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Biochimica et Biophysica Acta 1278 (1996) 68–72

BBA Biochimica et Biophysica Acta

Depth dependence of the perturbing effect of placing a bulky group (oxazolidine ring spin labels) in the membrane on the membrane phase transition

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Received 4 May 1995; revised 28 August 1995; accepted 7 September 1995

Abstract

Electron paramagnetic resonance (EPR) and differential scanning calorimetry (DSC) have been used to study the effect on the phase transition of dimyristoylphosphatidylcholine membranes of incorporating various stearic acid spin labels (SASL's) that contain the bulky oxazolidine ring at various positions along the stearyl chain. SASL's lowered the phase transition temperature and decreased the size of the cooperative unit, with the effects stronger in the order of 9-> 12-> 5-> 16-SASL > stearic acid (no label). Incorporation of stearic acid without the spin label slightly increases the phase transition temperature. Incorporation of 9-SASL (3 mol% of lipid) decreased the transition temperature by 1.8°C and the cooperative unit to 1/5 of that without the spin label, while the effect of 16-SASL was slight. The effect on transition enthalpy was small. It is concluded that the perturbing effect of placing a bulky group on the alkyl chain on phase transition is through inducing packing defects in the gel-phase.

Keywords: Phosphatidylcholine; Phase transition; Spin label; EPR

1. Introduction

The biological membrane has a three-dimensional architecture. Nevertheless, it is often treated as a two-dimensional structure. Even when interaction of molecules in the membrane or the effect of incorporating foreign molecules in the membrane is investigated, the 'depth'-dependent interaction of molecules in the membrane has not been the major subject of study in most cases. Previously, we reported two cases in which the 'depth'-dependent molecular interaction in the membrane plays critical roles in determining the two-dimensional organization of the membrane, such as microimmiscibility and transient molecular clustering: (1) mismatch in hydrophobic length between rhodopsin and the lipid alkyl chains in reconstituted membranes leads to molecular association of rhodopsin in the membrane, while at the best match found with palmitoyl chain, rhodopsin tends to be monomers [1]. This observation was later confirmed by Ryba and Marsh [2] and for Ca^{2+}-ATPase by Cornea et al. [3]. (2) In the membranes of cholesterol and *cis*-unsaturated phosphatidylcholine (PC), the fluid-phase immiscibility is prevalent and small cholesterol-rich (oligomeric) domains are forming and dispersing continually with a lifetime of 10^{-9} to 10^{-7} s [4,5]. This was induced by the non-conformability of the tetracyclic sterol backbone of cholesterol which reaches ≈ C10 of the alkyl chain and the rigid bend at the *cis* double bond at C9 = C10 when they are adjacently placed in the membrane.

Translational diffusion and permeability of small molecules such as molecular oxygen within and across the

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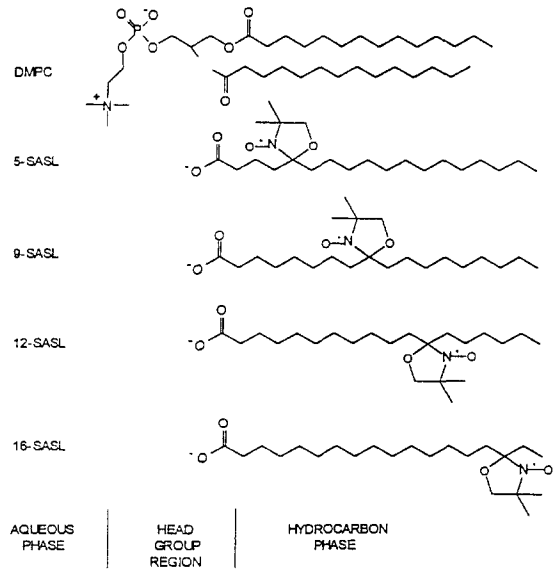

Fig. 1. Chemical structures of DMPC and SASL's used in this work. Their locations in the membrane, and the location of the nitroxide in the stearic chain are shown.

membrane are strongly affected by the 'depth'-dependent molecular interaction. Cholesterol decreased the local oxygen transport rate (the product of local oxygen concentration and diffusion coefficient) in the membrane near the membrane surface in the hydrophobic region but increased it in the central part of the bilayer of dioleoyl-PC and egg-yolk PC. This is probably caused by the bulky but short sterol backbone of cholesterol, which would create free volume in the membrane center [6,7]. The level of water penetration shows just opposite of the profiles of the oxygen transport rate across the membrane. Water penetration suddenly decreases about where the bulky sterol backbone of cholesterol ends in the direction normal to the membrane [8].

