Specific photoaffinity labelling of a ferripyoverdin outer membrane receptor of *Pseudomonas aeruginosa*

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Received 16 September 1996

Abstract In order to identify and characterize the receptors involved in pyoverdin-mediated iron transport in *Pseudomonas aeruginosa* ATCC 15692, a photoactivatable siderophore has been synthesized. In the dark, this probe is stable and is able to promote iron transport at the same rate as the native pyoverdin. Under irradiation at 312 nm, the molecule is photodecomposed and a clear inhibition of the iron transport is observed. With the radioactive form of this photoactivatable probe, we were able to visualize on a SDS-PAGE gel a labelled protein of approximately 90 kDa molecular mass, which is very likely the FpvA receptor or a yet unknown pyoverdin receptor.

Key words: Iron transport; *Pseudomonas aeruginosa*; Outer membrane receptor; Photoaffinity labelling; Pyoverdin; Azidopyoverdin

1. Introduction

When grown under iron-limited conditions, many aerobic bacteria synthesize and secrete into the environment iron chelators termed siderophores [1]. Siderophores are low-molecular-mass molecules capable of chelating and delivering iron to bacterial cells via specific high-affinity transport systems [2]. *Pseudomonas aeruginosa*, an important opportunistic pathogen of humans [3], produces at least two known siderophores when grown in iron-deficient conditions: pyochelin [4] and pyoverdin [5]. Pyoverdin is an octapeptide bound to a fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline [6] and pyochelin is a hydroxyphenylthiazolinylthiazolidine type of siderophore [7]. In addition, *P. aeruginosa* is able to utilize a number of heterologous siderophores, including pyoverdins produced by some other *Pseudomonas* [8], ferrioxamine B [9], aerobactin and enterobactin [10].

Two outer membrane receptors for ferripyochelin, of respectively 14 kDa [11] and 75 kDa [12] molecular mass, have been identified. The gene for the 75 kDa ferripyochelin receptor (*fptA*) has been cloned [13] and more recently sequenced [14]. Heinrichs et al. [12] suggested that the 14 kDa system operated in exponential phase cells, whereas the 75 kDa receptor transport was of greater importance in late exponential and early stationary phase. The receptor for ferripyoverdin has been identified as a 90 kDa outer membrane protein (FpvA) [15] and cloned [16]. A mutant deficient in expression of this protein still showed low uptake of ferripyoverdin, providing evidence for a second transport system [15]. As we mentioned above, *P. aeruginosa* is able to use heterologous siderophores like enterobactin [10]. Poole et al. [17] identified and cloned [18] a ferric-enterobactin receptor (PfeA) of 78 kDa molecular mass. It is now clear that *P. aeruginosa* has multiple iron transport systems, but the contribution of these systems to the growth in vivo is still unclear. The way all these receptors are activated is not yet known [19]. However, the presence in FpvA and in PfeA of sequences apparently conserved in TonB-dependent receptors [20] certainly suggests a TonB-like mechanism [16].

A method for studying the siderophore-receptor interactions during the iron transport and ultimately for isolating the protein receptors is photoaffinity labelling [21]. In this paper we describe the synthesis and the iron transport properties of a photoactivatable analogue of pyoverdin PaA, a peptidic siderophore of *P. aeruginosa* ATCC 15692. We also show how a photoactivatable radiolabelled pyoverdin analogue can be used to label specifically, when irradiated in the presence of whole cells of *P. aeruginosa* ATCC 15692, the outer membrane receptor involved in pyoverdin-mediated iron uptake.

2. Materials and methods

2.1. Growth condition

P. aeruginosa strain ATCC 15692 was grown under aerobic conditions in a succinate medium as described by Demange et al. [6].

2.2. Isolation and purification of the pyoverdins

The pyoverdins have been isolated and purified as described earlier by Albrecht-Gary et al. [22].

