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Spin-probes designed for measuring the intrathylakoid pH in chloroplasts

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Abstract

Nitroxide radicals are widely used as molecular probes in different fields of chemistry and biology. In this work, we describe pH-sensitive imidazoline- and imidazolidine-based nitroxides with p*K* values in the range 4.7–7.6 (2,2,3,4,5,5-hexamethylperhydroimidazol-1-oxyl, 4-amino-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-1-oxyl, 4-dimethylamino-2,2-diethyl-5,5-dimethyl-2,5-dihydro-1*H*-imidazol-1-oxyl, and 2,2-diethyl-5,5-dimethyl-4-pyrrolidyline-1-yl-2,5-dihydro-1*H*-imidazol-1-oxyl), which allow the pH-monitoring inside chloroplasts. We have demonstrated that EPR spectra of these spin-probes localized in the thylakoid lumen markedly change with the light-induced acidification of the thylakoid lumen in chloroplasts. Comparing EPR spectrum parameters of intrathylakoid spin-probes with relevant calibrating curves, we could estimate steady-state values of lumen pH_{in} established during illumination of chloroplasts with continuous light. For isolated bean (*Vicia faba*) chloroplasts suspended in a medium with $pH_{out}=7.8$, we found that $pH_{in}\approx 5.4–5.7$ in the state of photosynthetic control, and $pH_{in}\approx 5.7–6.0$ under photophosphorylation conditions. Thus, ATP synthesis occurs at a moderate acidification of the thylakoid lumen, corresponding to transthylakoid pH difference $\Delta pH\approx 1.8–2.1$. These values of ΔpH are consistent with a point of view that under steady-state conditions the proton gradient ΔpH is the main contributor to the proton motive force driving the operation of ATP synthesis, provided that stoichiometric ratio H^+/ATP is $n\geq 4–4.7$.

Keywords: Chloroplast; Electron paramagnetic resonance; Spin-probes; Transthylakoid proton gradient

1. Introduction

The transmembrane difference in electrochemical proton potentials, $\Delta\mu_{H^+}$, is one of the most important parameters of membrane bioenergetics [1–7]. There is general agreement that photosynthetic and oxidative phosphorylation is driven by the proton motive force $\Delta\mu_{H^+}$ as proposed by Peter Mitchell [1,2]. The transmembrane proton potential difference $\Delta\mu_{H^+}$ is not only the driving force for ATP synthesis but also serves as a regulatory factor controlling electron transport in energy transducing membranes (see for review [3–11]). The proton motive force $\Delta\mu_{H^+}$ consists of two components, the transmembrane difference in the activities of protons inside and outside the thylakoids (expressed as ΔpH) and the transmembrane electric potential difference ($\Delta\psi$) Both components of $\Delta\mu_{H^+}$ are competent as the driving force to actuate the operation of the ATP synthase machinery in chloroplasts [12–14]. It has become the textbook view that in chloroplasts, in contrast to mitochondria and bacteria, the transthylakoid proton gradient ($\Delta pH=pH_{out}-pH_{in}$) provides the main contribution to $\Delta\mu_{H^+}$ [3–7]. However, despite a general agreement that ΔpH is the main component of proton motive force in chloroplasts, there is no consensus in the literature about the ΔpH values established during steady-state photosynthesis in chloroplasts. The data available is often ambiguous, supporting either a moderate acidification of the intrathylakoid volume ($pH_{in}\sim 5.8–6.5$) or strongly acidic lumen ($pH_{in}<5$) (see [10] for review). The light-induced generation of significant transthylakoid pH difference ($\Delta pH>3–3.5$) associated with strong acidification of the thylakoid lumen in chloroplasts *in vitro* was reported in most of

Abbreviations: PS1 and PS2, Photosystem 1 and photosystem 2, respectively; P_{700}, primary electron donor of photosystem 1; PQ, plastoquinone; PQH_2, plastoquinol; EPR, electron paramagnetic resonance; MV, methylviologen

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earlier works [15–20]. Experimental evidence for moderate light-induced acidification of the thylakoid lumen appeared in subsequent works on ΔpH measurements in chloroplasts [21–23]. Analyzing the arguments *pro* and *contra* of high ΔpH values in photosynthetic systems *in vivo*, Kramer and co-workers suggested that pH_{in} should not drop below $pH_{in} \approx 5.8$ [10,11]. Convincing experimental evidence in favor of a moderate acidification of the intrathylakoid lumen pH came from pH_{in} estimates from $P_{700}^{\bullet+}$ re-reduction kinetics in higher plant chloroplasts *in vitro* [8,21,22] and *in situ* [24–26], as well as from measurements of ΔpH with spin-probes in isolated chloroplasts functioning under photophosphorylation conditions [23,27,28]. One of the reasons for appreciable scattering of ΔpH estimates in chloroplasts could be variations of experimental conditions, e.g., osmolarity and salt composition of a chloroplasts suspending medium [29–31].

There is also a peace of evidence that the contribution of electric potential difference $\Delta\psi$ to the proton potential $\Delta\mu_{H^+}$ in chloroplasts cannot be ignored, at least under some experimental conditions [32–34]. Another point of debates in the literature concerns the nature of the proton motive force driving ATP synthesis. In accordance with the orthodox Mitchell's point of view, only delocalized proton gradients (not constrained to membrane domains) are required for proton-driven ATP formation [1,2]. The alternative possibility of membrane-localized proton gradients was suggested by Williams [35,36]. There is evidence that under certain experimental conditions the essential portion of protons consumed by illuminated chloroplasts are constrained to localized domains within enclosed thylakoids as envisioned by chemiosmosis [37–41]. Thus, the question 'How acidic is the lumen?' [10] is still valid for bioenergetics.

Quantitative determination of ΔpH in chloroplasts is not a trivial task because of a small internal volume of thylakoids. There is a great deal of confusion concerning uncertainties over the accuracy and applicability of different methods for measuring ΔpH in energy transducing organelles. The two most frequently used methods for ΔpH measurements are based on pH-indicating probes (see for review [19,20]). These methods are (i) calculation of ΔpH from the partitioning of permeable amines between the vesicle interior and suspending medium, and (ii) measurement of the spectral response of pH-sensitive indicators loaded into the vesicles. 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, there are difficulties in the quantitative estimation of $\Delta\mu_{H^+}$ by molecular pH-probes because these may have secondary effects. For instance, many of widely used molecular pH-indicators (e.g., 9-aminoacridine, neutral red [42–46]) are not really adequate probes for quantifying the bulk-to-bulk phase ΔpH in chloroplasts because of their interaction with thylakoid membranes. According to [44], the binding of pH-indicating molecules to the membrane can lead to essential overestimations of ΔpH (up to 1–1.5 pH units). Another problem is the masking interference of the optical spectra of pH-probes and photosynthetic pigments.

