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REGULATION OF FRUCTOSE-1,6-BISPHOSPHATASE ACTIVITY IN INTACT CHLOROPLASTS

STUDIES OF THE MECHANISM OF INACTIVATION

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Summary

1. The aim of this work was to investigate the mechanism of dark inactivation of fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) in isolated intact chloroplasts of *Triticum aestivum*.

2. Dark inactivation of the enzyme, which was rapid under aerobic conditions, was prevented under anaerobic conditions when chloroplasts were incubated in the absence of an electron acceptor. Electron acceptors such as oxaloacetate readily brought about inactivation under anaerobic conditions whether chloroplasts were illuminated or in the dark. Inactivation of the enzyme also occurred if illuminated or darkened anaerobic chloroplasts were exposed to oxygen.

3. Pyocyanine, which catalyses a cyclic electron flow around Photosystem I, also caused inactivation of the enzyme in illuminated, anaerobic chloroplasts.

4. It is proposed that the activity of fructose-1,6-bisphosphatase is regulated by the availability of electrons, and thus by electron acceptors, and that dark inactivation may occur by a direct reversal of the activation process.

Introduction

Although there is evidence that both NADPH and ATP are made available to the chloroplast via shuttle systems during darkness [1], photosynthetic

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

CO_2 fixation is an entirely light dependent process. This is a consequence of the virtual inactivity of certain enzymes of the reductive pentose phosphate pathway in the dark. By this means futile cycles, such as might occur if phosphofructokinase and fructose-1,6-bisphosphatase were simultaneously active in the dark, are prevented. Changes in pH and Mg^{2+} concentration caused by illumination will create optimal conditions for catalysis, but certain enzymes are also reductively activated [2]. Although inactivity in the dark will obviously follow from the reversal of changes in pH and Mg^{2+}, the significance and extent of dark oxidation of enzymes, since it occurs to varying degrees with different enzymes [2], remains unclear.

Investigations into the mechanism of reductive activation of enzymes have implicated reductants generated by Photosystem I. The light effect mediator [3] and the thioredoxin : ferredoxin, thioredoxin reductase [4] systems have been proposed to mediate such reduction. Fructose-1,6-bisphosphatase is one of several enzymes which appear to be activated via the thioredoxin system, which derives its reducing power, in turn, from ferredoxin [4,5]. Although it has been suggested that dark inactivation of fructose-1,6-bisphosphatase might be mediated by oxidised glutathione [5], measurements of glutathione in chloroplasts show that it is largely present in the reduced state both in the light and the dark [6]. Other studies suggest that changes in the activity of the enzyme are influenced by the redox state of ferredoxin. These include studies using antimycin A in the reconstituted chloroplast system [7] and in intact chloroplasts [8,9], and the inhibition of photosynthesis by nitrite [10]. With intact chloroplasts, we have shown that fructose-1,6-bisphosphatase activity is diminished by the addition of electron acceptors and, under suboptimal conditions, is increased by the addition of antimycin A, in a manner which suggests that the activation state of the enzyme is very sensitive to the availability of electrons [11]. Changes in activity in the light and dark inactivation could thus occur by a direct reversal of the activation mechanism and this possibility is explored further in this paper.

Materials and Methods

Materials. Cellulase and macerozyme were purchased from Yakult Biochemicals Ltd., Nishinomiya, Japan. All other substrates, cofactors and enzymes were from Boehringer, Mannheim or from the Sigma Chemical Company.

Preparation of protoplasts and chloroplasts. Wheat (*Triticum aestivum* L., cv. Sappo) was grown in vermiculite under natural daylight and supplementary incandescent lamps ($30\ W \cdot m^{-2}$). For the isolation of protoplasts, leaf segments (0.5 to 1.0 mm wide) were cut by hand from 6- to 9-day-old material and incubated in the light at 28°C in 40 ml of a medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 2% (w/v) cellulase (Onozuka 3S), 0.3% (w/v) pectinase (Macerozyme R-10) and 5 mM 2-(*N*-morpholino)ethanesulphonic acid (Mes), pH 5.5. The leaf segments were incubated for 3 h and protoplasts were collected and purified as previously described [12]. Protoplasts were stored in the dark on ice for 2—3 h prior to use. Small aliquots of protoplasts (about 400 μg chlorophyll) were diluted with 0.5 M sorbitol, pelleted by centrifugation at $200 \times g$ for 2 min and resuspended in 0.5 ml 0.33 M sorbitol,

