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Effect of ultrasound on the function and structure of a membrane protein: The case study of photosynthetic Reaction Center from *Rhodobacter sphaeroides*

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ABSTRACT

Ultrasounds are used in many industrial, medical and research applications. Properties and function of proteins are strongly influenced by the interaction with the ultrasonic waves and their bioactivity can be lost because of alteration of protein structure. Surprisingly, to the best of our knowledge no study was carried out on Integral Membrane Proteins (IMPs), which are responsible for a variety of fundamental biological functions. In this work, the photosynthetic Reaction Center (RC) of the bacterium *Rhodobacter sphaeroides* has been used as a model for the study of the ultrasound-induced IMP denaturation. Purified RCs were suspended in i) detergent micelles, in ii) detergent-free buffer and iii) reconstituted in liposomes, and then treated with ultrasound at 30 W and 20 kHz at increasing times. The optical absorption spectra showed a progressive and irreversible denaturation in all cases, resulting from the perturbation of the protein scaffold structure, as confirmed by circular dichroism spectra that showed progressive alterations of the RC secondary structure. Charge recombination kinetics were studied to assess the protein photoactivity. The lifetime for the loss of RC photoactivity was 32 min in detergent micelles, ranged from 3.8 to 6.5 min in the different proteoliposomes formulations, and 5.5 min in detergent-free buffer. Atomic force microscopy revealed the formation of large RC aggregates related to the sonication-induced denaturation, in agreement with the scattering increase observed in solution.

1. Introduction

Ultrasonic treatments are widely utilized in research, industry and medicine [1]. A non-exhaustive list of possible uses includes many food processing applications (e.g. cleaning of equipment, homogenization of milk, inactivation of microorganisms, product modification [2–4]), pharmaceutical techniques (microencapsulation procedure, liposome preparation, drug release [5–7]), biotechnological processes (e.g. for extracting metabolites and bioactive compounds from plant or animal material [8,9]), and medicine applications (such as imaging, dentistry, liposuction, tumor ablation and kidney stone disruption [7]). Ultrasounds are also used in the investigation of protein conformational disorders such as Alzheimer's and prion diseases, where proteins are altered and misfolded to form aggregates [10,11].

Ultrasonic waves are elastic waves or stress waves with frequencies higher than 20 kHz. In a medium exposed to ultrasound, the formation of gas- or vapor-filled bubbles occurs, in a phenomenon called cavitation [7]. Bubbles can continuously oscillate in response to an oscillating pressure, with a radius that varies about an equilibrium value (Stable cavitation) [7,12], or oscillate with increasingly larger amplitudes until the outward expansion exceeds a limiting value, upon which the bubbles grow suddenly and then collapse violently (Inertial cavitation) [7,13]. The cavitation events, and the collapses of the bubbles in particular, produce shock waves, micro-streaming, and shear stresses [7,12]. Inertial cavitation also induces the formation of free radicals via thermal dissociation of water [14].

The interaction of the ultrasonic radiation with living material therefore, inevitably affects the structure and properties of biomolecules in a way that depend mainly on the duration and intensity

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of exposure. The damage that proteins can potentially receive from such extreme treatments include denaturation via two main mechanisms. The first one involves conformational perturbation, (e.g. unfolding process due to the rupture of the hydrogen bonds and Van der Waals interactions in the polypeptide chains and/or aggregation via noncovalent interactions); the second one occurs through chemical reactions (such as hydrolysis, oxidation, deamidation, β-elimination, etc.) [15]. In both cases sonication could denature proteins, through the formation of liquid-gas interfaces, local heating effects, mechanical/sheer stresses, and free radical reactions [1,16,17]. Bioactivity is lost as a result of alteration of protein secondary and tertiary structure [18].

Despite numerous applications of ultrasound in medical, pharmaceutical and industrial fields, the details of ultrasound-induced damage of proteins, and more generally of biomolecules, remain poorly characterized owing to the potentially complex mechanisms involved [1].

A certain number of studies on soluble proteins such as lysozyme [1,6], trypsin [18], bovine serum albumin (BSA), myoglobin, superoxide dismutase, Tm0979, hisactophilin [1], β2-microglobulin [19], whey protein [20] and on enzymes of biotechnological interest [21] was carried out. To date, to the best of our knowledge, no study was carried out on Integral Membrane Proteins (IMPs). This is undoubtedly due to the higher functional and structural complexity of IMPs as compared to water-soluble globular proteins. Moreover purified IMPs are not generally available on the market and they need to be reconstructed in a membrane-like environment to stabilize a correct spatial structure and to support their functional activity in solution [22]. IMPs, however, represent over 25% of the proteins encoded in the genome of higher animals and are responsible for a variety of fundamental biological functions [23,24]. They also play a crucial role in the cell communication, intercellular recognition, signal transduction, cell energetics, and transport processes through the membrane [22,24].

