Globins Scavenge Sulfur Trioxide Anion Radical*

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Background: Sulfite, an intermediate in sulfur metabolism, can be oxidized to the potentially toxic sulfur trioxide anion radical (STAR).

Results: Diverse globins efficiently reduced STAR in vitro, and flavohemoglobin protected yeast from STAR toxicity.

Conclusion: Globins can function as STAR scavengers.

Significance: The data suggest roles for diverse globins in protecting cells against sulfite stress.

Ferrous myoglobin was oxidized by sulfur trioxide anion radical (STAR) during the free radical chain oxidation of sulfite. Oxidation was inhibited by the STAR scavenger GSH and by the heme ligand CO. Bimolecular rate constants for the reaction of STAR with several ferrous globins and biomolecules were determined by kinetic competition. Reaction rate constants for myoglobin, hemoglobin, neuroglobin, and flavohemoglobin are large at 38, 120, 2,600, and $\geq 7,500 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively, and correlate with redox potentials. Measured rate constants for O$_2$, GSH, ascorbate, and NAD(P)H are also large at $\sim 100, 10, 130$, and $30 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively, but nevertheless allow for favorable competition by globins and a capacity for STAR scavenging in vivo. Saccharomyces cerevisiae lacking sulfite oxidase and deleted of flavohemoglobin showed an O$_2$-dependent growth impairment with nonfermentable substrates that was exacerbated by sulfite, a precursor to mitochondrial sulfite formation. Higher O$_2$ exposures inactivated the superoxide-sensitive mitochondrial aconitase in cells, and hypoxia elicited both aconitase and NADP$^+$-isocitrate dehydrogenase activity losses. Roles for STAR-derived peroxysulfate radical, superoxide radical, and sulfo-NAD(P) in the mechanism of STAR toxicity and flavohemoglobin protection in yeast are suggested.

Sulfite-derived free radicals may contribute to sulfite toxicity within cells (1). Transition metals, metalloenzymes including cyt c oxidase, O$_2$, 'O$_2$', photo-excited chromophores, and UV radiation oxidize (bi)sulfite ($pK_a = 7.2$) (1–11) forming the mild oxidant sulfur trioxide anion radical (STAR)$^4$-$\text{SO}_3^-$, which can initiate a O$_2$-Consuming free radical chain reaction (5, 10, 12, 13). STAR reacts rapidly with O$_2$ (12, 14) to form peroxysulfate radical, ‘SO$_5^-$, a strong oxidant that can be reduced to peroxymonosulfate, HSO$_5^-$, and peroxysulfate radical, ‘SO$_4^-$, a stronger oxidant (10). In addition, ‘SO$_4^-$ is generated as an unavoidable by-product of aerobic oxidations by STAR or ‘SO$_4^-$ (9), and STAR can form radical adducts (1, 15). More recently, neutrophil peroxidase and Cu,ZnSOD were shown to generate STAR in vitro (16–19) and ‘SO$_4^-$ oxidized and fragmented proteins in vitro (19).

Evidence is surprisingly absent for the formation or toxicity of STAR, ‘SO$_5^-$, or ‘SO$_4^-$ within cells despite the ubiquitous formation of sulfite via reductive sulfate assimilation, and oxidative catabolism of sulfur amino acids or sulfide, and the common exposure of humans to sulfite through diet and atmospheric SO$_2$. STAR formation has been judged relevant, but of uncertain significance, even in plants under photo-oxidative stress (20, 21). Erben-Russ et al. (22) suggested many years ago that the rapid reaction of STAR with antioxidants, such as GSH (23), would effectively detoxify STAR, if STAR ever formed. Sulfite oxidases and sulfite reductases are expected to maintain low intracellular sulfite levels (1, 24) and limit STAR formation.

While investigating the reactivity of (bi)sulfite with oxyMb, we observed the oxidation of deoxyMb to metMb by STAR. We now report that the reaction of STAR with ferrous globins is exceptionally rapid with STAR reacting 260- and ~1000-fold faster with Ngb and flavoHb, respectively, than with GSH, suggesting a STAR detoxification function. By studying STAR formation, STAR reactivities, and Saccharomyces cerevisiae flavoHb-deficient mutants, we identify targets and modes for STAR toxicity and sulfite stress that help define the STAR detoxification function.

Experimental Procedures

Materials—Crocin, Aspergillus niger glucose oxidase (600 units/ml), and all other reagents, unless otherwise specified, were obtained from Sigma-Aldrich. Crocin (catalog no. 17304), with a reported homogenous mass of ~999 g (M + Na$^+$) by electrospray-MS, showed an extinction equal to 19,500 ± 500 M$^{-1}$ cm$^{-1}$ at 432 nm in methanol with additional maxima at 323 and 457 nm indicative of a mixture of the 13-cis- and all-

S. cerevisiae; Ec, Escherichia coli; Ca, Candida albicans; Re, Ralstonia eutropha.
trans-crocin di(β-D-gentiobiosyl) esters (25). Bovine erythrocyte Cu,ZnSOD and bovine liver catalase were obtained from Roche Applied Science. Catalase was resuspended in acetate buffer (pH 5.8), concentrated, filter-sterilized, and stored at 4 °C. CO-saturated water stocks were prepared as described (26). Superdex 200 was obtained from GE Healthcare Life Sciences. Human HbA was from John Olson (Department of Biochemistry and Cell Biology, Rice University, Houston, TX). Recombinant rat cyt b₃ (mantle Ngb, Ec MnSOD, Ec flavoHb, Ca flavoHb, Sc flavoHb, and Re flavoHb) were prepared as described (27, 28). Sodium (bi)sulfite, pH 7.2, stock 1.0 M solutions were prepared by adding 1.0 M NaOH to an equal volume of 2.0 M sodium bisulfite and stored frozen at −20 °C. The dinitrosyl iron complex was freshly prepared as described (29). A 1 M KI solution was incubated under air to produce I₃⁻. I₃⁻ concentrations were determined by applying an extinction coefficient of 27.7 mM cm⁻¹ at a non-interfering 90° angle to the spectrophotometer light path. A peak emission at 462 nm with a bandwidth of 50 nm at half-maximal intensity and an illuminance of −20,000 lux at a 2-cm distance, was directed into a 1-ml cuvette at a non-interfering 90° angle to the spectrophotometer light path.

