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A Novel Type of Nitric-oxide Reductase

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***Escherichia coli* flavorubredoxin is a member of the family of the A-type flavoproteins, which are built by two core domains: a metallo-β-lactamase-like domain, at the N-terminal region, harboring a non-heme di-iron site, and a flavodoxin-like domain, containing one FMN moiety. The enzyme from *E. coli* has an extra module at the C terminus, containing a rubredoxin-like center. The A-type flavoproteins are widespread among strict and facultative anaerobes, as deduced from the analysis of the complete prokaryotic genomes. In this report we showed that the recombinant enzyme purified from *E. coli* has nitric-oxide reductase activity with a turnover number of ~15 mol of NO·mol enzyme^{-1}·s^{-1}, which was well within the range of those determined for the canonical heme b_3-Fe_B containing nitric-oxide reductases (*e.g.* ~10–50 mol NO·mol enzyme^{-1}·s^{-1} for the *Paracoccus denitrificans* NOR). Furthermore, it was shown that the activity was due to the A-type flavoprotein core, as the rubredoxin domain alone exhibited no activity. Thus, a novel family of prokaryotic NO reductases, with a non-heme di-iron site as the catalytic center, was established.**

Nitric oxide (NO) plays a key role in a wide variety of physiological and pathological processes in eukaryotes, notably cell signaling and host-pathogen responses. In prokaryotes, NO is encountered as an intermediate in the global nitrogen cycle, namely in the process of denitrification, *i.e.* reduction of nitrate/nitrite to N_2. Prokaryotes not directly involved in these metabolic pathways may nevertheless be exposed to high fluxes of NO, produced either abiotically or biotically (for example by macrophages (1)). In denitrifying bacteria, NO is reduced to N_2O by nitric-oxide reductase (NOR),[1] a membrane-bound enzyme containing at the catalytic subunit a heme b_3-Fe_B binuclear center (2, 3). NORs have been proposed to be evolutionarily related to the large family of heme-copper oxygen reductases on the basis of structural homologies (4, 5); consistently, two prokaryotic oxidases were recently reported to be endowed with NO reductase activity (6, 7). So far, only a second type of nitric-oxide reductase belonging to the cytochrome P450 family has been found in fungi (2).

The A-type flavoproteins are a large family of enzymes, widespread among Bacteria and Archaea, either strict or facultative anaerobes (8, 9). The major distinctive feature of this large family is the common core unit, built by two independent structural modules: the N-terminal one characterized by a β-lactamase-like fold and the second domain, having a short chain flavodoxin-like fold (10). Only a few members of this family have been so far purified from its natural host: the enzymes from *Desulfovibrio gigas* (11), *Rhodobacter capsulatus* (12), and *Methanobacterium thermoautotrophicum* (13). The first to be extensively studied was the rubredoxin:oxygen oxidoreductase from the sulfate-reducing bacterium *D. gigas*. This enzyme reduces O_2 to water receiving electrons from a type-I rubredoxin, which in turn is reduced by an NADH:rubredoxin oxidoreductase (14). The three-dimensional crystal structure of rubredoxin:oxygen oxidoreductase (10) revealed its modular architecture and, quite strikingly, showed that the β-lactamase domain harbors a non-heme di-iron site, consistent with the O_2 reductase activity of this enzyme. The flavodoxin domain contains one FMN molecule, which receives electrons from the rubredoxin (15). The homologous enzymes from cyanobacteria *Synechocystis* (8, 16) and *Anabaena* (17), from enterobacteria (*E. coli* (18), *Salmonella enterica* serovar Typhimurium LT2 (19), and *S. enterica* serovar Typhi CT18 (20)), and from *Clostridium perfringens* (21) contain one extra domain at the C terminus; whereas the cyanobacterial enzymes contain one NADH:oxidoreductase domain (condensing into a single protein the complete electron transfer chain from NADH to O_2), the enterobacterial enzymes have a rubredoxin-like domain (Rd), *i.e.* they bear the direct electron donor fused to the core catalytic domain. Interestingly, in these enterobacteria, downstream from the gene encoding for the A-type flavoprotein, there is a gene encoding for a NADH:rubredoxin oxidoreductase, with both genes possibly forming a dicistronic transcriptional unit.

