Control of porphyrin biosynthesis in *Rhodopseudomonas spheroides* and *Propionibacterium shermanii*

A direct ^{13}C nuclear-magnetic-resonance spectroscopy study

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The facultative anaerobes *Rhodopseudomonas spheroides* and *Propionibacterium shermanii* were grown under anaerobic and aerobic conditions. The effect of light was studied with the photosynthetic *R. spheroides*, and the adaptation of both species to dark anaerobic life was monitored by direct observation of 5-amino[5- ^{13}C]laevulinic acid metabolism by using ^{13}C nuclear-magnetic-resonance spectroscopy.

The recent upsurge in the development of high-resolution n.m.r. spectrometers and the increasing availability of ^{13}C -enriched compounds have made the possibility of using ^{13}C n.m.r. as a direct probe for the investigation of metabolic pathways a reality. Several recent publications have illustrated the utility of this technique for observing metabolic pathways (Ugurbil *et al.*, 1978; den Hollander *et al.*, 1979; Cohen *et al.*, 1979; Scott *et al.*, 1979) and enzyme-catalysed reactions (Burton *et al.*, 1979a,b) where unstable or transient species are involved.

The study of the dynamics of formation of tetrapyrroles in *Rhodopseudomonas spheroides* and *Propionibacterium shermanii* is admirably suited to this technique, since the intermediate porphyrinogens are highly O_2 -sensitive, whereas the presence of large amounts of pigments makes the determination of porphyrins and/or porphyrinogens difficult (Fuhrhop & Smith, 1975). Direct observation of porphyrinogen biosynthesis in intact *R. spheroides* and *P. shermanii* (Scott *et al.*, 1979) set the stage for a detailed evaluation of the metabolic flux of 5-aminolaevulinic acid into tetrapyrroles. In the present paper we discuss time-course experiments during adaptation of these organisms to different growth conditions. Previous studies carried out on the ability of *P. shermanii* to form corrins (Pearlman *et al.*, 1962) have concentrated on varying the fermentation conditions and monitoring the concomitant effect on the production of vitamin B_{12} . Similar work on the adaptation of *R. spheroides* (Lascelles, 1959) to form bacteriochlorophyll concerned the activity of 5-aminolaevulinic acid synthase and 5-aminolaevulinic acid dehydratase and their role in the control of the tetrapyrrole pathway.

In *R. spheroides* this pathway is of interest with respect to metabolic control since three major groups of tetrapyrroles are produced, namely haem, bacteriochlorophyll and corrins. In addition, the Athiorhodaceae, of which *R. spheroides* is a member, have the ability to grow in the dark or light either aerobically or anaerobically (Van Neil, 1944). Concentrations of bacteriochlorophyll may change 100-fold during changes in growth conditions, thus providing scope for the investigation of adaptive mechanisms associated with the change from photosynthetic to non-photosynthetic metabolism.

Materials and methods

Anaerobic experiments

For anaerobic experiments, cells of *R. spheroides* (A.T.C.C. 17023) were grown in the light by the method of Lascelles (1956) for 5 days, harvested by centrifugation and washed twice with phosphate buffer (0.05 M, pH 6.8). Wet packed cells (1 g) were suspended in 1 ml of the same buffer, containing the following constituents: $(\text{NH}_4)_2\text{HPO}_4$ (3 μmol), MgSO_4 (1 μmol), CaCl_2 (0.4 μmol), iron(III) citrate (0.001 μmol), MnSO_4 (0.001 μmol), sodium fumarate (20 μmol), glycine (5 μmol) and sodium 2-oxoglutarate (10 μmol). For aerobic experiments, cells of *R. spheroides* were grown as above both in the presence or absence of light and O_2/CO_2 (19:1) was bubbled through the medium continuously. Harvesting and preparation of the suspension were carried out as above.

Incubations were performed in n.m.r. tubes (10 mm diameter) for 36 h anaerobically in the dark at 28°C and typically contained cell suspension

(1.2 ml), $^2\text{H}_2\text{O}$ (0.1 ml) and 5-amino[5- ^{13}C]-laevulinic acid hydrochloride (2.5 mg), adjusted to pH 7.

^{13}C n.m.r. spectra were obtained at 20.0 MHz and 28°C in a Varian FT-80 n.m.r. spectrometer with $^2\text{H}_2\text{O}$ as internal lock. Spectral width was 5000 Hz, repetition rate 0.82 s and chemical shifts are referred to tetramethylsilane.

Aerobic experiments

Aerobic cultures of *P. shermanii* (A.T.C.C. 9614) were grown with illumination in Bernhauer's medium (Bernhauer *et al.*, 1959) for 4 days in a shake flask (150 rev./min), harvested by centrifugation (14 g) and washed twice with phosphate buffer (0.05 M, pH 7). Wet packed cells (2 g) were suspended in 1 ml of phosphate buffer (0.02 M, pH 7.5). Anaerobic cultures of *P. shermanii* (A.T.C.C. 9614) were grown under N_2 , harvested and suspended in phosphate buffer (0.02 M, pH 7.5).

