

# Nitric-oxide Dioxygenase Activity and Function of Flavohemoglobins

SENSITIVITY TO NITRIC OXIDE AND CARBON MONOXIDE INHIBITION\*

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**Widely distributed flavohemoglobins (flavoHbs) function as NO dioxygenases and confer upon cells a resistance to NO toxicity. FlavoHbs from *Saccharomyces cerevisiae*, *Alcaligenes eutrophus*, and *Escherichia coli* share similar spectra, O<sub>2</sub>, NO, and CO binding kinetics, and steady-state NO dioxygenation kinetics. Turnover numbers (V<sub>max</sub>) for *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs are 112, 290, and 365 NO heme<sup>-1</sup> s<sup>-1</sup>, respectively, at 37 °C with 200 μM O<sub>2</sub>. The K<sub>M</sub> values for NO are low and range from 0.1 to 0.25 μM. V<sub>max</sub>/K<sub>M</sub>(NO) ratios of 900–2900 μM<sup>-1</sup> s<sup>-1</sup> indicate an extremely efficient dioxygenation mechanism. Approximate K<sub>M</sub> values for O<sub>2</sub> range from 60 to 90 μM. NO inhibits the dioxygenases at NO:O<sub>2</sub> ratios of ≥1:100 and makes true K<sub>M</sub>(O<sub>2</sub>) values difficult to determine. High and roughly equal second order rate constants for O<sub>2</sub> and NO association with the reduced flavoHbs (17–50 μM<sup>-1</sup> s<sup>-1</sup>) and small NO dissociation rate constants suggest that NO inhibits the dioxygenase reaction by forming inactive flavoHbNO complexes. Carbon monoxide also binds reduced flavoHbs with high affinity and competitively inhibits NO dioxygenases with respect to O<sub>2</sub> (K<sub>I</sub>(CO) = ~1 μM). These results suggest that flavoHbs and related hemoglobins evolved as NO detoxifying components of nitrogen metabolism capable of discriminating O<sub>2</sub> from inhibitory NO and CO.**

Investigations of NO toxicity and defenses in *Escherichia coli* led to the isolation of an NO-inducible NAD(P)H, O<sub>2</sub>, and

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FAD-dependent NOD<sup>1</sup> activity. This enzyme activity was identified with the flavoHb encoded by the *hmp* gene (1, 2). More recently, we have shown that the flavoHb efficiently converts NO and O<sub>2</sub> to nitrate via a conventional two electron flavoenzyme mechanism (3). The proposed NOD reaction stoichiometry is described by Equation 1.



A function for flavoHbs in NO dioxygenation and detoxification is supported by a growing body of evidence. FlavoHbs are induced by NO, nitrite, nitrate, and NO-releasing agents in various bacteria (2, 4–9), and these flavoHbs protect bacteria, yeast, and *Dictyostelium discoideum* against growth inhibition and NO-mediated damage during exposures to authentic NO or NO releasing agents (1, 2, 6, 7, 10–12). Further, O<sub>2</sub> is required for the robust NO scavenging action of flavoHb both in *E. coli* and *in vitro*, and O<sub>2</sub> is required for the maximal protection of cells and NO-sensitive aconitases from NO-mediated damage (1, 2). Other flavoHb mechanisms may also protect cells against nitrosothiol or NO toxicity in the absence of O<sub>2</sub>. FlavoHbs may reduce NO or denitrosylate toxic nitrosothiols formed from NO, sequester NO or reactive heme, or catalyze other beneficial reactions (6, 10, 13, 14). These additional flavoHb activities and functions require consideration.

We have now investigated the NOD activities of flavoHbs isolated from *Saccharomyces cerevisiae*, *Alcaligenes eutrophus*, and *E. coli* and have compared these activities with other enzymic activities of flavoHbs including NO reductase, NADH oxidase, and FAD reductase activities. Spectra for the various O<sub>2</sub>, NO, and CO liganded flavoHbs and the transient kinetics for O<sub>2</sub>, NO, and CO binding are reported for flavoHbs and are discussed in relation to the susceptibility of NOD activity to NO and CO inhibition. The kinetics and stoichiometry of the NOD activity indicate a highly specific and efficient dioxygenase mechanism and function for the flavoHbs.

## MATERIALS AND METHODS

*Reagents*—NADPH, FAD, phenylmethylsulfonyl fluoride, and *Aspergillus niger* glucose oxidase (225 units/mg) were purchased from Sigma. NADH, *Aspergillus* nitrate reductase, and bovine liver catalase

<sup>1</sup> The abbreviations used are: NOD, nitric-oxide dioxygenase; flavoHb, flavohemoglobin; Hb, hemoglobin; Mb, myoglobin; HPLC, high pressure liquid chromatography.

(260,000 units/ml) were from Roche Molecular Biochemicals. Manganese-containing superoxide dismutase (2,700 units/mg) was isolated from *E. coli* strain DH5 $\alpha$  bearing the multi-copy plasmid pD11c (15, 16). Hemin (99.9%) was obtained from Fluka. Ultra-pure O<sub>2</sub> and CO were purchased from Praxair (Bethlehem, PA). NO was obtained from Aldrich.

**Purification of Flavohbs**—*S. cerevisiae* flavoHb was prepared from strain JM43. Cultures were grown on YPD-galactose medium and treated with antimycin A as described previously (17). Cells from 6 liters of culture (~72 g) were resuspended in 2 volumes of 20 mM potassium phosphate, pH 7.6, containing 1  $\mu$ M phenylmethylsulfonyl fluoride, and cells were lysed in a French press. Lysates were clarified by centrifugation at 16,000  $\times$  g, and cell-free extracts were applied to a 2.5  $\times$  10-cm DEAE-Sepharose CL-6B column (Sigma). After washing with 5 column volumes of 20 mM potassium phosphate, pH 7.6, and 50 mM KCl, flavoHb was eluted with a linear gradient of 50–150 mM KCl. Fractions with a 405 nm:280 nm ratio larger than 0.06 were pooled and concentrated on a YM10 membrane (Amicon). FlavoHb was then separated by gel electrophoresis in 10% polyacrylamide containing 25 mM Tris/192 mM glycine, pH 8.8, at 4  $^{\circ}$ C. The yellowish flavoHb band was excised and eluted with Electro-Eluter Model 422 (Bio-Rad). *A. eutrophus* flavoHb was purified from cell pellets provided by Dr. Bärbel Friedrich (University of Berlin, Germany) (14) as described above. *E. coli* flavoHb was purified from anaerobic nitrate-induced cultures of RB9060 containing the multi-copy plasmid pAlter + *hmp* (3). *E. coli* flavoHb was reconstituted with hemin as described (3). FlavoHb purities were judged to be greater than 95% by reverse phase HPLC and SDS-polyacrylamide gel electrophoresis analysis.