The purpose of the present work was to gain insight into the effects of ultrasound on IMPs. We studied the photosynthetic Reaction Center (RC) from the bacterium *Rhodobacter sphaeroides* as IMP model protein. Indeed, the availability of crystallographic structure at very high-resolution makes RC an ideal reference protein for the study of general principles of membrane protein architecture and structure-function relationships. Furthermore, in addition to absorbance signals related to tryptophan and tyrosine, RC features a rich electronic spectrum in the visible-NIR region due to the presence of several cofactors that facilitate the study of the protein. Finally, RC can be easily reconstituted in the membrane-mimicking environments represented by liposomes. All these features make the RC an ideal candidate to study sonication-induced denaturation phenomena on IMPs.

2. Materials and methods

2.1. Materials

All chemicals were purchased with the highest purity available and used without further purification. The reagent grade salts for the 50 mM K-phosphate, 100 mM KCl (pH 7.0) buffer solutions, sodium cholate (SC), N,N-dimethyldodecylamine-N-oxide (LDAO), cholesterol (CH), diphosphatidylglycerol (cardiolipin, CL), and G-50 Sephadex Superfine were purchased from Sigma. Phosphatidylcholine (PC) was from Lipoid.

2.2. RC protein purification

RCs were isolated from *Rhodobacter sphaeroides* strain R-26.1 grown photoheterotrophically under anaerobic conditions as previously described [25]. Protein purity was checked using the ratio of the absorbance at 280 and 802 nm (A_{280}/A_{802}), which was kept below 1.3, and the ratio of the absorbance at 760 and 865 nm (A_{760}/A_{865}), which was kept equal to or lower than 1 [26]. Protein was stored frozen at −20 °C in the final buffer Tris-HCl 20 mM, EDTA 1 mM, LDAO 0.025% (w/v), named TLE buffer.

2.3. RC reconstitution in liposomes

Reaction Center reconstitution in liposomes was accomplished by the micelle-to-vesicle transition (MVT) method as previously reported [27]. Briefly, 2.6 mg of total phospholipids were dissolved in a chloroform solution. Such solution was carefully dried on the walls of a conical vial under a gentle N_2 stream to form an evenly distributed film of phospholipids. A volume of 500 µl of a 4% sodium cholate solution in K-phosphate buffer (KPi) 50 mM, KCl 100 mM, pH 7.0 was added to the film and lipids were solubilized by 10–20 cycles one-second sonication to form a homogenous translucent solution. The appropriate amount of RC (45 µM stock solution) was added to this solution, vigorously shacked, stored for 15 min at 4 °C and finally loaded onto a 15 cm Sephadex G-50 column previously equilibrated with KPi 50 mM, KCl 100 mM, pH 7.0 for the size exclusion chromatography (SEC) step. The RC-proteoliposomes elute after the void volume of ∼1.5 mL in a 1 mL fraction.

2.4. Ultrasound treatment

A volume of 5 mL of micellar RC or RC-liposome suspensions, at total protein concentration of 1 µM, was sonicated in a glass vial, using an ultrasonic horn (3.5 mm diameter, Branson Sonifier® 250, Danbury, CT) operating at 20 kHz. The instrument, which can deliver a maximum power of 200 W, was set to 30 W in pulsed mode and duty cycle control 40%. During ultrasound treatment, the vial containing the sample was kept in an ice/water bath to prevent overheating phenomena.

2.5. UV–Vis–NIR spectroscopy

Absorption measurements were performed by means of a UV/vis/NIR Cary 5000 Spectrophotometer (Varian). Scattering contribution to absorption spectra was subtracted using the software "Spekwin32" (F. Menges, Version 1.72.2, 2016, http://www.effem-m2.de/spekwin/).

2.6. Charge recombination kinetics

Charge recombination (CR) kinetics were recorded at 865 nm using a kinetic spectrophotometer of local design implemented with an Hamamatsu R928 photomultiplier and an Hamamatsu Xenon flash lamp (pulse length ∼100 µs) used for RC photoexcitation. Data were collected onto a Digital Oscilloscope (Tektronics TKS3200) and trace deconvolution was performed using a C-code developed in our lab. All measurements were performed at 25 °C unless diversely indicated. The decay traces were recorded up to complete recovery of the photo-bleaching. The absorbance changes were measured assuming as starting value the baseline recorded before the flash [28].

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded at 25 °C using a Jasco J-810 spectropolarimeter equipped with a Peltier thermostat. Measurements were conducted with a 0.1 cm path length quartz cell, a 10 nm/min scan rate and a 0.2 nm bandwidth. Spectra were an average of 3 scans from 200 to 250 nm. The spectrum of 50 mM

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