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Short communication

Effect of dendrimers on pure acetylcholinesterase activity and structure

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Abstract

The effect of polyamidoamine (PAMAM) dendrimers on activity and fluorescence of pure acetylcholinesterase (EC 3.1.1.7.) was studied. It has been shown that all dendrimers studied decreased the enzymatic activity of acetylcholinesterase. This effect depended on the type of dendrimers. The data on the intrinsic fluorescence have shown that the dendrimers changed acetylcholinesterase conformation and the strongest effect was induced by PAMAM G3.5 dendrimer.

Keywords: Acetylcholinesterase; Enzyme activity; Conformation; PAMAM dendrimer; Fluorescence

1. Introduction

Dendrimers are new artificial polymers topologically based on the structure of the trees. Dendrimers combine typical characteristics of small organic molecules, having defined composition and monodispersity, with those of polymers possessing high molecular weight that result in multitude of physical properties [1–3]. Polyamidoamine (PAMAM) dendrimers are based on an ethylenediamine core and branched units built from methyl acrylate and ethylenediamine. The fourth generation of polyamidoamine dendrimers (PAMAM G4) used in the present work is water-soluble. It also possesses 64 amino groups on the surface, whereas PAMAM-OH G4 dendrimers have the same number of hydroxyl groups at the chain-ends. PAMAM G3.5 dendrimers possess 64 carboxyl groups on the surface. Molecular weights for PAMAM G3.5, G4 and PAMAM-OH G4, with similar diameters equal to 40 Å, are 12419, 14215 and 14279 Da, respectively [1,4].

Unlike classical linear polymers dendrimers possess empty internal cavities and many functional end groups, which are responsible for their high solubility and reactivity. These specific properties make dendrimers suitable for targeting, microarray systems, catalysis and drug delivery systems [4,5]. Except biomedical applications PAMAM dendrimers have different biotechnical applications, for example, they improve enzyme immobilization on potentiometric and amperometric sensors for pesticide determination [6,7]. However, the detailed molecular mechanisms of dendrimers' impact on proteins (i.e. enzymes) are still unknown. In our previous publications we have studied the effect of dendrimers on the conformation of bovine and human serum albumins and membrane acetylcholinesterase [8,9]. Acetylcholinesterase is a very efficient protein catalyst that hydrolyses its physiological substrate acetylcholine at one of the highest known catalytic rates [10]. It is a key enzyme in cholinergic neurotransmission and signal transduction [11]. Irreversible inhibition of AChE induces a constant excitation of the parasympathetic nervous system and muscle tissues which leads to death [12]. The effect of irreversible inhibition of acetylcholinesterase is also used in biosensors for environmental applications [6,7]. We found that the dendrimers act on membrane acetylcholinesterase decreasing its activity [9], but it is not clear whether it is a result of direct action of dendrimers on the enzyme or a result of indirect changes in lipid bilayer of membrane.

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This communication presents the data on the effect of dendrimers on the activity and conformation of pure acetylcholinesterase.

2. Experimental

2.1. Materials

Pure acetylcholinesterase (EC 3.1.1.7.), 5,5V-dithiobis (2-nitrobenzoic acid) (Ellman reagent [13]), acetylthiocholine iodide, PAMAM G4, PAMAM-OH G4 and PAMAM G3.5 dendrimers were obtained from Sigma-Aldrich (USA). All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

2.2. Acetylcholinesterase activity estimation

AChE activity was assayed by the method of Ellman et al. [13] with some modifications [14,21]. In this method acetylthiocholine was used as a substrate and the product, thiocholine, reacted with Ellman reagent to form a yellow anion 5-thio-2-nitrobenzoic acid. The formation of this product was an indicator of AChE activity. The reaction took place in a final volume of 3 ml of 0.15 M Na-phosphate buffer, pH 7.4, containing 0.025 units of AChE and 333 μM 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB). Dendrimers concentration ranging from 25 to 200 μM were added. The kinetics of acetylthiocholine iodide (0.53 mM) hydrolysis was recorded spectrophotometrically (Pharmacia LKB-Biochrom 4060, UK) at 37 °C, $\lambda = 412$ nm and the rate of the reaction was calculated from the equation:

$$v = \frac{\text{O.D.}^{412} \cdot F}{13,600 \cdot 1000} \left[\frac{\text{mol acetylthiocholine}}{\text{min} \cdot \text{mg of protein}} \right]$$

where O.D.^{412} is an increase of absorbance at 412 nm for 1 min, and F is a dilution coefficient in respect to the protein concentration.

