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LIGHT SCATTERING AND QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE AS INDICATORS OF THE PHOSPHORYLATION STATE OF THE ADENYLATE SYSTEM IN INTACT SPINACH CHLOROPLASTS

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Upon illumination of suspensions of intact chloroplasts, fluorescence of 9-aminoacridine was quenched, light scattering was increased, chlorophyll fluorescence was decreased after an initial increase, and chloroplast ATP/ADP ratios were increased. The response of 9-aminoacridine fluorescence quenching and light scattering to light intensity, anaerobiosis and inhibition of electron transport by DCMU was similar to that shown by chloroplast ATP/ADP ratios. It is discussed under what conditions 9-aminoacridine fluorescence quenching or light scattering can be used to monitor changes in the phosphorylation state of the chloroplast adenylate system.

Introduction

In chloroplasts a transthylakoid proton gradient is formed in the light. The observation was made that during continuous illumination of chloroplasts and leaves chlorophyll fluorescence tended to decrease when light scattering increased and vice versa. These phenomena, which are kinetically related to one another [1,2], appear to indicate the protonation of thylakoid components. In algae, ATP levels were observed to be high when light scattering was increased [3]. In leaves, scattering of green light has been observed to increase under illumination with red or far-red light as a complicated function of light intensity and composition of the surrounding gas phase [4]. When light scattering was decreased during illumination with red light by substituting nitrogen for air, both light scattering and the leaf ATP/ADP ratio decreased reversibly [5,6].

In the present study, light scattering is compared with chlorophyll fluorescence, 9-aminoacridine fluorescence quenching and ATP/ADP ratios of intact spinach chloroplasts. We wanted to know whether light scattering is a suitable indicator system that permits continuous monitoring of the energy status of the photosynthetic apparatus in the light.

Materials and Methods

Intact chloroplasts were isolated from spinach leaves, and CO_2-dependent oxygen evolution and adenylate contents of the chloroplasts were measured as described previously [7,8]. Ferricyanide reduction [9] indicated that in different chloroplast preparations between 70 and 82% of the chloroplasts had intact envelopes. Rates of photosynthesis ranged between 153 and 305 μmol CO_2-

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Abbreviation: DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

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dependent oxygen evolution per mg chlorophyll per h.

Light scattering of chloroplast suspensions (53 μg chlorophyll per ml, light path 0.5 cm) was recorded in transmission [4]. Absorbance changes seen together with scattering changes were small compared with scattering in the optical arrangement used and could be distinguished by their fast kinetics from the slow scattering changes (see insert in Fig. 2B). To avoid problems caused by baseline shifts, scattering was measured as the difference between transmission at the end of a 3 min illumination period and transmission in the dark after relaxation of the light-dependent scattering increase. Relaxation was complete after about 1 min in the dark.

9-Aminoacridine fluorescence was excited by a weak 415 nm beam. The concentration of 9-aminoacridine used in the chloroplast suspensions (4 μM) did not significantly inhibit CO_2-dependent oxygen evolution, but caused some change in the kinetics and some increase in the final steady-state level of both chlorophyll fluorescence and light scattering. Chlorophyll and 9-aminoacridine fluorescence were collected by fiber optics and recorded at 740 nm and 480 nm by a UDT photodiode and a photomultiplier. The diode and multipliers were protected against actinic light by suitable filter combinations. After filtering white light through a layer of water (130 mm), a K 65 filter from Balzers, Liechtenstein, a RG 610 filter from Schott, Mainz, and a heat-absorbing filter from Toshiba, Japan, the resulting actinic light had a half-bandwidth ranging between 626 and 675 nm.

Results

Fig. 1 shows simultaneous recordings of chlorophyll and 9-aminoacridine fluorescence changes observed upon illuminating a suspension of intact chloroplasts with short-wavelength red light. 9-Aminoacridine is a weak base that distributes according to the Henderson-Hasselbalch equation between compartments of different acidity [10]. It may also bind to energized membranes [11,12], but is nevertheless often used to indicate formation of a proton gradient [8,13,15].

When methylviologen and oxygen were present (Fig. 1A, left traces), red light caused considerable quenching of 9-aminoacridine fluorescence and, after an initial fast rise, of chlorophyll fluorescence. The kinetics of 9-aminoacridine fluorescence quenching and of the secondary decline in chlorophyll fluorescence were similar. They were dependent on light intensity. At 180 $W \cdot m^{-2}$, the half-time of the 9-aminoacridine fluorescence response and of the secondary decline in chlorophyll fluorescence were 4 and 10 s, respectively. When methylviologen was absent and only oxygen present (not shown), quenching of 9-aminoacridine fluorescence was slower and less extensive and the decline in chlorophyll fluorescence was also slower than in the presence of methylviologen.

