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Enhanced nitrite reductase activity associated with the haptoglobin complexed hemoglobin dimer: Functional and antioxidative implications

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Abstract

The presence of acellular hemoglobin (Hb) within the circulation is generally viewed as a pathological state that can result in toxic consequences. Haptoglobin (Hp), a globular protein found in the plasma, binds with high avidity the $\alpha\beta$ dimers derived from the dissociation of Hb tetramer and thus helps clear free Hb. More recently there have been compelling indications that the redox properties of the Hp bound dimer (Hb–Hp) may play a more active role in controlling toxicity by limiting the potential tissue damage caused by propagation of the free-radicals generated within the heme containing globin chains. The present study further examines the potential protective effect of Hp through its impact on the production of nitric oxide (NO) from nitrite through nitrite reductase activity of the Hp bound $\alpha\beta$ Hb dimer. The presented results show that the Hb dimer in the Hb–Hp complex has oxygen binding, CO recombination and spectroscopic properties consistent with an Hb species having properties similar to but not exactly the same as the R quaternary state of the Hb tetramer. Consistent with these observations is the finding that the initial nitrite reductase rate for Hb–Hp is approximately ten times that of HbA under the same conditions. These results in conjunction with the earlier redox properties of the Hb–Hp are discussed in terms of limiting the pathophysiological consequences of acellular Hb in the circulation.

Keywords

Nitrite reductase; Haptoglobin; Hemoglobin

Introduction

The presence of acellular hemoglobin within the circulation can trigger a cascade of processes with profound toxic consequences on surrounding tissues. Acellular Hb in the vasculature can occur due to acute or chronic hemolytic conditions that result in the release of free Hb from the red blood cell or from the infusion of Hb based oxygen carriers (HBOCs). The origins of the toxic effects are still not fully understood. One potential mechanism of toxicity is believed to be the result of the propensity of acellular Hbs to create or enhance oxidative stress largely due to the formation of globin-associated free radicals that are capable of initiating lipid peroxidation thereby triggering an inflammatory cascade

[1,2]. The other perspective is that acellular Hbs are highly effective at scavenging endothelial generated NO which can trigger or exacerbate pathological states through decreased tissue perfusion and decreased scavenging of reactive oxygen species (ROS) [3–6]. The toxic consequences of NO scavenging are likely to be especially serious when there is a backdrop of endothelial dysfunction that is already causing both a drop in NO bio-availability and an increase in oxidative stress [7].

Haptoglobin (Hp), an acellular globular protein found in the plasma plays a major role in mediating toxicity due to acellular Hb [8–10]. Hp is a hetero-dimer composed of α (9 kDa)- and β (33 kDa)-subunits and in humans exists in two allelic forms; Hp1 and Hp2, which differ only in their respective α chains. The α_1 -chain in Hp1 carries one free sulfhydryl group, while α_2 in Hp2 is longer and carries two sulfhydryl groups. Polymeric structures with variable numbers of associated Hp dimers are found in serum from individuals. Sera of individuals in which Hp molecules comprise either two $\alpha_1\beta$ units (Hp1–1), a variable number of $\alpha_2\beta$ units (Hp1–2), or two $\alpha_1\beta$ units and a variable number of $\alpha_2\beta$ units (Hp2–2) [11]. Hp seemingly binds with high avidity $\alpha\beta$ dimers derived from the dissociation of the Hb tetramer and thus participates in acellular Hb clearance without damage to the kidneys. Recently, it was demonstrated that Hp can play a more active role in minimizing acellular Hb derived toxicity by stabilizing reactive tyrosine radicals within the Hp bound globin thus reducing the likelihood of Hb-induced lipid peroxidation and inflammation [12].

The Hb–Hp complex might also have additional properties that could limit toxicity based on the functional properties of the Hp bound $\alpha\beta$ globin dimer. In solution, the functional properties of the $\alpha\beta$ dimer are similar to that of the R state tetramer [13–16]. There is however a phenomenon termed quaternary enhancement based on a modest enhancement of the oxygen affinity and ligand binding properties of the $\alpha\beta$ dimer when the dimer is incorporated into the R quaternary state of the Hb tetramer [17–24]. If the Hp bound dimer has R state functional properties, it is anticipated that the quaternary structure sensitive rate for the formation of NO from nitrite via a nitrite reductase reaction between a five coordinate ferrous heme (AKA deoxy heme) and nitrite should also be enhanced. Several studies show that an R state deoxy heme reacts much faster than a T state deoxy heme [25–30]. Increased production of NO by acellular Hb's with high oxygen affinity has been proposed as a mechanism to compensate for NO scavenging by acellular Hbs [31]. This mechanism has been proposed [31,32] as the basis for why certain PEGylated HBOCs are not vasoactive [33–35] despite an NO scavenging capacity comparable to other Hb based oxygen carriers (HBOCs) that promote vasoconstriction [33].

