Peptide Binding Domains Determined through Chemical Modification of the Side-Chain Functional Groups

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ABSTRACT A clear understanding of the specific secondary structure and binding domain resulting from the interactions of proteins and peptides with lipid surfaces will provide insight into the specific functions of biologically active molecules. We have shown in earlier studies that the stationary phases used in reverse-phase high-performance liquid chromatography represent a model artificial lipid surface for the study of induced conformational states of peptides on lipid interaction. We have now used reverse-phase high-performance liquid chromatography to determine the binding domains of peptides and, by extension, of proteins to a lipid surface. This approach consists of performing chemical modifications of specific amino acid side-chain functionalities after the interaction of the peptides with the reverse-phase high-performance liquid chromatography C_{18} groups. The susceptibility to oxidation was also studied after binding of the same peptides to liposomes. Oxidation of a single methionine residue "walked" through an amphipathic α-helical 18-mer peptide was selected to illustrate this approach. The extent of oxidation was found to be clearly dictated by the accessibility of the methionine residue to the aqueous mobile phase. The binding domain found for the peptide in its lipid-induced conformational state was unequivocally the entire hydrophobic face of the amphipathic α-helix.

INTRODUCTION

Hydrophobic environments, in particular in the presence of lipids, have been reported in many instances to induce specific conformations in peptides. The most thoroughly studied conformationally induced motif in such environments is the amphipathic α-helix, which is known to play a key role in a number of biologically active peptides (Kaiser and Kézdy, 1987; Dyson and Wright, 1993; Jones and Gierasch, 1994). Thus, artificial lipid surfaces have been used as model environments for biological membranes in order to study the potential for peptides to be induced into specific conformations. Recently, reverse-phase high-performance liquid chromatography (RP-HPLC) has proven to be a convenient and useful tool for the study of the secondary structure of peptides (Blondelle et al., 1992; Büttner et al., 1992; Purcell et al., 1993; Hodges et al., 1994; Sereda et al., 1994; Blondelle et al., 1995). Thus, the retention times of peptides capable of being induced into specific secondary structures in hydrophobic environments were found to be highly dependent on the specific structure induced. In turn, retention times that differ significantly from those expected from standard retention coefficient estimates (Meek and Rossetti, 1981; Sasagawa et al., 1982; Houghten and Ostresh, 1987; Zhou et al., 1990) are useful in the prediction of such induced structures. More specifically, the octadecyl groups of the commonly used C_{18} stationary phase represent a practical and convenient model for artificial lipid surfaces. Using multiple C_{18}-coated quartz plates in conjunction with circular dichroism (CD) spectroscopy, we have recently confirmed the similarity of the inducible properties of the C_{18} stationary phase of the RP-HPLC system to those of artificial lipids (Blondelle et al., 1995). In these studies, model peptides composed solely of lysine and leucine residues, but in different arrays, were found by CD spectroscopy to adopt a similar secondary structure (α-helical or β-sheet, according to the array) upon binding to artificial lipid micelles or to the C_{18}-coated plates. Such induced conformations were also found to be responsible for the different retention times seen for peptides having the same length and amino acid composition (therefore the same theoretical retention time (RT) calculated from retention coefficients) but different primary sequences. Because of the amphipathic character of the model peptides used in these studies, hydrophobic interactions between the leucine residues and the lipid surface can be expected to be the driving force resulting in the specific induced secondary structures found. Although these comparative studies using CD spectroscopy increase our understanding of the effect of the RP-HPLC stationary phase on peptide secondary structures, a more precise understanding of the exposed and buried domains of lipid-bound peptides has not been determined.

In the current study, we have taken a new approach to determine the spatial orientation of each residue in a lipid-bound peptide. This involves monitoring the chemical modification of specific side-chain functional groups while the peptide is bound to the lipid surface. This approach was initiated using single substitution analogs of a lysine/leucine-based model peptide described earlier to adopt an amphipathic α-helical conformation in hydrophobic environments (Büttner and Houghten, 1991; Büttner et al., 1992; Blondelle et al., 1995). This peptide is composed of nine

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lysine and nine leucine residues sequenced in a 3.6 residue periodicity (Ac-LKLLKKLLKKLKKLLKKL-NH_2, referred to as $[LK]^{3.6}$). Methionine was selected to successively replace each residue (for a total of 18 peptide analogs) in this sequence because of its ability to be chemically modified at the thioether side chain and its hydrophobic character, although methionine is less hydrophobic than leucine (leucine/methionine hydrophilicity ratios vary from 1.4 to 1.6 (Hopp and Woods, 1981; Fauchère and Pliška, 1983; Guo et al., 1986; Sereda et al., 1993)). Substitution of leucine by methionine was therefore not expected to impair the induced secondary structure (helical propensity parameter P_α: 1.20 for methionine and 1.34 for leucine; Chou and Fasman, 1974); free energy of helix formation $\Delta\Delta G^0 = -1.37$ and -1.60 for methionine and leucine, respectively (Chakrabartty et al., 1994); $\Delta\Delta G = -0.67$ and -0.81 for methionine and leucine, respectively (Zhou et al., 1994); or the hydrophobic binding domain of $[LK]^{3.6}$. The oxidation was carried out while the peptides were bound to either the RP-HPLC C_{18} stationary phase or to artificial liposomes.

