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The quaternary hemoglobin conformation regulates the formation of the nitrite-induced bioactive intermediate and the dissociation of nitric oxide from this intermediate

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ABSTRACT

Deoxyhemoglobin reduces nitrite to nitric oxide (NO). In order to study the effect of the hemoglobin quaternary conformation on the nitrite reaction, we compared T-state deoxyhemoglobin with R-state deoxyhemoglobin produced by reacting hemoglobin with carboxypeptidase-A prior to deoxygenation. The nitrite reaction with deoxyhemoglobin was followed by chemiluminescence, electron paramagnetic resonance and visible spectroscopy. The initial steps in this reaction involve the binding of nitrite to deoxyhemoglobin followed by the formation of an electron delocalized metastable intermediate that retains potential NO bioactivity. This reaction is shown by visible spectroscopy to occur 5.6 times faster in the R-state than in the T-state. However, the dissociation of NO from the delocalized intermediate is shown to be facilitated by the T-quaternary conformation with a 9.6 fold increase in the rate constant. The preferred NO-release in the T-state, which has a higher affinity for the membrane, can result in the NO diffusing out of the RBC and being released to the vasculature at low partial pressures of oxygen.

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Introduction

Nitric oxide as a vasodilator plays a major role in regulating blood flow and vascular tone [1]. The primary source for the synthesis of NO in the circulatory system involves endothelial nitric oxide synthase [2]. Since nitric oxide has a life time in plasma of <0.1 ms [3], effective delivery of NO to the vasculature would require that the NO is synthesized at the site where it is needed. The reported [4] reduced activity of nitric oxide synthase at reduced partial pressures of oxygen may limit the ability of endothelial nitric oxide synthase to supply NO to the microcirculation. To resolve this dilemma mechanisms for the transport of NO activity by RBCs have been proposed. A mechanism involving the transfer of NO to a thiol group producing S-nitrosylated hemoglobin (SNOHb) was originally proposed in 1996 by the seminal paper of Stamler and collaborators [5], with alternative hypotheses proposed by other investigators involving the hypoxic release of ATP [6] and reduction of nitrite to NO by deoxygenated hemoglobin in the RBC [7,8].

Any role for RBCs in transporting nitric oxide must, however, be able to avoid the very efficient scavenging of nitric oxide in RBCs by both oxyhemoglobin (oxyHb) and deoxyhemoglobin (deoxyHb). The hypothesis suggesting hypoxic release of ATP does not involve a pool of RBC NO that can react with hemoglobin [6,9]. The Stamler hypothesis bypassed this difficulty by transferring the NO to the β-93 thiol group with the formation of SNOHb [5]. This NO can then be transferred to endothelial cells through transnitrosation of membrane thiol groups [10]. The 2003 studies by Rifkind and Gladwin and their collaborators [7,8] proposed a mechanism that involved the reduction of nitrite back to NO by a reaction with deoxyHb. The attraction of this mechanism is that it reuses the NO produced by the endothelium, without requiring the transport of unstable nitric oxide. The feasibility of this mechanism, however, requires that the nitrite reduced back to NO is not quenched by hemoglobin.

Gladwin and collaborators originally suggested that the nitrite is reduced to NO by a metabolon (a complex of band 3, carbonic anhydrase, and deoxyhemoglobin reductase) localized on the membrane at the point where it is needed [11]. More recently [12] they have proposed that nitrite complexes with oxidized hemoglobin (metHb) formed during the reduction of nitrite by deoxyHb and that the reaction of the NO formed with this complex produces N_2O_3 that can diffuse out of the cell and/or react with thiols to form S-nitrosothiols. The difficulty with this mechanism is that it uses the final products (metHb and NO) formed by the

Abbreviations: DeoxyHb, deoxyhemoglobin; metHb, methemoglobin; oxyHb, oxyhemoglobin; CPA, carboxypeptidase A; IHP, inositol hexaphosphate; PBS, phosphate buffered saline; NO, nitric oxide; RBC, red blood cell; SNOHb, S-nitrosohemoglobin; NOA, nitric oxide analyzer.

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reaction and requires that the NO produced react with the low levels of nitrite reacted metHb instead of the readily available oxyHb and deoxyHb.

To explain how NO can be formed in RBCs without reacting with oxyHb or deoxyHb, Rifkind and collaborators have been able to demonstrate the formation of an intermediate with the electron shared between the NO, the heme iron and perhaps the β-93 thiol of hemoglobin [8,13–15]. This electron delocalization provides for a metastable NO that remains associated with hemoglobin, but which can under the proper conditions be released to the vasculature. These studies provide the basis for the accumulation, within RBCs, of a pool of potentially bioactive NO. It is, however, still necessary to explain how this NO can be released from the RBC.