EPR spin-probes have already demonstrated their potential for monitoring the intrathylakoid pH in chloroplasts (see [47] for references). A practical usage of chemically stable nitroxides as pH-probes is based on the sensitivity of their EPR spectra to the environment (solvent polarity, pH, and microviscosity of the local surroundings). Among a variety of different spin-probes, the nitroxide radicals with the proton-accepting groups (the amine-type [23,27,28,48–52], imidazoline- and imidazolidine-based nitroxides [53–57]) are of special interest as the reporters of the intrathylakoid pH. In particular, in our previous works [27,28], we described a new method for quantifying the ΔpH value in chloroplasts from partitioning 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempamine) between the thylakoid lumen and external volume. This approach is based on the threshold effect of concentration-depending broadening of the EPR signal from Tempamine accumulating in the lumen in response to generation of ΔpH. One of the advantages of a spin-probe technique for measuring pH is that EPR spectra of nitroxides have distinct features that enable one to discriminate them from EPR signals given by other paramagnetic species.

The main purpose of this paper is to provide the background and to develop a method for measuring the intrathylakoid pH in chloroplasts with pH-sensitive spin-probes which EPR spectra change with protonation/deprotonation. Previously, we reported about synthesis of a number of novel imidazoline- and imidazolidine-based pH-sensitive nitroxides [54–56] and investigated their interactions with chloroplasts [57]. In the present study, we describe how the nitroxides of this kind with appropriate pK_a values can be used for quantitative measurements of the lumen pH (pH_{in}) in chloroplasts.

2. Materials and methods

2.1. Chloroplasts

Bean plants (*Vicia faba*) were grown in a greenhouse at a growth temperature of 20–22 °C. Chloroplasts were isolated from 2–3 weeks old leaves as described earlier [21]. Isolated class B chloroplasts (ac. 4–5 mg chlorophyll/ml) were suspended in the medium containing 50 mM sucrose, 2 mM $MgCl_2$, 10 mM P_i, and 15 mM HEPES–NaOH (pH 8.0). 20 μM methyl viologen (MV) was used as a mediator of the linear (pseudocyclic) electron transport from water to oxygen ($H_2O \rightarrow PS2 \rightarrow PS1 \rightarrow MV \rightarrow O_2$). Reaction media used for EPR measurements usually contained additionally either 4 mM MgADP (metabolic state 3) or 20 μM MgATP (metabolic state 4). Concentrations of nitroxides used for pH_{in} measurements were not higher than 1.0 mM.

2.2. Spin-probes

Among a variety of pH-sensitive nitroxides described in [57], we have chosen four pH-sensitive spin-probes: 2,2,3,4,5,5-hexamethylperhydroimidazol-1-oxyl (HMI); 4-amino-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (ATI); 4-dimethylamino-2,2-diethyl-5,5-dimethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-4); and 2,2-diethyl-5,5-dimethyl-4-pyrrolidyline-1-yl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-5). Chemical structures of these nitroxides are shown in Fig. 1. These radicals should be suitable for monitoring the light-induced changes in the intrathylakoid pH_{in}, because their pK_a values fall in the physiologically important range from 4.7 to 7.6. Chemical aspects of spin-probe synthesis are described in [54–57]. The abbreviations for nitroxides shown in Fig. 1 are adopted from [57].

2.3. EPR measurements

EPR spectra were measured with a Varian E-4 X-band spectrometer. Suspension of chloroplasts was placed in a standard flat quartz cuvette (3 × 40 mm; internal thickness, 0.1 mm) positioned in the rectangular TE_{102} cavity of the

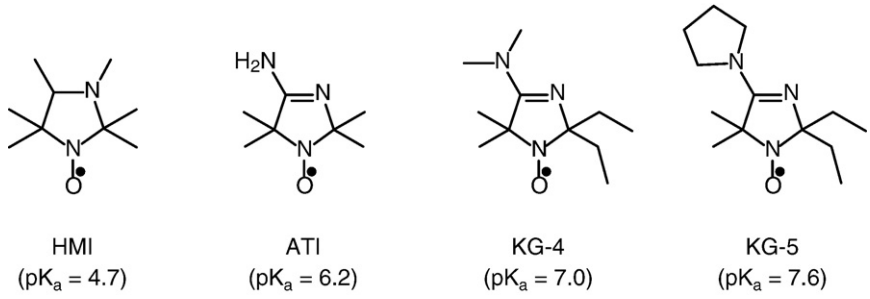

Fig. 1. Structures of imidazoline- and imidazolidine-based nitroxides used for measuring the intrathylakoid pH: 2,2,3,4,5,5-hexamethylperhydroimidazol-1-oxyl (HMI); 4-amino-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (ATI); 4-dimethylamino-2,2-diethyl-5,5-dimethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-4); 2,2-diethyl-5,5-diemethyl-4-pyrrolidyline-1-yl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-5).

EPR spectrometer. In order to maintain controlled aerobic conditions in a chloroplasts suspension, we also used oxygen-permeable plastic tubes (TFE tubes from Zeus Inc.: I.D. 0.635 mm, wall thickness 0.051 mm). Samples were illuminated with subsaturating white light from a 100 W tungsten lamp (~20 W/m^2 at the surface of EPR cuvette); infrared light was cut off with a 5-cm layer of water. Our control measurements demonstrated that during illumination of chloroplasts for 30 s (this illumination time was typical in our experiments) the temperature of a sample placed into a quartz cuvette was maintained constant (within the accuracy limit of 0.5 °C).

EPR signals from nitroxide radicals were recorded at room temperature (20–22 °C) at subsaturating microwave power 10 mW and modulation amplitude 0.25 G. EPR signal from $P_{700}^{\bullet+}$ of oxidized reaction centers of PS1 was recorded with subsaturating microwave power of 10 mW and a modulation amplitude of 4 G. For monitoring the time course of the light-induced redox transients of P_{700}, the magnetic field was fixed, as a rule, at the low-field extremum of the EPR signal from $P_{700}^{\bullet+}$ (signal I). The concentration of P_{700} in a chloroplast suspension used for EPR measurements of pH_{in} was ~ 4 μM (or ac. 2 mg/ml of chlorophyll).

