Nitrite Reductase Activity of Hemoglobin S (Sickle) Provides Insight into Contributions of Heme Redox Potential Versus Ligand Affinity*

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Hemoglobin A (HbA) is an allosterically regulated nitrite reductase that reduces nitrite to NO under physiological hypoxia. The efficiency of this reaction is modulated by two intrinsic and opposing properties: availability of unliganded ferrous hemes and R-state character of the hemoglobin tetramer. Nitrite is reduced by deoxygenated ferrous hemes, such that heme deoxygenation increases the rate of NO generation. However, heme reactivity with nitrite, represented by its bimolecular rate constant, is greatest when the tetramer is in the R quaternary state. The mechanism underlying the higher reactivity of R-state hemes remains elusive. It can be due to the lower heme redox potential of R-state ferrous hemes or could reflect the high ligand affinity geometry of R-state tetramers that facilitates nitrite binding. We evaluated the nitrite reductase activity of unpolymerized sickle hemoglobin (Hbs), whose oxygen affinity and cooperativity profile are equal to those of HbA, but whose heme iron has a lower redox potential. We now report that Hbs exhibits allosteric nitrite reductase activity with competing proton and redox Bohr effects. In addition, we found that solution phase Hbs reduces nitrite to NO significantly faster than HbA, supporting the thesis that heme electronics (i.e. redox potential) contributes to the high reactivity of R-state deoxy-hemes with nitrite. From a pathophysiological standpoint, under conditions where Hbs polymers form, the rate of nitrite reduction is reduced compared with HbA and solution-phase Hbs, indicating that Hbs polymers reduce nitrite more slowly.

The various redox reactions catalyzed by ferrous hemes of the heme-globins (primarily hemoglobin and myoglobin) have interested scientists for over 150 years (1). In 1901, while studying the mechanism of meat curing, John Haldane described the ability of nitrite to oxidize hemoglobin (Hb) to methemoglobin (met-Hb) and generate iron-nitrosyl-hemoglobin (iron-nitrosyl-Hb) in sections of meat not exposed to air (2), although he was most likely observing the analogous reaction with myoglobin (Mb). The reaction of nitrite with deoxyhemoglobin (deoxy-Hb) was further characterized by Brooks in 1937 (3) and by Doyle and colleagues in 1981 (4). Recent work has demonstrated that deoxy-Hb can effectively catalyze the reduction of nitrite to NO along physiological oxygen and pH gradients (5–9). This reaction has been implicated in nitrite- and deoxy-Hb-dependent vasodilation in vivo (5, 10–12) and in vitro (5, 13), as well as in nitrite-mediated cytoprotection following ischemia-reperfusion injury (14–17). An analogous reaction of nitrite with deoxymyoglobin (deoxy-Mb) has been associated with the regulation of cardiomyocyte respiration during physiological and pathological hypoxia (18, 19).

In an anaerobic environment nitrite is reduced to NO as deoxy-Hb is oxidized to met-Hb (Equation 1) (6, 20). NO generated in this reaction can then be bound by other ferrous deoxy-hemes to form iron-nitrosyl-Hb (Equation 2) (21).

\[ \text{Nitrite (NO}_2^-) + \text{deoxy-Hb (Fe}^{2+}\text{)} + \text{H}^+ \rightarrow \text{NO} + \text{met-Hb (Fe}^{3+}\text{)} + \text{OH}^- \]  
(Eq. 1)

\[ \text{NO} + \text{deoxy-Hb (Fe}^{2+}\text{)} \rightarrow \text{iron-nitrosyl-Hb (Fe}^{2+}\text{-NO)} \]  
(Eq. 2)

Importantly, this reaction is allosterically regulated, such that the maximal rate of nitrite reduction is observed at the midpoint of the reaction, rather than at the beginning when the concentration of deoxy-hemes is greatest (6, 13). The two products of the deoxy-Hb-nitrite reaction, met-Hb and iron-nitrosyl-Hb, can both stabilize the Hb tetramer in the R- or high oxygen affinity conformation (6). Notably, R-state hemes have been shown to have greater intrinsic nitrite reductase activity than T-state hemes, reflected by their higher bimolecular rate constants (8, 18, 22). Thus, as the fraction of ferric and iron-nitrosylated hemes (and hence the degree of R-state stabilization) increases as the reaction progresses, the apparent bimolecular rate constant for nitrite reduction increases as well. This is because there are in fact two true bimolecular rate con-

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5 The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; met-Hb, methemoglobin; Hbs, sickle hemoglobin.
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FIGURE 1. Hemoglobin is an allosteric nitrite reductase. Tetrameric T-state deoxy-Hb (2−) reduces nitrite to NO, generating a met-heme (3+) and an iron-nitrosyl-heme (2−-NO), on the same or different Hb tetramers, which stabilize the tetramer(s) in the R-state. Increasing R-state character is associated with a higher bimolecular rate constant. As a result, ferrous deoxy-hemes on these R-state-stabilized tetramers react with nitrite faster than those on T-state-stabilized tetramers, thereby exponentially propagating nitrite reduction and R-state stabilization. This process therefore represents a unique allosteric autocatalytic reaction mechanism.

