Biochimica et Biophysica Acta, 548 (1979) 608–615

BBA 47768

CHROMIUM OXALATE: A NEW SPIN LABEL BROADENING AGENT FOR USE WITH THYLAKOIDS

STEVEN P. BERG * and DONNA M. NESBITT

Department of Biological Sciences, University of Denver, Denver, CO 80208 (U.S.A.)

(Received April 12th, 1979)

Key words: Chromium oxalate; Thylakoid; Spin label broadening; Tempamine; Photophosphorylation; Electron transport; ESR; Chloroplast

Summary

Potassium tris(oxalato)chromate(III) trihydrate (chromium oxalate) has been shown to be a more useful broadening agent than potassium ferricyanide for the spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine (Tempamine) in thylakoid suspensions. Our data show that chromium oxalate is less permeable than ferricyanide, does not inhibit thylakoid electron transport or photophosphorylation, and is not photoreduced by thylakoids.

Introduction

Spin labels may be used to selectively probe the aqueous interior of cells and organelles. These techniques are based on the findings of Keith et al. [1] that Ni^{2+} cannot cross various biological membranes but that the spin label 2,2,6,6-tetramethylpiperidone-*N*-oxyl (Tempone) is freely permeable through the same membranes. Thus when Ni^{2+} and Tempone are added to suspensions of cells, both Ni^{2+} and Tempone are found in the extracellular space but only Tempone is found within the cells. Since Ni^{2+} broadens the external Tempone signal to near invisibility, the major observable Tempone signal is that arising from the intracellular space. Thus the microenvironment of the Tempone molecules in the cytoplasm of a cell may be studied by electron paramagnetic resonance (EPR). Morse [2] noted a number of difficulties with the Ni^{2+} Tempone methodology in red blood cell suspensions and reported an improved system utilizing potassium ferricyanide as a broadening agent for the spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine (Tempamine). Early work in

* To whom all correspondence should be addressed.

this area was reviewed by Eaton and Eaton [3]. Berg et al. have used the same ferricyanide/Tempamine technique to study the rotational motion of Tempamine in an organelle, the aqueous lumen of the spinach thylakoid [4]. However, the use of ferricyanide to broaden the external Tempamine signal in thylakoid suspensions is associated with several critical problems: (1) High concentrations of ferricyanide (80 mM) are required to completely broaden a Tempamine signal, and with this high concentration comes a necessarily high osmolarity which is much greater than the osmolarity to which thylakoids are usually subjected. (2) Thylakoids readily reduce ferricyanide in the light, thus lowering the concentration of the signal broadening agent and lending ambiguity to the measurement of Tempamine rotation. (3) Ferricyanide slowly crosses the thylakoid membrane making it necessary to work very quickly once ferricyanide is added to thylakoids. Long-term experiments are impossible.

Due to these problems with ferricyanide, much of our recent effort has been a search for more effective and practical broadening agents. These broadening agents should have the following characteristics: (1) They must not inhibit thylakoid electron transport or photophosphorylation. (2) They must not be reduced by thylakoids. (3) They must be stable in aqueous solution. (4) They must have net charge and other properties so that they will not readily cross thylakoid membranes. (5) They must possess optimal magnetic properties which allow complete spin label broadening at lower concentrations so that osmolarity may be minimized.

The experiments of Yager et al. [5,6] prompted us to re-examine the rotational motion of Tempamine in spinach thylakoids using a new spin broadening agent, potassium tris(oxalato)chromate(III) trihydrate, (chromium oxalate). Our data show that: (1) Chromium oxalate is a more effective broadening agent than ferricyanide. (2) It crosses the thylakoid membrane at a much slower rate than ferricyanide. (3) It does not inhibit thylakoid electron transport or photophosphorylation. (4) It is not photoreduced by thylakoids.

Materials and methods

Tempamine was synthesized by the methods of Rozantsev [7]. All Tempamine solutions were adjusted to pH 7.5 before use. Chromium oxalate was synthesized by the methods of Bailar et al. [8] and characterized by its visible spectrum [9] and EPR g-value [5,6,10].

