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Rose Bengal located within liposome do not affect the activity of *inside-out* oriented Na,K-ATPase

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Abstract

DPPC:DPPE–proteoliposomes (in which the enzyme is *inside-out* oriented) and DLOPC:DLOPE–proteoliposomes (in which the enzyme is only 40% *inside-out* oriented) is an excellent model for studying the selective effect of the reactive oxygen species, produced by the photo-activation of Rose Bengal. Both proteoliposomes used, when submitted to photo-irradiation with laser using 1200 mJ/cm^2 energy dose, in the absence of the Rose Bengal, did not shown any effect in the ATPase activity and in the integrity of its systems. Also, no effect was observed using 50 μM of Rose Bengal encapsulated in the interior of the DPPC:DPPE–proteoliposome system. But, when we use 50 μM of Rose Bengal, present only in the extravesicular environment, and photo-irradiation with a laser dose of 200 mJ/cm^2, it results in the loss of 40–50% of the ATPase activity, with damage of the DPPC:DPPE–proteoliposome integrity. Using a dose of 400 mJ/cm^2 the ATPase activity was totality lost. Consequently, these effects could be correlated with direct damage in the peptide structure. The photo-irradiation of the system constituted by DLOPC:DLOPE–proteoliposome in the presence of Rose Bengal, encapsulated only in the interior compartment or in the extra-liposomal environments, revealed a gradual decrease of the ATPase activity, maintaining it at 30% after a dose of 1200 mJ/cm^2 and losing total ATPase activity at 800 mJ/cm^2, respectively, with the loss of integrity of this vesicular system in both conditions studied. The generated singlet oxygen could attack the double linkage present in the fatty acid structure of the lipid instead of the amino acid in the protein structure and, in a second step, result in an indirect inactivation of the enzyme activity. In summary, these results indicated that singlet oxygen species produced by photo-oxidation of Rose Bengal using laser light could act in protein and lipid structure depending on its proportion or distribution.

Keywords: Rose Bengal; Na,K-ATPase; DPPC:DPPE–Liposome; ATPase activity

1. Introduction

Photodynamic effects are the basis of the series of clinical applications such as the photodynamic therapy (PDT). These effects occur in the presence of a photosensitizer, oxygen and light and membrane damage has been considered an important part of the photosensitized cellular modifications which lead to cell death [1–9].

The photochemical mechanism underlying these processes involves absorption of visible radiation by the dye molecules and subsequent formation of reactive intermediates, such as singlet oxygen ($^1O_2^*$), superoxide anion (O_2^-) and other reactive oxygen species and radicals of the dyes. These intermediates (or products formed from them) react rapidly with many cellular targets (proteins, lipids or nucleic acids) and induce a cascade of different effects [10–13]. One effect of great interest so far has been the loss of membrane functions, such as: direct damage of oxidative membrane (lipid peroxidation is an important example), the inactivation of membrane enzymes and the changes in the membrane channels or in many other pathways responsible for the transport of ions, amino acids or sugars [11,14–17].

Proteins are the major targets for photo-oxidation within cells due to their abundance, the presence of endogenous

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chromophores within protein structures (such as amino acid chain and prosthetic groups), their ability to bind exogenous chromophoric materials and their rapid rates of reaction with other excited state species [13].

Two important types of processes can occur during the exposure of proteins to UV or visible light: (i) the first involves direct photo-oxidation arising from the absorption of UV radiation by the protein structure (primarily side-chains), or bound chromophores, thereby generating the uptake of excited state species groups, which yields reactive excited states (singlet or triplets) or radicals as a result of photo-ionization; (ii) the second involves indirect oxidation of the protein via the formation and subsequent reactions of oxygen reactive species generated by the transfer of energy to ground triplet molecular oxygen by either protein-bound or other chromophores species [13].

Regardless of the type of process, the role of singlet oxygen acting as oxidant is one of the most important initial damages of protein on the side chains of Trp, Tyr, His, Met, Cys and cystine residues. Recent data also show that peroxide species (both endo- and hydro-peroxides) are important intermediates in the oxidation of Trp, Tyr and its residues, and that these species should mediate further damage. Unlike radical-mediated, backbone fragmentation does not appear to be a major process, whereas the formation of cross-linked process or aggregated species appears to have a major consequence of $^1O_2$-mediated protein oxidation [13]. In addition to that, indirect action in the protein could occur via lipid peroxidation. This is a complex process, in which poly-unsaturated fatty acids in biological membranes systems undergo changes in its structural and functional characteristics, resulting in the inactivation of various enzymes [11,18–20].

