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Conformational analysis of Pneumococcal Surface Antigen A (PsaA) upon zinc binding by fluorescence spectroscopy

ANA PAULA D. ANO BOM[1], IZABELLA B.S. CORRÊA[1], ANA PAULA C. ARGONDIZZO[2], MARCO ALBERTO MEDEIROS[2], ROGER B. DOS SANTOS[3], THEO LUIZ F. DE SOUZA[3] and JOSÉ G. DA SILVA JUNIOR[1]

[1]Lab. of Macromolecules, Bio-Manguinhos, Fiocruz, Av. Brasil, 4365, Room 205, Manguinhos, 21040-900 Rio de Janeiro, RJ, Brazil
[2]Lab. of Recombinant Technologies, Bio-Manguinhos, Fiocruz, Av. Brasil, 4365, Room 209, Manguinhos, 21040-900 Rio de Janeiro, RJ, Brazil
[3]Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Av Carlos Chagas Filho, 373, Room 21, Ilha do Fundão, 21941-901 Rio de Janeiro, RJ, Brazil

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ABSTRACT

PsaA (pneumococcal surface antigen A) is a *S. pneumoniae* virulence factor that belongs to the metal transport system. The Manganese PsaA binding has been associated with oxidative stress resistance becoming a pivotal element in the bacteria virulence. It has been shown that Zinc inhibits the Manganese acquisition and promotes bacteria toxicity. We have performed a PsaA conformational analysis both in the presence (Zn-rPsaA) and in the absence of Zinc (free-rPsaA). We performed experiments in the presence of different Zinc concentrations to determine the metal minimum concentration which induced a conformational change. The protein in free and Zn-binding condition was also studied in pH ranging 2.6-8.0 and in temperature ranging 25°C-85°C. pH experiments showed a decrease of fluorescence intensity only in acidic medium. Analysis of the heat-induced denaturation demonstrated that Zinc-binding promoted an increase in melting temperature from 55°C (free-rPsaA) to 78.8°C (Zn-rPsaA) according to fluorescence measurements. In addition, the rPsaA stabilization by Zinc was verified through analysis of urea and guanidine hydrochloride denaturation. Data showed that Zinc promoted an increase in the rPsaA stability and its removal by EDTA can lead to a PsaA intermediate conformation. These findings can be considered in the development of vaccines containing PsaA as antigen.

Key words: conformational analysis, rPsaA, zinc, stability, *Streptococcus Pneumoniae*.

INTRODUCTION

The *Streptococcus pneumoniae* is a gram-positive coccus, also known as pneumococcus, the most common human respiratory pathogen responsible for the annual death of millions of children worldwide (Kadioglu et al. 2008). The pneumococcus colonizes the upper respiratory tract producing severe diseases in humans including pneumonia, otitis media and meningitis (Hu et al. 2013). *S. pneumoniae* has two types of virulence

Correspondence to: Ana Paula Dinis Ano Bom
E-mail: adinis@bio.fiocruz.br

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determinants, the heterogenic capsule and the conserved surface and subsurface proteins (Rajam et al. 2008). Pneumococcal surface antigen A (PsaA) is a virulent factor, a 37 kDa lipoprotein which is hydrophobic, immunogenic and genetically conserved in *S. pneumonia* serotypes (Larentis et al. 2012).

The survival of pathogens inside their hosts depends on an efficient transport system of essential metals. Pneumococci possess an ATP-binding cassette (ABC) transporter cation permease encoded by the *psaBCA* locus (Dintilhac and Claverys 1997). This transporter is composed of the products of three genes, two units of an ATP-binding protein (PsaB) and two units of a hydrophobic membrane protein (PsaC), and a solute-binding lipoprotein (PsaA) organized in an operon with a gene encoding PsaD, a thiol peroxidase (Novak et al. 1998). The PsaA protein consists of two domains α/β arranged in a twofold pseudosymmetry axis. The domains are linked by a single helix (residues 165-189) acting as a 'backbone' to the structure, antiparallel to the fourth helix (residues 143–162) of the N-terminal domain as well as parallel to the fourth helix (residues 291–308) of the C-terminal domain. The β strands within each domain form parallel sheets and a metal-binding site is formed in the domain interface by the side chains of His67, His139, Glu205 and Asp280 (Lawrence et al. 1998).

The binding of Manganese to PsaA on the cell surface has been associated to oxidative stress resistance, during infections, and becoming a pivotal element for the bacteria growth, proliferation and virulence (Johnston et al. 2006, Ogunniyi et al. 2010). It has been shown that Zinc excess promotes toxicity to the bacteria because Zinc competes with Manganese for a binding to the same specific PsaA site, consequently inhibiting the acquisition of these essential metals (McDevitt et al. 2011).

Recent structural advances analyses of X-ray crystallography have revealed a set of high resolution structures of metal free and metal bound PsaA conformations. The metal free is found as an open state conformation in comparison to the Zinc bound PsaA that is a closed state conformation, consequence of ideal tetrahedral coordination (Couñago et al. 2014). Additionally, studies based on electron paramagnetic resonance (EPR) spectroscopy and molecular dynamics (MD) proposed structurally distinct protein conformations of metal-free PsaA, suggesting a greater flexibility (Deplazes et al. 2015).

Zinc uptake consists of AdcBC transporter with two Zinc binding proteins, AdcA and AdcAII, while the Zinc efflux is encoded by CzcD. It has been shown that high concentrations of Zinc promote a transcriptional regulation of psaBCA via PsaR. It creates insufficient Manganese uptake (Jacobsen et al. 2011). Recent studies have shown that Cadmium dysregulates metal homeostasis by perturbing the Manganese and Zinc uptake and efflux pathways (Begg et al. 2015). These studies about PsaA-metal-binding mechanism highlight the role of these metals in the cell.

