On the Light Dependence of Fatty Acid Synthesis in Spinach Chloroplasts

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ABSTRACT

The capacity of intact chloroplasts to synthesize long chain fatty acids from acetate depends on the stroma pH in *Spinacia oleracea*, U. S. hybrid 424. The pH optimum is close to 8.5. Lowering of the stroma pH leads to a reduction of acetate incorporation but does not suffice to eliminate fatty acid synthesis completely. Chain elongation from palmitic to oleic acid shows the same pH dependence. Fatty acid synthesis is activated in the dark upon the simultaneous addition of dihydroxyacetone phosphate and orthophosphate supplying ATP and oxaloacetate for reoxidation of NADPH in the stroma. Under these conditions both dark fatty acid synthesis and synthesis of oleate from palmitate show the same pH dependence as in the light. Dark fatty acid synthesis is further stimulated by increasing the stromal Mg^{2+} concentration with the ionophore A 23187. In contrast to CO_2 fixation, dark fatty acid synthesis is considerably reduced by dithiothreitol (DTT). This observation may be due to an acetyl-CoA deficiency, caused by a nonenzymic acylation of DTT, and a competition for ATP between DTT-activated CO_2 fixation and fatty acid synthesis. Because D,L-glyceraldehyde as inhibitor of CO_2 fixation compensates the DTT effect on dark fatty acid synthesis, reducing equivalents may be involved in the light dependence of acetate activation.

Long chain fatty acid synthesis from acetate in the stroma space of isolated intact chloroplasts (13) shows a strong light dependence (1, 11, 17, 24). This system requires ATP, NADPH, coenzyme A, Mg^{2+}, and HCO_3^- as substrates (23, 24). Under light conditions, energy and reducing equivalents are derived from photosynthetic reactions. Illumination of chloroplasts causes (a) a proton transport across the thylakoid membrane with a concomitant increase of the pH in the chloroplast stroma by about 1 pH unit (27), (b) an increase in the stromal Mg^{2+} concentration by 1 to 3 mM (14), and (c) an increase of the ATP/ADP ratio (20). It is very well established that the effect of light on the regulation of carbon fixation is mediated by these parameters (8). Furthermore, an activation of enzymes by SH groups, which are reduced by photosynthetic electron transport, has to be considered (2). To clarify the cause of the light dependence of fatty acid synthesis, we have analyzed the involvement of these parameters in the light control of fatty acid synthesis in isolated intact spinach chloroplasts.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*, U. S. Hybrid 424; Ferry-Morse Co., Mountain View, CA) was grown in hydroponic culture (9) and intact chloroplasts were prepared as in Werdan *et al.* (27) with an O_2 evolution rate in the range of 100 to 140 $\mu mol \cdot mg^{-1}$

$Chl \cdot h^{-1}$. In those experiments, in which Mg^{2+} dependence was to be assayed, EDTA (2 mM) and $MgCl_2$ (1 mM) were omitted from the reaction mixture described later on. The pH in the stroma was determined according to Heldt *et al.* (5). Extracts for determination of ATP were prepared by direct addition of 10% $HClO_4$ to the stirred chloroplast suspension. The ATP concentration in the neutralized stromal extracts was measured enzymically via 3-phosphoglycerate kinase according to (20). For fatty acid synthesis, chloroplasts (100 μg Chl/ml) were incubated under saturating white light at 25°C in a medium containing 0.33 M sorbitol, 50 mM Hepes (adjusted to the appropriate pH with NaOH), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.3 mM K_2HPO_4, 10 mM $NaHCO_3$, and 0.15 mM [1-^{14}C]acetate (57.2 $mCi \cdot mmol^{-1}$). Exceptions to these assay conditions are noted in the legends of figures and tables. For dark fatty acid synthesis, the reaction mixture additionally contained 5 mM dihydroxyacetone phosphate, 5 mM oxaloacetate, and 15 mM K_2HPO_4 according to Werdan *et al.* (27). CO_2 fixation from $NaH^{14}CO_3$ (1 $mCi \cdot mmol^{-1}$) was measured in parallel samples under the same conditions as described above. Reactions were terminated after 10 min by addition of 5 volumes methanol. Extraction and detection of labeled fatty acids was as described earlier (18).

