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Forum Review

Critical Redox and Allosteric Aspects of Nitric Oxide Interactions with Hemoglobin

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ABSTRACT

Nitric oxide (NO) is an important signaling molecule. Relatively long-lived NO adducts at the heme and SH groups of hemoglobin (Hb) could enable NO to carry out long-range signaling functions. In spite of significant advances, there remain as yet unresolved issues regarding the possible role of Hb in moderating NO-signaling events that affect blood pressure regulation. In this review, we summarize recent reports concerning the redox and allosteric aspects of NO/Hb interactions that have advanced our understanding of the physiological significance of NO binding to heme groups (forming NO-Hb) and of reactions promoting formation of *S*-nitrosated Hb (SNO-Hb). Allosteric mechanisms modify the bioactivity of NO/Hb complexes by altering the lifetime of NO-Hb and the properties of SNO-Hb. Redox reactions are significant because of the complex chemistry possible for NO and its oxidation products. Reactions at ferrous and ferric heme sites have differing consequences and affinities for interactions with NO. Moreover, redox changes at heme groups affect reactivity of SH groups and vice versa. In spite of low levels of NO-Hb and SNO-Hb found *in vivo*, recent findings do not rule out participation of NO-Hb or SNO-Hb in NO-dependent signaling reactions. *Antioxid. Redox Signal.* 6, 979–991.

INTRODUCTION

It was discovered relatively recently that nitric oxide (NO) is the elusive endothelium-derived relaxing factor (EDRF) that binds to the heme protein guanylate cyclase and stimulates the dilation of blood vessels (24, 40, 41, 60). This was a remarkable finding, because NO is a short-lived species whose free radical nature seemed inconsistent with its having a biological role. However, following the identification of NO as EDRF, the number of biological functions ascribed to NO has increased dramatically, including NO-dependent reactions that control smooth muscle relaxation, platelet inhibition, neurotransmission, and immune regulation (56, 72, 80). Today this reactive diatomic gas is recognized as one of the vital signaling molecules of living organisms.

The discovery of NO as EDRF poses some immediate questions with regard to NO-dependent blood pressure regulation, particularly in light of the fact that the heme protein hemoglobin (Hb) is present at high concentrations (~2.5 m*M*) in circulating red blood cells and has a higher affinity for NO than for other heme ligands. Key questions are: Is some of the NO generated by endothelial cells degraded, trapped, or carried to remote sites by interaction with Hb? If any of these events happen to a significant extent, how is NO-dependent normal blood pressure maintained in healthy individuals? These questions are fundamental to understanding NO-dependent regulation of blood pressure and in seeking means of dealing with adverse conditions of high and low blood pressure. In spite of significant advances in the field of NO and Hb biochemistry, these questions have only partially been answered.

Controversial aspects of the physiological consequences of interactions of NO and Hb have arisen, at least in part, because of technical difficulties associated with accurate detec-

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tion of the low levels of NO and NO derivatives found *in vivo*. Moreover, the earlier literature on NO and its interactions with transition metals testifies to the complex chemistry possible for NO and the multiple oxidation states available to nitrogen in NO_x (NO, NO_2, NO_2^-, etc.). This chemistry encompasses a wide range of possible oxidation or reduction products made possible by interaction of NO with transition metals and other redox-active materials. In addition to redox shifts, it is now clear that the interactions of NO with heme proteins like Hb are subject to allosteric control by metabolic intermediates. These redox and allosteric effects on NO/Hb interactions can have far-reaching consequences with respect to molecular signaling events. Because this field of study has been rapidly changing, we have attempted to summarize in this article the recent findings that pertain to the redox reactions and allosteric control mechanisms operative in interactions of NO with the heme and SH groups of Hb, and the possible physiological significance of these interactions.

Investigations of the reactions of NO with Hb have had a long history, beginning with studies of the similarities and differences of NO, carbon monoxide, and O_2 binding to heme proteins. These studies led to the conclusion that the heme groups of deoxy Hb bind NO very strongly, almost irreversibly, with a K_d near 4 p*M*. The second-order combination rate constant (k_{on}) of ~10^7 M^{-1} s^{-1} for NO is similar to that for oxygen combination, and is essentially diffusion-controlled (*i.e.*, for both ligands, the rate is controlled primarily by the heme-pocket barriers that slow diffusion to the heme binding site). The much higher affinity of NO relative to oxygen is due to differences in the rate of ligand dissociation (k_{off}), which is of the order of ~30 s^{-1} for oxygen and ~0.0001 s^{-1} for the fully liganded tetramer (with significant differences under varied conditions of temperature and effectors). The rate of NO dissociation from $Hb(NO)_4$ increases as the reaction progresses, indicating that partially NO-saturated, T-state, Hb has a lower NO affinity than fully liganded, R-state, Hb (58). On the basis of the kinetic data, it was predicted that a Hill coefficient of 1.6 would be observed if NO binding equilibria could be measured (58). Due to the technical difficulty of measuring equilibria with such a high-affinity ligand, with vanishingly small levels of free NO present when the heme sites are not fully saturated, these early authors correctly concluded that such curves "would be immune from experimental verification for some time."

In our review of recent findings, we will first address redox and allosteric control of interactions of NO with the heme groups of Hb where oxygen reversibly binds. Reactions between NO and the heme of normal human Hb occur at very high rates (3, 15, 27, 58, 70). These rapid reactions can scavenge NO, increase blood pressure, and pose considerable problems for development of cell-free Hb-based blood substitutes. Well documented cases of hypertension produced by introduction of cell-free Hb have been traced to its interactions with NO (51). Adverse blood pressure increases occur when the free NO concentration is decreased by formation of nitrosylated Hb (NO-Hb) (which occurs when NO interacts with deoxy Hb) and by formation of oxidized (met) Hb and nitrate (which occurs when NO interacts with oxy Hb). Due to these NO-scavenging reactions, cell-free Hb may cause perturbations of NO-signaling reactions such as NO-mediated platelet functions, macrophage behavior, cell and bacterial proliferation, endothelial adhesion, and smooth muscle relaxation (56).

An unresolved and somewhat controversial aspect of the interactions of NO with Hb heme groups is how conditions *in vivo* either avoid, or deal with, NO scavenging by Hb in red blood cells. Although this topic has been addressed in detail (47, 50, 79), it has yet to be resolved. The low degree of NO scavenging by red blood cells appears due to diffusion-related factors that effectively distance intracellular Hb from the epithelial walls where NO is generated. It has also been suggested that the red blood cell membrane is somewhat protective, and that NO autooxidation reactions (forming N_2O_3) will produce both nitrite and *S*-nitrosothiols (RSNO) in lipid membrane compartments where the relative hydrophobicity of NO would increase its local concentration (22). Primary *S*-nitrosation reactions within the red cell membrane are consistent with recent data showing that exposure of red cells to NO gas leads to *S*-nitrosation of red cell membrane proteins with minimal *S*-nitrosation of intracellular hemoglobin (62). Accordingly, efforts are being made to make blood substitutes based on cell-free Hb behave similarly to Hb in red blood cells (77). Cross-linking of cell-free Hb decreases its vasoactivity, possibly because cross-linking stabilizes Hb as tetramers or as supratetramer assemblies and decreases extravasation of Hb into intracellular spaces. Encouraging reports suggest that cell-free Hb that has been extensively modified by polyethylene glycol attachment does not show vasoactivity *in vivo* (78), and thus may overcome the hypertension-generating problems that have plagued other Hb-based blood substitutes.