This manuscript describes studies designed to determine whether the hemes in the Hb–Hp complex exhibit spectroscopic and functional properties consistent with the Hp complexed globin $\alpha\beta$ dimer having R state-like properties including an enhanced rate for the nitrite reductase reaction. Functional properties include measurements of oxygen affinity, geminate and solvent phase CO recombination and nitrite reductase activity (initial rates). The geminate recombination is additionally useful in that the geminate yield (fraction of the photodissociated population undergoing geminate recombination) for the dimer is lower than that of the R state tetramer (quaternary enhancement effect) [20] but still much greater than the near zero geminate yield for the low affinity T state tetramer [36]. Thus, the geminate recombination can help establish whether the Hp bound dimer exhibits that same reduction in geminate yield as seen for the unbound solution phase dimer. In addition to the functional studies, the visible (Q bands) [37,38] and near IR (Band III) [22,29,39–44] absorption spectrum of the fully deoxygenated ferrous derivative is also used to evaluate whether the deoxy hemes manifest spectra that are consistent with an R-like species. In all cases, the results are consistent with an R-like heme species both functionally and

spectroscopically. The geminate yield is consistent with an Hp bound dimer functionally similar to the unbound dimer in solution.

Materials and methods

Sample preparation

Human hemoglobin A (HbA)—Hp was a kind gift from Bio Products Laboratory (BPL), Hertfordshire, England. Typical size exclusion HPLC separation profiles of Hp samples used in this study shows the following molecular weight distribution (60% dimer, 21% trimer and 19% larger forms). In Hp1–1 individuals, Hp will be present in its simplest form, consisting of dimers (two α and two β -chains, connected by disulfide bridges) with a molecular weight of 98 kDa. In Hp2–1 and Hp2–2 individual's trimers, tetramers and polymers are present with molecular weights up to ~700 kDa. Adult human Hb (HbA₀) was prepared by ammonium sulfate precipitation, stripped of endogenous organic phosphates and purified by anion-exchange FPLC as previously described [45].

Hemoglobin–haptoglobin (Hb–Hp) complex preparation—Adult human Hb, purified by FPLC chromatography and stripped of anionic effectors as described in Methods, was passed through a superdex-200 column for removal of catalase using 50 mM potassium phosphate buffer at pH 7.4 using the protocol described earlier [45]. For complex formation, the purified Hb was mixed with Hp in a 1:1.5 ratio (60 μ M in heme). The complex was further purified using a BioSep 3000 column using 50 mM potassium phosphate buffer at pH 7.4 (Phenomenex, Torrance, CA). MALDE-MS was used to verify the Hb–Hp complex formation as described previously [46].

$\beta\beta$ XL P2K HbA—This high oxygen affinity cross linked derivative of the HbA tetramer (prepared and provided by members of Professor S. Acharya's research group at the Albert Einstein College of Medicine) is stabilized through a bifunctional maleimide-PEG (2K) linker (bis mal-PEG) that crosslinks the two β chains through a maleimide reaction with the two β Cys 93 sulfhydryls [47]. This cross linked Hb has similar functional properties to other Hbs (e.g. NEM Hb) that have the same thiol modification but without crosslinking [18,32,48,49]. The maleimide modification increases oxygen affinity and shifts the allosteric equilibrium towards the R state. The cross linked derivative is used to eliminate any possible issue of dimer formation thus allowing a comparison of the Hb–Hp complex with a high oxygen affinity tetramer that has been shown to be vasodilatory in the presence of low levels nitrite (Cabrales and Friedman, unpublished results).

