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EGTA, A CALCIUM CHELATOR, INHIBITS ELECTRON TRANSPORT IN PHOTOSYSTEM II OF SPINACH CHLOROPLASTS AT TWO DIFFERENT SITES

Rita Barr, Karen S. Troxel and Frederick L. Crane

Department of Biological Sciences
Purdue University
West Lafayette IN 47907

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Summary

A fifteen minute incubation of spinach chloroplasts with the divalent Ca^{2+} chelator, EGTA, in concentrations 50-250 μM, inhibits electron transport through both photosystems. All photosystem II partial reactions, including indophenol, ferricyanide and the DCMU-insensitive silicomolybdate reduction are inhibited from 70-100%. The photosystem II donor reaction, diphenyl carbazide → indophenol, is also inhibited, indicating that the inhibition site comes after the Mn^{2+} site, and that the first Ca^{2+} effect noted (site II) is not on the water oxidation enzyme, as is commonly assumed, but between the Mn^{2+} site and plastoquinone A pool. The other photosystem II effect of EGTA (Ca^{2+} site I), occurs in the region between plastoquinone A and P700 in the electron transport chain of chloroplasts. About 50% inhibition of the reaction ascorbate + TMPD → methyl viologen is given by incubation with 200 μM EGTA for 15 min. Ca^{2+} site II activity can be restored with 20 mM $CaCl_2$. Ca^{2+} site I responds to Ca^{2+} and plastocyanin added jointly. More than 90% activity in the ascorbate + TMPD → methylviologen reaction can be restored. Various ways in which Ca^{2+} ions could affect chloroplast structure and function are discussed. Since EGTA is more likely to penetrate chloroplast membranes than EDTA, which is known to remove CF_1, the coupling factor, from chloroplast membranes, and since Mg^{2+} ions are ineffective in restoring activity, it is concluded that Ca^{2+} may function in the electron transport chain of chloroplasts in a hitherto unsuspected manner.

Introduction

Fredricks and Jagendorf (1) found that Ca^{2+} salts stimulated, but Na^+ or K^+ salts inhibited Hill reaction rates in *Anacystis nidulans*. Piccioni and Mauzerall (2,3,4) found that the addition of Ca^{2+} ions increased Hill reaction rates twentyfold in cell-free extracts from *Phormidium luridum*. Since they had used high concentrations of EGTA

Abbreviations:

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC - diphenylcarbazide; DCIP - 2,6-dichloroindophenol; DBMIB - 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; EGTA - ethyleneglycol bis (α - aminoethyl ether)-N,N'-tetraacetic acid; FeCN - potassium ferricyanide; MV - methylviologen; PS I - photosystem I; PS II - photosystem II; SM - silicomolybdic acid; TMPD - N,N,N',N' - tetramethyl-p-phenylene diamine.

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(1 mM) in the original breaking of cells by French press treatment, it occurred to us that, maybe, they had devised an efficient way of depleting chloroplasts of Ca^{2+}. We in this study tried washing spinach chloroplasts with various concentrations of EGTA and found that electron transport was inhibited 50% or more. Further studies by means of various chloroplast partial reactions showed that not water oxidation itself, as postulated by Piccioni and Mauzerall (2,3,4) or Brand (5) was the site requiring Ca^{2+}, but 2 other sites further along the electron transport chain in PS II were found to be inhibited by the EGTA treatment, implying that Ca^{2+} may be required for their activity in vitro.

Materials and Methods.

Sucrose (0.4 M)- NaCl(0.05 M) chloroplasts (SN chloroplasts) were prepared by grinding 20 g deveined spinach leaves, obtained from the local market, for 15 sec. in 80 ml of SN grinding solution in a Waring blendor. The slurry was filtered through 8 layers of cheesecloth, then through a layer of Miracloth and centrifuged in 2 centrifuge tubes at 200 xg for 2 min. The supernatant was transferred to clean centrifuge tubes and recentrifuged at 600 xg for 10 min. to obtain a chloroplast pellet, which was gently suspended with a brush in 5 ml SN. Chlorophyll was determined according to Arnon (6). O_2 evolution or uptake on chloroplast partial reactions was measured with a Clark-type oxygen electrode, as reported previously (7). Reaction rates were recorded with a Sargent-Welch SRG recorder. Electron donation to PS II with the diphenyl carbazide/DCIP system was measured spectrophotometrically at 600 nm, using 21.2 as the extinction coefficient for DCIP (8).

