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LIGHT-INDUCED MOVEMENT OF MAGNESIUM IONS IN INTACT CHLOROPLASTS

SPECTROSCOPIC DETERMINATION WITH ERIOCHROME BLUE SE

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SUMMARY

The metallochromic indicator Eriochrome Blue SE was used to measure light-induced internal movement of Mg^{2+} in intact chloroplasts. By dual-wavelength spectroscopy (measuring wavelength 554 nm, reference 592 nm) a light-induced, dark-reversible absorbance increase of Eriochrome Blue in samples of isolated intact chloroplasts was observed. The light/dark difference spectrum of Eriochrome Blue between 550 and 590 nm (reference wavelength 562 nm) indicated that this absorbance increase was caused by an increased concentration of free Mg^{2+} in a neutral or slightly alkaline chloroplast compartment.

The signal was seen only with intact, but not with broken, envelope-free chloroplasts, which had lost most of their divalent cations. This is interpreted to show that the indicator responds to an increase of Mg^{2+} concentration in the chloroplast stroma, which represents an efflux of Mg^{2+} from the intra-thylakoid space caused by light-dependent proton pumping.

As calculated from corrected values of the absorbance increase of Eriochrome Blue, the light-induced internal release of Mg^{2+} was close to 100 nequiv per mg chlorophyll at pH 7.6 and 250 nequiv at pH 7.1. This corresponds to a light-dependent increase in the concentration of free Mg^{2+} in the stroma of about 2 and 5 mM, respectively.

INTRODUCTION

The light-induced proton uptake by thylakoids leads to secondary ion movements which electrically balance proton transfer across the membrane. In studies with isolated thylakoids the nature of the species involved in secondary ion transport seems to depend on experimental conditions. Counter transport of Mg^{2+} and K^+ with protons [1] as well as co-transport of Cl^- [2] have been reported. Hind et al. [3] observed that proton influx was balanced by an almost equivalent Cl^- influx and Mg^{2+} (or Ca^{2+}) efflux and to a minor extent by K^+ efflux. Chow et al. [4] found mainly Mg^{2+} efflux, but did not observe major Cl^- influx. In view of this con-

flicting evidence an estimate of secondary ion movements in intact, physiologically active chloroplasts seemed desirable. The movement of Mg^{2+} is of particular interest with regard to photosynthetic carbon metabolism. Since the chloroplast envelope acts as a barrier to free transfer of cations [5–8], Mg^{2+} efflux from the thylakoids would lead to a substantial increase of the Mg^{2+} concentration in the stroma region of intact chloroplasts. This increase may play an important role in the light activation of stroma enzymes such as ribulose-1,5-bisphosphate carboxylase and fructose-1,6-bisphosphatase [9–11]. The movement of Mg^{2+} in intact chloroplasts may also stimulate photophosphorylation and, furthermore, is thought to regulate the distribution of light energy between the two photosystems, thus increasing the efficiency of photosynthesis (see ref. 12).

From studies of the slow, energy-dependent quenching of chlorophyll *a* fluorescence [6, 7, 13–15] and light scattering changes at 535 nm [6, 15] light-induced movement of divalent cations in intact chloroplasts is, indeed, evident. Conceivably, the proton influx into the thylakoids liberates Mg^{2+}, which at the high pH in the dark is bound to negative groups on the inner face of the membrane. Mg^{2+} rather than Ca^{2+} seems to be the major moving ion species [16]. However, these observations provide only qualitative evidence for light-induced Mg^{2+} transfer. Recently, Scarpa [17] has described a method for determining free Mg^{2+} in biological systems by dual-wavelength spectroscopy with the metallochromic indicator Eriochrome Blue SE. Under proper conditions the indicator is highly specific toward Mg^{2+}. In the present study this method has been applied to intact isolated chloroplasts. Apparently, the indicator is able to penetrate the chloroplast envelope. In view of the high amounts of Mg^{2+} (and Ca^{2+}) found in intact chloroplasts (see ref. 5) it should be emphasized that Eriochrome Blue responds only to the free, thermodynamically active fraction of the total Mg^{2+} content of chloroplasts. This allows one to measure kinetically, and with high specificity, the light-induced increase of Mg^{2+} concentration in the stroma of the intact organelles, in contrast to other methods available for measuring Mg^{2+} movement, which require disruption of the chloroplast envelope.

