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Involvement of Molecular Oxygen in the Enzyme-Catalyzed NADH Oxidation and Ferric Leghemoglobin Reduction[1]

Lin Ji, Manuel Becana, and Robert V. Klucas*

Department of Biochemistry and School of Biological Science, University of Nebraska, Lincoln, Nebraska 68583 (L.J., R.V.K.); and Estación Experimental de Aula Dei, Apdo 202, 50080 Zaragoza, Spain (M.B.)

ABSTRACT

Ferric leghemoglobin reductase (FLbR) from soybean (*Glycine max* [L.] Merr) nodules catalyzed oxidation of NADH, reduction of ferric leghemoglobin (Lb^{+3}), and reduction of dichloroindophenol (diaphorase activity). None of these reactions was detectable when O_2 was removed from the reaction system, but all were restored upon readdition of O_2. In the absence of exogenous electron carriers and in the presence of O_2 and excess NADH, FLbR catalyzed NADH oxidation with the generation of H_2O_2 functioning as an NADH oxidase. The possible involvement of peroxide-like intermediates in the FLbR-catalyzed reactions was analyzed by measuring the effects of peroxidase and catalase on FLbR activities; both enzymes at low concentrations (about 2 μg/mL) stimulated the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction. The formation of H_2O_2 during the FLbR-catalyzed NADH oxidation was confirmed using a sensitive assay based on the fluorescence emitted by dichlorofluorescin upon reaction with H_2O_2. The stoichiometry ratios between the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction were not constant but changed with time and with concentrations of NADH and O_2 in the reaction solution, indicating that the reactions were not directly coupled and electrons from NADH oxidation were transferred to Lb^{+3} by reaction intermediates. A study of the affinity of FLbR for O_2 showed that the enzyme required at least micromolar levels of dissolved O_2 for optimal activities. A mechanism for the FLbR-catalyzed reactions is proposed by analogy with related oxidoreductase systems.

Molecular O_2 plays an important role in biological nitrogen-fixing systems. The nitrogenase complex is rapidly and irreversibly inactivated under aerobic conditions and, therefore, nitrogen-fixing organisms must exclude O_2 from the enzyme site. However, the resulting diminished diffusion fluxes of O_2 limit the availability of ATP and reductant for nitrogenase activity of diazotrophic microorganisms (7). Plants and bacteria have developed strategies for solving the O_2 dilemma of the deleterious effects of O_2 while preserving the energetic advantages of aerobic metabolism (20). The evolution of a variety of symbiotic structures, such as the root nodules of legumes, is probably the ultimate strategy. Within these complex structures, biochemical and physiological functions interact to achieve a dynamic balance between respiratory O_2 consumption and inward O_2 flux (21). The enzyme systems within these structures are directly or indirectly under the control and regulation of O_2.

O_2 is widely involved in biological oxidation and reduction systems. O_2 is strongly electrophilic, and, therefore, the reaction types in which it participates are a consequence of the amount of electronic charge that enzymes and their substrates present to O_2 (14). Products of the O_2-involved reactions in model biological systems are derived from various reactive O_2 species such as singlet O_2, superoxide radical, H_2O_2, and hydroxyl radical. Although the kinds of reactions in which O_2 participates in living organisms and the nature of the prosthetic groups and cofactors that catalyze them are well defined, the protein structures of enzymes mediating O_2 transfers, the structures of active sites, and the mechanisms by which O_2 is brought into reaction by these enzymes remain largely unsolved problems.

FLbR[2], an enzyme hypothesized (18) to maintain Lb in soybean nodules in the functional ferrous state (Lb^{+2}), was identified and purified to homogeneity (13). The FLbR was shown to be an FAD-containing multifunctional enzyme. The enzyme exhibited high activities for NAD(P)H-dependent DCIP and Lb^{+3} reductions and, in the absence of any other exogenous electron carriers, catalyzed NADH oxidation, functioning as NADH oxidase. An O_2 requirement for Lb^{+3} reduction by FLbR was observed while investigating properties of FLbR. Definitive evidence concerning the involvement of O_2 in NADH oxidation as well as in DCIP and Lb^{+3} reduction by this enzyme was previously nonexistent. The role of O_2 in these FLbR-catalyzed reactions is unknown. However, the superoxide anion does not appear to be involved in the FLbR-catalyzed Lb^{+3} reduction because superoxide dismutase has no effect on the reaction (6). The objectives of our study were to (a) obtain direct evidence that O_2 is required for FLbR-catalyzed NADH oxidation as well as Lb^{+3} and DCIP reductions, (b) determine the range of concentrations of O_2 required for FLbR activities, (c) study the stoichiometry between substrates and products for FLbR

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[2] Abbreviations: FLbR, ferric leghemoglobin reductase; Lb, leghemoglobin; DCIP, 2,6-dichloroindophenol; DCF, dichlorofluorescein; LDCF, dichlorofluorescin; Lb^{+3}, ferric Lb; Lb^{+2}, ferrous Lb, the reactive species that combines reversibly with O_2 to give $Lb^{+2}O_2$ (oxyferrous Lb); FAD, flavin adenine dinucleotide.

