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Lipids, Proteins, and Their Interplay in the Dynamics of Temperature-Stressed Membranes of a Cyanobacterium, *Synechocystis* PCC 6803[†]

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ABSTRACT: Proper responses to low- and high-temperature stresses are essential for the survival of many organisms. It has been established that at low-temperature stress the sufficient microviscosity of the lipids is decisive in this respect. In many organisms, adapting the level of lipid unsaturation to the low growth temperature regulates this feature. At high-temperature stresses, however, there are no unequivocal results concerning the role of the lipids. In these temperature ranges, the lipids are all disordered and fluid and their physical parameters change slowly with increasing temperatures, while biological organisms give characteristic stress responses in rather narrow temperature ranges. Therefore, one may speculate that other membrane parameters/components, which change sharply in the range of the high-temperature stress, may give a signal to initiate the general response of the cells. For such a role, proteins are the trivial candidates. To reveal the role of the lipids and the proteins in these processes, we used a genetically engineered system, based on a cyanobacterium, *Synechocystis* PCC 6803. In the wild-type cells of this bacterium, by altering the growth temperature, the polyunsaturated lipid content of the cell membranes can be varied considerably (as required by the homeoviscous adaptation principle). In the case of *desA*$^-$/*desD*$^-$ mutant cells, which can contain only monounsaturated fatty acyl chains in their lipids, homeoviscous adaptation of the lipids is not possible. Since *desA*$^-$/*desD*$^-$ mutation affects only the lipids, additional perturbations (e.g., altered protein content) should minimally disturb the comparison of the lipid behaviors in wild-type and mutant cells. Infrared spectra of thylakoid and cytoplasmic membranes isolated from wild-type and mutant cells were recorded in 3 °C steps between 8 and 92 °C. By analyzing the rates of protein structural changes, hydrogen−deuterium exchange, in-membrane lipid disorder, and water−membrane interfacial order/hydration as functions of the temperature, we conclude that (i) the gel-to-liquid crystalline phase transition of the lipids correlates with the growth temperature in the wild-type cells but not in the *desA*$^-$/*desD*$^-$ mutants, (ii) over the physiological temperature range, both protein and lipid dynamics are regulating/regulated, providing remarkably constant dynamics for both the thylakoid and cytoplasmic membrane, (iii) in the high-temperature stress region, protein structure and dynamics are changing sharply without any correlation with growth temperature and/or mutation, i.e., membrane protein stability does not seem to depend on the lipid composition of the membrane (this finding points to the possible primacy of proteins as initiators/targets of heat-shock alarms), and (iv) there are substantial differences between the dynamics of the proteins of the thylakoid and cytoplasmic membranes, reflecting their different protein complexes and lipid-to-protein ratios.

Biological membranes are delicate assemblies of proteins and lipids. For the functioning of a membrane (this may mean barrier properties, signal transduction, energy production, material transport, etc.), both the individual properties of the constituent proteins and lipids and their interactions are decisive. Proper interactions assume proper membrane dynamics, which is able to cope with altering external conditions (e.g., temperature, light/darkness in photosynthetic organisms, osmotic/salt stresses, etc.). For temperature adaptation, the homeoviscous adaptation (*1*) has been postulated as a correction mechanism and was proven mostly for poikiotherm organisms. Homeoviscous adaptation theory focuses on the role of lipids in maintaining membrane dynamics. Much less is known about the role of protein dynamics and even less about the role of protein−lipid interaction in maintaining membrane dynamics and, thus, the functionality of a biological membrane, especially around its physiological low- and high-temperature limits.

It has been shown that, at the physiological low-temperature limit and under cold-stress, maintaining high lipid dynamics (liquid-crystalline phase) in the membranes is a very important factor in survival and adaptation (*2*−*6*). At the other end, around the high-temperature physiological limit and under heat stress, a histidine kinase has been identified as a source of signaling for increased thermotolerance (*7*). For nonphotosynthetic membranes, heat-shock proteins were shown to participate in membrane protection (*8*). With regard to membrane lipids, on the analogy of the effects of membrane-fluidizing agents, the formation of "hyperfluid" lipid domains has been proposed as a possible source of the heat-shock alarm signal (*9*, *10*).

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On the basis of our earlier studies, it seems that around or slightly above the physiological high-temperature limit of an organism a rather steep membrane−protein denaturing starts (*11*), while lipid disorder in biological membranes at high temperatures is a very smoothly changing feature (*11*, *12*).

The data, discussed above, clearly show that lipid composition and dynamics have the primary role in low-temperature adaptation and cold stress. At the high-temperature end, however, the specific roles of lipids and proteins in preserving membrane structure and/or emitting stress signals are far from being understood. In addition, the interaction between lipids and proteins among high-temperature stress conditions has not been studied in detail so far.

In the present paper, the following features of thylakoid and cytoplasmic membranes will be examined by Fourier transform infrared (FTIR)[1] spectroscopy: (i) changes in the amide II region due to hydrogen/deuterium (H/D) exchange (a measure of protein dynamics), (ii) structural changes reflected by the amide I region, especially the evolution of a band around 1619 cm^{-1}, which is diagnostic for heat denaturing of proteins (*13*), (iii) the in-membrane average disorder of the lipid fatty acyl chains via the temperature dependence of the frequency of the $\nu_{sym}CH_2$ band (*14*), and (iv) changes in the membrane−water interfacial region via looking at the ester C═O band at 1750−1720 cm^{-1} (*15*).

