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EPR studies on the influence of chainlength on the segmental motion of spin-labelled fatty acids in dimyristoylphosphatidylcholine bilayers

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The rotational dynamics of spin-labelled fatty acids of different chainlengths (9, 10, 12, 14, 16 and 18 C-atoms) and different positions of labelling (5-C, 6-C and 7-C) have been studied in dimyristoylphosphatidylcholine bilayers using EPR spectroscopy. The segmental flexibility at a given label position is found to vary considerably with the length of the lipid chain, when this is less than that of the dimyristoylphosphatidylcholine host lipid. For both the charged and protonated forms of labelled fatty acids with chainlengths of 9, 10, and 12 C-atoms, the spectral anisotropy decreases steadily with decreasing chainlength in fluid phase bilayers. The differences become especially pronounced at the 7-C position of caprylic acid and the 6-C position of nonanoic acid, where the label is located close to the terminal methyl end of the chain. An unusually high degree of motional freedom is found for both these spin-labels, even in gel phase bilayers. There is relatively little effect of chainlength of the labelled fatty acid when this is longer or comparable to that of the host lipid (i.e., for fatty acid chainlengths of 18, 16 and 14 C-atoms), except if the label position is close to the terminal methyl end of the chain. The implications for the heterogeneous lipid chain composition in biological membranes are discussed.

Introduction

In addition to the different lipid polar headgroup species found in biological membranes, there is also a considerable heterogeneity in the fatty acid chain composition (see, for example, Ref. 1). This heterogeneity embraces a considerable range of lipid chainlengths and it seems appropriate to enquire what consequences this may have for the membrane dynamics. Direct systematic studies on this topic are relatively few. Far more attention has been paid to the mixing properties of binary lipid systems with differing chainlengths [1–3], and more recently to the chainlength asymmetry in a given phospholipid species [4,5]. Although of great value, such investigations do not address the situation in which the difference in chainlength is large, but nonetheless the chains mix well, as is likely often to be the case in natural membranes.

We have approached this problem by examining the segmental flexibility of spin-labelled fatty acids of different chainlengths in a homogeneous lipid model membrane. A range of chainlengths has been chosen that

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Abbreviations: 5-SASL, 2-tridecyl-4,4-dimethyl-3-oxy-2-oxazolidinebutanoic acid; 5-PASL, 2-undecyl-4,4-dimethyl-3-oxy-2-oxazolidinebutanoic acid; 6-SASL, 2-dodecyl-4,4-dimethyl-3-oxy-2-oxazolidinepentanoic acid; 6-PASL, 2-decyl-4,4-dimethyl-3-oxy-2-oxazolidinepentanoic acid; 6-MASL, 2-octyl-4,4-dimethyl-3-oxy-2-oxazolidinepentanoic acid; 6-NASL, 2-propyl-4,4-dimethyl-3-oxy-2-oxazolidinepentanoic acid; 7-SASL, 2-undecyl-4,4-dimethyl-3-oxy-2-oxazolidinehexanoic acid; 7-PASL, 2-nonyl-4,4-dimethyl-3-oxy-2-oxazolidinehexanoic acid; 7-MASL, 2-heptyl-4,4-dimethyl-3-oxy-2-oxazolidinehexanoic acid; 7-LASL, 2-pentyl-4,4-dimethyl-3-oxy-2-oxazolidinehexanoic acid; 7-CASL, 2-propyl-4,4-dimethyl-3-oxy-2-oxazolidinehexanoic acid; *n*-FASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)alkanoic acid (where F is used generically to indicate the different fatty acid chainlengths); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid; EPR, electron paramagnetic resonance.

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encompasses that of the host lipid bilayers and all are found to mix well with the host lipid at the concentrations used. The dynamic properties of the host lipid membrane remain constant, but the segmental flexibility at a given chain position is found to vary considerably with the length of the lipid probe chain when the position of labelling comes close to the terminal methyl group or when the chainlength is less than that of the host lipid.

Materials and Methods

Spin-labelled stearic acids (*n*-SASL) and myristic acid (6-MASL) were synthesized according to the methods of Hubbell and McConnell [6]. All other spin-labelled fatty acids were obtained from the Institute J. Stefan, University of Ljubljana, Jugoslavia. Dimyristoylphosphatidylcholine was obtained from Fluka (Buchs, Switzerland).

