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Comparative Oxidation of Hemoglobins A and S

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The mutant hemoglobin S (HbS) previously was reported to undergo accelerated autooxidation during incubation in vitro. However, subsequent observations have raised the possibility that this might be explained by adventitious association of molecular iron with HbS, rather than reflecting an inherent property of HbS. Using purified HbA and HbS obtained from genotypic HbAS donors, we found that the observed oxidation rate of HbS, but not of HbA, is indeed exaggerated by adventitious iron. This result suggests a preferential partitioning of molecular iron to HbS over HbA, which was further supported by experimentation. However, after elimination of this effect, there still remains a significant increase in inherent autooxidation rate for HbS. Physiologic oxidants (superoxide, peroxide, hydroxyl radical) and various Fe(III) chelates all stimulate oxidation of oxyHb, but they do so equivalently for HbA and HbS. Nevertheless, these mechanisms also would contribute to excessive biologic oxidation of HbS because the cytoplasm of sickle red blood cells, unlike that of normal cells, would be exposed to abnormal amounts of oxidants and low–molecular-weight iron compounds.
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THE MUTANT HEMOGLOBIN (Hb) that defines sickle cell anemia, HbS, is reported to exhibit an accelerated autooxidation rate during incubation in vitro.[1,2] Consistent with this, intact sickle red blood cells exhibit a correspondingly exaggerated generation of activated oxygen species during incubation in vitro.[3] On the other hand, no structural or biochemical explanation for this enhanced oxidation tendency has been forthcoming. In fact, the two Hbs are isomorphic at the level of x-ray crystallography, have multiple identical functions, and show only minor differences at the N-terminal end of their respective β-globin chains.[4] HbS does manifest one dramatic behavioral difference in the form of an abnormal surface denaturation tendency[4] that is derived from the mutant Hb's hydrophobic surface substitution ($\beta^{6Glu \rightarrow Val}$). While this probably underlies heme pocket disturbances during interfacial interactions between Hb and chemical denaturants[5] or phospholipids,[6] it comprises an uncertain proximate cause of abnormal Hb oxidation in solution.

Given this background, more recent observations require consideration of the possibility that observation of augmented HbS oxidation actually results from the presence of adventitious iron carried by the purified Hb. In first place, the cytosolic aspect of the membranes of sickle—but not of normal—red blood cells carries substantial amounts of molecular iron.[7] Despite its association with the membrane with very high avidity,[8] this Fe^{3+} is redox-active. For example, it can form a redox couple that, even while remaining membrane-bound, oxidizes soluble oxyHb to metHb.[9] This presumably corresponds to the known ability of Fe^{3+} chelates to promote oxidation of oxyHb.[10,11] Of particular relevance, binding studies demonstrated that Fe^{3+} binds to hemichrome,[12] denatured low-spin ferric Hb, should this be present; HbS is more likely than HbA to form hemichrome from metHb as a result of its surface denaturation behavior.[4,13]

Thus, the possibility exists that adventitious iron, whether physiologic (eg, derived from cytoplasm) or an outright contaminant (eg, derived from experimental buffers), could explain the previous impression that HbS has an abnormal tendency to autooxidize. This is important to resolve because a tendency for HbS to undergo enhanced oxidization (either because of intrinsic properties of the Hb or because of physiologic adventitious iron) has significant implications for the cellular pathobiology of sickle cell disease, in which red blood cells develop numerous membrane defects that are believed to be oxidative in etiology.[14]

MATERIALS AND METHODS

Materials. Chromatography supplies were obtained from BioRad (Hercules, CA), and reagents from Sigma Chemical (St Louis, MO). With the exception of Hb and iron itself, all reagents, solutions, and materials used in these experiments were treated for at least 3 hours before use to remove any contaminating iron using an iron-chelating resin, Chelex-100 (Sigma), at 1 g per 100 mL solution.