The purity of pyoverdins was monitored by electrophoresis on cellulose acetate films using a SEBIA horizontal electrophoresis tank. The electrophoreses were run in 100 mM pyridine-acetic acid pH 5.0 buffer, at a constant voltage (300 V), for 30 min.

2.3. Synthesis of aminopyoverdin-Fe(III) (PaA-NH_2-Fe(III)) complex

Pyoverdin PaA-Fe(III) complex (90 mg, 65 μmol) was dissolved in 500 μl of DMSO/H_2O (7:1, v/v). 34 mg (177 μmol) of EDCI (1-(3-dimethylaminopropyl)-3-ethycarbodiimide hydrochloride) (Aldrich Chemie, Steinheim, Germany) was added and the flask was stirred at room temperature for 3 h. 50 μl (615 μmol) of 1,2-diaminoethane was added and the solution stirred for 5 h at room temperature. The reaction was monitored by film electrophoresis on cellulose acetate. After evaporation of the solvent, the residue was dissolved in 500 μl

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Abbreviations: PaA, pyoverdin PaA; PaA-NH_2, aminopyoverdin; PaA-N_3, azidopyoverdin; HOHAHA, homonuclear Hartmann Hahn spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence

PII S0014-5793(96)01071-X

pyridine-acetic acid 50 mM pH 5.0 buffer, and applied on a CM-Sephadex C-25 column (1.5×20 cm) eluted with a linear gradient of pyridine-acetic acid pH 5.0 buffer (50–500 mM, 2×100 ml). 67 mg of aminopyoverdin-Fe(III) was obtained (74%).

Electrophoresis on cellulose acetate films: migration distance = 3.0 cm.

UV-Vis: λ_{max} = 400 nm, ε = 19 000 $M^{-1} \times L$; λ_{sh} = 460 nm, ε = 6200 $M^{-1} \times L$; λ_{sh} = 540 nm, ε = 2300 $M^{-1} \times L$ in pyridine-acetic acid pH 5.0 buffer.

FAB-MS: m/z = 1429 m.u. (M^+).

2.4. Decomplexation of aminopyoverdine-Fe(III) (PaA-NH_2-Fe(III))

The decomplexation of PaA-NH_2-Fe(III) was performed as for pyoverdin-Fe(III) according to Albrecht-Gary et al. [22].

Electrophoresis on cellulose acetate films: migration distance = 8 cm.

UV-Vis: λ_{max} = 380 nm, ε = 16 500 $M^{-1} \times L$; λ_{max} = 360 nm, ε = 16 000 $M^{-1} \times L$ in 50 mM pyridine-acetic acid pH 5.0 buffer.

FABS-MS: m/z = 1376 m.u. (M^+).

2.5. Synthesis of the azido derivative (PaA-N_3)

15 mg (10 μmol) of aminopyoverdin was dissolved in 75 μl of a mixture of dimethylformamide-water (7:1, v/v) and 18 μl of a 10% solution of triethylamine in dimethylformamide was added. After stirring the mixture for 5 min at room temperature, 3.5 mg (13 μmol) of 4-azidobenzoic acid *N*-hydroxysuccinimide ester dissolved in 200 μl of dimethylformamide was added. After 1 h at room temperature, 1 ml of ethyl acetate was added. The mixture was centrifuged and the pellet dissolved in 200 μl water. Excess reagent was removed by two successive extractions with 100 μl ethyl acetate.

Electrophoresis on cellulose acetate film: migration distance 1.0 cm.

UV-Vis: λ_{max} = 258 nm, ε = 41 500 $M^{-1} \times L$; λ_{max} = 360 nm, ε = 16 000 $M^{-1} \times L$; λ_{max} = 380 nm, ε = 16 500 $M^{-1} \times L$ in 50 mM acetic acid/sodium acetate pH 5.0 buffer.