Samples were prepared by dissolving 1 mg of DMPC with 1 mol% of spin-label in dichloromethane. The solution was evaporated with a stream of nitrogen and the residual traces of solvent removed under vacuum overnight. The dry lipid was then hydrated with 40 μl of the required buffer by warming above the phase transition and vortexing. The dispersion was then transferred to a 100 μl capillary and centrifuged at full speed in a Biofuge to obtain a tightly packed lipid pellet. The supernatant was then removed to minimize the aqueous spin-label signal and the capillary was flame sealed. The buffers used were 2 mM sodium acetate, 0.1 mM EDTA (pH 4.0), and 2 mM sodium borate, 0.1 mM EDTA (pH 9.0). For the pH titration, 2 mM Hepes with 0.1 mM EDTA was used.

EPR spectra were recorded on a Varian E-12 Century Line spectrometer equipped with nitrogen gas flow temperature regulation. Samples were contained in sealed 1 mm o.d. glass capillaries accommodated within a standard quartz EPR tube which contained silicone oil for thermal stability. Temperature was measured with a thermocouple situated in the silicone oil just above the top of the EPR cavity.

The apparent order parameters were calculated according to:

$$S^{app} = (A_{\parallel} - A_{\perp})/[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})] \cdot (a_0'/a_0) \quad (1)$$

where $2A_{\parallel}$ is identified with the outer maximum hyperfine splitting, $2A_{max}$, and $A_{\perp}$ is obtained from the inner, minimum hyperfine splitting, $2A_{min}$, using the following corrections [6,7]:

$$\begin{aligned} A_{\perp} &= A_{min}(G) + 0.85\,G & S^{app} &< 0.45 \\ A_{\perp} &= A_{min}(G) + 1.32G + (1.86G)\cdot\log[1 - S^{app}] & S^{app} &> 0.45 \end{aligned} \quad (2)$$

The apparent isotropic hyperfine splitting constant is given by:

$$a_0 = (1/3)(A_{\parallel} + 2A_{\perp}) \quad (3)$$

and that corresponding to the single-crystal environment in which the principal values of the hyperfine tensor, A_{xx}, A_{yy}, and A_{zz}, were measured [8] is given by: $a_0' = (1/3)\ (A_{xx} + A_{yy} + A_{zz})$. Detailed lineshape simulations have shown that the spectra such as those in Fig. 1 contain important contributions from slow molecular motions [9]. Thus the order parameter calculated using Eqn. 1, which assumes fast molecular motion, can only be considered as an effective value but is nonetheless useful for making intercomparisons between labels with different positions of labelling and/or different chain lengths, in the same lipid host. Comprehensive lineshape simulations for flexible spin-labelled lipid chains [9,14] have indicated that in the fluid phase the apparent order parameter so-calculated will be dominated by the ordering associated with the fast chain motions, viz. *trans-gauche* isomerism. In the gel phase, the apparent values calculated for S will tend to be dominated by the slower rate of *trans-gauche* isomerism, however [9,14].

Results

The EPR spectra of three positional isomers of the palmitic acid spin-label, 5-, 6- and 7-PASL, in DMPC bilayers at pH 4.0 and a temperature of 45 °C are given in Fig. 1. In common with previous results for the stearic acid spin-label isomers, *n*-SASL, of longer chainlength and the corresponding phosphatidylcholine derivatives, the spectra display a characteristic flexibility gradient (Ref. 10; Pates, Watts and Marsh, unpublished results). The spectral anisotropy, characterized by the difference between the outermost and innermost hyperfine splittings, decreases with increasing distance of the position of labelling from the polar headgroup. Thus decreasing the fatty acid chainlength of the label by two CH_2 units (from stearic acid to palmitic acid) does not appear to have a great influence on the segmental flexibility for chainlengths in this range. The experiments to be described in the following were designed to determine to what extent this flexibility gradient depends on the total length of the fatty acid spin-label chain.