OxyMb—Horse heart metMb (30 mg) was reduced with dithionite in a 1-ml volume of 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. Mb was separated on a Superdex 200 column, concentrated, and assayed for heme (26).

Reduced cyt c—Horse heart cyt c(III) (55 mg) was reduced with dithionite, separated on a Superdex 200 column, and concentrated. Cyt c(II) was measured using an extinction coefficient of 27.7 mM cm⁻¹ at 550 nm (31).

Mitochondrial Membranes—Mitochondria (1.2 g of wet weight) were isolated (32) from fresh chicken livers (900 g) purchased from a local market and were stored in 70-ml aliquots at −70 °C. Aliquots were sonicated in 0.5 ml of 50 mM sodium MOPS, pH 7.0, buffer at 4 °C, and membranes were washed twice by centrifuging at 25,000 g for 1 min. Membranes were resuspended in 0.5 ml of MOPS buffer and kept on ice. Cyt c oxidase activity was measured by following the oxidation of 20 μM cyt c(II) at 550 nm in 0.5 ml of 10 mM sodium MOPS buffer, pH 7.0, at 37 °C and by applying an extinction coefficient of 21.0 mM cm⁻¹ cm⁻¹ (31). Cyt c oxidase activity was 0.9 nmol s⁻¹ per mg mitochondria or ~2.5 μM when applying a kcat of 350 s⁻¹ (33) and a density of 1 g/ml.

STAR Generation—STAR was generated by adding (bi)sulfite-oxidizing catalysts to O₂-depleted buffer in 1-ml quartz cuvettes thermostatted at 37 °C. Unless otherwise indicated, buffer was depleted of O₂ with glucose oxidase (0.6 unit/ml), 5 mM glucose, and 15 mM catalase (350 units/ml) for 5 min prior to adding (bi)sulfite and crocin. Catalysts were added, and STAR was detected by the oxidative bleaching of crocin (22) with high bisulfite concentrations or O₂-depleted conditions, thus favoring oxidation by STAR over SO₃²⁻ or SO₂⁻. Initial rates of crocin oxidation were generally followed at 480 nm (E = 10.3 mM cm⁻¹ cm⁻¹), a noninterfering wavelength. Unless indicated otherwise, 50 mM potassium phosphate, pH 7.2, containing 0.5 mM EDTA (buffer A) was used as the buffer. Mitochondrial cyt c oxidase-catalyzed STAR generation was assayed by following crocin (60 μmol) oxidation in the presence of 200 μM O₂ in 10 mM sodium MOPS buffer, pH 7.0, containing 0.5 mM EDTA, 5 mM sodium (bi)bisulfite, and 10 μM cyt c(II) in a 0.5-ml volume at 37 °C. Rates were calculated by applying an extinction coefficient of 15.0 mM cm⁻¹ cm⁻¹ at 432 nm and by subtracting rates measured without added cyt c(II).

STAR Scavenging—Bimolecular rate constants for STAR oxidation of deoxyMb and deoxyHbA, k₅₅₅ (40) were calculated from the inhibition of globin oxidation by GSH, where the percentage of inhibition = 100 (k₅₅₅[globin]/k₅₅₅[globalin] + k₅₅₅[globalin]), and k₅₅₅ = 9.6 × 10⁻⁹ M⁻¹ s⁻¹ (22). Other bimolecular rate constants were calculated from inhibition of crocin oxidation, where the percentage of inhibition = 100(k₅₅₅[B]/(k₅₅₅[crocin][crocin] + k₅₅₅[B]), k₅₅₅ = 1.0 × 10⁹ M⁻¹ s⁻¹ (22), and B is the competitor. Cyt c(II) or NADH were also used to estimate STAR reaction rate constants for targets with lower reaction rate constants. The extinction coefficients and wavelengths applied for measuring cyt c(II) and NADH oxidation were 21.00 mM⁻¹ cm⁻¹ at 550 nm (31) and 6.20 mM⁻¹ cm⁻¹ at 340 nm, respectively. Glucose oxidase (0.6 unit/ml), glucose (5 mM), and catalase (350 units/ml) were used to deplete O₂ from reactions as indicated. Rate constants for the reaction of STAR with O₂ were estimated from the difference in the rates of crocin (60 μmol) bleaching in the absence or presence of 200 μM O₂.

Media, Cell Culture, Harvests, and Enzyme Assays—Starter cultures of S. cerevisiae parental strain BY4742 and the isogenic flavoHb-deficient yhbΔ·KanMX strain 15887 (34) were grown overnight in 3 ml of LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter of tap water, pH 7.0) supplemented with 20 μM glucose in 15-ml culture tubes with aeration in a rotary shaker at 250 rpm at 30 °C. Experimental cultures were grown in 50-ml Erlenmeyer flasks in 10 ml of LB medium with specified glucose and were initiated with 1% inocula from overnight cultures. Flasks were agitated at various speeds in an Innova 3100 elliptical rotary shaker water bath at 30 °C (New Brunswick, Edison, NJ) to achieve varying levels of aeration. All cultures were grown under low light. Growth was monitored by the turbidity at 600 nm. Cells were harvested by centrifugation and rapidly frozen in a −20 °C ethanol bath. Extracts were prepared by sonicating cell pellets in Y-Per S reagent (Thermo Scientific-Pierce) containing 2.5 mM sodium citrate and 0.6 mM MnCl₂ and centrifuging lysates at 25,000 × g for 20 s. Aconitase activity was immediately measured at 30 °C with the coupled IDH assay (35). IDH activity was measured by following 2 mM isocitrate-dependent NAD⁺ reduction. Protein was measured at 280 nm with an absorbance of 1 equal to 1 mg/ml.

