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Membrane dynamics as seen by Fourier transform infrared spectroscopy in a cyanobacterium, *Synechocystis* PCC 6803 The effects of lipid unsaturation and the protein-to-lipid ratio

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Abstract

The roles of lipid unsaturation and lipid-protein interactions in maintaining the physiologically required membrane dynamics were investigated in a cyanobacterium strain, *Synechocystis* PCC 6803. The specific effects of lipid unsaturation on the membrane structure were addressed by the use of desaturase-deficient (*desA*$^-$/*desD*$^-$) mutant cells (which contain only oleic acid as unsaturated fatty acid species) of *Synechocystis* PCC 6803. The dynamic properties of the membranes were determined from the temperature dependence of the symmetric CH_2 stretching vibration frequency, which is indicative of the lipid fatty acyl chain disorder. It was found that a similar membrane dynamics is maintained at any growth temperature, in both the wild-type and the mutant cell membranes, with the exception of mutant cells grown at the lower physiological temperature limit. It seems that in the physiological temperature range the desaturase system of the cells can modulate the level of lipid desaturation sufficiently to maintain similar membrane dynamics. Below the range of normal growth temperatures, however, the extent of lipid disorder was always higher in the thylakoid than in the cytoplasmic membranes prepared from the same cells. This difference was attributed to the considerable difference in protein-to-lipid ratio in the two kinds of membranes, as determined from the ratio of the intensities of the protein amide I band and the lipid ester C=O vibration. The contributions to the membrane dynamics of an ab ovo present 'structural' lipid disorder due to the protein–lipid interactions and of a thermally induced 'dynamic' lipid disorder could be distinguished.

Keywords: Desaturase deficient mutant; Fourier transform infrared spectroscopy; Lipid; Membrane dynamics; Protein-to-lipid ratio; (*Synechocystis* PCC 6803)

1. Introduction

The membranes, which comprise delicate assemblies of lipids and proteins, perform multiple functions in living organisms. They separate cells and cell compartments from their environment and provide an effective barrier against a large variety of compounds. On the other hand, the membranes also have to ensure communication with the same environment, by transporting materials either passively or actively into and out of the cell. An equally im-

Abbreviations: ESR, electron spin resonance spectroscopy; FTIR, Fourier transform infrared spectroscopy

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portant additional function of the membranes is to mediate to the cell machinery the external signals arriving at the cell surface. This permits the adaptation of the cells to the ever-changing external conditions. For all these functions, a biological membrane has to form a physically solid, yet dynamic entity, establishing dynamic equilibria between its constituents and between itself and its environment.

In cyanobacteria, the growth temperature determines the level of unsaturation of glycerolipids in the cell membranes, more unsaturated fatty acyl chains being present at lower growth temperatures. The physical and biochemical characteristics of membrane lipids depend on the level of unsaturation of their fatty acids, while the physical state of the lipids in biological membranes plays an important role in the various functions of the membranes [1]. For appropriate functioning, membrane constituents require the membrane lipids to be in a liquid-crystalline state where rotational transmembrane movements of the lipid and protein molecules are possible. It has been demonstrated in model membranes that the characteristic temperature of the liquid-crystalline → gel phase transition depends on the level of saturation of the membrane glycerolipids [2], and that the physiological activities change drastically at the phase transition temperature [3]. In photosynthetic membranes, the phase behavior of glycerolipids is similarly regulated by the level of their unsaturation, by fatty acid desaturases [4] which introduce double bonds directly into the fatty acids of the glycerolipids. It has been shown that the desaturase activity level plays a crucial role in the responses of the organisms to changes in the ambient temperature, such as an enhanced tolerance to low-temperature stress, by modulating the phase transition temperature of the lipids in the cytoplasmic membrane [5].

Synechocystis PCC 6803 is a transformable cyanobacterium strain that has been used extensively as a model for the chloroplasts of higher plants [4]. The photosynthetic (chloroplast) membranes of higher plants or cyanobacteria contain high levels of linolenic glycerolipids, and the phase transition of these lipids in a model system should therefore be far below room temperature. However, the photosynthetic membranes of cyanobacteria contain high amounts of proteins [6], which results in a higher phase transition temperature of lipids [7]. Moreover, Wada et al. [8] found that the phase transition temperature of cytoplasmic membranes is lower than that of thylakoid membranes.