EPR spectra were obtained on a Varian E-9 spectrometer using an E-231 cavity with a VT Dewar insert. The spectrometer is interfaced to a Varian 620L/103 computer permitting data manipulation using the CLASS language [11]. Rotational correlation times (τ_c) were calculated from the equation [12–15]:

$$\tau_c = kW_0 \left\{ \left[\frac{h_0}{h_{-1}} \right]^{1/2} - 1 \right\},$$

where k is a constant which depends on the principal values of the g and hyperfine tensors for the spin label and on the microwave frequency. The

other parameters are measured directly from the spectra. The first derivative apparent peak to peak midfield line width, midfield line height, and highfield line height are W_0, h_0, and h_{-1} respectively. For the purposes of this paper, the major limitation of this formula is that it requires isotropic tumbling of the nitroxide spin label.

Thylakoids were isolated from *Spinacea oleracea* as we have described elsewhere [4]. Chlorophyll concentrations were determined by the methods of Arnon [16]. Electron transport and photophosphorylation measurements were as described by Berg and Izawa [17]. Maximum illumination was 500 kerg · sec^{-1} · cm^{-2} but it was necessary to adjust illumination to compensate for light absorbed by the chromium oxalate. A 4 × 4 × 1 cm glass cuvette was filled with varying concentrations of chromium oxalate. The filtered actinic light was passed through the chromium oxalate solution before irradiating a 1 × 1 × 2 cm chamber containing a control thylakoid suspension with no chromium oxalate. The light intensity was then varied such that the electron transport rates were all identical regardless of the concentration of the filtering chromium oxalate. These light intensities were noted and used to illuminate thylakoid suspensions containing the corresponding concentrations of chromium oxalate. The visible absorption spectrum for chromium oxalate is characterized by low absorbance in the regions around 700 nm and 490 nm. These regions were used as windows for the actinic illumination of thylakoid suspensions.

Results

The effects of increasing ferricyanide and chromium oxalate concentrations on the signal of 5 mM Tempamine are compared in Fig. 1. Signal height is reduced to nearly zero by 80 mM ferricyanide as has been previously reported by Morse [2] and Berg et al. [4]. Chromium oxalate causes similar signal height reduction at only 40 mM, indicating that chromium oxalate is a much more effective broadening agent than ferricyanide for the spin label Tempamine. These results are in good agreement with those of Yager et al. [5,6].

Fig. 2A shows that thylakoid electron transport (measured as O_2 production) from water to ferricyanide is unaffected by concentrations of chromium oxalate up to about 66 mM. It can also be seen from the lower trace in the absence of the well known electron acceptor ferricyanide that chromium oxalate can not serve as an electron transport acceptor. Since the addition of chromium oxalate to a final concentration of 66 mM causes a large change in osmolarity, we maintained constant osmolarity in each sample by adding varying amounts of KCl. If KCl is not added, increasing concentrations of chromium oxalate appear to cause inhibition of electron transport. This inhibition is non-specific and can be duplicated with increasing concentrations of KCl or other salts.

Fig. 2B shows that the condition of the thylakoids is not significantly affected by increasing concentrations of chromium oxalate. The high ionic strength or osmolarity causes some reduction in photophosphorylation efficiency from 1.1 (control) to 0.8, but it is clear that the membranes are intact and functional.

