J Membrane Biol (2009) 227:133–140
DOI 10.1007/s00232-008-9152-2

Fluorescence Quenching as a Tool to Investigate Quinolone Antibiotic Interactions with Bacterial Protein OmpF

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Received: 2 June 2008 / Accepted: 19 December 2008 / Published online: 16 January 2009

Abstract The outer membrane porin OmpF is an important protein for the uptake of antibiotics through the outer membrane of gram-negative bacteria; however, the possible binding sites involved in this uptake are still not recognized. Determination, at the molecular level, of the possible sites of antibiotic interaction is very important, not only to understand their mechanism of action but also to unravel bacterial resistance. Due to the intrinsic OmpF fluorescence, attributed mainly to its tryptophans (Trp^{214}, Trp^{61}), quenching experiments were used to assess the site(s) of interaction of some quinolone antibiotics. OmpF was reconstituted in different organized structures, and the fluorescence quenching results, in the presence of two quenching agents, acrylamide and iodide, certified that acrylamide quenches Trp^{61} and iodide Trp^{214}. Similar data, obtained in presence of the quinolones, revealed distinct behaviors for these antibiotics, with nalidixic acid interacting near Trp^{214} and moxifloxacin near Trp^{61}. These studies, based on straightforward and quick procedures, show the existence of conformational changes in the protein in order to adapt to the different organized structures and to interact with the quinolones. The extent of reorganization of the protein in the presence of the different quinolones allowed an estimate on the sites of protein/quinolone interaction.

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Keywords Spectrofluorescence · Biophysics · Structure of membrane protein · Fluorescent probe

Introduction

Quinolone antibiotics are widely prescribed drugs because of their safety, good tolerance and broad antibacterial spectrum (McCaffrey et al. 1992; Park et al. 2002; Siporin 1989; Wolfson and Hooper 1989). Different generations of quinolone antibiotics have been developed (Fig. 1) in order to enlarge the spectrum of activity and to solve problems of resistance that have emerged (Ball 2000; Hirai et al. 1986; Vazquez et al. 2001). Quinolone activity is based on the inhibition of homologous type II topoisomerases, DNA gyrase and DNA topoisomerase Iς, enzymes that control DNA topology and are vital for chromosome function and replication (Mascaretti 2003; Pestova et al. 2000). In order to develop their activity, these antibiotics need to cross the bacterial barriers, the first being the outer membrane. Among the mechanisms of bacterial resistance is the decrease of outer membrane permeability that prevents the influx of antibiotics, reducing the access of quinolones to their target of action by nonexpression or structurally changed expression of outer membrane porins (Chevalier et al. 2000; Mascaretti 2003). OmpF is one of those porins which microbiological studies have related to the permeation of some quinolones through the outer membrane (Chapman and Georgopapadakou 1988; Chevalier et al. 2000; Hirai et al. 1986; Mascaretti 2003; Piddock et al. 1999). Indeed, porin-deficient mutants of *Escherichia coli* are resistant to fluoroquinolones, although the role of OmpF, either as a channel or as an enabler of quinolone diffusion at the OmpF–lipid interface, has not yet been elucidated. Moreover, these antimicrobial agents can also