Fourier transform infrared (FTIR) spectroscopy can be used successfully to study lipid conformational order in both model [9,10] and biological membranes [11,12]. As regards the very short time scale of molecular vibrations (10^{-14} s), it should be noted that the picture provided by FTIR spectroscopy is an average of all molecules present in the system in any conformation at that moment. There is no selectivity according to the dynamics of whole molecules, as can be observed with spin-labeled electron spin resonance (ESR) or fluorescence anisotropy techniques, for instance. Nevertheless, the ability to monitor endogenous molecules [13] or molecules that are isotopically labeled in situ [14], rather than chemically quite distinct probe molecules, is an important feature and in many cases a distinct advantage of FTIR spectroscopy.

In model systems, FTIR spectroscopy has been widely used to investigate acyl chain conformations in phospholipid bilayers [15]. The infrared spectroscopic parameter most often utilized in these systems is the frequency of the symmetric CH_2 stretching mode near 2851 cm^{-1}. This frequency has been shown to increase by 2–5 cm^{-1} at the gel → liquid-crystalline phase transition temperature. The temperature dependence of this frequency shift is a sensitive measure of lipid conformations [9]. We recently demonstrated that the underlying mechanism of the apparent shift of the $\nu_{sym}CH_2$ frequency is the competition of two close-lying bands at around 2851 and 2856 cm^{-1}, assigned to *trans* and *gauche* segments of the fatty acyl chains, respectively [16].

FTIR spectroscopy is one of the major techniques for the determination of protein secondary structures (for a review, see [17]). Thus, FTIR spectroscopy offers unique possibilities for the simultaneous study of protein and lipid structures and dynamics in biological membranes.

In this paper we analyze the changes that occur in both the lipid and the protein moieties of thylakoid and cytoplasmic membranes in consequence of changes in growth temperature and/or alterations in the level of lipid desaturation, by site-directed mutagenesis with the strain *Synechocystis* PCC 6803. The thermotropic response curves of the $\nu_{sym}CH_2$ fre-

quency revealed how wild-type cells change the level of unsaturation in the fatty acyl chains of the membrane lipids, in order to optimize the physical properties of their membranes at different growth temperatures. In mutant cells defective in lipid desaturation, it was further shown how the lack of polyunsaturated glycerolipids in the membranes is compensated to match the dynamics required for the membranes in wild-type cells. A comparison involving the behavior of thylakoid and cytoplasmic membranes pointed to the previously often neglected influence of the protein-to-lipid ratio on the membrane structure. The FTIR spectra of the membranes were utilized to determine the changes in the protein-to-lipid ratio.

2. Materials and methods

2.1. Sample preparations

Thylakoid and cytoplasmic membranes were prepared by the method of Murata and Omata [18]. The *desA*$^-$/*desD*$^-$ mutant was generated by Tasaka et al. [19]. Cells were grown at 25°C or 35°C. For simplicity, we applied the following abbreviations for the eight different membranes used in this paper: W, wild-type cells; M, *desA*$^-$/*desD*$^-$ mutant cells; T, thylakoid membrane; C, cytoplasmic membrane; 25, cells grown at 25°C; 35, cells grown at 35°C. Thus, WT25 means wild-type thylakoids from cells grown at 25°C; MC35 indicates cytoplasmic membranes from *desA*$^-$/*desD*$^-$ mutant cells grown at 35°C; etc.

2.2. FTIR measurements

For FTIR measurements, membrane suspensions were centrifuged in a Beckman TL100 centrifuge at 75 000 rpm for 20 min at 4°C, then resuspended in identical but D_2O-based buffer. The 0.4 unit shift between pH and pD was taken into account. FTIR measurements were carried out on a Nicolet Magna 560 and a Philips PU9800 FTIR spectrometer at 2 cm^{-1} spectral resolution. For each background and sample spectrum, 128 interferograms were accumulated. Temperature-dependent experiments were carried out by repeating the following measurement cycle: sample spectrum → background spectrum → new temperature setting → waiting for 10 min for the new thermal equilibrium to be attained → reading the actual temperature. With this sequence, the sample was kept in the dark until the new thermal equilibrium set in. The temperature was increased in 2–3°C steps by using a water-thermostated sample holder. The stability of the adjusted temperature was about 0.1°C.

Data analysis was carried out with SPSERV software (Bagyinka, Szeged, Hungary). Prior to data analysis, no manipulations were carried out on the spectra. Spectral regions of interest were fitted with Lorentzian components. All component parameters (frequency, bandwidth and intensity) were freely optimized by the program. The accuracy of component band frequency determination was better than 0.1 cm^{-1} in the C–H stretching region and about 2 cm^{-1} in the amide I and II regions.