Preparation of solution phase samples of HbA and Hb–Hp for kinetic and spectroscopic study—Deoxy heme derivatives of HbA and Hp–Hb for kinetic and spectroscopic solution studies (~0.2 mM heme) were prepared from the initial oxygenated derivatives by exhaustive purging with Ar in an oxygen free glove box, and adding a modest excess of dithionite (>1:1 dithionite:heme) as necessary. The visible to near IR optical absorption spectra of samples in Bis–Tris buffer, pH 7.0, were scanned to establish the ligation and redox status of the heme. COHb samples were prepared by exposing the deoxy samples to buffer saturated CO gas, until the absorption spectrum revealed complete conversion to the COHb derivative. Aquomet samples were prepared by oxidizing Hb using K₃Fe(CN)₆, which was subsequently removed using a spin column prepared from G10 Sephadex in the same buffer. In some instances L-cysteine was used to reduce the aquomet samples in order to compare deoxy heme populations generated from the oxy heme plus dithionite or aquomet heme plus L-cysteine.

Oxygen equilibrium measurements—Oxygen equilibrium binding studies were carried out using a Hemox Analyzer (TCS Scientific, New Hope, Pennsylvania, US) [50]. Sample deoxygenation and reoxygenation was performed using pure nitrogen gas and air, respectively. Oxygen tensions were measured using a Clark Model 5331 O₂ Probe (Yellow Springs Instrument Company, Yellow Springs, Ohio, US). Oxygen equilibrium curves (OECs) were generated by measuring O₂ fractional saturation by optical absorbance spectroscopy using a dual-wavelength spectrophotometer at varied O₂ partial pressures. Hb and Hb–Hp complex solutions (heme concentrations in the range of 60–75 μM) were used in 10 mM potassium phosphate/0.1 M NaCl, pH 7.4 at 37 °C. All solutions contained 4 μl of the Hayashi mixture [50] of reducing enzymes to minimize Hb oxidation during measurements. Data obtained from these studies were fit using the nonlinear least-squares method and Adair equations built-into the Hemox Analyzer software (P₅₀ Plus, Version 1.2).

Geminate and solvent phase CO recombination kinetics—CO recombination traces from the CO heme derivatives of HbA and Hb–Hp were generated and displayed on a log–log plot of normalized absorbance (NA) versus time as previously described and discussed in details [36,51–54]. Photodissociation of the CO from the heme is triggered using a 7 ns pulse at 532 nm from a Nd:YAG laser. The recombination trace over several decades in time starting from 10 ns out to 1 s is generated from a change in absorption using the several milliwatt 442 nm output of a CWHeCd laser as a probe of the evolving populations undergoing CO recombination.

Nitrite reductase activity at pH 7.0—Deoxygenated Hb samples were prepared in solution at pH 7.0 in 0.05 M Bis–Tris; ~0.2 mM in heme with the exception that an excess of dithionite was added to a concentration of 1 mM. Nitrite was added in a slight excess of 1.1:1(heme:nitrite) to deoxy Hb) to initiate the reaction. Under these conditions, the nitrite reductase reaction is modified in that the met (Hb⁺) product generated from the nitrite reductase reaction is reduced back to deoxy Hb by the excess dithionite (Eq. (1)):

The deoxy Hb binds NO yielding the ferrous NO derivative of Hb (NOHb). As a result, the observed reaction consists overwhelmingly of the loss of deoxy Hb and the formation of NOHb as previously described [29,55]. Dithionite at this concentration does not reduce nitrite to NO [55,56].

The change in absorption as a function of time at 430 nm (Soret band for deoxy HbA) was monitored on a Lambda 2 (Perkin Elmer, Norwalk, CT). The choice of the 430 nm and to follow the initial rate of decay of the starting deoxy species in solution is based on the relatively large extinction coefficient for deoxyHb at this wavelength, ~133,000 M⁻¹·cm⁻¹ compared to (NOHb, ~8400, and met Hb, ~4000). The initial linear portion was used to calculate initial rates.

The Q-band absorption spectra (~480–800 nm) was also scanned and deconvoluted into populations of products using authentic standard basis sets and a program in Mathcad, V14.0 (Parametric Technology Corporation, Needham, MA). The deoxy heme population was plotted as a function of time to provide an alternative approach to generate the initial nitrite reductase rates.

Initial rates for the nitrite reductase reaction were generated for the Hb–Hp complex, HbA and ββ XL P2K HbA.