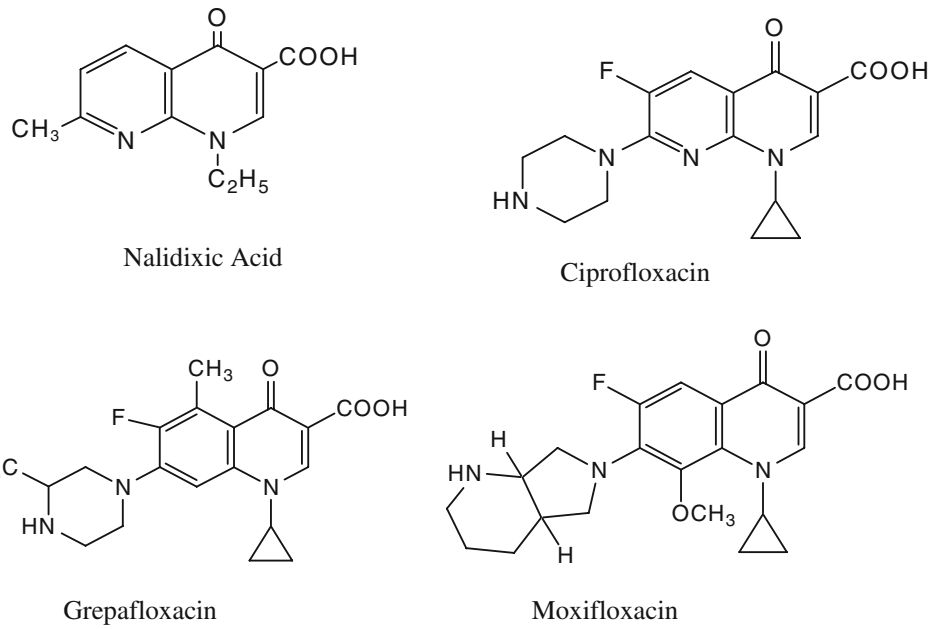

Fig. 1 Basic quinolone structure and chemical structures of the representative quinolones studied. Each of the quinolones studied is a representative member of the different generations: NA (first-generation), CP (second-generation), GR (third-generation) and MX (fourth-generation)

enter the cell by a lipid-mediated pathway (Bedard and Bryan 1989; Chapman and Georgopapadakou 1988; Chevalier et al. 2000; Denis and Moreau 1993; Mortimer and Piddock 1993). The relative importance and the different sites of uptake of quinolones by OmpF are issues of great importance in order to develop new molecules with fewer resistance problems.

OmpF is a trimer within the membrane, and it contains just two tryptophan residues per monomer (Fig. 2), Trp^{214} at the lipid–protein interface and Trp^{61} at the trimer interface (Lee 2003). The protein shows a maximum of fluorescence emission at relatively low wavelengths, which suggests that both tryptophans are in hydrophobic environments (Lakowicz 1999). This is confirmed not only by experiments involving OmpF mutants (O'Keeffe et al. 2000) lacking one or both Trp, proving the hydrophobicity of the environment of both Trps, but also by electron microscopic studies that show that Trp^{61} is in an environment more hydrophobic than that surrounding Trp^{214} (Cowan et al. 1992).

In this study intrinsic OmpF fluorescence was quenched by acrylamide or iodide and, by means of their different accessibility to the protein tryptophans, information on the possible interaction sites between quinolones and OmpF was gained. Iodide readily enters the hydrophobic regions of membranes or similar structures (Lakowicz 1999; Moro et al. 1993) but must be repelled by the negative charges of the OmpF ionic channel (Lehrer 1971, 1977) (OmpF is a porin with cationic selectivity [Nikaido and Rosenberg 1983], so it interacts with Trp^{214}). Acrylamide, a small polar molecule, cannot quench Trp^{214} as it is unable to quench fluorophores buried in the membranes. Furthermore, acrylamide has a quenching efficiency that is independent of the polarity of the microenvironment surrounding the fluorophore but is limited only by possible

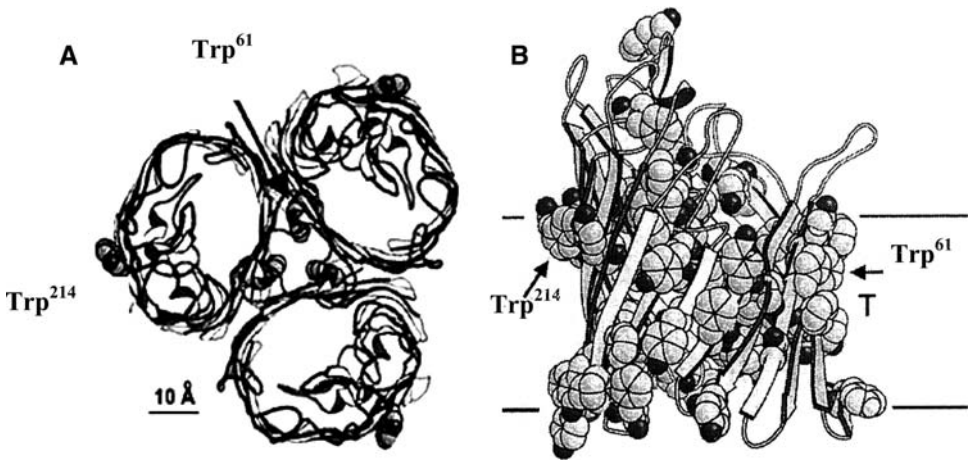

Fig. 2 Structure of the OmpF trimer. The positions of the two Trp residues (Trp^{214} and Trp^{61}) in each monomer are shown. Views of OmpF organization: top (**a**) and perpendicular to membrane axis (**b**). Trimer interface in **b** is represented by T

