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The pH-dependent conformational transition of β-lactoglobulin modulates the binding of protoporphyrin IX

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Abstract

We have investigated the interaction between PPIX and β-lactoglobulin (β-lg) as a function of the pH of the solution. β-lg is a small globular protein (MW ≈18 kDa) with a very well characterized structure that reveals several possible binding sites for ligands. The interaction with β-lg affects the photophysical properties of PPIX. The shift of PPIX emission maximum, excitation maximum and the increase of the fluorescence intensity is an indicator that binding between the porphyrin and β-lg occurs. The binding constant appears to be modulated by the pH of the solution. Spectroscopic measurements do not reveal any significant energy transfer between the Trp residues of β-lg and PPIX, however, fluorescence anisotropy decay measurements confirm the binding and the modulation introduced by the pH of the solution. Since β-lg has been shown to be stable within the range of pH adopted in our experiments (5.0–9.0), the results suggest that PPIX binds a site affected by the pH of the solution. Because of the crystallographic evidence an obvious site is near the aperture of the interior β-barrel however an alternative (or concurrent) binding site may still be present.

Keywords: Lactoglobulin; Porphyrin; Fluorescence spectroscopy; Binding

1. Introduction

The photobiological effect of molecules such as PPIX depends on their photophysical and physico-chemical properties that produce important light-mediated biological effects [1–8]. The action of PPIX in vivo and in vitro has been investigated for several decades, however, its interaction with proteins has not been studied in much detail. Many studies suggest that binding and/or irradiation of molecules such as PPIX and TSPP produce direct damage of proteins involved in the apoptotic pathway including pro-caspases 3 and 9, Bcl-2, Bcl-xL and Tubulin [9–11]. Direct photo-induced effects of porphyrin on proteins may produce a more sophisticated cellular damage that proceeds through conformational modifications of the polypeptide [12] and may specifically affect apoptosis [9]. Hence, the interaction of proteins with photoactive molecules is at the same time an interesting biophysical issue, where the irradiation of a photoactive ligand may affect the conformation of a polypeptide, and a useful biomedical model that may provide much needed improvement in the application of porphyrin-like molecules in clinical settings. In the past 5 years, we have carried out a number of preliminary investigations regarding PPIX interaction with large globular proteins [13,14]. With the results presented here, we started to focus on the interaction between PPIX and smaller globular proteins that are at the same time better molecular models and more relevant to biomedical applications. In this manuscript are presented the results obtained studying the binding of PPIX to β-lg. This poly-

Abbreviations: PPIX, Protoporphyrin IX; β-lg, β-lactoglobulin; GI, gastrointestinal tract; DMSO, Dimethylsulfoxide; KI, potassium iodide; S-V, Stern–Volmer; Trp, Tryptophan, $TPPS_4$, meso-tetra(sulphonatophenyl)-porphine; ANS, 1-anilinonaphtalene-8-sulfonate; FRET, Fluorescence Resonance Energy Transfer; CD, Circular Dichroism

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peptide is a 162 residues-long, small globular protein (approximately 18 kDa) [15] that, like all lipocalins [16], is characterized by an interior β-barrel [15,16] and at pH>2.5–3.0 is found in dimeric form [16]. Its physiological role is still unclear and, although PPIX is not a specific ligand, it has been demonstrated that β-lg binds a variety of hydrophobic molecules such as retinol, fatty acids, triglycerides, PPIX and ANS [17–22]. The location of the binding site for these molecules is, however, still controversial [17,19]. In addition, β-lg is associated with a substantial conformational change near pH 7.5–8.0 that modifies the access to the β-barrel [23]. The pH-dependent conformational change of β-lg introduces an element of modulation in the binding mechanisms offering an ideal model for the investigation of how molecules such as PPIX may bind to smaller globular proteins. At the same time, the investigation may help to establish whether proteins with structural properties such as β-lg could be used as carriers of photoactive molecules for more specific delivery of these molecules to abnormal cells.

In this study, we have employed several optical techniques that involve the detection of the fluorescence of either PPIX or β-lg. Binding of PPIX to proteins affects both the emission of PPIX and the protein [13]. We used these properties to investigate the stoichiometry, the dissociation constant and the location of the binding sites in the pH range between 5 and 9.

FRET measurements were carried out using the intrinsic Trp residues as donors, and the bound PPIX molecule as the acceptor [24].

To complete the investigation of the binding properties between PPIX and β-lg we carried out fluorescence anisotropy decay of PPIX and Circular Dichroism (CD). The photophysical properties of PPIX are ideal for the study of the binding of this molecule to a protein. The fluorescence lifetime of bound PPIX ranges between 12 and 16 ns and this allows the measurement of rotational correlation times ϕ typical of a ligand bound to a protein with the size of β-lg [25]. Values of ϕ are related to the apparent hydrodynamic radius of the protein [25,26], which is dependent on the location of the fluorescent probe within the protein itself. In the range of pH values included in this study, β-lg is in dimeric form [20] and the results obtained indicate the pH modulation of the binding which suggest a site near the β-barrel or in some way affected by the conformational change of the protein. The absence of CD signal of the bound PPIX confirms that the porphyrin molecule is not distorted by the interaction with the site and opens the possibility of other binding sites including one at the monomer–monomer interface of the β-lg dimer as suggested for other lipocalins [27–29].