Another critical aspect of how NO and Hb interact *in vivo* concerns the possible role of allosteric interactions in determining the fate of NO in blood. The allosteric control mechanisms that underlie the R- and T-state model of Hb function (57, 65) also play a significant role in interactions between NO and the heme groups of Hb (74), although not in the essentially diffusion-controlled initial stages of NO interactions with oxygenated or deoxygenated heme sites. Even these rapid reactions can be modified by site-directed mutagenesis in the heme pocket region, which can significantly reduce the rate of NO interactions with cell-free Hb (19). We review in the following the evidence for both homotropic (heme–heme) and heterotropic (anion–heme) allosteric control of interactions of NO and Hb that play a significant role in establishing the lifetime of NO-Hb, the fate of NO released from NO-Hb, and the migration of NO among α and β chains of the Hb tetramer.

The reactions of NO with heme-containing proteins like Hb were the subject of extensive study even before NO was recognized as a biologically significant molecule. NO is a distinctive, high affinity ligand of ferrous heme, with appreciable affinity for ferric and ferryl heme as well (3, 35). NO not only reacts with ferrous Hb with rate constants near the diffusion limit, it also exhibits the slowest dissociation rate of any heme ligand thus far characterized. Almost four decades ago, these features prompted the use of NO to block either the α or β hemes of Hb in order to obtain stable half-liganded intermediates in the study of the mechanism of ligand cooperativity (5). It soon became apparent, however, that among the various heme ligands, NO has unique features in addition to

its ability to form hexacoordinate complexes with ferrous, ferric, and ferryl heme. In the presence of allosteric cofactors that stabilize the low-affinity T-state of Hb, such as inositol hexaphosphate and, to a lesser degree, 2,3-diphosphoglycerate, the reaction between NO and the heme groups of deoxy Hb can lead to formation of pentacoordinate nitrosyl Hb, a form lacking the bond between the proximal His and the Fe(II) atom. The extent of this process, which occurs to a significant extent only with α-chain heme groups, is sensitive to environmental conditions (pH, temperature, anions, etc.) and can be followed by its distinctive spectroscopic features (20, 33, 37, 38, 74, 75, 84). Interestingly, the pivotal reaction between NO and the heme group of guanylate cyclase also produces pentacoordinate heme in a process that initiates the conformational changes required for enzyme activation and muscle relaxation (39). The affinity of NO for heme is higher in the pentacoordinate than in the hexacoordinate state (45), although, remarkably, the conversion to pentacoordinate heme geometry is promoted by conditions that lower the affinity of Hb for oxygen.

The second topic we address in this review concerns *S*-nitrosated Hb (SNO-Hb) that is formed by nitrosation reactions involving the SH groups of Hb at β93Cys. SNO-Hb is not formed by the direct interactions of NO with Hb, because our studies have shown that NO is not itself a nitrosating agent (*i.e.*, deoxy Hb exposed to excess NO to form $Hb(NO)_4$ does not form SNO-Hb even upon exposure to air if excess NO is removed by nitrogen flushing prior to air exposure). However, biological conditions can lead to nitrosating reactions that do result in SNO-Hb formation. We review data in this report on the allosteric properties of SNO-Hb and the allosteric control mechanisms found to be operative in transnitrosation reactions (transfer of NO^+ groups among RSNO compounds) and in nitrosative reactions (transfer of NO^+ groups from NO_x species to SH groups) that can lead to formation of SNO-Hb.

SNO-Hb is a modified form of Hb that has been postulated to be involved in a dynamic cycle of intravascular NO uptake, transport, and delivery (42, 54, 73). The potential role of SNO-Hb as an NO transporter is very attractive, a possible role referred to as the SNO-Hb hypothesis. As pointed out in the seminal study by Stamler and co-workers (42), the β93Cys residues at which NO can be bound are highly conserved in mammalian Hbs, suggestive of an important physiological role. Moreover, the environment of β93Cys is sensitive to the R ↔ T conformational equilibrium of Hb (6, 25, 44), and an internal electron-transfer pathway makes the SH-group environment sensitive to events at the heme and vice versa (12). Stamler and co-workers, who advanced the SNO-Hb hypothesis, suggested that the conformational transition from the R- to T-state could promote the allosteric delivery of both oxygen and NO to regions with low oxygen tension.

The two central observations put forward in support of the SNO-Hb hypothesis are (a) higher SNO-Hb levels in arterial than in venous blood, and (b) evidence that delivery of oxygen and delivery of NO are allosterically coupled events (30, 42, 73). As detailed in our review of Recent Results, both of these key observations have been challenged (11, 20, 28, 29, 61, 83), and recent studies have shown that SNO-Hb is relatively unstable in the reductive environment inside red blood cells and thus may never attain levels appropriate for an effective "reservoir" of bioactive NO (29). These findings argue against the SNO-Hb hypothesis in its original form. However, it can be argued that artificially increased levels of SNO-Hb in red blood cells do not adequately represent the *in vivo* situation. Future studies may show that SNO-Hb plays a role like that seen in some *in vitro* studies, where NO or RSNO species derived from cell-free preparations of SNO-Hb (42, 53, 73) or by SNO-Hb in red blood cells (62) were shown to promote vascular relaxation of aortic rings and at least partially offset the vasoconstrictive activities associated with NO scavenging by cell-free preparations of normal Hb.

In light of the complex chemistries possible for NO and NO derivatives in biological systems, and the evidence in support of formation and reactivity of SNO-Hb under physiological conditions, we suggest that it is premature to conclude that SNO-Hb does not play a significant role in NO-dependent blood pressure regulation.

RECENT RESULTS

Advances in knowledge of the properties of NO-Hb and SNO-Hb have greatly clarified the often controversial role of allostery in NO binding to Hb and in oxygen binding to partially nitrosylated Hb or partially *S*-nitrosated Hb. We review in this section recent results pertaining to the stability and lifetime of both NO-Hb and SNO-Hb, and how these are controlled by redox changes and by heterotropic and homotropic allosteric interactions. The stability of these NO-derived entities is relevant to their fates *in vivo* and *in vitro* and governs the extent to which formation of NO-Hb or SNO-Hb conserves the bioactivity of NO.

