Cytoglobin (Cygb) was investigated for its capacity to function as a NO dioxygenase (NOD) in vitro and in hepatocytes. Ascorbate and cytochrome b₅ were found to support a high NOD activity. Cygb-NOD activity shows apparent second order rate constants of 1000 M⁻¹ s⁻¹ and 3 × 10⁶ M⁻¹ s⁻¹, respectively. In rat hepatocytes engineered to express human Cygb, Cygb-NOD activity shows a similar k_{cat} of 1.2 s⁻¹, a K_{m}(NO) of 40 μM, and a k_{cat}/K_{m}(NO) (K’_{NOD}) value of 3 × 10⁷ M⁻¹ s⁻¹, demonstrating the efficiency of catalysis. NO inhibits the activity at [NO]/[O₂] ratios > 1:500 and limits catalytic turnover. The activity is competitively inhibited by CO, is slowly inactivated by cyanide, and is distinct from the microsomal NOD activity. Cygb-NOD provides protection to the NO-sensitive aconitase. The results define the NOD function of Cygb and demonstrate roles for ascorbate and cytochrome b₅ as reductants.

The functions of cytosolic O₂-binding hemoglobins in eubacteria, archaea/bacteria, yeast, fungi, plants, nematodes, protozoa, and mammals have remained largely enigmatic (1, 2). The discovery of a nitric-oxide dioxygenase (NOD)³ (EC 1.14.12.17) function for the inducible Escherichia coli flavohemoglobin suggested a primal and common function for hemoglobin and their associated reductases (3). Indeed, evolutionarily distant globins catalytically dioxygenate NO with high fidelity (4, 5). For a number of cytosolic hemoglobin, including the mammalian Cygb and Ngb, function remains largely unsettled (9–11).

Measurements of the NO reactivities of the oxy-complexes of Ngb and Cygb in vitro (12, 13) support a NOD function. However, O₂ transport-storage (14, 15), peroxidase (16, 17), oxidase (18), heterotrimeric Gα protein guanine nucleotide dissociation inhibitor (19), and other functions (11, 20, 21) have also been postulated, and it remains unclear which of the proposed functions is physiologically significant and under what circumstances. For example, several NO metabolic activities and sinks have been measured in various cells and organelles (22–24) that would make a NOD activity appear either redundant or inconsequential. In addition, measurements of NO metabolism by globins with autooxidizable electron donors in the absence of SOD (13) do not allow the discernment of catalytic NO dioxygenation from NO oxidation by O₂⁴.

Understanding globin function(s) continues to demand a synthesis of knowledge of structure, ligand affinities, reactivities, autooxidation rates, and electron donors. Moreover, to assess the NOD function of a globin, knowledge of its interactions with NO and O₂ within the cellular milieu is essential. Recently, Cygb-dependent NO dioxygenation and protection of NO-sensitive respiration within mouse NIH3T3 fibroblasts was reported (25), yet the relative capacity of Cygb for a NOD function with NO, O₂, and cellular reductant(s), as well as its relation to other cellular NO sinks, remains to be defined.

Here we describe interactions of Cygb with NO, O₂, and reductants that govern its capacity to function as a NOD in vitro and in rat hepatocytes. Investigations of the NOD activities of Cygb, Ngb, and Mb with ascorbate reveal a uniquely efficient redox coupling with Cygb that may be attributed to a unique Cygb structure. In addition, we report that the Cygb-NOD activity is physically and kinetically distinct from the microsomal NOD activity (23, 26, 27).

