

In situ* Assessment of Porphyrin Photosensitizers in *Propionibacterium acnes

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Porphyrins are known to be efficient photosensitizer molecules and the combined action of light and porphyrins in *Propionibacterium acnes* have a lethal action on the cells. Identification and quantification of *in situ* porphyrins in *P. acnes* have been done using an integrating sphere connected to an ordinary absorption spectrophotometer, and the amounts of porphyrins in the cells were quantified by measuring scattering free absorption spectra of the cell suspensions. The concentration of porphyrins in *P. acnes* cells were increased in either of two ways; by the addition of δ -aminolevulinic acid (ALA), which lead to the formation of coproporphyrin III under the incubation conditions used in these experiments, or by the addition of protoporphyrin IX (PPIX) to the cell suspension. In the latter case, PPIX molecules are taken up by the cells in a membrane-mediated uptake mechanism, and accumulate in the cells either on a monomeric or a particular aggregate form. The fraction of porphyrins on aggregate form increased with increasing PPIX additions. In the case of ALA induced porphyrin production, only monomeric porphyrins were stored in the cells. In both cases, the cells have a limited binding capacity of monomeric porphyrins, which is estimated to be 3×10^5 molecules/cell, or one porphyrin molecule to every 100th lipid molecule in the cell membrane.

Key words: *Propionibacterium acnes*, Porphyrins, Absorption Spectra

Introduction

The Gram-positive bacterium *Propionibacterium acnes* plays a central role in the acne disease (Cunliffe and Goulden, 2000; Itoh *et al.*, 2000). It produces endogenous porphyrins (Kjeldstad *et al.*, 1984; Romiti *et al.*, 2000; Ashkenazi *et al.*, 2003), like many other cell types (Juzenas *et al.*, 2001). In *P. acnes* the terminal product in the sequence of metabolic reactions is protoporphyrin IX (PPIX), while in plant cells, Mg^{2+} ions are further inserted in the center of PPIX to produce a chlorophyll molecule and in animal cells, the insertion of Fe^{2+} ions leads to the production of a heme. In *P. acnes* at anaerobic conditions, PPIX is accumulated, while at semianaerobic conditions, uroporphyrin III (UP III) and coproporphyrin III (CP III) are formed from the corresponding porphyrinogens, which are earlier steps in the reaction pathway leading to PPIX.

The porphyrins make *P. acnes* light sensitive. The sensitivity can be increased by using δ -aminolevulinic acid (ALA) to increase the production of endogenous porphyrins (Johansen *et al.*, 2003; Ashkenazi *et al.*, 2003). External porphyrins can also be added to increase light sensitivity (Melø, 1987). The bacterium can thus serve as a model

system in studies of photoinactivation of cells as used in so-called photodynamic therapy, PDT (Johansen *et al.*, 2003; Kjeldstad and Johnsson, 1986; Kjeldstad *et al.*, 1986). Most PDT studies published have been performed on eukaryotic cells (often tumor cells), however, PDT investigations on bacterial cells complete and extend the knowledge about light induced porphyrin photosensitization. PDT on *P. acnes in vitro* has been demonstrated (see below) and PDT in clinical tests using both red and blue light is now underway (Hongcharu *et al.*, 2000; Itoh *et al.*, 2000; Papageorgiou *et al.*, 2000).

Illumination of *P. acnes* will excite PPIX (or other porphyrins) in the cells into higher singlet states, which will give rise to both fluorescence emission (Johnsson *et al.*, 1987) and a population of the triplet state (3PPIX), due to intersystem crossing. 3PPIX is a metastable state, which can either donate the excited state energy to a ground state oxygen molecule, whereby a singlet oxygen ($^1O_2^*$) molecule is created, or abstract an electron from a neighbouring molecule, whereby radical species are formed. The first type of process is called a Type II reaction, while an electron transfer process is called a Type I reaction (Ack-

royd *et al.*, 2001). In both cases the result may be lethal to the bacterial cell, and *P. acnes* can be inactivated by the combined action of light and an endogenous photosensitizer (Melø and Reisæter, 1986; Kjeldstad *et al.*, 1988).

In order to investigate and quantify the reaction mechanisms by which *P. acnes* cells are inactivated, the concentrations of absorbing pigments need to be measured. Hence scattering-free absorption spectra of the endogenous porphyrins should be measured. We will show that the porphyrin status of the *P. acnes* cell can, in fact, be estimated from scattering-free absorption measurements using an optically integrating sphere set-up.

