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INHIBITION OF ATP SYNTHESIS IN CHLOROPLASTS BY LIPID SOLUBLE NITROXIDE RADICAL SPIN LABELS

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The influence of various stearic acid spin labels (SASLs) that contain a bulky oxazolidine ring at various positions along the steryl chain on electron transport, proton uptake and photophosphorylation in bean chloroplasts has been investigated using potentiometric methods. SASLs added at rather low concentration (about 10 μM) efficiently inhibit (50% of control) ATP synthesis. However, a decrease in light-induced uptake of protons accompanied by stimulation of the electron transport was observed only at much higher concentrations of SASLs (about 0.1 mM). The influence on electron and proton transport depends on the position of the oxazolidine free-radical moiety along the stearic chain, with effects stronger in the order of 16- > 12- > 5-SASL. Incorporation of stearic acid without the spin label or water-soluble nitroxide radicals in the same range of concentrations only slightly decreases ATP formation and proton uptake. It is concluded that direct interaction of the oxazolidine free-radical moiety with the membrane sector CF_0 of the ATP synthase and not the drop in transmembrane pH difference causes inhibition of photophosphorylation by SASLs.

INTRODUCTION

Biological membranes, including the coupling membranes of energy transducing organelles (chloroplasts, mitochondria and chromatophores), have a distinct three-dimensional structure (Nelson, Cox & Lehninger, 1999; Voet, Voet & Pratt, 1998). However, these membranes are often treated as two-dimensional structures, and the lateral domain organization of biological membranes has been the hottest issue of membrane biophysics during recent years (Edidin, 1997; Simons & Ikonen, 1997; Kawasaki, Yin, Subczynski, Hyde & Kusumi, 2001). Lipid soluble spin labels, as well as fluorescent dyes, are often used as molecular probes for testing the physical properties and structural changes in the lipid domains of chloroplast membranes (Kocherginsky & Swartz, 1995; Li, Knowles, Murphy & Marsh, 1990; Li, Knowles, Murphy, Nishida & Marsh, 1989; Ruuge, Subczynski & Tikhonov, 1977a). In most cases, however, the depth-dependent effect of incorporating "foreign" molecules (molecular probes, inhibitors, uncouplers, etc.) into the thylakoid membranes has not been a major subject of experimental study in membrane bioenergetics.

Previously, we reported two cases of depth-dependent effects that played a significant role in molecular interactions in the thylakoid membrane, determining the redox transitions (Ruuge *et al.*, 1977a) and the physical interaction of free-radical moiety of spin labels with molecular oxygen (Ligeza, Tikhonov, Hyde & Subczynski, 1998). Light-induced reduction of spin labels was fastest when the nitroxide radical was positioned in the polar headgroup region of the thylakoid membrane, while reoxidation of previously reduced spin labels was more effective for deeper localization of the nitroxide moiety in the hydrocarbon core of the lipid bilayer (Ruuge *et al.*, 1977a). These experiments indicated that the reduced headgroup of the plastosemiquinone molecule (the reducing agent for spin labels) was located in the polar headgroup region of the lipid bilayer portion of the thylakoid membranes. Our experiments also demonstrated that the translational diffusion of small molecules such as molecular oxygen, within the lipid bilayer portion of thylakoid membranes, was strongly depth-dependent, being highest in the core of the thylakoid membrane (Ligeza *et al.*, 1998). Additionally, oxygen transport (the product of local oxygen concentration and the local oxygen diffusion coefficient) was strongly affected by the depth-dependent molecular interactions in the membrane. For example, cholesterol decreased local oxygen transport in the membrane near its surface in the hydrophobic region, but increased it in the membrane center (Subczynski, Hyde & Kusumi, 1989; Subczynski, Hyde & Kusumi, 1991). Polar carotenoids, however, strongly decreased oxygen transport in the membrane center (Subczynski, Markowska & Sielewiesiuk, 1991).