Hb. Fresh red blood cells were obtained from donors with sickle trait (ie, having both HbA and HbS), so that both Hbs studied would be from the same intracellular environment. The two Hbs were isolated, purified, and stripped (of organic phosphates and counter ions) using four sequential chromatographic steps (DEAE-cellulose, Sephadex G100, AG11A8, and AG501×8), as recommended by Caughey and Watkins.[15] HbA and HbS were then dialyzed together against the same solution of experimental buffer: 140 mmol/L NaCl, 20 mmol/L HEPES, pH 7.0.

Hb oxidation. Hb at 1 mg/mL (15.5 μmol/L Hb tetramer, or 62 μmol/L in heme) and the various reactants, each formulated in experimental buffer (with pH adjusted as necessary to 7.0), were prewarmed separately and then admixed in a temperature-controlled spectrophotometer (37 ± 0.1°C). Absorbance at 577 nm was monitored every 30 seconds for 30 minutes to directly measure loss of oxyHb. Simultaneous monitoring of an isosbestic point for oxyHb and metHb (587 nm) showed no change in A_{587} over the course of these experiments, confirming that no species other than oxyHb and metHb appeared in the system to introduce artifacts. Measurements verified that pH remained stable at 7.0 ± 0.01 U during all of these experiments. At this pH, some metHb would be in the form of ferrihemoglobinOH, but this would comprise a small percentage of the total metHb (<10%) at this pH, and its extinction coefficients indicate that its effect on results measured in this fashion would be to blunt, not augment, apparent oxidation rate.[16]

To calculate results, the measured absorbance changes were expressed on a log scale and plotted against time, so that initial oxidation rates could be calculated from the slope of the resulting linear plots. Hb

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Submitted July 2, 1997; accepted December 17, 1997.

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0006-4971/98/9109-0007$3.00/0

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oxidation rates are expressed here as the disappearance of oxyHb in units of micromolar heme per minute (μmol/L/min).

Variables. Except as specifically noted later, all experiments were conducted with Hb at 1 mg/mL. To identify the inherent oxidation rate and show the presence of adventitious iron, the oxidation rate of the purified Hbs was compared with and without inclusion of 10 μmol/L DTPA, a hexadentate chelator that does not itself stimulate Hb oxidation (see Results).

To identify the oxidation-stimulating effect of iron chelates, we admixed Hb with iron chelates composed of 50 μmol/L Fe^{3+} and variable chelator concentrations chosen so that greater than 99% binding of iron was assured in each case, based on reported chelator affinities. Expressed as log of the cumulative stability constant,[8] these affinities are as follows: deferiprone (L1), 35.9; EDTA, 25.0; nitrilotriacetic (NTA), 24.3; citrate, 11.5; ATP, adenosine triphosphate, approximately 6; and adenosine diphosphate, approximately 4. Affinities for 2,3-disphosphoglycerate (23DPG) and glutathione (GSH) are not known. For these experiments, results are reported as the absolute increment in Hb oxidation rate measured in the presence of the iron chelate versus the presence of chelator without iron and thus eliminate any potential effects of the chelators themselves on absorbance measurements.

In other experiments, Hbs were exposed to enzymatically generated physiologic oxidants. Superoxide ($\cdot O_2^-$) was generated using 500 μmol/L xanthine, 1×10^{-4} U/mL xanthine oxidase, plus 10 μmol/L DTPA; the addition of catalase did not alter Hb oxidation rate in this system, so we did not routinely include it. H_2O_2 was generated using 500 μmol/L glucose, 4×10^{-3} U/mL glucose oxidase, plus 10 μmol/L DTPA. Hydroxyl radical ($\cdot$OH) was generated using glucose, xanthine, and both enzymes as earlier, but with DTPA replaced by EDTA/Fe^{3+} at 10 μmol/L each. For each of these experiments, the negative control sample consisted of all relevant reagents except the enzyme(s).