Sickles hemoglobin (HbS) contains a mutant form of the β-globin chain with a single amino acid substitution at the 6 position, where the hydrophilic glutamate is replaced by the hydrophobic valine (32, 33). When HbS concentration exceeds its solubility threshold deoxygenation induces polymerization of HbS tetramers, rigidifying and ultimately deforming red blood cells (34). Polymerization of HbS is affected primarily by Hb concentration and composition (relative amounts of HbS, HbF, and HbA forms), but is also modulated by pH, temperature, phosphate concentration, and ligand pressure (35–37). Interestingly, non-polymerized solution-phase HbS follows the same equilibrium ligand-binding curve as HbA (38) and demonstrates similar ligand-binding kinetics (39–41). Moreover, there is no apparent difference in kinetic cooperativity between...
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HbS and HbA, such that these species similarly transition from T- to R-state (41). These observations suggest that the HbS

\[ \beta_{66}^{66\text{Glu}} \to \text{Val} \]

mutation does not alter the ligand-binding geometric properties of the heme pocket.

However, HbS deviates significantly from HbA in the electronics of its heme pocket. Unpolymerized stripped HbS has a considerably lower redox potential than HbA, with \( E_{1/2} \) = 70 mV for HbS compared with \( E_{1/2} \) = 85 mV for HbA (42). In an aerobic environment unpolymerized HbS also oxidizes more easily than HbA (43–45) with reported rates of autoxidation nearing 0.05 h\(^{-1}\) for oxy-HbS versus 0.029 h\(^{-1}\) for oxy-HbA at 37 °C (1.7-fold faster) (46).

By uncoupling redox potential from ligand affinity, HbS presents a unique opportunity to study the contribution of heme electronics versus heme pocket geometry to the mechanism of nitrite reduction. We therefore characterized the kinetics of nitrite reduction by deoxy-HbS and compared it to the analogous reaction catalyzed by deoxy-HbA. We also examined the effects of polymerization on nitrite reduction kinetics.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hb Reagents**—All chemicals were purchased from Sigma unless specified otherwise.

**Anaerobic Reactions of Nitrite with HbA**—All reagents were prepared at 25 °C in 0.1 M phosphate buffer, pH 7.4, unless indicated otherwise. Purified human HbS was purchased from Sigma and reduced by incubation of an anaerobic HbS stock solution with 500 mM sodium hydrosulfite. Excess sodium hydrosulfite was removed by passage through two sequential Sephadex G-25 columns (Amersham Biosciences). Human normal adult hemoglobin (HbA) was prepared by hypotonic lysing erythrocytes, discarding membrane fractions after centrifugation, and dialyzing against 0.1 M phosphate buffer, pH 7.4, with storage of the hemolysate at −80 °C (6). Freshly isolated HbS was similarly prepared from a volunteer with high fractional HbS content (91.2% HbS, 4.2% HbF, 4.1% HbA,) or similar Hb distributions. Hb standard species for spectral deconvolution were prepared as previously described (23). Oxygen saturation and concentration of HbA and HbS heme species were measured by visible absorption spectroscopy (HP8453 UV-visible spectrophotometer; Hewlett-Packard or a Cary 50 spectrophotometer) followed by deconvolution of the spectrum into components from standard spectra of HbA using least-squares analysis (6). Standard reference spectra included deoxy-HbA, iron-nitrosyl-HbA, met-HbA, and nitrite-methemoglobin (nitrite-HbA). Oxyhemoglobin (oxy-HbA) was included only to confirm successful deoxygenation of the reaction. In some cases, Hb was concentrated using Centricon concentrating filters. All concentrations reflect heme, rather than tetrameric Hb, species.

**Anaerobic Reactions of Nitrite with HbS**—All reactions were run anaerobically at 37 °C in 0.1 M phosphate buffer, pH 7.4. HbA stock solution was deoxygenated by purging the headspace with helium gas with a channel for gas escape. Sodium nitrite was reacted with 22, 47, and 90 μM heme) or 0.2 cm (for reactions with 250 μM heme). Experiments with greater than 0.1% met-HbA or oxy-HbA prior to the addition of nitrite were discarded. The concentrations of heme species at each time point were determined by least squares spectral deconvolution using standard reference spectra as described above. The instantaneous reaction rates were measured as the instantaneous rate of deoxy-HbA consumption (negative change of deoxy-HbA concentration with respect to the time interval). The instantaneous bimolecular rate constants were calculated by dividing the instantaneous reaction rate at each time point by the remaining deoxyheme and nitrite concentrations.

