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QUANTITATIVE ANALYSIS OF THE BENZANTHRONE AMINODERIVATIVE BINDING TO AMYLOID FIBRILS OF LYSOZYME

K. O. Vus[1], V. M. Trusova[1], G. P. Gorbenko[1], E. Kirilova[2], G. Kirilov[2], I. Kalnina[2]

[1]V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077
[2]Department of Chemistry and Geography, Faculty of Natural Sciences and Mathematics, Daugavpils University, 13 Vienibas, Daugavpils LV5401, Latvia

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The accumulation of amyloid fibrils in different tissues is associated with a number of neurodegenerative diseases. Despite a huge variety of amyloid-specific probes, all of them suffer from many drawbacks, highlighting the necessity of searching for more preferable dyes. In the present work, the potential of new fluorescent probe AM3 for selective detection of fibrillar protein aggregates, formed from lysozyme, has been evaluated. To quantify the affinity of this dye for amyloid fibrils, the isotherms of dye binding to the fibrillar lysozyme have been derived from fluorimetric titration. Parameters of the dye-protein complexation: association constant, molar fluorescence and binding stoichiometry, calculated from the Langmuir adsorption model, revealed that AM3 interacts strongly with protein insoluble aggregates. High values of the binding parameters make AM3 an alternative to a widely-used amyloid-specific probe Thioflavin T. We also investigated the effects of polarity and viscosity on AM3 fluorescence properties. The binding of AM3 to the protein hydrophobic cavities has been followed by red shift of the dye emission spectra, which can be explained by H-bonding between proton-donating groups of the protein and carbonyl moiety of the probe. Long-wavelength shift of emission maximum was observed also upon increasing the excitation wavelength. This finding suggests that reorientation time of solvent molecules is higher, than the dye fluorescence lifetime. Fluorescence anisotropy studies revealed slow rotation diffusion of the probe, bound to amyloid fibrils being indicative of high viscosity of AM3 microenvironment. The observed photophysical properties of the new aminobenzanthrone derivative make AM3 a perspective probe for basic research and medical diagnostics.

KEY WORDS:, amyloid fibrils, benzanthrone fluorescent dye, dye-protein binding, lysozyme.

КОЛИЧЕСТВЕННЫЙ АНАЛИЗ СВЯЗЫВАНИЯ АМИНОПРОИЗВОДНОЙ БЕНЗАНТРОНА С АМИЛОИДНЫМИ ФИБРИЛЛАМИ ЛИЗОЦИМА

Е. А. Вус[1], В. М. Трусова[1], Г. П. Горбенко[1], Е. Кирилова[2], Г. Кирилов[2], И. Калниня[2]

[1]Харьковский национальный университет имени В.Н. Каразина, пл. Свободы, 4, Харьков, 61077
[2]Кафедра химии и географии, факультет естественных наук и математики, Даугавпилский университет, 13 Виенибас, Даугавпилс LV5401, Латвия

Отложение амилоидных фибрилл в различных тканях связано с развитием ряда нейродегенеративных болезней. Несмотря на большое количество специфичных к амилоидам зондов, им присуще множество недостатков, следовательно, актуален поиск более эффективных маркеров. В данной работе оценивалась способность нового флуоресцентного красителя АМ3 к селективному определению фибриллярных белковых агрегатов, приготовленных из лизоцима. Высокие значения параметров связывания флуорофора с белком (константы ассоциации, молярной флуоресценции и стехиометрии), определенных в рамках модели Ленгмюра, свидетельствуют о прочном связывании зонда с нерастворимыми агрегатами лизоцима, что делает АМ3 альтернативой часто используемому специфическому амилоидному маркеру Тиофлавину Т. В работе были также исследованы эффекты полярности и вязкости на флуоресцентные свойства АМ3. Связывание АМ3 с белком (предположительно, с его гидрофобными полостями) сопровождается красным сдвигом максимума спектра флуоресценции зонда, что можно объяснить образованием водородной связи протон-донорными группами белка и карбонилом АМ3. Длинноволновый сдвиг максимума эмиссии наблюдался и при увеличении длины волны возбуждения. Это означает, что время переориентации растворителя больше времени жизни

возбужденного состояния, т. е. имеет место медленная релаксация растворителя вокруг диполей флуорофора в возбужденном состоянии. Изучение анизотропии флуоресценции показало, что вращательная диффузия АМ3, связанного с фибриллами, существенно замедлена. Это свидетельствует о высокой вязкости микроокружения красителя. Флуоресцентные свойства новой производной бензантрона создают предпосылки для применения АМ3 в фундаментальных исследованиях и медицинской диагностике.
КЛЮЧЕВЫЕ СЛОВА: амилоидные фибриллы, бенантроновый флуоресцентный зонд, связывание красителя с белком, лизоцим.

