ELSEVIER

Biochimica et Biophysica Acta 1468 (2000) 262–272

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A transmembrane peptide from the human EGF receptor: behaviour of the cytoplasmic juxtamembrane domain in lipid bilayers

Simon Sharpe, Chris W.M. Grant *

Department of Biochemistry, University of Western Ontario, London, ON N6A 5C1, Canada

Received 18 January 2000; received in revised form 20 June 2000; accepted 20 June 2000

Abstract

Solid state ^{2}H NMR spectroscopy was employed to study peptides related to the transmembrane domain of the human epidermal growth factor receptor, for insight into the interaction of its cytoplasmic juxtamembrane domain with the membrane surface. Since such receptors have clusters of (+)charged amino acids in this region, the effect of (−)charged phosphatidylserine at the concentration found naturally in the cytoplasmic leaflet (15 mol%) was considered. Each peptide contained 34 amino acids, which included the hydrophobic 23 amino acid stretch thought to span the membrane and a ten amino acid segment beyond the 'cytoplasmic' surface. Non-perturbing deuterium probe nuclei were located within alanine side chains in intramembranous and extramembranous portions. ^{2}H NMR spectra were recorded at 35°C and 65°C in fluid lipid bilayers consisting of (zwitterionic) 1-palmitoyl-2-oleoylphosphatidylcholine, with and without 15 mol% (anionic) phosphatidylserine. The cationic extramembranous portion of the receptor backbone was found to be highly rotationally mobile on a time scale of 10^{-4}–10^{-5} s in both types of membrane – as was the α-helical intramembranous portion. Deuterium nuclei in alanine side chains (-CD_3) detected modest changes in peptide backbone orientation and/or dynamics related to the presence of 1-stearoyl-2-oleoylphosphatidylserine: in the case of the extramembranous portion of the peptide these seemed related to lipid charge. Temperature effects on the peptide backbone external to the membrane were qualitatively different from effects on the helical transmembrane domain – likely reflecting the different physical constraints on these peptide regions and the greater flexibility of the extramembranous domain. Effects related to lipid charge could be detected in the spectrum of CD_3 groups on the internally mobile side chain of Val^{650}, six residues beyond the membrane surface.

Keywords: ErbB-1; Epidermal growth factor receptor; Deuterium nuclear magnetic resonance; Peptide; Model membrane; Signal transduction; Receptor tyrosine kinase; Conformation; Bilayer; Phosphatidylserine

1. Introduction

Solid state ^{2}H NMR spectroscopy is a technique of choice for assessing spatial arrangement and behaviour of molecules in fluid membranes – phenomena considered to be key issues in signalling at cell surfaces. We have been attempting to extend ^{2}H NMR techniques, developed on bacterial and model peptides in a number of other laboratories (reviewed

Abbreviations: EGF, epidermal growth factor; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; SOPS, 1-stearoyl-2-oleoylphosphatidylserine; SOPC, 1-stearoyl-2-oleoylphosphatidylcholine; TFE, trifluoroethanol

* Corresponding author. Fax: +1 (519) 6613175;
E-mail: cgrant@julian.uwo.ca

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00267-4

in [1–4]), to human receptor tyrosine kinases in fluid membranes (e.g. [5–7]). In the present work we examined a transmembrane segment of the human epidermal growth factor (EGF) receptor, focusing on the peptide region immediately beyond the membrane surface. Coulomb forces seem likely to be relevant in this case since transmembrane proteins of higher animals have clusters of (+)charged amino acid residues immediately at and just beyond the cytoplasmic surface [8,9], which in turn is (−)charged by virtue of the presence of lipids (notably phosphatidylserine (PS)). PS is a relatively minor phospholipid; however, it is 3-fold enriched in the plasma membrane relative to internal organelles, and 80–90% of this is located in the cytoplasmic leaflet. It typically comprises some 15% of cytoplasmic surface lipids in the plasma membrane, and it is the dominant (−)charged lipid there [10,11]. PS is thought to be involved in signal transmission: suggested physical bases for such involvement generally invoke interaction of its (−)charge with species having a (+)charge [12]. More generally, receptor charge alteration by phosphorylation within the cytoplasmic domain is well known to accompany many examples of signal modulation [13–15]. There have been few direct measurements on the extramembranous portions of transmembrane peptides in bilayers.

The EGF receptor is a class I receptor tyrosine kinase, and a prototypic example of higher animal signal transduction systems [13,14]. It is a 170 kDa species possessing a glycosylated portion responsible for the earliest events in recognition at the cell surface, a single α-helical transmembrane domain, and an intracellular portion exhibiting protein kinase activity as well as phosphorylation and docking sites. Its amino acid sequence contains a cluster of three (+)charged residues at the cytoplasmic surface (Arg^{645}, Arg^{646}, Arg^{647}) and another such cluster several residues downstream (Arg^{651}, Lys^{652}, Arg^{653}). We hypothesized that certain lipid-based forces intrinsic to the cell cytoplasmic surface would be sensed by nuclear probes in the extramembranous portion of a natural receptor. Peptides examined in the present work contained the putative transmembrane region of the human EGF receptor (residues 622–644) plus a ten residue stretch of the cytoplasmic domain. The terminal amino acid of the peptide cytoplasmic extension was Thr^{654}, which is known to become charged by phosphorylation during EGF-mediated signal transduction – perhaps as a regulatory mechanism. The methyl side chain of alanine was chosen as a probe location in this study since it is directly attached to the peptide backbone and thus directly reflects peptide backbone behaviour. Deuterated alanine was placed at positions 650 and 652 (six and eight residues respectively downstream from the membrane 'cytoplasmic' surface), and at residue 637 (which is well within the relatively stable transmembrane helix). Substitution of amino acids by alanine is a common relatively 'benign' procedure in protein biochemistry [16]. Peptides were studied by wide-line NMR in unsonicated lipid bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), or in the same host matrix containing 1-stearoyl-2-oleoylphosphatidylserine (SOPS) at 15 mol%, since these are common naturally occurring forms of phosphatidylcholine and phosphatidylserine.