Little is known about which sites in membranes (lipid or protein) are the most sensitive to photosensitized damage and how the sensibility of these sites varies with the location or distribution of the dye molecules in the membrane. The knowledge of this behavior may lead to the development of a new drug delivery system targeting specific sites in membrane lipid or in protein structures. Many dye molecules clearly present one partition into cellular membranes structure: upon light activation, they may initiate photobiological responses. In some cases, the dyes are distributed into all of the membranous structures; and, in other cases, the photosensitizing dye may be selectively localized in a specific membrane structure or part of it [13,18].

The vesicular systems reconstructed with associated protein are excellent models to study the selective effect of light-activated dye on different regions (inside or outside) of the proteoliposome [5,17,21,22].

In our laboratory, we have been working with the $(\alpha\beta)_2$ form of Na,K-ATPase from the outer medulla of rabbit kidney [23,24]. It is well established that the enzyme complex consists of two polypeptide chains: the α-subunit or catalytic chain (MWr ~110 kDa) that is responsible for the enzymatic activity of the complex [25,26], and the β-subunit (MWr 35–50 kDa) that has a structural function, and may also play a functional role in the catalytic reaction and ion-pumping mechanism [26,27]. A third γ-subunit (found only in the kidney) is a small hydrophobic proteolipid (MWr 7–12 kDa) that is associated with the Na,K-ATPase which, although not essential either for catalytic activity or for ion transport, does act as a kinetic regulator [28,29].

Na,K-ATPase is a member of the P-type family of active cation transport proteins. It is found in the plasma membrane of virtually all animal cells and has been isolated in membrane bound form from tissues rich in the protein, such as mammalian kidney. The classical specific inhibitors of the Na,K-ATPase are the cardiac glycosides. They bind to the extracellular surface of the enzyme and the binding and inhibition are dependent on the ligands present being ouabain the most widely used cardiac glycoside. Another potent inhibitor of the enzyme is orthovanadate, an analogue of the phosphate state of transition, which, in contrast to the cardiac glycoside, binds to the cytoplasmatic side of the system and needs magnesium for binding. Since these inhibitors bind to specific sites located in the enzyme molecule, they have been used to study liposome reconstituted enzyme orientation [26,30–32].

Recently, we showed that in DPPC:DPPE liposomes systems, the Na,K-ATPase is reconstituted, preferentially, with the ATP hydrolysis site (or vanadate inhibitory binding site) located at the external side of the lipid bilayer vesicle, called *inside-out* orientation. Moreover, it was also shown that the proteoliposomes systems constituted by DPPC:DLOPE, DLOPC:DLOPE or DPPC:DPPE:cholesterol do not have homogeneity in the distribution of Na,K-ATPase orientation [33].

In the present study, we have conducted an investigation using these vesicular systems with defined protein orientation in association with Rose Bengal, a classical photosensitizer for mechanism studies. The proposal of using of the proteoliposome with perfect orientation of the Na,K-ATPase will allow the evaluation of the selective effect of the photo-oxidation process, induced by the photosensitizers when associated with the biological system.

2. Materials and methods

2.1. Materials

All solutions were made using Millipore Direct-Q ultra pure apyrogenic water and all reagents were of the highest purity commercially available: Trichloric Acetic Acid (TCA); tris[hydroxymethyl]aminomethane (Tris); N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES); Adenosine 5′-Triphosphate Tris salt (ATP); bovine serum albumin (BSA); Dodecyloctaethyleneglycol ($C_{12}E_8$) and Rose Bengal were from Sigma. Dipalmitoyl-

phosphatidylcholine (DPPC), dipalmitoylphosphatidyl-ethanolamine (DPPE), dilinoleoylphosphatidylcholine (DLOPC) and dilinoleoylphosphatidyl-ethanolamine (DLOPE) were from Avanti Polar Lipids. Ethylenediaminetetracetic acid (EDTA), potassium chloride, sodium chloride and magnesium chloride were from Merck. Biobeads were from BioRad.