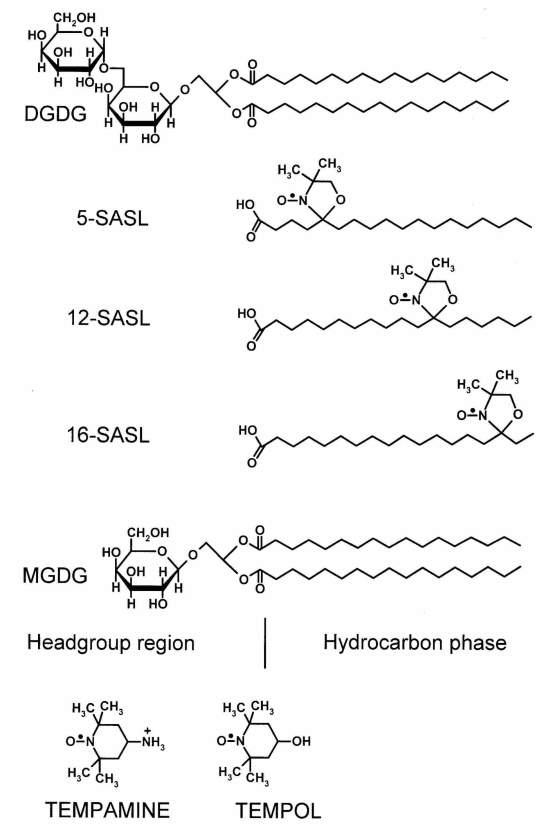

Fig. 1. Chemical structures of spin labels used in this work. SASLs location in the membrane and the location of the nitroxide moiety in the stearic chain are indicated. The lipid domain of the thylakoid membrane is symbolized here by galactolipids (monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG), which represent major components of the lipid portion of thylakoid membranes. These lipids account for about 70 mol% of total polar lipids in thylakoid membranes (Nishihara, Yokota & Kito, 1980).

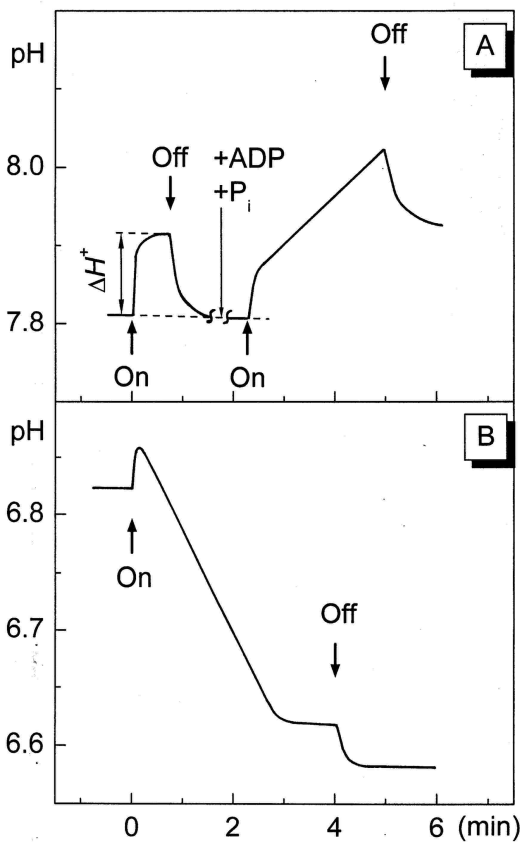

Fig. 2. Light-induced changes in the pH of chloroplast suspension. Reaction volume was 5 ml; chloroplast suspension (0.15 mg chlorophyll per ml) was incubated in the reaction medium containing 0.2 M sucrose, 2 mM $MgCl_2$ and 0.05 M NaCl. A) 1 μM methyl viologen was used as the mediator of electron transport from photosystem 1 to oxygen; 0.16 mM ADP and 1 mM P_i were added as indicated. B) 50 μM FeCy was used as an electron acceptor. "On" and "Off" indicate that light was turned on or off.

Depth-dependent phenomena observed in model membranes were reviewed in our recent paper (Subczynski & Wisniewska, 2000).

In this work, we studied the effects of the nitroxide radical on electron transport, proton uptake and photophosphorylation in bean chloroplasts and their dependence on the radical position in the lipid bilayer of the thylakoid membrane. We used stearic acid spin labels (SASLs) with different positions of the oxazolidine free-radical ring along the acyl chain of stearic chain (Fig. 1). For many years, these spin labels were used to probe structural changes, dynamics and functional activity of chloroplast membranes (Li *et al.*, 1990; Li *et al.*, 1989; Ruuge *et al.*, 1977a; Ligeza *et al.*, 1998; Weaver & Chon, 1966; Torres-Pereira, Mehlhorn, Keith & Packer, 1974; Ruuge, Subczynski & Tikhonov, 1977b). Nevertheless, systematic study of their perturbing effect in chloroplast membranes is very limited (Tsapin, Timofeev, Goldfeld, Filiushina & Krendeleva, 1974; Vozvishaeva, Goldfield, Hangulov & Tsapin, 1974; Maruyama & Ohnishi, 1974). The purpose of the present work was to evaluate the perturbing effects of various SASLs on the light-induced energy transducing processes in chloroplasts. Using potentiometric methods for monitoring the photochemical activity of chloroplasts, we demonstrated that SASLs, whose nitroxide radicals are located within the thylakoid membrane, act as potent inhibitors of photophosphorylation in chloroplasts. The difference in the action of SASLs and water-soluble spin labels, as well as the analysis of literature data, allowed us to speculate about the possible mechanism of the inhibitory action of SASLs on ATP formation. We concluded that the inhibitory

