Fluidity Changes in Thylakoid Membranes of Durum Wheat Induced by Oxidative Stress: A Spin Probe EPR Study

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Electron paramagnetic resonance (EPR) spectroscopy was applied to the study of fluidity in thylakoids of durum wheat (*Triticum durum* Desf. cv. Creso) treated with oxygen free radicals. The radicals were generated by using Fe^{2+}−EDTA and ascorbic acid and let to act either on thylakoid membranes isolated from leaves or on leaves themselves, from which thylakoids were subsequently extracted. The oxidative treatment was prolonged for periods ranging from 15 min to 3 h. Line shape analysis of EPR spectra recorded as a function of temperature on concentrated suspensions of thylakoids labeled with 5-doxylstearic acid (5-DSA) allowed to get information about the fluidity of differently treated membranes. An axially symmetric rotational diffusion model, defined by the components of the rotational tensor D_{par} and D_{perp} and by the diffusion tilt angle θ, was assumed for the dynamics of the spin probes. Good agreement with experimental spectra was obtained with a diffusion tilt angle continuously increasing with temperature and a high anisotropy ratio $N = D_{par}/D_{perp}$ of the spin probe rotational diffusion. High N values have been considered as an indication for the immobilization of the spin probes in the hydrophobic part of the membranes. The changes of θ with temperature have been interpreted in terms of chain isomerization within the probes. The differences found between the probe dynamics in the differently treated thylakoids have been discussed on the basis of the time and type (on the leaves or on the membranes) of oxidative treatment.

Introduction

In thylakoids, membrane lipids play an important role in regulating the spatial organization of intrinsic protein−pigment complexes involved in the electron transport and, in turn, the light reactions of photosynthesis.[1−3] Membrane fluidity is determined mainly by the length and the degree of unsaturation of alkyl chains in fatty acids of the constituent lipids and by the protein/lipid ratio.[4]

In response to several environmental stresses, such as freezing, dehydration and drought, the adaptation shown by many plants is related to changes in membrane composition and phase transitions, which optimize the fluidity.[5−10] In particular, in previous studies exposure of thylakoid membranes of durum wheat (*Triticum durum*) to various stresses resulted in changes of lipid compositions, lipid-to-protein ratios,[11] membrane fluidity,[12] and superoxide radical production.[13−15] Different responses were observed in thylakoids of differently sensitive cultivars.[11] The same qualitative changes were observed after exposing isolated membranes to systems generating oxygen free radicals in vitro.[15−18] These findings support the statement that several environmental stresses increase the production of oxygen free radicals, which mediate the degradation of membrane components.[19]

In this study, we employ the free radical generating system Fe^{2+}−EDTA and ascorbate[16,20,21] to investigate the effects of oxidative stress ($O_2^{\cdot-}$ and $HO^{\cdot}$ radicals are produced) on membrane fluidity of thylakoids of *T. durum* Desf. cv. Creso. The treatment is applied for periods ranging from 15 min to 3 h either to thylakoid membranes isolated from leaves or to leaves themselves, from which thylakoids are subsequently extracted. In fact a different response can be expected for leaves with respect to isolated thylakoids because of both the different penetration of reactive oxygen species in the two systems and the loss of water-soluble antioxidants in the extraction of thylakoids.

The changes in membrane fluidity are detected by EPR spectroscopy on concentrated suspensions of thylakoids using 5-doxylstearic acid (5-DSA)[12,22−24] as a spin probe in the temperature range between 0 and 70 °C. In 5-DSA, the doxyl (4,4-dimethyl-*N*-oxy-oxazolidinyl) group is located at the 5th carbon atom of the fatty acid chain, so that it probes the fluidity of the membrane regions close to the polar lipid headgroups,[25] which control the penetration of water soluble activated oxygen species. An accurate line shape analysis of the spectra on the basis of Freed's theory of slow motional EPR[26,27] is performed in order to quantitatively determine the kinetic parameters for the dynamics of the spin probes inside the different samples. The results are discussed in relation to the time and type (on the membranes or on the leaves) of the oxidative treatment.

Experimental Section

Materials. Wheat seedlings (*Triticum durum* Desf. cv. Creso) were grown for 15 days under well watered conditions in a controlled environment at a day/night temperature regime of 20/15 °C, with a photoperiod of 16 h and a photon flux density of 400 $\mu Em^{-2}s^{-1}$, and 80% relative humidity. All reagents, of analytical grade, were purchased from Aldrich or Carlo Erba.

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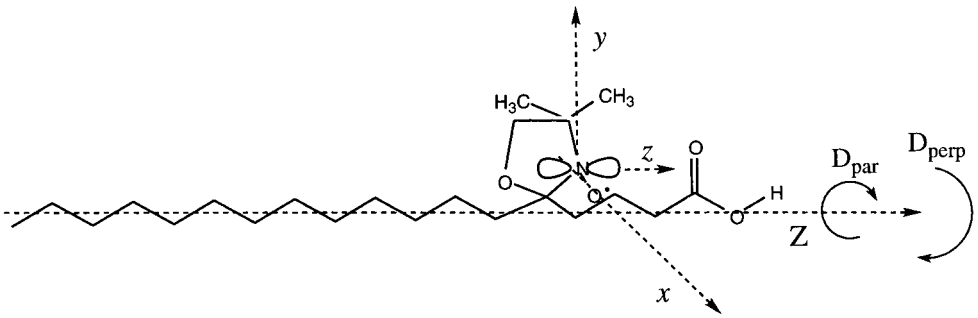

Figure 1. Molecular structure of 5-DSA spin probe and location of axis systems.