2.2. Preparation of Na,K-ATPase

Membrane-bound and solubilized Na,K-ATPase was obtained from the dark red outer medulla of the kidney of adult New Zealand white rabbits as described previously [23]. $C_{12}E_8$ solubilized enzyme was concentrated on YM-30 Amicon filter, dialyzed overnight at 4 °C against 5 mM Tris–HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl and 0.005 mg/mL $C_{12}E_8$. The enzyme was purified by gel filtration at 4 °C on a Sepharose 6FF column (26×200 cm), equilibrated and eluted in the same buffer, using a flow rate of 1 mL/ min. Finally, 1.0 mL aliquots, presenting about 600 U/mg of ATPase activity, were frozen in liquid nitrogen and stored at −20 °C. The MW obtained for the purified enzyme was 320 kDa which is consistent with the $(\alpha\beta)_2$ form.

2.3. Protein analysis

The protein concentration of the enzyme extracts was estimated in the presence of 2% (w/v) SDS as described by Santos et al. [23]. Protein determination of proteoliposome was performed according to the methodology described by Cornelius [34]. Bovine serum albumin was used as the standard.

2.4. ATPase activity measurement

ATPase activity was assayed discontinuously at 37 °C by the quantification of phosphate release as described by Heinonen and Lathi [35], adjusting the assay medium to a final volume of 1.0 mL. The reaction was initiated by the addition of the enzyme, stopped with 0.5 mL of cold 30% TCA at appropriate time intervals and centrifuged at $4000 \times g$ immediately prior to phosphate determination. Standard conditions were 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM $MgCl_2$ and 50 mM NaCl.

Assays were performed in triplicate, using three different enzyme preparations, and controls without added enzyme were included in each experiment to quantify non-enzymatic hydrolysis of the substrate. The initial rates of hydrolysis were constant for at least 30 min, provided that less than 5% of substrate was consumed in the reaction. One enzyme unit (U) is defined as the amount of enzyme which hydrolyzes 1.0 nmol of substrate per minute at 37 °C. For inhibition studies, the enzyme or proteoliposome were previously incubated in a reaction medium with 2 mM ouabain (for 2 h) or 3 μM vanadate (for 1 h) at 4 °C, and processed as previously described for the determination of residual ATPase activity.

2.5. Preparation of proteoliposomes with or without Rose Bengal

Proteoliposomes of DPPC:DPPE or DLOPC:DLOPE were prepared by co-solubilization of lipids, protein and detergent in a weight ratio of 1:1 lipid/lipid and 1:3 lipid/protein as previously described by Santos et al. [33]. The lipid mixture was dissolved in chloroform and dried with a nitrogen flow to deposit a thin lipid film on the wall of the glass tube. These films were then dried under vacuum for about 1 h. This lipid film was resuspended in 5 mM Tris–HCl buffer, pH 7.0, containing 150 mM KCl and 10 mg/mL $C_{12}E_8$, and after incubation at 60 °C for 1 h, it was vigorously stirred using a vortex at 10 min intervals. The mixture was sonicated using a microtip for 1 min at 200 W (VibraCell, model VC-600).

The purified Na,K-ATPase was added to the lipid/detergent mixture and the suspension kept on ice for 10 min. Subsequently, the $C_{12}E_8$ was removed by addition of 200 mg/mL Biobeads with changes at time intervals of 5, 10 and 45 min. The beads were removed by centrifugation at $4000 \times g$ for 10 min at 4 °C. Finally, the vesicle suspension was ultracentrifuged for 1 h, at $100000 \times g$ and 4 °C and the pellet was re-suspended in 5 mM Tris–HCl buffer, pH 7.0 containing 150 mM KCl. The diameters obtained by dynamic light scattering for DPPC:DPPE– and DLOPC:DLOPE–proteoliposome were 140 and 160 nm, presenting ATPase activity of about 500 and 200 U/mg, respectively.

Rose Bengal (1 mM) was prepared in 5 mM Tris–HCl buffer, pH 7.5 containing 1 mM EDTA and 150 mM KCl and added to the lipid film and processed as described above, to obtain the proteoliposome with the dye encapsulated. The inhibition study showed that DPPC:DPPE– and DLOPC:DOLPE–proteoliposome have 90 and 40% of *inside-out* orientation for the reconstituted enzyme [33].

The quantification of Rose Bengal encapsulated in proteoliposome was done after the separation of the aqueous phase from the mixture of the vesicle sample/chloroform 1:1 (v/v). The dye present in the aqueous solution was determined using an absorption calibration curve at 550 nm of Rose Bengal in the range of 1–20 μM. The presence of the Rose Bengal without laser photo-irradiation did not show any significant effect in the ATPase activity of the enzyme.

