O$_2$ and Reactive Oxygen Species Detoxification Complex, Composed of O$_2$-Responsive NADH:Rubredoxin Oxidoreductase-Flavoprotein A2-Desulfoferrodoxin Operon Enzymes, Rubperoxin, and Rubredoxin, in Clostridium acetobutylicum\textsuperscript{v†}

Shinji Kawasaki,* Yu Sakai, Tohru Takahashi, Ippei Suzuki, and Youichi Niimura

Department of Biosciences, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

Received 26 June 2008/Accepted 8 December 2008

Clostridium acetobutylicum, an obligate anaerobe, grows normally under continuous-O$_2$-flow culture conditions, where the cells consume O$_2$ proficiently. An O$_2$-responsive NADH:rubredoxin oxidoreductase operon composed of three genes (nror, fprA2, and dsr), encoding NROR, functionally uncharacterized flavoprotein A2 (FprA2), and the predicted superoxide reductase desulfoferrodoxin (Dsr), has been proposed to participate in defense against O$_2$ stress. To functionally characterize these proteins, native NROR from C. acetobutylicum, recombinant NROR (rNROR), FprA2, Dsr, and rubredoxin (Rd) expressed in Escherichia coli were purified. Purified native NROR and rNROR both exhibited weak H$_2$O$_2$-forming NADH oxidase activity that was slightly activated by Rd. A mixture of NROR, Rd, and FprA2 functions as an efficient H$_2$O-forming NADH oxidase with a high affinity for O$_2$ (the $K_m$ for O$_2$ is 2.9 ± 0.4 $\mu$M). A mixture of NROR, Rd, and Dsr functions as an NADH-dependent O$_2$ reductase. A mixture of NROR, Rd, and rubperoxin (Rpr, a rubrerythrin homologue) functions as an inefficient H$_2$O-forming NADH oxidase but an efficient NADH peroxidase with a low affinity for O$_2$ and a high affinity for H$_2$O$_2$ (the $K_m$s for O$_2$ and H$_2$O$_2$ are 303 ± 39 $\mu$M and ≤1 $\mu$M, respectively). A gene encoding Rd is dicistronically transcribed with a gene encoding a glutaredoxin (Gd) homologue, and the expression levels of the genes encoding Gd and Rd were highly upregulated upon exposure to O$_2$. Therefore, nror operon enzymes, together with Rpr, efficiently function to scavenge O$_2$, O$_2^-$, and H$_2$O$_2$ by using an O$_2$-responsive rubredoxin as a common electron carrier protein.

The genus Clostridium, consisting of bacteria that are typical obligate anaerobes, is of great interest due to its use for bioenergy fermentation and biodegradation. The members of this genus exhibit O$_2$-sensitive growth profiles (11, 34). The mechanism by which oxidative growth inhibition of these bacteria leads to cell death is believed to be due to a lack of enzymes such as catalase and superoxide dismutase (SOD), which can scavenge reactive oxygen species (ROS) (12, 26, 35, 37). It has been reported for one member of this species, Clostridium acetobutylicum, that although cell growth ceases during a short period of aeration, cell growth resumes once the aeration is stopped, without apparent cell damage (28). It was proposed that the observed growth cessation under aerated conditions was due to a decrease in intracellular reducing capacity caused by initiation of an NADH oxidase reaction. Subsequent to this study, we have shown that several species of Clostridium, such as C. butyricum, C. acetobutylicum, and C. aminovalericum, grow normally under O$_2$ flow culture conditions by efficiently consuming O$_2$ (15–18). Molecular approaches have been used to determine the enzymes required for the microaerobic growth of Clostridium species. These experiments have mainly been performed on C. acetobutylicum, an efficient acetone-butanol-fermenting bacterium whose genomic structure has been elucidated (27). In our previous study, two proteins, namely, rubperoxin and flavoprotein A1, were identified for the first time as O$_2$-inducible proteins in C. acetobutylicum by using two-dimensional electrophoresis, and the transcripts were rapidly upregulated following exposure to O$_2$ (17). Rubperoxin was identified as a rubrerythrin homologue and was later named rubperoxin based on its unique structure and on its functional characteristics (19). This protein was also identified as a heat-inducible protein by other investigators and was therefore named heat shock protein HSP-21 (24). These investigators reported that this protein was induced by heat, low temperature, pH, butanol, NaCl, H$_2$O$_2$, and O$_2$ (10, 24). Other O$_2$ stress-responsive genes, encoding nror operon enzymes (NROR, FprA2, Dsr, Orf2451, and flavodoxin), glutathione peroxidase homologues, bacterioferritin comigratory protein, and thiol peroxidase, were identified in our previous study with the induction of enzyme activities for O$_2$ and ROS scavenging (18). With the exceptions of rubperoxin and Dsr, the functions of most of these O$_2$-inducible proteins have not been determined. Rubperoxin has been shown to function as a novel type of NAD(P)H-dependent H$_2$O$_2$ reductase together with an unknown proximal electron donor protein (19). The recombinant Dsr protein has been purified and is proposed to function as a superoxide reductase using spinach ferredoxin NADP\textsuperscript+ reductase (30). However, there are still open questions concerning the identity of its proximal electron donor protein and concerning the kinetic details of the reaction.

Regarding O$_2$-consuming activity in the Clostridium spe-

\* Corresponding author. Mailing address: Department of Biosciences, Tokyo University of Agriculture, 1-1-1 Setagaya-ku, Tokyo 156-8502, Japan. Phone and fax: 81-3-5477-2764. E-mail: kawashin@nodai.ac.jp.
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cies, we have reported the purification and characterization of a H₂O-forming NADH oxidase in *C. acetobutylicum* (16). The gene encoding *C. acetovaricardum* NADH oxidase is rapidly upregulated upon exposure to O₂, and enzyme kinetic studies have revealed that this enzyme can function as an O₂-consuming enzyme in vivo (the Kₘ for O₂ is calculated as 61.9 μM) (16, 18). A H₂O-forming NADH oxidase homologue is not present in the *C. acetobutylicum* genome, though NROR does have a low homology (17% identity) to this enzyme (18). The *C. acetobutylicum* NROR was originally characterized as a NADH:rubredoxin oxi- doreductase, although a function of NROR in protection against oxidative stress has not been elucidated (9, 29). Although we have shown that *nrro* operon genes are responsive to O₂ stress, the NROR enzyme lacks a cysteine residue at the active center, which implies that NROR lacks an NADH-dependent O₂ reductase activity (18).