2. Materials and methods

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), 1-stearoyl-2-oleoyl-3-*sn*-phosphatidylserine (SOPS), and 1-stearoyl-2-oleoyl-3-*sn*-phosphatidylcholine (SOPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol was from Sigma (St. Louis, MO). Lipids were used without further purification. Deuteromethyl-*l*-methionine and perdeuterated *l*-alanine were from Isotec (Miamisburg, OH). Perdeuterated *l*-valine was from CIL (Andover, MA). FMOC-blocked amino acids were synthesized following standard procedures as described previously [6]. Peptides were produced by solid phase synthesis at Queens Peptide Synthesis Laboratory (Kingston, ON) or by Chiron Mimotopes (Clayton, Australia). Sequences were confirmed by mass spectroscopy: purity was >90–95% by HPLC and mass spectroscopy.

Liposomes were prepared by hydration of thin films made by drying down solutions of peptide and lipid in trifluoroethanol (TFE, Aldrich, NMR grade) containing 5% $CHCl_3$ (BDH, analytical grade) via the general protocol described previously [5]. Initial solubilization in TFE/$CHCl_3$ was with warming to 60°C with periodic bath sonication until complete dissolution was visually apparent. Hydra-

tion of the thin films was with 30 mM HEPES, 20 mM NaCl and 1 mM EDTA (pH 7.2) in deuterium-depleted water (CIL (Andover, MA) and Isotec (Miamisburg, OH)).

^{2}H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using a single-tuned Doty 5 mm solenoid probe. A quadrupolar echo sequence [3] (SSECHO from the Varian pulse library) was employed with full phase cycling. $\pi/2$ pulse lengths used were 5.1–6.8 μs with pulse spacing of 15–20 μs and a repetition time of 100 ms. The spectral sweep width was 100 kHz, and spectra were analysed with a line broadening of 50–100 kHz. ^{31}P NMR spectra were recorded at 161.7 MHz on a Varian Infinity Plus 400 spectrometer using a 2 μs $\pi/2$ pulse and ^{1}H decoupling. Spectra were run with temperature regulation to ± 0.1°C. Membrane samples for CD spectroscopy were prepared in a similar manner to NMR samples, but with bath sonication at 23°C to reduce liposome size: samples contained 25 μM peptide (6 mol% relative to phospholipid) in phosphate buffer, and spectra were read in a 1 mm path length cell at 23°C using a Jasco J-810 instrument.

3. Results and discussion

Fig. 1 provides amino acid sequences of the transmembrane peptides studied in the present work: locations of deuterium probe nuclei are indicated in bold font. Each sequence runs from Ile^{622} to Thr^{654} of the human EGF receptor, with lysine at position 621. The two cytoplasmic clusters of (+)charged amino acids are identified: net charge of the C-terminal extramembranous domain was +5 in each case. The uppermost sequence (*peptide A*) had selectively deuterated alanine at positions 650 and 637: thus Ala^{650} and Ala^{637} have -CD_3 side chains, providing one probe site in the extramembranous domain (Ala^{650}) and another in the hydrophobic transmembrane portion (Ala^{637}). In *peptide B*, Ala^{652} with a -CD_3 side chain replaced (+)charged Lys^{652}; and native Ala^{637} was again deuterated. In the latter case, in order to keep the overall charge of the cytoplasmic region the same, the carboxy terminus was amidated. *Peptide C* had no alanine substitutions for native amino acids, and contained -CD_3 groups at natural residues, Ala^{623}, Met^{644}, and Val^{650} in various combinations.

^{31}P NMR spectra of samples containing up to 6 mol% peptide were found to be powder patterns of width 45 ppm (Fig. 2). Such patterns reflect ^{31}P chemical shift anisotropy, and characterize phospholipids in fluid bilayer form ([17] and references therein) (Fig. 2). These results are in agreement with our previous freeze-fracture electron microscopy characterization of similar samples [7], which demonstrated only bilayer structures.

Elongated amphiphiles dispersed in fluid membranes tend to undergo rapid symmetric rotation

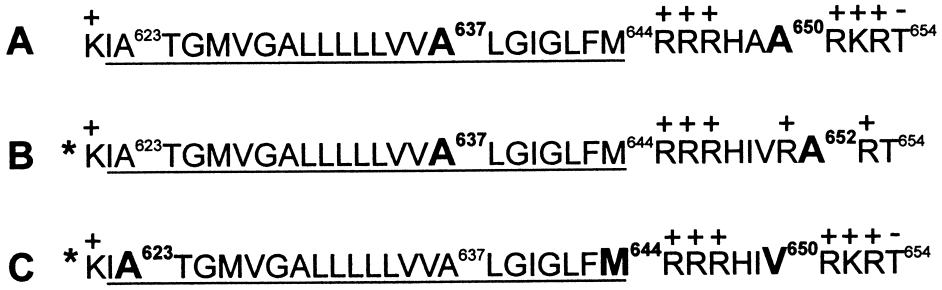

Fig. 1. Amino acid sequences of transmembrane peptides studied. Amino acids are cited by single letter code; bold format indicates the locations of deuterium probe nuclei. Sequences run from Ile^{622} to Thr^{654} of the human EGF receptor, with lysine at position 621. Overall charge on the C-terminal extramembranous domain was +5 in each case. In *peptide A* (uppermost), alanine residues at position 650 in the extramembranous domain (A^{650}) and at 637 in the hydrophobic transmembrane domain (A^{637}) each possess a deuterated (CD_3) side chain. This sequence has alanine substitutions for (uncharged) I^{649} and V^{650}. *Peptide B* contains a deuterated alanine substitution for (+)charged K^{652}: in order to keep the overall charge of the cytoplasmic region the same, the C-terminus was amidated. *Peptide C* is the natural sequence from Ile^{622} to Thr^{654} (no alanine substitutions) and is deuterated at Ala^{623}, Met^{644}, and Val^{650}. Peptides *B* and *C* were biotinylated at the amino terminus (asterisk).