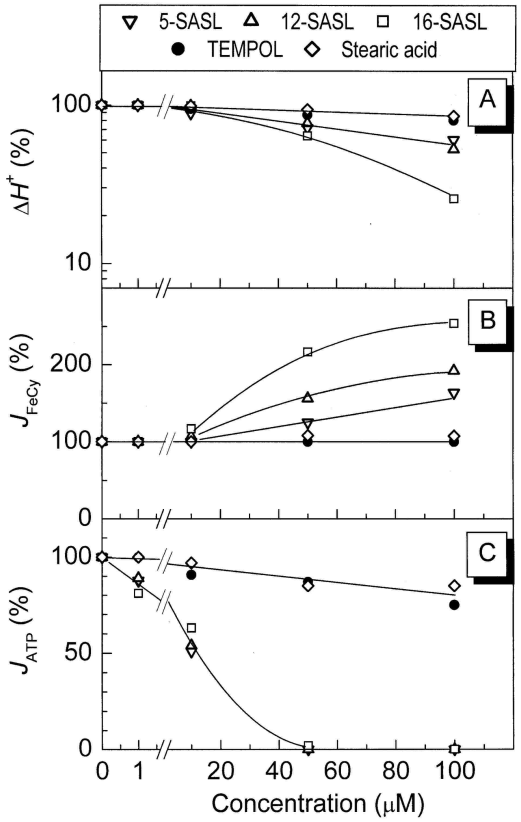

Fig. 3. Effects of spin labels on: the light-induced uptake of protons (ΔH^+), the rate of FeCy reduction (J_{FeCy}), and the rate of ATP formation (J_{ATP}). A) 100% of ΔH^+ corresponds to 120-150 hydrogen ions H^+ taken by thylakoids per one reaction centre P_{700}. B) 100% of J_{FeCy} corresponds to 80-100 electron equivalents per one second per one reaction centre P_{700}. C) 100% of J_{ATP} corresponds to 40-50 ATP molecules per one reaction centre P_{700} formed per one second. Experimental conditions as in Fig. 2A.

activity of SASLs could be caused by direct interaction of nitroxide moiety with the functional groups of the proton channel in the membrane sector CF_0 of the ATP synthase.

MATERIALS AND METHODS

Reagents

5-SASL, 12-SASL and 16-SASL were obtained from Molecular Probes (Eugene, OR). Other reagents were obtained from Sigma (St. Louis, MO).

Isolation of chloroplasts

Chloroplasts (class B) were isolated from bean leaves (*Vicia faba*) according to (Tikhonov, Khomutov, Ruuge & Blumenfeld, 1981). Isolated chloroplasts were suspended in a medium containing 0.2 M sucrose, 2 mM $MgCl_2$, and 0.01 M

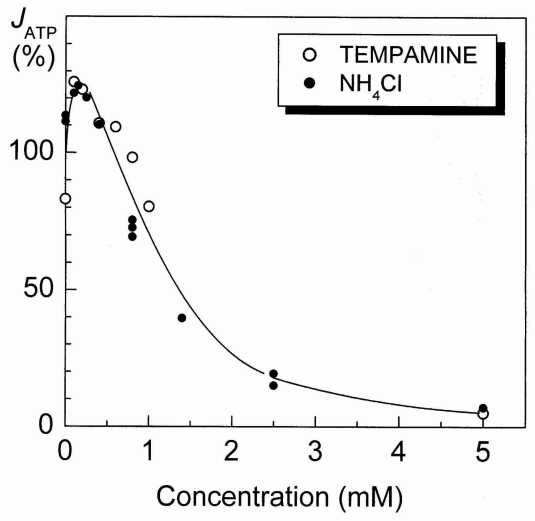

Fig. 4. Influence of TEMPAMINE and NH_4Cl on the rate of ATP formation. Experimental conditions as in Fig. 2A, except that the reaction medium contained 1 mM ADP, 2 mM P_i and 1 mM HEPES (pH 8.0).