activities, and (d) investigate the mechanism of the FLbR reactions.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Pyridine nucleotides (NADH, NADPH, NAD^+), DCIP, DTT, horseradish peroxidase, catalase, H_2O_2, Bis-Tris, and Tris base were purchased from Sigma (St. Louis, MO). 2,7-Dichlorofluorescin diacetate was purchased from Eastman-Kodak (Rochester, NY). Sodium acetate, potassium phosphate (dibasic and monobasic), and ammonium sulfate were from Mallinckrodt (St. Louis, MO). N_2, O_2, and carbon monoxide (highly pure, >99.99%) were from Liquid Carbonic Corp. (Chicago, IL). All other inorganic and organic chemicals were from Fisher (Pittsburgh, PA).

Preparations of FLbR and Lb

FLbR was purified from soybean (*Glycine max* [L.] Merr. cv Hobbit × *Bradyrhizobium japonicum* strains SR 123 or 89) root nodules. The protocols for the extraction and purification of the FLbR were as described by Ji et al. (13). Lb and Lb^{+3} were prepared as described by Saari (17). Lb^{+3} concentration was measured using an extinction coefficient of 8.5 mM^{-1} cm^{-1} at 495 nm (3).

Determination of the O_2 Requirement for FLbR Activities

O_2 requirement for DCIP reductase activity of FLbR was investigated in the absence and presence of O_2. Approximately 950 μL of reaction mixture containing 0.6 mM NADH, 0.1 mM EDTA, 0.4 mM DCIP, and 20 mM Tris-HCl (pH 8.3) were added to a 1.5-mL semimicrocuvette equipped with a stopper (Quaracell Products, Baldwin, NY). The cuvette was sealed with a rubber septum. The septum was fitted with two hypodermic needles to allow gases to circulate through the cuvette chamber. O_2 was removed from cuvette by gassing with O_2-free N_2 at a flow rate of 10 mL/min for 3 h. The O_2-free N_2 was obtained by sparging highly pure N_2 through an alkaline pyrogallol solution. Under these conditions, the partial pressure of O_2 in the free space of the cuvette approached zero, and the O_2 dissolved in the solution was nearly zero, as shown by measurements with an O_2 microelectrode (Microelectrodes, Inc., Londonderry, NH). Slight evaporation was also observed, and the volumes were readjusted to the original volume by the addition of O_2-free water. Fifty microliters of FLbR (about 5 μg) were injected into the cuvette to start the reaction. The initial rate of DCIP reduction was determined at 600 nm with a Spectronic 3000 array spectrophotometer (Milton Roy, Rochester, NY), and data were acquired using Rapidscan software.

The O_2 requirement for FLbR-catalyzed NADH oxidation was determined by kinetic analysis of the reaction in the absence and presence of O_2. About 950 μL of reaction mixture containing 40 mM potassium phosphate (pH 7.0) and 0.6 mM NADH were added to a 1.5-mL semimicrocuvette. The cuvette was sealed with a rubber septum, and O_2 was removed from the reaction medium as described previously. Fifty microliters of FLbR (about 5 μg) were injected into the cuvette to start the reaction.

The O_2 requirement for the NADH-dependent Lb^{+3} reduction by FLbR was studied by measuring spectral changes between 300 and 650 nm in the absence and presence of O_2. Absorption changes at 340 nm were used to monitor NADH oxidation. Because both Lb^{+3} and $Lb^{+2}O_2$ also absorb light at this wavelength (Table I), a simultaneous equation was used for calculations. Absorption changes at 556 nm are indicative of reduction of Lb^{+3} to Lb^{+2}. Absorption changes at 574 nm were used to determine Lb^{+3} reduction and ligation with O_2 to form $Lb^{+2}O_2$. About 900 μL of reaction mixture containing 40 mM potassium phosphate buffer (pH 7.0) and 0.6 mM NADH were added to a 1.5-mL semimicrocuvette, and O_2 was removed from the reaction medium as described previously. After O_2 was removed, 50 μL of Lb^{+3} (0.7 mM) and 50 μL of FLbR (about 5 μg) were injected into the reaction cuvette to start the reaction. A series of spectra between 300 and 700 nm versus time in the absence of O_2 and under atmospheric conditions (or by addition of O_2 to the O_2-free reaction medium) were recorded with a Spectronic 3000 array spectrophotometer equipped with spectral acquisition software.

Effects of Peroxidase and Catalase on FLbR Activities

The effect of peroxidase on FLbR activities was measured in reaction mixtures containing 0.6 mM NADH, 0.05 mM Lb^{+3}, 0.5 units/mL of horseradish peroxidase (about 2 μg), and 40 mM potassium phosphate buffer (pH 7.0). The effect of catalase was measured by adding 20 units/mL of catalase (about 2 μg) in place of peroxidase in the above reaction mixtures. Effects of peroxidase and catalase on the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction in the absence and presence of O_2 were investigated by the spectral methods as described before.

