The biosynthesis of the chromophore of phycocyanin

Pathway of reduction of biliverdin to phycocyanobilin

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The later stages in the pathway of biosynthesis of phycocyanobilin, the chromophore of phycocyanin, were studied by using radiolabelled intermediates. Three possible pathways from biliverdin IX- α to phycocyanobilin were considered. ^{14}C -labelled samples of key intermediates in two of the pathways, 3-vinyl-18-ethyl biliverdin IX- α and 3-ethyl-18-vinyl biliverdin IX- α , were synthesized chemically and were administered to cultures of *Cyanidium caldarium* that were actively synthesizing photosynthetic pigments in the light. Neither of these two compounds was apparently incorporated into the phycobiliprotein chromophore, suggesting that two of the three pathways were not operative. By elimination, the results imply that the third possible pathway, which involves phytychromobilin, the chromophore of phytyochrome, represents the route for biosynthesis of phycocyanobilin. Unfortunately, since ^{14}C -labelled phytychromobilin is not available, no direct proof of this pathway could be obtained. However, if correct, the present interpretation represents a unified pathway for biosynthesis of all plant bilins, via the intermediacy of phytychromobilin.

INTRODUCTION

The linear tetrapyrrole biliverdin is formed by macrocyclic ring cleavage of the haem of haemoglobin and cytochrome *P*-450 during the normal turnover of these proteins (Schmid & McDonagh, 1975; Brown & Troxler, 1982). In mammals this reaction represents a step in the catabolic elimination of unwanted haem and has no biosynthetic significance. However, in plants and cyanobacteria linear tetrapyrroles have important and purposeful functions, since they form the chromophore of the photosynthetic accessory pigments phycocyanin and phycoerythrin in certain algae and the chromophore of phytyochrome in higher plants. The structures of these compounds and their close relationship to that of biliverdin are shown in Fig. 1.

In recent years a number of different studies, using ^{14}C -labelling and ^{18}O -labelling, have shown that the biosynthesis of these plant tetrapyrroles occurs via the intermediacy of haem. Protoporphyrin IX is converted into haem, which is then broken down to biliverdin by exactly the same process as that used by mammals for haem catabolism (Troxler *et al.*, 1979; Brown *et al.*, 1980, 1981, 1982). For the formation of phycocyanin in the rhodophyte *Cyanidium caldarium*, studies employing the incorporation of ^{14}C -labelled materials (Brown *et al.*, 1984) and isolation of intermediates (Köst & Benedikt, 1982; Beale & Cornejo, 1983) clearly demonstrated the intermediacy of biliverdin in the biosynthetic pathway. However, the biosynthetic route from biliverdin to phycocyanobilin, the chromophore of phycocyanin, has not yet been determined. Overall, two reduction steps are necessary, at ring A and at the vinyl group of ring D of biliverdin, along with an isomerization of the vinyl group on ring A to ethylidene, as evident from Fig. 1. The isomerization step may occur as a thermodynamically

necessary consequence of ring A reduction and may not necessarily be under enzyme control (Simpson & Smith, 1988).

Three separate pathways (with minor variations) are possible, as shown in Scheme 1. These are reduction of ring A first, followed by isomerization and reduction of the ring D vinyl group (pathway I), or reduction of the ring A vinyl group first and the subsequent isomerization of a ring A double bond to the ethylidene group, followed by reduction of the vinyl group of ring D (pathway II), or reduction of the ring D vinyl group first, followed by reduction at ring A and isomerization (pathway III). The variation of pathway III involving mesobiliverdin (Scheme 1) has previously been shown not to occur, since administered mesobiliverdin is not incorporated into phycocyanobilin (Brown & Holroyd, 1984).

In the present work, we have attempted to distinguish between these various possibilities by the synthesis of ^{14}C -labelled compounds and their administration to the alga *C. caldarium*. The synthesis of these compounds presents a difficult chemical problem, but the preparation of small quantities of two of the key intermediates proposed in Scheme 1 has been described (Smith & Pandey, 1984) and we have adapted this method to prepare the ^{14}C -labelled derivatives. These are the 3-ethyl-18-vinyl biliverdin intermediate formed in pathway II and the corresponding 3-vinyl-18-ethyl intermediate formed in pathway III (see Scheme 1). We have used the same general procedures as in previous work, where administration of ^{14}C -labelled biliverdin to *C. caldarium* resulted in significant incorporation of radiolabel into phycocyanin (Brown *et al.*, 1984). Unfortunately, chemical synthesis of the two intermediates in pathway I, including phytychromobilin free acid, has not proved feasible in spite of considerable effort. Small quantities of phytychromobilin dimethyl ester have been synthesized

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Fig. 1. Structures of phycobilins and their relationship to biliverdin

Key to substituents: -Me, -CH₃; -P, -CH₂CH₂CO₂H.

