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THE INFLUENCE OF SPINACH THYLAKOID LUMEN VOLUME AND MEMBRANE PROXIMITY ON THE ROTATIONAL MOTION OF THE SPIN LABEL TEMPAMINE

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Phosphorylating electron-transport reactions, using either Photosystem I or Photosystem II, were found to be competent in driving the light-induced increase in rotational hindrance of the spin label Tempamine. The uncoupler gramicidin prevented the change induced by either photosystem while the electron-transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) only prevented the light-induced change associated with Photosystem II. The internal lumen microviscosity is termed 'η' and was found to be inversely proportional to the lumen volume. Substances which should compete for Tempamine-binding sites on the thylakoid membrane were found to be largely ineffectual in changing η. These results further support the hypothesis that the magnitude of η is directly related to the proximity of Tempamine to a membrane surface.

Introduction

We have been studying the rotational motion of Tempamine while it resides in the lumen of spinach thylakoids under a variety of physiological conditions [1–4]. We have learned that: (1) the rotational motion of the spin label in the thylakoid lumen is hindered 7–11-fold compared to the motion of the label in bulk water [1,2]; (2) when the thylakoid is illuminated, the rotational hindrance increases to as much as 30-fold, depending on the light intensity [3]; and (3) the light-induced hindrance of spin label motion is directly related to the ability of thylakoids to accumulate protons [4]. Others have observed that in the light, under coupling conditions, the thylakoid lumen shrinks and the opposing membranes come closer together [5,6]. Thus, we have interpreted our finding of the light-induced hindrance as being related to the membrane approach phenomenon. However, as the membrane faces approach, a cooperative increase in water order would be expected to occur and so our results remain consistent with our previous speculations that water order could be involved with the rotational hindrance of spin labels in the thylakoid lumen.

The questions which we deal with in this communication are: (1) Can the light-induced hindrance of rotational motion be produced separately by electron transport through either PS II or PS I? (2) Is the hindered rotational motion of the spin label in the lumen of thylakoids related to the close proximity of the membrane and, if so, can the rotational hindrance be varied by osmotically manipulating the thylakoids? (3) Can any significant portion of the rotational hindrance be attributed to the binding of the spin label to some thylakoid component?

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Tempamine, 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine; Tricine, *N*-tris(hydroxmethyl)methylglycine; Chl, chlorophyl; PS, photosystem.

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Materials and Methods

Tempamine and phenazine methosulfate were purchased from Aldrich (Milwaukee, WI) and used without further purification. Tris(oxalato)chromate(III) (chromium oxalate) was synthesized and characterized as described previously [2]. Pyocyanin was photochemically produced from phenazine methosulfate as described elsewhere [7]. DCMU was purchased from Pfaltz and Bauer (Stamford, CT) and was recrystallized prior to use. Diaminodurene was purchased from Research Organics/Inorganics (Cleveland, OH) and was recrystallized prior to use. DBMIB was a generous gift from Professor A. Trebst, Bochum. [^{3}H]H_2O, [^{14}C]insulin, and [^{14}C]sorbitol were obtained from ICN Chemical and Radioisotope Division (Irvine, CA). The silicone fluids, Versilube F-50 and SF96-50 were generous gifts of the General Electric Co., Waterford, NY.

EPR spectra were recorded on a Varian E-9 spectrometer using an E-231 cavity with the irradiation grid plate removed to allow actinic illumination. Rotational correlation times (t_c) of Tempamine residing in the aqueous lumen of the thylakoid were calculated from the Kivelson equation [8–11] as we have described previously [1,4]. The value of t_c is a precisely defined physical property of spin label motion only when all the assumptions incumbent on the equations are realized. For this reason, we do not place great emphasis on the actual value of t_c we calculate. Rather, we stress the relative changes in t_c which occur when we perturb the system. We have examined these relative changes by introducing the internal microviscosity (η), such that $\eta = t_c$(lumen)/t_c(bulk water) [3,4]. EPR signals arising from Tempamine located in the aqueous lumen were observed using techniques we have previously described [1,2]. Typical EPR samples of 100 μl were prepared as described elsewhere [4] except that electron acceptors other than methyl viologen were used at the concentrations indicated in the figure legends. The samples were not degassed. Modulation amplitude was kept below one-third of the narrowest line width or 0.5 G, whichever was smaller. Forward and reverse spectra from illuminated samples were averaged and used to calculate t_c as described previously [3,4].

