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PAPER

π-Cation interactions as the origin of the weak absorption at 532 nm observed in tryptophan-containing polypeptides†

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We have previously reported that bovine serum albumin (**BSA**) and other proteins that do not contain prosthetic groups exhibited a weak light absorption in the visible, only detectable by pulsed laser-induced optoacoustic spectroscopy (**LIOAS**). Human serum albumin (**HSA**) exhibited signals 25% higher than those observed with BSA. Signals comparable to those obtained with BSA were observed with poly(L-Trp, L-Lys), poly(L-Trp, L-Arg) or poly(L-Trp, L-Orn) at pH 7.0. No signals were obtained when tryptophan was replaced by other amino acids or when free tryptophan or the tripeptide Lys-Trp-Lys was assayed (pH 7.0). Tryptophan in HCl 5 N produced LIOAS signals similar to those produced by tryptophan-containing copolymers. Moreover, the absorption peak could be observed in a UV-VIS spectrophotometer. Therefore, the LIOAS signals obtained with BSA, HSA, and tryptophan-containing random copolymers may be attributed to a new transition of the indole moiety of their tryptophan residues when "protonated". Tryptophan residues of proteins are known to participate in π-cation interactions, which are important in protein stability and function. As a matter of fact, HSA and BSA contain an internal tryptophan in close proximity to lysine and arginine residues and therefore suitable for π-cation interactions. The strength of this type of interaction strongly depends on distances and relative orientations of both amino acid residues. Accordingly, these interactions should be highly sensitive to conformational changes. Based on preliminary results that have shown that LIOAS signal at 532 nm depended on the aggregation state of BSA and/or on the oxidation state of its Cys-34, we postulate that the LIOAS signal observed with proteins and tryptophan-containing polypeptides are related to Trp-Lys or Trp-Arg interactions and that the intensity of the signal depends on the strength of such interactions.

Introduction

As it has been previously reported, laser-induced optoacoustic spectroscopy (LIOAS) signals were obtained with different proteins without a prosthetic group when excited at 532 nm. Such signals were attributed to radiationless deactivation following weak absorption in the visible.[1] This weak absorption could not be detected by conventional techniques, since it cannot be easily separated from scattering effects which are important in solutions of macromolecules. Furthermore, LIOAS shows a much higher sensitivity than regular absorption spectroscopy for the detection of light absorption.[2]

Solutions of different aromatic amino acids yielded signals only slightly different than those obtained with the buffer alone. However, a random copolymer of tryptophan and lysine, produced signals ten times larger than observed with the amino acids. On those grounds, we postulated that a new electronic transition produced by charge transfer between the indole moiety of the tryptophan and either the polypeptide backbone or the lysine residues was responsible for the weak absorption detected by LIOAS.[1]

The so called π-cation interaction is enthalpically favourable between positively charged side chains and the π-electron cloud of aromatic side chains. Burley and Petsko[3] have performed a geometric analysis of the crystal structures of 33 proteins searching for side-chain amino groups (Lys, Arg, Asn, Gln, His) that are in close proximity to aromatic residues (Phe, Tyr, Trp). They consider that a favourable interaction could occur when the distance between both partners is longer than 3.4 Å and shorter than 6 Å. Gallivan and Dougherty[4] carried out a careful energetic analysis concluding that 26% of all the tryptophans from the data-set were involved in energetically significant π-cation interactions. The role of such interactions on protein stability[4–6] and function[5,7,8] is increasingly recognized.

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Here we report evidence that the weak light absorption detected by LIOAS at 532 nm is related to a new electronic transition of the indole moiety of "protonated" tryptophan, or to Arg or Lys residues noncovalently bound to tryptophan, by means of π-cation interactions.