steric shielding (Lakowicz 1999; Moro et al. 1993). For the trimer interface this is not a problem as it is known that the monomers are well separated over a distance of ~35°A (Cowan et al. 1992), which can perfectly accommodate the acrylamide molecule. Accordingly, this molecule allowed detection of changes in the environment of Trp^{61}.

The results obtained in the presence of quinolones allowed us to conclude that the uptake of these drugs changes with their generation, showing that nalidixic acid (NA), a first-generation quinolone, interacts near Trp^{214} but moxifloxacin (MX), a fourth-generation quinolone, interacts near Trp^{61}. This study assumes importance in the overall context of the increasing problem of bacterial resistance to antibiotics currently in use and the consequent need of understanding the processes involved in order to produce different molecules with increased antibacterial activity and fewer resistance problems.

Materials and Methods

Grepafloxacin (GR) was a gift from Glaxo-Wellcome (London, UK). Ciprofloxacin (CP) and MX were a gift but from Bayer (Newbury, UK). NA and *N*-(2-hydroxyethyl) piperazine-*N*'-ethanesulfonic acid (HEPES) were from Sigma (St. Louis, MO). Octylpolyoxyethylene (oPOE) was from Bachem (Torrance, CA), and all other chemicals were from Merck (Darmstadt, Germany; *pro analysi*). All solutions were prepared with 10 mM HEPES buffer (0.1 M NaCl, pH 7.4). OmpF was purified from *E. coli*, strain BL21 (DE3) Omp8, following published procedures (Garavito and Rosenbusch 1986). OmpF concentration was estimated using the bicinchoninic acid protein assay against bovine serum albumin as standard.

All absorption determinations were recorded with a Unicam UV-300 spectrophotometer equipped with a constant-temperature cell holder. Spectra were recorded at 37°C in 1-cm quartz cuvettes with a slit width of 1 mm in the range 230–350 nm. Fluorescence measurements were performed in a Varian (les Ulis, France) spectrofluorometer, model Cary Eclipse, equipped with a constant-temperature cell holder (Peltier single cell holder). All spectra were recorded at 37°C, under constant stirring, with a slit width of excitation and emission of 10 nm, 305–400 nm for emission and 290 nm for excitation.

Solutions

All antibiotic solutions and suspensions were prepared in 10 mM HEPES buffer (pH 7.4, I = 0.1 M NaCl). Solutions of 5 M acrylamide or potassium iodide, in deionized water, were always prepared on the day of the experiment. For the potassium iodide solution, as it becomes easily oxidized, extra care was taken by adding sodium thiosulfate (final concentration 10 mM). Tryptophan solution was also prepared in HEPES buffer. In the quenching studies an adequate amount of the concentrate solution of Trp was measured for the fluorescence cell and the concentration was adjusted, with buffer of the same composition of the corresponding insertion medium, to similar values as those present in the amount of OmpF used.

Mixed Micelles of oPOE/OmpF/DMPC or DMPG

Lipid film of dimyristoylphosphatidylcholine (DMPC) or dimyristoylphosphatidylglycerol (DMPG, ~5.7 μmol) was prepared by evaporation to dryness of a lipid solution in chloroform (or 1:1 mixture of chloroform/methanol in the case of DMPG) under a stream of argon and then under vacuum for a minimum of 3 h. The resulting dried lipid film was dispersed with a solution of HEPES buffer containing OmpF in 1% of oPOE. The molar ratio between lipid and protein was around 1,000. The oPOE concentration in the final suspension was always higher than the critical micellar concentration of this detergent (0.23%), and the weight ratio (w/w) between lipid and detergent was around 0.2.