Table I. *Extinction Coefficients Used for Calculations*

Molecule	Wavelength	Extinction Coefficient	Ref.
	nm	mM^{-1} cm^{-1}	
Lb^{+3}	340	24.3	
	496	8.5	3
	574	4.9	1
Lb^{+2}	556	14.5	1
$Lb^{+2}O_2$	340	24.8	
	574	15.1	1
NADH	340	6.2	10
NAD^+	340	0.1	10
FAD	375	9.3	10
FMN	373	10.4	10
DCIP	600	21.0	5

Table II. *Effects of O_2 on FLbR Activities for NADH Oxidation and for DCIP and Lb^{+3} Reductions*

Type of Enzymic Reaction	Specific Activity	
	With O_2	Without O_2
	nmol $min^{-1} \cdot mg^{-1}$ protein[a]	
DCIP reduction	4530	47.6
NADH oxidation	1850	55.6
Lb^{+3} reduction	388	2.0

[a] About 5 μg/mL of FLbR was used for each assay.

Detection of H_2O_2 Formed by FLbR-Catalyzed NADH Oxidation

The detection of H_2O_2 formed by the FLbR-catalyzed NADH oxidation was carried out using a sensitive fluorescence method described by Cathcart et al. (8) and Ferrer et al. (12). The assay is based on the fluorescence detection of DCF, which is formed by H_2O_2 oxidation of the nonfluorescent precursor LDCF. The assay medium contained 2 μM LDCF, 0.5 mM NADH, 50 mM Tris-HCl buffer (pH 7.5), and 10 μg/mL of FLbR to a total of 2 mL. The reaction was monitored using a Perkin-Elmer LS 50 spectrofluorometer (Perkin-Elmer, Norwalk, CT), which was interfaced to a computer. The excitation and emission wavelengths were 500 and 520 nm, respectively, with 2.5-nm slit widths.

Stoichiometry Analysis

The diode-array spectrophotometer provided an effective means to study the stoichiometry of FLbR-catalyzed NADH oxidation and Lb^{+3} reduction. The NADH oxidation and Lb^{+3} reduction were simultaneously measured as a function of time by acquiring spectra between 300 and 650 nm at a frequency interval of 15 s. The ratios of $[NAD^+]$ (the first product of NADH oxidation) to $[Lb^{+2}O_2]$ (the final product of Lb^{+3} reduction) were calculated from spectral data by simultaneous equations to demonstrate the stoichiometric relationships between FLbR-catalyzed NADH oxidation and Lb^{+3} reduction. Effects of NADH concentrations on this stoichiometry were also analyzed in the presence of O_2 by the same spectroscopic means. NADH concentrations of 0.022, 0.044, 0.088, 0.132, 0.176, 0.220, 0.264, and 0.340 mM were used.

Measurement of O_2 Levels Required for FLbR Activities

To determine the O_2 required for FLbR activities, 700 μL of 50 mM potassium phosphate buffer (pH 7.0) and 300 μL of distilled water were placed into nine semimicrocuvettes. Cuvettes were sealed, and O_2 was removed as described previously. Different volumes of pure O_2 were injected into the free space of each cuvette. Because the cuvette was sealed, the injection of various volumes of O_2 increased the partial pressure in the space and increased the O_2 dissolved in the solution. The concentration of O_2 dissolved in the solution in each case could be estimated using the standard solubility of O_2 (Bunsen coefficient $\alpha = 0.02831$ at 25°C) and the partial pressure correction. The O_2-injected cuvettes were equilibrated for 2 h before assaying. Fifty microliters of 0.56 mM Lb^{+3}, 50 μL of 6 mM NADH, and 10 μL of FLbR (10 μg) (all reagents were O_2 free) were injected into each of nine cuvettes to start the reaction. Kinetics of Lb^{+3} reduction by FLbR in the presence of various O_2 concentrations was measured by monitoring absorbance changes at 574 nm within 300 s with a kinetic acquisition program. Values of $[O_2]_{0.5}$, the concentration of O_2 at half of the maximal reaction rate, were determined from a plot of the initial velocity (in nmol/min) of the enzyme-catalyzed NADH oxidation and Lb^{+3} reduction versus the O_2 concentrations in solution (in μM).

The molar extinction coefficients used for calculations in this study are summarized in Table I. The extinction coefficients of Lb^{+3} and $Lb^{+2}O_2$ at 340 nm were calculated from spectra of Lb^{+3} and $Lb^{+2}O_2$ of known concentrations.

RESULTS

O_2 Requirement for DCIP Reductase Activity

The NADH-dependent DCIP reductase activity of FLbR was near zero in the absence of O_2 (Table II). Addition of O_2 to the reaction medium restored DCIP reductase-specific activity to about 4500 nmol $min^{-1} \cdot mg^{-1}$ protein (Table II). The DCIP reductase activity of FLbR was dependent on O_2.