2.6. Characterization of the proteoliposome system

The proteoliposome system with or without Rose Bengal (0.5 mg of total protein) was characterized by chromatography filtration on a Sepharose 6B column (0.5×144 cm) equilibrated and eluted in 5 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA and 150 mM KCl and a flow rate of

18 mL/h. Fractions of 3 mL were collected and were assayed for: presence of protein (at 280 nm); ATPase activity (at 355 nm); Rose Bengal (at 550 nm); and phospholipid (presence of phosphate obtained by previous acid hydrolysis, at 820 nm, according to the procedure described by Chen et al. [36].

2.7. Photo-irradiation studies

Studies of photo-irradiation of the solubilized-enzyme and proteoliposomes (with or without Rose Bengal) were done using the second harmonic of the ND-YAG Laser (Continuum, mode SURELITE I-10) in 532 nm with 8 ns. The beam diameter incident on the sample was 6 mm, and the repetition rate was 10 Hz. The pulse energy was typically 20 mJ per pulse with cumulative energy dose range from 200 to 1200 mJ/cm^2 measured with a Field Master power meter with L-30V head.

3. Results

$C_{12}E_8$-solubilized Na,K-ATPase was photo-irradiated in the presence of 50 mM of Rose Bengal using different energy doses (200–1200 mJ/cm^2) and the residual ATPase activity was determined as shown in Fig. 1. Is important to note that in the absence of the dye (or in the presence of 50 μM of the dye, but without laser photo-irradiation), no effect in the activity was observed. However, in the presence of the dye and 800 mJ/cm^2 energy dose, we observed the total loss of ATPase activity of the solubilized enzyme.

In order to fully understand the selective effect of the reactive oxygen species, produced by photo-activation of

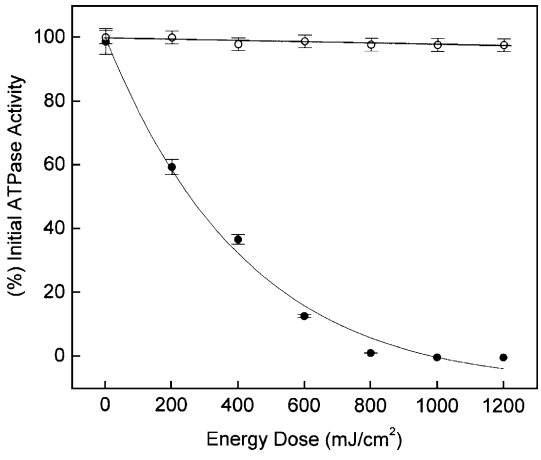

Fig. 1. Residual ATPase activity after photo-irradiation of $C_{12}E_8$-solubilized Na,K-ATPase: (●) in the absence of Rose Bengal (○) in the presence of 50 μM Rose Bengal. Studies of photo-irradiation were done using the second harmonic of the ND-YAG Laser (532 nm) with 8 ns of laser pulse through an 8 mm slit. Samples were irradiated in a quartz cuvette (1 cm) with gentle stirring, using laser power of 20 mJ/pulse with cumulative energy dose range from 200 to 1200 mJ/cm^2. Data are reported as the mean ± S.D. of the triplicate measurement of different enzyme preparations, which was considered to be statistically significant at $P \leq 0.05$.

Rose Bengal upon different regions of the protein structure, we standardized an experimental mimetic system, such as liposomes, which involves the transmembrane domains of the enzyme, allowing the dye encapsulation. Therefore, vesicular systems, named proteoliposomes, were previously made in our laboratory using different phospholipids: DPPC:DPPE and DLOPC:DLOPE. To determine the protein orientation of the Na,K-ATPase reconstituted in the liposome we used kinetic studies in the presence of vanadate and ouabain since these inhibitors bind to opposite specific sites located in the enzyme molecule. The Na,K-ATPase activity of solubilized and purified enzyme is inhibited in about 90% and 72.5% in the presence of 2 mM ouabain and 3 μM vanadate, respectively. On the other hand, a kinetic inhibition of ATP hydrolysis for the proteoliposome system constituted by enzyme and DPPC:DPPE, revealed a small inhibition (6%) for ouabain, which could be attributed to the non-oriented or *rightside-out* form. With the addition of detergent in the reaction medium, an opening of the vesicles and a progressive enhancement of the inhibition occurs, probably due to the exposure of the ouabain binding sites. Inhibition studies using 3 μM vanadate in the activity assays of the Na,K-ATPase reconstituted in DPPC:DPPE–liposome showed that, in the concentration range of 0.2–1.0 mg/mL of $C_{12}E_8$, vanadate inhibited about 78% of ATPase activity of the enzyme without an expressive variation. Thus, these studies reflect a preferential inside-out enzyme reconstitution because the ouabain inhibition increases only by addition of detergent or alamethicin. Together, inhibition studies of ATPase activity using ouabain and vanadate clearly suggest an *inside-out* orientation of the Na,K-ATPase reconstituted in DPPC:DPPE–liposomes. On the other hand, DLOPC:-DLOPE systems showed only 40% of the enzyme oriented *inside-out* (results not shown, but detailed in [33]).

