Biosynthesis of phycobiliproteins

Incorporation of biliverdin into phycocyanin of the red alga *Cyanidium caldarium*

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¹⁴C-labelled biliverdin IX α was administered to cultures of *Cyanidium caldarium* that were actively synthesizing photosynthetic pigments in the light. Between 9 and 12% of the phycobiliprotein chromophore produced in such cultures was derived from exogenous biliverdin. These results demonstrate that biliverdin is an intermediate in the biosynthesis of phycobiliproteins.

Phycocyanin and phycoerythrin are phycobiliproteins that function as photosynthetic antennae pigments in various types of algae (O'Carra & O'hEocha, 1977). Phytochrome, a related phycobiliprotein, plays an important role in photomorphogenesis in higher plants (Pratt, 1978). The chromophores of these proteins are bilins, which are closely related in structure to each other and to the mammalian blue pigments biliverdin and bilirubin. The biosynthesis of the phycobiliproteins is of interest both in terms of the metabolic pathway for formation of the chromophores and in terms of the linkage of chromophore to apoprotein.

In recent studies, we have focused attention on the biosynthesis of phycocyanobilin (Scheme 1), the chromophore obtained from phycocyanin, in the rhodophyte *Cyanidium caldarium* (Brown *et al.*, 1981). Dark-grown cells of this organism contain no photosynthetic pigments, but they may be readily induced to synthesize phycocyanin, allophycocyanin and chlorophyll *a* when they are resuspended in minimal medium and exposed to light (Bogorad *et al.*, 1963). By using this method for induction of pigment, it was shown that protohaem is a biosynthetic precursor of phycocyanin in *C. caldarium* (Brown *et al.*, 1981). The formation of the phycocyanin chromophore from haem requires two reduction steps in addition to the haem-cleavage step (see Scheme 1). If haem is cleaved directly to bile pigment before reduction, then biliverdin would be an intermediate in phycocyanin synthesis. If, however, one or both of the reduction steps occurs before haem cleavage, then biliverdin would not be an intermediate.

Previous experiments have shown that at least one of the possible haem-reduction products (mesohaem) is not an intermediate (Brown & Holroyd, 1984). We now report studies that directly assess the role of biliverdin as an intermediate by administration of exogenous biliverdin to cells of *C. caldarium*.

Experimental

Materials

All materials obtained commercially were of analytical-reagent grade where available, and solvents were redistilled before use. 5-Amino-[4-¹⁴C]laevulinic acid hydrochloride was obtained either from Amersham International, Amersham, Bucks., U.K. (sp. radioactivity approx. 40 mCi/mmol) or from New England Nuclear, Boston, MA, U.S.A. (sp. radioactivity approx. 50 mCi/mmol).

Unlabelled biliverdin IX α was prepared by coupled oxidation of pyridine haemochrome (O'Carra, 1975). This method gives a mixture of all four structural isomers (α , β , γ and δ). The biliverdins were methylated and the mixture was resolved by preparative t.l.c. (silica-gel G; heptane/butanone/acetic acid, 10:5:1, by vol.; O'Carra, 1975).

¹⁴C-labelled biliverdin IX α could be obtained in a similar way by using [¹⁴C]haem prepared as previously described (Brown *et al.* 1981). However, the following procedure was found to give a better yield. A high reticulocyte count was induced in a male New Zealand White rabbit by five consecutive daily injections (subcutaneous) of a 2.5% (w/v) solution of acetylphenylhydrazine (Craig *et al.*, 1976). The rabbit was rested for 2 days and then

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

Scheme 1. Possible pathways in the biosynthesis of phycocyanobilin from haem

Key to substituents: M, $-\text{CH}_3$; V, $-\text{CH}=\text{CH}_2$; P, $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$; E, $-\text{CH}_2\text{CH}_3$; A, $=\text{CH}-\text{CH}_3$.

