

9-Aminoacridine Binding to Chloroplast Membranes in Dark. Reversal by Mg^{2+}

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Chloroplast Membranes, Acridine Binding, Cation Effects

9-Aminoacridine (9AA) binds to photosynthetic membranes of unilluminated chloroplasts in low-salt media. The binding was insensitive to the uncouplers of photophosphorylation. The apparent binding constant was $140 \mu M$. The binding isotherm as a function of 9AA concentration was sigmoidal, and approximately 3 mol 9AA/mol chlorophyll was bound at saturating concentrations of 9AA.

Addition of Mg^{2+} partially reversed the binding of 9AA in chloroplasts in the dark as observed by a Mg^{2+} -induced increase of 9AA fluorescence as well as by spectrophotometric measurements of free 9AA. It appeared, however, that use of fluorescence techniques for measuring free 9AA introduced an error in the estimation of the magnitude of binding, particularly at low concentration of 9AA ($<75 \mu M$). This is probably due to change in fluorescence yield of membrane-bound 9AA on addition of cations. The nature of the binding of 9AA to the thylakoid membranes and the effects of Mg^{2+} thereupon suggest that both chemical binding of cations and screening of surface charge of the membranes should be considered in discussing the mechanism of cation action on chloroplast structure and function. Interpretation of these data with respect to heterogeneity of sites of cation action upon or within chloroplast membranes is discussed.

Introduction

In course of studies on the cation effects on the light-induced proton gradient in isolated chloroplasts as measured by 9-aminoacridine (9AA) fluorescence quenching [1] it was observed [2] that in low-salt chloroplasts a significant amount of 9AA fluorescence was quenched before actinic illumination compared to the chloroplasts in presence of divalent cations. This report describes some of the properties of the effects of Mg^{2+} on 9AA fluorescence quenching in unilluminated chloroplasts.

Materials and Methods

Class II chloroplasts were isolated from 2–3 week old dwarf peas (Greater Progress) grown in the greenhouse. The procedure of isolation was of Nobel [3] as modified by Rurainski and Hoch [4]. Approximately 10 gm of young leaves were ground in a chilled mortar with 10 ml of a medium containing 0.4 M sucrose, 10 mM NaCl, and 15 mM Tris-HCl (pH 7.5). The macerate was filtered through two layers of silk bolting cloth (125 mesh) and centrifuged for 50 seconds at $2000 \times g$. The pellet was taken up in a small volume of grinding medium

(2–3 mg Chl/ml) and stored in ice. The actual experiment was started immediately after isolation and continued for not more than four hours. Chlorophyll concentration was determined by the method of Arnon [5].

9AA fluorescence was measured in a relaxation spectrophotometer used in the fluorescence mode [6]. The emission was excited by a weak modulated (16.1 cps) monochromatic (362 nm) beam which was additionally passed through a 362 nm interference filter and a Corning glass filter No. 9874. The emission was isolated by Corning glass filter Nos. 3060 and 9782. The amplitude of the modulated component of fluorescence was measured with an Ithaco Phase-Lock Amplifier Model 353 and recorded on a Mosley 7100 BM strip chart recorder.

Binding of 9AA to chloroplasts was measured either spectrophotometrically or by fluorescence of 9AA. The spectrophotometric assay (suitable only for dark binding) was performed by incubating the chloroplasts with 9AA, centrifuging the chloroplasts out of the suspension, and then determining the difference in OD from the control (with no chloroplasts) at 362 nm in a Perkin Elmer Colman 124 double beam spectrophotometer.

Results

Fig. 1 illustrates the effects of Mg^{2+} on 9AA fluorescence level before and during actinic illumination. Two distinct effects were observed. Firstly,

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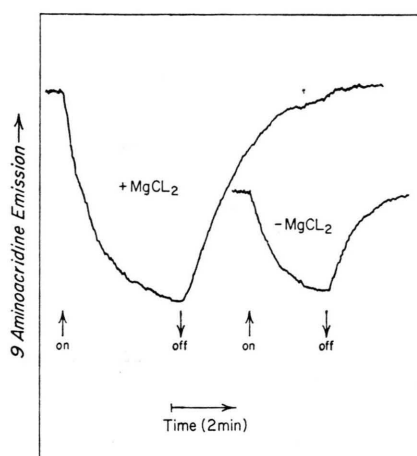


Fig. 1. Effects of Mg^{2+} on 9AA fluorescence before and during actinic illumination. Reaction mixture contained 15 mM Tricine buffer, pH 7.0, 10 mM NaCl, 20 μ g Chl/ml, 20 μ M 9AA, and 5 mM $MgCl_2$ when added.

the dark fluorescence level was increased significantly; secondly, the light-induced quenching of fluorescence was greater in presence of Mg^{2+} . The light-induced quenching, as has been shown previously [1], was abolished by uncouplers, and is generally regarded [1, 7] to be a measure of proton gradient across the thylakoid membranes. This report concerns only with the dark fluorescence level and describes the effects of Mg^{2+} on it.