about axes perpendicular to the bilayer. For such molecules containing deuterium nuclei, Eq. 1 is useful in relating ^{2}H NMR spectral splittings (ΔV_Q) to molecular orientation and motional characteristics.

$$\Delta V_Q = (3/8)\ (e^2Qq/h)\ S_{mol}\ |3\cos^2\ \Theta_i - 1| \qquad (1)$$

e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for a C-D bond) [1,3], S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the C-D bond relative to the bilayer normal, and Θ_i is the orientation of the C-D bond relative to an axis of rotation being considered. For deuterated methyl groups, which undergo rapid rotation about the attaching bond axis even at temperatures well below 0°C, it is convenient to consider a resultant C-D vector directed along the C-CD_3 bond. This can be dealt with by taking Θ_i to be the angle between the C-CD_3 vector and the molecule rotational axis, and introducing an additional factor of 1/3 to the right hand side of Eq. 1. Eq. 1 dictates that each -CD_3 group should give rise to a pair of peaks (a 'Pake doublet') whose separation, ΔV_Q, is related to orientation and motional behaviour of molecules to which deuterium is attached. Peak *shape* can also provide insight: for instance a central shift of spectral intensity is common for deuterons in molecules undergoing discontinuous rotation ('axially asymmetric' rotation) (reviewed in [2]).

^{2}H NMR spectra typifying those obtained for membranes containing deuterated *peptide A* are presented in Fig. 3. In each case the peptide was incorporated into unsonicated, fully hydrated fluid bilayers, well above the host matrix gel/fluid phase transition temperature. Spectra in the left hand column are for membranes containing 6 mol% and 1 mol% *peptide A* in membranes of the zwitterionic phospholipid, POPC (POPC gel/fluid phase transition temperature, −3°C [18]). As noted in Fig. 1, *peptide A* has deuterated alanine at position 650 (six residues beyond the 'cytoplasmic' membrane surface) and at position 637 (well within the α-helical intramembranous region). The ^{2}H NMR features of interest are the paired peaks and their separation, ΔV_Q, as described above. However, each spectrum also contains a sharp central peak which arises from residual deuterated water and can include a contribution from the presence of some vesicles

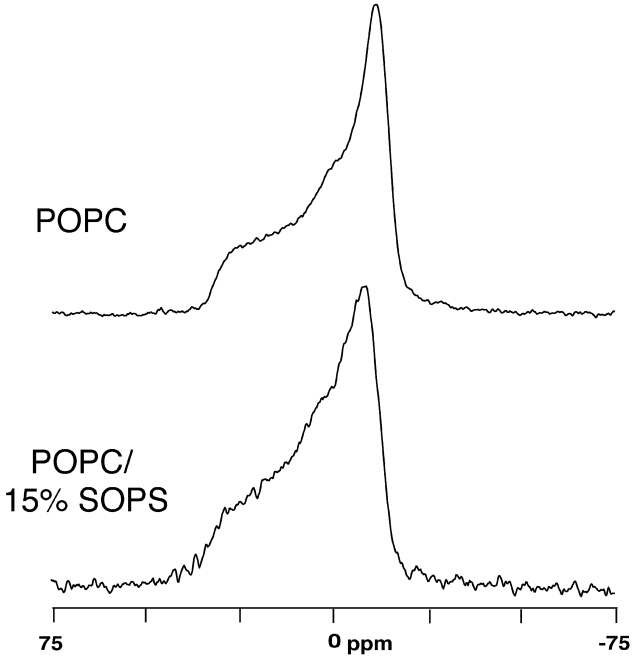

Fig. 2. Typical ^{31}P NMR spectra, demonstrating the bilayer nature of the samples. Upper spectrum: 6 mol% *peptide A* in POPC at 35°C. Lower spectrum: 6 mol% *peptide A* in POPC/SOPS in a molar ratio of 85/15 at 35°C. In each case samples were fully hydrated unsonicated liposomes suspended in 30 mM HEPES, 20 mM NaCl and 1 mM EDTA (pH 7.2). The number of accumulated transients was 38 800 (upper) and 12 000 (lower) at a sweep width of 40 kHz; a line broadening of 100 Hz was applied.

with high curvature (for which the quadrupole splittings are averaged to zero by isotropic reorientation). It may be noted however that Fig. 2 shows little evidence of an isotropic peak arising from small vesicles. The sharp central peak will not be further considered here.

