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Molecular basis of the inhibition of gentamicin nephrotoxicity by daptomycin; an infrared spectroscopic investigation

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Abstract

The lipopeptide daptomycin has been reported to reduce in vivo the nephrotoxicity of aminoglycoside antibiotics (Wood et al. (1989) Antimicrob. Agents Chemother. 33, 1280–1285; Beauchamp et al. (1990) Antimicrob. Agents Chemother. 34, 139–147). A recent dialysis study confirmed the existence of an electrostatic interaction between daptomycin and tobramycin (Couture et al. (1994) Antimicrob. Agents Chemother. 38, 742–749). The interaction of gentamicin with daptomycin and phosphatidylinositol (PI) dispersions was investigated by FTIR spectroscopy. We found no evidence of a direct interaction involving the neutralization of the aspartate groups of daptomycin by gentamicin and the amide I band of daptomycin did not reveal significant conformational changes of its peptidic moiety. On the other hand, daptomycin readily inserts within bilayers of PI, dimyristoylphosphatidylglycerol or dipalmitoylphosphatidylcholine, as judged from its influence on the fluidity of these bilayers. The incorporation of daptomycin into PI bilayers has no significant effect on the lipopeptide amide I band. Gentamicin also binds to PI bilayers and the associated modifications of the lipid bands are consistent with a tightening of the lipid network resulting from head group neutralization by gentamicin. The affinity of the aminoglycoside for PI is slightly increased in the presence of daptomycin, in agreement with the results of the dialysis study mentioned above. The lipid features indicate that its head group is still affected by gentamicin charges, but the thermotropic behavior of the hydrophobic portion becomes similar to that of the pure lipid. It is proposed that the contribution of daptomycin to the membrane charge density and its effect on the lipid packing both combine to counteract the inhibition of phospholipase activity due to aminoglycosides. Further work will attempt to determine how the peptide rings and gentamicin molecules are organized at the bilayer surface, how specific these interactions are and to confirm the influence of daptomycin on the phospholipid catabolism.

Keywords: Daptomycin; Gentamicin; Aminoglycoside; Nephrotoxicity; Phosphatidylinositol; Infrared spectroscopy

1. Introduction

The aminoglycosidic antibiotic gentamicin (Fig. 1A) is widely used to treat infections caused by Gram-negative bacteria [1]. Unfortunately, aminoglycosides may also yield oto-and nephrotoxic reactions. The higher susceptibility of kidney and inner ear cells seems to be related to the presence of high concentrations of phosphatidylinositols in their cytoplasmic membranes [2]. The existence of specific biochemical interactions of aminoglycosides with their target cells has been suggested [2]. Aminoglycosides are not metabolized and are eliminated from the body by the kidney only. The resulting high concentrations of these drugs in the glomerular filtrate promote their nephrotoxicity.

The first step in the mechanism leading to aminoglycoside-induced acute renal failure is the binding of the positively charged antibiotic to anionic lipids on brush border membranes [3,4] and possibly also on basolateral membranes. Phosphatidylinositides have been proposed to be the receptors for aminoglycosides on brush border membranes [5]. The reabsorption then occurs through pinocytosis, followed by fusion of the endocytotic vacuoles with primary lysosomes. The aminoglycoside accumulates within lysosomes and inhibits phospholipases and sphingomyelinase activity [6,7], causing the formation of

Abbreviations: DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol.

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myeloid bodies. The growth of these tightly apposed lipidic bilayers is believed to result in swelling of the lysosomes and ultimately in their bursting and the release of large amounts of aminoglycoside, lysosomal enzymes and phospholipids into the cytosol. Necrosis of proximal tubular cells follows, leading to impairment of renal function. In fact, a recent immunocytochemical study of the subcellular distribution of gentamicin showed that the drug distributed in the cytoplasm, in the mitochondria and in the nucleoli of necrotic proximal tubular cells [4]. Owing to the remarkable regenerative properties of kidney cells, this impairment is usually reversible upon cessation of treatment. Nevertheless, the reduction of aminoglycoside nephrotoxicity remains a concern among clinicians and various strategies are being explored to reduce it. Oral supplementation of calcium has been found to reduce gentamicin nephrotoxicity, the cation acting as a competitive inhibitor of the drug on renal membrane binding sites [8,9]. The applicability of calcium supplementation for clinical use is questionable since high amounts are required to get protection. Latamoxef, an oxacephem antibiotic, has been shown to protect rat from nephrotoxicity induced by the aminoglycoside tobramycin. Latamoxef reduces tobramycin intrarenal level [10] by inhibiting its binding to brush border membranes [11]. It has been shown to react chemically with tobramycin in vitro [12]. Pyridoxal 5′-phosphate (vitamin B-6) also protects against gentamicin induced nephrotoxicity by mechanisms similar to latamoxef [13]. On the other hand, a protection of renal function can also be afforded without any reduction of aminoglycoside cortical level. Hence, coadministration of polyaspartic acid with gentamicin prevented functional renal failure [14–18] in spite of up to 10-fold increase of the level of accumulated aminoglycoside [16]. A reduction of lysosomal phospholipidosis was noted and the usual post-necrotic aminoglycoside-induced increase in DNA synthesis was prevented [18]. The polyamino acid was found to bind gentamicin and to displace it from anionic phospholipids in vitro [19]. The clinical use of polyaspartic acid needs to be assessed.