КІЛЬКІСНИЙ АНАЛІЗ ЗВ'ЯЗУВАННЯ АМІНОПОХІДНОЇ БЕНЗАНТРОНУ З АМІЛОЇДНИМИ ФІБРИЛАМИ ЛІЗОЦИМУ

К. О. Вус[1], В. М. Трусова[1], Г. П. Горбенко[1], Е. Кірілова[2], Г. Кірілов[2], І. Калніня[2]

[1]Харківський національний університет імені В.Н. Каразіна, пл. Свободи, 4, Харків, 61077
[2]Кафедра хімії та географії, факультет природничих наук та математики, Даугавпілський університет, 13 Вієнібас, Даугавпілс LV5401, Латвія

Відкладання амілоїдних фібрил у різних тканинах пов'язано з розвитком ряду нейродегенеративних хвороб. Незважаючи на велику кількість специфічних до амілоїдів зондів, вони мають багато недоліків, отже, є актуальним пошук більш ефективних маркерів. У даній роботі оцінювалась можливість використання нового флуоресцентного барвника АМ3 для селективного визначення фібрилярних білкових агрегатів, отриманих з лізоциму. Високі значення параметрів зв'язування флуорофору с білком (константи асоціації, молярної флуоресценції та стехіометрії), визначених в рамках моделі Ленгмюра, свідчать про сильне зв'язування зонда с нерозчинними агрегатами лізоциму, що робить АМ3 альтернативою до часто використовуваного специфічного амілоїдного маркеру Тіофлавіну Т. У роботі були також досліджені ефекти полярності та в'язкості на флуоресцентні властивості АМ3. Зв'язування АМ3 з білком (з його гідрофобними порожнинами) супроводжується червоним зсувом максимуму спектру флуоресценції зонда, що може бути пояснено утворення водневих зв'язків між протон-донорними групами білка та карбонілом АМ3. Довгохвильовий зсув максимуму емісії спостерігався також при збільшенні довжини хвилі збудження. Це означає, що час переорієнтації розчинника більший за час життя збудженого стану, тобто має місце повільна релаксація розчинника навколо диполей флуорофора у збудженому стані. Вимірювання анізотропії флуоресценції показало, що обертальна дифузія АМ3, зв'язаного з фібрилами є уповільненою. Це свідчить про високу в'язкість мікрооточення барвника. Виявлені флуоресцентні властивості нової похідної бензантрону створюють передумови для застосування АМ3 у фундаментальних дослідженнях та медичній діагностиці.
КЛЮЧОВІ СЛОВА: амілоїдні фібрили, бенантроновий флуоресцентний зонд, зв'язування зонда з білком, лізоцим.

The so-called conformational diseases are induced by loss-of function and gain-of function mechanisms. The latter includes aggregation of misfolded proteins into amyloid fibrils, i. e. highly insoluble, ordered structures associated with a number of human diseases, such as Alzheimer's disease and type II diabetes, systemic amyloidosis, prion diseases, etc. [1,2]. Although the protein components of amyloid fibrils from various disorders differ considerably from each other in primary sequence, all aggregates of misfolded proteins share a common cross-β-structure, with β-strands perpendicular and β-sheets parallel to the fiber axis [2,3]. Mature fibrils have a diameter of 4-13 nm and usually contain from 2 to 6 laterally associated or twisted protofilaments, each 2-5 nm in diameter. Since the understanding of molecular mechanisms of protein fibrillogenesis is essential for development of strategies for inhibiting amyloid formation and treatment of neurodegenerative diseases, detection of such insoluble structures is of great importance. Classical amyloid-specific dyes are Congo Red (CR) and Thioflavin T (ThT) [4,5]. Congo Red is a cationic benzothiazole fluorophore, which upon binding to fibrils tends to reside in the cavities with its long axis parallel to the long axis of the fibril [1,6-8]. Thioflavin T is located in either cavities (with a diameter 8-9 Å) or regular grooves, formed by the β-strands along the wet side of the β-sheet extension direction [6]. The dye-protein association gives rise to the characteristic fluorescence [1,5]. Despite being widespread, CR and ThT suffer from several drawbacks: i) they affect the stability of