Hydrogen/deuterium (H/D) exchange has long been utilized to study protein structure (*16*−*18*). The method is based on the fact that H atoms bound to N or O are exchangeable with the H or D atoms of the surrounding H_2O or D_2O, respectively. For H/D exchange, the most sensitive region of the infrared spectrum is the amide II band around 1550 cm^{-1}, since it has a large contribution from N−H deformation of the peptide bond. Therefore, a large isotopic shift occurs upon replacing N−H groups (1550 cm^{-1}) by N−D ones (1450 cm^{-1}, amide II′) when a protein is dissolved in D_2O. Since the accessibility of the different protein domains depends on fluctuations of the protein structure, the rate of the H/D exchange is a direct measure of protein dynamics (*19*). By classifying the peptide protons according to their H/D exchange rates, a wealth of information has been obtained on the dynamics of proteins (*20*−*22*). For membrane proteins, H/D exchange is more complex (*23*). Here, certain regions of the proteins can be directly accessible from the water phase, and there are hydrophobic regions buried in the membrane interior. The hydrophobic phase restricts the access of the H_2O/D_2O molecules to the buried protein surfaces. In addition, the extent of this restriction may depend on the physical state (gel, liquid crystalline) of the lipid matrix.

The amide I region (1700−1600 cm^{-1}) is used for the determination of the secondary structure of proteins. Under the broad envelope of the amide I band there are component bands, which, when resolved by mathematical methods, can be used to characterize the α-helical, *β*-sheet, random, etc. content of the given protein. For reviews on infrared spectroscopy of soluble and membrane proteins see refs *23*−*26*. Regarding protein denaturing, in most cases it is accompanied with the aggregation of the proteins, and these aggregated forms exhibit a strong band at around 1619 cm^{-1} and a weaker one around 1685 cm^{-1}. These bands are characteristic of *β*-structures (*13*) and sensitively monitor the progress of protein denaturing even in complex biological systems (*11*).

Despite the fact that the structure of the lipid double layers has long been well understood (*14*), the link between the physical state of the lipids and the H/D exchange of membrane proteins has not yet been explored. Lipid polymorphism in model and biological membranes has been extensively studied by FTIR spectroscopy (*27*). In this respect, the most frequently utilized infrared spectroscopic parameter is the frequency of the symmetric CH_2 stretching mode ($\nu_{sym}CH_2$) near 2850 cm^{-1}. This frequency has been shown to increase by 2−5 cm^{-1} upon gel-to-liquid crystalline phase transition (for a review on $\nu_{sym}CH_2$ in lipids, see ref *28*). Using the 3050−2800 cm^{-1} C−H stretching region, the adaptation to growth temperatures and the corresponding changes in lipid disorder in cyanobacterium thylakoid membranes have sensitively been followed (*12*, *29*).

Another lipid-related band, the ester C═O stretching vibration at 1750−1720 cm^{-1}, is also sensitive for the structural changes in the biological membranes. This broad band can be resolved into two components around 1742 and 1727 cm^{-1}. First, these components were thought to reflect the *sn*-1 and *sn*-2 ester bonds of the glycerol backbone, but the present agreement on their assignment is that they are due to differently hydrated subpopulations of both carbonyls (*30*, *31*). Upon increasing temperatures, the intensity of the lower frequency component increases due to the increased chances of the C═O groups becoming hydrated in the more disordered membranes. (For a review on the membrane structure sensitivity of this band, see ref *15*.)

To separate the effects of the protein self-dynamics and of the lipid environment, we used a genetically engineered system, developed in a cyanobacterium, *Synechocystis* PCC 6803. The wild type of this bacterium is known to increase the proportion of the polyunsaturated fatty acids in its membranes via a desaturase enzyme cascade when its growth temperature is shifted from 35 to 25 °C (*32*). By growing wild-type cells at these temperatures and measuring the lipid disorder in their isolated thylakoid and cytoplasmic membranes over a wide temperature range, one can observe the functioning of homeoviscous regulation by providing the same membrane disorder (fluidity/microviscosity) at the respective growth temperatures (*12*) . By knocking out the genes of certain desaturase enzymes, a mutant ($desA^-/desD^-$) can be constructed, which contains only monounsaturated fatty acids (*33*). This mutant is very restricted in controlling its lipid dynamics; thus, only a small growth temperature effect on the lipid disorder can be expected.

We have studied the H/D exchange and the structural changes of the proteins, the in-membrane lipid disorder, and changes in the water−membrane interfacial region in thylakoid and cytoplasmic membranes prepared from wild-type and mutant *Synechocystis* PCC 6803 cells as functions of the temperature. Changes in the infrared absorption spectra were analyzed by singular value decomposition (*34*) in selected spectrum regions. Upon evaluating the data, we searched correlating changes in the selected spectral regions to demonstrate lipid−protein interactions.

MATERIALS AND METHODS

Samples. *Synechocystis* PCC 6803 cyanobacterium wild-type (W) and $desA^-/desD^-$ desaturase enzyme deficient mutant (M) cells were grown at 25 °C (25) and 35 °C (35); from cells, thylakoid (T) and cytoplasmic (C) membranes were prepared

[1]Abbreviations: FTIR, Fourier transform infrared; SVD, singular value decomposition.

as described earlier (*12*). The naming of the samples reflects their origin and growth temperature; e.g., MC35 is a cytoplasmic membrane isolated from mutant cells grown at 35 °C, WT25 is a thylakoid membrane prepared from wild-type cells grown at 25 °C, etc. All technical details concerning cell growth and membrane preparations were exactly the same as described in ref *12*.

Infrared Measurements. FTIR spectra were recorded on a Philips PU9800 Fourier transform infrared spectrometer, averaging 128 scans at 2 cm^{-1} spectral resolution by using a sample shuttle and a DTGS detector. Samples from frozen (−70 °C) stocks were brought to 4 °C immediately before measurement. The amount of the membrane suspension required for one measurement was taken out (≈50 μL), diluted with D_2O-based buffer (≈1 mL), centrifuged, resuspended in 1 mL of D_2O-based buffer, and centrifuged again. The pellet was immediately placed between CaF_2 windows. The whole preparation process took about 30 min until the sample was in place in the spectrometer. The temperature was increased in 35 steps between 8 and 92 °C, and at each temperature step two spectra were recorded that yielded 2 × 35 absorption spectra per temperature scan. For stabilizing the new temperatures, 7 min was left, and also 7 min was the time interval between the two spectra recorded at the same temperature. A water-thermostated cell holder controlled the temperature of the sample. The accuracy of the temperature setting was about 0.1 °C. Spectrum manipulations were carried out with the SPSERV software of Dr. Cs. Bagyinka (Biological Research Center, Szeged, Hungary).

