Action of Calcium Ions on Spinach (*Spinacia oleracea*) Chloroplast Fructose Bisphosphatase and Other Enzymes of the Calvin Cycle

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(Received 7 February 1980)

Thiol-treated spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase is powerfully inhibited by Ca^{2+} non-competitively with respect to its substrate, fructose 1,6-bisphosphate. $500\text{ }\mu\text{M}$ - Ca^{2+} causes virtually complete inhibition and the K_i is $40\text{ }\mu\text{M}$. Severe inhibition of sedoheptulose bisphosphatase is also caused by Ca^{2+} . A role for Ca^{2+} in regulation of the Calvin cycle in spinach chloroplasts is proposed.

Illumination of chloroplasts causes a movement of protons from the stroma into the thylakoids (Hind *et al.*, 1974). This is accompanied by an efflux of Mg^{2+} from the thylakoids (Krause, 1974; Barber *et al.*, 1974; Bulychiev & Vrendenberg, 1976; Portis & Heldt, 1976; Chow *et al.*, 1976), although the Mg^{2+} efflux at pH 8.0, the pH of the stroma in the illuminated chloroplast (Werdan *et al.*, 1975), is not sufficient to balance the proton movement (Krause, 1977; Ben-Hayyim, 1978). Ca^{2+} is also present within the chloroplast at concentrations of 15 mM (Nobel, 1969) or even higher, since a concentration of 25 mM may be calculated from the data of Portis & Heldt (1976) by assuming the internal chloroplast volume to be $24\text{ }\mu\text{l/mg}$ of chlorophyll. This Ca^{2+} does not appear to be involved in balancing proton movements (Hind *et al.*, 1974; Roeske *et al.*, 1974), and its function is unknown.

In the presence of the bivalent-cation ionophore A23187, Ca^{2+} at concentrations as low as 1 mM completely inhibits CO_2 fixation by type-A (Hall, 1972) spinach (*Spinacia oleracea*) chloroplasts (Portis & Heldt, 1976). The site of inhibition was suggested to be fructose bisphosphatase (Baier, 1976), since inhibition of this enzyme by Ca^{2+} was reported by Racker & Schroeder (1958). Portis & Heldt (1976) argued that all the Ca^{2+} in chloroplasts must be tightly bound to prevent such inhibition, although they provided no direct evidence of this.

However, most studies on chloroplast fructose bisphosphatase have been carried out with a form of the enzyme that cannot function *in vivo* (Charles & Halliwell, 1980). Only the reduced (thiol-treated) form of the enzyme is operative in the Calvin cycle. In view of this, we have reinvestigated the effect of Ca^{2+} on fructose bisphosphatase under the conditions most likely to exist *in vivo*, and we have

examined its effect on some other Calvin-cycle enzymes.

Materials and Methods

Materials

All reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Spinach (*Spinacia oleracea*) was purchased locally.

Determination of fructose bisphosphatase activity

The procedure used was as described previously (Charles & Halliwell, 1980) except that EDTA was omitted. Ca^{2+} at the concentrations used did not affect the coupled enzyme assay.

Determination of ribulose 5-phosphate kinase activity

Reaction mixtures contained, in a total volume of 2 ml, 100 mM-Tris/HCl buffer, pH 8.0, 10 mM- MgCl_2 , 0.2 mM-NADH, 1 mM-ATP, 1 mM-phosphoenolpyruvate, 6 units ($\mu\text{mol/min}$) of pyruvate kinase (EC 2.7.1.40), 10 units ($\mu\text{mol/min}$) of lactate dehydrogenase (EC 1.1.1.27) and chloroplast extract equivalent to $8\text{ }\mu\text{g}$ of chlorophyll. The reaction was started by the addition of 0.4 mM-ribulose 5-phosphate and followed by the ΔA_{340} due to the oxidation of NADH. The initial rate of ΔA was proportional to the amount of extract added. Dithiothreitol or Ca^{2+} at the concentrations used did not affect the coupled enzyme assay. One unit of enzyme was defined as the amount required to phosphorylate $1\text{ }\mu\text{mol}$ of ribulose 5-phosphate/min at 25°C .

Determination of glyceraldehyde 3-phosphate dehydrogenase activity

Reaction mixtures contained, in a total volume of 2 ml, 100 mM-Tris/HCl buffer, pH 8.0, 10 mM-

MgCl₂, 0.3 mM-NADPH, 3 mM-ATP, 3 units (μ mol/min) of phosphoglycerate kinase (EC 2.7.2.3) and chloroplast extract equivalent to 20 μ g of chlorophyll. This mixture was incubated for 5 min at 25°C, after which time the reaction was started by the addition of 4 mM-3-phosphoglycerate and followed by the ΔA_{340} due to the oxidation of NADPH. The initial rate of ΔA was proportional to the amount of extract added. Dithiothreitol and Ca²⁺ at the concentrations used did not affect the coupled enzyme assay. One unit of enzyme was defined as the amount required to hydrolyse 1 μ mol of 1,3-bisphosphoglycerate/min.