2.4. Visualization of EPR signals from spin-probes localized inside the thylakoids

In order to register EPR signals from nitroxides localized inside the thylakoids ('internal' EPR signals), we used traditional EPR line broadening technique [28,51,52]. Extraction of the 'internal' EPR signal from the total EPR signal was performed with the aid of chromium oxalate (CrOx), membrane-impermeable paramagnetic agent added to a chloroplast suspension. Colliding with spin-probe molecules localized outside the thylakoids, paramagnetic molecules CrOx markedly broaden the 'external' EPR signal, whereas the 'internal' EPR signal remained practically unaffected by membrane-impermeable CrOx molecules. The 'internal' EPR signal was obtained by subtracting the background signal (EPR signal of spin-probe molecules dissolved in a suspending medium containing CrOx) from the total EPR signal in a chloroplast suspension. In order to make correction for volume excluded by chloroplasts (thylakoid membranes + lumen), we normalized an amplitude of the broadened 'background' EPR signal (buffer + spin-probe + CrOx) with regard to broadened 'external' EPR signal observed in a chloroplast suspension (chloroplasts + spin-probe + CrOx). Note that the procedure of equalizing the amplitudes of 'external' and 'background' signals becomes possible due to a clear-cut distinction between broadened 'external' EPR signal and relatively narrow 'internal' signal which remains unaffected by CrOx.

In order to minimize a potential influence of CrOx on the 'internal' EPR signal, the final concentration of CrOx was ≤15 mM. As we have demonstrated earlier [28], at low concentrations of CrOx (≤15–30 mM) its penetration into the lumen is negligible, so CrOx should not disturb the line shape of the 'internal' EPR signal. Also, we have demonstrated that the addition of CrOx (at least up to 30 mM) did not influence the rate of ATP synthesis in chloroplasts (for more details see Section 3.4).

Previously, we have shown that the loss of paramagnetism of spin-probes HMI, ATI, KG-4, and KG-5 due to interactions of these nitroxides with the chloroplasts is not significant, provided that the efflux of electrons from PS1 to the terminal electron acceptor does not limit the noncyclic (pseudocyclic) electron transport in chloroplasts [57]. The latter condition is fulfilled in the presence of molecular oxygen — a terminal electron acceptor of PS1 in Class B chloroplasts. In order to avoid the light-induced depletion of oxygen, we added catalase (~ 10 U/ml) to chloroplasts. In order to make corrections for potential distortions of EPR signals that might arise from the light-induced reduction of spin-probes, we usually averaged EPR spectra recorded upon scanning magnetic field in two directions, backward and forward.

2.5. Assay of chloroplast activity

Photosynthetic activity of chloroplasts was assayed by measuring the light-induces transients of the EPR signal from $P_{700}^{\bullet+}$ as described earlier [21,22]. The rate of photophosphorylation, J_{ATP}, was measured either by conventional potentiometric method [58] and/or by 'kinetic' method. The latter approach is based on the dependence of the rate of electron transfer between PS2 and PS1 on the chloroplast metabolic state. Transition from metabolic state 3 (excess of ADP in the pool of adenine nucleotides) to the state of photosynthetic control (state 4, exhausted pool of ADP) can be detected from the peculiarities in the kinetics of P_{700} redox transients [21]. The rate of ADP phosphorylation was evaluated by measuring a characteristic time, $\Delta\tau$, preceding the metabolic transition state 3 → state 4. Taking into account that this transition occurs concomitantly with the exhaustion of most of added ADP, we could evaluate the rate of ATP synthesis as $J_{ATP}=[ADP]_0/\Delta\tau$, where $[ADP]_0$ is the concentration of MgADP added to chloroplasts. Details of measuring the kinetic parameter $\Delta\tau$ are described below (Section 3.3). Note that EPR measurements of P_{700} redox transients allowed us the monitoring of metabolic state to be performed under experimental conditions similar to that used for ΔpH measurements with spin-probes. Since a kinetic method is not suitable for estimating J_{ATP} in the presence of spin-probe molecules (because of superposition of EPR signals from $P_{700}^{\bullet+}$ and nitroxides), we assayed the effects of spin-probes on J_{ATP} by potentiometric method [58].

2.6. Reagents

Chromium oxalate was synthesized according to [59]. Other reagents were purchased from 'Sigma'.

3. Results and discussion

3.1. EPR properties of pH-sensitive nitroxides in bulk water

Fig. 2 illustrates how EPR signals from spin-probes HMI, ATI, KG-4, and KG-5 dissolved in a suspending medium change with variations of pH. In two extreme cases, $pH \ll pK_a$ or $pH \gg pK_a$, when most of nitroxide molecules exist either in protonated or in deprotonated form, each EPR spectrum represents a triplet given by rapidly tumbling nitroxides ($\tau_c \cong 4 \cdot 10^{-11}$ s). The triplet signal

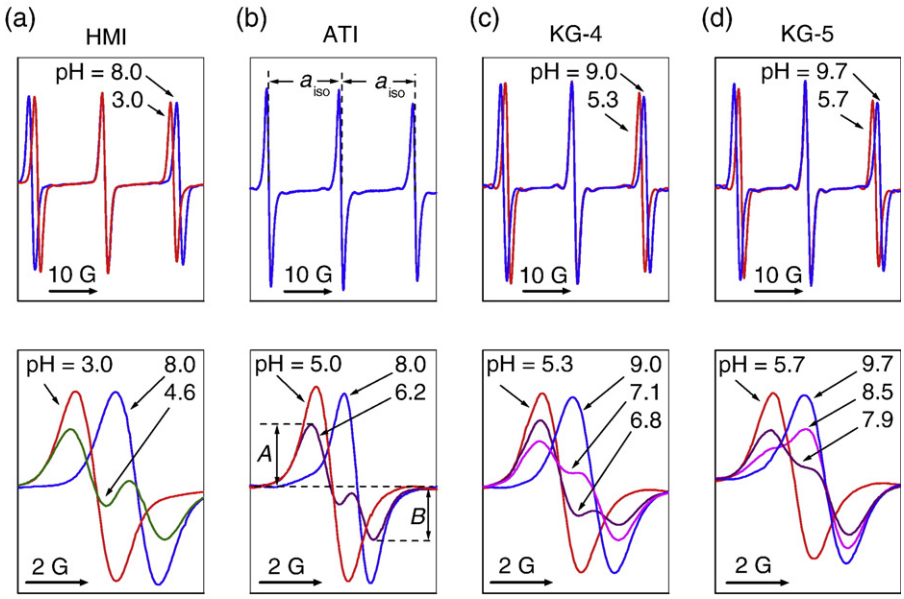

Fig. 2. Effects of pH on EPR spectra of pH-sensitive nitroxides in water solution. (a) 2,2,3,4,5,5-Hexamethylperhydroimidazol-1-oxyl (HMI). (b) 4-Amino-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (ATI). (c) 4-Dimethylamino-2,2-diethyl-5,5-dimethyl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-4). (d) 2,2-Diethyl-5,5-diemethyl-4-pyrrolidyline-1-yl-2,5-dihydro-1*H*-imidazol-1-oxyl (KG-5).