Table I shows the magnitude of fluorescence increase by Mg^{2+} , which varied from 20 to 50% from one preparation to another. It also shows that within the pH range used there was no significant effects of pH on the increase of fluorescence level. Quenching of 9AA fluorescence appears to be due to binding of the dye to the membranes. Upon addition of Mg^{2+} the dye is released back into the medium. The fluorescence quenching in the dark, unlike the light-induced quenching, was not sensitive

Table I. Effects of Mg^{2+} on 9AA fluorescence level in dark (% increase of 9AA fluorescence by 5 mM Mg^{2+}).

pH	Expt 1	Expt 2	Expt 3
6.2	20	34	53
6.8	23	31	52
7.6	21	32	58
8.5	19	30	53

Reaction mixture: 15 mM buffer-MES for pH 6.2 and 6.8, Tricine for pH 7.6 and 8.5; 10 mM NaCl, 20–25 μ g Chl/ml in 2.0 ml total. 20 μ M 9AA.

Table II. Effects of uncoupler and chlorophyll concentration on 9AA binding (% increase of 9AA fluorescence by 5 mM $MgCl_2$).

	25 μ g Chl/ml	50 μ g Chl/ml
Gramicidin 0 μ M	22	40
Gramicidin 0.1 μ M	21	38
CCCP 1.0 μ M	21	39

Reaction condition same as in Table I.

to the uncouplers such as gramicidin and CCCP (Table II), indicating that the dark binding occurred in the absence of a high energy state. Table II also illustrates that the binding was linearly related to the amount of membranes present, *i.e.* to the concentration of the binding sites.

The 9AA fluorescence increase on addition of Mg^{2+} is a measure of the amount of 9AA released by Mg^{2+} . To measure the amount bound to the membranes either in presence or absence of Mg^{2+} , we monitored the loss of fluorescence from a solution in which chloroplasts were incubated and then removed by centrifugation. Table III shows that

Table III. Binding of 9-aminoacridine by chloroplasts in the dark.

pH	$MgCl_2$	9AA fluorescence (arbitrary unit)			mol dye bound per mol Chl	Apparent Δ pH
		A	B	C		
7.0	—	169	134	21	.21	—2.1
	+	169	159	6	.06	—1.8
7.5	—	166	123	26	.26	—2.2
	+	166	158	5	.05	—1.7
8.0	—	143	103	28	.28	—2.3
	+	143	137	4	.04	—1.6
8.5	—	142	100	29	.29	—2.3
	+	142	138	3	.03	—1.5

Chloroplasts at 20 μ g Chl/ml were incubated with 20 μ M 9AA in presence or absence of 5 mM $MgCl_2$ for 10 min at 12 °C. Then after centrifuging (12000 $g \times 10$ min) the chloroplasts out the fluorescence of the clear supernatant was measured (column B). Fluorescence level of 20 μ M 9AA (without addition of chloroplasts) is shown in column A. Column C gives the percent decrease in B from A, *i.e.*, $C = (A - B)/A \times 100$. The apparent H^+ gradient (Δ pH) was calculated from the equation

$$\Delta pH = \log \left(\frac{Q}{1-Q} \cdot \frac{1}{V} \right)$$

where Q is the fraction of 9AA fluorescence quenched (column C \div 100), and V is the ratio of the chloroplast volume to the total volume of the suspension [1]. $V = 0.002$ and 0.001 in absence and in presence of Mg^{2+} ; the values being obtained from packed volume measurements according to ref. [9].

about 80–90% of 9AA bound (from a 20 μM 9AA solution) was released from the chloroplast membranes by Mg^{2+} (column C). The results also show that upon addition of 20 μM 9AA approximately 0.2–0.3 mol of the dye bound per mol of chlorophyll in the membrane.