Daptomycin (Fig. 1B) has been proposed as an alternative to vancomycin, [20,21] a glycopeptidic antibiotic used in combination with aminoglycosides to enhance their bactericidal activity but which also aggravates their nephrotoxicity [6,22,23]. Daptomycin, formerly known as LY146032, is a lipopeptidic antibiotic active against Gram-positive bacteria in which it inhibits lipoteichoic acid synthesis. Daptomycin has been found to prevent the development of aminoglycoside-induced functional renal failure [24]. Similar to polyaspartic acid, it inhibited lysosomal phospholipidosis and increased cellular regeneration in the presence of similar or even higher aminoglycoside levels [6]. The mechanism for this inhibition is still unknown and the present spectroscopic investigation is an attempt to address this question. We are thus searching for spectral indications of (i) a direct interaction between daptomycin and gentamicin in solution and of (ii) a possible influence of daptomycin on gentamicin binding to lipidic membranes. Bilayers of phosphatidylinositol are used as model membranes because gentamicin binds preferentially to that lipid.

2. Materials and methods

The sodium salt of L-α-phosphatidylinositol from soya bean was obtained from Sigma (St. Louis, MO). Gentamicin was kindly donated by Schering Canada (Pointe-Claire, Québec, Canada) whereas daptomycin was a generous gift of Eli-Lilly Canada (Scarborough, Ontario, Canada). Deuterated water was from MSD Isotopes (Montréal, Québec, Canada). The lipidic dispersions (10 wt%) were prepared in H_2O or ^2H_2O with at least three freeze-thaw cycles. Additional freeze-thaw cycles were also performed after additions of the required amount of 5% gentamicin solution and 10% daptomycin solution in H_2O or ^2H_2O, after appropriate pH adjustment by addition of minimal amounts of HCl or NaOH (or $^2HCl/NaO^2H$), as required.

Infrared spectra were obtained at room temperature on a Digilab FTS-40 Fourier-transform spectrometer. A total of 256 interferograms were co-added for each spectrum, with a spectral resolution of 2 cm^{-1}. Experiments at variable temperature were realized on a Digilab FTS-60 Fourier-transform spectrometer equipped with a thermostatted bath, with an equilibration period of 7 min after reaching each temperature. Data treatment was performed as described earlier [25].

3. Results

Infrared spectroscopy is an excellent technique to probe molecular interactions because the vibrational modes that are examined are often affected by changes in molecular segment conformation, charge and hydrogen bonding. The simplest hypothesis to explain the protection afforded by daptomycin against gentamicin nephrotoxicity is a direct interaction of the two oppositely charged species in solution, resulting in an impairment of the aminoglycoside binding to brush border membranes.

3.1. Direct interaction of daptomycin and gentamicin

Gentamicin (Fig. 1A) has a very simple infrared spectrum. Below 2000 cm^{-1}, it comprises a single well defined band, at approx. 1103 cm^{-1} (Fig. 2). This band contains contributions from many vibrations, in particular C-N stretching from the primary and secondary amine groups, C-O stretching and C-C stretching. This composite band remains the same at pH 7.5 and 5.4. This latter is the pH found in lysosomes, where gentamicin accumulates in kidney cells [26]. The contributions of C-N, C-O and C-C

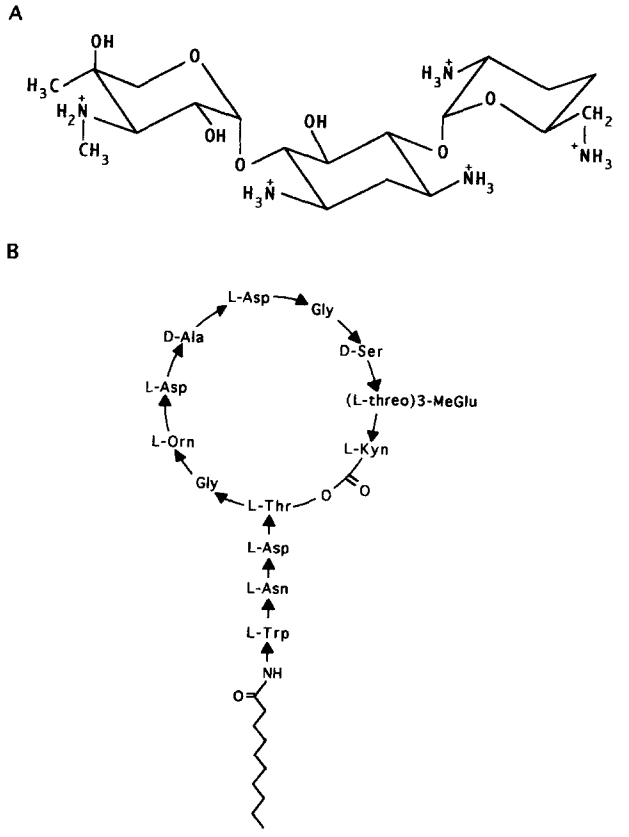

Fig. 1. Chemical structure of gentamicin C_{1A} (A) and of daptomycin (B).