Materials and Methods

Materials

The stem *P. acnes* culture, ATCC 6919, was grown anaerobically at 37 °C on blood agar plates, and inoculated on fresh plates once every week. Before each experiment, agar plates were inoculated and the cells were harvested 5 days later. The cells were grown semianaerobically by using Anaerocult A (1.13829 Microbiologie, Merck, Germany), which contains oxygen binding substances. After harvesting, the cells were washed twice with buffer solution, made of KCl (7.46 g, 100 mM), PIPES buffer (piperazine-N,N'-bis[2-ethanesulfonic acid], 6.05 g, 20 mM), distilled water (1 l) and finally adjusted to pH 7.2 by NaOH and sterilized at 120 °C for 20 min. The washing was done using centrifugation ($1400 \times g$) and resuspension in buffer solution until the optical density of the suspension was 1 (OD = 1), corresponding to a cell density of 5×10^8 cells/ml.

Incubation of the cells

The incubation of the cells with δ -aminolevulinic acid (ALA) was done by adding 320 μ g ALA to a 5 ml OD = 1 suspension of *P. acnes*. This was done by preparing a stock solution of 42 mg ALA in 2 ml PIPES buffer, and adding 15 μ l from a fresh stock solution to a 5 ml suspension of *P. acnes* cell in a test tube. The test tubes were then kept at 37 °C for 4 h, and the cells were afterwards washed twice, as above, in buffer solution. The cells were then ready for experiments. In some cases, which will be specified, the cell density was increased by centrifugation and resuspension of the cells in a smaller volume.

Incubation of the cells with PPIX was done by adding small volumes from a stock solution of PPIX to cell suspensions of 5 ml. The stock solution was prepared by adding 2 mg PPIX (Sigma Chemical Company) to 2 ml solution consisting of 0.1 mM NaOH and ethanol in a volume ratio of 1:1. Then the test tubes containing the cells were stored in darkness for 1 h, at room temperature, washed by centrifugation, and finally resuspended before the experiments.

Instrumentation

Absorption spectra were measured using a Hitachi U-3000 UV spectrophotometer equipped with a 15 cm diameter integrating sphere. A cell suspension, not loaded with ALA or PPIX, was used as a reference solution. The sample and reference solutions were given the same treatment, except for the ALA or PPIX loading.

The fluorescence measurements were performed using a Jobin Yvon-SPEX Fluorolog ISA (New Jersey, USA).

Results

In Fig. 1 is shown the absorption spectra from *P. acnes* cells loaded with ALA for four hours, afterwards washed and resuspended in buffer solution. The instrument was equipped with an integrating sphere, and another cell suspension, subjected to the same experimental protocol as the sample solution, except for the ALA-loading, was

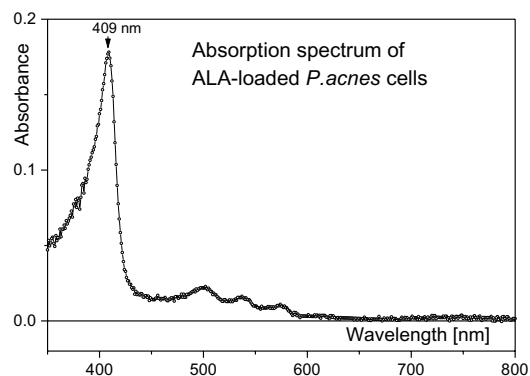


Fig. 1. A scattering free absorption spectrum of *Propionibacterium acnes* in solution, incubated with δ -aminolevulinic acid. As a reference solution was used a suspension of unloaded cells, and the instrument was equipped with an integrating sphere. It is seen that essentially coproporphyrin III was produced by the cells at these conditions.

used as a reference. The absorption spectrum is typical for a free base porphyrin, with a Soret peak at 409 nm. Three of the four Q-absorption bands, with peaks at 500, 539 and 576 nm, are clearly visible. The fourth Q-band with maximum at 611 nm, which is not so prominent, is associated with CP III.

Fig. 2 shows a set of absorption spectra of *P. acnes* cells loaded with different amounts of PPIX. As above, the use of an unloaded cell suspension as reference eliminated scattering and the measured spectra are due to absorption by PPIX in the cells. It will be shown below that any of the measured absorption spectra is a sum of two distinct absorption spectra; one from monomeric and another from aggregated PPIX in the cells, and that the proportion of the two component spectra vary with the amount of added PPIX.

