Regulation of Acetyl-Coenzyme A Carboxylase and Acetyl-Coenzyme A Synthetase in Spinach Chloroplasts

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Acetyl-CoA Carboxylase, Acetyl-CoA Synthetase, Light Dependence of Fatty Acid Synthesis in Chloroplasts

In analogy to chloroplast fatty acid synthesis from acetate the key enzymes of acetate fixation, acetyl-CoA synthetase and acetyl-CoA carboxylase, in rapidly Triton X-100 lysed spinach chloroplasts show an activation by light and deactivation in the dark. The stimulation of acetyl-CoA carboxylase by dithiothreitol in darkened chloroplasts points to an involvement of reducing equivalents in the light activation of this enzyme. But more than by alterations of the activation state per se, these enzymes appear to be effected by changes in their catalytic activity due to differences in the proton-, Mg^{2+}- and adenine nucleotide levels of the chloroplast stroma. Thus the pH dependence of both enzymes, as immediately extracted from Triton X-100 lysed chloroplasts, resembles that recently found for lipid incorporation of acetate into intact spinach chloroplasts in the light with an identical pH optimum of about pH 8.5 for the acetyl-CoA carboxylase. Moreover, in the same extracts both enzyme activities show the already postulated requirement for MgATP and free Mg and are competitively inhibited by free ATP and ADP with respect to MgATP. But on account of the fact, that the extractable acetyl-CoA synthetase as opposed to the carboxylase activities exceed by far the lipid incorporation rates of acetate by illuminated chloroplasts before disruption, acetyl-CoA synthetase will be excluded as rate limiting step in fatty acid synthesis from acetate. From key enzymes of acetate fixation only the carboxylase appears to be involved therefore in the light regulation of acetate incorporation into long-chain fatty acids.

Introduction

Chloroplast fatty acid synthesis from acetate appears to be regulated by light [1–6]. Like the Calvin cycle enzymes this effect of light seems to be mediated by light dependent variable parameters in the chloroplast stroma, *e.g.* the concentration of H^+, Mg^{2+} and ATP [6] which are known essential factors for acetate activation [3, 7]. Furthermore light-enhancement of fatty acid synthesis from acetate may be due to an enzyme interconversion mediated by changes in the redox poising [6, 8]. Acetyl-CoA carboxylase has been examined as possible site for such a light regulation of fatty acid synthesis [8]. In this study we have examined:

1. the acvtivation state of the key enzymes of acetate fixation in chloroplasts, *e.g.* acetyl-CoA synthetase and acetyl-CoA carboxylase in immediately lysed chloroplasts [9] during a light-dark transient, and
2. the kinetic dependence of these enzyme activities rapidly extracted from illuminated chloroplasts [10] on various stromal solutes and metabolites.

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Materials and Methods

Spinach (*Spinacia oleracea*, U.S. Hybrid 424, Ferry-Morse Co., Mountain View, CA, USA) was grown in hydroponic culture [11] and intact chloroplasts were prepared as in [12]. The chloroplasts (100 µg Chl · ml^{-1}) which showed an O_2-evolution rate in the range of 80–140 µmol · mg^{-1} Chl · h^{-1} were suspended in a reaction mixture containing 0.33 M sorbitol, 50 mM Hepes/KOH pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$ and 40 µg · ml^{-1} catalase from bovine liver (Boehringer, Mannheim), unless otherwise stated.

Assay of acetyl-CoA synthetase

The radioactivity assay of acetyl-CoA synthetase in chloroplasts by [1-^{14}C]acetate incorporation [13] was carried out with 100 mM Tricine/KOH (pH 8.0), 2 mM $MgCl_2$, 1 mM ATP, 0.5 mM CoA, 0.5 mM [1-^{14}C]acetate (specific activity: 57,2 mCi · $mmol^{-1}$) and 0.2% (w/v) Triton X-100 (final volume: 0.3 ml), if not stated otherwise. The reaction was stopped with 0.3 ml of an activated charcoal slurry: glacial acetic acid (1:1) mixture and labelled acetyl-CoA, adsorbed to charcoal, was

measured according to Kuhn *et al.* [13]. Control samples contained neither CoA nor ATP, both of which were essential for high rates of [1-^{14}C]acetate adsorption to the charcoal.