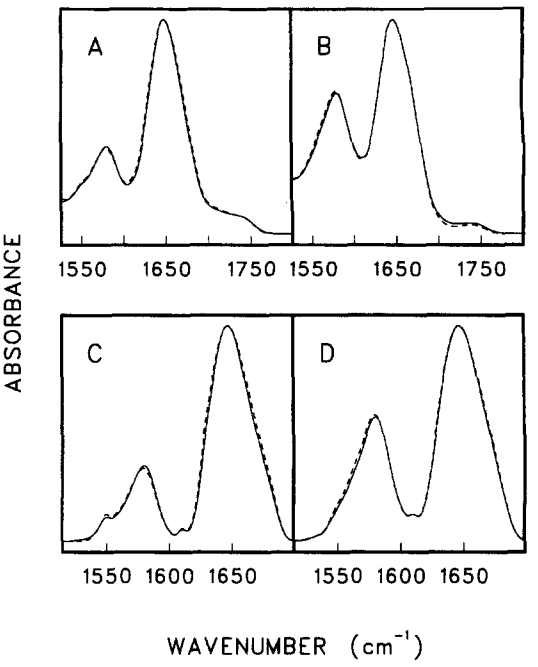

Fig. 3. Carbonyl stretching region of the infrared spectra of daptomycin alone (solid line) or with gentamicin (dashed line) in solution, at a gentamicin/daptomycin molar ratio of 2:1 in aqueous solutions, at pH 5.4 (left panels) and 7.5 (right panels). The original spectra (A and B) were deconvolved using a bandwith of 13 and a breakpoint of 1.75 (C and D).

stretching vibrations of daptomycin in that region do not give rise to a well defined band. The addition of daptomycin to gentamicin does not result in any significant alteration of the frequency or overall shape of gentamicin 1103 cm^{-1} band at either pH.

The lipopeptide daptomycin (Fig. 1B) contains many features that can be sensitive to changes in conformation or ionization of the molecules. The 3-methyl glutamate and the three aspartate side chains confer a net negative charge on the molecule at neutral pH. The carbonyl stretching vibrations of these groups give a band of moderate intensity at 1580 cm^{-1} at both pH 5.4 and 7.5, consistently with the prevalence of the ionized state in that pH interval (Fig. 3A, solid line). At lower pH, the carboxylate band loses intensity and the protonated acid band grows up at 1710 cm^{-1}. Another carbonyl stretching band is also found at approx. 1744 cm^{-1} at all pH values. This peak is probably due to the C = O stretching vibration of the ester function of the peptidic ring and of the aromatic ketone group of the L-kynurenine residue. This feature is of little diagnostic value. On the other hand, the amide I band seen at 1649 cm^{-1} is known to be sensitive to peptide backbone conformation. In proteins and polypeptides, its shape and frequency are used to characterize secondary structure. The cyclic structure of daptomycin peptidic portion severely restrains the backbone flexibility and impedes the formation of sufficiently long α-helices or β-sheets for example. Nevertheless, interactions at the side chains and changes in environment can still be reflected in spectral variations of the amide I band because they can affect the backbone geometry. The shape and frequency of the amide I band are not affected upon going from pH 5.4 to 7.5 and addition of gentamicin also fails to induce any significant changes (Fig. 3).

Although the neutralization of a fraction of the carboxylate groups produced upon lowering the pH from 7.5 to 5.4 was reflected in a marked decrease of the intensity of the band at 1579 cm^{-1}, no consistent change of that band was noted when gentamicin was added. If there is an

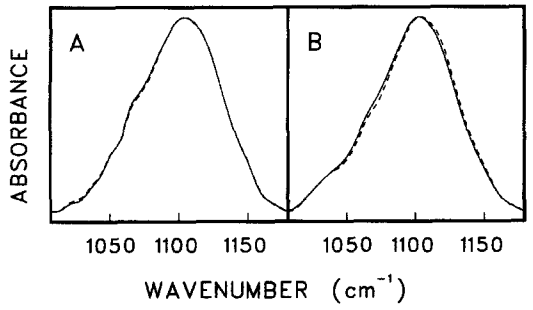

Fig. 2. Infrared spectrum of gentamicin in the C-O, C-C and C-N stretching region at pH 5.4 (A) and 7.5 (B). Solid lines represent aqueous solutions of gentamicin and dashed lines, aqueous solutions of gentamicin in the presence of daptomycin at a gentamicin/daptomycin molar ratio of 2:1. Spectral contributions of daptomycin have been subtracted.

electrostatic interaction between daptomycin and gentamicin, it can only be a loose one, extending through the hydration spheres of these molecules and causing no constraints on their conformation.

