pH GRADIENTS ACROSS THYLAKOID MEMBRANES MEASURED WITH A SPIN-LABELED AMINE

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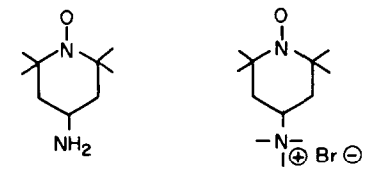
1. Introduction

In their uncharged form amines have been shown to be permeable across membranes and to establish concentration gradients proportional to H^+ gradients [1,2]. In their protonated form they may bind to negative moieties on the surface of proteins or lipid layers. Amines have therefore been used to study pH gradients across membranes [2–9] as well as the surface charge of membranes [10–13]. In particular the quenching of 9-aminoacridine fluorescence has been used to measure the pH gradient across thylakoid membranes [4,8], while that changes in the fluorescence of acridines are claimed [10,12,13] to be closely associated with their strong interaction with the membrane surface and that transport across the thylakoid membrane may not be required.

When using amine probes to measure pH gradients across membranes it becomes important to distinguish between the free and membrane bound populations of the probe; we have therefore used the spin-labeled amine 4-amino-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (Tempamine) with $pK = 9.5$, which shows distinct bound and free EPR signals. An impermeable analogue, 4-trimethyl ammonium-2,2,6,6-tetramethyl-piperidine-*N*-oxyl bromide (CAT_1) and the impermeable spin broadening agent $K_3Fe(CN)_6$ were used to demonstrate that it is the uncharged form of Tempamine that is permeable to the thylakoid membrane. The permeable uncharged spin label 2,2,6,6-tetramethyl-4-piperidone(^{15}N-Tempone) was used to show that at low concentrations of Tempamine (150 μM) virtually no swelling of the thylakoids occurred during illumination.



TEMPAMINE CAT_1 ^{15}N TEMPONE

Our studies have allowed us to determine the internal aqueous concentration of this amine in the thylakoid and to accurately measure the pH gradient across its membrane.

2. Materials and methods

Thylakoid membranes were prepared from spinach leaves in 0.4 M sucrose, 10 mM NaCl buffered with 10 mM tricine, at pH 8, and resuspended in this medium at 6 mg chl/ml [14]. For the present studies thylakoid suspensions (0.6 mg chl/ml) in 90 mM NaCl or KCl, tricine $>$ 1 mM (pH 8), in the presence of spin label and 0.1 mM methylviologen were placed in quartz EPR tubes of 1 mm internal diameter. Where indicated, samples were illuminated by a 400 W tungsten halogen lamp (GE 3476) at heat filtered saturating light intensities ($\sim$ 200 W/m^2) inside the microwave cavity and the spectra recorded in an E-109 E spectrometer at microwave power of 10 mW, time constant 0.128 s and scan time 9 min. $K_3Fe(CN)_6$, the spin broadening agent, was usually used at conc. $\leq$ 40 mM. Tempamine was from Aldrich Chemical Co., CAT_1 was synthesized [15] and ^{15}N-Tempone was a gift from A. D. Keith.

3. Results

3.1. *Tempamine does not bind to thylakoid membranes in the dark*

A spectrum of Tempamine in 90 mM NaCl is shown in fig.1 (upper spectra). The three lines are narrow and of about equal height. When thylakoids (0.6 mg chl/ml final conc.) are added to the solution in the presence of < 1 mM $K_3Fe(CN)_6$ to prevent spin reduction, spectral changes are negligible. Binding of this probe to either lipids or proteins would partially immobilize the label resulting in differential line broadening [16] and a corresponding decrease in line heights. Since this is not observed, we conclude that binding of Tempamine to thylakoid membranes in the dark is negligible.

3.2. *Uptake of Tempamine by thylakoids during illumination*

When a suspension of thylakoids and Tempamine is illuminated in the EPR cavity, changes in line heights, measured from the baseline, are observed depending on the probe concentration; at low concentrations of Tempamine, which do not uncouple the thylakoids, there is virtually no reduction of the line heights (fig.2 upper scan). Reduction of nitroxide radicals during illumination of thylakoid membranes is well known [17,18], but the presence of 1 mM ferricyanide may prevent such reduction during the time of illumination. At high Tempamine concentrations there is a reversible reduction of line heights. This is consistent with concentration dependent spin–spin broadening as the accumulation of Tempamine inside the thylakoids exceeds the critical concentration for the onset of exchange broadening [19]. Exchange broadening at these concentrations is expected to affect all three lines equally as observed.

