Uptake of protoporphyrin and continuous spectrophotometric assay for magnesium chelatase in *Rhodobacter spheroides*

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Uptake of protoporphyrin was shown by *Rhodobacter spheroides* under anaerobic conditions in the dark. The process was not energy-dependent but required EGTA and was markedly stimulated by Methyl Viologen. Kinetic studies were consistent with a saturable process with $K_d = 5\text{--}10\ \mu\text{M}$ and $B_{\text{max.}} = 2.2\ \text{nmol}$ of protoporphyrin bound/mg dry weight of cells. Bound protoporphyrin could be converted into magnesium protoporphyrin monomethyl ester under anaerobic conditions in the light or at low $p\text{O}_2$ (6.3 %) in the dark. This formed the basis of a sensitive

continuous spectrophotometric assay for magnesium chelatase, which avoids the need to extract the product into organic solvent, and may facilitate the development of a cell-free system for magnesium chelatase in photosynthetic bacteria. It is proposed also that the uptake mechanism shown for exogenous protoporphyrin may indicate the existence of a ligand or carrier system for endogenously produced protoporphyrin essential for magnesium chelatase activity.

INTRODUCTION

The first reaction in the tetrapyrrole pathway specifically committed to the synthesis of bacteriochlorophyll, magnesium chelatase, remains uncharacterized, and has not been demonstrated in a cell-free system from photosynthetic bacteria. Recent work with cell-free systems from pea seedlings indicated that both soluble and particulate protein fractions were required for activity [1], but with cucumber etioplasts, a membrane fraction alone was sufficient [2]. These differences may be due to solubilization of membrane components during the preparation of the extract from peas but not from cucumber. In bacteria, magnesium chelatase activity appears in addition to be closely linked with the next step in the pathway, methylation, resulting in the formation of magnesium protoporphyrin monomethyl ester [3].

Previous assay for magnesium chelatase in these micro-organisms was by incubating whole cells with exogenous protoporphyrin and measuring magnesium protoporphyrin monomethyl ester spectroscopically after its differential extraction into diethyl ether [3]. The object of the present experiments was to devise a continuous assay for magnesium chelatase in *Rhodobacter spheroides*, in order to increase our understanding of the reaction(s) and to facilitate the establishment of a cell-free system.

The assay described here allows continuous reaction rates to be determined, avoids the need for extractions into organic solvents and is highly sensitive. An uptake mechanism for exogenous protoporphyrin has been shown and some of its properties have been determined.

MATERIALS AND METHODS

Chemicals

Porphyrins and other compounds were prepared as previously described [3] or were obtained from readily available commercial sources.

Micro-organisms

R. spheroides, wild-type and Tn5 mutants were a gift from Dr. C. N. Hunter [4]. Wild-type cells were grown in M22+ medium under photosynthetic conditions or semiaerobically in the dark and mutants were grown semiaerobically in the dark essentially as previously described [5]. *Rhodobacter capsulatus* (wild-type SB 1003) was provided by Dr. B. Hundle and was grown photosynthetically or semiaerobically in the dark in RCV medium [6]. *Escherichia coli*, strain HB101 (NCIMB 11668), was obtained commercially and was grown aerobically in Luria–Bertani medium [7].

Spectroscopy

This was performed in an SP 1800 Pye–Unicam double-beam recording spectrophotometer.

Assays for protoporphyrin uptake and magnesium chelatase

To suspensions of micro-organisms (generally 2.5 mg dry weight) in Tris/EGTA buffer, pH 8.5, (final concentrations 23 mM and 5 mM for Tris and EGTA respectively) in cuvettes of 2 mm path length were added protoporphyrin (up to 15 nmol in dimethyl sulphoxide (DMSO)/aq. 5 M ammonia (2:1, v/v) (maximum volume added 4 μl), or in a lipid sol [3]) and other compounds as indicated to a final volume of 0.5 ml. Difference spectra were determined between cell suspensions with and without added protoporphyrin, or differing only in the content of the compound under investigation.

