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The Mechanism of the Control of Carbon Fixation by the pH in the Chloroplast Stroma

Studies with Acid Mediated Proton Transfer across the Envelope

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Abstract. The salts of several weak acids have been used to render the envelope permeable to protons. In order to investigate the role of stromal pH changes in the light regulation of CO_2 fixation, formate, octanoate, nitrite, and glyoxylate have been tried as tools to reverse the light-dependent alkalization of the stroma. For this purpose, the decrease of the stromal pH in illuminated spinach chloroplasts, as caused by the addition of these substances or by instantaneous lowering of the pH in the medium, has been compared with the corresponding decrease of CO_2 fixation and the change of stromal metabolite levels. It appears from out data that formate and octanoate are suited best to obtain a specific inhibition of CO_2 fixation by lowering the stromal pH. The measurement of the corresponding metabolite levels indicates that this inhibition is primarily due to an inhibition of fructose- and sedoheptulose bisphosphatase. It is concluded that these two enzymes are important regulatory steps for the light control of CO_2 fixation.

Key words: Chloroplast – CO_2 fixation – Fructose 1.6-bisphosphatase – Proton transfer – Sedoheptulose 1.7-bisphosphatase – *Spinacia*.

Introduction

Illumination of intact chloroplasts leads to an alkalization of the stroma by 0.8 to 1.0 pH units (Heldt et al. 1973). This is initially caused by light-dependent proton transport from the stroma across the thylakoid membrane into the thylakoid space. A consequence of this alkalization in the stroma is the formation of a proton gradient across the envelope. The maintenance of this gradient against passive proton fluxes across the envelope appears to be achieved by active proton extrusion from the stroma into the external space (Gimmler et al. 1975). The mechanism of this process is not known; it is likely to be an active transport requiring ATP.

It has been found previously that CO_2 fixation by intact chloroplasts is strongly dependent on the stromal pH (Werdan et al. 1975). Those pH changes occurring in the stroma during a dark-light transition appear to be sufficiently high to switch CO_2 fixation from zero to maximal activity. These findings suggest that the stromal pH is an important factor for the light regulation of CO_2 fixation.

This regulatory mechanism can be further elucidated by experimentally manipulating the stromal pH in intact chloroplasts. One way of doing this is to lower the pH in the medium, and another way is to render the envelope permeable to protons. The latter can be achieved by the addition of weak acids, of which both the dissociated and the non-dissociated form are able to penetrate the envelope. Thus protons can be transferred indirectly across the envelope by a shuttle of the dissociated and non-dissociated acid (Purczeld et al. 1978). So far, a reversal of the light-induced alkalization of the stroma, leading to an inhibition of CO_2 fixation, has been observed with chlorocarbonylphenylhydrazone (Werdan et al. 1975), nitrite (Purczeld et al. 1978) and also with high concentrations of acetate, bicarbonate, and octanoate (Purczeld et al. 1978; Werdan et al. 1975). Metabolite analysis in chloroplasts in which CO_2 fixation was inhibited by nitrite indicated that this inhibition was ultimately due to an inhibition of fructose- and sedoheptulose bisphosphatase (Purczeld et al. 1978). It was

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therefore concluded that these enzymes are important regulatory sites for the light regulation of the reductive pentose phosphate pathway.

It is possible that some of these substances employed for rendering the envelope permeable to protons may also influence CO_2 fixation in a secondary way. Thus, it is feasible that the inhibitory effect of nitrite may be partially due to a change of the reductive state of ferredoxin, *which would have then also affected the activation state of CO_2 fixation enzymes, since several of these are known to be activated by reduced ferredoxin through the mediation of thioredoxin* (Buchanan et al. 1979). For this reason, our studies on the experimental changes of the stromal pH in illuminated chloroplasts have been extended by employing other substances and by comparing their effect on the inhibition of CO_2 fixation.

Methods

For the preparation of spinach chloroplasts, the oxygen evolution was measured and the stromal metabolite levels were determined by chromatographic assay of ^{32}P labeled compounds (see Lilley et al. 1977). The measurements of the pH in the stroma and the thylakoid space of intact chloroplasts were performed according to the method of Heldt et al. 1973.

The chloroplasts (0.05 mg of chlorophyll ml^{-1}) were incubated in a medium containing 0.33 M sorbitol, 50 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), neutralized with NaOH to pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM K_2HPO_4, 5 mM $NaHCO_3$, and 2000 units ml^{-1} catalase from beef liver (Boehringer, Mannheim, FRG). Formate, octanoate, nitrite, and glyoxylate were added as sodium salts. The samples were illuminated with a tungsten halogen light source provided with a heat filter. The light intensity, measured with a RG 630 cut-off filter (Schott, Mainz, FRG), was 100 Wm^{-2}.