In each case the spectrum of *peptide A* consists of two Pake doublets having quadrupole splittings much smaller than the 40 kHz value expected for an immobilized alanine. Thus the peptide is undergoing 'rapid' symmetric rotational diffusion in the membrane. The time scale that determines 'fast' vs. 'slow' in these experiments is 10^{-4}–10^{-5} s (the inverse of the spectral width in Hz) [1,3]. The outer doublet was assigned to (intramembranous) Ala^{637} based on spectra of peptides deuterated only at this location (not shown here). From the non-zero splitting of the inner Pake doublet (associated with Ala^{650}), one can conclude that the extramembranous portion is reorienting in a non-random fashion with

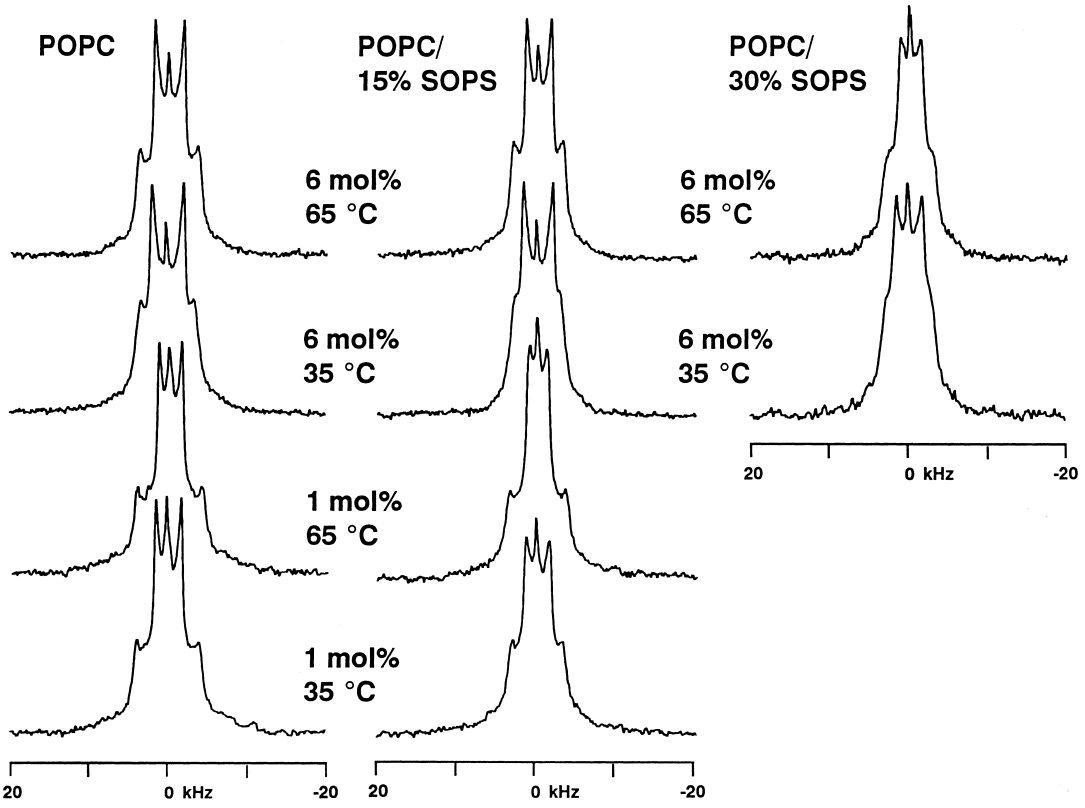

Fig. 3. ^{2}H NMR spectra of *peptide A* in fluid bilayers. Spectra correspond to the peptide shown in Fig. 1A, containing one deuterated amino acid positioned six residues external to the membrane surface (Ala^{650}), and one within the helical transmembrane portion (Ala^{637}). In the left hand column the membrane consisted of only the zwitterionic phospholipid, POPC, containing 6 or 1 mol% peptide. In the centre column the membrane also contained 15 mol% SOPS (relative to POPC). The right hand column shows spectra of the same peptide at 6 mol% in membranes containing 30 mol% SOPS (relative to POPC). Liposomes were suspended in 30 mM HEPES, 20 mM NaCl and 1 mM EDTA (pH 7.2) in deuterium-depleted water. Spectra were run at 65°C or 35°C as indicated. Each spectrum represents 400 000–600 000 accumulated transients.

a preferred axis of rotation (presumably associated with rotational diffusion of the peptide as a whole).

The NMR spectra described above characterize bilayer membranes and rapid axially symmetric rotation of deuterated amphiphiles inserted into bilayer membranes, and thus provide compelling evidence of the transmembrane nature of the peptide. We have previously demonstrated further such evidence by freeze-fracture electron microscopy of phospholipid bilayers prepared in the same fashion and containing *peptide C* [6,19]. In addition, CD spectra of the peptides at 6 mol% in POPC and in POPC/SOPS were characterized by negative peaks at 208 and 222 nm (spectra not shown), indicating predominantly α-helical secondary structure – as expected for transmembrane peptides [20] and as reported previously for this peptide in the same TFE solutions used to prepare the NMR samples [21]. 2-D ^{1}H NMR of the same peptide in TFE demonstrated that it is the transmembrane region which is helical [21].

Issues addressed in the present experiments relate to forces which might be exerted upon the cytoplasmic juxtamembrane domain of a receptor tyrosine kinase. One aspect of this is that the (−)charged phospholipid, PS, exists in the cytoplasmic leaflet of the cell membrane at up to 15 mol% [10,11]. Spectra in the centre column of Fig. 3 are paired with those in the left hand column, but are of *peptide A* at 6 mol% and 1 mol% in membranes of POPC containing 15 mol% phosphatidylserine. By utilizing nuclear probes attached covalently to the peptide, sensitivity to peptide phenomena is optimal. Despite this, and the intrinsic sensitivity of solid state ^{2}H NMR spectra to probe orientation and dynamics,

the spectra in the presence of SOPS are qualitatively very similar to those observed in the absence of this (−)charged lipid. Thus coulomb attraction between peptide and membrane surface has certainly not led to immobilization of the peptide intramembranous or extramembranous domains at physiologic concentrations of PS. There were, however, measurable quantitative differences in the presence of PS. Spectral splittings are listed in Table 1 for comparison with those observed in the matrix of zwitterionic POPC alone. In each case there was a decrease in quadrupole splitting upon addition of SOPS to the membrane. Higher concentrations of SOPS (beyond the range occurring naturally) caused proportionately greater increases in these changes in spectral splitting over the peptide concentration range studied (e.g. Fig. 3, right hand column) but were not systematically examined.