In the absence of both methylviologen and oxygen (Fig. 1A, right traces), both linear and cyclic electron transport were suppressed under red light, presumably by overreduction of the electron carriers, and there was scarcely any quenching of 9-aminoacridine fluorescence. The secondary decline in chlorophyll fluorescence was small, but still significant, and the level of fluorescence finally attained in the steady state was higher than in the presence of methylviologen and oxygen.

Fig. 1B shows a simultaneous recording of chlorophyll fluorescence and scattering of a weak beam of 540 nm light by chloroplasts illuminated with red light. In the presence of methylviologen and oxygen (Fig. 1B, left traces), the large secondary decline in chlorophyll fluorescence was accompanied by a large increase in light scattering which was very similar to the light-dependent increase in 9-aminoacridine fluorescence quenching. The half-time of the light scattering increase was about 1 s and that of the secondary decline in chlorophyll fluorescence 4 s. Half-times of the light scattering increase and of the quenching of 9-aminoacridine fluorescence and of chlorophyll fluorescence were influenced by chloroplast ageing, and the chloroplasts used for the experiment shown in Fig. 1A were older than those used for the experiment of Fig. 1B. When methylviologen and oxygen were absent (Fig. 1B, right traces), scattering was not increased by red light, the secondary decrease in chlorophyll fluorescence was small and the final fluorescence level was high. Occasionally, anaerobic conditions did not completely suppress light-dependent light scattering [16], perhaps, be-

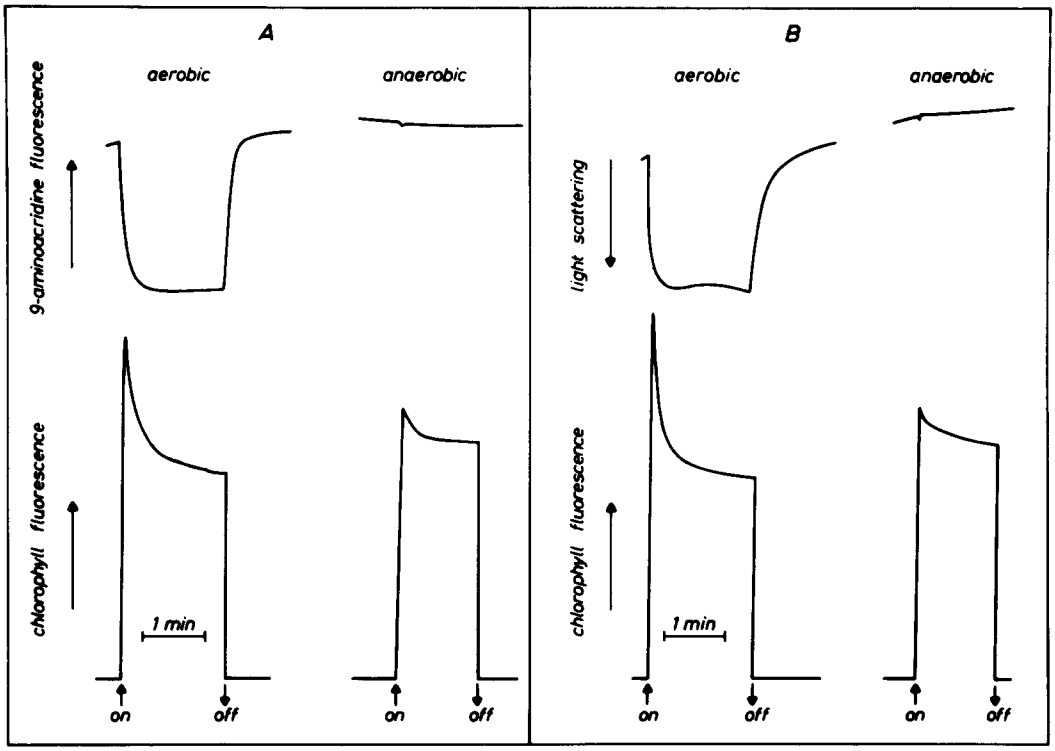

Fig. 1. A. Simultaneous recording of 9-aminoacridine and chlorophyll fluorescence of a chloroplast suspension illuminated with 180 $W \cdot m^{-2}$ red light in the presence (left traces) and in the absence (right traces) of 0.28 mM oxygen and 50 μM methylviologen. B. Simultaneous recording of chlorophyll fluorescence and of light scattering by a chloroplast suspension. Conditions as under (A). The anaerobic samples contained 20 mM glucose, sufficient glucose oxidase to rapidly reduce O_2 and 1300 units/ml catalase to decompose H_2O_2.

cause endogenous electron acceptors were still present.