MATERIALS AND METHODS

Peptide synthesis

Peptides were prepared by simultaneous multiple peptide synthesis using t-Boc chemistry, as described elsewhere (Houghten, 1985). The lysine side chains were protected with chlorobenzyloxycarbonyl, and the methionine side chains were protected with sulfoxide groups. Half of each peptide resin was deprotected using a low hydrogen fluoride procedure (HF) (Tam et al., 1983) to remove the sulfoxide-protecting group. All peptide resins were then treated with a high HF procedure using a 24-vessel cleavage apparatus (the chlorobenzyloxycarbonyl protecting groups were simultaneously removed while the peptides were cleaved from the resins) (Houghten et al., 1986). Omission of the low HF treatment on a portion of each peptide resin allowed the generation of methionine sulfoxide-containing peptides. Peptides were extracted with 10% acetic acid and lyophilized. Laser desorption time-of-flight mass spectroscopy (Kompact MALDI-TOF mass spectrometer; Kratos, Ramsey, NJ) and analytical RP-HPLC were used to determine the identity and purity of the peptides.

RP-HPLC analysis

Relative retention times were determined using a Beckman System Gold gradient HPLC (Beckman Instruments, Fullerton, CA). Samples (20 μl, 5 mg/ml in H_2O) were analyzed on a VYDAC 218TP54 C_{18} column (Alltech Associates, Los Altos, CA) (4.6 mm ID × 250 mm, 5 μ). Peptide elution was monitored at 215 nm. Solvent A consisted of 0.05% trifluoroacetic acid (TFA) in water and solvent B of 0.05% TFA in acetonitrile. The peptides were analyzed using a 1%/min increasing gradient. Variations in retention times of ±0.05 min were observed between multiple analyses.

CD measurements

All measurements were carried out on a J-720 CD spectropolarimeter (Jasco, Easton, MD) at 25°C, using an RTE 110 waterbath and temperature controller (NESLAB, Dublin, CA). The instrument was routinely calibrated with an aqueous solution of *d*-10-camphorsulfonic acid. CD spectra were acquired at a scan speed of 20 nm/min, and results were obtained by averaging three to six scans. CD spectra of the same buffer and lipid solutions without peptides were used as baseline in all the experiments. The concentrations of the peptides were determined by quantitative amino acid analyses (Biotechnology Resource Laboratory, New Haven, CT). The mean residue ellipticities ($[\theta]$ − deg · cm^2/dmol) were calculated using the equation $[\theta] = 100\theta/cnl$, where θ is the ellipticity (mdeg), c is the peptide concentration (mM), n is the number of residues, and l is the path length (cm). The limits of error of measurements at 222 nm were ±500 (deg · cm^2/dmol).

Oxidation of the thioether groups during RP-HPLC

The peptides were injected onto a C_{18} column using 5% solvent B at a 1 ml/min flow rate. Solvent B was increased to 20% using a 2%/min gradient and was then maintained at 20% during the oxidation step. A solution of 1 ml of 15% hydrogen peroxide (H_2O_2)/0.025% TFA/H_2O was then injected onto the column, and the flow rate was reduced to 0.01 ml/min. After a 20-min exposure of each peptide to the oxidizing agent, the resulting mixture of oxidized and nonoxidized peptides was eluted from the column using a 1%/min increasing gradient. Co-injection of the methionine analogs and their synthetic methionine-sulfoxide equivalents were similarly analyzed by RP-HPLC using a control injection of 0.025% TFA/H_2O instead of H_2O_2. This enabled an accurate determination of the specific retention times of the expected oxidized and nonoxidized forms of the peptides under these RP-HPLC conditions. For those cases in which the oxidized and nonoxidized forms of the peptides eluted at the same time, the peaks were collected after the oxidation step and analyzed by electrospray ionization mass spectrometry (University of California, Davis) (Fenn et al., 1990).

Oxidation of the thioether groups in the presence of artificial lipids

Egg phosphatidylcholine (EPC) and phosphatidylserine (PS) were purchased from Sigma (St. Louis, MO). Liposomes were prepared at a 10 mg/ml final concentration of 70% EPC/30% PS in 5 mM MOPS buffer using standard procedures (Dijkstra et al., 1987). Small unilamellar vesicles (SUVs) were obtained by a 20-min sonication using a Vibracell VC 375 sonicator (Sonics and Materials, Danbury, CT). A 5-mg/ml solution of peptide in H_2O (30 μl) was mixed with 95 μl SUVs; 15% H_2O_2/0.025% TFA/H_2O (50 μl) was then added. After 30 min at room temperature, the excess H_2O_2 was neutralized by a 1-h treatment with a platinum-coated disk. The pH was then brought down with 10 μl TFA, which resulted in partial denaturation of the SUVs. The peptide was then extracted from the lipidic phase using a H_2O/chloroform extraction system and was analyzed by RP-HPLC to determine the percent oxidation. As for the oxidation experiment during RP-HPLC described above, control treatments were also carried out using methionine sulfoxide-containing peptides as well as methionine-containing peptides with H_2O/0.025% TFA.