The enzyme activity at Fig. 1 is presented in % to control, i.e.

$$A = \frac{v_D}{v_0} \cdot 100\ [\%]$$

where A—activity in % to control, v_D—the rate of reaction in the presence of dendrimer, v_0—the rate of reaction in the absence of dendrimer.

2.3. Fluorescence measurements

Intrinsic fluorescence spectra for AChE were taken with a Perkin-Elmer LS-50B spectrofluorometer at room temperature (20 °C). The excitation wavelength was set at 295 nm and the emission range was set between 305 and 420 nm. Before fluorescent measurements it was checked whether dendrimers had not been excited by 295 nm wavelength and had not emitted fluorescence. The excitation and emission slit widths for all spectra were 5 nm and 2.5 nm, respectively. Samples were contained in 1 cm path length quartz cuvettes and were continuously stirred. Fluorescence intensities were corrected for dilution.

All results were expressed as a mean value of five experiments.

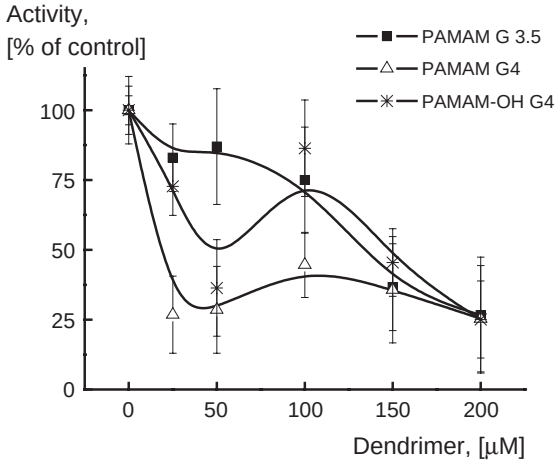

Fig. 1. The effect of PAMAM dendrimers on the acetylcholinesterase activity. Conditions: 3 ml of 0.15 M Na-phosphate buffer, pH 7.4, 37 °C, containing 0.025 units of AChE, 333 μM DTNB and dendrimers in studied concentrations. Acetylthiocholine iodide in final concentration of 0.53 mM was added and hydrolysis was recorded at $\lambda = 412$ nm.

3. Results and discussion

The results of the effect of dendrimers on the catalytic activity of AChE are presented in Fig. 1. In the presence of all types of dendrimers the decrease of the AChE activity was observed. This decrease was dependent on the type of dendrimers used. All dendrimers at low concentrations (<50 μM) induced the decrease of AChE activity. However, for higher concentrations of PAMAM G4 and PAMAM-OH G4 dendrimers (50–100 μM) the partial reactivation of AChE was observed. The consecutive addition of these dendrimers (>100 μM) decreased the AChE activity again. In contrast, the activity of AChE upon addition of PAMAM G3.5 dendrimer in a range of 0–200 μM continuously decreased without significant reactivation. The interactions between dendrimers and AChE (both enzyme and enzyme–substrate complex) have the character of non-competitive inhibition [15] because of the following reasons. At first, dendrimers cannot specifically bind to the same site of the enzyme as the substrate because they have a diameter of 40 Å while the active site has the gorge of 18–20 Å. Secondly, dendrimers can non-specifically bind to both free enzyme and enzyme–substrate complex. Lastly, dendrimers structurally differ from the substrate (acetylthiocholine) [15]. The addition of

dendrimers led to the decrease in 1.6 times of Michaelis constant K_M of pure AChE immobilized on PAMAM dendrimers [6,7] while the dependence of K_M of membrane-bound AChE had a complex character [9].

The effect of dendrimers on conformation of AChE macromolecule was evaluated by fluorescence analysis. The results are presented in Figs. 2 and 3. All dendrimers at low concentrations induced the quenching of AChE fluorescence (Fig. 2). However, this effect was different for all dendrimers. In case of PAMAM G4 and PAMAM-OH G4 dendrimers we observed the sharp decrease of fluorescence of AChE upon addition of low concentrations of dendrimers (<50 μM). The consecutive addition of these dendrimers in a range of 50–150 μM induced a partial increase of AChE fluorescence. In contrast, the fluorescence of AChE upon addition of PAMAM G3.5 dendrimer continuously decreased. The similar changes were observed for fluorescence emission maximum of AChE (Fig. 3). Upon addition of PAMAM G3.5 dendrimer the fluorescence emission maximum of AChE was continuously red-shifted (from 360 till 366 nm). In case of PAMAM G4 dendrimers the significant red shift of fluorescence emission maximum of AChE was observed upon addition of high dendrimer concentrations (>100 μM).