As a start, in Fig. 3 is shown the absorption spectrum of PPIX in ethanol (10 μ l PPIX from a stock solution to 2 ml ethanol) and that of *P. acnes* in suspension for the smallest PPIX addition (2 μ l of PPIX added to 2 ml suspension). The latter spectrum was scaled to equal height as the first in the Soret region. The spectrum of PPIX in ethanol has been displaced by 5 nm to longer wavelengths, and this shift, which was done in the worksheet, made the spectra of PPIX in ethanol and in the cells to coincide. It means that the absorption spectrum of PPIX in the lipid like environments of the cells is shifted by 5 nm to the red, as compared to that of PPIX in ethanol, and that PPIX in the cells is on monomeric form in this case. In

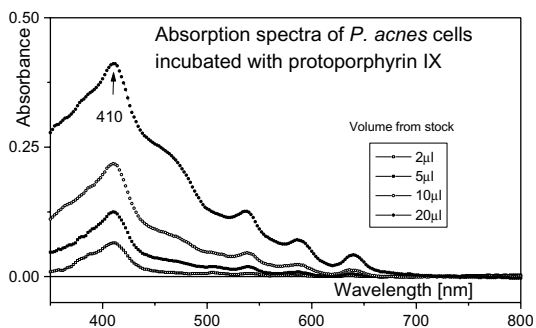


Fig. 2. Scattering free absorption spectra of *Propionibacterium acnes* cells in suspension, for increasing amounts of exogenously added protoporphyrin IX (see figure legend). For the smallest addition, the spectrum is essentially that of PPIX monomers, for higher additions, the overall spectrum is a sum of two spectra, one for PPIX monomers and another for PPIX in aggregate form. The final addition of PPIX, 20 μ l, corresponds to a concentration of 1.67×10^{-5} M.

the data treatment below the shifted spectrum of PPIX in ethanol will be used as a monomer spectrum (A_m).

To find the absorption spectrum of the PPIX aggregates, a difference between two absorption spectra measured from *P. acnes* suspensions (10 μ l minus 5 μ l loading, see Fig. 2) was made. When one of the two spectra was suitably scaled, the Soret peak of the difference spectrum vanished. It was taken to be the spectrum of the aggregates (A_a), and is shown in Fig. 3. It has been subjected to a scaling, so that the sum of the weights of the monomer and the aggregate spectrum in *P. acnes* will equal the amount of PPIX added to the suspension.

It was possible to reproduce any spectrum shown in Fig. 2 as a sum of the monomer and the aggregate spectrum introduced above:

$$A = \alpha A_m + \beta A_a$$

where α and β are the amounts of a monomer- and aggregate spectrum needed to reconstitute the measured absorption spectrum. The calculation of the α and β coefficients was done in a worksheet, using a Levenberg-Marquard iteration procedure, and are shown in Fig. 4 as a function of added PPIX. The residual spectra were in all cases not significant.

By using the scaled aggregate spectrum, introduced above, the sum of the coefficients could in

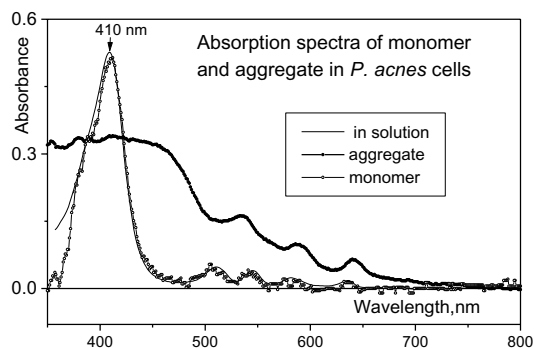


Fig. 3. Absorption spectra of protoporphyrin IX monomers and aggregates in *Propionibacterium acnes*, after exogenous addition of PPIX to the cell suspension. The PPIX monomer spectrum is the scattering free absorption spectrum of *P. acnes* in suspension, for the smallest addition of PPIX. The absorption spectrum of the PPIX aggregates is taken as a difference of the absorption spectra of a high and low loaded *P. acnes* suspension (see the text for further explanation). For a comparison, the absorption spectrum of a PPIX in ethanol is also shown (displaced by 5 nm on the wavelength scale).