3. Results

3.1. Analysis of the C–H stretching region of the FTIR spectrum

Fig. 1A shows the C–H stretching region of thylakoid membranes prepared from *Synechocystis* PCC 6803 grown at 35°C. The frequency of the indicated CH_2 symmetric stretching vibration was determined with high accuracy by curve fitting, and the thermotropic response of this frequency was used to characterize the lipid fatty acyl chain structure in the membranes.

At the bottom of Fig. 1A, the residual curve of the fit is depicted. At near 2850 cm^{-1}, the fit has a slight but systematic error around the $\nu_{sym}CH_2$ band when it is fitted with one component. The $\nu_{sym}CH_2$ band can be fitted more accurately with two components (Fig. 1B), and the assignments of these two component bands are important as concerns an understanding of the information provided by FTIR spectroscopy on the lipid structure of the membranes. The apparent upward shift observed in the $\nu_{sym}CH_2$ band as the temperature is raised is actually a result of the changes in intensity of the two component bands under its contour, as shown in Fig. 1B [16]. The lower-frequency component band at around 2851 cm^{-1} was assigned to CH_2 groups on *trans* (ordered) seg-

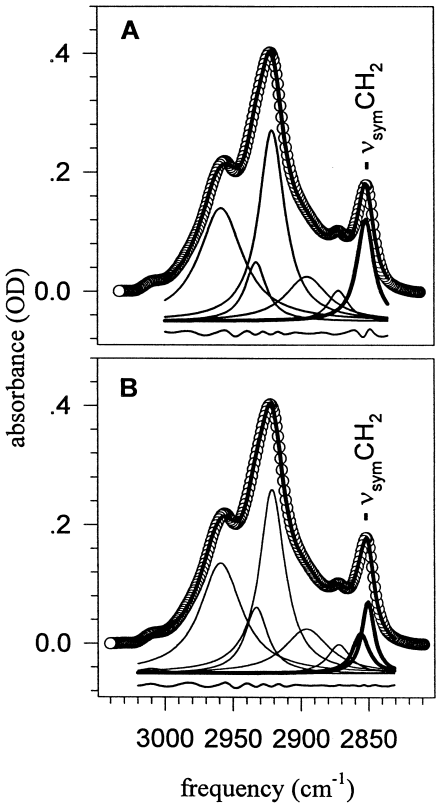

Fig. 1. Analysis of the C–H stretching region of the FTIR spectra of thylakoid membranes prepared from wild-type cells of *Synechocystis* PCC 6803 grown at 35°C. Circles indicate measured data points. The continuous line through the circles is the sum of the fitted Lorentzian components, which are displaced for clarity. The $\nu_{sym}CH_2$ components discussed in this paper are drawn with thicker lines. (A) Fit with one component for the $\nu_{sym}CH_2$ band (note the systematic error in the residual curve at the bottom of the figure). (B) The $\nu_{sym}CH_2$ band is fitted with two components (observe the disappearance of the systematic fitting error in the residual curve under the components). For details, see text.

ments of the fatty acyl chains, while the higher-frequency component band at around 2856 cm^{-1} was assigned to CH_2 groups situated on *gauche* (disordered) segments of the fatty acyl chains.

For example, if a temperature elevation causes the amount of *gauche* segments to increase, there is an accompanying increase in the intensity of the 2856 cm^{-1} component band. Since the total number of CH_2 groups in the sample is constant, the number of *trans* segments and consequently the intensity of the 2851 cm^{-1} component band decrease. As a result, the whole CH_2 symmetric band will shift upfield as the temperature increases.

The apparent frequencies of the symmetric CH_2 vibration obtained from peak frequency determinations [20–24] are most often used as a measure of lipid order in the literature; accordingly we too use this parameter to characterize the membrane lipid states, bearing in mind, however, that the higher the $\nu_{sym}CH_2$ frequency, the higher the proportion of *gauche* segments in the fatty acyl chains of the membrane lipids.

3.2. Membrane lipids in wild-type cells

The thermotropic responses of $\nu_{sym}CH_2$ of thylakoid and cytoplasmic membranes prepared from wild-type cells grown at 25°C or 35°C are illustrated in Fig. 2. A higher degree of disorder could be expected for both thylakoid and cytoplasmic membranes from the cells grown at 25°C (Fig. 2A), in