As described in association with Eq. 1, the spectral splitting, ΔV_Q, arising from a given alanine -CD_3 group reflects the average orientation (Θ_i) of that group. The splitting is further modulated by motional order: the order parameter, S_{mol}, varies from 1.0 for perfect orientational order to zero for random reorientation. Hence the splitting of a Pake doublet associated with a given probe is reduced by 'wobble' (disorder) of the portion of the molecule to which it is attached, but can be increased or decreased by orientation change. At 65°C and 35°C the introduction of 15 mol% SOPS into the POPC membrane led to a decrease in spectral splitting for Ala^{637} within the relatively stable hydrophobic α-helical region of the peptide, and also for Ala^{650} six residues downstream from the cytoplasmic membrane surface. The magnitude of this decrease ($\Delta(\Delta V_Q)^S$ in Table 1) was about 1 kHz for Ala^{637} (some 14% of the spectral splitting) at both 65°C and 35°C; and 0.4 or 0.2 kHz for Ala^{650} (12% or 6% of the spectral splitting) at 65°C and 35°C respectively. We cannot rule out the possibility that this effect of SOPS may arise partly from a decrease in the motional order of the peptide in the membrane rather than from a direct orientational effect induced by the (−)charged PS, although the higher phase transition temperature of hydrated SOPS (17°C [22]) might lead one to expect it to *increase* spectral splittings rather than decrease them if the changes were due to effects on peptide motional order.

A significant pattern of change in spectral splitting as a function of *temperature* ($\Delta(\Delta V_Q)^T$) is apparent in

Table 1
Spectral splittings, ΔV_Q, associated with Ala^{637}, Ala^{650}, Ala^{652}

Probe location and peptide concentration	Temperature	ΔV_Q (±0.3 kHz)		$\Delta(\Delta V_Q)^S$
		POPC	POPC+SOPS	
Ala^{637}				
6 mol%	65°C	7.86	6.78	−1.08
	35°C	7.23	6.15	−1.08
$\Delta(\Delta V_Q)^T$		−0.63	−0.63	
1 mol%	65°C	8.66	7.59	−1.07
	35°C	8.28	6.83	−1.45
$\Delta(\Delta V_Q)^T$		−0.38	−0.76	
Ala^{650}				
6 mol%	65°C	3.89	3.46	−0.43
	35°C	4.28	4.05	−0.23
$\Delta(\Delta V_Q)^T$		+0.39	+0.59	
1 mol%	65°C	3.11	2.67	−0.44
	35°C	3.41	3.21	−0.20
$\Delta(\Delta V_Q)^T$		+0.30	+0.54	
Ala^{652}				
6 mol%	65°C	2.22	1.77	−0.45
	35°C	2.65	2.29	−0.36
$\Delta(\Delta V_Q)^T$		+0.43	+0.52	

$\Delta(\Delta V_Q)^S$ is the change in spectral splitting for a given set of conditions upon addition of 15 mol% SOPS to the membrane.
$\Delta(\Delta V_Q)^T$ is the change in spectral splitting for a given sample upon dropping the temperature from 65°C to 35°C.

Table 1. Alanine in the portion of the peptide external to the membrane (Ala^{650}) exhibits decreased splitting as temperature is raised to 65°C. In contrast, the splitting associated with alanine in the helical transmembrane portion (Ala^{637}) does the opposite – i.e. increases as temperature is increased. This is apparent at both low and high concentrations of peptide in the membrane, and occurs in the presence and absence of SOPS. Thus in each case, as temperature is increased by 30°C, the spectral splitting for Ala^{650} *decreases* by about 0.5 kHz, while that for Ala^{637} *increases* by about 0.5 kHz. The behaviour of Ala^{650} is consistent with a simple increase in 'wobble' of the extramembranous portion of the peptide at higher temperature. This would suggest some freedom of motion for residue 650, exterior to the membrane surface, and is in keeping with the observation that its splittings are smaller than those associated with Ala^{637} – i.e. Ala^{650} being in a less ordered, less structured portion of the peptide. The effect on the intramembranous helical site implies that it remains highly ordered at both temperatures (consistent with values of S_{mol} near 0.9 measured for bacterial transmembrane peptides [23–25]), and that the probe on Ala^{637} in the transmembrane helix undergoes some orientational change when temperature is varied between 35°C and 65°C.

Results derived with a second peptide, having deuterated alanine as a probe at extramembranous residue 652 (*peptide B* in Fig. 1), are also listed in Table 1. Since very limited quantities of this peptide were obtained in pure form, only one concentration range is given. The results at this second extramembranous site reinforce the pattern described above for Ala^{650}.

In considering the effect of phosphatidylserine in the above experiments, results found with *peptide C* were examined. This peptide was produced for a separate study [6], and included deuterium probe nuclei on residues with more lengthy side chains (Fig. 1): the $-CD_3$ group at the end of the side chain in Met^{644} within the hydrophobic interior, and the two $-CD_3$ groups at Val^{650} in the cytoplasmic extramembranous domain (in addition to a fourth at Ala^{623} in a relatively less structured portion of the transmembrane α-helix near the 'extracellular' membrane surface [6]). Fig. 4A presents selected spectra of this peptide. As we have noted previously [6] its spectrum in POPC bilayers is a simple sum of four Pake doublets from the four CD_3 groups – one associated with Ala^{623}, one with Met^{644}, and two on the side chain of Val^{650}. Addition of 15 mol% SOPS to the membrane led to a striking collapse of the two doublets corresponding to Val^{650} to a broad central peak. Its effect on the doublets associated with Ala^{623} and Met^{644} was limited to very small splitting changes (within experimental uncertainty). This was demonstrated using peptides with identical sequences to *peptide C* but containing deuterium probe nuclei only on methyl groups of Ala^{623} or Met^{644} side chains (Fig. 4B). Using the same peptide *sequence*, *C*, but containing deuterium nuclei only on Val^{650}, it was confirmed that the spectral collapse associated with addition of physiological amounts of SOPS was due to the two Pake doublets of Val^{650}. The bottom spectral pair (Fig. 4C) was obtained with the amino acid sequence of *peptide C*, but in which only Val^{650} was deuterated on its two terminal methyls: it demonstrates that the same phenomenon of spectral collapse to a single broad line for Val^{650} also occurs when 15% SOPS is added to POPC/peptide membranes containing 33 mol% cholesterol.