Sulfonation of GSH and NADPH—S-Sulfoglutathione was synthesized by the Cu²⁺-catalyzed oxidation of (bi)sulfite essentially as described (36). NADPH (5 μmol) was sulfonated by 5 μmol Cu²⁺-catalyzed oxidation of (bi)sulfite (25 μmol) in a 1.1-ml volume, and the sulfo-NADPH derivative was isolated in ≥90% yield. Copper ions were removed by batch Dowex 50w-X8 treatment. Sulfate and sulfite were removed by precipitation with barium acetate. The sulfo-NADPH was fractionated...
SO₃⁻ Scavenging by Globins

FIGURE 1. Oxidation of Mb by (bi)sulfite. A–C, oxyMb (10 μM heme) was incubated with no additions (A), 3 μM Cu,ZnSOD (B), or 3 μM Cu,ZnSOD and 5 mM GSH (C) at 37 °C in 40 mM potassium phosphate buffer, pH 7.3. The spectra were recorded before (red lines) and at 2-min intervals after adding 20 mM sodium sulfite (black lines). D and E, oxyMb (5 μM) was deoxygenated in buffer A in the absence (D) or presence of 20 μM CO (E). Mb spectra were recorded prior to O₂ depletion (solid red line), following deoxygenation and the addition of 5 mM (bi)sulfite (blue lines), or immediately after the addition Cu,Zn-SOD (3 μM) (dashed red lines), and at 10-s intervals thereafter (solid black lines). E, potassium ferricyanide (10 μM) was added, and the spectrum was recorded after 20 s (dashed black line). Arrows indicate absorbance increases and decreases. OxyMb was deoxygenated with 0.1 unit/ml glucose oxidase, 350 units/ml catalase, and 5 mM glucose at 37 °C.

on AG1-X8 resin (Bio-Rad) with 0–2 m ammonium acetate. Barium acetate was added (40 mg/ml), and the barium salt of sulfo-NADP was precipitated with 5 volumes of ethanol at −20 °C, centrifuged, vacuum dried, and resuspended in water. Barium was replaced with ammonium (sulfate), and barium sulfate was removed by centrifugation. Sulfo-NADP and C₂₁H₂₉N₇O₂₀P₃S, showed an absorbance maximum of 264 nm where the course of 20–30 s. The initial rates were linearly dependent

Results

Oxidation of Ferrous Mb by (Bi)sulfite—In air-saturated buffer, (bi)sulfite simultaneously deoxygenated oxyMb (λₘₐₓ = 414 nm) forming deoxyMb (λₘₐₓ = 434 nm) and oxidized deoxyMb to metMb (λₘₐₓ = 409 nm) (Fig. 1A). The addition of Cu₉ZnSOD increased the rate and amount of metMb formed (Fig. 1B). MnSOD, Cu²⁺, and Fe²⁺/³⁺ also accelerated metMb formation (data not shown). GSH, a STAR scavenger (22), inhibited metMb formation catalyzed by Cu₉ZnSOD (Fig. 1C). In an O₂-depleted buffer (≤1 μM), deoxyMb (Kₘₐₓ = 0.9 μM) was similarly oxidized to metMb when Cu₉ZnSOD was added to (bi)sulfite (Fig. 1D). Binding of CO to form

MbFe²⁺CO (λₘₐₓ = 422 nm) blocked ferrous heme oxidation (solid black lines) but did not prevent ferrous heme oxidation by ferricyanide (dashed black line) (Fig. 1E). The data suggest STAR generation and ferrous heme binding and oxidation.

STAR Generation—In confirmation of the work of Rangue-lova et al. (19), (bi)sulfite reduced Cu(I),ZnSOD and also Mn(III)SOD (Fig. 2, A and B, respectively, lines 1). The addition of GSH (5 mM) to scavenge STAR and prevent Cu(I) and Mn(II) oxidation increased the rate and extent of reduction (lines 2). GSH did not reduce Cu(I),ZnSOD or Mn(III)SOD (lines 3).

Cu₉ZnSOD and MnSOD were directly assayed for catalysis of STAR formation from the bleaching of the water-soluble carotenoid crocin (22). The reaction rate constants are summarized in Table 1. MnSOD catalyzed STAR generation more rapidly than Cu(I),ZnSOD as expected from the faster rate of Mn(III)SOD reduction (Fig. 2, A and B, respectively, compare lines 1). The >1:1 stoichiometry of crocin oxidized to oxidant added suggests a chain reaction under the O₂-depleted condition because of residual O₂, crocin, and catalyst intermediates or intermediates in sulfate formation.

Other oxidants also catalyzed STAR generation (Table 1). Ferricyanide oxidized (bi)sulfite to STAR with a rate constant ~5 orders of magnitude smaller than that for the solvent-exposed heme propionate of Mb (37) arguing for a direct oxidation of MbFe²⁺CO by ferricyanide in Fig. 1E. Notably, neither oxyMb nor oxyHb oxidized (bi)sulfite. Photo-excited FMN and cyt c oxidase generated STAR with high rate constants, thus confirming the early data and interpretations of Fridovich and Handler (7, 8). Contrary to an earlier report (6), the flavoenzyme glucose oxidase produced STAR in the presence of glucose and O₂. STAR was also generated by I₂⁻/I⁻, dinitrosyl iron complex, and HNO₃, the latter agents of NO stress (38).

Cu₉ZnSOD and FMN/light showed favorable characteristics for use as STAR generators for kinetic competition assays (see below). These characteristics included catalytic amplification (Table 1) with initial rates of crocin bleaching measureable over the course of 20–30 s. The initial rates were linearly dependent upon Cu₉ZnSOD and (bi)sulfite concentrations (Fig. 3, A and B). FMN/light also showed a linear dependence with ≤0.4 μM.

\[ \text{SO}_3^\text{−} \text{Scavenging by Globins} \]

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The reaction with 50% inhibition observed at ~40 μM GSH (Fig. 4B).

STAR also rapidly oxidized deoxyHbA, but only by ~10% (Fig. 5) and in a low salt phosphate-(bi)sulfite buffer. Additional STAR exposures caused further rapid deoxyHbA oxidation; however, each subsequent STAR exposure oxidized only ~10% of the deoxyHbA (inset). For deoxyHbA, 50 and 200 μM GSH inhibited oxidation by 30.0 ± 2.5 and 61.3 ± 5.0% (n = 3 ± S.D.), respectively. Similar low oxidation yields were observed with deoxyHbA formed by depleting O2 with glucose oxidase (data not shown). By applying a rate constant of 9.6 x 10^6 M^-1 s^-1 for the reaction of STAR with GSH (22), rate constants of 3.8 x 10^7 M^-1 s^-1 and 1.2 x 10^8 M^-1 s^-1 are estimated for reaction of STAR with deoxyMb and deoxyHbA, respectively.