The recombinant enzyme from *E. coli* (named flavorubredoxin, FlRd), purified and characterized, was shown to contain one rubredoxin-like center, one FMN, and one di-iron center. It was further shown that indeed it receives electrons from the NADH:oxidoreductase (FlRd-red) encoded in the same gene locus (22). As for the enzyme from *D. gigas*, it was also proven that FlRd is able to reduce O_2 to water; moreover, the di-iron center was shown to bind NO (22). Recently, Gardner *et al.* (23)

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[1] The abbreviations used are: NOR, nitric-oxide reductase; FlRd, flavorubredoxin; FlRd-red, flavorubredoxin-reductase; Rd, rubredoxin.

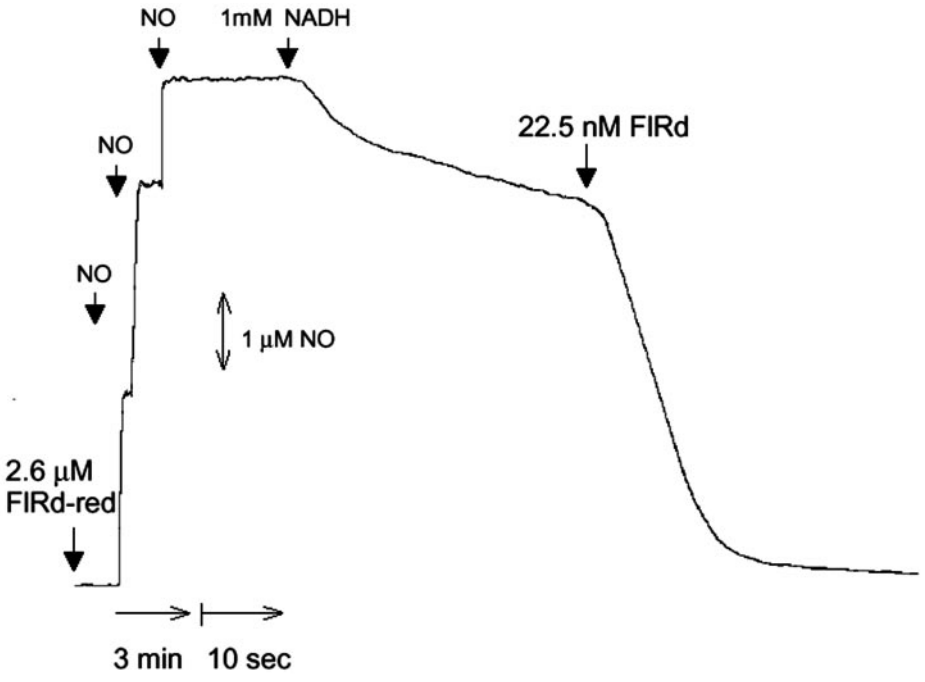

FIG. 1. **NO reductase activity of *E. coli* FlRd.** To an anaerobic buffer, containing 2.6 μM FlRd-red, three aliquots of NO (yielding a final concentration of 6.8 μM) were sequentially added. After raising the chart speed, 1 mM NADH was added. Following the addition of 22.5 nM FlRd at about 4.7 μM NO, a fast consumption of NO was observed whose time course followed a zero-order kinetics. Analysis of this trace yields a NO reductase activity for FlRd corresponding to 14.6 mol of NO·mol $FlRd^{-1}\cdot s^{-1}$.