10 mM EDTA, 25 mM Tricine (pH 8.4). Protoplasts were then broken by passing them three times through a 20-μm nylon mesh attached to a 1-ml plastic syringe. Chloroplasts were collected by centrifugation at 250 × *g* for 45 s, and the pellet was resuspended in the same medium. The percentage of intact chloroplasts as determined by ferricyanide-dependent O_2 evolution before and after osmotic shock [13] was greater than 90%. Chlorophyll was determined by the method of Arnon [14].

Illumination of chloroplasts and assay of photosynthesis. O_2 evolution was followed polarographically at 20°C in a Clark-type electrode system [15], purchased from Hansatech Ltd., Hardwick Industrial Estate, King's Lynn, Norfolk. Reaction mixtures were illuminated from 150-W quartz-iodine slide projectors provided with an I.C.I. Perspex Red 400 filter and Calflex C heat filter. The light intensity was 330 $W \cdot m^{-2}$. Chloroplasts were illuminated in a medium containing 0.33 M sorbitol, 10 mM EDTA, 25 mM Tricine (pH 8.4) with the addition of 10 mM $NaHCO_3$ and 0.2 mM P_i as indicated. The chlorophyll concentration was 50 to 100 μg/ml in a total volume of 1 to 2 ml. Reaction mixtures were made anaerobic by the addition of 10 mM glucose, 50 units/ml glucose oxidase and 1100 units/ml catalase.

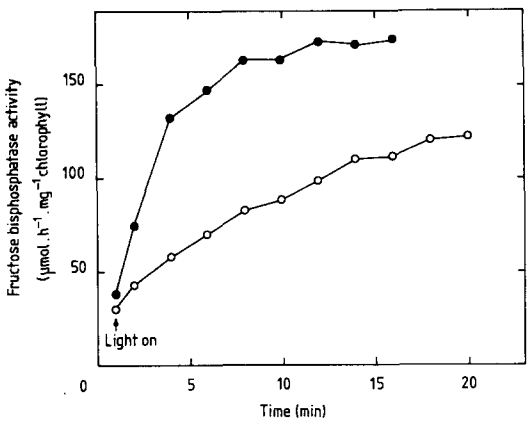
Fructose-1,6-bisphosphatase activity. Activity was measured at 20°C. Samples (2 to 5 μg chlorophyll in 50 μl) were withdrawn from the electrode cell and assayed immediately in a 1-ml reaction mixture containing 1 mM fructose-1,6-bisphosphate, 1 mM EDTA, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 4 units glucosephosphate isomerase and 2 units glucose 6-phosphate dehydrogenase in 100 mM Tris-HCl (pH 8.2) [16].

Results

Activity of fructose-1,6-bisphosphatase under anaerobic conditions

In order to determine the role of oxygen in the dark inactivation of fructose-1,6-bisphosphatase, experiments were done under anaerobic conditions. When chloroplasts were illuminated in the absence of oxygen and other electron acceptors, light activation of fructose-1,6-bisphosphatase was slower than in the aerobic control (also without an acceptor) (Fig. 1), consistent with the inhibition of non-cyclic electron flow under anaerobic conditions [10,17].

When aerobic chloroplasts were darkened or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added (Fig. 2), a rapid inactivation of fructose-1,6-bisphosphatase immediately followed. Under anaerobic conditions, in the absence of an acceptor, inactivation did not occur when chloroplasts were darkened (Fig. 3). When illumination was resumed, the activation process was continued. If oxygen was then passed over the surface of the medium, an immediate inactivation followed, with activity returning to the dark value. The fructose-1,6-bisphosphatase activity remained at this low value for 2 to 3 min and then re-activation occurred which was no faster than that observed under anaerobic conditions (compare Fig. 1), although the chloroplasts were by then aerobic (the glucose in the glucose oxidase system was by then exhausted and oxygen had returned to the air level). When the light was finally switched off, inactivation, typical of that observed under aerobic conditions, again occurred.