**STAR Scavenging by Ngb—**A mitochondrial NADH-cyt b5 reductase/cyt b5 system (32, 39) was reconstituted for reduction of metNgb under anoxic conditions. Mitochondrial membrane NADH-cyt b5 reductase efficiently reduced cyt b5 (Fig. 6A, inset) and reduced cyt b5 reduced metNgb at a constant rate of 1.5 μM min^-1 with an estimated bimolecular rate constant of ~10^9 M^-1 s^-1 and achieved ≥ 85% metNgb reduction at equilibrium (Fig. 6B, inset). DeoxyNgb was tested for its capacity to inhibit STAR-mediated crocin oxidation (Fig. 6C), and the percentage of inhibition of initial rates was calculated (Fig. 6D).

Using the bimolecular rate constant of 1.0 x 10^9 M^-1 s^-1 for the reaction of STAR and crocin (22) and correcting for fractional metNgb reduction, an average reaction rate constant of 2.6 x 10^8 M^-1 s^-1 is estimated.

**STAR Scavenging by flavoHb—**The STAR scavenging activity of reduced microbial flavoHb was measured from initial rates of STAR-mediated crocin oxidation in the presence of NADH. Inhibition was measured with increasing concentrations of Ec flavoHb (Fig. 7A, lines 1–7), and the percentage of inhibition of crocin oxidation was calculated (Fig. 7B, line 1). The effects of [Ca flavoHb] on crocin oxidation are also shown (line 2). Re flavoHb and Sc flavoHb were also tested at 1 μM. Reaction rate constants of 2.1 x 10^10, 7.5 x 10^9, 1.9 x 10^10, and 7.5 x 10^9 M^-1.

![Figure 3](https://example.com/figure3.png)

**Figure 3. Cu,ZnSOD and FMN/light-catalyzed STAR generation.** Initial rates of crocin oxidation were measured as a function of [Cu,ZnSOD] (dimer) with 5 mM (bi)sulfite (A), (bi)sulfite with 3 μM Cu,ZnSOD (B), [FMN] with 5 mM (bi)sulfite (C), or (bi)sulfite with 0.2 μM FMN (D). The rates were measured following Cu,ZnSOD addition (A and B) or illumination (C and D). The reactions were at 37 °C in 1-ml cuvettes in 1 ml of O2-depleted buffer A containing 15 μM crocin.

![Figure 4](https://example.com/figure4.png)

**Figure 4. STAR oxidation of deoxyMb and GSH competition.** A, deoxyMb (10 μM hemeproteins) was exposed to STAR generated by adding MnSOD (12 μM), and the spectra were recorded at 5-s intervals. B, deoxyMb was exposed to STAR in the presence of increasing GSH concentrations, and the percentage of inhibition of deoxyMb oxidation was calculated from the absorbances at 434 nm after 10 s exposures (n = 3 ± S.D.). The reactions were in 1.0 ml of buffer A containing 5 mM (bi)sulfite and 1 mM FMN in 1-ml cuvettes at 37 °C. Buffer was deoxygenated with a 1 min pre-exposure to light as described under “Experimental Procedures.” OxyMb was added to deoxygenated buffer, fully deoxygenated and reduced with 5 min of illumination, and exposed to MnSOD with only low (<500 lux) and intermittent (0.1 s) light exposure from the diode array spectrophotometer. Arrows indicate absorbance changes.

**Table 1**

Rate constants for STAR formation by (bi)sulfite oxidizing catalysts

| Catalyst/Oxidant | Concentration [μM] | Kₐ [m⁻¹ s⁻¹] | Total crocin oxidized/catalyst |
|------------------|-------------------|---------------|-------------------------------|
| CuII,ZnSOD      | ≤12               | 5.1          | 4.7                           |
| MnIII/SOD       | ≤15               | 10.9         | 2.0                           |
| Cu²⁺            | ≤4                | 41           | 9.9                           |
| Ferricyanide     | ≤50               | 10.7         | 3.9                           |
| oxyMb           | 10                | ND           |                                |
| oxyHbA          | 10                | ND           |                                |
| FMN/light        | ≤0.4              | 4,700        | 41                            |
| Cyt c oxidase    | ≤0.014            | 460          |                                |
| Glucose oxidase  | ≥0.5              | 43           |                                |
| I₂ (in 5 min KI)| ≤0.6              | 410          | 22                            |
| DNIC             | ≤3                | 31           | 2.4                           |
| HNO₂ (pKₐ = 3.4)| ≤5                | 4.1          |                                |

FMN (Fig. 3C) and the photo-excited FMN showed saturation by relatively low concentrations of (bi)sulfite (Fig. 3D).

**Oxidation of deoxyMb and deoxyHb by STAR—**The reaction of STAR with deoxyMb was measured in the absence of O2 and O₂-derived radicals (i.e. ‘SO₃⁻ and ’SO₄²⁻). FMN/light and (bi)sulfite were used to deplete O2 from buffers, reduce trace-containing metMb and produce a homogeneous deoxyMb spectrum (Fig. 4A). Under these conditions, light-generated FMNH² reduced metMb, and STAR, faster than STAR oxidized deoxyMb. STAR, generated by adding MnSOD under low lux, oxidized deoxy Mb by ~70% after 60 s, and GSH inhibited...
FIGURE 5. STAR oxidation of deoxyHbA. DeoxyHbA (10 μM heme) spectra were recorded at 5-s intervals following three successive additions of MnSOD (12 μM) to generate STAR (arrows). Inset, the percentage of deoxyHb oxidized to methHb was calculated from the change in absorbance at 430 nm with the successive additions of MnSOD and STAR generation (arrows). The reactions were in 1 ml of a 20-fold dilution of buffer A, with 5 mM sodium bisulfite, and 1 μM FMN. Reaction buffer was deoxygenated with 1 min of preillumination as described under “Experimental Procedures.” OxyHb was added to deoxyHbA (10 μM heme) spectra generated with photo-excited FMN. Differences in the values estimated using Cu,ZnSOD and FMN/light as STAR generation were in 1 ml of a 20-fold dilution of buffer A containing 5 mM glucose and depleted of O2 with 0.6 unit of glucose oxidase and 350 units of catalase prior to adding 50 μM NADH, 30 μM crocin, 5 mM (bi)sulfite, flavoHb, and initiating STAR formation with 3 μM Cu,ZnSOD.

s⁻¹ were estimated for the Ec, Ca, Re, and Sc flavoHb, respectively. 1 μM Ec flavoHb, a concentration that inhibited crocin oxidation by ~50% (Fig. 7C, compare lines 1 and 2), was tested for CO sensitivity. 10 μM CO decreased the inhibition of crocin oxidation (compare line 3 with line 2).