RESULTS AND DISCUSSION

pH Dependence of Fatty Acid Synthesis from Acetate in the Light. To investigate whether the alkalization of the chloroplast stroma caused by illumination has an influence on the rate of fatty acid synthesis from acetate, we varied the stroma pH by changing the pH in the medium. During this procedure the pH gradient across the thylakoid membrane is little affected (5).

The incorporation of [1-^{14}C]acetate into long chain fatty acids depended on the stroma pH and showed an optimum close to pH 8.5 (Fig. 1A). The rate of acetate incorporation into fatty acids declined about 4-fold between 8.5 and 8 but was relatively constant between pH 8 and 7. This shows that the pH shift, induced by a light-dark transition, is not able to switch off fatty acid synthesis completely. Nevertheless, its influence on the fatty acid synthesizing capacity of chloroplasts is evident.

Simultaneous analyses of the end products of fatty acid synthesis in chloroplasts, expressed by the ratio of radioactive oleate to palmitate synthesized from [1-^{14}C]acetate (Fig. 1B), suggest that the chain elongation from palmitic to oleic acid shows the same pH dependence (18). In this context, it seems of interest that the formation of malonyl-CoA as substrate for chain elongation reactions, catalyzed by the acetyl-CoA carboxylase, shows the same pH optimum (6).

Regeneration of Fatty Acid Synthesis from Acetate in the Dark. Fatty acid synthesis from acetate requires ATP for the formation of acetyl-CoA and malonyl-CoA, and NADPH for chain elongation and desaturation. These requirements are normally provided by photosynthetic electron transport. Provided
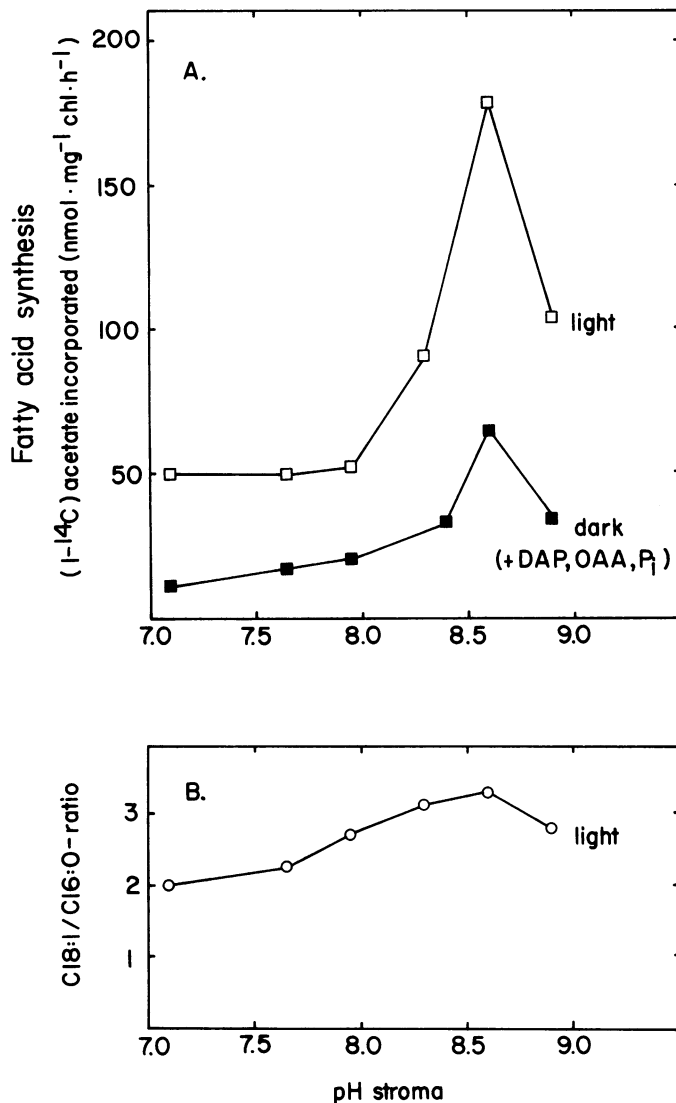
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FIG. 1. Dependence of the rate of fatty acid synthesis from [1-14C]acetate by intact spinach chloroplasts on the pH in the stroma under light and dark conditions. A, Dark fatty acid synthesis is driven by the addition of 5 mM DAP, 5 mM OAA, and 15 mM K_2HPO_4 (Pi) to the medium. Furthermore, the pH dependence of the chain elongation of palmitic acid in the light is demonstrated (B) as distribution of radioactivity between oleic- and palmitic acid ($C_{18:1}/C_{16:0}$ ratio). The data are mean values of five independent measurements.

