Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Magnetic Resonance 163 (2003) 257–269
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Determination of a transmembrane pH difference in chloroplasts with a spin label tempamine

Boris V. Trubitsin and Alexander N. Tikhonov*

Department of Biophysics, Faculty of Physics, M.V. Lomonosov Moscow State University, Moscow 119992, Russia

Received 27 December 2002; revised 17 March 2003

Abstract

We present a method for measuring the transmembrane pH difference ($\Delta pH = pH_{in} - pH_{out}$) in chloroplasts with a spin label TEMPAMINE (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) accumulating inside the thylakoids in response to generation of ΔpH. Experiments with chloroplasts suspended in the media of different osmolarity demonstrated that most of TEMPAMINE (TA) molecules taken up by chloroplasts were localized in the bulk of the thylakoid lumen. The ΔpH value was determined from the relationship $\Delta pH = \lg([H^+]_{in}/[H^+]_{out}) \cong \lg(C_{in}/C_{out})$, where C_{in} and C_{out} are the concentrations of TA inside and outside the thylakoids, respectively. To quantify the internal concentration C_{in}, we used the threshold nature of the concentration-dependent broadening of the EPR signal from TA. It was demonstrated that spin-exchange interactions between TA molecules caused an observable broadening of the signal only when the concentration of TA exceeded the threshold level, $[TA]_\vartheta \approx 2.0$–$2.2$ mM. The concentration dependencies of the signal parameters (the peak-to-peak amplitude, A_{pp}, and the linewidth, ΔH_{pp}) were described within a model of the non-homogeneous broadening of an unresolved hyperfine multiplet from the protons of TA molecule. If the concentration of TA inside the thylakoids went beyond the threshold level, the spin-exchange broadening of the EPR signal was accompanied by a reversible decrease in the signal height (parameter ΔA). By measuring the signal behavior at different levels of microwave power, we were able to discriminate between the line broadening effects caused by concentrating TA molecules inside the thylakoids or the light-induced changes in the concentration of oxygen. We developed a general algorithm for determination of the ΔpH value and the internal volume of thylakoids, V_{in}, from the non-linear dependence of parameter ΔA on the concentration C_0 of TA in a chloroplast suspension. Advantages of this method are: (i) it avoids the use of a broadening agent; (ii) it allows the internal volume of thylakoids to be evaluated; and (iii) the concentrations of TA used to measure the ΔpH are below the range of concentrations that could cause the uncoupling electron transport to ATP synthesis in chloroplasts. Results of our measurements are consistent with the literature data on ΔpH determinations by other methods.

Keywords: Spin labels; Chloroplasts; Proton gradient

1. Introduction

The transmembrane difference in electrochemical proton potentials, $\Delta\mu_{H^+}$, is one of the most important parameters of membrane bioenergetics [1–5]. The two components of the proton potential, ΔpH and $\Delta\varphi$, can serve the role of the driving force to actuate the operation of the ATP synthase machinery. The question whether these components are equivalent thermodynamically and kinetically is still open [6–9]. It is commonly believed that in chloroplasts, in contrast to mitochondria and bacteria, the ΔpH value ($\Delta pH = pH_{out} - pH_{in}$) provides the main contribution to the proton potential $\Delta\mu_{H^+}$ [1–5]. However, there is no consensus in the literature about the ΔpH values in chloroplasts [6–9]. Thus, the question 'How acidic is the lumen?' [8] is still valid for bioenergetics.

Quantitative determination of ΔpH in chloroplasts is not a trivial task because of small internal volume of thylakoids. The two most frequently used methods for ΔpH measurements in energy-transducing organelles (chloroplasts, mitochondria, and chromatophores) are based on pH-indicating probes. These methods

* Corresponding author. Fax: +7-095-9391195.
E-mail address: an_tikhonov@newmail.ru (A.N. Tikhonov).

1090-7807/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1090-7807(03)00110-1

are: (i) the calculation of ΔpH from the partitioning of permeable amines between the inner volume of the vesicles and the suspending medium [7,10–15] and (ii) the measurement of the spectral response of pH-sensitive indicators loaded into the vesicles [16,17]. An amine distribution technique is based on the assumption that the partitioning of probing molecules is determined by the ratio of hydrogen activities inside and outside the vesicles. Unfortunately, many of the widely used molecular indicators of ΔpH (e.g., 9-aminoacridine, neutral red [18–20]) are inadequate probes for measuring the bulk-to-bulk phase ΔpH in chloroplasts because of their interaction with thylakoid membranes. According to [19], the binding of pH-indicating molecules to the membrane can lead to essential overestimations of ΔpH (up to 1–1.5 pH units).

The purpose of this paper was to provide the background and to develop a new method for ΔpH measurements in chloroplasts with a spin label TEMPAMINE (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl). EPR spin-probes have already demonstrated their potential for monitoring the intrathylakoid pH (pH_{in}) in chloroplasts [13–15,21–29]. The water-soluble spin label TEMPAMINE (TA) can be used to measure the intrathylakoid pH_{in}, because TA molecules are taken up by chloroplasts in response to acidification of the thylakoid lumen. An obvious advantage of using pH-sensitive spin probes is that their EPR spectra usually are not masked by the EPR signals from electron transport components. There are indications that TA does not bind to the thylakoid membrane [13,21–23]. It should be noted, however, that the rotational motion of TA in the thylakoid lumen is slowed down by a factor of about 10 relative to the external bulk phase [21–29]. According to [22], the illumination of chloroplasts caused additional hindrance to TA rotation inside the thylakoids. This result might indicate the interaction of TA with the membranes and/or other constituents of thylakoids, being a challenge to TA as a probe for quantifying the bulk-to-bulk ΔpH. In this work, in order to prove that most of TA molecules taken up by chloroplasts were localized in the bulk of the thylakoid lumen, we investigated the interaction of TA molecules with chloroplasts suspended in media of different osmolarity. It has been demonstrated that the majority of TA molecules accumulated inside the thylakoids are localized in the osmotic volume of the thylakoids, i.e., in the bulk of the thylakoid lumen.

Similar to other penetrating amines [10–12], the steady-state ratio of TA concentrations inside and outside the thylakoids (C_{in} and C_{out}), should be determined by the ratio of hydrogen ion activities inside, $[H^+]_{in}$, and outside, $[H^+]_{out}$, the thylakoids:

$$C_{in}/C_{out} = ([H^+]_{in} + K_a)/([H^+]_{out} + K_a), \quad (1)$$

where K_a is the equilibrium constant of the reaction of TA protonation ($TH^+ \leftrightarrow T + H^+$). The pK_a value for TA molecules is about of 8.9 [30], while the external pH_0 is usually held below 8.0–8.5, i.e., $K_a \leqslant [H^+]_{out} \leqslant [H^+]_{in}$. Therefore, the ΔpH value can be determined as

$$\Delta pH = \lg([H^+]_{in}/[H^+]_{out}) = \lg(C_{in}/C_{out}). \quad (2)$$

In order to calculate the internal concentration C_{in}, it is necessary to quantify TA molecules inside the thylakoids and to determine the internal volume of thylakoids, V_{in}. A relatively small EPR signal from TA molecules localized inside the thylakoids can be visualized by broadening the background signal from external TA molecules with the membrane-impermeable paramagnetic agents [13,21–29]. Therefore, the light-induced uptake of TA by thylakoids could be detected as an enhancement of the 'internal' EPR signal. However, the accuracy of this method is restricted by very low concentrations of spin label ($\ll 2.5$–$5\,\mu M$) that should be used to avoid the concentration-dependent broadening of the 'internal' EPR signal [25]. It turns out, however, that the broadening effect provides the possibility to quantify the concentration of TA inside the thylakoids without using a broadening agent [26–28].

In the present report, we describe a method for measuring the internal concentration of TA (C_{in}) which is based on the effect of spin-exchange broadening of the EPR signal from TA molecules concentrated in the thylakoid lumen [27–29]. The measurements of the internal volume V_{in} are often complicated because thylakoids are good osmometers with variable volumes [10–12]. However, contrary to conventional methods for measuring ΔpH with penetrating amines, our method allows the value of ΔpH to be determined without knowing the internal volume of thylakoids. It is also important that concentrations of TA used to measure ΔpH are below the concentrations of TA that could cause an essential influence on photophosphorylation in chloroplasts [22,31].

2. Experimental

2.1. Chloroplasts

Bean plants (*Vicia faba*) were grown in a greenhouse at a growth temperature of 20 °C. Class B chloroplasts were isolated from 2- to 3-week-old leaves as described earlier [38]. Isolated chloroplasts (ac. 4–5 mg chlorophyll/ml) were suspended in a medium containing 50 mM sucrose, 2 mM $MgCl_2$, 5 mM P_i, and 15 mM Hepes–NaOH (pH 8.0). The osmolarity of the suspending medium was changed by variation of sucrose concentration (20–500 mM). 20 μM methyl viologen

(MV) was used as a mediator of non-cyclic (pseudo-cyclic) electron transport from water to oxygen, which involved two photosystems ($H_2O \rightarrow PS2 \rightarrow PS1 \rightarrow MV \rightarrow O_2$). Cyclic electron transport around photosystem 1 was mediated by an artificial electron cofactor phenazine methosulphate (PMS) in the presence of 3-(3,4)-dichlorophenyl-1,1-dimethylurea (DCMU), an inhibitor of photosystem 2.