arises due to the Fermi contact interaction of the unpaired electron with the nitrogen nucleus of the nitroxide fragment, which is characterized by the isotropic hyperfine splitting constant, a_{iso}. As one can see from Fig. 2, protonation of nitroxides induces a noticeable decrease in a_{iso}. Note that in aerated water solution, each component of the triplet signal represents a single line with unresolved multiplet structure, because the hyperfine splittings from the protons of spin-probe molecules are masked due to the broadening effect of paramagnetic O_2 molecules.

In the intermediate range of pH (pH ~ pK_a), each EPR spectrum represents the superposition of two signals, from protonated and deprotonated forms of nitroxides, indicating a slow proton exchange reaction [60–62]:

$$R^{\bullet}H^{+} + B^{-} \leftrightarrow R^{\bullet} + BH, \quad (1)$$

where $R^{\bullet}H^{+}$ and $R^{\bullet}$ stand for protonated and deprotonated forms of a spin-probe, B^{-} and BH denote deprotonated and protonated forms of buffer molecules in a water solution. For routine evaluation of a fraction of protonated nitroxides, one can use the ratio $f = A / (A + B)$, where A and B are the amplitudes of the high-field peaks related to protonated and deprotonated forms of a spin-probe (see Fig. 2b for definitions). From pH-dependences of the partition parameter f we have determined pK_a values for nitroxides used in this work: pK_a=4.7 for HMI, pK_a=6.2 for ATI, pK_a=7.0 for KG-4, and pK_a=7.6 for KG-5 (for more details see Ref. [57]).

3.2. Light-induced changes in the EPR spectra of nitroxides localized inside the thylakoids

Water soluble nitroxides penetrate the thylakoids, forming a partition between the intrathylakoid volume (lumen) and external solution. EPR signals from spin-probe molecules localized in the lumen ('internal' EPR signals) were visualized using the line broadening technique. Each 'internal' EPR signal was obtained as the difference between a total EPR signal (chloroplasts + spin-probe + CrOx) and corresponding 'background' EPR signal (buffer solution + spin-probe + CrOx). As we demonstrated earlier for spin-probe Tempamine [28], such a procedure yields the difference signal attributed to spin-probe molecules localized in the internal osmotic volume of thylakoids.

Fig. 3a–d compares the high-field lines of the difference EPR signals from HMI, ATI, KG-4, and KG-5 measured in dark-adapted thylakoids or during illumination of chloroplasts. In order to investigate effects of chloroplast illumination on the 'internal' EPR signals, we focused our attention on the high-field lines of these signals, because the high-field lines are most informative for measurements of pH_{in}. 'Internal' EPR signals of HMI, ATI, KG-4, and KG-5 (Fig. 3a–d) in dark-adapted chloroplasts resemble corresponding EPR signals from spin-probes dissolved in a chloroplast suspending medium with pH=7.8 (Fig. 3e–h). It is worth noting, however, that the 'internal' EPR signals in dark-adapted chloroplasts are somewhat broadened as compared to corresponding 'external' signals. The line broadening effect is caused by decelerated motion of nitroxides in the thylakoid lumen [28,51,52]. The addition of uncouplers (40 μM nigericine or 20 mM NH_4Cl) which dissipate the transthylakoid pH difference ($\Delta pH \approx 0$) does not influence the line shapes of the 'internal' signals (not shown). Therefore, we conclude that in dark-adapted chloroplasts the intrathylakoid pH_{in} is close to external pH_{out} ($pH_{in} \approx pH_{out}$).

Fig. 3a–d shows that illumination of chloroplasts causes distinctive changes in the shapes of the 'internal' EPR signals of all spin-probes, HMI, ATI, KG-4, and KG-5. The most significant spectral shifts were observed for spin-probes ATI, KG-4, and KG-5 characterized by pK_a=6.2, pK_a=7.0, and pK_a=7.6,

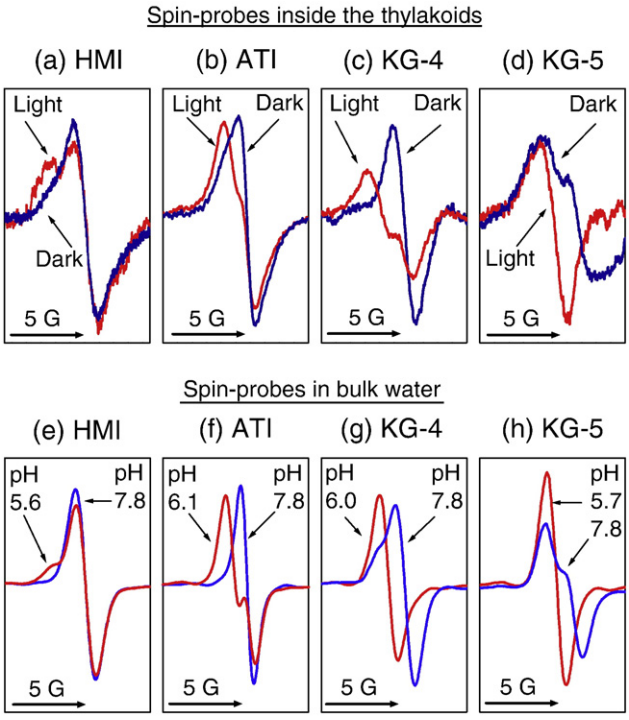

Fig. 3. Light-induced changes in the high-field component of the EPR signals from the spin-probes HMI (a), ATI (b), KG-4 (c), and KG-5 (d) localized inside the thylakoids. Effects of pH on the high-field component of the EPR signals of spin-probes HMI (e), ATI (f), KG-4 (g), and KG-5 (h) in bulk water.

respectively. One can see that the left extremes of the high-field peaks of ATI and KG-4 markedly shift towards the low-field regions of their spectra. For KG-5, we observed significant low-field shift of the right extreme. All these spectral changes are caused by a decrease in the apparent hyperfine splitting constants, reflecting the light-induced acidification of the lumen. In the case of HMI (pK_a=4.7), we observed only the appearance of a shoulder in the left side of the spectral line. The light-induced changes in the EPR spectra of spin-probes localized inside the thylakoids (Fig. 3a–d) are typical of spectral changes caused by protonation of nitroxides dissolved in bulk water (Fig. 3e–h). In the presence of uncouplers (gramicidin D, nigericin, or NH_4Cl, not shown), which dissipate the proton gradient, the illumination of chloroplasts did not affect the shapes of the "internal" EPR signals. Thus, we conclude that the light-induced spectral changes shown in Fig. 3a–d are really caused by a decrease in pH_{in}, i.e., spin-probes HMI, ATI, KG-4, and KG-5 can serve as molecular sensors for monitoring the acidification of the thylakoid lumen.