Singular Value Decomposition (*SVD*) *Analysis*. Temperature-dependent changes in the infrared spectra were analyzed with SVD analysis (*34*). The actual calculations were carried out with the SPSERV software. Briefly, if a system as a function of an external parameter (in the present case, temperature) contains $\mathbf{s}_i$ species, which are spectrally distinguishable, at any temperature the measured spectrum can be described as a linear combination of the spectra of these $\mathbf{s}_i$ species. The output of the SVD analysis is a reduced representation of the data matrix in terms of a set of $\mathbf{s}_i$ basis spectra, an associated set of temperature-dependent amplitude vectors ($\mathbf{v}_i$), and a diagonal matrix with the weights of the $\mathbf{s}_i$ species ($\mathbf{w}_i$). Since the set of output components is ordered by decreasing size, each subset consisting of the first ***j***-components provides the best ***j***-component approximation of the data matrix.

When spectra taken as a function of the temperature are to be analyzed, the $\mathbf{s_1}$ basis spectrum shows a temperature average; $\mathbf{s_2}$ shows the largest changes, which have to be combined with the $\mathbf{s_1}$ spectrum to get the actual spectrum at a given temperature. The $\mathbf{v_2}$ amplitude vector gives the temperature dependence of the largest change, manifested in the $\mathbf{s_2}$ spectrum, etc. In the present case, we restricted ourselves to the $\mathbf{s_1}$ and $\mathbf{s_2}$ spectra and the corresponding $\mathbf{v_1}$ and $\mathbf{v_2}$ vectors. Earlier, we had performed an analysis of the gel-to-liquid crystalline phase transition of dipalmitoylphosphatidylcholine with this technique. Details can be found there (*35*). In the SVD analysis, we have considered only the average ($\mathbf{s_1}$,$\mathbf{v_1}$) and the largest change ($\mathbf{s_2}$,$\mathbf{v_2}$) of the 35 × 2 spectra recorded upon increasing temperature (8−92 °C). This was an approximation but can be justified since the weights ($\mathbf{w}_i$) of the $\mathbf{s}_i$ basis spectra are decreasing very rapidly (e.g., for WT25 CH region in Figure 2: $\mathbf{w_1}$ = 1, $\mathbf{w_2}$ = 0.023, $\mathbf{w_3}$ = 0.014, $\mathbf{w_4}$ = 0.005, $\mathbf{w_5}$ = 0.001, etc.) In the figures, the rates of the changes, i.e., the first derivatives of the $\mathbf{v_2}$ vectors, will be plotted. We have found that the first derivatives, in spite of the higher noise, reflect the changes more visibly than the original $\mathbf{s_2}$ vectors. The first derivatives were obtained in the following way: the 35 × 2 points of the $\mathbf{v_2}$ vectors were first averaged, then the 35 points were interpolated by 300 points using a third-order polynomial, and then the first derivative on these 300 points was taken.

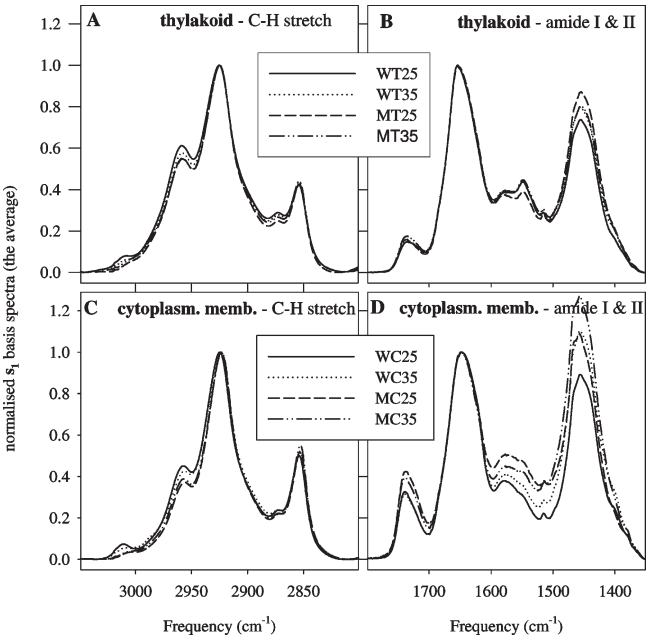

FIGURE 1: The C−H stretching (A, C) and amide I and II (B, D) regions of the studied thylakoid and cytoplasmic membranes, respectively. Note that, in the C−H stretching region, the intensity of the band around 3010 cm^{-1}, originating from −CH=CH− segments in the fatty acyl chains, is higher in the wild-type thylakoid (WT25) and cytoplasmic (WC25) membranes prepared from cells grown at 25 °C. The −CH=CH− segments are the results of the desaturase enzyme actions and are needed for low-temperature adaptation. Note also that the relative intensity of the ester C=O band around 1740 cm^{-1} is higher for cytoplasmic membranes (D), indicating a higher relative amount of lipids here as compared to thylakoids (B). Each plotted spectrum is an average in two senses: from each experiment, from the 35 × 2 spectra taken in the 8−92 °C range the $\mathbf{s_1}$ spectrum was calculated, which is an averaging in itself. Then the $\mathbf{s_1}$ spectra obtained from independent preparations of the same type of membranes (as indicated in Table 1) were averaged. Spectra are normalized at 2920 or 1650 cm^{-1}, respectively.

Conventional Curve Fitting. The actual frequencies of the $\nu_{sym}CH_2$ band (around 2850 cm^{-1}) were determined by fitting around its peak (2865−2840 cm^{-1}) with a Lorentzian curve using the SPSERV program. All details of such a fit can be found in ref *35*.

