Photodestruction of Endogenous Porphyrins in Relation to Cellular Inactivation of *Propionibacterium acnes*

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During growth of *Propionibacterium acnes* on Eagles medium protoporphyrin was accumulated inside the cells and coproporphyrin, both as a free base as metalcontaining, outside the cells. The photochemical processes in the endogenous porphyrins were studied by fluorescence spectroscopy during continuous irradiation of *Propionibacterium acnes* in suspension. The irradiation caused initially an increase in the content of protoporphyrin in the cells in comparison to that which had been accumulated during growth. Maximum light induced protoporphyrin production was achieved in 5 days old cultures. In old cultures where there was practically no initial protoporphyrin release, the fluorescence intensities from all the porphyrins present in the culture vanished exponentially with the irradiation time. The metal containing form of fluorescent coproporphyrin, with a maximum emission at 580 nm, was photobleached about ten times faster than the free base forms of coproporphyrin and protoporphyrin. Among these three fluorescent substances in the cell culture only the free base forms of the porphyrins have longer lifetimes than the cells themselves irradiated at the same conditions.

Introduction

Porphyrins are produced and accumulated in cultures of *Propionibacterium acnes (P. acnes)* growing at semianaerobic conditions [1]. It is established that in vivo fluorescence spectroscopy can be used to identify and quantify the amount of porphyrins in cell cultures [2, 3]. These endogenous porphyrins are involved in blue light photodestruction processes of *P. acnes* [4, 5] and these porphyrins will themselves be photooxidized by the irradiation [6]. In this paper the photooxydation of *P. acnes* porphyrins will be investigated and discussed in relation to photodestruction of P. acnes.

Methods and Materials

A laboratory culture, growing on blood agar, of *P. acnes* (serotype 2, CN 6278) was used. Eagles medium of pH = 6.7 was inoculated and the cells to be used in the experiments were growing at 37 °C in darkness in a semianaerobic atmosphere (about 2% O_2). Before the experiments the cells were harvested and suspended in phosphate buffered saline (PBS) with a pH = 6.7. The optical densities of the cell suspensions, in standard cuvettes, were always lower than 0.3 in order to ensure a nearly constant irradiation intensity in the cuvette.

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In the micelle experiments 25 mg of sodium dodecyl sulphate (SDS) was dissolved in 2 ml distilled water and the concentration of added porphyrin was 10^{-7} M. The porphyrins were from Porphyrin Product (Utah) and the purity of the samples was greater than 97%. The pH changes was brought about by adding small amounts of HCl. The pH value of the solution was measured by a Philips miniaturized pH electrode (type C14/02) in the cuvette.

The fluorescence spectra were recorded by a Spex Fluorolog 222. The emission spectra are uncorrected, however, the photomultiplier sensitivity is rather constant over the actual spectral range. The excitation part of this instrument, equipped with a 450 W Xenon lamp, was also used in the photobleaching experiments. The cells were irradiated at 400 nm and the intensity of the light was 65 $mW \cdot cm^{-2}$ with a bandwidth of 9 nm. The intensity of the lamp was constant during the irradiation experiments so that the actual fluence was simply the product of the intensity given above and the irradiation time (in seconds).

Experiments

Fluorescence from porphyrins in micelles

In order to identify the porphyrin in the bacteria (which will be studied below) with a fluorescence maximum at 612 nm, coproporphyrin III (CP III)

and protophorphyrin IX (PP IX) were incorporated into anionic detergent micelles which solubilize both the free base and the monobase form of the porphyrin [7]. In this system there is a rapid exchange of both porphyrin forms between the water and the lipid phase and the relative amount of the free base to the monobase in the lipid phase, where both these forms of the porphyrin are fluorescent, will depend upon the pH of the water phase [7]. In Fig. 1 the fluorescence spectra for CP III and PP IX in SDS-micelles are shown where both the free bases and the monobases were present. A pH titration of this system was done, and in Fig. 2 the fluorescence intensities of the monobase and the free base form are plotted as a function of pH. The pK values obtained from these experiments were 6.5 for both CP III and PP IX (see Fig. 2), which are somewhat higher than those values obtained by Dempsey [7] on the corresponding fully esterified prophyrins (5.9 for CP and 4.9 for PP).