Reactions of STAR with Biomolecules—The rate constants for reactions of STAR with biomolecules, determined with suitable competitors, are summarized in Table 2. The values were measured for GSH, ethanol, L-Trp, ascorbate, and O2 for comparison with published values. The values for several amino acids, NAD(P)H, and phenolic molecules including the antioxidant epigallocatechin-3-gallate were measured to further elucidate STAR reactivity. L-Cys rapidly reduced Cu(II),ZnSOD (data not shown), and a rate constant was determined only for STAR generated with photo-excited FMN. Differences in the values estimated using Cu,ZnSOD and FMN/light as STAR generators may be due to specific interfering reactions. For example, FMNH⁺ formed via photo-reduction reduces O2 to O₂⁻, and O₂⁻ can diminish or enhance the apparent O2 rate constant by acting as a facile STAR reactant or STAR generator (9, 11).

FIGURE 7. STAR scavenging by flavoHb and CO inhibition. A, effects of 0, 0.16, 0.31, 0.63, 1.3, 2.5, and 5.0 μM Ec flavoHb (heme) (colored lines 1–7, respectively) on STAR-mediated crocin oxidation. The data are staggered and extrapolated (dashed black lines) for presentation. B, percentage of inhibition of crocin oxidation by [Ec flavoHb] (line 1) and [Ca flavoHb] (line 2). C, crocin oxidation was measured in the absence (line 1) or presence of 1.0 μM Ec flavoHb alone (line 2) or with 10 μM CO (line 3) or without Cu,ZnSOD addition (line 4). Duplicate data are interpolated. The reactions were at 37 °C in 1.0 ml of buffer A containing 5 mM glucose and depleted of O2 with 0.6 unit of glucose oxidase and 350 units of catalase prior to adding 50 μM NADH, 30 μM crocin, 5 mM (bi)sulfite, flavoHb, and initiating STAR formation with 3 μM Cu,ZnSOD.

\[ \cdot\text{SO}_3^- \text{ Scavenging by Globins} \]
TABLE 2
Rate constants for reactions of STAR with biomolecules

Unless otherwise indicated, measurements were made in buffer A at 37 °C as described under “Experimental Procedures.” Crocin was generally used at 15 μM; when O₂ was the competing reactant, 60 μM was used. Measured kₓ values are the means of n ≥ 3 with standard deviations of <30%.

| Reactant | Reactant concentration | kₓ | STAR generator | Competitor | Reference |
|----------|------------------------|----|----------------|------------|-----------|
| Crocin   | ≤2,000                 | 1.0 × 10⁷ | Pulse radiolysis | None       | Ref. 22   |
| GSH      | ≤2,000                 | 8.9 × 10⁶ | Cu,ZnSOD       | Crocin     |           |
|          | ≥2,000                 | 9.6 × 10⁶ | FMN/light      | Crocin     |           |
| Ethanol  | 10⁶                    | 2.3 × 10⁸ | pulse radiolysis | Crocin     |           |
|          | 10⁶                    | 2.2 × 10⁸ | FMN/light      | Crocin     |           |
| Ascorbate| ≤1,000                 | 7.4 × 10⁶ | Cu,ZnSOD       | Crocin     |           |
|          | 200                    | 1.9 × 10⁸ | pulse radiolysis | Crocin     |           |
|          | 5,000                  | 0.9–3.1 × 10⁷ | flash photolysis | None       | Ref. 10   |
| O₂       | 200                    | 1.0 × 10⁵ | Cu,ZnSOD       | Crocin     |           |
|          | 500                    | 4.3 × 10⁵ | FMN/light      | Crocin     |           |
|          | 140–660                | 1.1–2.3 × 10⁹ | pulse radiolysis | None       | Ref. 12   |
| NADH     | ≤400                   | 1.9 × 10⁵ | Cu,ZnSOD       | Crocin     |           |
|          | 400                    | 4.4 × 10⁵ | FMN/light      | Crocin     |           |
| NADPH    | 400                    | 2.2 × 10⁵ | Cu,ZnSOD       | Crocin     |           |
|          | 400                    | 1.9 × 10⁵ | FMN/light      | Crocin     |           |
| Cyt c(II)| ≤10                    | 4.4 × 10⁶ | Cu,ZnSOD       | NADH       |           |
| l-Cys    | ≤2,000                 | 1.7 × 10⁶ | FMN/light      | Crocin     |           |
| l-Trp    | 500                    | 2.4 × 10⁶ | Cu,ZnSOD       | Cyt c(II)  |           |
| l-Met    | 1,000                  | 9.6 × 10⁵ | pulse radiolysis | None       | Ref. 86   |
| l-His    | 5,000                  | 6.4 × 10⁵ | Cu,ZnSOD       | Cyt c(II)  |           |
| Epi      | ≤600                   | 2.8 × 10⁵ | Cu,ZnSOD       | Cyt c(II)  |           |
|          | 600                    | 1.9 × 10⁵ | FMN/light      | Crocin     |           |
| EGCG     | ≤200                   | 3.2 × 10⁵ | Cu,ZnSOD       | Crocin     |           |
|          | ≤200                   | 8.9 × 10⁴ | FMN/light      | Crocin     |           |

(44), was employed to explore the capacity of a globin for STAR scavenging detoxification in a cellular milieu rich in STAR-reactive GSH, thiols, NAD(P)H, and varied O₂.