Assay of acetyl-CoA carboxylase

Acetyl CoA carboxylase activity in chloroplasts was measured as an acetyl-CoA-dependent fixation of $H^{14}CO_3^-$ into acid-soluble, heat-stable products [14]. The standard reaction mixture contained 100 mM Tricine/KOH (pH 8,5), 0.5 mM Acetyl-CoA, 1 mM ATP, 2 mM $MgCl_2$, 30 mM KCl, 7 mM $NaHCO_3$, 3 mM $NaH^{14}CO_3$ (specific activity: 1 mCi · mmol^{-1}) and 0.2% (w/v) Triton X-100 in a final volume of 0.3 ml. The reaction was terminated by adding 0.3 ml of 2 N HCl. The samples were dried at 70 °C in a scintillation vial and 0.2 ml 2 N HCl and 7 ml scintillator (6 g butyl-PBD, 600 ml toluene, 400 ml methylglycol) were then added and the acid-stable radioactivity was counted by liquid scintillation. Control samples without acetyl-CoA were included in each series of the assays.

Measurement of enzyme interconversion

For the study of the kinetics and the state of activation and inactivation of the enzymes chloroplasts in the standard medium were preilluminated or kept dark in the absence and presence of different DTT concentrations at 25 °C for the time intervals indicated (Figs. 1 and 2) and 50 µl aliquots were withdrawn and immediately lysed in 250 µl of a reaction mixture containing 0.2% w/v Triton X-100 [9, 15] and the essential unlabelled or radioactive substrates of the enzymatic reaction to be assayed. The final chlorophyll conc. was 20 µg/sample. This procedure allows an instantaneous start of enzyme activity measurements. The enzymic reaction, as initiated by the disruption of the chloroplasts, was terminated 30 s later by addition of hydrochloric acid, in order to keep the time of the assay short in relation to the kinetics of activation and inactivation [15] and to measure in the linear region of the reaction (up to 90 s).

Preparation of chloroplast crude extracts

In order to keep the investigated enzymes under approximate physiological conditions, modulation of their catalytic activity by ions (H^+, Mg^{2+}) and adenine nucleotides (ATP, ADP) has been measured in substrate free chloroplast crude extracts, prepared by rapid elution of Triton X-100 (final conc. 0.2% w/v) lysed preilluminated chloroplasts over a short Sephadex G25 column according to Laing and Roughan [10] and adjusted to 0.1 – 0.2 mg Chl · ml^{-1}.

In order to discriminate between the effect of free and adenine nucleotide bound Mg^{2+} on the activities of acetyl-CoA synthetase and carboxylase in above chloroplast extracts we calculated the free nucleotide concentration in the incubation by using the stability constants of Krämer [16].

Extraction and detection of labelled fatty acids was as described earlier [17].

Results and Discussion

Recent measurements of the endogenous acyl-acyl carrier protein pool sizes during steady-state fatty acid synthesis by isolated spinach chloroplasts [18] demonstrated the well known light dependence of fatty acid synthesis from acetate [1 – 3] at the level of its thioester intermediates. These results imply that the level of acyl-carrier protein thioesters reaches a "steady state" within 1 min of illumination and declines immediately after darkening of the chloroplasts. Because the latter compounds are direct substrates for specific acyl-thioesterases and transferases, the decline of the acyl-carrier protein pool in the dark leads to an immediate stop in the release of free fatty acids and reduced glycerolipid synthesis [5].