**FlavoHb Activity Assays**—NOD activity was measured using a NO electrode (World Precision Inst.) at 37  $^{\circ}$ C in 2 ml of 50 mM potassium phosphate, pH 7.8, containing 100  $\mu$ M EDTA, 1  $\mu$ M NO, 1  $\mu$ M FAD, 200  $\mu$ M O<sub>2</sub>, and 100  $\mu$ M NADH unless specified otherwise (3). Saturated NO (2 mM) was prepared as described previously (18). Saturated CO (1 mM) was prepared by vigorously stirring water in a septum-sealed vial under a stream of 99.99% CO (Praxair) at room temperature (19). Reaction mixtures were first scrubbed with 99.999% N<sub>2</sub> when the effects of O<sub>2</sub> concentration were measured. Saturated O<sub>2</sub> solution (1.14 mM) was prepared by vigorously stirring reaction buffer in a rubber septum-sealed vial under 99.99% O<sub>2</sub> (Praxair) at room temperature. The effect of FAD and CO on NOD activity was measured following repetitive additions of 2 nmol of NO and the stepwise removal of NO by 0.12–1.2 pmol of flavoHb in a 2-ml reaction at 37  $^{\circ}$ C. NOD activities were calculated with a correction for background rates of NO decomposition for each assay condition. NADH oxidase activity was followed at 340 nm in a 0.5-ml reaction mix containing 50 mM potassium phosphate, pH 7.8, 100  $\mu$ M EDTA, 100  $\mu$ M NADH, and 1  $\mu$ M FAD at 37  $^{\circ}$ C. NO reductase activity was measured using the NO electrode in an anaerobic 2-ml reaction at 37  $^{\circ}$ C containing 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 1  $\mu$ M FAD, 10 mM glucose, 8 units of glucose oxidase, 260 units of catalase, 100  $\mu$ M NADH, and 5  $\mu$ M NO. NO and flavoHb were added following 6 min of O<sub>2</sub> depletion, and NO decomposition rates were determined for 1  $\mu$ M NO. FAD reductase activity was assayed at 450 nm in a 1-ml reaction mixture prepared as described for the measurement of NO reductase activity except that NO was omitted and 20  $\mu$ M FAD was included.

**Cofactor and Protein Assays**—Heme was determined using the alkaline pyridine method (20). FAD was released from flavoHb by boiling for 3 min and was determined from the fluorescence at 520 nm with excitation at 460 nm (21). Alternatively, heme and FAD were adsorbed on the reverse-phase C18 HPLC column (SynChrom) in 0.1% trifluoroacetic acid in H<sub>2</sub>O and were separated with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Heme and FAD were eluted with 35–40% and 10–15% acetonitrile, respectively. FAD and heme standards were employed for the analysis.  $E = 6,220 \text{ m}^{-1} \text{ cm}^{-1}$  at 340 nm was used for the calculation of NAD(P)H concentration. The percentage of reduction of NAD(P)H preparations was determined from the absorbance ratio for 340 nm and 260 nm using  $14,250 \text{ m}^{-1} \text{ cm}^{-1}$  at 260 nm (22). NADH and NADPH were  $\geq 95\%$  reduced. Protein was measured using the dye binding assay with bovine serum albumin as the standard (23).

**Measurements of Rate Constants for Ligand Binding**—All measurements were made at 20  $^{\circ}$ C in 100 mM potassium phosphate, pH 7.0, and 0.3 mM EDTA for comparison with previous Mb and Hb studies. Recombinant sperm whale Mb was included as a control. FlavoHb and Mb ligand complexes were prepared, and stopped flow and laser photolysis measurements of ligand binding were made as described previously (3, 19).

**Nitrate, Nitrite, O<sub>2</sub>, and NADH Measurements**—Nitrate and nitrite were measured using the Griess reagent and nitrate reductase (24). O<sub>2</sub>

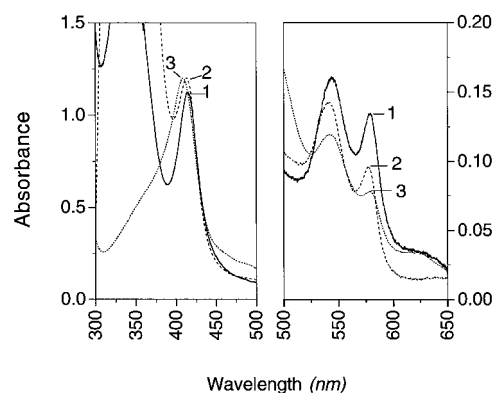


FIG. 1. Absorbance spectra of flavoHb(Fe<sup>2+</sup>)-O<sub>2</sub> complexes. Reduced *S. cerevisiae* (line 1), *A. eutrophus* (line 2), and *E. coli* (line 3) flavoHb(Fe<sup>2+</sup>)-O<sub>2</sub> complexes (10  $\mu$ M heme) were prepared with NADH and O<sub>2</sub> and were scanned as described under "Materials and Methods." The *E. coli* flavoHb was reconstituted with bovine heme and contained 0.95 mole fraction of heme. *S. cerevisiae* and *A. eutrophus* flavoHbs contained  $\geq 0.95$  mole fraction of heme as isolated. The mole fractions of FAD for the *E. coli*, *S. cerevisiae*, and *A. eutrophus* flavoHbs were 0.41, 0.44, and 0.44, respectively.

consumption was measured with a Clark-type O<sub>2</sub> electrode using a value of 200  $\mu$ M O<sub>2</sub> for air-saturated buffer at normal atmospheric pressure and 37  $^{\circ}$ C (25). NADH oxidation was monitored at 340 nm.