Results and discussion

We have previously reported that a random copolymer of L-tryptophan and L-lysine [poly (L-Lys, L-Trp)] yielded LIOAS signals similar to those obtained with BSA solutions.[1] Hence, we decided to apply LIOAS to solutions of different random copolymers of tryptophan with basic amino acids, *i.e.*, poly (L-Lys, L-Arg) and poly (L-Lys, L-Orn). These polypeptides yielded LIOAS signals similar to poly (L-Lys, L-Trp) (Fig. 1), whose magnitude depends on the nature of the basic amino acid (see Table 1). However, copolymers containing other aromatic amino acids and lysine [poly(L-Lys,L-Phe) and poly(L-Lys,L-Tyr)], yielded a LIOAS signal only slightly higher than that of the buffer alone (Table 1). Similarly, the tripeptide Lys-Trp-Lys did not yield a signal significantly different from the buffer.

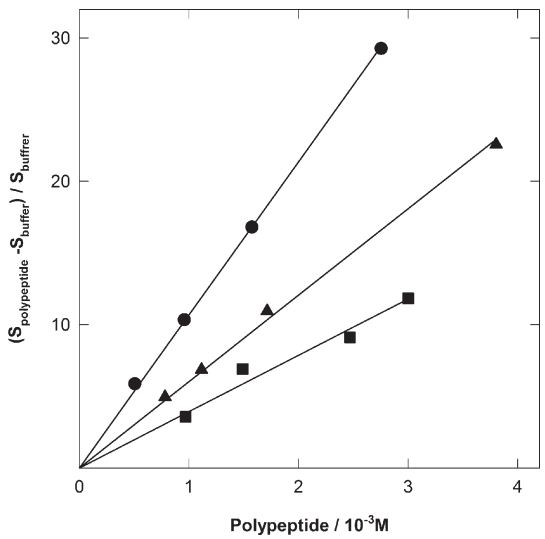

Fig. 1 *LIOAS signals of tryptophan-containing synthetic polypeptides*. The polypeptides were dissolved in 25 mM MES and 25 mM Tris (pH 7.0) and dialyzed as indicated under Experimental. For samples and buffer the amplitude of the first acoustic pulse (H) was determined at variable laser energy (E_L) The points represent the energy-normalized signals of (●) poly(Trp,Arg), (▲) poly(Trp,Lys) and (■) poly(Trp,Orn) estimated from linear $H_{polypeptide}$ *vs.* E_L plots obtained at different polypeptide concentration minus the energy-normalized signal of the buffer (S_{buffer}) and divided by the latter (see the equation under Experimental). Polypeptide concentration is expressed per tryptophan residue as described under Experimental. The lines represent the weighted linear regressions of the data.

Table 1 Molar absorption coefficients determined by LIOAS

Sample	$\varepsilon(532)/M^{-1}$ cm^{-1}
Poly(Trp,Lys)	2.3 ± 0.4
Poly(Trp,Arg)	4.0 ± 0.3
Poly(Trp,Orn)	1.6 ± 0.3
Poly(Phe,Lys)	< 0.1
Poly(Tyr,Lys)	< 0.1
Tryptophan in 0.01 N HCl	0.13 ± 0.03
Tryptophan in 1 N HCl	0.18 ± 0.05
Tryptophan in 5 N HCl	1.63 ± 0.35
Human serum albumin	48 ± 8

These results suggest that the weak absorption detected by LIOAS must be due to some interaction between tryptophan and basic amino acid residues, interaction that would not be possible in the tripeptide due to the predominantly *trans* configuration of the peptide bond. Interactions between tryptophan and arginine or lysine (π-cation interactions) have been described in proteins.[3,4]

We decided to mimic such interaction by preparing tryptophan solutions in HCl (high [H^+]) and determining the amplitude of the LIOAS signals (exciting at 532 nm). Tryptophan in 0.01 N HCl yielded signals that did not differ from those observed with tryptophan at pH 7. Conversely, tryptophan in 5 N HCl produced strong LIOAS signals when excited at 532 nm (Fig. 2 and Table 1).