The EGTA treatment of chloroplasts consisted of incubating chloroplasts (2.5 mg chlorophyll/40 ml EGTA solution) with 100-250 μM EGTA solutions for 15 min. at 4°C. Chloroplasts were pelleted by centrifugation at 600 xg for 10 min. Control chloroplasts were incubated with distilled water in place of EGTA solutions, but were treated alike otherwise. For rewashing treated and control chloroplasts, SN was used where indicated. EGTA was purchased from the Sigma Chemical Co. and recrystallized from a 50% solution of isopropanol.

Results and Discussion.

Studies of DCMU-insensitive silicomolybdate reduction by Photosystem II showed, that moderate concentrations of $CaCl_2$ ($\simeq$ 20 mM) stimulated this reaction at pH 6 and at pH 8 (Fig. 1), while Mg^{2+}, Sr^{2+} and Ba^{2+} ions inhibited it at similar concentrations. This lead us to investigate, how removal of Ca^{2+} from chloroplasts would affect various chloroplast partial reactions. As Fig. 2 shows, a 15 min. incubation of chloroplasts with

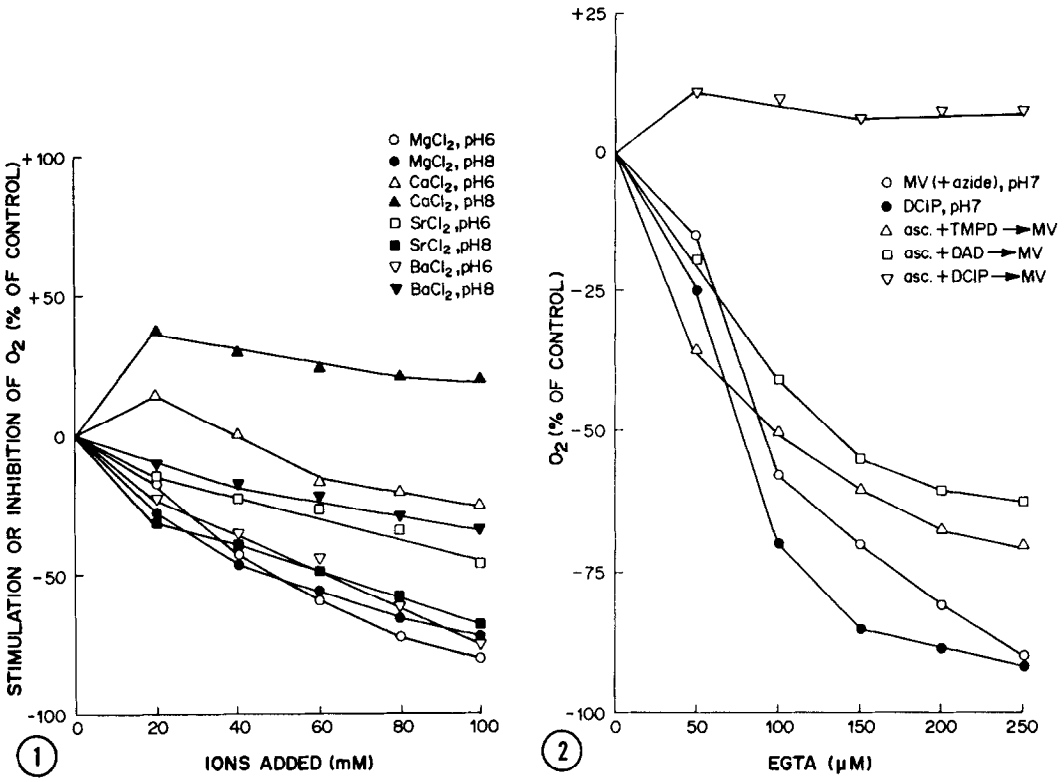

Fig. 1. The Effect of Ca^{2+} and Various Other Ions on the DCMU-Insensitive Silicomolybdate Reduction by Photosystem II of Spinach Chloroplasts. The control rate at pH 8 was 152 μequiv./mg chl · hr, at pH 6 - 188. The reaction mixture contained chloroplasts (50 μg chlorophyll), 25 mM Tris-Mes buffer, pH 6 or 8, 5 μM DCMU, silicomolybdic acid, 85 μM at pH 6, 250 μM at pH 8, and various ions in concentrations indicated; + indicates stimulation, - inhibition of rate in relation to control.