## RESULTS

**Spectra of Flavohbs**—Visible light absorbance by FAD and heme confers an orangish brown color on flavoHbs that is altered by reduction and ligand binding. The heme spectra and absorbance maxima are grossly similar for the ligand complexes and redox forms of various flavoHbs (3, 13, 21, 26–29). We measured absorbance spectra of purified *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs under identical conditions to evaluate the degree of spectral identity. With O<sub>2</sub> binding, the NADH-reduced flavoHbs display heme spectra that are typical of oxyHbs (Fig. 1). The flavoHbO<sub>2</sub> complexes show similar wavelength maxima for the Soret and  $\alpha,\beta$  bands (Table I). Notable differences are observed in the spectra attributable to the oxidized flavin (470 nm) and to the free NADH (340 nm). The *E. coli* flavoHb/NADH mixture shows greater absorbance at 470 nm and less absorbance at 340 nm; moreover, spectra must be recorded rapidly to observe the *E. coli* flavoHbO<sub>2</sub> complex because of the NADH oxidase activity (3, 28–30). The other flavoHbO<sub>2</sub> complexes do not have as high an oxidase activity (see Table V) but still deplete O<sub>2</sub> and NADH in minutes at the 1–10  $\mu$ M flavoHb concentrations used. *A. eutrophus* flavoHbO<sub>2</sub> shows a relatively low absorbance at 625 nm. Spectra for flavoHbNO and CO complexes and the reduced and oxidized forms reveal similar heme absorbance peaks (Table I). These spectra demonstrate the formation of both the flavoHbO<sub>2</sub> and flavoHbNO complexes that are required for flavoHb-catalyzed NO dioxygenation (2, 3, 31, 32) and NO reduction (13), respectively.

**O<sub>2</sub>, NO, and CO Binding Kinetics of Flavohbs**—O<sub>2</sub> and NO binding kinetics play a critical role in regulating the catalytic rate of the *E. coli* NOD activity (3). Thus, we examined rates of ligand binding to the *S. cerevisiae* and *A. eutrophus* proteins and compared these rates with those for *E. coli* flavoHb and recombinant sperm whale Mb. The NAD(P)H-reduced flavoHbs show rate constants for O<sub>2</sub> association in the range of 17–50  $\mu\text{M}^{-1} \text{ s}^{-1}$ , which are only slightly greater than those reported for mammalian Mbs (Table II). The O<sub>2</sub> dissociation rate constants for all three flavoHbs are very small, 0.2–0.6  $\text{s}^{-1}$ , compared with that for MbO<sub>2</sub>, 15  $\text{s}^{-1}$ . The net result is that all three flavoHbs show O<sub>2</sub> association equilibrium constants that are 20–200 times greater than that for Mb. The association

TABLE I  
Absorbance peaks for flavoHb ligand complexes

Spectra were measured in 100 mM potassium phosphate, pH 7.0, containing 0.3 mM EDTA. *S.c.*, *S. cerevisiae*; *A.e.*, *A. eutrophus*; *E.c.*, *E. coli*.

FlavoHb complex	Soret maximum	Visible maxima
	nm	nm
Fe <sup>2+</sup> -O <sub>2</sub> (oxy)		
<i>S.c.</i>	414	545, 578
<i>A.e.</i>	413	540, 578
<i>E.c.</i>	411	542, 578
Fe <sup>2+</sup> (deoxy)		
<i>S.c.</i>	431	557
<i>A.e.</i>	437	560
<i>E.c.</i>	425 (430) <sup>a</sup>	556
Fe <sup>3+</sup> (met)		
<i>S.c.</i>	402	488, 648
<i>A.e.</i>	400	485, 645
<i>E.c.</i>	404	491, 622
Fe <sup>2+</sup> -CO		
<i>S.c.</i>	421	537, 568
<i>A.e.</i>	424	540, 572
<i>E.c.</i>	422	539, 567
Fe <sup>2+</sup> -NO		
<i>S.c.</i>	419	543, 574
<i>A.e.</i>	419	548, 577
<i>E.c.</i>	419	549, 575
Fe <sup>3+</sup> -NO		
<i>S.c.</i>	419	534, 566
<i>A.e.</i>	419	535, 568
<i>E.c.</i>	420	533, 567

<sup>a</sup> There were multiple bands in the deoxy spectrum generated either by adding sodium dithionite or allowing complete reduction and oxygen consumption by NADH.

rate constants for NO binding to reduced flavoHb(Fe<sup>2+</sup>) and Mb(Fe<sup>2+</sup>) vary by <3-fold and are in the same range as the rate constants for O<sub>2</sub> binding, 10–30 μM<sup>-1</sup> s<sup>-1</sup>. Greater differences are seen in the ligand binding rate constants for NO binding to the oxidized flavoHb(Fe<sup>3+</sup>)s and for CO binding to the reduced proteins. All three flavoHb(Fe<sup>3+</sup>)s have unusually high NO association rate constants compared with that for Mb(Fe<sup>3+</sup>). The NO dissociation rate constants are also higher for flavoHb(Fe<sup>3+</sup>) than Mb(Fe<sup>3+</sup>). This result suggests that water is not bound or is only weakly coordinated to the Fe<sup>3+</sup> atom in the oxidized forms. This conclusion is supported by the positions of the Soret peaks of the Fe<sup>3+</sup>(met) forms that are all significantly blue shifted compared with those for human metHb and metMb.

