Phytochemistry, 1975, Vol. 14, pp. 1171–1173. Pergamon Press. Printed in England.

SUCCINYL COENZYME A AND PORPHOBILINOGEN FORMATION IN ISOLATED ETIO-CHLOROPLASTS AND GREENING LAMINAE

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(*Received* 18 *August* 1974)

Key Word Index—*Avena sativa*; Gramineae; succinyl CoA synthetase or thiokinase, δ-amino-laevulinic acid dehydratase, chlorophyll, etio-chloroplasts.

Abstract—A comparative study of succinyl CoA synthetase and δ-ALA dehydratase activities in greening *Avena* laminae and in isolated etio-chloroplasts showed the former enzyme activity to be non-plastidic in location during all stages of chloroplast morphogenesis and the latter to be mainly associated with the plastids. The maximum rate of formation of porphobilinogen in developing plastids was achieved after 24 hr of illumination.

INTRODUCTION

There are many reasons to believe that the initial step of chlorophyll synthesis, the synthesis of the δ-amino-laevulinic acid (δ-ALA), is a regulatory one and that the enzymes that convert δ-ALA to protochlorophyllide are present in non-limiting activities in higher plants [1, 2]. Glycine and succinyl CoA may be condensed to form α-amino-β-keto-adipic acid followed by decarboxylation to form δ-ALA by an enzyme known as δ-ALA synthetase and this has positively been identified in animal, yeast and bacterial tissues but not in higher plant tissues. One reason for this may be its apparent instability both *in vitro* and *in vivo* [2,3]. This is despite the fact that there is a useful technique of inhibiting δ-ALA dehydratase by laevulinic acid [4,5], which thereby allows an assessment of δ-ALA formation. Succinyl CoA synthetase or thiokinase thought to be responsible for the formation of succinyl CoA was initially reported in crude chloroplasts [6] but later was not found in chloroplasts but in a mitochondrial fraction [7]. It has been suggested that δ-ALA is formed in the mitochondria and then is transported to the chloroplasts for use in chlorophyll synthesis. In contrast, δ-ALA dehydratase has been shown to occur in chloroplasts from *Euglena* and higher plants [6,8–11] and is regarded as a good marker for the intracellular localization of porphyrin biosynthesis because porphobilinogen (PBG) does not cross the plastid envelopes in significant quantities [8]. Surprisingly little work on these early enzymes has been done with greening tissue, plastids especially, where the rate of chlorophyll formation is at its greatest. Etioplasts and developing chloroplasts may well show the presence of enzyme activity which is not detectable after plastids have matured. Furthermore, as a recent review suggests [12], in some of the published work on the early enzymes of chlorophyll biosynthesis, the high concentration of sucrose employed to isolate the plastids is liable to inactivate some of these enzymes before assay and a method using low sucrose should be employed before ruling out the presence of succinyl CoA synthetase in plastids.

RESULTS AND DISCUSSION

Macerates of etiolated or partially illuminated *Avena sativa* (var. Mostyn) laminae or preparations of isolated etio-chloroplasts from similar sources were assayed for succinyl CoA synthetase and δ-ALA dehydratase activity. These results

Table 1. Rates of formation of succinyl CoA, porphobilinogen and chlorophyll in *Avena* laminae

Hours of pre-illumination of seedlings	nmol product formed hr^{-1} $mg\ protein^{-1}$ Succinyl CoA	Porphobilinogen	Chlorophyll
0	1·62	1·46	N.S.
1	2·03	1·67	N.S.
2	2·25	1·79	N.S.
4	2·75	2·87	3·25
8	5·08	4·25	0·84
24	4·87	4·10	0·11
48	11·53	4·26	0·08
72	10·23	4·17	0·08
Light grown	12·44	3·07	N.S.

N.S. no significant rate recorded.

(Tables 1 and 2) clearly show that succinyl CoA synthetase activities as measured in plastids isolated by a low sucrose method are very low as compared to these activities in whole tissue and that the earlier reported absence of this enzyme from mature chloroplasts [7] also holds for greening plastids. δ-ALA dehydratase activity is clearly retained by developing plastids and that the rates for light-grown plants are comparable with earlier figures obtained by a non-aqueous technique [11]. It is also interesting to observe that in the intact tissue only a three-fold increase in activity was observed as greening proceeded but in isolated plastids this increase was more than ten-fold. Differential changes in the basis of expression, soluble protein, may account for this discrepancy or, alternatively, there may be δ-ALA dehydratase activity elsewhere in the cell in addition to that to be found in the plastids. Individual chlorophyll determinations [13] made at each stage of greening indicate that δ-ALA dehydratase activities *in situ* and *in vitro* and succinyl CoA synthetase activities *in situ* are not limiting to the formation of chlorophyll at any stage during greening. At most stages there is clearly an apparent excess of capacity to form succinyl CoA and PBG over that required. This is almost certainly due to the *in vitro* nature of enzyme assays. This cannot be avoided and no doubt the true *in vivo* rates are lower.

