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In situ detection of ALA-stimulated porphyrin metabolic products in *Escherichia coli* B by fluorescence line narrowing spectroscopy

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Abstract

In a recent work [Photochem. Photobiol. B: Biol. 50 (1999) 8] the successful photodynamic inactivation of *Escherichia coli* bacteria by visible light was reported based on δ-aminolevulinic acid (ALA)-induced endogenous porphyrin accumulation. In this work, the identification of these porphyrin derivatives in intact bacteria was performed by low-temperature conventional fluorescence and fluorescence line narrowing (FLN) techniques. Conventional fluorescence emission spectroscopy at cryogenic temperatures revealed the presence of the free-base porphyrins, identified earlier by high-performance liquid chromatography analysis of disintegrated bacterial cells after ALA induction; however, emission maxima characteristic for metal porphyrins were also observed. We demonstrated that the primary reason for this signal is that metal porphyrins are formed from free-base porphyrins by Mg^{2+} ions present in the culturing medium. Incorporation of Zn ions originating from the glassware could also be supposed. In the FLN experiment, the energy selection effect could be clearly demonstrated for (0,0) emissions of both the free-base and the metal porphyrins. The comparison of the conventional emission spectra and the bands revealed by the FLN experiment show that the dominant monomeric structural population is that of metal porphyrins. The intensity and the shape of the FLN lines indicate an aggregated population of the free-base porphyrins, beside a small monomeric population.

Keywords: Photodynamic inactivation; Bacteria; Fluorescence line narrowing; Inhomogeneous distribution function; Fluorescence spectroscopy; *Escherichia coli*

1. Introduction

It has been known for a long time that free-base porphyrin derivatives can be used as photosensitisers in photodynamic therapy (PDT). As haem biosynthesis involves free-base porphyrins, as metabolites, it has been suggested [2] to influence the cellular metabolism so that the amount of several endogenous porphyrin derivatives could be augmented in the cells and thus PDT can be based on their presence [3]. δ-aminolevulinic acid (ALA) was shown to effectively induce porphyrin synthesis [4], thus ALA-induced porphyrin photosensitisation became a method of PDT of cancerous tissues [5–7] and was also reported to be effective in the photodynamic inactivation (PDI) of bacterial cells [8–10].

Escherichia coli bacteria are resistant to direct photosensitised destruction by exogenous effects unless pre-treatment by chemical/biochemical agents makes the penetration possible across the cell wall [9,11,12].

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In a recent paper, we reported the successful inactivation of *E. coli* B bacteria by ALA-induced porphyrin photosensitisation and visible irradiation [1]. We also identified the free-base porphyrins by spectroscopy and high-performance liquid chromatography (HPLC) analysis of disintegrated cells after ALA induction. The efficiency of the PDI of cells strongly depends on the localisation of photoactive porphyrin derivatives inside the cells [13]. In the present studies we used the sensitivity of low-temperature fluorescence spectroscopy and fluorescence line narrowing (FLN) to obtain information about the structural organisation of the porphyrin derivatives inside intact *E. coli* cells.

The first FLN studies on biological systems were reported on haem proteins and photosynthetic pigments [14–16]. The possibility to measure in vivo biological tissues with the FLN method was first demonstrated by Avarmaa and co-workers [17] on etiolated leaves. Novikov reported line-narrowed fluorescence of porphyrins from rat muscle tissues detected in vivo [18]. FLN spectroscopy and its application for the study of proteins have been recently reviewed [19].

The FLN measurement is performed at cryogenic temperatures. The line narrowing effect is based on the idea that a specific chromophore in a given amorphous matrix (now cell compartments of bacteria) will experience a distribution of frozen environments at low temperature [20] that conserve the structural inhomogeneities of the matrix at the moment of freezing. This inhomogeneity is the reason for the significant broadening of the spectral bands of chromophores observed in amorphous matrices, even at low temperatures. The effect is verified if laser excitation is able to select a sub-population of chromophores of identical electronic transition energies at low temperature [21], and thus sharp lines of one specific sub-population can be detected. The resolved emission peaks should then simultaneously shift with the change of the excitation frequency, since at every excitation, another sub-population of identical chromophores (i.e., identical within the spectral width of the exciting laser light) in slightly different environments is selected for emission. Experimental evidence shows [19] that this is also the case of porphyrin derivatives embedded in proteins under isolated conditions (e.g., in monomeric haemoproteins). If, however, the chromophores are close to each other, energy transfer may broaden the spectral lines, so that line-narrowed spectra cannot be detected. This effect was used to monitor the structural organisation of protochlorophyllide forms in etiolated leaves [17,22]. If the energy selection effect can be demonstrated and line spectra are observed, these can be used to determine vibrational energies in the ground and first excited electronic state, and also to determine the inhomogeneous distribution function (IDF) of the (0,0) emission energies, that is, the true (0,0) band shape at low temperature, free of the distorting effect of phonon interaction and of temperature effects [19].