In this study, we investigated the function of O₂-responsive *nrro* operon enzymes by purification of the proteins and characteriza- tion of their enzymatic activity. We propose that the obligate anaerobe *C. acetobutylicum* possesses an efficient multienzyme complex that can scavenge O₂ and ROS by using NROR as a master electron donor protein.

**MATERIALS AND METHODS**

Reagents. The reagents were of the highest grade that is commercially avail- able. Riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide, horse heart cytochrome c, and bovine liver catalase were purchased from Sigma. Cow milk xanthine oxidase (XOD) was purchased from Roche.

Cloning and expression of NROR, Rd, FprA2, and Dsr in Escherichia coli. The genes CAC2448 (encoding NROR-N (5'-ATGATCATA TCATCATCATCATATAAAAGCACAATTTAATATTCAT-3') and NROR-N-C (5'-C CATCATCATCATCATATAAAAGCACAATTTAATATTCAT-3') and CAC2778 (encoding Rd) were amplified from the initial genes CAC2448 (encoding NROR), CAC2449 (encoding FprA2), and CAC2450 (encoding Dsr) and were ligated into the pET7Blue vector, respectively, as described previously (16, 18). The expression of the NROR-N, Rd, FprA2, and Dsr genes was driven by the strong T7 promoter.

**Purification of recombinant NROR and FprA2.** The *E. coli* CFE containing *rnrnor* or recombinant FprA2 was applied to a Talon metal affinity column (Clontech, Japan) and then sequentially washed with the same buffer containing 50 mM or 100 mM imidazole. The enzyme was eluted with a buffer containing 150 mM potassium phosphate buffer (pH 7.0) and eluted with sequential appli- cation of 190 mM and 230 mM potassium phosphate buffer (pH 7.0). The fractions were assayed by sodium dodecyl sulfate-polyacrylamide gel electro- phoresis (SDS-PAGE), and the fractions chosen for further purification were combined and applied to a gel filtration column (3.5 by 20 cm). The fractions containing NROR were concentrated with Amicon Ultra (30,000-Da cutoff; Millipore, Japan).

**Purification of recombinant Dsr.** The *E. coli* CFE in which the recombinant Dsr was overexpressed was fractionated by the stepwise addition of solid ammo- nium sulfate to give a final concentration of 40% (wt/vol). The precipitate obtained after centrifugation at 30,000 g × 15 min was resuspended in 0.5 M ammonium sulfate dissolved in the same buffer containing FeCl₃ (0.01 mM). The fractions were assayed by SDS-PAGE, and the fractions chosen for further purification were combined and applied to a gel filtration column (3.5 by 20 cm). The fractions containing FprA2 were concentrated with Amicon Ultra (30,000-Da cutoff; Millipore, Japan).

**Determination of the spectrum properties and protein concentration of the purified enzymes.** The purity of all of the purified enzymes was checked by SDS-PAGE and Coomassie brilliant blue staining. The identity of all of the purified proteins was confirmed by N-terminal amino acid sequencing. For each of the proteins, the sequence of the N-terminal 20 amino acids was completely identical to the sequence predicted by translation from the respective target gene. UV/visible absorption spectra of the purified proteins were recorded on a Beckman DU70 spectrophotometer using 1-cm-path-length quartz cuvettes. The molar absorption coefficient was determined for each protein by averaging the quantities of alanine, proline, valine, threonine, and tyrosine from quantitative amino acid analysis performed at Toray Research Center, Inc., using a Hitachi model L-8500 amino acid analyzer. The calculated molar extinction coeffi- cient was determined for Rd (ε₂₈₀ = 5,799.1 M⁻¹ cm⁻¹), rNROR (ε₂₈₀ = 12,870.6 M⁻¹ cm⁻¹), FprA2 (ε₃₅₀ = 14,318.7 M⁻¹ cm⁻¹), and Dsr (ε₃₅₀ = 18,667.9 M⁻¹ cm⁻¹).

For enzymatic analyses, purified enzyme solutions were desalted in an enzyme concentrator (Amicon Ultra, 3,000-kDa to 30,000-kDa cutoff; Millipore, Japan). Briefly, the buffer in which the purified enzyme was dissolved was changed to 50 mM potassium phosphate buffer, pH 7.0, by diluting the concentrated enzyme solution 100-fold with the new buffer, followed by centrifugation at 30,000 g × 15 min to give the original volume. This dilution and concentration process was then repeated, and the final concentrated enzyme solution was subjected to an enzyme assay. The flavin content of FprA2 was determined by high-performance liquid chromatography analysis according to a previously described method (16). Flavin
was identified using riboflavin, flavin adenine dinucleotide, and FMN as standards.

The specific activities shown are the averages of results from three independent measurements that varied by less than 10%.

**Measurement of the NADH oxidation activity of NROR in the presence or absence of Rd.** The NAD(P)H oxidation activity of NROR, and the effect of Rd on this activity, was assayed by monitoring the decrease in dissolved O2 concentration with an O2 electrode (YSI OH) at 37°C. The enzymatic reaction was started by the addition of purified native or recombinant NROR, or a mixture of native or recombinant NROR and Rd, into an air-saturated 50 mM potassium phosphate buffer containing NADH (150 μM) in a reaction cuvette. One unit of NADH- and NADPH-dependent O2 reductase activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol O2 per minute. The pH optimum of this enzyme reaction was determined with 50 mM potassium phosphate buffer over a pH range of 5.0 to pH 8.0. The pH optima for both native and recombinant NROR-Rd O2 reductase activity were pH 7.0, and this pH was used for the experiments described below.