The spectral effect of SOPS on Val^{650} is typical of that seen for deuterium probes whose overall rotational motions become axially asymmetric [2]. Our result is reminiscent of a recent observation by Koenig et al. [26] for a $-CD_3$ group on a membrane peptide from an HIV envelope glycoprotein associated with PS bilayers. In the latter case the $-CD_3$ group was on an isoleucine ('I_{16}') side chain on the part of the peptide extending beyond the surface: a 20°C temperature drop caused collapse of quadrupole splittings to a more central pattern suggested to be due to altered exchange amongst several conformations. In the case of Val^{650}, an intermediate exchange rate seems to be ruled out as the direct cause of pyramidal line shape since this line shape was not altered by varying the temperature over a range of 30°C. Further arguing against an 'intermediate time scale' explanation for the line shape is the fact that the PS effect was almost identical in membranes of POPC and POPC/cholesterol – membranes in which molecules have quite different rotation and diffusion rates. The change in rotational properties of the Val^{650} side chain seems best understood as resulting from a modest local change in orientation of the cytoplasmic portion of the EGF

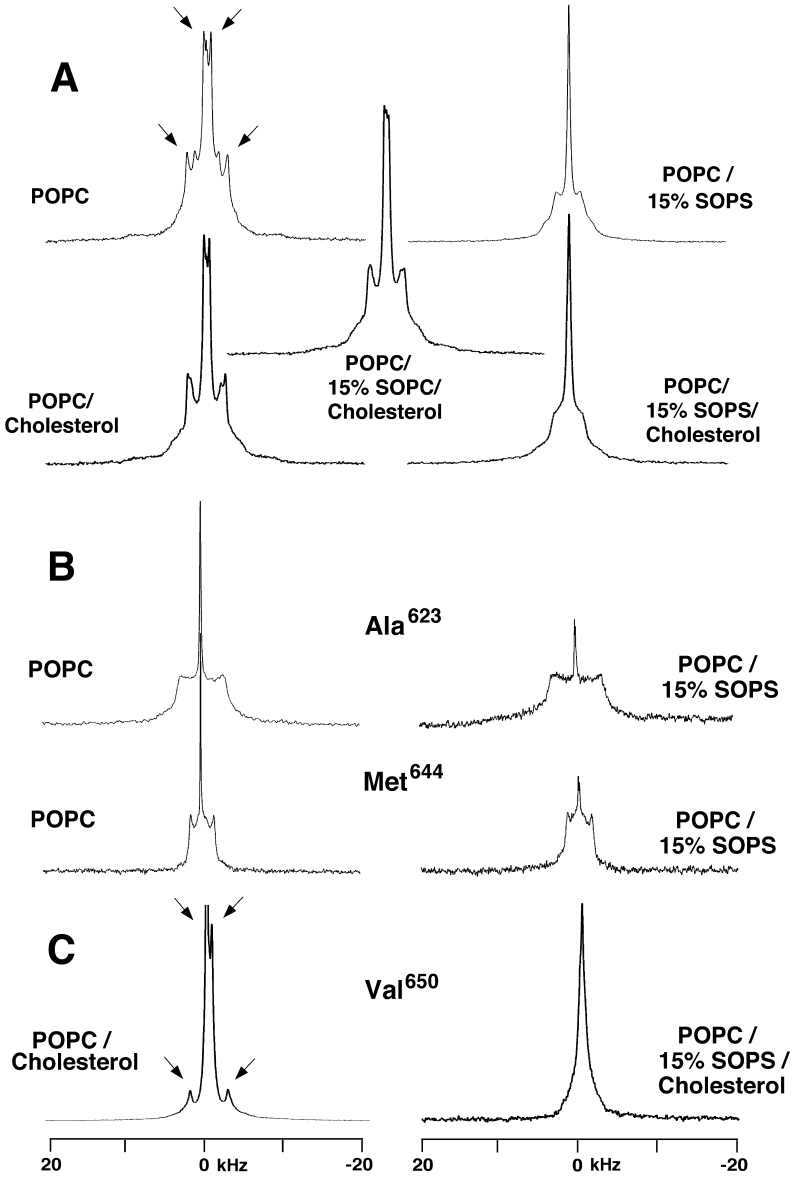

Fig. 4. ^{2}H NMR spectra corresponding to *peptide C* of Fig. 1. Spectra in A demonstrate the effect of adding 15 mol% SOPS to bilayer membranes containing *peptide C* with methyl deuteration at Ala^{637}, Met^{644}, and Val^{650}. The uppermost spectral pair in this group shows the effect of adding 15 mol% SOPS to the POPC host matrix containing *peptide C* (in which Val^{650}, Met^{644} and Ala^{637} all contain $-CD_3$ groups): collapse of two of the original four Pake doublets to a broad central peak. Shown immediately below these in the same spectral group (A), are equivalent spectra for samples upon addition of 33 mol% cholesterol to the host matrix, and a third spectrum demonstrating that replacement of 15% SOPS by SOPC did not lead to collapse of the Pake doublets. Spectra in B were obtained with the amino acid sequence of *peptide C*, but in which only Ala^{623} was deuterated (upper) and only Met^{644} was deuterated (lower): these demonstrate that the spectral collapse caused by 15 mol% SOPS is not associated with Ala^{623} or Met^{644}. The bottom spectral pair (C) was obtained with the amino acid sequence of *peptide C*, but in which only Val^{650} was deuterated: it demonstrates that the same phenomenon of spectral collapse to a single broad line for Val^{650} occurs when 15% SOPS is added to POPC/peptide membranes containing 33 mol% cholesterol. In each case, liposomes were suspended in 30 mM HEPES, 20 mM NaCl and 1 mM EDTA (pH 7.2) in deuterium-depleted water. Arrows indicate Pake features which collapse upon addition of 15 mol% SOPS. Each spectrum represents 290 000–360 000 accumulated transients obtained at 35°C.