In this comparison between the *in situ* and the intra-plastidic activities of these enzymes, it is unfortunate that it is impossible to obtain simultaneous measurements of other sub-cellular fractions with the same degree of accuracy. The reason is that whilst devising a technique to give good intact plastids there is a greater contamination of other fractions with broken plastids. Consequently the site of formation of the succinyl CoA in greening tissue is still unknown and presumed to be of mitochondrial origin by extrapolation of those results for fully greened tissue [7]. Better methods of mitochondrial preparation in low sucrose in

Table 2. Rates of formation of succinyl CoA, porphobilinogen and chlorophyll in etioplasts, etio-chloroplasts and chloroplasts isolated from *Avena*

Hours of pre-illumination of seedlings	nmol product formed hr^{-1} $mg\ protein^{-1}$ Succinyl CoA	Porphobilinogen	Chlorophyll*
0	0·09	2·05	N.S.
1	0·08	2·53	N.S.
2	0·11	4·15	N.S.
4	0·07	9·77	21·3
8	0·09	17·13	6·7
24	0·10	30·56	5·3
48	0·09	27·33	2·2
72	0·09	14·39	0·8
Light grown	0·08	9·71	N.S.

* Accumulated results from other experiments as the chlorophyll levels to be found in the particular preparations employed for enzyme assay were too small for estimation.

N.S. no significant rate recorded.

greening tissue, where the plastids are more similar in size to mitochondria and hence more difficult to separate, have yet to be devised.

It is possible that succinyl CoA could be formed by other enzymes such as α-ketoglutarate dehydrogenase or acyl-CoA transferase although this possibility has never been mentioned previously. This should be investigated in the future but the succinyl CoA synthetase assay measures the formation of succinyl CoA and sufficient endogenous substrates should have been present to indicate if alternative pathways to succinyl CoA synthetase were operative.

A great deal of effort was expended in attempts to determine simultaneous measurements of δ-ALA synthetase activity and δ-ALA formation in intact laminae and isolated plastids, in the presence and absence of laevulinic acid, only to end with inconclusive results. In a preliminary report of work with incubated isolated intact etioplasts we described the incorporation of radioactivity from ^{14}C-labelled δ-ALA succinate and glycine into chlorophyll [14]. However, relatively poor incorporations of label were observed and subsequently this label showed indications after hydrolysis that it was non-specific in its incorporation. Consequently although such preparations were able to form some chlorophyll our other intimation of the involvement of δ-ALA synthetase was incorrect. The nature and localization of δ-ALA synthetase which might link these two other enzymes still remains a mystery.

EXPERIMENTAL

Eleven-day old *Avena sativa* (var. Mostyn) seedlings were grown in the dark or partially illuminated in the later stages of growth in moist peat at 20°. Laminae from each batch were separately homogenized in the recommended media for either succinyl CoA synthetase [7] or the modified form [11] of that described by Steer and Gibbs [15] for δ-ALA dehydratase. The zero-time endogenous levels of succinyl CoA and PBG were determined by these methods and then remaining portions were incubated for 30 min at 30° before redetermination of succinyl CoA and PBG levels. Intact isolated etioplasts or etio-chloroplasts were prepared from the same batches of seedlings by the loosely-packed Sephadex method [16]. Endogenous levels of both succinyl CoA and PBG in sonicated (5 sec at 20 KHz) plastids were determined by the above methods followed by separate incubations for 30 min at 30° in the recommended media before the new levels of succinyl CoA and PBG were determined. The rates of enzyme activity in laminae homogenates and sonicated plastids were calculated by difference using protein as determined by the Folin method [17] as a basis of expression. Chlorophyll was determined at hourly intervals up to 4 hr of illumination by the method of Arnon [13] and thereafter at longer intervals and the differences between the values obtained and those immediately prior, recorrected to a rate expressed on an hourly basis.

Acknowledgements—I am indebted to the S.R.C. for a grant to assist me in this work and to Mrs. Mary West for her technical assistance.

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