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Correlation between porphyrin biosynthesis and photodynamic inactivation of *Pseudomonas aeruginosa* after incubation with 5-aminolaevulinic acid

Reinhard Sailer [a,*], Wolfgang S.L. Strauss [a], Karsten König [b], Angelika Rück [a], Rudolf Steiner [a]

[a] *Institut für Lasertechnologien in der Medizin und Meßtechnik an der Universität Ulm, Helmholtzstraße 12, 89081 Ulm, Germany*

[b] *Institut für Anatomie II, Universität Jena, Teichgraben 7, 07740 Jena, Germany*

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Abstract

The porphyrin biosynthesis of *Pseudomonas aeruginosa* was examined with respect to porphyrin and porphyrinogen formation using fluorescence spectroscopy and high performance liquid chromatography (HPLC) techniques. The porphyrin and porphyrinogen profiles, as well as the fluorescence spectra, were determined for different growth phases and after incubation with 5-aminolaevulinic acid (5-ALA). The fluorescence maxima were attributed to coproporphyrin (618 nm and 685 nm) or protoporphyrin (635 nm and 703 nm). Incubation of *Pseudomonas aeruginosa* with 5-ALA led to enhanced protoporphyrin accumulation as confirmed by fluorescence measurements and HPLC analyses. In addition to protoporphyrin synthesis, 5-ALA was mainly converted into non-fluorescing and photodynamically inactive porphyrinogens. Coproporphyrinogen was found to be the predominant substance. In addition, uroporphyrinogen, 7-carboxyporphyrinogen, 6-carboxyporphyrinogen and 5-carboxyporphyrinogen were determined. Consequently, the photodynamic inactivation of *Pseudomonas aeruginosa* was almost negligible after incubation with 5-ALA and irradiation with laser light at 630 nm. © 1997 Elsevier Science S.A.

Keywords: Fluorescence spectroscopy; HPLC; Photodynamic therapy; Porphyrinogens; Porphyrins; *Pseudomonas aeruginosa*

1. Introduction

The photodynamic inactivation of bacteria with exogenously applied photosensitizers was first described in 1904 by Jodlbauer and von Tappeiner [1]. In 1924, Passow and Rimpau [2] examined the photodynamic treatment of Gram-negative and Gram-positive bacteria with various photosensitizing dyes. These experiments showed a significantly lower inactivation of Gram-negative bacteria in comparison with Gram-positive bacteria. This observation was confirmed by several other workers [3,4]. The resistance of Gram-negative bacteria to photodynamic treatment as well as to many antibiotics was assigned to the outer membrane which serves as a permeation barrier towards lipophilic substances [5]. The disruption of the barrier function using the polycationic agent polymyxin B nonapeptide leads to an uptake of photosensitizers and, subsequently, to the inactivation of Gram-negative bacteria after photodynamic treatment [6]. In contrast, specific antibacterial photosensitizer immunoconjugates and cationic photosensitizers exhibit photodynamic activity against Gram-negative bacteria without additional disturbance of the outer membrane [7–10]. Moreover, endogenous porphyrins reduce the viability of bacteria after irradiation. *Propionibacterium acnes*, a porphyrin-accumulating Gram-positive bacterium, was inactivated considerably using near-UV light [11].

To enhance the biosynthesis of porphyrins, 5-aminolaevulinic acid (5-ALA), a naturally occurring intermediate of the biosynthetic pathway, has been applied. Recently, this compound has successfully been established as a new photosensitizing agent or prodrug for the photodynamic therapy of cancer [12]. Using 5-ALA, the rate-limiting enzyme in porphyrin biosynthesis, 5-ALA-synthetase, can be circumvented. 5-ALA is metabolized via several intermediates to the fluorescing, photodynamically active compound, protoporphyrin (PP), which itself serves as a precursor for cytochromes. For several Gram-positive [13,14] and Gram-negative [15,16] bacteria, enhanced porphyrin biosynthesis was shown after 5-ALA supplementation of the culture medium. For *Escherichia coli* strains, defective in 5-ALA synthesis, incubation with 5-ALA and subsequent irradiation with near-UV or blue light led to a pronounced

* Corresponding author.