2.6. Synthesis of the tritiated azido derivative-Fe(III) complex ([^{3}H]PaA-N_3-Fe(III))

50 μg (35 nmol, determined spectrophotometrically) of PaA-NH_2-Fe(III) were dissolved in 5 μl of dimethylformamide. 1 μl of a solution of triethylamine in dimethylformamide (diluted 50 times) and 5 μl (5 μCi) of a solution of 4-azido-3,5[^{3}H]benzoic acid *N*-hydroxysuccinimide ester (Dupont NEN, specific activity 45.7 Ci/mmol) in dimethylformamide were added and the tube was vigorously stirred at room temperature. The reaction was monitored by electrophoresis on cellulose acetate film. An aliquot of the reaction medium was removed, mixed with a concentrated solution of unlabelled PaA-N_3-Fe(III) and electrophoresed for 12 min. The film was then sliced into 5 mm wide sections and each slice was counted in 4 ml of AqualumaPlus scintillation liquid (LUMA, L.S.C., France). The counting was performed after 2 h.

After 1 h, 50 μl of distilled water was added and the aqueous phase was extracted with ethyl acetate (3×50 μl). The yield of the coupling reaction was 32% (calculated from the radioactivity counted) with a specific activity of 45.7 Ci/mmol.

2.7. Photolysis of PaA-N_3-Fe(III)

Photolysis was performed in 50 mM MOPS pH 7.0 buffer at 29°C with a bench lamp emitting at 312 nm (VL-M, 6W, Bioblock, France). The samples were kept in a quartz cell (Hellma, 454×12.5×9.5 mm), and irradiated with the light source maintained at a distance of 12 cm. A UV-visible spectrum of this solution was determined every 15 s.

2.8. P. aeruginosa growth in the presence of PaA-N_3

5 ml of a culture of *P. aeruginosa* ATCC 15692 harvested at the end of the exponential phase of growth was centrifuged (10 min at 12 000×*g*) under sterile conditions and the pellet was washed twice with a sterile succinate medium. After the last centrifugation the bacteria were suspended again in the culture medium at a concentration of 1.3×10^9 cfu/ml (OD_{470} = 1.3; OD_{600} = 0.81). 30 μl of this suspension was used to inoculate 3 ml of succinate medium and a solution of pyoverdin or its photoactivatable analogues was added at a final concentration of 25 μM. The cuvettes were stirred at 37°C in the dark, and the optical densities at 470 nm and 600 nm were measured as a function of time.

2.9. ^{55}Fe uptake in P. aeruginosa in the presence or absence of irradiation

10 ml of bacterial culture harvested at the end of the exponential phase of growth was centrifuged 10 min at 12 000×*g*. After having been washed with 50 mM MOPS pH 7.0 buffer, the pellet was suspended again in MOPS buffer and the optical density of the suspension at 600 nm was adjusted to 0.5. The cells were incubated for 15 min at 29°C before the beginning of the transport experiments.

The solutions of the siderophores complexed to ^{55}Fe (from $^{55}FeCl_3$, NEN, specific activity of 3 Ci/g) were prepared using a 1 mM solution of PaA or PaA-N_3 as a free ligand in 50 mM MOPS pH 7.0 buffer. To 200 μl of this solution was added 5 μl of a solution of $^{55}FeCl_3$ (1 μCi/μl) obtained by dilution of the stock solution, plus 800 μl of 50 mM MOPS pH 7.0 buffer. 500 μl of the radioactive complex solution thus formed were then added to 4.5 ml of the bacterial suspension. The mixture was stirred at 29°C in the dark or under irradiation (bench lamp maintained at 12 cm above the surface of the liquid). 500 μl aliquots were removed at different times and filtered on 0.45 μm porosity filters (Micronsep, France), pre-soaked in a 0.3% solution of polyethyleneimine. Each filter was rapidly washed twice with 2 ml 50 mM MOPS buffer at pH 7.0, and its radioactivity measured in 3 ml of scintillation liquid (AqualumaPlus) after 4 h incubation.

