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An ultraviolet photoacoustic spectroscopy study of the interaction between Lys49–phospholipase A_2 and amphiphilic molecules

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Abstract

We have used near ultraviolet photoacoustic spectroscopy (PAS) over the wavelength range 240–320 nm to investigate the complex formed between the homodimeric bothropstoxin-I, a lysine-49–phospholipase A_2 from the venom of *Bothrops jararacussu* (BthTx-I), with the anionic amphiphile sodium dodecyl sulfate (SDS). At molar ratios >10, the complex developed a significant light scatter, accompanied by a decrease in the intrinsic tryptophan fluorescence intensity emission (ITFE) of the protein, and an increase in the near UV–PAS signal. Difference PAS spectroscopy at SDS/BthTx-I ratios <8 were limited to the region 280–290 nm, suggesting initial SDS binding to the tryptophan 77 located at the dimer interface. At SDS/BthTx-I ratios >10, the intensity between 260 and 320 nm increases demonstrating that the more widespread tyrosine and phenylalanine residues contribute to the SDS/BthTx-I interaction. PAS signal phase changes at wavelengths specific for each aromatic residue suggest that the Trp77 becomes more buried on SDS binding, and that protein structural changes and dehydration may alter the microenvironments of Tyr and Phe residues. These results demonstrate the potential of near UV–PAS for the investigation of membrane proteins/detergent complexes in which light scatter is significant.

Keywords: Photoacoustic spectroscopy; Bothropstoxin-I; Difference spectroscopy; Fluorescence

Ultraviolet spectroscopy of aromatic amino groups using techniques such as absorbance and fluorescence spectroscopy have been extensively used in the characterization of structural changes in proteins. Although these techniques are extremely useful, they have proven to be of limited utility in the investigation of membrane proteins due to the errors associated with the high levels of light scatter that are typically encountered in mixtures of these proteins when associated with amphiphilic molecules. Therefore, the application of a technique that combines the benefits of ultraviolet aromatic spectroscopy with minimal artifacts from light scattering would be highly useful for the study of these systems.

Photoacoustic spectroscopy (PAS) [1] is derived from the photoacoustic effect in which relaxation processes after light energy absorption by a molecule releases heat that generates a pressure wave, which is detected by a sensitive microphone located within the sample compartment. Due to its versatility, PAS may be applied to address problems that cannot be solved by conventional spectroscopic methods, however its application to the study of biological materials has been limited to samples that absorb strongly

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in the visible range (400–600 nm). For example, it has recently been demonstrated that PAS is useful for monitoring the interaction between drugs and DNA [2,3].

With the aim of further understanding the structural basis of protein/membrane coupling of phospholipases A2 (PLA_2) we have studied the effects of the interaction between amphiphilic molecules and a sub-family of catalytically inactive group IIA PLA_2 in which the aspartic acid residue at position 49 in the active site region is substituted by a lysine [4,5]. The Lys49–PLA_2 subfamily includes the homodimeric bothropstoxin I (BthTx-I) from the venom of *Bothrops jararacussu* [6] which although lacks detectable hydrolytic activity [7], shows cytolytic [8] and Ca^{2+}-independent membrane damaging activities as demonstrated by the rapid release of liposome entrapped markers [9–13]. We have previously described the activation of the membrane damaging activity by the binding of ~4 SDS molecules to specific sites on the BthTx-I homodimer [14] and although this activation is essential for Ca^{2+}-independent membrane damaging activity, the permeabilization of phospholipid bilayer clearly requires the perturbation of significantly greater numbers of lipids. We have therefore proposed a micelle nucleation model [15] in which accumulation of amphiphilic molecules by BthTx-I drives a process in which protein structural changes are coupled to transitions in the aggregation state of the associated amphiphiles. The formation of BthTx-I/SDS complexes at molar ratios of between 10 and 30 is accompanied by an increase in the light scatter of the sample, which complicates the interpretation of absorbance and fluorescence spectra in the UV/visible region. Therefore, with the aim of understanding the changes in the protein structure under these conditions, we have used a novel configuration of nearUV–PAS to investigate the microenvironments of the aromatic residues on titration of the BthTx-I with SDS.

Materials and methods

Protein purification and sample preparation. Bothropstoxin-I (BthTx-I) was purified from whole *B. jararacussu* venom using a combination of cation-exchange and size-exclusion chromatography as previously described [14]. Protein purity was routinely evaluated by silver staining of SDS–PAGE gels, and aliquots of purified protein were stored at 4 °C and used within 10 days. For spectroscopic analyses the BthTx-I samples were prepared in 25 mM Tris–HCl buffer at pH 7.0 with 150 mM of NaCl at final protein concentrations of 37 μM for fluorescence and 2 mM for PAS. The titrations of SDS varied from 0 to 3.77 mM for fluorescence and 0–30.98 mM for PAS experiments, respectively.