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reduction in viability [17,18]. This indicates that endogenous porphyrins may be useful for the photodynamic inactivation of Gram-negative bacteria.

The aim of this study was to correlate porphyrin biosynthesis and photodynamic inactivation of the Gram-negative bacterium *Pseudomonas aeruginosa* after incubation with 5-ALA. Porphyrin biosynthesis was probed by high performance liquid chromatography (HPLC) analyses (considering porphyrinogen and porphyrin formation) and fluorescence spectroscopy.

2. Materials and methods

2.1. Chemicals

5-ALA was obtained from Fluka (Buchs, Switzerland). Solutions were freshly prepared in phosphate-buffered saline (PBS) and the pH was adjusted to 7.4. After sterilization by filtration using a 0.22 μm filter (Millipore, Eschborn, Germany), solutions were added aseptically to the culture medium.

2.2. Bacteria and culture conditions

Pseudomonas aeruginosa cells (DSM-no. 1117; kindly supplied by Prof. Dr. R. Marre, Abteilung für Medizinische Mikrobiologie und Hygiene, Universität Ulm) were grown aerobically in Trypticase Soy Broth (TSB) (Difco, Detroit, MI, USA) in 250 ml Erlenmeyer flasks at 28 °C. Cultures were agitated in a horizontally oscillating incubator (120 rev min^{-1}). Bacterial growth was determined by turbidity measurements using the transmission at 578 nm.

2.3. Fluorescence measurements

Bacteria were harvested by centrifugation (9000 rev min^{-1}; 10 min), washed twice with ice cold PBS and finally pelleted by centrifugation (9000 rev min^{-1}; 10 min) in plastic centrifuge tubes. Fluorescence excitation was performed using a Kr^+ laser (Spectra Physics, Mountain View, CA, USA) (407 nm; 8 mW cm^{-2}). Emission spectra were recorded using a fibre-optical sensor in combination with a polychromator and an optical multichannel analyser (ZWG, Berlin, Germany) [19].

2.4. HPLC

HPLC analyses were carried out using a Waters 600 multisolvent delivery system (Millipore Corporation, Milford, MA, USA) in combination with a spectrofluorometer (Kontron SFM 25, Neufahrn, Germany). Determinations were performed using a reverse-phase column (Nucleosil 120 $5C_{18}$; 4 mm × 250 mm; 5 μm particle size; Macherey und Nagel, Düren, Germany) and the ion pair technique (adapted from Ref. [20]). The column was eluted using a multilinear gradient beginning with 55% of solvent A (water, containing 40 mM sodium dihydrogenphosphate, adjusted to pH 5.6) and 45% of solvent B (methanol, containing 12.5 mM tetrabutylammonium phosphate (TBP), adjusted to pH 7.3), and ending with 2% of solvent A and 98% of solvent B after 40 min. The flow rate was 1.0 ml min^{-1}. The fluorescence was excited at 400 nm and detected at 625 nm. Individual porphyrins were quantified using an external standard mixture of free porphyrin carboxylic acids containing the I isomers of uroporphyrin (UP), 7-carboxyporphyrin (7-COOH), 6-carboxyporphyrin (6-COOH), 5-carboxyporphyrin (5-COOH) and coproporphyrin (CP) as well as mesoporphyrin IX. In addition, uroporphyrin III and coproporphyrin III isomers, as well as protoporphyrin IX (PP), were used as external standards. All porphyrins were obtained from Porphyrin Products (Logan, UT, USA). For this investigation, the I and III isomers of UP and CP were not separated.