Treatment of Leaves with Oxygen Free Radicals. Leaves were treated with oxygen free radicals generated by using the Fe^{2+}−EDTA chelate and ascorbic acid. Leaf strips (20 g) of 1 cm in length were incubated and shaken continuously at room temperature in 1 L of 10 mM phosphate buffer (pH 6.8) containing 2 mM ascorbic acid. The reaction was started by addition of 1 mM Fe^{2+}−EDTA. The treatment was performed in the light (photon flux density 11 μmol m^{-2} s^{-1}) and prolonged for 15 min, 30 min, 60 min, 2 h, or 3 h. Then leaf strips were sampled and carefully washed with distilled water. Control strips were incubated for 60 min in the same conditions, but without adding Fe^{2+}−EDTA.

Isolation of Thylakoid Membranes. Leaf strips were homogenized in an isolation medium (1:10 w/v) containing 0.33 M sucrose, 50 mM MES−NaOH (pH 6.6), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% bovine serum albumin and 5 mM ascorbic acid. The homogenate was filtered and then centrifuged at 200 *g* for 5 min, after which the supernatant was again centrifuged at 3000 *g* for 10 min. The resulting chloroplast pellet was suspended with a hypotonic buffer containing 10 mM MES−NaOH (pH 6.6), 10 mM NaCl, and 2 mM $MgCl_2$, and centrifuged at 12000 *g* for 10 min. The pelletted thylakoid membranes were suspended in 50 mM MES−NaOH (pH 6.6) and 0.33 M sucrose. All of the isolation procedures were carried out at 4 °C. The chlorophyll content was determined spectrophotometrically after addition of 80% aqueous acetone containing 2.5 mM sodium phosphate buffer (pH 7.8) and centrifugation at 35000 *g* for 10 min.[28]

Treatment of Thylakoid Membranes with Oxygen Free Radicals. Thylakoid membranes (0.3 mg Chl) were suspended in 500 μL of MOPS−KOH buffer (pH 7.5) and 250 μL of 8 mM ascorbic acid. Oxygen free radicals were generated by adding 250 μL of 40 mM Fe^{2+}−EDTA. The treatment was performed in the light (photon flux density 11 μmol m^{-2} s^{-1}) and prolonged for 15 min, 30 min, 60 min, 2 h, or 3 h. The resulting suspensions were centrifuged at 27000 *g* for 10 min. The pelletted thylakoid membranes were resuspended in 50 mM MOPS−KOH (pH 7.5) and centrifuged at 27000 *g* for 10 min.

Spin Labeling of Thylakoid Membranes and EPR Measurements. Samples of thylakoids were resuspended in 10 mM tricine−NaOH buffer (pH 6.5) containing 100 mM NaCl and then added to a glass tube with a film of 5-DSA (Sigma Chemicals, München, Germany). The applied concentration of spin probes was 1.5 μmol mg^{-1} chlorophyll. After 20 min of incubation at room temperature under nitrogen atmosphere, thylakoids were washed three times in 10 mM tricine−NaOH (pH 6.5) by centrifugation at 35000 *g* for 10 min. Free spin probes were not detected in the supernatant following the third washing. For EPR analysis, the concentrated membrane suspension was taken up into a 100 μL glass capillary, which was sealed and inserted into a quartz sample holder.

Spectra were recorded in a temperature range of 0 to 70 °C using a Varian E112 spectrometer (X-band) equipped with a Varian E257 temperature control unit. The spectrometer was interfaced to a 100-MHz personal computer by means of a homemade data acquisition system, consisting of an acquisition board[29] and a software package designed especially for EPR and ENDOR experiments.[30]

Spectra were recorded every 5 °C on temperature raising, waiting 15 min for equilibrating the temperature. For each spectrum, a field setting of 3265 G, a microwave power setting of 10 mW, and a modulation amplitude of 1.25 G were employed.

Dynamical Model and Magnetic Parameters of the Spin Probe. The rotational diffusion of doxylstearic acid spin probes was studied on the basis of an axially symmetric Brownian rotational diffusion model. The dynamics of the probe is described by the components of the rotational tensor D_{par} and D_{perp} along axes parallel and perpendicular, respectively, to the symmetry axis rotational diffusion tensor and by the diffusion tilt angle θ, which is the angle between the rotational diffusion symmetry axis (*Z*) and the *z* axis of the nitroxide axis system (*z* is the axis of the nitrogen $2p_z$ atomic orbital and *x* is along the N−O bond, see Figure 1). For each spectrum, the three parameters D_{par}, D_{perp}, and θ were determined by fitting the line shapes of the slow motional experimental EPR spectra with spectra calculated using the Slow-Motional EPR Line shape Calculation Program EPRLL, PC Version for Windows NT.[31] This program is included in an updated version of the Schneider−Freed set of programs[27] based on the stochastic Liouville equation that describes the time evolution of the macroscopic magnetization. Gaussian line shapes were assumed. Line widths ranged from 0.5 G at high temperatures to 2.5 G at low temperatures.

