HAEM SYNTHESIS BY ISOLATED CHLOROPLASTS

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Received July 14, 1967

It has been found that isolated chloroplasts of Euglena synthesise porphyrins when supplied with δ-aminolaevulinic acid or porphobilinogen (Carell & Kahn, 1964). Such porphyrins may be intermediates in the biosynthesis of chlorophylls, as suggested by mutant studies with Chlorella (Granick, 1948a,b, 1950) or in the biosynthesis of haem proteins.

The present communication reports that isolated spinach chloroplasts, purified by centrifugation on discontinuous gradients, possess the enzyme ferrochelatase: they form haems from protoporphyrin IX, mesoporphyrin IX and deuteroporphyrin IX when incubated with ferrous ions, under reducing conditions.

EXPERIMENTAL

Protoporphyrin IX and deuteroporphyrin IX were prepared as described by Falk (1964). Spinach was purchased in the market or grown in the greenhouse; maize was greenhouse grown. Crude chloroplasts were prepared after briefly homogenising leaves in 350mM NaCl, 100mM Tris, pH 7.0 or in 400mM sucrose, 70mM phosphate, pH 7.3, centrifuging out at 1000g for 12 min. and washing. Chloroplasts stripped of their outer membranes were prepared by centrifuging through glycerol (James & Das, 1957) and intact purified chloroplasts were prepared by centrifuging chloroplasts prepared in sucrose-phosphate through gradients of 32.3% and 38.3% Ficoll dissolved in the buffered sucrose.

RESULTS AND DISCUSSION

When crude chloroplasts were incubated with protoporphyrin and ^{59}Fe under N_2 in the presence of a reducing agent such as dithionite, mercaptoethanol or cysteine, [^{59}Fe]protohaem was formed and was assayed either

by extraction into cyclohexanone at pH 2, a modification of the method of Teale (1959), suggested by Dr. E. Goldwasser (personal communication), or by protohaem crystallisation after the addition of carrier protohaem as haemoglobin, as described by Porra & Jones (1963). This latter method avoids the extraction and counting of any non-haem form of cyclohexanone-soluble [^{59}Fe]-complexes that might be formed during incubation. However, as can be seen in Table 1 similar results are given by each assay system.

Table 1. Formation of [^{59}Fe] protohaem by crude spinach chloroplasts, assayed as cyclohexanone-soluble ^{59}Fe or as crystalline [^{59}Fe] protohaem.

Chloroplasts (1ml suspension, containing about 2 mg./ml. chlorophyll) were incubated in test tubes under N_2 at 25° in 4 ml. of a mixture containing Tris, pH 8.2 (25mM), protoporphyrin in 1% Tween 80 (25μM), [^{59}Fe]Cl_3 (4μM), and mercaptoethanol (25mM). After 10 min. incubation either A. 0.1 ml. N HCl and 4 ml. cyclohexanone were added, shaken, allowed to stand at 0°C for 2 hr., centrifuged and an aliquot of the upper phase taken for radioassay of [^{59}Fe] protohaem, or B. 20 ml. blood were added, the haem crystallised by the procedure of Nishida & Labbe (1957) and an aliquot dissolved in pyridine and assayed for radioactivity and protohaem.

Assay method	[^{59}Fe] Protohaem formed (mumoles/mg. chlorophyll/min.)
A. Cyclohexanone extraction	0.017
B. Protohaem crystallisation	0.015

The cyclohexanone asaay was therefore used to examine the porphyrin specificity of the chlorplast ferrochelatase (Table 2). This table also shows that where chloroplasts or porphyrins were omitted there was a relatively very low uptake of ^{59}Fe into the cyclohexanone phase, confirming that the haem assay method is valid not only for protohaem but also for meso- and deutero-haem. A requirement for a reducing agent in the incubation mixture was found. Simple anaerobic conditions or added glutathione (25mM) or ascorbate (5mM) were inadequate to activate the enzyme, but mercaptoethanol (25mM), cysteine (100mM) or dithionite (3mg./4 ml.) gave good activities.

It is known that in mammals mitochondria are a good source of ferrochelatase (Nishida & Labbe, 1959) and so the chloroplasts were purified by density gradient centrifugation to remove contaminating mitochondria. Microscopic examination confirmed that both the "stripped" and the intact purified chloroplasts were substantially free of mitochondria and bacteria. It was found that the ferrochelatase activity/

Table 2. Porphyrin specificity of spinach chloroplast ferrochelatase.

Incubation conditions were as described in Table 1, except that other porphyrins at 25μM were substituted for protoporphyrin, where shown ^{59}Fe was supplied at 30μM, and chloroplasts were omitted where indicated. The counts detected in 0.1 ml. cyclohexanone extract are given.

Incubation conditions	Radioactivity/ 0.1 ml. cyclohexanone (counts per min.)	Haem formed (mumoles/mg. chlorophyll/min.)
Chloroplasts + mesoporphyrin	5,900	0.376
omit chloroplasts + mesoporphyrin	58	
Chloroplasts + deuteroporphyrin	6,930	0.425
omit chloroplasts + deuteroporphyrin	60.6	
Chloroplasts + protoporphyrin	1,410	0.078
omit chloroplasts + protoporphyrin	58	
Chloroplasts, omit porphyrin	115	
boiled chloroplasts + mesorphyrin	54	

mg. chlorophyll was not significantly reduced by this purification (Table 3) and it seems reasonable to assume that the activity is a property of the chloroplasts themselves.

Table 3. Effect of purification by discontinuous gradient centrifugation upon ferrochelatase activity of spinach chloroplasts.

Intact chloroplasts were prepared in Ficoll; "stripped" chloroplasts prepared in glycerol (see Experimental). Incubation conditions as in Table 1.

Chloroplast preparation	Mesohaem formation (mumoles/mg. chlorophyll/min.)
Crude chloroplasts	0.141
Intact chloroplasts	0.125
"Stripped" chloroplasts	0.11

In addition to the assays described in this paper with intact chloroplasts it has been possible to detect ferrochelatase activity in acetone powders of spinach chloroplasts where the low chlorophyll content of the preparation has permitted the use of the pyridine haemochrome assay of Porra & Jones (1963,a) or a continuous double-beam spectrophotometric assay based upon porphyrin disappearance (Jones, unpublished results).

Chloroplasts prepared from maize were also found to contain ferrochelatase activity, although the activity/mg. chlorophyll was only 20% of that of spinach chloroplasts prepared at the same time

The results presented in this paper show that chloroplasts resemble mitochondria in the capacity to synthesise haem from porphyrins but the purification studies (Table 3) indicate that the activity is due to a true chloroplast enzyme, not to contaminating plant mitochondria. The ferrochelatase activity with different porphyrin substrates resembles that found in pig liver mitochondria, where the presumed natural substrate, protoporphyrin IX, also gives lower rates of haem synthesis than the synthetic porphyrins, mesoporphyrin IX and deuteroporphyrin IX. It is possible that if presented with a suitable magnesium complex the ferrochelatase preparation described would also form the magnesium porphyrins intermediate in chlorophyll synthesis, but attempts at Mg^{2+} incorporation have so far been unsuccessful and it is likely that the natural role of the chloroplast enzyme is the synthesis of cytochrome prosthetic groups. Its general similarity in properties to the pig liver mitochondrial enzyme (Porra & Jones, 1963a,b) supports this view. This capacity for haem synthesis further illustrates the autonomy of the chloroplast within the plant cell.

ACKNOWLEDGEMENTS

The author is grateful to Mr. K. R. E. Williams for his assistance. This work was supported by a Research Grant from the Science Research Council.

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