RESULTS

Peptide design and analyses

Two sets of analogs of the 18-mer $[LK]^{3.6}$ peptide were prepared simultaneously. One set consisted of 18 single substitution analogs derived by "walking" a methionine residue through the sequence, whereas the second set corresponded to their methionine sulfoxide equivalents. Simple synthetic procedures (i.e., low HF treatment or no treatment) allow the simultaneous generation of both sets of analogs. The methionine sulfoxide-containing peptides were prepared in order to clearly establish their retention times in RP-HPLC and in turn to serve as controls to monitor the oxidation reaction. The methionine-containing

peptides were stored under nitrogen at 4°C before their use in order to avoid air oxidation contaminants. All peptides were solubilized in water just before reaction for similar reasons.

The RP-HPLC retention times were first determined for each peptide in order to analyze the variations between the 18 different peptide analogs within one series (containing either a methionine or a methionine sulfoxide). It should be noted that if retention times were entirely dictated by the retention coefficients (Meek and Rossetti, 1981; Sasagawa et al., 1982; Houghten and Ostresh, 1987), only two distinct retention times should be observed (when leucine is substituted and when lysine is substituted). Variations between the retention times of the methionine-containing peptides and their equivalent sulfoxide form were also examined. These data permitted the optimization of the RP-HPLC gradient conditions necessary for the accurate determination of the percent oxidation. The variations in retention times observed for peptides of the same composition, as well as their significantly delayed elution relative to their theoretical retention times calculated from retention coefficients (theoretical retention times at 15.63 min when methionine replaces a leucine, 19.57 min when methionine replace a lysine), support the adoption of an amphipathic α-helical conformation for this set of peptides upon interaction with the C_{18} stationary phase (Table 1). Only three peptide nonoxidized and oxidized forms in this series did not separate clearly. These were for methionine substitution for lysines at position 9, 13, or 16 ($\Delta RT = |RT_{(non\text{-}oxidized)} - RT_{(oxidized)}|$ lower than 0.55 min) (Table 1). Co-elution runs for these peptides in the same gradient conditions led to two closely eluted peaks, which did not permit an accurate determination of percent oxidation. Similar results were obtained using other gradient systems (i.e., partly isocratic, 0.5%/min, or lower flow rate), as well as other solvent systems (i.e., H_2O/methanol/0.05% TFA; H_2O/isopropyl alcohol/0.05% TFA; and 0.25 M triethylammonium phosphate (pH 3)/acetonitrile). If one envisions the peptides in an amphipathic α-helical conformation (Fig. 1), then the lysine residues at positions 9, 13, and 16 are located in the middle of the hydrophilic face, i.e., the most distant from the hydrophobic/hydrophilic interface. These three lysine residues are therefore anticipated to be the most exposed to the solvent (i.e., mobile phase) and have little or no interaction with the C_{18} groups of the stationary phase. In these three cases, electrospray ionization mass spectrometry was used to determine the ratio of oxidized to nonoxidized methionine (Fenn et al., 1990).

TABLE 1 RP-HPLC retention times of the methionine- or methionine sulfoxide-containing peptides

Substituted residue	RT (min)*		
	Met form	Met(O) form	ΔRT (min)‡
L-1	34.12	30.70	3.42
K-2	37.64	36.80	0.84
L-3	34.12	30.13	3.99
L-4	33.18	25.33/25.58§	7.85/7.60
K-5	39.23	37.33	1.90
K-6	38.33	37.02	1.31
L-7	33.50	26.30/27.19§	7.20/6.31
L-8	34.13	29.53/29.81§	4.60/4.32
K-9	36.90	37.28	0.38
K-10	42.71	38.66	4.05
L-11	33.19	23.62/24.16§	9.57/9.03
K-12	42.00	37.44	4.56
K-13	36.78	37.28	0.50
L-14	33.30	28.88	4.42
L-15	32.91	27.50/28.11§	5.41/4.80
K-16	38.02	37.48	0.54
K-17	39.36	37.65	1.71
L-18	33.51	28.90	4.61

* Elution gradient: 5 min at 20% B, then 20–65% B in 45 min.
‡ $\Delta RT = |RT_{(non\text{-}oxidized)} - RT_{(oxidized)}|$.
§ The two peaks correspond to the two diastereomeric forms of the sulfoxide.