The *E. coli* flavoHb shows a much greater rate of CO binding than those observed for *A. eutrophus* and *S. cerevisiae* enzymes (Table II and Fig. 2). The majority of the absorbance change observed after flash photolysis of *E. coli* flavoHbCO occurs rapidly with an apparent bimolecular rate constant of ~20 μM<sup>-1</sup> s<sup>-1</sup>, whereas absorbance changes for CO rebinding to *A. eutrophus* and *S. cerevisiae* flavoHbs show a much smaller *k'*<sub>CO</sub> value equal to 0.1–0.5 μM<sup>-1</sup> s<sup>-1</sup>. As shown in Fig. 2, a slow phase is observed for CO binding to the *E. coli* enzyme and the *k'*<sub>CO</sub> value for this phase is similar to that of the other flavoHbs, ~0.5 μM<sup>-1</sup> s<sup>-1</sup>. In contrast to CO binding, all three flavoHbs have high O<sub>2</sub> association rate constants, low O<sub>2</sub> dissociation rate constants, and consequently high O<sub>2</sub> affinities for the flavoHb(Fe<sup>2+</sup>), properties shown to be important for the efficient NOD activity of the *E. coli* flavoHb (3). However, the differences in NO and CO binding suggest that the heme pockets of the flavoHbs differ.

*Steady-state Kinetic Constants for the NOD Activities of Flavohemoglobins*—Extensive amino acid sequence identities and spectral and ligand binding similarities among the flavoHbs strongly suggested a similar capacity for NO dioxygenation. The *S. cerevisiae* flavoHb was also recently shown to consume NO in

TABLE II  
Kinetic parameters for ligand binding to flavoHbs

Rate constants for O<sub>2</sub>, NO, CO association (*k'*), dissociation (*k*), and association equilibrium constants (*K*) were determined as previously described (3). *S.c.*, *S. cerevisiae*; *A.e.*, *A. eutrophus*; *E.c.*, *E. coli*; SW, sperm whale; ND, not determined.

Ligand pair	<i>k'</i>	<i>k</i>	<i>K</i>
	μM <sup>-1</sup> s <sup>-1</sup>	s <sup>-1</sup>	μM <sup>-1</sup>
O <sub>2</sub>			
<i>S.c.</i> flavoHb (Fe <sup>2+</sup> )	17	0.6 <sup>a</sup>	28
<i>A.e.</i> flavoHb (Fe <sup>2+</sup> )	50	0.2 <sup>a</sup>	250
<i>E.c.</i> flavoHb (Fe <sup>2+</sup> )	38	0.44	86
SW Mb (Fe <sup>2+</sup> )	17	15	1.2
NO			
<i>S.c.</i> flavoHb (Fe <sup>2+</sup> )	10–20 <sup>b</sup>	ND	ND
<i>A.e.</i> flavoHb (Fe <sup>2+</sup> )	10–20 <sup>b</sup>	ND	ND
<i>E.c.</i> flavoHb (Fe <sup>2+</sup> )	26 <sup>b</sup>	0.0002	130,000
SW Mb (Fe <sup>2+</sup> )	22	0.0001	220,000
NO			
<i>S.c.</i> flavoHb (Fe <sup>3+</sup> )	230 (0.7) <sup>c</sup>	~200	0.0033
<i>A.e.</i> flavoHb (Fe <sup>3+</sup> )	2.4	1,200	0.002
<i>E.c.</i> flavoHb (Fe <sup>3+</sup> )	44	~4,000	0.011
SW Mb (Fe <sup>3+</sup> )	0.08	12	0.007
CO			
<i>S.c.</i> flavoHb (Fe <sup>2+</sup> )	0.5	0.11 <sup>b</sup>	4.5
<i>A.e.</i> flavoHb (Fe <sup>2+</sup> )	0.11	0.08 <sup>b</sup>	1.4
<i>E.c.</i> flavoHb (Fe <sup>2+</sup> )	22 <sup>d</sup>	0.057 <sup>b</sup>	386
SW Mb (Fe <sup>2+</sup> )	0.51	0.019	27

<sup>a</sup> In the calculation of *k*<sub>O2</sub> for these proteins, it was assumed that the NADH oxidase activity of the proteins led to depletion of any free O<sub>2</sub> because the observed CO replacement rate was independent of the starting O<sub>2</sub> concentration and increased only to a small degree in going from ~600 to 50 μM starting O<sub>2</sub> concentration in the presence of ~600 μM NADH.

<sup>b</sup> The reactions were biphasic with ≥70% of the reaction occurring rapidly with the bimolecular rate constant shown.

<sup>c</sup> The reactions were markedly biphasic with ~50% of the reaction occurring very rapidly and 50% very slowly (rate constant in parentheses).

<sup>d</sup> As shown in Fig. 2, the reaction of CO with *E. coli* flavoHb (Fe<sup>2+</sup>) is biphasic with the majority of the reaction occurring very rapidly. The slow phase exhibits a bimolecular rate constant of 0.3–0.5 μM<sup>-1</sup> s<sup>-1</sup>.

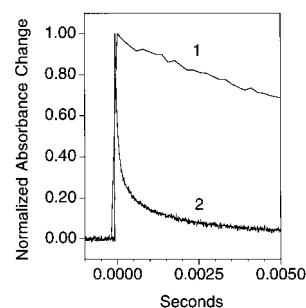


FIG. 2. CO rebinding to flavoHbs. NADH-reduced *A. eutrophus* (line 1) and *E. coli* (line 2) flavoHbs were equilibrated with 1 atm CO in 0.1 M phosphate, pH 7.0, at 20 °C and were photolyzed with a 300-ns excitation pulse.

an O<sub>2</sub>-dependent reaction in whole cells, thus supporting a common dioxygenase and NO detoxification function (11). Nevertheless, it remained to be determined whether various flavoHbs share a similar NOD activity. The flavoHbs show NOD activities with similar dependences upon O<sub>2</sub> concentration as

