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Interaction of glyceraldehyde-3-phosphate dehydrogenase and heme: The relevance of its biological function

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ABSTRACT

GAPDH was speculated to function as a transient trap to reduce the potential toxicity of free heme by a specific and reversible binding with heme. Up to now, there has been lack of studies focused on this effect. In this paper, the efficiency of GAPDH-heme complex on catalyzing protein carbonylation and nitration, the cross-linking of heme to protein formation, and cytotoxicity of GAPDH-heme were studied. It was found that the binding of GAPDH could inhibit H_2O_2-mediated degradation of heme. Peroxidase activity of GAPDH-heme complex was higher than that of free heme, but significantly lower than that of HSA-heme. Catalytic activity of heme corresponded complex toward tyrosine oxidation/nitration was decreased in the order of HSA-heme, heme and GAPDH-heme. GAPDH also inhibited heme-H_2O_2-NO_2^- induced protein carbonylation. No covalent bond was formed between heme and GAPDH after treated with H_2O_2. GAPDH was more effective than HSA on protecting cells against heme-NO_2^--H_2O_2 induced cytotoxicity. These results indicate that binding of GAPDH inhibits the activity of heme in catalyzing tyrosine nitration and protects the coexistent protein against oxidative damage, and the mechanism is different from that of HSA. This study may help clarifying the protective role of GAPDH acting as a chaperone in heme transfer to downstream areas.

1. Introduction

Hemoprotein is an indispensable enzyme in human bodies, performing key functions including electron transport, redox reaction catalysis, signal transduction, oxygen storage and transport, etc [1–4]. It is the last step and key step of hemoprotein synthesis that inserts heme into apoprotein in mitochondria [5]. In this case, heme needs to get access to apoprotein targets via endoplasmic reticulum and cytoplasm, so, it has been widely believed that protein carriers might be involved in this process to transport heme [5,6]. Searching of cytosolic heme binding proteins revealed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was responsible for heme transfer to inducible nitric oxide synthase (iNOS). Additionally, it was also reported that NO could inhibit heme inserting into iNOS, and NO as well as nitrosylation of GAPDH's Cys152 could regulate the transfer of heme [7,8].

Eukaryotic GAPDH takes part in various cell functions including transcription [9], oxidative stress [10,11], apoptosis [12,13] and autophagy [14]. Such functional diversity is mainly realized through changes in subcellular localization and/or post-translational modification [15]. Understanding of GAPDH had been continuously growing ever since GAPDH was successfully separated from rabbit muscle in 1964 [16]. Now it has been reported that GAPDH binds with heme [17,18], which might become a new clue to the exploration of unknown function of GAPDH. But GAPDH doesn't have standard heme binding sites which is different from the classic hemoprotein superfamily. Researches already showed that iron center of heme coordinated with two histidines (His) of GAPDH in GAPDH-heme complex [19]. Hence we speculated that the specific and reversible binding of GAPDH with heme might prevent free heme from binding with other proteins during heme transport. Free heme is cytotoxic due to its capability to catalyze the production of ROS that results in oxidative damage of DNA, protein, and lipid. When the key post-translational event of

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heme inserting into apoprotein by way of endoplasmic reticulum or cytoplasm happens, the above mentioned oxidative stress may take place. Therefore, the GAPDH-heme complex might also act as a transient trap to reduce the intracellular reactions and potential toxicity of free heme.

Overall, GAPDH may act as a molecular chaperone to regulate heme's potential toxicity before it reaches downstream delivery. However, the mechanism of GAPDH in reducing the potential toxicity of free heme is still unknown. Human serum albumin (HSA), as an important heme scavenger, is also capable to bind heme and protect nearby proteins against heme-induced oxidative damage. The binding constant of GAPDH with heme as reported was 1.9×10^{-8} M^{-1} [19], while that of HSA and heme was 9×10^{-9} M^{-1} [20]. GAPDH-heme and HSA-heme are both atypical heme binding proteins. In this paper, we will compare GAPDH-heme and HSA-heme in aspects of peroxidase activity and catalytic capability on protein nitration and oxidation. At the same time, the performance of GAPDH in decreasing heme's toxicity was studied as well.

2. Materials and methods

2.1. Materials

Ferriprotoporphyrin IX (hemin, iron(III) heme), human serum albumin (fatty acid and globulin free, Sigma A3782), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle lyophilized powder, 3,3′,5,5′- tetramethylbenzidine (TMB), 2,2′-amino-di- (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), enolase from Baker's yeast, 2,4-dinitrophenylhydrazine (DNPH), rabbit polyclonal antibody against 3-nitrotyrosine (3-NT) and dinitrophenol (DNP) were purchased from Sigma. Horseradish peroxidase-conjugated ImmunoPure goat anti-rabbit IgG was purchased from Pierce. HPLC grade acetonitrile and trifluoroacetic acid were purchased from Fisher Scientific. MilliQ water was used to prepare the water solution and all other solvents and other reagents were the highest purity and commercially available.

