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Inhibition of methemoglobin formation in aqueous solutions under aerobic conditions by the addition of amino acids

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ABSTRACT

Hemoglobin (Hb) as an important iron-containing oxygen-transport protein is easily oxidized to the ferric met-form, methemoglobin (metHb), and loses the capacity of binding oxygen during storage. In this study, the experimental data indicate that the presence of Tyr and Glu significantly suppress the metHb formation in the Hb solutions in aqueous environment under aerobic conditions at the temperature of 25 and 37 °C, respectively. At pO_2 of 144 Torr the metHb percentage in the Hb solutions was the lowest with less than 10% at day 7 after incubation with Tyr at the ratio of 24 at pH 9.5 at 25 °C. At 37 °C, the metHb percentage did not reach 5% after 12 h of incubation with Glu at the ratio of 24 at pH 9. Molecular simulation analysis suggest that the presence of Tyr or Glu may contribute to the formation of the breakwater network, the stabilization of distal histidine, the changes in the size of heme pocket, and eventually result in the inhibition of metHb formation. This study provides insight into a new design for Hb-oxygen based carriers with strongly inhibition of metHb formation in aqueous environment under aerobic conditions, even at physiological temperature *in vitro*.

1. Introduction

Hemoglobin (Hb), a tetrametric protein in red blood cells, is composed of four polypeptide chains (two α- and two β-chains), each of which containing a hydrophobic pocket bound to a prosthetic heme group. This group consists of a Fe(II) ion and a porphyrin ring as the O_2-binding site to carry and deliver oxygen. In the prosthetic heme group, the iron atom locating at the center of the porphyrin ring forms four bonds with porphyrin nitrogen, one covalent bond with the histidine residue and one bond with exogenous ligands (Fig. 1) [1].

The formation of metHb causes Hb to lose the capacity of binding oxygen under aerobic conditions. At a steady state the distal His58 stabilizes the bound dioxygen through a hydrogen bond. Upon the nucleophilic attack of water molecules, the FeO_2 center is open to the proton on the acidic pH side or hydroxide anion on the basic side. This results in an irreversible displacement of the bound dioxygen in the form of superoxide ion (O_2^-) and a replacement of the sixth coordination position originally binding with oxygen by water or hydroxide [2] and eventually leads to the formation of metHb [2,3]. Therefore, the inhibition of metHb formation is essential to preservation of Hb before clinical applications.

In vivo the percentage of metHb in red blood cells is less than 5% due to the presence of systematic reduction. However, *in vitro* due to the absence of the metHb reduction system which is removed during processes of Hb purification [4–7], the metHb formation and protein denaturation are the major problems in the Hb-containing systems and acellular-type Hb-based oxygen carriers [8]. These Hb-containing systems and Hb-based oxygen carriers, such as liposome-encapsulated Hb [9], Hb vesicles [10,11], and microparticles with Hb loading [12–15], are considered as potential candidates for red-blood-cell substitutes [16–19]. It is hence that the efficient inhibition of metHb formation in the Hb-containing systems and Hb-based oxygen carriers has far-reaching significance in clinical applications.

Various approaches including cryopreservation and freeze-drying have been investigated to stabilize Hb [20,21]. However, these methods are time-consuming and process-complicated. Hb preserved either alone or in oxygen carriers in aqueous environment is much more convenient for infusion in emergency situations. Therefore, the inhibition of metHb formation is the primary method for the stabilization of Hb during its preservation in aqueous environment. Previous reports have demonstrated that

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; Hb, hemoglobin; metHb, methemoglobin; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; Tris–HCl, Tris[hydroxymethyl-aminomethane]–HCl; TEMED, N,N,N′,N′-tetramethyl ethylenediamine.

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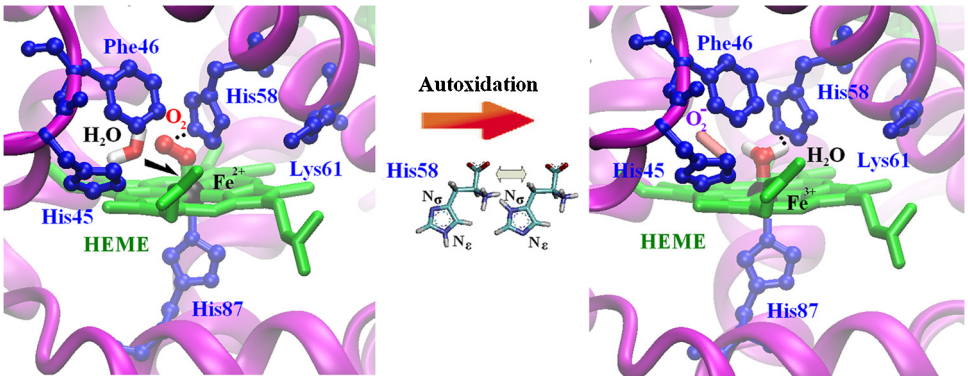

Fig. 1. Schematic representation of Hb autoxidation process in aqueous environment. At steady state the distal histidine (58) stabilizes the bound dioxygen through a hydrogen bond. Electron transfer from N to N at His58 weakens hydrogen bond between His58 and FeO_2 center. Nucleophilic attack of entering water molecule can result in the irreversible displacement of the bound dioxygen in the form of superoxide ion (O_2^-), and facilitate a full charge transfer from Fe (II) to O_2. This eventually causes the formation of metHb.

under anaerobic conditions the introduction of reductants or L-Tyr can suppress the metHb formation [6,22,23]. The thermal stability of Hb in aqueous environment can be improved in the presence of sarcosine and sorbitol [24]. MetHb formation can also be inhibited by the addition of glutamate [25] and antioxidant enzymes [26,27].

