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Research paper

Effect of carboxyl and amino groups in fluorescein molecules on their peroxidase-like activity

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ABSTRACT

Recently, an important and intriguing property of fluorescein to function as an artificial peroxidase was found. Although, as a new class of small molecule enzyme mimics, the catalytic activities should be highly associated with the composition and structure, little is known about the mechanism underlying the functional groups mediated enzyme-like activities. Herein, we report for the first time that fluorescein derivatives, *i.e.*, carboxyfluorescein (CF) and aminofluorescein (AF) possess peroxidase-like activity, and investigate the impact of different functional groups on the catalytic performance. By systematically evaluating absorption intensity, electron spin resonance (ESR) signals, enzyme kinetic parameters and activation energies, we obtain evidence that the carboxyl group can enhance catalytic activity and may serve as substrate-binding sites, but the amino group can even inhibit the catalytic reaction. Correspondingly, the theoretical study further identified these experimental results in molecular-level. These observations pave the way for identifying highly effective functional groups that promote the overall performance of fluorescein-based artificial peroxidase. Meanwhile, this work should facilitate the design and construction of tailor-made small molecule peroxidase mimics.

1. Introduction

Nature enzymes exhibit excellent activities under mild physiological conditions, but the high expense in preparation, purification and storage, and instability under harsh conditions impede their application dramatically [1–3]. Therefore, to compensate for these drawbacks of natural enzymes, a great deal of effort has been dedicated to develop artificial enzymes [4–6]. Artificial enzymes, the term initially coined by Ronald Breslow for enzyme mimetics [7], can be briefly described as examples of enzyme functions involving nonbiological materials [3]. To date, remarkable progress has been made in the field of artificial enzymes, for example, cyclodextrins, porphyrins, metal complexes and supramolecules have been investigated as artificial enzymes [1–6]. With the rapid development of nanoscience and nanotechnology, the emergence of nanomaterial-based artificial enzymes (nanozymes) receive more attention in various fields due to the remarkable merits of low price, high stability and potential of recovery and recycle [8,9]. Though promising, nanomaterials are metastable and spontaneous aggregation in aqueous solutions, especially under high salt conditions, which can result in losing the catalytic activity [9]. The synthesis of nanozymes need skilled technicians and involves rigid conditions and complicated, time-consuming operations, which limits the large-scale production and widespread applications. In addition, the poor reproducibility in activity between different batches and the biosafety of nanomaterials should also be taken into account [10]. Thus, developing new artificial enzymes to offset the disadvantages of nanozymes are highly appealing.

Fluorescein as a synthetic organic compound with photoluminescence properties [11] was first synthesized by Baeyer in 1871 [12]. Aside from large molar absorption coefficient and high fluorescence quantum yield, fluorescein is commercially available, cheap and convenient to use in many derivatives, such as isothiocyanate and fluorescein succinimidyl ester, that can be covalently attached to macromolecules and to amino acids, which should render them applicable in the area of biochemical research, health care applications and environmental science [11,13]. Notably, the fluorescein skeleton has been commonly utilized as a modular scaffold that is well suitable for elaboration to create various molecular tools, such as ion indicators [14], fluorescent labels for biomolecules and so on [15]. Alongside the exponential rise in the number of fluorescein-based molecular probes in the past few decades, the research for

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fluorescein as an artificial enzyme has only begun to emerge [16]. In 2016, we found that fluorescein possessed the peroxidase-like activity to catalyze the oxidation of chromogenic substrates by H_2O_2 [16]. Compared with natural enzymes, fluorescein as a small organic molecule is more stable against denaturation or protease digestion, robust to extremes of pH and temperature and cheap. In comparison to nanozymes, fluorescein has no batch-to-batch variations in the catalytic activity, but is highly stable against high concentration of salt. Unfortunately, the unsatisfactory catalytic activity may restrict its further applications. As a matter of fact, alteration of catalytic activity may derive from intrinsic structural changes which, therefore, should be predictable and controllable as much as possible. However, as a new class of enzyme mimics, the effects of functional groups on the peroxidase-like activity of fluorescein have not been investigated.

In the previous work [16], we found the peroxidase-like activity of fluorescein and revealed that —OH groups on xanthene rings were the catalytically active sites. Besides the active sites, the substrate-binding sites of enzyme mimics also play significant roles in the catalytic process [17]. Thus we try to find the substrate-binding sites of fluorescein and investigate group effects on the peroxidase-like activity. We describe the instinct peroxidase-like activity of carboxyfluorescein (CF) and aminofluorescein (AF), for which a ping-pong mechanism was proposed. By investigating the catalytic activity of fluorescein derivatives with different functional groups, we obtained sufficient experimental and theoretical evidence that the carboxyl group as binding site can enhance catalytic activity while the amino group can inhibit the catalytic reaction. To the best of our knowledge, for the first time, we clarify the effect of functional groups of fluorescein derivatives in the catalytic reactions. These observations pave the way for designing and tuning the catalytic activity of small molecule-based peroxidase mimics.