Quenching of OmpF Fluorescence by Acrylamide and Iodide

Fluorescence quenching studies were achieved by successive addition of a constant volume (10 μl) of acrylamide or KI (~5.0 M) solution to the cuvette (final concentration range 0.0–0.5 M) containing a constant amount of OmpF (~0.45 μM), in micellar suspension or inserted in liposomes, in the absence or presence of a certain constant quantity (final concentration 26–30 μM) of quinolone. The first UV-Vis and fluorescence spectrum was taken before the additions (with only protein suspension or protein suspension plus constant amount of quinolone), and then after each addition the fluorescence spectrum and absorption at the excitation wavelength (290 nm) were obtained. All determinations were performed at 37°C.

Data Analysis

Data were analyzed according to the Stern-Volmer equation for collisional quenching (Lakowicz 1999):

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, Q, and K_{SV} is the Stern-Volmer quenching constant. A plot of F_0/F vs. $[Q]$ gives rise to a linear plot with a y intercept equal to 1. However, this was only true for the quenching experiments

involving the free tryptophan in solution; in all studies involving the quenching of OmpF fluorescence the Stern-Volmer plot displayed a downward curvature (Fig. 3), representative of differing accessibilities of tryptophan residues to the quenchers. In those cases the quenching process is described by a modified Stern-Volmer equation:

$$F_0 - F = F_{0a} \cdot \left(\frac{K_a \cdot [Q]}{1 + K_a \cdot [Q]} \right) \quad (2)$$

where $(F_0 - F)$ refers to the change in fluorescence intensity on addition of the quencher, F_{0a} refers to the initial fluorescence (in the absence of quencher) accessible to the quencher and K_a is the Stern-Volmer constant of the accessible fraction of tryptophans. The value of the fraction of the initial fluorescence which is accessible to the quencher (f_a) is easily obtained from

$$f_a = \frac{F_{0a}}{F_0} \quad (3)$$

In all cases fluorescence intensities were corrected for dilution. For acrylamide quenching studies fluorescence measurements were further corrected for the absorption of the quencher at the wavelength of excitation of the protein, by application of the following formula (Coutinho and Prieto 1993):

$$F_{ex} = F \cdot \frac{A_T}{A_F} \cdot \frac{1 - 10^{-A_F}}{1 - 10^{-A_T}} \quad (4)$$

where F_{ex} is the fluorescence intensity corrected for absorbance at the excitation wavelength, F is the fluorescence intensity experimentally obtained, A_T is the absorbance of the sample containing the protein (or the protein more a constant amount of antibiotic) and a given concentration of the quencher and A_F is the absorbance of the sample containing only the fluorophore (OmpF or OmpF plus the amount of antibiotic).

After all the needed corrections, the spectral changes observed from the addition of the quenchers to the samples containing OmpF (or OmpF and antibiotic) were treated by nonlinear graphical methods, using the wavelength of maximum fluorescence intensity. Fitting of equation 2 to the experimental data was performed using the computer program Origin 6.1 (OriginLab, Northampton, MA).

Results and Discussion

Characterization of OmpF Intrinsic Fluorescence

The quenching studies were done with OmpF inserted in mixed micelles of detergent (oPOE) and phospholipid, DMPC or DMPG, a zwitterionic and an anionic lipid, respectively.

In order to determine the quenching efficiency and accessibility of the different quenchers to the tryptophan in distinct environments, solutions of free tryptophan in mixed micelles of DMPC or DMPG were prepared and fluorescence quenching experiments using acrylamide and iodide were performed (Table 1).

Table 1 shows the quenching results obtained with acrylamide and iodide for free Trp and OmpF in micellar media of distinct composition. The quenching of free Trp by iodide in micelles of DMPG is smaller than that observed in DMPC micelles, but for acrylamide, although the variation is of the same amplitude, the quenching is higher in DMPG micelles than in DMPC micelles. These variations are associated with the amphipathic nature of the free Trp, its preference for the interface region (Lee 2003) and its different partition in the different mixed micelles; partition of free Trp is higher in the negatively charged

Table 1 Stern-Volmer constants and accessible fraction of fluorescence quenching by acrylamide and iodide to the free tryptophan in solution and to the tryptophans of OmpF in different micellar media