It is important to stress that the light-induced spectral changes shown in Fig. 3a–d are determined by a decrease in pH_{in} rather than due to secondary effects, e.g., the concentration-dependent distortion of the 'internal' EPR signals that might be caused by ΔpH-driven accumulation of spin-probes in the lumen. Actually, Fig. 4 shows that the peak-to-peak widths of the high-field EPR lines of HMI and ATI in an aqueous solution increase with concentrations of spin-probes. However, this effect cannot be a reason for significant spectral changes of 'internal' EPR signals, because the light-induced uptake of nitroxides by thylakoids is negligible for spin-probes with $pK_a < pH_{out}$ [57]. Under our experimental conditions (pH_{out}=7.8), the relationship $pK_a < pH_{out}$ holds true for all spin-probes used in this work. It should be also noted that spectral changes shown in Fig. 3a–d cannot be attributed to light-induced distortions of EPR spectra due to the loss of nitroxide paramagnetism, because very similar spectral changes were observed for EPR signals recorded upon scanning magnetic field in both forward and backward directions (not shown). Thus it is safe to conclude that it the decrease in pH_{in}, rather than secondary effects, that is responsible for light-induced spectral changes shown in Fig. 3a−d.

3.3. pH-dependences of EPR spectrum parameters of spin-probe ATI

As we noted above, the light-induced changes in the 'internal' EPR signals of spin-probes HMI, ATI, KG-4, and KG-5 (Fig. 3a–d) resemble qualitatively spectral changes caused by protonation of these nitroxides dissolved in bulk water (Fig. 3e–h). However, as compared to 'external' EPR signals, corresponding 'internal' signals are somewhat broadened due to decelerated motion of nitroxides inside the thylakoids. Therefore, for quantitative determination of pH_{in} from 'internal' EPR spectra, it is necessary to have appropriate calibrating curves — pH-dependencies of spectral parameters measured for 'internal' EPR signals. Spin-probe ATI is of particular interest for measuring pH_{in}, because its pK_a value (pK_a=6.2) is close to steady-state pH_{in} values established during illumination of chloroplasts [8,10,11,21,28]. In this section, we consider how variations of pH_{out} in a suspension of uncoupled chloroplasts ($pH_{in} \approx pH_{out}$) influence spectral parameters of ATI molecules localized in the thylakoid lumen.

Fig. 5a shows a set of 'internal' EPR spectra (central and high-field components) of ATI in dark-adapted uncoupled chloroplasts ($pH_{in} \approx pH_{out}$) suspended in media with different

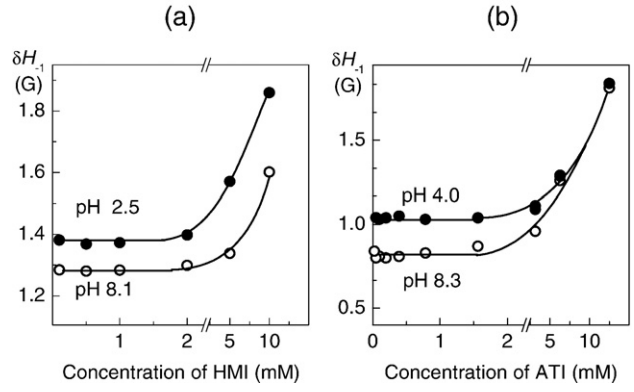

Fig. 4. Concentration-dependent broadening of EPR signals of HMI and ATI in water solution. The peak-to-peak line width of the high-field component (δH_{-1}) vs a spin-probe concentration in water solution: (a) HMI, (b) ATI. Solid and open symbols correspond to protonated and deprotonated forms of spin-probes, respectively. According to [78], protonation of ATI occurs at the nitrogen atom of the imidazoline ring.

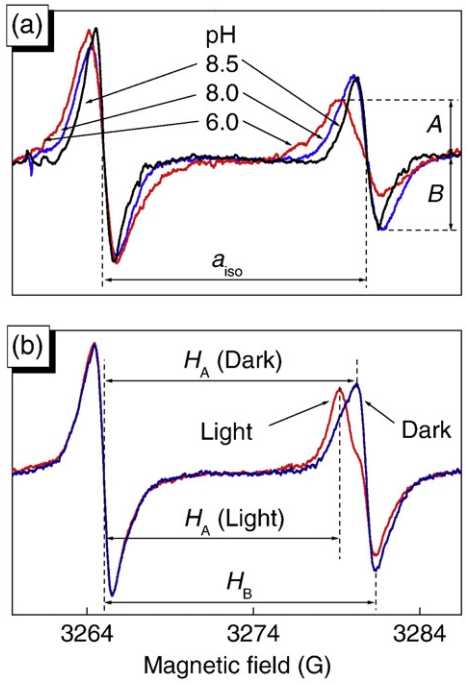

Fig. 5. Central and high-field lines of EPR signals given by ATI molecules localized inside the thylakoids. (a) Effects of pH on 'internal' EPR spectrum of ATI signal recorded in the dark. Chloroplasts were suspended in the media with different pH in the presence of 40 μM nigericine in order to equilibrate pH inside and outside the thylakoids ($pH_{in}=pH_{out}$). All EPR spectra are aligned relatively to their central lines. The amplitudes of the extremes *A* and *B* correspond to protonated and deprotonated forms of ATI, respectively. (b) Light-induced changes in 'internal' EPR spectrum of ATI (chloroplasts in state 4, $pH_{out}=7.8$).

pH_{out}. One can see that a decrease in pH_{out} is accompanied with a shift of the high-field extreme *A* towards the central line, demonstrating the acidification of ATI surroundings inside the thylakoids. Similar shift of the extreme *A* of spin-probe ATI is observed during the illumination of untreated (without added uncouplers) chloroplasts (Fig. 5b).