Nitrite reductase activity at pH 7.4 in the presence of L35, a potent allosteric effector

The nitrite reductase reaction rate in HbA has been shown to increase as the reaction progresses. The enhancement is directly attributable to the allosteric behavior of the Hb tetramer [27]. Initially at the start of the nitrite reductase reaction, the heme population for the Hb is fully deoxygenated which for HbA results in a population that is overwhelmingly T state. The T state has a ten fold slower rate than the R state. As the reaction progresses, the population becomes a mix of Hbs containing an increasing population of met hemes and NO ferrous hemes as well as a decreasing population of deoxy hemes. This population progression shifts the allosteric equilibrium towards the R quaternary state. The remaining deoxy hemes within the R state tetramers will manifest the approximately ten fold enhancement in the nitrite reductase rate. The introduction of an allosteric effector that stabilizes the T over R quaternary structure will inhibit or eliminate the above described progressive enhancement in rate. In contrast, the dimer is anticipated to be relatively insensitive to the addition of allosteric effectors and hence show minimal variation in the kinetic trace associated with the nitrite reductase reaction. L35 (2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid), is a potent allosteric effector [57] that is still fully operational at high pH [58] (unlike inositol hexaphosphate and diphosphoglycerate) which allows for the monitoring of its impact at pH 7.4 where the nitrite reductase reaction is slower thus allowing a more clear temporal window to assess allosteric effects.

Results

Oxygen affinity measurements

Fig. 1 shows representative oxygen equilibrium curves (OECs) together with their corresponding Hill plots for uncomplexed Hb and for the purified Hb–Hp complex. The Hb–Hp complex showed significantly increased O₂-binding affinity relative to uncomplexed Hb ($P_{50} = 4.59$ versus 13.51 mm Hg in 10 mM potassium phosphate/0.1 M NaCl, pH 7.4 at 37 °C (see also Table 1). Cooperativity of O₂ binding ($n_{1/2} = 2.37$ for HbA) was lost and subunit heterogeneity was evident in the Hill plots of the Hb–Hp complex ($n_{1/2} = 1.15$). We have recently reported and in accord with the elevated O₂ affinity, the apparent first order rate of O₂ dissociation from HbA and the Hb–Hp complex as captured in a stopped flow spectrophotometric method. Comparison of the process for Hb and the Hb–Hp complex, carried out under identical conditions, showed Hb to have an apparent rate constant for O₂ dissociation of 58 s^{−1} at the mid-point of the process, while that of the Hb–Hp complex was 25 s^{−1} (Banerjee et al., unpublished data). Both equilibrium and kinetic data are consistent with recently published work by our group [59] and with that of earlier studies using slightly different preparations of haptoglobin solutions [13,60].

Geminate and solution phase CO recombination

Fig. 2 displays on a log normalized absorbance/log time scale the normalized kinetic traces for the recombination of CO to HbA and Hb–Hp subsequent to photodissociation on a 7 ns time scale of the corresponding starting fully CO saturated derivatives. The recombination occurring on the sub-microsecond to second time scale is assigned to geminate recombination; whereas, the slower recombination is assigned to the solvent (bimolecular) recombination. The solvent phase recombination traces are consistent with R like species [18]. Under these experimental conditions (CO saturated buffer, low temperature and incomplete photolysis), there is limited evolution of the photoproduct population from R to T for the tetramer which accounts for the absence of the very much slower T state solvent phase. Both traces exhibit solvent phase recombination traces that are R-like. The tetramer exhibits a slightly faster rate for that process which might be due to quaternary enhancement. The geminate yield for the Hb tetramer is larger (~.6 versus .4) than for the

Hb/Hb complex. This difference in the geminate yield is consistent with the difference seen between R state COHA and the unbound CO $\alpha\beta$ dimer [20]. There are however multiple R states for the HbA tetramer [61–63] that exhibit similar differences in the geminate yield [19,58]; however, the known properties of the Hb–Hp complex overwhelmingly favor the dimer as the basis for the lower geminate yield seen for the complex. At the concentrations being used in the present study, the population of unbound dimers present in the COHbA sample is minimal. Under these conditions, T state CO derivatives of HbA derived from either mutants or sol–gel entrapment protocols exhibit a kinetic trace with a geminate yield at or below 0.1 and a solvent phase that is at least ten times slower than those shown in Fig. 2 [36,52,54], thus ruling out the possibility that the Hb–Hp complex might have T state properties when fully liganded. Although the reduced geminate yield and the R-like solvent phase recombination for the Hb–Hp sample are consistent with an $\alpha\beta$ dimer, a similar pattern is seen for maleimide modified tetramers [18].