To visualize the EPR signals from TA molecules localized inside the thylakoids, we used the broadening agent chromium oxalate. Chromium oxalate (CrO*x*) was synthesized according to [54]. It is noteworthy that most authors used fairly high concentrations ($\geqslant$40–80 mM) of a broadening agent (CrO*x* or potassium ferricyanide) to visualize intrathylakoid TA molecules [21–24,29]. However, it was demonstrated earlier [27,28] that penetration of CrO*x* molecules into the thylakoid lumen caused a certain broadening of the 'internal' signal when the concentration of CrO*x* exceeded 15–20 mM. To minimize the influence of CrO*x* on the 'internal' EPR signal, we added CrO*x* at concentrations not higher than 15 mM. In this case, the 'external' EPR signal was markedly broadened, whereas the 'internal' EPR signal was practically unaffected by CrO*x* [26,27].

2.2. EPR measurements and processing the EPR data

The conventional EPR spectra (first harmonic absorption in phase, 100 kHz field modulation) were measured with a Varian E-4 X-band spectrometer equipped with a variable temperature controller. Samples were placed into a thin-walled gas-permeable capillary made of methylpentane polymer (TPX, [34]) or into a standard Varian quartz cell. All the measurements were performed at room temperature (20–22 °C). EPR spectra of TA were recorded at subsaturating microwave power ($\leqslant$1–10 mW). The intensity of the EPR signal was determined by double integrating the digitized EPR spectrum. As a standard we used a spin label solution of a known concentration.

2.3. Rotational correlation time

Apparent correlation time τ_c was used to characterize the rotational mobility of TA, which EPR spectra revealed three relatively narrow symmetrical lines (corresponding nearly isotropic nanosecond rotation, $S \leqslant 0.4$). The empirical correlation time τ_c was calculated from the following formula [45]:

$$\tau_c = 0.673 \times 10^{-9} \Delta H_0 [\sqrt{I_0/I_{-1}} - 1] \text{ (s)}, \quad (3)$$

where ΔH_0 is the midfield peak-to-peak linewidth; I_0, and I_{-1} are the peak-to-peak heights of the mid- and high-field components of the spectrum, respectively.

3. Results

3.1. TEMPAMINE partitioning in thylakoids

TEMPAMINE molecules easily penetrate inside the thylakoids, partitioning between the thylakoid lumen ('internal' volume) and the bulk of chloroplast suspension ('external' volume). Spin label molecules localized inside the thylakoids were visualized by broadening the EPR spectrum of TA molecules outside the thylakoids ('external' EPR signal) with the membrane-impermeable paramagnetic compound chromium oxalate (CrO*x*). Closed thylakoid membranes maintain their barrier functions for CrO*x*, precluding the broadening of the EPR signal from TA inside the thylakoids.

The first-derivative EPR spectrum of TA in a chloroplast-suspending medium (Fig. 1, spectrum a)

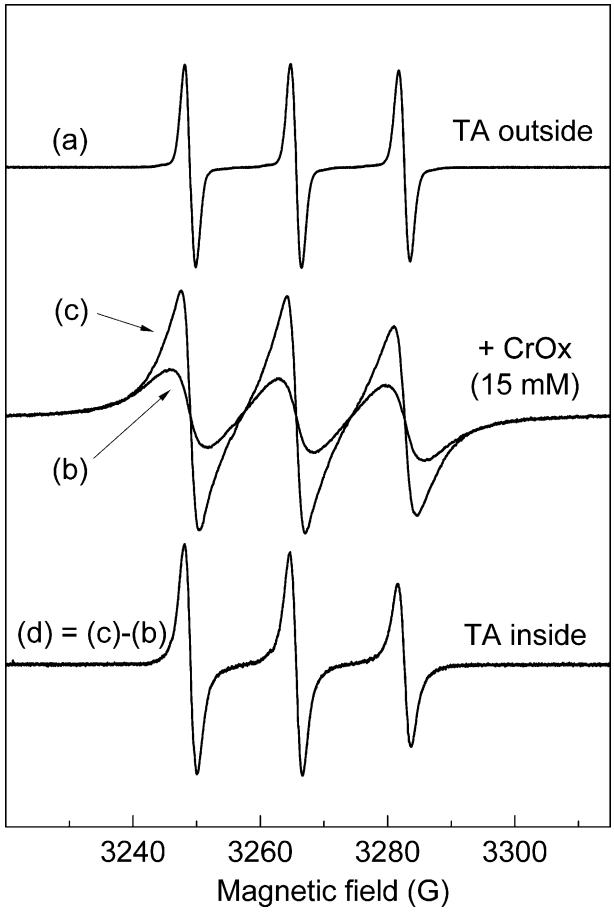

Fig. 1. EPR signals of TEMPAMINE: (a) suspending medium without a broadening agent; (b) suspending medium in the presence of 15 mM chromium oxalate; (c) chloroplast suspension in the presence of 15 mM chromium oxalate; and (d) difference EPR signal (spectrum c – spectrum b). Concentration of chloroplasts was equivalent to 10 μM reaction centers P_{700}. EPR signals of TEMPAMINE (1.0 mM) were recorded at microwave power 10 mW and modulation amplitude 0.5 G. Spectrometer receiver gain was equal to 10^2 (spectrum a) or 5×10^3 (spectra b and c).

represents a triplet given by the nitroxide radicals rapidly tumbling ($\tau_c \cong 4 \times 10^{-11}$ s) in polar surroundings. The isotropic hyperfine structure constant $a_{iso} = 16.9$ G is typical of TA radicals in aqueous solutions [32]. Addition of the paramagnetic compound (15 mM CrOx) caused an essential broadening of this signal (Fig. 1, spectrum b). When CrOx was added to a chloroplast suspension, the EPR signal (Fig. 1, spectrum c) represented the superposition of two signals, one of them was the broadened triplet from TA molecules localized outside the thylakoids, while the other signal was the triplet of narrow lines given by TA molecules localized inside the thylakoids ('internal' EPR signal). In order to obtain the 'internal' signal (Fig. 1, difference spectrum d), we subtracted the 'background' spectrum b (a buffer solution without chloroplasts) from the total spectrum c (chloroplasts + buffer). Before the subtraction procedure, the intensity of the broadened background spectrum (Fig. 1, spectrum b) was corrected, so that the line wings of the background signal were adjusted to the line wings of the total spectrum (Fig. 1, spectrum c). This protocol allows pure 'internal' spectra to be extracted without the contribution of the background 'external' signal. Actually, in the wing regions of the broadened background spectrum, the contribution of the narrow lines of the 'internal' signal was negligible. Therefore, the subtraction of the properly fitted background spectrum from the total spectrum should give three lines of the 'internal' signal without traces of the background signal. The residual EPR signal (Fig. 1, spectrum d) can be attributed to TA molecules localized in the aqueous phase of chloroplast osmotic volume (the thylakoid lumen). This conclusion follows from two observations: (i) the difference spectrum was characterized by the isotropic hyperfine splitting constant $a_{iso} = 16.9$ G, which was typical of the nitroxide radicals in a polar surroundings and (ii) the intensity of the difference signal decreased with the rise in the osmolarity of a suspending medium (Figs. 2 and 3).

The shape of the 'internal' signal (Fig. 1, spectrum d) differed from the shape of the 'external' signal (Fig. 1, spectrum a), indicating the hindrance to TA rotation in the thylakoid lumen. Similarly to earlier observations [13,21–29], we found that the rotational mobility of TA inside the thylakoids, described in terms of the apparent correlation time τ_c, was retarded by a factor of about 10, as compared to TA localized outside the thylakoids. There might be two reasons for slowing down the rotation of TA inside the thylakoids: (i) high viscosity of the aqueous medium inside the thylakoids and/or (ii) interactions of TA with the internal surface of the thylakoid membrane (e.g., TA binding or anchoring to the membrane). In order to discriminate between these factors, we investigated how variations of the internal volume of thylakoids could influence the rotational mobility of TA inside the thylakoids.

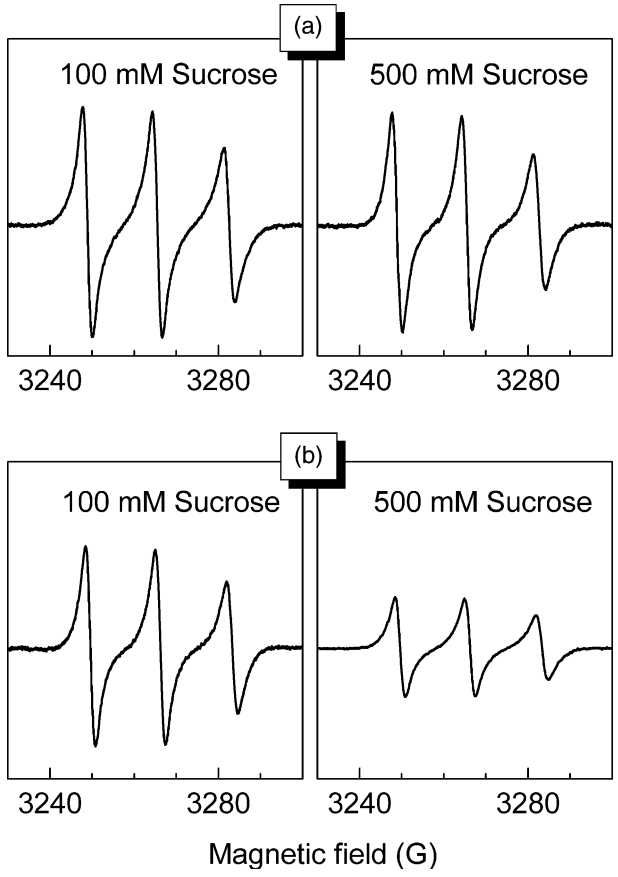

Fig. 2. Difference EPR signal of TEMPAMINE (1.0 mM) in a chloroplast suspension containing 100 or 500 mM sucrose as indicated. (a) Spin label was added after 15 min of chloroplast incubation at 4 °C. (b) Spin label was added after 12 h of chloroplast incubation at 4 °C. Other experimental conditions as in Fig. 1.