The first step in the fitting procedure is the proper choice of the principal components of the **A** and **g** tensors. Since these values cannot be measured from the low-temperature EPR spectra, because of the very broad lines, they have been refined starting from **A** and **g** components reported in the literature for 5-DSA in lipid bilayers.[22,32] The tensor components $\mathbf{A}_{xx} = 5.9$ G, $\mathbf{A}_{yy} = 5.4$ G, $\mathbf{A}_{zz} = 33.5$ G and $\mathbf{g}_{xx} = 2.0087$, $\mathbf{g}_{yy} = 2.0067$, $\mathbf{g}_{zz} = 2.0024$ have been determined following this procedure and used in the calculations of all EPR spectra.

Results and Discussion

Description of Spectra. EPR spectra of 5-DSA spin probes, inserted in differently treated thylakoid membranes (i.e., membranes incubated with the free radical generating system ascorbic acid and Fe^{2+}−EDTA for time periods of 15 min, 30 min, 60 min, 2 h, and 3 h or extracted from leaves incubated in the same conditions) and in untreated thylakoid membranes (i.e.,

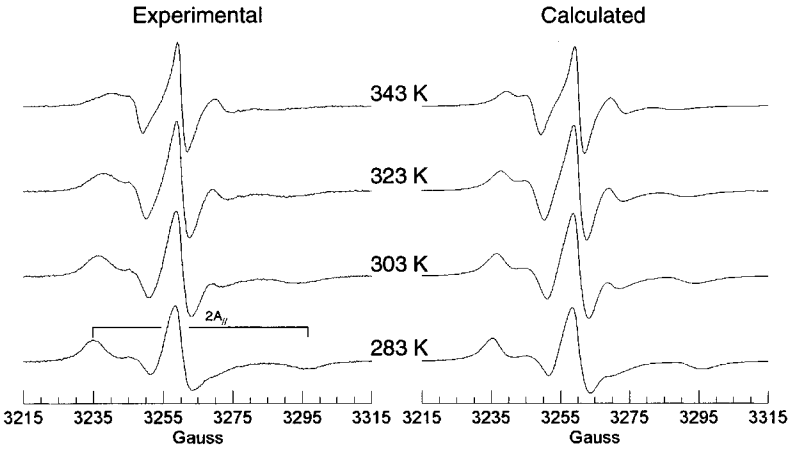

Figure 2. Experimental (left) and calculated (right) EPR spectra of 5-DSA in membranes extracted from untreated leaves of *Triticum durum* Desf. cv. Creso at the indicated temperatures.

membranes incubated for 60 min with ascorbic acid or extracted from leaves incubated for 60 min in the same conditions, indicated in the following as membrane control and leaf control, respectively) were recorded every 5 degrees on raising the temperature in the range 0−70 °C.

All samples show similar powder spectra with line shapes typical for an anisotropic rotation of the spin probe, as is generally observed in lipid bilayers.[22,33] As an example, a selection of EPR spectra obtained with 5-DSA probes in membranes extracted from leaf control is reported in Figure 2. A similar line shape evolution was observed for all samples as a function of temperature, with the maximal separation $2A_{||}$ and the line width becoming larger with decreasing the temperature (see Figure 3). The parameter $2A_{||}$, which can be considered as a measure of membrane fluidity, indicates that the motional freedom of the spin probe becomes more restricted at lower temperatures.

A direct comparison of spectra recorded on differently treated samples at the same temperature (see Figure 4) shows appreciable differences (experimental error in $2A_{||}$ measurements is ± 0.5 G). For instance, $2A_{||}$ values of 60.2, 59.5, 59.6, 57.2, and 57.8 G were measured in the spectra recorded at 25 °C on membranes treated with the Fe^{2+}−EDTA and ascorbate system for 15 min, 30 min, 60 min, 2 h, and 3 h, respectively, whereas $2A_{||}$ values of 58.6, 60.9, 60.5, 56.5, and 57.4 G were obtained from the spectra recorded at the same temperature on membranes extracted from leaves treated for 15 min, 30 min, 60 min, 2 h, and 3 h, respectively. The EPR spectra of 5-DSA in membrane and leaf controls at 25 °C have $2A_{||}$ values of 58.0 and 59.2 G, respectively. These results suggest that different membrane fluidity is associated with different oxidative treatments. In particular, the highest fluidity is detected for samples treated for 2 h, either on the leaves or on the membranes. In fact, an increase of fluidity is observed by increasing the incubation time of membranes with the Fe^{2+}−EDTA and ascorbate system from 15 min to 2 h, and a slight decrease when prolonging the incubation up to 3 h. A more complex behavior is shown by membranes extracted from treated leaves. Moreover, a lower fluidity of samples treated for 60 min is found with respect to the controls, notwithstanding the oxidative treatment was performed on leaves or on membranes. To quantitatively interpret the fluidity differences here sketched, the line shape analysis described in the following paragraph was performed on the EPR spectra.

Line Shape Analysis. The spectral line shapes were analyzed by applying a least-squares fitting procedure in which an axially symmetric Brownian rotational diffusion model for the molecular motions of the spin probes in the membranes was assumed.[27,32] As reported in the Experimental Section, two diffusion constants, D_{par} and D_{perp}, describe the reorientation of 5-DSA, whereas a diffusion tilt angle θ, which is the angle between the rotational diffusion symmetry axis and the z axis of the nitroxide axis system, is considered as a global parameter for the modeling of chain isomerization within the stearic acid chain. Being that the samples macroscopically unoriented, each spectrum results from the sum over all of the possible orientations of the rotational diffusion symmetry axis with respect to the magnetic field axis.