2.5. Determination of the porphyrinogen and porphyrin profiles

Cells were harvested by centrifugation (9000 rev min^{-1}; 10 min), washed twice with ice cold PBS and finally pelleted by centrifugation (9000 rev min^{-1}; 10 min). Samples were stored at −20 °C in an argon atmosphere to avoid unspecific oxidation of porphyrinogens by oxygen. Porphyrinogens and porphyrins were extracted almost anaerobically using 200 μl of methanol (containing 50 mM TBP) and acetone (1:1, v/v). Samples were treated with ultrasonic waves for 1 min and stored in the dark for 10 min. Finally, precipitated protein was separated by centrifugation (10 000 rev min^{-1}; 5 min). Porphyrins within the supernatant were quantified immediately after extraction. A second determination of the identical sample was performed after oxidation with 20 μl of Lugol solution [21]. The amount of porphyrinogens was calculated by subtracting the amount of porphyrins determined in oxidized samples from the amount of porphyrins in non-oxidized samples. The extraction efficiency ranged from 80% (UP) to 95% (CP) and was determined by a second extraction of identical samples. The dry weight was obtained from unprocessed samples after drying for 12 h at 105 °C.

2.6. Photodynamic treatment

Bacteria in the growth or stationary phase (18 h or 36 h after inoculation) were incubated with 5-ALA (0.2 mM) 2.5 h prior to irradiation. Aliquots were harvested by centrifugation (9000 rev min^{-1}; 10 min), washed twice with ice cold sterile PBS and finally transferred into sterile four-well multidishes (Nunc, Wiesbaden, Germany). Irradiation was performed at 630 nm using an Ar^+ laser-pumped dye laser system (Ar^+ laser, Meditec, Erlangen, Germany; dye laser, Spectra Physics, Mountain View, CA, USA) with a power density of 100 mW cm^{-2} and an energy density up to 400 J cm^{-2}. To determine the survival rates, aliquots of cell suspensions were taken before and after irradiation, diluted in

sterile PBS and spread out onto TSB agar plates. The number of colony forming units (CFU) was determined after an incubation period of 48 h at 28 °C. The survival fractions were calculated for each treatment according to the equation N/N_0 = survival fraction (where N is the number of CFU of irradiated cultures and N_0 is the number of CFU of untreated controls).

3. Results

3.1. Porphyrin biosynthesis of *Pseudomonas aeruginosa*

The porphyrin profiles of *Pseudomonas aeruginosa* cells were determined every 6 h after inoculation. Bacteria started to grow without a detectable lag phase; 30 h after inoculation, the optical density was rather constant, indicating the stationary phase of the cultures. During the early growth phase (up to 18 h after inoculation), increasing amounts of CP and PP (up to 5 pmol (mg dry weight)$^{-1}$) were found as determined by HPLC analyses of non-oxidized samples. Consistently, the fluorescence spectra recorded 12 h after inoculation exhibited maxima at 618 nm and 684 nm as well as at 635 nm and 703 nm corresponding to CP and PP (Fig. 1). During the stationary phase, the amounts of CP and PP decreased below 1.5 pmol (mg dry weight)$^{-1}$ and they were not detectable by fluorescence spectroscopy. In contrast, CP was the predominant porphyrin within oxidized samples during the whole period of cultivation, indicating the large amount of coproporphyrinogen within the bacteria. The largest amount of CP (47 pmol (mg dry weight)$^{-1}$) was found 6 h after inoculation. The amounts of PP within the non-oxidized and oxidized samples remained unchanged, indicating the absence of protoporphyrinogen in the samples. In addition, traces of UP, 7-COOH and 6-COOH (less than 1 pmol (mg dry weight)$^{-1}$) were detected within oxidized samples. The results of porphyrin determination are depicted in Fig. 2.

