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Conformation and activity alteration of horseradish peroxidase induced by the interaction with gene carrier polyethyleneimines

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Polyethyleneimine (PEI) has long been considered as "golden standard" for polymeric gene delivery carriers. However the molecular basis of the cytotoxicity of PEI is poorly understood. Little is known about the effects of PEI on the structure and functions of biomacromolecules. In this work, fluorescence, UV–vis absorption, circular dichroism spectroscopy were conducted to investigate the influence of PEI of average molecular weight 25, 10 and 1.8 kDa (denoted as PEI25k, PEI10k and PEI1.8k) on the conformation of horseradish peroxidase (HRP) and its catalytic efficiency. Zeta-potential measurement and isothermal titration calorimetry were used to reveal the mechanism of the interaction between PEIs and HRP. PEIs were found to bind onto the surface of HRP predominantly via hydrophobic interaction and hydrogen bond or van der Waals interaction. The complex formation between HRP and PEI induced a more compact conformation of the enzyme and an increased hydrophobicity of the microenvironment surrounding heme pocket. The conformational change of HRP had little impact on the affinity towards H_2O_2 and phenol. However, the increase in the non-planarity of porphyrin ring in the heme group led to an increase in the exposure degree of the active center and thus an enhancement of catalytic efficiency of HRP in the presence of high molecular weight PEIs (PEI25k and PEI10k). The polymer size played an important role in PEI-HRP interaction. PEI of low molecular weight (PEI1.8k) was less efficient to alter the conformation and catalytic activity of HRP in aqueous solutions.

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1. Introduction

Gene therapy has become an attractable subject due to its potential application in the treatment or prevention of inherited or acquired human diseases [1]. A major barrier for progress in human gene therapy is the lack of safe and effective vectors for gene transfer. Viral vectors, the most efficient vectors by successfully introducing foreign genes into cells by far, often induced severe toxicity and in some clinical situations their use has resulted in patient deaths [2,3], leading to surge in the design and engineering of polycation-based synthetic gene-transfer vehicle [4,5]. Polyethyleneimine (PEI) is a cationic polymer with dense amine groups prone to protonation. The high charge density and buffering capacity in endolysosome make it one of the most effective non-viral gene carriers and long been considered as "golden standard" for polymeric gene delivery systems [6–11].

Considerable reports have indicated that PEI can induce cytotoxicity, often defined by assessing the in vitro metabolic activity of cells. However, viability tests (e.g., MTT assay), usually conducted at 4–8 h following treatment with cationic macromolecules, may not provide a true representation of polymer safety in gene therapy trials. As shown by Moghimi et al. [11], the apoptosis in intact cell occurred at the later stage long after PEI-treatment [11]. By far, many efforts have been made to reduce the cytotoxicity and to promote the transfection efficiency of PEI [6–8]. However, the molecular basis of PEI cytotoxicity is still poorly understood. Knowledge about the influence of PEI on the structure and functions of proteins and its mechanism is limited. Mazzaferro et al. [12] suggested that the complex formation of PEI with proteins was predominated by electrostatic interaction, which contributed to the decrease in the thermal stability and secondary structure of acidic proteins, but had little effect on the conformation of basic proteins. However, the enhancement of catalytic activity, thermal stability and chemical stability of both muscle lactate dehydrogenase, a basic protein (pI 8.2) [13], and glucose dehydrogenase, an acidic protein (pI 6.0) [14], indicated that hydrophobic interaction, hydrogen bond or van der Waals interaction were likely to have an important role in the interaction between PEI and proteins. Previous work illustrated that the complex formation of PEIs with bovine serum albumin (BSA) induced a conformational change of the protein and greatly reduced its binding capability [15]. However, the electrostatic interaction between PEIs and BSA, an acid protein (pI 4.6) [16], made it difficult to manifest the influence of hydrophobic interaction, hydrogen bond or van der

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Waals interaction on the structure and functions of the protein. It is of interest to study the interaction between PEI and neutral proteins, such as horseradish peroxidase (HRP).

HRP is a representative member of the plant peroxidase superfamily extracted from horseradish (*Armoracia rusticana*) roots which catalyzes the oxidation of a broad range of substrates by hydrogen peroxide or by organic peroxides, providing an efficient way for removal of toxic hydrogen peroxide from the intracellular region [17]. The remarkable catalytic activity, availability and high stability in aqueous solvent make it a model enzyme for exploring the underlying structure-function relationships and thus the most extensively studied member of peroxidase superfamily, widely applied in chemical synthesis, medical diagnostics and bioremediation [17,18]. Fifteen peroxidase isoenzymes have been isolated from horseradish root using classical protein purification methods and are generally referred to by codes based on their pI values. HRP C, the predominant form of the isoenzymes, is a “neutral” monomeric glycoprotein of pI value around 7.0 [19] and thus a possible model to minimize the influence of electrostatic interaction and reveal the influence of hydrophobic interaction, hydrogen bond or van der Waals interaction between PEI and proteins.

In this work, fluorescence, UV–vis absorption, circular dichroism (CD) spectroscopy was conducted to investigate the influence of PEI on the conformation of HRP. Zeta-potential measurement and isothermal titration calorimetry (ITC) were used to reveal the mechanism of the interaction between PEI and HRP. Catalytic activity of HRP was evaluated by monitoring the oxidation of phenol in the presence of hydrogen peroxide. Furthermore, steady-state kinetic studies were carried out to get a further insight into the mechanism of functional alteration of HRP upon the binding with PEI. PEI of different molecular weight (MW 25, 10 and 1.8 kDa) was chosen in order to clarify the structure-activity relationship, which would facilitate the design of degradable PEI-Based polymeric matrix.