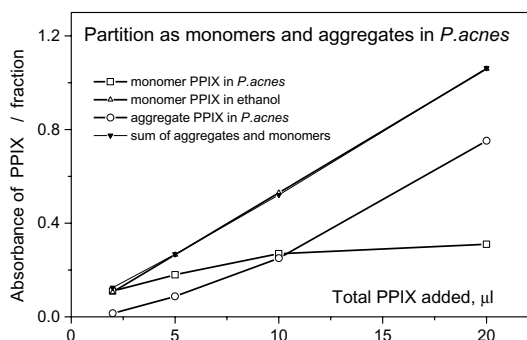


Fig. 4. The amounts of protoporphyrin IX on monomer and aggregate form in *P. acnes* as a function of total PPIX added to the cell. The sum of the amounts is also shown and compared to the total amount of PPIX added to the suspension. The final volume of PPIX added from the stock solution, 20 μl , corresponds to a concentration of $1.67 \times 10^{-5} \text{ M}$ in the cell suspension.

all cases be made equal to that of total PPIX added to the suspension. The amount of PPIX found in the cells was compared to added PPIX according to the following arguments: When PPIX from the stock solution was dissolved in ethanol, only PPIX monomers were formed. In Fig. 4 is the absorbance at the Soret peak of a solution of constant volume (2 ml ethanol, as the cell suspensions) to which increasing volumes of PPIX from the stock solution (same as those added to the cell suspensions) have been added. It is seen that the absorbance of the solution increases linearly with the volume added. Furthermore, Fig. 4 shows that the sum of the amounts of monomer and aggregate PPIX (α and β) coincides with the amount of monomeric PPIX in the ethanol solution in all cases. All PPIX in the cells are hence accounted for.

For increasing PPIX loadings, the scattering efficiency of the *P. acnes* cells became significantly smaller. The scattering spectrum was taken as the absorbance of the cells against buffer solution. When the scattering spectrum was added to the above equation and its weight found by the iteration procedure, it was seen that the scattering efficiency of the cells decreased linearly with total PPIX addition, indicating that the size of the cells became somewhat smaller by the PPIX loading.

Discussion

The scattering free absorption spectrum of *P. acnes* cells incubated with ALA under conditions used is mainly that of coproporphyrin III (CP III).

The biosynthesis pathway of the porphyrinogens from ALA is a sequence of enzyme controlled reactions, and each of the porphyrinogens in the sequence can be non-enzymatically oxidized into the corresponding porphyrin. The distribution of porphyrins among uroporphyrin III (UP III), CP III and PPIX will therefore depend upon the oxygen tension during incubation as well as the length of the incubation period. The semianaerobic incubation conditions used in the present investigations seems optimal for CP III production, judging from the spectra recorded.

The efficiency of PDT is dependent upon several factors; among which the absorption rate of the sensitizer is crucial (Konan *et al.*, 2002). The absorption rate depends both upon the exciting light intensity as well as the concentration of the sensitizer. The lethal action of the sensitizer is dependent upon the presence of a target molecule in the cell, which will be chemically modified by the sensitizer so that the vital cell function of the target molecule is impaired. Hence the sensitizer must be close to the target molecule, and the localization of the photosensitizer is important too. Since the action of the sensitizer is lethal, the knowledge of the number and location of CP III molecules in each cell is important for the understanding of the survival curves of *P. acnes* cells to irradiation.

For red light illumination, only absorbed by the porphyrins, the fraction of surviving cells is exponential versus illumination dose (Kjeldstad and Johnsson, 1986). This shows that only one quantum absorption in a porphyrin molecule is necessary for a lethal action.

The number of porphyrins in a cell can be estimated in the following way. According to Beers law, the concentration of the CP III is:

$$c = \frac{A}{\epsilon \cdot l} = \frac{0.05}{10^5 (\text{M}^{-1} \text{cm}^{-1}) \cdot 1 \text{ cm}} = 5 \cdot 10^{-7} \text{ mol/l} = 5 \times 10^{-10} \text{ mol/ml}.$$

A , the absorbance, is taken at the Soret peak of the ALA-loaded cells (see Fig. 1) and ϵ is the extinction coefficient of CP III at that wavelength. Since the density of cells in the suspension is 10^9 cells/ml, it means that the number of CP III molecules in each cell is:

$$N_{\text{CP III}}(\text{cell}) = \frac{5 \times 10^{-10} \text{ mol/l}}{10^9 \text{ cells/ml}} = 5 \times 10^{-19} N_{\text{A}}/\text{cell} = 3 \times 10^5 \text{ molecules/cell},$$

where N_A is the Avogadro number ($N_A = 6.023 \times 10^{23} \text{ mol}^{-1}$).

