БІОФІЗИКА КЛІТИНИ

УДК 577.37

INTRINSIC FLUORESCENCE OF LYSOZYME IN MODEL PROTEIN-LIPID SYSTEMS

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Submitted April 21, 2009

Accepted May 7, 2009

Fluorescence spectroscopy is one of the most powerful tools providing new insights into the structural, dynamic and functional behavior of biological macromolecules, being particularly useful in investigating the molecular details of protein-lipid association. Complete and accurate information about the conformational dynamics of protein molecules can be obtained using tryptophan (Trp) residues as intrinsic fluorescence probes. The fluorescence of indole chromophore is extremely sensitive to environment making it an ideal choice for reporting protein conformational transitions upon membrane interactions. Hen egg white lysozyme (Lz) is a multi-tryptophan protein which is extensively used in elucidating fundamental aspects of protein-lipid interactions. The main emitters responsible for 80% of lysozyme fluorescence are Trp62 and Trp108. The present study was undertaken to ascertain the alterations in lysozyme structural state upon association with model membranes composed of zwitterionic lipid 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and anionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). Fluorescence lifetime measurements showed that intensity-averaged lifetime ($<\tau>$) of Trp residues in lysozyme decreased upon the protein binding to model membranes. Furthermore, $<\tau>$ reduction from 1.94 to 1.74 ns was observed at decreasing lipid-to-protein molar ratio (L:P) from 1130 to 120. It was suggested that Trp specific interactions with certain amino acid residues in its surroundings is the main factor responsible for the recovered decrease in tryptophan lifetime and the observed contradictions between lifetime, quenching and steady-state experiments. Since Lz is a stable protein whose conformation is reported to change insignificantly upon the formation of protein-lipid contacts, it can be assumed that the processes behind the drop in $<\tau>$ involve Lz self-association in membrane-bound state. Trp62 and Trp108 are located in the protein active site which reportedly participates in Lz aggregation. Moreover, Cys76-Cys94 disulfide bridge capable of efficient quenching of Trp fluorescence and reducing the lifetime of protein fluorophores, also resides in the active site cleft. Thus, it may be supposed that interactions between Trp62 and Trp108 of one Lz monomeric molecule with disulfide bridge of another monomeric molecule during the protein aggregation result in reduction of $<\tau>$ values. The $<\tau>$ dependence on L:P can be explained by the fact that lysozyme self-association is apparently coverage-dependent process controlled by both electrostatic and hydrophobic protein-lipid interactions. Additional arguments in favor of the assumption on Lz aggregation come from the time-resolved anisotropy measurements. Lysozyme rotational correlation time which reflects the motion of the whole protein molecule, was found to exhibit twofold increase at increasing L:P values. The recovered membrane ability to modulate Lz aggregation behavior may largely determine the bactericidal and amyloidogenic propensities of this protein.

KEY WORDS: lysozyme, intrinsic fluorescence, protein-lipid interactions, protein aggregation

Fluorescence spectroscopy is one of the most powerful analytical tools extensively used in the biophysical and biochemical investigations, and related to them fields of science. Complex properties of biomacromolecules require sophisticated but at the same time simple techniques which would provide the accurate information about the structure, microenvironment and distribution of biological assemblies. Different kinds of fluorescence method, namely steady-state and time-resolved fluorescence, fluorescence polarization and fluctuation spectroscopy, microscopy, stopped-flow and laser-induced fluorescence, represents attractive alternative for such studies due to high sensitivity, specificity, stability and speed, involvement of rather inexpensive instrumentation and suitable time scales, relative simplicity in methodology, etc. [1] Experimental, analytical and quality control investigations, including, specifically, environmental monitoring, clinical chemistry (blood