MATERIALS AND METHODS

Intact chloroplasts were isolated from fresh spinach leaves using a modification [18] of the method described by Jensen and Bassham [19]. Usually, 70–80 % of the chloroplasts had retained intact envelopes, as routinely determined by the ferricyanide method [20]. The assay medium used for measuring Mg^{2+} fluctuations contained (in mM) sorbitol (330), NaCl (10), KH_2PO_4 (0.5), sodium acetate (20), and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) (40), adjusted to pH 7.6 or 7.1 with NaOH. Samples (3 ml) contained chloroplasts equivalent to 50 μg of chlorophyll. Eriochrome Blue SE concentration was 0.1 mM. No artificial electron acceptor was added. In the absence of bicarbonate, the Mehler reaction [21, 22] was the predominant electron transport reaction of the illuminated chloroplasts. Broken chloroplasts were prepared by osmotic rupture of intact chloroplasts in water and subsequent addition of an equal volume of double strength assay medium.

For detection of absorbance changes of Eriochrome Blue SE, as caused by Mg^{2+} binding, an Aminco DW-2 spectrophotometer was used. The sensitivity and

specificity toward Mg^{2+} of the experimental setup was tested as described by Scarpa [17] by adding small amounts of cations to a sample of assay medium containing 0.1 mM Eriochrome Blue. For induction of ion movements in the chloroplast samples a saturating beam of red light (300 W/m^{-2}) was used. The light passed through a 20 cm water layer, a RG 630 cutoff filter (3 mm, Schott and Gen., Mainz) and a 1 mm infrared absorbing filter Calflex C (Balzers, Liechtenstein). The exciting red beam entered the sample chamber of the spectrophotometer at right angles to the measuring beams. The multiplier of the photometer was protected from red light by filters 9782 (4 mm, Corning, New York) and BG 18 (2 mm, Schott and Gen., Mainz). The measuring beams had a spectral bandpass of 3 nm. The sample cuvette (light path 1 cm) was kept at 20 °C. Further details are given in the legends to the figures.

In order to evaluate the sieve effect of intact chloroplasts, the absorption band of reduced cytochromes b_6 and *b*-559 LP of chloroplasts was recorded in the presence and absence of Triton-X-100 as described by Heber et al. [23], using the same spectrophotometer as above. Sample and reference cuvette contained in 3 ml assay medium of pH 7.6 chloroplasts equivalent to 225 μg chlorophyll, glucose (10 mM) and Triton X-100 (0.1 % when present). To avoid autoxidation of cytochrome *b*-559 the suspensions were kept anaerobic by adding glucose oxidase and catalase. In the sample cuvette the cytochromes were reduced by $Na_2S_2O_4$ (10 mM).

Fluorescence of chlorophyll *a* and 9-aminoacridine, CO_2-dependent and 3-phosphoglycerate- dependent oxygen evolution by intact chloroplasts, and light induced H^+ uptake by broken chloroplasts in the presence of 5 mM $MgCl_2$ were monitored in control samples as described previously [24–26].

RESULTS AND DICUSSION

Light-dependent Eriochrome Blue absorbance changes

Fig. 1a depicts a characteristic dark-light transient as obtained by dual- wavelength spectroscopy of a sample containing intact chloroplasts and Eriochrome Blue SE (0.1 mM) at pH 7.6. The wavelength pair 554–592 nm was chosen, since it allows the most sensitive detection of free Mg^{2+} with a high specificity [17]. Under the experimental conditions of Fig. 1, addition of 1 μM Mg^{2+} to the medium produced a ΔA of $1.4 \cdot 10^{-3}$. The sensitivity toward Ca^{2+} was lower by a factor of about 15. Fig. 1b shows that a smaller and kinetically different light/dark signal is also seen in the absence of Eriochrome Blue under otherwise identical conditions. This appears to represent an energy-dependent change in light scattering rather than a true absorbance change [27]. Subtraction of this curve from that in Fig. 1a results in the corrected absorbance change of the dye (Fig. 1c). Kinetically, this signal resembles that of light-induced H^+ movement as measured with isolated thylakoids. If 592 nm is taken for reference and 554 nm for the sample wavelength, the light-induced absorbance increase, as depicted in Fig. 1c, indicates an increase in Mg^{2+} concentration, which is reversed in the dark. Since the chloroplast envelope is largely impermeable toward divalent cations [5–8], release of Mg^{2+} from the thylakoids would not increase the magnesium concentration in the medium. Therefore one has to conclude that the envelope allows permeation of Eriochrome Blue into the stroma where the indicator responds to changes in the concentration of free Mg^{2+}. Fig. 2 shows that the light-induced absorbance change of Eriochrome Blue depends, in fact, on the integrity