RESULTS

There are two kinds of variability, which may make the comparison of the infrared spectra of biological membranes of different origin difficult: (i) The first is variations due to the specific temperature dependences of these samples. We have overcome this problem by calculating the $\mathbf{s_1}$ SVD spectrum of each sample; thus we could compare a kind of "average" of all physical states present over the whole temperature range (8−92 °C) in each sample. (ii) The second is variations due to the differences between independent membrane preparations. Here, this variability was reduced by averaging the $\mathbf{s_1}$ spectra of the independent samples of the same type. Such "double averaged" infrared spectra in the regions of interest of this work are depicted in Figure 1. It should be noted here that the amide I and ester C=O regions of the $\mathbf{s_1}$ spectra of the same type of membranes

prepared from different cell cultures were very similar to each other (Figure 1B,D). The amide II′ regions, however, exhibited intensity variations, whose magnitudes were close to the differences seen between different types of membranes for this region. The reason for the larger variability in the amide II′ region is that upon H/D exchange the protons leaving the biological sample form H−O−D "hybrid" water molecules, which have their deformation band also here. With the extinction coefficient of the H−O−D band being much higher than that of the amide II′, and concerning the difficulties of having exactly the same membrane suspension/D_2O ratio in the samples, the larger uncertainty in the amide II′ + H−O−D deformation intensities is understandable. Being free from disturbing overlapping, the C−H stretching regions of the different $\mathbf{s_1}$ spectra exhibited small variations (Figure 1A,C).

Analysis of the C−H Stretching Region (3050−2800 cm^{-1}) of the Infrared Spectra: Lipid-Related Changes. Upon increasing temperatures, lipids exhibit characteristic phase transitions, which can be sensitively monitored by the frequencies of the $\nu_{sym}CH_2$ vibration in model lipid membranes (*14*). But in a biological membrane, both proteins and lipids contain CH_2 and CH_3 groups. However, the ratio of the CH_2/CH_3 groups in the lipid fatty acyl chains is far higher (15−17:1) than in protein amino acid side chains (2:1). We have calculated this latter ratio by averaging 17 membrane proteins; the actual values were about 1.2 CH_2 and 0.6 CH_3 groups per amino acid residue. Therefore, one may expect that lipid contribution dominates in the $\nu_{sym}CH_2$ band in biological membranes as well. Indeed, this is the case; for illustration, see Figure 4 in ref *36*. Thus, by analyzing the frequency shift of the $\nu_{sym}CH_2$ band (*14*), we can get information concerning mainly the fatty acyl chains even in biological membranes. The higher the $\nu_{sym}CH_2$ frequency, the higher the amount of the gauche segments in the fatty acyl chains. Since we needed absolute values for the comparison of different membranes, we used conventional curve fitting to determine the actual $\nu_{sym}CH_2$ frequencies instead of the SVD analysis used throughout this paper, which provides the temperature dependence of the changes only.

We fitted the $\nu_{sym}CH_2$ band between 2865 and 2840 cm^{-1} with a single Lorentzian curve (*12*) allowing a free linear baseline (in the present case, this limited region was enough since we were interested only in the peak positions) and plotted the obtained frequencies as a function of the temperature. The middle temperature (T_m) of the gel-to-liquid crystalline phase transition was determined by fitting the thermotropic responses with an exponential transition between the two states. (The applied function and the result of the statistical analysis of the fits on all samples studied are given in Table 1.)

Table 1 shows that while there is a large upshift in the T_m values upon increasing the growth temperature from 25 to 35 °C in wild-type cells, there is practically no change in the T_m of the *desA*$^-$/*desD*$^-$ mutants. In the mutant cells, the T_m of the cytoplasmic membranes is considerably higher than that of the thylakoids. This might be explained by the higher relative amount of lipids in the mutant cytoplasmic membranes (see the ester C═O bands around 1740 cm^{-1} in Figure 1B,D). In the mutant cytoplasmic membranes, the lipids are less perfectly matching the requirements of the optimal lipid−protein interactions; therefore, more lipids are needed to properly embed the proteins for maintaining the barrier properties of the membranes. On the other side, due to their higher relative amount, the lipids of the *desA*$^-$/*desD*$^-$ cytoplasmic membranes have a higher chance to form pure lipid domains, where the fatty acyl chains, free from disturbing lipid−protein interactions, may be more ordered and may have higher phase transition temperatures (*12*).

Table 1: Middle Values (T_m) of the Gel-to-Liquid Crystalline Phase Transition Temperatures Calculated from the Frequencies of the $\nu_{sym}CH_2$ Band

	T_m[a] (°C)		
	average[b]	SD	samples
WC25	14.2	1.0	2
WC35	22.4	1.7	3
WT25	14.8	1.6	6
WT35	19.6	1.4	5
MC25	26.8	2.4	2
MC35	28.2	2.0	4
MT25	20.8	2.7	4
MT35	20.4	1.3	5

[a]For the determination of T_m, the $y = A/(1 + \exp(-(x - x_0)/\Gamma))$ (where A is the amplitude and Γ is the width of the transition) function was used. [b]Typical thermotropic responses of the $\nu_{sym}CH_2$ band are shown in Figures 3B,D and 4B,D and will be discussed there.

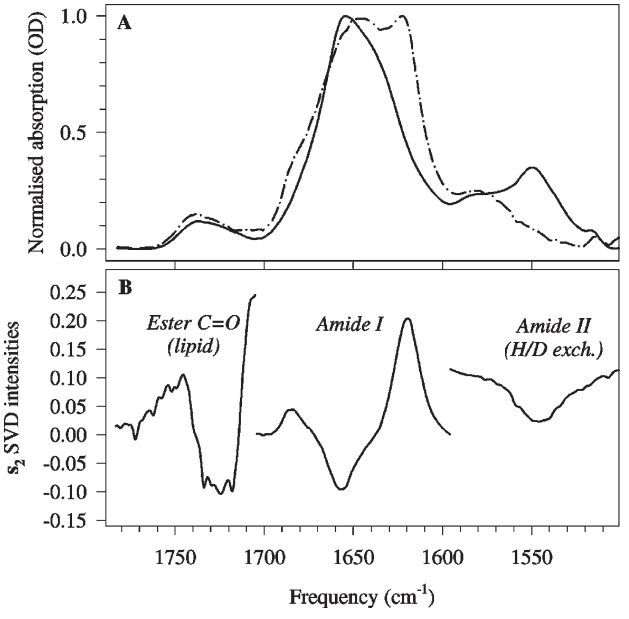

FIGURE 2: Infrared absorption spectra (A) in the 1800−1500 cm^{-1} region of wild-type *Synechocystis* PCC 6803 thylakoid membranes (WT25) recorded at 8 °C (——) and 92 °C (— · —). Singular value decomposition (SVD) analysis (B) of the 35 × 2 spectra recorded between 8 and 92 °C. Basis spectra of the largest changes ($\mathbf{s_2}$) accompanying the temperature increase are depicted for the ester C═O, the amide I, and the amide II regions.