Assuming that the dye binds to some functional groups in the membranes and that Mg^{2+} competes successfully with the dye for these groups, an attempt was made to titrate the unknown groups with 9AA. The amount of 9AA bound per mg chlorophyll was determined by the increase of fluorescence upon adding Mg^{2+} as a function of 9AA concentration added to the medium (Fig. 2). A linearity was observed at least up to 100 μM 9AA at which point one mole 9AA was bound per mole chlorophyll. This indicated that a large supply of these groups must be available.

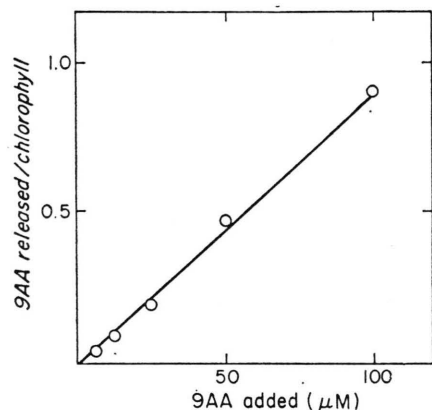


Fig. 2. 9AA binding as a function of 9AA concentration. Reaction mixture was same as in Fig. 1 except that the pH was 7.5 and concentration of 9AA as indicated. Amount of 9AA released was calculated from the increase of fluorescence on addition of 5 mM MgCl_2 to the chloroplast suspension.

We have used spectrophotometric techniques to monitor 9AA binding at high 9AA concentrations; this was accomplished by monitoring the removal of 9AA from the supernatant after centrifuging the chloroplasts out of solution. By this technique we were able to titrate the groups using a higher range of added 9AA. (In the experiment of Fig. 2 we had avoided very high levels of 9AA to eliminate the possible artifact of self quenching of fluorescence occurring at high absorbances.) Fig. 3 and Fig. 4 show the results at pH 8.5 and 6.1 respectively. Several features of these binding isotherms are to be noted. Firstly, as many as three 9AA/Chl can bind

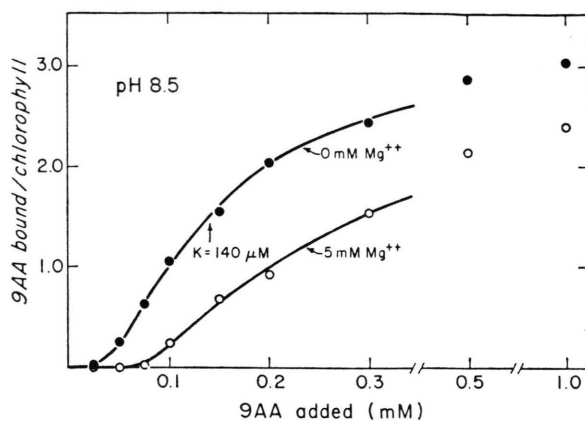


Fig. 3. 9AA binding measured spectrophotometrically (see Materials and Methods). Reaction mixture same as in Fig. 1 except that the pH was 8.5 and 9AA concentration as indicated.

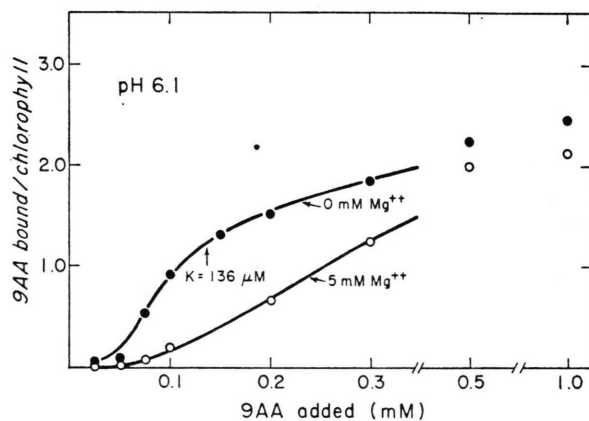


Fig. 4. 9AA binding measured spectrophotometrically. Reaction mixture same as in Fig. 1 except that MES buffer was used.

to the membrane in absence of Mg^{2+} . Secondly, in the lower range of 9AA concentration, bound 9AA was released almost entirely by 5 mM Mg^{2+} ; this observation being consistent with the fluorescence technique of determining 9AA binding (Table III). Thirdly, the apparent binding constant was approximately 140 μM . Fourthly, neither the nature of the binding isotherm nor the apparent binding constant altered significantly when the pH was changed from 8.5 to 6.1.