Determination of sedoheptulose biphosphatase activity

Reaction mixtures contained, in a total volume of 2 ml, 100 mM-Tris/HCl buffer, pH 8.0, 10 mM-MgCl₂ and chloroplast extract equivalent to 40 μ g of chlorophyll. The reaction was started by the addition of 0.4 mM-sedoheptulose 1,7-bisphosphate and incubated at 25°C for 20 min, after which time 1 ml of 10% (w/v) trichloroacetic acid was added. The precipitated extract was removed by centrifugation and P_i measured in the supernatant as follows. To 2 ml of the supernatant, 2 ml of water was added along with 0.5 ml of ammonium molybdate solution (1 g/100 ml of 0.05 M-H₂SO₄). The reaction was initiated by the addition of 0.5 ml of ascorbate solution (1 g/100 ml of water) and incubated at room temperature for 10 min, after which time the blue colour formed was measured at 650 nm. The assay was calibrated with analytical-grade KH₂PO₄. The blue colour formed after 10 min was proportional to the amount of extract added. One unit of enzyme was defined as the amount required to hydrolyse 1 μ mol of sedoheptulose 1,7-bisphosphate/min.

Purification and thiol treatment of spinach chloroplast fructose biphosphatase

The procedures used were as described previously (Charles & Halliwell, 1980).

Preparation and thiol treatment of intact chloroplasts

Intact chloroplasts were prepared by the method of Nakatani & Barber (1977). Chloroplasts used in the present study were at least 70% intact as determined by the ferricyanide test (Cockburn *et al.*, 1967; Heber & Santarius, 1970).

The chloroplasts were thiol-treated as follows. The preparation was diluted 1:3 with water and then adjusted to a final concentration of 20 mM by the addition of solid dithiothreitol. This mixture was incubated on ice for at least 1 h before assay of enzyme activities. Chlorophyll was determined by the method of Arnon (1949).

Results

In agreement with Racker & Schroeder (1958) and Baier (1976), freshly purified fructose biphosphatase is severely inhibited by Ca²⁺ (Fig. 1). Inhibition is almost complete at 1 mM-Ca²⁺. Thiol treatment of the enzyme as described in the Materials and Methods section increased the inhibitory effect of Ca²⁺ so that virtually complete inhibition is seen at 500 μ M-Ca²⁺ (Fig. 1).

Fig. 2 shows a Lineweaver-Burk (1934) plot of the fructose 1,6-bisphosphate saturation curve of thiol-treated fructose biphosphatase and the effect of two concentrations of Ca²⁺. The affinity for substrate is unaffected by Ca²⁺, but $V_{\max}$ is decreased 71% by 100 μ M-Ca²⁺. Ca²⁺ therefore seems to be a non-competitive inhibitor of fructose biphosphatase with respect to substrate, the K_i being 40 μ M (results not shown).

Fig. 3 shows the effect of Ca²⁺ on the Mg²⁺ saturation curve of thiol-treated fructose biphosphatase at pH 7.0, 7.5 and 8.0, the upper and lower pH values being those thought to exist in the stroma in the light and dark respectively (Werdan *et al.*, 1975). Throughout the pH range, Ca²⁺ exerts most inhibition at the lower Mg²⁺ concentrations, which suggests that Ca²⁺ is a competitive inhibitor of thiol-treated fructose biphosphatase with respect to Mg²⁺. The affinity of the enzyme for Mg²⁺ is obviously altered as the kinetics change from hyperbolic to sigmoidal. In the absence of Ca²⁺, thiol-treated fructose biphosphatase is completely inactive at pH 7.0 at an Mg²⁺ concentration of 4 mM. In the presence of 1 mM-Ca²⁺ at pH 7.0, the enzyme is completely inactive at Mg²⁺ concentrations as high as 16 mM (Fig. 3).

Fig. 1. Effects of Ca²⁺ on the activity of fructose biphosphatase

Enzyme activity was determined as described in the Materials and Methods section, except that Ca²⁺ was added and the concentration varied as shown. ●, Freshly purified fructose biphosphatase; ■, thiol-treated fructose biphosphatase.

Fig. 2. Effect of Ca^{2+} on the substrate saturation curve of thiol-treated fructose biphosphatase plotted as described by Lineweaver & Burk (1934)

Enzyme activity was determined as described in the Materials and Methods section, except that the concentration of fructose 1,6-bisphosphate was varied and Ca^{2+} was added as shown. ●, Control; ▲, +50 μM - Ca^{2+} ; ■, +100 μM - Ca^{2+} . V_{max} values were as follows: control, 167 units/mg of protein; +50 μM - Ca^{2+} , 91 units/mg of protein; +100 μM - Ca^{2+} , 50 units/mg of protein. v represents fructose biphosphatase activity in units/mg of protein and s represents [fructose 1,6-bisphosphate] in μM .