In fact, the incidence and severity of airway infections is related to dietary Zinc deficiency (Brooks et al. 2004). Moreover, Zinc deficiency increases risk of pneumococcus infection and death in mice (Couñago et al. 2014). Studies have shown Zinc supplementation combined with vaccination significantly reduced the severity of diarrhea caused by rotavirus (Telmesani 2010), improved the efficacy of oral cholera vaccine in children (Albert et al. 2003) and promoted higher seroconversion rate when compared to placebo after receiving the 23-valent pneumococcal conjugate vaccine thus suggesting Zinc influence in the vaccinal protection (Braga et al. 2015).

The present work performed a conformational analysis of PsaA recombinant protein in the presence (Zn-rPsaA) or absence of Zinc (free-rPsaA) to

describe the stability of rPsaA in the presence of Zinc. Effects of chemical agents and temperature on free-rPsaA and Zn-rPsa were investigated by fluorescence spectroscopy, and the thermodynamic parameters of both zinc binding and free rPsaA were obtained. Taken together, our results showed an rPsaA conformational change upon Zinc binding characterized by an increased stability of the protein and the structural consequences of the zinc removal.

MATERIALS AND METHODS

CHEMICALS

All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. The bis-ANS probe was purchased from Molecular Probes (Eugene, OR). Experiments were performed using the following buffers: 20 mM Tris-HCl; pH 8.0; Citrate-Phosphate (pH range 2.6 to 6.6) were prepared from stocking solutions 0.1 M Citric Acid and 0.2 M Dibasic Sodium Phosphate; and Phosphate Buffer (pH range 7.0 to 9.0) was prepared from stocking solutions 0.1 M Monobasic Sodium Phosphate and 0.1 M Dibasic Sodium Phosphate.

ANALYTICALS

The PsaA recombinant protein (comprising amino acid residues 22–310) was expressed and purified according to Larentis (Larentis et al. 2011). The rPsaA protein homogeneity was ascertained by SDS-PAGE 12% using increased protein concentration, while rPsaA quantification was estimated by BCA assay using BSA (1 mg/ml) as standard.

FLUORESCENCE SPECTROSCOPY MEASUREMENTS

The tryptophan and bis-ANS fluorescence spectra were recorded using a Jasco FP-6500 spectrofluorimeter (Jasco Corp., Tokyo, Japan). Tryptophan fluorescence emission spectra were obtained by setting the excitation wavelength at 280 nm, and the emission spectrum was recorded from 295 to 415 nm. Tryptophan fluorescence spectra were quantified as the center of the spectral mass ($<\upsilon>$) in Equation 1,

$$<\upsilon> = \Sigma\, \upsilon_i .\, F_i / \Sigma F_i$$

where *Fi* is the fluorescence emission at wavenumber υi, and the summation is carried out over the range of measured values of *F*.

The dissociation constant, K_D, was determined by fitting the $<\upsilon>$ differences against the concentration of Zinc. Data were fitted using a one site saturation ligand binding curve through SigmaPlot software.

Melting temperature (T_m), $G_{1/2}$ and $U_{1/2}$ values were obtained after chemical and thermal data fitting and first derivative measure from the respective fits. $G_{1/2}$ and $U_{1/2}$ corresponds to the concentrations of Guanidine (GndCl) and Urea, respectively, which caused 50% PsaA denaturation as verified using fluorescence spectroscopy.

Assuming a 2-state model, the molar fraction of unfolded PsaA (f_D) was calculated from the equation $f= ([<\upsilon>] - [<\upsilon_N>],)/([<\upsilon_D>], - [<\upsilon_N>],)$, where $[<\upsilon>]$ is the observed spectral center of mass at any particular GndCl concentration; $[<\upsilon_N>]$, and $[<\upsilon_D>]$ are the spectral center of mass of the native (folded) and denatured (unfolded) states, respectively. The free energy of folding at each GndCl concentration (ΔG) was calculated from the equation $\Delta G = RT\ln(f_D/1 - f_D)$ and then plotted against [GndCl]. Estimates of the free energy of folding in the absence of GndCl (ΔG^0) can then be obtained by linear extrapolation method (Pace 1986).

For experiments in the presence of bis-ANS, the excitation wavelength was fixed at 360 nm, and the fluorescence emission was collected from 400 to 600 nm. The bis-ANS measurements were expressed by spectral area ratio of bis-ANS

(bis-ANS spectra area values (A)/initial spectra area value (Ao)). Experiments followed by light scattering data had an excitation wavelength at 320 nm, and the emission was collected from 300 to 340 nm. The LS measurements were expressed by the spectral area value. Experiments were carried out with different protein preparations and each data point is representative of three independent measurements. The temperatures used are described for each experiment.

CHEMICAL AND PHYSICAL DENATURATION

The rPsaA in the 0.08 mg/mL concentration was incubated at increasing concentrations of Zinc (5-500 μM) to determine the metal minimum concentration which induces a conformational change. The (Zn-rPsaA or free-rPsaA) protein stability was observed in the presence of different Urea (1–7.5 M) and GndCl (1–6.5 M) for 18 h. So as to observe the effect of pH or temperature under rPsaA, the protein samples were incubated in pH range (2.6 at 8.0) as well in temperatures from 25ºC to 85ºC.

RESULTS

CONFORMATIONAL CHANGES IN rPsaA UPON THE ZINC BINDING

In order to evaluate the role of Zinc on the structural stability of rPsaA, this protein was expressed and purified (Larentis et al. 2011). Protein homogeneity was ascertained by denaturing gel electrophoresis (MW 37,700) (Supplementary Material – Figure S1). Conformational state of the purified rPsaA was verified by fluorescence spectroscopy in absence or presence of increasing Zinc concentrations (Fig. 1a, b). rPsaA fluorescence emission spectra, in the absence of Zinc, exhibited maximum at 330 nm, which indicates a protein native fold. In the presence of Zinc, the fluorescence spectra presented a reduction of intensity, but we did not verify a significant red-shift (Fig. 1a), probably due to conformational changes close to metal binding site (Deplazes et al. 2015) or due to fluorescence quench caused by Zinc (Dunning Hottop et al. 2003).