2. Experimental section

2.1. Reagents and chemicals

5(6)-carboxyfluorescein (CF, HPLC grade, ≥95%), fluorescein (F, Reagent Ph. Eur. grade) and 3,3′,5,5′-tetramethylbenzidine (TMB) (99%) were purchased from Sigma-Aldrich. 5(6)-Aminofluorescein (AF, HPLC grade, >94%) and sodium acetate anhydrous (99.99% metals basis) was obtained from Aladdin Industrial Corporation (Shanghai, China). H_2O_2 (30%) was purchased from Chongqing Chuandong Chemical Co., Ltd. (Chongqing, China). Acetate (HPLC grade, ≥99.9%) was obtained from Shanghai Mackin biochemical Co. Ltd. (Shanghai, China). All other reagents were of analytical grade and were used directly without further purification. Ultrapure water (18.2 M) was prepared with a Milli-Q system (USA) and used throughout the experiments.

2.2. Electron spin resonance (ESR)

DMPO as the •OH trap was used to detect the DMPO/•OH spin adduct. Experiments were carried out in 0.20 M HAc-NaAc buffer solutions (pH 3.0) containing 50 mM DMPO, 10 mM H_2O_2 and 0.10 mM F, AF or CF, and then the samples were put in a quartz capillary tubes and placed in the ESR cavity. All ESR measurements were carried out using a JES FA200 ESR Spectrometer (JEOL, Japan) at an ambient temperature.

2.3. Peroxidase-like activity

In a typical experiment, 100 μL of F, AF or CF (2.0 mM in ethanol anhydrous solution), 200 μL of TMB solution (6.0 mM) and 200 μL of H_2O_2 (0.10 M) were successively added into 1.0 mL HAc-NaAc buffer solution (0.20 M, pH 3.0) and the mixture was diluted with ultrapure water to a volume of 2.0 mL. After incubation for 180 min at 35 °C, the absorbance change at 652 nm was recorded using a UV-2450 UV–vis spectrophotometer (Shimadzu, Japan).

2.4. Robustness and stability

To examine the robustness of the peroxidase-like activity of fluorescein derivatives, F, AF or CF was first incubated at a range of temperatures from 4 to 90 °C and a range of pH values from 1 to 12 for 2 h, respectively, and then their activities were measured under optimal conditions.

2.5. Measurement of apparent activation energy

The apparent activation energies (E_a) values for F, AF and CF were calculated according to the Arrhenius equation: $\ln\nu = \ln A - \frac{E_a}{RT}$, in which A was the Arrhenius constant; R was the gas constant (8.314 J/mol·K); and T was the absolute temperature in K. When measuring the *E*a, the concentration of catalyst (F, AF or CF), TMB and H_2O_2 were 0.20, 0.60 and 10 mM, respectively. To obtain the Ea, we measured the absorbance within reaction time (t) of 5 min at temperatures ranging from 25 °C to 37 °C, and plotted $\ln(\Delta A/\Delta t)$ (initial reaction rate) against 1/T. The measurements of apparent activation energy were carried out by monitoring the absorbance change at 652 nm on a microplate reader (Infinite 200 PRO, TECAN, Austria).

2.6. Kinetic assay

The reaction kinetics for the catalytic oxidation TMB was studied by recording the absorbance change at 652 nm with a 1 min interval. Experiments were carried out in 5.0 mL of 0.20 M NaAc buffer (pH 3.0, 35 °C) using 0.10 mM fluorescein derivatives by varying concentrations of H_2O_2 at a fixed concentration of TMB or vice versa. The Michaelis-Menten constant was calculated using the Lineweaver-Burk plot: $1/\nu = K_m/(V_{max}[S]) + 1/V_{max}$, where ν is the initial reaction rate, V_{max} is the maximal reaction rate, and [*S*] corresponds to the substrate concentration. Catalytic constant (K_{cat}) is calculated according to equation: $K_{cat} = V_{max}/[E]$, [*E*] is the molar concentration of artificial enzyme.

2.7. Computational methods

The quantum theoretical calculations were done with the Gaussian 09 package [18]. All the geometries have been fully optimized using B3LYP calculations in conjunction with aug-cc-pVDZ basis set [19,20].

3. Results and discussion

3.1. Intrinsic peroxidase-like activity of fluorescein derivatives

TMB, one of the most classical horseradish peroxidase (HRP) substrates [21] in various bioassays, was used to investigate the peroxidase-like activity of fluorescein derivatives (*i. e.* AF and CF). As shown in Fig. S1, both AF and CF can catalyze the oxidation of TMB in the presence of H_2O_2 to produce a colored reaction with maximum absorbance at 652 and 370 nm. In contrast, no obvious color change occurred in the absence of fluorescein derivative, suggesting an intrinsic peroxidase-like activity of fluorescein derivative.

Although we used high pure reagents in the assays, the possibility that the catalytic reactions are caused by metals need to be ruled out. To verify this, we first determined the metal contents in fluorescein, AF and CF by an Agilent 7700ce inductively coupled plasma mass-spectrometry (ICP-MS) and the results were listed in Table