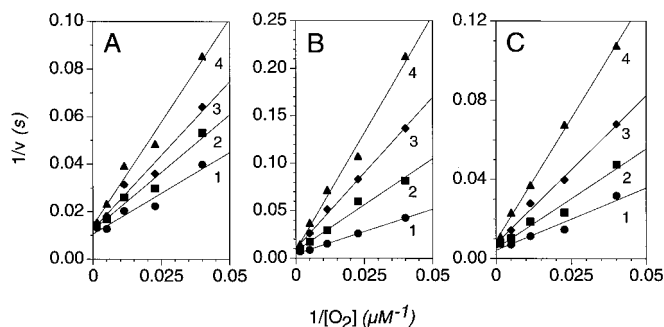


FIG. 3.  $O_2$  dependences and CO sensitivities of the NOD activities of flavoHbs. NOD activity of *S. cerevisiae* (A), *A. eutrophus* (B), and *E. coli* (C) flavoHbs was measured at 37 °C with various  $O_2$  concentrations in the presence of 0  $\mu M$  CO (line 1), 0.5  $\mu M$  CO (line 2), 1  $\mu M$  CO (line 3), or 2  $\mu M$  CO (line 4) in reactions containing 0.75  $\mu M$  NO, 200  $\mu M$  NADH, and 1  $\mu M$  FAD.

expected for similar  $O_2$  ligand binding constants (Table II). At 0.75  $\mu M$  NO, 37 °C and with 200  $\mu M$  NADH as reducing substrate, the *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs show apparent  $K_M$  values for  $O_2$  of 60, 80, and 90  $\mu M$ , respectively (Fig. 3, A–C, lines 1). Further, each flavoHb is competitively inhibited by CO as expected for a competition between  $O_2$  and CO for binding the reduced heme iron atom (Table II). The  $K_I$  values for CO for the *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs are approximately 1, 0.5, and 1  $\mu M$ , respectively (Fig. 3). Inhibition by CO is rapid and reversible as demonstrated by a constant rather than progressive inhibition of NOD activity during repetitive catalytic turnover (Fig. 4). The data further support a mechanism of NO dioxygenation involving the reaction of NO with a flavoHb $O_2$  complex (3) and suggest greater similarities of CO affinities of flavoHbs during catalytic turnover than those measured by rapid kinetics (Table II).

Each of the flavoHbs shows a normal saturation with NO at 200  $\mu M$   $O_2$  (Fig. 5, A–C, lines 1). The apparent  $K_M$  values for NO for the *S. cerevisiae*, *A. eutrophus*, and *E. coli* NOD activities are 0.13, 0.10, and 0.25  $\mu M$ , respectively (Table III). However, at 25 and 50  $\mu M$   $O_2$ , inhibition by NO is apparent at  $\geq 0.25$  and  $\geq 0.5$   $\mu M$  NO concentrations, respectively (Fig. 5, A–C, lines 2 and 3), and inhibition by NO appears more pronounced for the *S. cerevisiae* and *A. eutrophus* flavoHbs. The ability of NO to inhibit NOD activities at NO: $O_2$  ratios greater than 1:100 should elevate the values of  $K_M(O_2)$  determined from the data in Fig. 3. However, analyses of the  $O_2$  dependence of the *E. coli* NOD activity at low NO concentrations have indicated that the effect of NO inhibition on  $K_M(O_2)$  determination under these conditions is small (3).

The flavoHbs show large differences in their dependence upon NADH and NADPH for NOD catalysis (Table III). The maximal turnover rates ( $V_{max}$ ) calculated from plots of  $1/v$  versus  $1/[NAD(P)H]$  with saturating NADH or NADPH are also summarized in Table III. It is noteworthy that the *A. eutrophus* flavoHb does not utilize NADPH as a reducing substrate and that the *S. cerevisiae* and *E. coli* flavoHbs utilize either NADH or NADPH. The results are consistent with earlier reports of the specificity of various *A. eutrophus* flavoHb activities for NADH (27). Importantly, none of the NOD activities was significantly affected by superoxide dismutase (1 mg/ml) under standard assay conditions indicating a limited role for free  $O_2^-$  in flavoHb-catalyzed decomposition of NO.

We also examined the effect of temperature on the NOD activities. The *E. coli* NOD activity decreases  $\sim 3$ -fold in a shift from 37 to 20 °C, whereas the *S. cerevisiae* and *A. eutrophus* activities decrease by 21- and 16-fold, respectively (Table IV). These results indicate large effects of temperature on NOD catalysis and suggest possible differences in the rate-limiting

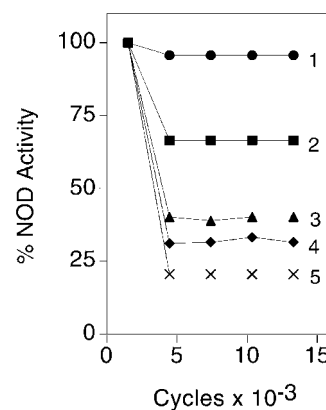


FIG. 4. CO inhibition of NOD activities during turnover. NOD activity of *E. coli* flavoHb was measured with 0  $\mu M$  CO (line 1), 0.5  $\mu M$  CO (line 2), 1  $\mu M$  CO (line 3), 2  $\mu M$  CO (line 4), and 4  $\mu M$  CO (line 5) following repetitive additions of 1  $\mu M$  NO as described under "Materials and Methods." One catalytic cycle represents the two-electron reaction measured by the decomposition of two NO molecules and was calculated relative to the heme content of flavoHb.

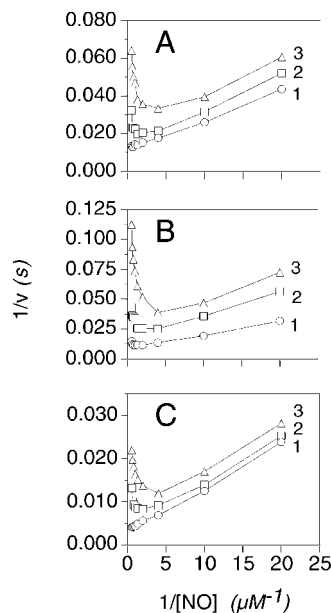


FIG. 5. NO dependences of the NOD activities of flavoHbs. NOD activity of *S. cerevisiae* (A), *A. eutrophus* (B), or *E. coli* (C) flavoHb was measured at 37 °C with 100  $\mu M$  NADH, 1  $\mu M$  FAD, and 200  $\mu M$   $O_2$  (line 1), 50  $\mu M$   $O_2$  (line 2), or 25  $\mu M$   $O_2$  (line 3) with varying concentrations of NO as described under "Materials and Methods."

flavin to heme electron transfer step between the flavoHbs (3). Differences in temperature effects may also be explained in part by relative differences in NO or  $O_2$  saturation.