Enzyme interconversion

In order to localize the light dependent steps in the reaction sequence from acetate to long-chain fatty acids we started to investigate to what extent the activities of the key enzymes in acetate fixation, *e.g.* acetyl-CoA synthetase and acetyl-CoA carboxylase, are controlled by light.

Fig. 1 shows a time course for the light activation and dark inactivation of acetyl-CoA synthetase and acetyl-CoA carboxylase of chloroplasts *in situ*. After the onset of illumination the activity of the acetyl-CoA synthetase increases about 2-fold and that of acetyl-CoA carboxylase increases about 3-fold. Darkening leads to a decrease of both enzyme activities. Compared to the rapid reduction of acyl-acyl carrier protein synthesizing capacity of chloro-

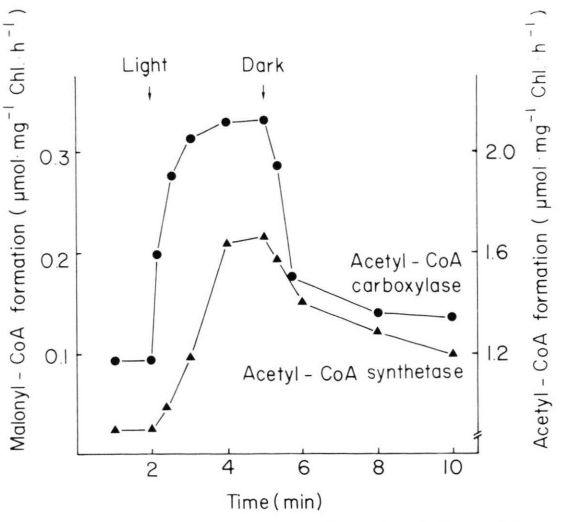

Fig. 1. Changes in the level of malonyl-CoA (●) and acetyl-CoA (▲) formed from [1-^{14}C]acetate in spinach chloroplasts during a light-dark transition. The time of light activation and dark inactivation of intact chloroplasts before their disruption in the Triton X-100 containing reaction mixture is plotted on the abscissa. In order to keep the time of the assay short in relation to the kinetics of activation and inactivation and to measure in the linear region of the reaction, the test was terminated after 30 s by addition of acid.

plasts after turning off the light [18] the deactivation of acetyl-CoA synthetase and acetyl-CoA carboxylase appears relatively low (Fig. 1). Latter comparison suggests, alterations of the activation state of these enzymes, as caused for example by reducing equivalents, to play a subordinate role in the light-enhancement of plastidary fatty acid synthesis from acetate. However, recent reinvestigations on the effect of the sulfhydryl reagent dithiothreitol (DTT) on dark activation of acetate incorporation into long-chain fatty acids of isolated chloroplasts [6], which appeared contradictory in the literature [4, 19], led to the conclusion, that beside changes in stromal solutes (H^+, Mg^{2+} and ATP) reducing equivalents may be in somehow involved in the light control of fatty acid synthesis. Last notion directed our attention to the localization of the DTT-sensible step within the reaction sequence of the plastidary acetate fixation, particularly because of the recent finding, light to be essential only for acetyl-CoA carboxylase activity in fatty acid synthesis of spinach chloroplasts [8].

Therefore, in analogy to the enzyme interconversion measurements (see methods), intact darkened chloroplast suspensions were incubated for 10 min with different DTT concentrations in the dark (Fig. 2), before they were lysed in the Triton X-100 containing reaction mixture. In spite of a five-fold dilution of DTT in the reaction mixture this method was not fit for a quantitative detection of the acetyl-CoA synthetase activity by adsorption of ^{14}C-labelled acetyl-CoA (from [^{14}C]acetate) to charcoal [13], because DTT undergoes a non-enzymic acetylation with acyl-CoA thioesters [19]. On the other hand carboxylase activities, determined by $^{14}CO_2$-fixation to acetyl-CoA, could be determined quantitatively if one allowed for adequate acetyl-CoA concentrations (0.5 mM) in the reaction mixture, to ensure that the formation of radioactive malonyl-CoA was not limited by DTT-binding [19] of the substrate acetyl-CoA. Furthermore through the acid stop in this test only the reaction products were measured while unreacted CO_2 was released. The observed stimulation of the acetyl-CoA carboxylase by DTT, shown in Fig. 2, appears to reflect therefore an involvement of reducing equivalents in the light activation of this enzyme.