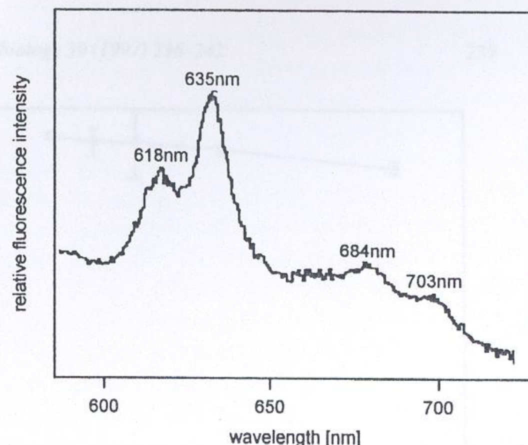

Fig. 1. Fluorescence spectrum of *Pseudomonas aeruginosa* during the early growth phase (12 h after inoculation; λ_{ex} = 407 nm).

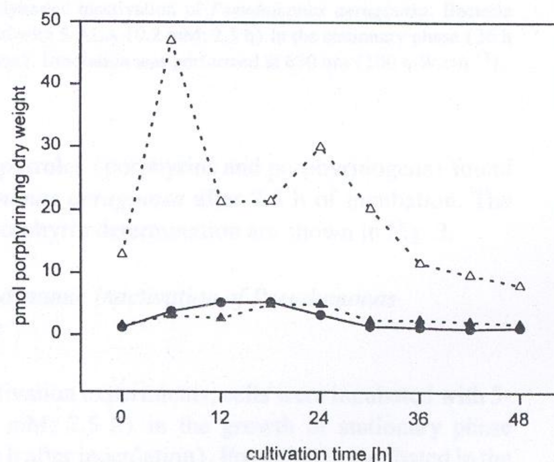

Fig. 2. Porphyrin biosynthesis of *Pseudomonas aeruginosa* during the growth phase (0–30 h) and the stationary phase (30–48 h): ▲, CP determined in non-oxidized samples; ●, PP determined in non-oxidized and oxidized samples; △, CP determined in oxidized samples.

3.2. Porphyrin biosynthesis of *Pseudomonas aeruginosa* during incubation with 5-ALA

The porphyrin profiles of *Pseudomonas aeruginosa* were determined every 30 min up to an incubation period of 2.5 h. Incubation (0.2 mM 5-ALA) started 18 h after inoculation. HPLC analyses of non-oxidized samples revealed increasing amounts of PP with increasing incubation time. After 2.5 h, about 27 pmol PP (mg dry weight)$^{-1}$ was determined compared with 2.5 pmol (mg dry weight)$^{-1}$ for the unincubated controls (Figs. 3 and 4(a)). In addition, minor amounts of CP and traces of UP, 7-COOH, 6-COOH and 5-COOH were found (Fig. 4(a)). Consistently, all cultures examined by fluorescence spectroscopy exhibited fluorescence maxima at 635 nm and 703 nm. The fluorescence intensity increased with increasing incubation time up to 2.5 h. Cultures observed up to 6 h retained constant fluorescence intensities until the

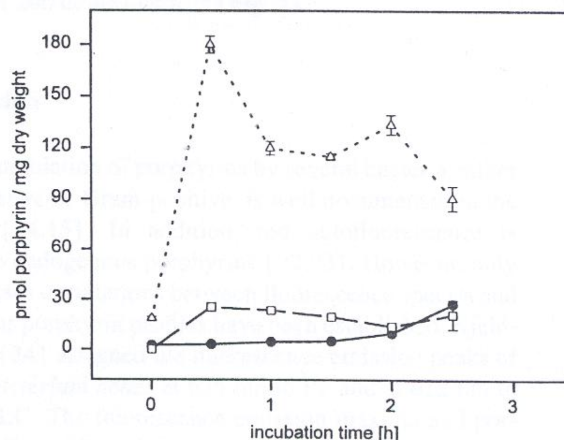

Fig. 3. Porphyrin biosynthesis of *Pseudomonas aeruginosa*; bacteria were incubated with 5-ALA (0.2 mM) in the growth phase (18 h after inoculation): △, CP determined in oxidized samples; □, UP determined in oxidized samples; ●, PP determined in non-oxidized and oxidized samples.