Even though kinetic details differed, the data of Fig. 1 show that the light-dependent formation of a proton gradient as indicated by 9-aminoacridine fluorescence quenching is closely related to an increase in light scattering by the chloroplasts. The decline in chlorophyll fluorescence, on the other hand, was still significant when red light was under anaerobic conditions incapable of causing much 9-aminoacridine fluorescence quenching and increased light scattering.

Fig. 2 shows the extent of 9-aminoacridine fluorescence quenching (A) and light scattering changes (B) by intact chloroplasts and stromal ATP/ADP ratios (C) as a function of light intensity. Values were taken after 3 min illumination. Light was very efficient in promoting formation of the proton gradient under aerobic conditions. When CO_2 was present, the half-maximum response of 9-aminoacridine fluorescence quenching was at about 7 $W \cdot m^{-2}$; when it was absent, at about 11 $W \cdot m^{-2}$. Light scattering was somewhat less sensitive to light than 9-aminoacridine fluorescence quenching and half-maximum responses were observed at about 15 $W \cdot m^{-2}$ with and 22 $W \cdot m^{-2}$ without CO_2. The rise of the ATP/ADP ratio exhibited half-saturation at only 5 $W \cdot m^{-2}$. In spite of these differences, the general shape of the curves shown in Fig. 2A, B and C is remarkably similar. Both light scattering and quenching of 9-aminoacridine fluorescence were somewhat decreased by CO_2. Although photosynthetic CO_2 reduction consumes ATP, this decrease was not apparent in the ATP/ADP ratios of intact chloroplasts. It is likely that experimental scatter had obscured actual ex-

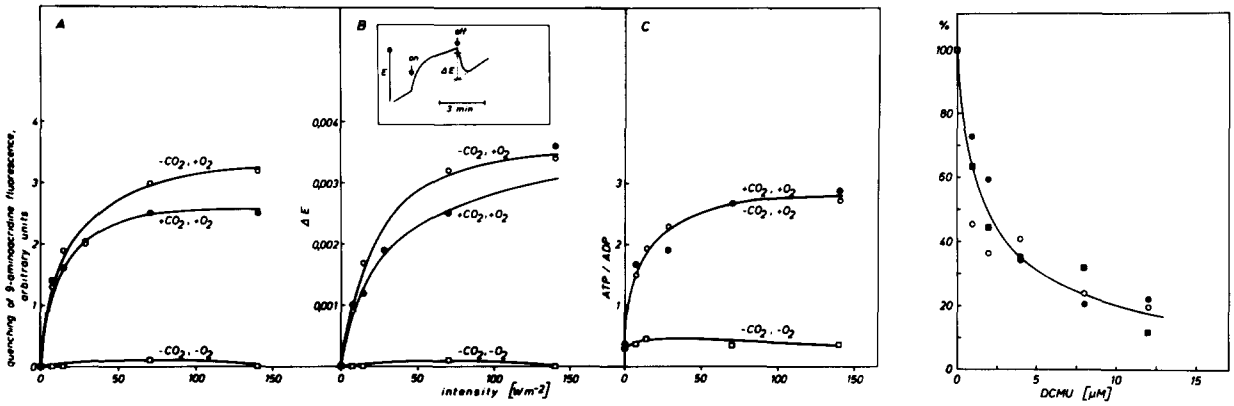

Fig. 2. Quenching of 9-aminoacridine fluorescence (A) and light scattering (B) by a suspension of intact chloroplasts and chloroplast ATP/ADP ratios (C) as a function of light intensity. Aerobic samples contained about 0.28 mM oxygen and 1300 units catalase/ml. 2 mM bicarbonate served as a source of CO_2 where indicated. The anaerobic samples were freed of oxygen as indicated in the legend to Fig. 1. Values were taken after 3 min illumination or 8 min in darkness. The inset in (B) indicates how light scattering measurements were taken.

Fig. 3. Scattering of 540 nm light (○) and quenching of 9-aminoacridine fluorescence (■) by a suspension of illuminated intact chloroplasts and chloroplast ATP/ADP ratios (●) as a function of the concentration of DCMU. Intensity of red light was 140 $W \cdot m^{-2}$. Values were taken after 3 min illumination. Samples contained 0.28 mM O_2 and 1300 units/ml catalase.

isting differences in ATP/ADP ratios. In leaves, CO_2 decreased both light scattering and ATP/ADP ratios (data not shown).