The shape of the survival curves versus irradiation dose is exponential (Melø, 1987), which tells that one hit (absorption of one quantum) is needed for cell inactivation. A typical survival time, which is the irradiation time needed for inactivation of an $e^{-1} = 0.37$ fraction of the cells, is:

$$T = 250 \text{ s.}$$

This means that in order to get one hit, the product of k_h , the probability per unit time for a hit, and T should be unity:

$$k_h \cdot T = 1, \quad \text{or} \quad k_h = \frac{1}{T} = 0.004 \text{ s}^{-1}.$$

The probability for a hit can be compared to the probability for an absorption in a porphyrin molecule to take place, which is given by:

$$k_\alpha = (\ln 10) \cdot (I_\alpha) \cdot (\epsilon \cdot 1000 \text{ cm}^2) \cdot (1/N_A) = 2.3 \times 10^3 \times 2.7 \times 10^{17} \times 10^3 / 6.023 \times 10^{23} = 1.03 \times 10^{-2} \text{ s}^{-1}$$

where I_0 , the fluence rate of the illuminator system used in the inactivation experiments on *P. acnes*, was measured to be $2.7 \times 10^{17} \text{ photons/cm}^2\text{s}^{-1}$. Furthermore, the illuminator has a constant intensity over its spectral range, which in the present case was from 585 to 740 nm, set by a dichroic mirror. Hence an averaged extinction coefficient of $10 \text{ M}^{-1}\text{cm}^{-1}$ is used, which is found as the average value of the spectral extinction coefficient of CP III over the illuminator range.

The calculation of k_α is an estimate, however, the two numbers are in accordance with one another. It means that the efficiency of cell inactivation following an absorption event is high:

$$\eta = \frac{k_h}{k_\alpha} = 0.4.$$

It can also be that one out of several porphyrins in *P. acnes* can cause cell inactivation, and in this case the efficiency of each porphyrin is smaller. This means that, if the DNA strand is the target molecule, and if CP III are bound to the DNA molecule at random positions, CP III can act as a photosensitizer at many locations along the strand.

The number of CP III is related to the total number of lipid molecules in the cell can also be

estimated. The number of lipid molecules (N_{lip}) can be assumed to equal twice the surface area of the cell (A_{cell}), which is a double layer, divided by the area occupied both one lipid molecule (A_{lip}):

$$N_{lip} = \frac{2 \cdot A_{cell}}{A_{lip}} = \frac{4\pi \times (10^{-6}\text{m})^2}{0.40(\text{nm})^2} \approx 3 \times 10^7.$$

The cell surface is considered to be that of a sphere with a radius of $1 \mu\text{m}$ and the area occupied by a lipid molecule is set to 0.4 (nm)^2 . It means that the maximum binding capacity of CP III to total lipid molecules is roughly 1:100.

It has been shown earlier that *P. acnes* cells has a limited binding capacity of PPIX monomers (Melø, 1987). The maximum monomer absorbance of PPIX is larger by a factor of three, as compared to CP III, indicating that the binding capacity is larger by the same factor. In contrast to CP III, excess PPIX accumulate in the cells, in the form of aggregates.

When the distances between monomers decrease, and aggregates are formed, interaction between monomer units increases, with the consequence that the energy levels of the monomers split and new energy levels and absorption bands arise. The absorption spectrum of the aggregates formed in this case is well defined, indicating that they consist of a well defined number of monomers with a well defined structure. However, the number of aggregates changes when the total amount of added PPIX increases and, furthermore, the aggregates accumulate inside the cells. The more the cells are loaded with PPIX, the less they scatter in relation to the unloaded cells in the reference solution. Hence, there is a reduction in cell size for increasing additions of total PPIX.

In this report we have quantified the endogenous porphyrins in the *P. acnes* cell under conditions that are of interest in photo inactivation of the cells. This is a part of a continuous study of the photobiophysics of this bacterium that is essential in the acne disease. A better understanding of the photoreactions will lead to a better knowledge of the molecular reactions (Ramstad *et al.*, 1997; Kjeldstad *et al.*, 1991) in the cell leading to inactivation and to an optimization of a clinical use of PDT treatment in the acne disease.

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