2.10. Analysis of the labelled outer membrane proteins

The bacteria were prepared as above in 50 mM MOPS pH 7.0 buffer.

5 μCi of [^{3}H]PaA-N_3-Fe(III) was added to the suspension, and after 2 min incubation, the tubes were irradiated for 8 min at 312 nm. In parallel the same experiment was performed in the presence of 1 μCi of [^{3}H]PaA-N_3-Fe(III) plus 5 μM PaA-Fe(III). Both suspensions were centrifuged 5 min at 12 000×*g*, and the pellet washed with 50 mM MOPS pH 7.0 buffer.

The analysis of the labelled outer membrane proteins was performed according to Mizuno and Kageyama [23] and Hancock and Nikaido [24] with some modifications. The two bacterial pellets were suspended in a 25 mM Tris-HCl pH 6.8 in the presence of 0.5 mg lysozyme. The cells, maintained in an ice bath were then sonicated (Bransonic 12, Branson Co., Shelton, CT, USA) for 6×30 s, with 30 s cooling between each sonication. Addition of sodium *N*-lauryl sarcosinate (sarkosyl) at a final concentration of 2% (v/v), solubilized the cytoplasmic membranes leaving the outer membranes intact. After incubation at 37°C for 10 min, 200 μl of a solution containing 1 mg DNase and 1 mg RNase per ml was added. The mixtures were reincubated for 5 min at 37°C. Centrifugation for 5 min at 5000×*g* removed the unbroken cells, and the supernatant was then centrifuged 60 min at 20 000×*g*. The pellets were washed with 50 mM Tris-HCl pH 6.8 and centrifuged as before.

The membranes were loaded on a 12% SDS-PAGE gel. After staining, the gel was sliced in 2.5 mm wide bands, each band being then placed in a counting vial and digested by hydrogen peroxide (300 μl) for 48 h at room temperature. 100 μl of SDS 1%/urea 8 M were added to each counting vial and the radioactivity counted after 12 h in 4.5 ml of scintillation liquid (AqualumaPlus).

3. Results and discussion

3.1. Properties of the photoactivatable pyoverdin analogue

Arylazides are known to be useful photoreactive moieties of photoaffinity probes [21]. They are chemically inert, but when photolysed, they produce aryl nitrenes, extremely reactive species forming stable bonds with practically all kinds of compounds [25,26]. Nitrenes are sufficiently reactive to insert even into C-H bonds.

The site chosen for the binding of the photoactivatable group to pyoverdin (PaA) was the succinyl moiety linked to the carbon C-3 of the chromophore. The first step was the introduction of a nucleophilic amino group on this spacer arm. For this purpose PaA-Fe(III) was coupled with a large excess of 1,2-diaminoethane, in the presence of EDCI. The reaction, monitored by film electrophoresis, gave one major derivative. The yield after purification was 74%. Using FAB-

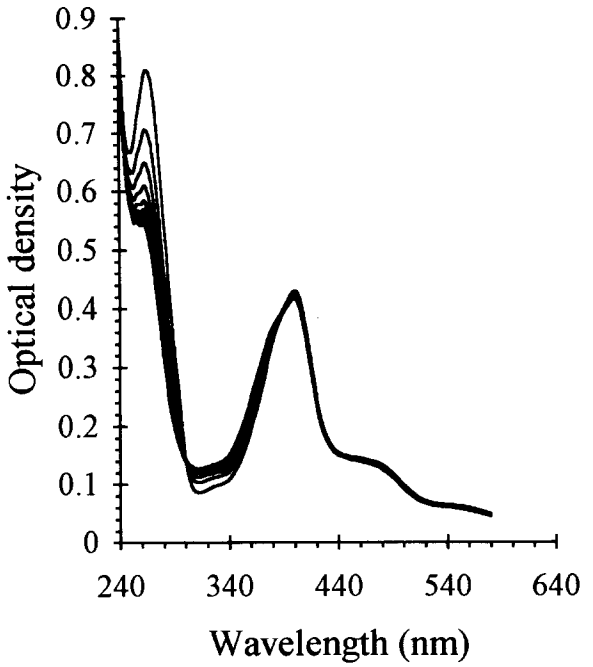

Fig. 1. Photodecomposition of PaA-N_3-Fe(III) under irradiation at 312 nm. PaA-N_3-Fe(III) was dissolved in 50 mM MOPS pH 7.0 buffer at 29°C at a concentration of 22 μM and irradiated at 312 nm. A UV spectrum of this solution was determined every 15 s.