O_2 Requirement for FLbR-Catalyzed NADH Oxidation

In the absence of O_2, FLbR activity for NADH oxidation was very low (Table II). In the presence of O_2, NADH was rapidly oxidized. The specific activity was 1850 nmol $min^{-1} \cdot mg^{-1}$ protein and was about 30-fold higher than that in the absence of O_2 (Table II). The kinetic data of the FLbR-catalyzed NADH oxidation are shown in Figure 1. The rate of anaerobic NADH oxidation in the absence of exogenous electron acceptors was near zero but significant in the pres-

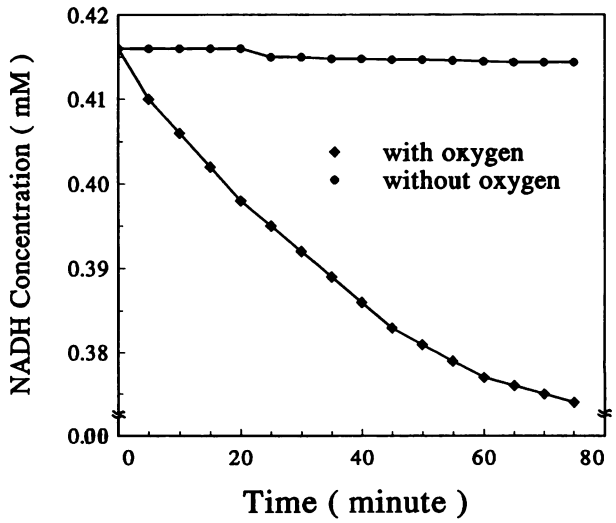

Figure 1. Kinetics of the FLbR-catalyzed NADH oxidation in the absence and presence of O_2. NADH oxidation was recorded as changes in absorbance at 340 nm. Each reaction contained about 5 μg of FLbR in a total volume of 1 mL.

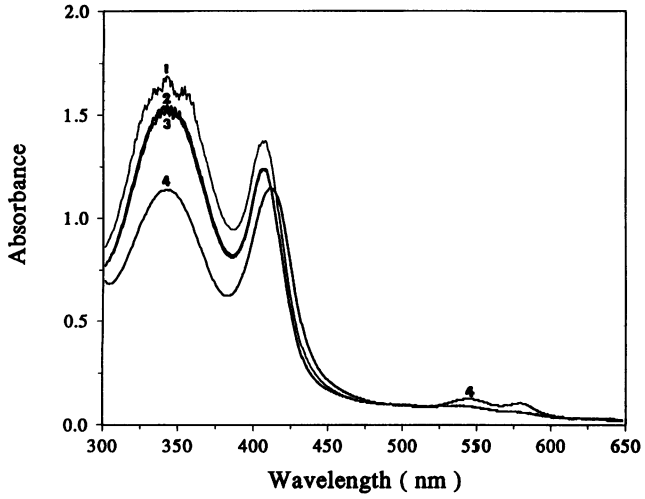

Figure 2. Spectral changes of FLbR-catalyzed NADH oxidation and Lb^{+3} reduction in the absence and presence of O_2. Line 1, The spectrum obtained when the reaction medium contained NADH and Lb^{+3} but not FLbR; line 2, the spectrum at time zero in the presence of FLbR and in the absence of O_2; line 3, the spectrum after 30 min in the presence of FLbR and in the absence of O_2; line 4, the spectrum 50 min after O_2 is added back to the reaction medium. NADH oxidation and Lb^{+3} reduction are shown as the spectral changes in a range of 300 to 400 nm and in a range of 520 to 600 nm, respectively. The reaction mixture contained 40 mM potassium phosphate (pH 7.0), 0.6 mM NADH, and 5 μg/mL of FLbR.

ence of O_2. These results indicated that FLbR possesses NADH oxidase activity in the absence of its substrate, Lb^{+3}.

O_2 Requirement for NADH-Dependent Lb^{+3} Reduction

The O_2 requirement for the FLbR-catalyzed Lb^{+3} reduction with NADH as the reductant was studied by measuring spectral changes between 300 and 650 nm (Fig. 2). When O_2 was removed from the reaction, absorption changes were not observed at 340 nm or at 556 nm after 30 min, and spectra were identical with the initial spectrum. Neither NADH oxidation nor Lb^{+3} reduction occurred, and thus, FLbR was inactive in the absence of O_2. Upon addition of O_2, both the absorption at 340 nm and absorptions at 541 and 574 nm changed, suggesting that NADH was oxidized and Lb^{+3} was reduced by the enzyme in the presence of O_2.

The kinetic data of FLbR-catalyzed NADH oxidation and Lb^{+3} reduction are shown in Table II. The maximal initial rate in the presence of O_2 is 1850 nmol of NAD^+ formed $min^{-1} \cdot mg^{-1}$ protein for the NADH oxidation and 388 nmol of $Lb^{+2}O_2$ formed $min^{-1} \cdot mg^{-1}$ protein for the Lb^{+3} reduction. Initial rates for both NADH oxidation and Lb^{+3} reduction in the absence of O_2 are negligible in comparison with those in the presence of O_2. The spectral and kinetic evidence strongly suggests that FLbR required O_2 for oxidation of NADH and reduction of Lb^{+3}.