Another transition in the fluorescence spectra from one form to another occurred during the pH titration experiments. For both CP III and PP IX the positions of the free base fluorescence maxima were shifted by 5 nm towards shorter wavelengths for a small increment in pH at 7. In Figs. 2A and 2B the relative amounts of each of these free base forms (denoted free base 1 and 2) are plotted as a function

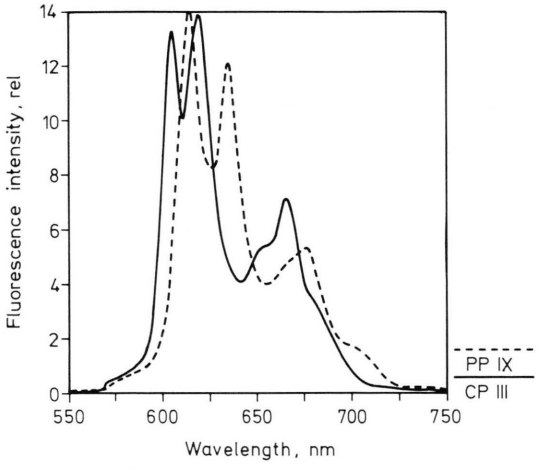

Fig. 1. The fluorescence spectra from coproporphyrin III and protoporphyrin IX in sodium dodecyl sulphate micelles. The pH of the micelle solution was chosen so that both the monobase and the free base porphyrin were present (5.6 for PP and 6.1 for CP). The free base porphyrin had emission maximum at longer wavelength compared to the monobase.

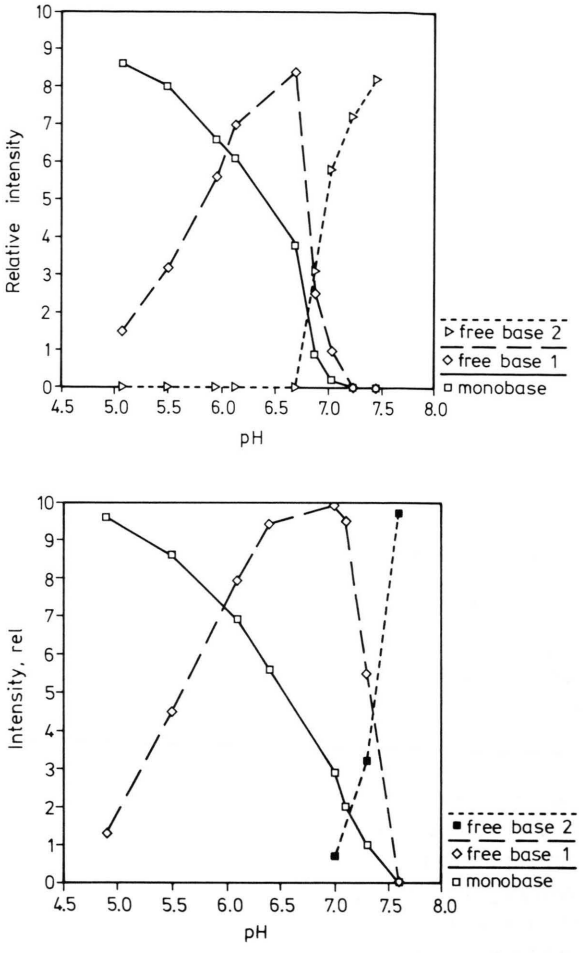

Fig. 2. A pH-titration of coproporphyrin III (2A) and protoporphyrin IX (2B) in sodium dodecyl sulphate micelles. The pK values of the monobases were close to 6.5 in both cases. At about pH = 7 the positions of the free base fluorescence maxima were shifted by 5 nm to shorter wavelengths indicating another transition in the system.

of pH. A possible explanation for the change in the free base fluorescence properties may be that the carboxylic acid side chain of the porphyrin becomes ionized at this pH value.