**Measurement of the NAD(P)H-dependent Rd reductase activity of NROR.** The NAD(P)H-dependent Rd reductase activity of NROR was assayed by monitoring the reduction of Rd that was detected as a decrease in absorbance at 492 nm using a Shimadzu U-160 spectrophotometer (Shimadzu, Kyoto, Japan) and 1-cm-path-length anaerobic quartz cuvettes at 25°C. All experiments were performed under an atmosphere of O2-free argon prepared by passing 99.9999% argon through a gas-clean column (O2 trapper; Nikka Seiko Co., Tokyo, Japan). The anaerobic enzyme samples and NAD(P)H solutions were prepared in an all-glass apparatus by sequential evacuation and reequilibration with O2-free argon. Enzyme and NAD(P)H solutions were introduced into anaerobic glass cuvettes that contained anaerobic reaction buffer (50 mM potassium phosphate buffer, pH 7.0) through a gas-tight syringe. One unit of NADH- and NADPH-dependent Rd reductase activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol Rd (440 μM) per minute.

**Measurement of the NAD(P)H-dependent O2 reductase activity of a mixture of NROR and Rd or NROR, Rd, and FprA2.** NAD(P)H-dependent O2 reductase activity was assayed by monitoring the decrease in dissolved O2 concentration with an O2 electrode (YSI OH) at 37°C. Reactions were started by the addition of purified native or recombinant NROR and Rd, or of NROR, Rd, and FprA2 proteins to a reaction cuvette containing 50 mM potassium phosphate buffer (pH 7.0). One unit of NADH- and NADPH-dependent O2 reductase activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol O2 per minute.

**Measurement of the NADH-dependent O2− reduction activity of a mixture of NROR, Rd, and Dsr.** NADH-dependent superoxide anion reductase activity was assayed by monitoring the decrease in absorbance at 340 nm with a Shimadzu U-160 spectrophotometer at 37°C. Reactions were started by the addition of NROR, a mixture of NROR and Rd, a mixture of NROR and Dsr, or a mixture of NROR, Rd, and Dsr proteins in air-saturated 50 mM potassium phosphate buffer (pH 7.0) into a reaction cuvette. A flux of superoxide anion was then initiated by the addition of a precalibrated amount of XOD and xanthine (0.5 mM each). The reactions were started before and after every experiment by measuring the rate of reduction of horse heart ferricytochrome c (20 μM or 100 μM) at 550 nm (εoxygen = 21 μM cm−1) (25). One unit of NADH- and NADPH-dependent superoxide anion reductase activity was defined as the amount of NROR that catalyzes the oxidation of 1 μmol NADH per minute. The small, background NADH oxidation that was detected prior to the addition of XOD was subtracted from that measured after the addition of XOD.

**Measurement of the NADH-dependent O2− and H2O2 reductase activity of a mixture of NROR, Rd, and Rpr.** Recombinant Rpr protein was purified from E. coli as described previously (19) with the exception of the FeSO4 (0.1 mM) into the E. coli culture medium, which ensured incorporation of the Fe atom into Rpr (1.8 ± 0.3 Fe/Rpr monomer). NADH-dependent O2 reductase activity was assayed by monitoring the decrease in dissolved O2 concentration with an O2 electrode at 37°C. One unit of NADH- and NADPH-dependent O2 reductase activity was defined as the amount of NROR that catalyzes the reduction of 1 μmol O2 per minute. NADPH-dependent H2O2 reductase activity was assayed by monitoring the decrease in absorbance at 340 nm with a Shimadzu U-160 spectrophotometer using 1-cm-path-length anaerobic quartz cuvettes at 37°C. All experiments were performed under an atmosphere of O2-free argon prepared by passing 99.9999% argon through a gas-clean column (O2 trapper; Nikka Seiko Co., Tokyo, Japan). The anaerobic enzyme samples, NADH, NADPH, and H2O2, were prepared in an all-glass apparatus by sequential evacuation and reequilibration with O2-free argon. The reactions were started by the addition of a mixture of NROR and Rd or NROR, Rd, and Rpr proteins in 50 mM potassium phosphate buffer (pH 7.0) through a gas-tight syringe. After the addition of all of the purified enzymes into a reaction cuvette, H2O2 (0.1 mM) was added using a gas-tight syringe. One unit of NADH- and NADPH-dependent H2O2 reductase activity was defined as the amount of NROR that catalyzes the oxidation of 1 μmol NADH per minute.

**Measurement of SOD activity.** SOD activity was assayed by monitoring the inhibition of nitroblue tetrazolium reduction (560 nm) by superoxide anion that is generated by xanthine and XOD at 37°C as described previously (2). One unit of SOD activity was defined as the amount of protein that inhibits the rate of nitroblue tetrazolium reduction by 50%.