Allosteric control of NO binding to Hb

In our recently published study on the fate of varied levels of NO introduced into samples of deoxy Hb, we showed that at equilibrium all NO introduced could be accounted for by the sum of hexacoordinate and pentacoordinate forms of NO-Hb. We showed that pentacoordinate NO-Hb has spectral features that resemble met Hb, with the consequence that at low Hb:NO ratios, where the pentacoordinate form is favored, it may appear that met Hb has formed when in fact, pentacoordinate NO-Hb has formed (20). Although NO-Hb can spontaneously degrade into authentic met Hb, we find that this process is very slow for purified Hb in the presence of metal chelators and the total absence of oxygen, with no detectable degradation for several days at neutral pH at 20°C. In accord with results of earlier studies (75, 84), we found that anions and low pH that favor the T-state preferentially promote formation of pentacoordinate heme geometry at low NO:Hb ratios.

The stoichiometric formation of NO-Hb we observed is consistent with earlier studies that showed NO to be a very high-affinity heme ligand, but differs from earlier data interpreted by Gow and Stamler (30) as indicating that low NO:Hb ratios promote met Hb formation under anaerobic conditions and an oxygen-dependent equilibrium between NO bound to heme in the T-structure and to the β-chain thiol in the R-structure. As evidence for this, among other findings, they showed changes in the level of the NO-Hb spectrum, seem-

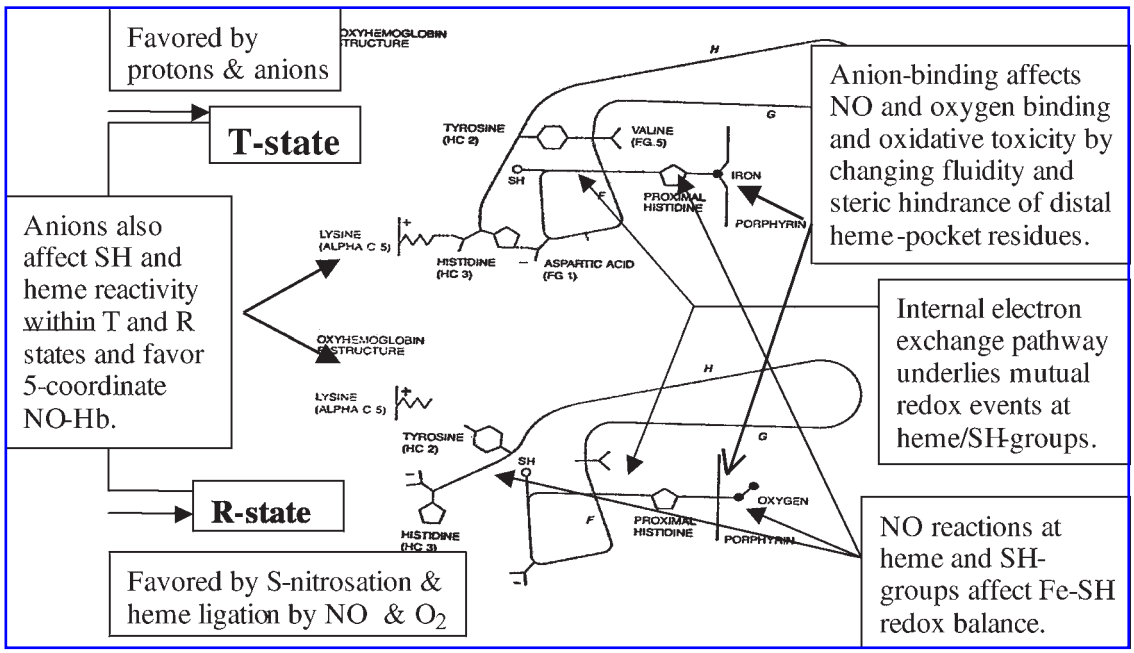
ingly coupled with changes in met Hb and SNO-Hb levels (30, 31). We provided an alternative explanation, based on parallel electron paramagnetic resonance (EPR) and visible spectroscopic studies, in which changes of the NO-Hb spectrum at low NO:Hb ratios under a number of buffer conditions can be largely understood in light of reversible shifts from hexacoordinate to pentacoordinate NO-Hb (20). It should be noted that experimental conditions in the later study (20) were not identical in all respects to those used previously (30, 31). As described in subsequent sections of this report, oxygen-mediated reactions with NO can, under some conditions, lead to SNO-Hb formation.

We showed that there are visible spectral changes that correlate with EPR changes that occur as NO levels are increased in solutions containing deoxy Hb. The visible spectral changes are similar to those that occur upon the two-step process of NO-driven reduction of met Hb (1), but actually reflect the transition of pentacoordinate NO-Hb to hexacoordinate NO-Hb as the R-state is attained (20). These visible spectral changes (observed above 50% heme saturation under equilibrium conditions) are a manifestation of the transition of NO-Hb from partially pentacoordinate to almost fully hexacoordinate heme geometry and represent a new addition to the NO-Hb literature. Our observations provide direct evidence that homotropic (heme–heme) allosteric interactions are operative in NO binding. NO ligation levels that trigger the transition to the R-state diminish the levels of pentacoordinate NO-Hb. Similar decreases in the fraction of pentacoordinate NO-Hb in a partially nitrosylated Hb sample can be brought about by oxygen addition, as more fully described elsewhere (20).

In our equilibrium studies with purified Hb, all NO present can be fully accounted for, without conversion to nitrate, nitrite, SNO-Hb, or other reactions, *even after oxygen addition*. The distribution of NO among pentacoordinate and hexacoordinate states changes very quickly (minutes) after oxygen addition. Only after long-term (hours) exposure to oxygen did the total NO-heme (sum of pentacoordinate and hexacoordinate states) decrease under our experimental conditions. The slow decrease of total NO-heme was coupled to increases in levels of met Hb and SNO-Hb (20). As the increases in met Hb and SNO-Hb were not synchronous with exposure of samples to oxygen, we concluded that low NO:Hb ratios do not *necessarily* lead to formation of met Hb or SNO-Hb in either the absence or presence of oxygen. The formation of appreciable amounts of SNO-Hb at low NO:Hb ratios as previously reported (30) are thus associated with other, nonobligatory, nitrosative and oxidative pathways. It is well documented that NO that is released into an oxygenated environment can form NO_x species that can drive nitrosation reactions (7, 22).

Although not a factor in the articles by Gow and co-workers cited above, artifactual generation of SNO-Hb, not linked to changes in protein quaternary structure, has been recently demonstrated to occur under experimental conditions where NO-saturated solutions (~2 m*M*) are added (as a "bolus") to oxy Hb solutions (32, 36, 43). Under these conditions, reactions take place at the interface between the NO and the Hb solution, due to the high local concentration of reactants before the system has reached homogeneity. These processes include the rapid formation of met Hb, its reaction with NO that produces the nitrosating species NO^+, as well as the reaction between NO and O_2, with production of nitrosating NO_2 and N_2O_3. A detailed review on the subject has been recently published (34).