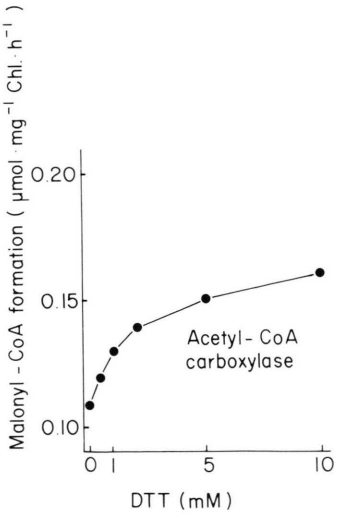

Fig. 2. Effect of dithiothreitol (DDT) on the activity of acetyl-CoA carboxylase measured as malonyl-CoA formation in spinach chloroplasts in the dark. Intact chloroplast suspensions were preincubated in the dark for 10 min with the DDT concentrations indicated and then disrupted in Triton X-100 containing reaction-mixture (further procedure as in Fig. 1).

pH Dependence

As shown in Fig. 3, in chloroplast extracts the optimal activity of acetyl-CoA synthetase is observed at about pH 8.0 and that of acetyl-CoA carboxylase at about pH 8.5. The latter pH-optimum appears identical with the stroma pH determined for optimal acetate incorporation into the lipid fraction of intact spinach chloroplasts [6]. During a light-dark transition a decrease of the pH from 8.4 to 7.4 was measured in intact chloroplasts incubated at pH 7.9 [6, 12]. But lowering of the stroma pH by 1 unit led only to a partial inhibition of the activities of acetyl-CoA synthetase and acetyl-CoA carboxylase (Fig. 3). The latter observation suggests that light-induced pH changes in the stroma may control the activities of both enzymes, a suggestion that is consistent with recent findings on the pH dependence of total fatty acid synthesis from acetate in intact chloroplasts [6].

Effect of Mg

The absolute requirement for Mg^{2+} in the initial steps of fatty acid synthesis from acetate in chloroplasts [14, 20, 21] has been confirmed in dark activation experiments of fatty acid synthesis in intact chloroplasts [6]. Furthermore a dual requirement of Mg^{2+} for the formation of MgATP and for enzyme activation has been established [14, 21].

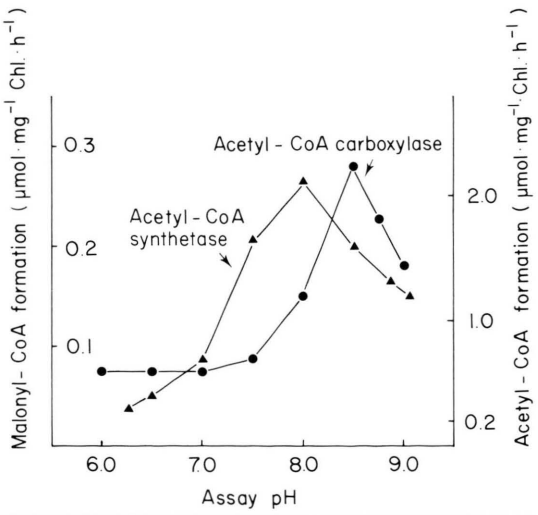

Fig. 3. Effect of pH on the activities of acetyl-CoA carboxylase measured as malonyl-CoA formation and acetyl-CoA synthetase in spinach chloroplast extracts.