In the present investigation, we studied the depth-dependent effect of placing a bulky group in the membrane on the phase transition of dimyristoylphosphatidylcholine (DMPC) bilayer. For this purpose, various stearic acid spin labels (SASL's) were used, i.e., the effect of the bulky oxazolidine ring attached to C5, 9, 12, or 16 (5-, 9-, 12-, or 16-SASL, respectively, Fig. 1) of the stearic acid was investigated using electron paramagnetic resonance spectroscopy (EPR) and differential scanning calorimetry (DSC). Another purpose of this study is to evaluate the perturbing effect of various SASL's in the membrane. Although these spin labels have been used for many years to study phase transition of membranes, systematic study on the perturbing effect of these spin labels is very limited [9–11]. Segmental order parameters estimated by deuterium magnetic resonance are quite different from those determined by spin labeling [12], which can be due to both difference in the sensitive time scale (ns versus μs) and the larger perturbing effect of the oxazolidine ring of SASL.

2. Materials and methods

2.1. Materials

DMPC and stearic acid were obtained from Sigma (St. Louis, MO), and stearic acid spin labels were obtained from Molecular Probes (Eugene, OR). The buffer used was 0.1 M sodium borate at pH 9.5. This high pH value ensures that all carboxyl groups of SASL's are ionized in PC membranes [13–16]. The structure of PC membranes is not altered at pH 9.5 [15–18].

2.2. Membrane preparation and EPR measurements

The membranes used in this work were multilamellar dispersions of lipids containing various amounts of SASL and were prepared as described by Kusumi et al. [15,18]. The lipid dispersion (10^{-5} mol of total lipid/ml) was centrifuged briefly, and the loose pellet ($\approx 20\%$ lipid w/w) was used for EPR measurement. The sample was placed in a 0.9 mm i.d. gas permeable TPX capillary [19]. The capillary was placed inside the EPR dewar insert and equilibrated with nitrogen gas, which was used for temperature control. The sample was thoroughly deoxygenated at a temperature well above the phase transition of the lipid bilayer to obtain correct EPR lineshape. This is especially important at phase transition, because oxygen concentration-diffusion product changes abruptly at the phase transition of the membrane [6,15,20]. EPR spectra were obtained at X-band. A Varian E-109 and Varian E-3 spectrometers with an E-231 Varian multipurpose cavity (rectangular TE_{102} mode) were used. Temperature was regulated by passing the nitrogen gas through the coil placed in a water bath, and was monitored using a copper-constantan thermocouple that was placed in the sample at the center of the microwave cavity. Temperature was regulated better than ± 0.1°C (this was possible because the temperature range used was close to the room temperature). Temperature was always lowered by adding small amount of cold water in the water bath with rapid agitation. Special care was taken not to raise the temperature during the cooling experiment.

2.3. DSC measurements

Multilamellar dispersions of lipids were formed as described above (but at a half concentration). DSC measurements were performed with a Seiko SSC5000-DSC100 differential scanning calorimeter (Seiko Instruments, Chiba, Japan). Temperature was raised at a rate of 0.5°C/min. The membrane suspension was sealed in a silver metal container of 6 mm$\phi \times$ 10 mm, and weighed within an accuracy of ± 1 μg. To balance the heat capacities between the sample and the reference holders, the buffer solution was used as a reference. In repeated measurements of the suspension, the temperature values at the peak of the transition could be reproduced to ± 0.1°C, and the

enthalpy determined from the numerical integration of the area under the DSC curve could be reproduced to within ±0.2 kcal/mol.