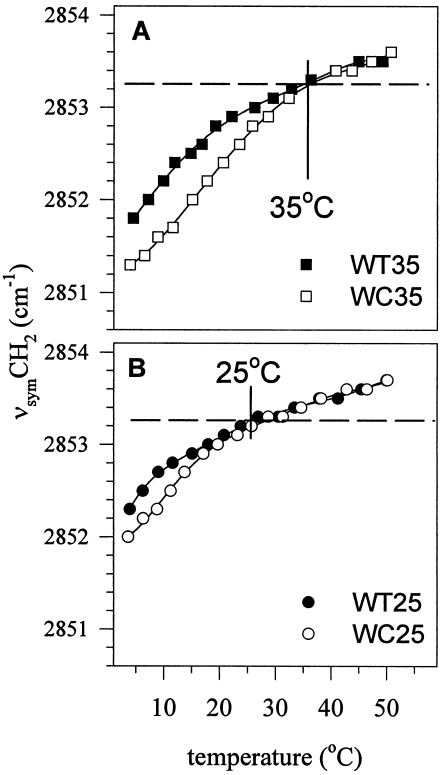

Fig. 2. Thermotropic responses of the $\nu_{sym}CH_2$ frequencies in thylakoid (WT) and in cytoplasmic (WC) membranes from wild-type cells. (A) Cells grown at 35°C; (B) cells grown at 25°C. the dashed line indicates the level of lipid disorder found at around the corresponding growth temperature. This level is identical in the membranes prepared from cells grown at either 25°C or 35°C.

accordance with the higher amount of polyunsaturated fatty acyl chains in these membranes as compared with that in the cells grown at 35°C (Fig. 2B).

At around and above the growth temperatures, the cytoplasmic and thylakoid membranes had very similar and converging degrees of fatty acyl disorder, as was expected at temperatures high enough to 'fluidize' the fatty acyl chains in the membranes more and more.

It is surprising, however, that at temperatures lower than the growth temperatures the fatty acyl chains of the cytoplasmic membranes were more ordered (as reflected by their lower $\nu_{sym}CH_2$ frequencies) than those of the thylakoid membranes prepared from the same cells (Fig. 2). Since the fatty acid compositions of the cytoplasmic and thylakoid membranes in *Synechocystis* PCC 6803 are very similar [25], the observed difference in lipid order cannot be a result of the different levels of unsaturated fatty acyl chains in the cytoplasmic and thylakoid membranes. The situation is further confused by spin-labeled ESR spectroscopic data from which it was concluded that the cytoplasmic membrane has a lower gel-to-liquid-crystalline phase transition temperature than the thylakoid membrane of the same cell [8]. Evidently, factors other than the fatty acid composition of the membranes have to be taken into account to resolve this discrepancy (e.g., protein-to-lipid ratios, vide infra).

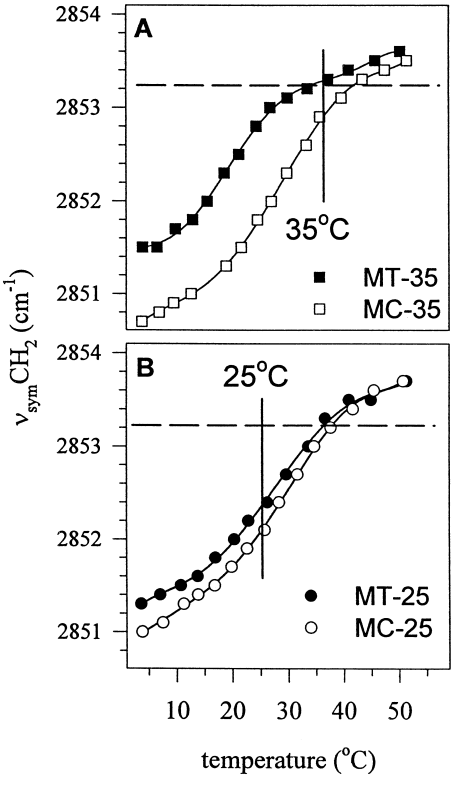

Fig. 3. Thermotropic responses of the $\nu_{sym}CH_2$ frequencies in membranes prepared from *desA*$^-$/*desD*$^-$ mutant (M) cells. (A) Mutant cells grown at 35°C; (B) mutant cells grown at 25°C. For the meaning of the abbreviations of the sample names, see Section 2. The dashed lines indicate the level of disorder found in membranes prepared from wild-type cells, as shown in Fig. 2.

It must be noted, however, that at around the corresponding growth temperatures the $\nu_{sym}CH_2$ frequency, i.e., the level of lipid disorder (the proportion of *gauche* segments to *trans*) has the same values for the thylakoid and cytoplasmic membranes prepared from cells grown at either 25°C or 35°C. In Fig. 2A and B, this identical level of disorder is indicated by a horizontal dashed line. This agreement may mean that these membranes require the same levels of lipid disorder for their functioning, and that in this temperature range the changes in lipid fatty acid composition achievable through acyl lipid desaturases are sufficient to maintain the physiologically required membrane dynamics.