Intrinsic tryptophan fluorescence emission spectroscopy (ITFE). ITFE spectra of BthTx-I in the presence of increasing concentrations of SDS were measured between 305 and 450 nm with an SLM 8100 Series 2 spectrofluorimeter, using an excitation wavelength of 295 and 10 mm optical path length quartz cuvettes. The excitation and emission slit widths were set to 4 nm, with a photomultiplier voltage of 650 V. Experiments were performed at 25 °C, and all protein emission spectra were corrected by subtraction of the spectrum from the equivalent buffer alone.

Photoacoustic measurements. The photoacoustic (PA) spectrometer was constructed in our laboratory and the details of this have been previously presented [2]. For the photoacoustic measurements in the nearUV region (240–320 nm), the SDS was added to the BthTx-I solution using a Hamilton microliter syringe in order to ensure the precise control of concentration and volume of the sample, and during the titration of the SDS, the sample volume was maintained at 200 μL. All measurements were performed at 25 °C, and each point in the spectrum is the average of 50 data collection repetitions. All PAS experiments were repeated at least three times and the normalization of the spectra was performed using the PA spectrum of black carbon [1].

Photoacoustic data analysis. Absorbance of the modulated light that impinges on the sample results in modulated heat generation, which after diffusion to the sample surface generates a modulated pressure signal in the transfer gas inside the PA cell. The PA signal depends inversely on the modulation frequency of the light, and may be represented as a vector with amplitude and phase characteristics. The amplitude signal is proportional to the amount of light absorbed, which depends on the optical absorption coefficient, $\beta = 2.303 \cdot \varepsilon \cdot C$, where is the molar extinction coefficient and C is the concentration [16]. Thus, the photoacoustic amplitude signal, measured from 240 to 320 nm reflects the absorbance of the aromatic amino acid species: $A_{PAS} = \varepsilon_{Trp} C_{Trp} + \varepsilon_{Tyr} C_{Tyr} + \varepsilon_{Phe} C_{Phe}$, where $\varepsilon_{Trp,Tyr,Phe}$, represents the respective molar extinction coefficients for each amino acid (Trp, tryptophan; Tyr, tyrosine and Phe, phenylalanine), and $C_{Trp,Tyr,Phe}$ represents the number of each aromatic amino acid. The molar extinction coefficients of the substance also depend on the wavelength (λ) of the illuminating light, and the correlation between absorption and wavelength results in a profile of the PA spectrum that is similar to the absorption spectrum. The PA amplitude signal also depends on the amount of absorbed energy converted into heat through nonradiative relaxation processes, and the phase behavior of the PA signal can be used to provide information related to the nonradiative relaxation time and thermal properties of the sample [1].

Results and discussion

The BthTx-I contains a single tryptophan residue at position 77 located at the dimer interface, and the fluorescent properties of this residue have been exploited to gain insights as to the dynamics and stability of the homodimeric form of the protein [13–15,17–19]. The main panel in

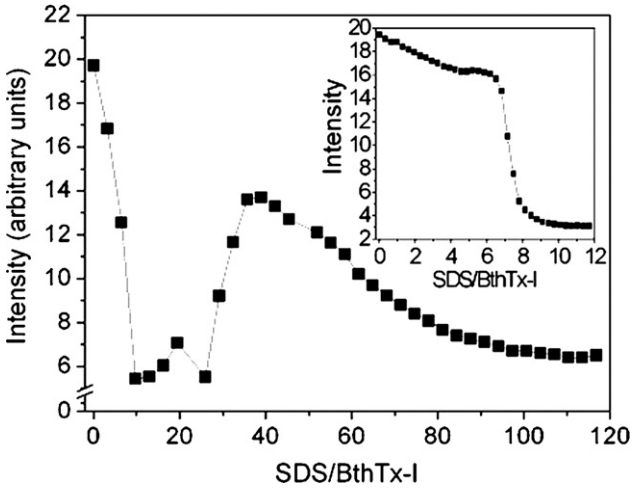

Fig. 1. The effect of SDS on the ITFE of BthTx-I. The intrinsic tryptophan fluorescence emission intensity of the BthTx-I was measured at increasing SDS/protein molar ratios between 0 and 120 as described in Materials and methods. The inset shows the changes in the ITFE of the BthTx-I at SDS/protein molar ratios between 0 and 12 measured under the same conditions. The experiments were performed in the presence of 25 mM Tris–HCl, pH 7.0, 150 mM NaCl at a protein concentration of 38 μM.