Ultrasonic irradiation

This was carried out with an MSE 150 W sonicator fitted with a titanium microprobe. Micro-organisms suspended at a concentration of 5 mg dry weight/ml in the Tris/EGTA mixture in 1.5 ml conical Eppendorf centrifuge tubes were irradiated at an

Figure 1 Spectral changes in suspensions of *R. spheroides* incubated with protoporphyrin

A suspension of cells was incubated under anaerobic conditions in the dark as described in the Materials and methods section. The difference spectrum was recorded immediately after addition of 13.2 nmol of protoporphyrin to the sample cuvette and mixing by inversion. Increase in A_{410} was continuously recorded and spectra were obtained at various intervals. The arrow indicates the addition of Methyl Viologen.

amplitude of 10–12 μm for period of 5 s. The tubes were immersed in ice/water during the procedure.

Preparation of spheroplasts

Micro-organisms, 5 mg dry weight/ml, were suspended in buffer [0.25 M sucrose, 50 mM Tris/HCl (pH 8.2), 2 mM EGTA] and incubated for 15–20 min at 20–22 °C after addition of 10–20 μg of lysozyme/ml. The spheroplasts were harvested by centrifuging with gentle acceleration to 2000 g for 5 min and resuspended in the same medium but without lysozyme.

Environmental conditions for assays

Under 'anaerobic' conditions in the dark, no special precautions were used to exclude oxygen, but the shape of the cuvette (2 mm $\times$ 40 mm $\times$ 10 mm) limited its diffusion into the bulk of the suspension. The spectrophotometer beam provided the only source of illumination. Other conditions were obtained by passing a stream of air, nitrogen or oxygen through the cuvette by means of a fine polythene tube. Illumination outside the spectrophotometer was obtained by placing the cell carriers 15–20 cm from a 60 W tungsten light bulb. Illuminance was 3000–4000 lx. The assays were generally carried out at 20–22 °C.

RESULTS

Protoporphyrin uptake or binding

On adding protoporphyrin to Tris/EGTA solution, a relatively flat spectrum was obtained, but when it was added to a suspension of *R. spheroides* anaerobically in the dark, a time-dependent increase in absorbance at 410 nm was observed (Figure 1). The change occurred whether the protoporphyrin was added in a lipid sol or in DMSO. The effect was stimulated by EGTA (1–20 mM) or a related chelator [3], with 90 % of its maximum

Figure 2 Relationship between increase in A_{410} and protoporphyrin concentration: effect of Methyl Viologen

Protoporphyrin in aq. ammonia/DMSO was added to photosynthetically grown *R. spheroides* (2.5 mg dry weight) suspended in 0.5 ml of Tris/EGTA buffer, as described in the Materials and methods section. Absorbance change at 410 nm was continuously measured relative to an identical suspension with no protoporphyrin added in the reference cuvette (a). (b) Methyl Viologen was included at a final concentration of 40 μM . The rates of increase in A_{410} in the plots were obtained graphically from tangents drawn to the initial rate of the progression curves. For convenient presentation, the values are shown calculated over 20 min. The points were fitted to the equation $Y = AX/(B + X)$, where $A = B_{\text{max}}$ and $B = K_d$, using commercially available software (Graphpad Inplot 4). The data are expressed also as Lineweaver–Burk plots (insets).

Figure 3 Relationship between increase in A_{410} and concentration of cells

A constant amount of protoporphyrin, 15 nmol in 4 μ l of aq. ammonia/DMSO, was added to different quantities of micro-organisms suspended in 0.5 ml of Tris/EGTA buffer as described in the Materials and methods section. The absorbance change at 410 nm was continuously recorded using an identical suspension without protoporphyrin in the reference cell. Both sample and reference cuvettes contained 40 μ M Methyl Viologen. The values shown are the actual increases in absorbance measured from the time of addition to the point when the progression curves flattened out. The relationship is linear but does not pass through the origin. The y intercept (0.024, 95% confidence intervals 0.015–0.033) represents the immediate increase in A_{410} observed when the protoporphyrin was added to Methyl Viologen in Tris/EGTA buffer in the absence of micro-organisms.

rate reached at a concentration of 2 mM. The rate of increase at 410 nm was further markedly increased by the addition of Methyl Viologen (Figure 1), with 95% of its maximum effect obtained at 40 μ M. In the absence of EGTA, Methyl Viologen stimulated the uptake of protoporphyrin at approximately one-

fifth the rate obtained in the presence of EGTA. A pH optimum was observed at approximately 8.5, with the uptake being reduced to 45% at pH 7.5. Exposure to high pO_2 or addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) did not impair the uptake of protoporphyrin, showing that this was not inhibited by oxygen and that energy was not required. After centrifugation, the peak at 410 nm was associated with the resuspended cells, whereas the supernatant had a flat spectrum similar to that originally obtained on adding protoporphyrin to the reaction mixture without cells.