Absorption spectra

The oxygen binding measurements and the CO recombination studies indicate that the hemoglobin species associated with the Hb–Hp complex is consistent with an Hp bound stabilized $\alpha\beta$ dimer. If this assessment is indeed correct one would expect that this species would be reflected in the structure sensitive features of the heme absorption spectrum. Two features of the absorption spectrum of the “deoxy” heme of Hb are especially revealing. The so called Q band centered at 555 nm for the deoxy hemes from the equilibrium ligand free T state Hb population shifts to ~558 nm or higher for deoxy hemes associated with ligand free R state Hbs generated using mutagenic/chemical modifications, sol–gel trapping, and time resolved spectroscopy of photodissociated R state COHb’s. Similarly, the proximal strain sensitive near IR band (Band III) at ~755 nm for equilibrium deoxy T state hemes is known to shift to 760 nm and higher (765 nm for the nanosecond R state photoproduct of COHbA) for the corresponding R state deoxy hemes. Fig. 3 compares the Q band and Band III spectra of the deoxy derivatives of HbA and Hb–Hp under similar conditions. Both the Q band and Band III show the spectral shifts indicative of an R-like environment for the hemes in the Hb–Hp complex. The identical shifts were observed for deoxy samples generated using either sodium dithionite or L-cysteine [29] to generate the deoxy species from either nitrogen purged ferrous samples (starting as oxy) or aquomet derivatives, respectively. The peak positions for the fully deoxygenated derivative of $\beta\beta$ XL P2K HbA were 555 and 758 nm for the Q band and the near IR band, respectively. This cross linked Hb has enhanced oxygen affinity but still manifests cooperativity in its oxygen binding albeit at a reduced level compared to HbA [48] and very similar to other PEG-maleimide modified Hbs [32,48,49].

Nitrite reductase activity

A representative comparison between HbA and Hb–Hp of the initial rate of decay of the starting fully deoxy heme population subsequent to addition of nitrite (1:1.1 heme:nitrite) is shown in Fig. 4. It can be seen that the initial rate for the Hb–Hp complex is considerably faster than that for HbA. The kinetic traces are generated from the change in the absorbance at 430 nm which preferentially reports on the deoxy population. Under the conditions of this measurement (a slight excess dithionite is added to insure no build up of met Hb during the course of the reaction), the presented time course exclusively probes the decay of the deoxy population at the expense of a build up of the ferrous NO heme species. Parallel measurements in which the entire spectrum is scanned, confirms and unambiguously demonstrates that the deoxy population decays as the ferrous NO heme population builds up. In the absence of the added dithionite, there is also a progressive build up of met heme and ferrous NO heme as anticipated based on the nitrite reductase reaction in question and the many previously reported results. The rates from the single wavelength kinetic

measurements and from analysis of the overall spectral changes are all consistent in showing that under the same conditions the initial rate for the Hb–Hp complex is roughly ten times that of the HbA sample as displayed in Table 1 along with the corresponding P_{50} and $n_{1/2}$ values described above. The shown rates are the average for two measurements for each sample. A very similar order of magnitude difference for the rates is seen for similar samples (both at pH 7.4 and at pH 7.0) probed using the full Q band absorption as the basis for deriving the initial decay of the deoxy population (not shown). Table 1 also contains the initial rate for $\beta\beta$ XL P2K HbA which is comparable to that of the Hb–Hp complex. An enhancement of this magnitude is also reported for other maleimide modified Hbs both with [32,49] and without [27] attached PEG. We compared a large series of PEGylated Hbs to maleimide modified Hbs and find that modification of $\beta 93$ through the maleimide reagent induces comparable rate enhancements either with or without attachment of PEG (unpublished results).