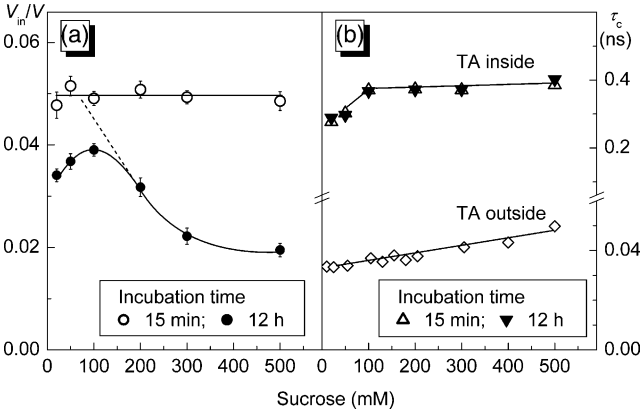

Fig. 3. Effects of sucrose on the internal volume V_{in} of thylakoids (a) and the apparent rotational correlation time τ_c of TEMPAMINE inside the thylakoids (b). Experimental conditions as in Fig. 2.

3.2. Effects of osmolarity on the internal volume of thylakoids

The intensity of the 'internal' EPR signal should be proportional to the osmotic volume of thylakoids, V_{in}.

The ratio of the internal volume V_{in} to the total volume of a chloroplast suspension, V, was determined as $V_{in}/V = I_{dif}/I_t$, where I_{dif} denotes the double-integral intensity of the 'internal' signal and I_t is the intensity of the total EPR signal in a chloroplast suspension without CrOx. Fig. 2 demonstrates that after extended incubation of chloroplasts (12 h at 4 °C) in the high-osmotic medium (500 mM sucrose) the intensity of the 'internal' signal decreased substantially compared to chloroplasts incubated in the low-osmotic medium (100 mM sucrose). Fig. 3 shows that the internal volume of thylakoids V_{in} accessible for TA molecules decreased by a factor of 2.5. Similar changes in the osmotic volume of spinach thylakoids were observed earlier with the use of penetrating radioactive amines or ^3H_2O [10–12]. The long-term incubation of chloroplasts at 100 mM sucrose caused only an insignificant decrease in the V_{in}. A certain decrease in the intensity of the 'internal' signal observed at low concentrations of sucrose (<100 mM) can be accounted for by the loss of the thylakoid membrane integrity due to osmotic shock. After short incubation of chloroplasts (15 min at 4 °C), the intensity of the signal was the same in the wide interval of sucrose concentrations (from 20 to 500 mM). This result can be explained by a rather slow shrinkage of thylakoids in response to the rise in osmotic pressure.

3.3. Effects of osmolarity on the mobility of TEMPAMINE

For TA molecules located outside the thylakoids, we observed a certain increase in the rotation correlation time τ_c with the rise in the concentration of sucrose (Fig. 3b). This is explained by the increase in the viscosity of a suspending medium. In contrast to TA in the external bulk phase, the rotational mobility of TA inside the thylakoids was practically independent of a sucrose concentration (100–500 mM) in the suspending medium. Fig. 2 demonstrates that the shape of the 'internal' signal did not change even after the incubation of chloroplasts for 12 h at 500 mM sucrose, when the osmotic volume of thylakoids was decreased by a factor of 2–2.5 (Fig. 3a). In the rather wide interval of sucrose concentrations (100–500 mM), the τ_c values were equal within error ($\tau_c \approx 4 \times 10^{-10}$ s) for chloroplasts incubated either for 15 min (Fig. 3b, open triangles) or 12 h (Fig. 3b, solid triangles). Thus, we did not observe essential slowing down of TA rotation with osmotically induced shrinkage of thylakoids. Under the low-osmotic conditions (<50 mM sucrose) we observed a certain decrease in the τ_c value that might be associated with the partial loss of the thylakoid integrity due to osmotic shock.

3.4. Light-induced uptake of TEMPAMINE by thylakoids

The light-induced accumulation of TA inside the thylakoids can be detected either from the enhancement of the 'internal' signal [21–23,25,26] or from the line broadening effect caused by the increase in the internal concentration of TA [24–28]. In this work, we used the latter approach because it enabled us to quantify the internal concentration of TA in the absence of external broadening agents and without knowing the internal volume [26–28]. Fig. 4a shows that the illumination of chloroplasts caused the broadening of the EPR line, characterized by decreasing the peak-to-peak amplitude (parameter ΔA) and a concomitant gain of the line wings. Similar broadening of the signal was observed with the rise in the concentration of TA in water (Figs. 4b and c). The light-induced broadening of the EPR signal in a chloroplast suspension (Fig. 4a) was associated with the uptake of TA by thylakoids. Actually, this effect was not observed in the presence of uncouplers

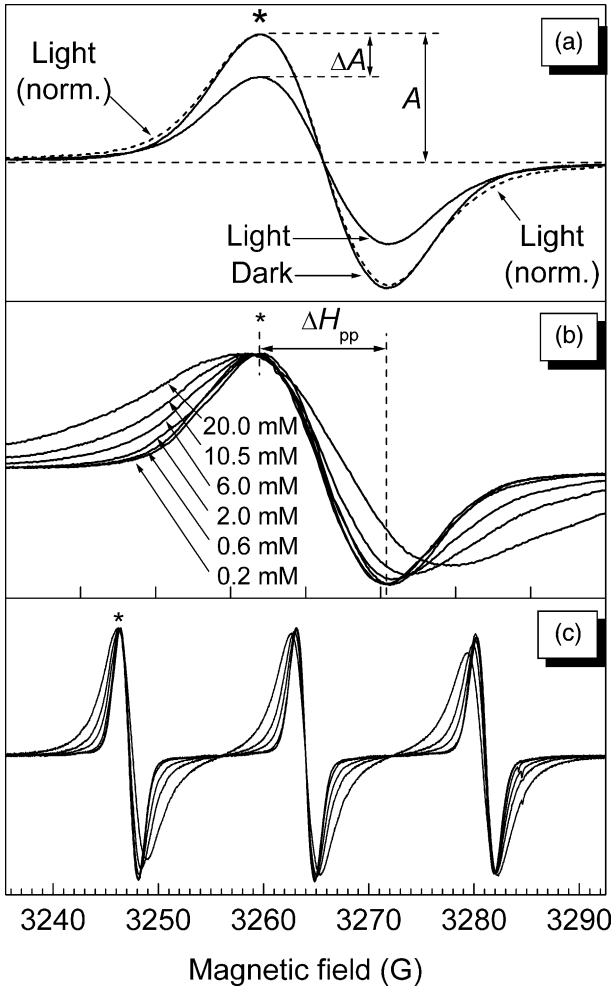

Fig. 4. (a) The low-field line of the EPR signal of TEMPAMINE (0.3 mM) in a bean chloroplast suspension. Solid lines show spectra recorded either in the dark or during illumination, as indicated. Dashed line represents the normalized EPR signal recorded during illumination of chloroplasts: the amplitude of this signal is increased up to the amplitude of the signal recorded in the dark. (b, c) Normalized EPR signals recorded at different concentrations of TEMPAMINE in water.

(e.g., NH_4Cl or nigericin) which destroyed the ΔpH (data not shown).

It is convenient to monitor the broadening effect by measuring the kinetics of the light-induced changes in the height of the EPR signal, fixing magnetic field at the peak of the spectral line. However, it is necessary to discriminate between different factors that can influence the EPR signal: (1) line broadening due to concentrating TA molecules inside the thylakoids [13–15,25–28], (2) redox transients of TA [32], and (3) light-induced changes in oxygen concentration in a chloroplast suspension [33–37]. In order to discriminate between these factors, we compared the signal behavior in 'control' chloroplasts (capable to generate ΔpH) and in de-energized chloroplasts (unable to maintain ΔpH) at different levels of microwave power, 1 or 10 mW.