2. Materials and methods

2.1. Chemicals

PPIX (disodium salt) and bovine β-lg (mixture of variants A and B) were purchased from Sigma-Aldrich (Dorset, United Kingdom and St. Louis, MO, USA) and used without further purification. Spectroscopic grade DMSO was also purchased from Sigma-Aldrich and used without further purification.

2.2. Buffers

Aqueous phosphate buffers were prepared using deionized water. One tablet of phosphate buffered saline tablets (Sigma-Aldrich) was diluted in 200 ml of water to produce a 10 mM buffer at pH 7.4 (as directed by the manufacturer). Buffers were subsequently adjusted at the correct pH by the addition of a 0.5-N solution of HCl or a 0.1-M solution of NaOH. In order to investigate the effects of the β-lg conformational transition, which occurs around pH 8 [30], buffers were prepared from pH 5 to pH 9 at increments of one pH unit.

2.2.1. Sample preparation

2.2.1.1. PPIX fluorescence. Since PPIX is not easily dissolved in aqueous solutions, an aliquot of the porphyrin was first dissolved in a small volume of DMSO. The concentration of this PPIX stock in DMSO was adjusted to 500 μM using absorption spectroscopy of a diluted solution and assuming that the optical density at 405 nm is $\varepsilon = 2.42 \times 10^5\ M^{-1}\ cm^{-1}$ [31]. A 10-μl volume of the 1-mM solution of PPIX in DMSO was then dissolved into 1.5 ml of buffer to yield a 3.3-μM solution of PPIX in buffer with less than 1% DMSO. In these aqueous samples, the optical density of PPIX at 405 nm was <0.2. The fluorescence of PPIX was recorded with excitation at 405 nm and emission in the 550–750 nm range. A stock solution of β-lg at the same pH as the solution of PPIX was prepared at a concentration of 54 μM by dissolving it directly in buffer. Increasing aliquots (from 10 to 100 μl) of the protein solution were added directly into a 3-ml (1×1 cm) quartz cell containing 1.5 ml of the aqueous solution of PPIX. The fluorescence spectrum of PPIX was recorded after each addition of the protein. The absorption at 405 nm of the same solution was also recorded and used to normalize the emission spectra for the absorbed energy [32]. Since the addition of the protein into the PPIX solution produces a dilution of PPIX a control experiment was carried out on samples where the same aliquots of buffer without β-lg were added to a solution of PPIX at the same concentration and volume as the one that was used to add the protein.

2.2.1.2. β-lg fluorescence quenching. The solutions for the PPIX-induced quenching of β-lg contained 15 μM of the protein. At this concentration, the optical density due to the absorption of β-lg is ≈0.12 at 295 nm. Excitation of protein fluorescence was carried out at 295 nm in order to selectively excite the Trp residues of β-lg. A stock solution of PPIX in buffer was prepared from the 500 μM solution of PPIX in DMSO. The concentration of PPIX in buffer for this portion of experiments was 12 μM. An aliquot of 1.5 ml of the β-lg solution was transferred to a 3-ml (1×1 cm) quartz cell. The intrinsic fluorescence of the protein was recorded between 299 nm and 450 nm. Increasing small aliquots (10–100 μl) of the 21 μM solution containing PPIX were added to the 1.5 ml aqueous solution of β-lg and the fluorescence of the protein was recorded after each addition. Since the addition of the PPIX produces a dilution of the protein, a control experiment was carried out by adding the same volume of buffer without PPIX to a second 1.5 ml solution containing 15 μM β-lg and recording the fluorescence of the protein. Absorption values at 295 nm were recorded for all samples and used to normalize the emission spectra of the protein [32].

2.2.1.3. FRET and fluorescence anisotropy decay. Experiments were carried out at a single β-lg/PPIX molar ratio. The molar ratio was pre-determined through the steady state fluorescence experiments described above and is reported later in the manuscript. The molar ratio was a function of pH and was chosen so that the maximum or near maximum amount of PPIX was bound to the protein. FRET experiments were carried out both using steady state and time-resolved fluorescence.

2.2.2. Instrumentation

2.2.2.1. Spectrophotometers. For the steady state experiments absorption spectra were recorded on a dual beam Hitachi 3210-A spectrophotometer (Hitachi Instruments Inc., Wokingham, UK). For FRET, fluorescence lifetime and fluorescence anisotropy decay absorption spectra were recorded using a Thermo Electron Evolution 300 dual beam spectrophotometer (Thermo Electron Corp., Madison, WI, USA). PPIX spectra were recorded between 250 and 700 nm at a rate of 240–300 nm/min and a spectral resolution of 2 nm. β-lg absorption spectra were recorded between 220 and 450 nm at the same rate