The impact of L35 on kinetic traces associated with the nitrite reductase reaction

Fig. 5 shows the time dependence of the Soret band at 430 nm over the course of the nitrite reductase reaction at pH 7.4. In panel A it can be seen that in the absence of the allosteric effector, HbA exhibits the characteristic time dependent acceleration in rate attributable to the progressive build up of met heme and ferrous NO heme resulting in a shift in the allosteric equilibrium from T to R. In the presence of L35, a very potent allosteric effector, the allosteric transition is clearly no longer in evidence on the time scale of the reaction. The trace remains slow (T-like) over the full duration of the observed process. In contrast, as can be seen in panel B, L35 has no discernible effect on the fast kinetic trace from Hb–Hp. The fast kinetics are not only consistent with the hemes in the Hb–Hp complex being R like but also not subject to significant allosteric regulation as anticipated for the hemes within the allosterically inert dimer.

Discussion

The above results are all consistent with the hemes in the Hb–Hp complex having largely unaltered functional properties attributable to a $\alpha\beta$ dimer of HbA. Most significantly with respect to bioactivity related to the toxicity of acellular HbA, the complex exhibits an enhanced rate for the production of NO from nitrite via the nitrite reductase reaction of deoxy heme with nitrite. The approximately ten fold enhancement of the initial rate over HbA, is similar to the enhancements seen for high oxygen affinity PEGylated HBOCs that are not vasoactive (vasoconstriction, increased mean arterial blood pressure) when infused into animal models. We (unpublished results with Cabrales, UCSD) and others [32] see similar enhancements in the rate for both PEGylated and non-PEGylated high oxygen affinity Hbs that have been modified at the Cys beta 93 position with the introduction of a maleimide group on the beta 93 thiol. All of these modified Hbs exhibit a shift in stability towards the R quaternary state. Importantly, based on its functional similarity to the PEGylated Hbs with respect to nitrite reductase activity, the Hb–Hp complex generates heme populations that should have favorable vasoactivity properties without the need for chemical or mutagenic modifications. The increased production of NO from nitrite should act synergistically with the capacity of the complex to inhibit free radical damage from acellular Hb. Results obtained using IV infused nanoparticles that release low levels of NO over a sustained period within the circulation [64], show that at low levels, NO in the vasculature inhibits HBOC induced vasoconstriction [65], reduces inflammation [64] and limits the inflammatory cascade/macrophage activation that leads to hemorrhagic shock [66]. Together all of the results point to the Hb–Hp complex having a pivotal role in maintaining vascular homeostasis in the presence of the pro-inflammatory and

provasoconstrictive stimulus associated with the presence of acellular HbA in the circulation.

Recent published animal studies showed that the infusion of Hb and after complexation with Hp prevented oxidative injuries to kidneys which normally accompany the infusion in animals of free and chemically modified Hbs [12]. Additionally, and unexpectedly the normal hypertensive responses seen in response to the infusion of free Hb were completely eliminated in two animal models of exchange transfusion [59]. Biochemically, we and others have also shown unequivocally that complexation with Hp shields key amino acids on the Hb molecule from oxidative damage triggered by the presence of oxidants such as H_2O_2 [12,67]. In contrast to uncomplexed Hb, the ferryl species formed in the Hb–Hp complex solution persisted throughout the experiment. In follow up EPR, photometric and spectroelectrochemical studies carried out by our group we showed that Hp stabilizes ferryl and its radical and kinetically rendering these damaging species inactive (Cooper et al.). Haptoglobin binding stabilizes hemoglobin ferryl iron and the globin radical on tyrosine β -145, *Antioxidant and Redox Signaling* (under revision). The redox potential ($E_{1/2}$) as measured by spectroelectrochemistry showed that the Hb–Hp complex was +54 mV, showing it to be much more easily oxidized than uncomplexed Hb ($E_{1/2} = +125$ mV). The ~70 mV shift of the reduction potential for Hb–Hp system resulting in a less redox active molecule (Banerjee et al., Haptoglobin alters oxygenation and oxidation of hemoglobin and decreases propagation of peroxide-induced oxidative reactions *Radical Biology and Medicine* (under revision)).