In contrast, the largest ΔRT between the nonoxidized and oxidized forms was observed when methionine replaced leucine at position 4, 7, or 11 (Table 1). These three leucine residues are located in the middle of the hydrophobic face of the helix (Fig. 1) and are therefore anticipated to have the strongest interaction with the C_{18} groups of the stationary phase. Any modification in the hydrophobicity at these positions should have the greatest effect, as observed here for the methionine sulfoxide analogs, on the retention behavior as compared with $[LK]^{3.6}$ (RT of $[LK]^{3.6}$ was 15.46 min under the gradient conditions described in Table 1). In addition, two distinct peaks were seen for each of the oxidized forms of these three peptides, as well as for the two analogs in which leucines at positions 8 and 15 were replaced. The two peaks that were found correspond to the expected two diastereomeric forms of the sulfoxide. A separate synthesis of the three peptide analogs, in which leucine-4, -7, or -11 was substituted with methionine sulfoxide using the isomer Boc-Met(O) having the configuration *SS* (Lavine, 1947), indicated that the *SS* form of the sulfoxide corresponded to the second peaks found in the "racemic" peptide sulfoxides (data not shown). From these results, a 1%/min RP-HPLC increasing gradient using an acetonitrile/TFA/H_2O solvent system was chosen to quantify the percent oxidation of all of the methionine-containing peptides when the peptides interact with a lipid surface, except for the three analogs in which the lysines at position 9, 13, or 16 were substituted, as mentioned above.

Oxidation of the thioether groups while bound to the C_{18} RP-HPLC stationary phase

Oxidation of the thioether groups of the methionine residues was carried out using a solution of 15% H_2O_2 in 0.025% TFA/H_2O. The peptides were injected onto a C_{18} RP-HPLC column and subsequently reacted with a later injection of H_2O_2. The percent oxidation was then calculated on the basis of the RP-HPLC peak area of the two eluted forms. Representative RP-HPLC spectra are shown in Fig. 2 for the two

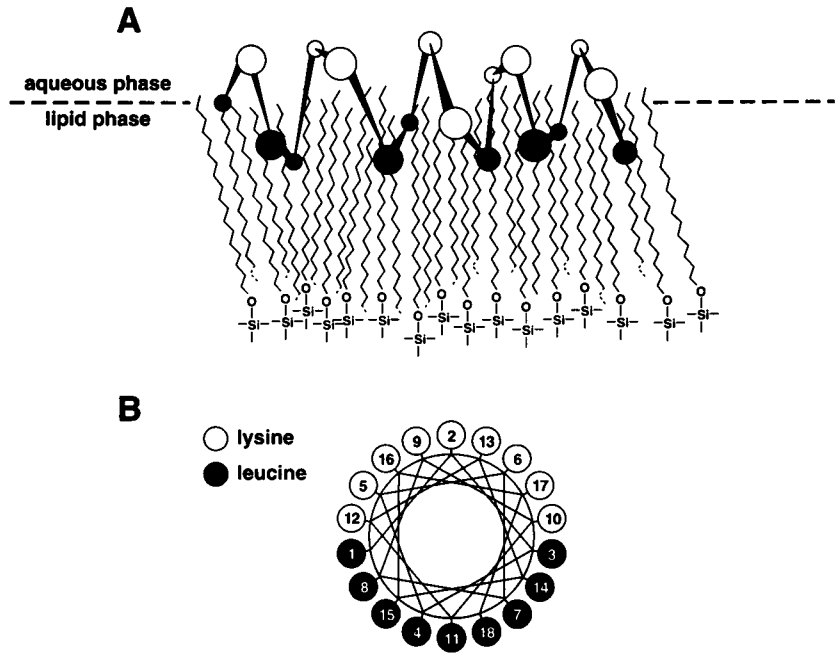

FIGURE 1 (*A*) Schematic representation of $[LK]^{3.6}$ upon interacting with C_{18} alkyl groups. The drawing illustrates the proposed induced conformation and resulting binding domain for $[LK]^{3.6}$. The lysine residues are represented by ○, and the leucine residues are represented by ●. (*B*) Helical wheel representation of $[LK]^{3.6}$. The numbers indicate the position of each residue within the sequence.

analogs in which methionine replaced leucine-4 or lysine-6. In order to ensure that the oxidative treatment, as well as the gradient used for this reaction monitoring, did not affect the retention times of the two forms of the peptides (oxidized and nonoxidized), the retention times of the methionine analogs and their synthetic methionine-sulfoxide forms were determined using similar gradient conditions but with an equivolume injection of 0.025% TFA/H_2O without H_2O_2. When compared with the control conditions described above, no differences in the separations between the oxidized and nonoxidized forms were found under the gradient conditions used for the oxidation study.