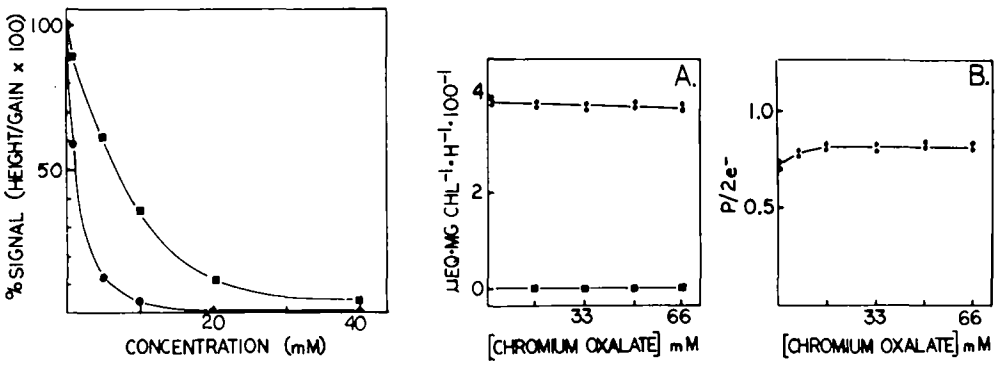


Fig. 1. Per cent signal height (h_0) of 5 mM Tempamine as a function of broadening agent concentration. Spectra were recorded from 65 μl samples contained in 1 X 100 mm Kimax capillary tubes in 4 mm quartz NMR sample tubes. Capillary tubes were sealed with vinyl putty. The samples were not degassed. Modulation amplitude was kept below 10^{-1} of the narrowest line width or 0.125 gauss, whichever was smaller. Spectra were recorded at 5 mW power and 20 G/min. pH was 7.5. Chromium oxalate, •; $K_3Fe(CN)_6$, ▪.

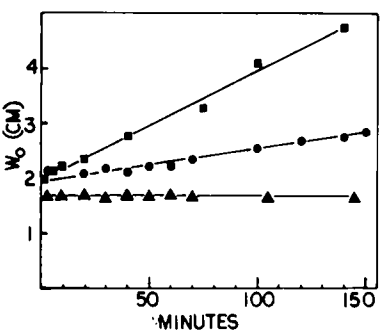
Fig. 2. Effect of chromium oxalate on electron transport (A) and photophosphorylation (B). Samples of 1.7 ml contained: 100 mM sucrose, 30 mM *N*-2-hydroxyethylpiperazine propane sulfonic acid (HEPPS/NaOH) pH 8.0, 3 mM $MgCl_2$, 5 mM $NaH_2\ ^{32}PO_4$, 0.75 mM ADP, and chloroplasts equivalent to 50 μg chlorophyll (Chl). Chromium oxalate was present at the indicated concentrations and KCl was added as needed to maintain the osmolarity at 380 mosM. Control (25 mM KCl and no chromium oxalate) electron transport rates and phosphorylation efficiencies were 375 μequiv · mg^{-1} Chl · h^{-1} and $P/2e$ = 1.1, respectively. $P/2e$ is the ratio of the moles of ATP produced per pair of electrons transported. With 0.4 mM $K_3Fe(CN)_6$, •; Without added $K_3Fe(CN)_6$, ▪.

Chromium oxalate, like ferricyanide [4], causes a slow time-dependent broadening of the internal Tempamine signal as shown in Fig. 3. However, the rate at which chromium oxalate broadens the internal Tempamine signal is much less than that for ferricyanide. This result is consistent with the interpretation that chromium oxalate may cross the thylakoid membrane as does ferricyanide but at a much slower rate.

The EPR spectrum of 5 mM Tempamine in water (Fig. 4A) indicates rapid isotropic tumbling. The rotational correlation time is calculated to be 4×10^{-11} s and the hyperfine coupling constant is 16.9 gauss. The spectrum of 5 mM Tempamine in the presence of 20 mM chromium oxalate at the same instrument gain is broadened so that no measurable signal height is present (Fig. 4A, arrow). However, when the instrument gain is increased to the level used in measuring spectra from thylakoid suspensions, the broadened peaks are clearly visible (Fig. 4B). The signal from a thylakoid suspension containing 5 mM Tempamine and 20 mM chromium oxalate (Fig. 4C) has characteristics of both the spectrum of Tempamine in the internal thylakoid space and of the chromium oxalate-broadened spectrum seen in Fig. 4B. The elements of the chromium oxalate-broadened Tempamine spectrum were removed by computer subtraction. A suspension such as that used for Fig. 4C was centrifuged to pellet the thylakoids. The signal from the supernatant (Fig. 4D) which was indistinguishable from the signal in Fig. 4B was then computer subtracted from the suspension signal of Fig. 4C. The resulting signal (Fig. 4E), representing the portion of the signal in Fig. 4C which arises from the thylakoid interior,

is nearly identical to the signal of 5 mM Tempamine in a glycerol-water solution with a bulk viscosity of 10 centipoise [4]. The rotational correlation time of Tempamine in Fig. 4C is $4.17 \cdot 10^{-10}$ s and is the same within error as Fig. 4E. This is the expected result since the maximum amplitude of the broadened signal (Fig. 4D) does not align itself with the maximum amplitude of the more narrow signal arising from the thylakoid interior (Fig. 4C or 4E). Thus the effect on h_0 and h_{-1} is small and the effect on the square root of the ratio becomes unimportant. For these reasons, our routine measurements of rotational correlation time are usually made from uncorrected suspension spectra similar to Fig. 4C.