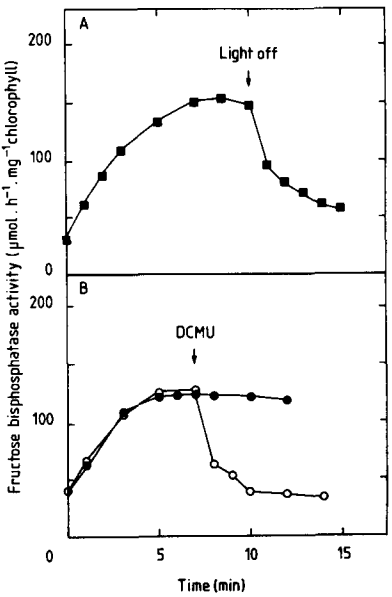


Fig. 1. Light activation of fructose-1,6-bisphosphatase in chloroplasts incubated under aerobic (•) and anaerobic (○) conditions. Chloroplasts were incubated in the absence of an added acceptor.

Fig. 2. Activity of fructose-1,6-bisphosphatase in aerobic chloroplasts. (A) shows dark inactivation and (B) the effect of addition of 10^{-5} M DCMU to illuminated chloroplasts (○). Chloroplast incubations contained 10 mM $NaHCO_3$ and 0.2 mM P_i in addition to reaction medium (see Materials and Methods). 1 μl of ethanol was added to the control (•) simultaneously with the addition of DCMU.

If oxygen is behaving simply as an electron acceptor in such circumstances, then inactivation should occur if an electron acceptor is added to anaerobic chloroplasts. This prediction was borne out when oxaloacetate was added to illuminated anaerobic chloroplasts in which activation of fructose-1,6-bisphosphatase was largely complete. As Fig. 4 (A) shows, there was then an immediate decline to an activity only slightly above that of the dark value. Unlike the response to O_2 (Fig. 3) however, no further activation of the enzyme occurred. Recovery was not aided by the addition of antimycin A, which enhances rates of oxaloacetate reduction in anaerobic spinach chloroplasts [18]. In contrast to the results obtained with oxaloacetate, no inactivation of fructose-1,6-bisphosphatase occurred when bicarbonate was added to illuminated, anaerobic chloroplasts (Fig. 4 (B)). This accords with previous observations that bicarbonate is a poor electron acceptor under anaerobic conditions [10].

Similar inactivation by electron acceptors was also observed in darkened anaerobic chloroplasts (Fig. 5). Electron acceptors were added immediately after the light was switched off. Whereas in the control there was no inactivation of fructose-1,6-bisphosphatase, admission of oxygen or addition of oxaloacetate or nitrite (which reacts directly with reduced ferredoxin in a reaction catalysed by nitrite reductase) caused inactivation. The inactivation brought about by oxygen was as rapid as that which occurred in illuminated anaerobic

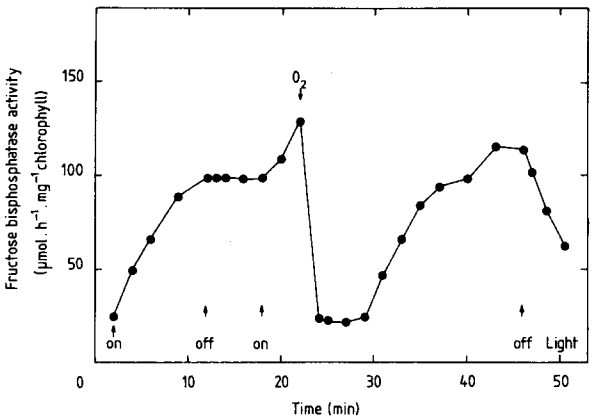


Fig. 3. Effect of light and dark on fructose-1,6-bisphosphatase activity in chloroplasts under anaerobic and aerobic conditions. The chloroplasts were initially illuminated under anaerobic conditions without an added acceptor, and were subjected to a dark interval. Oxygen was then admitted sufficient to return the oxygen concentration to air level.