The paramagnetic ion ferricyanide broadens the Tempamine spectrum in a collision dependent exchange interaction. This is shown in fig.1 (upper

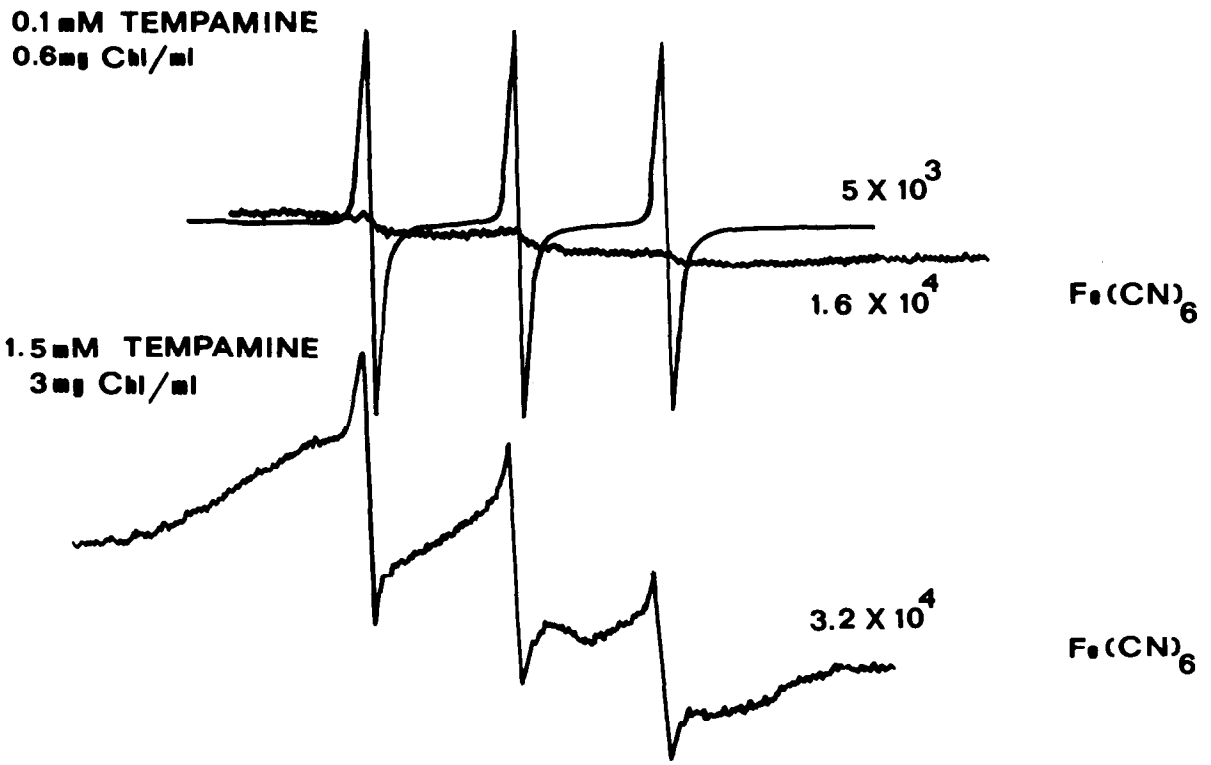


Fig.1. EPR spectra of Tempamine. Conditions as in section 2 with 30 mM $K_3Fe(CN)_6$ where indicated.