It is inferred that the spectral changes observed when protoporphyrin is added to cell suspensions under anaerobic conditions in the dark are due to an environmental change of the protoporphyrin from a predominantly polymeric suspension in the aqueous medium to a state with a higher proportion in monomeric 'true' solution, presumably in lipid or lipoprotein membrane-bound form.

Kinetic studies of the rate of increase in absorbance at 410 nm against the concentration of protoporphyrin indicated a rectangular hyperbola or binding isotherm relationship consistent with a saturable mechanism (Figure 2). Methyl Viologen increased the maximum binding nearly 20 times (from B_{max} 0.021 to 0.41), but had less effect on the apparent binding constant, K_d (6.56 μ M and 8.59 μ M). Values for K_d in the presence of Methyl Viologen were 5–10 μ M in different cultures, and up to 2.2 nmol of protoporphyrin could be bound/mg dry weight of cells (B_{max}), calculated using $\epsilon_{408\text{nm}}$ 262 as the absorbance value for protoporphyrin in 2.7 M HCl [8,9].

The quantity of protoporphyrin bound under anaerobic conditions in the dark, when conversion into magnesium protoporphyrin did not occur, was proportional to the quantity of cells in the reaction mixture, which is consistent also with a saturable process (Figure 3).

After ultrasonic irradiation of cells in Tris/EGTA for 5 s, there was an initially more rapid increase in A_{410} on addition of protoporphyrin, compared with that of intact cells, which lasted

Figure 4 Relationship between increase in A_{410} and protoporphyrin concentration in *R. spheroides* sonicated for 5 s

Cell suspensions in 1.0 ml of Tris/EGTA buffer were sonicated for 5 s and divided into 0.5 ml portions in the sample and reference cuvettes. Experiments were then carried out as described in the legend to Figure 2. (a) The traces compare the progression curves at 410 nm after addition of 13.2 nmol of protoporphyrin to a sonicated (s) and non-sonicated (ns) suspension of the same culture. Methyl Viologen was then added as shown by the arrows. (b) The initial rates, without Methyl Viologen, at different concentrations of protoporphyrin were determined and plotted as described in the legend to Figure 2.

Figure 5 Protoporphyrin uptake by spheroplasts of *R. spheroides*

Spheroplasts were prepared as described in the Materials and methods section. After addition of 15 nmol of protoporphyrin in aq. ammonia/DMSO, the increase in absorbance was measured continuously at 410 nm with an identical suspension of spheroplasts in the reference cuvette. Spectra between 370 nm and 450 nm were obtained at intervals before and after the addition of Methyl Viologen (MV).

for approx. 5 min and was followed by a gradual decrease in rate which tailed off over the next 15–20 min (Figure 4a). The enhancing effect of Methyl Viologen was less in sonicates. Addition of different concentrations of protoporphyrin to 5 s sonicates (Figure 4b) confirmed differences in uptake properties compared with intact cells, with $K_d = 34 \mu\text{M}$ consistent with decreased affinity, but maximum binding, B_{max} , of 0.33, representing a 15-fold increase in the number of binding sites (cf. Figure 2a).

The uptake of protoporphyrin by spheroplasts in the absence of Methyl Viologen was rapid with a rate approaching that of intact cells with Methyl Viologen, and, in contrast with sonicated cells, addition of Methyl Viologen after the rate of increase in A_{410} had tailed off still resulted in marked stimulation (Figure 5).

These findings could be explained by the brief (5 s) sonication damaging the cell wall and allowing greater access of protoporphyrin to binding sites on internal membranes. The effect of Methyl Viologen on protoporphyrin uptake would be lost if its effect was also mediated at the level of structural organization of the cell wall. Sonication may also disrupt internal membranes with loss of biologically relevant binding sites. The increase in permeability to protoporphyrin after exposure to lysozyme is presumably associated with a more selective effect on the cell wall than ultrasonic irradiation and no direct damage to the internal membrane system.