The results described above support and extend previous studies that showed the existence of allosteric regulation of NO/Hb interactions similar to those that occur in binding of lower affinity ligands. Scheme 1 provides a representation of the



SCHEME 1. A conceptual model of allosteric control points and heme-SH links that control NO binding to Hb and thereby affect NO-dependent signaling events. Modified from ref. (63).

interplay between heme and SH groups in homotropic and allosteric controls of Hb function. This conceptual model incorporates the well-known equilibrium between T and R quaternary conformational states of Hb and shows how anions and SH modifiers affect this equilibrium. It also incorporates recent findings that heme ligation binding is regulated not only by the T↔R equilibrium, but also by anion-dependent conformational fluctuations *within* states (10). The SH and heme positions illustrated are based on published crystal structures (63).

In spite of many publications documenting changes of NO-Hb in response to pH, anions, temperature, and degree of heme ligation, the literature contains contradictory statements asserting the absence of allostery in interactions of NO with Hb. Erroneous statements to the effect that NO-Hb is locked in either high (R) or low (T) affinity conformations, or lacks cooperativity or anionic regulation, disregard publications showing clear differences in the interactions of NO with varied conformational states of Hb. Importantly, the allosteric controls represented in Scheme 1 make the reactions between Hb and NO at low NO:Hb ratios, where the T-state predominates, different from those at higher ratios where the R-state predominates.

Allosteric control of NO-Hb stability

The range and duration of signaling capabilities of NO, limited by its high reactivity and short lifetime, can be greatly extended by adduct formation at the heme groups of the Hb tetramer. In the absence of organic phosphate effectors, NO-Hb is remarkably stable, even in the presence of oxygen. A sample of fully nitrosylated Hb (NO-Hb) under a full atmosphere of oxygen becomes oxidized to met Hb very slowly (over many hours) at neutral pH in Tris buffer at 20°C. This process is under allosteric control, with the result that dramatic increases in the rate of oxidation occur in the presence of organic phosphate cofactors, which decrease the half-life of NO-Hb to minutes. The oxidative degradation of NO-Hb is also highly chain-selective, which gives rise to a time dependence that can lead to apparently contradictory results. The rate observed when oxygen is added to a sample immediately after generation of partially nitrosylated Hb is different from that observed if one waits until NO distribution among subunits reaches equilibrium.

Chain-specific aspects of NO binding to the heme groups of Hb are under allosteric control. As shown by earlier EPR studies (75) and more recently by our combination of EPR and visible spectroscopic investigations (20), there is a slow heme concentration- and cofactor-dependent migration of NO to the α chain hemes that is favored by conditions that promote formation of pentacoordinate heme geometry. In general, the longer the equilibration of a partially nitrosylated sample under deoxy conditions, the less NO-Hb will be lost upon exposure to air. The bioactivity of NO is not totally lost from the system, however, because subsequent reductive processes could potentially result in the regeneration of NO and/or formation of NO-Hb from the oxidation products.

Allosteric control of oxygen binding by SNO-Hb

SNO-Hb has been reported to promote vasorelaxation under hypoxic conditions (53, 73). These reports illustrate a key feature of the SNO-Hb hypothesis, that release of NO (as NO gas or as an RSNO species) and release of oxygen from SNO-Hb are allosterically coupled events (42, 73). Although complex *in vivo* conditions may differ, studies to date with purified Hb have not substantiated this hypothesis. There is no appreciable release of NO from SNO-Hb upon deoxygenation of purified Hb solutions. When oxygenation/deoxygenation cycles with purified SNO-Hb at concentrations ranging from 0.06 m*M* to 10 m*M* are carried out in the absence of other reductants, the *S*-nitrosyl levels are maintained even in the presence of equimolar glutathione (10) or Band 3 protein (14). The reactions that occur at much higher glutathione:Hb ratios are discussed in the section of this report on allosteric control of SNO-Hb formation and degradation.

Although Hb's normal deoxy conformation cannot be assumed with NO on β93Cys, the protein has sufficient conformational flexibility to accommodate the S-NO linkage when oxygen is fully removed. The Hill plots of oxygen binding by SNO-Hb (11) reflect the R-state stabilization associated with *S*-nitrosation, with shifts toward higher affinity most evident in initial stages of oxygen binding. These features of altered oxygen binding by SNO-Hb are similar to those reported for other SH-modified Hb forms. The cooperativity of oxygen binding is typically reduced by SH modification (ranging from small reductions to complete elimination of cooperativity with some SH reagents). The lower asymptotes of oxygen-binding curves are left-shifted toward higher oxygen affinity, suggesting the presence of destabilized T-states. The SH modifications of Hb have little effect on the protein's responses to allosteric effectors. Significantly, the effect of the physiologically important effector, 2,3-diphosphoglycerate, is not diminished by *S*-nitrosation of Hb. When this allosteric response is overlooked, one can reach incorrect conclusions about the relative affinity of normal and *S*-nitrosated Hb for heme ligands, *i.e.*, that SNO-Hb does not exhibit an exceptionally high affinity under physiological conditions. This is important to note in light of arguments to the effect that the elevated affinity of SNO-Hb for heme ligands helps conserve NO bioactivity under hypoxic conditions (53).

SNO-Hb does not constitute a significant fraction of Hb present *in vivo,* so that the oxygen binding curves for partially *S*-nitrosated Hb are useful in considerations of possible NO binding and release, rather than in consideration of oxygen transport by red blood cells. To address more fully the *in vivo* situation, the effects of *S*-nitrosation on oxygen binding by purified adult human Hb (Hb A_0) were measured at concentrations approximating those found within red blood cells. Modification of previously published procedures was required to generate *S*-nitrosated Hbs without excessive met Hb formation. Concentrated samples of Hb A_0 and sickle cell Hb (Hb S) with varied levels of *S*-nitrosation and low met Hb content (<5% oxidized heme) were obtained by carrying out all preparative procedures at 4°C, minimizing light exposure, and using metal chelators to avoid metal-catalyzed decomposition of the S-NO linkage. An important issue settled by these studies was that high Hb concentration is not sufficient to cause loss of NO from the SNO-Hb derivative during deoxygenation. Spectral deconvolution assays before and after dithionite addition (as described in reference 11) on sample aliquots before and after a deoxy/oxy cycle showed no significant loss of NO from SNO-Hb in 10 m*M* heme solutions (levels of ±5% for the level

of *S*-nitrosation before and after oxygen binding determinations were found in six separate assays, and this variance is within experimental error). This finding agrees with reports of work using intact red blood cells and purified Hb showing that NO once bound to Hb heme groups or thiols remains stable even following repeated cycles of oxygenation/deoxygenation (83), but contrasts with reports of SNO-Hb instability within red blood cells (29) and reports of oxygen-dependent intramolecular NO transfer between heme and thiols (30, 31, 53). We consider that the differences between these results may well lie in differences in the extent to which low-molecular-weight thiols, reductants, and "sinks" for NO were present in the experimental conditions used in these studies.