Results

It has been previously shown that a number of other carboxylates such as propionate, glyoxylate, and formate also inhibit CO_2 fixation (Enser et al. 1980). Of these, glyoxylate was found to be an especially powerful inhibitor, since 0.08–0.25 mM of this substance were already sufficient for 50% inhibition of CO_2 fixation. In the experiment shown in Table 1, it was investigated whether this inhibition was due to a change of the stromal pH. In this experiment 0.8 mM glyoxylate produced a marked lowering of the stromal pH as well as a strong inhibition of CO_2 fixation. With 0.2 mM glyoxylate, however, CO_2 fixation was already inhibited by 40%, whereas the change of the stromal pH was only very small. From these findings, the conclusion is obvious that the inhibitory effect of these low concentrations or glyoxylate cannot be attributed entirely to stromal pH changes.

Table 1. Inhibition of CO_2 fixation by glyoxylate. The chloroplasts were preincubated with glyoxylate for 1 min in the dark. Measurements of pH and of oxygen evolution were performed in parallel samples after a subsequent illumination of 5 min

Addition	O_2 evolution in the presence of HCO_3^-		pH		
	$\mu mol\ mg^{-1}$ chlorophyll per h	Inhibition %	Stroma	Thylakoid	Δ
–	185	–	7.68	5.54	2.14
Glyoxylate (0.2 mM)	111	40	7.65	5.48	2.17
Glyoxylate (0.8 mM)	53	71	7.24	4.99	2.25

In the experiments shown in Table 2, the correlation between a change of the stromal pH and the inhibition of CO_2 fixation was further studied. In experiment A, the stromal pH was changed by a rapid decrease of the pH in the medium through the addition of protons. In a parallel experiment, a selected formate concentration was added in order to obtain about the same stromal pH shift. In both experiments CO_2 fixation was inhibited to the same extent. In experiment B, similar pH changes brought about by the addition of octanoate and formate were also accompanied by similar inhibition of CO_2 fixation. These findings strongly indicate that the inhibition of CO_2 fixation by formate and octanoate is due to the shift of the stromal pH caused by these substances.

When nitrite or glyoxylate were added in concentrations yielding a similar inhibition of CO_2 fixation as that observed with formate or glyoxylate, the corresponding pH changes in the stroma were found to be smaller. It seems possible, therefore, that not only in the case of glyoxylate but also with nitrite the inhibition of CO_2 fixation is not solely due to the observed shift of the stromal pH. In order to avoid these possible secondary effects on CO_2 fixation, octanoate and formate seem to be more suitable agents for studying the effect of stromal pH changes on CO_2 fixation.

Identification of the pH-Dependent Step Limiting CO_2 Fixation

Table 2 also shows an analysis of the major metabolites of the reductive CO_2 fixation cycle contained in the stroma. The inhibited step limiting the metabolic flux through this cycle should be recognized from

Table 2. Relationship between the inhibition of CO_2 fixation and the pH and metabolite levels in the stroma

A: The chloroplasts (0.05 mg chl ml^{-1}) were preilluminated in parallel samples for assay of metabolites, pH measurements and oxygen evolution for 4 min. Then 20 mM HCl were applied to lower the pH in the medium to 6.65, or sodium formate (27 mM) were added. Samples for the assays were taken after additional 5–6 min of illumination. Measurement of O_2 evolution were preformed at the same time

B: The chloroplasts were preincubated with the inhibitors for 1 min in the dark and then illuminated for 6 min before taking of samples and measurement of O_2 evolution

	pH medium	Inhibitor (mM)	pH stroma	CO_2 dependent O_2-evolution (μmol/mg chlorophyll per h)	Metabolites (nmol/mg chlorophyll) stroma						
					FBP	SBP	HMP	RuBP	PMP	DHAP +GAP	PGA
A											
L141	7.60	–	7.79	107	6	6	105	12	4	5	143
	6.65	–	7.0	33	16	6	40	12	4	10	43
	7.60	formate (27)	6.9	35	28	7	37	17	5	12	27
B											
L136	7.60	–	7.60	170	13	6	79	16	4	9	143
	7.60	formate (23)	7.24	61	28	8	28	11	2	11	16
	7.60	octanoate (19)	7.27	55	20	13	32	8	5	11	40
	7.60	nitrite (2.8)	7.43	60	26	13	10	7	2	13	7
	7.60	glyoxylate (0.14)	7.52	66	28	6	23	10	2	12	23