that the intact chloroplast is supplied with ATP and NADPH from external sources, it should be able to synthesize fatty acids from acetate in the dark if the pH in the stroma is adjusted to the appropriate value. But a direct transport of ATP into the chloroplast is very slow and the inner envelope membrane is impermeable for pyridine nucleotides (4). Therefore, an indirect transport of ATP and NADPH by a shuttle of DAP[1] and PGA in the presence of high Pi concentrations combined with an addition of OAA for reoxidation of the NADPH (catalyzed by the malate dehydrogenase in the stroma) according to Heldt et al. (27) has been used to drive fatty acid synthesis. Whereas the shuttle of DAP and PGA is mediated by the phosphate translocator, OAA and malate are transported by the dicarboxylate translocator (27). In order to adjust a similar stroma pH as under

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[1] Abbreviations: DAP, dihydroxyacetone phosphate; PGA, phosphoglycerate; OAA, oxaloacetate; GA, D,L-glyceraldehyde.

Table I. Effect of Mg^{2+} in the Presence of the Ionophore A23187 (2 µM) on Fatty Acid Synthesis (from [1-14C]Acetate) and CO_2 Fixation in Intact Spinach Chloroplasts under Alkaline Conditions (about pH 8.5) in the Dark (Fig. 1A)

Under comparable pH conditions in the stroma, the rates of fatty acid synthesis and CO_2 fixation in the light were 176 nmol·mg⁻¹ Chl·h⁻¹ and 108 µmol CO_2·mg⁻¹ Chl·h⁻¹, respectively. The data are mean values of at least five measurements.

Additions	+Mg^{2+}	[1-14C]Acetate Incorporation	CO_2 Fixation
	mM	nmol·mg⁻¹ Chl·h⁻¹	µmol·mg⁻¹ Chl·h⁻¹
None		<1	<1
DAP, Pi	1	<1	<1
OAA, Pi	1	<1	<1
DAP, OAA, Pi	1	65	4
DAP, OAA, Pi, A23187	1	12	1
	1	99	5
	2	107	5
	5	114	5
	10	123	5

appropriate optimized light conditions, a relative strong alkalization (≧pH 9) of the medium was necessary, because the stroma pH in the dark decreases by about 1 pH unit (27). Following these conditions, in contrast to other authors (7), a considerable rate of fatty acid synthesis from acetate was observed in the dark if all components of the system (DAP, Pi, and OAA) were present, but there was almost no reaction if any one of the compounds was omitted (Fig. 1A; Table I). Fatty acid synthesis showed the same pH dependence and a similar ratio of labeled oleate to palmitate (of about 3) synthesized from [1-14C]acetate under optimal pH conditions as observed in the light (Fig. 1B). CO_2 fixation was measured in parallel samples in order to examine whether the shuttle of DAP and PGA was active and showed a small regeneration as well (Table I).