The pH titration of the 7-SASL stearic acid spin-label in fluid phase DMPC bilayers is indicated by the EPR spectra in Fig. 2. The pK_a of the phosphatidylcholine headgroup lies well outside this pH range [1] and therefore its protonation state remains unaffected. As found previously for the 5-SASL spin-label [11], the EPR spectra in the titration region of the fatty acid consist of two components. One component has a smaller spectral

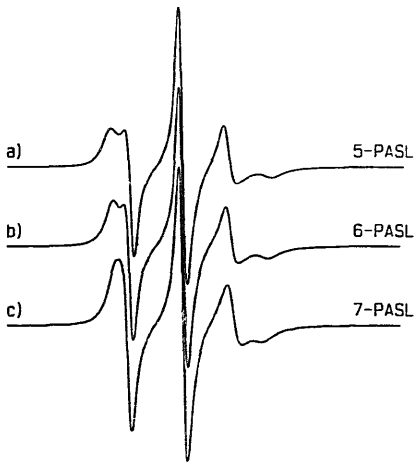

Fig. 1. EPR spectra of positional isomers of the palmitic acid spin-label in dimyristoylphosphatidylcholine bilayers at pH 4.0. $T = 45°C$. a, 5-PASL; b, 6-PASL; c, 7-PASL. Total scan width = 100 gauss.

anisotropy characteristic of the protonated form of the fatty acid observed at low pH (Fig. 2e), and the other component has a larger spectral anisotropy characteristic of the ionized form of the fatty acid at high pH (Fig. 2a). The midpoint of the titration is approximately at a pH 6–7. The fatty acid is fully protonated at pH 4, and almost completely deprotonated at pH 9. For this reason, these two pH values were chosen for the subsequent investigations, hence allowing the exploration of different ranges of segmental flexibility corresponding to the different vertical locations of the fatty acid within the bilayer (cf. Ref. 12).

The EPR spectra of the 7-positional isomer of spin-labelled fatty acids with chainlengths of 18 to 10 carbon

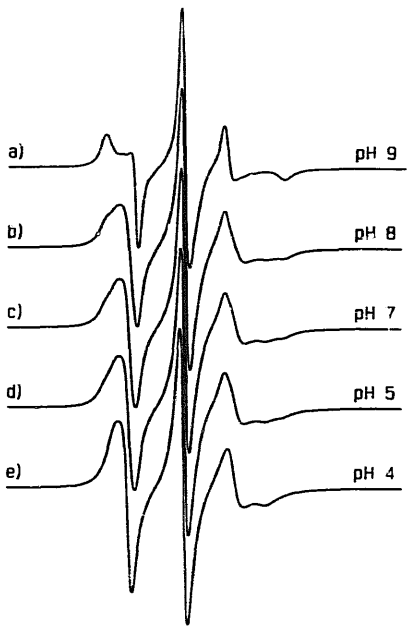

Fig. 2. pH dependence of the EPR spectra of the 7-SASL stearic acid spin-label in dimyristoylphosphatidylcholine bilayers at 45 °C. a, pH = 9.0; b, pH = 8.0; c, pH = 7.0; d, pH = 5.0; e, pH = 4.0. Total scan width = 100 gauss.

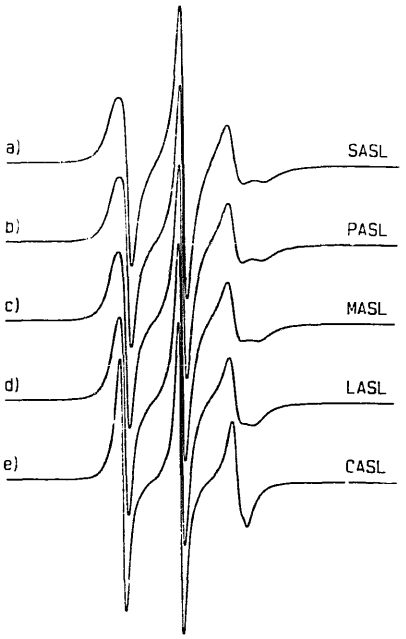

Fig. 3. EPR spectra of the 7-FASL fatty acid spin-labels of different chainlengths in dimyristoylphosphatidylcholine bilayers at pH 4.0. $T = 45°C$. a, Stearic acid, 7-SASL; b, palmitic acid, 7-PASL; c, myristic acid, 7-MASL; d, lauric acid, 7-LASL; e, caprylic acid, 7-CASL. Total scan width = 100 gauss.

