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Localization of Ferrochelatase and of Newly Synthesized Haem in Membrane Fractions from *Rhodopseudomonas spheroides*

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Cells of *Rhodopseudomonas spheroides*, strains R-26 or GVP, were grown photosynthetically, disrupted and two particulate fractions separated by sucrose-density-gradient centrifugation. The upper particulate fraction, enriched in bacteriochlorophyll, was identified as containing the chromatophores; the lower particulate fraction had the characteristics of the cell envelope. The two fractions differed in cytochrome content and cytochrome spectra. Ferrochelatase was found almost exclusively in the chromatophore fraction and was located on the outer face of the chromatophores, i.e. in contact with the cytosol in intact cells. The addition of $^{59}FeCl_3$ to cells growing in low-iron media resulted in labelling of the protohaem fraction (probably arising from cytochrome *b*) of the membranes. The specific radioactivity of the haem of the chromatophores rose more rapidly than that of the envelope fraction and then after 2h declined to approximately the same value, suggesting that haems of the chromatophore may act as precursors of haem of the envelope.

The purple non-sulphur bacterium *Rhodopseudomonas spheroides* can grow either aerobically in the dark or photosynthetically if illuminated under anaerobic conditions. Under the latter conditions the organism becomes highly pigmented as a result of the synthesis of bacteriochlorophyll and carotenoids (Cohen-Bazire *et al.*, 1957), and many of the cytochromes found in the aerobically grown form appear to be used in the synthesis of a photosynthetic electron-transport system (Connelly *et al.*, 1973). Electron microscopy shows that the photosynthetic form contains many convoluted membranes that apparently arise from the cytoplasmic membrane (Vatter & Wolfe, 1958), and it has been suggested that the photosynthetic membranes arise by a process of extrusion of the basal respiratory membrane following the addition of new photosynthetic components (Cohen-Bazire, 1963; Kosakowski & Kaplan, 1974). Such a process of membrane development appears possible since it has been shown that cyclic photosynthetic electron flow can be reconstituted in membranes from aerobically grown bacteriochlorophyll-free cells of *R. spheroides* by treatment *in vitro* with pure reaction-centre protein (Jones & Plewis, 1974) or, more effectively, with reaction-centre protein together with light-harvesting protein complex (Hunter & Jones, 1976).

It is possible to separate by density-gradient centrifugation the photosynthetic membranes (or chromatophores) of *R. spheroides* from a cell-envelope fraction that contains some cytoplasmic membrane (Niederman & Gibson, 1971; Niederman *et al.*, 1972; Ding & Kaplan, 1976). We have used this method to determine the subcellular localization of the enzyme ferrochelatase in this organism. Ferrochelatase is a membrane-bound enzyme that catalyses the insertion of ferrous iron in porphyrins to make haems; in all probability the one and only normal product is protohaem (see Jones, 1978, for a discussion of the specificity of ferrochelatase) and this protohaem is utilized in the synthesis of cytochromes *b* and *c* of the respiratory and the photosynthetic chain. The site of the combination reaction between cytochrome apoprotein and the prosthetic group is not known. One cytochrome at least, cytochrome c_2, is located between the cell membrane and the cell wall, in the periplasmic space (Prince *et al.*, 1975), and so a knowledge of the distribution of ferrochelatase within membrane fractions may assist in understanding how the late stages in cytochrome biosynthesis and the organization of electron-transport carriers are achieved.

We have also used pulse labelling with ^{59}Fe in an attempt to discover if the haems of one membrane fraction are labelled before other haem fractions. This sort of experiment may help to identify a region of membrane growth or extrusion from a pre-existing membrane, since such a region will be expected to contain much of the newly formed haem pigment and so become highly labelled.

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Materials and Methods

Growth of cells and preparation of extracts

The blue-green mutant R-26 of *R. spheroides* was a gift from Dr. R. K. Clayton, Department of Development and Physiology, Cornell University, Ithaca, NY 14853, U.S.A., and a green mutant of *R. spheroides*, GVP, was isolated as described by Connelly *et al.* (1973). Photosynthetic cells were grown anaerobically in the medium of Sistrom (1960) for 72h, illuminated by incandescent lamps. Cells were disrupted either by pressing twice through a French pressure cell and the resulting crude chromatophore particles prepared by differential centrifugation as described previously (Connelly *et al.*, 1973) or by the preparation of spheroplasts, followed by disruption in 0.1% Brij-58 at 4°C for 30min and separation of crude chromatophore particles (Siekevitz, 1973). Crude particle preparations were loaded on a linear 5–60% (w/w) sucrose gradient (Niederman *et al.*, 1972) and centrifuged in a swinging-bucket rotor at 138000*g* for 12h. The centrifuge tubes contained a volume of 20ml, and 1ml fractions were collected for analysis at the end of the centrifuge run.