Thylakoids were prepared as previously described [1] from deveined leaves of *Spinacea oleracea*. Chlorophyll concentrations were determined by the methods of Arnon [12]. Control thylakoids prepared as described elsewhere [13] were resuspended to 0.3 mg Chl/ml in buffer containing 200 mM sucrose, 20 mM Tricine-NaOH (pH 8.0), 3 mM $MgCl_2$, 0.05 mM K_3FeCN_6, 0.2% bovine serum albumin, 50 mM KCN, and 0.1 mM $HgCl_2$. This preparation was incubated at 4°C until withdrawn aliquots were unable to reduce methyl viologen (about 90 min). The inhibited suspension was centrifuged, washed once in the above buffer without KCN or $HgCl_2$ or albumin and resuspended to 5 mg Chl/ml for use in the EPR experiments. These thylakoids are termed KCN treated.

Electron transport was measured as oxygen production when the electron acceptor was ferricyanide or diaminodurene and as oxygen uptake when the acceptor was methyl viologen. Changes in oxygen concentration were measured with a Clark-type oxygen electrode [13]. The white light intensity just outside the water-jacketed 1.7 ml cell was about 650 kerg/s per cm^2. Complete reaction mixtures are indicated in the figure legends. The internal volume of the thylakoid samples were determined by the methods of Ort and Dilley [14,15]. Volume was calculated from the amount of [^{3}H]H_2O which was centrifuged through a layer of silicone fluid. [^{14}C]Inulin and [^{14}C]sorbitol were used to estimate the amount of [^{3}H]H_2O which was excluded by the external surface of the thylakoids.

Results

Fig. 1 shows EPR spectra typical of those used to produce the data contained in this paper. Each tracing A–D shows both a forward and a reverse scan through the 40 G magnetic field allowing for the temporal averaging of spectral parameters and the calculation of the lumen microviscosity as we have described elsewhere [3,4]. The hyperfine coupling constants of A to D are 16.7, 16.7, 16.7 and 16.4, respectively, indicating [1] that the Tempamine is residing in a highly polar solution, presumably in water, and that it is not partitioning significantly into the hydrophobic portions of the membrane.

In order to determine whether PS I or PS II electron transport is alone sufficient to drive the light-induced hindrance of rotational motion of Tempa-