3.2. Effect of gentamicin on phosphatidylinositol bilayers

To better judge the effect of daptomycin on gentamicin–PI interaction, we must first characterize this latter. The lipid spectrum contains two useful features in this regard: its carbonyl stretching band and its phosphate antisymmetric stretching band, both occurring in spectral regions free of gentamicin contributions.

The carbonyl stretching band of the ester functions of phosphatidylinositol in aqueous suspension is presented in Fig. 4. The original spectrum, consisting of an asymmetric band, reveals two components after resolution enhancement using Fourier self-deconvolution (Fig. 4A and B, solid line) [25]. The high frequency component, at 1745 cm^{-1}, pertains to carbonyl groups that are not hydrogen bonded and the peak at 1725 cm^{-1} corresponds to hydrogen bonded carbonyl groups. The addition of gentamicin to PI suspension in a molar ratio of 2 PI/1 gentamicin does not result in any significant frequency shift of these two components, but an increase of the intensity ratio of non bonded to bonded carbonyl bands is observed (Fig. 4, small dashes). Gentamicin thus induces an increase in the proportion of carbonyl groups that are not hydrogen bonded. Replacing the solvent ^2H_2O by H_2O resulted in a 1 cm^{-1} increase in the frequency of hydrogen bonded carbonyl band while the free carbonyl component remained unaffected (not shown). This frequency shift indicates that the carbonyl hydrogen bonding involves solvent molecules. The decrease in the population of hydrogen bonded carbonyl groups shows that gentamicin reduces the penetration of water to the bilayer interface region. It is

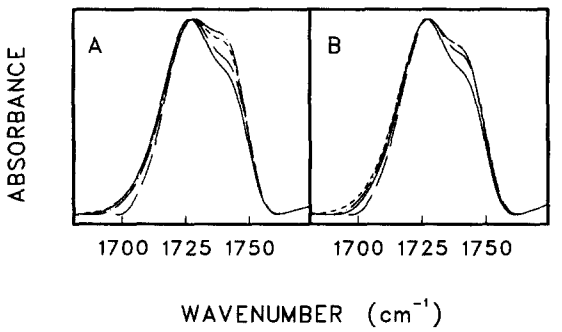

Fig. 4. Carbonyl stretching region of the infrared spectra of dispersions of phosphatidylinositol in ^2H_2O at p^2H 5.4 (A) and 7.5 (B). The spectrum of a dispersion of the pure lipid (solid trace) is compared to that of dispersions containing gentamicin (small dashes) at a PI/gentamicin molar ratio of 2:1, daptomycin (long dashes) at a PI/daptomycin molar ratio 4:1 or both gentamicin and daptomycin (dot-long dash) at a PI/gentamicin/daptomycin molar ratio 4:2:1. The spectra were obtained after resolution enhancement using Fourier self-deconvolution with a bandwith of 13 and a breakpoint of 1.5.

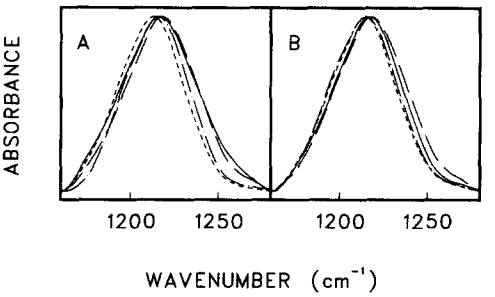

Fig. 5. Phosphate antisymmetric stretching region of the infrared spectra of aqueous dispersions of phosphatidylinositol alone (solid line) or with gentamicin (small dashes) at a PI/gentamicin molar ratio of 2:1, daptomycin (long dashes) at a PI/daptomycin molar ratio of 4:1 or both gentamicin and daptomycin (dot-long dash) at a PI/gentamicin/daptomycin molar ratio of 4:2:1, at pH 5.4 (A) and 7.5 (B).

consistent with a tightening of the lipidic network following neutralization of PI molecules by gentamicin. The same behavior is observed at p^2H 5.4 and 7.5. Ramsammy and Kaloyanides observed a gentamicin-induced decrease of glycerol permeability of DPPC/PI liposomes at pH 7.0 and related it to such a tightening of the lipidic network [27]. They also reported an increase of the activation energy for glycerol permeation and suggested that it might result from hydrogen bonding between some amino groups of gentamicin and the lipid carbonyl ester groups. That explanation is not consistent with our finding that gentamicin induces a decrease of the proportion of hydrogen bonded carbonyl groups.

