Activation of spinach chloroplast acetyl-coenzyme A carboxylase by coenzyme A

W.A. Laing and P. Grattan Roughan

Plant Physiology Division, DSIR, Palmerston North, New Zealand

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1. INTRODUCTION

Acetyl-CoA carboxylase catalyses the rate limiting step in the synthesis of fatty acids in animal systems [1]. To date, the reported activities of acetyl-CoA carboxylase from spinach [2–4] have been inadequate to support the observed rates of fatty acid synthesis either in isolated chloroplasts or in spinach leaves [2,5–9] and therefore no such regulatory role can be assumed for the chloroplast enzyme. In this paper we report physiological rates of acetyl-CoA carboxylase and show that the activity is regulated by CoA.

2. METHODS

2.1. *Materials*

Avidin, biotin, CoA and dithiothreitol were purchased from Sigma, ATP from Boehringer. $NaH^{14}CO_3$ and [2-^{14}C]malonyl-CoA were purchased from the Radiochemical Centre, Amersham. The HCO was filtered through a 0.025 μm filter to reduce background counts in the assays. All other chemicals were of analytical grade. Acetyl-CoA was prepared from acetic anhydride and CoA [10], treated with diamide [11] and separated on a Dowex 1 × 8 column with a 0 to 0.1 M HCl gradient. Fractions containing the acetyl-CoA were combined and lyophilized. The acetyl-CoA was dissolved in water, and neutralized. Acetyl-CoA was determined by enzymatic analysis [12] and total CoA was calculated from absorption at 260 nm [13]. Preparations generally contained 30% free CoA.

Spinach plants were grown hydroponically and chloroplasts isolated from young leaves [2] were resuspended at about 0.5 to 1 mg/ml chlorophyll in photosynthesis media (0.33 M sorbital, 0.05 M Hepes (pH 7.6), 1 mM $MgCl_2$ and $MnCl_2$, 2 mM EDTA, 0.2 mM $KHPO_4$, 5 mM $NaHCO_3$ and 50 U/ml of catalase) at 0°C. Bicarbonate dependent oxygen evolution by the chloroplasts was in the range of 80 to 170 μmol/mg chlorophyll/h.

2.2. *Experimental*

Chloroplasts were incubated in photosynthesis media in the light at 0.5 to 1 mg/ml chlorophyll for 5 min before adding Triton X-100 to 0.1% (w/v) to disrupt the chloroplasts [14]. After a further minute, the preparation was placed on a Sephadex G25 column (17 × 0.9 cm) equilibrated with 0.1 M tricine (pH 8.0), at 4°C and eluted under slight positive pressure. The green eluate was stored on ice and used within 1 h. The dilution of both the chlorophyll [15] and the RuBP carboxylase [16] on the Sephadex column were the same so chlorophyll was used routinely to estimate column dilution. Acetyl-CoA carboxylase was assayed at 25°C by adding pre-incubated chloroplast extracts to reaction mixtures containing 0.1 M tricine (pH 8.0), 50 mM KCl, and concentrations of $MgCl_2$, ATP, $NaHCO_3$ (at approximately 5 mg/mol), and acetyl-CoA as specified in the results. Assays were stopped with 0.5 M HCl, dried either at room temperature under vacuum or at 40°C and acid stable radioactivity determined by scintillation counting. Blank incubations contained no acetyl-CoA but were otherwise treated identically. Further details are found in the figure legends. The method of Huang [17] was used to authenticate the reaction product.

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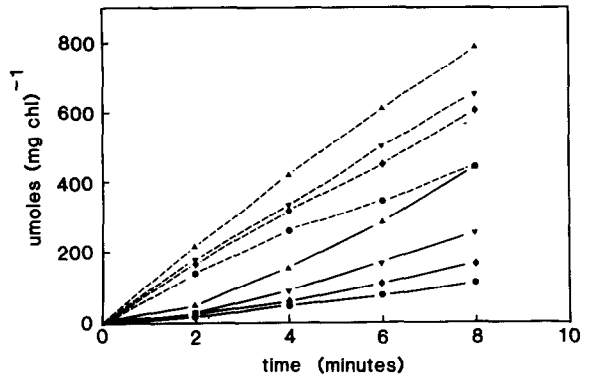