atoms, in DMPC bilayers at pH 4.0 are given in Fig. 3. The spectra were recorded at a temperature of 45 °C which is well above the gel-to-fluid phase transition of DMPC. The spectra of the stearic and palmitic acid derivatives, 7-SASL and 7-PASL, are very similar. That of the myristic acid derivative, 7-SASL, displays a slightly smaller anisotropy; that of the lauric acid derivative, 7-LASL, has a more markedly reduced anisotropy; and the caprylic acid derivative, 7-CASL, has an extremely small anisotropy.

The corresponding spectra of the various fatty acid spin labels recorded from DMPC dispersions at pH 9.0 are given in Fig. 4. For a particular label, the spectral anisotropy is much greater than at pH 4.0, but the qualitative trends with fatty acid chainlength are similar to those observed in Fig. 3. The spectra of the caprylic acid spin-label are partly obscured by the sharp peaks from the aqueous spin-label (Fig. 4e). This is unavoidable because of the low partitioning of this short-chain fatty acid in the ionized state. However, it is still clear that the spectral anisotropy of this label is very much reduced relative to that of the fatty acids of longer chainlength.

The data on the spectral anisotropy at pH 4.0 of the various spin-label positional isomers of different chainlengths are summarized in Fig. 5. The apparent spin-label order parameters, defined by Eqn. 1, are plotted as a function of temperature. The corresponding data at pH 9.0 are given in Fig. 6. In the gel phase at 15 °C, the differences in spectral anisotropy between the different labels at a given pH, and between the same labels at the

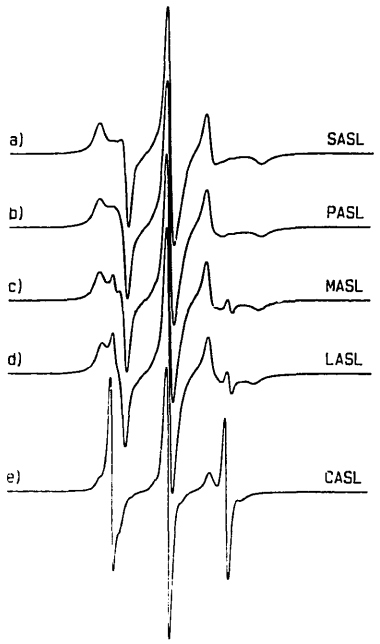

Fig. 4. EPR spectra of the 7-FASL fatty acid spin-labels of different chainlengths in dimyristoylphosphatidylcholine bilayers at pH 9.0. $T = 45\,^{\circ}C$. a, Stearic acid, 7-SASL; b, palmitic acid, 7-PASL; c, myristic acid, 7-MASL; d, lauric acid, 7-LASL; e, caprylic acid, 7-CASL. Total scan width = 100 gauss.

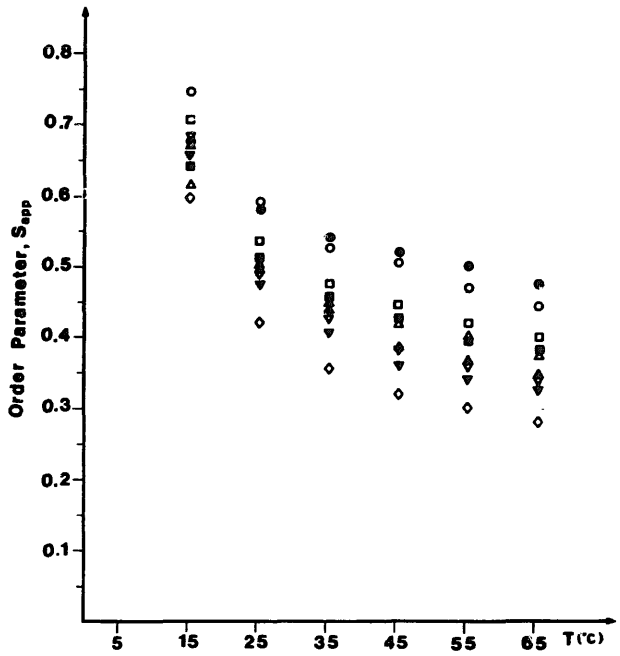

Fig. 6. Temperature dependence of the apparent order parameters, S^{app}, of the *n*-FASL fatty acid spin-labels of different chainlengths in dimyristoylphosphatidylcholine bilayers at pH 9.0. ○, 5-SASL; ●, 5-PASL; □, 6-SASL; ■, 6-PASL; △, 6-MASL; ▲, 7-SASL; ▽, 7-PASL; ▼, 7-MASL; ◇, 7-LASL.