The phosphate region must be studied in the absence of ^2H_2O to avoid interferences from its bending mode in the 1200 cm^{-1} region. The frequency of the phosphate antisymmetric stretching vibration usually varies between 1220 and 1270 cm^{-1}, depending on the extent of hydrogen bonding [28]. A lipidic dispersion of PI yielded a broad band with a maximum at 1216 cm^{-1} (Fig. 5A and B, solid line), for both pH 5.4 and 7.5. The addition of gentamicin resulted in a decrease of approx. 2 cm^{-1} of the frequency of the maximum of that band, irrespective of pH (Fig. 5A and B, small dashes). Actually, this frequency shift seems to result mostly from a marked decrease of the intensity on the high frequency side of the broad band, corresponding to lipid molecules with weaker hydrogen bonds. The frequency shift was smaller for a molar ratio of 8 PI/1 gentamicin instead of 2:1. The strengthening of phosphate hydrogen bonds is also consistent with a decrease of PI intermolecular distance resulting from charge neutralization by gentamicin.

3.3. Effect of daptomycin on phosphatidylinositol bilayers

Daptomycin bears a net negative charge which precludes electrostatic binding to PI bilayers. There should rather be electrostatic repulsion. On the other hand, the long decanoyl chain of the lipopeptide will also give rise

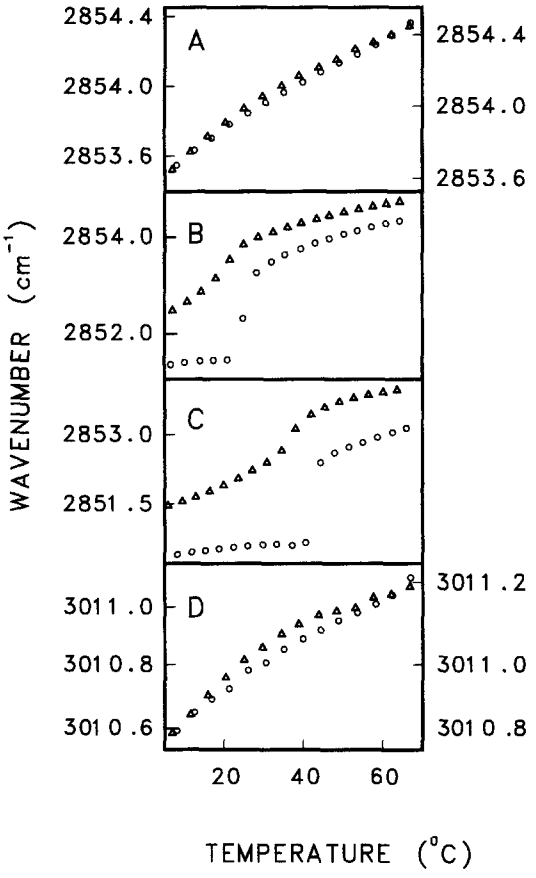

Fig. 6. Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of PI (A), DMPG (B) and DPPC (C) and of the frequency of the C-H stretching vibration of the olefinic groups of PI (D) in aqueous dispersions, in the absence (circles) and in the presence (triangles) of daptomycin at a lipid/daptomycin molar ratio of 2:1 at preparation, p^2H 5.4. The coordinates on the right side of panels A and D apply to triangles.

to an entropy-driven hydrophobic effect. In other words, the lipidic character of daptomycin could dominate the thermodynamic balance of forces and lead to the insertion of the decanoyl chain within the hydrophobic core of PI bilayer, in spite of repulsion between the peptidic ring and PI head groups.

If the decanoyl chain of daptomycin does insert within the hydrophobic core of PI bilayers, it should affect the properties of this latter. Soya bean PI contains a mixture of various acyl chains, the most common being palmitate (16:0) and linoleate (18:2) [29]. This heterogeneity abolishes cooperativity in the gel-to-fluid transition: the fluidity of the hydrophobic core increases gradually with temperature. The frequency of the C-H stretching modes can be used to probe membrane fluidity. It can be seen on Fig. 6A (circles) that the frequency of the symmetric C-H stretching vibration of the methylene groups of PI liposomes increases gradually upon raising the temperature from 5 to 60° C. The frequency of that mode changes dramatically at the gel-to-fluid transition for bilayers of lipids containing homogeneous acyl chains, like dimyristoylphosphatidylglycerol (DMPG) (Fig. 6B, circles) and dipalmitoylphosphatidylcholine (DPPC) (Fig. 6C, circles).

If the driving force in the interaction of daptomycin with PI is a hydrophobic effect, then the lipopeptide is likely to undergo the same type of interaction with any type of phospholipidic bilayers. Indeed, daptomycin induces a 5° C decrease of the transition temperature of DMPG bilayers (Fig. 6B, triangles) and a 7° C decrease of that of DPPC bilayers (Fig. 6C, triangles), as well as a decrease of the cooperativity and amplitude of that transition. The decanoyl chain of daptomycin is shorter than myristoyl and palmitoyl chains, so its insertion into the bilayer hydrophobic core impedes a perfect packing of the acyl chains on their entire length and consequently destabilizes the gel phase. Owing to the broadness of PI transition, the disordering effect of daptomycin insertion is more subtle and difficult to detect in the temperature profile of the methylene symmetric stretching vibration (Fig. 6A). The ordinate scales have been adjusted to match both ends of the profiles in order to facilitate comparisons of overall shapes. In the presence of daptomycin, PI gets disordered faster, that is to say at a lower temperature. Interestingly, the effect of daptomycin is more pronounced when monitored using the frequency of the C-H stretching mode of alkenyl groups. The double bonds of linoleyl chains are found at positions 9 and 12, which are most likely located below the terminal methyl group of daptomycin. That inner region of the bilayer core experiences the greatest disordering effect because the space between PI acyl chains is larger at that level when daptomycin is present in the upper part. Moreover, the acyl chain portion adjacent to the decanoyl chain of daptomycin is being compressed and its order is actually increased. The methylene stretching frequency reflects an average of the properties of upper and deeper regions whereas the alkenyl stretching frequency probes only the inner region, where the disturbing influence of daptomycin is maximal.