MATERIALS AND METHODS

Reagents—L-Ascorbic acid, sodium citrate, glucose, NADP⁺, NADPH, DPI, Me₃SO, sodium cyanide, 98.5% NO, Brilliant Blue G, recombinant human CYPOR (27 units/mg), spinach ferredoxin-NADP⁺ oxidoreductase (3.9 units/mg), porcine heart isocitrateg dehydrogenase, horse heart cytochrome c, Aspergillus niger glucose oxidase, bovine serum albumin, EDTA, HEPEs, Tris, glycine, dithiothreitol, chloramphenicol, and ampicillin were from Sigma-Aldrich. Sodium diphosphate and sodium monophosphate were obtained from Fisher. NADH, bovine liver catalase (260,000 units/ml), bovine pancreas deoxyribonuclease I (2000 units/mg), bovine erythrocyte...
copper- and zinc-containing SOD (5000 units/mg), and nitrate reductase (10 units/mg) were obtained from Roche Applied Science. DNA restriction and modifying enzymes were obtained from New England Biolabs, Inc. DNA primers, G418, and SeeBlue™ prestained protein molecular weight standards were purchased from Invitrogen. AG 1-X8 ion exchange resin (acetate form) was purchased from Bio-Rad. 99.993% O₂, 99.999% CO, 99.998% N₂, and 99.99% argon were from Praxair (Bethlehem, PA). Recombinant human NCB5OR (29) was provided by Drs. H. Zhu and F. Bunn (Brigham and Women’s Hospital, Boston, MA). Sperm whale Mb (4, 30) was obtained from Dr. J. Olson (Rice University, Houston, TX). E. coli MnSOD (2400 units/mg) was supplied by Miami Valley Biotech (Dayton, OH). Microsomes containing 10 mg/ml of protein and 70 milliunits/mg of membrane-bound CYPOR activity were isolated from Caco-2 cells (23).

Expression and Purification of Cygb and Ngb—Human Cygb cDNA Image clone 5193583 (ATCC number 7498923) was obtained from the American Type Culture Collection (Manassas, VA). Cygb cDNA was PCR-amplified with the respective sense and antisense primers GGAGCTGCATATGGAGA and CCTCAAGCTTCTTGCCAGAATGCAGCCAGCAGGA and CCTCAAGCTTCTTGCCAGAATGCAGCCAGCAGGA and were expressed and isolated from E. coli BL21(DE3)pLysS. Mouse Ngb cDNA in pET3A was provided by Dr. Thorsten Burmester and Thomas Hankeln (Johannes Gutenberg University of Mainz, Mainz, Germany) and was expressed and isolated from E. coli BL21(DE3)pLysS (31). E. coli expressing isopropylthio-β-D-galactoside-inducible globin were inoculated in 2 liters of Luria-Bertani medium (32) containing 50 μg/ml ampicillin, 1 μM hemin, and 5 units/ml catalase in 2.8-liter Fernbach flasks and grown in a 37 °C gyrorotatory shaker at 175 rpm. Globin expression was induced with 0.1 mM isopropylthio-β-D-galactoside when the cell density, as measured by absorbance at 550 nm, was 1.2. The cells were treated with isopropylthio-β-D-galactoside for 16 h and were harvested by centrifugation. The cell pellets were measured with an analytical balance.

Construction of Cygb-expressing Hepatocytes—The 995-bp EcoRI-XbaI fragment of cytoglobin cDNA Image clone 5193583 was isolated and ligated into the EcoRI-XbaI-restricted polylinker region of pcDNA3 v. 1.1 (Invitrogen) generating pcDNA3-hCygb. Rat hepatocytes were transfected with pcDNA3-hCygb or pcDNA3 DNA using FuGENE™ 6 transfection reagent (Roche Applied Science), and stable transfectants were selected with 0.3 mg/ml G418. The clones were isolated and screened for Cygb expression by Western blot analysis.

NO Consumption Assays—NO consumption was measured with ISO-NOP and microchip NO electrodes (WPI Instruments, Inc.) as previously described (34). NO scavenging activities of globins were routinely measured at 37 °C in 2 ml of 100 mM sodium phosphate buffer containing 0.3 mM EDTA and 1.0 mg of MnSOD (11 μM of the dimer). MnSOD was routinely added to NO consumption assays to competitively scavenge interfering O₂ generated by reductants and reductases (34). Copper- and zinc-containing SOD reacted with NO and was thus unsuitable. Cell NO metabolism was measured at 37 °C in Dulbecco’s phosphate-buffered saline containing 5 mM glucose and 100 μg/ml cycloheximide (27, 36). NO consumption measurements were corrected for background rates. NO-saturated water (1.94 mM) was prepared over AG 1-X8 resin as previously described (34). CO-saturated water (1.0 mM) was prepared under 99.5% CO (34). O₂-saturated buffer (1.14 mM) was prepared, and O₂ concentrations were varied in reactions as previously described (27, 34, 36). O₂ was depleted from buffers by bubbling with 99.99% argon and by preincubating for 5 min with 4 units of glucose oxidase and 260 units of catalase. O₂ concentration was measured with an O₂ electrode (YSI Co., Yellow Springs, OH). [O₂] was 200 and 260 μM in air-saturated buffers at 37 and 20 °C, respectively. Buffer conductivity was measured at 37 °C with an inductive conductivity monitor (GE Healthcare).