The shift in Hb's oxygen affinity associated with *S*-nitrosation is somewhat greater for samples at 10 m*M* heme (13) than observed at lower (0.1 m*M* heme) concentrations (11, 61). This result shows that, at concentrations like those in red blood cells, the modification of SH groups by NO interferes not only with assumption of the normal T-state, but also affects tetramer–tetramer interactions. This finding extends findings of earlier workers who have shown that tetramer–tetramer interactions at high (intracellular) Hb concentrations can shift the P_{50} for oxygen binding to both Hb A and Hb S to higher oxygen levels than required for half saturation at lower protein concentrations. Interestingly, above the minimum gelling concentration for Hb S, the concentration-dependent shifts for Hb S are much greater than those observed for Hb A (9). Our results confirmed these earlier studies, and showed that *S*-nitrosation has an enhanced effect on Hb S at Hb concentrations like those in erythrocytes. This R-state stabilization of Hb S was shown to have a significant inhibitory impact on the aggregation of Hb S that triggers sickle cell disease (13). Other SH modifications of Hb S have been reported to have similar antisickling effects (26), presumably also as a result of stabilization of the R-state conformation that has much lower propensity for Hb S aggregation.

Allosteric control of SNO-Hb formation and degradation

Both oxygen-mediated and transnitrosative pathways for SNO-Hb formation are sensitive to the availability of Hb's SH groups, which are known to be under allosteric control due to steric shifts associated with the quaternary T↔ R equilibrium of Hb. Functional and crystallographic studies have shown that the β93Cys residues at which NO is bound in SNO-Hb are more accessible in the high-affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb (3, 64). Many studies have confirmed the fact that there is a greater ease of formation of SNO-Hb in oxygenated Hb relative to deoxygenated Hb. The primary area of debate seems to be on the extent to which the destabilized T-state of SNO-Hb enhances NO release or transnitrosation reactions that have a bearing on blood pressure regulation. As noted in the following sections, the levels of reductants and low-molecular-weight thiols relative to SNO-Hb in a given setting are primary determinants of the stability of SNO-Hb. Consequently, many apparently contradictory findings may be reconciled if these determinants and their effects are specified.

Relevant reactions of NO with the SH groups of Hb are summarized in the equations below. In addition to transnitrosation reactions (Eq. 1) and copper-catalyzed RSNO decomposition (Eq. 2), RSNOs can *S*-thiolate sulfhydryl groups (Eq. 3), resulting in formation of a mixed disulfide of Hb and the NO donor [e.g., *S*-nitrosylated cysteine (CysNO) and Hb can form cystinyl-Hb]. SNO-Hb itself can serve as the RSNO (Eq. 3) and react with other thiols, creating Hb-S-SR with release of RS-NO or NO^-. NO^- could react with $HbFe^{III}$ to form $HbFe^{II}NO$. Recent studies have shown that NO has a highly negative reduction potential, whereby the reduction to NO^- is not favored under physiological conditions, and that, when formed, nitroxyl exists almost exclusively in its protonated form, HNO ($pK_a = 11.6 \pm 3.4$) (8).

RSNOs can function as NO^+ donors in the presence of an electron acceptor, where the reaction would be RS-NO + Hb-SH → Hb-S-SR + NO^+ + H^+ + $2e^-$ (Eq. 4). Additionally, Spencer and co-workers (71) reported a two-step process whereby *S*-nitrosoglutathione (GSNO) reacts first with deoxy Hb to produce met Hb, reduced glutathione, and NO free radical (Eq. 5), with the $NO^\bullet$ subsequently binding to deoxy Hb or reacting with oxy Hb. Finally, as shown in Eq. 6, apart from these NO-transfer reactions, $NO^\bullet$ can interact with oxygen to form effective nitrosating compounds, generally denoted as NO_x (22).

$$\text{RS-NO} + \text{Hb-SH} \rightarrow \text{Hb-S-NO} + \text{RSH} \quad (1)$$

$$2\text{RS-NO} \xrightarrow{Cu^{II}} \text{RSSR} + 2NO^\bullet \quad (2)$$

$$\text{RS-NO} + \text{Hb-SH} \rightarrow \text{Hb-S-SR} + NO^- \text{ (or HNO)} \quad (3)$$

$$\rightarrow NO^- \text{ (or HNO)} + HbFe^{III} \rightarrow HbFe^{II}NO$$

$$\text{RS-NO} + \text{Hb-SH} \rightarrow \text{Hb-S-SR} + NO^+ + H^+ + 2e^- \quad (4)$$

$$\text{RS-NO} + HbFe^{II} + H^+ \rightarrow HbFe^{III} + \text{RSH} + NO^\bullet \quad (5)$$

$$\rightarrow NO^\bullet + HbFe^{II} \rightarrow HbFe^{II}NO$$

$$\rightarrow NO^\bullet + HbFe^{II}O_2 \rightarrow HbFe^{III} + NO_3^-$$

$$NO_x + \text{Hb-SH} \rightarrow \text{Hb-S-NO} \text{ (}e.g.\text{, Hb-SH} + N_2O_3 \rightarrow \text{Hb-SNO} + HNO_2\text{)} \quad (6)$$

The relative significance of oxygen-mediated reactions (via NO_x compounds as in Eq. 6) and transnitrosative mechanisms (via low-molecular-weight RSNO donors as in Eq. 1) for generation of SNO-Hb *in vivo* is still unclear. Transnitrosative mechanisms that lead to SNO-Hb formation (Eq. 1) are favored by removal of metal chelators, and without the cystinylation reactions enhanced by metal contaminants (13). A recent report that metal chelators can partially inhibit *S*-nitrosation of Hb (68) is indicative of this still changing view of SNO-Hb biochemistry. It had previously been shown that metal ions can catalyze the decomposition of *S*-nitrosated compounds (52). It is becoming increasingly apparent that it is hard to predict the outcome of metal-catalyzed processes in the complex biological milieu based on simplified *in vitro* model studies.

Once formed, SNO-Hb has proven to be a remarkably stable RSNO compound in controlled *in vitro* situations. In the absence of light (which can photodissociate NO from SNO-Hb) and reductants (including redox-active metals), SNO-Hb levels in a purified Hb sample decrease only slightly over 24 h at 4°C. The sensitivity of SNO-Hb to metals and light can clearly lead to anomalous results. Metal contaminants can have several effects on the reaction of Hb and CysNO. At one level, the metal-catalyzed disruption of the RSNO linkage of

CysNO liberates NO· (Eq. 2), which can be captured at deoxy heme sites or cause formation of met Hb and nitrate upon reaction with oxy Hb.

The sensitivity of SNO-Hb to reductants merits attention, and use of varied levels and types of reductants in studies with SNO-Hb has no doubt contributed considerably to present controversies in the field. Studies with simple RSNO compounds such as CysNO have shown that RSNO stability is decreased in the presence of reductants such as NADPH. For example, the half-life of CysNO (at 37°C in the absence of light and metals) is decreased by a factor of two by addition of NADPH. This sensitivity to reductants makes it unlikely that high levels of SNO-Hb can accumulate in the reductive environment of red blood cells. SNO-Hb levels, artificially elevelated in red blood cells, were reported to fall rapidly (29). However, as pointed out elsewhere (53), an involvement of SNO-Hb in blood pressure regulation may not require SNO-Hb to be present at high levels.