The fluorescence Zinc titration assay showed that the changes in rPsaA conformation started at a concentration of 5 μM of Zinc and ranged up to 50 μM (Fig. 1b). The dissociation constant (K_D) calculated for 1:1 complexes of PsaA with Zn was 2.4 ± 0.4 μM. After this concentration, the spectral area values were maintained indicating stabilization of the structural modifications in the presence of metal (Fig. 1b).

ZINC BINDING INTERFERES IN rPsaA THERMAL DENATURATION

To reach a better understanding of the PsaA folding pathway, we analyzed the effect of temperature on the stability of free-rPsaA or Zn-rPsaA. We used a temperature range of 25-85ºC to verify the rPsaA conformational changes by intrinsic/extrinsic fluorescence spectroscopy and light scattering. Free-rPsaA conformational changes started at 43ºC and continued until 80ºC as verified by the decreasing values of spectral center of mass (Fig. 2a). Zinc binding to rPsaA promotes an expansion of the temperature range in which the protein maintains its stability. The analysis of the denaturation curve of Zn-rPsaA shows that the center of mass values changes as of temperatures above 70°C, and this denaturation process presented a minor total center of mass (CM) variation when compared to free protein (Fig. 2a). The melting temperature (Tm) obtained for the Free-rPsaA (55ºC) and Zn-rPsaA (78.8ºC) showed that Zinc induces a more thermo-stable structure. In order to investigate whether this protein would be able to return to its native conformation after thermal denaturation we measured the fluorescence intensity at 25ºC. Under watch, the free and bound rPsaA did not return to the initial conformation (Fig. 2a). Also observed was that free-rPsaA presented increasing values

of light scattering according to the increase in temperature, a fact evidenced by the temperatures above 50°C. On the other hand, in the presence of Zinc, a small variation of light scattering values was observed only around 80°C (Fig. 2b), which is consistent with the increase thermal stability of rPsaA verified by the fluorescence analyses (Fig. 2a). We also used bis-ANS that have been the most frequently used extrinsic fluorescent dyes for protein characterization. This dye is hardly fluorescent in aqueous environment, but become highly fluorescent in apolar, organic solvents or upon adsorption to solid phases (Rosen and Weber 1969). Considering the bis-ANS effects on free- and bounded protein, we observed the reduction of the spectral area (40-60°C) for Zn-rPsaA in comparison to free-PsaA. This indicates differences in the hydrophobic surface exposure for Zn-rPsaA when compared to free-PsaA although the curve profile was very similar (Fig. 2c).

COMPARISON OF FREE-RPSAA AND Zn-rPsaA UNFOLDING BY GNDCL AND UREA

GndCl or Urea effects on Zinc-free and Zinc-bound forms of rPsaA at 25°C demonstrated that a different unfolding profile were assumed for each protein as verified by center of mass values. The free-rPsaA was more sensitive to a small concentration of caotropic agents than Zn-rPsaA which can also be observed by the $[G]_{1/2}$ and $[U]_{1/2}$ values extracted from the denaturation curve (Fig. 3a, b). The $[G]_{1/2}$ of free-rPsaA and Zn-rPsaA were 0.67 M and 1.61 M, respectively. The $[U]_{1/2}$ values were also higher for Zinc-bound protein compared to free protein. The $[U]_{1/2}$ of free-rPsaA and Zn-rPsaA were 2.15 M and 4.63 M, respectively. These data indicate higher protein stability in the presence of Zinc. Although for both free- and Zn-rPsaA there are different concentrations of GndCl that result in protein conformational modifications, the final center of mass value for both free- and bound form of rPsaA protein were very similar

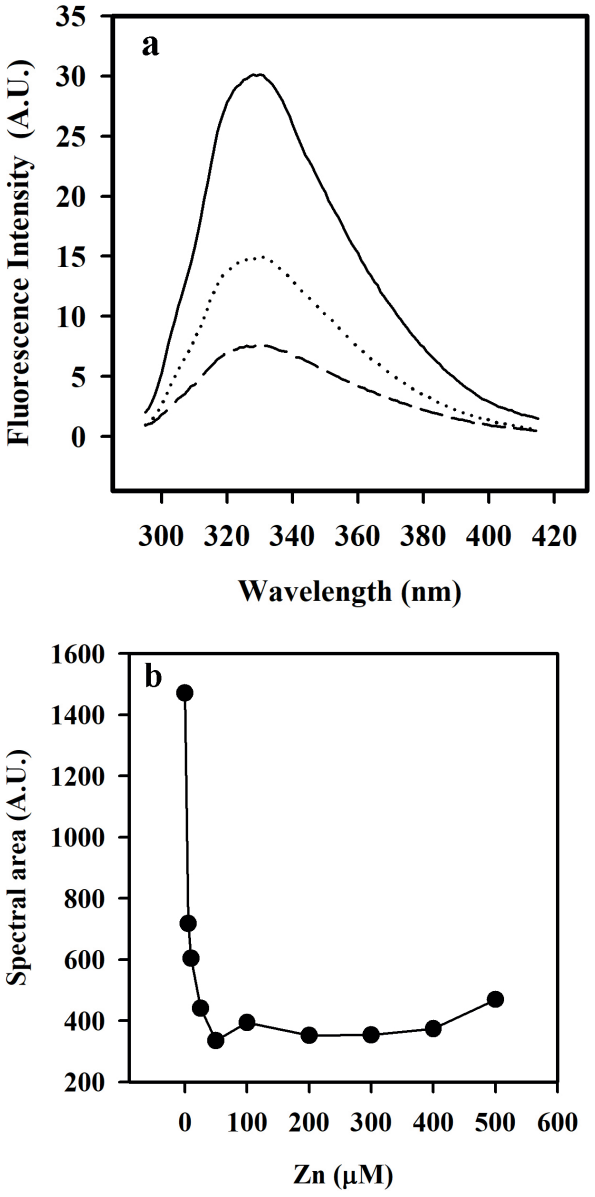

Figure 1 - Effect of Zinc on rPsaA. **a**) rPsaA protein fluorescence emission spectra in absence of Zinc (solid line), in presence of 5 μM (dotted line) and 50 μM of Zinc (dashed line). **b**) Zn-rPsaA fluorescence spectral area at different Zinc concentrations (5-500 μM). The samples were excited at 280 nm and emission collected from 295-415 nm at 25°C.