Under carefully maintained anaerobic conditions, and in the absence of electron acceptors, neither 9-aminoacridine fluorescence quenching nor light scattering or the ATP/ADP ratio were significantly increased under illumination of chloroplasts (Fig. 2).

Fig. 3 shows light scattering, quenching of 9-aminoacridine fluorescence and ATP/ADP ratios of chloroplasts illuminated with 140 $W \cdot m^{-2}$ red light as a function of the concentration of DCMU which inhibits electron transport between Photosystems I and II. All three parameters were decreased to a similar extent as the concentration of DCMU was increased.

The data from the figures suggest that under the conditions of the experiments, light scattering indicates the state of membrane energization and can be used in a qualitative manner to monitor the state of the chloroplast adenylate system.

Discussion

As a permeating amine, 9-aminoacridine can enter intact chloroplasts [8]. Its protonation product is trapped in the thylakoid compartment when this becomes acidic during illumination. Owing to concentration-dependent quenching, trapped amine does not emit fluorescence. For an ideal amine that does not bind to membranes and does not enter into secondary reactions, the concentration gradient of protonated amine is at equilibrium equal to the proton gradient between the intrathylakoid space of chloroplasts and the medium in which the chloroplasts are suspended [10].

Binding of the probe to the energized membrane complicates the situation. Hence, it is debated by a number of authors, to what extent 9-aminoacridine fluorescence quenching is a meaningful method for the quantitative estimation of ΔpH [10–12,14,15,17]. Even if binding is not considered, the relationship between proton gradient and quenching of 9-aminoacridine fluorescence in a chloroplast suspension is nonlinear. Fig. 4 shows quenching of an ideal fluorescent amine which is nonfluorescent in the trapped state as a function of the accumulation factor, i.e., the ratio of the concentration of protons inside an acidified compartment to the concentration outside. This relationship is shown for different

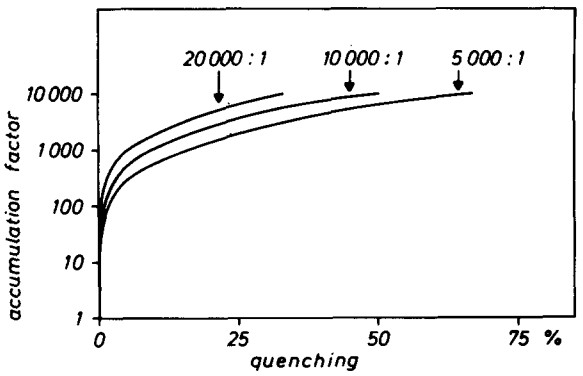

Fig. 4. Accumulation of 9-aminoacridine in the intrathylakoid space versus extent of fluorescence quenching for three different volume ratios of reaction medium to intrathylakoid space. For a chloroplast suspension containing 30 μg chlorophyll/ml the volume ratio is about 10000:1 [18]. Assumptions: 9-aminoacridine is an ideal amine; accumulated amine does not emit fluorescence; and binding of amine to membranes is insignificant.

volumes of the acidified compartment (the thylakoid space) relative to the volume of the outside medium. Ratios of the volume of the fluorescent medium to the nonfluorescent acidic compartment between 5000:1 and 20000:1 may be considered to be a realistic estimate for the concentration of chloroplasts used in the experiments of Figs. 1 and 2 [18]. It is apparent that in such a system there is scarcely any quenching of fluorescence at low $[H^+]_i/[H^+]_o$ (i and o denote inside and outside). Quenching does not become significant up to a $[H^+]_i/[H^+]_o$ ratio of about 100, i.e., to a ΔpH of 2. A further increase in the accumulation ratio or in $[H^+]_i/[H^+]_o$ then results in progressive quenching. Obviously, in dilute chloroplast suspensions quenching becomes considerable only when the proton gradient is large.

During transfer of protons into the thylakoids, electroneutrality is maintained mainly by countertransfer of Mg^{2+}. It has been shown by Krause and others that chlorophyll fluorescence quenching follows the Mg^{2+}-H^+ exchange [19–21]. Though a linear relationship was found when chlorophyll fluorescence was compared with intrathylakoid acidification, which was measured by 9-aminoacridine fluorescence quenching [22,23], the fluorescence yield was also influenced by the redox state of the acceptor Q [24] and by State 1-State 2 changes [25,26].