3. Results and discussion

3.1. EPR measurement

The main phase transition of DMPC membranes was first monitored by observing the intensity of the central line of the EPR spectra of SASL's. The intensity changes significantly at the phase transition; decrease in signal amplitude approaching 50% has been observed in a cooling experiment. Fig. 2A shows the changes in the normalized signal amplitude of 12-SASL. As the concentration of 12-SASL increases (from 1 to 3 mol%), the phase transition shifts toward lower temperatures and the width broadens. Fig. 2B shows the effect on the phase transition of various SASL's at 3 mol%. The influence on the transition temperature and on the sharpness of the transition depends on the location of the oxazolidine free-radical moiety along the stearic chain. The greatest effect was observed for 9-SASL. The effect is stronger in the order of 9-> 12-> 5-> 16-SASL. For 16-SASL, a small broadening with a negligible shift of T_m (less than 0.1°C) was observed. Therefore, we used 0.5 mol% of 16-SASL to monitor the effect of stearic acid (parent molecule without nitroxide) (Fig. 2C).

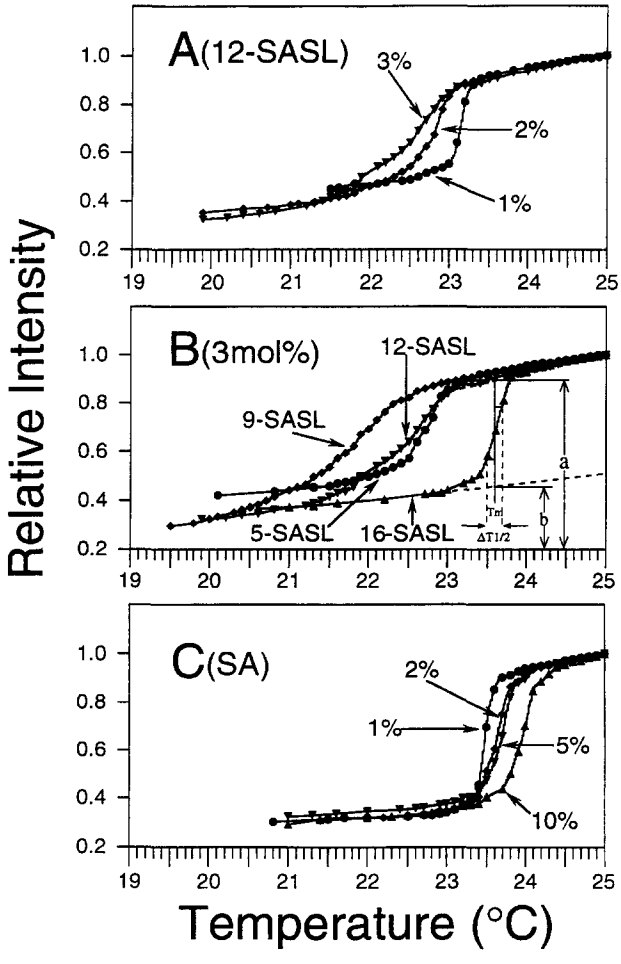

Fig. 2. Normalized intensity of the central peak of the EPR spectra plotted as a function of temperature (cooling experiments). (A) 12-SASL, shown as a function of 12-SASL concentration (1, 2, and 3 mol%). (B) Effect of 3 mol% of 5-, 9-, 12-, and 16-SASL. The definition of T_m is shown. T_m is the midpoint temperature at which the EPR signal amplitude equals $(a+b)/2$, where a and b are, respectively, the normalized intensity at a given temperature in the extended linear portions of the upper and lower ends of the transition curve. As the sharpness of the transition, we employ the width $\Delta T_{1/2}$, which is defined by two temperatures at which the EPR signal amplitude is $(a+3b)/4$ and $(3a+b)/4$. (C) Effect of stearic acid monitored with 0.5 mol% 16-SASL.

Phase transition temperature, T_m, and the width of transition, $\Delta T_{1/2}$, were operationally defined from the EPR data as shown in Fig. 2B as was previously done for Raman data [21]. Namely, T_m is the midpoint temperature at which the normalized intensity equals $(a+b)/2$, where a and b are, respectively, the intensities at a given temperature in the extended linear portions of the upper and lower ends of the transition curve, while $\Delta T_{1/2}$ is defined by two temperatures at which the normalized EPR signal intensities are $(a+3b)/4$ and $(3a+b)/4$. These give values close to the transition temperature and width defined by DSC for pure DMPC membranes [22]. $\Delta T_{1/2}$ ranged between 0.2°C and 1.2°C, the former for 0.5 mol% 16-SASL, while the latter for 3 mol% 9-SASL in the membrane.