As shown in Fig. 3, the methionine residues in this series were found to be oxidized to different degrees. This was clearly dependent on their location in the induced amphipathic α-helix. The susceptibility to oxidation varied with a 3.6 residue period, indicating that the peptides adopted an α-helical conformation upon binding to the C_{18} alkyl groups. Little oxidation occurred when methionine replaced a leucine residue, whereas extensive oxidation generally occurred when a lysine was replaced by methionine. These results, in particular the relative low percentage of oxidation found when methionine replaced leucine at position 1 or 18, indicate that all of the leucine residues in $[LK]^{3.6}$ are imbedded into the C_{18} surface. However, the N-terminal region seemed to be slightly more exposed to the solvent than did the C-terminal region. This can be explained by the proximity of leucine-1 to the aqueous/lipid interface in contrast to the C-terminus leucine residue (see helical wheel representation in Fig. 1). In a similar manner, the lowest amount of oxidation was observed for those analogs in which lysine-10 and -12 were replaced by a methionine, compared with that of the other lysine residues. As shown in the helical wheel representation (Fig. 1), lysine-10 and -12 are the closest to the aqueous/lipid interface. These lysine residues can thus be envisioned as being partially embedded in the lipid surface.

In the case of those analogs for which the oxidized and nonoxidized forms eluted at the same time (when methionine replaced lysine at position 9, 13, or 16), the peaks were collected after the H_2O_2 treatment and analyzed by electrospray ionization mass spectrometry. Two control mixtures made up of 33%/66% and 60%/40% methionine-containing peptide/methionine sulfoxide-containing peptide (with substitution of lysine-16), respectively, were used as references for quantitative mass spectral analyses. These control mixtures were found by mass spectral analyses to be represented at 36%/64% and 58%/43%, which supports the use of electrospray ionization mass spectral analyses for the determination of percent oxidation of closely related peptides (Fig. 4*A* and *B*). For the three cases mentioned above (substitution of lysine-9, -13, and -16), oxidation was found to be greater than 99%, along with 1–5% sulfone formation (as illustrated in Fig. 4 *C*).

Oxidation of the thioether groups while bound to liposomes

Earlier studies have shown that the C_{18} stationary phase of the RP-HPLC can mimic artificial lipid surfaces (Blondelle et al., 1995). We have also used artificial liposomes not only to confirm the validity of RP-HPLC as a useful means for the study of such interactions at aqueous/lipid interfaces, but also to evaluate the generality of chemical modification of specific functional groups for such studies. Four methionine substitution analogs were selected for this study: methionine replacing leucine-4 or lysine-6 and methionine replacing

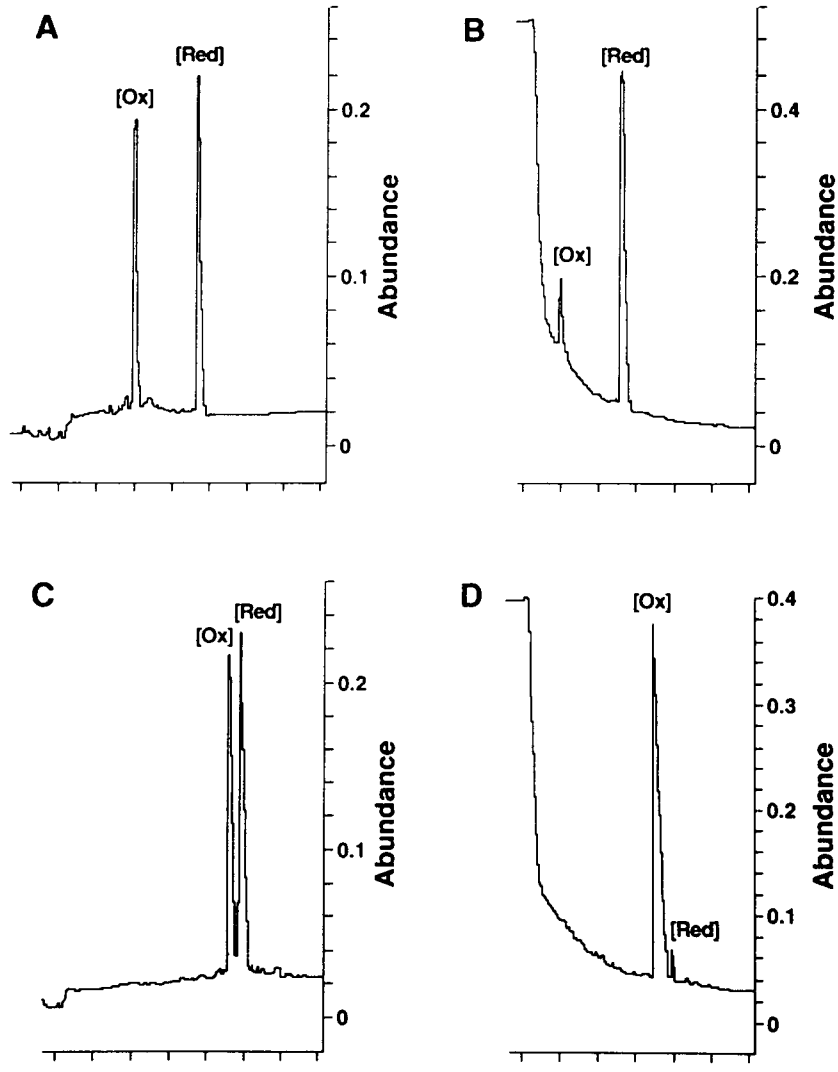

FIGURE 2 RP-HPLC chromatograms of the nonoxidized and oxidized forms of subL4M and subK6M. Co-injection of the nonoxidized (Red) and oxidized (Ox) forms of subL4M (*A*) and subK6M (*C*) are shown. The chromatograms of subL4M and subK6M after oxidation reaction are shown in *B* and *D*, respectively. The experimental conditions are described in Materials and Methods.