SVD Analysis of Temperature-Induced Changes in the 1800−1500 cm^{-1} Region. (A) Thylakoid Membranes. Figure 2 illustrates the extremities of the spectral changes upon increasing the temperature from 8 to 92 °C for a WT25 membrane. To separate the protein- and the lipid-related changes, three independent SVD's were performed in the 1800−1500 cm^{-1} spectrum range as shown in Figure 2B. The three regions corresponded to the ester C═O band (lipid related, sensitive for the water−membrane interfacial region) between 1800 and 1700 cm^{-1}, to the amide I region (protein secondary structure) between 1700 and 1600 cm^{-1}, and to the amide II region (H/D exchange ≈ protein dynamics) between 1600 and 1500 cm^{-1}. The obtained $\mathbf{s_2}$ spectra are shown in Figure 2B.

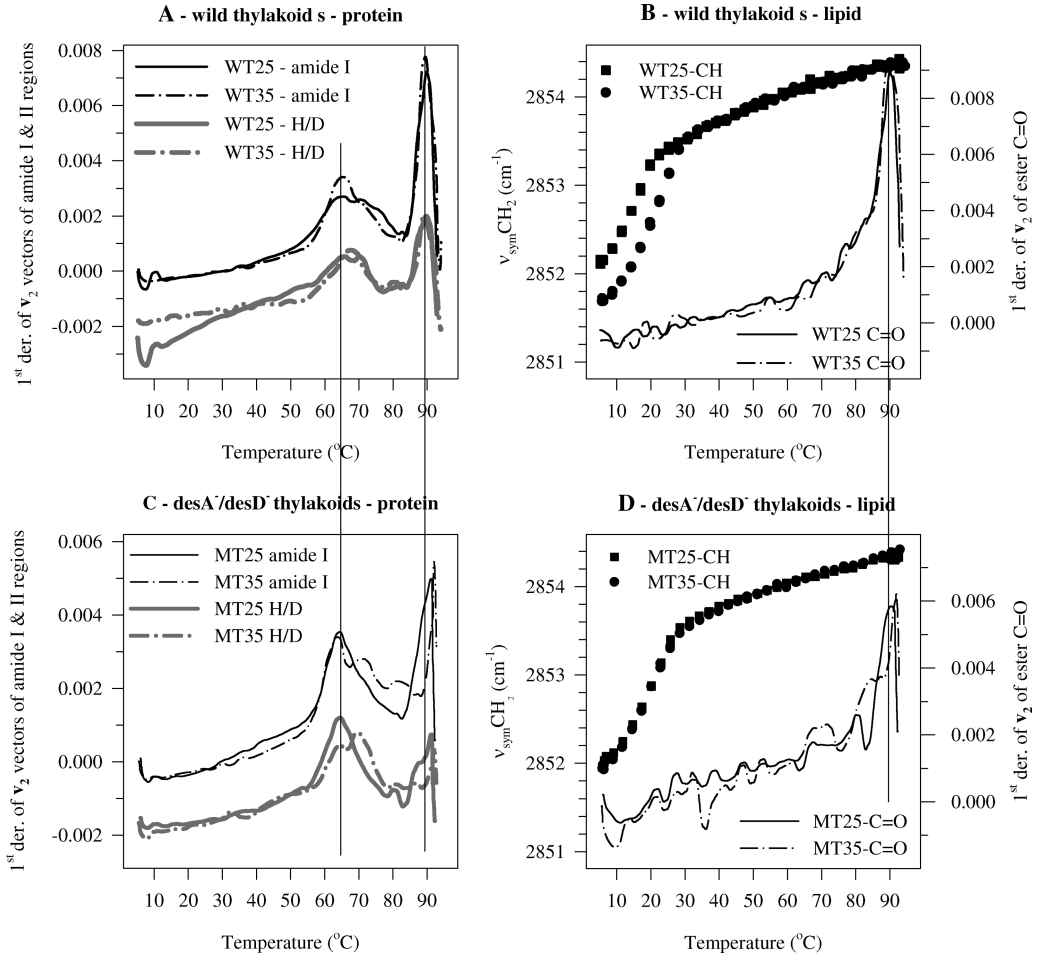

FIGURE 3: Singular value decomposition (SVD) analysis of the infrared spectra of thylakoid membranes prepared from wild-type (WT25, WT35) and *desA*$^-$/*desD*$^-$ mutant (MT25, MT35) *Synechocystis* PCC 6803 cells, grown at 25 and 35 °C. (A, C) Protein changes. The first derivatives of the $\mathbf{v_2}$ vectors indicate the rates of protein structural changes in the amide I and the rates of the H/D exchange in the amide II regions, respectively. (B, D) Lipid changes. Dots represent conventional curve-fitting analysis of the $\nu_{sym}CH_2$ band: higher frequencies indicate higher fatty acyl chain disorder. The continuous curves are the first derivatives of the $\mathbf{v_2}$ vectors of the ester C=O region; thus they represent the rates of the changes at/close to the membrane−water interface. (In panels A and D, the curves of the H/D exchange rates were displaced for clarity.)