To examine for any effect of Hb concentration, we compared oxidation rates for Hbs at several concentrations: 1 mg/mL (our standard condition), 10 mg/mL, and 100 mg/mL. For these experiments, we preserved the same Hb to DTPA ratio used for our standard conditions to ensure that all adventitious iron would be inactivated. Although incubations were performed at the higher Hb concentrations, for analysis, the samples were carefully diluted to [Hb] = 1 mg/mL for reading in the spectrophotometer. Therefore, the oxidation rate results (expressed in μmol/L/min) can be directly compared with those obtained in experiments at low Hb concentration. In similar fashion, we also examined the effect of one chelate, iron/ADP, at Hb and iron and ADP concentrations 10-fold higher than in our standard condition.

Iron binding to HbS. To determine if added iron would bind preferentially to HbS over HbA, purified preparations of both were treated with 10 μmol/L DTPA, followed by extensive dialysis (against 1.2×10^5 vol of experimental buffer) to remove DTPA and all adventitious iron as iron/DTPA. Then, Hbs were exposed to iron by incubation with Fe^{3+}/ADP (50 μmol/L/1 mmol/L) as earlier, since the poor solubility of iron in water precludes meaningful incubation with free iron per se. After 30 minutes, the Hbs were dialyzed together against 1.2×10^5 vol of experimental buffer to remove ADP and any Fe^{3+} not bound to Hb, and the oxidation rate was then determined with and without 10 μmol/L DTPA.

RESULTS AND DISCUSSION

Inherent oxidation rate. The comparative Hb oxidation rates we observed here under our standard condition (Hb at 1 mg/mL) before removal of any adventitious iron showed an average 52% rate augmentation for HbS (Table 1). The rates in the present report are expressed in the fashion in which the raw data were collected, in units of μmol/L/min. Conversion of these results to pseudo first-order rate constants (units of h^{-1}) yields corresponding values for HbA and HbS of 0.027 h^{-1} and 0.041 h^{-1}, respectively, which are consistent with those previously reported.[1,2]

Table 1. Rate of Hb Oxidation With and Without Removal of Adventitious Iron

Experimental Condition	n	Hb Oxidation Rate (μmol/L/min) HbA	HbS	P
Hb at 1 mg/mL				
Hb	6	.028 ± .003	.042 ± .004	$<10^{-4}$
Hb + 10 μmol/L DTPA	6	.029 ± .004	.035 ± .001	.022
dialyzed Hb	3	.030 ± .002	.035 ± .002	.042
Hb at 100 mg/mL*				
Hb	6	.029 ± .003	.042 ± .005	.019
Hb + 1 mmol/L DTPA	6	.028 ± .002	.034 ± .002	.002

Conditions were as follows: 140 mmol/L NaCl, 20 mmol/L HEPES, pH 7.0, [Hb] variable as indicated. Results are expressed as μmol/L heme oxidized per minute (mean ± SD). ''Hb'' refers to purified Hb prepared per Materials and Methods. ''Hb + DTPA'' indicates that experiment was conducted in presence of the indicated concentration of DTPA. ''Dialyzed Hb'' refers to purified Hb that was additionally treated with DTPA, followed by extensive dialysis against iron-free buffer to remove DTPA and any iron.

*Results for samples at [Hb] >1 mg/L are expressed after dilution to [Hb] = 1 mg/mL, which allows rates to be directly compared.

Inclusion of 10 μmol/L DTPA resulted in a significant decrement in the observed oxidation rate for HbS (Table 1). However, even after any adventitious iron was thus entirely inactivated by DTPA, a significantly increased oxidation rate is still evident for HbS (Table 1), substantiating the existence of an inherent increase in an autooxidation tendency for HbS. Interestingly, even though the two Hbs were derived from the same intracellular environment, addition of DTPA did not reduce the rate of HbA oxidation. These oxidation rates recorded in presence of DTPA represent the minimal rate observable under these conditions, as evidenced by the fact that higher DTPA concentrations exerted no additional protective benefit (data not shown). Also, oxidation rates dropped no further even after removal of all DTPA and iron/DTPA by extensive dialysis against iron-free buffer, confirming that the observed rate in the presence of DTPA really represents a minimal value (Table 1).