leucine-1 or leucine-18. The first two peptides were chosen as representative examples of low and high oxidation. They were found during the oxidative RP-HPLC treatment to result in 4% and 80% oxidation, respectively (Fig. 3). The two analogs at positions 1 and 18 were chosen to examine the end effects while being bound to a less rigid lipid layer (i.e., liposomes versus C_{18} packing).

All experiments reported in these studies were carried out under experimental conditions in which peptides are quantitatively bound to EPC/PS (70/30%) liposomes based on binding affinity studies (lipid/peptide ratio R_{lp} = 20) (Fig. 5A). Furthermore, all of the peptide analogs were found to adopt an α-helical conformation upon binding to the liposomes (Fig. 5 *B*). These results confirm our premise that substitution of a leucine or lysine residue by methionine does not impair the adoption of an α-helical conformation as compared with $[LK]^{3.6}$. Using an R_{lp} of 20, the four selected peptide/lipid mixtures were treated with H_2O_2 for 30 min. Neutralization of the excess H_2O_2, as well as extraction of the resulting peptides (mixture of both the oxidized and nonoxidized forms), was necessary to monitor the reaction by RP-HPLC. Because SUVs are denaturated on contact with the C_{18} stationary phase, the excess H_2O_2 that is present could result in additional oxidation of the released peptide before elution and in alteration of the percent oxidation. Injection of large amounts of SUVs onto the C_{18} column was found to affect the performance of the column in the gradient system used, which rendered peptide extraction necessary before injection. For the analog in which leucine-4 was replaced by methionine, 17% oxidation was found, whereas complete oxidation was seen when methionine replaced lysine-6. The similarity between these results and the preceding ones supports that the approach described here for the study of binding domains is likely to be of general use for a wide range of lipid surfaces.

The effects at the C- and N-terminal (i.e., degree of binding and accessibility to the solvent) were found to differ between binding to the C_{18} column and binding to liposomes. Thus, in the presence of liposomes, 90% oxidation was found for the analog in which methionine replaced leucine-1 versus 34% using the RP-HPLC procedure, and 100% was found (versus 12%) when leucine-18 was re-

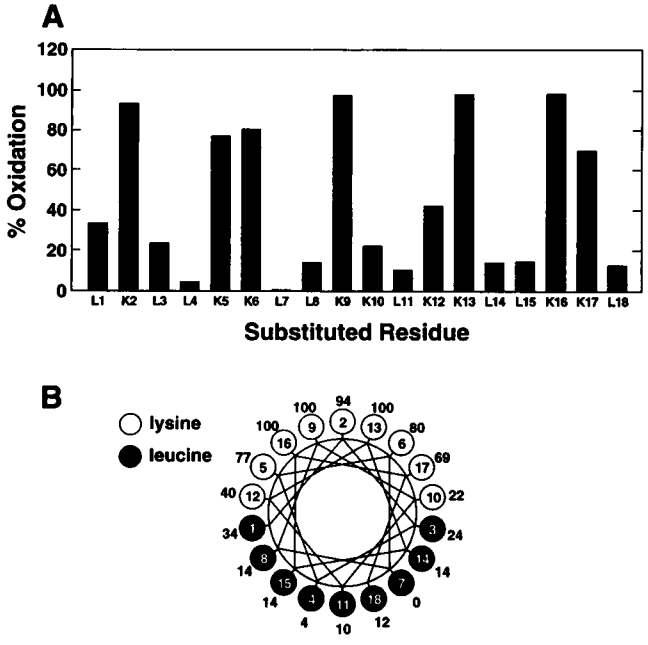

FIGURE 3 Oxidation of methionine using the RP-HPLC procedure. (*A*) Each bar represents the percent oxidation as determined by RP-HPLC. The values obtained from different oxidative runs varied ±3%. (*B*) The helical wheel is represented here to show the percent oxidation (numbers adjacent to the *circles* that represent the residues as described in Fig. 1) as a function of the location of methionine relative to the binding domain.

placed by a methionine. These variations can be explained if the C_{18} groups are envisioned as a static "rigid" surface as compared with the more flexible, and least homogeneous, liposome lipid layer.