Fig. 6 shows pH-dependences of spectral parameters H_A, H_B, and a_{iso} measured for 'internal' ATI molecules (see Fig. 5 for definitions). We have used these dependences as calibrating curves for estimations of pH_{in}. Spectral parameter H_A (a distance between the central line and the high-field extreme *A*) appears to be the most convenient one for quantitative determination of the light-induced changes in pH_{in} in physiologically important range of pH_{in} ($5.8<pH_{in}<6.8$). Explicit changes in the values of other spectral parameter (H_B and apparent splitting constant a_{iso}) are virtually observed only after more significant acidification of the thylakoid lumen ($pH_{in}<5.8$). Note that from the formal point of view pH-dependences of parameters H_A, H_B, and a_{iso} might be characterized by different pK_a values, which deviate from $pK_a \approx 6.2$ determined from sigmoid-like pH-dependence of the partition parameter $f=A/(A+B)$ for ATI dissolved in bulk water [57]. This circumstance can be explained by the fact that parameters H_A, H_B, and a_{iso}, which values unequivocally reflect the protonation of "internal" spin-probe, are not the characteristics of the degree of protonation of ATI molecules. Nevertheless, pH-dependences of these spectral parameters can be used as calibration curves for measuring pH_{in}.

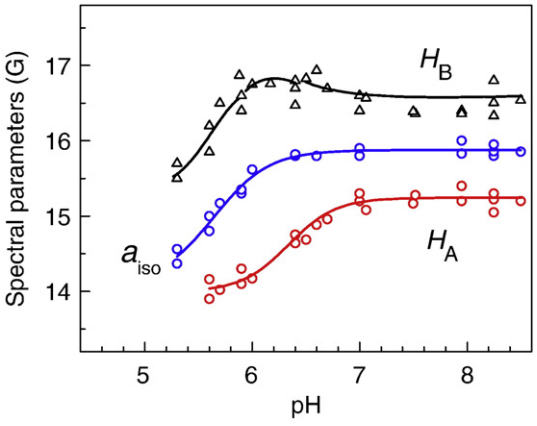

Fig. 6. Effects of pH on spectral parameters H_A, H_B, and a_{iso} of spin-probe ATI localized inside the thylakoids.

3.4. Effects of spin-probes and chromium oxalate on photosynthetic activity of chloroplasts

The method for measuring pH_{in} with pH-sensitive nitroxides described above is valid only if the spin-probes themselves and

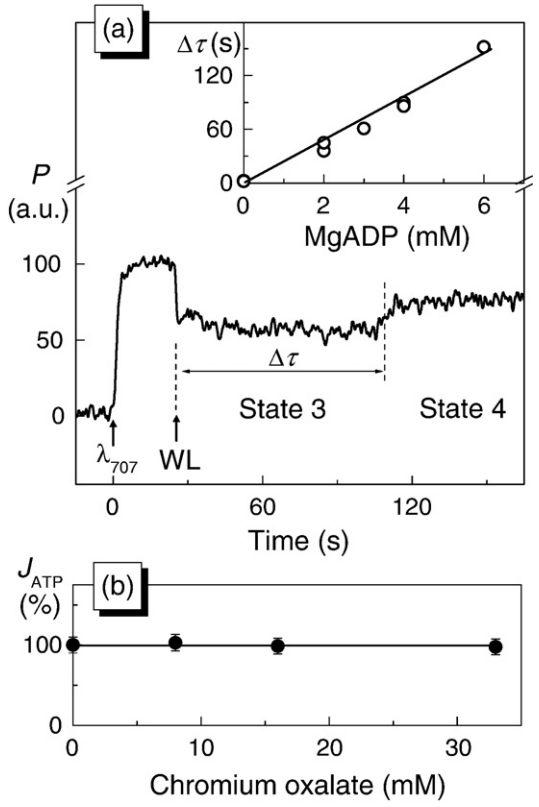

Fig. 7. (a) Kinetics of P_{700} redox transients in bean chloroplasts induced by switching on far-red light (λ_{707}) and white light (WL) in the presence of 4 mM of MgADP added to chloroplasts before illumination. Insert shows the plot of parameter $\Delta\tau$ vs a concentration of MgADP ($[ADP]_0$) added to chloroplasts. (b) Effect of chromium oxalate on the rate of ATP synthesis determined as $J_{ATP}=[ADP]_0/\Delta\tau$. The 100% value of J_{ATP} corresponds to photophosphorylation rate $J_{ATP}=25$ (ATP·s^{-1} per P_{700}).

CrOx would not disturb the normal functioning of chloroplasts. In this section, we consider the influence of CrOx and spin-probes on electron transport and photophosphorylation in chloroplasts. Fig. 7a shows the typical pattern of light-induced redox transients of P_{700} in chloroplasts with added 4 mM MgADP. Illumination of chloroplasts with far-red light (λ_{max}=707 nm) exciting predominantly PS1 induces the rise of the EPR signal from oxidized centers $P_{700}^{\bullet+}$. After switching on the white light (WL), this signal sharply decreases due to electrons donated by PS2 to $P_{700}^{\bullet+}$ via the intersystem electron transport chain. However, after a certain lag-phase ($\Delta\tau$), the EPR signal from $P_{700}^{\bullet+}$ elevates (Fig. 7a). Such a rise of the signal occurs due to deceleration of intersystem electron transport after substantial exhaustion of the ADP pool (metabolic transition state 3 → 4), because more significant acidification of lumen in state 4 causes a slowing down of b_6f complex turnover. The lag-phase $\Delta\tau$ characterizes a duration of state 3, parameter $\Delta\tau$ is proportional to initial concentration of MgADP ($[ADP]_0$) added to chloroplasts (see inset in Fig. 7a). The average rate of ATP synthesis can be evaluated as $J_{ATP}=[ADP]_0/\Delta\tau$. Fig. 7b shows that CrOx does not inhibit ATP synthesis in a rather wide range of CrOx concentrations (0–30 mM). Thus, the addition of CrOx at concentrations used in this work to visualize spin-probes inside the thylakoids ([CrOx] ≤ 10–15 mM) does not inhibit the photosynthetic activity of chloroplasts.