The hyperfine coupling constant of both the suspension spectrum and the computer subtracted spectrum is 16.7 gauss, close to the value of 16.9 gauss found for Tempamine in water (Fig. 4A). The similarity of these coupling constants indicates that Tempamine in a thylakoid suspension is sampling a region



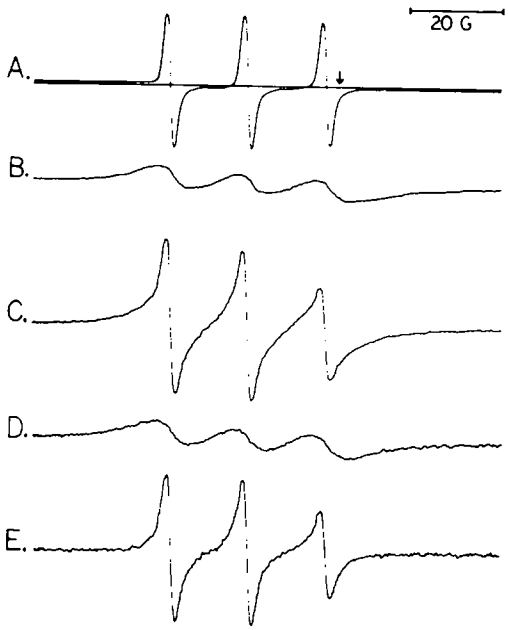


Fig. 3. Change in the apparent peak to peak midfield line width (W_0) with time measured from spectra similar to those in Fig. 4C. Samples contained 5 mM Tempamine, 180 mM sucrose, 18 mM *N*(tris-hydroxymethyl)methyl)glycine (tricine/NaOH) pH 7.5, 3 mM $MgCl_2$, 18 mM KCl, thylakoids equivalent to 5 mg chlorophyll/ml, and either 40 mM chromium oxalate, ●; 80 mM $K_3Fe(CN)_6$, ■; or 80 mM KCl, ▲. Instrument settings were as described in Fig. 1.

Fig. 4. EPR spectra of control and thylakoid samples. (A) 5 mM Tempamine alone in water. The apparently flat 'baseline' spectrum (arrow) is the broadened spectrum of 5 mM Tempamine and 20 mM chromium oxalate. Both spectra are at gain = 400. (B) 5 mM Tempamine and 20 mM chromium oxalate at gain = 8000. (C) Suspension of thylakoids like that described in Fig. 3 containing 5 mM Tempamine and 20 mM chromium oxalate (gain = 8000). (D) Supernatant from a sample such as that used for Fig. 4C after centrifuging for 10 min, 4°C, and 7500 × *g* (gain = 8000). (E) Signal obtained by computer subtraction of 91.5% of spectrum 4D from spectrum 4C. This spectrum is characteristic of Tempamine tumbling in a medium with a bulk viscosity of 10 centipoise (see ref. [3]). All instrument settings were as described in Fig. 1.

which is nearly as polar as water. If Tempamine were sampling a membrane microenvironment, the hyperfine coupling constant would be much lower [18,19,20] than the values observed here or previously [4]. The aqueous region sampled by Tempamine and unavailable to chromium oxalate in Figs. 4C and 4E is most likely the lumen of the thylakoid.

When spectra are generated by computer subtraction, the aim is to derive the Tempamine signal arising from the lumen of the thylakoid. This was accomplished by subtracting the chromium oxalate-broadened Tempamine spectrum arising from the space surrounding the thylakoids from the spectrum of the total thylakoid suspension. The amount to be subtracted depends on the percentage of the thylakoid-excluded volume in the suspension. When correlation times from computer subtracted spectra are plotted against the percent subtraction, there is little change in τ_c. Thus, fairly large errors in calculated excluded volume do not correspond with large errors in the calculation of τ_c. The excluded volume of our thylakoids at 380 mosM was calculated from the data of Rottenberg et al. [21] and the correct subtraction percentage was found to be about 91%. The resulting spectrum gives our best estimate for τ_c arising from Tempamine in the thylakoid lumen. The τ_c is about $4 \cdot 10^{-10}$ s indicating an approximate 10-fold hindrance in rotational freedom over that of bulk water.