**Anaerobic Reactions of Nitrite with HbS**—All reactions were run anaerobically at 37 °C in 0.1 M phosphate buffer and monitored by absorption spectroscopy in a 1-cm path length glass cuvette, except for reactions with 250 μM HbS that were carried out in a 0.2-cm path length cuvette. Additional modifications are described below for experiments performed in the presence of sodium dithionite. All experiments were conducted using HbS purchased from Sigma, except for studies comparing nitrite reductase activities of HbS and HbA and those conducted in the presence of dithionite, in which both Sigma HbS and freshly isolated HbS were used. Oxygenated HbS was added to an anaerobic reaction solution containing 0.1 M phosphate buffer, 10 mM 3,4-dihydroxybenzoic acid, and 5 units of 2 ml of protocatechuate 3,4-dioxygenase from *Pseudomonas* species (PCDA); HbS was then rapidly deoxygenated by application of positive helium pressure with a channel for gas escape. Sodium nitrite was added to initiate the reaction upon confirmation of HbS deoxygenation by spectral analysis and deconvolution, and the channel for gas escape was removed to preclude oxygen leak. Experiments with greater than 7% met-HbS or 0.3% oxy-HbS prior to the addition of nitrite were discarded. Effect of heme concentration was studied by reacting a range of HbS concentrations (25, 50, 100, or 250 μM heme) with 5 mM sodium nitrite at 37 °C and pH 7.4. Effect of nitrite concentration was studied by reacting 50 μM HbS with a range of sodium nitrite concentrations (0, 0.25, 1, 5, 10, or 20 mM nitrite) at 37 °C and pH 7.4. The effect of pH was studied by reacting 50 μM HbS with 5 mM sodium nitrite at 37 °C and a range of solution pH (pH of phosphate buffer and 3,4-dihydroxybenzoic acid adjusted to pH 6.73, 7.04, 7.3, 7.67, or 7.82). Instantaneous reaction rates and bimolecular rate constants were calculated as described above. Normalization of instantaneous bimolecular rate constants was done by multiplying the bimolecular rate constant at each time point by a correction factor of inverse log of \( -\Delta \text{pH} \) (where \( \Delta \text{pH} \) was the pH of the reaction −6.73) (6). These factors were calculated to be 2.04 (pH 7.04), 3.72 (pH 7.3), 8.71 (pH 7.67), and 12.3 (pH 7.82).

**Reactions of Nitrite with HbA and HbS in the Presence of Dithionite**—All reactions were conducted with excess sodium dithionite in 0.1 M phosphate buffer at pH 7 at room temperature and analyzed by global analysis using Specfit (Spectrum Software Associates). One may think that sodium dithionite would reduce nitrite to NO itself but it does not or does so very slowly as shown in Fig. 2. As long as the concentration of sodium dithionite is kept below 10 mM, dithionite does not effectively reduce nitrite. Higher concentrations of dithionite...
were found to affect nitrite (such as 50 mM dithionite, data not shown). The data were analyzed using singular value decomposition followed by fitting of the reduced data matrix to a zero-order kinetic model at all collected wavelengths and time points. Specfit was also used to obtain bimolecular rate constants when excess Hb was reacted with nitrite, fitting to a pseudo-first order process.

Studies of Polymerized HbS—HbS samples were concentrated to greater than 15 mM and then put under positive argon pressure overnight for deoxygenation. The samples were kept on ice to prevent polymerization during this process. The degree of deoxygenation was monitored by near infrared absorption spectroscopy examining the peak of deoxy-Hb at 760 nm in a 0.1-cm path length cell. Once the sample was at least 90% deoxygenated, sodium dithionite was added and the sample was temperature jumped to 37 °C to induce polymerization. Nitrite was then added to the sample and the kinetics of the nitrite/Hb reaction was monitored using near infrared absorption spectroscopy at room temperature.

Statistical Analysis—Data were analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and are reported as mean ± S.E. Unpaired t test was used to compare maximal rates of nitrite reduction by deoxy-HbS and deoxy-HbA. Linear regression analysis of the rates of nitrite reduction (shown in Figs. 4 and 5) is reported as mean ± 95% CI. For Figs. 7 and 8, standard deviations were calculated and Student’s t test was used to calculate a p value. Results were considered statistically significant with p < 0.05.