The physical basis of the light-scattering increase seen during formation of the proton gradient across the thylakoid membranes is also complex. Components appear to be conformational membrane changes resulting from the protonation of anionic groups of membrane proteins and, probably to a minor extent, osmotic volume changes of the thylakoid compartment [2,4,27,28]. Light scattering and chlorophyll fluorescence are correlated phenomena in chloroplasts and intact leaves [1,2].

It is important to note that, since light scattering follows 9-aminoacridine fluorescence quenching (Figs. 2 and 3) there is also a nonlinear relationship between the increase in light scattering and the formation of the proton gradient across the thylakoids. It follows that a significant proton gradient may be formed in the light which is not revealed by much increase in light scattering. Further increases in the proton gradient may then cause significant responses of light scattering (cf. Fig. 4). Since the thermodynamic driving force of the proton gradient

$$\Delta G_{H^+} = -RT \ln \frac{[H^+]_i}{[H^+]_o}$$

is logarithmically related to the magnitude of the proton gradient, both 9-aminoacridine fluorescence quenching and light scattering appear to be particularly sensitive indicators of the energy state of the photosynthetic apparatus within a range of membrane energization that is meaningful for biological energy conservation, i.e. at ΔpH values which are larger than 2.

There is the question to what extent and under what conditions the continuous recording of either 9-aminoacridine fluorescence quenching or light scattering, both of which are technically easy to perform, reflects the phosphorylation state of the chloroplast adenylate system which is believed to be related to the proton gradient and an electrical potential across the thylakoids (for a review see Ref. 29).

In intact chloroplasts, adenylate levels cannot continuously be recorded, and the point-to-point extraction and measurement of adenylates is

laborious and time-consuming. Fig. 2 shows that the light-intensity dependencies of 9-aminoacridine fluorescence quenching, of light scattering and of chloroplast ATP/ADP are similar in general, although there are differences in detail. Differences are seen particularly in the slopes of the light-dependency curves. The ATP/ADP ratio exhibits the steepest slope. It is followed by the curve of 9-aminoacridine fluorescence quenching and then by the light scattering curve. Fig. 3 shows that chloroplast ATP/ADP ratios, 9-aminoacridine fluorescence quenching and light scattering respond in a comparable manner to inhibition of chloroplast electron transport by DCMU. The phenomena recorded in Figs. 2 and 3 were measured after illuminating chloroplasts for 3 min. After long dark times, and with bicarbonate as electron acceptor, the secondary decline of chlorophyll fluorescence was often much slower than shown in Fig. 1 for methylviologen reduction. Under these conditions, the half-time of the rise in light scattering was increased to about 6 s at 115 $W \cdot m^{-2}$ red light, while the half-time of 9-aminoacridine fluorescence quenching was about 3 s and that of the decline in chlorophyll fluorescence even about 50 s.

The relationship between 9-aminoacridine fluorescence quenching, light-scattering changes and ATP/ADP ratios shown in Fig. 2 was not observed in the presence of NH_4Cl, which is a reputed uncoupler of phosphorylation from electron flow. At concentrations between 1 and 3 mM, NH_4Cl decreased 9-aminoacridine fluorescence quenching and increased chlorophyll fluorescence without significantly decreasing chloroplast ATP/ADP ratios or rates of CO_2 reduction by isolated chloroplasts [8,13,30].

Still, in the absence of NH_4Cl and during steady-state photosynthesis, the general agreement between 9-aminoacridine fluorescence quenching, light scattering and chloroplast ATP/ADP ratios is impressive. It appears to be sufficiently close to warrant the use of physical measurements such as light scattering or 9-aminoacridine fluorescence quenching as qualitative indicators of membrane energization and of the phosphorylation state of chloroplast adenylates under specified conditions such as the absence of significant levels of ammonium salts. Because the chlorophyll fluorescence decline is caused not only by acidification of the intrathylakoid space but also by partial reoxidation of the quencher Q [24] and by State 1-State 2 changes [25,26], it seems to be a less suitable indicator of the energy state of chloroplasts than the other parameters (cf. Fig. 1).

While in intact chloroplasts there is no special advantage in using light scattering instead of 9-aminoacridine to monitor changes in the energy state, this dye is unable to penetrate into cells. In green cells or tissues, light scattering is the method of choice to monitor the state of energization of the photosynthetic apparatus [1,4,6]. Further experiments are in progress to compare changes in light scattering by leaves with changes in the phosphorylation state of the leaf adenylate system.

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