It is also worth noting that chloroplasts retain the capability for ATP synthesis in the presence of spin-probes at concentrations used in this work for measuring pH_{in} (≤1 mM). Fig. 8 shows that ATI and HMI do not suppress the ATP synthesis even at higher concentrations (up to 3 mM). A certain increase in J_{ATP} observed at relatively high concentrations of ATI (1–3 mM) can be explained by stimulation of noncyclic electron flow from the chloroplast electron transport chain to ATI. A similar effect of stimulation of photophosphorylation by low concentrations of uncoupling agents was reported in [63–65]. Thus, we conclude that chloroplasts retain their photosynthetic activity in the presence of spin-probes and CrOx, at least under experimental conditions used in this work used for measuring pH_{in}.

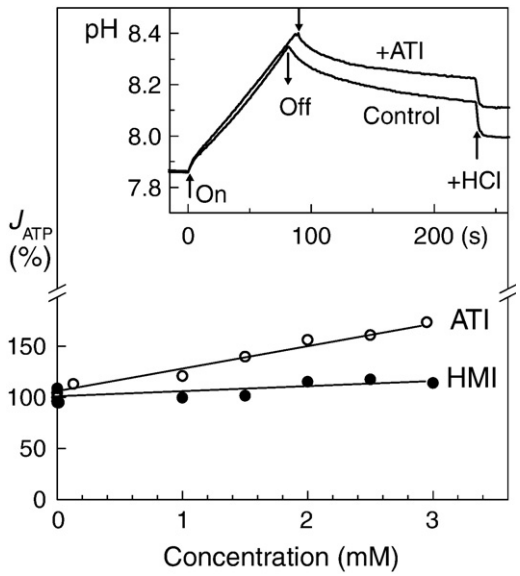

Fig. 8. Effects of HMI and ATI on the rate of ATP synthesis in bean chloroplasts. The inset shows the effect of ATI (1 mM) on the kinetics of the light-induced pH changes in weakly buffered chloroplast suspension.

3.5. Light-induced acidification of the lumen in metabolic states 3 and 4

Using the appropriate calibration curve (Fig. 6), we have determined the steady-state lumen pH_{in} established during the illumination of chloroplasts. Under experimental conditions favorable for intensive ATP synthesis (pH_{out}=7.8, excess of MgADP), the pH_{in} value decreases to pH_{in} ≈ 5.7–6.0 (data obtained for several batches of bean chloroplasts). This means that in metabolic state 3 the transthylakoid pH difference ($\Delta pH=pH_{out}-pH_{in}$) is equal to ΔpH ≈ 1.8.–2.1, which value is in a good agreement with our previous estimates of ΔpH under similar experimental conditions with a spin-probe Tempamine [28]. In the state of photosynthetic control (metabolic state 4), when the efflux of protons from thylakoids through the ATP synthase is reduced, we found more significant acidification of lumen (pH_{in}=5.4−5.7).

Consider how these estimates of ΔpH agree with the thermodynamic requirements for ATP synthesis. The actual free energy ΔG_{ATP} required to synthesize ATP from ADP and P_i is given by the following relationship:

$$\Delta G_{ATP} = \Delta G^0_{ATP} + RT\ \ln\frac{[ATP]}{[ADP]\cdot[P_i]}. \quad (2)$$

According to [66,67], under our experimental conditions (pH_{out}=7.8, $[Mg^{2+}]$=5 mM), the standard Gibbs free energy change of ATP formation from ADP and P_i is equal to 7.6 kcal/mol. In the course of chloroplast illumination, concentrations of ADP and inorganic phosphate ($[P_i]$) decrease proportionally to a concentration of ATP formed, $[ADP]=[AdN]_0-[ATP]$ and $[P_i]=[P_i]_0-[ATP]$. Thus, Eq. (2) can be rewritten as follows:

$$\Delta G_{ATP} = \Delta G^0_{ATP} + RT\ \ln\frac{[ATP]}{([AdN]_0-[ATP])\cdot([P_i]_0-[ATP])}. \quad (3)$$

Here, $[AdN]_0$ is the total concentration of adenine nucleotides in the system, $[P_i]_0$ is an initial concentration of inorganic phosphate, and [ATP] is a current concentration of ATP. Under our experimental conditions, $[AdN]_0 \approx [ADP]_0$, where $[ADP]_0$ is a concentration of MgADP added to chloroplasts before illumination. Fig. 9a shows the plots of ΔG_{ATP} vs [ATP] calculated from Eq. (3) for $[P_i]_0$=10 mM, and $[AdN]_0$=4 mM (these concentrations were used in most of our experiments). Assuming that the ATP synthase reaction is near equilibrium, we can estimate the threshold value of the proton motive force, $\Delta\mu^{th}_{H^+}$, that must be generated to sustain a steady-state ATP synthesis: $\Delta\mu^{th}_{H^+} = \Delta G_{ATP}/n$, where $n=H^+/ATP$ is the stoichiometry of protons pumped through the ATP synthase per ATP synthesized.

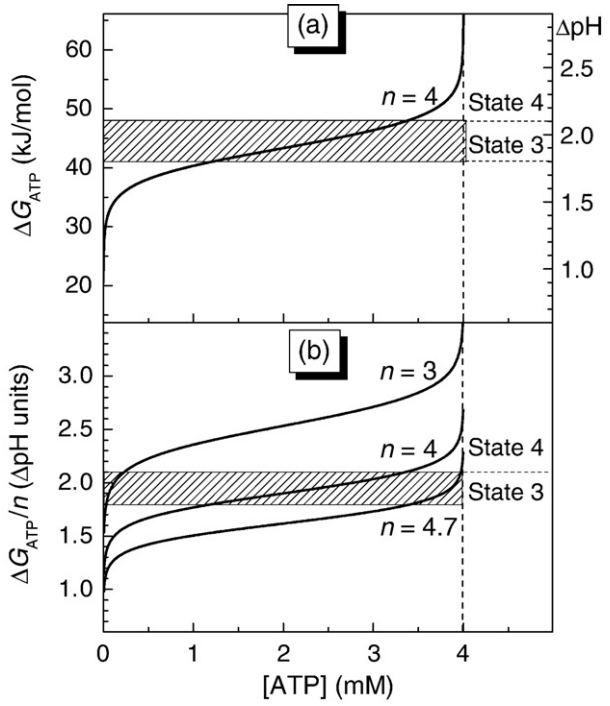

Fig. 9. The plots of ΔG_{ATP} (a) and $\Delta\mu^{th}_{H^+}$ (b) vs ATP concentration calculated from Eq. (3) for $[P_i]_0 = 10$ mM, and $[AdN]_0 = 4$ mM. Threshold values of proton motive force, $\Delta\mu^{th}_{H^+} = \Delta G_{ATP}/n$, are calculated for stoichiometric ratio $n = 3$, 4, and 4.7, as indicated.