Assays

Ferrochelatase was assayed by the dual-wavelength spectrophotometric method of Jones & Jones (1970) with deuteroporphyrin and Co^{2+} as substrates. Protein was determined by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard. Bacteriochlorophyll was estimated *in vivo* from its A_{870} (Clayton, 1963), or after extraction into organic solvents by the procedure of Clayton (1963). Succinate dehydrogenase was assayed by the method of King (1967). ^{59}Fe was determined by counting at infinite thinness in an end-window Geiger counter.

Results

As discussed by Niederman & Gibson (1978) chromatophores of *R. spheroides* can be separated from a more-dense cell-wall–cytoplasmic membrane complex by centrifugation in gradients prepared from aqueous sucrose. In such gradients the chromatophore fraction is recognized from its high specific bacteriochlorophyll content (see Fig. 1), whereas the denser envelope fraction has a relatively low bacteriochlorophyll content, although it retains succinate dehydrogenase activity, presumably arising from the attached cytoplasmic membrane fragments (Niederman & Gibson, 1971). The specific activity of the succinate dehydrogenase of bulked, lower, cell-envelope fraction was 39nmol/min per mg of protein; that of the chromatophore fraction was 52nmol/min per mg of protein. Both bands contained some bacteriochlorophyll and the bacteriochlorophyll spectra of the two bands were almost identical, and neither did the fourth derivative spectra (Butler & Hopkins, 1970) differ significantly. Since the bacterium used to obtain the gradient shown in Fig. 1 is carotenoid-free it was possible to obtain cytochrome spectra without the interference that frequently causes problems in wild-type cells. Spectra at liquid-N_2 temperatures showed that the lower band contained more of a long-wavelength cytochrome *b*, absorbing around 559nm at 77K, relative to cytochrome *c* (Fig. 2). The total cytochrome content of the lower, envelope, band was, however, lower than that of the chromatophore fraction (see Table 1) and the cytochrome *c* absorption maxima at 549nm and 547nm were less well defined.

Localization of ferrochelatase

It can be seen in Fig. 1 that ferrochelatase was accumulated in the chromatophore (upper) fraction. The specific activity of ferrochelatase in fraction 8 was 1.35nmol/min per mg of proetin; in fraction 13 it was 0.22nmol/min per mg of protein. The ferrochelatase activity of the chromatophore fraction, measured in the absence of detergent, was not increased by the addition of 1% of Triton X-100 or 1% sodium cholate, known to disrupt chromatophore

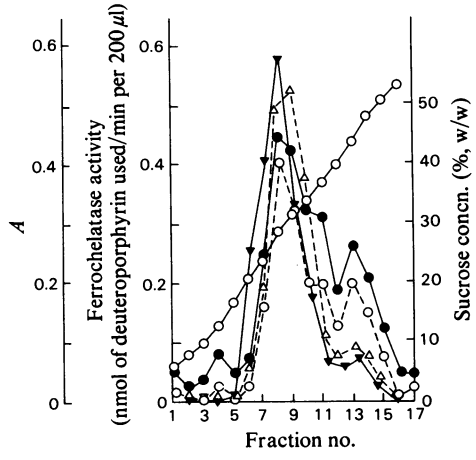

Fig. 1. *Representation of the distribution of ferrochelatase and bacteriochlorophyll after centrifugation on a* (1ml) *gradient of sucrose* (5–60%, *w/w*)

Crude chromatophores from *R. spheroides* R-26 were placed on a gradient (18ml) of sucrose (○—○) and centrifuged at 138000*g* for 12h. Fractions (1ml) were then collected and A_{260} (●), A_{280} (○) and A_{870} (△) (to indicate bacteriochlorophyll) were measured after diluting samples 40-fold. Ferrochelatase was assayed on 200µl portions (▲).

structure (e.g. Prince *et al.*, 1975). This indicates that the active centre of ferrochelatase is localized on the outer face of the chromatophore where it is accessible to the added porphyrin and metal, i.e. that surface that is in contact with the cytosol in intact cells. Such a conclusion must be based on the assumption that permeability of the membrane to substrates is rate-limiting in the chromatophore. This localization of ferrochelatase appears similar to that found in rat liver mitochondria, where the enzyme is located on the inner face of the inner mitochondrial membrane (Jones & Jones, 1970). The orientation of the chromatophores was confirmed in experiments where proton movement induced by light was monitored by a method dependent on the quenching of 9-aminoacridine fluorescence (Deamer *et al.*, 1972). Proton movement in the chromatophores had a polarity opposite to that of whole cells.