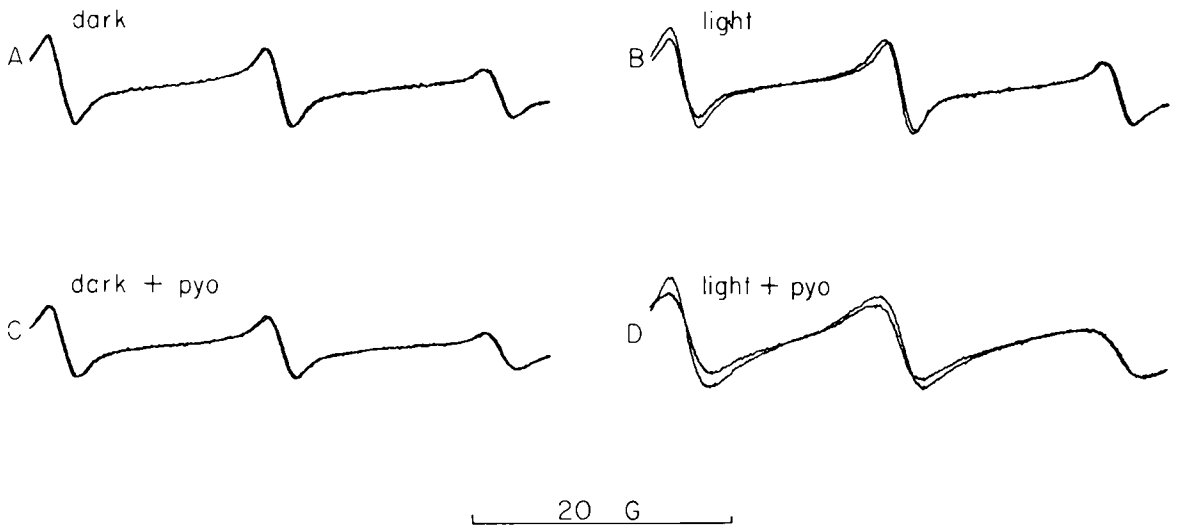

Fig. 1. Typical forward and reverse EPR spectra of Tempamine residing in the aqueous lumen of spinach thylakoids [1–4]. Forward and reverse spectra are shown so that spectral parameters may be averaged to correct for the disproportionation produced by the temporal reduction of the spin label during forward and reverse scans of the magnetic field as described in Ref. 4. Various reaction components and conditions of illumination are represented in the spectra shown. Typical EPR samples were prepared as follows: 83 μl of chloroplasts at 5 mg Chl/ml were added to a test tube containing 1 μl of 100 mM ADP, 1 μl 500 mM sodium phosphate, variable amounts of 100 mM pyocyanin, and 1 μl of 10 mM DCMU, all at pH 7.5. Then 3 μl of 100 mM Tempamine and 10 μl of 400 mM chromium oxalate (both at pH 7.5) were added. Spectra were recorded from 65-μl samples contained in 1 X 100 mm Kimax capillaries in 4-mm quartz NMR sample tubes. The modulation amplitude was 0.125 G, the microwave power 5 mW and the scan rate 20 G/min. The actinic light intensity was 400 kerg/s per cm^2 at the illumination face plate. Sample A is the same sample as B except the forward and reverse spectra were recorded prior to the illumination. Sample C is the same as sample A except it contains 75 μM pyocyanin (pyo) to catalyze cyclic electron transport around PS I. Sample C is the same sample as D except the forward and reverse spectra in the dark were recorded prior to the illumination of the sample. The hyperfine coupling constants of the four spectra are: A, 16.7 G; B, 16.7 G; C, 16.7 G; D, 16.4 G, indicating that the Tempamine is in an aqueous environment. The averaged and calculated spectral parameters from the spectra above are: A, W_0 = 2.09 G, h_0 = 5.95, h_{-1} = 3.82, η = 8.4; B, W_0 = 2.21 G, h_0 = 7.13, h_{-1} = 4.83; η = 7.7; C, W_0 = 2.13 G, h_0 = 6.78, h_{-1} = 4.48, η = 8.0; D, W_0 = 3.45 G, h_0 = 6.40, h_{-1} = 3.61, η = 18.6.

mine, we utilized pyocyanin-mediated electron transport around PS I in the presence of the PS II inhibitor DCMU. Fig. 2 shows that increasing concentrations of pyocyanin allow the observation of the light-induced hindrance of rotational motion. This hindrance appears maximal at about 150 μM pyocyanin. In the presence of both ADP and phosphate the hindrance observed is slightly less than can be observed when no phosphate is present. In dark samples, the pyocyanin causes no change in the rotational motion of Tempamine over the concentration range tested. Since DCMU was present, the electron transport occurring in this reaction system was solely through PS I.

Similar results were obtained with a PS II reaction system mediated by ferricyanide-oxidized diaminodurene in the thylakoids where PS I had been inhibited by treatment with KCN/Hg. These results, and those for PS I and for sequential electron transport through both photosystems, are summarized in Table I. It can be seen that either photosystem is independently capable of producing the light-induced hindrance of rotational motion. The level of hindrance produced by either photosystem alone is essentially like that observed when electrons are transported sequentially through both photosystems to methyl viologen. DCMU inhibits the light-induced hindrance in both the PS II reaction system and the sequential system as expected, but the pyocyanin-mediated cyclic system associated with PS I is not affected by DCMU. All reactions tested were sensitive to gramicidin, an uncoupler which dissipates the light-induced proton gradient in thylakoids and which has

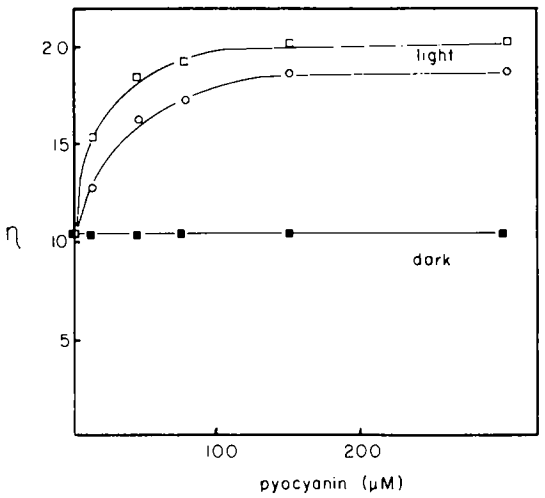

Fig. 2. Increase in the value of η in illuminated thylakoids with increasing concentrations of pyocyanin. The PS II electron-transport inhibitor DCMU was present in all samples at a concentration of 100 μM. Forward and reverse EPR spectra were obtained as described elsewhere [3] and the various parameters were averaged to allow calculation of rotational correlation times and the microviscosity (η). Typical EPR samples were prepared as described in Fig. 1.

been shown [4] to largely prevent the observation of the increased light-induced hindrance of rotational motion of spin labels.