Fig.1. Time course of the extent of enzyme action per mg chlorophyll after pre-incubation with either water (continuous lines) or 0.93 mM CoA (dashed lines) at four concentrations of chlorophyll. Enzyme was pre-incubated with CoA or with an equal volume of water in 0.1 M tricine buffer (pH 8.0), 2.3 mM $MgCl_2$ and 2.7 mM dithiothreitol at 25°C for 15 min. An aliquot of extract was added to an assay tube containing 1 mM ATP, 0.28 mM acetyl-CoA and 7.9 mM $NaHCO_3$ and aliquots were removed to acid at 2-min intervals. Extra CoA was added to reaction mix for enzyme pre-incubated with water to compensate for CoA carried over by the CoA pre-incubated enzyme. The pre-incubation chlorophyll concentrations were 33 (▪), 83 (◆), 150 (▾) and 349 (▴) μg/ml during pre-incubation in the case where the enzyme was pre-incubated with water and 33, 67, 166 and 366 respectively where the enzyme was pre-incubated with CoA.

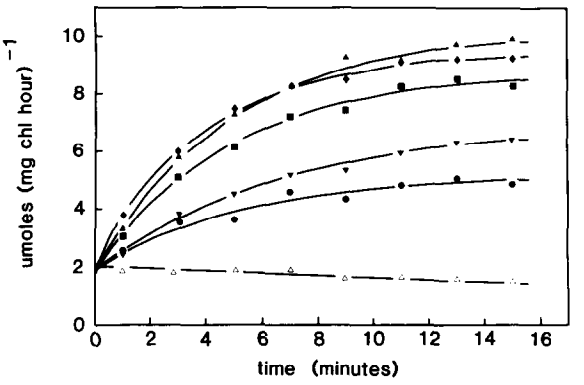

Fig.2. Time course of activation of acetyl-CoA by various concentrations of CoA. Extract (0.48 mg chlorophyll/ml) was pre-incubated in 0.1 M tricine (pH 8.0), 2.5 mM $MgCl_2$, 3.3 mM DTT and various concentrations of CoA. At intervals shown, 10 μl aliquots were removed into 50 μl of assay mix containing 1 mM ATP, 2.3 mM $MgCl_2$, 9.7 mM $NaHCO_3$, and 0.36 mM acetyl-CoA. After 1 min the reactions were stopped with acid. The data was fitted to an equation of the form: $Y = N_o + N_{eq}{}^* (1-\exp(-k_{obs}{}^* t))$, where Y is the activity observed at time t; N_o is the zero time activity; N_{eq} is the final activity and k_{obs} is the pseudo first order rate constant. Data was fitted as described by Laing and Christeller [19]. The lines on the graph are the fits to the above equation. (△), 0; (●), 0.05; (▾), 0.1; (▪), 0.3; (▴), 1.0; (◆), 3.33 mM CoA in pre-incubation.

3. RESULTS

The activity of acetyl-CoA carboxylase increased with time when extract was added to a complete assay medium (fig.1). This suggested that a component of the assay medium was stimulating enzyme activity. Free CoA was identified as the probable activator. After pre-incubation of the extract in the presence of 0.93 mM CoA, time courses were linear or curved slightly downwards (fig.1). Enzyme activity also increased with increased concentration of extract in the assay but CoA stimulation was still observed at the highest extract concentration used (fig.1). $MgCl_2$ was necessary for the full activation by CoA (data not shown) although about 50% activation was found in the absence of added $MgCl_2$. The activation by CoA was completed in about 15 min and appeared to follow a pseudo first order time course (fig.2). Pre-incubations in the absence of CoA resulted in slight but

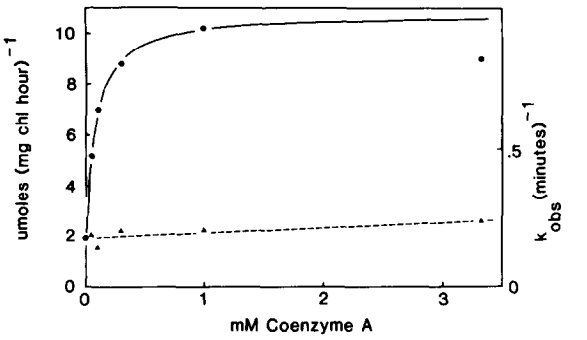

Fig.3. Response of the calculated k_{obs} and $N_{eq}+N_o$ (from fig.2) to CoA concentration. The fitted line to the $N_{eq}+N_o$ data points (●) is a hyperbola with the equation: $N_o + N_{eq} = N_o + N_{max} * CoA/(K_m(CoA) + CoA)$, where N_{max} is the predicted maximal value of N_{eq} at infinite CoA and $K_m(CoA)$ is the CoA concentration giving half maximal stimulation. N_{max} was 8.95 μmol/mg chl/h, N_o 1.87 and $K_m(CoA)$ 80 μM. K_{obs} is represented by (▴)

progressive decreases in activity. Although the pseudo first order rate constant was only slightly dependent upon CoA concentration, the extent of activation was markedly increased by CoA (fig.3). The data fitted a hyperbola and half maximal activation was reached by 80 μM CoA.