Generation of Peroxide in the FLbR-Catalyzed NADH Oxidation and Lb^{+3} Reduction

Because the FLbR exhibited NADH oxidase activity in the presence of O_2, a reaction mechanism that includes the generation of peroxide was suggested. The effect of peroxidase and catalase on FLbR activities for NADH oxidation and Lb^{+3} reduction was studied to determine whether H_2O_2 or other peroxide-like compounds are involved in the reaction. Peroxidase at a concentration of 2.3 μg/mL stimulated both NADH oxidation and Lb^{+3} reduction (Table III) but showed a greater effect on NADH oxidation than on Lb^{+3} reduction. Catalase at 1.8 μg/mL also stimulated both the NADH oxidation and the Lb^{+3} reduction (Table III). These results are indicative of the generation of H_2O_2 or some hydroperoxide intermediates during the FLbR-catalyzed NADH oxidation.

The formation of H_2O_2 or peroxide intermediate from the FLbR-catalyzed NADH oxidation was confirmed using a sensitive fluorescent DCF assay method (8, 12). The characteristic excitation/emission spectra of the fluorescent DCF oxidized from nonfluorescent LDCF by enzymically produced H_2O_2 were recorded (Fig. 3). The progress of H_2O_2 formation by the FLbR-catalyzed NADH oxidation was reflected on fluorescence increases. In the absence of the FLbR, no reaction took place, and the spectra remained unchanged after a 40-min incubation (Fig. 3, A and A′ versus B and B′). Upon addition of the FLbR, the increase in fluorescence was obvious after 5 min (C and C′) and 30 min (D and D′). This demonstrated that H_2O_2 or peroxide intermediates were generated and involved in the FLbR-catalyzed NADH oxidation.

Stoichiometry between the FLbR-Catalyzed NADH Oxidation and Lb^{+3} Reduction

The stoichiometry between the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction and the effects of various concentrations of NADH on those ratios are shown in Figure 4. The stoichiometry ratios are not constant throughout the reaction but increased with time and with NADH concentration in the reaction medium. The stoichiometry data also suggest that, although the reduction of Lb^{+3} is dependent on NADH oxidation, the two processes are not directly coupled.

O_2 Levels Required for FLbR Activities

To describe the requirement for O_2 more quantitatively, the effect of various O_2 concentrations on the enzyme-cata-

Table III. *Effects of Peroxidase and Catalase on FLbR Activities for NADH Oxidation and Lb^{+3} Reduction*

Treatment	Specific Activity			
	NADH oxidation[b]		Lb^{+3} reduction[c]	
	nmol $min^{-1} \cdot mg^{-1}$ protein[a]	%	*nmol $min^{-1} \cdot mg^{-1}$ protein*[a]	%
Without peroxidase	2110	100	400	100
With peroxidase[d]	5320	252	530	134
Without catalase	1920	100	410	100
With catalase[e]	6180	322	590	146

[a] About 3 μg/mL of FLbR was used for each assay. [b] The activity is defined as nmol of NADH oxidized min^{-1} mg^{-1} of protein. [c] The activity is defined as nmol of LbO_2 formed min^{-1} mg^{-1} of protein. [d] 0.5 Sigma units mL^{-1} (2.3 μg mL^{-1}) of peroxidase was used for the assays. [e] Twenty Sigma units mL^{-1} (1.8 μg mL^{-1}) of catalase were used for the assays.

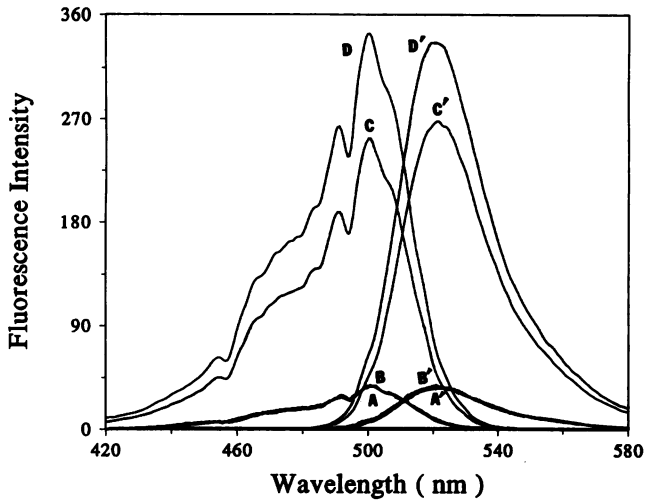

Figure 3. Generation of H_2O_2 in the FLbR-catalyzed NADH oxidation. Excitation and emission spectra demonstrate the fluorescent property of DCF that resulted from the oxidation of LDCF by H_2O_2 during the FLbR-catalyzed NADH oxidation. Curves A, A' and B, B' are excitation and emission spectra of controls in the absence of the FLbR, at time zero and after 40 min, respectively. Curves C, C' and D, D' are excitation and emission spectra in the presence of the FLbR after 5 min and 30 min incubation, respectively. The assay medium contained 50 mM Tris-HCl buffer (pH 7.5), 2 μM LDCF, and 0.5 mM NADH in a total volume of 2 mL. About 20 μg of FLbR was used for each reaction.