Fig. 2. The Effect of Various Concentrations of EGTA on Electron Transport in Spinach Chloroplasts after a Fifteen Minute Treatment. The control rate for the reaction $H_2O \rightarrow$ MV (+ azide) was 838 μequivalents/mg chl · hr; for the $H_2O \rightarrow$ DCIP reaction - 481; for the ascorbate + TMPD → MV - 1124; for the ascorbate + DCIP → MV - 981. The reaction mixture for the $H_2O \rightarrow$ MV (+ azide) reaction contained chloroplasts (50 μg chlorophyll), 25 mM Tris-Mes, pH 7, 0.5 mM MV, 0.5 mM Na azide and 5 mM NH_4Cl; for the $H_2O \rightarrow$ DCIP reaction chloroplasts, buffer and NH_4Cl as above plus 0.5 mM DCIP; for the reaction ascorbate + TMPD → MV, chloroplasts, 25 mM Tris-Mes, pH 8, 5 μM DCMU, 0.5 mM Na azide, 0.5 mM MV, 50 μM TMPD, and 1 mM Na ascorbate; for the ascorbate + DCIP → MV reaction everything as for the TMPD reaction except 0.5 mM DCIP in place of TMPD. + indicates stimulation, - inhibition of rate in relation to control.

200 μM EGTA inhibited electron transport in both photosystems from 50-90%. The only PS I donor reaction unaffected by the treatment was the ascorbate plus DCIP → MV reaction, indicating that the EGTA inhibition sites occur before P700, the reaction center chlorophyll complex in PS I.

Localization of the EGTA-created lesions in the electron transport chain was attempted by trying to restore the activity of various PS II partial reactions. As Fig. 3A shows, the DCMU-insensitive silicomolybdate reduction in EGTA-treated chloroplasts responded to the addition of exogenous Ca^{2+} ions both at pH 6 and at pH 8, while the same reaction in control chloroplasts was not changed more than 50%. The 750% stimulation by $CaCl_2$ shown in the EGTA-treated chloroplasts in Fig. 3A may be misleading without knowing, that the rate of silicomolybdate reduction without $CaCl_2$ was 10 µequiv O_2 evolved/mg chlorophyll · hr. It increased to 75 µequivalents in presence of 45 mM $CaCl_2$.

The reduction of indophenol by PS II in EGTA-treated chloroplasts also responded to the addition of $CaCl_2$ (Fig. 3B), but the reduction of methylviologen did not respond as well, indicating that another component of the PS II → PS I electron transport pathway was affected in EGTA-treated chloroplasts. The missing component may be plastocyanin. Only the joint addition of plastocyanin and $CaCl_2$ restored the H_2O → MV pathway by >50% (data not shown for Ca^{2+} site I).

Ferricyanide reduction by PS II showed differences in the restoration of electron transport rates in EGTA-treated chloroplasts (Fig. 3C): Ca^{2+} ions could restore the rate of pH 6, but not at pH 8, when dibromothymoquinone was present. These data, along with data in Fig. 3D, where restoration of activity by Ca^{2+} is shown on the PS II donor reaction, diphenylcarbazide → DCIP, offer proof, that the Ca^{2+} effect at site II is between the Mn^{2+} site, where diphenyl carbazide donates electrons, and between the plastoquinone pool, but not on water oxidation itself, as is commonly assumed. It may be, that there is a separate effect of Ca^{2+} ions on water oxidation, as assumed by Piccioni and Mauzerall (2,3,4) or by Brand (5) in algae, but the Ca^{2+} sites due to EGTA treatment appear to be past the water oxidation site, as shown by data presented in this study. Specificity for Ca^{2+} in the EGTA-treated chloroplasts is strongly suggested by restoration studies of the Ca^{2+} site II