	Iodide		Acrylamide	
	K_a (M^{-1})	f_a	K_a (M^{-1})	f_a
Trp MicMixDMPC	8.0 ± 0.1	1.0	11.0 ± 0.1	1.0
Trp MicMixDMPG	7.2 ± 0.1	1.0	12.0 ± 0.1	1.0
OmpF MicMixDMPC	2.8 ± 0.2	0.4 ± 0.1	3.8 ± 0.1	0.6 ± 0.1
OmpF MicMixDMPG	1.4 ± 0.1	0.6 ± 0.1	4.2 ± 0.1	0.7 ± 0.1

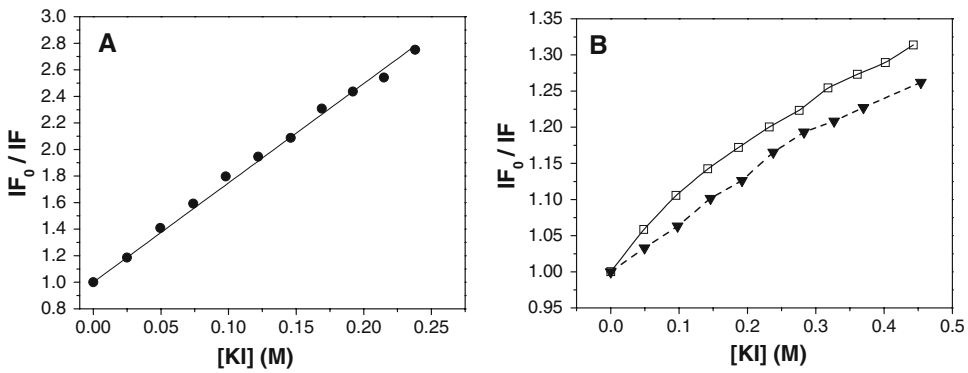

Fig. 3 Stern-Volmer (**a**) and modified Stern-Volmer (**b**) plots for iodide quenching of free tryptophan in solution (●) and tryptophan of OmpF structure, with the protein inserted in mixed micelles of DMPC (□) or DMPG (▼)

structures. The changes sensed by each of the quenchers are small but can be explained by increased contact in the case of acrylamide and by decreased contact of iodide, possibly by repulsion effects.

Comparing the results of free Trp with OmpF, it is possible to state that the variations occurred with the same orientation but with a very different intensity. Exposure of the protein to negative charges led to a different structural organization, protecting the negatively charged extremity of the channel from repulsion with the charges from the surrounding environment.

These results show that micellar surface charge can modulate the conformation and dynamics of OmpF, which is extremely relevant to understanding the role of the surface charge of bacterial membranes in the interaction of this protein with antibiotics.

The quenching by acrylamide was unaffected by the lipid environment, whereas the quenching constant in the presence of iodide ions strongly decreased. This behavior suggests that acrylamide must access Trp^{61} in the core of the trimer interface not being affected by the surrounding lipids and that iodide has preferential access to Trp^{214} near the protein–lipid interface (Lee 2003). When negatively charged lipids surround the protein, the structural conformation of OmpF is forced to rearrange and Trp^{214} will be deeply buried in the hydrophobic core of the membrane as dynamic quenching depends on contact (Lakowicz 1999) and k_a will decreases due to a higher steric shielding of iodide. These conclusions are further confirmed by the higher f_a values in presence of acrylamide as Trp^{61} has a higher fluorescence intensity because it is located in a more hydrophobic environment.

These results, based on lipids with different head groups, allowed confirmation of the regions that each of the quenchers access. For iodide, which is repelled by the negative charges, we observed a decrease in the quenching constant, which shows that the quencher is less effective when the protein is in a negative environment. For acrylamide, there is only a slight increase in the quenching constant, showing that the protein region that is accessed by this quencher is unaffected by lipid composition.

The different quenching behavior shown by iodide and acrylamide confirms that these quenchers can be used to probe access toward the different OmpF Trps, with the Trp in the hydrophobic core of the trimer interface detected only by acrylamide and the Trp near the protein–lipid interface detected by iodide.

Quenching Studies in the Presence of Drugs

Interaction of the drugs with the protein is described by the formation of a complex in the ground state (Neves et al. 2005): The quenching is static and the lifetimes of Trp, in a specific organized structure, do not change in the presence of the drugs. Therefore, the quenching parameters observed in the presence of acrylamide and iodide are comparable with those obtained in the absence of the drugs (Lakowicz 1999). The results are depicted in Table 2.