Fluorescence spectra from bacterial suspensions

The fluorescence spectra from a 2, 4 and 6 days old cell culture are shown in Fig. 3. The fluorescence spectrum from the 6 days old culture is mainly due to PP IX free base in hydrophobic environments, since the emission maximum is at 634 nm. The spectrum with an emission maximum at 612 nm from the

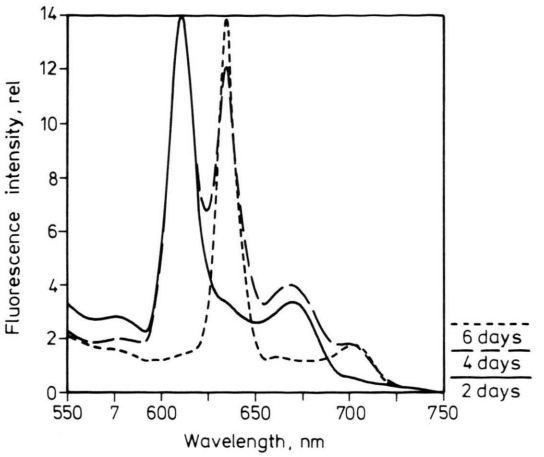

Fig. 3. The fluorescence spectra from a 2, 4 and 6 days old cell culture of *Propionibacterium acnes*. The cells were grown semianaerobically at 37 °C in darkness. During measurements the cells were suspended in phosphate-buffered saline with a pH of 6.7. The young cells contained mainly coproporphyrin III, which had peak fluorescence at 612 nm. Protoporphyrin IX, with fluorescence maximum at 634 nm, was the dominant porphyrin in old cell cultures. The fluorescence spectrum of the supernatant was equal to that of the 2 days old culture.

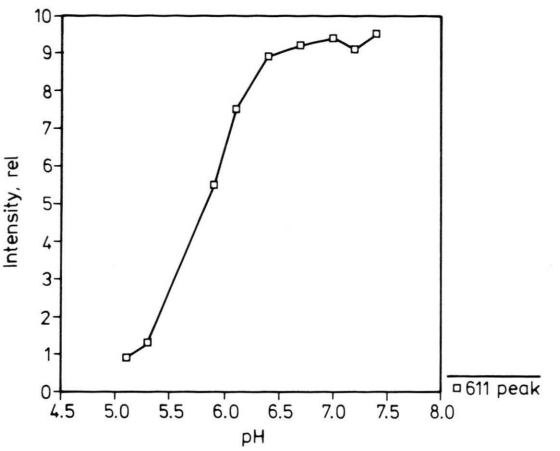

Fig. 4. A pH-titration of a 3 days old cell suspension. The pK value of this system was 5.8 and no monobase fluorescence appeared.

2 days old culture is due to a porphyrin located outside the cells because the same spectrum is obtained from supernatants of the cell suspensions (Fig. 3). According to the micelle experiments above, an emission with a maximum at 612 nm may either be due to CP III free base or to the PP IX monobase, since only these species have fluorescence maxima at approximately this wavelength (Fig. 1). A pH titration of the 2 days old culture in suspension showed that a change in the base form of the present porphyrin took place in the pH range between 5 and 6 (see Fig. 4), where transitions between free bases and monobases are known to take place. This observation excludes the PP IX monobase, mentioned above, as a candidate responsible for the bacterial fluorescence at 612 nm, since no monobase-dibase transition occurs in this pH-interval [7]. The 612 nm fluorescence from the bacteria must therefore be due to the free base form of CP III. The monobase fluorescence of CP III in this case was, however, not fluorescent as in the micelles, since no CP III monobase fluorescence appeared as the pH was lowered.