bled by cardiac puncture under phenobarbitone anaesthesia. 5-Amino[4- ^{14}C]laevulinic acid was incubated with approx. 100ml of whole blood (1 $\mu\text{Ci}/\text{ml}$ of blood) at 37°C for 24 h as previously described (Brown *et al.*, 1981). Red blood cells were collected by centrifugation (bench centrifuge, 10 min), and washed with 0.9% (w/v) NaCl solution. Cell lysis was achieved by suspension in water (volume equal to the original blood volume) for 10 min and a cell-free haemolysate was obtained by centrifugation (bench centrifuge, 10 min). Biliverdin was obtained by direct coupled oxidation without intermediate isolation of haem by addition of the haemolysate to 3 litres of 0.1 M-sodium phosphate buffer, pH 7.4, containing 30 g of sodium ascorbate and incubating the mixture at 37°C for 2 h. After addition of 900 ml of acetic acid and 2.5 litres of 5 M-HCl, the mixture was cooled at 4°C for 30 min. The biliverdins were extracted, methylated and purified by t.l.c. as described above. This procedure gave only biliverdins IX α and IX β , in the approximate ratio 2:1, and yielded 6 mg of biliverdin IX α dimethyl ester from 100 ml of rabbit blood.

Immediately before use in biosynthetic studies,

biliverdin IX α free acid was prepared from the dimethyl ester by alkaline hydrolysis as follows. A 3 mg portion of biliverdin IX α dimethyl ester was dissolved in 30 ml of methanol/1.0 M-NaOH (:1, v/v) and the hydrolysis was allowed to proceed at 37°C in the dark under N_2 for 30 min. Acetic acid (1.2 ml) was added and the pigment was extracted with chloroform (2 $\times$ 25 ml). The chloroform extract was washed with water (50 ml) and the biliverdin free acid was obtained by removal of solvent on a rotary evaporator. When applied to the t.l.c. system used for separation of biliverdin dimethyl ester isomers (O'Carra, 1975), the preparation of free acid remained at the origin and no trace of dimethyl ester was observed, confirming complete hydrolysis. When a sample of the free acid preparation was re-methylated and applied to the same t.l.c. system, a single band corresponding to biliverdin IX α dimethyl ester was observed, indicating that hydrolysis had not caused additional chemical modification of the pigment.

Culture conditions

The culture of *C. caldarium* mutant III-D-2 and the induction of pigment synthesis in dark-grown

cells were as previously described (Brown *et al.*, 1981). In earlier studies, uptake of protohaem and mesohaem into *C. caldarium* cells occurred rapidly when the haem was incubated with cells in the dark at pH 8 (Brown *et al.*, 1981; Brown & Holroyd, 1984). However, when biliverdin was incubated with *C. caldarium* under identical conditions, no uptake of pigment was observed either in terms of pigmentation of cells or in terms of loss of pigment from the medium. In order to ensure maximum exposure of exogenous biliverdin to greening cells, therefore, the pigment was dissolved directly in the culture medium. In some experiments, cultures were illuminated in the usual medium at approx. pH 2 (Brown *et al.*, 1981), but other experiments were performed at pH 6, when the cells appeared to form pigments equally well. Biliverdin was added to the cultures in the light when greening became just detectable visually.

Purification of pigments

After the biosynthesis of phycobiliproteins, the chromophore was isolated and purified by using one of two procedures. In Procedure 1, phycocyanobilin was cleaved from an unpurified trichloroacetic acid precipitate, methylated and purified by preparative t.l.c. (silica-gel G; chloroform/methyl acetate, 2:1, v/v) as previously described (Troxler *et al.*, 1979). The phycocyanobilin dimethyl ester was then further purified by a second preparative t.l.c. system, with silica-gel G and elution with chloroform/ethyl acetate/cyclohexane (32:9:4, by vol.). Three developments were usually carried out to ensure good separation of pigments. In Procedure 2, phycocyanin was purified before cleavage of chromophore from apoprotein by chromatography on a hydroxyapatite column (Brown *et al.*, 1975). Fractions containing phycocyanin were pooled, concentrated by ultrafiltration, and the pigment was further purified by chromatography

on a column of Sephadex G-200, eluted with 10 mM-potassium phosphate buffer, pH 7.0. For preparation of phycocyanobilin dimethyl ester from this purified phycocyanin, the protein was precipitated with trichloroacetic acid and the precipitated material was treated as previously described (Troxler *et al.*, 1979), which involves purification of phycocyanobilin dimethyl ester by preparative t.l.c. (silica-gel G; chloroform/methyl acetate, 2:1, v/v). This pigment was not subjected to further purification.