Metal-catalyzed redox effects that are spectrally silent can be detected by mass spectrometry. State-of-the-art electrospray ionization mass spectrometers provide an unparalleled opportunity to follow the simultaneous changes in an extremely wide range of molecular weight compounds. This technique makes it possible to quantify directly the time-dependent formation of SNO adducts on α or β chains of mixtures of Hb and low-molecular-weight thiols. Electrospray ionization mass spectrometry is thus proving to be a valuable method for verifying the extent of *S*-nitrosation and cystinylation in Hb samples. This method has revealed that contaminating metals can result in appreciable disulfide formation between SH groups of Cys and Hb. Modification of internal SH groups of Hb, as noted by Ferranti *et al*. (23), was confirmed in mass spectrometric analysis when Hb was exposed to a 10–20× excess of CysNO over heme. These internal modifications may not be of physiological significance, because they are improbable at the intracellular concentrations reported for Hb and red blood cell thiols. However, *in vitro* studies are frequently done at lower Hb concentration and with much higher ratios of CysNO or other NO donors. In such studies, the nitrosation of internal thiols could complicate studies of SNO-Hb biochemistry due to the functional repercussions of interfering with the interfacial regions of the Hb tetramer. Use of carefully authenticated SNO-Hb (without excessive met Hb or cystinylation products) is clearly a requirement for further studies on the mechanisms operative *in vivo* in both forming SNO-Hb and recovering bioactive NO from SNO-Hb.

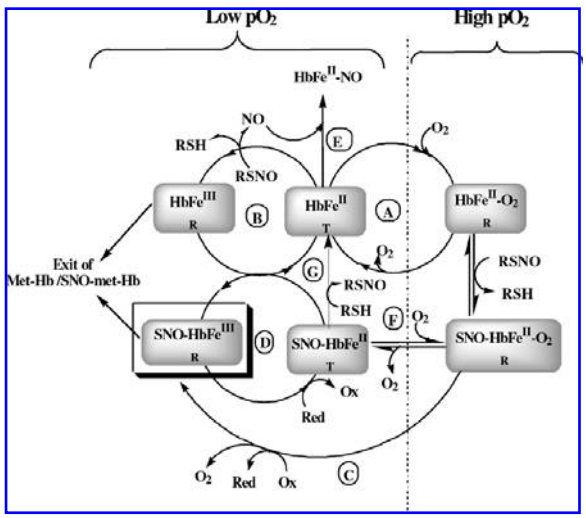
Redox behavior of Hb in relation to its NO derivatives

The heme redox potential is critical to Hb function. Loss of electrons from ferrous Hb occurs spontaneously *in vivo*, and the oxygen-binding state is restored enzymatically by electron-donating systems (46). Activated-oxygen species can be formed in the process, with potentially adverse physiological effects (2, 55, 81). Quantitative studies of the heme redox potential in Mb and Hb were begun by Taylor and Hastings (76). As reviewed elsewhere (3), the oxidation–reduction equilibrium of Hbs has been the subject of many earlier investigations. There remain many unanswered questions with regard to how globin structure controls the redox potential of the active metal sites of Hb. Earlier inconsistencies in redox studies of Hb (3, 4, 17, 18) are being resolved through use of an improved spectroelectrochemical approach to studies of Hb oxidation (21).

As shown in the earlier section on NO-Hb stability, the lifetime and fate of NO that is sequestered as NO-Hb are governed by temperature and by allosteric factors that alter the geometry and stability of the NO-Hb linkage. In this section, we discuss the redox characteristics of SNO-Hb and provide evidence that these characteristics are also subject to allosteric control.

It has now been well documented that the S-NO linkage in SNO-Hb is remarkably stable in the absence of light or reductants. We and others have shown that SNO-Hb can, for example, be cycled between deoxygenated, oxygenated, or oxidized forms without degradation of the S-NO linkage (11, 13, 83). Although the conformational shifts that occur as Hb is deoxygenated, oxygenated, or oxidized do not force the release of NO, allosteric effects and an internal electron transfer pathway (12) serve to link *S*-nitrosation to both oxygenation and oxidation processes. Further studies of the internal electron transfer pathway and its effects on heme oxidation were reported in a recent publication (14). It was determined that *S*-nitrosation and other SH modifications of Hb generally result in an increased ease of anaerobic heme oxidation, whereas anions cause redox shifts in the opposite direction. These allosteric effects on the redox behavior of normal and *S*-nitrosated Hb are represented in Scheme 2.

It has been shown that mixed thiosteric (SH-based steric effects) and anionic effects and increased met Hb formation occur when SNO-Hb interacts with the negatively charged groups of the cytoplasmic domain of Band 3 protein (anion-exchanger protein AE1). Although formation and deoxygenation of a SNO-Hb/Band 3 protein assembly do not alone cause significant NO release, even in the presence of equimolar glutathione, other biomaterials interacting with this assembly may favor the migration



SCHEME 2. Redox cycles of Hb and SNO-Hb. R- and T-states are noted in the gray boxes. Symbolic representations that emphasize the iron oxidation states are as follows: $HbFe^{II}$-O_2, oxy Hb; SNO-Hb^{II}-O_2, β93Cys-NO modified oxy Hb; $HbFe^{II}$, deoxy Hb; SNO-$HbFe^{II}$, β93Cys-NO modified deoxy Hb; $HbFe^{III}$, met Hb; SNO-$HbFe^{III}$, β93Cys-NO modified met Hb; $HbFe^{II}$-NO, Hb heme-NO adduct. From ref. (14).

of NO out of red blood cells, and may be linked to Heinz body formation within red blood cells (14). Heinz bodies, made of oxidized, denatured Hb, are formed under oxidizing conditions, bind to Band 3 protein, and are common in some pathological states (such as sickled blood cells). The lack of a strong glutathione effect on SNO-Hb in either the presence or absence of Band 3 protein is in accord with the findings of Patel and coworkers (61) that glutathione is both ineffective and slow in its transnitrosation reactions with SNO-Hb. These and other results led to generation of a conceptual model of the interrelationships between the deoxy, oxy, and met forms of normal and *S*-nitrosated Hb. Scheme 2 shows this conceptual model.