Many efforts have been made to standardize the construction of the vesicular systems in the presence of Rose Bengal as a photosensitizer dye. The proteoliposome or liposomes construction were monitored by chromatography filtration to evaluate the yield of encapsulation of the dye in the biomimetic system. Rose Bengal in buffer solution was eluted in a single peak around the fraction 30 to 40, which is detected at 550 nm (Fig. 2A). On the other hand, a proteoliposome (DPPC:DPPE or DLOPC:-DLOPE) preparation also revealed a single peak, excluded at the column V_o, which corresponds to the fraction number 10. This peak, when analyzed for the dye, phospholipid and protein contents (Fig. 2B), revealed the coexistence of all constituents in the same fraction, validating the procedure for encapsulation of the Rose Bengal in the vesicular system. These fractions were pooled and the aqueous fraction (after chloroform extraction to eliminate the lipids) was used to determine the amount of the dye encapsulated in the system. The proteoliposome systems studies revealed that 95% of the Rose Bengal used in the initial formulation have been efficiently encapsulated. Note that, in these

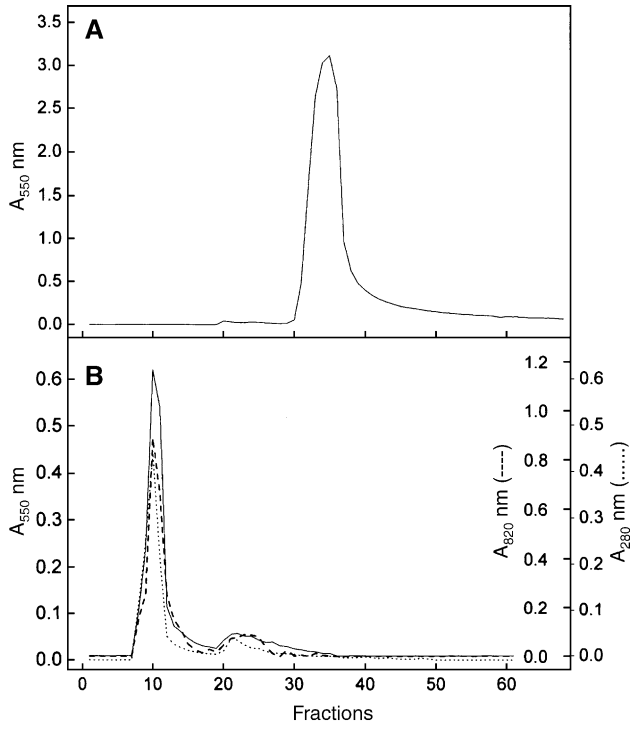

Fig. 2. Chromatographic analysis in Sepharose 6B (0.5 × 144 cm) column equilibrated and eluted with 5 mM Tris–HCl, pH 7.0, containing 150 mM KCl. Fractions of 3.0 mL were collected and assayed. A: Rose Bengal solution and B: DPPC:DPPE–proteoliposomes prepared in the presence of 50 μM Rose Bengal.