Fig. 5 (left box) demonstrates that the illumination of 'control' chloroplasts caused a relatively sharp decrease in the amplitude of the EPR signal due to TA uptake by thylakoids. After switching the illumination off, the leak of TA molecules from thylakoids was accompanied by the signal recovery. Reversible light-induced changes in the height of the signal, characterized by parameter ΔA, were determined by ΔpH [13–15,25–28]. A relatively small irreversible loss of the signal, characterized by parameter $\Delta I = A_0 - A$, can be explained by the reduction of nitroxide radicals due to their interactions with the chloroplast electron transport chain [25–27,32]. The addition of uncouplers, which destroyed the proton gradient in chloroplasts (e.g., 20 mM NH_4Cl), inhibited the reversible decrease in the signal amplitude related to the line broadening caused by concentrating TA inside the thylakoids (Fig. 5, right box). In this case, the light-induced response of the signal was determined by redox transients of TA and/or by the light-induced changes in the concentration of oxygen in a chloroplast suspension.

We could discriminate between the loss of TA paramagnetism and the influence of oxygen on spectral characteristics of the EPR signal. The sign of the effect caused by modulation of oxygen concentration depends on microwave power [33,34]. At subsaturating microwave power, oxygen acts mainly as a paramagnetic broadening agent. Therefore, de-oxygenation of a chloroplast suspension should lead to an increase in the signal amplitude due to narrowing the line. At saturating microwave power, oxygen reveals itself as a paramagnetic relaxator. In this case, the light-induced uptake of oxygen should lead to the decrease in the signal height [33–37].

Fig. 5 (right box) shows that at low microwave power ($P = 1$ mW) the illumination of de-energized chloroplasts initially led to an insignificant rise in the signal amplitude that could reflect the light-induced uptake of oxygen. The following decrease in the signal height was caused by the loss of TA paramagnetism characterized by parameter ΔI. In contrast, at higher

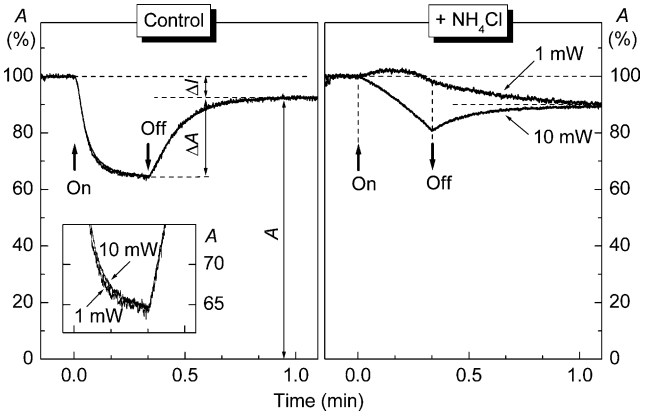

Fig. 5. Light-induced changes in the height of the EPR signal from 0.3 mM TEMPAMINE in a suspension of bean chloroplasts. Magnetic field was fixed at the low-field peak of the signal shown in Fig. 4 by asterisk. Microwave power was either 1 or 10 mW, as indicated. The initial amplitude of the signal recorded at 1 mW was normalized to the signal amplitude at 10 mW. Chloroplasts were suspended in the medium contained 50 mM sucrose, 2 mM $MgCl_2$, 20 mM Hepes (pH 7.8), and 20 μM methylviologen. Left box—control chloroplasts; right box—uncoupled chloroplasts (in the presence of 20 mM NH_4Cl). Concentration of chloroplasts was equivalent to 0.5 μM P_{700}.

microwave power ($P = 10$ mW) illumination of chloroplasts from the very beginning led to the decrease in the signal height. After switching the illumination off, the amplitude of the EPR signal either slowly decreased (at 1 mW) or slowly increased (at 10 mW). The signs of these changes indicated the regeneration of oxygen in the dark (e.g., due to decomposition of H_2O_2 formed in the course of illumination of de-energized chloroplasts).

It should be mentioned that in control chloroplasts the time-courses of the signal height were the same at 1 and 10 mW (Fig. 5, left box). This means that in control chloroplasts the light-induced changes in the concentration of oxygen were negligible. The light-induced uptake of oxygen observed in de-energized chloroplasts (Fig. 5, right box) can be accounted for by higher rate of electron transport in comparison with control chloroplasts. It is well-known that in control chloroplasts electron transport is slowed down due to acidification of the thylakoid lumen [7,8,38].

Fig. 6 shows the time-courses of the ratio $Q = \Delta A/A$, where A is the steady-state level of the signal after switching off illumination. The relative parameter Q characterizes the broadening effect corrected for the irreversible loss of the EPR signal (parameter ΔI). The steady-state value of parameter Q, which is determined by a number of TA molecules taken up by chloroplasts, depended on the metabolic state of chloroplasts and osmolarity of a suspending medium. Parameter Q was higher in the state of photosynthetic control (State 4, without added ADP) than under phosphorylating conditions (State 3, in the presence of ADP). Chloroplasts

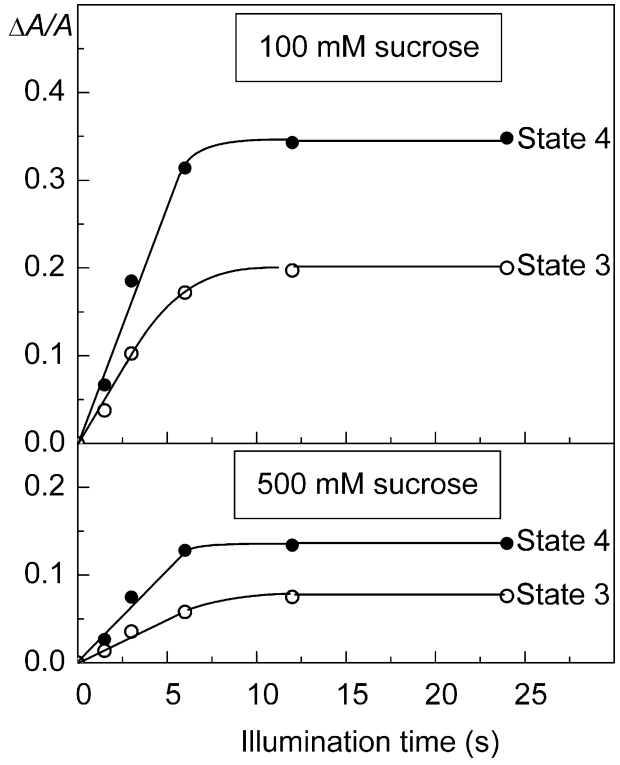

Fig. 6. Kinetics of the light-induced rise of parameter $Q = \Delta A/A$ measured for 0.3 mM TEMPAMINE in a chloroplast suspension. Parameter Q characterizes reversible changes in the signal height corrected for spin label reduction in the course of illumination. Chloroplasts were suspended in the media contained 100 or 500 mM sucrose, as indicated. EPR signals were recorded at microwave power 10 mW and modulation amplitude 0.5 G. Concentration of chloroplasts was equivalent to 0.5 μM P_{700}. State 3—chloroplasts in the presence of 4 mM Mg–ADP; State 4—chloroplasts in the presence of 20 μM Mg–ATP.

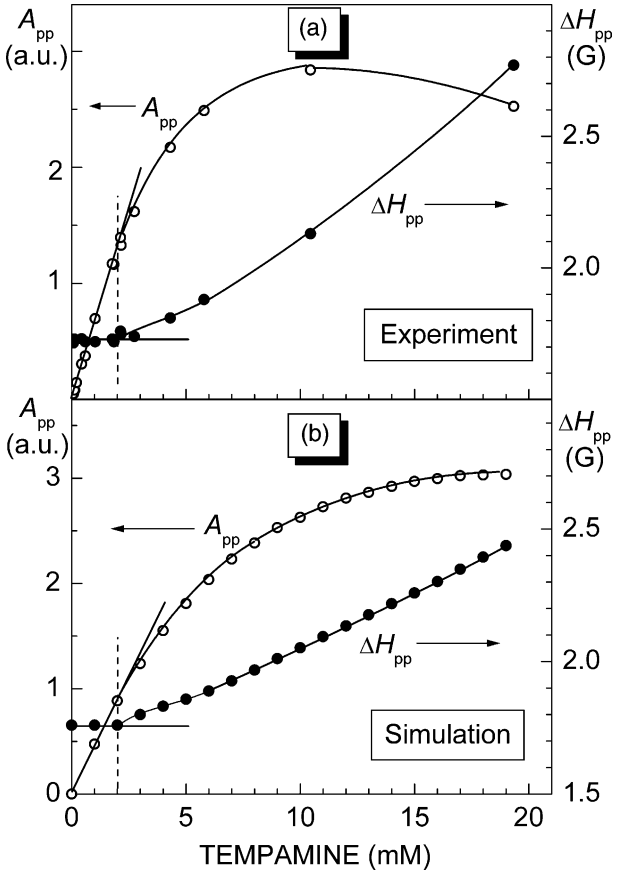

Fig. 7. Concentration dependencies of spectral parameters A_{pp} and ΔH_{pp} of the low-field component of the EPR signal of TEMPAMINE. (a) Experimental data (TEMPAMINE in oxygenated water solution). EPR signals were recorded at microwave power 5 mW and modulation amplitude 0.5 G. (b) Computer simulation; a model of eight protons (six equivalent protons with coupling constant $a_1 = 0.439$ G and two equivalent protons with coupling constant $a_2 = 0.45$ G) was used to simulate EPR signals at different concentrations of TEMPAMINE. The peak-to-peak linewidths of individual superhyperfine components of Lorentzian lineshape were varied from $\Delta H^i_{pp} = 0.647$ G to $\Delta H^i_{pp} = 1.8$ G.

suspended in the high-osmotic medium (500 mM sucrose) were characterized by lower values of parameter Q as compared to chloroplasts suspended in the low-osmotic medium (100 mM sucrose). This result demonstrated that the osmolarity-controlled swelling of thylakoids led to an increase in a number of TA molecules taken up by chloroplasts during illumination.