3.3. Membrane lipids in *desA*$^-$/*desD*$^-$ mutant cells

In order to study the effect of the unsaturation in the glycerolipids on the membrane structure, we used a mutant strain generated by insertional mutagenesis. In the *desA*$^-$/*desD*$^-$ strain, the *desA* and *desD* genes for Δ^{12} and Δ^6 desaturases, respectively, were disrupted by an antibiotic resistance cartridge [19]. In *desA*$^-$/*desD*$^-$ cells, therefore, oleic acid is the only unsaturated fatty acid that the cells can use in combination with saturated fatty acid molecular species so as to regulate the dynamic properties of their membranes.

Thermotropic responses obtained for mutant cells grown at 25°C or 35°C are depicted in Fig. 3. When the membranes prepared from the mutant cells (Fig. 3) are compared with the membranes from the wild-type cells (Fig. 2), it can be seen that MT35 and MC35 (Fig. 3A) exhibit similar degrees of fatty acyl chain disorder at 35°C to those of the corresponding WT35 and WC35 (Fig. 2A) membranes. In contrast, MT25 and MC25 (Fig. 3B) fail to reach

the level of disorder of their wild-type counterparts at around 25°C (Fig. 2B). The degree of disorder observed for the membranes from the wild-type cells is indicated by the same dashed line in Fig. 3A and B.

It should be added that the growth rate of the mutant cells is lower than that of the wild-type cells at 25°C [19] and growth is not possible below this temperature. Additionally, these cells are very sensitive to light at low temperatures (for details of these phenomena, see [19,26]). It has been shown that during low-temperature photoinhibition the regeneration cycle of the D1 protein, a component of the photosynthetic reaction center, is blocked at the step of processing the newly synthesized preD1 to D1 protein in the photosynthetic membrane. The presence of polyunsaturated glycerolipids in the membrane seems to be the key factor for the restoration of functioning D1 proteins in the thylakoid membranes of *desA*$^-$/*desD*$^-$ mutant cells growing at 25°C [27].

It may be seen in Fig. 3B that the lipid fatty acyl chain dynamics of the membranes prepared from the mutant cells grown at 25°C is very different from that for the membranes obtained from the wild-type cells grown at the same temperature. A joint consideration of our membrane dynamics data, the findings from the low-temperature photoinhibition experiments and the physiological difficulties of culturing *desA*$^-$/*desD*$^-$ mutant cells at 25°C suggests that the MT25 and MC25 membranes are functioning at their 'dynamic limit'. If anything such as the necessary exchange or processing of D1 proteins were to require major local rearrangements, even temporarily, these membranes could provide no 'structural buffer' for any extra dynamics.

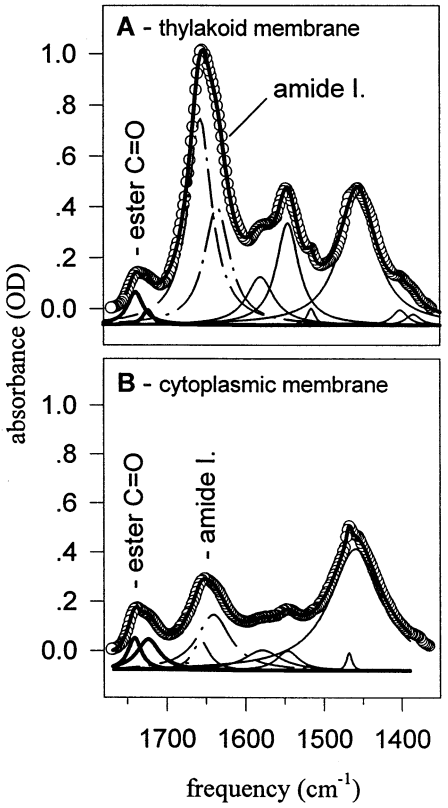

Fig. 4. Amide I region of thylakoid (A) and cytoplasmic (B) membranes prepared from wild-type *Synechocystis* PCC 6803 grown at 35°C. Note the much higher amide I to ester C=O band intensity ratio for the thylakoid membrane. Circles indicate experimental data points (for better visibility, only every second point is displayed). The continuous curve through the circles is the result of the fit obtained by using the depicted component bands. Component bands are displaced for clarity. Component bands drawn with dashed-dotted lines correspond to protein amide I bands, and those drawn with thicker lines to ester C=O vibrations. The ratio of these two types of component bands was used to determine the 'spectroscopic' protein-to-lipid ratio.