chloroplasts (Fig. 3). The kinetics of inactivation caused by oxaloacetate and nitrite were comparable to those observed under aerobic conditions in the presence of an acceptor (Fig. 2 and Ref. 11). With regard to the lack of inactivation of fructose-1,6-bisphosphatase observed when anaerobic chloro-

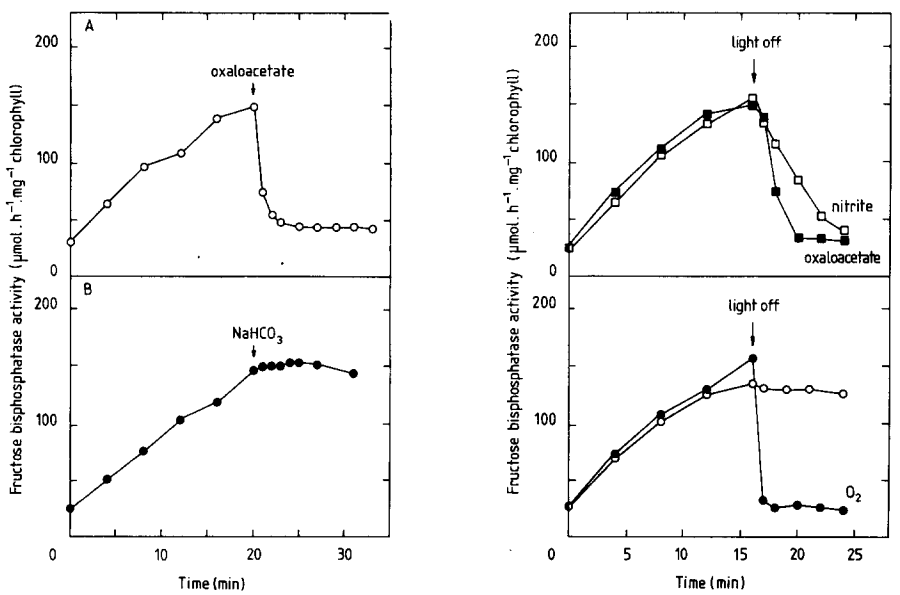


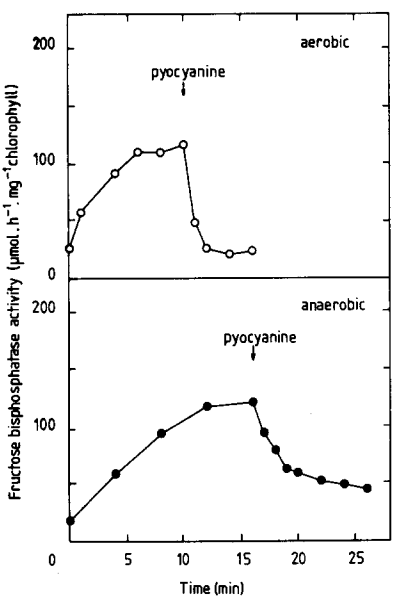
Fig. 4. Effect of oxaloacetate (1 mM) (A) and $NaHCO_3$ (2 mM) (B) on fructose-1,6-bisphosphatase activity when added to illuminated anaerobic chloroplasts.

Fig. 5. Influence of electron acceptors on fructose-1,6-bisphosphatase activity when added to darkened, anaerobic chloroplasts. Chloroplasts were previously illuminated under anaerobic conditions in the absence of an added electron acceptor for 16 min. Nitrite (1.33 mM), oxaloacetate (1.33 mM) were added or oxygen was admitted. 1 μl of water was added to the control (○).

plasts were darkened in the absence of an acceptor, it is evident that the reduction status of the enzyme was not in itself affected by the changes in pH and Mg^{2+} concentration which would have occurred during the transition from light to dark (cf. Ref. 10).