In this work, we report the conventional fluorescence spectra of intact *E. coli* bacteria at 77 K after ALA treatment. We show that beside the free-base porphyrins detected earlier by HPLC analysis, metal porphyrins are also present in the bacteria. We give a possible interpretation of the origin of such spectra based by comparative analysis: complex formation with Mg/Zn ions of the culturing medium could be supposed. We also report the results of FLN studies of the bands in the conventional spectra. The FLN spectra also yielded the vibrational energies of the porphyrins; the agreement with literature data supports the validity of the present studies.

2. Materials and methods

2.1. Sample preparation

The porphyrin derivatives were purchased from Porphyrin Products (Logan, UT). Stock solutions were prepared by dissolving the porphyrin powders in methanol (spectral grade, Merck, Germany). Porphyrin solutions were prepared by diluting the stock solutions in phosphate buffer (PBS, containing 0.013% $MgSO_4$, 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4 w/w, pH 7.4) and stirring at room temperature for a few minutes. The solutions were stored at 4°C. Prior to the measurements, glycerol was added (1:1 v/v) to the porphyrin solutions and then placed into a cylindrical cuvette for the measurements.

The induction of porphyrin synthesis by δ-aminolevulinic acid (ALA) in *E. coli* bacteria was performed as described earlier [1]. *E. coli* B cells were grown aerobically at 37°C in brain heart infusion

broth (Difco) completed with 0.5% NaCl. The overnight culture was transferred into a fresh medium and was grown to log phase. This culture was centrifuged and transferred into a synthetic medium (PBS supplemented with 0.3% glucose and 0.3% vitamin assay casamino acids (Difco), pH 7.4) with a final concentration of 10^7 cells ml^{-1} and grown. The growth of the bacterial culture was detected by turbidimetry based on the apparent optical density at 500 nm caused by light scattering. When the cell concentration reached 2×10^7 cells ml^{-1}, ALA was added to the culturing medium in a concentration of 5×10^{-3} mol l^{-1} from a stock solution dissolved in PBS, pH 7.4, and the cells were incubated at 37°C for 30 min. At the end of the incubation period, the bacterial cultures were harvested by centrifugation ($2000\times g$ for 10 min) and the cells were separated from the culturing medium. The bacterial cells were suspended in glycerol of spectroscopic grade, placed into a cylindrical cuvette and kept at −30°C until the spectroscopic measurements took place.

2.2. Conventional spectroscopy

Conventional fluorescence spectra were obtained with a FS900CD fluorimeter (Edinburgh Analytical Instrument, UK) equipped with a Xenon-lamp and a cooled Hamamatsu R955 photomultiplier tube as detector. The resolution of the fluorimeter was 0.5 nm. The fluorescence emission spectra were corrected for the spectral sensitivity of the detector. Temperature was adjusted by a closed-cycle He cryostat M22 (Cryophysics, Geneva, Switzerland).

2.3. Fluorescence line narrowing spectroscopy

High-resolution emission spectra were recorded with a fluorescence line narrowing (FLN) set-up. Excitation was achieved by a Coherent 899-01 tuneable dye laser with Rhodamine-560 and Rhodamine-590 dyes, pumped by a continuous-wave Coherent Innova-307 argon-ion laser (Palo Alto, CA). The power of the laser beam was attenuated to 1–2 mW by neutral filters. The spectral width of the laser was 0.5 cm^{-1} (0.02 nm at 570 nm). The emission spectra were measured at 90° from the excitation light by a THR-1000M monochromator (Jobin–Yvon, Longjumeau, France). The detector was a cooled GaAs photomultiplier R943-02 (Hamamatsu Photonics, Japan). The resolution of the whole spectrometer was 2–3 cm^{-1}. The samples were cooled to 10 K by the cryostat described above.