Similar uptake properties were obtained with the related photosynthetic micro-organism *R. capsulatus*, in contrast with much less uptake using *E. coli* strain HB 101.

Figure 6 Synthesis of magnesium protoporphyrin monomethyl ester from protoporphyrin previously taken up by *R. spheroides*

Cells loaded with protoporphyrin as described in the Materials and methods section were centrifuged and resuspended in distilled water. Oxygen was bubbled through the sample cuvette for 5 s periods and the absorbance change at 424 nm was recorded continuously until the response flattened out. The increased rate of change at 424 nm apparent just before the curve flattened out, shown here for two cycles of oxygenation, was a constant finding. Spectra between 370 nm and 450 nm were recorded before (1) and after (2–5) each period of oxygenation.

Magnesium chelatase: continuous spectrophotometric assay

R. spheroides cells preloaded with protoporphyrin by prior incubation in the dark as described above, and resuspended in buffer or in distilled water, showed spectral changes on illumination under anaerobic conditions or on exposure to low partial pressure of oxygen in the dark without further addition of EGTA or Methyl Viologen.

Absorbance at 424 nm increased with a corresponding decrease at 410 nm (Figure 6), consistent with synthesis of magnesium protoporphyrin monomethyl ester from the protoporphyrin previously taken up. This was confirmed by spectroscopy (Figure 7) and t.l.c. of organic solvent extracts of such cell suspensions. Similar quantitative values were obtained by applying the absorption coefficient available for the ether solution ($\epsilon_{419\text{nm}} 308$) [9,10] to the *in vivo* spectrum (Table 1).

A continuous assay was obtained by low-level aeration of cell suspensions in the dark. The $p\text{O}_2$ in the experiment shown (Figure 8) was 6.3 %, measured in the suspension with an oxygen electrode. Activity was rapidly arrested by CCCP as previously described [11], or by high $p\text{O}_2$. The final yield of magnesium protoporphyrin monomethyl ester was greater if the cells were not separated from the original suspending medium, presumably because uptake of protoporphyrin continued.

As the equipment available did not allow spectral determination and illumination to be carried out simultaneously,

Figure 7 Spectrum of a suspension of *R. spheroides* and of its ether extract after synthesis of magnesium protoporphyrin monomethyl ester

(a) Difference spectrum of a cell suspension of *R. spheroides* (0.5 ml, see the Materials and methods section) allowed to take up protoporphyrin and then incubated at low pO_2 (cf. Figure 6). The shoulder at 410 nm indicated by the arrow is due to residual protoporphyrin. For quantification (see Table 1), the peak height at 424 nm was measured from the apex to a line between the shoulder at 410 nm and the inflection at 440 nm. (b) Spectrum of the washed ether extract [3] of the cells shown in (a) (total volume 5.5 ml). The peak height at 419 nm was measured from the apex to a line between A_{370} and A_{440} . The spectrum of the visible bands (c) was determined after concentrating the ether extract to 1 ml by evaporation in a stream of N_2 . The bands are of similar height and $A_{419/551}$ or $A_{419/589}$ are similar to values previously reported for magnesium protoporphyrin methyl ester (approx. 15.8 compared with 16.9 [9,10]).

assay under anaerobic conditions in the light was performed by determining the spectral changes after successive 5–10 min periods of illumination. Under these conditions synthesis of magnesium protoporphyrin monomethyl ester was also readily demonstrated.

When protoporphyrin was added to both the sample and reference cuvettes of the spectrophotometer and a stream of air was passed through the sample cuvette only, the difference spectrum showed the time-related synthesis of magnesium protoporphyrin monomethyl ester (Figure 9). A corresponding negative peak at 410 nm was apparent, also reflecting the continuing uptake of protoporphyrin in the reference cuvette. Several other time-related absorbance peaks of smaller amplitude were found also at longer wavelength (not shown here), consistent with the synthesis of other bacteriochlorophyll intermediates.