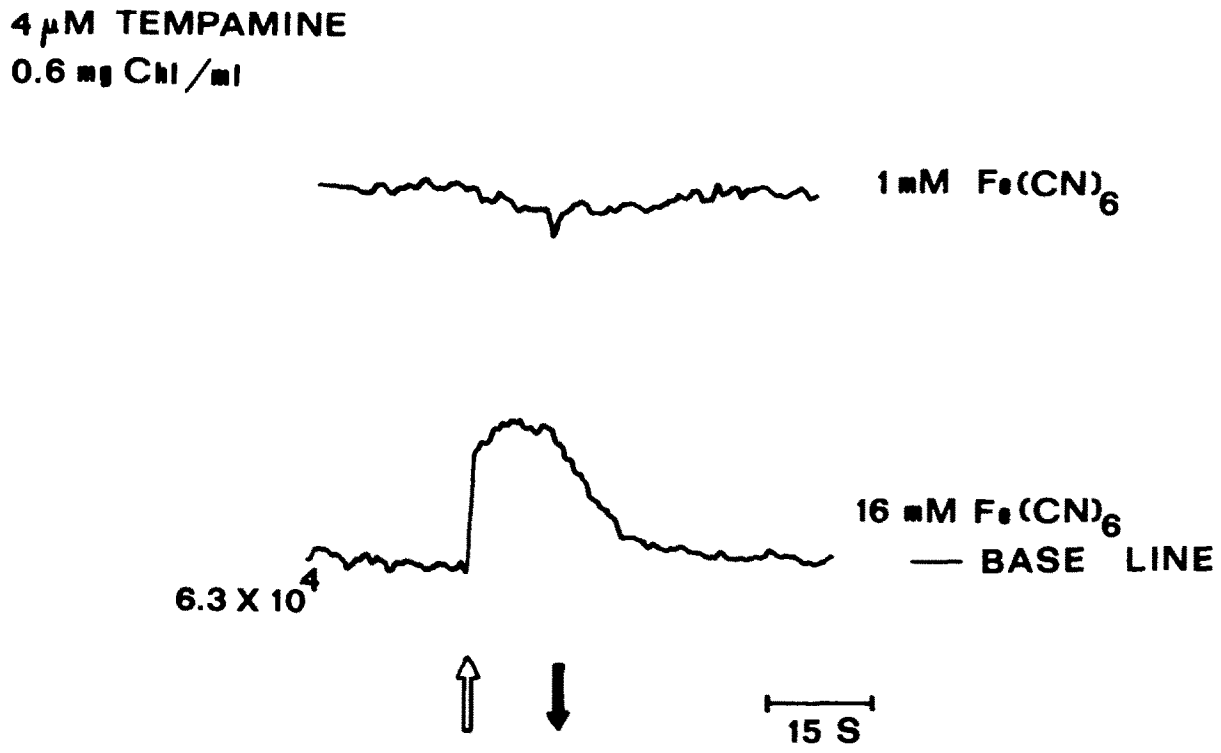


Fig.2. Tempamine low field line height changes during illumination. Light on and off is indicated by arrows.

spectra) where, at low Tempamine concentrations, the broadening of the signal causes its virtual disappearance; at high concentrations of Tempamine and thylakoids, the internal free signal can be clearly seen superimposed on the broadened component that arises from the external population of the spin probe. Ferricyanide is therefore impermeable to the thylakoid membranes (in the time scale of our experiments) and was used to quench the Tempamine signal arising from the exterior of the thylakoids [20]. The uptake of Tempamine into the thylakoids during illumination was observed directly as an increase in the remaining signal as shown in fig.2 (lower scan). The effect is reversible in the dark. At 0.6 mg chl/ml and < 10 μM Tempamine, about 45–55% total probe is taken up into the thylakoids during illumination (fig.3). The total amount of Tempamine taken up during illumination is linearly proportional to the chlorophyll concentrations up to values of ~ 1 mg chl/ml. In the presence of 30 mM NH_4Cl, 100 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) or 1 μM nigericin + 1 μM valinomycin, no uptake of Tempamine could be detected.

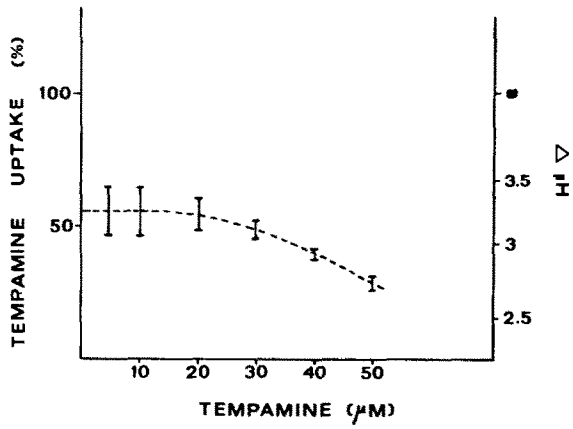


Fig.3. Tempamine uptake and corresponding calculated ΔpH. Chlorophyll concentration 0.6 mg chl/ml.

