Ferrochelatase activity in the photosynthetic alga *Cyanidium caldarium*

Development of the enzyme during biosynthesis of photosynthetic pigments

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Dark-grown cells of the photosynthetic alga *Cyanidium caldarium* were shown to contain ferrochelatase activity, which increased markedly when the cells were induced to form pigments by exposure to light. K_m values for the crude enzyme preparation were $14.8\mu\text{M}$ and $6.5\mu\text{M}$ for binding of Co^{2+} and deuteroporphyrin IX respectively.

The biosynthesis of haem in animal cells has been extensively studied and reviewed (O'Carra, 1975; Schmid & McDonagh, 1979; Brown & Troxler, 1982). In such cells, haem represents the functional end product of metabolism. Cleavage of the macrocyclic porphyrin ring by haem oxygenase produces the bile pigments biliverdin and bilirubin, which have no function other than providing a pathway for removal of unwanted haem. In photosynthetic algae and higher plants, however, bilins very similar in structure to the mammalian bile pigments play essential roles in photosynthesis and in photomorphogenesis. Recent experiments with the red alga *Cyanidium caldarium* have shown that the biosynthesis of phycocyanobilin, the bilin chromophore of the photosynthetic antenna protein phycocyanin, proceeds via synthesis and subsequent degradation of haem (Troxler *et al.*, 1978; Brown *et al.*, 1980, 1981). *C. caldarium* is a convenient organism for study of phycobiliprotein synthesis, since it may be cultured heterotrophically in the dark, where it grows without photosynthetic pigments. Such dark-grown cells may then be resuspended in minimal medium and illuminated. After an initial lag phase of several hours, rapid greening occurs, owing to formation of phycocyanin and chlorophyll *a* (Troxler, 1972).

Since phycocyanin is an abundant protein in these cells, and since it may be synthesized relatively quickly, rapid formation and degradation of haem must occur. This implies that the enzymes for formation of haem (ferrochelatase, protohaem ferro-lyase, EC 4.99.1.1) and degradation of haem

[haem oxygenase (decyclizing), EC 1.14.99.3] must be active in greening *C. caldarium* cells. Neither of these enzymes has yet been directly demonstrated in organisms synthesizing phycobiliproteins, although indirect evidence for ferrochelatase was obtained when it was shown that *N*-methylprotoporphyrin IX, a potential inhibitor of the enzyme, inhibited phycocyanin formation in *C. caldarium* (Brown *et al.*, 1982; Beale & Chen, 1983).

In the present paper we report direct measurements of ferrochelatase activity in *C. caldarium* and its variation with the pigment status of the cells.

Experimental

Culture of *C. caldarium*

The details of the growth medium and other culture conditions for *C. caldarium* have been described previously (Brown *et al.*, 1981). Dark-grown cells were collected by centrifugation (5000*g* for 15 min) and washed twice with water. For preparation of fully greened cells, dark-grown cells were resuspended in minimal medium (pH 1.7, 20× packed cell volume; Brown *et al.*, 1981) and incubated at 37°C for approx. 80 h. Illumination was provided by two 40 W fluorescent tubes (approx. 5400 lx). For experiments in which the progress of greening and enzyme activity was monitored, dark-grown cells (16 ml packed cell volume) were resuspended in 320 ml of minimal medium (pH 1.7), divided equally between four 250 ml conical flasks and incubated at 37°C. Illumination was carried out as described above. Portions (5 ml) were removed from each culture at appropriate intervals and pooled to give a 20 ml

Abbreviation used: Mops, 4-morpholine-propanesulphonic acid.

combined sample. From each combined sample, a small portion of suspension (100 μ l) was removed and diluted with 1.5 ml of water for spectral measurement, whereas the bulk of each sample was retained for cell disruption and measurement of enzyme activity.

After illumination, cells were collected by centrifugation (5000 *g* for 15 min). The cell pellet was resuspended in 20 mM-Mops/100 mM-KCl buffer, pH 7 (approx. 3 ml of buffer per *g* wet weight of cells), and the cells were disrupted by using a French pressure cell (Aminco American Instrument Co., Silver Springs, MD, U.S.A.) at 138 MPa (20000 lb/in²) internal pressure. The homogenate was centrifuged (2000 *g* for 5 min) and the supernatant was used immediately or stored at -40°C until required.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Spectral measurements

The progress of phycocyanin synthesis was monitored spectrophotometrically at 620 nm by using portions of each sample as described above. Measurements were made on a Pye-Unicam SP. 8-100 spectrophotometer, cells without pigment being used in the reference cuvette to correct for turbidity as described previously (Brown *et al.*, 1981).