reported that *E. coli* grown anaerobically and exposed (for a certain period of time) to NO develops a NO reductase activity, which is lost by knocking out the gene encoding for FlRd. However, the same authors were unable to show that the flavorubredoxin is responsible for NO reduction, attributing this failure to enzyme instability. In this article, we show that recombinant *E. coli* flavorubredoxin is a NO reductase, with a turnover number (14.9 ± 6.7 mol of NO·mol $FlRd^{-1}\cdot s^{-1}$) similar to that of canonical heme b_3-Fe_B containing NOR enzymes (2, 3). Thus we demonstrate the existence of a novel family of NO reductases, which is likely to be widespread among strict and facultative anaerobes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant FlRd and FlRd-red were expressed and purified as described previously (8, 22). Amplification of the Rd domain of *E. coli* FlRd was achieved by means of a PCR reaction using *Pfu* polymerase (Stratagene), the recombinant plasmid pME2543 (that contains the complete *E. coli* orf479 (8)), and the following oligonucleotides: sense primer, (5′-GCTCATATGGGC-CCACGGAT-3′), which introduced a *Nde*I site changing the codon of the residue 422 to the initiation codon ATG, and antisense primer, T7 22-mer (5′-GTAATACGACTCACTATAGGGC-3′). The amplicon was digested with *Nde*I and *Hind*III, ligated into compatible sites on pT7-7 (24), and transformed into *E. coli* DH5α cells. The cloned insert was then sequenced in both strands by using vector-specific oligonucleotide primers and fluorescent dideoxy terminators and an ABI model 377 DNA sequencer. After confirming that the correct nucleotide sequence was amplified, the recombinant plasmid was transformed in BL21-Gold(DE3). Overnight cultures, grown aerobically at 37 °C in minimal medium M9 (24) containing ampicilin (100 μg/ml), were diluted 1/100 into the same medium, grown to an A_{600} of 0.8, induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and harvested by centrifugation after 4 h. After cell disruption in a French press at 7000 p.s.i. (in two cycles), and soluble extract separation from the membranes through 16-h ultracentrifugation at 100,000 × *g* (at 5 °C), the rubredoxin domain of flavorubredoxin was purified in a two-step procedure, in a HiLoad Pharmacia system. The soluble extract was dialyzed overnight against 10 mM Tris-HCl, pH 7.6 (buffer A), and then loaded into a 60-ml Q-Sepharose column previously equilibrated with buffer A. The rubredoxin domain of flavorubredoxin eluted at ~400 mM NaCl was concentrated in a Diaflo cell with a YM3 cutoff membrane and further applied to a Superdex S-75 gel filtration column, previously equilibrated with 150 mM NaCl in buffer A. The eluted protein was found to be pure, as assayed by SDS-gel electrophoresis (25).

Total protein was determined using the BCA procedure (26) and iron quantitated by the TPTZ (2,4,6-tripyridyl-1,3,5-triazine) method (27). Total FMN content of FlRd was assessed after protein denaturation with 8% trichloroacetic acid, using an extinction coefficient of 12500 $M^{-1}\cdot cm^{-1}$ at λ = 450 nm to quantify the flavin. Recombinant FlRd was found to contain 1 mol of flavin/mol of protein and ~3.3 mol of iron/mol of protein, while FlRd reductase contained 1 mol of FAD/mol of protein, and the FlRd rubredoxin domain contained 1 mol of iron/mol of protein. The detailed characterization of the rubredoxin domain will be published elsewhere.

Kinetic Assays—Nitric oxide consumption measurements were carried out using a World Precision Instruments ISO-NOP 2 mm electrode, at room temperature. Assays were carried out under anaerobic conditions in 50 mM Tris-HCl buffer at pH = 7.6, in the presence of EDTA (20 μM), glucose (3 mM), glucose oxidase (4 units/ml), and catalase (130 units/ml). Oxygen consumption rates were measured using a Yellow Springs micro-O_2 electrode, in 50 mM Tris-HCl buffer at pH 7.6 and room temperature.

RESULTS AND DISCUSSION

Flavorubredoxin Is a Novel NO Reductase with High Affinity for NO—The NO reductase activity of *E. coli* FlRd was investigated measuring NO consumption in the presence of the physiological partner, FlRd reductase, and NADH. It was observed that FlRd catalyzes efficiently the NO consumption (Fig. 1); the rate of 14.9 ± 6.7 mol of NO·mol $FlRd^{-1}\cdot s^{-1}$ was estimated by averaging 26 independent measurements carried out at different concentrations of FlRd-red (Fig. 2) and at saturating NADH concentration (> 200 μM). The activity is linearly dependent on FlRd concentration, but is essentially independent of NO concentration from ≤1 μM (approximately the physiological levels) to ~10 μM. This shows that the enzyme has high affinity for NO (K_m < 1 μM), consistent with the zero-order kinetics of the observed NO consumption (Fig. 1).