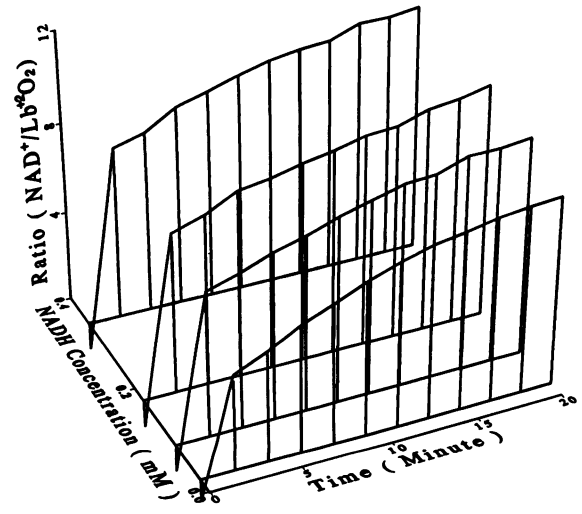

Figure 4. Effects of NADH concentrations and reaction time on stoichiometry of the FLbR-catalyzed NADH oxidation to Lb^{+3} reduction. Stoichiometry was calculated from the ratios of $[NAD^+]$ (the first product of NADH oxidation) to $[Lb^{+2}O_2]$ (the final product of Lb^{+3} reduction). The concentrations of NADH were 0.022, 0.088, 0.176, and 0.340 mM, respectively. Each reaction contained about 5 μg of FLbR in a total volume of 1 mL.

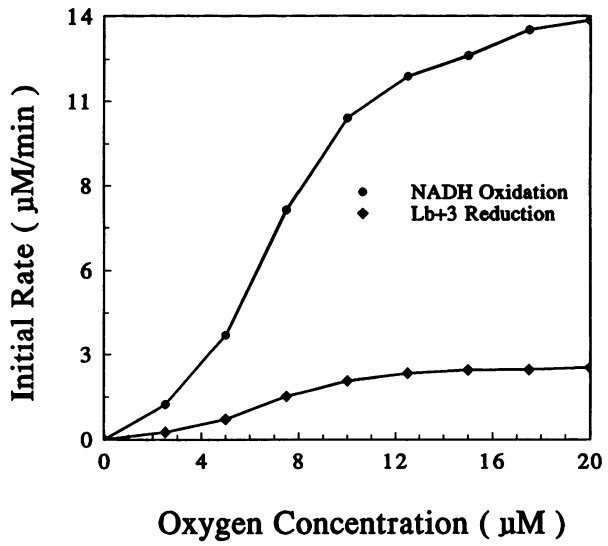

Figure 5. Effects of O_2 concentrations on the kinetics of the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction. Each reaction contained 10 μg of FLbR in a total volume of 1 mL.

lyzed NADH oxidation and Lb^{+3} reduction was measured. The initial rate of NADH oxidation and Lb^{+3} reduction as a function of O_2 concentration is illustrated in Figure 5. Enzyme activities for both NADH oxidation and Lb^{+3} reduction appear to increase as O_2 concentration was increased. This suggests that O_2 participates in these redox reactions either as an activator that stimulates enzyme activities or as a substrate that is consumed for NADH oxidation. For lower concentrations of O_2 (2.5–5.0 μM), the initial rate of both NADH oxidation and Lb^{+3} reduction rapidly increased to the maximum in about 10 s after enzyme was injected into the reaction medium (data not shown). This possibly resulted from the consumption of O_2 under such low concentrations of O_2. If we assume that O_2 was totally consumed at such low concentrations of O_2, the ratio of O_2 uptake to NADH oxidation can be calculated. Such calculations revealed that the O_2 uptake and NADH oxidation occurred stoichiometrically, giving a molar ratio of about 1. As concentrations of O_2 increased, both NADH oxidation and Lb^{+3} reduction increased but at different rates (Fig. 5). Rates of Lb^{+3} reduction and NADH oxidation approached a maximum at 10 to 12 μM and at 18 to 20 μM of O_2, respectively.

More specifically, $[O_2]_{0.5}$, the concentration of O_2 at half of the maximal rate for the FLbR-catalyzed Lb^{+3} reduction, was used to describe the O_2 requirement of FLbR. Although the concentrations of O_2 for the maximal rate are different for the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction, the values of $[O_2]_{0.5}$ are approximately the same, i.e. about 7 μM. This value of $[O_2]_{0.5}$ may be presented as a characteristic constant of the FLbR for O_2. These results indicate that the FLbR requires microaerobic conditions for activity and support the previous suggestions that O_2 is required for NADH oxidation.

DISCUSSION

This study provides evidence that in the presence of excess NADH reductant, O_2 was required for the FLbR-catalyzed

NADH oxidation, DCIP reduction, and Lb^{+3} reduction. When O_2 was removed from the medium, these enzymic activities were very low or not detectable, but, upon readdition of O_2 into the reaction medium, all were restored significantly. Although the Lb^{+3} reduction was dependent on the presence of NADH, the stoichiometry between the enzyme-catalyzed NADH oxidation and Lb^{+3} reduction was not constant but varied with time as well as concentrations of NADH and O_2 in the reaction medium. Also, the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction exhibited different kinetics. These results indicated that NADH oxidation and Lb^{+3} reduction were not directly coupled.