Under normal circumstances, the endothelial cell layer, RBC-free plasma zone, unstirred plasma layer around RBC, and RBC membrane provide diffusion barriers, which impair rapid, unhindered interactions between RBC Hb and endothelial NO [68]. Upon hemolysis or infusion of cell-free Hb, these diffusion barriers are no longer effective in preventing free Hb from reaching and reacting with endothelial NO. The vasoactivity of cell-free Hb-based blood substitutes is attributed to NO scavenging by free Hb which triggers vasoconstriction and subsequent elevation of blood pressure. There are a number of biochemical scenarios in which the Hb–Hp complex is envisioned to provide a protection against vascular toxicity and blood pressure controlling mechanisms. First, the molecular size of the Hb–Hp complex (~180–200 Å) unlike smaller Hb dimers (~15 Å) [68] can provide a physical separation away from RBC free zone thus reducing the interaction of NO with the complex. Additionally, our current data clearly show that following Hp binding, the heme active sites of the Hb subunits have high reactivity with O_2 and nitrite. The increase in nitrite reductase activity for the Hb–Hp complex is likely due to both an enhanced access of nitrite to the heme as seen with the other ligands (CO and O_2) and a decrease in redox potential stemming from the R-like properties of the dimer. If there is adequate nitrite present, the Hb–Hp complex should be effective at regenerating NO under conditions where NO is being scavenged. Studies with NO releasing nanoparticles have shown that even minor sustained production of NO can counter inflammation in the circulation. It would appear that functional properties of the Hb–Hp complex all favor minimizing the inflammatory cascade that can be triggered by the introduction of acellular Hb.

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Fig. 1.

Representative plots showing O₂ binding equilibrium curves (OECs) for the 60 μM (in heme) solutions of the Hb–Hp complex (---) and for uncomplexed Hb (—) measured 10 mM potassium phosphate/0.1 M NaCl, pH 7.4 at 37 °C. The average P_{50} and n_{50} values derived from three separate experiments are reported in Table 1. Insert for this figure shows the respective Hill plots for each curve.

Fig. 2. CO recombination traces displayed on a log normalized absorbance (NA) versus log time plot for COHbA (trace B) and CO Hb–Hp (trace A). The heme concentration for both samples was on the order of 0.45 mM in CO saturated 50 mM BisTris buffer at pH 7. The kinetics were generated from samples maintained at 3.5 °C. The kinetic phase occurring on the sub-microsecond time scale is geminate recombination whereas the slower recombination is attributed to solvent phase (AKA bimolecular) recombination.

Fig. 3. Comparison of the visible (Q band) and near IR (Band III) absorption spectra of deoxy hemes from HbA and Hb-Hp. The deoxy derivatives were prepared by purging the oxy derivatives with Ar and then adding dithionite; (>2:1 heme (0.24 mM) in 0.05 M Bis-Tris, pH 7.0). Very similar spectra were obtained by using L-cysteine to reduce aquomet derivatives.

Fig. 4.

Representative time course for the decay of the deoxy heme populations (as monitored by the absorbance at 430 nm) of HbA and Hb-Hp subsequent to the addition of a slight excess of nitrite to the corresponding deoxy heme populations (1.1:1). The heme concentration for both samples was 0.24 mM in BisTris pH 7.0 buffer heme concentration. The initial rates (Table 1) were calculated based on the best linear fit to the presented data.

Fig. 5.

Time course for nitrite reductase reaction of: A. ■ deoxy HbA + 3:1 nitrite:heme; ○ deoxy HbA + 3:1 nitrite:heme + L35; and B. ■ Hb-Hp 3:1 nitrite:heme; ○ Hb-Hp 3:1 nitrite:heme + L35; all in 0.05 M Bis-Tris, pH 7.4 with signal being monitored at 430 nm.

Table 1

Comparison of initial nitrite reductase rates for HbA and Hb-HP. Conditions: 0.2 mM heme, 1:1.1 heme:nitrite; 0.05 M Bis-Tris, pH 7.0 + dithionite (>1:1 heme). Rates derived from changes in the 430 nm deoxy band monitored as a function of time. The rates for HbA and Hb-HP are averages of two independent runs. The conditions for the oxygen affinity and cooperativity measurements are given in the text. The P_{50} and n_{50} (Hill coefficient) values for the crosslinked Hb are taken from an earlier work [48].

Sample	Rate ($\mu\text{M s}^{-1}$)	P_{50} (mm Hg)	n_{50}
Hb-HP	0.111 ± 0.001	4.79 ± 0.21	1.06 ± 0.081
Hb	0.022 ± 0.01	13.63 ± 0.11	2.38 ± 0.015
XL P2K Hb	0.087	6.5	2.1