RESULTS

Deoxy-HbS Is an Allosteric Nitrite Reductase—Analogous to the reactions of nitrite with deoxy-HbA and deoxy-Mb, deoxy-HbS effectively reduces nitrite to NO, producing equimolar ratios of met-HbS and iron-nitrosyl-HbS (Equations 1 and 2). As shown in Fig. 3A, the anaerobic reaction of 50 μM HbS with 5 mM nitrite generates 25 μM total met-HbS (sum of met-HbS and nitrite-bound met-HbS) and 25 μM iron-nitrosyl-HbS. Notably, this reaction demonstrates a sigmoidal kinetic profile.

FIGURE 3. Anaerobic reaction of nitrite with deoxy-HbS. A, reaction of 50 μM deoxy-HbS and 5 mM nitrite at 37 °C and pH 7.4 monitored by visible absorption spectroscopy. There is some met-HbS present in the beginning of the reaction due to the high rate of HbS autoxidation. B, instantaneous bimolecular rate constants over time for the reaction in panel A, calculated as the instantaneous reaction rate divided by the concentrations of deoxy-HbS and nitrite at that instant. Note that these are global bimolecular rate constants, which represent a weighted average of the pure T-state and pure R-state Hb rate constants. C, instantaneous reaction rates over time for the reaction in panel A, calculated as the change in deoxy-HbS concentration over time.
characterized by an early lag (slow) phase, followed by a rapid phase, and finally another slow phase. The observed reaction rate is determined both by the concentration of deoxy-hemes available to reduce nitrite and by their reactivity represented by the bimolecular rate constant (recall that the apparent global rate constant actually changes as the hemes become more R-state stabilized). The lag phase occurs at the beginning of the reaction when all deoxy-hemes are in the T-state and have low inherent reactivity with nitrite (i.e. low instantaneous bimolecular rate constant; Fig. 3B); the observed reaction rate is therefore low (Fig. 3C). As the reaction progresses, formation of met-HbS and iron-nitrosyl-HbS increases the degree of R-state stabilization, and the bimolecular rate constant, of the remaining free ferrous hemes (Fig. 3B). However, whereas the reactivity of these hemes increases, their availability decreases because they are consumed in the reaction. The observed rate of nitrite reduction is consequently highest when approximately half of deoxy-hemes have been converted to met-hemes and iron-nitrosyl-hemes, and the number of unliganded ferrous hemes stabilized in the R-state is maximized (Fig. 3C). The reaction ultimately slows down upon continued consumption of unliganded ferrous hemes even as their instantaneous bimolecular rate constant increases (Fig. 3, B and C). The reaction of deoxy-HbS with nitrite is therefore an allosteric autocatalytic reaction, catalyzed by conformational shifts induced by heme oxidation and nitrosylation.

Due to the low redox potential of HbS and its facile autoxidation to met-HbS upon deoxygenation, the presence of a small fraction of met-HbS at the onset of the reaction could not be avoided despite the use of a robust oxygen scavenging system (Fig. 3A). The initial reaction rates and bimolecular rate constants calculated for these reactions therefore do not reflect the true T-state deoxy-HbS reaction rates and rate constants, as some R-state stabilization is already present. Nevertheless, the maximal bimolecular rate constant, calculated when all HbS tetramers are in the R-state configuration, is not altered by met-HbS present at the beginning of the reaction. The magnitude of maximal reaction rates is not affected either, although this maxima will be observed earlier in the course of the reaction than if no autooxidation would have occurred.

The observed rate of nitrite reduction is directly proportional to the concentration of both deoxy-HbS and nitrite, as predicted by Equation 1. Thus, increasing the initial deoxy-HbS (Fig. 4A) or nitrite (Fig. 4B) concentration results in a linear increase in reaction rate. When maximum rates of nitrite reduction are plotted as a function of deoxy-HbS concentration and are extended by linear regression analysis to include 0 μM deoxy-HbS (y intercept), the direct dependence of reaction rate on deoxy-heme concentration is apparent (y intercept is −0.05996 ± 0.04542 μM/s; Fig. 4A). An analogous direct dependence of reaction rate on nitrite concentration is seen in Fig. 4B, with a y intercept of −0.005882 ± 0.02405 μM/s. As expected, the instantaneous bimolecular rate constants are not affected by increasing deoxy-HbS and nitrite concentrations (data not shown).

**Nitrite Reduction Is Modulated by Reaction pH**—The reduction of nitrite by deoxy-HbS requires a proton to form the reactive nitrous acid species (Equation 1), and it is therefore expected that the observed initial (Fig. 5A) and maximum (Fig. 5B) reaction rates increase with decreasing pH. Because the relative concentration of met-HbS detected at the beginning of these reactions is approximately equal in each of these reactions, the extent of their R-state stabilization can be assumed to be the same, allowing the comparison of their initial reaction rates and rate constants (in addition to the maximum rates and constants).