Illumination of intact chloroplasts also causes an increase in the stromal Mg^{2+} concentration of 1 to 3 mM (14). The influence of such changes of the stroma Mg^{2+} on dark fatty acid synthesis of intact chloroplasts described above was studied with the aid of the divalent cation ionophore A23187. This ionophore increases the permeability of the envelope for Mg^{2+} and consequently allows variation in the stromal Mg^{2+} level in the dark by increasing the Mg^{2+} concentration in the medium (15). As shown in Table I, dark fatty acid synthesis under the conditions described was considerably reduced by the release of Mg^{2+} if A23187 was given alone but was further restored (up to 70% of the light activity) with an increasing Mg^{2+} content in the reaction mixture. It has been recently shown (16) that in a sorbitol medium the stromal Mg^{2+} efflux, caused by the presence of A23187, is already inhibited by less than 0.1 mM Mg^{2+} in the medium, while higher concentrations lead to a net uptake, which remains constant at about 1 mM Mg^{2+} (150 nmol·mg⁻¹ Chl). The observation, that dark fatty acid synthesis was stimulated most up to 1 mM Mg^{2+} in the medium (Table I) supports this notion. The results further confirm the absolute Mg^{2+} requirement, which has been already postulated at least for the initial steps of fatty acid synthesis in chloroplasts (25), catalyzed by the acetyl-CoA synthetase and the acetyl-CoA carboxylase.

Effect of DTT. It is well known that the sulfhydryl reagent DTT has a stimulatory effect on the activity of various enzymes of the Calvin cycle (2). This suggests a possible involvement of light for the reductive formation of SH groups by photosynthetic electron transport. The stimulation of CO_2 fixation in the dark by DTT could presumably be associated with the light-dependent activation and inactivation of several enzymes of the reductive
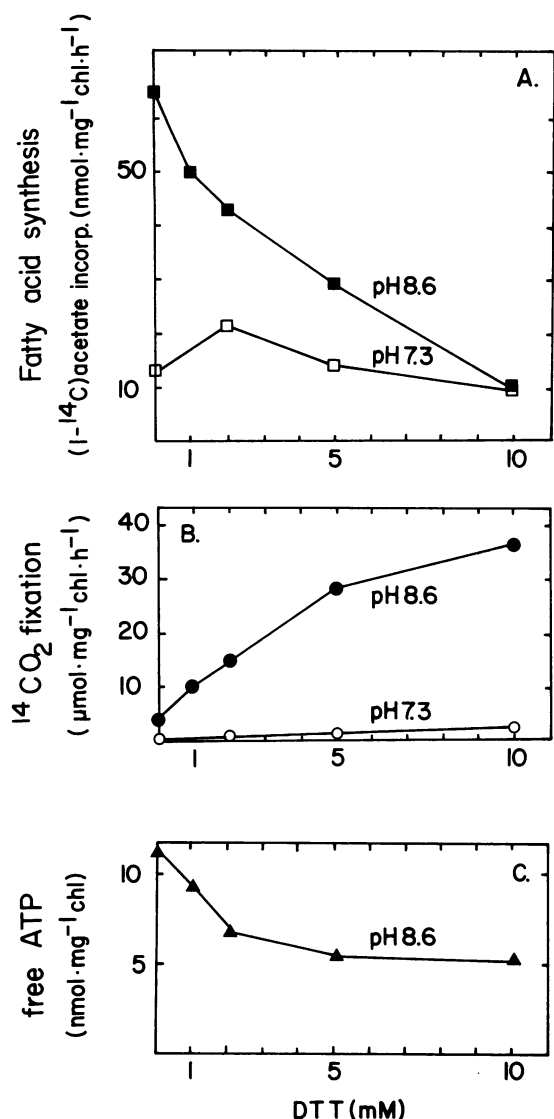
FIG. 2. Effect of increasing DTT concentrations in the medium on the rate of fatty acid synthesis (A) and CO_2 fixation (B) and on the level of free ATP (C) of intact spinach chloroplasts in the dark (conditions as in Fig. 1) under optimal and suboptimal pH conditions in the stroma. The data are mean values of four independent measurements.

pentose-P cycle mediated by reducing equivalents in the form of thioredoxin (2, 3). Conflicting results have been described concerning the influence of DTT on chloroplast fatty acid synthesis from acetate. Nakamura and Yamada (11) reported a reduction of the synthetic activity of intact chloroplasts in the presence of DTT. In contrast, Stumpf et al. (24) observed an enhanced incorporation of acetate into fatty acids by the addition of DTT. The latter findings agree with our results in experiments where broken chloroplasts were provided with the cofactors and substrates necessary for optimal fatty acid synthesis (unpublished data). In order to determine whether a light-activated 'thioredoxin-like' system is involved in the conversion of acetate into fatty acids, the effect of DTT on lipid incorporation of acetate has been reinvestigated.