The data show that dendrimers directly affect AChE, changing its conformation and catalytic activity. The similar situation was observed for 4,4′-bipyridine and coumarine derivative [14]. This effect can be explained by electrostatic [16] as well as hydrophobic interactions between dendrimers and protein macromolecule [7–9]. The AChE active centre is a narrow aromatic side chain lined gorge, some 18–20 Å in depth [17]. A negative charge of this centre is the reason why AChE activity may be inhibited by cationic ligands [16,20–22]. The dendrimers having 40 Å in diameter cannot penetrate the active centre but they can block its entrance by attaching to the protein surface near the gorge. When the low dendrimer concentrations were used, the biggest decrease in an enzyme activity was observed for cationic PAMAM G4 dendrimers and the least, for negatively charged PAMAM G3.5 dendrimers. It means that anionic dendrimers can be electrostatically repelled from the protein surface near the active centre. Presented data show that the local interactions near the centre play a key role for concentrations less than 50 μM. For concentrations above 150 μM the loss of AChE activity was the same for all types of dendrimers. It seems that in this case the decrease in the enzyme activity is a consequence of conformational changes of the protein. Dendrimers can bind to many regions of AChE surface [16–21] tightening the protein structure and restricting the molecular motions of the macromolecule, which are necessary for effective work of the catalytic centre [17–19]. First of all, it may be the binding to peripheral site of AChE [22]. The existence of conformational changes was proved by alterations of intrinsic fluorescence. The observed red shift and the decrease in the fluorescence intensity indicated that upon dendrimers tryptophan residues of AChE were exposed to more polar environment, thus the protein was partially unfolded. The reactivation of AChE activity, which was observed in case of PAMAM G4 and PAMAM-OH G4 dendrimers for concentration 100 μM, can be explained by their binding to peripheral site of AChE. Similar effect was observed earlier when cationic substrates bound to this site [22].

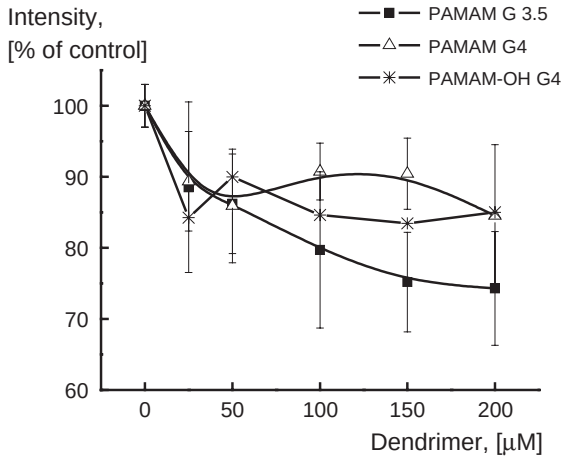

Fig. 2. The effect of PAMAM dendrimers on the acetylcholinesterase fluorescence. Conditions: 1.5 ml of 0.15 M Na-phosphate buffer, pH 7.4, 20 °C, containing 0.0125 units (15.7 μM) of AChE and dendrimers in studied concentrations. $\lambda_{ex.}$=295 nm, $\lambda_{em.}$=360 nm.

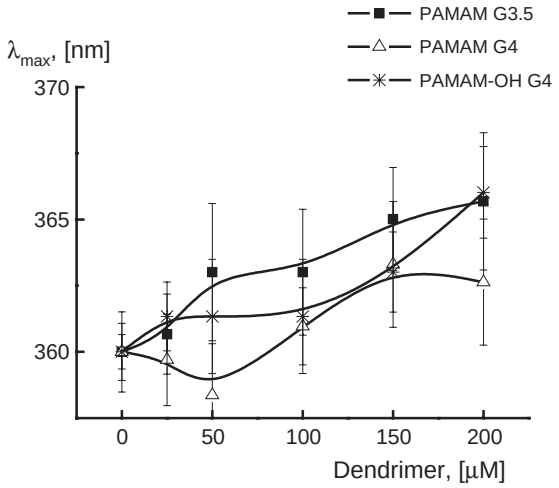

Fig. 3. The effect of PAMAM dendrimers on emission maximum of acetylcholinesterase fluorescence. Conditions: 1.5 ml of 0.15 M Na-phosphate buffer, pH 7.4, 20 °C, containing 0.0125 units (15.7 μM) of AChE and dendrimers in studied concentrations. $\lambda_{ex.}$=295 nm.

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