Control experiments showed that the observed NO consumption is exclusively attributable to FlRd-mediated catalysis: (*i*) in the absence of FlRd, NO consumption mediated by the NADH/FlRd-red pair was much lower (even at different FlRd-red concentrations) than in the presence of FlRd; (ii) FlRd denaturation by boiling the protein for 10 min in the presence of 10% SDS totally abolishes NO consumption, thus excluding that contaminant inorganic compounds in the medium are responsible for the observed NO degradation. Altogether, these results clearly establish that *E. coli* FlRd operates as a *bona fide* NOR, with a measured turnover number comparable with

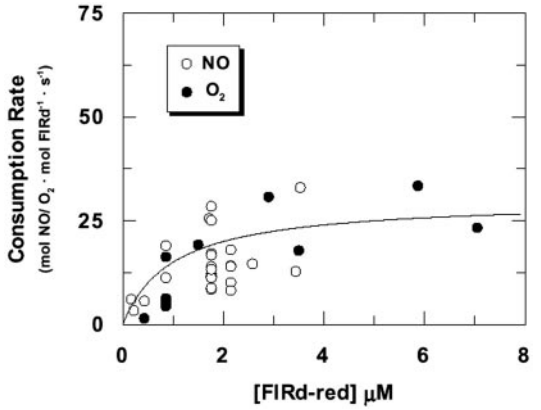

FIG. 2. ***E. coli* FlRd is a bifunctional NO and O_2 reductase.** O_2 and NO reductase activity of *E. coli* FlRd (7.5 or 22.5 nM) were measured in the presence of NADH (>200 μM), at increasing concentration of FlRd-reductase. NO consumption was measured at [NO] = 1 ÷ 10 μM, whereas O_2 consumption was estimated from initial rate at high [O_2], *i.e.* in air-equilibrated buffer.

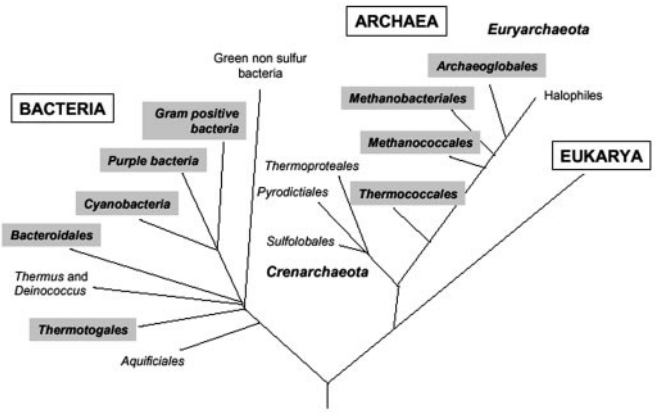

FIG. 3. **Distribution of A-type flavoproteins among prokaryotes.** Schematic representation of the 16 S RNA tree of life, showing the presence of A-type flavoproteins among bacterial and archaeal groups. *Gray shaded boxes*, *Archaeoglobales* (*e.g. Archaeoglobus fulgidus*), *Methanobacteriales* (*e.g. M. thermoautotrophicum*), *Methanococcales* (*e.g. M. jannaschii*), *Thermococcales* (*e.g. Pyrococcus furiosus*), *Thermotogales* (*e.g. Thermotoga maritima*), *Bacteroidales* (*e.g. Porphyromonas gingivales*), Cyanobacteria (*e.g. Synechocystis sp.*), Purple bacteria (*e.g.* the proteobacteria *E. coli*, *Salmonella thyphimurium*, *D. gigas*), Gram-positive bacteria (*e.g. C. perfringens*).

that published for NOR from *P. denitrificans* (in the range ~10–50 mol of NO·mol NOR^{-1}·s^{-1} (2, 3)).

The Di-iron Cluster Is the Active Site—The *E. coli* FlRd contains two different metal centers, integrated into distinct structural domains: the rubredoxin (Rd) center in a rubredoxin-like fold and the non-heme di-iron site. Several evidences show that the di-iron center is the site of NO reduction in FlRd. We previously observed by EPR spectroscopy that the di-iron center of FlRd is able to bind NO (22). In this work we have tested a truncated form of FlRd, consisting solely of the rubredoxin domain, that proved to be unable to process NO (data not shown), despite being still efficiently reduced by the NADH/FlRd-reductase couple.[2] Furthermore, the NO reductase activity is not inhibited by cyanide (even after 1-day incubation, both at 4 °C or at room temperature, and 3 mM cyanide), which is consistent with the fact that the di-iron site has low affinity for cyanide.