**Steady-state kinetic analyses.** Kinetic parameters of the purified enzymes were determined by the following experiments. Initial rates were determined from linear plots of product formation (or substrate disappearance). For the NADH-dependent O2 reductase assay, buffer solutions containing different concentrations of dissolved O2 were prepared by purging a N2-basal gas with different O2 concentrations. The final dissolved O2 concentration in the reaction cuvette was checked with an O2 electrode prefitted to a cuvette by a rubber seal to prevent O2 contamination from outside the cuvette. The Km value for Rd of NROR was determined using O2 as an electron acceptor by varying the concentration of Rd with fixed concentrations of NRR (0.01 μM), NADH (150 μM), and O2 (air saturated; 213 μM). The Kcat value for NADH and NADPH in the NAD(P)H-dependent O2 reductase reaction catalyzed by a mixture of rNROR and Rd was determined by varying the concentration of NADH or NADPH with fixed concentrations of NRR (0.01 μM), Rd (2 μM), O2 (air saturated, 213 μM). The Kcat value for FprA2 or Rpr in the NADH-dependent O2 reductase reaction catalyzed by rNROR, rNROR and Rd, rNROR together with Rd and FprA2, or rNROR together with Rd and Rpr was determined by varying the concentration of O2 with fixed concentrations of NRR (0.01 μM), Rd (2 μM), FprA2 (2 μM), Rpr (2 μM), and NADH (150 μM). The Kcat value for Dsr in the NADH-dependent superoxide reductase reaction catalyzed by a mixture of rNROR, Rd, and Dsr was determined by varying the concentration of Dsr with fixed concentrations of rNROR (0.01 μM), Rd (2 μM), NADH (150 μM), O2 (air saturated, 213 μM), xanthine (0.5 mM), and XOD (at a superoxide flux of 8.1 ± 0.3 μM/min). The Kcat value for H2O2 in the NADH-dependent H2O2 reductase reaction catalyzed by a mixture of rNROR, Rd, and Rpr was determined under anaerobic conditions by varying the concentration of H2O2 with fixed concentrations of rNROR (0.01 μM), Rd (2 μM), Rpr (2 μM), and NADH (150 μM). The Kcat value for Rpr in the NADH-dependent H2O2 reductase reaction catalyzed by a mixture of rNROR, Rd, and Rpr was determined by varying the concentration of Rpr with fixed concentrations of rNROR (0.01 μM), Rd (2 μM), Rpr (2 μM), and NADH (150 μM). Michaelis-Menten parameters (Km and Vmax) were determined by nonlinear regression analysis using Enzyme Kinetics Module 1.3 (Sigma Plot 10.0.1; Systat Software, Chicago, IL). All values reported are the means ± standard errors for three independent experiments. The maximum turnover number (kcat) was calculated on the basis of moles of substrate oxidized or reduced per second per NROR (monomer). The errors for kcat/Km were calculated with the following formula:

\[ \frac{k_{cat}}{K_m} = \frac{S}{S+E_{SE}K_m} + E_{SE}K_m \]

where SE and S are the standard errors for kcat and Km, respectively.

**Northern hybridization.** Northern hybridization was performed as described previously (17, 18). The RNA (15 μg) was loaded in 1.0% agarose gels and blotted onto nylon membranes (Hybond N+; Amersham, Japan). The membranes were probed with the entire coding sequence for glutaredoxin (Gd gene; GenBank accession no. CAC2777) or rubredoxin (Rd gene; GenBank accession no. CAC2778). The Gd and Rd genes were amplified by PCR using chromosomal DNA from C. acetobutylicum as a template and the following oligonucleotide primer pairs: 5′-ATGTTAAGAATGATATCCACA-3′ and 5′-TTATTTTAAATTAAAAGGT-3′ (Gd gene) and 5′-ATGAAAAATATGTGTTGTT-3′ and 5′-TTATTTTCAAGATGGCCTAA-3′ (Rd gene).

**RESULTS**

Purification of recombinant rubredoxin from E. coli and native NROR:rubredoxin oxidoreductase from C. acetobutylicum. In this study, in order to characterize the function of O2-inducible NROR in oxidative stress protection, native NROR was purified. We first purified recombinant C. acetobutylicum

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rubredoxin (Rd gene; GenBank accession no. CAC2778) by using an E. coli recombinant expression system. The purified Rd protein exhibited a red color, as two major peaks at 380 nm and 492 nm (see Fig. S1B in the supplemental material). This observed spectrum, consisting of two major peaks originating from the Fe-S cluster of Rd, is in good agreement with previously published values for rubredoxin from Clostridium pasteurianum (22). The purity of purified Rd was assessed by gel electrophoresis and Coomassie blue staining (see Fig. S1A in the supplemental material). The molar absorption coefficient of purified Rd was determined by quantitative amino acid analysis.

Using Rd as an electron acceptor in NADH-dependent rubredoxin reductase assays, we then purified the native NADH:rubredoxin oxidoreductase from microaerobically grown C. acetobutylicum cell extracts. After the first step of column chromatography, NROR activity eluted together with an NADH oxidase activity as a major single peak. This NADH oxidase activity was significantly decreased after passage through a hydroxyapatite column, which was the second column chromatographic purification step. High NADH oxidase activity was restored to the NROR fractions by the addition of purified Rd and FprA2. These data suggested that the observed decrease in NADH oxidase activity was due to the chromatographic separation of these associated proteins. Electrophoresis of the purified NROR on an SDS-polyacrylamide gel followed by Coomassie brilliant blue staining yielded a single band of approximately 43 kDa (see Fig. S1A in the supplemental material). The N-terminal amino acid sequence of the purified enzyme was determined, and the sequence of the N-terminal 20 amino acids was completely identical to that for the previously characterized C. acetobutylicum NROR (GenBank accession no. AAK08126). The purified NROR was yellow and showed two absorption peaks at 380 nm and 452 nm (see Fig. S1B in the supplemental material). The purified rNROR used NADH and NADPH as electron donors. The specific activity of rNROR in the NAD(P)H oxidase reaction (indicated by the arrow). Catalase catalyzes the stoichiometric conversion of 1 mol H2O2 to 1 mol H2O and 1/2 mol O2. The addition of catalase to the H2O2-forming NADH oxidase produces 50% O2 to the total amount of O2 consumed by the NADH oxidase reaction. This reaction is accomplished within 1 to 2 s after the addition of catalase. The addition of catalase to the H2O-forming type NADH oxidase produces no O2.