Fig. 3. Effect of Ca^{2+} on the Mg^{2+} saturation curve of thiol-treated fructose biphosphatase at three pH values

Enzyme activity was determined as described in the Materials and Methods section, except that the concentration of MgCl_2 and the pH were varied and Ca^{2+} was added as shown. pH values of 7.5 and 7.0 were obtained by using 100mM-imidazole/HCl buffer in place of 100mM-Tris/HCl buffer. The concentration of fructose 1,6-bisphosphate was 0.4 mM. ●, pH 8.0; ○, pH 8.0 + 100 μM - Ca^{2+} ; ▲, pH 7.5; △, pH 7.5 + 100 μM - Ca^{2+} ; ■, pH 7.0; □, pH 7.0 + 1 mM- Ca^{2+} .

Intact chloroplasts were prepared and the activities of sedoheptulose biphosphatase, ribulose 5-phosphate kinase and glyceraldehyde 3-phosphate dehydrogenase determined as described in the

Materials and Methods section. Significant activities of the kinase and dehydrogenase enzymes (0.17 and 0.72 unit/mg of chlorophyll respectively) were observed before thiol treatment. However, little sedo-

heptulose biphosphatase activity was found (0.024 unit/mg of chlorophyll). This low activity was increased more than 10-fold (to 0.272 unit/mg of chlorophyll) after thiol treatment of the chloroplast extract as described in the Materials and Methods section. Ca^{2+} (1 mM) had no significant effect on the kinase or dehydrogenase activities before or after thiol treatment, but it caused an 83% inhibition of thiol-treated sedoheptulose biphosphatase assayed at pH 8.0. At pH 7.5, 1 mM- Ca^{2+} inhibited thiol-treated sedoheptulose biphosphatase by 91% and at pH 7.0 completely inhibited the enzyme.

Discussion

Much attention has been given to the role played by Mg^{2+} in regulating the enzymes of the Calvin cycle. However, Ben-Hayyim (1978) has provided evidence that the Mg^{2+} released in the light does not enter the stroma after traversing the thylakoid membrane but becomes bound to the outside of the thylakoids, which are known to contain sites for the binding of bivalent cations. These sites are probably ionized carboxy groups on membrane proteins (Prochaska & Gross, 1975; Davis & Gross, 1975). In view of the striking effects of even 1 mM- Ca^{2+} on fructose and sedoheptulose biphosphatases, it is perhaps time to re-examine the evidence for a possible role of this ion in regulation of the Calvin cycle. If Ca^{2+} alone were to regulate the cycle, then it should be completely bound in the light, but release of 1 mM- Ca^{2+} (one-fifteenth or less of the total Ca^{2+} present) into the stroma in the dark would stop the cycle completely at the level of fructose and sedoheptulose biphosphatases, enzymes that are known to occupy key sites of regulation (Halliwell, 1978). Even smaller changes in the concentration of free Ca^{2+} might suffice, since the pH change that occurs in the stroma in a light-dark transition itself severely decreases the activity of fructose biphosphatase (Charles & Halliwell, 1980).

Miginiac-Maslow & Hoarau (1977) studied the effect of EDTA and the ionophore A23187 on the release of Ca^{2+} and Mg^{2+} from intact spinach chloroplasts. In the presence of EDTA in the external medium, the ionophore A23187 accelerated the release of Ca^{2+} from the chloroplasts, the increased release over a 6-min period corresponding to a stromal Ca^{2+} concentration of 6 mM [Fig. 6 in Miginiac-Maslow & Hoarau (1977)]. EDTA does not affect CO_2 fixation by intact chloroplasts (Avron & Gibbs, 1974) and so cannot penetrate to remove stromal cations. Hence Ca^{2+} must leave the chloroplast in the presence of the ionophore A23187 if the external Ca^{2+} concentration is lowered to zero by EDTA, and so it must be free, or very loosely bound, inside the stroma. There are many problems in experiments of this kind, as illustrated by the

attempts to use ionophores in determining the free stromal Mg^{2+} concentration [values range from 1–3 mM up to 23 mM; see Krause (1977) and Miginiac-Maslow & Hoarau (1977)], but these results provide a reason for supposing that some free Ca^{2+} exists in chloroplasts.

Let us further suppose that, in the light, all chloroplast Ca^{2+} is bound to thylakoid membrane CO_2^- groups. When the light is turned off and the pH of the stroma falls, a small fraction of these groups will become protonated and unable to bind Ca^{2+} . Thus a small part of the Ca^{2+} will be released and could inhibit the Calvin cycle. Such a protonation might also displace the bound Mg^{2+} proposed by Ben-Hayyim (1978), allowing its uptake into the thylakoids in exchange for the released protons.

We propose that Ca^{2+} plays an important role in 'switching off' the Calvin cycle during darkness and that the function of bivalent cations in regulating chloroplast metabolism requires a complete re-examination using much more sensitive techniques.

Note Added in Proof

(Received 26 February 1980)

Since the present paper was submitted, it has been shown (Barr *et al.*, 1980) that the addition of the specific Ca^{2+} chelator EGTA to a suspension of broken spinach chloroplasts causes severe inhibition of electron transport. Such results further illustrate the potential importance of Ca^{2+} in chloroplast metabolism.

We are grateful to the Wellcome Foundation and to the Central Research Fund of the University of London for financial support and to Miss Bernice Archard for her invaluable assistance in preparation of the manuscript. S. A. C. thanks the Science Research Council for a Research Studentship.

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