3.4. Protein-to-lipid ratios

A further important factor affecting the membrane structure and dynamics is the amounts of proteins and lipids in these membranes. Conventionally, these amounts are determined by biochemical techniques, which give absolute values but with rather large experimental errors, inherent to the methods applied.

From the FTIR spectrum, a precise protein-to-lipid ratio can be derived by dividing the relative intensity of the amide I protein band at around 1650 cm^{-1}, by that of the carbonyl stretching bands of the ester bonds connecting the fatty acids to the glycerol backbone of the lipids at around 1725–1740 cm^{-1}. Since the absorption coefficients may be different for the two bands, their intensity ratios cannot give absolute values. These changes in their relative intensities, however, do furnish realistic measures of differences in protein-to-lipid ratios from one membrane to another.

To illustrate the process of 'spectroscopic' protein-to-lipid ratio determination, the 1800–1350 cm^{-1} regions of the WT35 (Fig. 4A) and WC35 (Fig. 4B) membranes are shown. Both spectra can be fitted

very well with Lorentzian component bands. Here, a sufficient fit was sought which could be achieved by using only two components for the amide I region. Similarly, two components were used for the fit of the ester carbonyl band. Prior to curve fitting, the background due to the CaF_2 windows was subtracted. Subsequently, only a constant baseline was allowed during component band fitting.

This approach implies the neglect of the very broad but weak combination band of D_2O which also contributes to this region. The problem with D_2O spectrum subtraction is that the shape of the deformation band at around 1210 cm^{-1} (which could otherwise be a good candidate for the calibration of subtraction) is different in pure D_2O from that found in the extremely concentrated membrane suspensions (not shown), and therefore subtraction cannot be perfect. Since the broad D_2O combination band overlaps with the whole amide I region, in this case we cannot trace even the changes in its band shape. A reliable subtraction of the D_2O combination band by calculating its contribution via a pure D_2O spectrum by curve fitting is therefore not possible. Thus, we rather have chosen to compromise by retaining the slight and invariant distortion effect of the broad D_2O combination band on the protein-to-lipid ratio.

Table 1
Spectroscopically measured and calculated weight/weight protein-to-lipid ratios in wild-type and *desA*$^-$/*desD*$^-$ mutant cell membranes

	Protein-to-lipid ratio (spectroscopic) $I_{(\text{amide-I})}/I^{a}_{(\text{ester C=O})}$		Proportion of proteins (weight/weight), Conv. fact = 6.1[b]	
	Thylakoid	Plasma	Thylakoid	Plasma
WT35	11.9		67%	
WC35		2.4		29%
MT35	10.5		63%	
MC35		2.2		29%
WT25	13.1		68%	
WP25		2.6		29%
MT25	13.0		68%	
MC25		3.0		33%

[a]The largest error of the ratio determination is 15%. The errors of the ratios were calculated from the fitting errors of the relevant component bands shown in Fig. 4.

[b]The conversion factor between the spectroscopic and the weight/weight protein-to-lipid ratios was calculated on the basis of the biochemical protein/lipid ratio determinations of Tasaka et al. [19]. For details see text.

'Spectroscopic' protein-to-lipid ratios were calculated by dividing the sum of the amide I component band intensities by the sum of the intensities of the lipid ester carbonyl component bands. These data are shown in the first two columns of Table 1 for thylakoid and cytoplasmic membranes prepared from wild-type and mutant cells grown at 25°C or 35°C. It may be noted that the protein-to-lipid ratios are very similar in all thylakoid and in all cytoplasmic membranes, regardless of the growth temperatures and the origin of the membranes (wild-type or mutant cells).

For 'spectroscopic' protein-to-lipid ratios to be linked to absolute weight/weight values, the protein-to-lipid ratio must be known from a biochemical determination for at least one type of these membranes. Tasaka et al. [19] found weight/weight protein-to-lipid ratios of 2.3 and 2.0 for WT25 and MT25 membranes (see Table 1). From these data, we calculated conversion factors between spectroscopically and biochemically determined protein-to-lipid ratios for the two membranes. We obtained 5.7 for WT25 and 6.5 for MT25. Assuming that the lipid and protein absorption coefficients do not vary from membrane to membrane, we took the average (6.1) of these two values as a conversion factor. The contents of proteins in weight/weight percentages are given in Table 1 for the eight different membranes studied.