We were unable to observe selective growth inhibition of a Yhb1p-deficient yeast mutant relative to its isogenic parent by adding (bi)sulfite to cultures (data not shown) perhaps because of multiple mechanisms for sulfite removal (e.g. cytosolic sulfite reductase and sulfite efflux pumps) and toxicity (e.g. sulfitolysis, nucleophilic additions, and extracellular radical chains). Nevertheless, the Δyhb1 strain was observed to grow slower and to lower densities than its isogenic parent following glucose depletion and the shift to oxidative metabolism (Fig. 8, red square; compare dashed lines with solid lines), suggesting STAR-mediated damage to mitochondria. Lower culture aeration and a lower [O₂] (black square, partially relieved the growth inhibition of the mutant (dashed line), demonstrating an O₂-dependent toxicity. In support of sulfite/STAR involvement, supplementation of cultures with sulfide (white square), a metabolic precursor to mitochondrial sulfite (41, 42), further impaired the growth of the Yhb1p-deficient mutant (dashed line) but not the parent (solid line). Neither aeration (black circle) nor sulfide (white circle) affected the growth of the Yhb1p-deficient strain, as observed after 7 h of glucose-limiting growth (red square), when sufficient glucose was provided for fermentation (compare dashed lines with solid lines), further demonstrating critical damage by STAR, or its metabolites, to oxidative metabolism.

In a limited search, two critical sites of damage to oxidative metabolism were identified. At moderate culture aeration achieved at 200 rpm (Fig. 8B, top panel), the yhb1Δ strain expressed ~50% lower aconitase activity than the parent strain (Fig. 8B, top panel), whereas NADP⁺-IDH activity, measured as a control, was unaffected (Fig. 8B, bottom panel). Further, aconitase was activated by ~50% following 20 min of culture stasis and respiratory-induced anoxia (open bars), thus demonstrating the accumulation of a large fraction of reactivatable [3Fe–4S]− containing aconitase in the Δyhb1 strain, similar to that observed with O₂ stress (35, 45). Aconitase activity differences...
were less, or negligible, in cells grown under hypoxic conditions (≤150 rpm). Unexpectedly, however, respiratory-induced anoxia caused aconitase activity losses in cells grown under hypoxia, and greater losses were observed in yhb1Δ cells. Moreover, IDH also showed activity losses following hypoxic growth and respiratory-induced anoxia (Fig. 8B, bottom panel), albeit less marked (≤30%), suggesting hypoxic mechanisms for STAR toxicity.

Sensitivity of Aconitase and IDH to STAR and Sulfonates—STAR, generated by MnSOD or Cu²⁺-catalyzed (bi)sulfite oxidation, did not directly inactivate aconitase in the coupled NADP⁺-IDH assay (data not shown). Nor was aconitase inactivated by S-sulfoglutathione, a product of STAR and GSH (see below), but rather aconitase was activated (Fig. 9A, line 1). In contrast, GSH caused progressive aconitase inactivation (line 2) presumably because of enhanced O₂ sensitivity. Similar effects of GSH on aconitase stability have been reported for NO (46). The data suggest indirect mechanisms for STAR-mediated damage to aconitase within the yhb1Δ strain involving O₂⁻ (35, 45).

The product of the rapid reaction of STAR and NADPH, sulfo-NADPH (see below), competitively inhibited the yeast NADP⁺-IDH activity with respect to NADP⁺ as measured during the initial 2 min of catalysis (Fig. 9B). The extract activity showed an apparent $K_m$ (NADP⁺) of ~5 μM and was inhibitable to ~50% with an estimated $K_i$ (sulfo-NADPH) of ~10 μM, suggesting a sensitivity of either the mitochondrial, or the cytosolic, NADP⁺-IDH isozyme to sulfo-NADPH. Following 20 min of catalysis, inhibition by sulfo-NADPH (Fig. 9C, line 1) was not reversed by adding a competitive excess of NADP⁺ (line 2), thus revealing a progressive or suicide inactivation of IDH by sulfo-NADP and supporting a role for STAR in the loss of IDH activity within yeast.

Discussion

Reduced globins reacted rapidly with STAR. Reaction rate constants ranged over nearly 3 orders of magnitude from $3.8 \times 10^{-7} \text{M}^{-1} \text{s}^{-1}$ for deoxyMb to a remarkable $2.1 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$ for Ec flavoHb, a value exceeding that for NO dioxygenation by ~9-fold (47, 48). We can use the conventional hemispherical formula to approximate the diffusion limit for the reaction,

$$k_{ox} = 2\pi a D_{\text{STAR,37}}(N/1000) \quad \text{(Eq. 1)}$$

where $\alpha$ equals the ~14 Å radius of a positively charged cavity for STAR capture near the heme formed by Lys-11, Arg-134, Arg-184, Lys-373, Lys-381, His-386, His-393, and Lys-394 (Protein Data Bank code 1GVH) plus the radius of STAR ($D_{\text{STAR,37}} = 5.5 \times 10^{-12} \text{cm}^2 \text{s}^{-1}$) for NO₃⁻ (49), a ~0.2 Å smaller isomorphic trigonal planar anion, in place of an unknown value for STAR, we calculate $k'_{ox} = 1.5 \times 10^{-10} \text{M}^{-1} \text{s}^{-1}$, a value not too far from the value measured. As pointed out by Chou and Jiang (50) and Chou and Zhou (51), the equation ignores diffusion along the protein surface from >180° and electrostatic forces in flavoHb, the cavity is complex with projections and crevices and a net charge of approximately +4. The cavity, or short channel, may also serve in the efflux of the product SO₃⁻ and the ‘NO dioxygenation product NO₅⁻ (38, 52).

CO inhibited STAR reduction by deoxyMb and Ec flavoHb (Figs. 1E and 7C). STAR, with a dismutation rate constant of ~5.5 × 10⁸ M⁻¹ s⁻¹ (1, 10), and high reactivity with crocin, did not accumulate to sufficient levels to outcompete 10–20 μM CO with respective $k_{ox}$ values of 0.5–2 × 10⁶ M⁻¹ s⁻¹ for ferrous Mb and Ec flavoHb (47, 48). In further support of binding reduction of STAR by the ferrous iron, deoxyHbA in a tense conformation with subunits bearing predominantly out of plane iron (53, 54) largely resisted oxidation by STAR (Fig. 5). Only the smaller fraction of deoxyHbA subunits with an in-plane iron, similar to that in Mb, appear accessible to STAR binding reduction. O₂ binding also apparently inhibited the STAR reaction; heme oxidation and STAR scavenging was only observed with the deoxy forms. Together, the results suggest a mechanism for globin-catalyzed STAR reduction (Scheme 1) in which ferric heme is reduced by 1e⁻⁻ (Reaction 1), ferrous heme transiently interacts with STAR (Reaction 2), reduces STAR to

![SO₃⁻ Scavenging by Globins](image-url)
sulfite (Reaction 3), and releases sulfite (Reaction 4). In the catalytic cycle, O₂ or CO can bind the ferrous heme and inhibit the STAR reductase activity. Indeed, the linear rates of crocin bleaching inhibition observed with low [flavoHb] (Fig. 7) demonstrate enzymatic turnover. We suppose that turnover would be limited by heme reduction (Reaction 1) as described for nitric-oxide dioxygenase catalysis (48, 52).