DISCUSSION

Evidence of specific induced secondary structures of peptides at aqueous/lipid interfaces is expected to provide insight into the nature of interactions of peptides or proteins with biological receptors, as well as into protein folding processes. In particular, an understanding of the induced binding domains should provide information on the active sites and specific functions of biomolecules, as well as on the de novo design of new acceptors. The insertion of spectroscopic probes into peptides or protein fragments, such as fluorescent or spin labels and radiolabeled or fluorescent amino acids, has been used for the study of peptide and protein interactions with lipid surfaces (Utsumi et al., 1984; De Kroon et al., 1990; Chung et al., 1992; Mahaney et al., 1992; Oomen and Kaplan, 1992). We have recently developed an approach to evaluate the preferred binding domains of peptides at aqueous/lipid interfaces by the chemical modification of specific functional groups. The amount of chemical modification is dictated by the accessibility of the functional groups to the aqueous phase containing the chemical reagents. Thus, calculation of the percent modification allows the evaluation of the spatial location of each residue of the peptide that binds to a lipid surface. In contrast to other methods used to study peptide/lipid interactions, the current approach involves the use of a simple and widely available technique, RP-HPLC, to monitor the chemical modifications. The practicality of RP-HPLC for such studies was determined on the basis of earlier work, which demonstrated that the C_{18} groups of the RP-HPLC stationary phase could be used as a mimic for lipid membranes in a manner similar to artificial liposomes.

The approach presented here is similar in concept to the selective oxidation of methionine residues reported for proteins such as ovine hormone (Houghten and Li, 1976), human choriomammotropin (Houghten and Li, 1977), and human somatotropin (Houghten et al., 1977). Such chemical modifications have been carried out in order to determine the accessibility of methionine residues and the change in physicochemical properties after such chemical treatment. In these earlier studies, the degree of chemical modification that was observed gave additional information on the specific role that methionine residues play in the tertiary structure of the proteins as well as on their biological activities. In such cases, the hydrophobic core of the proteins can be likened to a "micro" C_{18}/silica particle with surface-accessible methionine susceptible to chemical modification, whereas the methionine buried inside the hydrophobic core is not. Thus, one can envision an extension of the use of RP-HPLC to perform similar studies for the evaluation of accessible residues in proteins.

The present study involves the straightforward oxidation of the thioether group of methionine residues. However, a similar approach can be envisioned involving a range of other chemical modifications on methionine or other sulfide-containing amino acids such as thioproline (e.g., alkylation of sulfide groups using iodoacetic acid or iodoacetamide, etc.), as well as on other functional groups such as the thiol group in cysteine, the indole ring in tryptophan, or the ϵ-amino group of lysine residues (reviewed in Means and Feeney, 1990). It should be noted that as for all binding studies, the insertion of or substitution of a particular amino acid by a different one containing a chemically modifiable functional group should not impair the binding properties of the peptides studied. Methionine was chosen for this study because it has physicochemical characteristics similar to those of leucine (i.e., nonpolar and of similar side-chain length). As anticipated, methionine substitution did not affect the induced α-helical conformation upon interaction of the peptides with a lipid surface, such as the C_{18} groups during RP-HPLC, as shown by the significantly delayed elution times compared with those predicted by using retention coefficients. This result is supported by the adoption of an α-helical conformation in the presence of liposomes as shown by CD. The variation in the percent oxidation between the different analogs indicates that the entire 18-residue peptide is folded into an α-helical conformation, and that all the leucine residues are interacting with the C_{18} groups. The leucine residues located in the middle of the hydrophobic face seemed to be more deeply embedded into the lipidic chains than those located at the C- and N-

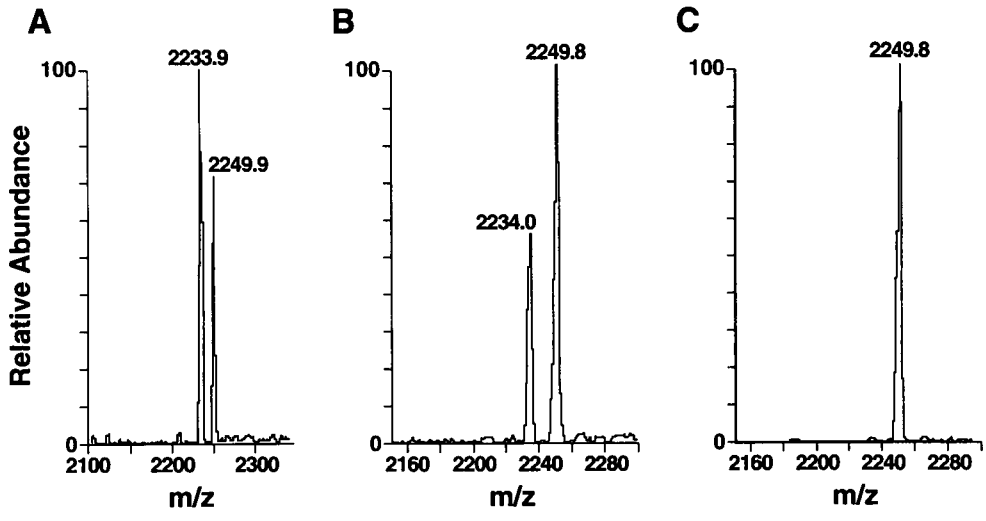

FIGURE 4 Electrospray ionization mass spectra of (*A*) a mixture of 60% subK16M and 40% of its oxidized form; (*B*) a mixture of 33% subK16M and 66% of its oxidized form; (*C*) subK16M after RP-HPLC oxidative treatment. Theoretical molecular weights are 2234.6 for subK16M and 2250.6 for its oxidized form.