fibrillar intermediates; ii) spectral properties of ThT are dependent on pH and morphology of fibrils; iii) binding affinity of CR to amyloid fibrils of some proteins is lower than that for native protein [1,5,9]. In view of this a variety of new fluorescent probes are continuously evaluated for their ability to serve as the markers of amyloid fibrils [2,5]. In the present study a novel aminobenzanthrone derivative AM3 has been tested for its sensitivity to the amyloid fibrils of cationic protein lysozyme (Lz). Lysozyme is a small multidomain protein with high content of secondary structure [10]. The choice of this protein was dictated by crucial role of Lz amyloidogenesis in the development of such severe disease as systemic amyloidosis [3]. AM3 is a lipophilic probe, displaying weak fluorescence in polar solvents, large Stokes shift (*ca.* 105 nm in ethanol) and high extinction coefficient (*ca.* 13200 $M^{-1}cm^{-1}$). More specifically, our goal was severalfold: i) to characterize quantitatively AM3 complexation with lysozyme fibrils; ii) to compare AM3 binding parameters with those of ThT; iii) to analyze spectral characteristics of AM3 (fluorescence anisotropy, emission maximum shift and red edge excitation shift (REES)) in the presence of Lz fibrillar aggregates.

MATERIALS AND METHODS

Chicken egg white lysozyme was purchased from Sigma (St. Louis, MO, USA). AM3 (3N-(N', N'- dimethylformamidino) benzanthrone)) was synthesized, as described in more detail previously [11]. The reaction of lysozyme fibrillization was initiated using the approach developed by Holley and coworkers [3]. Protein solution (3 mg/ml) was prepared by dissolving lysozyme in deionized water with subsequent slow addition of ethanol to a final concentration 80%. Next, the samples were subjected to constant agitation at ambient temperature. This resulted in the formation of lysozyme fibrils over a time course of about 30 days. Next, amyloid aggregates were diluted to reach the concentration of 0.6 mg/ml. Steady-state fluorescence spectra were recorded with LS-55 spectrofluorometer (Perkin–Elmer Ltd., Beaconsfield, UK). AM3 emission spectra were recorded at 20 ˚C with excitation wavelength 470 nm. AM3 concentration was determined spectrophotometrically, using extinction coefficient ε_{470}= 13200 $M^{-1}cm^{-1}$. All spectra were corrected for dilution and light scattering effects.

RESULTS AND DISCUSSION

Shown in Fig. 1 are AM3 emission spectra recorded at increasing concentration of fibrillar lysozyme. As seen from this figure, the dye fluorescence intensity increases considerably with the protein concentration, suggesting that AM3 strongly binds to Lz aggregates. For instance, the probe fluorescence intensity (I) in the presence of 2.8 μM lysozyme was 11 times higher than that for the free dye (I_0) (Table 1).

At the first step of our study quantitative parameters of the dye–protein interactions – association constant (K_a), binding stoichiometry (n) and molar fluorescence of the bound dye (a_{bound}), have been determined by analyzing the changes in AM3 fluorescence (ΔI) upon increasing protein concentration (C_P), represented in Fig. 2 in terms of the Langmuir adsorption model:

$$\Delta I = 0.5 a_{bound}\left[Z_0 + nC_P + 1/K_a - \sqrt{(Z_0 + nC_P + 1/K_a)^2 - 4nC_P Z_0}\right] \quad (1)$$

where Z_0 is total concentration of the dye.

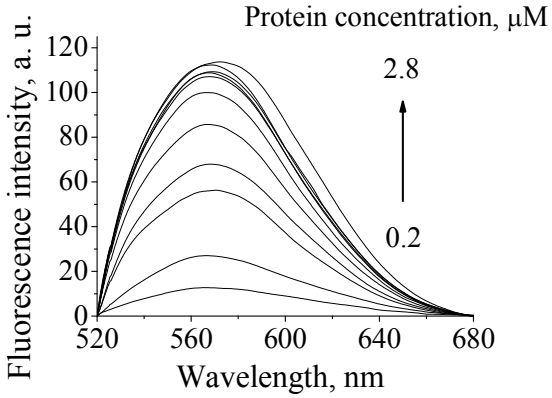

Fig. 1. AM3 fluorescence spectra in the presence of fibrillar lysozyme. The dye concentration was 1.3 μM.