Effect of Hb concentration. Since the degree of dimerization increases as the Hb concentration decreases, our standard experimental conditions would entail substantially greater dimerization than under physiologic Hb concentrations (~320 mg/mL in heme). Therefore, we also determined oxidation rate at a 100-fold higher Hb concentration that would diminish the proportion of dimers by an order of magnitude.* Results of this

*For example, for our standard experimental condition of Hb at 1 mg/mL (62 μmol/L heme) and 37.0°C, we calculate that the *proportion* of heme in dimers is 12.5%, compared with 4.1% at 620 μmol/L heme, 1.3% at 6,200 μmol/L heme, and 0.7% at the physiologic 20 mmol/L heme. Yet the corresponding *absolute* dimer concentrations would be 2.9×10^{-6} mol/L, 12.8×10^{-6} mol/L, 41.0×10^{-6} mol/L, and 74.1×10^{-6} mol/L, respectively. This calculation assumes that the tetramer-dimer equilibrium can be expressed by $D^2/T = K$, where $T = H/4 - D/2$, H = [heme], D = [dimer], T = [tetramer], and $K = 1.1 \times 10^{-6}$ mol/L at 37.0°C. This value for K is calculated from a value of 1.5×10^{-6} mol/L at 21.5°C assuming a ΔH of −3,900 cal (1 cal = 4.18 J).[18]

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experiment (Table 1) were identical to those observed under our standard conditions, establishing that our data are indeed relevant to physiologic Hb concentrations.

Iron partitioning to HbS. Since the previous data suggest that adventitious iron associates preferentially with HbS, we designed an experiment to test this directly (Table 2). We obtained iron-free preparations of Hbs (by addition of DTPA and subsequent removal of iron/DTPA by dialysis) and determined their oxidation rates (Table 2). Then, we reexposed them to Fe^{3+}/ADP, after which the two Hbs were extensively dialyzed together to remove ADP and any Fe^{3+} not bound to Hb. Subsequent examination of the oxidation rate showed reinstitution of a greater increase for HbS than for HbA (Table 2). Since direct exposure of HbA and HbS to the same concentration of iron/ADP exerts exactly the same effect on oxidation rate (as shown later), this experiment suggests preferential binding of iron to HbS. Consistent with this, final addition of DTPA restored the basal oxidation rate identified in the iron-free preparations in the first place (Table 2).

Effect of iron chelates. Any molecular iron in red blood cells that is not associated with Hb or the membrane is likely to be in the form of low–molecular-weight chelates. Since these are known to promote Hb oxidation,[10,11] we tested whether HbS was abnormally susceptible to this effect. All of the tested Fe^{3+} chelates significantly stimulated Hb oxidation, but in each case this incremental effect was equivalent for HbA and HbS (Table 3). This excludes an abnormal inherent susceptibility for HbS to be oxidized by Fe^{3+} chelates. However, the abnormal decompartmentalization of iron characteristic of the sickle red blood cell nevertheless predicts that this is a mechanism relevant to biology, because the sickle cell cytoplasm may have low–molecular-weight Fe^{3+} chelates not present in normal red blood cells.

The magnitude of oxidation effected by the different Fe^{3+} chelates varied enormously in these experiments, although discerning the reasons why is far beyond the scope of this investigation. Clearly, our expectation, based on earlier and more limited data,[10] that oxidation rates would be simply related inversely to chelator affinity for Fe^{3+} is not substantiated by our data. The reason for the observed variation must be related to the multiplicity of factors that are known to influence rate of chelate-induced oxidation.[10] Significantly, the incremental change in oxidation rate seen in presence of Fe^{3+}/ADP was the same for Hbs examined at 1 mg/mL (Table 3) and at 10 mg/mL (.342 ± .020 for HbA and .332 ± .018 for HbS; n = 3).