Incubations were carried out in n.m.r. tubes for a total of 46 h, anaerobically in the dark at 22°C, and contained cell suspension (1.5 ml), $^2\text{H}_2\text{O}$ (0.15 ml) and 5-amino[5- ^{13}C]-laevulinic acid hydrochloride (5.0 mg), adjusted to pH 7.5.

^{13}C n.m.r. spectra were obtained at 50.0 MHz and 22°C in a Varian XL-200 n.m.r. spectrometer with a spectral width of 10 000 Hz and pulse repetition rates of 0.723 s (for Fig. 2a) and 0.6 s (for Fig. 2b). Chemical shifts are referred to tetramethylsilane.

5-Amino[5- ^{13}C]-laevulinic acid hydrochloride was synthesized from K^{13}CN and ethyl 3-formylpropionate (Battersby *et al.*, 1973).

Incubation mixtures were analysed by esterification [methanolic 5% (v/v) H_2SO_4] of freeze-dried cells and subsequent t.l.c. [benzene/ethyl acetate/methanol, 20:4:1 (by vol.)].

The isomer ratio of uroporphyrinogens and coproporphyrinogens were determined by high-pressure liquid chromatography of the purified porphyrin esters. Uroporphyrin esters were resolved on two μ -porasil columns (Waters) with heptane/acetone/acetic acid/water (600:300:200:0.1, by vol.) as the developing solvent, and coproporphyrin esters were chromatographed on a μ Bondapak C18 column (Waters) with methyl cyanide/water (7:3, v/v) as the developing solvent.

The presence of extracellular porphyrins was determined by centrifugation of the incubation mixture and n.m.r. spectral analysis of the supernatant solution.

Results and discussion

The ^{13}C n.m.r. spectrum of anaerobically grown *R. spheroides* incubated with 5-amino[5- ^{13}C]-laevulinic acid (1) (Fig. 1) indicates that most of the substrate has been incorporated into porphyrino-

Fig. 1. ^{13}C n.m.r. spectrum of anaerobically grown *R. spheroides* incubated with 5-amino[5- ^{13}C]-laevulinic acid, after 46 000 pulses with a repetition rate of 0.81 s. Abbreviations used: C, coproporphyrinogen; ALA, 5-aminolaevulinic acid.

gens with no accumulation of the intermediate porphobilinogen (2). Examination of the signals at 20–24 p.p.m. and 123–125 p.p.m. indicates the presence of type III porphyrinogens as judged by the expected couplings and signal heights from labelling in C-4, -5, -9, -10, -14, -15, -16, and -20 (Scott *et al.*, 1979). Subsequent isolation and t.l.c. of the cell extract showed that the major porphyrinogen formed was coproporphyrinogen III (80%) (3b), together with some uroporphyrinogen III (3a) and protoporphyrinogen IX (5%) (3c), some of which had been excreted extracellularly. A time-course experiment (24 h) failed to show the appearance of porphobilinogen, suggesting that the enzymes porphobilinogen deaminase and uroporphyrinogen III cosynthetase were not present in rate-limiting quantities. Parallel studies with crude cell-free extracts of *R. spheroides* and porphobilinogen as substrate gave similar results, indicating that in light-adapted anaerobically grown cells the rate-determining reactions of porphyrinogen biosynthesis are those catalysed by 5-aminolaevulinic acid synthase and coproporphyrinogen III oxidase.

This can be contrasted with the results for anaerobically and aerobically cultivated *P. shermanii* when incubated with 5-amino[5- ^{13}C]-laevulinic acid. The metabolic sequence is illustrated by the spectra (Figs. 2a and 2b), where it can be seen that a large amount of porphobilinogen (2) (C-11, $\delta = 35.23$ p.p.m.; C-2, $\delta = 116.93$ p.p.m.) is accumulated before porphyrinogens are produced by the action of deaminase and cosynthetase. A time-course experiment over the incubation period (46 h) reveals that the adaptation process is rapid, 5-amino[5- ^{13}C]-laevulinic acid being consumed within the first 16 h. T.l.c. of cell extracts of both incubations gave, as the major components, uroporphyrin III and coproporphyrin III. Quantitative u.v.-visible spectral analysis using known extinction coefficients (Smith, 1975) gave a ratio for uroporphyrin III to coproporphyrin III of 1:1 in

Fig. 2. ^{13}C n.m.r. spectra of *P. shermanii*, grown (a) anaerobically and (b) aerobically, incubated with 5-amino[5- ^{13}C]laevulinic acid for 46 h

Spectrum (a) is the result of 29 875 pulses and (b) of 26 674 pulses. Abbreviations used: PBG, porphobilinogen; C, coproporphyrinogen; U, uroporphyrinogen.

anaerobic growth conditions. This is also reflected in the ratio of intensities of the two doublets at δ 124.92 and 125.40 p.p.m. (Fig. 2a), which correspond to the α -pyrrolic carbon atoms of uroporphyrinogen III and coproporphyrinogen III respectively. Aerobic growth conditions (Fig. 2b) gave a final uroporphyrinogen/coproporphyrinogen ratio of 3:1.