Figures 4 and 5 show the activity of the two key enzymes of acetate fixation in chloroplast extracts on the concentration of free and ATP-bound Mg^{2+}. The primary stimulation of both enzyme activities in incubations of a constant ATP content (1 mM), caused by increasing Mg^{2+} up to equimolar concentrations (1 mM), would be due to an accumulation of MgATP [22] at the expense of free ATP (Fig. 4). The relatively small secondary stimulation of acetyl-CoA synthetase and carboxylase, found at higher Mg^{2+} concentrations (≧ 1 mM), when the ATP concentration for complexation with Mg is limited, can be ascribed to free Mg^{2+} (Fig. 5). To find an explanation for the activating influence of free Mg^{2+}, the dependence of the K_m and V_{max} of both enzymes on the concentration of free Mg^{2+} (Fig. 6) has been investigated under a constant MgATP level (1 mM), calculated according to Krämer [16].

While the K_m of the carboxylase for MgATP (K_m(MgATP) = 0.24 mM) is significantly reduced by free Mg^{2+}, its V_{max} (V_{max}(MgATP) = 0.36 μmol · mg^{-1} Chl · h^{-1}) for the same substrate remains unchanged. In contrast, free Mg^{2+} increases the V_{max} (V_{max} (MgATP) = 1.85 μmol · mg^{-1} Chl · h^{-1})

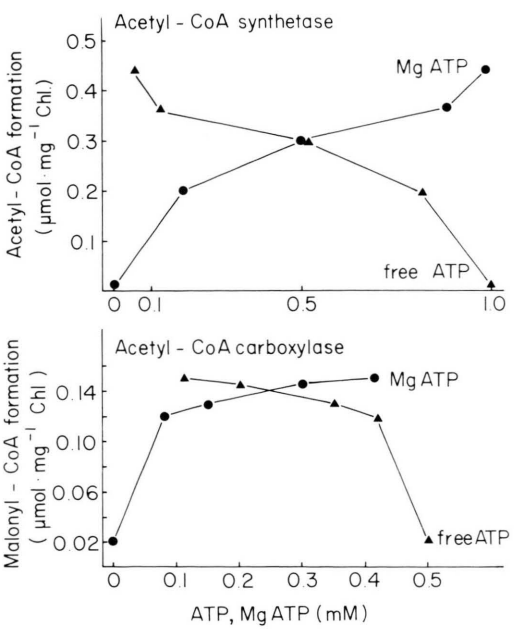

Fig. 4. Effect of free ATP and MgATP on the activities of acetyl-CoA synthetase and acetyl-CoA carboxylase in spinach chloroplast extracts. The free nucleotide concentration during incubation was calculated by using the stability constants of Krämer [16].

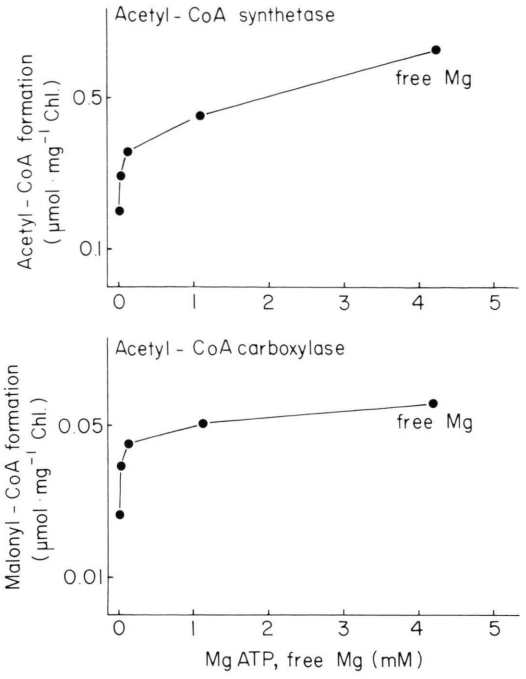

Fig. 5. Effect of Mg^{2+} on the activities of acetyl-CoA synthetase and acetyl-CoA carboxylase in extracts of spinach chloroplasts under a constant MgATP level (1 mM), calculated according to Krämer [16].