A series of FLN spectra were recorded to determine the inhomogeneous distribution function (IDF) by tuning the frequency of the laser in the range of 16 595–17 921 cm^{-1} (603–558 nm). The intensities of the peaks were read in function of their shifting position upon tuning the excitation energy. Correction for the baseline of the spectra due to the strong light scattering of the samples and for the intensity of exciting light was performed prior to evaluation. To obtain the IDF, we fitted the data points with Gaussian distribution functions of the form: $G(\nu) = \nu(0) + c*\exp-(\nu-\mu)^2/2\sigma^2$, where ν is the (0,0) transition wavenumber, μ is the mean transition wavenumber and σ is the standard deviation of the distribution.

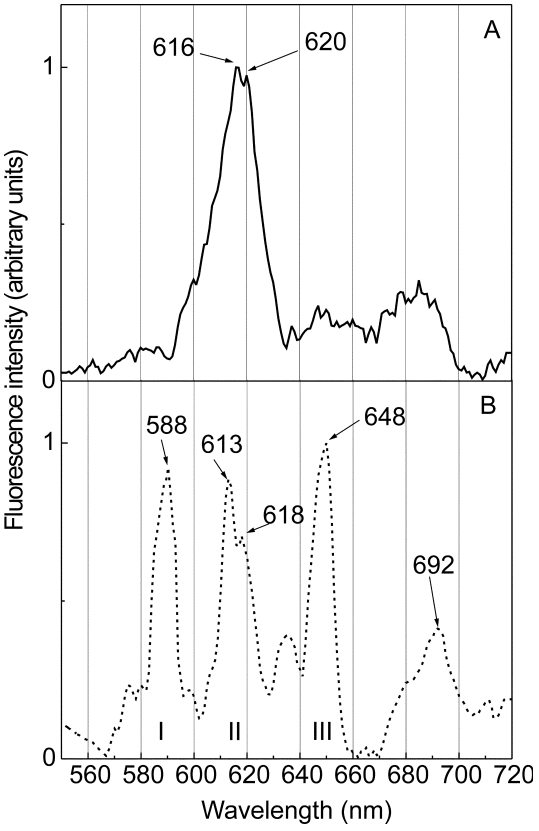

Fig. 1. Conventional fluorescence emission spectra excited at 400 nm of intact *E. coli* cells at 293 K (A) and at 77 K (B). The positions of the maxima of the main bands are indicated.

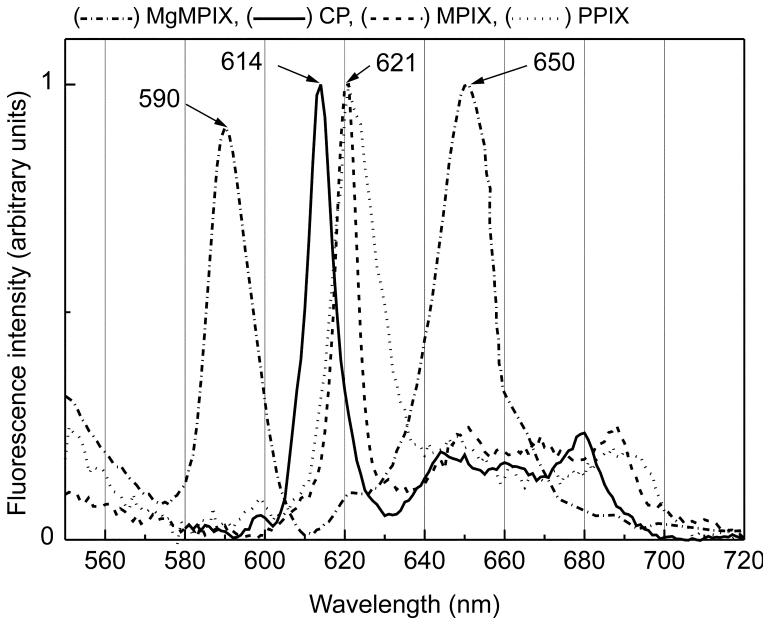

Fig. 2. Conventional fluorescence emission spectra of free-base porphyrins (excited at 400 nm) and of Mg-MPIX (excited at 410 nm) dissolved in Mg-free phosphate buffer (PBS) at 77 K. The positions of the maxima of the main bands are indicated. The emission spectra of CPIII, MPIX, PPIX and Mg-MPIX are drawn with solid line, dashed line, dotted line and dotted-dashed line, respectively.