MS techniques and 2D NMR correlations (HOHAHA, ^{1}H-^{13}C correlations, HMQC and HMBC, all performed on the free ligand with a 500 MHz Bruker spectrometer) we could assign all the signals corresponding to the protons and the carbon atoms (data not shown) and obtain the proofs that the 1,2-diaminoethane moiety is bound to the succinyl group (^3J correlation between the CH_2CO carbonyl of the succinate moiety at 177.2 ppm and the $NHCH_2$ protons of the 1,2-diaminoethane group at 3.60 ppm, and ^2J correlation between the $NHCH_2$ proton of the 1,2-diaminoethane group at 8.28 ppm and the CH_2CO carbonyl of the succinyl group at 177.2 ppm). All the other resonances were found to be very similar to those reported for pyoverdin PaA [6].

The introduction of the arylazido groups on the amino derivative of PaA was performed by treatment with a slight excess of 4-azidobenzoic acid *N*-hydroxysuccinimide ester, in the presence of triethylamine. One major product was observed by electrophoresis on cellulose acetate films. Due to its fairly low stability as a free ligand, PaA-N_3 was used without further purification in the experiments described in this paper. Further characterization of the products is under investigation.

The UV-visible absorption spectra of the ferric complex of PaA-N_3 (Fig. 1) shows two maxima, at 258 nm and at 400 nm corresponding respectively to the absorption of the arylazido group and of the complexed chromophore.

The photodecomposition of PaA-N_3-Fe(III) in 50 mM MOPS pH 7.0 buffer under irradiation at 312 nm is characterized by the decrease of the absorption band of the arylazido group at 258 nm and by the unaltered maximum absorption of the chromophore. The half-life of the compound deduced from this photodecomposition is about 1 min, but is expected to be longer in the presence of bacteria, due to light scattering.

3.2. Iron transport properties of PaA-N_3

^{55}Fe transport measurements, mediated by pyoverdin PaA or PaA-N_3, have been performed according to Knosp et al. [27], with slight modifications. The rates of uptake of ^{55}Fe presented in Fig. 2A show that PaA-N_3 behaves exactly like pyoverdin PaA at the same concentration. PaA-N_3 has still the properties required for the recognition of a pyoverdin by its receptors and for the transport of iron in *P. aeruginosa* ATCC 15692.

The photodecomposition product obtained after pre-irradiation of a solution of PaA-N_3 at 312 nm, and its addition to the uptake medium, gave a ^{55}Fe uptake rate similar to that of PaA (Fig. 2A). This proves that the photolyzed analogue can still be recognized by the receptors.

Before measuring the inactivation of the ^{55}Fe uptake by irradiation in the presence of the photoactivatable pyoverdin analogue during transport, it was essential to determine the effect of UV light itself on the bacterial iron transport. In two separate experiments the bacteria were or were not subjected to irradiation at 312 nm. ^{55}Fe complexed to pyoverdin PaA was then added and its uptake measured. No difference in uptake was seen (results not shown), indicating that irradiation at 312 nm has no effect on pyoverdin-mediated iron uptake.