3.3. *Only the uncharged probes permeate the thylakoid membrane*

A permanently charged analogue of Tempamine, CAT_1, was used to show that the thylakoids are impermeable to the charged form of the probe. After incubation for 2 h in the dark at 25°C no uptake of CAT_1 could be observed; the same was true during illumination, as expected, since the light-driven potential should extrude permeable cations. The ionic radius of charged Tempamine is slightly smaller than that of CAT_1, but the two molecules are sufficiently similar in structure to allow the conclusion that thylakoids are impermeable to the charged amine.

Thylakoids were also illuminated in the presence of both Tempamine and ^{15}N-Tempone. The latter probe, being relatively nonpolar and uncharged, is expected to be permeable to the thylakoid membranes but is not expected to accumulate inside in response to a pH gradient; it has a spectrum which can be clearly resolved from that of Tempamine. During illumination (in the presence of 160 mM ferricyanide to quench the external signal of both Tempamine and ^{15}N-Tempone) the line heights of ^{15}N-Tempone changed negligibly while those of Tempamine increased dramatically, providing evidence that light induced swelling of the thylakoids produces negligible changes in volume when the Tempamine concentration is low (< 150 μM), in agreement with results for other amines [4].

An estimate of the ferricyanide impermeable space was necessary to calculate the internal concentration of Tempamine. This was obtained using high concentrations of chlorophyll (3 mg chl/ml) and Tempamine (1.5 mM) in the dark; the spectrum is shown in fig.1 (lower spectrum). A simple comparison of line heights gives us a value of ~ 1 μl/mg chl for the ferricyanide impermeable space; the same result is obtained using ^{15}N-Tempone either in the dark or during illumination. This volume is smaller than the osmotic volume measured [3,8,21] with radioactive sorbitol, which under our conditions should vary from 3–8 μl/mg chl; the unstirred aqueous layers may be responsible for the differences. In addition, some of the thylakoid membranes we obtain in our preparations may be leaky to $K_3Fe(CN)_6$, but the light-dependent uptake of Tempamine is measured at similar concentrations of ferricyanide so that 1 μl/mg chl is the operational volume in our studies.

3.4. *The pH gradient established during illumination*

It has been shown that amines will equilibrate across membranes in the same ratio as that of protons if the uncharged form of the amine is much more permeable than the charged form [3]. With this assumption, the quantitative data on Tempamine accumulation can be used to calculate the transmembrane pH gradient. At 0.6 mg chl/ml the ferricyanide impermeable space is 0.6 μl/mg of suspension; when the concentration of Tempamine is 8 μM, 45–55% total Tempamine is taken up during illumination. This will correspond to an internal concentration in the range of 6.0–7.33 mM and a corresponding external concentration in the range of 4.4–3.6 μM. The ratio of internal to external Tempamine concentrations is therefore in the range of 1364–2036, and the pH gradient, given by the logarithm of the above ratio, is in the range of 3.13–3.31 units. Volume changes during illumination would only significantly affect the internal concentrations of Tempamine and, as clearly seen from a calculation similar to the above, a volume change as large as 50% would only introduce an error of 0.2 pH units into our result. It is well known that significant volume changes only occur at amine concentrations greater than those necessary for uncoupling [3,4]; our results have been obtained for concentrations of Tempamine that do not uncouple proton uptake.

Binding of Tempamine to the thylakoids would introduce considerable uncertainties into our estimation of the light-driven pH gradient. Significant binding has also been ruled out by estimating the pH gradients from the Tempamine gradients at different thylakoid concentrations (< 1 mg chl/ml); we found the same value for ΔpH and we therefore infer that virtually all the Tempamine remains in the aqueous environment.

4. Conclusions

Using spin-labeled probes similar in structure and size, but differing in that one of them can be protonated (Tempamine) while the other is permanently charged (CAT_1) it has been possible to demonstrate the uptake of Tempamine into the ferricyanide inaccessible space of the thylakoids during illumina-

tion and the impermeability of CAT_1 across the thylakoid membrane. We have also been able to demonstrate that at low concentrations of Tempamine ($< 20\ \mu M$) and fairly high concentrations of chlorophyll (0.6 mg chl/ml) virtually no binding of Tempamine to the thylakoid membrane is detectable either in the dark or in the light. Furthermore, this technique allows unambiguous determinations of inside and outside aqueous concentrations of Tempamine in a thylakoid suspension and therefore accurate determinations of the pH gradient across these membranes during illumination. Our results give ΔpH values of the order of 3.2 ± 0.2 in good agreement with the data in [22].

Acknowledgements

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