(Fig. 3a, b). After dilution of the unfolded rPsaA to subdenaturing concentrations, or the removal of GndCl by ultrafiltration, we verified that the spectral center of mass returned to a value close to the initial one (data not shown). rPsaA stability increase can also be observed by comparing the

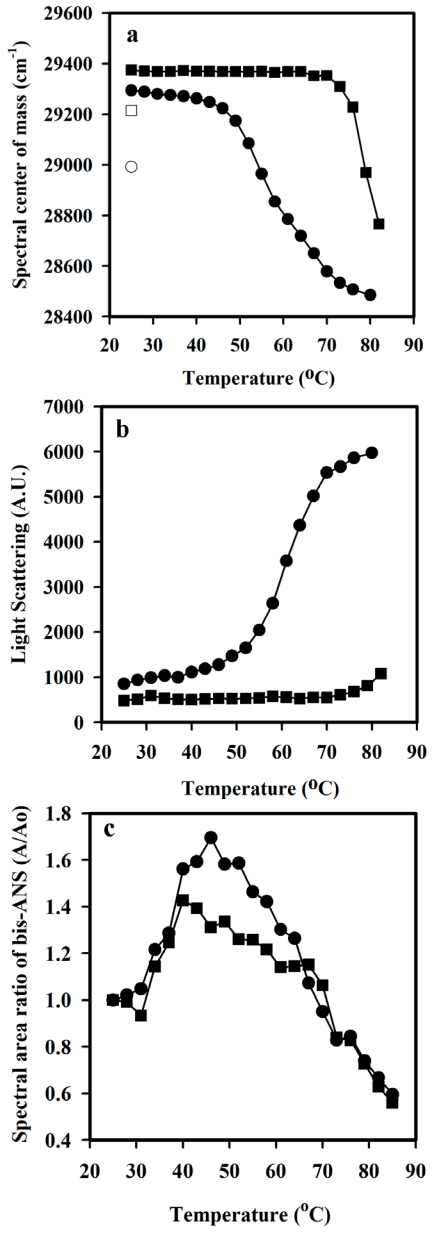

Figure 2 - rPsaA temperature effect in presence of 50 μM Zinc (square) and absence of Zinc (circle). The samples were monitored at intervals of 3°C, kept on 10 minutes at each temperature. **a**) The free or Zinc-bound rPsaA was submitted to increasing temperatures and the unfolding was followed by center of mass values based on tryptophan fluorescence emission. The samples were excited at 280 nm and the emission was measured from 295 to 415 nm. **b**) The thermal curves were monitored by light scattering (LS), the samples were illuminated at 320 nm and the LS was collected from 300 nm to 340 nm. **c**) Normalized bis-ANS fluorescence spectral area of free-rPsaA (circle) or Zinc-bound rPsaA (square). The sample was excited at 360 nm, and the emission was measured from 400 to 600 nm. The open symbols correspond to CM values after returning to room temperature.

ΔG° values in the absence (-3.28 kcal/mol) and in the presence of Zinc (-6.35 kcal/mol).

THE EFFECT OF pH ON THE rPsaA STRUCTURE

Because pH can be crucial to the growth of *Streptococcus Pneumoniae* (Moscoso et al. 2009), we verified the behavior of rPsaA in different pHs. We compared the pH curve profile of this protein in the absence or presence of Zinc. It was noted that at more acidic pHs (2.6 to 4.0) rPsaA protein shows reduced fluorescence intensity when compared to pH 7.0. From the pH range 4.5 to 8.0 the protein presents similar fluorescence spectra to those obtained at pH 7.0 (Fig. 4a). The pH experiments analyzed by center of mass values for free rPsaA and Zn-rPsaA practically showed similar curve profiles (Fig. 4b).

Taking into account the protein at acidic pH, we detected different values of bis-ANS spectral area when compared free protein and Zinc bound protein. The bis-ANS fluorescence is superior in a free-rPsaA, similar to thermal denaturation data (Fig. 4c).

EFFECTS OF GLYCEROL ON rPsaA AND EDTA ON Zn-rPsaA

In order to verify if the stabilization produced by the PsaA Zinc binding could be simulated or abolished, we performed a heat denaturation aiming to show the effect of glycerol and EDTA on free-rPsaA and Zinc bound recombinant protein, respectively. Although glycerol (20% v/v) conferred some stabilization to free-rPsaA, the thermal stabilization produced by the metal on the protein was superior, as per values of center of mass obtained (Fig. 5a). Besides, treatment with EDTA produces an intermediate denaturation curve between the free and Zinc bound protein, even showing similarity to the final CM value of Zn-rPsaA in the presence or absence of EDTA (Fig. 5a). In fact, the Tm value extracted from the EDTA curve (55.2ºC) was very similar to the Tm value extracted from the free-