3. Results

3.1. Conventional fluorescence emission spectra measured at 293K and 77 K

In the conventional spectra determinations the sample was excited in the Soret band of the chromophores at 400 nm, by using a wide slit of 7 nm to excite all kinds of chromophores at the same time. In Fig. 1, the fluorescence emission spectra of *E. coli* cells are compared at 293 (Fig. 1A) and 77 K (Fig. 1B). At room temperature, a broad (20 nm half-width) emission band was observed, with a double maximum with maxima at 616 and 620 nm. The complex (0,0) emission band can be interpreted as the contribution of free-base porphyrin derivatives, identified by HPLC analysis of disintegrated bacterial cells as CPIII, MPIX and PPIX in our previous study [1].

In the emission spectrum of *E. coli* cells at 77 K (Fig. 1B) additional components (Bands I and III), not found in HPLC analysis, can also be clearly distinguished besides Band II attributed to free-base porphyrins. The comparison of the two spectra shown in Fig. 1A,B suggests that the additional bands (I and III) are also present in the room temperature spectrum, and they are better seen due to the higher resolution at low temperature. In Fig. 2, we show the emission spectrum of free base and MgMPIX in PBS at 77 K. It is seen that the emission maxima are well comparable with Bands I–III in Fig. 1B, found in the intact bacterial cells at 77 K.

The culturing medium of the bacteria, PBS, contains K- and Mg-salts. It is known that Mg-ions are able to form stable complexes with free-base porphyrins under certain experimental conditions [24]. To test whether the Mg-content of PBS can be accounted for the spectra observed at 77 K in the case of the bacterial cells, the spectra of MPIX was determined in PBS at both temperatures. The room-temperature emission spectrum is shown in Fig. 3A by continuous line. It was a typical MPIX spectrum with a (0,0) band maximum at 624 nm. The 77 K emission spectrum, shown by dashed line in Fig. 3A, besides the typical MPIX spectrum, exhibits also Bands I and III. The result of a control experiment is shown in Fig. 3B. In Fig. 3B the continuous line shows the spectrum of MPIX in Mg-free PBS at room temperature, and the dashed line shows the spectrum at 77 K. Ordinary two-band emission spectra were obtained at both temperatures. The same test was performed with the two other metal-free

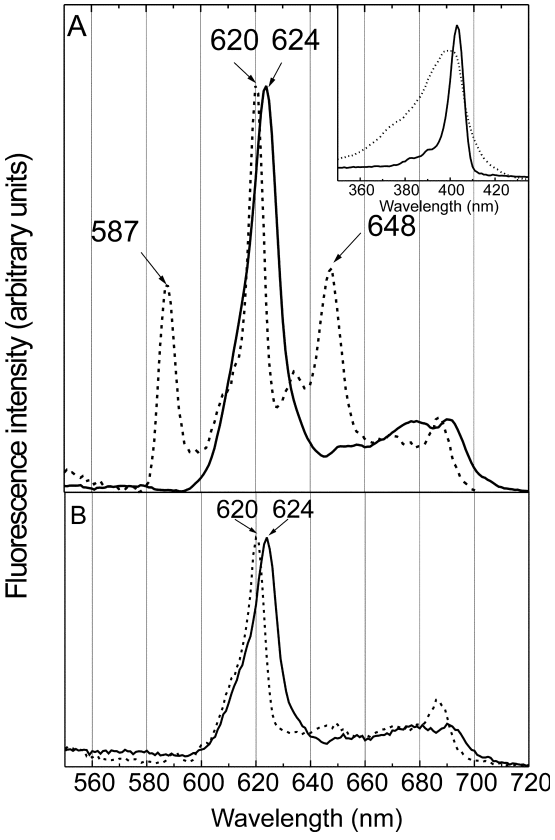

Fig. 3. Conventional fluorescence emission spectra of MPIX dissolved in phosphate buffer (PBS) (A) and in Mg-free PBS (B) at 293 (solid lines) and 77 K (dashed lines). The positions of the maxima of the main bands are indicated. In the inset, the fluorescence excitation spectra of MPIX, dissolved in PBS, are shown at 77 K. The spectra were taken at emission wavelengths of 588 (solid line) and 611 nm (dashed line), respectively.

porphyrins, but they did not show additional bands indicative of Mg-incorporation (spectra not shown).