Scheme 1. Possible pathways for conversion of biliverdin into phycocyanobilin

The asterisk (*) indicates ¹⁴C labelled compounds. —X— indicates pathway excluded by a previous study (Brown & Holroyd, 1984). Key to substituents: -Me, -CH₃; -P, -CH₂CH₂CO₂H.

(Weller & Gossauer, 1980) but this material cannot be hydrolysed to the free acid without rearrangement, and the dimethyl ester would not be appropriate for biosynthetic work. It is not possible at present, therefore, to test pathway I directly.

EXPERIMENTAL

Materials

The monoethylmonovinyl biliverdins (Scheme 1) have previously been prepared in unlabelled form (Smith & Pandey, 1984), from di-butyl *b*-bilene dicarboxylates.

¹⁴C-labelled monoethylmonovinyl biliverdins were prepared in an identical fashion, except that the Vilsmier formulation of pyrromethanes to give 5-formylpyrromethanes was carried out with [¹⁴C]dimethylformamide (Amersham International) and phosphoryl chloride. The resulting ¹⁴C-labelled monoethylmonovinyl biliverdins therefore were ultimately isotopically labelled at the central methene carbon atom.

Culture conditions

C. caldarium is a very convenient organism for study of phycobilin biosynthesis, since it may be grown

heterotrophically in the dark without formation of photosynthetic pigments. Such cultures may then be induced to synthesize chlorophyll *a* and phycocyanin by exposure to light.

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). Dark-grown cells (5 ml packed cell volume) were washed twice with water and resuspended in 100 ml of medium at pH 1.7. The cell suspension was then exposed to light at approx. 5400 lx at 37 °C. When greening became just evident visually, the cells were resuspended in medium at pH 6 (see Brown *et al.*, 1984) and the radiolabelled intermediate was added (1.1 mg in the case of the 3-ethyl-18-vinyl derivative and 1.5 mg in the case of the 3-vinyl-18-ethyl derivative). In each case incubation was continued in the light until greening was complete (Brown *et al.*, 1984).

Purification of pigments

After the biosynthesis of phycobiliprotein, the chromophore was isolated and purified by using one of two procedures. In procedure 1, phycocyanobilin was cleaved from an unpurified trichloroacetic acid precipitate of phycocyanin and the pigment was 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 200 mm × 100 mm precoated silica-gel G plates (supplied by BDH Chemicals, Poole, Dorset, U.K.) 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 first purified by chromatography on a hydroxyapatite column (Brown *et al.*, 1975). Fractions containing phycocyanin were pooled, then 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.

For chlorophyll isolation, the pellet obtained from centrifugation after cell disruption (see above) was treated with 6 vol. of acetone/water (5:1, v/v). After centrifugation (bench centrifuge) for 10 min the green supernatant was retained and a second identical extraction of the pellet was carried out. The supernatants were pooled and to the combined extract saturated NaCl (0.25 vol.), water (0.25 vol.) and diethyl ether (1 vol.) were added. After shaking, the diethyl ether extract (greenish yellow) was removed and the aqueous layer was extracted three times with diethyl ether (1 vol.). The combined diethyl ether layers were concentrated in a stream of N₂, when a water layer again separated. The dense-green diethyl ether layer was removed by using a Pasteur pipette, evaporated to dryness under a stream of N₂ and assayed for radioactivity.

Radioactivity assays

Phycocyanobilin dimethyl ester was determined spectrophotometrically ($\epsilon_{369} = 54.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and assayed for radioactivity by the procedures used in previous work (Brown *et al.*, 1981). The radioactivity of chlorophyll was similarly determined by using ϵ_{664} (diethyl ether) = $65 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. In view of the importance of obtaining precise specific radioactivities, the counting efficiency of each sample was determined individually by adding a known volume of n-[¹⁴C]hexadecane (sp. radioactivity $0.868 \times 10^6 \text{ d.p.m./ml}$) and re-counting.

RESULTS

Specific radioactivity of reduced biliverdins

A small sample of the 3-vinyl-18-ethyl biliverdin was dissolved in 1.75 ml of chloroform and its absorbance at 370 nm was measured. Samples (1.0 ml and 0.5 ml) were then assayed for radioactivity. By using a value of $\epsilon_{374} = 48.4 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Stoll & Gray, 1977), the mean specific radioactivity of the two samples was $6.3 \times 10^{12} \pm 0.1 \times 10^{12} \text{ d.p.m./mol}$. The mean specific radioactivity of two samples of the 3-ethyl-18-vinyl isomer was similarly determined as $1.07 \times 10^{11} \pm 0.02 \times 10^{11} \text{ d.p.m./mol}$ ($\epsilon_{376} = 57.9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Stoll & Gray, 1977).