Phycocyanobilin dimethyl ester was determined spectrophotometrically ($\epsilon_{369} = 54.2 \times 10^3$ litre·mol⁻¹·cm⁻¹) and assayed for radioactivity by the procedures used in previous work (Brown *et al.*, 1981).

H.p.l.c.

H.p.l.c. was performed on a Bio-Rad liquid chromatograph; a reversed-phase column, eluted with methanol/water (39:11, v/v; Schoch *et al.*, 1978) was used, with a flow rate of 0.6 ml/min. Pigments were detected at 370 nm. Samples of pure phycocyanobilin dimethyl ester and biliverdin IX α dimethyl ester were run separately and gave well-resolved peaks with distinct retention times (19.9 min and 16.7 min respectively). Furthermore, synthetic mixtures of the two pigments were readily resolved in this system.

Results and discussion

Two experiments were carried out with ¹⁴C-labelled biliverdin IX α . In the first (Expt. 1, Table 1), the cells were illuminated in pH 2 medium and the pigment was purified by Procedure 1 described above (i.e., without previous purification of phycocyanin). Significant radioactivity was found in the phycocyanobilin dimethyl ester, indicating incorporation of label from biliverdin. From the known

Table 1. Incubation of ¹⁴C-labelled biliverdin with *C. caldarium* in the light

Dark-grown cells of *C. caldarium* were incubated in the light at pH 2 as described previously (Brown *et al.*, 1981). When greening became just evident visually, either ¹⁴C-labelled biliverdin was added directly (Expt. 1) or the cells were resuspended in medium at pH 6 and ¹⁴C-labelled biliverdin was added. In both cases, incubation was continued in the light until greening was complete. For Expt. 1, phycocyanobilin dimethyl ester was prepared as described in Table 1, without previous purification of phycocyanin and purified via two t.l.c. steps (see the Experimental section). For Expt. 2, phycocyanin was first purified, and phycocyanobilin dimethyl ester was obtained from purified protein as described in the Experimental section. Specific radioactivities were determined by spectrophotometric measurements ($\epsilon_{369} = 54.2 \times 10^3$ litre·mol⁻¹·cm⁻¹) and by liquid-scintillation counting as previously described (Brown *et al.*, 1981).

Expt.	Culture vol. (ml)	pH	[¹⁴ C]Biliverdin added (mg)	10 ⁻¹⁰ × Sp. radioactivity of added biliverdin (d.p.m./mol)	10 ⁻¹⁰ × Sp. radioactivity of phycocyanobilin dimethyl ester (d.p.m./mol)	Fraction of phycocyanobilin from added biliverdin (%)
1	100	2	3.0	11.5	1.46	12.7
2	200	6	6.0	2.16	0.20	9.4

specific radioactivity of the biliverdin used, these data suggested that 12.7% of the phycocyanobilin formed was derived from biliverdin. In the second experiment (Expt. 2, Table 1), the cells were incubated at pH 6. From this and other experiments without use of radiolabel it was clear that pigmentation occurred equally well at pH 6 as in the more usual medium at pH 2. The phycocyanin from this experiment was purified as described in the Experimental section and phycocyanobilin was obtained by cleavage from apoprotein and t.l.c. as described for Procedure 2. Significant radioactivity was also found in this sample, indicating incorporation of biliverdin corresponding to 9.4% of the phycocyanobilin formed.