**FAD Dependence of the NOD Activities of Flavohemoglobins**—The *A. eutrophus* flavoHb structure shows one FAD and one heme per protein molecule (33); however, some preparations of flavoHb are deficient in one or both cofactors following isolation (2, 3, 21). Our preparations of *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs contained 28–44% of the predicted FAD. A 2-min preincubation with FAD increases the initial NOD activity for each flavoHb by  $\sim 7$ –30% (Fig. 6). The effect is largest for *E. coli* flavoHb, which is the most FAD-deficient of the three (0.28 mole fraction). In the absence of added FAD, each of the flavoHbs loses  $\geq 50\%$  of its NOD activity within  $\sim 5,000$  two-electron catalytic cycles (Fig. 6, lines 4–6). For *E. coli* flavoHb, this activity loss is even faster with lower but still saturating concentrations of NADH or with NADPH (1–3). In the presence of 1  $\mu M$  FAD, the *E. coli* and *S. cerevisiae* NOD activities are

TABLE III  
Steady-state constants for the NOD activities of flavoHbs

Assays of NOD activity were at 37 °C as described under "Materials and Methods."

FlavoHb	$K_M$ (NADH) <sup>a</sup>	$K_M$ (NADPH) <sup>a</sup>	$V_{max}$ (NADH/NADPH) <sup>b</sup>	$K_M$ (NO) <sup>d</sup>	$K_M$ (O <sub>2</sub> ) <sup>e</sup>
	$\mu\text{M}$	$\mu\text{M}$	$\text{s}^{-1}$	$\mu\text{M}$	$\mu\text{M}$
<i>S. cerevisiae</i>	28	390	112/93	0.13	60
<i>A. eutrophus</i>	70		290/<0.2 <sup>c</sup>	0.10	80
<i>E. coli</i>	3	140	365/200	0.25	90

<sup>a</sup> Measured with 1.8  $\mu\text{M}$  NO and 200  $\mu\text{M}$  O<sub>2</sub>.

<sup>b</sup> Calculated for saturating NADH or NADPH with 1.8  $\mu\text{M}$  NO and 200  $\mu\text{M}$  O<sub>2</sub>.

<sup>c</sup> Measured with 1 mM NADPH at 37 °C with 1.8  $\mu\text{M}$  NO and 200  $\mu\text{M}$  O<sub>2</sub>.

<sup>d</sup> Determined from the data in Fig. 5.

<sup>e</sup> Estimated from the data in Fig. 3.

TABLE IV

Temperature dependence of the NOD activities of flavoHbs

NO dioxygenase activities were measured with 100  $\mu\text{M}$  NADH, 1  $\mu\text{M}$  NO, and 200  $\mu\text{M}$  O<sub>2</sub>. Values are expressed relative to heme content.

FlavoHb	37 °C	20 °C	Ratio
	$\text{s}^{-1}$	$\text{s}^{-1}$	
<i>S. cerevisiae</i>	80	3.9	21
<i>A. eutrophus</i>	120	7.4	16
<i>E. coli</i>	240	90	2.7

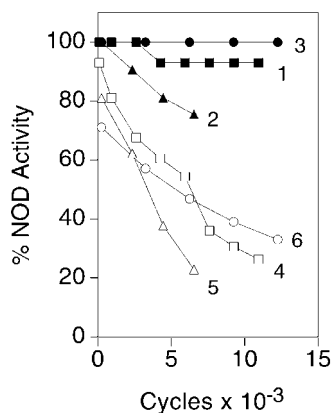


FIG. 6. Effects of FAD on the NOD activities of flavoHbs. NOD activity of 1.2 pmol of *S. cerevisiae* flavoHb (lines 1 and 4), 0.48 pmol of *A. eutrophus* flavoHb (lines 2 and 5), 0.79 pmol of *E. coli* flavoHb (lines 3 and 6) was measured at 37 °C with (lines 1-3) or without (lines 4-6) 1  $\mu\text{M}$  FAD as described under "Materials and Methods." Reactions were initiated with 1  $\mu\text{M}$  NO, following a brief 2-min preincubation of flavoHbs with or without FAD plus 100  $\mu\text{M}$  NADH and 200  $\mu\text{M}$  O<sub>2</sub>. *S. cerevisiae*, *A. eutrophus*, and *E. coli* flavoHbs contained 0.44, 0.44, and 0.28 mole fractions of FAD and  $\sim 1.0$ ,  $\sim 1.0$ , and 0.42 mole fractions of heme, respectively. Cycles were calculated relative to flavoHb heme content.

more stable (lines 1 and 3). In contrast, the *A. eutrophus* enzyme slowly loses activity even with added FAD (line 2).

The results demonstrate the requirement of a free pool of FAD for sustained and maximal NOD activity of flavoHbs during the course of a typical NOD activity assay and suggest dissociation and reassociation of bound FAD during enzyme turnover. These results, together with the FAD deficiency of the flavoHb preparations, warranted the inclusion of FAD in all flavoHb activity assays. In addition, the data indicate that NO inhibition of the NOD activity is effectively reversible, at least for the *E. coli* and *S. cerevisiae* flavoHbs, because there is no significant loss of activity following repetitive NO additions (1  $\mu\text{M}$ ) and a total of >10,000 two electron turnover cycles (Fig. 6, lines 1 and 3).