Tris-HCl buffer (pH 7.5). For potentiometric measurements, an aliquot of chloroplasts from the stock solution was resuspended (final concentration of 0.15 mg chlorophyll per ml) in the reaction medium (5 ml) containing 0.2 M sucrose and 2 mM $MgCl_2$. Methyl viologen (MV) or potassium ferricyanide (FeCy) was used as the electron acceptors, depending on the type of experiment (see details in the figure legends). Chlorophyll concentration was determined according to Arnon (1949). The concentration of photosystem 1 reaction centers, P_{700}, was determined according to (Tikhonov *et al.*, 1981) from the intensity of the EPR signal 1 given by oxydized centers P_{700}^+. All manipulations with chloroplasts were performed in dark or dim light at 0°C.

Potentiometric measurements of chloroplast photochemical activity

The functional characteristics of chloroplasts were studied by potentiometric methods according to (Karlish & Avron, 1968; Telfer & Evans, 1972). Light-induced changes in pH of the reaction medium were measured with a combined glass-calomel electrode and recorded with a Kipp-micrograph recorder. Five ml of chloroplast suspension was placed in the glass cell and stirred. Chloroplasts were illuminated with white light from a 100 W tungsten lamp; infrared light was cut off with a 5 cm layer of water. Light intensity on the cell wall was 120 W/m^2. SASLs and stearic acid were added to the chloroplast suspension from the ethanol solution. The final concentration

of ethanol in the reaction medium did not exceed 1% v/v. All the measurements were performed at room temperature.

Fig. 2 shows the typical pattern of light-induced changes of pH in the chloroplast suspension. To quantify the pH changes, appropriate amounts of 0.01 M HCl were added at the end of each experiment to return the suspension pH to its initial value. The experimental conditions indicated in the figure legends were chosen based on separate experiments carried out to optimize the yield of the measured parameters. Photophosphorylation was measured as the irreversible light-induced uptake of protons according to the equation

$$MgADP + P_i + nH^+ \rightarrow MgADP + H_2O, \quad (1)$$

where n = 0.96 at pH 8 (Chance & Nishimura, 1967). Along with potentiometric methods, in some experiments we also used the luciferine-luciferase method for measuring ATP synthesis (Strehler, 1968). The results of photophosphorylation assays obtained by these methods were practically the same.

RESULTS AND DISCUSSION

Effects of stearic acid spin labels on the photochemical activity of chloroplasts

Fig. 1 shows the chemical structures of the lipid soluble spin labels, derivatives of stearic acid. SASLs are intercalated in the membrane with the hydrophilic part (left-hand side) in the polar headgroup region of the membrane. Earlier we demonstrated that SASLs dissolved in thylakoid membranes gave EPR spectra that were typical of the EPR spectra of SASLs located in a hydrophobic membrane environment (Ruuge *et al.*, 1977a; Ligeza *et al.*, 1998; Ruuge *et al.*, 1977b). Chemical structures of water-soluble spin labels used in this investigation are also shown in Fig. 1.

Fig. 3 demonstrates the dependence of light-induced uptake of protons (ΔH^+), the rate of FeCy reduction (J_e), and the rate of ATP synthesis (J_{ATP}) on concentrations of SASL, stearic acid and the water-soluble spin label TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl). Addition of SASLs caused a decrease in uptake of protons (Fig. 3A). The effect depended on the concentration of SASLs and the position of the nitroxide moiety along the acyl chain. At the maximum concentration used in our experiments (0.1 mM), addition of 5-, 12-, and 16-SASL led to a decrease in the ΔH^+ value of 35, 45 and 70%, respectively. This effect is caused by the oxazolidine free-radical fragment of SASL which is buried inside the membrane. Actually, neither stearic acid itself (without the oxazolidine free-radical fragment) nor the water-soluble spin label TEMPOL markedly affected the light-induced uptake of protons.