Influence of pyocyanine

Pyocyanine catalyses a cyclic electron flow around photosystem I by accepting electrons from the reducing side of the photosystem and returning them to a point near to *P*-700 (Fig. 7), rates of associated photophosphorylation being very high [19]. It might be predicted that pyocyanine would bring about the inactivation of fructose-1,6-bisphosphatase. When pyocyanine was added to aerobic chloroplasts in the presence of an acceptor, inactivation was indeed rapid (Fig. 6). This experiment does not distinguish whether pyocyanine exerted its effect by oxidising the enzyme or, like DCMU, by inhibiting non-cyclic electron flow, allowing inactivation by bicarbonate and oxygen. When pyocyanine was added under anaerobic conditions, and in the absence of



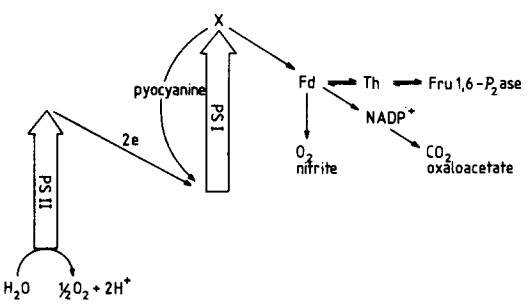


Fig. 6. Effect of pyocyanine (50 μM) on fructose-1,6-bisphosphatase activity in aerobic and anaerobic chloroplasts. Aerobic chloroplasts were incubated with 10 mM $NaHCO_3$ and 0.2 mM P_i. Anaerobic chloroplasts contained no added acceptor.

Fig. 7. Proposed mechanism for inactivation of fructose-1,6-bisphosphatase. Photosynthetic electron transport reduces fructose-1,6-bisphosphatase via the Z-scheme, ferredoxin (Fd) and thioredoxin (Th) and its reductase. An appropriately active electron flow is needed to maintain this activated state. If it is diminished by decreased light, the addition of inhibitors (such as DCMU) or if electrons are diverted to alternative acceptors (such as oxaloacetate) the activity of the enzymes declines because of the reversible nature of the steps between it and ferredoxin. Similarly, when electron flow to ferredoxin ceases in the dark, electrons return from fructose-1,6-bisphosphatase via ferredoxin, to oxygen. In the absence of O_2 and alternative acceptors this route is closed and the enzyme remains active. Conversely, alternative acceptors allow dark inactivation under anaerobic conditions or speed inactivation under aerobic conditions by acting as electron sinks.

an acceptor, it could be seen to act in its own right as an electron acceptor which oxidised the enzyme (Fig. 6), although inactivation was slower compared to that achieved by other electron acceptors.

Discussion

The results of this work can be explained by a scheme in which the activation state of fructose-1,6-bisphosphatase is readily altered by changes in the availability of electrons from ferredoxin and that the activation process is readily reversible (Fig. 7). We have shown [11] that addition of electron acceptors to aerobic chloroplasts results in partial inactivation of fructose-1,6-bisphosphatase and the present results clearly show that addition of electron acceptors results in enzyme inactivation in the absence of oxygen. Under aerobic conditions, oxygen appeared to be a rather poor electron acceptor, in agreement with previous findings [17]. The present results show that under anaerobic conditions, oxygen inactivation of fructose-1,6-bisphosphatase was much more rapid and extensive than under aerobic conditions both in the light and the dark (Ref. 11. and Figs. 2 and 3). While certain carriers in the electron transport chain might become abnormally reduced under anaerobic conditions and then react with oxygen, it is not apparent how this would cause such a marked increase in the rate and extent of oxidation of fructose-1,6-bisphosphatase. One feasible explanation is that reaction of oxygen with such reduced carriers causes a transient increase in the concentrations of superoxide and H_2O_2 in the chloroplast, which would in themselves enhance enzyme inactivation (see, for example, Ref. 20), and also possibly account for the slow recovery of fructose-1,6-bisphosphatase activity in the light (Fig. 3), whereas under aerobic conditions, with oxygen as the only acceptor, superoxide and H_2O_2 formed by reduction of oxygen would probably be immediately reduced [6].