A major problem in assessing the possible role of biliverdin in phycocyanin biosynthesis is the difficulty in distinguishing between genuine incorporation and contamination of the product phycocyanobilin by biliverdin carried over during purification procedures. The ability of the t.l.c. system usually used for phycocyanobilin dimethyl ester (silica-gel G; chloroform/methyl acetate, 2:1, v/v; Troxler *et al.*, 1979) to discriminate between the two pigments was therefore tested, by using a synthetic mixture of biliverdin IX α dimethyl ester and phycocyanobilin dimethyl ester. These experiments clearly showed that biliverdin could not adequately be distinguished from phycocyanobilin by using this t.l.c. system, since both pigments had the same R_f value when run separately and the synthetic mixture was not resolved. However, in the second preparative t.l.c. system used (silica-gel G; chloroform/ethyl acetate/cyclohexane, 32:9:4, by vol.), biliverdin dimethyl ester and phycocyanobilin dimethyl ester had different R_f values, and it would be expected that any biliverdin contamination would be removed at this stage. This was confirmed by applying a sample of phycocyanobilin dimethyl ester prepared by Procedure 1 to a small-scale analytical t.l.c. system (silica-gel G; heptane/butanone/acetic acid, 10:5:1 by vol.; O'Carra, 1975), which also readily separates phycocyanobilin dimethyl ester and biliverdin IX α dimethyl ester. No biliverdin IX α was detected. By using this method, it was estimated that a biliverdin impurity of 2–3% in phycocyanobilin would be detectable. These experiments show that the results obtained with Procedure 1 (Expt. 1, Table 1) cannot be explained on the basis of biliverdin contamination of phycocyanobilin dimethyl ester.

The electronic spectrum of the purified protein prepared in Expt. 2 was identical with that of a sample prepared from cells not incubated with biliverdin. Spectra of synthetic mixtures of pure phycocyanin and biliverdin IX α showed clear evidence of the presence of biliverdin at biliverdin

levels of less than 5% (mol of biliverdin/mol of phycocyanin chromophore). Clearly, therefore, the phycocyanin sample obtained in Expt. 2 contained no biliverdin in excess of this level and the radioactivity observed must be due to genuine incorporation rather than contamination by biliverdin.

Finally, a sample of phycocyanobilin dimethyl ester prepared by Procedure 2 from cells that had been incubated with biliverdin was subjected to h.p.l.c. as described in the Experimental section. This procedure showed the phycocyanobilin dimethyl ester to be free from biliverdin under conditions where less than 1% of biliverdin impurity would have been detected.

The present results show that exogenous biliverdin is incorporated into the phycocyanobilin chromophore of the phycocyanin of *C. caldarium*. Comparison between Expts. 1 and 2 shows that the extent of incorporation does not depend appreciably upon the pH of the culture medium. It is unlikely, therefore, that the observed incorporation could be due to any chemical modification of biliverdin occurring during the prolonged exposure to acid medium at 37°C.

The incorporation of biliverdin in the present work (approx. 9–12% of the phycocyanobilin formed) was somewhat lower than that previously observed for haem incorporation (Brown *et al.*, 1981; Schuster *et al.*, 1983). In those studies, however, cells rapidly accumulated the administered pigment, whereas in the present studies there was no evidence that cells could accumulate biliverdin. It appears, therefore, that the relatively low incorporation of biliverdin into phycocyanobilin is probably due to poor cellular uptake and hence the inability of the exogenous pigment to compete strongly with the endogenous pathway.

From the present results and those of other recent studies (Köst & Benedict, 1982; Beale & Cornejo, 1983; Brown & Holroyd, 1984), it is now clear that biosynthesis of the chromophore of phycocyanin proceeds via formation of haem and biliverdin in exactly the same way as formation of mammalian bile pigments (Schmid & McDonagh, 1975). The reduction steps necessary for phycocyanobilin formation must therefore occur subsequent to biliverdin formation. Although there is little experimental evidence, it is likely that biosynthesis of the chromophores of phycoerythrin and phytochrome proceed by an identical route so far as biliverdin. Only the final steps in the biosynthesis of the phycobiliproteins now remain to be elucidated.

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