receptor: although Val^{650} is itself uncharged, it is surrounded by amino acids with large (+)charged side chains.

The concept that potential fields can induce conformational changes in dipolar molecules at membrane surfaces has been extensively developed by others. Most such experiments have utilized solid state ^2H NMR to monitor deuterium probes located within phospholipid head groups ([18,27–29] and references therein). Resultant charge-based intermolecular associations have been reported to be rapidly reversible on the NMR time scale (e.g. [27,28,30]). Consistent with this, in the present work no separate spectrum of immobilized peptide was observed. The magnitude of splitting changes observed here for deuterated alanine probes in the extramembranous peptide domain could be accounted for by a change of as little as a few degrees in *average* orientation of the extramembranous portion at physiological concentrations of SOPS (although they became substantially larger at SOPS concentrations beyond the physiological). Watts and colleagues [31] recorded similarly small changes (0.1–0.2 kHz) in *lipid* head group $-CD_3$ probes upon binding of the protein, myosin, to lipid bilayers: these were interpreted in terms of conformational change in lipid probes brought about by coulomb forces arising from interaction with a cluster of (+)charged lysine residues in the protein. Struppe et al. [32] used deuterium probes on myristic acid to detect electrostatic interaction between anionic phospholipids and lysine residues in a myristoylated peptide.

The origins of the spectral effects recorded here are potentially complex, as noted by Seelig et al. [27] regarding membrane protein charge interactions with lipids in general. Alterations in head group charge are also known to alter membrane thickness and lipid packing [33], although systematized comparisons of the lipids used here are not available. In the present work POPC and SOPS were selected as common natural species. Since small quantities of the (−)charged lipid were added to the host matrix, and since the samples have been studied well above the phase transitions of the lipids, one may anticipate a relatively homogeneous lipid matrix and modest changes in host matrix properties. The main phase transition of SOPS [22] is 17°C, vs. −3°C for POPC [18], and thus it might be expected to slightly *increase* spectral splitting (via the S_{mol} term in Eq. 1 if its major effect upon the peptide arises from changes in lipid order. In fact, the spectral splittings did the opposite – i.e. *decreased* upon addition of SOPS. It is certainly possible though that the anticipated small change in lipid packing and membrane thickness has some effect on peptide orientation. In order to derive some idea of the relative contributions of such phenomena, the zwitterionic lipid, SOPC (phosphatidylcholine with the same fatty acid composition as SOPS), was added in place of SOPS to several samples. This was done for instance by adding 30% SOPC vs. 30% SOPS to POPC membranes containing 1 mol% *peptide A*. Interestingly, at 65°C SOPC had an effect opposite to that of SOPS on alanine probes in the extramembranous environment of *peptide A*: SOPC lead to a slightly *increased* spectral splitting of 3.53 kHz at Ala^{650} whereas 30% SOPS caused a *decrease* to 2.21 kHz. Similar results were obtained at 35°C, with an increase in splitting to 4.03 kHz in SOPC-containing membranes and a decrease to 2.96 kHz caused by SOPS. On the other hand, the effect of SOPC on Ala^{637} in the membrane *interior* was qualitatively the same as that seen upon SOPS addition, although the quantitative result of SOPC addition was smaller (e.g. SOPC only caused a decrease in Ala^{637} splitting to 7.28 kHz at 65°C vs. SOPS causing a decrease to 6.54 kHz). These observations echo the pattern of the temperature-induced changes noted above – that the cytoplasmic and intramembranous portions of the peptide can respond differently to a given perturbation. They suggest that the effects of SOPS on the hydrophobic region of the peptide (Ala^{637}) have a significant basis in changes to membrane thickness and/or lipid order, while SOPS effects on the extramembranous portion (Ala^{650}) reflect a greater influence of charge. In keeping with such a view, addition of 15% SOPC did not induce the dramatic qualitative spectral changes caused by SOPS in the extramembranous deuterated valine residue (Val^{650}) of *peptide C* (Fig. 4A).

It has been emphasized that the magnitude of surface potential effects is sensitive to vertical location of charges relative to the membrane surface [28]. Roux et al. [34] concluded for instance that the (+)charged peptide, pentalysine, induces relatively small effects on the quadrupole splittings of deuterated phosphatidylcholine head groups because the

peptide is located outside the head group region (and see [27]). Arêas et al. [31] concluded that there were relatively smaller coulomb field effects between deuterated PS lipid head groups and (+)charged peptide domains *beyond* the lipid head group region. Such results seem relevant to transmembrane proteins such as those studied in the present work since only a fraction of the (+)charged amino acids will be within the lipid head group region.