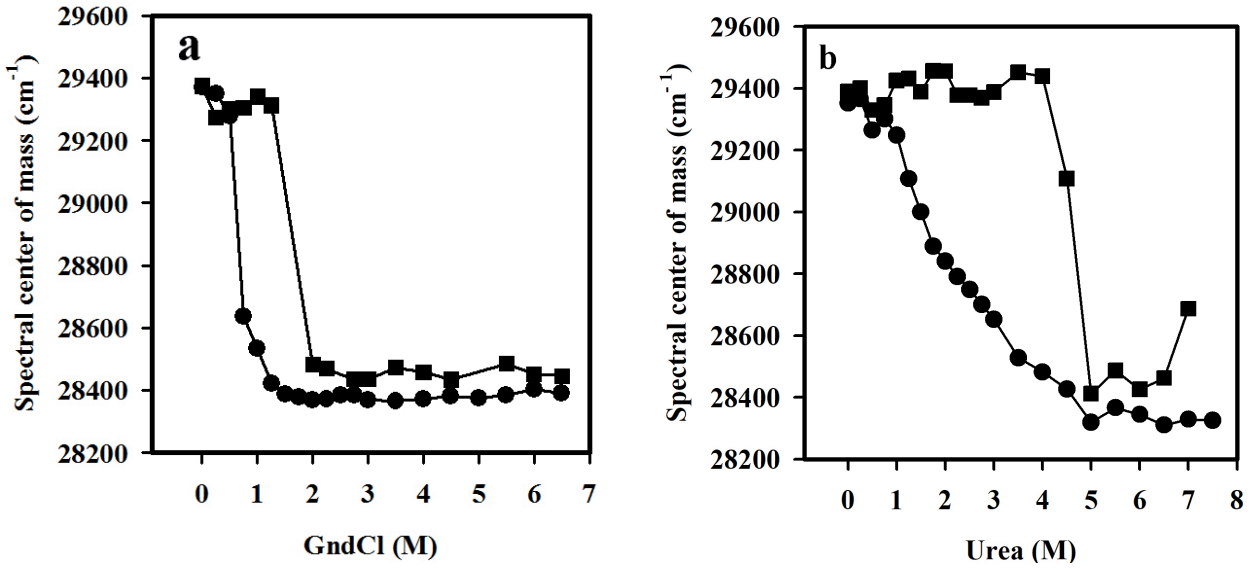

Figure 3 - GndCl and Urea effects on the rPsaA protein structure in the presence of 50 μM Zinc (square) and absence of Zinc (circle). rPsaA (0.08 mg/ml) was treated with different concentrations of GndCl (0.25 to 6.5 M) or Urea (0.25 to 7.5 M) during overnight incubation. (**a**) GndCl and (**b**) Urea conformational changes were followed by center of mass values based on tryptophan fluorescence emission. The samples were excited at 280 nm and the emission was measured from 295 to 415 nm at 25ºC.

PsaA curve (55ºC). Data suggest that EDTA-treated PsaA has lower stability than Zn-rPsaA.

Light scattering experiments showed that only free-rPsaA presented higher variation of LS values when submitted to increasing temperatures when compared to free protein treated with glycerol and Zn-rPsaA in the presence and absence of EDTA (Fig. 5b).

DISCUSSION

Pathological for humans, *Streptococcus pneumoniae* possesses an adhesin protein (PsaA) necessary in the initial stage of infection (Tai 2006). This lipoprotein binds divalent metals such as Manganese, Zinc and Cadmium. The Manganese complex is fundamental to avoid the bacteria cell oxidative stress (Ogunniyi et al. 2010). Zinc competes with Manganese by the same site in PsaA structure and carries out the protein for a more closed conformational state than Manganese (Couñago et al. 2014). *S. pneumoniae* transition metal ions uptake pathway has been investigated resulting in discovery of a crescent number of conserved protein involved in this mechanism (Deplazes et al. 2015, Shafeeq et al. 2013, Bayle et al. 2011). Recently, the role of PsaA and the transcriptionally PsaA repressor (psaR) in the metal transport have been demonstrated (Li et al. 2014, Lisher et al. 2013).

Zinc has been a WHO-recommended supplement for children with respiratory infections and in the management of diarrhea (Lassi et al. 2016). A systematic study of conformation changes induced by Zinc metal was investigated by present work. Literature is the source when it comes to the selection of Zinc concentration. Such concentrations are reported to range from 5 μM in the nasopharynx to ~300 μM in lung tissue during the course of an infection of a mammalian host by the pathogen *Streptococcus pneumoniae* (Jacobsen et al. 2011). Our findings have shown the enhancement of rPsaA structural stability promoted by Zinc. We observed conformational changes between the free-rPsaA and Zn-rPsaA based on fluorescence spectra studies (Fig. 1a, b). The decrease in fluorescence intensity

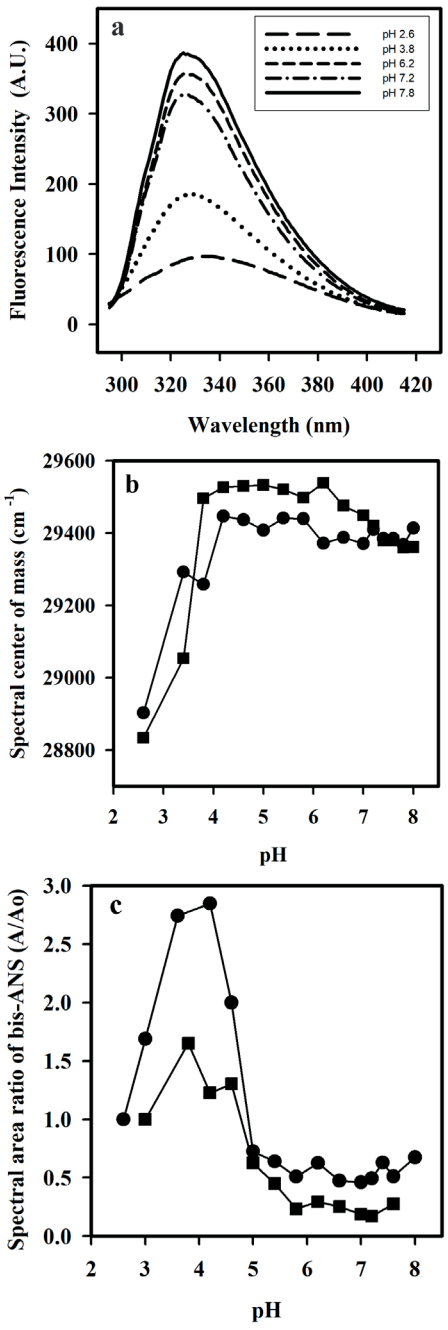

Figure 4 - pH effect on rPsaA in presence of 50 μM Zinc (square) and absence of Zinc (circle). (**a**) Spectra of intrinsic tryptophan fluorescence emission of rPsaA protein in different pHs (2.6 to 8.0) [pH 2.6 (long dashed line), pH 3.8 (dotted line), pH 6.2 (short dashed line), pH 7.2 (dash-dotted line), pH 7.8 (solid line)]. (**b**) pH-induced conformational changes of rPsaA protein were followed by center of mass based on tryptophan fluorescence emission in different pHs. The excitation occurred at 280 nm and the emission was collected from 295 to 415 nm at 25ºC. (**c**) bis-ANS fluorescence spectral area of free- (circle) or Zinc-bound rPsaA (square), (excitation 360 nm; emission from 400 to 600 nm).