Incorporation of ^{59}Fe into the protohaem components of membrane fractions of R. spheroides

Cultures of *R. spheroides* GVP were grown photosynthetically in the standard medium from which iron salts had been omitted. The low concentration of iron in the medium was shown by the profuse excretion of free porphyrin into the medium. *R. spheroides* (12 litres) was grown anaerobically to mid-exponential phase (24h), and a small mass of $^{59}FeCl_3$ (60μCi, 14μg of Fe) was injected into the exponential-phase culture, which was then re-placed in the light. Portions (3 litres) were withdrawn at 20, 40, 80 and 120min, ferrous sulphate (30mg) was added immediately on collection of each portion, and chloramphenicol (100mg) was also added to halt growth. Cells were harvested and fractionated as shown in Fig. 1. Bacteriochlorophyll and carotenoids were extracted from the heavy and light fractions by repeated washing with a mixture of 1% NH_3 and acetone (1:9, v/v) containing 1mg of 2,6-bis-(t-butyl)-*p*-cresol/ml. The protohaem was then extracted from the membrane fractions with 80% (v/v) acetone/water containing 0.5% (w/v) HCl to separate the haem from any apoprotein. This procedure was repeated and these extracts were combined. The haems were taken into diethyl ether and the diethyl ether solution was washed repeatedly with 1% (w/v) HCl to remove inorganic iron. The ether was evaporated and the haems were taken up with a small volume of pyridine. Portions from each sample were taken for spectrophotometric determination of the protohaem. Samples of the pyridine solutions (0.2ml) were made up to 5.0ml with a solution mixture containing 1 part of pyridine to 2 parts of 0.1M-NaOH. The resulting haem solution was divided between two cuvettes. Sodium dithionite was added to the sample cuvette and a reduced-minus-oxidized difference spectrum

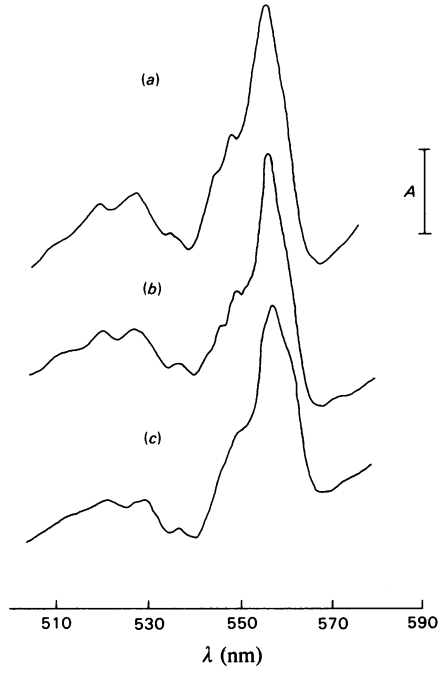

Fig. 2. *Reduced-minus-oxidized difference spectra at 77K of particulate fractions from R. spheroides R-26*
Particles were suspended in 10% (w/v) sucrose and either reduced with a few grains of sodium dithionite or oxidized with 200μM-potassium ferricyanide. The sensitivity for recording spectra differed in each case and is given below as the distance, *A*, between the bars on the Figure. (*a*) Unfractionated chromatophores (2mg of protein/ml), $A=0.05$; (*b*) upper band from gradient (0.92mg of protein/ml), $A=0.025$; (*c*) lower band from gradient (0.41mg of protein/ml), $A=0.001$.

Table 1. *Bacteriochlorophyll and cytochrome content of the upper (chromatophore) band and lower (cell-envelope) band separated by density-gradient centrifugation of a crude particle fraction from R. spheroides strain R-26*
The membrane bands were separated as shown in Fig. 1. Cytochromes *b* and *c* were determined from reduced-minus-oxidized spectra at $A_{560}-A_{570}$ and $A_{551}-A_{540}$ respectively, with $\Delta A_{mM}=20$.