The insertion of daptomycin into PI bilayers may also affect the lipid head group and interface. The frequency of the two components of PI carbonyl stretching band, obtained after derivatization of the original spectra, are not affected by daptomycin. A small increase of the proportion of non hydrogen bonded carbonyl groups is observed at both pH 5.4 and 7.5 (Fig. 4A and B, long dashes), suggesting that the insertion of daptomycin results in a tightening of the bilayer at the interface level.

The phosphate antisymmetric stretching band is slightly shifted to higher frequencies (Fig. 5A and B, long dashes), by approx. 1 cm^{-1}. This could be due to steric constraints put on the head group by the bulky peptidic moiety of daptomycin. Actually, an apparent narrowing of the amide I band of this latter is observed in the presence of PI (Fig. 7C and D, long-short dashes), but the subtraction of the spectral contribution of the C = C stretching mode of PI linoleyl chains (dot-long dash) yields a band (long dashes)

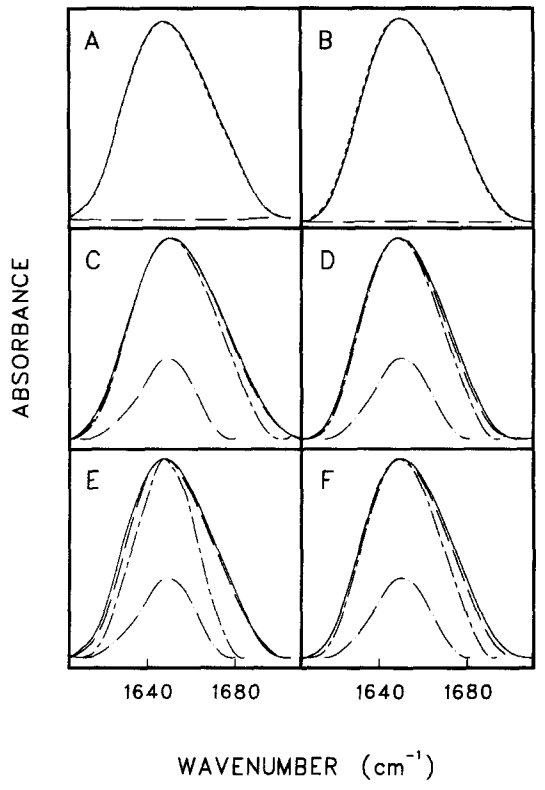

Fig. 7. Amide I region of the infrared spectra of daptomycin (solid line) in ^2H_2O at p^{2}H 5.4 (left panels) or 7.5 (right panels). Gentamicin has no contribution in that spectral region (A and B, long dashes). The amide I band of daptomycin in the presence of gentamicin at a gentamicin/daptomycin molar ratio of 2:1 appear in small dashes (A and B). The spectra of daptomycin in the presence of PI, at a PI/daptomycin molar ratio of 4:1, are given in C and D (short-long dashes). However, PI does contribute in that region (C-F, dot-long dash) and this spectral contribution has been subtracted to give the actual amide I band of daptomycin in the presence of PI (C and D, long dashes). The original spectra of PI/gentamicin/daptomycin at a molar ratio of 4:2:1 (E and F, short-long dashes) has also been corrected for PI contribution and the corrected daptomycin spectra are given in long dashes (E and F).

that is almost superimposable to that of daptomycin alone (solid line).

3.4. *Effect of daptomycin on gentamicin-phosphatidylinositol interaction*

Gentamicin acts as a polycation with PI bilayers. Its addition to a dispersion of this negatively charged lipid results in a fast and massive aggregation. The formation of these big, flaky aggregates is due to the binding of gentamicin to bilayers from different liposomes. Daptomycin also causes PI precipitation, but the aggregates are not bulky. A clear supernatant, free of PI, is also obtained. We checked for the presence of the antibiotics in the supernatant, using the C-O and C-N stretching band (1103 cm^{-1}) as a rough estimate of gentamicin concentration and the amide I band for daptomycin monitoring. It was an easy test to see if the presence of daptomycin within PI bilayers would impede or affect in any way gentamicin binding or if premixing of daptomycin and gentamicin would prevent the fixation of the aminoglycoside on the lipidic surface. Surprisingly, we consistently found slightly less gentamicin in the supernatant in samples containing daptomycin (not shown). All mixtures presented spectral evidences of large amounts of both antibiotics in the precipitate. Therefore, daptomycin *does not* impede the binding of gentamicin to phosphatidylinositol. Infrared spectroscopy can be used to characterize the ternary complex of gentamicin-daptomycin-PI.