The weak light absorption at 532 nm of proteins and polypeptides could not be observed in a UV-VIS spectrophotometer mainly because such weak absorption is masked by the strong light scattering due to their high molecular weight. However, tryptophan has a much lower molecular weight and consequently it will be a much weaker light scatterer than proteins and polypeptides. Therefore, the visible spectra of tryptophan in 0.01 and 5 N HCl (Fig. 3A) were analysed. The spectra obtained with the 0.01 N HCl tryptophan solution is representative of the tail of tryptophan absorption in the UV and the light scattering in the visible range. When this spectrum is subtracted from that obtained with a 5 N HCl tryptophan solution, a weak absorption peak could be observed centred at 532 nm (Fig. 3B). The absorption coefficient estimated from the differential spectra shown in Fig. 3B, $\varepsilon(532)$ = 2 M^{-1} cm^{-1}, does not significantly

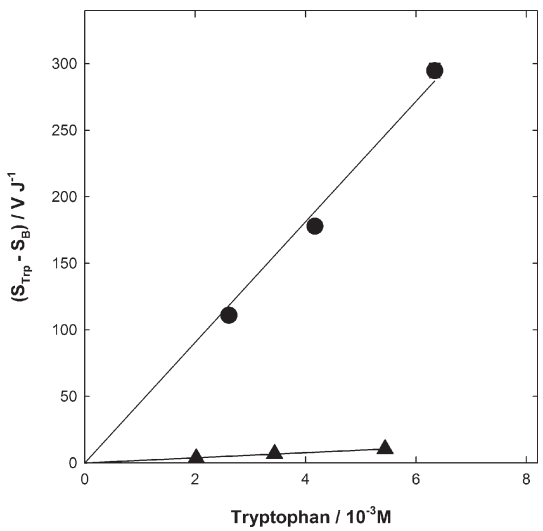

Fig. 2 *LIOAS signals of hydrochloric acid solutions of tryptophan*. The experimental conditions are similar to those described in the legend to Fig. 1. The points represent the energy-normalized signals obtained from linear $H_{tryptophan}$ *vs.* E_L plots obtained at different [tryptophan] minus the energy normalized signal of the buffer. Tryptophan was dissolved in (●) 5 N and (▲) 0.01 N HCl. The lines represent the weighted linear regression of the experimental data.

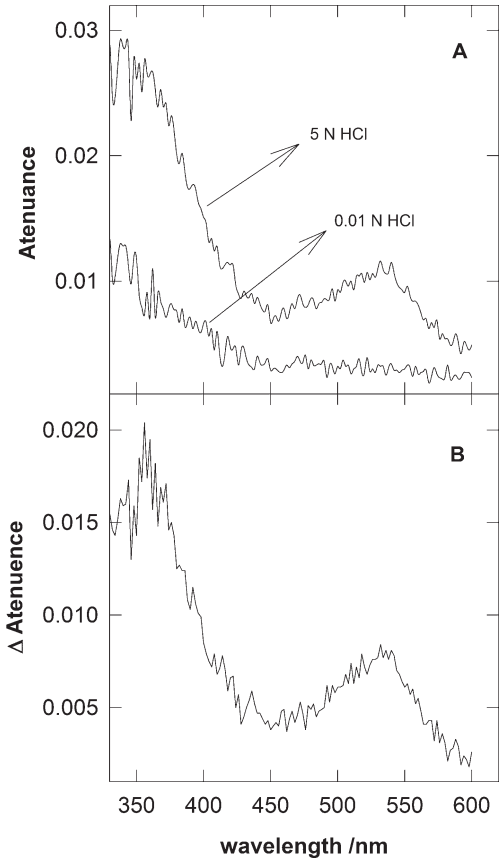

Fig. 3 *UV-VIS spectra of hydrochloric acid solutions of tryptophan*. The UV-VIS spectra of 4 mM tryptophan in 0.01 and 5 N HCl was determined in a dual beam Shimadzu UV-2102PC spectrophotometer (**A**). The differential spectrum is shown in panel **B**.