Electrons involved in NADH oxidation and O_2 reduction are probably transferred by intermediate carriers rather than by a direct interaction between NADH and Lb^{+3}. Because exogenous electron carriers were not added to the reactions, actual electron carriers might have been the enzyme itself or endogenously formed intermediates. Possibly, the enzyme is converted into, or maintained in, a catalytically active state by O_2-dependent NADH oxidation. The activated, NADH-reduced form of FLbR mediated the electron transfer to the substrate and catalyzed the consequent Lb^{+3} reduction. The enzyme-FAD-hydroperoxide could be an intermediate in mediating this electron pathway (15, 19), considering the structural similarity to the enzyme family of flavin-nucleotide disulfide oxidoreductases (13).

FLbR could also catalyze NADH oxidation in the presence of O_2 and in the absence of any other electron mediators, functioning as an NADH oxidase. This reaction required formation of H_2O_2 and/or other peroxide-like intermediates. Because FLbR also exhibited NADH-oxidizing activity in the presence of O_2 with the concomitant generation of H_2O_2, this enzymic reaction could be generalized as

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2.$$

Both peroxidase and catalase at low concentrations (about 2 μg/mL) stimulated the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction, providing evidence that H_2O_2 or enzyme-peroxide intermediates were involved. These higher rates may have resulted from the removal of H_2O_2 or other hydroperoxides as products of NADH oxidation. Hydroperoxides can reoxidize FLbR-reduced Lb (Lb^{+2}) to Lb^{+3} or to even higher valence states such as Lb(IV), a very stable but inactive form of Lb (4). Thus, H_2O_2 may either decrease the availability of Lb^{+3} or inactivate Lb in vivo and in vitro. In the case of flavoprotein monoxygenases, NADH reduces the enzyme-bound flavin, which then reacts with O_2 to produce the flavin (C4a)-hydroperoxide (15). The hydroperoxide is very stable in the absence of the substrate, but, upon addition of the third substrate, the hydroperoxide can rapidly transfer its terminal O_2 to the substrate, which then loses H_2O to generate the oxidized flavoprotein. A similar mechanism might also contribute to the accelerations of the FLbR-catalyzed NADH oxidation and Lb^{+3} reduction by catalase and peroxidase.

In contrast to the stimulation effects reported here, we previously observed that higher concentrations of catalase (>10 μg/mL) modestly inhibited Lb^{+3} reduction (6), suggesting that H_2O_2, in addition to its involvement in the NADH oxidation, may be an intermediate affecting Lb^{+3} reduction. The inhibitory effects of higher concentrations of catalase on Lb^{+3} reduction may have resulted from the binding of these enzymes to the active site of FLbR. The diverse and paradoxical effects of catalase might reflect the diversity of roles played by various O_2 species derived from the O_2-involved reactions. The redox state of enzymes, substrates, and intermediates, as well as the availability and coordination of metals, all influence the roles of those O_2 species.

The partial N-terminal amino acid sequence analysis of FLbR has shown that FLbR is highly related to other flavin dinucleotide disulfide oxidoreductases, especially to lipoamide dehydrogenase (13). Both FLbR and lipoamide dehydrogenase are enzymes consisting of two identical subunits, each having a molecular mass of 52 to 54 kD, one molecule of FAD as prosthetic group, and one active disulfide center in the N-terminal region (13). These common characteristics suggest that FLbR may be a flavin dinucleotide-disulfide oxidoreductase and that the mechanism of FLbR is analogous to those of other flavin dinucleotide oxidoreductases and, particularly, to that of lipoamide dehydrogenase.

Lipoamide dehydrogenase is a multifunctional flavoenzyme with significant oxidoreductase activity (9). The electrons flow sequentially from NADH to FAD to the active disulfide, and then one or two electrons are transferred to different substrates or acceptors. Lipoamide dehydrogenases can be reduced to a four-electron state, but the two-electron reduced form is the catalytically active species involved in the oxidation of dihydrolipoamide and in the reduction of lipoamide with NADH over a broad range of pH (16). Lipoamide dehydrogenases can also function as NADH oxidases. The NADH-dependent oxidase activity of lipoamide dehydrogenase from porcine heart amounts to about 8% of its activity, generating O_2^- and H_2O_2 in a ratio of 1:9 under aerobic conditions (5). The four-electron state of lipoamide dehydrogenase preferentially reacts with O_2 and displays high NADH oxidase activity.

By analogy with what is known about the mechanism of lipoamide dehydrogenase and of other flavin dinucleotide oxidoreductases (15), we propose that, in the absence of substrate, the fully reduced FLbR form preferentially reacts with O_2 as an NADH oxidase. In the presence of substrate (Lb^{+3}), the rapid reoxidation of the enzyme to the catalytically active form would then occur with the concomitant generation of H_2O_2, either by a direct one-electron transfer or via the FLbR-hydroperoxide intermediate; the resulting active FLbR form would catalyze Lb^{+3} reduction. In the reduction of Lb^{+3} under aerobic conditions, the hydroperoxide could also rapidly transfer its terminal O_2 to the accompanying reduced Lb (Lb^{+2}) to give $Lb^{+2}O_2$.