different pH values, are much smaller than those at higher temperatures in the fluid phase, consistent with the tighter chain packing in the gel phase. Exceptions are the short labels 7-CASL and 6-NASL. These labels, which are separated by only two C-atoms from the terminal methyl group, possess a much greater degree of motional freedom even in the gel phase than do their longer chain counterparts.

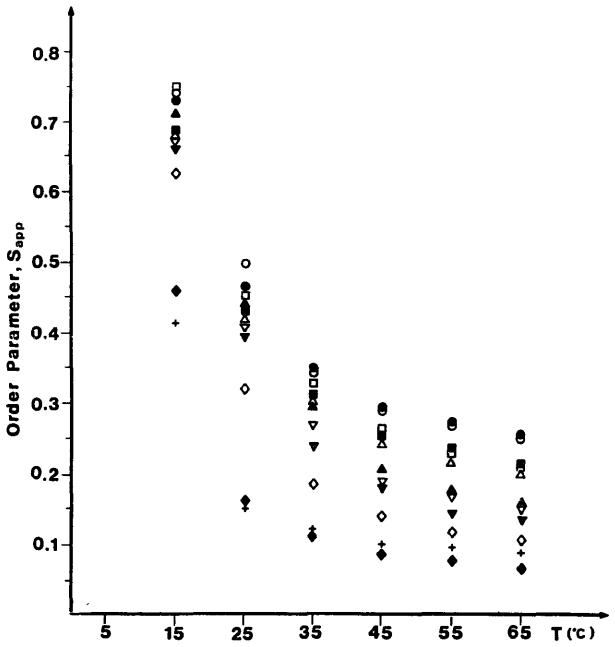

Fig. 5. Temperature dependence of the apparent order parameters, S^{app}, of the *n*-FASL fatty acid spin-labels of different chainlengths in dimyristoylphosphatidylcholine bilayers at pH 4.0. ○, 5-SASL; ●, 5-PASL; □, 6-SASL; ■, 6-PASL; △, 6-MASL; +, 6-NASL; ▲, 7-SASL; ▽, 7-PASL; ▼, 7-MASL; ◇, 7-LASL; ◆, 7-CASL.

In the fluid phase, the spectral anisotropy of a given label at pH 4.0 is much less than that at pH 9.0, corresponding to a deeper location in the bilayer of the protonated fatty acid than of the charged form (see, for example, Refs. 11 and 12). This effect is observed irrespective of the chainlength of the label, but the chainlength dependent effects in the fluid phase are observed consistently at both pH values.

Generally, the differences between the spectral anisotropy of fatty acids with the same label position but different chainlengths become more pronounced on proceeding toward the terminal methyl end of the chain, from the 5-position to the 7-position. More marked, however, is the dependence on chainlength for a given position of labelling. The 18-C, 16-C and 14-C atom chainlengths have similar anisotropies, with those of the 7-MASL label being somewhat smaller. As soon as the chainlength of the label becomes less than that of the host lipid (14-C), however, the dependence on chainlength for a given label becomes very marked (6-NASL, 7-LASL and 7-CASL). For the very short-chainlength 6-NASL, the decrease in spectral anisotropy is much greater than that on increasing the label position by one CH_2 group from 6-MASL to 7-MASL (Fig. 5).