Analysis of Eq. (3) allows us to evaluate the interval of ΔpH values required to hold ΔG_{ATP} at different concentrations of ATP and stoichiometric ratio n. Fig. 9b shows the plot of $\Delta\mu^{th}_{H^+}$ (expressed in $\Delta\mu^{th}_{H^+}/2.3RT$) vs [ATP] calculated for $n = 3$, 4, and 4.7. Parameter $n = 3$ was accepted in earlier works (see [68,69] for review); however, resent measurements of stoichiometric ratio H^+/ATP in chloroplasts lead to $n = 4$ [70,71]. Assuming that under steady-state conditions all the proton motive force $\Delta\mu_{H^+}$ is in the form of ΔpH, and that $n = 4$, we obtain that the transthylakoid proton gradient ΔpH = 1.8–2.1, which has been measured in this work under photophosphorylation conditions (shown as a shadowed interval in Fig. 9b), should be enough to sustain the steady-state ATP synthesis at $\Delta G_{ATP} \approx 41$–48 kJ/mol. According to Giersch et al. [72], the ΔG_{ATP} in intact chloroplasts is 40–50 kJ/mol. Mechanistic approach to estimation of the H^+/ATP ratio, based on structural studies of CF_1–CF_0 ATP synthase [73], gives a fractional stoichiometry $n \approx 4.7$. In this case, the transthylakoid proton gradient ΔpH = 1.8–2.1 should be enough to maintain the ATP synthesis at $\Delta G_{ATP} \approx 48$–55 kJ/mol.

The dramatic rise of ΔG_{ATP} with the exhaustion of ADP (Fig. 9) corresponds to metabolic transition state 3 → state 4. According to [28], under experimental conditions used in our work, a steady-state proton gradient establishes no longer than after 6–12 s of chloroplast illumination. When 4 mM MgADP is added to chloroplasts, a metabolic transition state 3 → state 4 occurs in 90 s after switching on the illumination (Fig. 7). In this work, EPR spectra of spin-probes were usually recorded in the time interval of 12–24 s after switching on the light. Therefore, we could be sure that under our experimental conditions the pH_{in} values were determined in the true metabolic state 3, i.e., before a substantial depletion of the ADP pool. Thus, a simple analysis of Eq. (3) shows that experimentally determined proton gradient ΔpH ≈ 1.8–2.1 is quite enough to sustain the ATP synthesis in a broad range of ATP concentrations (Fig. 9b). It is also safe to conclude that under experimental conditions used in this work the transthylakoid pH difference can serve as the main component of proton motive force, which value is sufficient for supporting efficient ATP synthesis in chloroplasts.

4. Concluding remarks

(1) We have described a group of pH-sensitive imidazoline- and imidazolidine-based nitroxides (HMI, ATI, KG-4, and KG-5) with pK_a values in the interval of 4.7–7.6, which proved themselves as the adequate probes for measuring the intrathylakoid pH in chloroplasts. EPR spectra of these nitroxides localized inside the thylakoids are sensitive to the light-induced acidification of the thylakoid lumen. Although all spin-probes described above demonstrate their potential for monitoring the intrathylakoid pH, in our particular case ($pH_{in} \sim 6$), spin-probe ATI with appropriate pK_a value ($pK_a \approx 6.2$) appears to be the most suitable sensor for quantifying pH_{in}. In the meantime, spin-probes KG-4, KG-5, or HMI would have the advantage for measuring pH_{in} in other metabolic states (e.g., $pH_{in} \sim 7$ or $pH_{in} < 5$).

(2) Having determined relevant calibrating curves (EPR parameters of spin-probe ATI localized in the lumen vs pH_{in}), we could quantify the intrathylakoid pH_{in} in isolated bean chloroplasts (*V. faba*) established in two metabolic states: $pH_{in} \approx 5.7$–6.0 (photophosphorylation conditions) and $pH_{in} \approx 5.4$–5.7 (state of photosynthetic control). These results come to a good agreement with our previous data obtained under similar experimental conditions with a spin-probe Tempamine [28].

(3) Our estimates of pH_{in} in state 3 show that efficient synthesis of ATP can be driven by a moderate proton gradient, $\Delta pH = pH_{out} - pH_{in} \approx 1.8$–2.1, determined at external $pH_{out} = 7.8$. These values of ΔpH are consistent with a point of view that ΔpH is the main contributor to the proton motive force driving the operation of CF_0–CF_1 ATP synthase under the steady-state conditions, provided that stoichiometric ratio H^+/ATP is $n = 4$–4.7.

(4) Results of our *in vitro* measurements of pH_{in} in chloroplasts under photophosphorylation conditions are consistent with previous estimates of pH_{in} based on measuring the post-illumination reduction of $P^{\bullet+}_{700}$ in chloroplasts *in situ* [24] and in isolated intact chloroplasts [25]. Note that upon a moderate acidification of the lumen ($pH_{in} \approx 6$) the rate of plastoquinol oxidation by the cytochrome b_6f complex remains rapid ($\tau_{1/2} \leq 20$–25 ms) [8,10,21,22,25,26,74–77]. More significant acidification of the lumen in the state of photosynthetic control ($pH_{in} \leq 5.4$–5.7) causes a noticeable deceleration of electron flow from the plastoquinol pool to $P^{\bullet+}_{700}$ ($\tau_{1/2} \geq 40$ ms), which can be explained by the feed-back pressure of intrathylakoid protons on the turnover of the cytochrome b_6f complex [5,8–11]. According to [10,24,25],

in intact leaves of different species functioning under normal experimental conditions, the half-time for $P_{700}^{\bullet+}$ reduction is about 20-28 ms, indicating that lumen pH is maintained at moderate levels ($pH_{in} \approx 6$), which only insignificantly retard the b_6f complex turnover.

(5) Concerning methodological aspects of this work, we would like to stress that simplicity of the method, negligible interference with the photosynthetic activities of chloroplasts, and spectral distinction of nitroxides from other metabolically active paramagnetic species convince us of the advantages of the present approach to membrane bioenergetics. We are currently working to develop the next generation of pH-sensitive spin-probes that could be used for selective measuring the hydrogen ion activity in different domains of the thylakoid lumen (bulk phase and membrane/lumen interface). Such site-specific pH-sensors might be used to scrutinize the localized mechanisms of proton transport coupled to ATP synthesis in chloroplasts and other energy transducing systems.

Acknowledgements

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