Nitrate and Nitrite Assays—Nitrate was reduced to nitrite with NADPH and nitrate reductase. Nitrite was assayed using the Griess reagent (34).

Globin Reduction Assays—Reduction of ferric Cygb, Ngb, and Mb was measured by following the formation of the ferrous-CO complex at 422, 416, and 422 nm, respectively, in a 1-cm thermostatted quartz cuvette at 37 °C. The reactions were

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Mammalian Cell Culture—The rat hepatocyte, K9 (CRL-1439), the human lung adenocarcinoma A549 (CCL185), and the human colorectal carcinoma Caco-2 (HTB-37) were obtained from the American Type Culture Collection. The cells were grown, passaged, and harvested as previously described (27). The cells were counted with a hemacytometer, and the weights of the cell pellets were measured with an analytical balance.

Western Blot Analysis—The cells were lysed with an equal volume of detergent buffer containing 20 mM Tris-Cl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1% Triton X-100. The extract protein was assayed (33), separated by reducing SDS-PAGE in 1.0-mm Precast 8–16% gradient gels (Invitrogen), and transferred to nitrocellulose membranes. The membranes were washed, blocked with 5% milk, probed with rabbit anti-Cygb antibodies (15, 35), and detected using peroxidase-conjugated goat anti-rabbit IgG (Pierce) and the SuperSignal® West Dura extended duration substrate kit (Thermo Scientific) on Kodak X-Omat™ film. Cygb was measured by Western analysis and densitometry with purified human Cygb standards.
initiated by adding 10 μM of globin (heme) to an anaerobic 1-mL
reaction mix containing buffer, 20 μM CO, and the indicated
concentrations of ascorbate. O$_2$ was depleted from reactions by
scrubbing the reaction mix with N$_2$ and reacting 5 mM glucose
with 2 units of glucose oxidase and 260 units of catalase for 10
min prior to adding 4–8 μL of the concentrated globin.

**Cell NO Exposures and Aconitase Assays**—Freshly harvested
K9neo and K9Cygb hepatocytes were resuspended at a density
of 3.2 × 10$^6$ cells/mL in 3-mL of serum-free F12K medium buf-
tered to pH 7.4 with 50 mM sodium HEPES and containing 100
μg/mL cycloheximide. O$_2$, N$_2$, and NO gas mixtures were deliv-
ered at 30 mL/min using three-way gas proportioners (27). The
cells were harvested, and the extracts were assayed for aconi-
tase activity and protein as previously described (27).

**Data Analysis**—The Tukey-Kramer (Honestly Significant
Difference) statistical analysis method in the program JMP
(SAS Institutes Inc.) was used for the analysis of significance
($p < 0.05$). The data presented are representative of the results
of two or more trials.

**RESULTS**

**Ascorbate-driven NO Metabolic Activity of Cygb, Ngb, and
Mb**—Ascorbate, a potential electron donor for plant hemoglo-
bins (37, 38) and Mb (39), was investigated for its ability to
support enzymic NO scavenging by Cygb, Ngb, and Mb. With
10 μM ascorbate, the enzymic NO scavenging activity is linearly
dependent upon globin concentration. Human Cygb shows a
turnover rate of 0.25 NO/heme/s with 100 nM NO at 37 °C (Fig.
1A, ●). An ∼4-fold slower turnover is seen at 20 °C (Fig. 1A, ▲).
Mouse Ngb (Fig. 1B, ●) and sperm whale Mb (Fig. 1C, ●) show
respective 150- and 70-fold lower activities than Cygb.

The Cygb activity shows an apparent $K_m$(ascorbate) value of
2 mM (Fig. 2A), an apparent $K_m$ for NO of 40 nM, and $k_{cat}$ of 0.5
s$^{-1}$ with saturating ascorbate and O$_2$ in neutral phosphate
buffer (Fig. 2B). With 100 nM NO, half-maximal activity is seen
at ∼20 μM O$_2$ (Fig. 2C). Inhibition of the activity occurs at
[NO]/[O$_2$] ratios >1:500 (see below), thus complicating the
determination of a $K_m$(O$_2$) value. Similar to other NOD activi-
ties (3, 23, 27, 40), the activity is inhibited by CO and inactivated
cyanide. With 20 μM O$_2$, 10 μM CO inhibits the Cygb activity by
∼50% (Fig. 2D). NO progressively inhibits the activity during
turnover with 400 nM NO and 200 μM O$_2$ (Fig. 2E, ●), and the
activity is slowly inactivated by 250 μM cyanide (Fig. 2E, ○).
Preincubation of Cygb with cyanide for 7 min does not cause a
greater loss of activity (Fig. 2E, compare ○ and △), thus demon-
strating the importance of turnover for cyanide-mediated
inactivation.