The three $\mathbf{s_2}$ spectra reflect the following changes upon increasing temperatures: (i) the intensity of the lower frequency component of the ester C=O band decreases around 1727 cm^{-1}, while that of the higher frequency component around 1742 cm^{-1} increases; (ii) two new bands appear in the amide I region, around 1619 and 1685 cm^{-1}, respectively, at the cost of the α-helix and partly of the β-sheet content, indicating protein denaturing; (iii) the amide II band disappears due to H/D exchange.

In Figure 3A, the first derivative of $\mathbf{v_2}$ (the rate of the changes) is shown for the amide I and II regions of wild-type thylakoids prepared from cells grown at 25 or 35 °C (WT25, WT35). Note that protein denaturing takes place in two steps, around 65 and 87−90 °C, respectively. The rates of the H/D exchange (i.e., the rate of the disappearance of the amide II band around 1550 cm^{-1}) change together with the structural rearrangements of the proteins. There is no difference between the temperature dependence of the protein-related events in the WT25 and WT35 thylakoids. This is surprising, since these membranes have different lipid compositions exhibiting different thermotropic responses of the $\nu_{sym}CH_2$ band at low temperatures (Figure 3B). WT35 has its gel-to-liquid crystalline phase transition at $T_m = 21 \pm 0.5$. In WT25, the phase transition is at a considerably lower temperature ($T_m = 15 \pm 0.5$) due to the higher amount of polyunsaturated fatty acyl chains produced by the desaturase enzymes (*32*). (These data are valid only for the depicted curves; the averages and the standard deviations for all samples are given in Table 1.)

In the mutant thylakoid membranes, the protein behavior is the same as in the wild-type thylakoids (Figure 3A,C). Since the *desA*$^-$/*desD*$^-$ desaturase-deficient mutants contain only monounsaturated fatty acids, they have little room for changing the level of unsaturation of their membranes upon temperature adaptation. This limitation is well reflected by the practically identical thermotropic responses of the $\nu_{sym}CH_2$ frequencies in MT25 ($T_m = 20 \pm 0.5$ °C) and in MT35 ($T_m = 19 \pm 0.5$ °C) (Figure 3D). Due to the low level of lipid unsaturation, MT25 and MT35 exhibit similar phase transition temperatures as the "naturally saturated" WT35 thylakoid (Table 1).

The ester C=O band (close to water−membrane interface) does not show any major change in the low-temperature region (Figure 3B,D), where the frequency of the $\nu_{sym}CH_2$ band (reflecting in-membrane disorder) has upshifted significantly. But at high temperatures (87−90 °C), in correlation with the second protein structural transition, the ester C=O band is exhibiting a major change. That may mean that while changes in lipid−protein interactions caused by heat denaturing of proteins (Figure 3A,C) are not affecting significantly the in-membrane fatty acyl chain disorder, they considerably change the water−membrane interfacial region (Figure 3B,D). The fact that