Purification and characterization of recombinant NROR in E. coli. To further characterize the function and kinetic properties of the NROR protein, a large amount of protein was required. For this purpose, rNROR was expressed in E. coli and purified. The purified rNROR showed almost the same characteristics as those of the native enzyme, including spectral analysis results (see Fig. S1B in the supplemental material), specific activity of NAD(P)H oxidase, and reaction properties. The purified rNROR used NADH and NADPH as electron donors. The specific activity of rNROR in the NAD(P)H oxidase assay in air-saturated buffer was 7.1 U/mg protein or 7.4 U/mg protein when 150 μM of NADH or NADPH, respectively, was used as an electron donor. The NADH- and NADPH-dependent O2 reductase reaction catalyzed by rNROR also stoichiometrically reduced O2 to H2O2 (Fig. 1), and the pH optimum of this reaction was pH 7.0.

Unless otherwise indicated, the following enzymatic characterization of NROR was performed using rNROR. Under anoxic conditions, rNROR (0.7 nM) catalyzed a hyperreducing activity of Rd (70 μM), calculated as 1,940 U/mg rNROR (kcat = 1.3 × 103 [s−1]) when NADH (150 μM) was used as an electron donor and 408 U/mg rNROR (kcat = 288 [s−1]) when NADPH (150 μM) was used as an electron donor at pH 7.0. Under aerobic conditions, rNROR catalyzed both NADH- and NADPH-dependent O2 reductase activity, but the affinity for NADPH was very low compared to that for NADH (the Km's for NADH and NADPH were 0.7 ± 0.1 μM and 124.3 ± 9.1 μM, respectively, when O2 [air-saturated buffer in which the dissolved-O2 concentration is 213 μM at 37°C] was used as an electron acceptor). The following enzymatic assay and kinetic studies were performed using NADH as an electron donor. The Km of rNROR for O2 and the Vmax of the NADH oxidase reaction are listed in Table 1. The affinity of this reaction for O2 was increased by the addition of Rd. The Km's for Rd of rNROR was 0.54 ± 0.1 μM when O2 (air-saturated buffer in which the dissolved-O2 concentration is 213 μM at 37°C) was used as an electron acceptor. Investigation of the enzymatic
reaction of rNROR with other associated proteins will be described in a later section.

**Purification of recombinant FprA2 and Dsr.** As described in our previous study, *nror* operon genes are transcribed tricistronically with *fprA2* and *dsr* (18). In addition, the *dsr* gene can also be regulated independently of the *nror* promoter (18). The FprA2 protein has a homology with FprA homologues found in *Desulfovibrio gigas* (GenBank accession no. AAG34792) (7) and in *Moorella thermoacetica* (GenBank accession no. Q9FDN7) (5) (28.3% and 28.5% identity, respectively). These proteins have been shown to function as Rd-dependent oxidases or nitric oxide reductases. Dsr is homologous (36% identity) to the Dsr protein of *Treponema pallidum* (GenBank accession no. AAC65791), which functions as a superoxide reductase (14, 21). To characterize the function of FprA2 and Dsr, these proteins were overexpressed in *E. coli* and purified. The purity of the recombinant enzymes was determined by SDS-PAGE. Following electrophoresis, staining of the SDS-polyacrylamide gel with Coomassie brilliant blue yielded single bands of approximately 13 kDa and 13 kDa for purified FprA2 and Dsr, respectively (see Fig. S1A in the supplemental material). N-terminal amino acid sequencing of each of these purified enzymes revealed a perfect match to the sequence predicted by translation from the respective target gene.

The spectrum of FprA2 was dominated by the flavin moiety showing two major peaks at 377 nm and 450 nm (see Fig. S1B in the supplemental material). Gel filtration of the purified FprA2 protein yielded a single peak whose elution volume corresponded to an estimated molecular mass of 180 kDa. These data demonstrate that the purified FprA2 protein is a homotetramer. Flavin analyses confirmed a cofactor content of 0.9 ± 0.2 FMN per FprA2 monomer (data are the means ± standard deviations of results from three independent analyses).

Purified *Clostridium acetobutylicum* Dsr showed nearly no significant absorption spectrum except at 280 nm (see Fig. S1B in the supplemental material). When the purified protein was treated with K4[Fe(CN)6] as an oxidant, the obtained spectrum showed an increased absorption centered at 631 nm (see Fig. S1B, inset, in the supplemental material), which is attributed to the ferric form of the iron center (21). These results indicated that the Dsr protein was purified in its reduced form. The spectra that we obtained for Dsr are in good agreement with those reported for Dsr of *T. pallidum* (21).

**Characterization of the reaction of rNROR with Rd and FprA2.** To characterize the function of *nror* operon enzymes, we analyzed the enzymatic properties of a mixture of purified recombinant proteins of NROR, Rd, FprA2, Dsr, and Rpr. When FprA2 was added to a reaction mixture containing rNROR and Rd, the NADH oxidase activity was significantly enhanced. The *Kₘ* for FprA2 in this reaction was 0.12 ± 0.01 μM in the presence of 0.01 μM rNROR and 2 μM Rd. In the absence of FprA2, the reaction product of the NADH-dependent O₂ reduction catalyzed by rNROR or rNROR mixed with Rd was H₂O₂ (Fig. 1). This reaction product changed to H₂O when FprA2 was added to the mixture (Fig. 1). The small amount of H₂O₂ detected corresponds to the amount of H₂O₂ produced from the reaction of rNROR and Rd. Therefore, the NADH-dependent O₂ reductase activity catalyzed by a mixture of rNROR, Rd, and FprA2 reduces O₂ by four reducing equivalents to H₂O, as summarized in the following equation:

\[ 2\text{NADH} + 2\text{O}_2 \rightarrow 2\text{NAD}^+ + 2\text{H}_2\text{O} \]

A steady-state kinetic analysis of this reaction was performed, and the *Kₘ* for O₂ was 2.9 ± 0.4 μM (Table 1; also see Fig. S2 in the supplemental material). This level of O₂ corresponds to the concentration of saturated, dissolved O₂ that is present in an atmosphere containing 0.3% O₂ at 37°C. The *Vₘₐₓ* value was calculated at 62.7 ± 0.8 U/mg rNROR. Taking into account the final product of O₂ reduction, as well as the kinetic properties of the reaction, such as *kₗₑₚ/Kₘ*, these data indicate that the NROR-Rd-FprA2 system must be a key complex of O₂ scavenging.