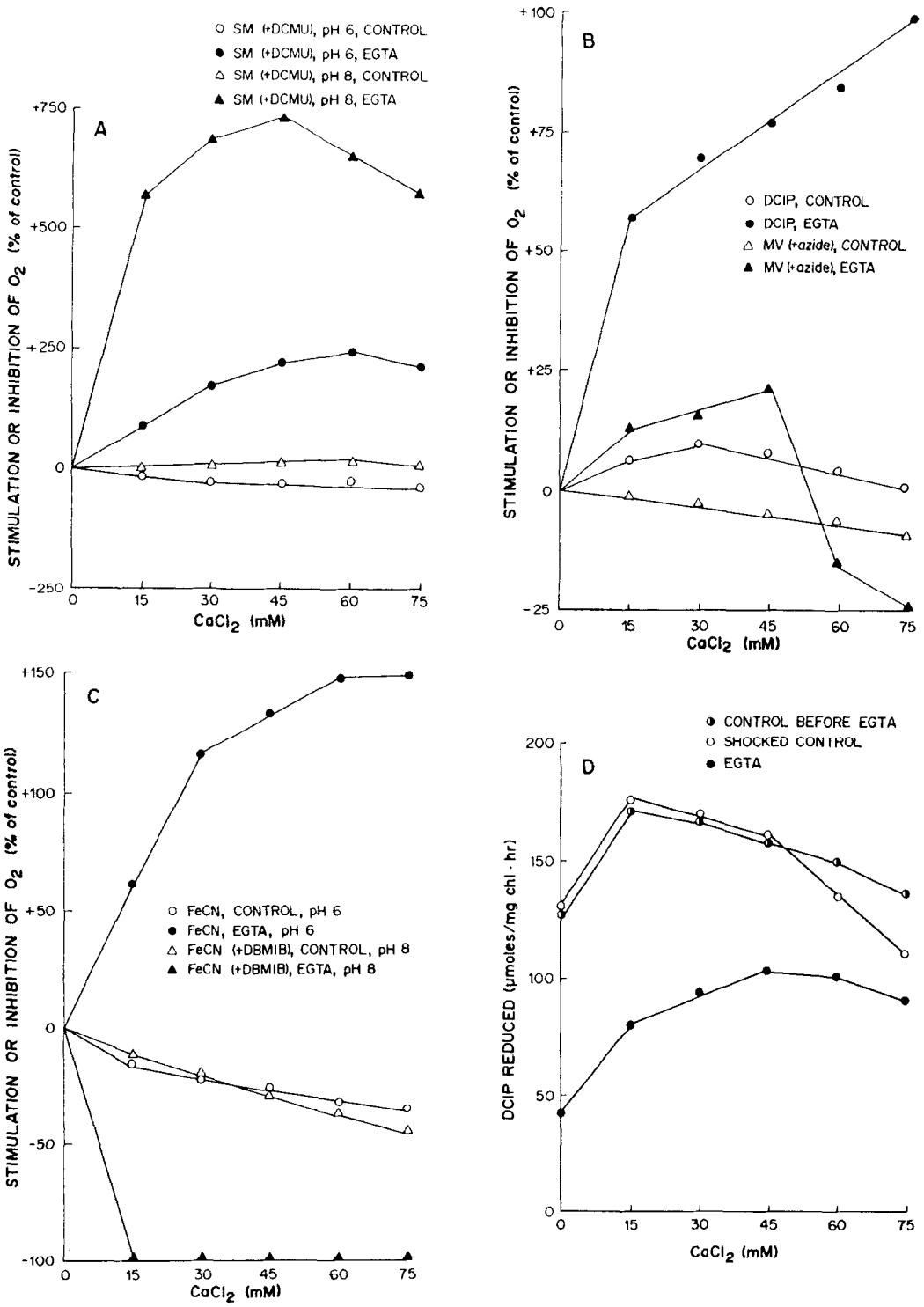

Fig. 3. Restoration of Electron Transport in Various Partial Reactions of Photosystem II and I by Ca^{2+} Ions in EGTA-Treated Chloroplasts. A- in the reaction $H_2O \rightarrow$ SM (+DCMU), at pH 6 and 8; B- in the reactions