**Other Enzymic Activities of FlavoHbs**—We investigated three other enzymic activities of the flavoHbs and compared their turnover rates with those for NO dioxygenation. Each flavoHb shows the anaerobic NO reductase activity reported for

*E. coli* flavoHb (6, 13) (Table V). The *E. coli* flavoHb NO reductase activity is  $\sim 4$ -fold higher than that of the other flavoHbs but is still several orders of magnitude lower than that of the NOD activity. Each flavoHb also displays the NADH oxidase activity reported for *E. coli* flavoHb (28–30). The *E. coli* NADH oxidase activity is  $\sim 7$ -fold higher than that of the other flavoHbs. This higher NADH oxidase activity accounts for the rapid depletion of NADH by *E. coli* flavoHb observed in spectra of flavoHbO<sub>2</sub> (Fig. 1). The data also reveal comparable anaerobic FAD reductase activities for the flavoHbs (34).

**Stoichiometry of the FlavoHb Reaction**—The stoichiometry of the flavoHb-catalyzed decomposition of NO was measured using the reaction conditions now established for optimal NOD activity. FlavoHb converted 40 nmol of NO, 39.1 nmol of O<sub>2</sub>, and 19.9 nmol of NADH to 39.6 nmol of nitrate. The 2-ml reaction contained an initial 100  $\mu\text{M}$  NADH, 200  $\mu\text{M}$  O<sub>2</sub>, and 1  $\mu\text{M}$  FAD in 50 mM potassium phosphate buffer, pH 7.8, and 0.1 mM EDTA at 37 °C with 8 pmol of *E. coli* flavoHb and was primed with 10 sequential additions of 2  $\mu\text{M}$  NO. Nitrite was not a significant product of the reaction.

## DISCUSSION

Various functions for Hbs, Mbs, and the two-domain flavoHbs have been postulated based upon their intrinsic physical properties (35–38). By definition, members of this Hb superfamily are globular, contain heme, and bind O<sub>2</sub> reversibly (39). Partial transfer of the ferrous heme electron to O<sub>2</sub> strengthens the Hb(Fe<sup>2+</sup>)O<sub>2</sub> bond and may especially suit Hbs for O<sub>2</sub> transport, storage and sequestration functions (40). The Hb(Fe<sup>2+</sup>)O<sub>2</sub> complex may also be optimized to catalyze the oxidation or oxygenation of suitable substrates (36). Indeed, many oxyHbs catalyze a variety of (per)oxidations (38, 41–44) and rapidly dioxygenate NO (31, 32). A (per)oxidase or peroxygenase function for Hbs is attractive because a dehalo-peroxygenase/oxidase activity isolated from a marine polychaete annelid has been found to share 20.6% amino acid sequence identity with sea hare Mb (45). Recent proposals of specialized functions have also included a reductive removal of toxic O<sub>2</sub> by the periericent *Ascaris suum* Hb (46).

A function for flavoHb and Hbs with associated reductases was suggested with the identification of the efficient NOD activity in *E. coli* with flavoHb (1–3). We have now demonstrated similar NOD activities of the *A. eutrophus* and *S. cerevisiae* flavoHbs. The results extend the range of the NOD function of the ancient flavoHbs from bacteria to yeast (17). These NOD activities have high turnover rates and low  $K_M$  values for NO (Table III).  $V_{max}/K_M(\text{NO})$  ratios for NOD activities at 37 °C and a normoxic O<sub>2</sub> level of 200  $\mu\text{M}$  are 900–2,900  $\mu\text{M}^{-1} \text{s}^{-1}$ . These apparent bimolecular rate constants for flavoHb-catalyzed NO dioxygenation are only 2–6-fold lower than the diffusion-limited second order rate constant for the reaction of NO with free superoxide (47, 48) and are more than 20-fold higher than those reported for the mammalian oxyMb and oxyHb (32). Further, maximal NOD turnover rates exceed the

TABLE V  
Turnover rates for flavoHb activities

Activities were assayed at 37 °C with 100  $\mu\text{M}$  NADH as described under "Materials and Methods." NO dioxygenase activities were measured with 1  $\mu\text{M}$  NO and 200  $\mu\text{M}$  O<sub>2</sub>. Turnover rates for NO dioxygenation, NO reduction, NADH oxidation, and FAD reduction are expressed relative to the heme content of individual flavoHbs.

FlavoHb	NO dioxygenase	NO reductase	NADH oxidase	FAD reductase
	$s^{-1}$	$s^{-1}$	$s^{-1}$	$s^{-1}$
<i>S. cerevisiae</i>	91	0.02	0.021	0.09
<i>A. eutrophus</i>	141	0.03	0.025	0.05
<i>E. coli</i>	232	0.12	0.171	0.07

turnover rates measured for NO reduction, NADH oxidation, and FAD reduction by several orders of magnitude (Table V). The reaction stoichiometry supports the proposed NO dioxygenase mechanism described by Equation 1 (2, 3).

Previous approximations of the flavoHb reaction stoichiometry have also supported a dioxygenase mechanism for NO metabolism (6). However, the high concentrations of NO (100  $\mu\text{M}$ ) and flavoHb ( $\sim 0.5$   $\mu\text{M}$ ) used in these early studies are likely to have provided conditions for NO inhibition, NO reduction, reactions leading to the formation of nitrite, and nonstoichiometric nitrate, NADH and O<sub>2</sub> ratios. NO dioxygenation thus appears to be a highly specific and well adapted enzymic function for the flavoHbs. FlavoHbs may significantly control NO toxicity and signaling functions in a variety of organisms.

Competition between NO and O<sub>2</sub> for binding to reduced flavoHb (Table II) inhibits NOD activities at NO:O<sub>2</sub> ratios  $\geq 1:100$  (Fig. 5). CO shows inhibitory effects comparable with those of NO with 50% inhibition occurring at CO:O<sub>2</sub> ratios of  $\leq 1:60$ . Given the sensitivity of the NOD reaction to NO and CO inhibition, NO and CO may inhibit NOD activity *in vivo*. NOD activity inhibition may affect the survival of organisms exposed to toxic NO and CO produced by the cells of many tissues of higher organisms (49–51). Furthermore, because NO and CO are abundantly produced by organic combustion and decay reactions, NO and CO inhibition of the NOD activity of the ancient flavoHbs may have been an important factor in the evolution of the ligand discrimination properties of Hbs.