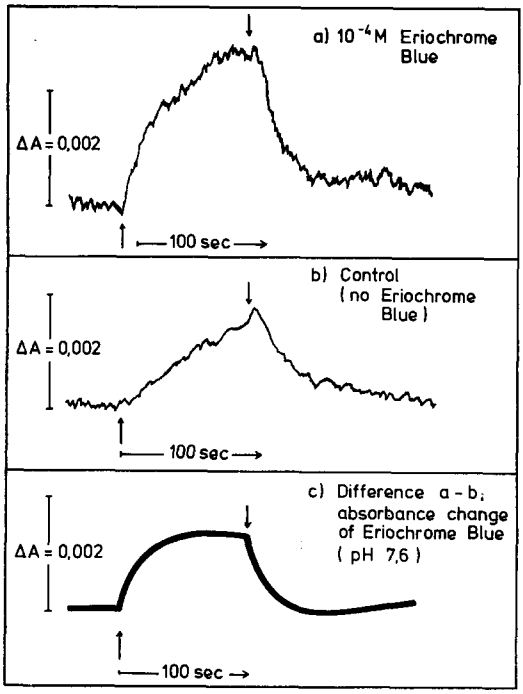

Fig. 1. Absorbance changes of Eriochrome Blue SE at 554 nm, indicating light-induced Mg^{2+} movement in intact chloroplasts. Reference wavelength 592 nm. Samples in (a) and (b) contained 16.7 μg chlorophyll per ml assay medium, pH 7.6, and Eriochrome Blue as noted. Light path, 1 cm. Exciting light, 300 W/cm^{-2}. Light on, upward arrows; light off, downward arrows. The Eriochrome Blue-independent signal (b) was subtracted to obtain the true absorbance change (c).

of the chloroplasts and is not produced by broken chloroplasts contaminating the preparation. Osmotically ruptured chloroplasts lose most of their internal Mg^{2+} when suspended in a medium free of divalent cations, as used in our experiments [6]. The light response of Eriochrome Blue is absent in samples of these envelope-free chloroplasts (Fig. 2). In samples of intact chloroplasts the light response of the indicator was eliminated (as was the Eriochrome-independent signal depicted in Fig. 1b) by uncoupling concentrations (10^{-6} M) of carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), which demonstrates that the internal Mg^{2+} release depends on energized proton transport.

When the medium was adjusted to pH 7.1, the light/dark signals of Eriochrome Blue were smaller but kinetically similar to that shown for pH 7.6 in Fig. 1. At pH 7.1 the sensitivity of the dye toward Mg^{2+} was lower (1 μM Mg^{2+} corresponding to $\Delta A = 0.4 \cdot 10^{-3}$) but discrimination between Ca^{2+} and Mg^{2+} stricter than at pH 7.6. An increase of Ca^{2+} in the medium by 25 μM gave no detectable absorbance change.

Difference spectrum of Eriochrome Blue – Mg^{2+}

Evidence that the absorbance changes of Eriochrome Blue (Fig. 1c) are indeed caused by changes in the concentration of free Mg^{2+} in intact chloroplasts is provided