Recent studies have shown that the nitrite reaction depends on the hemoglobin quaternary conformation [16–18]. We have previously developed a kinetic method to distinguish between the initial nitrite reaction and the dissociation of NO from the electron-delocalized metastable nitrite reacted hemoglobin [14]. Since the dissociation step and not the initial nitrite reaction would be involved in the release of NO from RBCs, we wanted to analyze the effect of the quaternary conformation on the two steps involved in the nitrite reaction. To investigate the effect of the quaternary conformation on the two distinct steps involved in the nitrite reaction, we have in this paper used carboxypeptidase A (CPA) modified hemoglobin, which retains the R-state even when fully deoxygenated. We were then able to perform a detailed analysis of the nitrite reaction for fully deoxygenated T and R state hemoglobins utilizing a combination of chemiluminescence, visible spectroscopy, electron paramagnetic resonance and filtration methods. Based on this analysis, we have shown that while the nitrite reaction is enhanced by R state hemoglobin as previously reported [16] the dissociation of NO from the delocalized intermediate is actually enhanced in T-state hemoglobin. These results can explain the allosteric release of NO to the vasculature by combining the accumulation of potentially bioactive NO in the R quaternary conformation with the release from hemoglobin in the T quaternary conformation when the hemoglobin is also bound to the membrane.

Materials and methods

Preparation of hemoglobin

Hemoglobin was prepared from fresh RBCs as described earlier [8].

Preparation of R-state deoxyhemoglobin

Hemoglobin was incubated with CPA obtained from Sigma Chemical Co., St. Louis, Missouri for 2 h at 37 °C in 0.1 M Tris–HCl buffer, pH 8.0. and then passed through a Sephadex G-100 column to remove the enzyme [19]. This reaction cleaved the terminal histidine and tyrosine in the β-chains stabilizing the R–state even when hemoglobin is fully deoxygenated.

Preparation of methemoglobin

Methemoglobin was prepared by oxidizing oxyhemoglobin with a 1.2 fold excess of potassium ferricyanide. The oxidized hemoglobin was then passed down a G-25 Sephadex column to remove the excess ferricyanide and the ferrocyanide formed during the reaction.

T-state methemoglobin was prepared by adjusting the pH to 6.5 and adding a molar equivalent of inositol hexaphosphate (IHP) [20].

Deoxygenation of hemoglobin

Hemoglobin in 50 mM NaCl and 4 mM phosphate buffer, pH 7.4 (PBS) was deoxygenated in an anaerobic Coy glove box. The glove box uses hydrogen and a palladium catalyst to remove any residual oxygen resulting in <1 ppm oxygen. Multicomponent fitting of the deoxyHb sample obtained by this procedure did not detect any oxyHb and no oxyHb accumulation was detected during the course of the experiment.

Determination of nitrite consumption

About 1 mM deoxyHb was reacted with ~0.1 mM nitrite in PBS, pH 7.4 for 60 min at room temperature (22 °C) in the anaerobic Coy glove box. To quantitate the consumption of nitrite, the reaction was carried out in a petri dish containing 5 ml of R or T state deoxyHb. The reaction solution was placed on a shaker and, at specific time intervals, 0.5 ml aliquots were transferred to microfilterfuge tubes (Rainin Instrument Co., Inc. filters with a 10,000 mol. wt. cutoff) and centrifuged for 2 min at 6500 rpm using a microfuge (LK Scientific) inside the anaerobic glove box. After centrifugation, 5 μl of the filtrate was injected into the Nitric Oxide Analyzer (NOA) purge vessel to determine nitrite by chemiluminescence (see below). At zero time the free nitrite indicates the actual concentration of nitrite added.

Chemiluminescence method to determine nitrite

The Model 280 NOA from Sievers Instruments was used. The sample was injected into the NOA purge vessel containing 7 ml of glacial acetic acid and 1 ml of 0.5 M ascorbic acid in order to determine the concentration of nitrite as previously described [21].

Chemiluminescence method to determine total heme-NO

The Model 280 NOA from Sievers Instruments was used in accordance with previously published procedures [8]. With the purge vessel containing 5.5 ml of 100 mM sulfanilamide dissolved in 87.5% glacial acetic acid, 1.0 ml of 1.0 M potassium ferricyanide in water and 0.1 ml antifoam reagent at 37 °C, the chemiluminescence measures the concentration of total heme-NO (Hb(II)NO, Hb(III)NO and any heme based intermediates) in a hemoglobin sample [15].

Electron paramagnetic resonance (EPR) determination of Hb(II)NO

Samples were transferred anaerobically to 4 mm clear fused Quartz EPR tubes (707 SQ 250 M – WILMAD) and frozen immediately by submerging the EPR tubes in liquid nitrogen and stored at 77 K until EPR measurements were performed. EPR spectra were measured using a Bruker EMX spectrometer with 100 kHz modulation as previously described [8].

The reaction of nitrite with deoxyhemoglobin followed by visible spectroscopy

After hemoglobin (100 μM) or CPA reacted hemoglobin (57.3 μM) were deoxygenated, the nitrite reaction was initiated by using a gas tight syringe to add nitrite in a septum sealed cuvette with a 1:1 final molar ratio of nitrite to heme. Spectra of hemoglobin from 490 to 640 nm were continuously recorded on a Perkin Elmer Lambda 35 spectrophotometer for 35 min. The resultant spectra obtained in any experiment were analyzed using a least squares multicomponent fitting program (Perkin Elmer Spectrum QuantC v 4.51) to obtain concentrations of deoxyHb, metHb, Hb(II)NO, oxyHb and nitrite bound metHb. Our spectral analysis