Table I. The Effect of Ca^{2+} and Other Ions on the Photosystem II Reaction, Diphenylcarbazide → Indophenol, in Spinach Chloroplasts

		Electron Transport Rate[1]			
ION ADDED	CONC. (mM)	CONTROL DCIP REDUCED	%[2]	EGTA-TREATED DCIP REDUCED	%
None	-	100	100	50	-50
KCl	15	200	+100	60	-40
$MgCl_2$	7.5	200	+100	55	-45
$CaCl_2$	15	175	+ 75	105	+ 5
$MnCl_2$	7.5	175	+ 75	25	-75
$CuCl_2$	7.5	75	- 25	40	-60
$FeCl_2$	7.5	85	- 15	45	-55
$ZnCl_2$	1.5	110	+ 10	48	-52

[1] μmoles DCIP reduced/mg chl · hr.

[2] + indicates stimulation, - inhibition of rate in relation to control.

Reaction mixture for the reaction, DPC → DCIP contained chloroplasts (50 μg chlorophyll), 25 mM Tris-Mes, pH 7.5, 5 mM NH_4Cl, 0.5 mM DPC and 0.5 mM DCIP.

with other ions (Table I). It can be seen that only Ca^{2+} could fully restore DCIP reduction in the diphenylcarbazide → DCIP pathway in EGTA-treated chloroplasts.

Data for the Ca^{2+} site I, which has been narrowed down to the plastocyanin region, is presented in detail elsewhere. Restoration studies with Ca^{2+} on the ascorbate + TMPD → MV reaction in EGTA-treated chloroplasts showed that full restoration of activity required the addition of both Ca^{2+} and plastocyanin.

The exact role in which Ca^{2+} influences electron transport in spinach chloroplasts is unknown at present. Previous studies by Gross and associates

Fig. 3 (continued)
H_2O → DCIP and H_2O → MV (+ azide); C- in the reaction H_2O → FeCN, pH 6 and H_2O → FeCN (+DBMIB), pH 8. D- in the donor reaction DPC → DCIP. Various concentration of Ca^{2+} added as indicated. The control rate in A of H_2O → SM (+DCMU), pH 6, was 248, in EGTA-treated chloroplasts 23; control at pH 8 - 164, EGTA - 10; in B the control rate for the reaction H_2O → DCIP was 817, for EGTA - 158; the control rate of H_2O → MV (+ azide) was 1139, in EGTA - 158; in C the control rate for H_2O → FeCN, pH 6 was 344, in EGTA, pH 6 it was 62; control H_2O → FeCN (+DBMIB), pH 8 - 152, EGTA 0; in D the control rate of DPC → DCIP was 130 in shocked control chloroplasts, in EGTA chloroplasts - 43. All rates are expressed as μequiv./mg chl · hr. + indicates stimulation, - inhibition of rate in relation to control.

(9,10,11) have emphasized its participation as a non-specific divalent cation in the distribution of excitation energy between the two photosystems termed "spillover". However, Gross and Hess (9) found 2 specific Ca^{2+} binding sites in untreated chloroplasts. They deduced from their data that Ca^{2+} plays a role in "spillover". The present study with EGTA-treated chloroplasts, along with the restoration studies, implies that Ca^{2+} plays a more direct role in electron transport at 2 sites in PS II. Ca^{2+} site II may be related to the organization of the PS II reaction center, since Bose and Arntzen (12) found that the reaction center was inactive in absence of divalent cations. The Ca^{2+} site I, the site in the plastocyanin region, has not been described before. The role of Ca^{2+} ions at this site is totally unknown. It may be that Ca^{2+} organizes the way, in which plastocyanin is attached to the chloroplast membranes to make it function. Further studies are underway to distinguish between various alternatives in which Ca^{2+} functions in the electron transport chain of chloroplasts.

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