The results demonstrate $K_m$ values for NO and O$_2$ within phys-
ologically relevant concentration ranges. However, the $K_m$(ascor-
bate) value of 2 mM is greater than the $K_m$ values of 0.4–0.9 mM
reported for ascorbate-utilizing enzymes (41, 42), and the ascob-
tate concentrations measured in some tissues (43).

**Anions Competitively Inhibit Activity**—Salts were tested for
effects on the $K_m$(ascorbate) value and steady-state behavior of
Cygb. Lowering the buffer sodium phosphate concentration from
100 to 10 mM decreases the $K_m$(ascorbate) value to 0.25 mM (Fig.
3A, line 1). Salts, including potassium chloride (lines 2–4) or
sodium chloride (line 5), competitively inhibit the activity with
respect to [ascorbate]. Increasing the sodium phosphate concen-
tration or including sodium phosphate or NaCl in a 25 mM sodium
citrate buffer also competitively inhibits the activity and increases
the $K_m$(ascorbate) value (Fig. 3B). Furthermore, the competitive
effect of buffer salt depends more on the anion than the ionic
strength. Salts do not significantly affect the $k_{cat}$ achieved with
saturating ascorbate (Fig. 3A) or the $K_m$(NO) (data not shown).
The results suggest electrostatic interactions in the binding of the
negatively charged ascorbate anion.

**Cygb Reduction by Ascorbate**—The rates of ferric globin
reduction were measured under conditions similar to those for
ascorbate-driven NO metabolism (Fig. 2A). Globin reduction
was measured in the presence of excess CO and the absence of

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**FIGURE 1.** Catalytic NO metabolism by globins in vitro. Human Cygb (A), mouse Ngb (B), and sperm whale Mb (C) were measured for NO metabolic activity at 37 (●) or 20 °C (▲) in a 2-mL reaction volume containing 10 mM L-ascorbate, 100 mM sodium phosphate, pH 7.0, 0.3 mM EDTA, and 11 μM MnSOD. The reactions were initiated with 400 nM NO, and the activities were determined for 100 nM NO. The concentrations of globins are relative to heme content.

**FIGURE 2.** Cygb-catalyzed NO metabolism with ascorbate. A, Cygb activity was measured by varying concentrations of ascorbate with 200 μM O$_2$ and 200 mM NO B, activity was measured with 200 μM O$_2$ for different [NO]. C, activity was measured at different [O$_2$] with 100 nM NO. D, activity was measured with 20 μM O$_2$, 150 nM NO, and varying [CO]. E, activity was measured for 100 nM NO with no addition (●), the addition of 250 μM sodium cyanide (○), or following a 7-min preincubation with 250 μM sodium cyanide (△). All of the measurements were made with 22.5 μM Cygb (heme) at 37 °C in 100 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM EDTA, 11 μM MnSOD, and 10 mM ascorbate, unless otherwise specified. The reactions were initiated with 400 nM NO.
O₂ to stabilize the reduced heme from reoxidation. The rate of Fe²⁺(CO) complex formation was measured by following the absorbance increase at 422 nm as described under “Materials and Methods.” Under these conditions, the Cygb reduction rate shows a linear dependence upon [ascorbate] up to 8 mM (data not shown). The apparent second order rate constant for ascorbate-mediated reduction of ferric Cygb is 1.3 ± 0.2 M⁻¹ s⁻¹. The corresponding rate constants for Ngb and Mb are 0.10 and 0.13 M⁻¹ s⁻¹, respectively. The results demonstrate an ∼10-fold faster reduction rate for Cygb than for Ngb and Mb. However, these differences do not account for the larger differences in turnover rates measured in Fig. 1.