Ferrochelatase assay

The enzyme was assayed at 30°C by the dual-wavelength spectroscopic method of Jones & Jones (1969, 1970), with deuteroporphyrin (25 μM) and Co^{2+} (40 μM) as substrates.

Results and discussion

When samples from either dark-grown or fully greened cells were assayed for ferrochelatase with Co^{2+} and deuteroporphyrin IX as substrates, substantial activity was immediately apparent. The observed activity of pigmented cells was greater than that of unpigmented cells for the same wet weight of cells. Activity was abolished on addition of *N*-methylprotoporphyrin IX (1 μM), a specific inhibitor of ferrochelatase (De Matteis *et al.*, 1980; Tephly *et al.*, 1981). Further experiments were therefore carried out to obtain a preliminary kinetic characterization of the enzyme and also to assess its development during cell pigmentation.

Stability and specific activity of the enzyme

Various volumes of a fresh crude cell extract (protein content approx. 30 mg/ml) were assayed for ferrochelatase activity immediately after preparation. Reaction rates were linear at least up to a

protein concentration (in the assay mixture) of 1.5 mg/ml.

Values for the specific activity of ferrochelatase in such a freshly prepared cell extract and after storage for 24 h at 2°C , for 24 h at -40°C and for 5 days at -40°C were determined with respect to Co^{2+} and deuteroporphyrin IX as substrates. The results showed that the enzyme activity is not grossly affected by storage at -40°C or by freezing and thawing. The data also showed that the enzyme would be expected to be stable while stored on ice during kinetic and other studies.

In common with findings for ferrochelatase from other organisms (Jones, 1983), protoporphyrin IX (specific activity determined to be approx. 0.015 nmol/min per mg) was a much poorer substrate *in vitro* than deuteroporphyrin IX.

Kinetic studies

Reciprocal plots for the variation in reaction velocity with Co^{2+} concentration and deuteroporphyrin concentration showed that the data are consistent with simple Michaelis-Menten kinetics for each substrate, the values of K_m being 6.5 μM for deuteroporphyrin IX and 14.8 μM for Co^{2+} .

Development of ferrochelatase during greening

The progress curve for biosynthesis of phycocyanin during illumination of dark-grown *C. caldarium* cells is shown in Fig. 1. After an initial lag period of 5–6 h, rapid pigment synthesis ensued, in agreement with previous studies (Brown *et al.*, 1981). Measurements at 675 nm showed that chlorophyll synthesis occurred in parallel with phycocyanin synthesis, i.e. the ratio A_{675}/A_{620} remained constant with time, also in agreement with previous findings (Brown *et al.*, 1982).

Measurements of ferrochelatase activity during pigmentation are also shown in Fig. 1. The activity rapidly increased on exposure of cells to light until a maximal level was approached after about 70 h. Clearly, therefore, ferrochelatase specific activity increased substantially during the pigmentation process. Equally significant, however, is the fact that the ferrochelatase activity appeared to increase rapidly initially, i.e. without an apparent lag phase, so that the enzyme activity increased in advance of pigmentation.

General discussion

The observation of ferrochelatase activity in *C. caldarium* represents the first direct demonstration of the enzyme in organisms synthesizing phycobiliproteins via haem. As might be expected, the order of magnitude of specific activity found is much higher than that of green plant cells (typically 0.08 nmol/min per mg of protein; Jones, 1983), where only small amounts of haem are required for

Fig. 1. Development of ferrochelataase activity and phycocyanin during pigmentation of *C. caldarium*

Dark-grown cells of *C. caldarium* were illuminated at 37°C as described in the text. After appropriate time intervals, samples were removed and measurements of enzyme activity and phycocyanin (A_{620}) were made. Further details are given in the text. ○, Phycocyanin synthesis; ●, ferrochelataase activity.

synthesis of cytochromes and other haemoproteins. The K_m values for the enzyme reported here for the crude homogenate are somewhat lower than those for the pure mammalian enzyme (Dailey & Fleming, 1983), but resemble those for the enzyme obtained from green plants (Jones, 1983).

The finding that ferrochelataase levels in *C. caldarium* increase substantially during pigmentation of dark-grown cells might be expected on the basis that the latter have no large-scale requirement for haem production. It also seems logical that the increase in ferrochelataase should precede pigmentation, as found in the present work, since formation of phycocyanin depends upon the prior

synthesis of ferrochelataase. It is not clear from the present work, however, whether the increase of the enzyme activity is due to a specific induction of ferrochelataase or whether it is solely a reflection of the generalized increased increase in plastid membrane formation during the adaptation of *C. caldarium* cells to light.

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