The drop in proton uptake was accompanied by acceleration in electron transport from water to FeCy (Fig. 3B). The strongest effect (by a factor of 2.5) was observed for 16-SASL, while 12- and 5-SASL accelerated electron transport 1.9 and 1.7 times respectively. Stearic acid or TEMPOL did not affect the rate of FeCy reduction. Acceleration of electron transport can be caused by release of photosynthetic control, i.e., by stimulation of basal electron flow due to a decrease in the proton gradient across the thylakoid membrane. The difference in efficiencies of 5-, 12-, and 16-SASL can be explained by the different mobilities of their nitroxide radical fragments within the thylakoid membrane. It is well known that 16-SASL exhibits the highest rotational motion, wobbling and rate of vertical displacement in the lipid domain of membranes, when compared to 5-SASL and 12-SASL (Ligeza *et al.*, 1998; Yin & Subczynski, 1996).

It is remarkable, however, that SASLs caused much stronger inhibitory effects on the rate of ATP formation (Fig. 3C) than on proton uptake and the rate of electron transport to ferricyanide. At concentrations of about 0.01-0.015 mM, addition of SASLs decreased the rate of photophosphorylation by a factor of two. As one can see from Figs. 3A and 3B, at these concentrations SASLs had little effect on the ΔH^+ and J_e values. At a concentration of about 0.05 mM, ATP formation was completely inhibited, while the light-induced uptake of protons was reduced only by 20-30%. Neither stearic acid nor the water-soluble spin label TEMPOL caused inhibition of photophosphorylation at this concentration (Fig. 3C). These results indicate that the strong inhibitory effect of SASLs on ATP synthesis can be explained by direct action of the nitroxide radical on the ATP synthase (the "local" mechanism of uncouping) rather than by a relatively small drop in the transmembrane bulk-to-bulk phase pH difference ("delocalized" mechanism).

Effect of water-soluble spin label TEMPAMINE on the rate of ATP formation

The water-soluble spin label TEMPAMINE (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) demonstrated another mode of photophosphorylation inhibition in chloroplasts, which can be explained by the protonophoric action of the amino group of this molecule. Fig. 4 shows the influence of TEMPAMINE on the initial rate of ATP syn-

thesis in chloroplasts. We observed non-monotonous dependence of the ATP formation rate versus the TEMPAMINE concentration: low concentrations of TEMPAMINE (up to 0.1 mM) stimulated ATP synthesis, while higher concentrations caused a gradual decrease in the rate of photophosphorylation. As can be seen from Fig. 4, the same concentration dependence was also obtained for NH_4Cl. The 50% drop of J_{ATP} was obtained at a concentration of amines (TEMPAMINE or NH_4Cl) of about 1.1 mM, which was two orders of magnitude higher than the concentration of SASLs that caused the same change in J_{ATP}.

Stimulation of ATP synthesis by small amounts of uncouplers (amines and nigericin) was first observed by (Giersch, 1981). Activation of photophosphorylation was explained by a kinetic factor (Giersch, 1981; Giersch, 1983; Pick & Weiss, 1988). At low concentrations, an uncoupler causes acceleration of electron transport due to certain decreases in the transmembrane pH difference (ΔpH), which is the main factor controlling the rate of the noncyclic electron transport in chloroplasts (Tikhonov *et al.*, 1981; Rottenberg, 1993; Rumberg & Siggel, 1969; Blumenfeld & Tikhonov, 1994; Kramer, Sacksteder & Cruz, 1999). With further rise in the concentration of amines (TEMPAMINE or NH_4Cl), their uncoupling action dominates the stimulation effect, thus leading to a reduction in the rate of ATP synthesis. Since TEMPAMINE and NH_4Cl have virtually the same uncoupling ability, we can conclude that it is the amine group rather than the nitroxide group of the TEMPAMINE molecule that is responsible for its influence on the rate of ATP formation. Thus, in contrast to SASLs, the action of TEMPAMINE on photophosphorylation in chloroplasts can be related to the effects of conventional amino-type uncouplers.

Possible mechanisms of photophosphorylation inhibition by lipid soluble spin labels