**Characterization of the reaction of rNROR with Rd and Dsr.** We have previously proposed that the function of Dsr is that of a superoxide reductase based on its structural similarity to the Dsr of *Treponema pallidum*. In a recent report, recombinant *Clostridium acetobutylicum* Dsr (Strep tagged for purification) was suggested to function as an NADPH-dependent superoxide reductase using Spinach ferredoxin NADP⁺ reductase (30). In the present study, the native-form Dsr protein was newly characterized with regard to its enzymatic and spectral properties and its enzymatic kinetics in the presence of its proximal electron donor protein. The spectral properties of Dsr are described in earlier paragraphs. The purified Dsr protein exhibited low SOD activity (83.7 ± 6.1 U/mg protein) in the absence of an electron donor. An SOD activity has been reported for the Dsr protein from *T. pallidum* (35 U/mg protein) (21), *P. furiosus* (200 U/mg protein) (13), and a *Desulfovibrio* species (20 to 70 U/mg protein) (20). To determine the function of Dsr as an NROR- and Rd-dependent superoxide anion reductase, the effect of Dsr on NADH oxidation was analyzed in the presence of xanthine and XOD as the superoxide anion generator (Fig. 2; also see Fig. S3 in the supplemental material). The reaction of xanthine and XOD gener-

### Table 1. Steady-state kinetic parameters for the NADH-dependent O₂ reductase activities

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<tr>
<th>Protein mixture*</th>
<th>Final product</th>
<th><em>Kₘ</em> for O₂ (μM)</th>
<th><em>Vₘₐₓ</em> (U/mg NROR)</th>
<th><em>kₗₑₚ</em> (s⁻¹)</th>
<th><em>kₗₑₚ/Kₘ</em> (μM⁻¹ s⁻¹)</th>
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<tbody>
<tr>
<td>rNROR</td>
<td>H₂O₂</td>
<td>280 ± 41</td>
<td>15.8 ± 1.3</td>
<td>10.9 ± 0.9</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>rNROR-Rd</td>
<td>H₂O₂</td>
<td>109 ± 9</td>
<td>16.4 ± 0.5</td>
<td>11.3 ± 0.3</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>rNROR-Rd-Rpr</td>
<td>H₂O₂</td>
<td>303 ± 39</td>
<td>43.7 ± 3.4</td>
<td>30.1 ± 2.3</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>rNROR-Rd-FprA2</td>
<td>H₂O₂</td>
<td>2.9 ± 0.4</td>
<td>62.7 ± 0.8</td>
<td>43.2 ± 0.6</td>
<td>14.9 ± 2.1</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> NADH oxidaseb</td>
<td>H₂O₂</td>
<td>61.9</td>
<td>119</td>
<td>97.7</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* The rNROR concentration was 0.01 μM. The concentrations of recombinant Rd, Rpr, and FprA2 were 2 μM.

b *Clostridium acetobutylicum* H₂O₃-forming NADH oxidase (16).
activated. The NADH-dependent O2 reductase activity, cata-
lyzed by the mixture of rNROR, Rd, and Dsr proteins. Air-saturated 50 mM potassium phosphate buffer, pH 7.0, containing 150 μM of NADH, was introduced into a reaction cuvette at 37°C. The indicated protein mixture was added into the reaction cuvette at the time indicated by the arrow, and NADH oxidation was measured at A405. Fifty seconds after the measurement was started, xanthine and/or XOD, 5 μM to 50 μM H2O2 (data not shown), or 100 μM H2O2 (line 4) was added. The time course curve for each concentration of H2O2 (5 to 50 μM) was fitted to that of 100 μM H2O2 (line 4). The superoxide anion fluxes were 8.1 ± 0.3 μM/min (lines 1 to 5) and 16.4 ± 0.5 μM/min (line 6).

oxidation of O2− and H2O2 from the reduction of O2 (6). The superoxide anion flux was determined as equal to the rate of reduction of cytochrome c (1, 25). The calculated rate of the superoxide anion flux generated by 0.5 mM xanthine and 1 μl of purchased XOD added to 1 ml of a reaction mixture was 8.1 ± 0.3 μM/min. In the presence of rNROR and Rd, Dsr activated NADH oxidation in the presence of xanthine and XOD. In the absence of Rd or Dsr, NADH oxidation was not activated. The NADH-dependent O2 reductase activity, catalyzed by the mixture of rNROR and Rd, was not influenced by the addition of 0.1 μM to 10 μM Dsr (data not shown). When various amounts of H2O2 were added to the reaction mixture (5 μM, 20 μM, 50 μM, and 100 μM), no increase of NADH oxidation was observed (Fig. 2). These results indicated that the substrate of Dsr is superoxide anion. The specific activities of the NADH-dependent O2− reductase activity catalyzed by the mixture of rNROR, Rd, and Dsr were 17.9 ± 0.13 U/mg rNROR and 27.3 ± 0.6 U/mg rNROR at superoxide anion fluxes of 8.1 ± 0.3 μM/min and 16.4 ± 0.5 μM/min, respectively. Kinetic parameters of the NADH-dependent O2− reductase reaction are shown in Table 2.