The lipid carbonyl stretching region of the infrared spectrum of PI-gentamicin-daptomycin at a molar ratio of 4:2:1 is presented in Fig. 4C. At both pH 5.4 and 7.5, there is a more pronounced decrease of hydrogen bonded PI carbonyl groups than in the presence of gentamicin only. The result is essentially the same if daptomycin is added after or before gentamicin, or if they are mixed prior to addition to PI dispersion. As for PI-daptomycin and PI-gentamicin mixtures, there is no significant shift of the frequency of the two carbonyl stretching components. It thus appears that hydrogen bonding of PI carbonyl groups still involves solvent molecules, but the proportion of bonded groups is smaller because gentamicin and daptomycin work in conjunction to tighten up the lipidic network, squeezing out more water molecules. The basic factor, of course, is charge neutralization of PI head groups.

As described above, daptomycin causes a high frequency shift of the antisymmetric stretching bands of PI phosphate group whereas gentamicin induces a decrease of its frequency and bandwidth at both pH 5.4 and 7.5 (see Fig. 5). In the presence of both gentamicin and daptomycin, there is a low frequency shift similar to that obtained with gentamicin alone but of a smaller magnitude (Fig. 5A and B, dot-long dash).

The spectral changes analyzed up to this point are consistent with an additivity of effects of daptomycin and gentamicin. The presence of the bulky peptidic loop of daptomycin at the bilayer surface does not prevent subsequent binding of gentamicin and conversely, daptomycin interacts with the bilayer in spite of prior treatment with gentamicin. The slower aggregation observed when premixed gentamicin and daptomycin are added to PI dispersions suggests that achieving appropriate spatial organization of the three components is an arduous task. It may explain also why the spectra are not as reproducible for the ternary mixtures. The steric constraints between the gentamicin molecules adsorbed onto the lipidic surface and the peptidic rings extending out of the bilayer can possibly result in conformational changes of the peptidic portion. The amide I region of the infrared spectrum of daptomycin is presented in Fig. 7 (solid line). As discussed above, gentamicin alone does not affect the amide I band of daptomycin and this latter is not significantly affected when daptomycin inserts in a lipidic environment. However, the width of the amide I band is slightly reduced in the ternary complex, once the lipid contribution has been subtracted (Fig. 7E and F, long dashes). This shallow

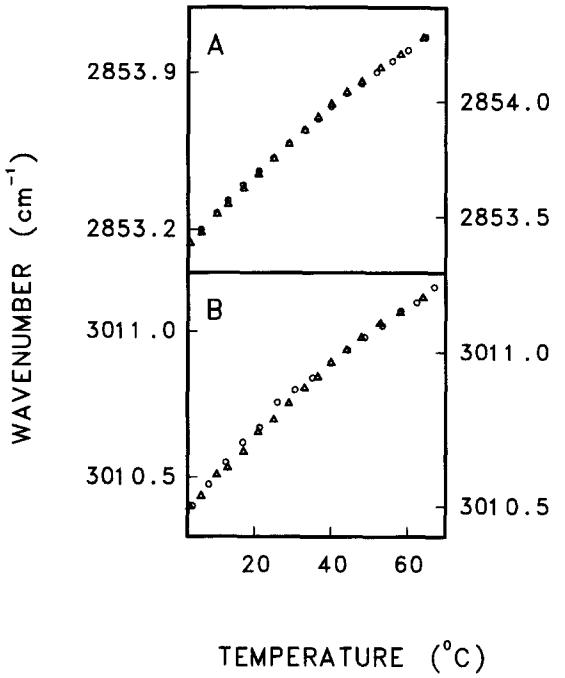

Fig. 8. Temperature dependence of the frequency of the symmetric C-H stretching vibration of the methylene groups of PI (A) and of the frequency of the C-H stretching vibration of the olefinic groups of PI (B) in aqueous dispersions, in the absence (circles) and in the presence (triangles) of both gentamicin and daptomycin at a lipid/gentamicin/daptomycin molar ratio of 4:2:1 at preparation, p^2H 5.4. The coordinates on the right side of panels A and B apply to triangles.

effect probably reflects the accommodation of the peptide to the steric constraints imposed by gentamicin on daptomycin.

As described earlier, the insertion of the decanoyl chain of daptomycin into the hydrophobic core of PI bilayers results in an increased order of the portion of the acyl chain that is adjacent to the lipopeptide decanoyl chain, whereas the terminal portion of the long lipidic acyl chain becomes more fluid. Gentamicin induces a rigidification of PI bilayers. A decrease in the fluidity of the membrane was also observed in the presence of the aminoglycoside drug (data not shown), in agreement with observations made with PC/PI mixtures by differential scanning calorimetry [27] or with brush-border membranes, using electron spin resonance [30]. When both daptomycin and gentamicin are present, the effect disappears and the temperature dependence of the frequency of the methylene symmetric stretching mode of the lipid acyl chains in samples with and without the mixture of both antibiotics is identical, as seen in Fig. 8.