There may be several reasons for the inhibitory action of SASLs on ATP formation in chloroplasts:
(i) direct interaction of the nitroxide radical with the membrane sector of ATP synthase (the "local" mechanism of uncoupling); (ii) competition for electrons between electron carriers and nitroxide radicals; (iii) spin label-induced decrease in the bulk-to-bulk phase transmembrane pH difference, $\Delta pH = pH_{out} - pH_{in}$, ("delocalized" mechanism of uncoupling); (iv) structural modification of thylakoid membranes by SASLs. Analysis of our results and comparison with literature data show that it is the "local" mechanism of uncoupling which is the dominant factor of SASL action on ATP synthesis. It is known that spin labels can accept electrons from the chloroplast electron transport chain (Ruuge *et al.*, 1977a; Weaver & Chon, 1966; Tikhonov, Khomutov & Ruuge, 1980). Earlier, we demonstrated that light-induced redox transients of the photosystem 1 reaction centre P_{700} were not inhibited by SASLs (Ruuge *et al.*, 1977b). In the present work, we also demonstrated that the addition of SASLs did not slow the rate of non-cyclic electron transport, but even accelerated electron flow to ferricyanide ions when SASLs were added at rather high concentrations (Fig. 3B). We therefore conclude that SASLs do not inhibit non-cyclic electron flow in chloroplasts.

Stimulation of electron transport by SASLs can be explained by a certain decrease in the ΔpH value, which manifests itself as a decrease in the light-induced uptake of protons. However, this effect cannot explain such a strong inhibition of ATP synthesis at much lower concentrations of SASLs. Actually, complete inhibition of ATP formation at 0.05 mM of SASL was accompanied by a relatively small decrease (20-30 %) in the ΔH^+. Such a drop of ΔH^+ is equivalent to the relatively small decrease in the ΔpH value (no more than 0.2 pH units). Therefore, at the concentrations of SASLs that substantially suppress photophosphorylation in chloroplasts, the "delocalized" mechanism of uncoupling should be negligible.

Among SASLs, 12-SASL has the strongest effect on the structure of lipid membranes. Five-SASL shows a much lesser effect, and 16-SASL, where the radical moiety is located in the centre of the lipid bilayer, virtually does not influence the membrane (Wisniewska, Nishimoto, Hyde, Kusumi & Subczynski, 1996). As can be seen from Figs. 3A and 3B, the action of SASLs on light-induced uptake of protons and electron flow does not correlate with the possible structural effects of SASLs. This result indicates that SASLs can interact directly with the ATP synthase. Water soluble spin labels (TEMPOL and TEMPAMINE), had lower effects on ATP synthesis compared to SASLs. Therefore, we suggest that the effects of SASLs were realised by the interaction of nitroxide radicals with the membrane sector (CF_0) rather than the water exposed complex (CF_1) of the ATP synthase.

There are two possible mechanisms of SASL influence on the chloroplast ATP synthase: (i) redox modulation of the ATP synthase activity, and, (ii) uncoupling action of SASL. In the first, the high efficiency of SASLs as inhibitors of ATP synthase might be associated with the chemical