The rate constants reported in Table 1 describe methods for generating STAR but also provide a basis for modeling STAR generation within cells. Low \( K_{m} \) (20 \( \mu M \)) and large \( k_{cat}/K_{m} \) (\( \sim 2 \times 10^{6} \) \( M^{-1} \) \( s^{-1} \)) values reported for sulfite oxidase (55) suggest a slow steady-state [sulfite] and low rate of STAR formation in most cells under normal conditions. For example, we can estimate that the \( \sim 2.5 \) \( \mu M \) of cyt c oxidase measured in chicken liver mitochondria reacting with 20 \( \mu M \) (bi)sulfite with a bimolecular rate constant of 460 \( M^{-1} \) \( s^{-1} \) (Table 1), or \( \sim 10,000 \)-fold less efficiently than sulfite oxidase, would generate STAR at a relatively low rate of \( \sim 1.4 \) \( \mu M \) \( min^{-1} \). However, the availability of co-substrate cyt c(III), molybdenum cofactor (43), and inhibitory anions (e.g., chloride, \( K_{i} = 4 \) \( mM \), and phosphate (55), and sulfite biosynthesis or influx rates must be considered when approximating the sulfite removal capacity of sulfate (55), and sulfite biosynthesis or influx rates must be considered when approximating the sulfite removal capacity of sulfate.

The rate constants for reactions of STAR with several molecules were measured to validate the kinetic competition methods and to evaluate the STAR reactivity of globins. Rate constants determined for GSH, ethanol, and L-Trp were similar to those previously reported (Table 2). However, ascorbate and O₂ values differed beyond that expected for small pH and temperature differences and require resolution. The \( \sim 10 \)-fold smaller ascorbate rate constant (12, 23) may be partly resolved by accounting for the back reaction, namely ascorbyl radical reduction by (bi)sulfite. The \( \sim 100 \)-fold lower O₂ value we report is particularly significant because the STAR scavenging detoxification capacity of a globin becomes feasible at physiological [O₂]. The larger rate constant for O₂ was initially derived from models of multiple reactions and data fitting including the smaller ascorbate rate constant and with other cited possible complications (12) that may have produced an overestimate. Subsequent O₂ rate constants determined using large \( \gamma \) rays to generate \( >10^{-4} \) \( M \) STAR and to measure O₂-enhanced STAR decay rates (14, 56) have also produced overestimates. Thus, \( \cdot O_{2} \) generation from e−(aq) and O₂ in a favorable competition with \( N_{2}O \) (57), and the rapid reaction of \( \cdot O_{2} \) with STAR (9) (\( k_{11} = \sim 4 \times 10^{10} \) \( M^{-1} \) \( s^{-1} \)) (11) would have rapidly depleted O₂ (58) and dominated the O₂-dependent component of STAR decay. In addition to the data in Table 2, we can estimate a rate constant of \( \sim 4 \times 10^{8} \) \( M^{-1} \) \( s^{-1} \) for the back reaction of STAR with the Cu(I),ZnSOD monomer from the net rate of Cu(II),ZnSOD reduction by (bi)sulfite where the theoretical rate at 0.5 \( mM \) Cu(II),ZnSOD is 25.5 \( \mu M \) \( s^{-1} \) using a \( k'_{apparent} \) of 5.1 \( M^{-1} \) \( s^{-1} \) (Table 1) and where rates of 0.05 \( \mu M \) \( s^{-1} \) and 8.3 \( \mu M \) \( s^{-1} \) are measured in the absence and presence of 5 \( mM \) GSH, respectively (Fig. 2A).

STAR is a moderately strong oxidant (Fig. 10A) and can form radical adducts (1). A consensus STAR reduction potential of \( +0.73 \) V was reported for neutral pH (59). Fig. 10B shows a summary plot of the log of rate constants versus reduction potential differences for reactions of STAR. The hypothetical dashed blue line represents the linear relationship expected for barrier-free electron transfer and highlights the large \( \Delta E^{'} \) required for a bona fide diffusion-limited scavenger. FlavoHb with a reported heme reduction potential of \( \sim 0.125 \) V (60) showed a 4–8-fold larger rate constant than Ngb at \( \sim 0.129 \) V (61), which may be due to hindrance of STAR access by the distal His E7 \( Fe^{2+} \) ligand. The analysis also reveals an ascorbate rate constant more consistent with a calculated \( \Delta E^{'} = +0.448 \) V. On the other hand, the analysis (Fig. 10B) shows that STAR reacts too fast with GSH, L-Cys, NAD(P)H, and crocin for an electron transfer. We suppose that the reactions with thiols and NAD(P)H proceed via inner sphere bonding forming a sulfo adduct and reducing anion radical (Reaction 1) (15) that is oxidized by O₂ to form a sulfonate (Reaction 2). Indeed, STAR conjugates with GSH or NADPH in the copper-catalyzed synthesis of S-sulfoglutathione (36) and a sulfo-NADP⁺, respectively. Conversely, bonding of STAR to the polyunsaturated crocin molecule generates a sulfo adduct and an oxidizing radical (Reaction 3). Reduction by sulfite would generate another STAR (Reaction 4) and may partly account for the >1:1 stoichiometry of crocin oxidized measured for Cu(II),ZnSOD, Mn(III)SOD, Cu²⁺, ferricyanide, and dinitrosyl iron complex under the O₂-depleted conditions of the STAR generation assay (Table 1). A zero sum STAR reaction is consistent with the negligible effect of crocin on the O₂-depleting free radical chain oxidation of sulfite (data not shown).