and tissue screening, photodynamic therapy, immunohistochemical studies and medical applications), DNA/RNA assays, microarrays and sequencing, genetic analysis, just to name a few, can all benefit from the application of fluorescence detection. An important area of the use of fluorescence spectroscopy involves tracking of membrane processes with particular reference to investigation of molecular details of protein-lipid association. Biological membranes are complex assemblies, where protein molecules are surrounded by a bilayer composed of different types of lipids. Physicochemical properties of the bilayer may influence protein structure, folding and function, while specific interactions with lipid molecules can also contribute towards the biological activity of the proteins. A great number of recent works provide evidence for the fact that formation of protein-lipid complexes may exert influence on protein conformation and its oligomerization state. The biological significance of protein deposition has been shown to be much higher than formerly thought. First, because the formation of insoluble protein deposits, amyloid aggregates, in extracellular space of human tissues correlates with the development of some debilitating human disorders, e.g. Alzheimer's, Parkinson's disease, type II diabetes, transmissible spongiform encephalopathies, etc [2]. Second important manifestation of protein aggregation relates to the intracellular accumulation of overexpressed proteins into inclusion bodies which are thought to be responsible for such disorders as cataract, retinitis pigmentosa, Huntington's, etc. A vast majority of studies suggests that lipid bilayer provides an environment where protein molecules adopt conformation and orientation promoting their assembly into supramolecular complexes. However, despite the extensive research of lipid-controlled protein self-association, the exact molecular mechanisms underlying this process are not fully understood. Complete and accurate information about protein structural and dynamical properties may be obtained using tryptophan (Trp) residues as intrinsic, site-specific fluorescent probes [3]. The well-known sensitivity of tryptophan fluorescence to environmental factors (e.g. polarity), spectral relaxation processes, rotational motions within the protein molecule or protein as a whole, presence of the nearby quenching groups, etc. makes Trp fluorescence a valuable tool in examination of protein structure and its interactions with lipid molecules. In the present study different kinds of fluorescence spectroscopy have been utilized for tryptophan-based tracing of the changes in lysozyme (Lz) structural state upon its association with model membranes composed of zwitterionic lipid 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and anionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). Lz is a multi-tryptophan protein possessing high aggregation and membrane-associating ability. The choice of Lz as protein component of the examined model system was dictated by wide spectrum of its biological functions, viz. bactericidal, antitumor, immunomodulatory and amyloidogenic activities, which are believed to be determined to a great extent by membranotropic properties of Lz. Involvement of different fluorescent approaches may bring significant breakthroughs in understanding the *i)* molecular details of protein structural modifications upon membrane association, and *ii)* general principles of protein aggregation behavior in membrane environment.

MATERIALS AND METHODS

Chicken egg white lysozyme and HEPES were purchased from Sigma (St. Louis, MO, USA). 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and anionic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of analytical grade. Large unilamellar lipid vesicles composed of SOPC and its mixtures with POPG in molar ratio 4:1 were prepared by the extrusion method [4]. Steady-state fluorescence spectra were recorded with LS-50B spectrofluorometer equipped with a magnetically stirred, thermostated cuvette holder (Perkin-

Elmer Ltd., Beaconsfield, UK). Fluorescence measurements were performed at 25 °C and using 10 mm path-length quartz cuvettes. Emission spectra of lysozyme were recorded with excitation wavelength of 296 nm. Excitation and emission band passes were set at 5 nm. Fluorescence quenching experiments were carried out with the neutral water soluble quencher, acrylamide. Emission spectra of lysozyme were recorded with excitation at 296 nm, using 5 nm band passes for both excitation and emission. Time-resolved fluorescence measurements were performed with a commercially available laserspectrometer (Photon Technology International, Ontario, Canada). Fluorescence lifetimes were derived from the non-linear least squares approximation of the emission decay curves by two exponents using the software provided by the instrument manufacturer. Instrument response functions were measured separately using aqueous glycogen solution. The validity of a particular model was judged by the value of the reduced χ^2.

RESULTS AND DISCUSSION

Monitoring the structure, organization and dynamics of the proteins and peptides utilizing fluorescence spectroscopic approaches represent a convenient and sensitive tool with suitable time resolution and minimum perturbation of the examined system. A particular advantage of approaches based on fluorescence spectroscopy is the multiplicity of measurable parameters which complement each other in terms of their information content. Tryptophan serves as intrinsic, site-specific fluorescent probes for protein structure and dynamics due to high sensitivity of these residues to the alterations in their local environment. Presence of Trps as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analysis. The present paper is directed toward the tryptophan-based investigation of lysozyme-lipid interactions. Lysozyme contains six tryptophans (residues 28, 62, 63, 108, 111 and 123). Trp62, Trp63, Trp108, and Trp123 are reported to be exposed to solvent, while Trp28 and Trp111 are buried into the hydrophobic interior of the protein. Based on steady-state examination of behavior of lysozyme derivative [5], Trp62 and Trp108 were reported to be the major emitters accounting for ~80% of lysozyme fluorescence with their emission maxima being observed at 352 and 342 nm, respectively. Therefore, during the analysis of the present results, the changes in Lz spectral behavior were attributed mainly to these residues. Remaining fluorescence originates from Trp28 and Trp111 whose emission maximum is around 323 nm. Superposition of spectral contributions from several fluorophores differing in their local environment results in a relatively broad fluorescence spectrum for lysozyme as it is shown in Fig. 1, A. The maximum position corresponds to 337 nm. Lz binding to the SOPC:POPG (4:1) model membranes exerted no influence on protein intrinsic fluorescence (Fig. 1, A) indicating insignificant alterations in the polarity of Trp environment upon protein association to the liposomes. Importantly, these findings are consistent with the observations of Sankaram and Marsh which reported Lz to retain its globular form in the complexes with lipids [6]. However, this statement is somewhat in conflict with the data of fluorescence quenching experiments. Acrylamide quenching was found to be accompanied by a blue shift of emission maximum, indicating that the individual Trp residues are differently accessible to the quencher. Accordingly, Stern-Volmer plots show dependence on emission wavelength (Fig. 1, B). Analysis of the results of quenching experiments within the framework of the simplest model, which yields wavelength-dependent Stern-Volmer constants averaged over all Trp residues contributing to lysozyme fluorescence, showed that the most effective quenching of Trp fluorescence was observed for lysozyme in aqueous environment. Formation of protein-lipid complexes resulted in approximately two-fold decrease in Stern-Volmer constants suggesting the reduced accessibility of Trp residues to the solvent in lipid-bound Lz (Fig. 1, B). This effect may be explained by the participation