DISCUSSION

Protoporphyrin uptake

Although the present findings have been obtained using exogenous protoporphyrin, it seems possible that they are indicative of a naturally occurring process of intracellular binding of endogenously synthesized protoporphyrin, perhaps necessary for favourable presentation to the magnesium-chelating enzyme system. Previous determination of protoporphyrin in *R. spheroides* measured by extraction into organic solvents and then into HCl [11] gave values of 0.03–0.10 nmol/mg dry weight of cells under photosynthetic conditions but increased levels of 0.30 nmol/mg dry weight when photosynthesis was inhibited,

Table 1 Measurement of magnesium protoporphyrin monomethyl ester (MgprotoMe) synthesis in *R. spheroides* *in vivo* and after extraction into diethyl ether

The absorption coefficient for magnesium protoporphyrin in diethyl ether was used in the calculation for the whole cells.

	A_{419}	MgprotoMe (nmol)
Direct assay	0.63	2.02
Ether extract	0.56	1.82

Figure 8 Synthesis of magnesium protoporphyrin monomethyl ester by *R. spheroides*: continuous aeration in the dark

Cells were incubated with protoporphyrin anaerobically in the dark for 15 min and spectra were recorded immediately after addition of the protoporphyrin (spectrum 1), 5 min later (spectrum 2), and 5 min after adding Methyl Viologen (spectrum 3). The sample cuvette was then aerated through a fine polyethylene tube connected to a peristaltic pump drawing in atmospheric air. The pumping rate chosen gave a pO_2 in the cuvette of 6.3%, measured with a Clark-type oxygen electrode. Absorbance was continuously recorded at 424 nm during aeration. The width of the trace resulted from the stream of air bubbles going through the cuvette. Spectra 4–6 were measured at intervals during the reaction.

consistent with the existence of intracellular protoporphyrin-binding sites. The experiments described here do not allow firm conclusions to be drawn about the existence of different types of binding sites or receptors for protoporphyrin. The apparently different dissociation constant (K_d) obtained in the experiment with cells sonicated for 5 s compared with intact cells can only be considered to be suggestive. There may be a relationship with the long-postulated carrier protein for intermediates of the bacteriochlorophyll pathway [12], and extension of this work may provide

Figure 9 Synthesis of magnesium protoporphyrin monomethyl ester and uptake of protoporphyrin

Protoporphyrin and Methyl Viologen were added to a suspension of *R. spheroides* in Tris/EGTA buffer, which was then divided between the sample and reference cuvettes. The sample cuvette was aerated as described in the legend to Figure 8, to give a pO_2 of 6.3%. A_{424} was continuously recorded and spectra between 370 nm and 450 nm were determined at intervals. The arrows indicate the direction of the spectral changes with time, starting from the first essentially flat difference spectrum obtained before aeration.

a means of characterizing it. Recent studies have identified porin [13] as the macromolecule associated with excreted intermediates, which is an unlikely candidate for the postulated carrier protein. These findings do not, however, exclude the existence of a more specific metabolically active carrier.

Preliminary studies with mutants that are photosynthetically incompetent [4] because of defects of enzymes at the magnesium-insertion step or at other sites of bacteriochlorophyll synthesis show decreased protoporphyrin uptake compared with wild-type organisms (A. Gorchein, unpublished work). This may be because these mutants lack the structure or organization found in chromatophores or internal membranes of photosynthetically competent wild-type organisms. These findings indicate a need for caution in interpreting negative results of assays using

exogenous protoporphyrin in such mutants [14], unless uptake of protoporphyrin has been shown to be non-limiting.

Magnesium chelatase

It has been shown here that uptake or binding of protoporphyrin by the cells and the subsequent formation of magnesium protoporphyrin (methyl ester) can be separated. Neither EGTA nor Methyl Viologen seemed to be directly required for magnesium insertion, but EGTA or a related chelator was essential for the uptake of protoporphyrin, and Methyl Viologen further increased the rate and amount of uptake.

The mode of action of Methyl Viologen in increasing the uptake of protoporphyrin is not readily explained. Effects caused by changes in the redox potential of the electron-transport chain appear unlikely. It seems possible that it increases the permeability of the cell wall or cytoplasmic membrane to protoporphyrin by free-radical damage (see ref. [15]).

The development of this continuous spectrophotometric assay has provided further information on a step involved in the mechanism of action of magnesium chelatase in whole cells using exogenous protoporphyrin. It may provide the means to obtain further insight into the existence of a carrier system for endogenously synthesized protoporphyrin and other intermediates of the bacteriochlorophyll pathway, and also enable determination of the requirements for cell-free activity of magnesium chelatase in the photosynthetic bacteria.

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