**Characterization of the reaction of rNROR with Rd and Rpr**. Ruberopin, a reverse-type ruberythrin homologue, was previously determined to function as an efficient scavenger of H2O2, preferentially using NADH together with an unknown proximal electron donor protein (19). We speculated that NOR might be an electron donor protein for this reaction since ruberopin has a conserved rubredoxin motif in its N terminus (17, 19). In the presence of O2, the mixture of rNROR, Rd, and Rpr proteins catalyzes NADH-dependent H2O2-forming oxidase activity (Fig. 2). The Km for O2 of this reaction (303 ± 39 μM) is higher than the dissolved-O2 concentration in air-saturated medium (approximately 210 μM at 37°C). The affinity of this reaction for O2 was 100-fold lower than that of the mixture of rNROR, Rd, and FprA2. Furthermore, the kcat/Km value indicated that the oxidase activity of this reaction was significantly inferior to the oxidase activity generated by the mixture of rNROR, Rd, and FprA2 and to that of the *Clostridium aminovalericum* H2O2-forming NADH oxidase (Table 2) (16). Purified rNROR exhibited NADH-dependent H2O2 reductase activity in the presence of Rd and Rpr (Fig. 3). This reaction showed a high affinity for H2O2, but this affinity for H2O2 was too high for the Km to be accurately measured. This is because NADH oxidation after the addition of H2O2 follows zero order kinetics down to the detection limit of NADH (2 μM) at 340 nm. Therefore, the Km for H2O2 was estimated to be less than 1 μM (Table 2; also see Fig. S4 in the supplemental material). The NADH-dependent H2O2 reductase activity increased as the concentration of Rpr increased, whereas the NADH-dependent O2 reductase activity did not (Table 3). The Km for Rpr in the NADH-dependent rNROR: Rd:Rpr O2 reductase reaction was 0.19 ± 0.01 μM in air-saturated buffer, and the Km for Rpr in the NADH-dependent rNROR:Rd:Rpr H2O2 reductase reaction was 35.9 ± 5.6 μM under the saturated H2O2 condition (0.1 mM). The Vmax of the NADH-dependent H2O2 reductase reaction was estimated to be 1.089 ± 0.123 U/mg rNROR, and the kcat/Km was 20.9 ± 4.0 μM−1 s−1 (Table 2). These results indicated that the rNROR-

![FIG. 2. Detection of NADH-dependent O2 reductase activity catalyzed by a mixture of rNROR, Rd, and Dsr proteins. Air-saturated 50 mM potassium phosphate buffer, pH 7.0, containing 150 μM of NADH, was introduced into a reaction cuvette at 37°C. The indicated protein mixture was added into the reaction cuvette at the time indicated by the arrow, and NADH oxidation was measured at A405. Fifty seconds after the measurement was started, xanthine and/or XOD, 5 μM to 50 μM H2O2 (data not shown), or 100 μM H2O2 (line 4) was added. The time course curve for each concentration of H2O2 (5 to 50 μM) was fitted to that of 100 μM H2O2 (line 4). The superoxide anion fluxes were 8.1 ± 0.3 μM/min (lines 1 to 5) and 16.4 ± 0.5 μM/min (line 6).](image)

**TABLE 2. Steady-state kinetic parameters of the enzymatic reactions that scavenge ROS in the rNROR-Rd protein mixture**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cosubstrate</th>
<th>Km (μM)</th>
<th>Vmax (U/mg rNROR)</th>
<th>kcat (s−1)</th>
<th>kcat/Km (μM−1 s−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2 b</td>
<td>Rpr</td>
<td>35.9 ± 5.6</td>
<td>1089 ± 123</td>
<td>750 ± 84.7</td>
<td>20.9 ± 4.0</td>
</tr>
<tr>
<td>Rpr</td>
<td>H2O2</td>
<td>≤1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O2 c</td>
<td>Dsr</td>
<td>1.0 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsr</td>
<td>O2−</td>
<td>27.7 ± 4.5</td>
<td>75.6 ± 8.6</td>
<td>52.1 ± 5.9</td>
<td>1.9 ± 0.4</td>
</tr>
</tbody>
</table>

a The rNROR concentration was 0.01 μM. The Rd concentration was 2 μM.
b The H2O2 concentration was 0.1 mM.
c The Rpr concentration was 2 μM. Kinetic parameters (Vmax, kcat, and kcat/Km) are not provided.
d At an O2− flux of 8.1 ± 0.3 μM/min. Kinetic parameters (Vmax, kcat, and kcat/Km) are not provided.
e The Dsr concentration was 2 μM.
f O2− production (μM/min).
Recombinant O2 7.1

Table 3. Specific activities of O2 and ROS-scavenging enzyme reaction for native NROR and recombinant NROR

<table>
<thead>
<tr>
<th>NROR</th>
<th>Protein mixture</th>
<th>Substrate</th>
<th>Sp act(^{c}) (U/mg NROR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Rd</td>
<td>O2</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Rd-FprA2</td>
<td>O2</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Rd-Rpr (2 µM)</td>
<td>O2</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>Rd-Rpr (2 µM)</td>
<td>H2O2</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td>Rd-Dsr</td>
<td>O2</td>
<td>26.4</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Rd</td>
<td>O2</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>Rd-FprA2</td>
<td>O2</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>Rd-Rpr (2 µM)</td>
<td>O2</td>
<td>63.3</td>
</tr>
<tr>
<td></td>
<td>Rd-Rpr (2 µM)</td>
<td>H2O2</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td>Rd-Dsr</td>
<td>O2</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>Rd-Dsr</td>
<td>H2O2</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td>Rd-Dsr</td>
<td>H2O2</td>
<td>203</td>
</tr>
</tbody>
</table>

\(^{a}\) The NROR concentration was 0.01 µM. The protein concentration of native NROR was calculated using the molar extinction coefficient of NROR (\(\varepsilon_{280} = 12,870.6 \text{ M}^{-1} \text{ cm}^{-1}\)) originating from protein-bound FAD.

\(^{b}\) The concentrations of Rd, FprA2, and Dsr were 2 µM.

\(^{c}\) The concentration of O2 was 213 µM. The concentration of H2O2 was 0.1 mM. The O2 flux was 8.1 ± 0.3 µM/min.

\(^{d}\) One unit was defined as the amount of NROR that catalyzes the oxidation of 1 µmol O2 per minute with O2 used as a substrate or the oxidation of 1 µmol NADH per minute with H2O2 or O2 used as a substrate. Data are averages of results from three independent measurements that varied by less than 10%.