T_m values are shown as a function of n (for n-SASL, $n = 5, 9, 12, 16$) and the mol fraction of SASL (and stearic acid) in Fig. 3. The perturbing effect is largest at C9 and decreases as the depth in the membrane becomes either shallower or deeper (Fig. 3A). In addition, up to 3 mol%, T_m changes linearly with SASL concentration (Fig. 3B). In the case of stearic acid, its incorporation increases T_m. This observation is consistent with previous reports showing that long chain hydrocarbon acids and alcohols cause an increase of the transition temperature [23,24].

3.2. DSC experiments

DSC experiments were performed at 0, 3, and 10 mol% SASL concentrations. Typical DSC curves are presented in Fig. 4 for pure DMPC suspension and DMPC membranes containing 10 mol% of 5-, 9-, 12-, and 16-SASL. Only a single peak appeared in the presence of 5-, 12- and 16-SASL, while, for 9-SASL, two peaks appeared, suggesting occurrence of phase separation.

These peaks give the midpoint of phase transition (T_{cal}), and by integrating these curves, total enthalpy for the transition (ΔH_{cal}) was evaluated. T_{cal} and ΔH_{cal} are summarized in Table 1 along with T_m and $\Delta T_{1/2}$. T_{cal} and T_m are similar to each other. The effect is stronger in the order of 9-> 12-≈ 5-> 16-SASL.

Similar observations were reported on the effect on T_{cal}

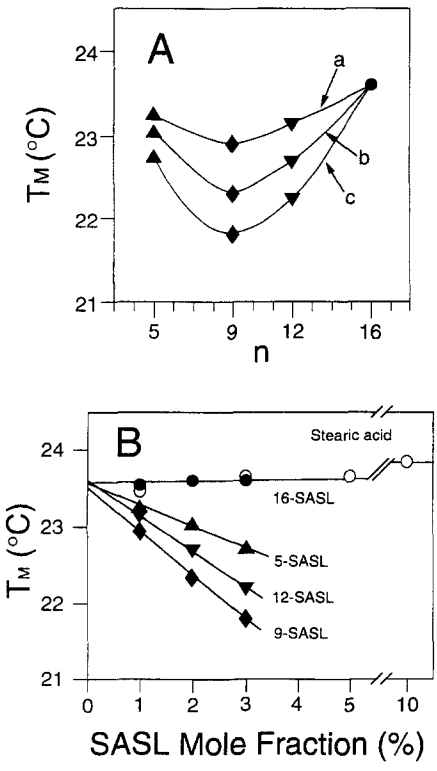

Fig. 3. (A) T_m plotted as a function of n (n-SASL) for DMPC membranes containing (a) 1, (b) 2, and (c) 3 mol% SASL. (B) T_m plotted as a function of concentration of SASL and stearic acid.

of placing a single double bound along the stearoyl chain of distearoylphosphatidylcholine [25–27]. The effect was strongest when the location of the unsaturated *cis* double bond was in the middle of the lipid alkyl chain.

ΔH_{cal} is less sensitive to the depth-dependent effect of the perturbing group. The effect is large for 5-, 9-, 12-SASL, and stearic acid. The reason for the large decrease for stearic acid is not clear.

3.3. The size of the cooperative unit

The van 't Hoff enthalpy ΔH_{vH} can be evaluated from T_m and $\Delta T_{1/2}$ obtained from the EPR experiment (shown in Table 1) according to the equation,