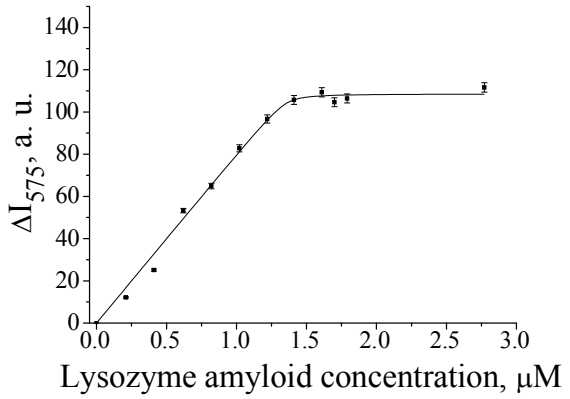

Fig. 2. The isotherm of AM3 binding to lysozyme fibrils. Solid line represents theoretical curve providing the best fit of experimental data.

As evident from Table 1, AM3 association constant is *ca*. 2 orders of magnitude higher than that of ThT. Likewise, the molar fluorescence of AM3 bound to Lz exceeds the analogous parameter of ThT by a factor of ~4 [4]. The molar fluorescence of free AM3 derived from the dye spectrum in buffer solution ($a_{free}=I_{575}/Z_{tot,}$ where I_{575} – fluorescence intensity at emission maximum 575 nm) proved to be *ca.* 9 times lower than that evaluated for fibril-bound dye (Table 1).

As follows from the estimates reported elsewhere, ThT affinity for the misfolded transthyretin was characterized by apparent association constant (*ca.1.5* μM^{-1}), and for Aβ peptide (1-40) and apolipoprotein ApoII fibrils it has the same order, being more pronounced than that for lysozyme (*ca.* 0.31 μM^{-1}) [1,4,6]. The apparent association constant is reverse to the protein concentration, corresponding to the half-height of the maximum emission intensity at a certain dye concentration [1]. Its value depends on the dye and protein concentrations, while the corresponding parameter, calculated from the Langmuir model is invariable for a given fluorophore-protein complex. It is clear that the dependence of ThT-amyloid binding on the amino-acid sequence may reveal lower sensitivity to a variety of misfolded assemblies. Additionally, fibril molecular packing, being dependent on sample preparation method, may obscure the dye-protein binding, decreasing the binding stoichiometry and affinity [12]. It appeared that upon AM3 complexation with lysozyme fibrils, the dye fluorescence changes reach the plateau faster than in the case of ThT, revealing greater sensitivity to small amyloid concentrations. ThT stoichiometry was approximately 1.5 *mol/mol* for lysozyme, and 0.03 *mol/mol* for Aβ (1-40) peptide fibrils, as reported previously [1,4]. These estimates were obtained as dye/protein ratio, taking the protein concentration corresponding to a saturation level of the binding isotherm. The mean value of these parameters was about 1 *mol/mol*, obtained for "peptide self-assembly mimic" (PSAM) scaffold by Biancalana and coworkers, being the same to that of AM3 (Table 1), calculated, however, in terms of Langmuir model for lysozyme fibrils [13]. The differences are presumably arise from the distinct accessibility of amyloid binding sites to the dye, defined by the fibril morphology. As follows from the available data, ThT-protein binding is generally characterized by the 0.1 – 1 stoichiometry values, rarely exceeding the unit.

Structural formula of AM3 and ThT probes are shown in Fig. 3. AM3 is a polar lipophilic molecule, possessing a large Stokes shift due to solvent relaxation, induced by increased dipole moment of the excited-state fluorophore. Generally, if solvent is polar, then probes

with charge separation (between carbonyl and amino groups in the case of AM3) may become the lowest energy state, giving rise to red shift of emission spectrum [14].

Table 1. Parameters of the AM3-lysozyme complexation

n	K_a, μM^{-1}	a_{bound}, μM^{-1}	a_{free}, μM^{-1}	I/I_0	Anisotropy	λ_{em} shift, *nm*	REES, *nm*
1±0.07	21±6	92±20	10±2	11	0.22±0.02	9	5.5

The increase of AM3 fluorescence observed upon its binding to Lz may be explained by at least two factors: i) dye transfer to the protein environment of lower polarity, and ii) immobilization of the probe molecule within the protein matrix resulting in strongly hindered fluorophore rotation [6,14].