FBP = fructose-1.6-bisphosphate; SBP = sedoheptulose-1.7-bisphosphate; HMP = mixture of hexose- and heptose monophosphates; RuBP = ribulose-1.5-bisphosphate; DHAP = dihydroxyacetone phosphate; GAP = glyceraldehyde phosphate; PGA = 3-phosphoglycerate; PMP = pentose monophosphatases

an accumulation of substrates and decrease of the products of this particular step. With all four inhibitors, the inhibition is accompanied by an increase of the levels of fructose bisphosphate and a decrease of hexose- and heptose monophosphates (these compounds are usually not well separated by our chromatographic procedure and therefore determined in total). These results clearly indicate that the hydrolysis of fructose bisphosphate was limiting the diminished rate of CO_2 fixation observed in the presence of all inhibitors employed in these studies. In experiment A, the addition of formate also caused an increase in the RuBP level. Such a rise of the RuBP, occasionally observed when CO_2 fixation was moderately inhibited by stromal pH changes, may reflect the pH-dependent activation state of this enzyme (Lorimer et al. 1978). As was shown earlier, phosphoglycerate reduction, being a partial reaction of CO_2 fixation, is not sensitive to stromal pH changes (Werdan et al. 1975). According to previous findings, the enhanced stromal triosephosphate/phosphoglycerate ratio observed during inhibited CO_2 fixation reflects an increase of the stromal ATP/ADP and NADPH/NADP ratios (Portis et al. 1977). Thus, a diminished rate of CO_2 fixation ultimately leads to an extensive photoreduction of the phosphoglycerate formed. Furthermore, the decreased pH gradient across the envelope also results in a stimulation of 3-phosphoglycerate efflux from the chloroplasts (Heldt et al. 1978; Kaiser et al. 1979). Thus, both these effects may explain the marked increase of the stromal triosephosphate/phosphoglycerate ratio observed here. We have recently reported on those changes of the metabolite levels caused by the addition of nitrite, as shown here (Purczeld et al. 1978). In these earlier studies these metabolite changes were even more pronounced, since a much higher degree of inhibition was introduced. It may be noted that with such a higher inhibition also a marked increase of the levels of sedoheptulose bisphosphatase was observed, which appears to be less pronouced when a lower degree of inhibition is employed.

Discussion

Very recently, evidence has been presented from enzyme-activity measurements in freshly ruptured chloroplasts that several enzymes of the reductive CO_2 fixation cycle, such as fructose- and sedoheptulose – bisphosphatase, phosphoribulokinase, glyceraldehydephosphate dehydrogenase, and ribulose bisphosphate carboxylase, are activated by light. Whereas, the activation of RuBP carboxylase was found to be dependent on the pH, the Mg^{2+}, and the metabolite levels in the stroma (Lorimer et al. 1978); the activations of the other enzymes are now, at least in part, understood as being derived from the reduc-

tion of S-S groups by reducing equivalents from ferredoxin, as generated in the light (Buchanan et al. 1979). Since the activation reaction itself (Heber et al. in press) as well as the activity of the activated enzymes appear to be strongly pH dependent (Baier et al. 1975; Preiss et al. 1967), the dramatic effect of the stromal pH on the rate of CO_2 fixation can now be well explained. Since there appear to be so many enzymes regulated by light, the question arises over the relative contribution of these enzymes in limiting the CO_2 fixation cycle. The relative role of these several enzymes for the light regulation of CO_2 fixation may of course vary with different metabolic conditions. Nevertheless, this question may be answered in the case of isolated spinach chloroplasts under standard conditions, which have been mostly used so far as a model system for studying CO_2 fixation.

As has been shown in the preceeding paragraphs, nitrite, which has been previously used as a tool for experimentally changing the stromal pH, may introduce a secondary effect on CO_2 fixation. For this reason, our earlier conclusion on the role of *pH changes in the light regulation of fructose bisphosphatase* may be open to criticism. The data of the present report clearly show that also when using formate or octanoate as a tool, or when decreasing the pH of the medium in order to change the stromal pH, the resulting inhibition of CO_2 fixation is accompanied by an increase of the stromal fructose bisphosphate and a decrease of the corresponding monophosphate. These results demonstrate that CO_2 fixation of chloroplasts can be regulated via the activity of fructose bisphosphatase by light-dependent transport of protons across the thylakoid membrane.

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