If protons modulated the reaction rate by their concentration effect alone, analogous to deoxy-heme and nitrite, increasing proton concentration should accelerate the deoxy-HbS-nitrite reaction rate by the inverse log of −ΔpH (so decreasing the pH by 1 unit, or increasing proton concentration by 10-fold, should
reactions were carried out at 37 °C in 0.1M phosphate buffer adjusted to pH 6.73, 7.04, 7.3, 7.67, or 7.82. The concentration. Adjustment was made by multiplying the calculated rate constants by the following correction panel C normalized by the inverse log of nitrite-met-HbS over total HSb) for reactions in constants as a function of heme ligand and oxidation states (the fraction of iron-nitrosyl-HbS, met-HbS, and (oxidation) Bohr effect, which results in a dampened rise of thetronics and heme pocket geometry. This constitutes the redox with nitrite by either of the two potential regulatory mechanisms discussed later in the article, they alter both heme electronics and heme pocket geometry. This constitutes the redox (oxidation) Bohr effect, which results in a dampened rise of the instantaneous bimolecular rate constant over time with decreasing reaction pH (6). An analogous redox Bohr effect is observed in the reduction of nitrite by deoxy-HbS, such that the maximum rate of nitrite reduction is controlled both by the concentration of protons (increases the rate) and by proton-mediated T-state stabilization (decreases the rate). Analysis of maximum reaction rates as a function of proton concentration reveals that the y intercept is 0.1004 ± 0.03755 μM/s (does not approach 0 μM/s), indicative of reaction rate modulation by factors other than proton concentration alone (Fig. 5B).

The calculated initial instantaneous bimolecular rate constants increase as reaction pH is lowered from 7.82 to 6.73 (Fig. 5C). This primarily reflects the proton concentration effect, with minor, if any, contribution by the redox Bohr effect, as almost all of the deoxy-hemes are T-state stabilized (the reaction has not yet generated met-hemes and iron-nitrosyl-hemes to promote R-state stabilization). The instantaneous bimolecular rate constants change as a function of proton concentration because they are calculated by dividing the instantaneous reaction rate by the instantaneous concentrations of deoxy-HbS and nitrite, whereas proton concentration is not included in the denominator despite its involvement in the reaction mechanism. Decreasing reaction pH results in an increased reaction rate and consequently a higher computed bimolecular rate constant. The initial bimolecular rate constants were calculated to be 0.44 ± 0.01 M⁻¹ s⁻¹ at pH 7.82, 0.48 ± 0.05 M⁻¹ s⁻¹ at pH 7.67, 1.27 ± 0.09 M⁻¹ s⁻¹ at pH 7.3, 2.11 ± 0.17 M⁻¹ s⁻¹ at pH 7.04, and 4.36 ± 0.22 M⁻¹ s⁻¹ at pH 6.73 (Fig. 5C).

The redox Bohr effect does, however, affect the maximum (final) bimolecular rate constant and the rate at which it is achieved. To clearly demonstrate the redox Bohr effect on nitrite reduction and distinguish it from the direct proton concentration effect, we normalized the calculated rate constants to correct for the difference in proton concentration between reactions (6). This correction now reveals a dampening of allosteric autocatalysis by the redox Bohr effect, such that the normalized maximum bimolecular rate constants actually decrease with decreasing pH, as the T-to-R shift is retarded and allosteric autocatalysis is limited. These normalized maximum constants are 42.65 ± 1.04 M⁻¹ s⁻¹ at pH 7.82, 43.71 ± 7.28 M⁻¹ s⁻¹ at pH 7.67, 34.05 ± 1.29 M⁻¹ s⁻¹ at pH 7.3, 28.17 ± 2.15 M⁻¹ s⁻¹ at pH.
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7.04, and 25.58 ± 4.81 \text{m}^{-1}\text{s}^{-1} at pH 6.73 (n = 4 each; Fig. 5D). Importantly, the normalized initial bimolecular constants are not altered by the change in reaction pH, as the redox Bohr effect has no effect on T-state hemes; they were calculated to be 5.45 ± 0.15 \text{m}^{-1}\text{s}^{-1} at pH 7.82, 4.19 ± 0.46 \text{m}^{-1}\text{s}^{-1} at pH 7.67, 4.70 ± 0.33 \text{m}^{-1}\text{s}^{-1} at pH 7.3, 4.31 ± 0.34 \text{m}^{-1}\text{s}^{-1} at pH 7.04, and 4.36 ± 0.22 \text{m}^{-1}\text{s}^{-1} at pH 6.73 (n = 4 each; Fig. 5D).