Satisfactory agreement between calculated and experimental spectra (see for example Figures 2 and 4) was obtained with a diffusion tilt angle continuously increasing with temperature and a high anisotropy ratio $N = D_{par}/D_{perp}$ of the spin probe rotational diffusion (N ranges from about 60 at high temperature to more than 1000 at low temperature). High N values are an indication of the immobilization of the spin probes in the hydrophobic part of the membranes where the doxyl group of 5-DSA is located.[25] The D_{perp} values are low ($\leq 5\ 10^6\ s^{-1}$), suggesting an environment that strongly hinders the rotation along directions perpendicular to the long axis of the spin probes. The increase of θ with increasing the temperature (see Figures 5c and 6c) can be interpreted in terms of a greater number of distortions in the alkyl chain of the spin probes, which becomes more and more different from the fully extended all-trans configuration characterized by $\theta = 0°$.

Diffusion coefficients were determined in the fitting program as $\mathrm{Log}D_{par}$ and $\mathrm{Log}D_{perp}$. Errors up to 0.3% were obtained for $\mathrm{Log}D_{par}$ values at all investigated temperatures, giving errors up to 5% on D_{par} parameters. Similarly, errors lower than 0.5% were found for $\mathrm{Log}D_{perp}$ values at temperatures between 30 and 70 °C, which correspond to errors up to 7% on D_{perp} coefficients. On the contrary, an accurate determination of D_{perp} values was not possible for some samples at temperatures between 0 and 30 °C because the rigid motional limit of the EPR technique was approached. In particular, for D_{perp} higher than $10^5\ s^{-1}$, the errors were up to 25%, whereas the procedure was poorly sensitive to lower D_{perp} values, for which often only an upper limit could be determined. Errors lower than 1% were found for θ values in all of the calculations. Correlation coefficients lower than 0.810 in absolute value were found between $\mathrm{Log}D_{par}$ and both $\mathrm{Log}D_{perp}$ and θ, whereas correlation coefficients

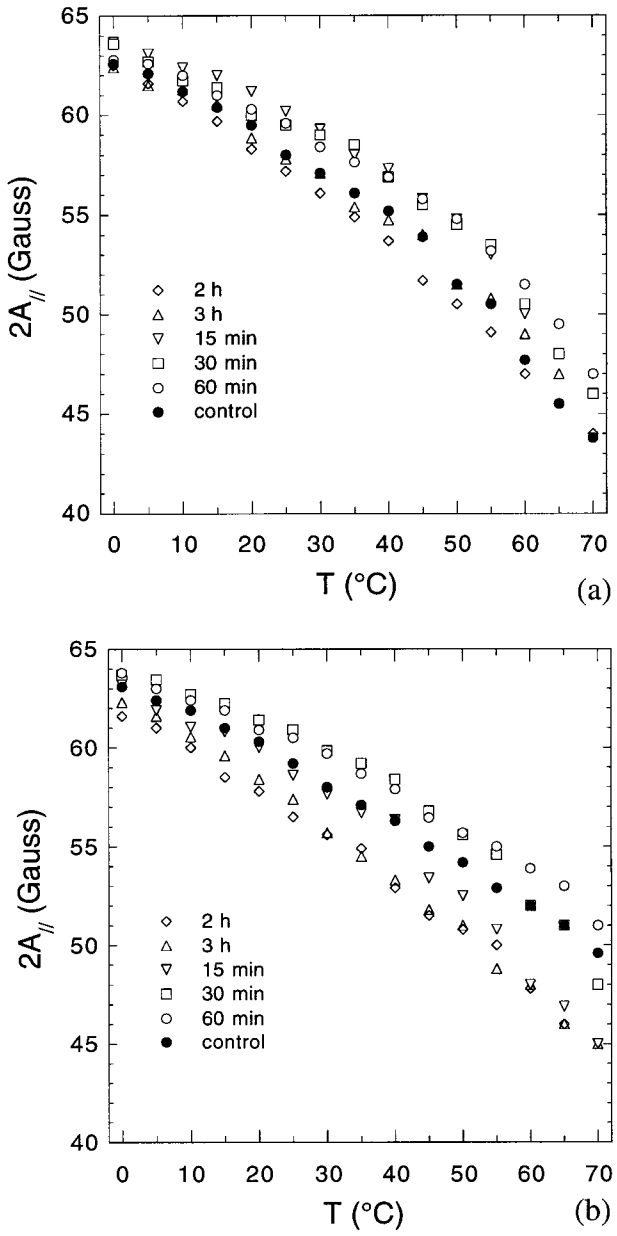

Figure 3. $2A_{||}$ measured in the experimental EPR spectra of 5-DSA in thylakoid membranes: (a) membranes of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}−EDTA/ascorbic acid for the indicated periods; (b) membranes extracted from leaves of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}−EDTA/ascorbic acid for the indicated periods.

between LogD_{perp} and θ reached values of −0.995 in few cases. These results indicate that the determination of the parameter D_{par} is substantially independent from that of the other fit parameters; on the other hand, an individual determination of the fit parameters D_{perp} and θ is hindered in some cases, probably because of a correlation between the chain segmental motions and the rotational diffusion of the spin probe long axis.

The calculated D_{par} values showed increasing trends with increasing the temperature for all samples examined (see Figures 5a and 6a), which could be described in terms of the Arrhenius equation $D_{par} = D_{par}^0 \exp(-E_a^{par}/RT)$ obtaining the activation energies reported in Table 1. On the other hand, no regularly increasing trends with increasing the temperature were observed for D_{perp} values (see Figures 5b and 6b). Two different Arrhenius equations of the type $D_{perp} = D_{perp}^0 \exp(-E_a^{perp}/RT)$ holding in two different temperature ranges (namely, 0−30 °C and 30−70 °C) were employed to describe these trends. The obtained activation energies are reported in Table 1.