After pre-incubation in CoA, the carboxylase activity was 90% inhibited by avidin and added biotin prevented this inactivation. The products of the reaction, with and without CoA pre-incubation, co-chromatographed on TLC with authentic [2-^{14}C]malonate after alkaline hydrolysis, and remained on the origin before hydrolysis. This is consistent with the behaviour of [^{14}C]malonyl-CoA [17]. Incubations containing phosphoenol pyruvate formed no acid stable radioactive product, and the product formed in the presence of ribulose bisphosphate remained on the origin of the chromatogram, even after treatment with alkali.

4. DISCUSSION

Previous attempts to measure acetyl-CoA carboxylase from spinach leaves have reported low activities relative to the rate of [^{14}C]acetate incorporation into long chain fatty acids. Roughan et al. [2] reported activities of 0.15 to 0.25 μmol/mg chlorophyll/h and a rate of approximately 0.3 μmol/mg/h can be calculated from the purification protocol of Mohan and Kekwick [3]. An activity of about 0.12 μmol/mg/h can be calculated from Kannagara and Stumpf [4] assuming the HCO_3 concentration was 0.6 mM in equilibrium with air at pH 7.9. However, this was in the presence of added transcarboxylase from *E. coli*. Spinach chloroplasts have been reported to incorporate acetate into fatty acids at rates of up to 2.1 μmol/mg/h [2,7–9] and spinach leaves incorporated $^{14}CO_2$ into fatty acids at similar rates [8,9]. The maximum CoA stimulated rate of acetyl-CoA carboxylase reported in this study was 10 μmol/mg/h (fig.3). However, the $K_m(HCO_3)$ for the carboxylase has been reported as 3 mM [3] similar to the value of 4.6 mM found in this laboratory (Laing, unpublished results). Using a $K_m(HCO_3)$ of 3.8 mM and assuming air levels of CO_2 in the chloroplast at pH 8.0, then the above maximum rate of 10 is corrected to 2.0 μmol/mg/h. Thus the fully CoA activated acetyl-CoA carboxylase found in these chloroplasts has a rate similar to the maximal rates of fatty acid synthesis reported in spinach leaves and chloroplasts. Assuming that the intrinsic capacity for fatty acid synthesis by spinach leaves is near 2 μmol/mg/h, then in leaves the rate of fatty acid synthesis may be limited by the rate of acetyl-CoA carboxylase activity due to the low concentration of CO_2 in air. However, fatty acid synthesis in spinach chloroplasts is usually measured in the presence of 10 mM HCO_3 so that the carboxylase would no longer limit fatty acid synthesis. As the acetyl-CoA synthetase activity has been reported to be up to 7.8 μmol/mg/h [18], and acetate is supplied to chloroplasts at saturating concentrations, either the supply of ATP or reducing power or the rates of the fatty acid synthetase would limit fatty acid synthesis. There is no data on the concentration of free CoA in the chloroplast. However, the concentration of 80 μM necessary for half activation may well be physiological and CoA could be a significant regulator of fatty acid synthesis in vivo. High activity, up to 30 μmol/mg/h, of acetyl-CoA carboxylase has been reported in developing Maize leaves [19]. However no studies of whether CoA activated the enzyme were reported.

Recent studies with rat liver acetyl-CoA carboxylase have shown that it is also activated by CoA [20]. The mammalian system is thought to undergo complex aggregation upon activation [1]. This results in non-linear responses of reaction rate to enzyme concentration. Perhaps the stimulation of the activity per mg chlorophyll with increased amounts of chloroplast extract per ml of assay also reflects a similar activation by aggregation. Further analysis of this phenomenon would best be done with purified enzyme. However it should be noted that the assay system used here was very dilute compared to the situation in a chloroplast, and that the activity per mg chlorophyll was still increasing at the highest concentrations of extract used here.

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