HbS Is a More Efficient Nitrite Reductase Than HbA—Finally, we compared the efficiencies of nitrite reduction by deoxy-HbA and deoxy-HbS in an effort to distinguish between the potential contributions of heme pocket geometry and heme electronics to this reaction. Both HbA and HbS demonstrate faster rates of nitrite reduction upon R-state stabilization, which can occur secondary to either the lower redox potential of R-state hemes or to the altered heme pocket geometry associated with transition to the R-state conformation. However, because HbA and HbS have comparable ligand-binding affinities and kinetics (i.e. similar heme pocket geometries), but HbS has a significantly lower \( E_{ox} \), comparison of their maximal reaction rates can discriminate between these properties. As noted earlier, comparison of initial reaction rates would not be useful, as all deoxy-HbA present in the beginning of the reaction is in the T-state (no met-HbA present), whereas some deoxy-HbS is already R-state stabilized by the low amounts of met-HbS produced by heme autoxidation.

Fig. 6 contrasts the maximal rates of nitrite reduction by deoxy-HbA and deoxy-HbS. A range of deoxy-HbA and deoxy-HbS concentrations (~22, 90, and 250 \text{\mu M} deoxy-heme) was reacted with 5 mM nitrite at 37 °C and pH 7.4. To enable the comparison of maximal reaction rates, we matched these reactions by their initial deoxy-Hb, rather than total Hb, concentrations. In all experiments deoxy-HbS reduces nitrite significantly faster than deoxy-HbA (\( n \geq 4 \) at each concentration; \( p < 0.03 \)), suggesting that the difference in the rate of nitrite reduction between HbS and HbA is modulated primarily by the heme redox potential rather than its ligand-binding affinity (Fig. 6A). These results were confirmed by analogous experiments conducted using freshly isolated human Hb with high fractional HbS content (91.2% HbS, 4.2% HbF, and 4.1% HbA\(_2\), confirmed by high pressure liquid chromatography analysis). As shown in Fig. 6B, HbS reduces nitrite significantly faster than HbA at all heme concentrations (~47, 90, and 260 \text{\mu M} deoxy-heme; \( n = 4 \) each; \( p < 0.02 \)).

Reaction of Hb and Nitrite in the Presence of Dithionite—It has long been known that one can make iron-nitrosyl-Hb by mixing Hb with nitrite and sodium dithionite, which some have attributed to dithionite-mediated reduction of nitrite to NO. However, as shown in Fig. 2, dithionite in fact does not effectively reduce nitrite, and the formation of iron-nitrosyl-Hb is therefore the result of deoxy-Hb-mediated reduction of nitrite (generating met-Hb and iron-nitrosyl Hb; Equations 1 and 2) followed by the reduction of newly formed met-Hb to deoxy-Hb. We used this discovery to more stringently compare the reactions of nitrite with HbS and HbA, without the possible confounding effect of variable methemoglobin levels at the beginning of these reactions. Fig. 7A shows the concentration of deoxy-HbS and met-HbS as a function of time measured by spectral deconvolution after 100 \text{\mu M} deoxy-HbS was reacted with 5 mM nitrite in the presence of 2.5 mM dithionite at room temperature. The shape of the curves are similar to those obtained under anaerobic conditions in the absence of dithionite, such that both are approximately zero order with notable sigmoidal character. Thus, in both the presence and absence of dithionite, the instantaneous rate is governed by the competing effects of deoxy-heme availability and the overall fraction of R-state tetramers (stabilized by conversion to iron-nitrosyl-Hb). This is illustrated in Fig. 7B, where the characteristic upside down U-shape is obtained when the instantaneous rate is plotted as a function of time. The approximate pseudo-zero order nature of the reaction is also seen in Fig. 7C, where the absorbance at 555 nm as a function of time is compared with a global fit to a zero order process. Although the similarity between the data and the fit are apparent, deviation suggests that the actual data are slower than the theoretical fit early and late in the reaction, consistent with sigmoidal kinetics.

Comparison of maximum rates of the reaction of 5 mM nitrite with 100 \text{\mu M} HbS or HbA in the presence of 2.5 mM dithionite revealed that HbS is faster (same as in the reactions carried out