Oxaloacetate also caused inactivation which was much more marked than under aerobic conditions, being similar to the enhanced inhibition of fructose-1,6-bisphosphatase caused by addition of an electron acceptor to aerobic chloroplasts in which electron flow was inhibited by DCMU [11]. This pronounced effect can be attributed to the fact that under anaerobic conditions electron flow would also be much reduced [17]. Continued illumination of anaerobic chloroplasts in the presence of oxaloacetate (Fig. 4) presumably failed to increase the activity of the enzyme because electron flow was still inhibited by anaerobiosis and oxaloacetate reduction was far from saturated.

The inactivation of fructose-1,6-bisphosphatase in darkened anaerobic chloroplasts by electron acceptors also accords with the scheme illustrated in Fig. 7. It is worth noting that oxaloacetate acts as an electron acceptor despite the fact that the enzyme which catalyses its reduction, $NADP^+$-dependent malate dehydrogenase, is light-activated in chloroplasts [3]. It is possible that reduction of oxaloacetate could be effected by NAD-specific malate dehydrogenase utilising NADPH via a transhydrogenase but, since the electron flow involved is not large, it is more likely that the NADP-specific malic dehydrogenase still retains enough activity during darkening to drain electrons from fructose-1,6-bisphosphatase to oxaloacetate. The inactivation of fructose-

1,6-bisphosphatase caused by pyocyanine agrees with the scheme in that electrons normally available for reduction of the enzyme are drained into an artificial cyclic electron flow catalysed by pyocyanine. It thus contrasts with the effect of antimycin A, which stimulates fructose-1,6-bisphosphatase activity under certain limiting conditions [11] by inhibiting natural cyclic electron flow.

Clearly the simplest explanation of such results is that the ferredoxin : thioredoxin system is readily reversible and could therefore catalyse both reduction and oxidation of fructose-1,6-bisphosphatase (Fig. 7). (Recently a protein-mediated, ferredoxin-independent mechanism of activation has been identified [21] but could clearly be accommodated by appropriate modification of the scheme shown in Fig. 7). Although electron acceptors such as oxaloacetate and nitrite would be expected to accept electrons from fructose-1,6-bisphosphatase via ferredoxin, it is possible that oxygen inactivation may arise by a more direct interaction. Studies of the activation of fructose-1,6-bisphosphatase and $NADP^+$-dependent malate dehydrogenase [22] and of coupling factor ATPase [23] by purified thioredoxin and its reductase in vitro showed an absolute requirement for anaerobic conditions. Similarly, inactivation of $NADP^+$-malate dehydrogenase from maize in the presence of 'regulatory protein' was mediated by oxygen [24].

It is pertinent to ask what the oxidant might be in vivo. In protoplasts, dark inactivation of fructose-1,6-bisphosphatase is somewhat slower and less extensive than in chloroplasts (Leegood, R.C. and Walker, D.A. unpublished data). The operation of shuttle devices across the chloroplast envelope in protoplasts could restrict inactivation. For example, any import of triose phosphate into the chloroplast [1] might tend to slow down the rise in the ratio $NADP^+$/NADPH which occurs when the light is switched off [1]. Thus electron acceptors such as oxygen and nitrite might be the predominant oxidants in protoplasts.

Such a mechanism of regulation could have wider implications for the regulation of photosynthesis. Modulation of enzyme activity by electron acceptors occurs in the light under aerobic conditions [11] as well as in the dark. An obvious example is photosynthetic induction, in which the level of the natural acceptor only gradually increases [25]. Thus fructose-1,6-bisphosphatase activity might be in relative excess at an early stage of induction. This would be a possible mechanism for the retention of triose phosphate within the stroma. (Such retention, as distinct from export, is required if autocatalysis is to bring about an increase in photosynthetic intermediates necessary to sustain high rates of CO_2 fixation). It also seems likely that control by electron availability is not restricted to fructose-1,6-bisphosphatase and that other enzymes in the chloroplast are similarly modulated. It may be noted that photoregulation of fructose-1,6-bisphosphatase is entirely eliminated by dithiothreitol (as the above results would suggest) and that subsequent work [11] has shown that the influence of competing electron acceptors is dependent on electron availability.

Acknowledgements

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