3.5. Broadening vs spin label concentration

Under conventional experimental conditions (oxygenated suspension, modulation amplitude 0.5 G) we usually observed three EPR lines with unresolved hyperfine structure (Figs. 1 and 2). At low concentrations of TA ($\leqslant$2 mM), the peak-to-peak linewidth, ΔH_{pp}, was independent of concentration (Fig. 4b). In accordance with our earlier observations [25–28], spin-exchange interaction between TA molecules caused an increase in the ΔH_{pp} value only when the concentration of TA exceeded the threshold level $[T]_\vartheta \approx 2.0$–2.5 mM. Fig. 7a shows that the peak-to-peak amplitude of the EPR signal, A_{pp}, increased proportionally to TA concentration up to $[T]_\vartheta \approx 2.0$ mM, while it declined from the linear law as the concentration of TA became higher than the threshold concentration $[T]_\vartheta$. The deviation from the linear law and further drop in the A_{pp} value were accompanied by the increase in the linewidth ΔH_{pp} (Fig. 7a).

The threshold character of the concentration dependencies of spectral parameters A_{pp} and ΔH_{pp} could be explained by peculiarities of broadening the EPR lines with unresolved hyperfine structure. Under experimental conditions used to measure the ΔpH (an aerated suspension of chloroplasts), we registered three lines (Fig. 4). Each of these lines represented an unresolved multiplet of superhyperfine lines. Actually, EPR spectrum of TA molecules in deoxygenated deuterated water (Fig. 8a) demonstrated well resolved superhyperfine

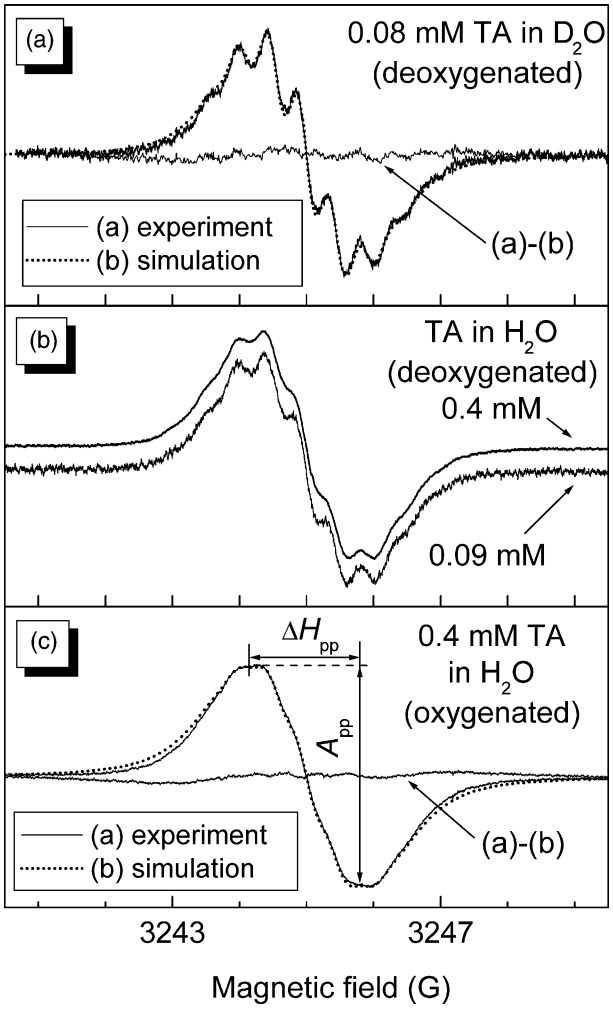

Fig. 8. EPR spectra of TA in water solution. (a) Deoxygenated solution of 0.08 mM TEMPAMINE in D_2O. (b) Deoxygenated solutions of 0.09 or 0.4 mM TEMPAMINE in H_2O, as indicated. (c) Oxygenated solution of 0.4 mM TEMPAMINE in H_2O. Deoxygenated samples were prepared by blowing Ar gas through a water solution of spin label for 10 min. EPR signals were recorded at microwave power 5 mW and modulation amplitude 0.05 G. Simulated EPR spectra shown by dotted lines (a, c) were calculated using a model of eight protons (six protons with coupling constant $a_1 = 0.439$ G and two protons with coupling constant $a_2 = 0.45$ G) and the following linewidths of the individual Lorentzian lines of the hyperfine multiplet: (a) $\Delta H^i_{pp} = 0.439$ G (TEMPAMINE in deoxygenated water) and (c) $\Delta H^i_{pp} = 0.647$ G (TEMPAMINE in oxygenated water).

structure arising due to interaction of the unpaired electron with the protons. With increasing the concentration, collisions of spin labels with each other became more frequent, leading to broadening the hyperfine lines (compare spectra in Fig. 8b). However, in the aerated (oxygenated) solution of TA, due to interplay between broadening superhyperfine lines, the peak-to-peak width ΔH_{pp} of the envelope of the unresolved multiplet did not change with concentration (in the concentration interval below the threshold concentration of TA, see Figs. 4b and 7a).

3.6. Simulation of concentration-dependent broadening

Computer simulation of EPR spectra supported the idea that the threshold nature of the concentration dependencies of spectral parameters ΔH_{pp} and A_{pp} can be explained by interplay between superhyperfine lines. According to [40–42], there are two groups of equivalent protons that give the main contribution to superhyperfine splitting in TA molecule: six protons of two methyl groups (coupling constant $a_1 = 0.436$–0.462 G) and two protons bound to carbon atoms of the piperidine ring (coupling constant $a_2 = 0.45$ G). The contribution of other protons is less significant. Taking into account the interaction of the free electron with eight protons, we simulated the multiplet EPR spectrum of TA in deoxygenated water (Fig. 8a). In the presence of oxygen (Fig. 8c), collisions of TA with molecular oxygen additionally broadened hyperfine lines, leading to the disappearance of the superhyperfine structure. Both simulated spectra, resolved (Fig. 8a) and unresolved (Fig. 8c), were in agreement with the experimental ones.

The threshold character of the concentration dependencies of spectral parameters A_{pp} and ΔH_{pp} can be described using the assumption that a linewidth ΔH^i_{pp} of each individual superhyperfine line linearly depends onthe concentration C of radicals involved in spin-exchange interactions [39,42]:

$$\Delta H^i_{pp}(C) = \Delta H^i_{pp}(0) + K_{ex}C. \quad (4)$$

Here, $\Delta H^i_{pp}(0)$ is the intrinsic peak-to-peak width of the ith component, the coefficient K_{ex} is the exchange rate determined by the diffusion coefficient of spin label and the efficiency of spin-exchange interactions. Varying the Lorentzian widths ΔH^i_{pp} of individual components ($i = 1, 2, \ldots, 8$) of the multiplet according to Eq. (4), we could simulate the threshold character of the concentration dependencies of spectral parameters A_{pp} and ΔH_{pp} (Fig. 7b). Similar to experimental plots (Fig. 7a), when the concentration of TA was below the threshold level, the calculated parameter A_{pp} increased linearly with concentration, while the ΔH_{pp} remained constant (Fig. 7b). Certain difference between the simulated and experimental plots of spectral parameters A_{pp} and ΔH_{pp} observed at high concentrations can be explained by violating the linear relationship between ΔH^i_{pp} and C [39].

It should be noted that spin-exchange frequency depends on molecular mobility of spin labels [39]. Therefore, one could expect that TA molecules localized inside the thylakoids might reveal the broadening effect at higher concentrations compared to TA outside the thylakoids. However, earlier we demonstrated that the threshold concentrations for TA localized inside or outside the thylakoids were practically the same. According to [27], inside the thylakoids TA molecules were characterized by $[T]_\vartheta \approx 2.0$–$2.5$ mM.