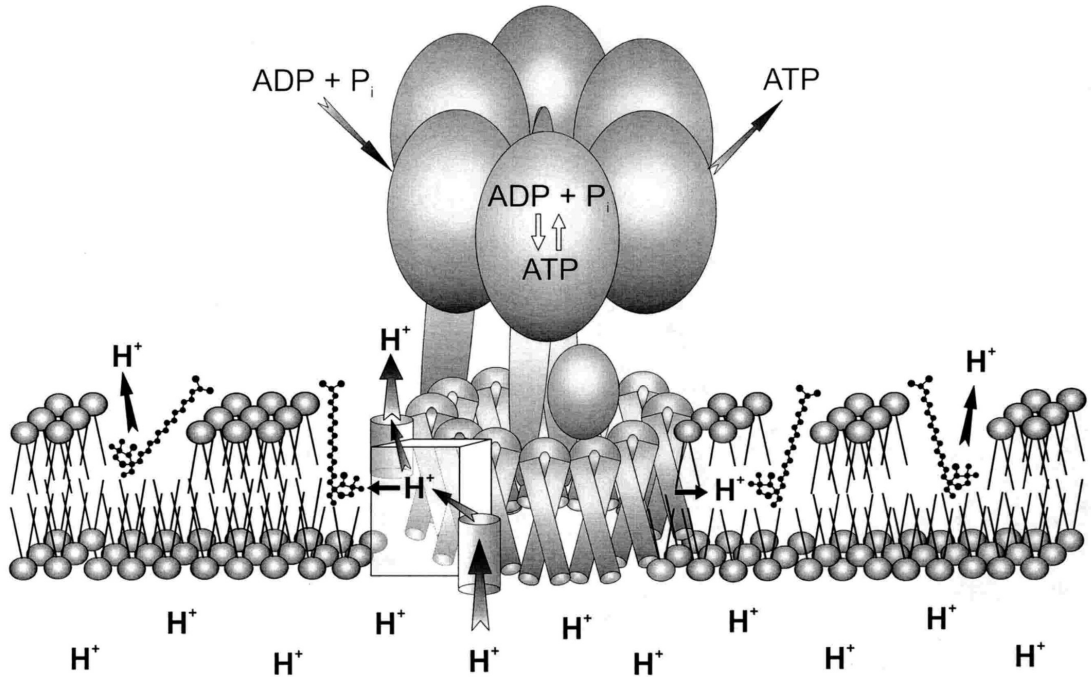

Fig. 5. Scheme of SASL interactions with the chloroplast ATP synthase. The nitroxide group of SASL provides an intramembrane pass road for protons from CF_0 to the bulk phase.

reactivity of the nitroxide radical. It is well known that the enzyme activity of chloroplast ATP synthase is controlled by the redox status of subunit γ thiol groups (Mills & Mitchell, 1982; Moroney, Andreo & McCarty, 1984; Haraux & de Kouchkovsky, 1998; McCarty, Evron & Johnson, 2000). If the nitroxide radicals had access to these groups, they could oxidise them, decreasing the ATP synthase activity. The subunit γ of CF_1 contains four cystein residues, γCys89, γCys199, γCys205 and γCys322 (McCarty *et al.*, 2000). Two of them, γCys199 and γCys205, are likely involved in redox regulation of chloroplast ATP synthase by thioredoxins (McCarty *et al.*, 2000; He, Miginiac-Maslow, Sigalat, Keryers & Haraux, 2000). According to recent structural data (Groth & Pohl, 2001), the three-dimensional structure of CF_1 is similar to that of the mitochondrial coupling factor F_1 (Abrahams, Leslie, Lutter & Walker, 1994; Bianchet, Hullihen, Pederson & Amzel, 1998; Stock, Leslie & Walker, 1999). Therefore, residues γCys199 and γCys205 (which are responsible for the redox modulation of chloroplast ATP synthase) should be positioned in the central cavity of the $\alpha_3\beta_3$-core of CF_1, located well off the membrane buried nitroxide radicals of SASLs. At the same time, water-soluble spin labels, which might attack the thiol groups of subunit γ, were much less efficient in suppressing ATP synthase activity than SASLs. Therefore, we assume that the redox-type mechanism of the ATP synthase inhibition by SASLs seems to be unrealistic.

In the second mechanism, the nitroxide radical of SASL, while moving in the lipid domain of the membrane, can intercept protons directly from the functional groups involved in proton translocation across the membrane sector of the ATP synthase complex. The crucial role in proton translocation through the membrane complex F_0 belongs to the conserved acidic residues (Glu61 in spinach thylakoids, Asp61 in *E. coli*, Glu59 in *Saccharamyces cervisiae*) of the membrane-embedded oligomer (Haraux & de Kouchkovsky, 1998). In chloroplasts, the oligomer consists of 14 subunits (Seelert, Poetsch, Dencher, Engel, Stahlberg & Muller, 2000) known as subunits III, which are arranged in a cylindrical ring connected to the outward end of subunit γ. Subunits III of chloroplast ATP synthase are equivalent to subunits *c* in bacteria and mitochondria. Each individual subunit *c* (and likely subunit III) consists of two transmembrane α-helices connected by a short loop on the F_1 side of the membrane. The C-terminal transmembrane segment of subunit *c* contains the conserved residue Asp61. The molecular architecture of the transmembrane F_0 sector, recently determined from crystals of a yeast F_1c_{10} complex (Stock, Leslie & Walker, 1999), suggests that the C-terminal α-helices of subunits *c* are placed at the periphery of the cylindrical *c*-oligomer. According

to molecular models for *E.coli* enzyme (Groth & Walker, 1997; Rastogi & Girvin, 1999; Dmitriev, Jones & Fillingame, 1999; Groth, 2000), the carboxyl groups of Asp61 are exposed to the periphery of the oligomer ring. Being located approximately in the center of the C-terminal α-helix, the protonated carboxyl group should be accessible from the lipid phase of the membrane. Therefore, it is reasonable to assume that nitroxide radicals of SASLs, while moving in the lipid core of the thylakoid membrane, could intercept protons directly from CF_0, thus inhibiting operation of the ATP synthase machinery (the "local" mechanism of uncoupling).