2. Experimental

2.1. Materials

Branched PEI with different molecular weight (average molecular weight 25, 10 and 1.8 kDa, labeled as PEI25k, PEI10k and PEI1.8k, respectively.), HRP (Type VI, RZ $= A_{403}/A_{208} \geq 3.0$, activity $\geq 250\ U \cdot mg^{-1}$) were obtained from Sigma-Aldrich (USA). Analytical grade phenol, 4-aminoantipyrin (4-AAP) and hydrogen peroxide (30%) were from Sinopharm Chemical Reagent Co. Ltd. (China). All of the materials were used as received. All of the stock solutions and the solutions for analysis were prepared in 10 mM phosphate buffered solution (PBS) (pH 7.4).

Stock solutions of PEIs ($5.0\ mg \cdot mL^{-1}$) were prepared by dissolving the products in PBS buffer and adjusted to pH 7.4 using HCl and NaOH solution. The stock solutions were stored at 0–5 °C and diluted to the desired concentration when used. It should be noticed that molarity in M was adopted for the concentration of the materials used in this work except PEIs, to which mass volume concentration in $mg \cdot mL^{-1}$ was applied, for the sake of comparison to display the role of polymer size in the PEI-protein interaction.

Stock solution of HRP was prepared by dissolving the product in PBS buffer and adjusted to pH 7.4 using HCl and NaOH solution. The concentration of HRP was determined based on Soret absorption at 403 nm on a UV-2501PC UV–vis spectrophotometer (Shimadzu), at which the molar absorption coefficient was taken as $102\ M^{-1} \cdot cm^{-1}$ [20]. The stock solution was stored at −20 °C and diluted to the desired concentration when used. Solutions for spectra analysis and zeta-potential measurement, with final HRP concentration of 7.5 μM, were prepared by diluting the stock solution into PBS buffer containing PEI of various concentration (0–1.0 $mg \cdot mL^{-1}$) and incubated in a water bath thermostated to 25 °C for 15 min before performance.

2.2. Methods

2.2.1. UV–vis Absorption

UV–vis absorption spectra of HRP were recorded on an UV-2501PC UV–vis spectrophotometer (Shimadzu, Japan) equipped with a 1 cm × 1 cm quartz cell in a jacketed holder connecting to a water bath thermostated to 25 °C. PBS buffer containing PEI of the same molecular weight and of the same concentration was used as reference. The absorption spectra were recorded from 200 to 700 nm.

2.2.2. Circular Dichroism (CD) Measurement

CD spectroscopy analysis was carried out on a MOS-450 spectropolarimeter (Bio-Logic, France). The spectra were collected from 190 to 260 nm in nitrogen atmosphere, using a corvette of 1 mm path length in a jacketed holder connecting to a water bath at 25 °C. All of the CD spectra were obtained by averaging 3 runs with a step interval 0.5 nm and acquisition duration 0.1 s. A blank for PEI-containing PBS buffer was measured under the same experimental conditions and subtracted from the data.

The secondary structure contents were calculated by using CDSSTR software in the DICHROWEB web server (http:dichroweb.cryst.bbk.ac.uk/html/home.shtml) [21].

2.2.3. Intrinsic Fluorescence

Fluorescence measurements, including fluorescence emission of tryptophan ($\lambda_{ex} = 295$ nm) and synchronous fluorescence for tyrosine ($\Delta\lambda = \lambda_{em} - \lambda_{ex} = 15$ nm), were performed on a RF5301PC spectrofluorimeter (Shimadzu, Japan) using a 1 cm × 1 cm quartz cell in a jacketed holder connecting to a water bath thermostated to 25 °C. The fluorescence spectra were recorded from 250 to 450 nm. The excitation and emission slit widths were set to 3 nm.

2.2.4. Zeta-potential Measurement

Zeta-potential of HRP was determined on a Zetasizer Nano-ZS dynamic scattering spectrometer (Malvern, UK) by using disposable folded capillary cell at 25 °C. Zeta-potential was calculated using the Smoluchwski mathematical model. The measurement was performed at least three times.

2.2.5. ITC

The microcalorimetric measurements were carried out using a Microcal ITC_{200} isothermal titration calorimeter (GE, USA). PEI solution ($5.0\ mg \cdot mL^{-1}$) and HRP solution (100 μM) was prepared, adjusted to pH 7.4 and degassed just before performance. The titration was carried out by successive injections of 2 μL of PEI solution from a 40 μL syringe into HRP solution filled in the measurement cell at 25 °C. Interval between injections was 120 s to allow complete equilibration and agitation speed was 600 rpm. A background titration, consisting in injecting the same PEI solution into PBS was subtracted from each experimental titration to account for the dilution effects.

The data were collected automatically and subsequently analyzed with sequential two-step binding model proposed in the origin software package supplied by Microcal. The heat flow was recorded as a function of time. The concentration of the titrant and the sample were used to fit the heat flow per injection to an equilibrium binding equation, providing best fit values of apparent binding constant K_a, change in enthalpy ΔH, change in entropy ΔS and free energy ΔG.

2.2.6. Peroxidase Activity Assay

The enzyme activity was assayed by modified Worthington method [22]. Stock solutions of phenol, 4-AAP, hydrogen peroxide, prepared in PBS and adjusted to pH 7.4 using HCl and NaOH solution, were combined with PEI solutions into a reaction mixture containing 80.0 mM phenol, 1.1 mM 4-AAP, 1.0 mM hydrogen peroxide and PEIs of a predefined concentration (0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 $mg \cdot mL^{-1}$). Enzyme solution containing 0.001 $mg \cdot mL^{-1}$ HRP and

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