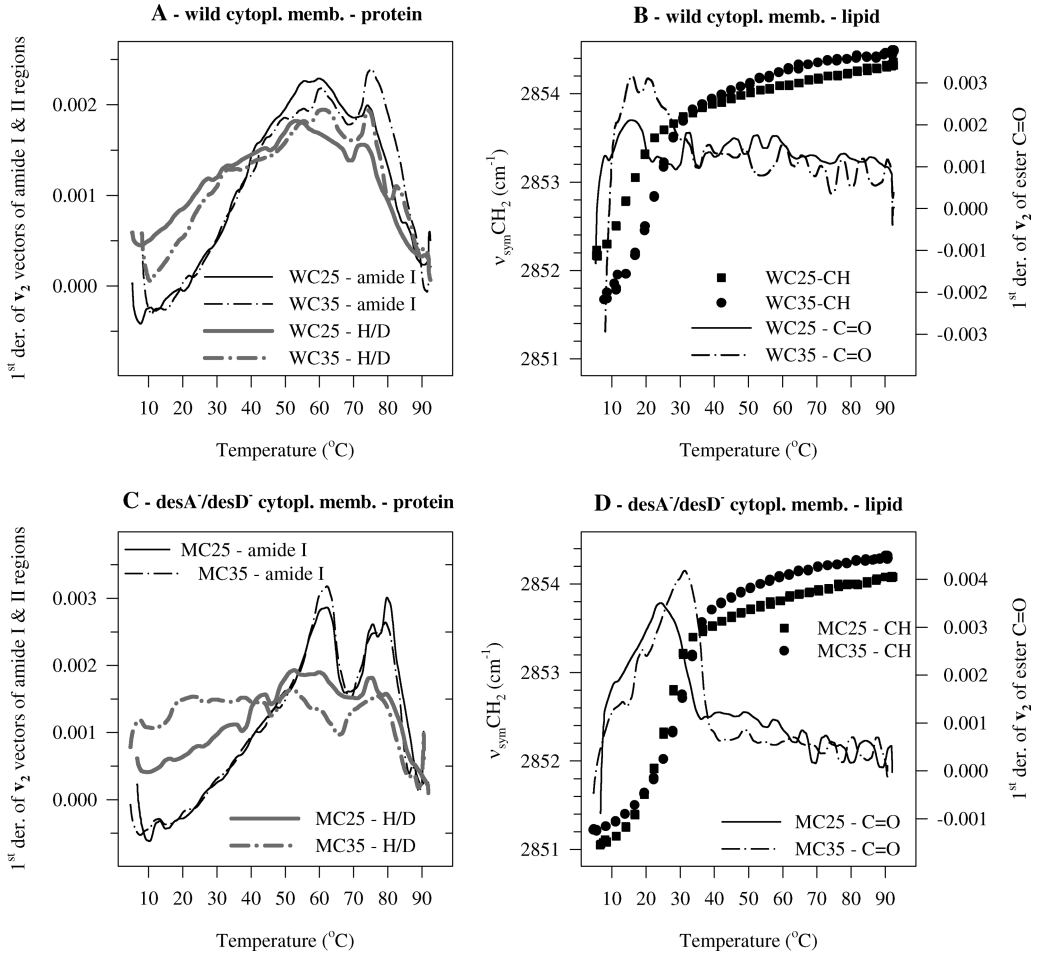

FIGURE 4: Singular value decomposition (SVD) analysis of the infrared spectra of cytoplasmic membranes prepared from wild-type (WC25, WC35) and *desA*$^-$/*desD*$^-$ mutant (MC25, MC35) *Synechocystis* PCC 6803 cells, grown at 25 and 35 °C. (A, C) Protein changes. The first derivatives of the ν_2 vectors indicate the rates of protein structural changes in the amide I and the rates of the H/D exchange in the amide II regions, respectively. (B, D) Lipid changes. Dots represent conventional curve-fitting analysis of the $\nu_{sym}CH_2$ band: higher frequencies indicate higher fatty acyl chain disorder. The continuous curves are the first derivatives of the ν_2 vectors of the ester C=O region; thus they represent the rates of the changes at/close to the membrane−water interface.

the first structural transition of the proteins (60−65 °C) does not correlate with any lipid-related parameter may indicate that this transition involves mostly self-movements of the proteins.

(*B*) *Cytoplasmic Membranes*. In wild-type cytoplasmic membranes (Figure 4A), instead of the two distinct transitions, one broad feature can be seen, which, nevertheless, is keeping some analogy with the thylakoids: It can be interpreted as a first transition at somewhat lower temperature, ~60 °C (versus ~65 °C in thylakoids), and as a second, overlapping transition around 75−80 °C (versus 87−90 °C in thylakoids). The separation of the two transitions is more evident in the *desA*$^-$/*desD*$^-$ cytoplasmic membranes (Figure 4C).

There is a major difference between thylakoids and cytoplasmic membranes in the temperature dependence of the events taking place in the water−lipid interfacial region. In contrast to thylakoids, the rates of the ester C=O band change largely upon the gel-to-liquid crystalline phase transition of the fatty acyl chains in the low-temperature regime (Figure 4B,D) and remain rather constant at high temperatures. Thus, in the cytoplasmic membranes, there is no correlation between changes of the ester C=O band and the protein structure at high temperatures (Figure 4B,D) as seen in thylakoids.

The temperature dependence of protein denaturing and dynamics does not correlate with the growth temperature either in the wild-type or in the *desA*$^-$/*desD*$^-$ cytoplasmic membranes. The thermotropic responses of the lipids, however, in the same way as in thylakoids, exhibit growth temperature sensitivity in the wild type and insensitivity in the *desA*$^-$/*desD*$^-$ citoplasmic membranes (Table 1).

DISCUSSION

Fourier transform infrared spectroscopy can provide simultaneous information about proteins and lipids since the characteristic regions of these components are well separated in the infrared spectrum (1700−1500 cm^{-1} for the proteins; 3050−2800 and 1740−1720 cm^{-1} for the lipids). By recording infrared spectra upon increasing temperatures, both the individual characteristics and the interactions of the membrane constituents can be determined. For the analysis of the temperature dependence of the changes in the above regions, the method of singular value decomposition (SVD) was applied.

We were interested to learn whether lipids/lipid desaturation or proteins are dominant in maintaining membrane dynamics among physiological conditions and under low/high-temperature stress.

Our system, based on a cyanobacterium, *Synechocystis* PCC 6803, made possible to study the effect of lipid unsaturation in

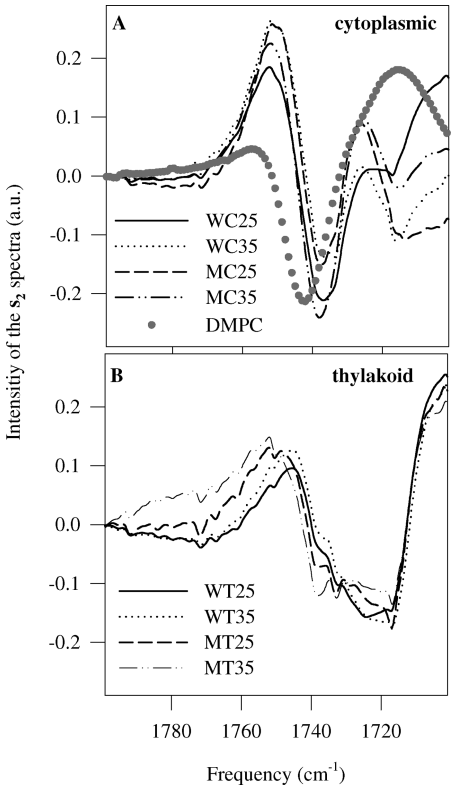

FIGURE 5: SVD analysis of the 1800−1700 cm^{-1} region (ester C═O stretch, lipid-related). (A) $\mathbf{s_2}$ spectra of cytoplasmic membranes and a model lipid, dimyristoylphosphatidylcholine (DMPC). (B) $\mathbf{s_2}$ spectra of thylakoid membranes.

otherwise largely identical organisms. The wild-type *Synechocystis* can produce polyunsaturated lipids to enable sufficient membrane lipid dynamics (fluidity/microviscosity) at temperatures as low as 22 °C (*32*). In contrast, the desaturase enzyme deficient mutant (*desA*$^-$/*desD*$^-$) of *Synechocystis* PCC 6803 can contain only monounsaturated lipids and has serious difficulties to grow already at 25 °C (*12*, *33*). In the present work, we have compared the thylakoid and the cytoplasmic membranes of both wild-type and *desA*$^-$/*desD*$^-$ mutant cells, grown at 25 or 35 °C.

Lipids. The thermotropic responses of the $\nu_{sym}CH_2$ frequencies for wild-type cells nicely reproduced the earlier results (*12*). Supporting the homeoviscous adaptation principle, the absolute level of the lipid disorder, as reflected by the $\nu_{sym}CH_2$ frequency, was practically the same at 25 and 35 °C in the membranes of the cells grown at respective temperatures (Figures 3B and 4B). Due to the cold-induced lipid desaturation, T_m's of the gel-to-liquid crystalline phase transition were lower in the membranes of the cells grown at 25 °C than in those grown at 35 °C (Figures 3B and 4B). In the *desA*$^-$/*desD*$^-$ mutant cells, this adaptation was blocked (Figures 3D and 4D, Table 1).