We have suggested previously that the light-induced hindrance of rotational motion may be related to concomitant thylakoid lumen volume changes [4]. Since lumen volume may be readily manipulated by varying the thylakoid's suspending osmoticum, we show in Fig. 3 that at osmolarities less than about 300 mosM the internal thylakoid volume is inversely proportional to the external osmolarity. At osmolarities above 300 mosM the internal volume seems independent of media osmolarity. The internal microviscosity (η) can also be seen to be inversely related to the internal volume of the thylakoid. Thus, as thylakoids swell and the internal volume increases, the internal microviscosity decreases.

One of the major possibilities which we have entertained to explain the hindered rotation of the spin label is that Tempamine binds to some thylakoid component. We present here data from competition experiments which suggest that even at infinite concentrations, competitive species cannot reduce η to the levels measured from bulk water samples. Fig. 4A

TABLE I

THE EFFECTS OF VARIOUS ELECTRON-TRANSPORT SYSTEMS ON THE ABILITY TO OBSERVE THE LIGHT-INDUCED HINDRANCE OF ROTATIONAL MOTION IN THE SPINACH THYLAKOID LUMEN

Reaction conditions were like those described in Fig. 1, except that where indicated, the following electron acceptors and inhibitors were present: diaminodurene (DAD), 4 mM; $K_3Fe(CN)_6$, 12 mM; gramicidin, 20 μg/ml; pyocyanin, 200 μM; DCMU, 100 μM; methyl viologen, 250 μM. EPR spectra were obtained and η was calculated as described in Fig. 1. KCN treatment was as described in Materials and Methods.

Photosystem	Thylakoid treatment	Addition	η	
			Dark	Light
II	KCN	none	11.0	11.0
II	KCN	DAD + $K_3Fe(CN)_6$	11.4	18.1
II	KCN	DAD + $K_3Fe(CN)_6$ + DCMU	11.2	11.4
II	KCN	DAD + $K_3Fe(CN)_6$ + gramicidin	11.1	11.2
II + I	none	none	10.8	13.1
II + I	none	methyl viologen	10.7	19.6
II + I	none	methyl viologen + DCMU	11.1	11.3
II + I	none	methyl viologen + gramicidin	10.7	11.0
I	none	none	10.9	13.9
I	none	DCMU	10.5	10.9
I	none	pyocyanin + DCMU	10.9	22.4
I	none	pyocyanin + DCMU + gramicidin	10.7	12.2

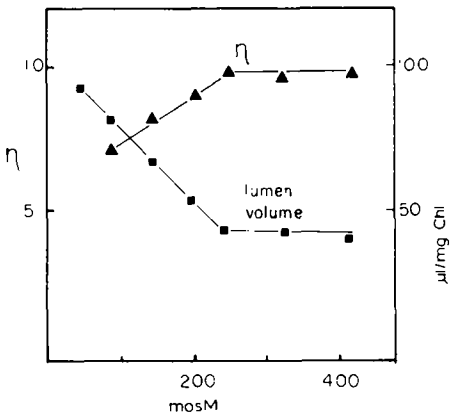

Fig. 3. The value of η and the internal thylakoid volume as a function of osmolarity. Thylakoids were prepared as described in Material and Methods and then washed three times in sucrose solutions of increasing concentrations. The osmolarity was calculated from thylakoid samples containing 3 mM Tempamine, 20 mM chromium oxalate and the corresponding sucrose concentrations. The internal volume was determined by the method of Ort and Dilley [14,15]. EPR spectra were obtained and η was calculated as described in Fig. 2.

shows the effects of increasing methylamine concentrations on η in dark thylakoids. The value of η is reduced from about 9.7 in the absence of methylamine to about 7.5 at 120 mM. The double-reciprocal plot of the same data (Fig. 4B) allows extrapolation to infinite methylamine concentration. At this point, η is only reduced to 7.4. Methylamine would be expected to compete effectively for amine-binding sites,

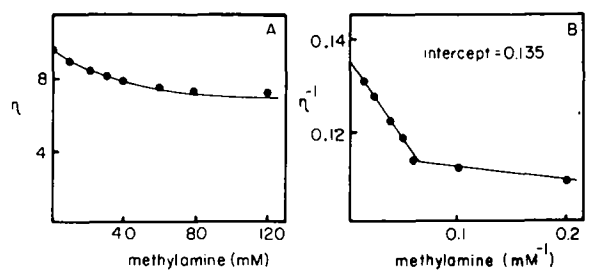

Fig. 4. The competitive effects of methylamine on the calculated microviscosity, η, in the lumen of the spinach thylakoid. Preparations were like those described in Fig. 2 except no electron-transport acceptors or inhibitors were present. Spectra were recorded in the dark and η was calculaed as described in Materials and Methods.