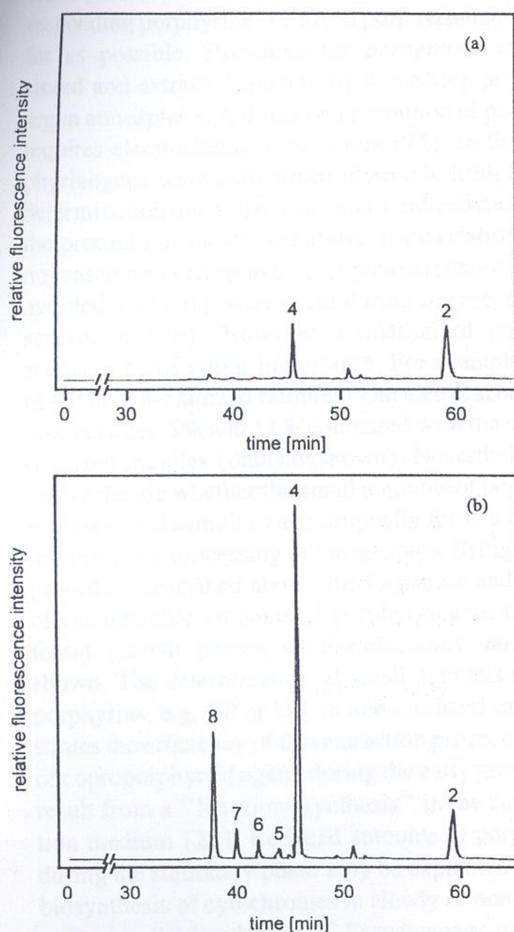

Fig. 4. High performance liquid chromatogram of a non-oxidized (a) and oxidized (b) extract of *Pseudomonas aeruginosa*; bacteria were incubated with 5-ALA (0.2 mM; 2.5 h) in the growth phase (18 h after inoculation): 8, UP; 7, 7-COOH; 6, 6-COOH; 5, 5-COOH; 4, CP; 2, PP.

end of the incubation period. Concomitantly, no spectral changes were observed (data not shown).

In contrast, CP was again the main component within oxidized samples during the whole incubation period. The highest amount of CP was determined after 30 min of incubation (180 pmol (mg dry weight)$^{-1}$). In addition, considerable amounts of UP were detected with a maximum (25.3 pmol (mg dry weight)$^{-1}$) after 30 min of incubation. Moreover, minor amounts of 7-COOH, 6-COOH and 5-COOH, less than 10 pmol (mg dry weight)$^{-1}$, were found. Fig. 4(b) shows the high performance liquid chromatogram of the oxidized sample from cells incubated for 2.5 h. Taken together, the biosynthesis of large amounts of several porphyrinogens was demonstrated. A similar temporal pattern was found for all porphyrinogens, with a maximum accumulation after 30 min of incubation and a moderate decrease until the end of the incubation period. The amounts of PP within non-oxidized and oxidized samples remained unchanged, again indicating the absence of protoporphyrinogen in the samples. The portion of photodynamically active PP was 19% at best of all the

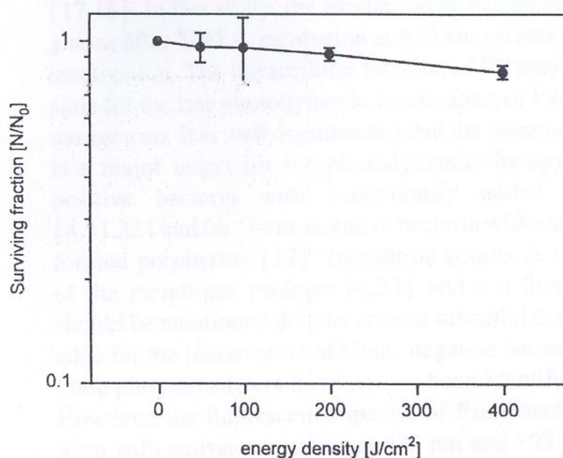

Fig. 5. Photodynamic inactivation of *Pseudomonas aeruginosa*. Bacteria were incubated with 5-ALA (0.2 mM; 2.5 h) in the stationary phase (36 h after inoculation). Irradiation was performed at 630 nm (100 mW cm^{-2}).

cyclic tetrapyrroles (porphyrins and porphyrinogens) found in *Pseudomonas aeruginosa* after 2.5 h of incubation. The results of porphyrin determination are shown in Fig. 3.