R. spheroides, when grown aerobically in the dark, produces essentially no carotenoid pigments

and is cream coloured, in contrast with the dark-reddish-brown coloration of anaerobic cultures. Under the former conditions no bacteriochlorophyll can be detected. When the organism grown in this way was incubated with 5-amino[5- ^{13}C]laevulinic acid in the n.m.r. tube as before (anaerobically in the dark) for 36 h, 15% of the starting 5-amino-laevulinic acid remained and the only intermediate formed in significant quantities was porphobilinogen, amounting to approx. 80% of the ^{13}C signal (Fig. 3a). Only trace quantities of porphyrinogens are visible; some of the porphobilinogen may form porphyrinogens non-enzymically (Mauzerall, 1960). Further incubation (49 h total) at 28°C resulted in partial consumption of the remaining 5-amino[5- ^{13}C]laevulinic acid, the porphobilinogen signals increased slightly, whereas porphyrinogen synthesis was minimal compared with the anaerobic light-grown cultures (Fig. 3b). Furthermore, the isomer ratio of porphyrinogens formed was estimated from the peak heights and from high-pressure-liquid-chromatographic analysis to be 60:40 for type I/type III. Under these conditions uroporphyrin and coporphyrin were also formed, as judged by t.l.c. of the esters.

When aerobic dark-grown cells of *R. spheroides* were subjected to anaerobic-light conditions and the cells harvested between 12 and 48 h to monitor any changes in the flux of 5-amino[5- ^{13}C]laevulinic acid into tetrapyrroles during the adaptation from non-photosynthetic to photosynthetic metabolism, no accumulation of porphobilinogen or porphyrins was observed, suggesting that any intermediates were being carried through to bacteriochlorophyll and were not accumulating either intra- or extracellularly. 5-Aminolaevulinic acid was consumed

Fig. 3. ^{13}C n.m.r. spectra of *R. spheroides* grown aerobically in the dark, incubated with 5-amino[5- ^{13}C]laevulinic acid for (a) 36 h and (b) 49 h

Spectrum (a) is the result of 12 500 pulses and (b) of 33 500 pulses. Abbreviations used: ALA, 5-aminolaevulinate; PBG, porphobilinogen; C, coproporphyrinogen; U, uroporphyrinogen.

Fig. 4. ^{13}C n.m.r. spectrum of *R. spheroides* grown aerobically in the light, incubated with 5-amino[5- ^{13}C]laevulinic acid after 33 500 pulses

Abbreviations used: PBG, porphobilinogen; C, coproporphyrinogen; U, uroporphyrinogen.

very slowly, indicating that the adaptation was not immediate and that adaptation probably involved enzyme synthesis *de novo* as previously suggested (Lascelles, 1959). When cells were grown aerobically in the light and incubated with 5-amino[5- ^{13}C]laevulinic acid (for 36 h in the dark under N_2), accumulation of type III porphyrinogen was evident after 36 h (Fig. 4). It is noteworthy that some porphobilinogen remains and that decarboxylase activity is incomplete as judged by the presence of uroporphyrinogen III and coproporphyrinogen signals at 123–125 p.p.m.

The cumulative results of this work suggest that in addition to 5-aminolaevulinic acid synthase, coproporphyrinogen III oxidase is also a key regulating enzyme in tetrapyrrole biosynthesis in *R. spheroides*, since coproporphyrinogen III accumulates in large quantities under anaerobic light conditions. Aerobic growth conditions, however, in the case of *P. shermanii*, affect an earlier enzyme in tetrapyrrole biosynthesis, uroporphyrinogen III decarboxylase, which causes a larger accumulation of uroporphyrinogen III relative to coproporphyrinogen III, whereas uroporphyrinogen III decarboxylase activity is affected in *R. spheroides* by aerobic culture in the light.

On changing from anaerobic to aerobic respiratory metabolism in the dark, the enzymes of *R. spheroides* most markedly affected appear to be porphobilinogen deaminase and, to an even greater extent, uroporphyrinogen III cosynthetase, resulting in accumulation of porphobilinogen, an observation not recorded in previous studies. The enzymes 5-aminolaevulinic acid dehydratase and 5-aminolaevulinic acid synthase have to date been assumed to be the major control points in the tetrapyrrole biosynthetic pathway, as both enzymes are four to five times less active in cell-free extracts from aerobic dark-grown cells compared with those from semi-anaerobic light-grown cells. However, the build-up of large amounts of porphobilinogen not only in *R. spheroides* but in *P. shermanii* tends to suggest that greater control lies with porphobilinogen deaminase and uroporphyrinogen III cosynthetase in whole cells. The point is significant in that 5-aminolaevulinic acid dehydratase, traditionally accepted as being the most O_2 -sensitive of the enzymes of the tetrapyrrole pathway, still remains substantially active under aerobic growth conditions. In the whole cell the effect of O_2 is clearly diminished, pointing to the danger of correlating results with whole cells and cell-free extracts and highlighting the uniqueness of the non-invasive n.m.r. approach as a powerful experimental tool for studying the normal cellular machinery at work.

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