4. Discussion

Because of the lack of satisfactory alternative drugs, aminoglycosides are still essential for the treatment of severe infections. Various strategies are currently evaluated to reduce the clinical occurrence of aminoglycoside-induced acute renal failure. The aim of this spectroscopic investigation is to shed some light on the mechanism by which daptomycin inhibits gentamicin nephrotoxicity.

A recent dialysis study indicated a direct interaction of daptomycin with the aminoglycoside tobramycin [31]. The dependence on ionic strength confirmed the electrostatic character of this interaction. Interestingly, the results showed an increased binding of tobramycin after incorporation of daptomycin into liposomes containing PI. In the present study, we found no spectral evidence of a direct interaction of daptomycin with gentamicin in solution. The absence of significant spectral changes and the fact that both compounds remain in solution after mixing preclude tight interactions and direct neutralization of daptomycin's carboxylates. Nevertheless, the formation of a loose complex cannot be excluded, it would justify the dialysis results and still be compatible with our spectroscopic study.

The postulated electrostatic interaction of daptomycin with gentamicin in solution does not prevent the binding of the aminoglycoside with PI. Its inhibitory mechanism is thus different than that of latamoxef [11] and pyridoxal 5′-phosphate [13]. In contrast with these previous results, our results indicate that daptomycin promotes gentamicin binding to PI bilayers. Once again, this finding is in agreement with the dialysis study mentioned above [31]. It also relates to in vivo results showing that daptomycin protects proximal tubular cells from tobramycin induced nephrotoxicity without reducing tobramycin cortical levels [6,24]. Beauchamp and coll. found that despite similar or higher amounts of aminoglycosides in the lysosomes, daptomycin inhibited aminoglycoside-induced lysosomal phospholipidosis [6]. The dialysis results indicate that the interaction between tobramycin and membranes containing daptomycin and PI was actually stronger at lysosomal pH, that is 5.4, than at pH 7.4. This raises one question: if the sequestration of PI by gentamicin prevents the lipid from activating phospholipases and consequently leads to phospholipidosis, then how could PI play its role of activator when it is involved in a tighter complex with daptomycin and the aminoglycoside? The organization of the complex with daptomycin must be clarified. A recent deuterium nuclear magnetic resonance study reported that the most toxic aminoglycosides induce a greater perturbation of the acyl chains in PC-PI mixtures [32]. The results were consistent with the formation of PI enriched domains after binding of the most toxic aminoglycosides, whereas less toxic aminoglycosides would not lead to lipid phase separation. Both the formation of PC enriched areas and the neutralization of PI charges can interfere with the activity of phospholipases. The binding of phospholipase A_2 to phospholipidic membranes requires the presence of negatively charged lipids [33,34] and perturbations of the charge distribution can result in a dramatic reduction of their binding [35].

The spectroscopic data presented here revealed a few features about the interactions of daptomycin with PI and PI + gentamicin. Owing to the hydrophobic character conferred by its decanoyl chain, daptomycin does incorporate into lipidic membranes, notwithstanding the repulsion between its peptidic moiety and the negatively charged head groups of DMPG or PI. The insertion of the short decanoyl chain of daptomycin molecules in the hydrophobic core of the bilayer results in an increased order of the portion of the acyl chains that is adjacent to the decanoyl chains, whereas the terminal portion of the long lipidic acyl chains (mostly palmitate and linoleate) becomes more fluid. On the other hand, the conformation of the peptidic moiety of daptomycin experiences no significant alteration, as seen from its amide I band (Fig. 7). When gentamicin is also present, this latter indicates a slight change of the peptide conformation. With the data available, it is impossible to determine how the peptide rings and gentamicin molecules are organized at the bilayer surface. Obviously, their interaction is stronger in a membrane environment than when both are free in solution. The nephroprotection afforded by daptomycin may involve two elements. The negative charges located on the hydrophilic peptide moiety increase the charge density at the membrane surface, thus promoting the binding of the phospholipase molecules. Secondly, the rate of hydrolysis of these enzymes is very sensitive to the lipid packing in the bilayer and falls drastically as the temperature departs from the gel-to-fluid phase transition temperature [36]. Gentamicin induces an increase of the transition temperature of pure lipids and rigidifies PI bilayers, but daptomycin increases the disorder in the inner core of the hydrophobic region and thus affects the lipid packing. This could also promote substrate binding to the phospholipases.

5. Conclusions

The nephroprotection provided by daptomycin does not involve a reduced affinity of the aminoglycoside for PI but it can be related to changes in the physical properties of the bilayer. We propose that daptomycin counteracts the inhibition of phospholipase activity due to gentamicin by restoring the critical charge density and distribution required for optimal enzyme activity and by affecting the lipid packing.

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