Cygb NO Metabolic Activity Produces Nitrate—Slow and gradual addition of 32 nmol of NO-saturated water (16-µl) with a gas tight syringe to a 2-ml reaction mix containing 50 mM Tris-Cl, pH 7.5, 2 mM ascorbate, 200 µM O₂, 11 µM MnSOD, and 1.0 µM Cygb at 37 °C yields 28.7 ± 1.0 nmol of NO₃⁻ and 2.8 ± 0.5 nmol of NO₂⁻ or 91 ± 3% NO₃⁻ and 9 ± 2% NO₂⁻. The results demonstrate a nitrate-generating NOD mechanism for Cygb (4, 5). However, the data also indicate secondary NO oxidation reactions, possibly representing residual reactions of O₂⁻ with NO to form peroxynitrite and NO₂⁻ (44).

Cygb-NOD Activities with NADH, NADPH, cytochrome b₅, and Cellular Reductases—Several cellular reductants and reductases with globin reducing capacity (5, 13, 45, 46) were tested for their ability to support the NOD activity of Cygb in the presence of a high [SOD].

NADH and NADPH support a low Cygb-NOD turnover that is >15-fold slower than with ascorbate under otherwise comparable conditions (Table 1), demonstrating a preference for ascorbate. Reduced cytochrome b₅ also supports the NOD activity of Cygb and shows saturation with half-maximal activity at 0.3 µM (Fig. 4A). Ferredoxin reductase and NADPH produce a low background activity in the cytochrome b₅ reducing system. CyPOR can also support a low activity of Cygb-NOD (Fig. 4B). NC5B5OR, an enzyme with a flavin-containing reductase domain and a cytochrome b₅ domain that is found in the lumen of the endoplasmic reticulum (29), also supports the NO metabolic activities of Cygb (Fig. 4C), Ngb (Fig. 4D, △), and Mb (△). Cygb shows the highest activity of the three (Fig. 4D, compare △ with □ and △). Microsomes containing membrane-bound CyPOR (14 milliunits) and incubated with 100 µM NADPH can supply electrons for the NO metabolic activity of Cygb. With 300 nM Cygb, microsomes show greater activity than that observed with NADPH alone and catalyzed by the microsomal NOD activity (Fig. 4E) (23).

The results demonstrate that, in addition to ascorbate, cytochrome b₅, and NC5B5OR, cytochrome b₅ can effectively support Cygb-NOD activity. We can estimate apparent second order rate constants for cytochrome b₅ and NC5B5OR-mediated reduction of Cygb of 3 × 10⁴ and 6 × 10⁶ M⁻¹ s⁻¹, respectively. None of the electron donors is as effective in supporting the NO scavenging activity of either Ngb or Mb, thus suggesting electron donor specificity for Cygb.

![Diagram](https://via.placeholder.com/150)

**FIGURE 4.** Cygb-catalyzed NO metabolism with cellular reductants and reductases. A, activity of 15 nM Cygb in the presence of varying concentrations of rat cytochrome b₅, 100 µM NADPH, and 20 milliunits of ferredoxin-NADP⁺ oxidoreductase. B, activity of 15 nM Cygb with varying concentrations of soluble human CyP5OR (2 milliunits/nmol) and 20 µM NADPH. C, activity of 30 nM Cygb with varying concentrations of NC5B5OR and 100 µM NADH. D, activity of 15 nM Cygb with varying concentrations of Cygb, Ngb, and Mb with 28 nM NC5B5OR and 100 µM NADH. Globin concentrations represent heme content. The reactions in A–D were in 2-ml of 100 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM EDTA, 11 µM MnSOD, and 200 µM O₂ at 37 °C. The error bars represent the S.D. of three measurements.
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**FIGURE 5. Cygb expression in cultured rat hepatocytes (K9), A549, and Caco-2 cells.** Human Cygb was measured in K9, A549, and Caco-2 cell extracts (A) and in K9neo and K9Cygb cell extracts (B) by Western blot analysis as under "Materials and Methods." The gel lanes were loaded with 200 (A) or 100 μg (B) of cell-free extract protein or the indicated amounts of human Cygb. The Cygb monomer (mono) and dimer (di) signals are labeled.

**NO Consumption and Cytoglobin Expression by Cultured Hepatocytes and A549 and Caco-2 Cells**—Cultured rat hepatocytes (K9) and human A549 lung and intestinal Caco-2 cells catalytically consume NO at 1.2 ± 0.1, 5.7 ± 0.4, and 22.6 ± 1.2 nmol of NO/min/10^7 cells (± S.D., n = 3), respectively, with 1 μM NO and 200 μM O_2. None of the cells express Cygb as measured by Western blot analysis (Fig. 5A). Human Cygb migrates as a monomer (20.9 kDa) on reducing SDS-PAGE gels. A weak signal from the disulfide-linked dimeric Cygb is detected with >25 ng of purified Cygb/lane after long film exposures. The results are consistent with immunohistochemical assays of Cygb in rat hepatocytes (15, 16).