of Zn-rPsaA in comparison to free-rPsaA could be related to fluorescence quench by Zinc or due to conformational changes close to the metal binding region and the helix that act as a spring according to "spring-hammer" like mechanism for metal ion binding (Couñago et al. 2014). This model displays a combination of metal ion geometry and the distortion of helix linking the N- and C-terminal lobes of the protein (Begg et al. 2015). These findings are also corroborated by Deplazes et al. (2015), who point to the higher flexibility and conformational dynamics of metal-free PsaA in solution. Therefore, our study corroborates the literature data in relation to the conformational change caused by Zinc in PsaA evidencing the importance of Zinc in metal transport by PsaA and toxicity to bacteria.

Since the conformation of a protein is intrinsically related to its stability, we have used physical and chemical agents to induce rPsaA denaturation. Here we show that Zinc binding leads to stabilization of PsaA against Urea, GndCl and temperature-induced unfolding (Figs. 2, 3). We verified that Zinc binding promotes an increase in Tm from 55ºC (free-rPsaA) to 78.8ºC (Zn-rPsaA) according to fluorescence spectral measurements and light scattering (Fig. 2a, b). Consistent to our study, thermal stability from 62ºC to 72ºC for Zn-rPsaA in comparison with free-rPsaA was also demonstrated by Mc Devitt (McDevitt et al. 2011) using differential scanning calorimetry (DSC). Additionally, our light scattering data showed that PsaA aggregation in high temperatures is inhibited in the presence of Zinc (Fig. 2b).

Similarly, Zn-rPsaA incubated with the same concentrations of Urea and GndCl showed more resistance to conformational changes than free protein. In this context, Zn-rPsaA supported higher urea and GndCl concentrations ($U_{1/2}$ = 4.63 M; $G_{1/2}$ = 1.61 M) than the free rPsaA ($U_{1/2}$ = 2.15 M; $G_{1/2}$ = 0.67 M) (Fig. 3a, b). Probably, the great stability reached by the complex Zn-rPsaA might be due to the

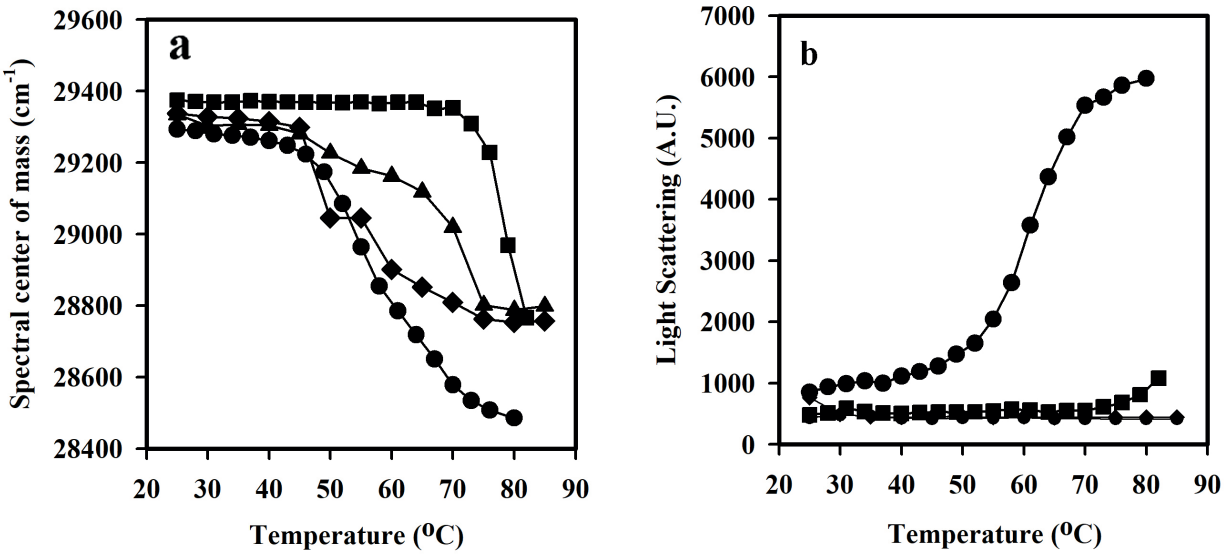

Figure 5 - Comparison of Zinc, EDTA and Glycerol effects on rPsaA. Increasing temperatures (25ºC to 85ºC) were used. Measurements were performed in the presence of 50 **µM** Zinc (square), in the absence of Zinc (circle), in the presence of 50 µM Zinc plus 5 mM of EDTA (diamond), and in the presence of 20% glycerol (triangle). The denaturation curve was determined through center of mass (**a**) and light scattering (**b**).

ideal tetrahedral metal coordination demonstrated by Couñago in their X-ray crystallography studies (Couñago et al. 2014).

We have noted that both free-rPsaA and Zn-rPsaA were not sensitive to the pH range 4.5–8.0, both showing similar fluorescence spectra (Fig. 4a, b), whereas in acidic pH, both proteins presented different fluorescence spectra, but the native protein conformational state was maintained at neutral and basic pH (Fig. 4a, b). Our results are in agreement with those obtained by Iyer (Iyer et al. 2012) that showed a change in the content of PsaA secondary structure at acidic pH (pH 3 and 4). We also observed that in room temperature and acidic pH (pH 3 and 4), the rPsaA treated with bis-ANS showed an increase in exposure of hydrophobic sites in rPsaA in relation to neutral and basic pHs, indicating a conformational change of this protein in those pHs (Fig. 4c).