In the spectra presented here, as well as in previously published ones [3], a third fluorescent component emitting maximally at 580 nm, was present. The

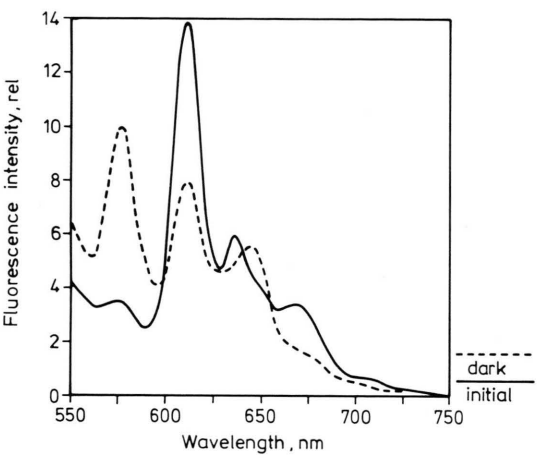

Fig. 5. The fluorescence spectra from a 5 days old cell culture recorded 10 min (−) and 3 h (---) after transfer to PBS-suspension from Eagles medium. The sample was kept in darkness. During that time interval the 612 nm peak had decreased and the 580 nm peak increased.

fluorescence intensity of this component and that of CP III changed in an antiparallell way when the suspensions were kept in darkness (Fig. 5). This indicated that the substance fluorescent at 580 nm is a derivative of CP III.

In Fig. 6 the amounts of CP III and PP IX, measured as the fluorescence intensities at 612 nm and 634 nm, are plotted as a function of the age of the culture.

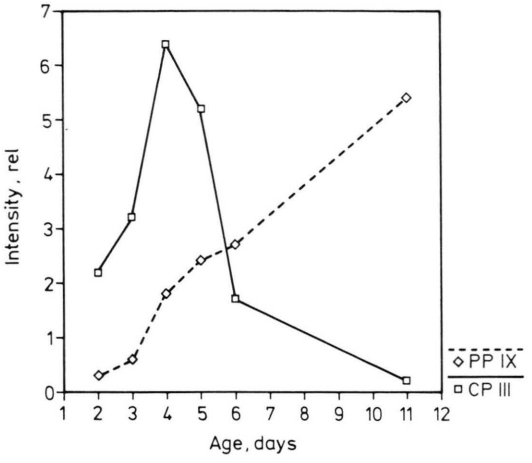

Fig. 6. The amounts of coproporphyrin III and protophorphyrin IX in the culture as a function of age. The fluorescence intensities were divided by the optical density of the suspensions in order to correct for varying cell densities.

Photobleaching of P. acnes porphyrins

Cell cultures of different ages were suspended in PBS and continuously irradiated. The spectrum with the peak at 612 nm then decayed exponentially with time (see Fig. 7). The irradiation caused an initial increase in the 634 nm spectrum which indicates that some PP was released by the light (see Fig. 8). Maximum increase in PP fluorescence was achieved in 5 days old cultures. Prolonged illumination caused

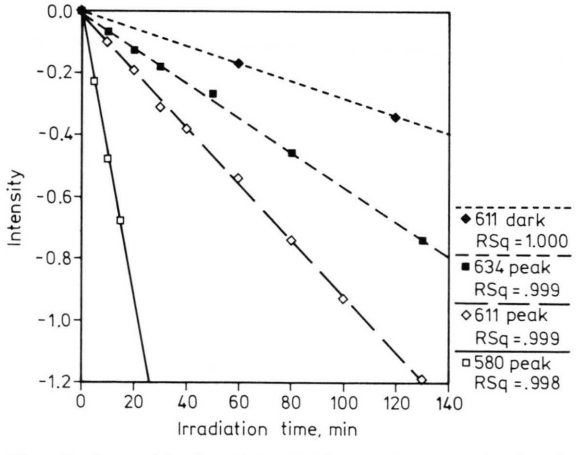

Fig. 7. Logarithmic plot of the various peaks in the fluorescence spectrum from a cell suspension as a function of irradiation time a) decay at 611 nm peak in darkness, b) the 634 nm peak in an old culture, c) the 611 nm peak. The irradiation conditions were in all cases the same.