terminal. In addition, partial interaction was observed for the lysine residues located at the hydrophobic/hydrophilic interface. Because of their long alkyl side chains, these lysines can be seen as being partly embedded into the hydrophobic surface and extended toward the polar face to insert their charged moieties into the aqueous environment (Mishra et al., 1994). Furthermore, the accessibility to oxidation of the methionine residue when replacing lysines, compared with little or no accessibility when replacing leucines, shows that the helical axis of the peptide is parallel to the lipid plane.

CONCLUSION

The similarity in percent oxidation initially obtained for selected analogs when the peptides were in contact with the C_{18} stationary phase and in the presence of artificial liposomes is in agreement with our earlier work on probing the use of RP-HPLC for peptide/lipid interaction studies. The performance of chemical modifications in the presence of liposomes described here also suggests the generality of the approach to lipid surfaces. In addition, the development of new procedures for the specific chemical modification of proteins has been greatly expanded (reviewed in Means and Feeney, 1990). This increases their possible pharmaceutical application as well as the understanding of their biochemical structures and functions. Thus, one can also envision taking advantage of the binding properties of peptides and proteins to lipid surfaces to perform specific side-chain modifications during RP-HPLC on residues exposed to the solvent. The resulting induction of the biological conformation of proteins and peptides in the presence of artificial lipids such as the C_{18} stationary phase should limit the perturbation of their secondary structures, which can occur during the chemical modification procedures. This feature may render the RP-HPLC approach described here useful for the ready introduction of radiolabeled or fluorescent probes into peptide or protein sequences.

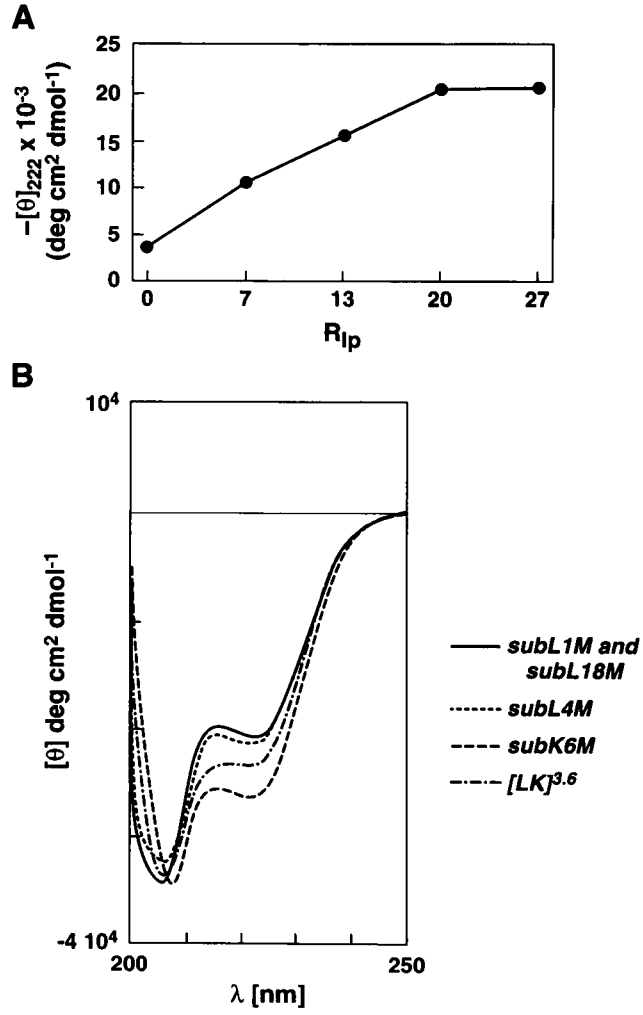

FIGURE 5 (*A*) Binding affinity curve for subL4M. $[\theta]_{222}$ values were calculated for R_{lp} (EPC:PS/peptide) varying from 0 to 27. (*B*) CD spectra in the presence of liposomes. The CD spectra were recorded at a peptide concentration of 40 μM in 5 mM MOPS buffer in the presence of EPC/PS with R_{lp} = 20 for (—) subL1M and subL18M, (· · · ·) subL4 M, (- - -) subK6 M, and (-·-·-) $[LK]^{3.6}$.

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