3.3. Photodynamic inactivation of *Pseudomonas aeruginosa*

For inactivation experiments, cells were incubated with 5-ALA (0.2 mM; 2.5 h) in the growth or stationary phase (18 h or 36 h after inoculation). For cultures incubated in the growth phase, as well as for unincubated controls, no reduction of CFU was observed after irradiation with energy densities up to 400 J cm^{-2}. Only cultures incubated in the stationary phase exhibited a weak inactivation to 75% compared with incubated and unirradiated controls using energy densities of 200 or 400 J cm^{-2} (Fig. 5).

4. Discussion

The accumulation of porphyrins by several bacteria, either Gram-negative or Gram-positive, is well documented in the literature [14,15]. In addition, red autofluorescence is assigned to endogenous porphyrins [22,23]. However, only in a few cases correlations between fluorescence spectra and intracellular porphyrin profiles have been established. Kjeldstad et al. [24] assigned the fluorescence emission peaks of *Propionibacterium acnes* at 635 nm to PP and at 612 nm to CP by HPLC. The fluorescence emission maxima and porphyrin profiles of *Pseudomonas aeruginosa* observed in this study correspond well to these results. However, the correlation between the fluorescence emission spectra and HPLC analyses of porphyrins requires caution. It must be taken into consideration that biological samples may contain fluorescing porphyrins as well as non-fluorescing porphyrinogens simultaneously. Commonly used extraction procedures include the

esterification of porphyrins, multisolvent partitioning or solid phase extraction [25,26]. These time-consuming procedures are expected to enhance porphyrinogen oxidation to the corresponding porphyrins. To avoid porphyrinogen oxidation as far as possible, *Pseudomonas aeruginosa* samples were stored and extracted quickly by a one-step procedure in an argon atmosphere. A direct determination of porphyrinogens requires electrochemical detectors [27]. In this study, porphyrinogens were determined indirectly from the porphyrin determination of oxidized and non-oxidized samples. Despite the precautions mentioned above, the oxidation of porphyrinogens to the corresponding porphyrins cannot be completely avoided. Oxidation may occur during storage, extraction and separation [26]. However, oxidation of porphyrinogens seems to be of minor importance. For example, the amount of CP in non-oxidized samples of an identical bacteria culture was between 5% and 11% compared with the corresponding oxidized samples (data not shown). Nevertheless, it is difficult to decide whether the small amounts of porphyrins in the non-oxidized samples were originally formed by the bacteria or during the processing of the samples. Using the extraction procedure described above, the formation and accumulation of considerable amounts of porphyrinogens during the different growth phases of *Pseudomonas aeruginosa* were shown. The determination of small amounts of hydrophilic porphyrins, e.g. CP or UP, in non-oxidized samples demonstrates the efficiency of this extraction procedure. High levels of coproporphyrin(ogen) during the early growth phase may result from a "luxurious synthesis" in the complex cultivation medium [28]. Reduced amounts of porphyrin(ogens) during the stationary phase may be explained by the reduced biosynthesis of cytochromes in slowly or non-dividing cells.