Mixed Micelles of oPOE/OmpF/DMPC

For studies performed in the presence of the drugs, in DMPC, the major change to the quenching of OmpF fluorescence by iodide is observed in the presence of NA, a marked decrease in the value of k_a being observed. The presence of CP gives only a slight decrease in the value of k_a, and GP did not bring any changes. However, MX caused slight increases in k_a and f_a. Taking into consideration the different access of iodide to Trp^{214} and the alterations introduced by the presence of each of the antibiotics, it is possible to deduce that NA interacts with the protein in the proximity of this tryptophan, this assumption being in agreement with the higher hydrophobicity of this antibiotic characterized by higher values of partition in hydrophobic environments (Piddock et al. 1999) and with conductance studies performed in black lipid bilayer that showed that NA did not block the OmpF channel (Mach et al. 2008). The results for CP seem to show that this antibiotic can interact also near this Trp, but GP seems to develop its interaction without interfering with the environment of Trp^{214}. For MX a slight interference is observed but in a different direction: It favors quenching by iodide.

Table 2 Stern-Volmer constants and accessible fraction of fluorescence quenching by acrylamide and iodide to the tryptophans of OmpF inserted in the micellar system

	No drug		NA		CP		GR		MX	
	K_a (M^{-1})	f_a	K_a (M^{-1})	f_a	K_a (M^{-1})	f_a	K_a (M^{-1})	f_a	K_a (M^{-1})	f_a
Acrylamide/DMPC	3.8 (±0.1)	0.6 (±0.1)	3.5 (±0.2)	0.6 (±0.1)	3.1 (±0.1)	0.6 (±0.1)	3.6 (±0.1)	0.6 (±0.1)	5.1 (±0.1)	0.6 (±0.1)
Acrylamide/DMPG	4.2 (±0.1)	0.7 (±0.1)	4.3 (±0.1)	0.6 (±0.1)	4.5 (±0.3)	0.6 (±0.1)	2.4 (±0.1)	0.7 (±0.1)	3.6 (±0.2)	0.7 (±0.1)
IP^{-}/DMPC	2.8 (±0.2)	0.4 (±0.1)	1.5 (±0.3)	0.5 (±0.1)	2.2 (±0.1)	0.4 (±0.1)	2.7 (±0.2)	0.3 (±0.1)	3.1 (±0.2)	0.4 (±0.1)
IP^{-P}/DMPG	1.4 (±0.1)	0.6 (±0.1)	2.1 (±0.1)	0.4 (±0.1)	2.8 (±0.1)	0.4 (±0.1)	4.4 (±0.1)	0.3 (±0.1)	4.7 (±0.2)	0.4 (±0.1)

First column describes the quencher and the phospholipid used. The antibiotic concentrations used were 27.9 μM (NA), 26.9 μM (CP), 27.9 μM (GP) and 27.9 μM (MX)

This change can be explained by a destabilization on the lipid order in the presence of MX (Bensikaddour et al. 2008), facilitating iodide's access to this Trp, or by a conformational change of OmpF accommodating the translocation antibiotic and exposing the Trp^{214} closer to the bilayer surface. Recent results from molecular modeling studies confirm this last hypothesis (Mach et al. 2008).

Fluorescence quenching by acrylamide does not show any significant change in the presence of NA, CP or GP; but MX causes an increase in k_a and f_a. This behavior combined with the results obtained for iodide can be explained considering that the extension of the interaction MX–OmpF is greater than the one for CP and GP or considering that it simply happens in a distinct manner with a possible conformational change that does not happen in these conditions for the other drugs. These results are corroborated by fluorescence energy transfer studies performed for CP and by conductance and molecular modeling studies performed for MX, which show that CP interacts at the protein–lipid interface and MX at the OmpF channel (Fernandes et al. 2007; Mach et al. 2008).