Discussion

It appears that 9AA binds to the thylakoid membranes in absence of Mg^{2+} . Since the thylakoid membranes have net negative charge at physiological

pH and 9AA bears a positive charge ($pK \sim 10$), most likely electrostatic binding takes place, and Mg^{2+} competes successfully releasing the dye out. We exclude the possibility of an existence of ΔpH across the membranes responsible for the disappearance of the dye for two reasons. Firstly, the disappearance was insensitive to uncouplers; secondly, calculations show (Table III, last column) that a ΔpH of approximately two units should exist to account for the disappearance of the dye, which seems very unlikely because there exists no driving force in the dark to maintain such a high proton gradient.

A high solubility of a substance in the membrane may result in its disappearance from the medium into the membranes. Fig. 2 and Fig. 3 show that approximately one 9AA/chlorophyll was "lost" from a solution containing $20 \mu g$ Chl/ml and $100 \mu M$ 9AA. This result can be accounted for solely in terms of solubility, if 9AA has 100 fold higher solubility in the membranes than in the buffer. Although the partition coefficient of 9AA between thylakoid membranes and water is unknown, a value of 1.2 has been reported between olive oil and water [8]. This suggests that increased membrane solubility is not sufficient to account for the observed disappearance of the dye. Moreover, if the loss of 9AA from solution were due to increased solubility of the dye in the membrane, it is difficult to visualize how Mg^{2+} could possibly decrease the solubility by about 100 fold.

The release of the dye back into the medium on addition of Mg^{2+} cannot be due to osmotic shrinking of chloroplast volume. The fractional increase of 9AA fluorescence level on addition of Mg^{2+} was found to be practically independent of pH between 6.0 and 8.5 (Table I), while the shrinking of chloroplast volume by Mg^{2+} was insignificant at low pH and increased with increasing pH [2, 1].

Recently, Barber *et al.* [10] have proposed that the cation effects on chloroplast structure and function can be explained in terms of diffuse electric double layer at the surface of the thylakoid membrane. Searle *et al.* [11], analyzing the cation induced increase of 9AA fluorescence in terms of diffuse double layer, have concluded that the cation-induced displacement of 9AA from the diffuse layer adjacent to the negatively charged membrane surface resulted in the observed increase of 9AA fluorescence by cations. Our observations, however, can-

not exclude binding of 9AA to certain specific sites in the membranes. The observed sigmoidicity of the binding isotherms (Figs 3 and 4) indicates some kind of cooperative binding. Moreover, an apparent binding constant of $140 \mu M$ appears to be too low to be explained in terms of electrical double layer alone. It is to be noted that low binding constants (approximately $50 \mu M$ and $5 \mu M$) for Ca^{2+} to the thylakoid membranes have been reported previously [12].

Mg^{2+} -induced release of 9AA showed a linear relationship with 9AA concentration (Fig. 2) when the release was measured by the increase of 9AA fluorescence. However, the same relation was sigmoid (Fig. 5) when the release was measured

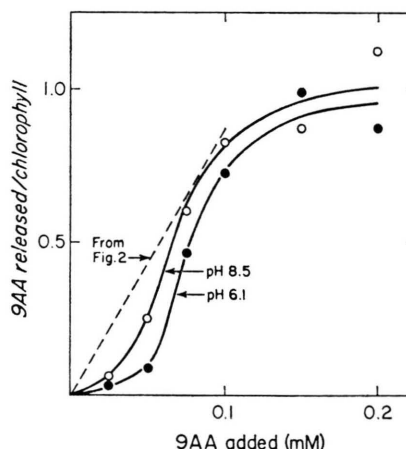


Fig. 5. 9AA release by Mg^{2+} as a function of 9AA concentration From Figs 3 and 4.

by absorbance change. Since the latter technique gives a direct determination of 9AA bound to the membrane, while the former technique depends on the fluorescence yields of the bound and free 9AA under various conditions, the apparent discrepancy may be explained by assuming that 9AA binds to two kinds of sites and the fluorescence yield of the residual bound 9AA in presence of Mg^{2+} was highly increased for one kind of binding site. It is further assumed that in the lower concentration range of 9AA ($< 100 \mu M$) the dye is preferentially bound to this kind of site. This explains why we obtained more fluorescence increase on addition of Mg^{2+} in the lower 9AA concentration than could be expected from the actual amount of 9AA released. In other words, the increase of fluorescence may not be a

true measure of 9AA released, and the observed linearity of fluorescence increase as a function of 9AA concentration (Fig. 2) may be fortuitous. The above interpretation suggest that the lifetime of 9AA fluorescence in presence of chloroplast membranes

should be measured to test if addition of cations alters the lifetime.

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