TABLE 1: Activation Energies Determined Analyzing the Trends of Diffusion Coefficients D_{par} and D_{perp} as a Function of Temperature in Terms of the Arrhenius Equation

treatment time	E_a^{par} (kJ/mol)	E_a^{perp} (kJ/mol)[a]	E_a^{perp} (kJ/mol)[b]
samples treated on membranes			
15 min	13.52 ± 0.29	33.27 ± 4.07	126.70 ± 18.23
30 min	12.53 ± 0.24	26.82 ± 3.65	91.06 ± 10.60
60 min	11.41 ± 0.20	25.77 ± 3.24	158.79 ± 11.11
2 h	14.02 ± 0.26	3.23 ± 2.29	50.52 ± 7.92
3 h	14.08 ± 0.39	13.53 ± 2.58	40.28 ± 4.44
samples treated on leaves			
15 min	12.65 ± 0.23	22.51 ± 2.76	97.96 ± 4.95
30 min	13.95 ± 0.28	34.82 ± 4.27	112.62 ± 11.64
60 min	10.04 ± 0.20	41.83 ± 2.48	*c*
2 h	9.93 ± 0.34	4.10 ± 1.76	38.29 ± 4.65
3 h	12.40 ± 0.21	4.47 ± 1.40	55.00 ± 4.30
control samples			
on membranes	12.77 ± 0.23	14.15 ± 2.94	62.54 ± 6.37
on leaves	11.35 ± 0.19	19.44 ± 1.56	102.90 ± 23.11

[a] Determined from D_{perp} values in the temperature range between 30 and 70 °C. [b] Determined from D_{perp} values in the temperature range between 0 and 30 °C. [c] Not determined because of the lack of D_{perp} values in this temperature range.

Activation energy values between 10 and 14 kJ/mol were determined for the rotational diffusion of the spin probe around its long axis in all examined samples, whereas a higher variability was found for the activation energy associated with the rotational diffusion of the long axis itself. In fact, E_a^{perp} values ranging from about 3 to 42 kJ/mol were obtained between 30 °C and 70 °C, and from about 38 to 127 kJ/mol between 0 °C and 30 °C, depending on the sample. The latter values are affected by higher errors, due to the poor determination of D_{perp} coefficients at low temperatures, and, in the case of thylakoid membranes extracted from wheat leaves treated for 60 min, the availability of only two D_{perp} values in the 0−30 °C temperature range prevented the determination of E_a^{perp}. Notwithstanding the uncertainty affecting the E_a^{perp} values, we can state that for all samples a marked increase in the sensitivity of the reorientation motion of the long molecular axis of 5-DSA, governed by D_{perp}, at temperatures between 0 and 30 °C with respect to higher temperatures is found.

Effects of Oxidative Stress on Membrane Fluidity. The results of the line shape analysis indicated an analogous rotational dynamics of 5-DSA in all investigated samples, i.e., a highly anisotropic rotational diffusion in an environment that strongly hinders the rotation along directions perpendicular to the long axis of the spin probes. The combination of rotational parameters leads to a higher anisotropy at lower temperatures, associated to a location of the probe in a tighter environment. Moreover, a greater temperature dependence of the rotation along directions perpendicular to the long axis of the spin probes was found below 30 °C (see Figures 5b and 6b), which can be associated to a change in the lipid structure of thylakoid membranes. Finally, an increase of θ with increasing the temperature (see Figures 5c and 6c) was obtained for all samples, which can be interpreted in terms of a greater number of distortions in the alkyl chain of the spin probes.

Besides the above-reported analogies, significant differences in the simulation parameters for the probe dynamics at the same temperature were detected in the differently treated thylakoids (see Figures 5 and 6), which were associated to differences in the activation energies E_a^{par} and E_a^{perp} (see Table 1). As far as the directly treated thylakoid membranes are concerned, the