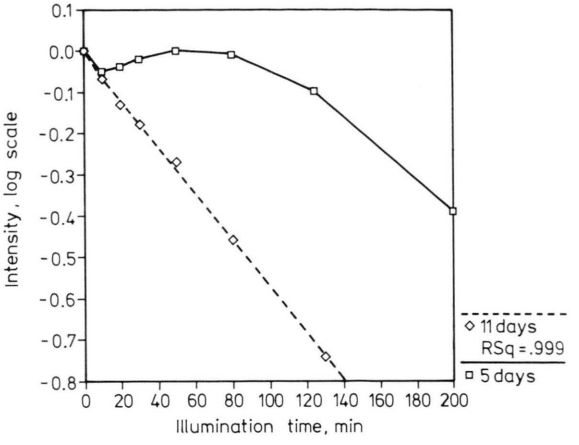

Fig. 8. The amount of fluorescent PP in the cells *versus* irradiation time for a 5 and 11 days old cell culture.

also a photodecomposition of the lightinduced PP and in old cultures, where there was practically no initial photorelease of PP, the amount of PP decayed exponentially with time (see Fig. 7). The 580 nm peak, investigated in an earlier paper [3], was under these irradiation conditions reduced to a fraction of one to ten in 20 min (see Fig. 7).

Discussion

Protoporphyrin was constantly produced and accumulated in cultures of *P. acnes* during growth (see Fig. 6). In the lag phase and early exponential phase CP III was predominantly produced. There was a leakage of synthesized CP III out of the cells and the free base form was bound to SDS-like structures in the cell surroundings. Upon a pH-lowering the free base fluorescence disappeared as in anionic detergent systems, but the monobase fluorescence did, however, not appear, which indicates that the monobase remains in the water phase.

The incorporation of a metal ion in the center of the porphyrin molecule may explain the changes in the fluorescence properties of CP III during darkness. The metal ion increases the probability for intersystem crossing and hence photooxydation as well as removes the degeneracy in the energy levels which explains the shift in the emission maximum from 612 nm to 580 nm [8, 9].

The relation between the light induced production of fluorescent PP and irradiation time has a sigmoidal character (see Fig. 8). The initial delay between produced PP and irradiation time may either indicate

that two or more reaction steps are involved in this photoproduction process or that a diffusion step is involved. A related phenomenon was observed in erythrocytes where PP bound to hemoglobin subsequent to a photorelease migrated out of the cells [10]. In our case the photooxydation may probably release PP bound to the production site in the cells and free PP can diffuse towards the lipid membrane where it becomes highly fluorescent.

The photodecompositions were for all the three porphyrins purely exponential, showing that these reactions are unimolecular. This involves that the singlet oxygen produced by the irradiation attacks the same porphyrin molecule as in which the absorption took place. The participation of oxygen in the photodecomposition has been shown earlier [3, 4]. The reactions leading from absorption to photooxydation of a porphyrin are the following:

$$h\nu + P \xrightarrow{k_a} {}^1P^* \xrightarrow{k_{isc}} {}^3P^* + {}^3O_2 \xrightarrow{k_t} P + {}^1O_2^* \xrightarrow{\alpha} PO_2.$$

The processes are: absorption, intersystem crossing, energy transfer and oxydation. The rate constants for these processes are denoted; k_a, k_{isc}, k_t, respectively, and the probability for oxygenation is α.

Singlet oxygen produced in this way may, alternatively, attack a target molecule denoted T in the cell and the effect of this oxygenation is lethal [4]:

$$T + {}^1O_2^* \xrightarrow{\beta} TO_2.$$

The probability for this process to occur may be denoted β.