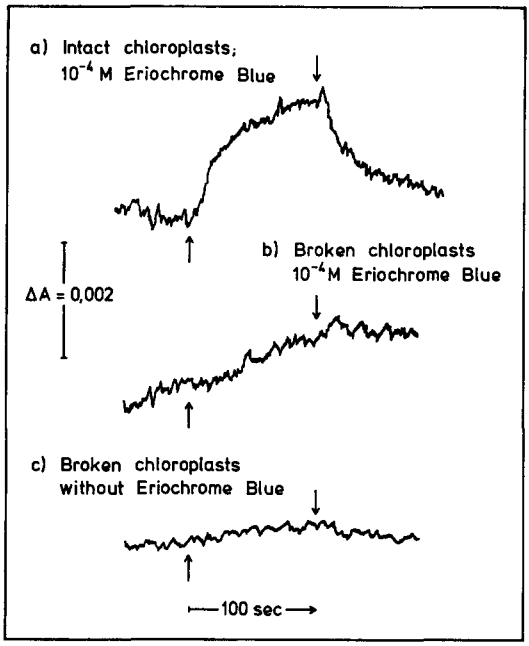

Fig. 2. Light-induced absorbance changes of Eriochrome Blue SE in a sample of intact chloroplasts (a) and absence of absorbance changes in a sample of osmotically ruptured chloroplasts (b). As a control the trace recorded with broken chloroplasts in the absence of Eriochrome Blue is shown (c). Experimental conditions as for Fig. 1.

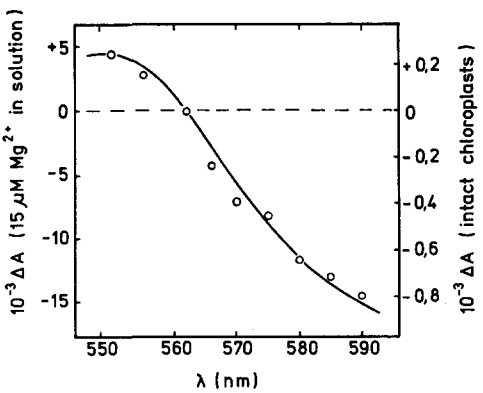

Fig. 3. Difference spectrum of the Eriochrome Blue – Mg^{2+} complex (solid line) and light/dark difference spectrum of Eriochrome Blue in samples of intact chloroplasts (circles). The solid line is the spectrum of Eriochrome Blue (0.1 mM) plus 15 μM $MgCl_2$ versus Mg^{2+}-free Eriochrome Blue, dissolved in assay medium, pH 7.6, as recorded in split beam mode. Circles represent light-induced absorbance changes of Eriochrome Blue in the presence of intact chloroplasts, as seen after 100 s in the light. Values for the respective wavelengths were obtained as shown in Fig. 1, but with 562 nm for reference. Experimental conditions as for Fig. 1.

by the experiment shown in Fig. 3. The solid line in Fig. 3 depicts the difference spectrum of the Eriochrome Blue – Mg^{2+} complex (versus Mg^{2+}-free Eriochrome Blue) as recorded in the absence of chloroplasts. The circles show individual measurements of light-dependent absorption changes of Eriochrome Blue in the presence of intact chloroplasts at different wavelengths (reference wavelength 562 nm, the isosbestic point of Eriochrome Blue – Mg^{2+} [17]). The values represent the steady state, 100 s after onset of illumination, and were corrected for absorbance changes in the absence of the dye, in the manner shown in Fig. 1. Essentially, the light/dark difference spectrum so obtained is identical with the difference spectrum of Eriochrome Blue – Mg^{2+} versus Eriochrome Blue in the absence of chloroplasts.

Calculation of Mg^{2+} concentration changes in the chloroplast stroma

A quantitative determination of Mg^{2+} fluctuations in chloroplasts with Eriochrome Blue relies on several assumptions:

(1) The free dye equilibrates across the chloroplast envelope which results in about equal concentrations in the medium and chloroplast stroma. Permeability of the envelope to Eriochrome Blue is suggested by the experiments shown in Figs. 1 and 2. The Eriochrome Blue – Mg^{2+} complex, on the other hand, must be unable to penetrate the envelope. Otherwise, Eriochrome Blue would deplete the chloroplasts of their Mg^{2+} by transfer to the medium. This does not happen as shown by unaffected CO_2 fixation by the chloroplasts after addition of the dye (see below). Binding of Mg^{2+} by Eriochrome Blue in the stroma would lead to some accumulation of total dye in this compartment; but because of the small proportion of chloroplast volume in the sample (about 1/2000) the concentration of free dye would remain practically constant. Equal distribution is disturbed to some extent by the Donnan potential of the envelope. However, this effect is minimized by the presence in the medium of a high concentration of sodium acetate (20 mM). Both acetic acid and acetate can penetrate the chloroplast envelope. Acetate therefore suppresses the Donnan potential and has indeed been observed to practically abolish the Δ pH across the envelope [28].