4. Discussion

4.1. The effect of the proteins on the lipid structure

The effect of the presence of different amounts of proteins to the lipid chain order has been studied earlier in model systems. The incorporation of increasing amounts of glycophorin into dimyristoylphosphatidylcholine multilayers abolished the pretransition, broadened the gel-to-liquid-crystal transition and increased the bandwidths of the νCH_2 vibrations of the lipids [28]. Similar results were obtained with Ca^{2+}-ATPase and bacteriorhodopsin, under the phase transition the amount of gauche isomers augmented with the presence of increasing amount of proteins in dipalmitoylphosphatidylcholine vesicles [29].

The protein-to-lipid ratios in the thylakoid and the cytoplasmic membranes differed markedly (Fig. 4A,B). The protein content is around 68% in the thylakoid and 29% in the cytoplasmic membranes of *Synechocystis* PCC 6803 (Table 1). These values are in good agreement with those obtained by Omata and Murata [6] (1983) for another cyanobacterium strain, *Synechococcus* PCC 6301 (*Anacystis nidulans*). The difference in protein-to-lipid ratio in the thylakoid and cytoplasmic membranes has important consequences as concerns the interpretation of the lipid disorder data. A higher amount of membrane proteins means that there is a higher proportion of lipids in the close vicinity of proteins with somewhat broken fatty acyl chains to maintain optimal lipid–protein interactions. There are therefore more *gauche* conformers in the lipids of the membranes with high protein content.

Since these *gauche* segments come from the structural constraints of the biological membranes, we refer to them as 'structural' disorder. At low temperatures, this 'structural' disorder prevails; it maintains a population of *gauche* segments in the fatty acyl chains of the lipids in the biological membranes. At the same low temperatures, these *gauche* segments would disappear from a pure lipid system where bulk lipids can freeze into a gel phase with *trans* segments along the fatty acyl chains. Consequently, the apparent frequency of the $\nu_{sym}CH_2$ band, which is a good measure of the *gauche* segment population in the fatty acyl chains [16], is higher in biological membranes than in pure lipids. Thus, the higher $\nu_{sym}CH_2$ frequencies observed at low temperatures can be attributed to the 'structural' disorder imposed on the lipids by the protein–lipid interactions in the membranes. This parameter should therefore exhibit a strong correlation with the protein-to-lipid ratio.

The fatty acid compositions of the thylakoid and cytoplasmic membranes prepared from the same cells are similar [25]; accordingly the higher protein-to-lipid ratio (Table 1) may explain the higher frequencies observed for the $\nu_{sym}CH_2$ bands at low temperatures in both WT25 and WT35 thylakoids as compared with the corresponding WC25 and WC35 cytoplasmic membranes (Fig. 2). The situation is similar in the case of mutant cells (Fig. 3).

All wild-type cell membranes exhibited similar $\nu_{sym}CH_2$ frequencies at their growth temperatures (Fig. 2). The increase from the different low-temperature starting values to these uniform ones is due to the growing number of temperature-induced *gauche* segments in the fatty acyl chains as the temperature is elevated. This additional upshift of the $\nu_{sym}CH_2$ frequency is considered a consequence of increasing 'dynamic' lipid disorder.

We are aware that this argument would likewise hold on taking into account $\nu_{sym}CH_2$ frequencies at temperatures lower than that of the gel → liquid-crystalline phase transition of the given membrane. Since we wished to avoid the addition of any freeze-protecting material to the membrane suspensions and the risk of fracturing membrane lipids from the proteins, we accepted a 'mixed' starting state at around 5°C.

4.2. Membrane dynamics

It is of interest that the extent of lipid disorder is roughly identical in all membranes at their physiologically relevant growth temperature, regardless of their protein-to-lipid ratio (Fig. 2). As discussed above, this lipid disorder is comprised of two components: (i) the disorder due to protein–lipid interactions ('structural' disorder) and (ii) that due to the temperature-induced lipid dynamics ('dynamic') disorder. This could mean that there is an absolute level of lipid disorder in functioning membranes, to which all lipids contribute, either at protein–lipid interfaces or in bulk lipid matrices. For functioning, a membrane requires certain barrier properties, a certain mobility for the proteins, and certain physical characteristics. It is probable that, although in different manners by the different lipid classes, all these requirements are met by an overall lipid disorder, which is well characterized by the $\nu_{sym}CH_2$ frequency.