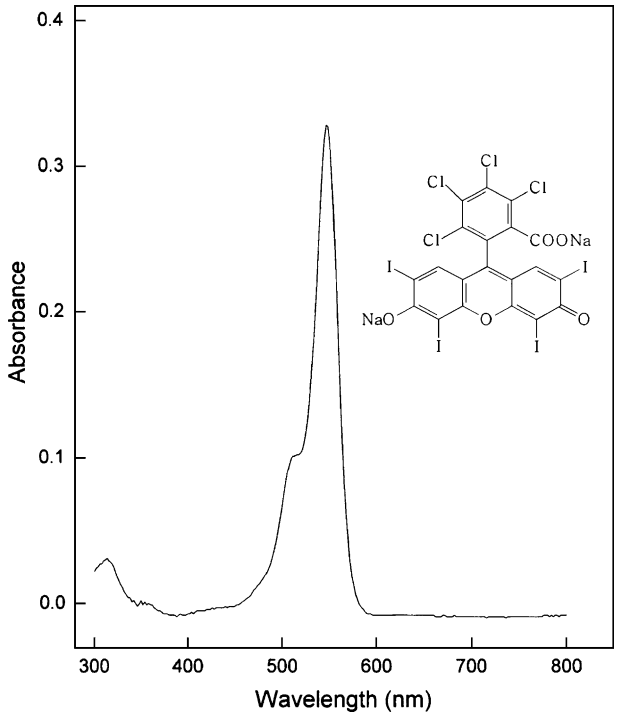

Fig. 3. Absorption spectra of 4.0 μM Rose Bengal in Tris–HCl 5 mM buffer, pH 7.5, containing 150 mM KCl. Insert: Rose Bengal structure.

vesicular systems, neither Na,K-ATPase or the free dye was observed, since the $C_{12}E_8$-solubilized enzyme was eluted in fraction number 25 when applied in the column (0.005 mg/mL of the detergent in this elution buffer is needed, results not shown). Also, the presence of the dye in DPPC:DPPE–proteoliposomes without laser photo-irradiation did not show any significant effect in the ATPase activity and the enzyme recovered 86.5% of the initial activity, after chromatography elution.

Fig. 3 shows the characteristic absorption spectra of Rose Bengal (4 μM) in 5 mM Tris–HCl buffer, pH 7.5, containing 150 mM KCl present in the aqueous phase after chloroform extraction of the proteoliposome preparation. It is worth emphasizing that the process to obtain the proteoliposome does not affect the absorption properties of this xanthene dye, maintaining its absorption band in the range of 450–600 nm.

DPPC:DPPE–proteoliposome, when submitted to photo-irradiation with laser using 1200 mJ/cm^2 energy dose in the absence of the Rose Bengal, did not shown any effect in the ATPase activity (Fig. 4). In addition to that, the effect of vanadate (71%) and ouabain (6%) inhibitors does not change when compared to before the photo-irradiation, suggesting that the integrity of the system was not affected. The same results were observed employing 50 μM of Rose Bengal encapsulated in the interior of the proteoliposome system (Fig. 4). But, when we use 50 μM of Rose Bengal present only in the extravesicular environment and photo-irradiation with a laser dose of 200 mJ/cm^2, it results in the loss of 40–50% of the ATPase activity. Using a dose of 400 mJ/cm^2, the ATPase activity was totality lost (Fig. 4).

The systems which present 40% of the residual activity (after photo-irradiation of a dose of about 200 mJ/cm^2

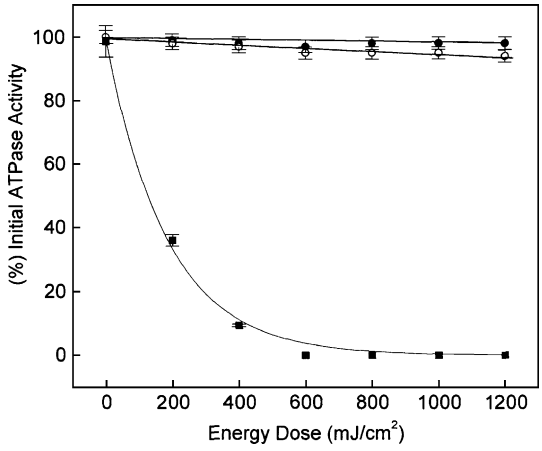

Fig. 4. Residual ATPase activity after photo-irradiation of DPPC:DPPE–Na,K-ATPase vesicular systems: (●) in the absence of Rose Bengal; (○) with 50 μM the Rose Bengal encapsulated in proteoliposome; (■) in the presence of 50 μM Rose Bengal in extravesicular medium. Studies of photo-irradiation were done using the second harmonic of the ND-YAG Laser (532 nm) with 8 ns of laser pulse through an 8 mm slit. Samples were irradiated in a quartz cuvette (1 cm) with gentle stirring, using laser power of 20 mJ/pulse with cumulative energy dose range from 200 to 1200 mJ/cm^2. Data are reported as the mean ± S.D. of the triplicate measurement of different enzyme preparations, which was considered to be statistically significant at $P \leq 0.05$.