Rat hepatocytes offered a cell model for measuring the capacity of Cygb to function as a NO scavenger. Rat hepatocytes express negligible Cygb and relatively low levels of NO metabolic activity, synthesize ascorbate (47, 48), and contain microsomal detoxification enzymes including CYPOR and cytochrome b_5. Transfection of hepatocytes with pcDNA3-hCygb expressing the human Cygb gene under control of the cytomegalovirus promoter produced several stable cell lines with elevated Cygb expression (data not shown). A representative clone, K9Cygb, was selected for further investigation. K9Cygb expressed 50 ± 10 ng of Cygb/100 μg of soluble protein, whereas the control cell line, K9neo, which was stably transfected with the pcDNA3 vector, showed no detectable Cygb protein (Fig. 5B). If we assume that ~90% of the cell weight is water, we can estimate that K9Cygb hepatocytes express 1.3 ± 0.2 μM Cygb, because the wet weight of 10^7 K9 cells is 21 ± 3 mg (± S.D., n = 3), and 10^7 cells release 1.10 ± 0.21 mg of soluble protein (± S.D., n = 3) with detergent lysis.

**NO Metabolism in K9Cygb and K9neo Hepatocytes**—K9Cygb cells show ~7-fold greater NO metabolic activity than K9neo cells with 200 nM NO and 200 μM O_2 (Fig. 6A). With 10 nM NO, the activity difference is larger. The Cygb-NOD activity is ~13-fold greater for 200 μM O_2 and ~9-fold higher at a more physiological O_2 concentration of 10 μM (Fig. 6B).

The NO metabolic activity shows an apparent K_m(NO) value of 40 nM with 200 μM O_2 (Fig. 7A). Half-maximal activity is observed with ≤5 μM O_2 with 20 nM NO (Fig. 7B, ). NO inhibits the activity at higher NO:O_2 concentration ratios precluding the determination of a true K_m(O_2) value. CO also inhibits the activity, and inhibition is also less pronounced with higher O_2 concentrations (Fig. 7B, O). Similar to in vitro reactions (Fig. 2E), the NO metabolic activity is weakly yet progressively inactivated by 250 μM cyanide during turnover (Fig. 7C, compare O with the control ). In contrast, the basal NO metabolic activity in K9neo cells is rapidly inactivated by 250 μM
cyanide (Fig. 7C, compare □ with the control ■). NO metabolism by K9Cygb cells is also insensitive to the CYPOR and flavoenzyme inhibitor, DPI. DPI (50 μM) inactivates <10% of the total activity (Fig. 7D, compare ○ with the control ●). The results demonstrate the capacity of Cygb to function as a NOD. Further, the data show that the activity is distinct from the cyanide and DPI-sensitive microsomal NOD (23, 27). The results also suggest that the cyanide-sensitive NO metabolic activity previously attributed to Cygb in NIH3T3 fibroblasts (25) is due to the ubiquitous microsomal NOD.

Sensitivity of Aconitase to NO-mediated Inactivation in Cygb-expressing Hepatocytes—Exposure of K9neo and K9Cygb hepatocytes to an atmosphere containing 480 ppm of NO balanced with 21% O2 and N2 for 60 min inactivates the NO-sensitive aconitase in rat hepatocytes. The metabolic activity of Cygb in hepatocytes is DPI-resistant, slowly inactivated by cyanide (Fig. 7C), and inhibited by NO at [NO]/[O2] ratios of >1:500 (Fig. 2F).

Our results demonstrate that ascorbate and cytochrome b5 both act as efficient electron donors for the Cygb-NOD activity and, moreover, suggest that the preference for electron donors in cells will be dependent upon their relative abundance. The low Km(ascorbate) value of 0.25–0.67 mM measured at physiological salt concentrations (Fig. 3B) argues for a role as an electron donor in ascorbate-utilizing collagen-synthesizing hepatic stellate cells and fibroblasts and ascorbate-rich neurons (15, 16). We have not measured ascorbate or cytochrome b5 levels in cultured rat (K9) hepatocytes. However, hepatocytes synthesize ascorbate and are a major source of ascorbate in rats (43, 48). Ascorbate concentrations of 6 nmol/mg of protein (~1.2 mM) and 2 mM have been measured in cultured primary rat hepatocytes (47, 48).