Conjugate to the apparent order parameters are the apparent isotropic hyperfine splitting factors given by Eqn. 3. In the absence of overwhelming contributions to

the spectral splittings from slow molecular motions, these should be determined principally by the polarity of the environment in which the spin-label group is situated. The near constancy of the observed values of a_0 with temperature supports this view. The mean values of a_0, over the measured range of temperatures in the fluid phase, are given in Table I. Data are given for the different spin-label positional isomers and for the different chainlengths, and at the two pH values of measurement. A systematic dependence on the isomer position is seen at a given pH, and a systematic difference is also seen between given isomers at the two pH values. The a_0 values for a given positional isomer at a given pH do not display a systematic dependence on the chainlength of the fatty acid. This indicates that the spin-label position in the bilayer does not change systematically as does the segmental flexibility, although it cannot be excluded that some of the shorter chainlength spin-labels are located slightly deeper in the bilayer than their longer chain counterparts.

Discussion

The spin-labels employed in this study have chainlengths (18-C to 9-C atoms) differing maximally by 9 C-atoms and which extend to 4–5 C-atoms on either side of that of the host DMPC lipid bilayers (14-C atoms in length). They thus may be taken as representative of the range of chainlength variation of the lipids in biological membranes (cf. Ref. 1). Although the chainlengths of natural lipids are generally longer than those of DMPC, the bilayer thickness will be more comparable due to the presence of *cis*-unsaturated bonds in the natural systems.

The present results show that the flexibility of the fatty acid chains increases considerably as soon as the chainlength becomes shorter than that of the host lipid, even when the position of labelling is still considerably removed from that of the terminal methyl group (7-LASL). These differences are observed for both the charged and protonated forms of the various fatty acids which have different respective vertical locations in the bilayer. In contrast there is relatively little effect when the chainlength is longer or comparable to that of the host lipid and the label position is not close to the terminal methyl end of the chain (7-SASL, 7-PASL and 7-MASL). As the chainlength is decreased further, the differences become even more pronounced, especially when the labelling position, although remote from the terminal methyl groups of the host lipid, becomes close to the terminal methyl ends of the labelled chains. In the latter case, an unusually high degree of motional freedom can be found even in relatively rigid gel phase lipid bilayers.

Although all the present measurements have been performed with fatty acids in which the position of labelling is situated well above the terminal methyl groups of the host DMPC lipids, some information is available from our previous studies which relate to the opposite situation. The anisotropy of the spectra of stearic acid spin-labelled at positions 13-, 14- and 16- in DMPC bilayers decreases systematically on approaching the terminal methyl end of the labelled chain [10,13]. By contrast, however, the anisotropy in DMPC bilayers of myristic acid labelled on the 13-position [14], i.e., adjacent to the terminal methyl group, is even less than that of the 16-position labelled stearic acid which is located one C-atom removed from the terminal methyl group. This suggests that the mobility of chains longer than those of the host lipid is relatively decreased at those segments extending beyond the terminal methyl region of the host bilayer. As noted above, essentially the reverse is true for shorter chains.

These findings have interesting implications for the chain motions in natural membranes, the lipids of which have a considerable range of different chainlengths. The effect of chainlength heterogeneity in fluid phase bilayers can be predicted to be one of smearing out of the flexibility gradient that is characteristic of single component lipid bilayers (see, for example Refs. 2, 14 and 15). The shorter chains have an intrinsically higher segmental flexibility in the region of overlap with the longer chains. In this sense, the chain heterogeneity may serve to 'buffer' the membrane fluidity and confer upon the upper sections of the lipid chains a degree of motional freedom that is closer to that in the region of the chain ends. It could be that these effects, combined with the necessary chain interdigitation that must take place at the centre of the lipid bilayer, provide a profile of

TABLE I

Isotropic hyperfine splitting constants, a_0 (gauss), of the different fatty acid spin labels, n-FASL, in dimyristoylphosphatidylcholine bilayers at pH 4.0 and pH 9.0, as a function of label position, n, and fatty acid chainlength

n	*n*-SASL		*n*-PASL		*n*-MASL		*n*-LASL		*n*-CASL		*n*-NASL
	pH 4	pH 9	pH 4	pH 9	pH 4	pH 9	pH 4	pH 9	pH 4	pH 9	pH 4
5	15.1	15.25	14.95	15.15	–	–	–	–	–	–	–
6	15.0	15.2	14.95	15.25	14.85	15.2	–	–	–	–	14.8
7	14.85	15.15	14.75	15.1	14.8	15.15	14.65	15.15	14.7	–	–

chain flexibility that is best matched to the requirements of integral membrane proteins.

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