From earlier experiments it is known [4] that the fraction of surviving *P. acnes* cells is exponentially related to the blue light irradiation dose. An exponential dependence is typical of a one hit case, which here means that only one photooxygenation of a special target molecule is necessary to kill the cell.

From the first of these equations it is seen that the amount of non-bleached porphyrin will be:

$$P(t) = P_0 \cdot e^{-kt},$$

where P_0 is the initial amount of porphyrin and k denotes the product of the various rate constants involved including the intensity of the exciting light.

When the amount of porphyrin in the cell decreases, because of photooxydation, the probability pr. unit time for photodestruction of a target molecule also decreases. According to the second of the above equations one has:

$$dN_{TO_2}(t)/dt = \beta \cdot {}^1O_2^* = \beta/\gamma \cdot k/\alpha \cdot P_0 \cdot e^{-kt},$$

where γ is the total rate constant for singlet oxygen quenching including α and β. The average number of oxygenated target molecules after a time T of irradiation will be:

$$N_{TO_2}(T) = \int_0^T dN_{TO_2}(t) = \beta/\alpha \cdot \gamma \cdot P_0 \,(1\text{-}\exp(-kT)).$$

It is seen from this relation that in order to have a number greater than one (necessary to kill the cell) the initial concentration of the porphyrin must exceed a critical value, even for infinite irradiation times:

$$\beta/(\alpha \cdot \gamma) \cdot P_0 \geq 1$$

or, for a fixed concentration, that the ratio between the probabilities for porphyrin oxydation to target cell oxydations must be less than a critical value. Furthermore, a deviation form pure exponential survival curves can be predicted for small $\beta P_0/(\alpha\gamma)$ values, and if the concentration is below this critical value only a partial bleaching of the culture is obtained.

The measured lifetime of *P. acnes* cells is greater than that of the endogenous CP III fluorescent at 580 nm irradiated at the same conditions. It is therefore little likely that CP III in this form participates in the photodestruction process of the cells. This substance is simply bleached out before the lethal hit would occur. The two remaining porphyrins can, judged from their bleaching rates compared to rate of cell destruction, may be responsible for the cell killing. Other experiments to be published establish a linear relationship between the intensity of the 634 nm fluorescence and the slope of the survival curves. Such a relation is in line with the simple model outlined above when the amount of porphyrin exceeds a critical value. Furthermore, the existence of a linear relation between the angular slope of the survival curve and the amount of fluorescent PP in each cell confirms that the probability for lethal action (β) is independent of PP concentration. In other words, the migration distance of a singlet oxygen molecule from the creation site to the target is independent of the density of PP entities. This will be the case when the absorbing molecules are located in the cell membrane and that the target molecule is inside the cell so that the migration distance is approximately equal for all singlet oxygen molecules created in the membrane.

[1] W. S. Lee, A. R. Shalita, and M. B. Poh-Fitzpatrick, J. Bacteriol. **133,** 811–815 (1978).
[2] T. B. Melø and M. Johnsson, Dermatologica **164,** 167–174 (1982).
[3] T. B. Melø, G. Reisæter, and A. Johnsson, Z. Naturforsch. **40c,** 125–128 (1985).
[4] B. Kjeldstad, Z. Naturforsch. **39c,** 300–302 (1983).
[5] B. Kjeldstad and A. Johnsson, Photochem. Photobiol. **43,** 67–70 (1986).
[6] G. S. Cox, Ch. Bobillier, and D. G. Whitten, Photochem. Photobiol. **36,** 401–407 (1982).
[7] B. Dempsey, M. B. Lowe, and J. N. Phillips, in: Haematin Enzymes (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), **Vol. I,** p. 29, Pergamon, Oxford 1961.
[8] F. Richelli and L. I. Grossweiner, Photochem. Photobiol. **40,** 599–606 (1984).
[9] R. Margalit and S. Cohen, J. Inorg. Biochem. **25,** 187–195 (1985).
[10] A. Brun and S. Sandberg, Photochem. Photobiol. **41,** 535–541 (1985).