Owing to the very small quantities of ¹⁴C-labelled materials available, only one experiment with each isomer was possible.

Administration of the ¹⁴C-labelled 3-vinyl-18-ethyl isomer

For the experiment in which ¹⁴C-labelled 3-vinyl-18-ethyl isomer was administered, sufficient material was obtained such that phycocyanin and phycocyanobilin dimethyl ester could be purified by procedure 2 (see the Experimental section).

Results of the radioactivity assay of phycocyanin and phycocyanobilin following administration of this isomer are shown in Table 1, which reveals that for the purified phycocyanin sample measurable radioactivity was obtained, leading to an apparent incorporation of 0.36% (based on the known specific radioactivity of the labelled 3-vinyl-18-ethyl biliverdin). However, when phycocyanobilin was obtained from the purified phycocyanin sample, it revealed much less radioactivity, corresponding to an apparent incorporation of only 0.044%.

In this experiment, chlorophyll was also isolated as described in the Experimental section. The sample revealed only very small radioactivity, corresponding to an apparent incorporation of 0.041%, almost identical with that of the phycocyanobilin.

Administration of the ¹⁴C-labelled 3-ethyl-18-vinyl isomer

Because of the lower specific radioactivity of this isomer, it was possible to assay only a small quantity (1.1 nmol) of phycocyanin, and it was necessary to prepare phycocyanobilin dimethyl ester via procedure 1, to ensure maximum yield for radioactivity counting. The results are shown in Table 2, which reveal only a very low amount of radioactivity in phycocyanobilin dimethyl ester and no radioactivity counts above background in phycocyanin.

Table 1. Administration of ^{14}C -labelled 3-ethyl-18-vinyl biliverdin

For experimental details see the text. Radioactivities of duplicate samples of each compound were counted and gave consistent results within experimental error.

Samples	Radioactivity measured (d.p.m.) (after background correction)	Quantity of sample whose radioactivity was counted (nmol)	$10^{-10} \times$ Specific radioactivity (d.p.m./mol)	Apparent incorporation (%)
Phycocyanin	566	24.8	2.29	0.36
Phycocyanobilin dimethyl ester	134	47.9	0.28	0.044
Chlorophyll <i>a</i>	37	14.3	0.26	0.041

Table 2. Administration of ^{14}C -labelled-3-vinyl-18-ethyl biliverdin

For experimental details see the text. Radioactivities of duplicate samples of each compound were counted and gave consistent results within experimental error.

Samples	Radioactivity measured (d.p.m.) (after background correction)	Quantity of sample whose radioactivity was counted (nmol)	$10^{-10} \times$ Specific radioactivity (d.p.m./mol)	Apparent incorporation (%)
Phycocyanin	0	1.1	0	0
Phycocyanobilin dimethyl ester	13	92.7	0.014	0.13

DISCUSSION

For the administration of ^{14}C -labelled 3-vinyl-18-ethyl biliverdin (Table 1), there was a small, but significant, incorporation of radioactivity into the purified phycocyanin, corresponding to 2.29×10^{10} d.p.m./mol of chromophore. However, when the chromophore was cleaved from apoprotein, purified by t.l.c. and assayed for radioactivity, the specific radioactivity was dramatically decreased to 0.28×10^{10} d.p.m./mol, on the basis of the small total radioactivity recovered (134 d.p.m.). Clearly, therefore, the greater part of the radioactivity in the phycocyanin samples must have resided in the protein moiety of the phycocyanin. Its origin is not clear, but may be due to a small radiochemical impurity in the ^{14}C -labelled 3-vinyl-18-ethyl biliverdin or possibly even to some metabolic breakdown of the labelled material by *C. caldarium* cells. The important issue in the present work is whether the very small incorporation of label into phycocyanobilin is significant in terms of the possible intermediacy of 3-vinyl-18-ethyl biliverdin. The very low level of apparent incorporation (0.044%) suggests that this is not the case. Previous work has shown that similar administration of compounds now known to be intermediates (haem and biliverdin itself) led to incorporations greater than 30% and 10% respectively. Since the 3-vinyl-18-ethyl biliverdin would be nearer the end product (phycocyanobilin), if it were an intermediate then comparable or greater incorporation might be expected. Although it is necessary to consider the possibility that uptake of exogenous 3-vinyl-18-ethyl bili-

verdin into *C. caldarium* cells might be difficult, this seems unlikely in comparison with biliverdin, which is very similar structurally and in terms of its solubility properties.