The molecular dimensions of AM3 (1×0.7 nm) are close to those of ThT (1.5×0.6 nm). Thus, it can be supposed that protein binding sites for these probes are similar, these may be cavities or grooves, being specific for the classical amyloid marker ThT. Cavities have the diameter ~1 nm and are localized between the protofilaments when in protofibrils and mature fibrils, whereas grooves are formed by the side chains of amino-acid residues participating in the formation of β-strands on the surface of the fibril. These structures have lower polarity and higher viscosity, compared to a bulk phase, and binding of AM3 to these protein regions may result in the increase in dye emission. The AM3-protein binding is presumably stabilized by both Van der Waals and hydrophobic interactions. ThT benzothiazole and benzamine rings freely rotate around the shared C=C bond in the solution. Fluorophore bound to fibrillar aggregates, displays enhanced emission accompanied by the red shift of fluorescence maximum from 445 to 482 nm. It should be noted, that environmental viscosity alone does not induce the characteristic fluorescence of ThT, since no red shift in emission maximum is observed, and tighter packing of ThT in the cavities cannot explain it spectral behaviour. It was demonstrated that the red shift of ThT may depend on the solvent [6,7]. For the sake of comparison, two AM3 spectra, normalized to intensity maxima, are shown in Fig. 4. Concomitant emission shifts may reflect the same electrical properties of the charged ThT and polar AM3 dyes, bound to amyloid fibrils (λ_{em} shift, Table 1). AM3 may associate with protein aggregates via different mechanisms, depending on the component peptide, as was suggested for Congo Red [12]. Particularly, its carbonyl group may serve as hydrogen bond acceptor.

Early diagnostics of amyloid diseases, i. e. detection of misfolded prefibrillar structures in vitro is of great importance for effective treatment of these disorders. To exemplify, such fluorophore as bis-ANS or DCVJ have been used for this purpose [1,3]. The marker for protofilaments, protofibrils or mature aggregates must be insensitive to native protein, which may be present in assay samples. Therefore, it is desirable to examine AM3 sensitivity to native lysozyme and to its prefibrillar structures. It should be pointed out, that the dye staining alone is rarely used for amyloid fibril detection in vitro. If you use a classical fluorophore for this purpose, a false-negative result may occur as a consequence of low accessibility of protein binding sites to the dye due to fibril morphology and different probe affinity, depending on the component peptide. A false-positive result is possible if the marker has high affinity for native protein, being present in fibril sample, and even for bacteria (i. e. Congo Red), which may grow simultaneously with insoluble aggregates under favourable conditions, when samples are incubated in phosphate buffer at physiological pH and temperature to induce amyloid formation. For correct detection and full characterization of amyloid

aggregates in vitro, a transmission electron microscopy and Fourier infrared spectroscopy should be used in addition to the dye staining [12].

At the next step of our study we evaluated fluorescence anisotropy for the probe in the presence of fibrillar lysozyme. Fluorescence anisotropy is determined as:

$$R = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where I_{VV}/I_{VH} corresponds to vertically polarized excitation and vertically/horizontally polarized emission respectively; G is a factor, determining ratio of the sensitivities of detection system for vertically and horizontally polarized light [14]. The absolute error of the mean anisotropy value (calculated from 5 measurements) was evaluated, using a *t*-criterion for confidence level of 0.95. In non-viscous solutions the anisotropy is primarily determined by rotational motion of the fluorophore. For proteins these motions are dependent upon the size and shape of the molecule, as well as its extent of aggregation. Depolarization by rotational diffusion of spherical rotors is described by the Perrin equation:

$$r_0/r = 1 + \theta/\tau \quad (3)$$

where τ is the fluorescence lifetime, θ is the rotational correlation time. If the correlation time is much larger than the lifetime ($\theta >> \tau$), the measured anisotropy (r) is equal to the fundamental anisotropy (r_0). For the spherical molecule θ is given by:

$$\theta = \eta V/RT \quad (4)$$

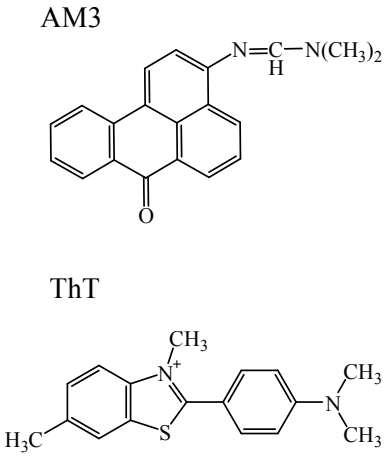

Fig. 3. Structural formula of AM3 and ThT fluorescent probes.