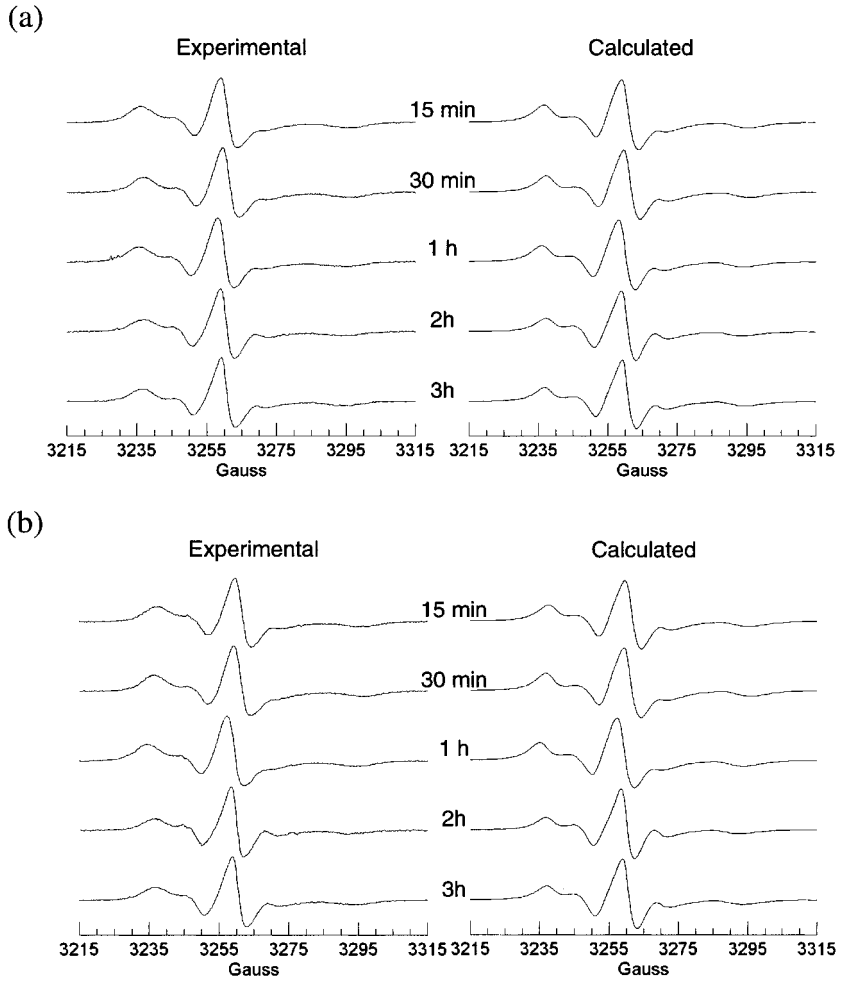

Figure 4. Experimental (left) and calculated (right) EPR spectra of 5-DSA in thylakoid membranes at 25 °C: (a) membranes of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}−EDTA/ascorbic acid for the indicated periods; (b) membranes extracted from leaves of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}−EDTA/ascorbic acid for the indicated periods.

maximum rigidity was found for the 15 min treatment (see Figure 5 and Table 1), and an increasing rotational mobility was observed by prolonging the time of membrane incubation in the Fe^{2+}−EDTA and ascorbate system (see Figure 5). In particular, small increments of both D_{par} and D_{perp} were detected when the incubation time rose from 15 to 60 min. When we prolonged the treatment period up to 2 h and then to 3 h, we observed first an increase and then a decrease of D_{par} (see Figure 5a). A higher mobility along directions perpendicular to the long axis of the spin probes was also found for membranes treated for 2 and 3 h, as revealed by the higher D_{perp} values determined at low temperature (see Figure 5b) and, especially, by the sensibly lower E_a^{perp} values (see Table 1), which indicate a less hindered rotational motion.

A higher rotational mobility was also found for thylakoid membranes extracted from leaves treated for periods of 2 and 3 h with respect to those extracted from leaves treated for times up to 60 min, the lowest fluidity being observed for treatment periods of 30−60 min (see Figure 6 and Table 1). In fact, both D_{par} and D_{perp} values and E_a^{perp} values suggest a decrease in the fluidity when the treatment period is increased from 15 to 60 min (see Figure 6 and Table 1).

When compared with the respective controls (i.e., membrane or leaf samples incubated with ascorbic acid for 60 min), both membranes directly treated with the free radical generating system for 60 min and membranes extracted from leaves treated for 60 min show a decrease of the fluidity of the lipid environment surrounding the probe. If we assume that the controls are valid for samples treated for periods different from 60 min, as previously observed,[34] oxidative treatments up to 60 min bring to membranes more rigid than the control, while a fluidity higher than that of the control is observed for treatments of 2 and 3 h (see Figure 6 and Table 1).

On the basis of these findings, the first response of membranes to oxidative treatments seems to be a decrease of fluidity in the peripheral region of the hydrophobic core close to lipid headgroup zone, as previously observed for thylakoid membranes from plants grown in excess copper.[12] This change in membrane fluidity can be related to a decrease in lipid content in the membrane, with a consequent increase of the protein-to-lipid ratio, which was observed by some of the authors[15] for thylakoids extracted from wheat (*T. durum* Desf. cv. Ofanto) leaves treated with Fe^{2+}−EDTA and ascorbate for 30 min.

The initial fluidity decrease can be regarded as a protection against the penetration of oxygen free radicals into the membrane hydrophobic interior where the majority of the fatty-acid double bonds, which are a target for the destructive action of these species, are located. However, when the oxidative treatment is performed for longer periods, the attacks of free radicals to membrane lipids result in a decreased packing of the lipid bilayer and then in a lower membrane microviscosity. A similar