The structural causes of the differences in CO binding between the flavoHbs and the heterogeneity seen in the *E. coli* flavoHb traces are not clear. There are two possibilities. The proteins may exist in alternative conformations, one rapidly reacting and the other slowly reacting. Based on the low  $K_I$  for CO seen in the NOD steady-state assays, the more rapidly reacting conformer appears to occur during catalysis. The cause of the rate differences could be explained by differing extents of hydration in the distal pockets or changes in iron reactivity, but no structural data are available to allow discrimination between these possibilities. Alternatively, the differences may be related to the extent of reduction of the CO complexes. If only 1 or 2 electrons are present, they may redistribute to the flavin after photolysis, causing both the fraction of reduced iron and, hence, reactivity toward CO, to decrease markedly. A similar mechanism could explain the high  $K_M$  values for O<sub>2</sub> observed in the steady-state assays, where the enzyme cycles between the two, one, and oxidized enzyme species (3). The competition between the flavin and iron for electrons could cause a significantly lower reactivity toward O<sub>2</sub> as well. In our transient kinetic measurements with the reduced flavoHbO<sub>2</sub> complex, the enzymes are almost certainly in the three-electron reduced state. The initial reduced state will contain two electrons from NADH. When autooxidation creates the one electron reduced species, flavoHb is immediately reduced by the excess of NADH present. In the case of CO

complexes, NADH reduction in the absence of O<sub>2</sub> can only create the two-electron reduced species.

The reasons for the FAD deficiency of flavoHb preparations and the apparent loss of bound FAD during catalytic turnover remain to be elucidated. Nevertheless, these and other data suggest that FAD readily dissociates from the flavoHbs. Substoichiometric increases in the NOD activities of the FAD-deficient flavoHbs with added FAD (Fig. 6) may be explained by an incomplete complement of heme or the introduction of trace amounts of FAD with NADH that has been prepared from biological sources. The low stimulation of activity may also reflect nonoptimal conditions for FAD association.

FlavoHb and Hb genes and proteins are found in diverse microorganisms (5, 8–10, 12, 17, 52–59) including *D. discoideum* (AB025583 and AB025584), *Pyricularia grisea* (AA415144), *Erwinia chrysanthami* (O47266), *Xylella fastidiosa* (AE003859.1), *Deinococcus radiodurans* (AE001863), *Salmonella typhimurium* (AF020388.1), *Bacillus subtilis* (P49852), *Bacillus halodurans* (AB024563), *Campylobacter jejuni* (AL139079), *Schizosaccharomyces pombe* (AL132779.2), *Vibrio parahaemolyticus* (P40609), *Fusarium oxysporum* (AB016807), *Pichia norvegensis* (Q03331), *Botrytis cinerea* (AL116429.1 and AL115888.1), *Vitreoscilla stercoraria* (P04252), *Aquifex aeolicus* (AE000678), and *Mycobacterium bovis* (AF130980 and AF213450). Hbs with poorly defined functions are also expressed in various plant and animal tissues (35, 60). Trademarks of flavoHbs, including amino acid sequences, spectra, and ligand binding kinetics, may point to a NOD function for this type of heme protein. Highly conserved and unique flavoHb and Hb heme pocket residues Tyr(B10) and Gln(E7) may especially signify a NOD function. The large O<sub>2</sub> association rate constants and small O<sub>2</sub> dissociation rate constants of flavoHb, conferred by the Tyr(B10) hydroxyl, provide the relatively stable Fe<sup>2+</sup>-O<sub>2</sub> intermediate required for the enzymic NO dioxygenation (3).

NO reductase, nitrosothiol denitrosolase, NADH oxidase, FAD reductase, and other proposed activities of the flavoHbs (6, 13) also require consideration in the evaluation of flavoHb function. Thus, although an O<sub>2</sub> requirement for protection against NO damage has been demonstrated for flavoHb in *E. coli* (1, 2), flavoHbs have provided comparable growth protection against NO donor compound and nitrosothiol toxicity in anoxic *S. typhimurium* and *S. cerevisiae* cultures (10, 11). These anaerobic protective effects of flavoHb have suggested the participation of additional flavoHb activities, functions, and mechanisms. An anaerobic NO reduction function has been suggested (6, 10, 11, 13, 14). However, the prevalence of separate and efficient NO-inducible anaerobic NO reductases in *E. coli* and other organisms including *A. eutrophus* (1, 61) coupled with the extremely low NO reductase activities of flavoHbs (Table V) suggests that the anaerobic protective effects of flavoHbs are not related to the reduction of NO to the nitroxyl anion. Moreover, given the nanomolar O<sub>2</sub> dissociation equilibrium constants of flavoHbs (Table II) and the high NOD turnover rates, it is clear that trace O<sub>2</sub> contamination may produce a significant NOD activity in growth protection experiments. In addition, greater knowledge of the differences between authentic NO, nitrosothiol, acidified nitrite, and NO donor compound toxicities and the physiological significances of these toxicities is required for a full understanding of the NO detoxification function of flavoHbs.

The finding that NOD activity is conserved among the 1.8 billion-year-old flavoHbs (17) suggests that the ancestral Hb originated to help protect cells from the damaging effects of ambient NO and O<sub>2</sub> by combining them to form nontoxic nitrate. A nitrate production function may have also been advantageous during the  $\sim 1.5$  billion-year period between the origin

of oxygen-releasing photosynthesis and the advent of energy-yielding aerobic respiration. Nitrate would have yielded energy by serving as an anaerobic terminal electron acceptor at the same time that toxic NO and O<sub>2</sub> were removed. More stable O<sub>2</sub>-transporting Hbs and O<sub>2</sub>-storing Mbs would have only evolved much later when the atmosphere became rich in O<sub>2</sub> and large multicellular animals became possible because of the energy O<sub>2</sub> provided *via* an efficient O<sub>2</sub> supply system and respiration (62). The earliest Hbs may have served primarily as detoxifying agents along with superoxide dismutases, catalases, peroxidases, and cytochrome P-450s (60).

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