structure. Thus, the acylated regions do not appear to have a common defined structural motif. Our results indicate that effects of acylation on structure are variable. In the case of 11R, there is no drastic structural difference from the nonacylated peptides, even in hydrophobic environment. In 14R, myristoylation results in conformational heterogeneity. In 31R, acylation results in a slight increase in helical content in SDS micelles, whereas in 12R, palmitoylation seems to stabilize a β-turn, but only in TFE. No increased partitioning

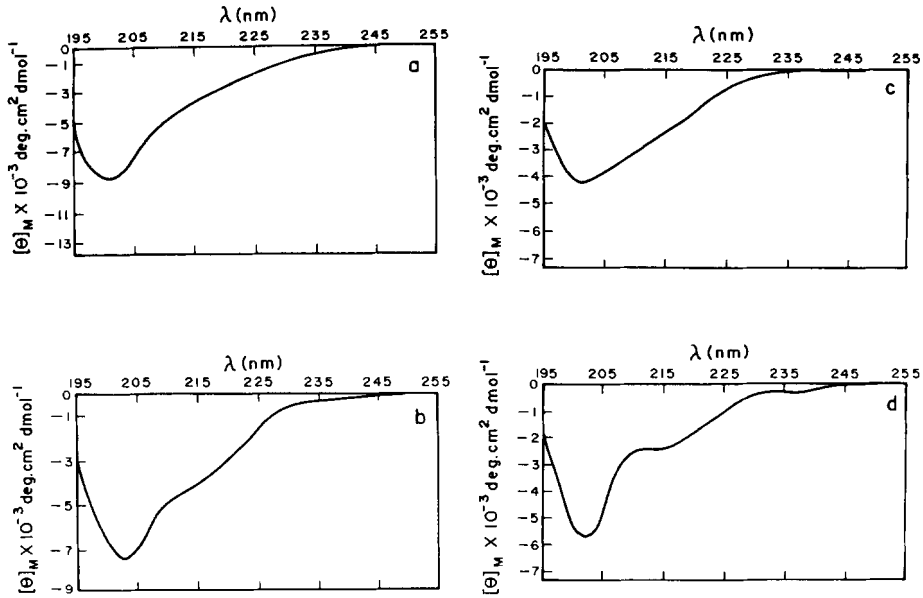

FIG. 8. **CD spectra of peptides 11R and 11Myr.** *a*, 11R in TFE; *b*, 11R in SDS micelles; *c*, 11Myr in TFE; *d*, 11Myr in SDS micelles. Peptide = 0.1 mM.

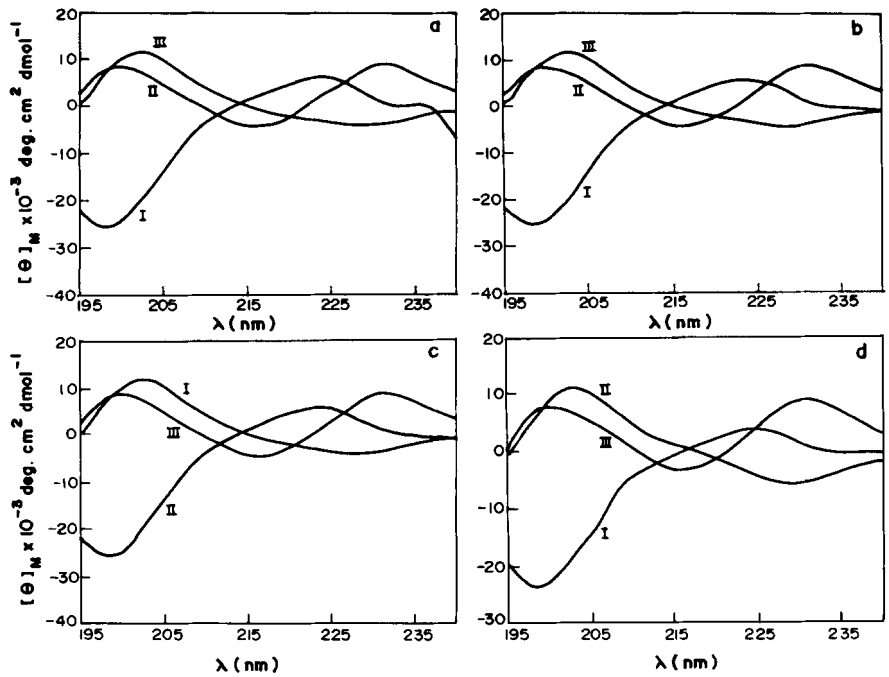

FIG. 9. **Pure components obtained after convex constraint analysis of the CD spectra of peptides 11R and 11Myr.** *a*, 11R in TFE; *curve I*, 46%; *curve II*, 27%; *curve III*, 21%. *b*, 11R in SDS; *curve I*, 45%; *curve II*, 18%; *curve III*, 19%. *c*, 11Myr in TFE; *curve I*, 25%; *curve II*, 45%; *curve III*, 19%. *d*, 11Myr in SDS; *curve I*, 51%; *curve II*, 23%; *curve III*, 17%.

into micelles is discernible.

The crystal structures of the myristoylated catalytic subunit of cAMP-dependent protein kinase has been determined recently (32) and in this structure, the myristoyl chain is intimately associated with the polypeptide chain of the protein. The surface of the enzyme surrounding the myristoyl moieties attached to the N-terminal glycine is very hydrophobic. The recombinant enzyme obtained from *Escherichia coli*, lacking the myristoyl group, was more labile to heat denaturation, indicating that the myristoyl group has an important role in conferring structural stability to the protein. A structural role is also evident in the poliovirus capsid protein, VP4, where the myristoyl group provides a hydrophobic anchor between subunits on the coat surface (33). Our results suggests that the myristoyl chain or even the palmitoyl chain does not appear to have the ability to force a hydrophilic peptide segment into a hydrophobic micellar environment. Thus, the presence of a single myristoyl chain may not be sufficient for membrane association of a globular protein. In fact, the Gibbs free energy for binding of a myristoylated protein to the lipid bilayer indicates that it is not sufficient to stably anchor a myristoylated protein to the lipid bilayer (34). In a class of G proteins involved in vesicular trafficking, the proteins switch between a cytosolic form in which the myristoyl fatty acid chain is in association with a protein and a form where the fatty acyl chain helps in membrane association (35). This switching phenomenon would argue against the myristoyl chain providing a stable membrane anchor. Thus, it is unlikely that the myristoyl group has a primary role in providing an anchor for membrane association.

The increased helical content when 31Pal is associated with micelles indicates that palmitoylation increases affinity of 31Pal to micelles. Thus, in proteins where palmitoylation occurs in the vicinity of a transmembrane segment, it may favor greater partitioning of hydrophobic segment into the membrane and may have a role in the assembly of membrane proteins. Very little is known as to how palmitoyl groups modulate peptide structure especially in non-membrane-associated proteins. In G protein-coupled receptors that have covalently

attached palmitoyl group, signal transduction proceeds through acylation-deacylation cycles (36, 37), indicating a dynamic role for palmitoyl modification. The palmitoyl group could also have a structural role similar to that of the myristoyl group as the palmitoyl chain too does not appear to force a hydrophilic peptide segment into a hydrophobic mileu. Thus, the mere presence of a fatty acid moiety may not be sufficient for membrane binding especially in proteins which do not have a hydrophobic peptide segment in the vicinity of the acylation site. There must clearly be additional factors that have a role in membrane association of fatty acylated proteins, as there is increasing evidence that this modification is a dynamic one with acylation-deacylation cycles, especially in proteins involved in signal transduction (37).

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