3.7. Determination of ΔpH from the light-induced broadening effect

The internal concentration of TA (C_{in}) can be calculated from the equation of material balance for TA molecules distributed between the inner compartment of thylakoids and the outer bulk phase:

$$C_{in}V_{in} + C_{out}(V - V_{in}) = C_0V, \quad (5)$$

where V_{in} is the internal volume and V is the total volume of a chloroplast suspension, C_{in} and C_{out} are the steady-state concentrations of TA inside and outside the thylakoids during illumination, and C_0 is the concentration of TA in a chloroplast suspension in the dark. Taking into account the relationship $V_{in}/V \ll 1$, which holds true in most experiments with chloroplasts, from Eqs. (2) and (5) we obtain

$$C_{in} = C_0 \frac{10^{\Delta pH}}{1 + (V_{in}/V)10^{\Delta pH}}. \quad (6)$$

Eq. (2) can be re-arranged as

$$\Delta pH = \lg(C_{in}/C_{out}) = \lg(C_{in}/C_0) + \lg[1 + (V_{in}/V)10^{\Delta pH}]. \quad (7)$$

If the relationship $(V_{in}/V)10^{\Delta pH} \ll 1$ holds true, the light-induced changes in the concentration of TA outside the thylakoids are negligible ($C_{out} \approx C_0$). By diluting the chloroplasts suspension sufficiently, the internal volume V_{in} can be decreased to meet the $(V_{in}/V)10^{\Delta pH} \leqslant 1$ condition. In this case, the ΔpH value can be determined by means of the following approximation

$$\Delta pH \approx \Delta pH_{app} = \lg(C_{in}/C_0). \quad (8)$$

The threshold nature of the broadening effect provides the basis for quantifying the internal concentration C_{in} [7,26–28]. Actually, the light-induced accumulation of TA in the thylakoid lumen should lead to the concentration-dependent broadening of the EPR line only if the internal concentration of TA overcomes the threshold level, i.e., if $C_{in} \geqslant [T]_\vartheta$. It should be noted, however, that Eq. (8) gives underestimated ΔpH values if the light-induced uptake of TA by thylakoids leads to essential changes in the concentration of TA outside the thylakoids (C_{out}). Therefore, a rigorous procedure for determination of ΔpH should also take into account the light-induced decrease in C_{out}, as well as the broadening effect caused by slowing down the rotation of TA inside the thylakoids. Below we consider a general algorithm for measuring the ΔpH value which accounts both these factors. It is also important to note that our method is valid at any ratio V_{in}/V. An advantage of this approach is that it allows the evaluation of two parameters, ΔpH and V_{in}, without using a broadening agent.

Let the concentration of TA in the chloroplast suspension in the dark be equal to C_0. Since $\Delta pH = 0$, the initial concentrations of TA inside and outside the thylakoids are the same, $C_{in} = C_{out} = C_0$. The light-induced generation of ΔpH causes the redistribution of spin label molecules between the external volume and the thylakoid lumen. The concentrations of TA in a chloroplast suspension, C_0, usually do not exceed 0.3–0.4 mM (Fig. 9). Therefore, a line shape of the 'external' EPR signal does not alter with illumination of chloroplasts, because the concentration of TA outside the thylakoids always remains below the threshold level ($C_{out} \leqslant C_0 < [T]_\vartheta$). On the other hand, spectral parameters of the 'internal' EPR signal can change with concentrating TA molecules inside the thylakoids. According to the protocol used in this work, we measured the light-induced changes in the height A of the EPR signal, fixing magnetic field at the peak of the low-field line (Fig. 4). Let $F(C_{in})$ is a function characterizing a decrease in the amplitude of the signal of TA molecules translocated into the thylakoid lumen. Then the light-induced decrease in the height of the EPR signal at fixed magnetic field will be equal to $\Delta A = A_{dark} - A_{light}$:

$$\Delta A = k[C_0(V - V_{in}) + F(C_0)C_0V_{in}] - k[F(C_{in})C_{in}V_{in} + C_{out}(V - V_{in})] = kC_0V_{in}\frac{[1 - F(C_{in})] \times 10^{\Delta pH} - [1 - F(C_0)]}{1 + (V_{in}/V) \times 10^{\Delta pH}}, \quad (9)$$

where k is the coefficient of proportionality between the number of TA molecules and the intensity of the EPR signal. The C_{out} value is determined by Eq. (6).

The function $F(C_{in})$ can be presented as the product of three terms which describe the influence of different factors on the light-induced response of the EPR signal:

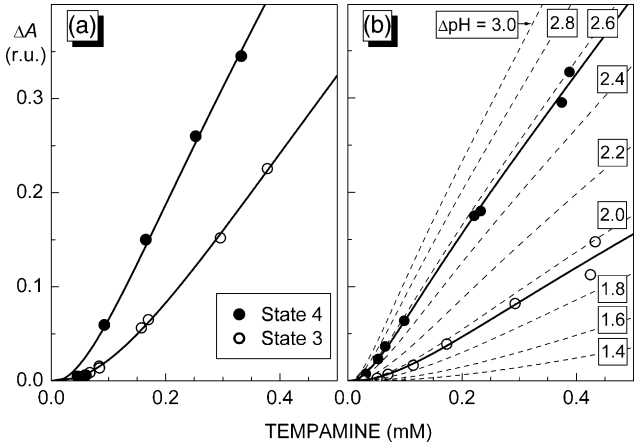

Fig. 9. The light-induced decrease in the signal height (parameter ΔA) versus the concentration of TEMPAMINE in a chloroplast suspension. Solid lines—the non-linear curve fit of experimental data; dashed lines—theoretical plots of ΔA vs C_0 simulated for $V_{in}/V = 0.003$ and different ΔpH values, as indicated. (a) Noncyclic electron transport (in the presence of 7.5 μM methylviologen). (b) Cyclic electron transport (in the presence of 2 μM DCMU and 100 μM PMS). Experimental conditions were the same as in Fig. 5.

$$F(C_{in}) = \alpha(C_{in})\beta(C_{in})f_0. \quad (10)$$

The first term $\alpha(C_{in})$ describes the concentration-dependent deviation of the signal amplitude A from the linear law (Fig. 10a). This term is a dominant factor which determines a decrease in the function $F(C_{in})$ at relatively high concentrations of TA. The second term, $\beta(C_{in})$, describes changes in the signal height A caused by shifting the position of the signal extreme due to the line broadening effect (Fig. 4b). The contribution of $\beta(C_{in})$ to the decrease in the function $F(C_{in})$ is insignificant compared to $\alpha(C_{in})$ (Fig. 10b). The coefficient f_0 characterizes a decrease in the line height due to slowing down rotations of TA inside the thylakoid. The latter factor can reveal itself even at low concentrations of TA, when the contribution of the concentration-dependent broadening effect is negligible. It should be noted, however, that the slowing down of TA rotation gives a minimal contribution to the line broadening when magnetic field is fixed at the low-field peak of the signal. This is because the low-field line of the 'internal' EPR signal (Fig. 1, spectrum d) is less broadened compared to other lines.

Analytical approximations of the functions $\alpha(C_{in})$ and $\beta(C_{in})$ can be found by fitting experimental data (Fig. 10):

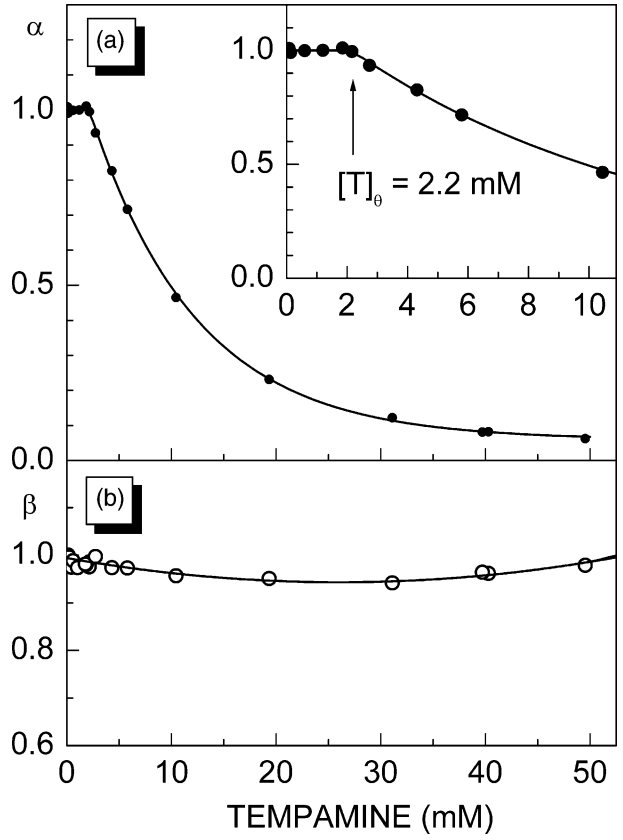

Fig. 10. Concentration dependence of parameters α and β (see details in text) for TEMPAMINE in water solution.

$$\alpha = 1 + \frac{0.94\{\exp[(2.2 - C_{in})/10.2] - 1\}}{\exp[(2.2 - C_{in})/0.001] + 1}, \quad (11)$$

$$\beta = 1 + \frac{7.5 \times 10^{-5} C_{in}^2 - 0.004 C_{in}}{\exp[(C_{in} - 52)/0.5] + 1}, \quad (12)$$

where C_{in} is the milimolar (mM) concentration of TA inside the thylakoids. The coefficient f_0 can be found as

$$f_0 = A_{pp}^{in}/A_{pp}^{out} = (\Delta H_{pp}^{out}/\Delta H_{pp}^{in})^2, \quad (13)$$

where ΔH_{pp}^{out} and ΔH_{pp}^{in} are the peak-to-peak linewidths of the EPR lines for TA molecules localized outside and inside the thylakoids, respectively. In order to rule out the possible influence of the broadening agent on the ΔH_{pp}^{in} value [27], we measured the 'internal' EPR signals at different concentrations of CrOx. For the low-field line, the extrapolation of the plot ΔH_{pp}^{in} vs [CrOx] to the low limit of CrOx concentration gives $\Delta H_{pp}^{in} = 1.81$ G (Fig. 11). Taking into account that $\Delta H_{pp}^{out} = 1.74$ G, we obtained that $f_0 = 0.92$.