Recent findings regarding SNO-Hb contributions to vasodilation

The initial observation of vasodilatory activity of SNO-Hb was made with cell-free Hb samples added to an aortic ring preparation using rather complex mixtures of buffers and gases (42, 73). In these studies, NO or an RSNO moiety derived from SNO-Hb was apparently released from SNO-Hb, because vasorelaxation (rather than vasoconstriction) was promoted by SNO-Hb addition. It remains unclear what triggered NO release in these studies, which to some extent approximated *in vivo* conditions. In our SNO-Hb studies, we found no significant degradation of SNO-Hb in the presence of red blood cell lysates when incubated for 24 h in either oxygenated or deoxygenated conditions. Other *in vitro* studies have shown that neither the presence of equimolar glutathione nor deoxygenation is an effective trigger for NO release (11, 61). Contrasting results, showing glutathione-induced decreases of SNO-Hb levels, have come from studies that included cellular reductants and higher ratios (100:1 to 1,000:1) of glutathione to Hb. As mentioned previously, when glutathione is present at elevated concentration, it can participate in transnitrosative reactions that reduce the SNO-Hb level. When this occurs, the GSNO formed has been shown to have a vasorelaxant effect (53). Whether this is the mode of reaction underlying export of bioactivity from red cells with elevated SNO-Hb levels remains unresolved. The experiments in which vasorelaxant effects were observed may have included as yet undefined biologically active reductive materials that favored SNO-Hb decomposition. Another possibility is the generation of NO from reduction of nitrite, an unavoidable contaminant of NO-containing solutions. This process is mediated by deoxygenated Hb, as was recently reported (16, 59, 83). Clarification of these interactions of NO and RSNO species with Hb are relevant to formulating blood substitutes that lack adverse vasoconstrictive properties.

Considerable controversy surrounds the determination of levels of SNO-Hb *in vivo* and the role of SNO-Hb in blood pressure regulation *in vivo*. Many aspects of the SNO-Hb hypothesis, at least in its original form, have been brought into question by recently published work by Gladwin and collaborators (28, 29, 83). Lower erythrocytic RSNO values in humans were found than the approximately micromolar levels reported in rats (42) and humans (54). The average levels found in blood of eight human volunteers were 26 n*M* (0.00026% SNO per heme subunit) *S*-nitrosated compounds in human arterial erythrocytes and 73 n*M* (0.00073% SNO per heme) in venous erythrocytes. These values not only are much lower than those previously reported (42, 54), but differ significantly in that they do not show the previously reported arterial/venous gradient with higher levels of SNO-Hb in arterial blood that supported the concept of a dynamic cycle in which SNO-Hb is formed in the lungs and degraded in the microvasculature. However, as pointed out here and elsewhere (53) nanomolar levels of SNO-Hb may be sufficient for the role in blood pressure regulation put forward by the SNO-Hb hypothesis. Moreover, a small arterial/venous gradient would be technically difficult to detect.

To evaluate the effects of Hb oxygen saturation on the stability of intraerythrocytic SNO-Hb and to evaluate further the hypothesis that NO release from the erythrocyte is linked to oxygen release, Gladwin and co-workers performed experiments on human red cells in which the levels of SNO-Hb were elevated to 10,000 times the normal *in vivo* levels (29). It should be noted that the artifical elevation of SNO-Hb levels may not accurately mimic the *in vivo* situation. In these studies, the elevated levels of intraerythrocytic SNO-Hb fell rapidly, independent of oxygen tension and Hb saturation. Most of the NO released during this process was oxidized to nitrate. A fraction (25%) was exported as RSNO, but this fraction was not increased at low oxygen tension that favors the deoxy (T-state) conformation. Results of these studies show that, within the reductive erythrocyte environment, the β93Cys is largely maintained in a reduced state (low levels, however, are not precluded by these findings). These findings, and the evidence summarized in earlier sections showing that deoxygenation of Hb does not trigger NO release from SNO-Hb, suggest that SNO-Hb is incapable of significant participation in oxygen-linked NO storage and delivery. However, in light of the complexities thus far revealed, we doubt that we have reached the end of the story.

Even at the low steady-state *in vivo* levels referenced above, SNO-Hb may still prove to be a vital link in the complex story of NO-dependent blood pressure regulation or of other NO-dependent signaling events. Lipton *et al*. (48) have elegantly shown that injections of plasma from deoxygenated blood close to the brain regions controlling ventilation induce an increased rate of ventilation similar to that seen in response to hypoxia. This response, which is specifically mediated by a metabolite of GSNO, strongly suggests a fundamental role of SNO-Hb in oxygen-dependent transnitrosation reactions, which moreover would require subnanomolar levels of SNO and would not result in significant (*i.e.*, detectable) arterial–venous differences in SNO-Hb content, features consistent with the results of Gladwin and coworkers cited above. Additionally, although it is unclear whether or to what extent it occurs in mammals, the SNO-Hb respiratory cycle may be active in other vertebrates. Rassaf *et al.* (66) have recently reported that the higher SNO yield in rodent Hb than in human Hb correlates to a higher thiol reactivity. Several turtle species, showing exceptional resistance to hypoxia and with high erythrocytic glutathione concentrations (~2 m*M*) (67), have up to 16–20 cysteine residues per Hb tetramer, which makes these animals excellent candidates for further investigations.

DISCUSSION

NO is an important signaling molecule. It can lead to a wide range of NO-derived materials *in vivo*, determined

largely by the presence of redox-active compounds and allosteric modifiers of the compounds with which it interacts. As a result of signaling events, NO can promote vascular relaxation, and studies approximating *in vivo* conditions have shown that Hb nitrosated on its sulfhydryl groups (SNO-Hb) can also act as a vasodilator, transmitting the vascular relaxation possibilities and other aspects of NO biochemistry far away from the site of NO generation (40, 73). Hb-based NO transport thus has the potential to greatly extend the range of NO-dependent signaling reactions. SNO-Hb, which has the potential to both stabilize and transport NO, can be formed via interaction with nitrosating agents formed upon interaction of NO and oxygen (NO_x) and by NO-exchange reactions (transnitrosations) with nitrosated forms of low-molecular-weight thiols such as cysteine and glutathione. Conversely, low-molecular-weight thiols can act as NO acceptors in transnitrosation reactions where NO is donated by *S*-nitrosated proteins such as SNO-Hb. Unlike SNO-Hb, free NO is a very reactive molecule, whose lifetime in the complex cellular milieu would be expected to be very short apart from its ability to form adducts that preserve its bioactivity.

As knowledge of the involvement of NO in biological systems increases (22, 47, 49, 51, 56, 69, 80), the interactions of NO with Hb become more intriguing. It has been shown that NO interacts with Hb in three distinct types of reactions: (a) radical–radical reactions ($NO^{\bullet}$ with Fe), (b) redox reactions ($NO^{\bullet}$ to NO^{+} or NO^{-}; SNO-Hb to $NO^{\bullet}$ and S^{-}), and (c) NO-transfer reactions (R-SNO to SNO-Hb). Redox and NO-transfer reactions are both potentially involved in SNO-Hb formation and degradation *in vivo,* and are potentially subject to allosteric control.

Biologically important reactions of NO may occur without the release of the free radical NO, because NO can be specifically transferred from one NO-carrying group to another in transnitrosation reactions (6, 7). The physiologically significant reactions of NO may involve facilitated transfer of the NO moiety from one carrier to another. This might occur directly or by transient release and uptake pathways, rather than through the diffusion and reaction of free NO.