In fact, *Streptococcus pneumoniae* faces a range of potentially acidic conditions in the middle and late stages of growth in vitro and in several human fluids during the infection process and in biofilms present in the nasopharynx of the carriers (Donlan and Costerton 2002). *Streptococcus pneumoniae* has different expression patterns in response to low pH that indicate acid tolerance response (Martin-Galiano et al. 2005). A similar expression profile could occur *in vivo* during the infection or in the transmission processes in the carrier. Moreover, physiological changes due to pH decrease have been reported for *Streptoccocus mutants* during biofilm growth in the dental plaque formation process (Svensäter 1997).

We also tested the effect of the glycerol as a stabilizer on rPsaA and the metal chelator EDTA on the Zn-rPsaA. Indeed, the intracellular environment is densely packed with macromolecules and this is referred to as macromolecular crowding (Mourão et al. 2014). The excluded-volume effect promoted by crowding has been demonstrated to interfere with the stability of macromolecules (Minton 2005). For that reason, it was verified whether there would be differences in the stability of PsaA in the presence of glycerol when compared to PsaA and Zn-PsaA. Our fluorescence and light scattering

data demonstrated that the treatment with glycerol was partially able to protect the rPsaA from thermic denaturation, presenting a lower center of mass and light scattering variation than that observed with the protein in the absence of Zinc (Fig. 5a, b). The thermal stability reached by Zinc was higher than glycerol probably because the glycerol acts in macromolecular crowding of the protein, i.e. on protein environ while the Zinc binding can change the protein conformation. The results obtained by rPsaA metal chelation analysis performed by fluorescence, using 5 mM EDTA (McDevitt et al. 2011) showed an intermediate fluorescence curve between free-rPsaA and Zn-rPsaA, probably due to the partial recovery of the denaturation profile of the free-PsaA, exemplified by the close values of Tm. One possibility would be the effect of EDTA on PsaA structure, preventing the complete recovery of the denaturation profile of free-PsaA, this fact was observed in CBP/p300 C/H1 domain study (Matt et al. 2004) (Fig. 5a). The same light scattering variation of Zn-rPsaA and of this protein incubated with 5 mM EDTA are indicative that the chelating agent was not sufficient to reverse the effect of the divalent transition metal on rPsaA (Fig. 5b) which indicates the formation of an intermediate conformation of the rPsaA. These findings could be considered in vaccine development containing PsaA as antigen once the PsaA conformational change caused by zinc removal could lead to the unpredictable immunogenic consequences such as the loss of immunization efficiency against *Streptococcus pneumonia*.

Data related to rPsaA conformational analysis is significant to improve the structural understanding of this protein when bound to Zinc and its biological implications. Additionally, due to a continuing increase of *S. pneumoniae* antibiotic resistance, the search for a better vaccine or other drug target against the microorganism is ongoing. In this context, PsaA remains a potential candidate for the development of a protein-based vaccine against the infectious bacteria. Overall, our studies raise the discussion about PsaA structure analysis and Zinc binding and removal effects on rPsaA.

REFERENCES

ALBERT MJ ET AL. 2003 Supplementation with zinc, but not vitamin A, improves seroconversion to vibriocidal antibodyin children given an oral cholera vaccine. J Infect Dis 15 187(6): 909-913.

BAYLE L, CHIMALAPATI S, SCHOEHN G, BROWN J, VERNET T AND DURMORT C. 2011. Zinc uptake by Streptococcus pneumoniae depends on both AdcA and AdcAII and is essential for normal bacterial morphology and virulence. Mol Microbiol 82(4): 904-916.

BEGG SL ET AL. 2015. Dysregulation of transition metal ion homeostasis is the molecular basis for cadmium toxicity in Streptococcus pneumoniae. Nat Commun 6: 6418.

BRAGA CB, SANTOS IK, PALMEIRA P, PERIA FM, RIBEIRO SM, MARTINEZ EZ, DA ROCHA JJ AND DA CUNHA SF. 2015. Effect of Zinc Supplementation on Serological Response to Vaccination Against Streptococcus Pneumoniae in Patients Undergoing Chemotherapy for Colorectal Cancer. Nutr Cancer 67(6): 926-932.

BROOKS WA, YUNUS M, SANTOSHAM M, WAHED MA, NAHAR K, YEASMIN S AND BLACK RE. 2004. Zinc for severe pneumonia in very young children: double-blind placebo-controlled trial. Lancet 363: 1683-1688.

COUÑAGO RM ET AL. 2014. Imperfect coordination chemistry facilitates metal ion release in the Psa permease. Nat Chem Biol 10(1): 35-41.

DEPLAZES E, BEGG SL, VAN WONDEREN JH, CAMPBELL R, KOBE B, PATON JC, MACMILLAN F, MCDEVITT CA AND O'MARA ML. 2015. Characterizing the conformational dynamics of metalfree PsaA using molecular dynamics simulations and electron paramagnetic resonance spectroscopy. Biophys Chem 207: 51-60.

DINTILHAC A AND CLAVERYS JP. 1997. The adc locus, which affects competence for genetic transformation in Streptococcus pneumoniae, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcus adhesins. Res Microbiol 148: 119-131.

DONLAN RM AND COSTERTON JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15(2): 167-193.

DUNNING HOTOPP JC, AUCHTUNG TA, HOGAN DA AND HAUSINGER RP. 2003. Intrinsic tryptophan fluorescence as a probe of metal and alpha-ketoglutarate binding to TfdA, a mononuclear non-heme iron dioxygenase. J Inorg Biochem 93(1-2): 66-70.

HU D ET AL. 2013. Roles of virulence genes (PsaA and CpsA) on the invasion of Streptococcus pneumoniae into blood system. Eur J Med Res 18(1): 14.