Effect of oxy radicals. All three physiologic oxidants ($\cdot O_2^-$, H_2O_2, and $\cdot OH$) significantly accelerated Hb oxidation, but no difference in susceptibility between HbA and HbS was noted in this regard (Table 4). Again, however, this mechanism remains relevant to biologic HbS oxidation, since the sickle red blood cell generates excessive amounts of these activated oxygen species compared with normal red blood cells.[3]

Discrepancy of GSH results. Because our observation of minimal oxidation promotion by the Fe^{3+} chelate with GSH (Table 5) differs from the large GSH effect reported earlier,[17] we examined this further. Our data demonstrate that GSH has a hemoglobin oxidizing effect even in the absence of iron (Fig 1). However, this effect is dose-dependent, and is only seen at GSH to heme ratios of 2:1 and greater, ratios that far exceed physiologic conditions (ratio of ≃1:4). In fact, these unusual conditions actually were used in that earlier report,[17] so this explains why our results differ. Additional experiments performed at two different Hb concentrations substantiate the importance of the GSH to Hb ratio (Table 5).

Table 2. Evidence for Preferential Association of Iron With HbS

Experimental Condition	Hb Oxidation Rate (μmol/L/min) HbA	HbS
1. Standard Hb preparation	.028 ± .001	.047 ± .001
2. #1 with iron removed	.029 ± .001	.034 ± .003
3. #2 reexposed to iron	.094 ± .003	.145 ± .012
4. Incremental rate (#3 minus #2)	.065 ± .003	.114 ± .014*
5. #3 with DTPA added	.029 ± .002	.035 ± .003

Results are shown as the mean ± SD (n = 3 for each) and expressed as for Table 1. Experimental conditions: see text. Note that reexposure to iron (line 3) involved 50 μmol/L iron with 1 mmol/L ADP, so the resulting incremental oxidation rate (line 4) can be compared directly with the ADP experiment shown in Table 3.

*(*P* = .004 for HbA *v* HbS, indicating preferential association of iron with HbS.

Table 3. Increase in Hb Oxidation Rate Caused by Iron Chelates

Experimental Condition	n	Increase in Hb Oxidation Rate (μmol/L/min) HbA	HbS
Hb + Fe^{3+} + L1	4	.025 ± .008	.026 ± .003
Hb + Fe^{3+} + NTA	8	.825 ± .045	.850 ± .047
Hb + Fe^{3+} + EDTA	9	.130 ± .007	.138 ± .014
Hb + Fe^{3+} + citrate	9	.026 ± .005	.031 ± .012
Hb + Fe^{3+} + ATP	8	.349 ± .028	.341 ± .029
Hb + Fe^{3+} + ADP	8	.340 ± .018	.340 ± .024
Hb + Fe^{3+} + 23DPG	3	.121 ± .025	.096 ± .010
Hb + Fe^{3+} + GSH	3	.016 ± .006	.010 ± .011

Conditions were as follows: same as for Table 1, except for the addition of 50 μmol/L Fe^{3+} in the form of a chelate. Results (mean ± SD) are shown as the absolute increase in Hb oxidation rate over that measured in presence of Hb plus chelator, but without added iron. For this set of experiments, these basal rates of oxidation were .029 ± .002 for HbA and .042 ± .003 for HbS. Chelator to iron ratios were 1:1 for EDTA, 2:1 for NTA, 4:1 for GSH, 10:1 for ATP and 23DPG, 20:1 for citrate and ADP, and 40:1 for L1. All oxidation rate increments were significant; but for any given chelator, there was no significant difference for HbA *v* HbS.

Table 4. Increase in Hb Oxidation Rate Due to Physiologic Oxidants

Experimental Condition	n	Increase in Hb Oxidation Rate (μmol/L/min) HbA	HbS
Hb + $\cdot O_2^-$	6	.095 ± .008	.089 ± .008
Hb + H_2O_2	6	.103 ± .012	.092 ± .008
Hb + $\cdot OH$	6	.134 ± .014	.120 ± .019

Conditions were as follows: same as for Table 1, except for presence of oxidant generating systems (see Methods). Results are shown as the absolute increase in Hb oxidation rate (mean ± SD) exhibited by the complete system over that measured for the controls that deleted the enzymes. For this set of experiments, basal rates of oxidation were .029 ± .002 for HbA and .041 ± .004 for HbS. All oxidation rate increments were significant, but there were no significant differences for HbA *v* HbS.