It should be mentioned that ESR and fluorescence anisotropy spectroscopy indicated a higher probe mobility in cytoplasmic membranes as compared to thylakoids [8], and it was concluded from the data that cytoplasmic membranes have a higher fluidity than that of thylakoids. Accordingly, lower phase transition temperatures have been demonstrated for cytoplasmic membranes [8,30,31]. It should be remembered, however, that FTIR spectroscopy involves a very different energy range from that for

labeling ESR or fluorescence spectroscopy. In the FTIR spectra, elementary movements of segments of all molecules present in the system can be seen, whereas movements of whole labeled molecules are measured with the other two techniques. Accordingly, in consequence of the much higher protein-to-lipid ratios, the rotation of a 'lipid-like' labeled molecule is liable to be more limited in thylakoids (even among more disordered lipids) than in the large (and possibly more ordered) lipid matrices of cytoplasmic membranes with low protein content. There is therefore no discrepancy between our conclusions and the data obtained by other techniques; the conditions of the experiments should merely be specified more thoroughly. (A systematic study of the relationship of the information provided by FTIR and spin-labeling ESR spectroscopy on the state of lipids in biological membranes is under way in our laboratory.) Our present study highlights that the actual protein-to-lipid ratios in the system under investigation should be known whenever techniques are employed that are designed to furnish data on membrane structures and dynamics.

4.3. Membranes at extreme temperatures

The extreme temperatures at both ends of the physiological temperature range pose a lethal threat for cells. At the low-temperature end, the acyl-lipid desaturase system is induced [1]. However, the question remained open as to whether modulation of the level of unsaturation in the membrane lipids alone is sufficient for protection, or other factors, e.g., proteins and pigments, are also necessary.

Our structural investigations demonstrate (Figs. 2 and 3) that the changes initiated in the level of lipid unsaturation by acyl–lipid desaturases are sufficient to protect the cells at low temperatures. In wild-type cells, via tuning of the fatty acid composition, a similar membrane dynamics is maintained over the whole physiological temperature range (Fig. 2). Any major role of proteins in the acclimation can be excluded, since the protein-to-lipid ratio was the same within experimental error in any type of membrane studied, independently of the growth temperature and mutation (Table 1).

The crucial role of the membrane dynamics in the acclimation of cells is most evident in the case of the lipid desaturase-deficient mutant, which could not attain the required membrane dynamics at low temperatures because of the lack of polyunsaturated lipids in its membranes (Fig. 3A). The importance of lipid desaturation diminishes toward higher temperatures, where temperature-induced motions in the saturated fatty acid chains can substitute for the higher 'fluidity' of the unsaturated fatty acids (Fig. 3B).

In the high-temperature range, the thermotropic curves of all membranes converge toward similar values (Figs. 2 and 3). This is to be expected, since at higher temperatures more lipids will melt, and the melted lipids will be more similar to each other, regardless of their differences at the level of fatty acyl chain saturation/unsaturation. As regards the alarming of the cells at high temperatures, several possible signaling mechanisms have been proposed, one involving changes in the physical state of the bulk lipids. Our experiments suggest that 'fluidity' changes in the bulk lipid parts of the membranes do not act as primary sensors of the temperature. If there is any signaling mechanism in the membrane at all, it could be triggered either by alterations in specific microdomains of the lipids, or by changes in the lipid–protein interactions, in the proteins, or in other components of the membranes.

In conclusion, it may be stated that the lipid disorder in the thylakoid and cytoplasmic membranes, as represented by the upshifted frequency of the $\nu_{sym}CH_2$ vibration, is a resultant of the cumulating 'structural' and 'dynamic' lipid disorder. The contribution of the 'structural' disorder to the upshift in the $\nu_{sym}CH_2$ frequency correlates strictly with the protein content of the membranes. In the wild-type cells, regardless of the protein-to-lipid ratio, the $\nu_{sym}CH_2$ frequencies were similar at around the growth temperatures in the thylakoid and cytoplasmic membranes, indicating that a certain membrane dynamics is required for the membrane functions. When all polyunsaturated lipids were eliminated from the membrane by using lipid desaturase-deficient mutant strains, it was shown that the regulation of the level of unsaturation is more important in the low-temperature acclimation than in that at high temperatures.

On introduction of a precise protein-to-lipid ratio determination method involving the FTIR spectra of

the membranes, it was found that the protein-to-lipid ratio is about five times higher in the thylakoid membranes than in the cytoplasmic membranes. It was concluded that the actual protein-to-lipid ratio in the membrane should be taken into account for a correct interpretation of data obtained by any technique applied to study membrane structure/dynamics. Accordingly, in the future, membrane proteins as entities and as partners in protein–lipid interactions must be investigated in a more detailed way if the aim is a deeper insight into the dynamics of biological membranes.

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