**DISCUSSION**

In this study, we aimed to determine the function of the O2-responsive proteins in *C. acetobutylicum*. The enzyme NAD(P)H:rubredoxin oxidoreductase has been proposed to have a central role in the protection of anaerobic bacteria, such
as sulfur reducers and a hyperthermophile. In *Desulfovibrio gigas*, NROR is composed of two subunits, with molecular masses of 27 kDa and 32 kDa (4). *D. gigas* NROR specifically binds NADH but not NADPH and has been shown to possess hyperreactivity of Rd reduction. Rd has been shown to function as an electron carrier protein in the reduction of rubredoxin:oxygen oxidoreductase (Roo), and the serial interaction of NROR, Rd, and Roo has been proposed as a central reaction in the reduction of O$_2$ to H$_2$O (7, 36). In *P. furiosus*, a hyperthermophilic archaeon, the NROR enzyme, exists as a monomer with a molecular mass of 45 kDa (8, 23). The *P. furiosus* NROR is homologous with *C. acetobutylicum* NROR (29% identity) but differs from the latter in that it is more specific to NADPH (8, 23). Purified recombinant *P. furiosus* NROR was shown to catalyze an Rd-dependent superoxide reductase reaction together with a recombinant *P. furiosus* superoxide reductase (8). The existence of an NROR-dependent O$_2$ detoxification system is unclear in *P. furiosus*.

In this study, both native and recombinant *C. acetobutylicum* NROR proteins were purified. Both proteins exhibited NADH- and NADPH-dependent H$_2$O$_2$-forming oxidase activity. However, their affinity for NADPH was significantly lower than that for NADH. The addition of Rd weakly activated the H$_2$O$_2$-forming NADH oxidase reaction but did not significantly increase the affinity for O$_2$. The further addition of FprA2 significantly enhanced its affinity to O$_2$, with a high turnover number, and furthermore, the reaction was converted from a H$_2$O$_2$-forming to a H$_2$O-forming reaction. FprA homologues in *Moorella thermoacetica* and *Desulfovibrio* species have been described, and the kinetic parameters of these FprA homologues with regard to oxygen metabolism have been investigated (32, 33). FprA of *M. thermoacetica* shows an NADH:O$_2$ reductase activity when coupled with Hrb (high-molecular-weight rubredoxin that contains a rubredoxin motif) as an electron donor protein (32). FprA had a higher affinity for NO, with an apparent $K_m$ of 4 $\mu$M, than for O$_2$, for which the apparent $K_m$ was 26 $\mu$M. The activity of FprA from *D. vulgaris* was also investigated using the Hrb protein from *M. thermoacetica* as an electron donor protein, and this FprA protein also had a higher $K_m$ for NO (the apparent $K_m$ was 19 $\mu$M) than for O$_2$ (the apparent $K_m$ was 24 $\mu$M) (33). The conclusion from these studies was that these two FprA homologues are involved in NADH-dependent NO reduction. The FprA homologue from methanogenic bacteria, F$_{420}$H$_2$ oxidase, was also shown to have a high affinity for O$_2$ (the apparent $K_m$ was 2 $\mu$M) in an assay that measured the oxidation of protein-bound flavins by O$_2$ (31). It was proposed that F$_{420}$H$_2$ oxidase catalyzes O$_2$ reduction to H$_2$O by coupling with Frh (F$_{420}$-reducing hydrogenase) and Mtd (F$_{420}$-dependent methyl- enetetrahymelenanthoerin dehydrogenase) as proximal electron donor proteins (31). In the present study, the *C. acetobutylicum* NROR-Rd-FprA2 system was estimated to be enough to scavenge a trace of O$_2$ under hypoxic conditions. We have shown that rubperoxin can also catalyze the H$_2$O-forming NADH oxidase reaction in the presence of NROR and Rd. However, the kinetic parameters of the NROR-Rd-Rpr reactions indicated that the NROR-Rd-Rpr system is able to reduce O$_2$ under highly aerated conditions but is not an efficient O$_2$ scavenger under hypoxic conditions. Due to the high affinity of the NROR-Rd-Rpr system for H$_2$O$_2$, and its extremely high

![FIG. 5. Scheme of the NADH-dependent O$_2$, O$_2^-$, and H$_2$O$_2$ detoxification complex composed of NADH:rubredoxin oxidoreductase (NROR), rubredoxin (Rd), flavoprotein A2 (FprA2), desulfoferrodoxin (Dsr), and rubperoxin (Rpr) in the obligate anaerobe *C. acetobutylicum*.](http://aem.asm.org/)

$V_{max}$ and $k_{cat}/K_m$ values (Table 2), we propose that the main function of Rpr in vivo is that of an NADH-dependent H$_2$O$_2$ scavenger.

Rd was originally identified as an electron carrier protein with an unknown function and is widely distributed among anaerobes. Recently, Rd was shown to be involved in superoxide reduction, and a rubredoxin:oxygen oxidoreductase activity was described to occur in a variety of anaerobes (1, 3, 4, 13). To our knowledge, little is known concerning the transcriptional response of rubredoxin to oxygen. In this study, we have shown that a gene encoding rubredoxin is strongly, and rapidly, upregulated in response to oxygen, indicating that the role of rubredoxin in *Clostridium* is in protection against oxidative stress. The role of the small redox protein (glutaredoxin-like protein) that is cotranscribed with Rd is still unclear.

In summary, we propose that *Clostridium acetobutylicum* possesses the NADH-dependent O$_2$, O$_2^-$, and H$_2$O$_2$ detoxification complex shown in Fig. 5. Many O$_2$-inducible proteins have been identified in *C. acetobutylicum*, such as A-type flavoprotein A1, bacterioferritin conimulatory protein, thiol peroxidase, glutathione peroxidase-like proteins, flavodoxin, and a small redox protein, glutaredoxin (18). While these proteins remain to be characterized in detail, it seems very likely that these proteins play a role in cellular defense against oxidative stress.

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**REFERENCES**