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Table 5. Increase in Hb Oxidation Rate Due to GSH

Experimental Condition	n	Hb Oxidation Rate (μmol/L/min)
Hb at 1 mg/mL (62 μmol/L heme)		
No GSH	6	.029 ± .002
+ 125 μmol/L GSH	6	.034 ± .005
+ 250 μmol/L GSH	6	.041 ± .011
+ 500 μmol/L GSH	6	.071 ± .022
Hb at 10 mg/mL (620 μmol/L heme)		
No GSH	6	.028 ± .001
+ 125 μmol/L GSH	6	.029 ± .005
+ 250 μmol/L GSH	6	.027 ± .004
+ 500 μmol/L GSH	6	.028 ± .002
+ 1250 μmol/L GSH	3	.033 ± .008
+ 2500 μmol/L GSH	3	.057 ± .002
+ 5000 μmol/L GSH	3	.085 ± .004

Results are shown as the mean ± SD. Conditions are as for Table 1, with the inclusion of 10 μmol/L DTPA in each case and with variable [GSH] and [Hb] as shown.

Conclusion. These results document that purified HbA and HbS preparations, even from the same cellular source (ie, genotypic HbAS donors), exhibit different autooxidation rates. The described data suggest that this is accounted for by differential association of adventitious molecular iron so that sufficient amounts to augment the Hb oxidation rate are carried on HbS, but not on HbA (although our data set does not include direct measurement of such adventitious iron). However, elimination of this effect still leaves an exaggerated oxidation rate for HbS, clarifying the earlier reports of this fact.[1,2] In addition to this inherent autooxidation tendency, oxidation of HbS would also be influenced by exogenous stimuli, such as low–molecular-weight Fe^{3+} chelates and physiologic oxidants (ie, oxygen radicals), as shown here. Our data indicate that HbS has no exaggerated inherent susceptibility to such stimuli. However, these mechanisms are still relevant to sickle disease pathobiology, because sickle cells would be subjected to abnormal levels of such stimulation derived from the abnormal iron decompartmentalization[7] and excessive oxidant generation[3] characteristic of red blood cells in this disease.

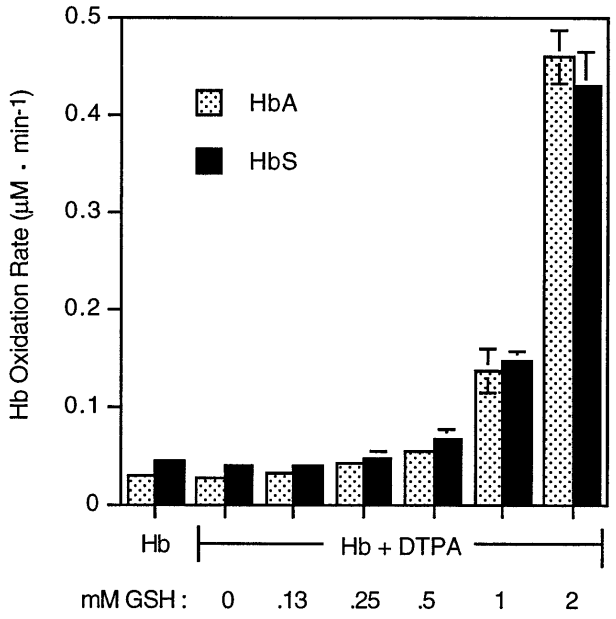

Fig 1. GSH in the absence of iron accelerated Hb oxidation, but only at very high GSH to Hb ratios. Experiments were performed in the presence of DTPA to ensure that observed effects were iron-independent. The [Hb] was 62 μmol/L in heme, and oxidation was accelerated only when the GSH to heme ratio was ≥2:1; the physiologic ratio is closer to 1:4.

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