Discussion

The different properties of Tempamine and ferricyanide, together with the recently reviewed ability of ferricyanide and other transition metal ions to broaden nitroxyl signals [3] are the foundations for techniques designed to measure parameters associated with aqueous regions surrounded by biological membranes. Tempamine and ferricyanide have been used to study the aqueous lumen of spinach thylakoids [4] and pH gradients across thylakoid membranes [22]. Tempamine is freely permeable to the thylakoid membrane as evidenced by its ability to produce amine-type uncoupling of photophosphorylation [4] and by the accumulation of Tempamine in response to a light driven pH gradient across the thylakoid membrane [22]. The relationships between amines, the uncoupling of ATP synthesis, and the thylakoid pH gradients have recently been reviewed [23]. Tempamine remains the spin label of choice for thylakoid membranes because it is freely permeable to the membrane without partitioning significantly into the membrane.

The transition metal Ni^{2+} was used as a broadening agent for Tempone for a number of different cellular systems [1]. Ferricyanide proved to be a more desirable broadening agent than Ni^{2+} in red blood cells [2], but it has been associated with a number of problems in our thylakoid studies [4]. We present evidence in this paper that chromium oxalate is a spin label broadening agent which substantially reduces the problems associated with ferricyanide.

Chromium oxalate is a more effective broadening agent for Tempamine than is ferricyanide. Fig. 1 shows that only about half the molar concentration of chromium oxalate is required to achieve the same broadening effects which ferricyanide produces. This is in agreement with prior reports of Yager et al. [5,6] who also demonstrated that the optimal broadening conditions are at

pH values less than 8 with low buffer concentrations. The pH optimum for photophosphorylation is around pH 8 [24] and thylakoids are normally isolated and maintained between pH 7 and 8. Therefore, one can achieve optimal chromium oxalate broadening in a pH range appropriate for thylakoid studies.

We have shown elsewhere [4] that 80 mM ferricyanide (required for optimal Tempamine broadening) tends to cause some inhibition of thylakoid electron transport and some reduction in phosphorylation efficiency. Above 80 mM there is a sharp increase in ferricyanide inhibition and uncoupling of the thylakoids [4]. Fig. 2 shows that chromium oxalate causes no measurable inhibition or uncoupling of the thylakoids up to and beyond the optimal broadening concentration of 40 mM. Unlike ferricyanide, chromium oxalate can not be reduced by the thylakoids even at high concentrations (66 mM). Thus there is no ambiguity about the concentration of the broadening agent and, more importantly, we expect that one may choose any of a variety of electron transport acceptors in the presence of chromium oxalate making it possible to monitor changes in the lumen which may occur under various conditions of thylakoid electron transport.

An additional characteristic which makes chromium oxalate preferable to ferricyanide is that the thylakoid membrane is much less permeable to chromium oxalate. As broadening agents cross the membrane and enter the lumen, they begin to affect the internal Tempamine signal. If the rate of entry, and thus broadening, is fast relative to the scanning rate of the instrument, the high-field line will be broadened to a greater extent relative to the midfield line giving rise to an unjustifiably large τ_c. Thus the slower entry of chromium oxalate allows improved accuracy of the measurement of τ_c.

Using chromium oxalate as a broadening agent, we have measured τ_c of Tempamine residing in the lumen of thylakoids to be about $4 \cdot 10^{-10}$ s. The correlation time of Tempamine in water is $4 \cdot 10^{-11}$ s, thus the rotation of the spin label in the thylakoid is hindered by a factor of about ten relative to bulk water. Since chromium oxalate is effective at lower concentrations, the osmolarity of the suspending medium has been reduced from 540 mosM [4] to a more reasonable 380 mosM (Fig. 4E). With the decreasing osmolarity there is no concomitant change in τ_c. This is consistent with our preliminary result that there is no significant increase in swelling associated with this osmolarity range. We know that the motion of Tempamine in the lumen of the thylakoid does respond to changes in osmolarity when the appropriate range of osmolarities is observed. Tempamine shows a linear increase in τ_c between 50 and 250 mosM at which point τ_c reaches a limiting value. This is in contrast to red blood cells which show a linear increase in τ_c up to about 600 mosM (Morse, P.D., Lusczakoski, D.M. and Simpson, D.A., unpublished).