Having determined the function $F(C_{in})$, we could describe numerically experimental dependencies of parameter ΔA on the concentration of TA in a chloroplast suspension C_0 (Fig. 9). It should be mentioned that thylakoids are good osmometers, therefore, their internal volume could change in the course of chloroplast illumination. In order to avoid data scattering due to the light-induced swelling or shrinkage of thylakoids, we always used the plots ΔA vs C_0 for steady-state values of parameter ΔA that corresponded to the condition $V_{in} = \text{const}$. Theoretical curves (ΔA vs C_0) were calculated according to Eqs. (9)–(12). Using the Levenberg–Marquardt minimization algorithm [44] for fitting

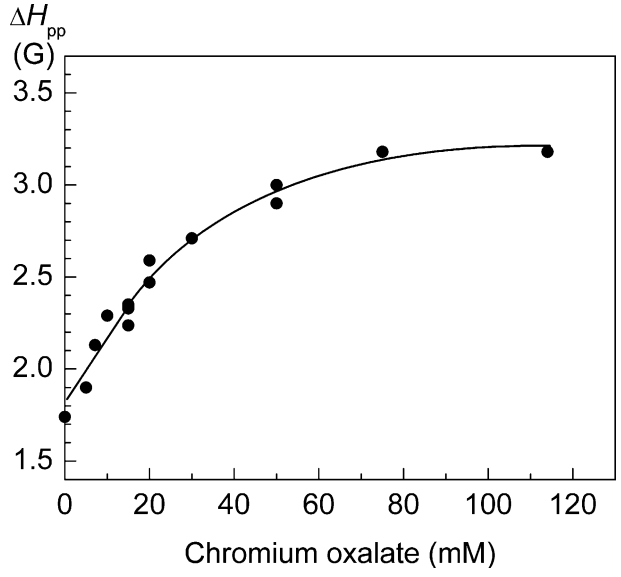

Fig. 11. Effect of chromium oxalate on the linewidth of the low-field component of the difference EPR signal of TEMPAMINE inside the thylakoids.

Table 1

Metabolic state	Non-cyclic electron transport		Cyclic electron transport	
	ΔpH	$(V_{in}/V) \times 100$	ΔpH	$(V_{in}/V) \times 100$
State 3 (+4 mM Mg–ADP)	1.96 ± 0.02	0.93 ± 0.09	1.95 ± 0.16	0.29 ± 0.14
State 4 (+20 μM Mg–ATP)	2.54 ± 0.05	0.68 ± 0.17	2.54 ± 0.06	0.31 ± 0.05

theoretical curves to experimental data (Fig. 9), we found parameters ΔpH and V_{in}/V for chloroplasts functioning in different metabolic states (Table 1).

4. Discussion

Results of our experiments with chloroplasts suspended in the media of different osmolarity demonstrated that an essential body of intrathylakoid TA molecules could be attributed to spin labels dissolved in the aqueous osmotic volume of chloroplasts, i.e., in the thylakoid lumen. The amounts of TA molecules localized inside the thylakoids in dark adapted chloroplasts (Fig. 3) or taken up by illuminated chloroplasts (Fig. 6) were sensitive to variations of a sucrose concentration in the suspending medium. This result showed that most of the intrathylakoid TA molecules were localized in the osmotic volume. The rotational mobility of TA inside the thylakoids was almost ten times slower ($\tau_c \approx 0.4$ ns) compared to TA outside the thylakoids ($\tau_c \approx 0.04$ ns). The restricted motion of TA inside the thylakoids demonstrated that the viscosity of the aqueous medium in the bulk of the lumen was markedly higher compared to bulk water outside the thylakoids. This characteristics of the thylakoid lumen seems to be one of the basic properties typical of the cell interior [43]. The ordered state of the intrathylakoid water was considered as one of the possible reasons for maintaining the viscous environment of TA inside the thylakoids [21–23]. A relatively high viscosity of TA surroundings could be also explained by the influence of different constituents of the thylakoid (membranes, biopolymers) confined to a rather small volume [21,25]. Certain peculiarities of the 'internal' EPR signal (higher amplitude of the low-field line compared to the central and high-field lines, Fig. 1) might indicate [45] that TA molecules perform slightly anisotropic rotations inside the thylakoids. It was suggested earlier [21,25] that an anisotropy of TA rotations inside the thylakoids might be explained by TA interactions with the inner surface of the thylakoid membrane.

If TA binding to the membrane were the main reason for the hindrance to TA rotation inside the thylakoids, then the apparent correlation time τ_c should strongly depend on the osmotic volume of thylakoids. Actually, in this case the 'internal' EPR signal would represent the superposition of two signals, one signal from TA molecules rapidly rotating in the bulk of the lumen and another signal from immobilized membrane-bound TA molecules. It means that the shape of the 'internal' EPR signal will depend on the ratio N_{mem}/N_{bulk}, where N_{mem} is the number of TA molecules immobilized on the inner surface of the thylakoid membrane and N_{bulk} is the number of unbound TA molecules moving in the bulk phase of the lumen. It is easy to demonstrate that the ratio N_{mem}/N_{bulk} should be reciprocal to the osmotic volume of thylakoids, V_{in}. Let the interaction of TA molecules with the membrane, $(TA)_{bulk} + M \leftrightarrow (TA)_{mem}$, is described by the equation of Langmuir adsorption:

$$\frac{N_{mem}}{MC_{bulk}} = K, \tag{14}$$

where M is the surface density of unoccupied groups on the membrane that can bind TA molecules, C_{bulk} is the concentration of TA in the bulk of the lumen, and K is the equilibrium constant. The number of free (unbound) TA molecules in the bulk phase equals to $N_{bulk} = C_{bulk}V_{in}$. The number of membrane-bound TA molecules equals to $N_{mem} = M_0 - M$, where M_0 is the surface density of TA binding groups on the inner surface of the membrane. From Eq. (14) we obtain

$$\frac{N_{mem}}{N_{bulk}} = \frac{KM_0}{(1 + KC_{bulk})V_{in}}. \tag{15}$$

According to Eq. (15), a portion of slow rotating spin labels interacting with the membrane should increase with lowering the internal volume V_{in}. Therefore, if TA binding to the membrane were the main reason for slowing down the rotation of spin label then one could expect that osmotically induced shrinkage of thylakoids would lead to an increase in the apparent correlation time τ_c. However, we showed that the rotational mobility of TA inside the thylakoids did not change (within experimental error) with the essential decrease in the internal volume V_{in} (Fig. 3). This result means that TA binding to the membrane cannot entirely explain the restricted rotational motion of TA in the thylakoid lumen. Another evidence that the slowing down of TA rotation cannot be attributed entirely to interactions of spin labels with the membrane or other thylakoid constituents were presented in [13,21]. Thus, we conclude that the vast majority of TA molecules inside the thylakoids are localized in the bulk of the lumen. It means that TA can serve the role of an adequate probe for measuring the bulk-to-bulk ΔpH.

A general method for measuring the ΔpH in chloroplasts developed in this work is based on the effect of

the concentration-dependent broadening of the EPR spectrum of TA. Measuring the signal behavior at different levels of microwave power (Fig. 5), we could discriminate between the line broadening effect caused by TA concentrating inside the thylakoids, the loss of TA paramagnetism, and the influence of oxygen on the EPR signal. Reversible light-induced changes in the height of the signal (Fig. 5a, parameter ΔA) were attributed to the line broadening due to frequent collisions of TA molecules inside the thylakoids. The threshold nature of the broadening effect (Fig. 7) was used to quantify the concentration of TA molecules accumulated in the thylakoid lumen. The non-linear curve fit of experimental data (ΔA vs C_0) based on the Levenberg–Marquardt minimization algorithm [44] enabled us to find two important bioenergetic parameters, ΔpH and V_{in} (Table 1).

In the metabolic State 4 (basal electron transport, without added ADP) we obtained $\Delta pH = 2.54$ (at the external $pH = 7.8$). The same value of ΔpH in the thylakoids of bean chloroplasts was obtained earlier by so called 'kinetic' method [7,38], or by measuring the intrathylakoid pH_{in} with pH-sensitive imidazoline radicals [46]. This result is also consistent with earlier measurements of ΔpH by conventional methods based on the use of penetrating amines [10–12,47–49]. For instance, Giersch et al. [49] reported the value $\Delta pH = 2.24$ (external $pH = 7.62$) across the thylakoid membrane of intact chloroplasts calculated from the distribution of ^{14}C-labeled methylamine and dimethyloxazolidinedione. Under photophosphorylation conditions (electron transport coupled to ATP synthesis, State 3), we determined lower pH gradient ($\Delta pH \cong 1.96$). This result can be explained by the efflux of protons from the thylakoid lumen through operating ATP synthase complexes. It is important that concentrations of spin label used to determine ΔpH are not high ($\leqslant$0.3–0.4 mM), so that the uncoupling effect of TA can be neglected [21,31].