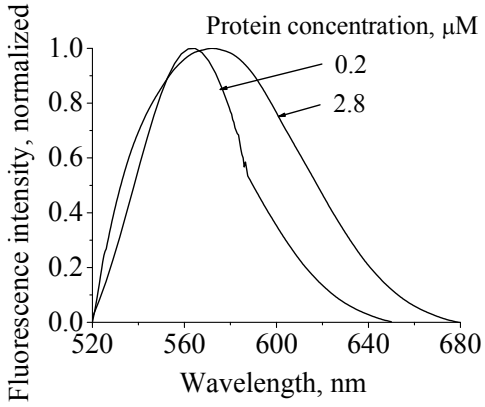

Fig. 4. Normalized fluorescence spectra of AM3, bound to fibrillar lysozyme. The dye concentration was 1.3 μM.

where η is the viscosity, T is the temperature in $^{\circ}K$, R is the gas constant, and V is the volume of the rotating unit [14]. The recovered anisotropy value was found to be 0.22±0.02, showing AM3 slow rotational diffusion in the presence of amyloid fibrils.

At the last step of the study we estimated REES of the dye, bound to amyloid structures. AM3 is known to change its emission maximum upon changing the excitation wavelength, similar to other probes in motionally restricted media. In this case solvent reorientation time is greater than excited-state lifetime (*ca.* ~ 10^{-9} s), and the fluorescence of the dye occurs from the higher vibrational level of the first excited electronic state of the molecule. The REES occurs when excitation at the red edge of adsorption band selectively excites those fluorophores, which interact more strongly with the solvent molecules in the excited state [14,15]. The dye emission spectra shown in Fig. 5 were obtained with the excitation wavelength ranging from 410 to 440 nm and normalized to their fluorescence intensity

maxima. As seen in Fig. 6, the dependence of emission maximum on excitation wavelength is close to linear.

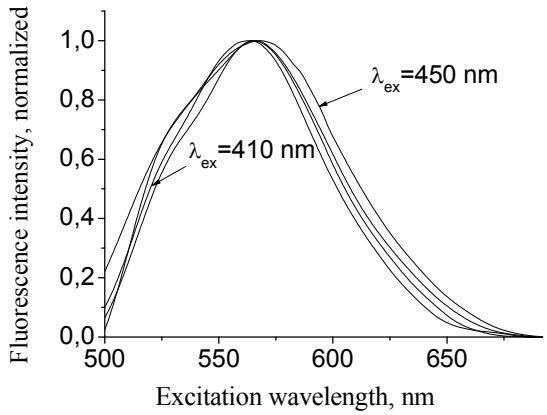

Fig. 5. AM3 excitation spectra in the presence of 2.8. μM fibrillar lysozyme. The dye concentration was 1.3 μM.

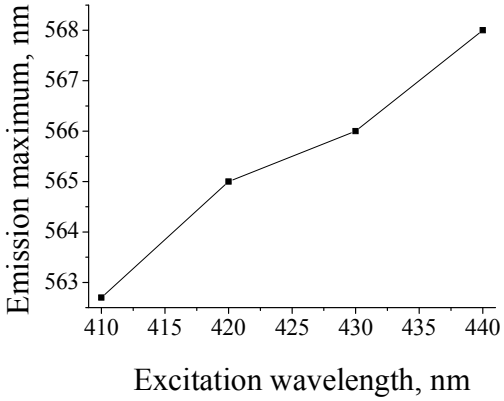

Fig. 6. AM3 emission maximum as a function of excitation wavelength.

REES and anisotropy measurements reveal high viscosity of AM3 microenvironment in its binding sites within fibrillar lysozyme.

CONCLUSIONS

To summarize, we obtained an increased knowledge of the fluorescence properties of the new aminobenzanthrone derivative, particularly of its feature to bind fibrillar protein aggregates. Specifically, it was revealed that AM3 has more pronounced amyloid-sensing abilities than classical amyloid marker Thioflavin T, i.e. higher association constant and molar fluorescence. REES, red shift of emission maximum and high anisotropy value of this dye in the presence of lysozyme aggregates confirm its high sensitivity to environmental polarity and viscosity [11]. This fact also proves that fluorophore binds selectively to amyloid structures, followed by its characteristic fluorescence appearance. Further investigation of AM3 fluorescence in different biological environments can expand the application area of this new probe.

ACKNOWLEDGEMENTS

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