\[
\begin{align*}
\text{RH} + \cdot \text{SO}_3^- & \rightarrow R\cdot\text{SO}_3\text{H}^- \\
R\cdot\text{SO}_3\text{H}^- + O_2 & \rightarrow R\cdot\text{SO}_3^- + O_2^- + H^+ \\
R + \cdot \text{SO}_3^- & \rightarrow R\cdot \text{SO}_3^- \\
R\cdot\text{SO}_3^- + \text{SO}_3^- + H^+ & \rightarrow \text{RHSO}_3^- + \cdot \text{SO}_3^- \\
\end{align*}
\]

Reactions 1–4

While the kinetic data argue for STAR formation and toxicity and STAR scavenging by globins within cells, yeast offered a tractable cell model for exploring STAR formation, toxicity and the capacity of a globin for STAR detoxification. S. cerevisiae express an ‘NO-inducible flavoHb, Yhb1p (62), with an established nitric-oxide dioxygenase activity (34, 47) and ‘NO detoxification function (44, 63) and an undefined protective activity against O₂ and oxidative stress (44, 64, 65). Within yeast, Yhb1p localizes to the cytosol and mitochondria (44) at \( \sim 5 \mu M \).
Although yeast have the capacity to detoxify sulfite via a cytoplasmic sulfite reductase and an inducible sulfite efflux pump that is co-regulated with Yhb1p (24, 62), yeast uniquely lack the mitochondrial sulfite reductase and an inducible sulfite efflux pump (36, 37). Nonfermentative mitochondria-dependent growth of most yeast strains amounts to significant sulfite formation (66). Increased STAR formation, and STAR-mediated mitochondrial damage as a consequence of basal mitochondrial (bi)sulfite, increased STAR formation, and STAR-mediated toxicity (Scheme 1). For Ngb, reducing systems (e.g. cyt b5) would appear adequate, and O2 inhibition would appear minimal because <12% of ferrous Ngb binds O2 at the low retinal O2 concentrations (69, 70). Further investigations of STAR generation, STAR reactivity, and STAR metabolite (e.g. sulfoneNAD(P), S-sulfoglutathione, and S-sulfocysteines) formation and interactions will be required to fully understand STAR toxicity and the STAR detoxification function of globins.

Author Contributions—P. R. G. coordinated the experiments, collected data, and wrote the paper. D. P. G. designed experiments for measuring STAR formation and scavenging and collected data for Figs. 2, 3, and 10 and Tables 1 and 2. A. P. G. prepared mitochondrial membranes, designed and performed experiments describing cyt c oxidase activities and Ngb reduction, and acquired data for Tables 1 and 2 and Figs. 6 and 10. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE 10. Redox potentials and STAR reaction rate constants. A, bisulfite and sulfate are oxidized to STAR. STAR is reduced to sulfite through an electron transfer or to bisulfite via an H-atom abstraction. B, STAR rate constants (kcat) determined here (red squares) or previously reported (black circles) are plotted on a log scale against redox potential differences (ΔE°) where a STAR reduction potential of +0.73 V is applied. Multiple rate constant estimates are grouped (blue dashed line). The hypothetical dependence of log kcat on ΔE° is shown by the dashed blue line. The values highlighted by the red box exceed the expected values. The reduction potentials (relative to NHE) applied are: NAD (NAD/P)/H (0.138 (71); l-methionine radical (Met), +1.19 (72); l-histidine radical (His), +1.17 (73); L-tryptophan radical (Trp), +1.05 (74); 1-hydroxyethyl radical (EtOH), +0.98 (75); l-glutathionyl radical (GSH), +0.92 (76); l-cysteinyl radical (Cys), +0.92 (77); crocin radical, +0.74 (78); epinephrine radical (Epi), +0.345 (79); ascorbyl radical (Asc), +0.282 (80); cyt c (red line), +0.254 (81); l-epigallocatechin-3-gallate radical (EGCG), +0.43 (82); metHbA (HbA), +0.144 (83); Cu2+,ZnSOD (SOD), +0.102 (84); metMb (Mb), +0.046 (85); EC, Re, Sc, and Ca flavoHbs (flavoHb(s)), −0.125 (60); and metNgb (Ngb), −0.129 (61).

SO2−3 Scavenging by Globins

Although yeast have the capacity to detoxify sulfite via a cytoplasmic sulfite reductase and an inducible sulfite efflux pump that is co-regulated with Yhb1p (24, 62), yeast uniquely lack the molybdenum cofactor (43) and a mitochondrial sulfite oxidase and are thus expected to experience elevated mitochondrial (bi)sulfite, increased STAR formation, and STAR-mediated mitochondrial damage as a consequence of basal mitochondrial sulfur amino acid and sulfide metabolism to sulfite, which in most yeast strains amounts to significant sulfite formation (66). Indeed, nonfermentative mitochondria-dependent growth of the Yhb1p-deficient mutant strain was impaired, and metabolic loading of mitochondria with sulfite, through sulfite supplementation, selectively decreased mutant growth (Fig. 8A). Formation of SO2−3 from the reaction of STAR with O2 and formation of O2−2 (9) (Reactions 1 and 2), HSO3−, and SO2−3 from the reactions of STAR, SO2−3, and HSO3− with cellular reductants provide one mode for STAR toxicity that can explain the greater growth inhibition of yhb1Δ cells exposed to a greater [O2]. Although appearing as a small and indirect measure of STAR toxicity, the 25–50% inactive fraction of aconitase measured in yhb1Δ cells indicates large 5–10-fold increases in mitochondrial O2−2 levels (35, 45) and severe oxidative stress. Formation of sulfonates in reactions with STAR with NAD(P)/H (Reactions 1 and 2) and inhibition inactivation of NADP+/IDH and other dehydrogenases by sulfo-NAD(P)/H by hypoxia and other low NAD(P)+ conditions provide an additional mode for STAR toxicity and increased O2−2 generation.

The data in Table 2 and a rate equivalence of kox(Ngb)[Ngb] [STAR] to kox(GSH)[GSH][STAR] or kox[ascorbate][ascorbate] [STAR] allow us to estimate a STAR scavenging capacity for the ~100 μM Ngb expressed in the light-susceptible retinal rod cell inner segment and mitochondria (67, 68) well beyond that of physiological [GSH] and equal to ~2 mM ascorbate. Similar approximations can be made for all globins but estimates must consider globin oxidation and reduction rates and O2 competition (Scheme 1).
‘SO\textsubscript{3}\textsuperscript{−}’ Scavenging by Globins

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