Similar values of ΔpH were obtained for chloroplasts functioning during non-cyclic or PMS-mediated cyclic electron transport (Table 1). On the other hand, the volumes of internal compartments, where TA molecules accumulated, were essentially different (Table 1). Under the conditions of non-cyclic electron transport, we obtained the ratio $V_{in}/V \approx 0.007$–0.009, whereas under the conditions of cyclic electron transport this ratio was estimated as $V_{in}/V \approx 0.003$. Such a difference can be explained [7,26,50] by the lateral heterogeneity of thylakoids in higher plant chloroplasts. It is well-known that most of photosystem 1 complexes are embedded into the membranes of stroma-exposed thylakoids, while the vast majority of photosystem 2 complexes are localized in the thylakoids of grana [51–53]. Therefore, during linear (non-cyclic) electron transport, driven by simultaneously collaborating photosystem 1 and photosystem 2, TA molecules were actively taken up by thylakoids of both kinds, stroma- and grana-exposed thylakoids. On the other hand, the contribution of grana-exposed thylakoids to TA uptake was reduced during cyclic electron transport, mediated by PMS around the photosystem 1 in the presence of DCMU—an inhibitor of photosystem 2. Because most of photosystem 2 complexes localized in the thylakoids of grana were inactive during cyclic electron transport, the efficient volume of compartments accumulating TA molecules was smaller ($V_{in}/V \approx 0.003$) as compared to non-cyclic electron transport ($V_{in}/V \approx 0.007$–0.009) driven by both photosystems.

5. Conclusions

We developed a general method for measuring the transmembrane pH difference in chloroplasts based on the effect of the concentration-dependent broadening the EPR signal of water soluble spin label TA accumulating inside the thylakoids. It was demonstrated that most of TA molecules taken up by chloroplasts were localized in the bulk of the thylakoid lumen, i.e., TA could serve the role of a probe for the bulk-to-bulk ΔpH. Using the threshold nature of the broadening effect, we suggested a method for quantifying the concentration of TA inside the thylakoids. This approach allowed two important bioenergetic parameters, ΔpH and intrathylakoid volume V_{in} to be determined.

Acknowledgments

This work was supported by Grant 03-04-48981 from the Russian Foundation for Basic Researches, and Grants 99-1086 and 01-483 from the INTAS. The authors thank Dr. M.K. Aliev and Dr. S.K. Chamorovsky for critical reading of the manuscript.

References

[1] P. Mitchell, Biol. Rev. 41 (1966) 445–502.
[2] D.G. Nickolls, Bioenergetics. An Introduction to the Chemiosmotic Theory, Academic Press, New York, 1982.
[3] V.P. Skulachev, Membrane Bioenergetics, Springer, New York, 1988.
[4] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of Biochemistry, John Wiley and Sons, New York, 1998.
[5] R.E. Blankenship, Molecular Mechanisms of Photosynthesis, Blackwell Science, 2002.
[6] R.A. Dilley, Curr. Top. Bioenergetics 16 (1991) 265–318.
[7] L.A. Blumenfeld, A.N. Tikhonov, Biophysical Thermodynamics of Intracellular Processes. Molecular Machines of the Living Cell, Springer, New York, 1994.
[8] D.M. Kramer, C.A. Sacksteder, J.A. Cruz, Photosynth. Res. 60 (1999) 151–163.
[9] G. Kaim, P. Dimroth, EMBO J. 18 (1999) 4118–4127.

[10] H. Rottenberg, T. Grunwald, M. Avron, Eur. J. Biochem. 25 (1972) 54–63.
[11] S. Schuldiner, H. Rottenberg, M. Avron, Eur. J. Biochem. 25 (1972) 64–70.
[12] H. Rottenberg, T. Grunwald, Eur. J. Biochem. 25 (1972) 71–74.
[13] A.T. Quintanihla, R.J. Mehlholrn, FEBS Lett. 91 (1978) 161–165.
[14] D.S. Cafiso, W.L. Hubbel, Biochemistry 17 (1978) 3871–3877.
[15] R.J. Mehlhorn, P. Candau, L. Packer, Methods Enzymol. 88 (1982) 751–762.
[16] W. Junge, W. Auslander, A.J. McGeer, T. Runge, Biochim. Biophys. Acta 546 (1979) 121–141.
[17] W. Junge, Curr. Top. Membr. Transp. 16 (1982) 431–463.
[18] J.S. Lolkema, K.J. Hellingwert, W.N. Konigs, Biochim. Biophys. Acta 681 (1982) 85–94.
[19] A.B. Hope, D.B. Matthews, Aust. J. Plant Physiol. 12 (1985) 9–19.
[20] F.A. De Wolf, B.H. Groen, L.P.A. Van Houte, F.A.L.J. Peters, K. Krab, R. Kraayenhof, Biochim. Biophys. Acta 809 (1985) 204–214.
[21] S.P. Berg, D.M. Lusczakoski, P.D. Morse II, Arch. Biochem. Biophys. 194 (1979) 138–148.
[22] D.M. Nesbitt, S.P. Berg, Biochim. Biophys. Acta 593 (1980) 353–361.
[23] D.M. Nesbitt, S.P. Berg, Biochim. Biophys. Acta 679 (1982) 169–174.
[24] B.A. Melandri, R.J. Mehlhorn, L. Packer, Arch. Biochim. Biophys. 235 (1984) 97–105.
[25] A.N. Tikhonov, A.A. Timoshin, Biol. Membranes (USSR) 2 (1985) 608–622.
[26] S.G. Magnitsky, A.N. Tikhonov, Biophysics (Russia) 40 (1995) 347–358.
[27] S.G. Magnitsky, M. Masarova, A.N. Tikhonov, Curr. Top. Biophys. 20 (1996) 25–30.
[28] S.G. Magnitsky, A.N. Tikhonov, Biophysics (Russia) 43 (1998) 69–76.
[29] B. Wille, Biochim. Biophys. Acta 936 (1988) 513–530.
[30] X. Wan, T.-C. Fu, A. Funk, R.E. London, Brain Res. Bull. 36 (1995) 91–96.
[31] A. Wasniowska, W.K. Subczynski, A.N. Tikhonov, Curr. Top. Biophys. 26 (2002) 83–91.
[32] N. Kocherginsky, H.M. Swartz, Nitroxide Spin Labels: Reactions in Biology and Chemistry, CRC Press, Roca Raton, 1995.
[33] S. Belkin, R.J. Mehlhorn, L. Packer, Arch. Biochem. Biophys. 252 (1987) 487–495.
[34] J.S. Hyde, W.K. Subczynski, in: L.J. Berliner, J. Rueben (Eds.), Biological Magnetic Resonance, vol. 8, Spin Labeling. Theory and Applications, Plenum, NY, 1989, pp. 399–425.
[35] K. Strzalka, T. Walczak, T. Sarna, H.M. Swartz, Arch. Biochem. Biophys. 281 (1990) 312–318.
[36] A. Ligeza, A. Wisniewska, W.K. Subczynski, A.N. Tikhonov, Biochim. Biophys. Acta 1186 (1994) 201–208.
[37] A. Ligeza, A.N. Tikhonov, J.S. Hyde, W.K. Subczynski, Biochim. Biophys. Acta 1365 (1998) 453–463.
[38] A.N. Tikhonov, G.B. Khomutov, E.K. Ruuge, L.A. Blumenfeld, Biochim. Biophys. Acta 637 (1981) 321–333.
[39] Y.N. Molin, K.M. Salikhov, K.I. Zamaraev, Spin Exchange: Principles and Applications in Chemistry and Biology, Springer-Verlag, Berlin, 1980.
[40] J.J. Labsky, J. Pilar, J. Lovy, J. Magn. Reson. 37 (1980) 515–522.
[41] J.J. Windle, J. Magn. Reson. 45 (1981) 432–439.
[42] B. Bales, in: L.J. Berliner, J. Rueben (Eds.), Biological Magnetic Resonance, vol. 8, Spin Labeling. Theory and Applications, Plenum, NY, 1989, pp. 77–130.
[43] A.D. Keith, W. Snipes, Science 183 (1974) 666–668.
[44] J.J. More, in: G.A. Watson (Ed.), Lecture Notes in Mathematics, vol. 630, Springer-Verlag, Heidelberg, 1995, pp. 105–116.
[45] A.M. Vasserman, A.N. Kuznetsov, A.N. Kovarskii, A.L. Buchachenko, Z. Struct. Chim. 12 (1971) 609–616.
[46] B.V. Trubitsin, I.A. Grigor'ev, A.N. Tikhonov, Abstracts of 3rd International Conference on Nitroxide Radicals, Kaiserslautern, Germany, 2001, p. 17.
[47] R.E. McCarty, in: Encyclopedia of Plant Physiology, New Series, Transport in Plants III, Springer-Verlag, Heidelberg, 1976, pp. 347–376.
[48] H.W. Heldt, K. Werdan, M. Milovancev, G. Geller, Biochim. Biophys. Acta 314 (1973) 224–241.
[49] C. Giersch, U. Heber, Y. Kobayashi, Y. Inoue, K. Shibata, H.W. Heldt, Biochim. Biophys. Acta 590 (1980) 59–73.
[50] A.N. Tikhonov, L.A. Blumenfeld, Biophysics (USSR) 30 (1985) 527–537.
[51] J.M. Anderson, W. Haehnel, FEBS Lett. 146 (1982) 13–17.
[52] J.M. Anderson, A. Melis, Proc. Natl. Acad. Sci. USA 80 (1983) 745–749.
[53] P.-Å. Albertsson, Trends Plant Sci. 6 (2001) 349–354.
[54] J.C. Bailar Jr., E.M. Jones, Inorg. Synth. 1 (1939) 35–38.