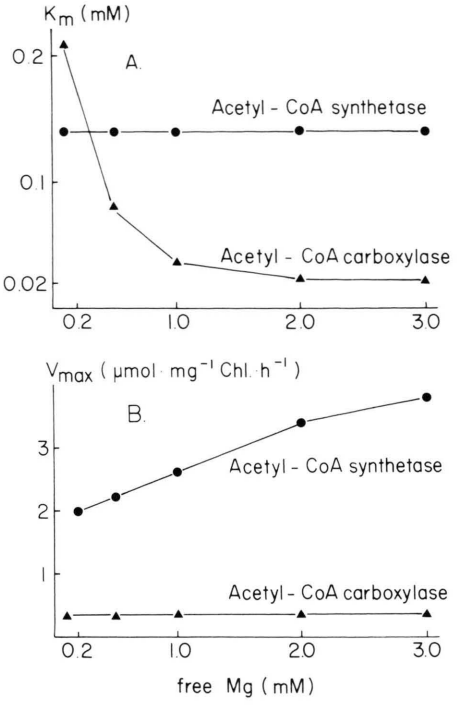

Fig. 6. Effect of Mg on K_m (Fig. A) and V_{max} (Fig. B) of acetyl-CoA synthetase and acetyl-CoA carboxylase in spinach chloroplast extracts. The concentration of MgATP, calculated according to Krämer [16], was a constant 1 mM.

of the synthetase but does not change the K_m for MgATP (K_m(MgATP) = 0.14 mM; Fig. 6). The decrease of the Michaelis-Menten constant of the carboxylase for MgATP by free Mg^{2+} may explain the difference in K_m observed here with that of the enzyme (K_mATP = 0.039 mM) recently found in crude carboxylase preparations from spinach [23]. An evaluation of the data by Hill plots (not shown) clearly indicated that there is no cooperativity between the catalytic binding sites of both enzymes for the substrates examined. The results of this kinetic study support the unique relationship between MgATP as substrate and free Mg^{2+} as an activator of acetyl CoA carboxylase activity as well as the notion, that both ions probably add to this enzyme in an equilibrium ordered manner [14]. Furthermore the kinetic constants determined for acetyl CoA carboxylase in the applied crude extracts (Fig. 6, Table I) resemble that recently found for partially purified enzyme preparations

Table I. Kinetic constants of acetyl-CoA synthetase and acetyl-CoA carboxylase from spinach chloroplast extracts (mean values).

		K_m [mM]			V_{max} [µmol · mg^{-1} Chl · h^{-1}]			K_i [mM]	
Enzymes	Substrate	MgATP	Free Mg	Acetyl-CoA	MgATP	Free Mg	Acetyl-CoA	Free ATP	ADP
acetyl-CoA synthetase		0.14	0.14	–	1.85	–	–	1.50	0.71
acetyl-CoA carboxylase		0.24	0.08	0.06	0.36	0.36	0.10	0.44	0.10

from spinach chloroplasts [21]. The data support earlier findings [20], that free and ATP-bound Mg^{2+} are required for optimal activity of acetyl CoA synthetase even though the kinetic mechanism for the activation of this enzyme by free Mg^{2+} appears to differ from that of the carboxylase (Fig. 6). Free Mg^{2+} seems to increase the affinity of the catalytic centre for MgATP in carboxylase. In the synthetase it accelerates the enzyme catalyzed reaction possibly by a conformational change of either the enzyme or the enzyme substrate complex. The observed dependence of the kinetic constants of acetyl CoA synthetase and carboxylase on free Mg^{2+} may be of regulatory significance for compensating changes in the level of chelating adenine nucleotides, which has been found to influence the activities of both enzymes [24]. Latter assumption is supported by the observation, that an activation of fatty acid synthesis in darkened chloroplasts by supplying cofactors (ATP, NADPH) *via* a dihydroxyacetone phosphate shuttle [12] was considerably stimulated by increasing the stromal Mg concentration beyond the supposed dark value (1–3 mM; [25]), although the calculated increase of the simultaneously available ATP level (from 0.2–0.4 mM) was comparably low. Thus, the observed activation by free Mg^{2+} may be ascribed to a compensation of the inhibitory effect of increasing ADP concentrations in the dark [23, 25].