$$\Delta H_{vH} = 4RT_m^2/\Delta T_{1/2} \quad (1)$$

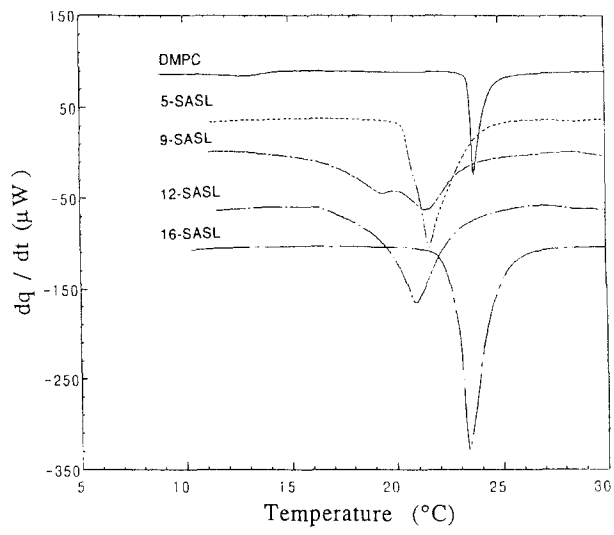

Fig. 4. DSC curves (heating experiments) for multilamellar dispersion of DMPC containing no or 10 mol% of 5-, 9-, 12-, and 16-SASL. These curves were not corrected for lipid concentration.

The ratio $\Delta H_{vH}/\Delta H_{cal}$ gives the number of molecules in a cooperative unit ([22,28], also listed in Table 1), although this number cannot be equated explicitly to the number of lipid molecules or hydrocarbon chains undergoing transition simultaneously. However, it can give a convenient measure of the cooperativity of the transition. The cooperative unit size obtained here for pure DMPC membrane is greater than those estimated previously [22,28,29]. This may be due to the cooling experiment for estimation of ΔH_{vH} and/or the low rate of temperature change (2 C°/h) in our measurements.

The cooperative unit size is far more sensitive to addition of SASL's than the enthalpy change (ΔH_{cal}) (Table 1). The effect is stronger in the order of 9- > 12- > 5- > 16-SASL > stearic acid. At the position of the oxazolidine ring, the cross-section of a stearic acid spin label is approximately twice that of a normal chain (Fig. 5, the cross-sections were obtained for the space filling models created using Alchemy III software). If such a molecule is then incorporated into a bilayer, the neighboring chains are pushed apart and free volume is created, by which cooperativity may be lost.

Fig. 5 shows a schematic drawing of geometrical rela-

Table 1
Properties of the phase transition of multilamellar dispersion of DMPC membranes in the presence of 3 mol% of various SASL's [a]

Addition	T_m (°C) EPR	$\Delta T_{1/2}$ (°C) EPR	T_{cal} (°C) DSC	ΔH_{vH} (kcal/mol) EPR	ΔH_{cal} (kcal/mol) DSC	Cooperative unit (No. of molecules)
No [b]	23.6	0.20	23.8	3080	5.5	560
5-SASL	22.8	0.40	22.2	1530	5.0	306
9-SASL	21.8	1.15	21.1 [d]	530	5.0 [e]	106
12-SASL	22.2	0.75	22.4	813	5.0	163
16-SASL	23.6	0.25	23.2	2460	5.5	448
Stearic acid [b, c]	23.7	0.30	24.3	2054	4.5	456

[a] Accuracies of measurements are the following: T_m, ±0.1°C; $\Delta T_{1/2}$, ±0.2°C; T_{cal}, ±0.1°C; ΔH_{cal}, ±0.2 kcal/mol. [b] EPR measurements performed with 0.5 mol% 16-SASL. [c] Effect of 5 mol% of SA. [d] Indicating the major peak. [e] Estimated from the total area under the DSC curve.

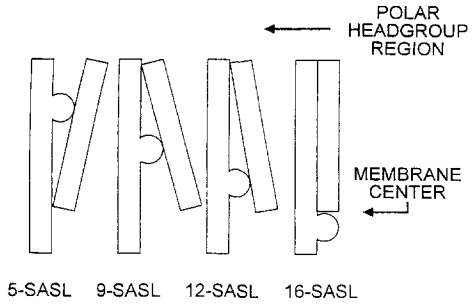

Fig. 5. Schematic drawing showing geometrical relationship between SASL's and neighboring myristoyl chains in the gel-phase membrane. All chains are assumed to have all *trans* conformations. At the position of the oxazolidine ring, the cross section of a stearic acid spin label is 2.1-times greater than that of a normal chain as estimated from the shadows of the space filling models using a software Alchemy III.

tionship between SASL's and neighboring myristoyl chains in the gel-phase membrane. All chains are assumed to have all *trans* conformations. Packing defects (free volume in the membrane) induced by placing the bulky group is largest when it is placed in the middle of the chain (Fig. 5), which we propose is the reason that 9-SASL has the largest effect on phase transition.