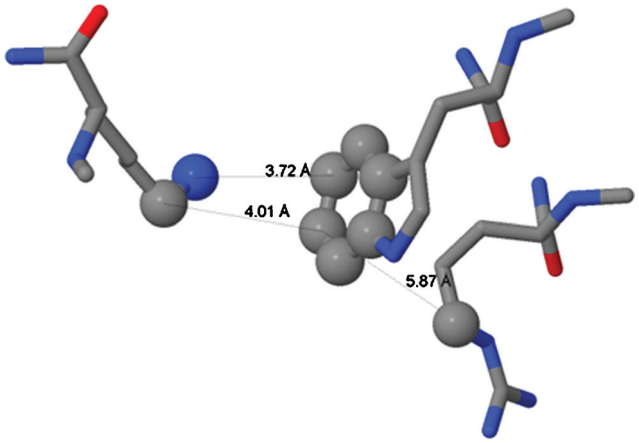

Fig. 4 *Putative π-cation interactions between Trp 214 and Lys 199 or Arg 218* (*HSA*). The distances between Trp 214 and Lys 199 or Arg 218 were measured using the Jmol from the Protein Explorer site, using the HSA structure (1AO6,[12] Protein Data Bank).

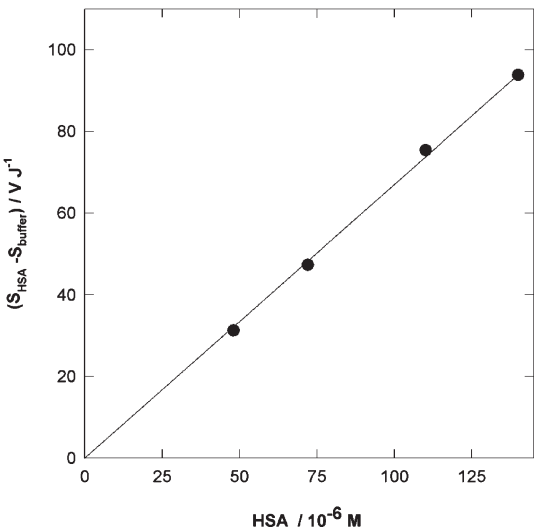

Fig. 5 *Energy-normalized LIOAS signal of human serum albumin* (*HSA*). The experimental conditions are similar to those described in the legend to Fig. 1. The points represent the energy-normalized signals estimated from linear H_{HSA} *vs.* E_L plots obtained at different [HSA] minus the energy normalized signal of the buffer. The line represents the weighted linear regression of the data.

differ from that estimated by LIOAS (see Table 1). This absorption is thus attributed to a Trp-H^+ ground state charge transfer complex.

The not well-resolved absorption observed around 350 nm can be interpreted either as a new absorption band corresponding to a transition to a higher energy level of the Trp-H^+ complex or as an extended red tail of tryptophan in strong acid solutions, similar to that observed by Truong with tryptophan in aqueous 4.5 M $CaCl_2$.[11] This author has attributed the longer red tail to a solute to solvent charge transfer state and not to direct interaction between tryptophan and the salt since it was not observed at lower $CaCl_2$ concentrations (10^{-2} M).[11] However, the data reported by Truong does not exclude the possibility of a weak Trp-Ca^{2+} interaction.

As a matter of fact, human serum albumin (HSA), whose tertiary structure is known, has an internal tryptophan (Trp-214) capable of participating in a π-cation interaction with Lys-199 and Arg-218 that are in its close vicinity (see Fig. 4). HSA also exhibited LIOAS signals when excited at 532 nm. The energy normalized LIOAS signal depended linearly on [HSA] (Fig. 5). From the slope of the plot shown, an absorption coefficient (48 M^{-1} cm^{-1}) similar to those obtained from different BSA preparations could be estimated (Table 1). As it has been previously reported[1] different commercially available BSA preparations yielded different absorption coefficients, namely: BSA (essentially fatty acid free), $\varepsilon(532)$ = 35 $M^{-1}cm^{-1}$; BSA (essentially globulin free), $\varepsilon(532)$ = 17 $M^{-1}cm^{-1}$. It is well known that BSA preparations are heterogeneous due to aggregation and to the non-uniform oxidation state of the Cys-34, the only BSA thiol that does not participate in internal disulfide bridges.[9,10]

Previous results have shown that the absorption coefficients estimated by LIOAS were related to the aggregation state[1] and the amount of free SH groups.[13] The BSA dimer yielded a signal three times larger than the BSA monomer. BSA-Cys, a BSA preparation in which Cys34 is participating in a disulfide bond with cysteine, yielded a signal similar to the BSA dimer.