The uncoupling action of a lipid soluble spin label could be promoted by the light-induced reduction of the nitroxide radical ($>N^{\bullet}-O + e^- \rightarrow >N-O^-$), which is followed by protonation of the reduced fragment ($>N-O^- + H^+ \rightarrow >N-OH$). Earlier (Ruuge *et al.*, 1977a), we demonstrated that SASLs can reversibly lose their paramagnetism in the course of their interactions with the chloroplast electron transport chain. It is likely that there are plastosemiquinone molecules on the acceptor side of photosystem 2 that serve the role of major electron donor for nitroxide radicals (Tikhonov *et al.*, 1980). Under normal physiological conditions, these plastosemiquinones operate in anion forms ($Q_A^{\bullet-}$ and $Q_B^{\bullet-}$), being able to donate an electron to the nitroxide radical (e.g., $Q_A^{\bullet-} + >N^{\bullet}-O \rightarrow Q_A + >N-O^-$). The reduced fragment of a spin label, which initially appears in its anion form (>N-O$^-$), should have an increased affinity for a proton. Being localized in the lipid bilayer of the thylakoid membrane, the anion $>N-O^-$ could intercept a proton ($>N-O^- + H^+ \rightarrow >N-OH$) from one of the functional groups involved in proton translocation across the membrane sector of ATP synthase. Reduced spin labels can also be reoxidised by chloroplasts (Ruuge *et al.*, 1977a). Reoxidation of the protonated fragment >N-OH would be accompanied by proton dissociation into the aqueous bulk phase: >N-OH $\rightarrow$ $>N^{\bullet}-O + e^-$ to electron transport chain) + H^+ (to bulk phase). Thus, the nitroxide group of SASLs might serve the role of intramembrane proton shuttle, providing a bypass for protons from the CF_0 to the bulk phase and suppressing operation of the ATP synthase machinery (Fig. 5).

In principle, the protonation/deprotonation cycle might also occur without the redox transients of the nitroxide radical:

$$>N^{\bullet}-O + H^+ \text{ (from } CF_0) \rightarrow >N^+{}^{\bullet}-OH \rightarrow >N^{\bullet}-O + H^+ \text{(to bulk phase)}. \quad (2)$$

On first glance, this mechanism might seem unrealistic. Nitroxide radicals localized in polar surroundings are characterised by low pK values (pK ≤ 1-3, see (Khramtsov & Volodarsky, 1998), therefore, the radical protonation should not occur under physiological conditions. However, in the hydrophobic environment inside the membrane, the pK value of the nitroxide radical might shift to substantially higher values. If this is the case, the protonated radical $>N^{\bullet}-OH$ should be expelled from the hydrophobic moiety of the membrane core to the membrane surface where the proton could easily dissociate into the aqueous bulk phase.

CONCLUDING REMARKS

SASLs have been widely used to study model and biological membranes, including the coupling membranes of chloroplasts. However, our present study clearly demonstrated that the use of SASLs for testing the photochemical activity of chloroplasts or probing membrane conformation and dynamics requires certain caution in the interpretation of experimental data. For chloroplasts, the guideline for acceptable SASL concentration can be estimated from Fig. 3 in this report.

Experimental results presented above demonstrate that lipid soluble spin labels (SASLs) act as efficient inhibitors of photophosphorylation in chloroplasts. They suppress ATP synthesis at much lower concentrations than water-soluble nitroxide radicals (TEMPAMINE and TEMPOL). Analysing the effects of spin labels on the rate of ATP formation, electron transport and proton uptake, we conclude that lipid soluble spin labels act as "local" uncouplers, which could interact directly with the proton channel in the membrane sector CF_0 of ATP synthase. We theorize that SASLs might behave similarly to the group of uncouplers, characterised by Rottenberg (1993) as "decouplers," which can selectively release occluded protons from the intramembrane proton pools. Among the phenomenological characteristics of decouplers, Rottenberg mentioned:

i) stimulation of basal electron transport ("state 4"), up to the level of a phosphorylating condition ("state 3");
ii) inhibition of ATP synthesis;
iii) relatively small effects on membrane ion permeability and "bulk-to-bulk" transmembrane differences in proton electrochemical potentials, $\Delta\mu_{H+}$.

Actually, the results presented in Fig. 3 demonstrate that SASLs efficiently inhibited ATP forma-

tion and stimulated basal electron transport without a significant reduction in light-induced uptake of protons. Providing a direct escape route for protons from the core of the coupling membrane, SASLs efficiently inhibit ATP synthesis in chloroplasts.

Acknowledgements

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