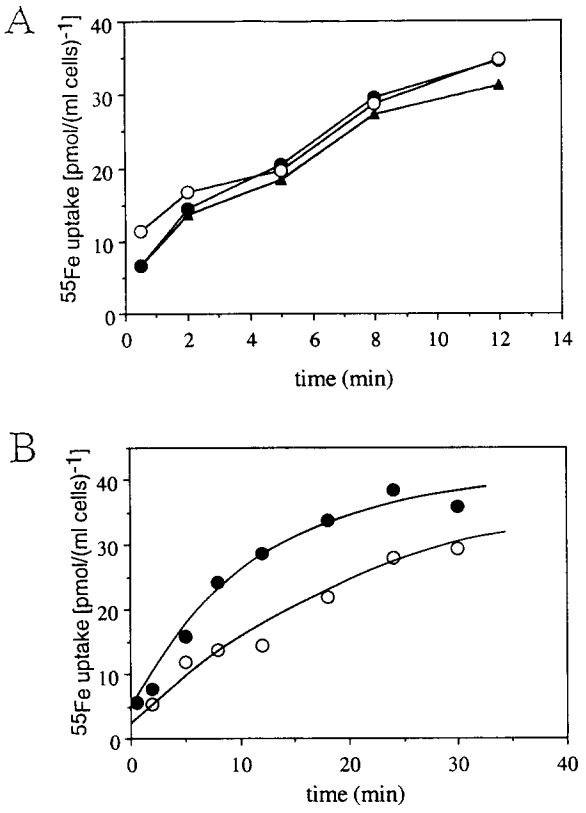

Fig. 2. ^{55}Fe uptake by *P. aeruginosa*. *P. aeruginosa* ATCC 15692 at a concentration of 1.3×10^9 cfu/ml was incubated 15 min in 4.5 ml 50 mM MOPS pH 7.0 buffer at 29°C before the beginning of the transport assays. A: After this incubation, 500 μl PaA-^{55}Fe(III) (▲), PaA-N_3-^{55}Fe(III) (●) or pre-irradiated PaA-N_3-^{55}Fe(III) (○) was added to the cells at a final concentration of 10 μM. 500 μl aliquots were removed at different times, filtered and counted. B: After this incubation, 500 μl PaA-N_3-^{55}Fe(III) (10 μM) was added and 500 μl aliquots were removed at different times, in the dark (●) or under continuous irradiation at 312 nm (○).