In the view of lipid adaptation, it is interesting that changes in the structure/dynamics of the proteins show no correlation with the growth temperature either in the wild-type or in the mutant cells (Figures 3A,C and 4A,C). From that, one may conclude that the self-motions/stability of the proteins is more decisive in the "fate" of proteins than the interactions with the neighboring lipids.

In thylakoids, the large-scale change of the ester C═O band appears together with the final, high-temperature protein structural change (Figure 3B,D). In cytoplasmic membranes, it changes during the gel-to-liquid crystalline phase transition of the lipids at low temperatures (Figure 4B,D). This latter behavior would be expected in a pure lipid system as well. Indeed, the $\mathbf{s_2}$ basis spectra of the ester C═O stretching region of cytoplasmic membranes and of dimyristoylphosphatidylcholine (DMPC) multibilayers are similar to each other (Figure 5A) but not to the $\mathbf{s_2}$ spectra of thylakoids (Figure 5B). The very different ester C═O $\mathbf{s_2}$ spectra found for the heat-induced disintegration of thylakoids can be considered as a sign that they do not represent an ordered → disordered lipid phase transition in that sense, as it happens in a model lipid system or in the cytoplasmic membranes.

May one extrapolate from this result that in thylakoid membranes, before the heat-induced segregation of lipids and proteins, there is no extended lipid bilayer matrix in such a form, as it exists in lipid multibilayers? In cytoplasmic membranes, which have much higher lipid/protein ratios (compare Figure 1D to Figure 1B), the lipid amount should be enough for forming extended lipid-bilayer domains in the membranes. We are aware that a firm conclusion in this question needs further studies; the present results are only indications for a possible origin of the differences.

In cytoplasmic membranes, the lipid/protein ratio is higher than in the thylakoids. See the higher relative intensities of the ester C═O bands (around 1740 cm^{-1}) versus the amide I bands (around 1650 cm^{-1}) in Figure 1B,D. The higher relative amount of lipids is more evident in the *desA*$^-$/*desD*$^-$ cytoplasmic membranes; it is the highest in the MC25 membrane. This finding can be rationalized in the following way: If, due to the *desA*$^-$/*desD*$^-$ mutation, the unsaturated lipids, optimized for protein−lipid interactions at low growth temperature, are not available, the lipid−protein interactions can be crucial. The greater the discrepancy between the needed and the available lipids, the more "poorly sealing" lipid necessary to maintain membrane functionality/integrity: This is reflected by the highest ester C═O/amide I intensity ratio found for the MC25 membrane (Figure 1D).

Proteins. Heat denaturing of the proteins takes place in two steps in both types of membranes, only that the second step is at considerably higher temperature in thylakoids (Figures 3A,C and 4A,C). For these two steps, our tentative explanation is as follows: The first step, around 60−65 °C, is due to direct H/D exchange between the protein and the surrounding D_2O and involves some irreversible protein structural change as well. The second step, around 75−90 °C, should be the final denaturing involving protein aggregation and the dissolution of the membrane structure.

This idea is supported also by the two-step heat denaturing of lysozyme, when dissolved in D_2O (*36*). In lysozyme, the first step (around 50 °C) is the H/D exchange, but in this water-soluble protein the structural oscillations, which accompany the H/D exchange, are small, transient, and fully reversible. This is not so in the case of our membrane proteins since in the membranes the lipids can immediately counteract even the smallest protein movement; thus the membrane proteins cannot find back to their original structures after any single H/D exchange event; the small irreversible steps are accumulating into a visible structural change. In lysozyme, the second step is the irreversible thermal denaturing at 65−70 °C. This value correlates well with that found for the protein in the cytoplasmic membranes.

The first structural transition of the proteins is only at slightly lower temperature in cytoplasmic membranes (∼60 °C) than in thylakoids (∼65 °C), in agreement with the hypothesis that they

are connected to direct H/D exchange. The difference is larger in the case of the second, high-temperature transition: 75−80 °C in cytoplasmic membranes versus 87−90 °C in thylakoids. This difference can be accounted for by the different protein content/organization of the cytoplasmic and the thylakoid membranes. In thylakoids, the protein-to-lipid ratio is higher, and the proteins are organized into larger protein complexes, in which more, relatively strong, polar, electrostatic, etc. interprotein connections can be formed. In cytoplasmic membranes, not only the amount of the proteins is less but also the protein complexes are smaller (*37*). Therefore, there is less strong interprotein interaction, and more, weak, hydrophobic lipid−protein interaction in the cytoplasmic membranes, resulting in less stable protein complexes as shown by the lower temperatures of the final denaturing. Concerning denaturing of their proteins, other membranes looked upon so far, yeast vacuole, rat liver mitochondria (data not shown), were similar to the cytoplasmic membranes.

Stress Alarms. With regard to the role of lipid and protein dynamics under low-temperature stress (15−22 °C), at physiological temperatures (22−39 °C), and under high-temperature stress (40−45 °C), we may conclude the following: At the low end of the temperature range where the cells can still survive, the proper membrane dynamics is maintained by regulating the level of lipid desaturation (at least in these cyanobacteria). Lipid fatty acyl chains, which exhibit large and steep increase in their disorder during the gel-to-liquid crystalline phase transition, produce only a small and smooth further increase in their dynamics in the physiological temperature range (∼25−40 °C) and above (Figures 3B,D and 4B,D). In contrast, protein dynamics is little affected by the lipid phase transition; it remains rather constant up to the upper physiological temperature limit. However, in the high-temperature stress range and above, both the dynamics and the structure of the proteins are changing rapidly (see the H/D curves in Figures 3A,C and 4A,C). Thus, we think that proteins or protein-related changes might be responsible for alarming and initializing heat-shock responses, and these are the features which should be kept under control by the stress response of the cells.

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