Cell fraction	Cytochrome *b* (nmol/mg of protein)	Cytochrome *c* (nmol/mg of protein)	Bacteriochlorophyll (nmol/mg of protein)
Upper (chromatophore) band	2.2	1.3	35.0
Lower (cell-envelope) band	0.54	0.41	3.8

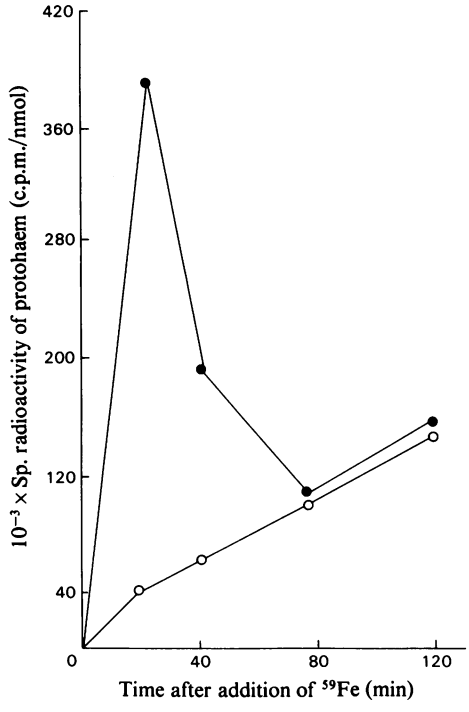

Fig. 3. *Incorporation of ^{59}Fe into protohaem components of membrane fractions of R. spheroides GVP*

Low-iron culture (12 litres) was grown photosynthetically to mid-exponential phase, when 60μCi of $^{59}FeCl_3$ (14μg of Fe) was injected and the cells were re-placed in the light. Cells (3-litre portions) were harvested at intervals, and carrier ferrous sulphate (30mg) and chloramphenicol added. Cells were disrupted and membranes fractionated as shown in Fig. 1. Protohaem was extracted, purified and counted as described in the Results section. Symbols: ●, protohaem from upper band; ○, protohaem from lower band.

recorded over the region 510–570nm. Protohaem contribution was calculated with $A_{557}-A_{540}=20.7$. Samples of the pyridine solutions were dried on planchettes and the ^{59}Fe was determined with a gas-flow counter.

As may be seen from Fig. 3 the ^{59}Fe behaves as a pulse in these low-iron cells. It is rapidly incorporated into the haem of the light membrane fraction and with passage of time the specific radioactivity increases in the haem of the heavy fraction, with an accompanying diminution of the specific radioactivity of the light fraction.

Discussion

Density-gradient centrifugation of crude particles from *R. spheroides* shows that two membrane fractions can be clearly separated. The lower band has a relatively low bacteriochlorophyll content but retains succinate dehydrogenase activity, in agreement with its identification as cell envelope plus some attached cytoplasmic membrane. The cytochrome composition of this band was not identical with that of chromatophores; in chromatophores the reduced cytochrome *c* at 77K has a characteristic absorption maximum at 549nm, with a second peak at 547nm (Fig. 2). In the envelope fraction this second peak of cytochrome *c* is less well defined, suggesting that the cytochrome *c* either is different or is attached differently to the membrane. The cytochrome *b* absorption in the β-peak region is broad in the envelope fraction, probably arising from a mixture of components, whereas the chromatophore fraction is enriched in a *b*-cytochrome with a sharp absorption maximum at 557nm at 77K. The two membrane fractions differed clearly in their content of ferrochelatase. The low ferrochelatase specific activity of the envelope fraction suggests that haem synthesis is associated exclusively with the chromatophores, or with a membrane closely associated with the chromatophores. Similarly the distribution of ^{59}Fe-labelled protohaem after the addition of small amounts of labelled iron to photosynthetically growing cultures suggests that haem pigments are first incorporated into the chromatophore fraction. The protohaem of chromatophores is very rapidly labelled, presumably because porphyrin substrate is already accumulated in iron-deficient cells (Burnham & Lascelles, 1963) and the supply of iron is rate-limiting, but the haem of the envelope fraction is labelled much more slowly. Indeed the time course shown in Fig. 3 suggests that the protohaem observed in the chromatophore fraction could be a precursor of the haem of the envelope.

The method of haem extraction does not enable us to distinguish between the haem already attached to proteins, such as cytochromes of the *b*-type, and a possible pool of newly formed membrane-bound haem, as yet unattached to a specific apoprotein. Our results suggest that, if there is a series of regions in the cytoplasmic membrane from which the chromatophore membranes are extruded, then the haem-synthesizing system is associated with this region and becomes detached from the envelope during the process of pinching off the intracytoplasmic membrane to form the chromatophores. Newly synthesized haem pigments are incorporated first into chromatophores, perhaps because the apoproteins move to the haem formed by the ferrochelatase, and move later to the envelope membrane.

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