Inhibitors

It has been shown that free ATP caused a considerable decrease of acetyl CoA carboxylase activity [14, 21]. This effect has been recently attributed to comtaminations of the carboxylase by ATPase and adenylate kinase generating significant amounts of ADP and AMP, which are inhibitors of the carboxylase [23]. This interpretation cannot be applied in the present investigation because the disruption of chloroplasts in the presence of Triton X-100 and subsequent gel filtration [10] eliminate cofactors and substrates for ATPase and adenylate kinase. Further, during the short assay times of only 5–10 min, ATP hydrolysis in the extracts will play only a minor role, as suggested by simultaneous measurements of the phosphorylation potential (data not shown). Therefore, the reduction of acetyl-CoA carboxylase and synthetase activities at ATP concentrations exceeding the adjusted Mg^{2+} content in the reaction mixture (Fig. 7), which is superimposed on the stimulating effect of MgATP on both enzyme activities (Figs. 4 and 5), seems to be related more to a competitive inhibition by free ATP than to an inhibition by ATP hydrolysis [23]. The K_i values for free ATP obtained for acetyl CoA carboxylase and acetyl CoA synthetase were 0.44 and 1.5 mM respectively (Table I). But the relatively high stromal Mg^{2+} concentrations present in intact chloroplasts (0.4–1 $\mu mol \cdot mg^{-1}$ Chl) [26], suggest that an inhibition by free ATP is unlikely to be of physiological significance. On the other hand, the level of ADP is known to increase in darkened chloroplasts [25]. Therefore the above mentioned evidence for the inhibitory effect of ADP [23] has been extended to both key enzymes of acetate fixation in the chloroplast extracts. The K_m of both enzymes for MgATP increases with ADP concentration (Table II). This indicates that ADP is not only a competitive inhibitor for acetyl CoA carboxylase ($K_i = 0.1$ mM) but also for acetyl CoA synthetase ($K_i = 0.71$ mM) with respect to MgATP (Table I). In

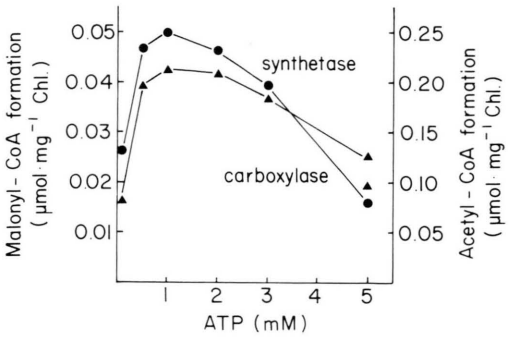

Fig. 7. Effect of ATP at a constant Mg^{2+} (1 mM) in the assay medium on the activities of acetyl-CoA synthetase and acetyl-CoA carboxylase in spinach chloroplast extracts.

Table II. Effect of ADP on the K_m of acetyl-CoA synthetase and acetyl-CoA carboxylase in spinach chloroplast extracts for MgATP.