In the above discussion we have assumed that the restriction of Tempamine motion is caused by water within the lumen which is more viscous than the bulk water of the suspending medium. This may not be the only explanation for the increased rotational correlation time of Tempamine, and in an earlier paper we discussed at length a number of alternative explanations [4].

In summary, we have found that chromium oxalate is a more effective broadening agent than ferricyanide thus allowing us to measure τ_c at a lower osmolarity. Chromium oxalate is also found to be less deleterious to thylakoid

biological activities and less permeable to thylakoid membranes than ferricyanide. The only drawback of chromium oxalate is that it is a colored substance which requires control of light intensities if actinic illumination is required.

Acknowledgements

This research was supported by a grant from the United States Department of Agriculture (No. 5901-0410-8-0118-0) to SPB. The authors are grateful to Professor Gareth Eaton, Department of Chemistry, University of Denver for providing references [4] and [5] prior to publication, for technical advice in performing these experiments and for reading this manuscript.

References

1 Keith, A.D. and Snipes, W. (1974) Science, 183, 666—668
2 Morse, II, P.D. (1977) Biochem. Biophys. Res. Commun. 77, 1486—1491
3 Eaton, S.S. and Eaton, G.R. (1978) Coord. Chem. Rev. 26, 207—262
4 Berg, S.P., Lusczakoski, D.M. and Morse, II, P.D. (1979) Arch. Biochem. Biophys. 193, 138—148
5 Yager, T.D., Eaton, G.R. and Eaton, S.S. (1978) J. Chem. Soc. Chem. Commun. 944—945
6 Yager, T.D., Eaton, G.R. and Eaton, S.S. (1979) Inorg. Chem. 18, 725—727
7 Rozantsev, E.G. (1970) in Free Nitroxyl Radicals, Plenum Press, New York
8 Bailar, J.C., Jr. and Young, E.M. (1939) Inorg. Syn. 1, 35
9 Malati, M.A. and Abdul Azim, A.A. (1959) Egypt. J. Chem. 2, 47; Chem. Abstr. 54 (1960) 2085F
10 Singer, L.S. (1955) J. Chem. Phys. 23, 379
11 Klopfenstein, C.H., Jost, P. and Griffith, O.H. (1972) Comput. Chem. Biochem. Res. 1, 176
12 Kivelson, D. (1960) J. Chem. Phys. 33, 1094—1106
13 Kivelson, D. (1972) in Electron Spin Relaxation in Liquids (Muus, L.T. and Atkins, P.W., eds.), pp. 213—278, Plenum Press, New York
14 Stone, T.J., Buckman, T., Nordio, P.L. and McConnell, H.M. (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 1010—1017
15 Keith, A.D., Bulfield, G. and Snipes, W. (1970) Biophys. J. 10, 618—629
16 Arnon, D.T. (1949) Plant Physiol. 24, 1—15
17 Berg, S.P. and Izawa, S. (1977) Biochim. Biophys. Acta 460, 206—219
18 Griffith, O.H. and Jost, P.C. (1976) in Spin Labeling Theory and Applications (Berliner, L.J., ed.), pp. 454—524, Academic Press, New York
19 Wu, S.H.W. and McConnell, H.M. (1974) Biochemistry 14, 847—854
20 Yoshioka, H. (1979) J. Am. Chem. Soc. 101, 28—32
21 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54—63
22 Quintanilha, A.T. and Mehlhorn, R. (1978) FEBS Lett. 91, 104—108
23 Good, N.E. (1977) in Photosynthesis I: Photosynthetic Electron Transport and Photophosphorylation (Trebst, A. and Avron, M., eds.), pp. 429—436, Springer-Verlag, Berlin
24 Saha, S., Ouitrakul, R., Izawa, S. and Good, N.E. (1971) J. Biol. Chem. 246, 3204—3209