of Trp62 and Trp108 in the formation intermolecular contacts between lysozyme monomer molecules aggregating in membrane environment. Principle possibility for this process was illustrated by the data of calorimetric, circular dichroism, and ^{13}C-NMR studies [7], where Trp62 and other residues located in Lz active site were found to participate in protein self-association.

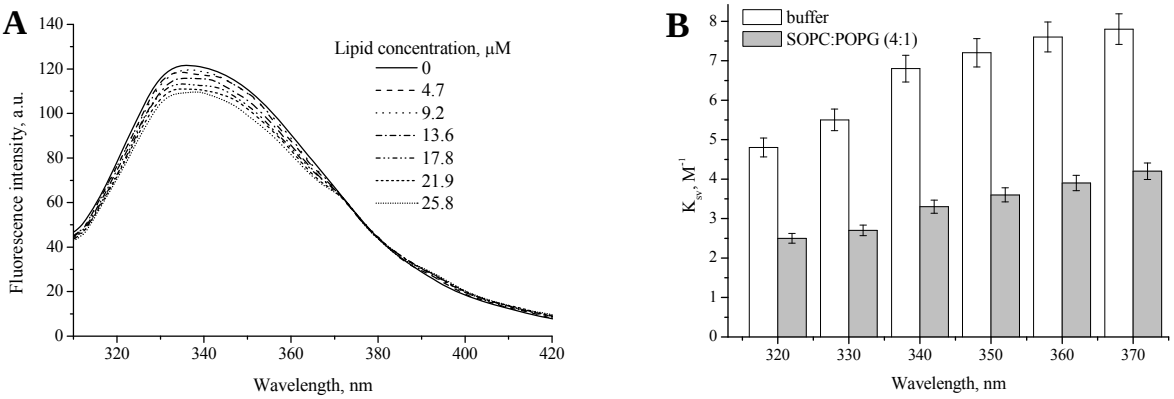

Fig. 1. Emission spectra (A) and Stern-Volmer constants for acrylamide quenching (B) of free and lipid-bound lysozyme

To obtain additional arguments for or against this assumption, time-resolved studies have been carried out. The fluorescence decay parameters of lysozyme Trp residues were determined in the absence and presence of SOPC:POPG vesicles. The fluorescence curves were optimally fitted using a two-exponential decay function with the longest lifetime component (τ_1) assigned to Trp62 and the shortest one (τ_2) – to Trp108 [8].

Table 1. Parameters of lysozyme fluorescence decay

Lz concentration, μM	α_1	τ_1, ns	α_2	τ_2, ns	<τ>, ns	χ^2
In buffer						
0.04	0.074	2.749	0.180	0.285	2.28	1.23
0.4	0.078	2.781	0.170	0.279	2.3	1.51
In the presence of SOPC:POPG lipid vesicles. Lipid concentration – 48 μM						
0.04	0.097	2.525	0.285	0.306	1.94	1.41
0.08	0.121	2.475	0.321	0.310	1.92	1.13
0.13	0.199	2.423	0.506	0.350	1.87	0.81
0.17	0.221	2.371	0.595	0.42	1.74	1.25
0.21	0.249	2.194	0.745	0.474	1.55	1.01
0.25	0.263	2.182	0.839	0.505	1.46	1.60
0.29	0.285	2.172	0.976	0.611	1.42	1.27
0.33	0.298	2.159	1.25	0.664	1.39	1.28
0.37	0.328	2.133	1.47	0.682	1.35	1.29
0.4	0.341	2.111	1.63	0.714	1.28	1.46