IYER V, HU L, LIYANAGE MR, ESFANDIARY R, REINISCH C, MEINKE A, MAISONNEUVE J, VOLKIN DB, JOSHI SB AND MIDDAUGH CR. 2012. Preformulation characterization of an aluminum salt-adjuvanted trivalent recombinant protein-based vaccine candidate against Streptococcus pneumoniae. J Pharm Sci 101(9): 3078-3090.

JACOBSEN FE, KAZMIERCZAK KM, LISHER JP, WINKLER ME AND GIEDROC DP. 2011. Interplay between manganese and Zinc homeostasis in the human pathogen Streptococcus pneumoniae. Metallomics 3: 38-41.

JOHNSTON JW, BRILES DE, MYERS LE AND HOLLINGSHEAD SK. 2006. Mn2-Dependent Regulation of Multiple Genes in Streptococcus pneumoniae through PsaR and the Resultant Impact on Virulence. Infection and Immunity 74: 1171-1180.

KADIOGLU A, COWARD W, COLSTON MJC, HEWITT CRA, ANDREW PW AND PATON JC. 2008. The role of Streptococcus pneumonia virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 72: 2689-2697.

LARENTIS AL, ARGONDIZZO APC, ESTEVES GS, JESSOURON E, GALLER R AND MEDEIROS MA. 2011. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in Escherichia coli and recombinant protein stability during long-term storage. Protein Expr Purif 78(1): 38-47.

LARENTIS AL, MONTEIRO JF, NICOLAU Q, ARGONDIZZO APC, GALLER R, RODRIGUES MI AND MEDEIROS MA. 2012. Optimization of medium formulation and seed conditions for expression of mature PsaA (pneumococcal surface adhesin A) in Escherichia coli using a sequential experimental design strategy and response surface methodology. J Ind Microbiol Biotechnol 39: 897-908.

LASSI ZS, MOIN A AND BHUTTA ZA. 2016 Zinc supplementation for the prevention of pneumonia in children aged 2 months to 59 months. Cochrane Database Syst Rev 4(12): CD005978.

LAWRENCE MC, PILLING PA, EPA VC, BERRY AM, OGUNNIYI AD AND PATON JC. 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure 12: 1553-1561.

LI N, YANG XY, GUO Z, ZHANG J, CAO K, HAN J, ZHANG G, LIU L, SUN X AND HE QY. 2014. Varied metal-binding properties of lipoprotein PsaA in Streptococcus pneumoniae. J Biol Inorg Chem 19(6): 829-838.

LISHER JP, HIGGINS KA, MARONEY MJ AND GIEDROC DP. 2013. Physical characterization of the manganese-sensing pneumococcal surface antigen repressor from Streptococcus pneumoniae. Biochemistry 52(43): 7689-7701.

MARTÍN-GALIANO AJ, OVERWEG K, FERRÁNDIZ MJ, REUTER M, WELLS JM AND DE LA CAMPA AG. 2005. Transcriptional analysis of the acid tolerance response in Streptococcus pneumoniae. Microbiology 151: 3935-3946.

MATT TD, MARTINEZ-YAMOUT MA, DYSON HJ AND WRIGHT PE. 2004. The CBP/p300TAZ1 domain in its native state is not a binding partner of MDM2. Biochem J 381: 685-691

MCDEVITT CA, OGUNNIYI AD, VALKOV E, LAWRENCE MC, KOBE B, MCEWAN AG AND PATON JC. 2011. A Molecular Mechanism for Bacterial Susceptibility to Zinc. PLoS Pathogens 9: 1-9.

MINTON AP. 2005. Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations. J Pharm Sci 94: 1668-1675.

MOSCOSO M, GARCÍA E AND LÓPEZ R. 2009. Pneumococcal biofilms. Int Microbiol 12(2): 77-85.

MOURÃO MA, HAKIM JB AND SCHNELL S. 2014. Connecting the Dots: The Effects of Macromolecular Crowding on Cell Physiology. Biophys J 107: 2761-2766.

NOVAK R, BRAUN JS, CHARPENTIER E AND TUOMANEN E. 1998. Penicillin tolerance genes of Streptococcus pneumoniae: the ABC-type manganese permease complex Psa. Mol Microbiol 29: 1285-1296.

OGUNNIYI AD, MAHDI LK, JENNINGS MP, MCEWAN AG, MCDEVITT CA, GOULD KA AND PATON JC. 2010. Central role of manganese in regulation of stress responses, physiology, and metabolism in Streptococcus pneumoniae. J Bacteriol 192: 4489-4497.

PACE NC. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol 131 266-280.

RAJAM G, ANDERTON JM, CARLONE GM, SAMPSON JS AND ADES EW. 2008. Pneumococcal Surface Adhesin A (PsaA). Critical Reviews in Microbiology 34: 131-142.

ROSEN CG AND WEBER G. 1969. Dimer formation from 1-anilino-8- naphthalenesulfonate catalyzed by bovine serum albumin. A new fluorescent molecule with exceptional binding properties. Biochemistry 8: 3915-3920.

SHAFEEQ S, KUIPERS OP AND KLOOSTERMAN TG. 2013. The role of Zinc in the interplay between pathogenic streptococci and their hosts. Mol Microbiol 88(6): 1047-1057.

SVENSÄTER G, LARSSON UB, GREIF EC, CVITKOVITCH DG AND HAMILTON IR. 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol 12(5): 266-273.

TAI SS. 2006. Streptococcus pneumoniae protein vaccine candidates: properties, activities and animal studies. Crit Rev Microbiol 32(3): 139-153.

TELMESANI AMA. 2010. Oral Rehydration Salts, Zinc Supplement and Rota Virus Vaccine in the Management of Childhood Acute Diarrhea. J Family Community Med 17.2: 79-82.

SUPPLEMENTARY MATERIAL

Figure S1 - Denaturing gel electrophoresis SDS-PAGE of rPsaA. Molecular weight protein standards Precision Dual Color (Bio-Rad) (lane 1); rPsaA after purification (lanes 2, 3, 4, 5).