ADP Concentration [mM]	K_m [mM]	
	Acetyl-CoA carboxylase	Acetyl-CoA-synthetase
0	0.14	0.02
0.2	0.18	0.12
0.5	0.24	0.26
1.0	0.32	0.52

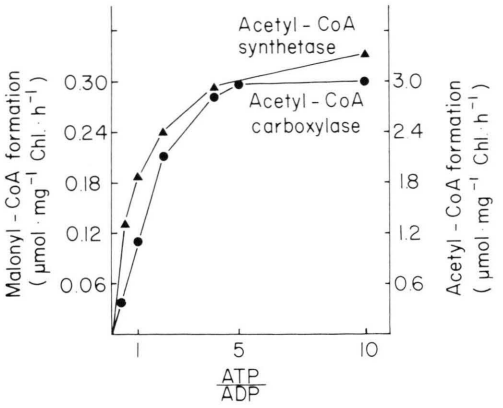

Fig. 8. Effect of the ATP/ADP quotient in the assay medium on the activities of acetyl-CoA carboxylase and acetyl-CoA synthetase in spinach chloroplast extracts. The samples were incubated for 10 min in the standard medium with 2 mM $MgCl_2$ and 1 mM ATP at different ADP-concentrations. The ATP used for the ATP/ADP quotient is total ATP.

order to determine the regulatory significance of the stromal adenine nucleotide levels on the activities of both enzymes during a light-dark transition, the dependence of their activities on the ATP/ADP quotient in incubations of chloroplast extracts has been compared with corresponding values determined in the stroma of illuminated (ATP/ADP = 2–3) and darkened (ATP/ADP = 0.4–0.6) isolated chloroplast [25, 27]. The results (Fig. 8) suggest that the calculated decrease of the stromal ATP/ADP quotient during a light-dark transient, at least in isolated chloroplasts, led to a strong reduction of the activities of acetyl CoA synthetase and carboxylase and could therefore exert a regulatory function in fatty acid synthesis [6, 23]. The relatively higher stromal ATP/ADP quotient ($\geqq 1$) found in chloroplasts from wheat leaf protoplasts [27] in the dark, which appears to be maintained by ATP import from the cytosol, may further explain, why leaf discs [5] and wheat leaf protoplasts (unpublished results) as opposed to isolated chloroplasts [5, 18] were still capable of fatty acid synthesis (about 20% of the corresponding light rates) in the dark.

The above evidence suggests that a cooperation of enzyme interconversions and light dependent changes in the H^+- and Mg^{2+}-concentration and ATP/ADP quotient affect the catalytic activities of acetyl-CoA synthetase and carboxylase in the chloroplast stroma. Evidently these play an important role in the light activation of acetate incorporation into long-chain fatty acids [1–6]. The rates of lipid incorporation of acetate by illuminated chloroplasts before disruption were 100–360 nmol · mg^{-1} Chl · h^{-1}. In chloroplast extracts under optimized conditions, malonyl-CoA was synthesized with similar rates (up to 360 nmol · mg^{-1} Chl · h^{-1}), while acetyl-CoA formation showed significantly higher values (3 µmol · mg^{-1} Chl · h^{-1}). Even allowing for the effect of stromal parameters (*e.g.* H^+, Mg^{2+} and ATP/ADP quotients) on enzyme activity in illuminated chloroplasts the high activity of acetyl-CoA synthetase measured suggests that it is unlikely a rate limiting step in the light regulation of fatty acid synthesis from acetate. This conclusion is consistent with the findings that no substantial difference in acetyl-CoA formation was observed between illuminated and darkened chloroplasts [28], although fatty acid synthesis in the dark was nearly totally inhibited [1–6]. In contrast, the activity of acetyl-CoA carboxylase in chloroplast extracts, calculated with respect at the known levels of above stromal solutes (H^+, Mg^{2+} and ATP/ADP quotient) in illuminated chloroplasts [27], was similar to the rate of lipid incorporation of acetate in intact chloroplasts in the light. This suggests that, as proposed [8, 23], the carboxylase is the key enzyme of acetate fixation that would appear to be involved in the light control of acetate incorporation into long-chain fatty acids in chloroplasts.

Acknowledgements

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