Compared with earlier results (27), our intact chloroplast preparations showed, at pH 8.6, a marked stimulation in the regeneration of CO_2 fixation by DTT (Fig. 2B), but the simultaneous rate of fatty acid synthesis from acetate was inhibited (Fig. 2A). Although dark CO_2 fixation of the same chloroplast suspension was almost completely eliminated by lowering the stroma

pH to 7.3, substantial activity of fatty acid synthesis remained (Fig. 1A), which was even slightly stimulated by the addition of DTT in concentrations up to 2 mM (Fig. 2A). However, higher concentrations of DTT caused a decrease of the activity.

Because DTT undergoes a nonenzymic acetylation with acyl-CoA-thioesters (22), especially at alkaline pH (optimum at about 9.0), the observed inhibitory effect of DTT on dark fatty acid synthesis could be linked to a lack of acetyl-CoA as fatty acid intermediate caused by the acetylation reaction with DTT. The reduced formation of acylated DTT derivatives under neutral or weak acidic conditions (22) would probably account for the slight stimulation of dark fatty acid synthesis by 2 mM DTT at pH 7.3 in the chloroplast stroma (Fig. 2A). Furthermore, if one assumes that only about 1% of the total CO_2 fixed by isolated intact spinach chloroplasts is utilized for lipid biosynthesis (10), another explanation for the observed inhibitory effect of DTT on fatty acid synthesis in the dark (Fig. 2A) would be a limited availability of certain substrates (e.g. ATP), for which the enzymes of the dominant Calvin cycle compete with those of fatty acid synthesis. Thus, the obviously low level of ATP in darkened chloroplasts (3) is apparently further withdrawn from fatty acid synthesis as a consequence of its utilization by DTT-induced CO_2 fixation (Fig. 2C). This assumption is supported by the fact, that the ATP measured in chloroplasts is only partially available for metabolic reactions, because 3 to 4 nmol ATP is bound to the ATP synthase in the dark (19). A decrease of the ATP content in darkened chloroplasts to 5 nmol·mg^{-1} Chl, as a consequence of increasing DTT concentrations in the medium (Fig. 2C), will therefore result in an available stromal ATP level of only about 1 nmol·mg^{-1} Chl. Assuming a stromal volume of 25 μl·mg^{-1} Chl (3), this would give a minimal value for free ATP of about 60 μM. This concentration is twice the K_m determined for phosphoribulokinase (8) but far below the K_m of acetyl-CoA carboxylase (K_m: 240 μM; Sauer and Heise, unpublished results) for ATP, as assayed with these enzymes extracted from illuminated chloroplasts (according to Laing et al. [8]). Assuming that both these enzymes have a key function in the light regulation of either the Calvin cycle (Ru5P kinase [3]) or fatty acid synthesis (12), our results indeed support the involvement of reducing equivalents in the light regulation of enzymes of the pentose-P cycle (2). However, their regulatory functions in fatty acid synthesis remain to be determined.