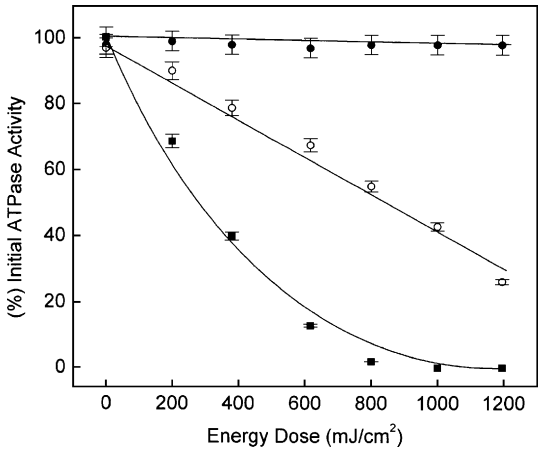

Fig. 5. Residual ATPase activity after photo-irradiation of DLOPC:-DLOPE–Na,K-ATPase vesicular systems: (●) in the absence of Rose Bengal; (○) with 50 μM Rose Bengal encapsulated in proteoliposome; (■) in the presence of 50 μM Rose Bengal in extravesicular medium. Studies of photo-irradiation were done using the second harmonic of the ND-YAG Laser (532 nm) with 8 ns of laser pulse through an 8 mm slit. Samples were irradiated in a quartz cuvette (1 cm) with gentle stirring, using laser power of 20 mJ/pulse with cumulative energy dose range from 200 to 1200 mJ/cm^2. Data are reported as the mean±S.D. of the triplicate measurement of different enzyme preparations, which was considered to be statistically significant at $P \leq 0.05$.

energy) were studied in the presence of specific inhibitors. The behavior of the vanadate inhibition was not affected (75% of inhibition), but ouabain enhanced the inhibition of the ATPase activity (reaching up to 95%), suggesting the exposition of the binding sites of this compound, previously inaccessible.

The photo-irradiation of the system constituted by DLOPC:DLOPE–proteoliposome in the absence of Rose Bengal did not affect the ATPase activity (Fig. 5). However, the photo-irradiation of this proteoliposome in the presence of Rose Bengal, encapsulated only in the interior compartment or in the extra-liposomal environments, revealed the gradual decrease of the ATPase activity, maintaining it at 30% after a dose of 1200 mJ/cm^2 and losing total ATPase activity at 800 mJ/cm^2, respectively (Fig. 5). But, the increase in ATPase inhibition by both compounds, vanadate and ouabain, suggests the loss of integrity of this vesicular system in both conditions studied.

4. Discussion

Applications of photosensitization in medicine and biology would benefit from greater efficiency and selectivity for targeting cellular and subcellular sites. Many of the photosensitizers used in biology and medicine are located either in cellular membranes, the plasma membrane or intracellular membranes [4,6,7,9,37–45]. Membrane photosensitization has been shown to decrease the plasma membrane potential, inhibit transport of molecules across the plasma membrane and both inhibit and activate membrane associated enzymes [14,37,46]. Cellular responses induced by plasma membrane photosensitization include apoptosis, necrosis [47] and expression of early response genes [48].

Among the various photosensitive agents used in PDT are the merocyanine derivatives, xanthenes phtalocyanines and hematoporphyrin dyes, the first two used in basic research and the last two in vivo and clinical trial [6,7,39]. In PDT studies, Rose Bengal (a xanthene derivate) has been used as a model compound, with excellent photophysical properties, although not indicated for clinical use. This dye has an absorption in the region of 500 nm and a good quantum yield of singlet oxygen production [17,45].

Our results of photo-oxidation of solubilized and purified Na,K-ATPase (Fig. 1) sustain the direct action of the singlet oxygen species upon protein, resulting in the loss of its activity. Different mechanisms were proposed to the action of the singlet oxygen as inactivator of enzyme activity: S—S bond or amino acid oxidation, break of peptide or hydrogen bounds, etc., resulting in changes in conformational states of the protein [13,15,17,19,49].

Some authors showed that the Na,K-ATPase and Ca-ATPase photo-inactivation occurs in two pathways: the fast process originating from photomodification of the cytoplasmic domains by singlet oxygen from the aqueous phase and the second, the slow one, which is caused by a modification of the membrane domain of the ion pump. It remains to be confirmed whereas the slow process is due to photosensitized lipid peroxidation, i.e., due to the effect of the lipid environment on the enzyme [17,50,51].