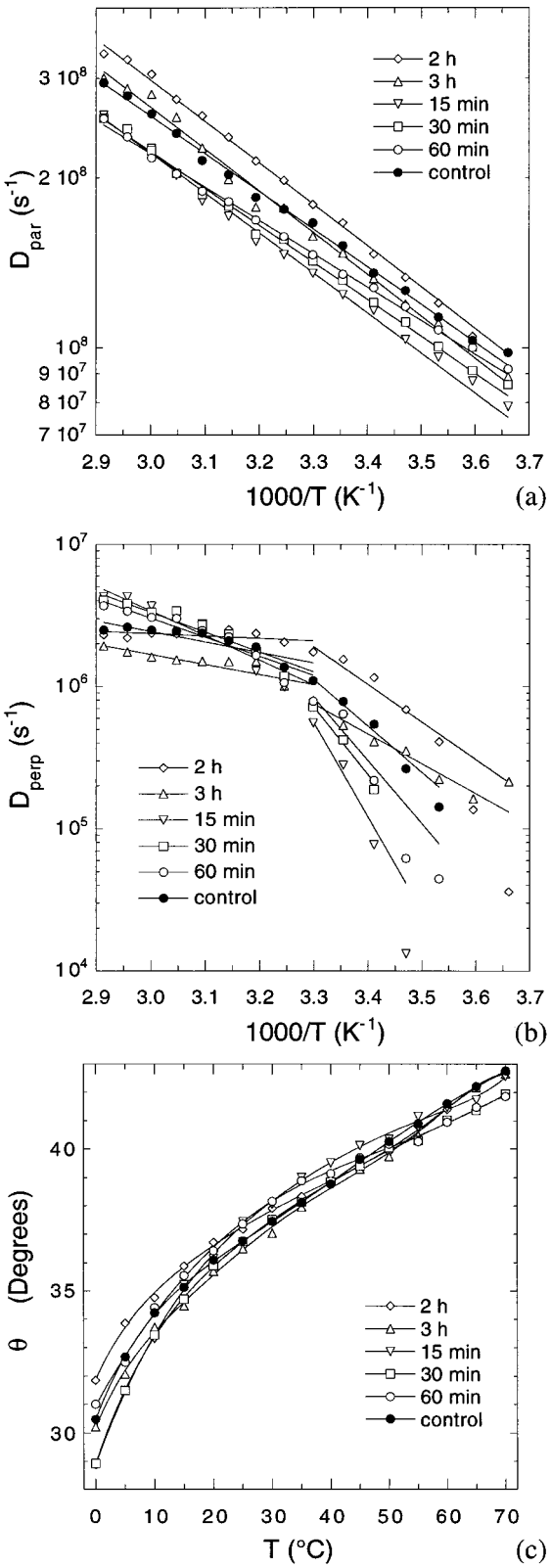

Figure 5. Rotational diffusion constants, D_{par} (a) and D_{perp} (b), and diffusion tilt angle θ (c) for the motion of 5-DSA in thylakoid membranes of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}–EDTA/ascorbic acid for periods indicated in the legend, vs 1000/*T*. In plots a and b, lines represent fits of the trends of rotational diffusion constants as a function of temperature in terms of the Arrhenius equation as described in the text, whereas in plot c lines are to guide the eyes.

behavior was observed by other authors in human erythrocyte membranes.[34]

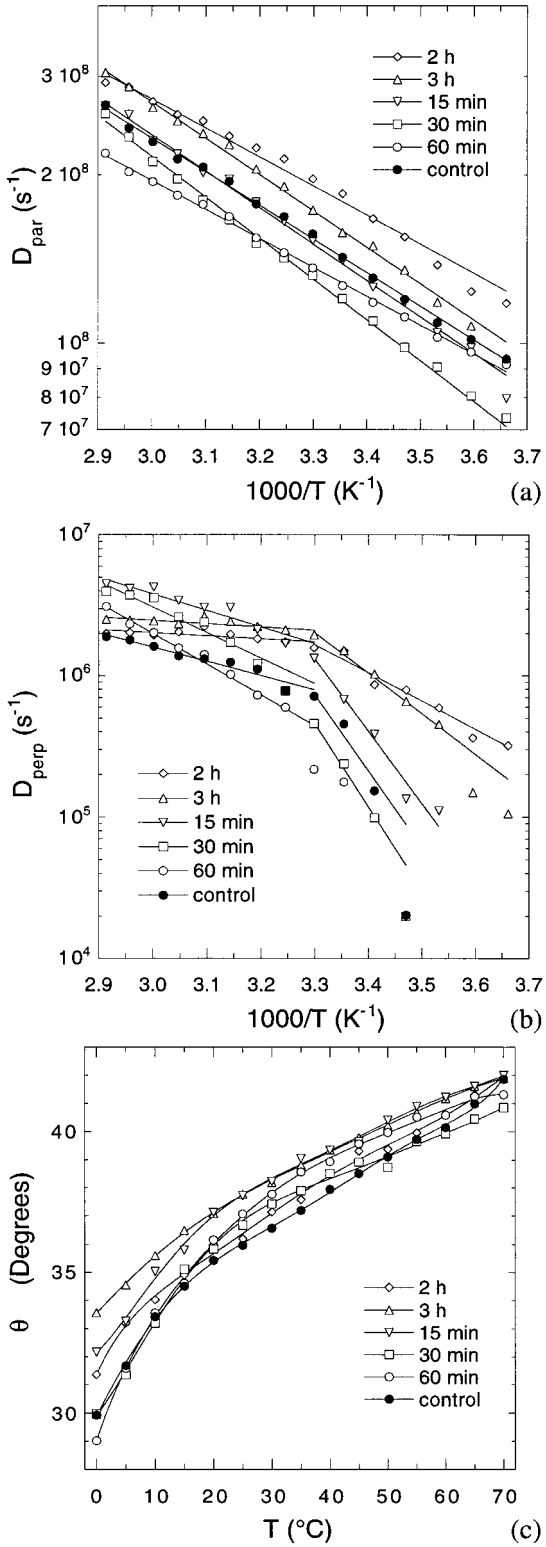

Figure 6. Rotational diffusion constants, D_{par} (a) and D_{perp} (b), and diffusion tilt angle θ (c) for the motion of 5-DSA in thylakoid membranes extracted from leaves of *Triticum durum* Desf. cv. Creso treated with Fe^{2+}-EDTA/ascorbic acid for periods indicated in the legend vs 1000/*T*. In plots a and b, lines represent fits of the trends of rotational diffusion constants as a function of temperature in terms of the Arrhenius equation as described in the text, whereas in plot c, lines are to guide the eyes.