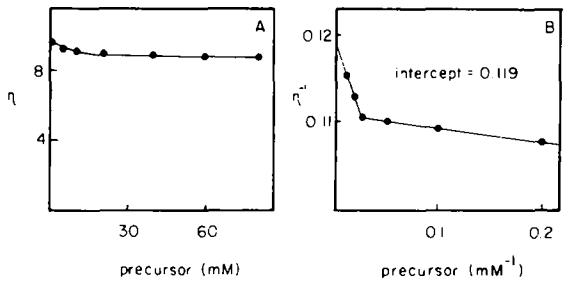

Fig. 5. The competitive effects of 4-amino-2,2,6,6-tetramethylpiperidine (precursor) on η. Reaction condition and calculations were like those described in Fig. 4.

but Tempamine is a far more complicated molecule which might bind in other ways. Thus, we were prompted to explore the competitive effects of 4-amino-2,2,6,6-tetramethylpiperidine. This molecule differs from Tempamine only in the substitution of a hydrogen for an oxygen and so it is likely that 4-amino-2,2,6,6-tetramethylpiperidine would be among the most effective competitors possible. Fig. 5A shows that the effects on η are even less than those observed for methylamine. The double-reciprocal plot (Fig. 5B) shows that at infinite concentrations of 4-amino-2,2,6,6-tetramethylpiperidine, η approaches a value of only 8.4.

Discussion

We have previously reported that Tempamine residing in the lumen of spinach thylakoid yields EPR spectra which are characteristic of a spin label tumbling isotropically in a medium which is about 7–11-times more viscous than bulk water [1,2]. These findings are again confirmed in Fig. 1 which shows spectra of Tempamine residing in the lumen of the thylakoid in the presence of DCMU and pycocyanin, under regimes of both light and dark. We have also reported a light-induced hindrance of spin label motion [3] which was found to be associated with the light-driven accumulation of protons in the thylakoid lumen [4]. We speculated that the hindrance observed in the thylakoid lumen might be due to close membrane proximity. In this report we have measured η and (in parallel samples) the lumen volumes (Fig. 3) and we find that η is related to lumen volume. As the membranes move closer together during the

osmotic shrinking of the thylakoids, η increases to the levels normally seen in dark thylakoids at more normal osmolarities. This notion is further supported by the values of η obtained from thylakoids uncoupled by methylamine [4]. Methylamine, which induces thylakoid swelling in the light [16], actually reduces η in the light to levels below those observed in the dark. This is the expected result if η is related to membrane proximity.

In our earlier reports [4] we provided evidence that proton accumulation and not thylakoid electron transport was required for the observation of light-induced change in η. If this was correct, then any electron-transport reaction which is coupled to ATP synthesis should be able to produce the light-induced increase in hindrance. This hypothesis was tested by measuring η in thylakoids transporting electrons through only PS II and only PS I. As expected, both reaction systems were fully competent in producing the light-induced change, and these changes were almost completely inhibited by gramicidin, in all reaction systems tested. Thus, it seems very clear that there is a light-driven protonation of the lumen and a concomitant lumen volume change which together are important for the observation of the light-induced change in spin label motion.

The pyocyanin-mediated PS I reaction is of particular interest because it is a cyclic reaction system and so there is no net oxidation or reduction occurring. Thus, one need not be concerned about the possibility of exhausting all the electron acceptor before both the forward and reverse EPR scans are complete.

A final explanation of the restriction of Tempamine motion remains elusive. But here we do report that molecules which should compete very effectively with Tempamine for any specific binding sites have very little ability to reduce η in the dark. The spin label precursor, 4-amino-2,2,6,6-tetramethylpiperidine, which most closely resembles Tempamine, is only capable of reducing η from about 9.6 to 8.4 at infinite concentration. Methylamine appears to cause a little more reduction in η but this may be due to some dark accumulation of this amine and the concomitant slight swelling of the thylakoids, Therefore, the hindrance of rotation which we measure does not seem to be the simple result of binding to a thylakoid component(s). Ordered water in the lumen could explain our results and would be consistent with the proton translocation function of the lumen and with an expected cooperative increase in the fraction of ordered water as membranes come into closer juxtaposition. On the other hand, the motionally restrictive environment could be due to random, nonspecific collisions between the spin label and the hydrophilic head groups and proteins which are exposed on the membrane surface. These random, nonspecific collisions would also be expected to increase in frequency as the lumen volume decreases and membrane proximity increases.

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