3.2. High-resolution fluorescence spectra of intact E. coli cells

The IDF of the porphyrins inside the bacteria was determined by measuring a series of emission spectra when tuning the laser in the frequency range of 16 595–17 921 cm^{-1} (603–558 nm). Resolved emission lines could be observed superimposed on a very strong background compared to the weak line spectra shown in Fig. 4A. The series shown in Fig. 4B are after background subtraction (the excitation range corresponds with those of vibronic excitations, as indicated). If one subtracts the energy of a sharp (0,0) emission line from the applied excitation frequency, the excited state vibrational mode of excitation can be determined. Some characteristic excited state vibrational wavenumbers are indicated in Fig. 4B. These sharp (0,0) lines shift together with the excitation wavenumber and their intensities change according to the population distribution function [20]. Thus, these intensities could be used to determine the IDF of the electronic origins of the porphyrin system. Note that the narrow lines became broader and very weak below about 16 900 cm^{-1}.

The IDF of the porphyrins in *E. coli* bacteria is shown in Fig. 5. It is a wide, complex band. The

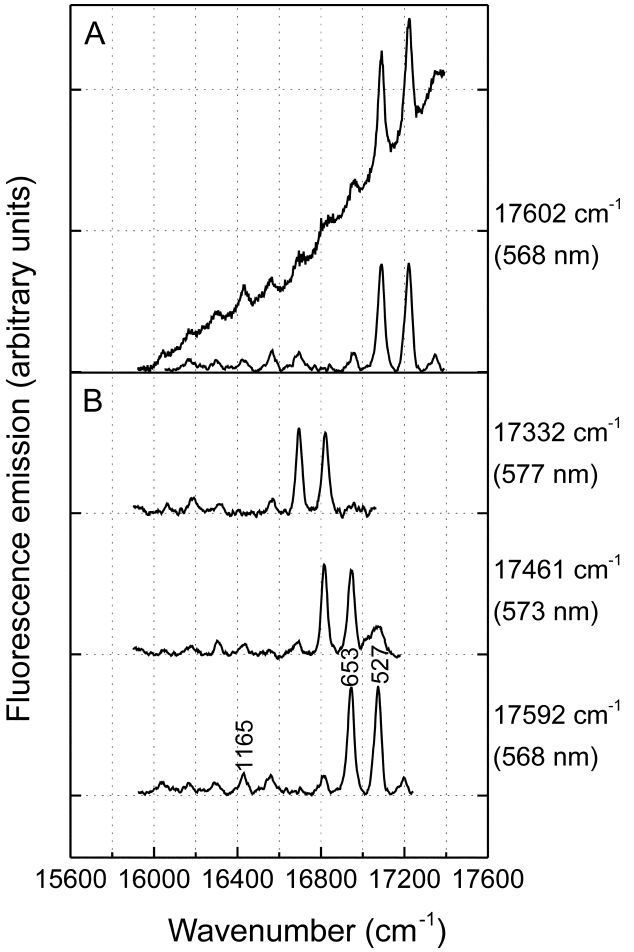

Fig. 4. (A) FLN spectra of intact *E. coli* cells before (upper curve) and after background correction (lower curve). The excitation wavenumber was set at 17 602 cm^{-1}. (B) (0,0) emission lines as a function of excitation wavenumbers in the FLN spectra of *E. coli* cells after background correction. Excitation wavenumbers/wavelengths are given on the right of the figure. Some excited state vibrational wavenumbers are indicated.