(2) The presence of 20 mM sodium acetate also suppresses the light-induced alkalization of the chloroplast stroma [28]. The extent of light-induced Eriochrome Blue absorbance change was found to be slightly decreased when sodium acetate was omitted. Since the Mg^{2+} sensitivity of Eriochrome Blue [17] as well as the absorbance spectrum of the free dye are strongly pH dependent, one would expect that the pH changes in the stroma, which do occur in the absence of sodium acetate, exert a complex effect on the light-induced signal of the indicator. Therefore, only the values obtained in the presence of sodium acetate have been used for calculating Mg^{2+} concentration changes.

(3) The sieve effect, or the strong light scattering by intact chloroplasts do not significantly affect the signal produced by the indicator. In other words, the absorbance change by binding of Mg^{2+} to the dye in the chloroplast stroma is essentially the same as when the Mg^{2+} were homogeneously distributed in the medium. We tried to estimate the magnitude of the sieve effect by spectroscopy of reduced chloroplast cytochromes [23]. After reduction of cytochromes b_6 and b-559 LP with dithionite, the absorption band of intact chloroplasts at 563 nm was compared with that seen after adding 0.1 % Triton-X-100 (Fig. 4). This concentration of detergent mini-

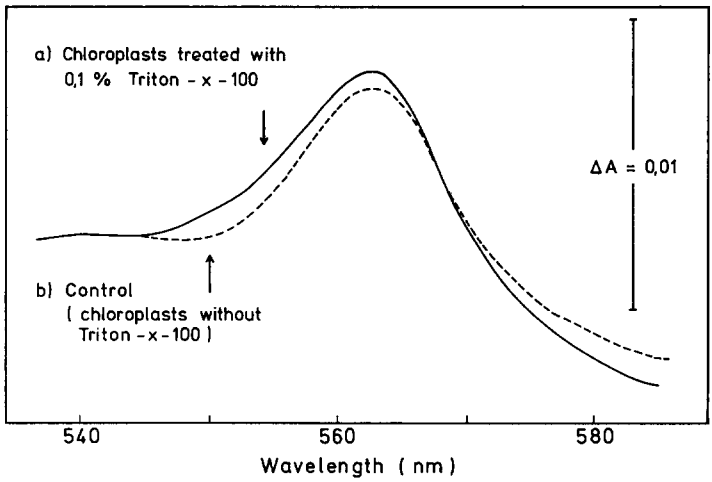

Fig. 4. Difference spectra of cytochromes b_6 and *b*-559 LP in isolated chloroplasts after reduction with 10 mM dithionite, versus dithionite-free references. (a), difference spectrum in the presence of 0.1 % Triton X-100; (b), difference spectrum of intact chloroplasts in the absence of Triton-X-100.

mized light scattering by the sample and dissolved almost totally the chloroplast structures detectable with the light microscope. Addition of detergent did not markedly increase the height of the cytochrome absorption band indicating that the influence of the sieve effect can be neglected in the spectral region of the measurements.

(4) Eriochrome Blue (0.1 mM) and sodium acetate (20 mM) do not exert detrimental side effects on proton and Mg^{2+} transfer in the chloroplasts. When 0.1 mM Eriochrome Blue was added to intact chloroplasts fixing CO_2 at a rate of about 65 μmol · mg chlorophyll^{-1} · h^{-1}, no inhibition was observed. This suggests that essential chloroplast activities were similar with and without the dye. Surprisingly, Eriochrome Blue (0.1 mM) did inhibit phosphoglycerate-dependent O_2 evolution significantly. Since CO_2 fixation was not diminished by the dye, this may be due to inhibition of phosphoglycerate transfer across the envelope. The sodium acetate present in the assay medium (20 mM) inhibited CO_2 fixation to some extent (up to 25 %), as was expected from the effect of sodium acetate on the stroma pH [28]. The effect on internal H^+ pumping and counter transport of Mg^{2+} seems, however, to be negligible, because sodium acetate did not significantly diminish the extent of light-induced 9-aminoacridine fluorescence quenching and energy-dependent chlorophyll *a* fluorescence quenching, indicators of internal proton and metal cation distribution, respectively, in intact chloroplasts [25, 6, 15]. Finally, the question may arise, whether binding of Mg^{2+} by the indicator would disturb the cation distribution within the chloroplasts. This appears unlikely because of the high dissociation constant of the Eriochrome Blue-Mg^{2+} complex. In our assay medium (pH 7.6) a K_D of 0.5 mM was determined spectroscopically (Fig. 5). If the concentration of free dye is 0.1 mM, the ratio of free to indicator-bound Mg^{2+} is 5, i.e. 83 % of the Mg^{2+} remain free. At pH 7.1 (K_D = 1.6 mM [17]) this ratio is even higher.