In vitro FLbR requires at least micromolar levels of O_2 for optimum Lb^{+3} reduction. Because the internal concentrations of free O_2 in intact legume nodules are in the range of 10 to 30 nM (2, 11), the question arises as to the physiological relevance of FLbR in soybean nodules. We suggest that $Lb^{+2}O_2$ could act as an O_2 donor in reactions mediated by oxidoreductases. $Lb^{+2}O_2$ may donate O_2 to O_2-requiring enzymes or enzymic substrates with its concomitant deoxygenation. Thus, $Lb^{+2}O_2$ could efficiently provide O_2 for O_2 acceptors and be maintained in the functional ferrous state and

also could maintain a low free O_2 environment for optimum nitrogenase activity in nodules.

LITERATURE CITED

1. **Appleby CA** (1969) Properties of leghemoglobin in vivo, and its isolation as ferrous oxyleghemoglobin. Biochim Biophys Acta **188:** 222–229
2. **Appleby CA** (1984) Leghemoglobin and *Rhizobium* respiration. Annu Rev Plant Physiol **35:** 443–478
3. **Appleby CA, Nicola NA, Hyrrell JGR, Leach SJ** (1975) Characterization and improved separation of soybean leghemoglobins. Biochemistry **14:** 4444–4450
4. **Aviram I, Wittenberg BA, Wittenberg JB** (1978) The reaction of ferrous leghemoglobin with hydrogen peroxide to form leghemoglobin (IV). J Biol Chem **253:** 5685–5689
5. **Bando Y, Aki K** (1991) Mechanisms of generation of oxygen radicals and reductive mobilization of ferritin iron by lipoamide dehydrogenase. J Biochem **109:** 450–454
6. **Becana M, Klucas RV** (1990) Enzymatic and nonenzymatic mechanisms for ferric leghemoglobin reduction in legume root nodules. Proc Natl Acad Sci USA **87:** 7295–7299
7. **Bergersen FJ** (1984) Oxygen and the physiology of diazotrophic microorganisms. *In* C Veeger, WE Newton, eds, Advances in Nitrogen Fixation Research. Martinus Nijhoff/Dr. W Junk Publishers, New York, pp 171–180
8. **Cathcart R, Schwiers E, Ames BN** (1983) Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal Biochem **134:** 111–116
9. **Danson MJ** (1988) Dihydrolipoamide dehydrogenase: a "new" function for an old enzyme? Biochem Soc Transact **16:** 87–89
10. **Dawson RMC, Elliott DC, Elliott WH, Jones KM**, eds (1986) Data for Biochemical Research, Ed 3. Oxford Science Publications, New York
11. **Denison RF, Layzell DB** (1991) Measurement of legume nodule respiration and O_2 permeability by noninvasive spectrophotometry of leghemoglobin. Plant Physiol **96:** 137–143
12. **Ferrer AS, Santema JS, Hilhorst R, Visser AJWG** (1990) Fluorescence detection of enzymatically formed hydrogen peroxide in aqueous solution and in reversed micells. Anal Biochem **187:** 129–132
13. **Ji L, Wood S, Becana M, Klucas RV** (1991) Purification and characterization of soybean root nodule ferric leghemoglobin reductase. Plant Physiol **96:** 32–37
14. **Keevil T, Mason HS** (1978) Molecular oxygen in biological oxidations: an overview. Methods Enzymol **52:** 3–40
15. **Massey V, Schopfer LM, Anderson RF** (1988) Structural determinations of the oxygen reactivity of different classes of flavoproteins. *In* R Alan, ed, Oxidase and Related Redox Systems. Liss, New York, pp 147–166
16. **Massey V, Veeger C** (1961) Studies on the reaction mechanism of lipoyl dehydrogenase. Biochim Biophys Acta **48:** 33–47
17. **Saari LL** (1982) Leghemoglobin reductase. PhD Thesis. University of Nebraska-Lincoln
18. **Saari LL, Klucas RV** (1984) Ferric leghemoglobin reductase from soybean nodules. Arch Biochem Biophys **231:** 102–113
19. **Williams CH Jr** (1991) Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase and mercuric ion reductase—family of flavoenzyme transhydrogenases. *In* F Müller, ed, Chemistry and Biochemistry of Flavoenzymes, Ed 1, Vol 3. CRC Press, Boca Raton, FL, pp 121–211
20. **Witty JF, Minchin FR, Sheehy JE, Minguez IM** (1984) Acetylene-induced changes in the oxygen diffusion resistance and nitrogenase activity of legume root nodules. Ann Bot **53:** 13–20
21. **Witty JF, Minchin FR, Skøt L, Sheehy JE** (1986) N_2 fixation and oxygen in legume root nodules. Oxford Surv Plant Mol Cell Biol **3:** 275–314