In order to differentiate between the DTT inhibition of dark fatty acid synthesis by acyl-DTT formation on one hand and by the limited availability of ATP on the other, the same experiments were repeated in the presence of increasing concentrations of GA (Fig. 3), a known inhibitor of CO_2 fixation (21). As shown in Figure 3B, dark CO_2 fixation (under above optimized conditions) was indeed progressively reduced with increasing GA concentrations. The decrease was less pronounced than in the light (Fig. 3B) and depended on the DTT concentration in the medium, but reached nearly the same value at 5 mM GA (Fig. 3B). In the absence of DTT, dark fatty acid synthesis showed a slight stimulation up to 1 mM GA (Fig. 3A), which supports the above mentioned competition of fatty acid synthesis and CO_2 fixation for the stromal ATP pool. With higher GA content in the medium, it decreased again. The reason for such an inhibition of fatty acid synthesis at higher GA concentrations, which was confirmed in light control experiments (Fig. 3A), is not known. While the DTT-stimulated CO_2 fixation in the dark was inhibited by increasing GA (Fig. 3B), the DTT-inhibited fatty acid synthesis (Figs. 2A and 3A), in contrast, was stimulated by increasing GA (Fig. 3A). Thus, the inhibition of dark fatty acid synthesis by DTT appears to be due not only to acetyl-DTT formation (22) but also to the ATP demand of CO_2 fixation. Compared to the light control (Fig. 3A), which is considerably reduced at higher GA conents (by about 60% at 5mM GA), the latter
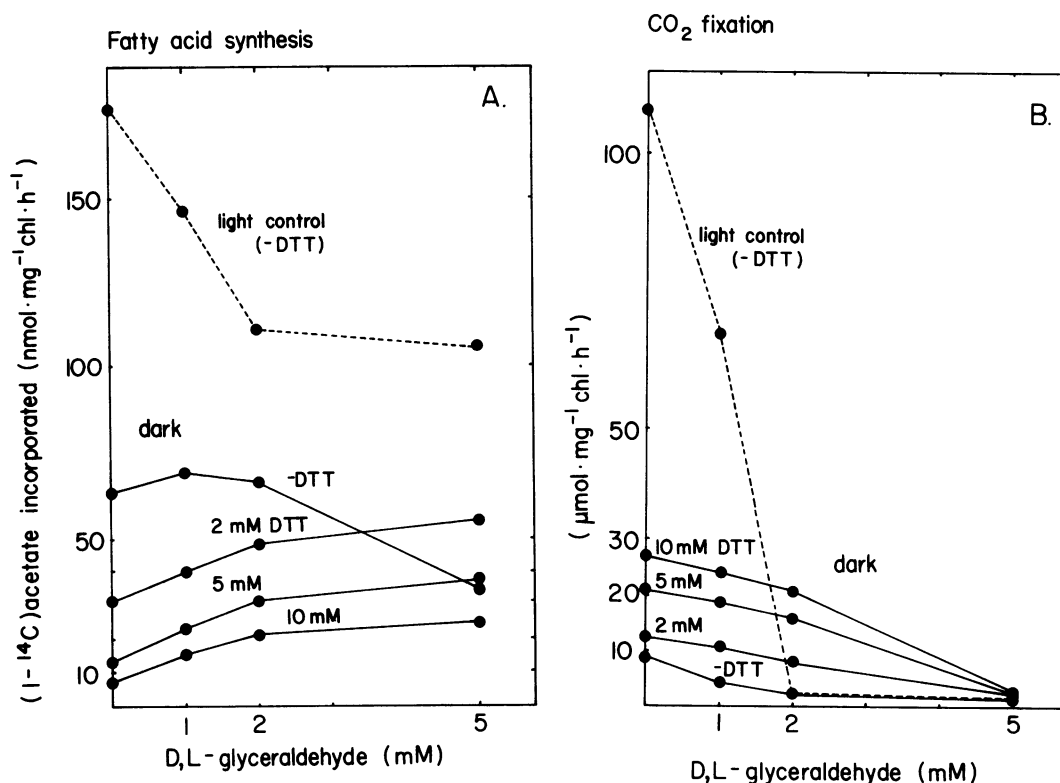
FIG. 3. Effect of increasing GA concentrations in the medium on the rate of fatty acid synthesis (A) and CO_2 fixation (B) of intact spinach chloroplasts under optimal pH conditions in the stroma (see Fig. 1) in the light (●---●) and in the dark (●——●) in the absence (−DTT) and presence of different DTT concentrations (2, 5, and 10 mM DTT). The data are mean values of four independent measurements.

observations indicate that there is a considerable stimulation of dark fatty acid synthesis by DTT and suggest, therefore, an involvement of reducing equivalents in the light activation of acetate incorporation into long-chain fatty acids.

We concluded from our data that the effect of light on the enzymes of acetate activation within chloroplasts is mediated by several parameters, such as the ATP/ADP ratio, the pH, and the Mg^{2+} concentration in the stroma, which are significantly changed during a light-dark transient (3) and by reducing equivalents as well. It should be noted that the decrease of the stromal ATP level in the dark is accompanied by an increasing level of ADP, which has been shown to be a strong competitive inhibitor of the acetyl-CoA carboxylase (26).

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