During the incubation of *Pseudomonas aeruginosa* with 5-ALA, an increase in PP fluorescence was measured. HPLC analyses of non-oxidized samples confirmed that PP was the most abundant porphyrin. In addition, minor amounts or traces of hydrophilic porphyrins were detected. As deduced from determinations of oxidized samples, high amounts of porphyrinogens, mainly coproporphyrinogen and uroporphyrinogen, and minor amounts of 7-carboxyporphyrinogen, 6-carboxyporphyrinogen and 5-carboxyporphyrinogen were found. An accumulation of protoporphyrinogen was not observed. This indicates that 5-ALA was mainly converted into non-fluorescing and photodynamically inactive compounds. The accumulation of porphyrin intermediates in *Pseudomonas aeruginosa* cells after incubation with 5-ALA has previously been published [15], but porphyrinogens and porphyrins were not analysed separately. The conversion of porphyrinogens to PP seems to be ineffective. One explanation may be that the conversion of coproporphyrinogen to protoporphyrinogen is a rate-limiting step in bacterial porphyrin biosynthesis [29]. Furthermore, the excretion of hydrophilic porphyrinogens into the culture medium has been suggested [15,30].

Previous studies have shown the successful inactivation of Gram-negative bacteria using endogenous sensitizers and near-UV light. *Escherichia coli* cells with deficient porphyrin biosynthesis were incubated with the porphyrin precursor 5-ALA and, subsequently, a pronounced inactivation was found [17,18]. In this study, the irradiation of *Pseudomonas aeruginosa* after 5-ALA incubation at 630 nm caused only minor inactivation. The intracellular location of PP may be responsible for the low photodynamic inactivation of *Pseudomonas aeruginosa*. It is well documented that the plasma membrane is a major target for the photodynamic therapy of Gram-positive bacteria with exogenously added porphyrins [4,31,32] and for Gram-negative bacteria with endogenously formed porphyrins [17]. Irradiation results in crosslinking of the membrane proteins [4,31] and ion fluxes [33]. It should be mentioned that the critical subcellular sites responsible for the inactivation of Gram-negative bacteria with cationic photosensitizers have not yet been identified [10,34]. However, the fluorescence spectra of *Pseudomonas aeruginosa* with emission maxima at 635 nm and 703 nm indicate that PP is located within a lipophilic environment [35], e.g. the plasma membrane.

One reason for the weak inactivation may be the weak absorption of PP in the red spectral region relative to the absorption around the Soret band. Even for *Propionibacterium acnes*, which is known to accumulate high amounts of PP [24], inactivation with red light requires high light doses to obtain a significant reduction in viability [36]. For *Escherichia coli*, similar results were obtained [37]. Moreover, a rapid photobleaching of porphyrins, which has been described for metalloporphyrins in *Propionibacterium acnes* [35], may lead to a fast deprivation of porphyrins within the cells. However, the photobleaching rates of PP correspond to the rate of cell destruction of *Propionibacterium acnes* [35]. In addition, small amounts of photosensitizer have been suggested to result in a sublethal damage of bacteria and tumour cells [35,38]. If small amounts of photosensitizer and photobleaching coincide, it is expected that inactivation will become negligible.

Therefore the formation of large amounts of photodynamically inactive porphyrinogens, together with a weak accumulation of photodynamically active PP, seems to be responsible for the weak inactivation of *Pseudomonas aeruginosa*. To obtain significant inactivation on incubation with ALA, variations in the culture conditions to enhance PP formation may be useful in vitro. Alterations of pH, oxygen pressure, availability of iron and energy or carbon sources are known to affect porphyrin biosynthesis [11,28,39]. Another possibility may be the use of "porphyrinogenic" agents, e.g. thiols [40].

Bacterial inactivation after incubation with 5-ALA can be assigned to endogenous porphyrins. Fluorescence spectroscopy seems to be a useful tool to monitor porphyrin formation. This method can be applied in vitro and in vivo. Moreover, accompanying in vitro investigations of porphyrin biosynthesis, considering both fluorescing porphyrins and non-fluorescing porphyrinogens, are desirable. The two-step procedure described above, successive porphyrin

determination of non-oxidized and oxidized extracts of the same sample, fulfils this requirement sufficiently. Therefore the formation and accumulation of photodynamically active porphyrins and photodynamically inactive porphyrinogens are determined quantitatively.

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