However, after treatment with thioglycolate that only reduces Cys34, the LIOAS signal obtained is similar to that of the BSA monomer (data not shown). These results suggest that the intensity and/or maximal wavelength of such light absorption would depend on rather small conformational changes.

Conclusions

The most likely explanation for the weak light absorption detected at 532 nm by LIOAS with: (i) proteins containing tryptophan and arginine; (ii) polypeptides containing tryptophan and arginine or lysine or ornithine; and (iii) strong acid solutions of tryptophan, is an electronic transition characteristic of π-cation interactions of tryptophan.

The variability of $\varepsilon(532)$ obtained with different BSA commercially available preparations that differ in aggregation state and in the oxidation state of Cys34 are likely to be due to subtle conformational changes. This should be expected if the LIOAS detected signal is a manifestation of a π-cation interaction, whose strength will strongly depend on the distance and orientation of the aromatic and amino group side chains.

Finally, using conventional techniques it is rather difficult to discriminate between a weak absorption and light scattering, which is important for solutions of macromolecules. Therefore, the light-scattering insensitive LIOAS should be the technique of choice for detecting those small changes in conformation.

Experimental

The experimental setup for LIOAS has already being described.[1,14] The second harmonic (532 nm) of a Nd:YAG laser (15 ns pulse width) was used as excitation beam. The energy of the laser pulses ranged from 4×10^{-5} to 8×10^{-4} J. The energy-normalized LIOAS signals were calculated from the dependence of the amplitude (H) of the first acoustic pulse with the laser energy (E_L) for sample and buffer solutions of the first acoustic pulse with the laser energy (E_L) for sample ($S_{sample} = H_{sample}/E_L$) and buffer solutions ($S_{buffer} = H_{buffer}/E_L$). The absorption coefficients, $\varepsilon(532)$, were estimated as follows:[1]

$$\varepsilon(532) = \frac{\left(\frac{S_{sample} - S_{buffer}}{S_{buffer}}\right) \alpha(532)_{water}}{\ln 10\ [sample]} = \frac{(S_{sample} - S_{ref})A_{ref}}{S_{ref}[sample]}$$

where $\alpha(532)_{water}$ is the water linear absorption coefficient (4.27×10^{-4} cm^{-1}) reported by Tam and Patel,[15] A_{ref} the absorbance and S_{ref} the energy normalized LIOAS signal of a calorimetric reference (bromocresol green or potassium dichromate).

The polypeptides were dissolved in MES–Tris buffer (25 mM each) at pH 7.0, and exhaustively dialyzed against the same buffer previously treated with Chelex. The concentration of the tryptophan-containing polypeptides was determined spectrophotometrically using $\varepsilon(280) = 5600$ M^{-1} cm^{-1} and expressed on the basis of tryptophan residues.[16]

HSA was dissolved (1 to 10 mg mL^{-1}) at pH 7.0 in a buffer (MES–Tris) containing 25 mM Tris and 25 mM 2-*N*-morpholinoethanesulfonic acid (MES). The human serum albumin concentration was determined spectrophotometrically using $\varepsilon(278) = 36\,600$ M^{-1} cm^{-1} in aliquots withdrawn directly from the cuvette.[17] The solutions were filtered through 0.01 μm cellulose nitrate filters (Sartorius) prior to the optoacoustic measurements. The purity of the samples was checked by polyacrylamide gel electrophoresis (PAGE) performed under native and denaturing (SDS) conditions with a Phast-System (Pharmacia).

General

Tryptophan, poly(L-Lys,L-Phe)1 : 1, poly(L-Lys,L-Tyr)4 : 1, poly(L-Lys,L-Trp)4 : 1, poly(L-Arg, L-Trp)4 : 1, poly(L-Orn, L-Trp)4 : 1 and the tripeptide Lys-Trp-Lys were obtained from Sigma Chem. Co. Human serum albumin (essentially fatty acid free), bovine serum albumin (monomer, dimer and BSA-Cys) were also obtained from Sigma Chem. Co. All the other reagents used were of analytical grade.

Absorption spectra were recorded with a Shimadzu UV-2102PC spectrophotometer.

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