Confirmation that the low level of apparent incorporation does not imply the intermediacy of 3-vinyl-18-ethyl biliverdin came from consideration of the results for chlorophyll *a* (Table 1). Since direct incorporation of 3-vinyl-18-ethyl biliverdin into chlorophyll *a* is not possible, any apparent incorporation would have to be due to a general labelling of the porphyrin-biosynthetic pathway, presumably by metabolic breakdown of the labelled 3-vinyl-18-ethyl biliverdin. Table 1 shows that there was a very small amount of radioactivity recovered in chlorophyll, and that in terms of specific radioactivity this corresponded almost exactly to that found in phycocyanobilin dimethyl ester. It appears that there may well be a very low level of labelling of the porphyrin-biosynthetic pathway and that this accounts for the very small amounts of radioactivity recovered from both phycocyanobilin dimethyl ester and chlorophyll.

For administration of the alternative isomer, 3-ethyl-18-vinyl biliverdin, no significant radioactivity counts above background were found either for the phycocyanin or for the phycocyanobilin dimethyl ester, prepared by procedure 1. Although the small quantity of phycocyanin whose radioactivity was counted (1.1 nmol) may cast some doubt on the validity of the negative result obtained, the sample of phycocyanobilin dimethyl ester assayed was relatively large (92.7 nmol) and the low and statistically insignificant radioactivity observed above back-

ground clearly demonstrates that this compound is not an intermediate in phycocyanobilin biosynthesis.

Our results therefore demonstrate that pathways II and III (Scheme 1) are not operative in the synthesis of phycocyanin in *C. caldarium*. By elimination, we are led to conclude that pathway I is the most likely route of synthesis, i.e. that the chromophore of this phycobili-protein is synthesized from biliverdin via the immediate reduction of ring A, followed by vinyl-ethylidene isomerization, which may or may not be enzyme-mediated. It is noteworthy that the compound thereby produced is phytochromobilin, which is the chromophore of the plant regulatory protein phytochrome.

It must be emphasized that there is presently no direct proof for pathway I, and such proof must await the development of a chemical synthesis for either of the intermediates in pathway I or, alternatively, the isolation of one of these intermediates from an algal system. Nevertheless, if pathway I is correct, the implications for the biosynthesis of plant bilins in general should be recognized. Since phytochromobilin is an intermediate on this pathway, it seems possible that this represents a single unified pathway for synthesis of all plant bilins. In higher plants phytochromobilin formed by pathway I would be incorporated directly into phytochrome, whereas in various algae phytochromobilin could be an intermediate that may be converted either into phycocyanobilin by a single reduction step at the vinyl group on ring D or into phycoerythrobilin (the chromophore of phycoerythrin) by a single reduction step at a methene bridge.

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REFERENCES

- Beale, S. I. & Cornejo, J. (1983) *Arch. Biochem. Biophys.* **227**, 279–286
- Brown, A. S., Foster, J. A., Voynow, P. V., Franzblau, C. & Troxler, R. F. (1975) *Biochemistry* **14**, 3581–3588
- Brown, S. B. & Holroyd, J. A. (1984) *Biochem. J.* **217**, 265–272
- Brown, S. B. & Troxler, R. F. (1982) in *Bilirubin*, Vol. 2 (Metabolism) (Heirwegh, K. P. M. & Brown, S. B., eds.), CRC Press, Boca Raton
- Brown, S. B., Holroyd, J. A. & Troxler, R. F. (1980) *Biochem. J.* **190**, 445–449
- Brown, S. B., Holroyd, J. A., Troxler, R. F. & Offner, G. D. (1981) *Biochem. J.* **194**, 137–147
- Brown, S. B., Holroyd, J. A., Vernon, D. I., Troxler, R. F. & Smith, K. M. (1982) *Biochem. J.* **208**, 487–491
- Brown, S. B., Holroyd, J. A. & Vernon, D. I. (1984) *Biochem. J.* **219**, 905–909
- Köst, H.-P. & Benedikt, E. (1982) *Z. Naturforsch. C. Biosci.* **35**, 763–769
- Schmid, R. & McDonagh, A. F. (1975) *Ann. N.Y. Acad. Sci.* **244**, 533–552
- Simpson, D. J. & Smith, K. M. (1988) *J. Am. Chem. Soc.* **110**, 2854–2861
- Smith, K. M. & Pandey, R. (1984) *Tetrahedron* **40**, 1749–1754
- Stoll, M. S. & Gray, C. H. (1977) *Biochem. J.* **163**, 59–101
- Troxler, R. F., Brown, A. S. & Brown, S. B. (1979) *J. Biol. Chem.* **254**, 3411–3418
- Weller, J.-P. & Gossauer, A. (1980) *Chem. Ber.* **113**, 1603–1611

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