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**FIGURE 6.** HbS reduces nitrite faster than HbA. A, maximum rates of nitrite reduction achieved in the reactions of 5 mM nitrite with a range of deoxy-HbA and deoxy-HbS concentrations (\( n \geq 4 \) each; \( p < 0.03 \)). B, an analogous set of experiments conducted using freshly isolated Hb with 91.2% HbS (\( n = 4 \) each; \( p < 0.02 \)). All reactions were carried out at 37 °C and pH 7.4, using similar initial concentrations of deoxy-heme.
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in the absence of dithionite shown in Fig. 6). The maximum rate for HbS was found to be 0.21 ± 0.01 μM/s compared with 0.14 ± 0.02 μM/s for HbA (p < 0.01, n = 3, Table 1). There was no significant difference between the initial rates; 0.10 ± 0.03 μM/s for HbS compared with 0.093 ± 0.003 μM/s for HbA (p = 0.36, n = 3, Table 1), which confirms that there is no difference in the reaction rates of the two T-state hemes. This result was confirmed in the reactions of high concentrations of HbS and HbA with 5 mM nitrite. In these studies (Table 1) the concentration of HbS was high but still below that necessary for polymerization, the T-to-R allostERIC shift did not occur because of the large excess of hemes to nitrite, and the bimolecular rate constant was 0.18 ± 0.08 M⁻¹ s⁻¹ for HbS compared with 0.20 ± 0.04 M⁻¹ s⁻¹ for HbA (p = 0.13, n = 5). Thus, whereas the initial rates of the HbS and HbA reactions with nitrite are not significantly different, their maximal rates are different. Moreover, the overall reaction rate of 5 mM nitrite was faster with 100 μM HbS than with 100 μM HbA, as indicated by the rates obtained using a global fit to a zero order process as in Fig. 7C. The observed zero order rate constants were 0.0019 ± 0.0001 s⁻¹ for HbS compared with 0.0013 ± 0.0002 for HbA (p < 0.01, n = 3).

Finaly, we compared the rates of nitrite reduction by polymerized HbS and HbA. Fig. 8A shows the absorption spectra of the reaction of 17 mM HbA with 5 mM nitrite; the decrease in absorbance at 760 nm is indicative of the conversion of deoxy-Hb to iron-nitrosyl-Hb. A comparable reaction was carried out between 20 mM HbS and 5 mM nitrite (Fig. 8B) and was fitted to a pseudo-first order process to obtain a bimolecular rate constant. Because polymerized HbS scatters light making the samples turbid (Fig. 8B), the raw data were adjusted to a baseline at the isosbestic point (786 nm; Fig. 8C). As shown in Table 1, the bimolecular rate constant for the reaction of polymerized HbS was significantly lower than the analogous reaction with HbA: 0.13 ± 0.04 M⁻¹ s⁻¹ for HbS compared with 0.20 ± 0.04 M⁻¹ s⁻¹ for HbA (p < 0.01).

DISCUSSION

Human HbS is unique among the heme-globins in that its ligand-binding affinity and kinetics approximate those of HbA (in the unpolymerized state) (38–41), yet its considerably lower heme redox potential renders it more prone to oxidation (42–46). However, the solubility of the deoxygenated form of HbS is ~17 g/dl at 37 °C, whereas it is 70 g/dl for HbA (49). In vivo, HbS exceeds its solubility threshold when deoxygenated and polymerizes to form a gel (50), which is associated with a drastic decrease in its ligand-binding affinity (51–56). Importantly, all heme concentrations used in studies summarized in Figs. 3–7

TABLE 1

Rate of reactions of hemoglobin with nitrite in the presence of dithionite

| Description | HbS | HbA | Units |
|-------------|-----|-----|-------|
| Maximum rate, 100 μM Hb, 5 mM nitrite | 0.21 ± 0.01 (n = 3) | 0.14 ± 0.02 (n = 3) | μM s⁻¹ |
| Initial rate, 100 μM Hb, 5 mM nitrite | 0.10 ± 0.03 (n = 3) | 0.093 ± 0.003 (n = 3) | μM s⁻¹ |
| Observed apparent zero-order rate constant, 100 μM Hb, 5 mM nitrite | 0.0019 ± 0.0001* (n = 3) | 0.0013 ± 0.0002 (n = 3) | s⁻¹ |
| Bimolecular rate constant, excess Hb | 0.18 ± 0.08 (n = 5) | 0.20 ± 0.04 (n = 5) | M⁻¹ s⁻¹ |

* For reaction of 7 mM HbS with 1.75 mM nitrite and 17 mM HbA with 5 mM nitrite, the pseudo-first order rate constant (generated from specfit) divided by the initial concentration of Hb was used.

** p < 0.01 (compared to 100 μM HbA + 5 mM nitrite).
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FIGURE 8. Reaction of 5 mM nitrite with 17–20 mM HbA or HbS. The samples were deoxygenated overnight prior to addition of 10 mM sodium dithionite. The mixtures were then incubated at 37 °C for 10 min prior to addition of nitrite. The reactions were followed by absorption spectroscopy in a 0.1-cm path length cell in a Cary 50 spectrophotometer, collecting absorption spectra every 15 s. All reactions were conducted under anaerobic conditions. A, reaction of 17 mM HbA with 5 mM nitrite. B, reaction of 20 mM HbS with 5 mM nitrite. The displacements of spectra relative to each other is due to scattering of the HbS polymers that move in and out of the observation beam. C, reaction of 20 mM HbS with 5 mM nitrite after baselining at the isosbestic point 786 nm.

fall significantly below the solubility threshold and all HbS are present in the non-polymerized state. The data shown in Fig. 8 explores the effects of polymerization on the kinetics of the nitrite-deoxy-Hb reaction.