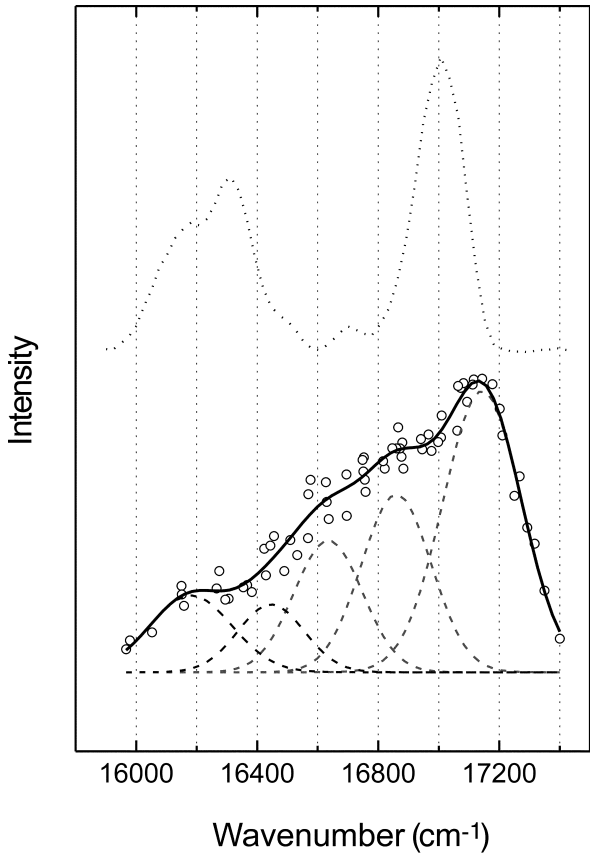

Fig. 5. The inhomogeneous distribution function (IDF) of endogenous porphyrins in the *E. coli* bacteria. The data were fitted with Gaussian curves (dashed lines), their sum is shown with a continuous line. The parameters of the Gaussian components are listed in Table 1. The (0,0) band region of the conventional 77 K emission spectrum of *E. coli* cells is shown also for comparison (dotted line).

IDFs of the individual porphyrins were determined by Gaussian fitting. The calculations showed that at least three components are needed to fit the data points; however, five components gave a much better fit. These Gaussians have maxima at 16 181, 16 446, 16 635, 16 860 and 17 144 cm^{-1}; their half-values are 160, 129, 133, 136 and 146 cm^{-1}, respectively (Table 1).

We have determined the excited state vibrational wavenumbers of the porphyrins inside the *E. coli* cells (Table 2). We also show comparable literature data [25] for free-base (PPIX) and a Mg-containing porphyrin (Mg-PPIX). It is seen from Table 2 that between the excited state vibrational wavenumbers of *E. coli* bacteria lines of both the free-base and of metal porphyrins can be found.

4. Discussion

The room-temperature fluorescence emission spectrum of *E. coli* B cells, shown in Fig. 1A, can be interpreted as the resultant spectra of the constituent porphyrins (see Fig. 2), in a less polar media. The emission spectrum agrees with the presence of all three free-base porphyrins that was supposed to be present, based on our previous analysis of disintegrated bacterial cells [1].

The low-temperature spectra showed two additional emission bands compared with the room-temperature emission spectra: Band I at 588 nm and Band III with maximum at 648 nm (Fig. 1B). The wavenumber difference between these two maxima was found around 1500 cm^{-1}, which corresponds to the difference between the (0,0) band and its vibronic envelope of regular porphyrins [26]. The positions of the maxima show close resemblance to the (0,0) band and vibronic envelope in the spectra of closed shell metal porphyrins [26]. Similar phenomena could be observed in the spectrum of MPIX dissolved in PBS (containing Mg^{2+}): bands at 587 and 648 nm appear at 77 K, that were not observed at room temperature (Fig. 3A). The fluorescence excitation spectra, shown in the inset of Fig. 3A, measured at

Table 1
Parameters of the Gaussian decomposition of IDF (see Fig. 5)

Band position (μ)		HWHH (cm^{-1})	Area (%)	Comparable band positions in the spectrum at 77 K
cm^{-1}	nm			
16 181	618	160	11	618
16 446	608	129	8	613
16 635	601	133	16	588
16 860	593	136	24	588
17 144	583	146	40	588

Table 2
Excited-state vibrational frequencies of endogenous porphyrins in intact *E. coli* B bacteria (given in cm^{-1}) compared with the excited state vibrational frequencies of PPIX and Mg-PPIX in myoglobin (Mb) (see [25])