The Km(ascorbate) and kcatal values for the Cygb-NOD activity (Figs. 2 and 3) allow us to estimate apparent second order rate constants for Cygb reduction ranging from 125 to 1000 M⁻¹ s⁻¹ and dependent upon anion concentrations, whereas the ferric Cygb was reduced by ascorbate with an ~100-fold smaller rate constant under similar buffer conditions. The disparate rate constants indicate that ferric Cygb, per se, is not an obligate intermediate in the catalytic cycle. The rate of Cygb reduction may be greater for the high potential Fe²⁺ (OONO⁻) intermediate (4, 5) like the fast ascorbate-mediated reduction of the ferryl form of leghemoglobin (52) and Compound I or II in ascorbate peroxidase (53–55). The high reduction rate constants estimated for cytochrome b5 and NCB5OR may be similarly explained. The steady-state kinetic results suggest caution when evaluating roles of potential electron donors based solely on transient measurements of ferric globin reduction (12, 13, 45, 56, 57).

The Michaelis-Menten behavior of the Cygb-NOD activity with ascorbate and the competitive inhibition by anions (Figs. 2A and 3) strongly suggest a positively charged binding site for ascorbate. Cygb has unique invariant ArgE10–84 and LysFG2–116 residues near a solvent-exposed heme propionate carboxylate (58, 59) that may form a binding site similar to that in ascorbate peroxidase (60). In the peroxidase-ascorbate complex (Protein Data Bank code 1OA0) (Fig. 8, top panel), Arg-172 facilitates ferric heme reduction by hydrogen bonding and stabilizing ascorbate ene-diolate (53, 55, 60), and a Lys-30 amine hydrogen bonds the ascorbate 6-hydroxyl. In the dimeric Cygb structure (Protein Data Bank code 1UMO) (58), the heme propionate, ArgE10 and LysFG2 residues are located in different positions in the A and B subunits (Fig. 8, compare bottom left and bottom right panels), indicating a large conformational shift, and a possible inducible ascorbate-binding site. Moreover, the A and B subunit conformations are associated with changes between hexa- and pentacoordinate iron (58), imply-
ving communication between the iron and the putative ascorbate-binding site during catalysis. It is noteworthy that the B subunit carboxylate O-atom and LysFG2 N-atom are separated by 10.7 Å (Fig. 8, bottom right panel) as seen in the ascorbate peroxidase-ascorbate complex (Fig. 8, top panel). In the mono-meric Cygb structure (Protein Data Bank code 1V5H) (59), the ArgE10 guanidine nitrogen is 6.6 Å from the carboxylate O-atom and 8.5 Å from the LysFG2 N-atom, the latter two being only 2.9 Å from each other, further illustrating the large structural flexibility of the site.

Elevated Cygb expression in activated hepatic stellate cells, chondroblasts, osteoblasts, and hypoxic neurons (15, 16, 21, 61) suggests an important role for Cygb in the protection of these cells against NO toxicity; however, quantitative knowledge of Cygb expression levels and the capacity for NO detoxification has been lacking. We have determined that ~1.3 μM Cygb confers a NOD activity equal to ~1.6 μM NO s⁻¹ (k_{cat} [Cygb]) to cells at saturating [O2] and [NO]. This NOD activity protected the NO-sensitive aconitate by a modest ~14% during a continuous exposure of cells to ≤0.45 μM NO at 200 μM O2. It should be noted, however, that sustained [NO]/[O2] ratios of >1:500 progressively inhibit the NOD activity. Cells would require a higher [Cygb] to achieve a lower steady-state [NO] and afford greater protection. By scavenging NO, Cygb would also decrease peroxynitrite formation (44) and protect respiration (25) and enzymes important to fibrogenesis such as the prolyl 4-hydroxylase (61, 62). Cygb could also regulate the nanomolar [NO] found in tissues (63) and feedback regulate O2 delivery to tissues (8, 64). The large effects of [O2] on activity (Fig. 7B) may also explain the benefit of hypoxic induction of Cygb (15, 65).

Experiments can now be directed toward determining the roles of an ascorbate and cytochrome b5-driven Cygb-NOD activity during fibrosis (16) and other conditions and elucidating the electron transfer mechanisms for ascorbate and cytochrome b5.

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