2.2. Preparation of HSA-heme and GAPDH-heme

Heme stock solution was prepared by dissolving solid hemin in dimethyl sulfoxide, and the concentration was measured using UV–Visible absorbance with an extinction coefficient of 5.84×10^4 cm^{-1} M^{-1} at 410 nm [21]. Heme stock solution was diluted in 100 mM phosphate buffered saline (PBS, pH 7.4) immediately before use. HSA-heme was prepared by incubating heme stock and HSA in a 1:1 molar ratio in 100 mM PBS (pH 7.4) at 37 °C for 15 min. Similarly, GAPDH-heme complex was prepared by incubating heme stock and GAPDH tetramer in a 1:2 molar ratio in PBS for 15 min [17].

2.3. Decomposition of heme induced by H_2O_2

To determine the decomposition of heme induced by H_2O_2, excess H_2O_2 (100 μM) was added to initiate heme (20 μM) oxidation in the presence of HSA (20 μM) or GAPDH (40 μM). The kinetic change of absorbance at Soret band peaks (410 nm) were recorded every 30 s.

2.4. Measurement of peroxidative activities

TMB, a classical substrate for peroxidases, was employed for peroxidative activity assays. The reaction was initiated by the addition of H_2O_2. The assay mixture contained 400 nM heme or heme complex [at 1:1 HSA: heme (400 nM) ratio and 2:1 GAPDH tetramer: heme (400 nM) ratio], 0.42 mM TMB, and 3 mM H_2O_2 in 50 mM citric acid buffer (pH 5.0) (all the concentrations were final concentration in the mixture). Then absorbance of TMB oxidation product at 652 nm ($\varepsilon_{652} = 3.9 \times 10^4$ cm^{-1} M^{-1}) was measured to determine the peroxidative activities of heme and its related complexes [22]. Meanwhile, ABTS was also employed to confirm the peroxidative activity of heme and its related complexes. The assay mixture contained 1 μM heme or heme complex [at 1:1 HSA: heme (1 μM) ratio and 2:1 GAPDH tetramer: heme (1 μM) ratio], 5 mM ABTS, and 3 mM H_2O_2 in 50 mM PBS (pH 5.0). The reaction was initiated by adding heme or the complexes. The corresponded peroxidase activity was determined by measuring the increase in absorbance at 405 nm ($\varepsilon_{405} = 3.68 \times 104$ cm^{-1} M^{-1}).

2.5. Fluorescence measurement

Dityrosine fluorescence was excited at 325 nm, and monitored at 350–500 nm using gap width of 5 nm. Fluorescence intensity was measured at 414 nm every 5 s. Heme-induced dityrosine formation was monitored upon the addition of 200 μM H_2O_2 to a solution of 1 μM heme or heme complex [at 1:1 HSA: heme (1 μM) ratio and 2:1 GAPDH tetramer: heme (1 μM) ratio] and 200 μM tyrosine in 100 mM PBS (pH 7.4) at room temperature.

2.6. Detection of protein nitration and carbonylation by western blot

A heme (or HSA-heme/GAPDH-heme)-H_2O_2-NO_2^- system was employed to study the modification of enolase, an enzyme that is a target of protein nitration in many diseases [23,24]. Samples were prepared by incubating 2 μM heme or its related complexes with 500 μM NO_2^-, 500 μM H_2O_2 and 0.1 mg ml^{-1} enolase in 100 mM PBS (pH 7.4) at 37 °C for 60 min.

To detect protein carbonylation, samples were denatured and derivatized with 10 mM DNPH in the presence of 3% SDS. After incubation at room temperature for 30 min, an equal volume of neutralization solution (2 M Tris, 30% glycerol) was added to terminate the reaction. Next, samples were mixed with loading buffer and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For detection of protein tyrosine nitration, samples were mixed with loading buffer and subjected to 12% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus. Then proteins were immunoblotted first with a rabbit polyclonal antibody against 3-NT (1:1000) or DNP (1:3000) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000). Finally, chemiluminescence was measured using the ECL system (Pierce) to identify corresponded proteins.

2.7. Measurement of heme to protein cross-linking

The formation of covalent heme: protein cross-link was determined by reversed phase HPLC as described previously [25]. GAPDH-heme (100 μM) was reacted with H_2O_2 (100 μM) at 37 °C, 25 mM sodium acetate buffer (pH 5.0) containing 100 μM diethylenetriaminepentaacetic acid (DTPA), for 30 min. Samples were analyzed on an Agilent HP1100 HPLC fitted with a diode array spectrophotometer. A Zorbax Stable Bond300 C3 reverse phase column was used (150 × 4.6 mm, 5 μm pore size). Solvents used were: A, 0.1% trifluoroacetic acid (TFA); B, acetonitrile containing 0.1% TFA. Elution profile was 0–10 min, 35% B; 10–15 min, 35% B increasing to 37% B; 15–16 min, 37% B increasing to 40% B; 16–20 min, 40% B increasing to 43% B; steady for 5 min then increasing to 95% B for 25–30 min. Column temperature was 25 °C, and pump flow rate was 1 ml · min^{-1}.