In Table I values from different experiments are listed for the light-induced increase of Mg^{2+} concentration in the stroma compartment of intact chloroplasts,

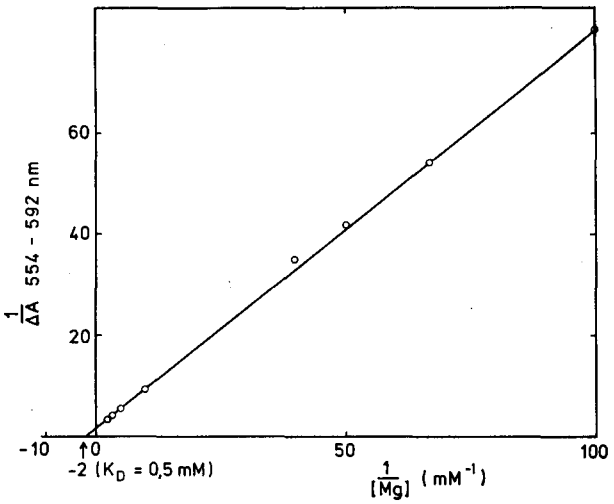

Fig. 5. Lineweaver-Burk plot of Eriochrome Blue absorbance changes versus Mg^{2+} concentration. Measuring wavelength 554 nm, reference 592 nm. Increasing amounts of $MgCl_2$, as given in the figure, were added to the sample cuvette containing 0.1 mM Eriochrome Blue in assay medium, pH 7.6. The dissociation constant of the Eriochrome Blue – Mg^{2+} complex (K_D) under these conditions was 0.5 mM.

TABLE I

LIGHT-INDUCED CHANGE OF Mg^{2+} CONCENTRATION IN THE STROMA OF INTACT CHLOROPLASTS

ΔA denotes the light-induced absorbance changes of Eriochrome Blue as measured 100 s after onset of illumination (measuring wavelength 554 nm, reference 592 nm). The average of several light/dark signals of one sample was corrected for the absorbance change occurring in the absence of Eriochrome Blue, as determined with a parallel sample (cf. Fig. 1). Values of Mg^{2+} concentration increase were corrected for the contamination of the sample by broken chloroplasts. The stroma volume was assumed to be 25 μl per mg chlorophyll [29].

Experiment No.	$\Delta A \times 10^{-3}$	Integrity of chloroplasts (%)	Increase in free Mg^{2+} (nmol per mg chlorophyll)	$\Delta[Mg^{2+}]$ in the stroma (mM)
(a) pH 7.6				
1	1.04	75	58	2.3
2	0.83	70	50	2.0
3	0.74	82	38	1.5
4	0.87	67	54	2.2
5	1.08	71	64	2.5
6	0.69	67	43	1.7
7	0.76	76	42	1.7
Average:			50±4	2.0±0.14
(b) pH 7.1				
1	0.57	72	146	5.8
2	0.78	70	208	8.3
3	0.35	82	79	3.2
4	0.30	67	85	3.4
5	0.39	71	103	4.1
Average:			124±24	5.0±1

as determined with Eriochrome Blue. Basis of the calculation are the corrected light/dark absorbance changes of the indicator in the presence of intact chloroplasts (cf. Fig. 1). From the known Mg^{2+} sensitivity of the system (see above) the total increase of free Mg^{2+} (in nmol per mg chlorophyll) was calculated. Considering that this change is confined to the osmotic volume of the chloroplast stroma, about 25 μl per mg chlorophyll [29], the concentration increase in the stroma at pH 7.6 is approx. 2 mM. The space of the thylakoids has not been considered in the calculation. It accounts for only about 12 % of the total osmotic volume and even in the dark is more acidic than the stroma [29] which would diminish Mg^{2+}-mediated absorbance changes of the indicator. Light-dependent changes of the stroma volume may also be regarded as negligible [29].