4. Conclusions

Little is known about the behaviour of the cytoplasmic juxtamembrane domain of receptor tyrosine kinases; yet this region is a control point in their function. In the present work, solid state NMR of transmembrane peptides related to the EGF receptor offered insight into some of the forces that may be involved. Probes attached to the peptide backbone, 6–8 residues from the membrane 'cytoplasmic' surface, demonstrated that the cytoplasmic portion exhibited rapid axially symmetric motion – as did the helical transmembrane portion. These motional characteristics were also present when a physiologic concentration (15 mol%) of the (−)charged lipid, phosphatidylserine, was incorporated into the membrane. The extramembranous domain had more internal flexibility than did the transmembrane helix, but retained a preferred axis of rotation. Physiological concentrations of phosphatidylserine appeared to induce modest orientational changes in the peptide extramembranous domain – presumably reflecting surface potential effects on the clusters of positively charged amino acids near the cytoplasmic surface. There was a clear difference in temperature sensitivity between probes attached directly to the peptide backbone in the extramembranous portion vs. the transmembrane helical portion: this seems likely to reflect greater flexibility of the extramembranous portion and temperature-induced reorientation of the transmembrane portion. There was evidence that membrane thickness and lipid packing effects had a greater influence on the intramembranous portion of the peptide.

Acknowledgements

This research was supported by an operating grant to CWMG from the MRC of Canada. SS holds an NSERC PGSB award. NMR spectroscopy was carried out in the McLaughlin Macromolecular Structure Facility, established with joint grants to the department from the London Life Insurance Co., the R.S. McLaughlin Foundation, the MRC Development Program, and the Academic Development Fund of UWO; and in the UWO NMR Facility.

References

[1] J. Seelig, Q. Rev. Biophys. 10 (1977) 353–418.
[2] S.J. Opella, Methods Enzymol. 131 (1986) 327–361.
[3] J.H. Davis, in: E. Buncel, J.R. Jones (Eds.), Isotopes in the Physical and Biomedical Science, vol. 2, Elsevier, Amsterdam, 1991.
[4] D.J. Siminovitch, Biochem. Cell Biol. 76 (1998) 411–422.
[5] A.C. Rigby, K.R. Barber, G.S. Shaw, C.W.M. Grant, Biochemistry 35 (1996) 12591–12601.
[6] D.H. Jones, A.C. Rigby, K.R. Barber, C.W.M. Grant, Biochemistry 36 (1997) 12616–12624.
[7] D.H. Jones, K.R. Barber, C.W.M. Grant, Biochim. Biophys. Acta 1371 (1998) 199–212.
[8] G. von Heijne, J. Mol. Biol. 225 (1992) 487–494.
[9] R.A.F. Reithmeir, C.M. Deber, in: P. Yeagle (Ed.), The Structure of Biological Membranes, CRC Press, Boca Raton, FL, 1992, pp. 337–393.
[10] A.J. Schroit, R.F.A. Zwaal, Biochim. Biophys. Acta 1071 (1991) 313–329.
[11] R. Lipowsky, Curr. Opin. Struct. Biol. 5 (1995) 531–540.
[12] M. Mosior, R.M. Epand, J. Biol. Chem. 269 (1994) 13798–13805.
[13] W.J. Fantl, D.E. Johnson, L.T. Williams, Annu. Rev. Biochem. 62 (1993) 453–481.
[14] P. van der Geer, T. Hunter, Annu. Rev. Cell Biol. 10 (1994) 251–337.
[15] N.E. Hynes, D.F. Stern, Biochim. Biophys. Acta 1198 (1994) 165–184.
[16] C. Landolt-Marticorena, K.A. Williams, C.M. Deber, R.A.F. Reithmeier, J. Mol. Biol. 229 (1993) 602–608.
[17] P.G. Scherer, J. Seelig, Biochemistry 28 (1989) 7720–7728.
[18] P.J. Davis, K.M.W. Keough, Biophys. J. 48 (1985) 915–918.
[19] D.H. Jones, K.R. Barber, C.W.M. Grant, Biochim. Biophys. Acta 1371 (1998) 199–212.
[20] T.C. Chang, C.-S.C. Wu, J.T. Yang, Anal. Biochem. 91 (1978) 13–31.
[21] A.C. Rigby, C.W.M. Grant, G.S. Shaw, Biochim. Biophys. Acta 1371 (1998) 241–253.

[22] D. Bach, E. Wachtel, N. Borochov, G. Senisterra, R.M. Epand, Chem. Phys. Lipids 63 (1992) 105–113.
[23] R.E. Koeppe II, J.A. Killian, D.V. Greathouse, Biophys. J. 66 (1994) 14–24.
[24] R.S. Prosser, S.I. Daleman, J.H. Davis, Biophys. J. 66 (1994) 1415–1428.
[25] F.M. Marassi, A. Ramamoorthy, S.J. Opella, Proc. Natl. Acad. Sci. USA 94 (1997) 8551–8556.
[26] B.W. Koenig, J.A. Ferretti, K. Gawrusch, Biochemistry 38 (1999) 6327–6334.
[27] P.G. Seelig, P.M. MacDonald, P.G. Scherer, Biochemistry 26 (1987) 7535–7541.
[28] M. Roux, M. Bloom, Biochemistry 29 (1990) 7077–7089.
[29] K.J. Crowell, P.M. Macdonald, J. Phys. Chem. B 102 (1998) 9091–9100.
[30] C. Dempsey, M. Bitbol, A. Watts, Biochemistry 28 (1989) 6590–6596.
[31] J.A. Arêas, G. Gröbner, C. Glaubitz, A. Watts, Biochemistry 37 (1998) 5582–5588.
[32] J. Struppe, E.A. Komives, S.S. Taylor, R.R. Vold, Biochemistry 37 (1998) 15523–15527.
[33] R.P. Rand, V.A. Parsegian, Biochim. Biophys. Acta 988 (1989) 351–376.
[34] M. Roux, J.-M. Neumann, R.S. Hodges, P.F. Devaux, M. Bloom, Biochemistry 28 (1989) 2313–2321.