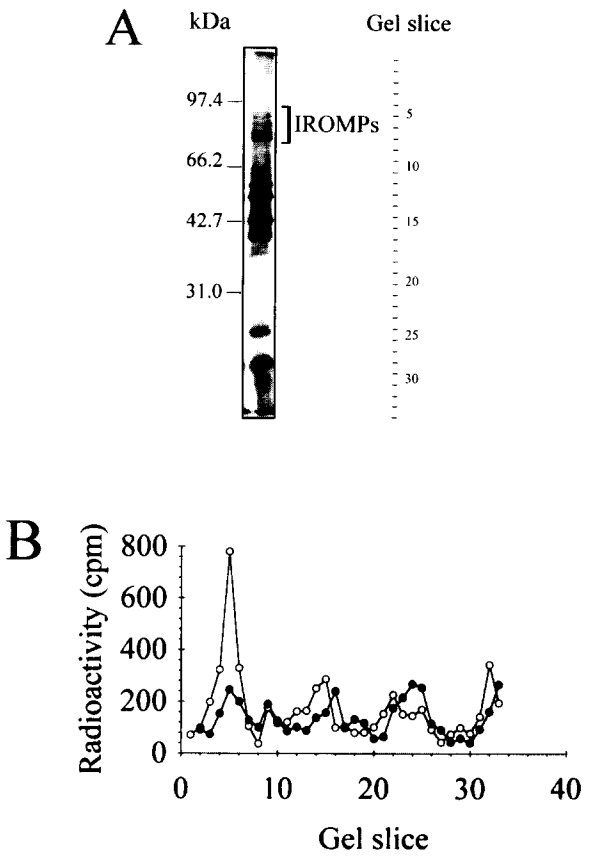

Fig. 3. Analysis of the labelled outer membrane proteins by SDS-PAGE. *P. aeruginosa* ATCC 15692 at a concentration of 1.3×10^9 cfu/ml in 50 mM MOPS pH 7.0 buffer at 29°C was irradiated at 312 nm in the presence of [^{3}H]PaA-N$_3$-Fe(III) (22 nM) and in the presence or absence of PaA-Fe(III) (5 μM). The outer membrane proteins of this labelled bacteria were loaded on a SDS-PAGE gel. A: The Coomassie-stained gel; B: the corresponding radioactive tracing of the sliced gel (1 slice corresponds to 2.5 mm), for the bacteria irradiated in presence of [^{3}H]PaA-N$_3$-Fe(III) (○) or in the presence of [^{3}H]PaA-N$_3$-Fe(III) plus PaA-Fe(III) (●).

The rates of ^{55}Fe uptake in the presence of PaA-N$_3$ in the dark and under continuous irradiation at 312 nm are represented in Fig. 2B. An inhibition of 15% is observed between both curves. Since the pre-irradiated photolyzed analogues can promote the uptake of ^{55}Fe with the same rate as natural PaA or PaA-N$_3$, the effect of inhibition can be assigned exclusively to the inactivation of some receptor(s) involved in the iron transport, and not to the modification of the affinity of the analogue after photolysis. Therefore, this inhibition would be due to the photolabelling of the proteins occurring in the transport, by the nitrene generated by photoactivation.

3.3. *Analysis of the labelled outer membrane*

In order to identify on the wild strain which proteins on the outer membrane are the receptors of pyoverdin, photoaffinity labelling experiments have been performed on bacteria in the presence of tritiated PaA-N$_3$-Fe(III) or in the presence of [^{3}H]PaA-N$_3$-Fe(III) plus a large excess of PaA. The outer membrane proteins of these labelled bacteria were analyzed by SDS-PAGE. The Coomassie-stained gel and the corresponding radioactive trace are shown in Fig. 3. The position of the labelled band corresponds to a protein of approximately 90 kDa molecular mass. A characteristic feature of the photoaffinity labelling process is that the natural substrate, in our case pyoverdin PaA, should protect against irreversible labelling [21]. The competition experiment, irradiation in the presence of [^{3}H]PaA-N$_3$-Fe(III) plus an excess of PaA, shows that the radioactive peak is abolished in the presence of PaA during irradiation. These data indicate that the labelled protein is a specific receptor of PaA of approximately 90 kDa involved in the transport of iron via the pyoverdin system. These data are consistent with binding of the azido-pyoverdin to the FpvA receptor, although we cannot preclude that a yet unknown pyoverdin-specific receptor different from FpvA lies in the same molecular mass range. Further experiments using blocking antibodies would be required to identify the receptor unambiguously. Attempts to determine the N-terminal sequence of *P. aeruginosa* high molecular mass iron regulated outer membrane proteins have not been successful (A.W. Smith, unpublished data).

4. Conclusion

The results presented here indicate that PaA-N$_3$ is a successful photoaffinity probe for the pyoverdin-mediated iron transport system in *P. aeruginosa* ATCC 15692. In the dark this molecule behaves like PaA, but under irradiation it inhibits efficiently, irreversibly and specifically the pyoverdin-mediated iron transport. A photolabelled outer membrane receptor, which is very likely FpvA or a yet unknown pyoverdin receptor, has been visualized on SDS-PAGE gel.

PaA-N$_3$ should be a good probe to localize and identify some other proteins involved in the transport of PaA-Fe(III) in the other cell compartments of *P. aeruginosa*.

Acknowledgements: We wish to thank Professor Maurice Goeldner for his kind help in providing us the possibility of performing some of the radiolabelling experiments in his laboratory, and for his very stimulating discussions as well. We also wish to thank Mr. Bachir Machi and Mr. Rui Ventura for their expert technical assistance.

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