In addition, the metHb formation is strongly pH-dependent, and the nucleophilic attack to FeO_2 during the metHb formation is more significantly enhanced under acidic conditions than alkaline [3,28]. Temperature should be another important factor to affect protein stability. However, the report about the effect of temperature on metHb formation is rare. Although it is contributive to the stabilization of Hb by pH adjustment and temperature control, the methods for suppressing the metHb formation in aqueous environment under aerobic conditions are still needed to be improved.

The aim of this study was to suppress the metHb formation in aqueous environment under aerobic conditions. Due to the high stability, low cost and good safety, amino acids are the potential stabilizers of proteins. This study was the first report to examine the effects of five different amino acids on the inhibition of metHb formation at different pHs and different temperatures, and further the effect of the mole ratio of amino acid to Hb on the metHb formation. The possible mechanism behind the inhibiting effect of amino acids on the metHb formation was discussed based on the molecular simulation.

2. Experimental procedures

2.1. Materials

Donated red blood cells were obtained from the Anzhen Hospital (Beijing, China). Tris[hydroxymethyl-aminomethane]–HCl (Tris–HCl), acetonitrile, methanol, glycine and NaCl were purchased from Sinopham Chemical Reagent (China). Acrylamide, N,N′-methylene bisacrylamide, coomassie blue G-250, acetic acid/ethyl alcohol and ammonium persulfate were purchased from Xinglong (China). N,N,N′,N′-tetramethyl ethylenediamine (TEMED) was purchased from Merck (UK). L-Trp, L-Cys, L-Lys, L-Tyr, sodium glutamate and bovine serum albumin (BSA, MW66000) were purchased from Sigma–Aldrich (USA). Anion exchangers and Q Sepharose Fast Flow (Q-SFF) resin used in this study were produced from GE Healthcare (USA). Dialysis membrane with cut-off molecular weight of 3500 Da was purchased from Union Carbide (USA).

2.2. Preparation and purification of Hb from red blood cells

Hb was isolated from the donated blood using hemolysis. The red blood cells were suspended in 20 mM Tris–HCl buffer (pH 8.0) at the ratio of 1:2 (v/v) at 4 °C for 24 h. After hemolysis, the suspension of lysed cells was centrifuged at 2300 × *g* for 1 h at 4 °C. The supernatant was purified using the ion exchange chromatography method with Q-SFF resin at room temperature [29,30]. Briefly, the supernatant was loaded with an injector and eluted with a linear gradient of buffer from 100% buffer A (20 mM Tris–HCl, pH 8.0) to 75% buffer B (20 mM Tris–HCl plus 0.2 M NaCl, pH 8.0) in 5-fold volume of column. The elution was monitored with a UV detector at 280 nm. The Hb solution after purification was concentrated to 150 g/l using a dialysis membrane with the cut-off molecular weight of 3500 Da.

2.3. Storage

150 g/l of Hb in 20 mM Tris–HCl buffer was diluted to 50 g/l using Tris–HCl buffer (pH 7.4). The Hb diluted solutions were incubated at pH 7, 8, 9, 9.5 and 10 (20 mM Boric acid sodium hydroxide buffer for pH 10) at pO_2 of 144 Torr at 25 or 37 °C. The stock solutions of Glu, Lys, Tyr, Cys and Trp were prepared at the concentration of 0.04 M at pH 7. For Tyr, the stock solution was prepared at around pH 10 due to the low solubility of Tyr below pH 10. Glu, Lys, Cys, Tyr or Trp was introduced to the Hb solutions at the mole ratio of 24, respectively, and the pH was adjusted to the required pH using the Tris–HCl buffer (20 mM). Then the Hb solutions were incubated at 25 °C for up to 7 days or 37 °C for up to 21 h at pO_2 of 144 Torr at pH 8, 9 and 9.5, respectively. In addition, Tyr and Glu were introduced in the Hb solutions at the mole ratio of 8, 16 and 24 at pH 9 and 9.5, respectively, and incubated at 25 °C for up to 2 days. The pH at the end point for all the tested conditions was measured using a pH meter (Sartorius, Germany).

2.4. Measurement of metHb content

The Hb solutions were diluted from 50 g/l to 0.5 g/l before assay. The pH of the diluted Hb solutions measured using a pH meter was in the range of 6–8 for all the tested conditions. The absorption of the diluted Hb solutions (0.5 g/l) at 560, 576 and 630 nm was measured using a UV-Vis spectrometer (Shanghai Spectrum, China). The content of metHb was determined using the following equations described by Benesch et al., which was valid between pH 6.2 and pH 8.8 [31].

$$A_{560} = \varepsilon_{560} C_{\text{deoxyHb}} + \varepsilon_{560} C_{\text{oxyHb}} + \varepsilon_{560} C_{\text{metHb}} \quad (1)$$

$$A_{576} = \varepsilon_{576} C_{\text{deoxyHb}} + \varepsilon_{576} C_{\text{oxyHb}} + \varepsilon_{576} C_{\text{metHb}} \quad (2)$$

$$A_{630} = \varepsilon_{630} C_{\text{deoxyHb}} + \varepsilon_{630} C_{\text{oxyHb}} + \varepsilon_{630} C_{\text{metHb}} \quad (3)$$