E. coli B	PPIX-Mb	Mg-PPIX-Mb
392	396	
408		410
484	485	482
494–498		492
503	503	505
509		
519		
527–529		
532–536	532	532
609		
614		618
621		
628–631		
636		634
644–646	640	
651–655	656	
660–664	681	667
774		770
783		780
788		
1028		
1035		
1044	1045	
1054	1051	1055
1158	1198	1155
1165	1168	1141
1170		1171
1177		1192
1270	1272	1248
1278		
1292		
1297–1300	1298	
1308	1320	1306
1437	1406	1435
1567	1533	1568
1552	1552	

588 and 611 nm, respectively, also confirm that there are metal porphyrin derivatives besides the free-base porphyrin in the sample. These results support our hypothesis that Mg porphyrins are formed from free-base porphyrins in the Mg-containing buffer. Further support came from the results of an opposite experiment: the spectra of MPIX solutions, dissolved in Mg^{2+}-free PBS, did not show any of Bands I or III, either at room temperature or at 77 K (Fig. 3B). If we kept the solution at room temperature for a longer time, then Bands I and III appeared again in the low-temperature fluorescence emission spectrum, however with low intensity. This showed that Mg is not the only factor leading to the effect. Literature data prove that Zn, Cu and Co ions, originating from the used glassware or reagents, into free-base porphyrins may also form complexes with free-base porphyrins [28–30]. On the basis of our measurements and of the findings of Sommer et al. [30] we can conclude that mostly Mg- and also Zn porphyrins are formed in the bacteria. It may also be that Cu- and Co porphyrins are present, but they are neither fluorescent nor photosensitisers, so they did not affect our spectra and the photodynamic efficiency of the ALA-induction.

The incorporation effect was not observed for PPIX or CPIII, only for MPIX. The results show that the Mg^{2+} content of the PBS, and also the Zn contamination of the solutions kept in glassware and the presence of MPIX in the cells, together are responsible for the appearance of Bands I and III at 77 K.

During the incubation of the cells at 37°C, the Mg^{2+} ions, present in the culturing medium, may form Mg porphyrin complexes. The complex of the porphyrin ring with the Mg^{2+} ion is rather weak; Bardos et al. observed the demetallisation of the ring in aqueous solution at room temperature during ageing under illumination through a monochromator [27].

The structural forms of the porphyrins inside the *E. coli* cells were studied with FLN techniques. The photodynamic inactivation of *E. coli* cells was attributed mainly to monomeric porphyrins [13,31]. The detection of FLN lines in the (0,0) range of chromophores indicates the presence of monomers in the sample. The FLN spectra of *E. coli* cells had resolved lines both in the (0,0) region of free-base porphyrins and Mg/Zn porphyrins (Fig. 4), however, the line spectra were very weak and of broader lines in the range of the (0,0) bands of the free-base porphyrins. There can be two reasons of the loss of resolution and broadening: stronger phonon coupling and intrinsic line broadening by additional relaxation effects like, e.g., energy transfer. Both effects can be expected in highly interacting environments that may be the result of binding to cell compartments or to aggregated structural forms.

We compared the relative area of the components in the IDF, which are proportional to the relative amount of chromophores in the sample. The dominant component is the bimodal distribution associated with Mg/Zn porphyrins. The relative intensity of the free-base components is much weaker in the IDF than in the conventional spectrum. This may be the consequence of the fact that among the chromophores leading to the intense band at 613 nm in Fig. 1B, only a small fraction is contributing to the line spectrum. Those porphyrins that are able to bind Mg/Zn ions seem to be in specific structural environments, isolated from each other.

In the IDF, the five components have half-widths around 130–160 cm^{-1}, which are similar to those reported for porphyrins in amorphous solids [23] or for Mg-PPIX in myoglobin [25]. In well-ordered protein pockets, such as cytochrome *c* [32,33] or horseradish peroxidase [34–36], much narrower half-widths were observed, in the range of 20–40 cm^{-1}. Comparing our results with these data we concluded that, in *E. coli* bacteria, the porphyrin molecules do not have a uniform, strictly packed structure with respect to their immediate environments.

The wide energy range of the spectral components in the IDF (see Table 1) means that the porphyrins in the *E. coli* cells can transfer energy in a wide photon energy range to their environment. This is advantageous from the point of view of photodynamic inactivation of the bacterial cells. These results could give an explanation to the efficient inactivation of *E. coli* cells, performed by us, after exogenous ALA induced accumulation of endogenous porphyrins.

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