At pH 7.1 the standard error was larger due to the lower Mg^{2+} sensitivity of Eriochrome Blue. Still, it can be seen from Table I that the calculated increase in Mg^{2+} concentration, in the average about 5 mM, was higher than at pH 7.6. This is consistent with the higher proton uptake of thylakoids at the lower pH. As measured with a glass electrode, our chloroplast preparations showed, after osmotic rupture of the envelopes, a light-dependent H^+ uptake of about 250 nequiv per mg chlorophyll at pH 7.6, and 380 nequiv at pH 7.1. In the average, Mg^{2+} increase in the intact chloroplasts (Table I) was 100 and 250 nequiv Mg^{2+} per mg chlorophyll, respectively. Although in intact chloroplasts H^+ transfer may differ somewhat from that observed with free thylakoids, these results show that to a considerable extent proton transfer is balanced by Mg^{2+} movement in intact chloroplasts. In this context it should be noted that the higher thermodynamic activity of Mg^{2+} in the light is expected to cause increased binding of Mg^{2+} to membrane surfaces and proteins in the stroma. Since the indicator responds only to free Mg^{2+}, the measured internal Mg^{2+} release is a minimum value for actual magnesium transfer from the thylakoids to the stroma space.

CONCLUSIONS

The experiment depicted in Fig. 3 shows that the Mg^{2+}-complexing agent Eriochrome Blue SE indicates light-induced concentration increases of free Mg^{2+} in samples of intact chloroplasts. The absence of light-dependent absorbance changes of the dye in samples of broken chloroplasts which had lost most of their Mg^{2+} suggests that the dye moves across the envelope into the chloroplast stroma. After proper correction, the light-dependent absorbance changes reveal reversible fluctuations of Mg^{2+} in intact chloroplasts (Fig. 1), as produced by active internal proton transfer. Ca^{2+} movement does not contribute significantly to the observed signals. As has been discussed above, a number of assumptions that must be made in order to calculate the extent of Mg^{2+} movement, appear justified. One premise is that the pH in the stroma is close to that in the medium and is not changed by illumination. This is assured due to the presence of sodium acetate which facilitates transfer of protons between stroma and medium [28] but obviously does not markedly affect intrachloroplast ion movements. Likewise, side effects of the Eriochrome Blue on internal ion transport seem to be insignificant. Furthermore, it appears that the sieve effect of the intact chloroplasts can be neglected. The validity of the results obtained by spectroscopy of Eriochrome Blue is supported by coincidental experiments by

Portis and Heldt [30], who determined Mg^{2+} fluxes by flame spectroscopy subsequent to fast separation of the thylakoids from stroma components. Their values for light-dependent Mg^{2+} transfer, 26–60 nmol per mg chlorophyll (at pH 8.0), are in the same range as our data obtained with Eriochrome Blue at pH 7.6 (Table I).

The calculated values listed in Table I show that larger Mg^{2+} fluctuations take place at the lower pH (7.1), as would be expected from the proton-pumping capacity of isolated thylakoids. The increase in the number of free magnesium equivalents in the stroma of intact chloroplasts was lower than the active proton uptake, as observed when isolated thylakoids were illuminated. However, such a direct comparison between different experimental systems may be misleading. That part of the Mg^{2+} transferred from the thylakoids to the stroma of intact chloroplasts will be bound again in the stroma compartment and therefore is not detectable with Eriochrome Blue, has to be taken into account. Thus, the magnitude of the observed increase in the level of free Mg^{2+} strongly suggests that Mg^{2+} movement represents the major part of secondary intrachloroplast ion transfer.

At pH 7.6, which is close to natural physiological conditions, illumination would still increase the concentration of free Mg^{2+} in the chloroplast stroma by about 2 mM. This is a higher value than that calculated on theoretical grounds by Hope et al. [31]. The concentration increase is sufficient for a significant role of Mg^{2+} in the light activation of the photosynthetic carbon reduction cycle [9, 10], provided the dark level of free Mg^{2+} in the stroma is low. So far, however, the thermodynamic activity of Mg^{2+} in the chloroplast stroma in the dark remains unknown.

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