Flavorubredoxin Is a Bifunctional NO and O_2 Reductase, albeit with Distinct Affinities—The FlRd di-iron site is capable of reducing O_2 to water, in the presence of NADH and FlRd-red (22). Within experimental errors, the NO and O_2 consumption rates are similar (Fig. 2), as estimated from the initial rate at high O_2 concentration, *i.e.* in air-equilibrated buffer. NO and O_2 consumption may be thus rate-limited by the same event (for instance by internal electron transfer to the di-iron site). However, whereas NO is processed with a high affinity, O_2 reduction does not follow a zero-order kinetics, as the apparent rate starts to slow down at ~200 μM O_2. This points to a relatively low affinity for O_2, compared with the much higher affinity for NO (K_m < 1 μM). It is interesting that a similar observation has been described for the *P. denitrificans* NOR (see Fig. 7 in Ref. 28).

CONCLUSIONS

The results reported in this article demonstrate that *in vitro* *E. coli* FlRd has NO reductase activity, comparable with that of canonical heme b_3-Fe_B containing NORs. Although at this stage it was not possible to measure this activity in other A-type flavoproteins, the large overall similarity in amino acid sequence among most members of this family, and the conservation of residues binding the di-iron site (29) strongly suggests that the NO reductase activity will be found in many other (if not all) A-type flavoproteins. These enzymes seem therefore to display a bifunctional activity, in so far as they are also able to catalyze the reduction of O_2 to water. In this respect we notice a similarity between the heme-copper oxygen reductases and the NO reductases, providing evidence for a widespread bifunctional versatility in the prokaryotic response to environmental conditions, notably detoxification of O_2 or NO.

This enzyme family is present in a large variety of prokarytotes, phylogenetically and metabolically as diverse as hyperthermophilic anaerobic archaea (such as *Pyrococcus* species, *e.g.* Ref. 30), strict and facultative anaerobic bacteria (including enterobacteria, ((19, 20)), photosynthetic cyanobacteria (16, 17), nitrogen-fixing organisms (such as *R. capsulatus* (12)), sulfate-reducing archaea (31) and bacteria (11, 15), and *Clostridia* species (21, 32) (Fig. 3). The genomic organization of the A-type flavoproteins is however quite diversified. In fact, among the known genomes, only enterobacteria have a genomic organization, whereby the gene encoding for the FlRd is adjacent to that encoding for its reductase (18, 22), and both are close to a putative NO regulator (23). In other microorganisms, the genes encoding for A-type flavoproteins are close to sequences encoding for quite diverse or still unknown proteins. Especially interesting cases are those found in the genomes of *C. perfringens* (21) and *acetobutylicum* (32), *Methanobacterium thermoautrophicum* (33), *Methanococcus jannaschii* (34), *Pyrococcus horikoshi* (30), and *Moorella thermoacetica* (35), in which the A-type flavoproteins are in clusters containing genes coding for proteins involved in oxidative stress responses, such as alkyl hydroperoxide reductases, superoxide reductases, or superoxide dismutases. Thus, although there is no simple and direct correlation between the genomic organization of these enzymes and their physiological functions, examination of the available gene clusters suggests strong physiological links between O_2 and NO metabolisms. Furthermore, while in organisms such as *D. gigas*, *R. capsulatus*, and *M. thermoautotrophicum*, the A-type flavoproteins are expressed constitutively under sulfate-reducing, nitrogen-fixing, and methanogenic growth conditions, respectively, in *E. coli* the FlRd endowed with NO reductase activity were proposed to be induced by exposure to NO (23).

In summary, we have shown that the purified recombinant *E. coli* A-type flavoprotein has a high NO reductase activity, which allows to propose a novel family of NO reducing enzymes

[2] C. M. Gomes, J. B. Vicente, L. M. Saraiva, and M. Teixeira, unpublished observations.

that may play a key role in NO detoxification by prokaryotes. The relationships between the two functions of this family related to NO and O_2 detoxification, and the understanding of the intricate regulation of the expression and activities of these enzymes, are challenges for the near future.

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