A great number of these works were not executed with the isolated enzyme, but were done using membrane extracts, resulting in the variety of effects by reactive species in lipid, proteins or others endogenous compounds present in the membrane structure. The target of singlet oxygen species in these experimental systems is difficult to be determined due to the many possibilities of direct or indirect oxidation processes, which result in enzyme inactivation.

Conventional or modified liposomes are actually used as an excellent carrier (and delivery system) for both the hydrophobic photosensitizers (interacting in the lipid bilayer) or the hydrophilic dyes (in the internal aqueous cavity) for the treatment of tumors in PDT therapy [4–7,9,43]. In addition to that, liposomes or proteoliposomes are also a mimetic model of lipid membranes (different composition of lipid and proteins could be used) to study the effect and the target of the reactive species produced during light photo-inactivation [22,52–54].

The compartmentalization of sensitizers affects the production, lifetime and mobility of oxygen reactive species. Indeed, unilamellar liposomes consist of distinct regions which need attention: aqueous phase around the vesicle; intravesicular part constituted by the phospholipids region and the aqueous phase entrapped inside the vesicle.

When singlet oxygen is generated, several pathways of diffusion could appear, depending on its location [22].

Using proteoliposome constituted by DPPC:DPPE–Na,K-ATPase (remember that in these systems the Na,K-ATPase is oriented *inside-out* in the liposome as described by Santos et al. [33]), the lipoperoxidation effects were discarded due the absence of unsaturated fatty acid. Consequently, the loss of the ATPase activity, when Rose Bengal is present only in the extravesicular environment, could be correlated with the direct damage in the peptide structure (Fig. 4). Either the isolated or combined effects have been described for the action of the singlet oxygen into Na,K-ATPase: direct action in hydrolysis, metal (Na^+, K^+, Mg^{2+}) or nucleotide binding site; obstruction of enzyme phosphorylation site; difficulties in the movement of N and P domains, formation of cross-links between different peptide chains, among others, all important to complete the enzyme catalytic cycle [15]. Also, Kourie [49] showed that the physical changes in the structure of the channel and pump proteins modify the function of the transporting proteins and/or availability of regulatory sites on these proteins.

The results obtained for constituted vesicular systems of DLOPC:DLOPE–Na,K-ATPase (in the system where the enzyme is reconstituted 40% *inside-out* and 60% *rightside-out* and/or not oriented) are more complex to be interpreted due to the presence of unsaturation in the phospholipids chain as well as to the mix of enzyme orientation. In these systems, in the presence of extravesicular Rose Bengal were submitted to laser photo-irradiation, the gradual decrease of the ATPase activity suggests the direct action of the singlet oxygen species upon protein exposed in the external side of the proteoliposome (note that a reduction occurs in smaller energy doses). After the loss of integrity of this vesicular system, the singlet oxygen species act also in the ATP hydrolysis sites oriented in the interior of the proteoliposomes. In the same way, this process occurs *inside-out* when the dye is entrapped only in the interior compartment (Fig. 5).

Besides that, the evaluation of the residual activity of the proteoliposome revealed that they lost their integrity after the photo-oxidation, as shown for the saturated systems. It is difficult to detect if these effects happen to arise from an action of the reactive species present in the protein structure or still in the phospholipid structure (by a lipoperoxidation process).

A fact that should be commented is that the activity measured decreases more quickly when the photosensitizers dyes are located outside, in spite that 60% of the hydrolyzed sites are orientated to the inside of the vesicular system. This probably happens due to a larger molar concentration of lipid/protein present in the proteoliposome. In fact, the molecular rate of lipid/protein is around 150 [33], which results in greater probability of the generated singlet oxygen to attack the double linkage present in the fatty acid structure of the lipid instead of the amino acid in the protein structure. In a second step, this process can result in an indirect inactivation of the enzyme, as also described by other authors [17].

In summary, these results indicated that singlet oxygen species produced by photo-oxidation of Rose Bengal, using laser light in 532 nm, could act in protein and lipid structure depending on its proportion or distribution. Since Na,K-ATPase is an important enzyme, and critical for cellular viability, studies where the transport activity is monitored simultaneously with the hydrolyze activity of ATP using proteoliposome systems can bring new information about the PDT effects, as well as the mechanisms of action for the reactive oxygen species and their preferred target.

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