The values of amplitudes (α_1 and α_2), lifetimes and calculated intensity-averaged lifetime $<\tau>$ are summarized in Table 1. $<\tau>$ for lysozyme in buffer was found to be *ca.* ~2.3 ns independently on protein concentration. Upon addition of the model lipid membranes α_1, α_2 and τ_2 exhibited an increase by 25, 70 and 10%, respectively, while the τ_1 underwent a decrease by 9%. These changes resulted in overall $<\tau>$ decrease followed the Lz-lipid binding. Moreover, $<\tau>$ reduction from 1.94 to 1.28 ns was observed at decreasing lipid-to-protein molar ratio (L:P) from 1130 to 120 (Table 1). One could impartially note some discrepancy in the data – constancy in Trp quantum yield along with essential changes in lifetimes upon protein binding to the membranes. Its should be noted that steady-state measurements do not always accurately reflect the exact alterations in protein structural and dynamical properties since they provide intensity average of the underlying decay processes. Steady-state signals are often proportional not to the most populated state but simply to the state that emits the most light. Time-resolved studies, however, can provide detailed information concerning the population distribution of molecular species in the excited state. Furthermore, fluorescence intensity may be affected by fluorophore concentration and photobleaching. In our case such concentration effects are likely to manifest themselves in superposition of spectral responses of free and bound lysozyme leading finally to invariability of protein emission spectra in aqueous and lipid environment. Lifetime, in turn, is inherent fluorophore characteristic providing more precise information on the conformational flexibility of the molecule [1].

Table 2. Fluorescence anisotropy decay of Trp residues in lysozyme

Lz concentration, μM	ϕ_1, ns	ϕ_2, ns	r_∞	χ^2
In buffer				
0.08	0.181	5.91	0.032	0.57
In the presence of SOPC:POPG lipid vesicles. Lipid concentration – 48 μM				
0.08	0.269	6.2	0.047	0.14
0.17	0.288	8.3	0.061	0.29
0.25	0.366	10.4	0.073	0.36
0.33	0.382	13.1	0.098	0.74

The most probable explanations for the revealed decrease of Trp lifetime in the lipid environment involves: *i)* increased polarity of Trp microenvironment; *ii)* changes in the local environment of the indole ring (rotation about Trp side chain); *iii)* Trp interactions with neighboring amino acid residues (e.g. Cys); *iv)* intermolecular energy transfer from Trp62 to Trp63 [1]. However, the first possibility is ruled out by above quenching and steady-state fluorescence experiments. Rotation about Trp side chain may occur, but this process cannot satisfactorily explain $<\tau>$ dependence on L:P. Intermolecular energy transfer is not reflected in Lz excitation spectra which are unchangeable upon protein-lipid interactions (data not shown). Motivated by the above rationales, we suggested that Trp specific interactions with certain amino acid residues in its surroundings is the main factor responsible for the recovered decrease in tryptophan lifetime and the observed contradictions between lifetime, quenching and steady-state experiments. Such interactions may be intramolecular contacts with sulfur-containing residues which are known to be effective collisional quenchers of indole fluorescence [1]. These considerations point to the scenario in which interactions between Trp62 and Trp108 of one Lz monomeric molecule with Cys76-Cys94 disulfide bridge (also

residing in active site) of another monomeric molecule during the protein aggregation result in reduction of $<\tau>$ values.

Lz binding to the lipid membranes influenced also its dynamical behavior. As shown in Table 2, protein anisotropy decay is described by two-exponential decay function. The shortest rotational correlation time (ϕ_1) can be assigned to the local motions of Trp62 within the protein molecule [8] while the longest one (ϕ_2) is consistent with that expected fo r the whole-body rotation of lysozyme. Formation of Lz-membrane complexes resulted in more than two-fold increase in both correlation times with the effect being dependent on L:P ratio. Furthermore, residual anisotropy (r_∞) was also found to increase upon protein binding to the liposomes (Table 2). These results points to the increased constraints for Lz motions in lipid-bound state. Along with immobilization in membrane plane, protein rotation can be impeded by the formation of Lz aggregates. Trp participation in protein self-association explains the changes in ϕ_1, while higher volume of protein assembly relative to monomer species governs the increase in ϕ_2.

CONCLUSIONS

Overall, the results obtained strongly suggest Lz aggregation in membrane environment. The dependence of lifetime and rotational correlation time on L:P is evidently explained by the fact that lysozyme self-association is controlled by the amount of surface coverage, balance between electrostatic and hydrophobic protein-lipid interactions and, consequently, protein orientation in membrane plane. The recovered membrane ability to modulate Lz aggregation behavior may largely determine the bactericidal and amyloidogenic propensities of this protein.

ACKNOWLEDGEMENTS

The author is sincerely grateful to Drs. Domanov Ye.A. and Sood R. for technical assistance during data acquisition, Prof. Kinnunen P.K.J. for providing the laboratory facilities for time-resolved measurements, and Prof. Gorbenko G.P. for helpful discussions, constructive comments and critical review of the manuscript. The work was supported by the grant № 4534 from the Science and Technology Center in Ukraine and Fundamental Research State Fund (project number F28.4/007).

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