SASL's have been widely used for studying biological and artificial membranes. However, the present study clearly showed that the use of these probes in investigation of phase transition of membranes and in the studies of lipid conformation and dynamics in the gel phase requires caution in their interpretation of data. The guideline for the acceptable SASL concentrations can be estimated from Fig. 3 in this report.

Acknowledgements

This work was supported in part by U.S. Public Health Service Grants GM22923 and RR01008 and by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan.

References

[1] Kusumi, A. and Hyde, J.S. (1982) Biochemistry 21, 5978–5983.
[2] Ryba, N.J.P. and Marsh, D. (1992) Biochemistry 31, 7511–7518.
[3] Cornea, R.L. and Thomas, D.D. (1994) Biochemistry 33, 2912–2920.
[4] Pasenkiewicz-Gierula, M., Subczynski, W.K. and Kusumi, A. (1990) Biochemistry 29, 4059–4069.
[5] Subczynski, W.K., Antholine, W.E., Hyde, J.S. and Kusumi, A. (1990) Biochemistry 29, 7936–7945.
[6] Subczynski, W.K., Hyde, J.S. and Kusumi, A. (1989) Proc. Natl. Acad. Sci. USA 86, 4474–4478.
[7] Subczynski, W.K., Hyde, J.S. and Kusumi, A. (1991) Biochemistry 30, 8578–8590.
[8] Subczynski, W.K., Wisniewska, A., Yin, J.-J., Hyde, J.S. and Kusumi, A. (1994) Biochemistry 33, 3670–3681.
[9] Tinoco, J., Ghosh, D. and Keith, A.D. (1972) Biochim. Biophys. Acta 274, 279–285.
[10] Cadenhead, D.A. and Müller-Landau, F. (1973) Biochim. Biophys. Acta 307, 279–286.
[11] Cadenhead, D.A. and Müller-Landau, F. (1974) J. Colloid Interface Sci. 49, 131–134.
[12] Seelig, J. and Niederberg, W. (1974) Biochemistry 13, 1585–1588.
[13] Sanson, A., Ptak, M., Rigaud, J.L. and Gary-Bobo, C.M. (1976) Chem. Phys. Lipid 17, 435–444.
[14] Egret-Charlier, M., Sanson, A., Ptak, M. and Bouloussa, O. (1978) FEBS Lett. 89, 313–316.
[15] Kusumi, A., Subczynski, W.K. and Hyde, J.S. (1982) Proc. Natl. Acad. Sci. USA 79, 1854–1858.
[16] Kusumi, A., Subczynski, W.K. and Hyde, J.S. (1982) Fed. Proc. 41, 1394, Abst. 6571.
[17] Träuble, H. and Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71, 214–219.
[18] Kusumi, A., Subczynski, W.K., Pasenkiewicz-Gierula, M., Hyde, J.S. and Merkle, H. (1986) Biochim. Biophys. Acta 854, 307–317.
[19] Hyde, J.S. and Subczynski, W.K. (1989) In Biological Magnetic Resonance, Vol. 8, Spin-Labeling: Theory and Applications (Berliner, L.J. and Reuben, J., eds.), pp. 399–425, Plenum Press, New York.
[20] Subczynski, W.K. and Hyde, J.S. (1981) Biochim. Biophys. Acta 643, 283–291.
[21] Huang, C., Lapides, J.R. and Levin, I.W. (1982) J. Am. Chem. Soc. 104, 5926–5930.
[22] Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3652–3866.
[23] Lee, A.G. (1976) Biochemistry 15, 2448–2454.
[24] Ben Yashar, V., Menashe, M., Biltonen, R.L., Johnson, M.L. and Barenholz, Y. (1987) Biochim. Biophys. Acta 904, 117–124.
[25] Barton, P.G. and Gunstone, F.D. (1975) J. Biol. Chem. 250, 4470–4476.
[26] Marsh, D. (1990) Handbook of Lipid Bilayers, pp. 135–162. CRC Press, Boca Raton.
[27] Cevc, G. (1991) Biochemistry 30, 7186–7193.
[28] Fumero, J., Bammel, B.P., Hopkins, H.P. and Smith, J.C. (1988) Biochim. Biophys. Acta 944, 164–176.
[29] Parente, R.A. and Lentz, B.R. (1984) Biochemistry 23, 2353–2362.