The anaerobic reaction of solution-phase deoxy-HbS with nitrite generates equimolar quantities of met-HbS and iron-nitrosylated HbS, consistent with Equations 1 and 2 and analogous reactions catalyzed by deoxy-HbA and deoxy-Mb. The rate of nitrite reduction is determined by the concentrations of deoxy-
heme, nitrite, and proton, again consistent with the proposed reaction mechanism. Deoxy-HbS, similar to deoxy-HbA, manifests allosteric autocatalysis, with maximal reaction rates observed in the middle of the reaction, when the concentrations of free ferrous hemes (decrease as the reaction progresses) and R-state stabilized ferric and iron-nitrosylated hemes (increase as the reaction progresses) are approximately equal. Finally, nitrite reduction is dually modulated by reaction pH, whereby protons increase the rate of the reaction due to their concentration effect on nitrous acid formation, yet simultaneously decrease the bimolecular rate constant and dampen allosteric autocatalysis via the redox Bohr effect. The net result of decreasing reaction pH is therefore a greater rate of nitrite reduction, although this increase is smaller than would have been caused by the proton concentration effect alone.

Importantly, deoxy-HbS is a more potent nitrite reductase than deoxy-HbA. Comparison of maximal rates attained under identical reaction conditions revealed that HbS reacts with nitrite significantly faster than HbA. Because unpolymerized HbS displays the same equilibrium and kinetics in ligand binding as HbA (heme pocket ligand-binding geometry) but has a lower \( E_{1/2} \) (heme redox potential), the faster rate of nitrite reduction by HbS indicates that heme pocket electronics, rather than geometry, are responsible for the difference between HbS and HbA, and also shows that generally, redox potential plays a role in allosteric control of nitrite reduction and NO generation.

Notably, we have also demonstrated that the nitrite-deoxy-Hb reaction can be effectively carried out in the presence of sodium dithionite, facilitating its study in laboratories where the necessary degree of complete deoxygenation is not easily accomplished. Moreover, these studies confirmed that 1) the allosterically controlled kinetics of the reaction of deoxy-HbS with nitrite, and 2) the maximal and overall rate of the reaction of deoxy-HbS with nitrite is faster than that with HbA. We also found that the initial reaction rate of nitrite with unpolymerized HbS is not significantly different from that with HbA, whereas when HbS polymerizes, the reaction rate is slower. Generally, the observed reaction rate can be written as,

\[
-d[\text{deoxy-Hb}]/dt = [\text{nitrite}][k_P[R] + k_T[T] + k_P[P]] \quad \text{(Eq. 3)}
\]

where \( R, T, \) and \( P \) refer to deoxy-hemes in molecules that are in the R-, T-, or polymerized state, respectively. Each species has its own specific bimolecular rate constant. For samples containing no polymers (including all HbA samples), \([P]\) is zero. We have shown that that \( k_P \) is the same for HbS and HbA (observed in the initial reaction rates), whereas \( k_T \) for HbS is larger than for HbA (observed in the maximal reaction rates). When the concentration of HbS exceeds its solubility threshold, HbS polymerizes whereby some T-state HbS remains in solution with the remainder locked in the polymer phase. Experiments using polymerized HbS revealed that \( k_P \) is smaller than \( k_T \). Previous work has found that \( k_P \) is likely to about 60 times larger than \( k_T \), and our data suggests that this is at least part due to redox potential differences.

HbS provided a unique experimental system in which changes in heme electronics are divorced from changes in heme ligand-binding affinities. Our data suggest that HbS polymeri-
zation would diminish nitrite reductase activity of HbS. Perturbations of the nitrite pathway in the physiological context of sickle cell disease are likely also due to NO scavenging by cell-free Hb released into the plasma by hemolysis (58, 59). There may also be impaired conversion of nitrite to NO as a result of competition for nitrite between plasma Hb (oxidizes NO to nitrate; NO$_3^-$) and ceruloplasmin (oxidizes NO to nitrite; NO$_2^-$) (60). These physiological considerations will have to be studied further and at this point remain a conjecture.

In conclusion, the characterization of the anaerobic reaction of HbS with nitrite provides additional insights into the allosteric nature of nitrite reduction and into the effects of proton concentration and redox Bohr effect on reaction rate. HbS also elaborates on the role of heme pocket geometry, in the modulation of nitrite reductase activity of HbS. Perturbation would diminish nitrite reductase activity of HbS. These physiological considerations will have to be studied further and at this point remain a conjecture.

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