The different response of directly treated membranes to the oxidative treatment with respect to membranes extracted from treated leaves is most probably due to a different effective concentration of active oxygen species in the two systems, which

should be higher in isolated thylakoid membranes. In fact, during the leaf treatment a lower access of reactive oxygen species generated outside the leaf tissue to thylakoids localized inside the chloroplasts has to be expected. Moreover, the extraction of thylakoid membranes causes a loss of water-soluble antioxidants, so that a reduced radical scavenger activity must be considered in isolated membranes.

The changes in fluidity here reported can be related to the results obtained in a parallel study performed in our laboratory[35] on lipid peroxidation products formed in the attack of thylakoid membranes of *T. durum* Desf. cv. Creso with the Fe^{2+}−EDTA and ascorbate mixture. In that work, the oxidative treatment was performed in the same conditions adopted in the present study. Both isolated thylakoids and leaves were treated for incubation times of 15, 30, and 60 min, and the degree of lipid peroxidation was evaluated by means of the thiobarbituric acid (TBA) test,[16,36,37] i.e., detecting aldehydic breakdown products of peroxidation, which form red/yellow complexes with TBA (these products are thus termed thiobarbituric acid reactive substances or TBARS). Isolated thylakoid membranes subjected to the oxidative treatment showed a TBARS content increasing with incubation time (from 15.8 nmol mg^{-1} chlorophyll at 15 min to 18.2 nmol mg^{-1} chlorophyll at 60 min) and higher than that of the control (14.7 nmol mg^{-1} chlorophyll). On the other hand, for membranes extracted from treated leaves a TBARS content essentially constant and equal to that of the control (14.8 nmol mg^{-1} chlorophyll) was found for incubation times up to 30 min, whereas after 60 min of treatment a higher TBARS production was observed (16.4 nmol mg^{-1} chlorophyll). Moreover, directly treated membranes showed TBARS contents higher than the respective membranes extracted from treated leaves for all incubation times investigated.

Although a direct correlation between TBARS content and membrane fluidity is hard to draw, these data support the idea that the initial decrease of fluidity in the peripheral region close to lipid headgroup zone of thylakoid membranes, observed during the oxidative treatment, limits the access of the reactive oxygen species to the membrane core, thus avoiding a massive lipid peroxidation. Moreover, leaves are more protected against oxygen free radicals than are isolated membranes, so that a lower production of aldehydes is observed during their treatment with the oxygen radical generating system.

Comments on the Simulation Model. We have presented an analysis of experimental EPR spectra based on a rotational model that requires only three input parameters (D_{par}, D_{perp}, and θ). This model is somewhat similar to the VAR (very anisotropic rotation) model[38−40] because values of D_{par}, the rotation rate along the symmetry axis of the rotational tensor, as high as 3×10^8 s^{-1} were found. A good agreement between experimental and calculated spectra was obtained with this model. In particular, it allowed the most significant spectral features to be well reproduced. In some cases, a refinement of the model including a distribution of θ values could have improved the reproduction of widths without changing the line positions,[41] but we judged such complication inessential to our purpose of directly comparing spectra of differently treated samples. As for the possible application of the MOMD (microscopic order macroscopic disorder) model, a model proposed for ordered systems,[38,39] it could be used for describing the motions of 5-DSA in thylakoid membranes, in which a microscopic orientational order could exist, due to the lipid aggregation. In fact, the MOMD model has already been employed to study molecular dynamics in model bilayers.[38] However, thylakoid membranes differ from model systems having a quite heterogeneous composition that includes a variety of lipids in which proteins are inserted.[4] Moreover, 5-DSA spin probes are located at the membrane polar interface, so that they suffer of the high ordering imposed by both lipid aggregation and lipid−protein interactions. In this situation, the results of VAR and MOMD models are quite similar.[38] Thus, having to choose between a model that introduces an ordering potential as an additional parameter (as in MOMD model) and a simpler anisotropic diffusion in an anisotropic medium model, we opted for the latter.

Conclusions

In this study we presented quantitative details on the fluidity changes in thylakoid membranes induced by oxidative stress, by simulating the EPR spectra of the 5-DSA spin probe. The stress was realized in vitro by the Fe^{2+}−EDTA and ascorbate system in aqueous aerated solutions. The dynamics of the probes was simulated on the basis of an axially symmetric rotational diffusion model defined by the components of the rotational tensor, D_{par} and D_{perp}, and by the diffusion tilt angle θ. Satisfactory agreement between calculated and experimental spectra was obtained for all samples with a diffusion tilt angle continuously increasing with temperature and a high anisotropy ratio $N = D_{par}/D_{perp}$ of the spin probe rotational diffusion, indicating an immobilization of the spin probes in the membranes. Small but significant differences were found in the simulation parameters for differently treated membranes. In particular, a decrease of membrane fluidity was observed for short treatment periods, whereas treatments prolonged up to 2−3 h brought an increase of fluidity. The decreased fluidity of the membrane region adjacent to polar lipid headgroups could reflect a mechanism to limit the penetration of activated oxygen species into the membrane hydrophobic interior. To this respect, leaves showed a longer resistance period with respect to isolated membranes, probably due to both the presence of a higher content of antioxidants and the protection of thylakoids against reactive oxygen species by several barriers, such as cell wall, plasmalemma, and plastid envelope.

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