It has now been extensively documented that nitrosated low-molecular-weight thiols (RSNO species) will react with Hb and can transfer NO to its thiol groups. The nitrosation of β93Cys is favored over reaction with internal (hidden) thiols, but cystinylation occurs along with nitrosation if metal contaminants are not excluded from the reaction. The metal-enhanced formation of SNO-Hb and cystinylated derivatives appears to follow oxygen-mediated pathways.

Cystinylation along with nitrosation of Hb was noted in some studies of SNO-Hb (82) and was considered a necessary part of *S*-nitrosation events associated with exposure of Hb to nitrosated glutathione (GSNO). The "SNO-Hb" characterized by these authors had 43% of the β-chain SH groups mononitrosated and 28% cystinylated. It is important to note that the size and nature of the SH modifier alters Hb function (44), so that this "SNO-Hb" is not equivalent to the SNO-Hb characterized by ourselves and others (11, 61) in which the β93SH group was the only SH group nitrosated and the cystinylation reaction was avoided.

We have shown that SNO-Hb is remarkably stable, even in the deoxy state, in the absence of light and in the absence of NO^{+} acceptors or reductants. It is relevant that nitrosylated thiols, such as SNO-Hb or CysNO, are very susceptible to copper-catalyzed decomposition (7, 22). The short half-life usually found for CysNO is often due to artifactual contamination of buffers with copper or other redox-active trace metals. Free copper and iron in off-the-shelf buffers may frequently be in the nanomolar range, levels sufficient to alter reactions from the pathways they would take in the absence of redox metals.

The sensitivity of RSNO compounds to metals complicates discussions of SNO-Hb formation. For example, metal chelators *promote* transnitrosation reactions leading to SNO-Hb formation by avoiding secondary metal-catalyzed cystinylation reactions. Metal chelators *reduce* SNO-Hb formation, however, under conditions where the major pathway is that of SH-group reaction with NO_x compounds. In aerobic conditions, metals aid in the formation of NO_x compounds by promoting the release of NO from CysNO or other RSNO donor compounds so that the reactions with oxygen that result in NO_x can occur. Simple tests, therefore, for involvement of the NO_x pathway in SNO-Hb formation are (a) to remove oxygen and (b) to add metal chelators. Both modifications of the experimental condition should largely disallow NO_x-mediated SNO-Hb formation, while having no inhibitory effect on transnitrosative SNO-Hb formation.

The rate and extent of transnitrosation reactions in forward or reverse directions are clearly dependent on the nature of the experimental conditions. Moreover, constituents of erythrocytes may alter the rates of SNO-Hb generation and degradation. Conformational shifts that alter SH-Hb group accessibility are clearly significant as part of the allosteric control of SNO-Hb formation (*vide infra*).

Many of the unresolved issues regarding the roles of NO-Hb and SNO-Hb concern the levels of these materials *in vivo*. The reported levels of SNO-Hb and albumin vary from 50 n*M* to 2.5 μ*M* and from 30 n*M* to 7 μ*M*, respectively (83). The obstacles to accurate measurement revolve around: (a) the instability of SNO-Hb in the presence of millimolar glutathione, (b) the levels of nitrite in blood and in experimental solutions, and (c) the difficulty of distinguishing NO bound to heme and SH sites on Hb.

Nitrite is a potential reservoir of NO bioactivity, because it is readily reduced to NO. This reductive interconversion has, however, added to technical difficulties of NO measurements at physiologically relevant levels. New methods for separately quantifying NO-Hb and SNO-Hb levels (29, 83) appear to have overcome at least some technical difficulties, and data obtained using these new methods are leading to an altered view of the interactions of NO and Hb *in vivo*. These new methods give measured levels of ~25–75 n*M* RSNO in human red blood cells. These low levels could be obtained from low-level NO autooxidation reactions, although more complex intramolecular redox-dependent NO transfers have not been ruled out. It has been reported that the NO autooxidation reaction (forming N_2O_3) will produce both nitrite and RSNOs, particularly in lipid membrane compartments where the relative hydrophobicity of NO would increase its local concentration (22). Primary *S*-nitrosation reactions within the red cell membrane are consistent with recent data showing that exposure of red cells to NO gas leads to *S*-nitrosation of red cell membrane proteins with minimal *S*-nitrosation of intracellular Hb (62). It should be noted, however, that data obtained using these new methods for quantifying NO-Hb and SNO-

Hb do not rule out the SNO-Hb hypothesis, because it has been argued that nanomolar levels of SNO-Hb could play a significant role in blood pressure regulation (53).

Perspective

NO is a short-lived signaling molecule. The interactions of NO with Hb generate relatively long-lived Hb adducts at heme and SH groups that could enable NO to carry out long-range signaling functions. In spite of recent advances, there remain as yet unresolved issues of the extent to which the interactions between NO and Hb play a role in blood pressure regulation. These issues involve critical redox and allosteric aspects of the interactions between NO and Hb that are central to understanding and dealing with clinical problems associated with NO-dependent high or low blood pressure. Moreover, understanding and controlling the interactions of NO and Hb are also pivotal to development of effective Hb-based blood substitutes, because cell-free Hb molecules appear to interact more directly with the endothelial tissues where NO is generated than occurs for Hb carried within red blood cells. Certainly, the potential for extravasation of cell-free Hb exists, and it appears that extravasation can be reduced or largely ameliorated by cross-linking and PEGylation reactions.

Recent findings were reviewed with regard to redox and allosteric processes of relevance to the interactions of NO with Hb. These encompass three distinct types of reactions: (a) radical–radical reactions ($NO^{\bullet}$ with Fe), (b) redox reactions ($NO^{\bullet}$ to NO^{+} or NO^{-}; SNO-Hb to $NO^{\bullet}$ and S^{-}), and (c) NO-transfer reactions (R-SNO to SNO-Hb). Redox reactions are centrally involved in NO/Hb interactions, with differing affinities and reaction pathways for formation and degradation of adducts with ferrous and ferric heme. Redox and NO-transfer reactions control rates of SNO-Hb formation and degradation *in vitro* and possibly *in vivo*. Conceptual schemes that may aid in future research along these lines were presented for allosterically controlled Hb reactions (Scheme 1) and for redox-mediated interactions involving SNO-Hb (Scheme 2). The reaction cycles shown in these schemes may prove to be of critical importance to understanding the interactions of NO and Hb both *in vitro* and *in vivo*.

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ABBREVIATIONS

CysNO, *S*-nitrosylated cysteine; EDRF, endothelium-derived relaxing factor; EPR, electron paramagnetic resonance; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; Hb A, purified adult human hemoglobin; Hb S, sickle cell hemoglobin; NO, nitric oxide; NO-Hb, NO bound to heme of hemoglobin; RSNO, *S*-nitrosothiol; SNO-Hb, *S*-nitrosated hemoglobin.

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