Mixed Micelles of oPOE/OmpF/DMPG

For studies performed in the presence of drugs, with OmpF inserted in mixed micelles of oPOE and DMPG, the fluorescence quenching of OmpF tryptophans by iodide showed significant changes in the presence of all the antibiotics, with an increase of the k_a value for all of the drugs. For NA, the slight increase of k_a could be related to a normal destabilization of the micelle around the protein in consequence of the presence of the antibiotic, which would facilitate the access of iodide through neutral areas; however, this effect is always equilibrated by steric shielding from the presence of antibiotic molecules on the pathway of the quencher and resulting in a smaller increase. The most significant increase in k_a for iodide quenching is felt in the presence of GP and MX and is the result of the interaction of these drugs at two different levels: First, they can neutralize the negative charges and destabilize the micellar structure by binding in the interface and, in this way, facilitate iodide access to Trp^{214}; second, if their interaction with OmpF is at the hydrophilic channel level, they cause conformational changes on the protein that may lead to better access of the quencher in other regions of the protein. Destabilization of the micelle happens as a consequence of electrostatic interactions of the drugs (partially ionized at pH 7.4) with the negative charges of DMPG. This assumption is confirmed by the partition coefficients of these drugs in liposomes of DMPG, values considerably higher than the ones obtained for the same drugs in liposomes of DMPC and by their preferential location near the lipid head group (Neves et al. 2007; Rodrigues et al. 2002). Conformational changes can occur as already stated for MX in DMPC medium. The fluorescence quenching by acrylamide showed no changes in the presence of NA or CP, but the presence of GP and MX caused a marked decrease to the k_a of this quencher. The behavior in the presence of NA is once more a proof that the interaction of this antibiotic with OmpF is restricted to the lipid–protein interface and has no consequence in the environment of Trp^{61}, which agrees with the results obtained previously for CP (Fernandes et al. 2007). The opposite behavior observed for GP and MX predicts an interaction with the hydrophilic channel of OmpF, which consequently changes near the environment of Trp^{61}.

Concluding Remarks

Aqueous soluble quenchers like iodide and acrylamide are widely used to provide information on the gross location of tryptophan residues in the complex three-dimensional structure of soluble and membrane-bound proteins (Alston et al. 2008; Eftink 1991; Lima et al. 2008; Raja et al. 1999). In our study this simple methodology proved to be very useful, to identify the two different Trps in OmpF. Taking into account the knowledge of the OmpF trimer structure, the fluorescence quenching studies performed in mixed micelles of DMPC and DMPG allowed us to identify that iodide quenches preferably the fluorescence that arises from Trp^{214} and acrylamide identifies only the fluorescence that arises from Trp^{61}. This result can be extended for DMPC proteoliposomes; moreover, it can be concluded that OmpF conformation in the two organized media is clearly different.

With these conclusions in mind and the knowledge that quinolones interact by static quenching with OmpF, it was possible to assess the location of quinolone interactions on the different organized structures. The results in the presence of the antibiotics show that quinolones have different affinity for distinct areas of the protein, with NA clearly interacting in the surrounding area of Trp^{214}, CP interacting also near this Trp and MX clearly interacting in the interior of the hydrophilic channel near Trp^{61}. The importance of this protein for these quinolone translocations is also different; in the presence of NA there was no conformational change detected, so it is possible to infer that for NA there is only a profit of the lipid–protein interface but not a real need for binding to the protein. For the other antibiotics there was always evidence of conformational changes and evidence of binding to the protein. These results achieved based on quenching studies were corroborated by other techniques showing that a preliminary conclusion on the interaction of antibiotics with outer membrane proteins can be gained using a simple

fluorescence methodology. Moreover, as the use of model membranes is becoming more popular among cell biologists, this work demonstrates that fluorescence quenching can be a practical and straightforward alternative to verify/confirm drug–membrane protein interaction. A final consideration must be made regarding the differences observed in the presence of negatively charged lipids: Not only did electrostatic attraction of the drugs account for a significant proportion but also the protein acquired a different conformation which obviously modulates the permeation of the quinolones. This kind of result gives clear evidence of the importance of cell surface charge and consequently of membranes in the permeation through the bilayers.

Acknowledgments Partial financial support for this work was provided by Fundação para a Ciência e Tecnologia (FCT, Lisbon) through project POCI/SAU-FCF/56003/2004 and by the EU-Marie-Curie Research training network (MRTN-CT-2005-019335 Translocation). P. N. thanks FCT for a fellowship.

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