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RUBREDOXIN OXIDASE, A NEW FLAVO-HEMO-PROTEIN, IS THE SITE OF OXYGEN REDUCTION TO WATER BY THE "STRICT ANAEROBE" Desulfovibrio gigas

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SUMMARY A rubredoxin-oxygen oxidoreductase, a homodimer with a molecular weight of 43 kDa per monomer, was found to be a component of an electron transfer chain that couples the reduction of oxygen to water with NADH oxidation. This FAD-containing protein appears to contain a new type of heme group. The electron transfer chain is not inhibited by cyanide and azide. In contrast, CO decreases NADH oxidation rate and also induces release of the prosthetic groups from the native terminal reductase.

The search for a terminal oxygen reductase in sulfate-reducing bacteria has been prompted by a series of recent observations all pointing to the fact that O_2 may play a physiological role in these so-called "strict anaerobes". Firstly, several new data show that some strains can actually utilize oxygen and generate ATP (1), remarkably, they are even capable of reversing sulfate reduction by using reduced sulfur compounds as energy and electron sources for the reduction of oxygen (2). The terminal reductase responsible for this particular metabolism is not known, but it has been observed that its activity is not inhibited by cyanide and azide (1). Secondly, a recent study of the rRNA sequences of several Desulfovibrio species and their comparison with the rRNA of some

Abbreviations used: **RNO**: rubredoxin-NAD$^+$ oxidoreductase; **ROO**: rubredoxin-oxygen oxidoreductase; **Rd**: rubredoxin; **SOD**: superoxide dismutase; **TCA**: tri-chloric acid; **SDS-PAGE**: sodium dodecylsulfate-polyacrylamide gel electrophoresis. **DEAE**: diethyl aminoethyl.

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aerobic bacteria showed that these organisms appeared to have evolved from aerobic bacteria, thus suggesting stronger relationships within these organisms than it was thought before (3). Finally, our own unpublished observations, showing that Desulfovibrio gigas was able to utilize internal polyglucose for the formation of ATP while reducing O_2 through a chain of reactions not inhibited by cyanide, were determinant for this research.

We want to report here the complete purification of a rubredoxin-oxygen oxidoreductase and its identification as a flavo-hemo-protein. Unlike cytochrome-oxidase, the activity of this new terminal reductase is not inhibited by cyanide or azide.

MATERIALS AND METHODS

Organism and Growth Conditions: The growth of Desulfovibrio gigas (ATCC 19364) and the preparation of cell-free extracts were as described previously (5).

Enzymatic Activity Determinations: Enzymatic activities were determined by the oxidation rate of NADH following the decrease of absorption at 340 nm. The reaction mixture contained 60 nmol NADH, 10 pmol D. gigas RNO, and 3 nmol D. gigas rubredoxin (6) in a total volume of 1 ml. The consumption of oxygen was measured by a Clark electrode (YSI model 5300, Yellow Springs Instrument Co., Inc.). Hydrogen peroxide was determined by the method of Childs and Badsley (7).

Purification of the Terminal Reductase: The dialyzed D. gigas crude extract was loaded onto a DEAE-cellulose column, the fraction containing APS reductase was detected as having terminal reductase activity. This fraction was treated by several chromatographic steps including DEAE-Biogel, hydroxylapatite, and HPLC molecular sieve. The purity of the protein was determined by SDS-PAGE. Rubredoxin-NAD^+ oxidoreductase (RNO), prepared in (4), was further purified until a specific activity of 12 µmol NADH oxidized/min/mg was reached. The characterization and properties of this flavoprotein will be reported elsewhere.

Analytical Procedures: Molecular weight was determined by SDS-PAGE (8). A Pharmacia FPLC-gel filtration column (Superdex-75, 1 x 30 cm) was used for the determination of the molecular weight of the native protein. Protein concentration was measured by the method of Bradford (9). Plasma emission spectroscopy, Jarrell-Ash model 750 atom comp, was used for the determination of the metal content of the protein. Absorption measurements were performed with a Shimadzu spectrophotometer (UV 265). The flavin determination was done according to the method described previously (10).

RESULTS

Enzymatic Activity: As shown on fig. 1, the addition of ROO to RNO and Rd increased twenty-fold the rate of NADH oxidation over a

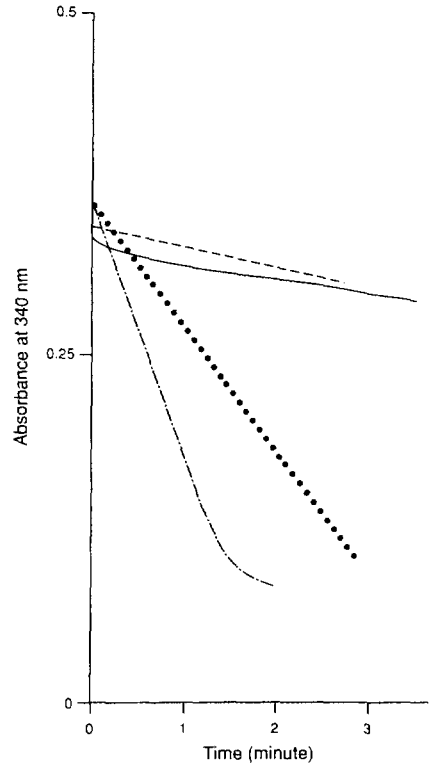
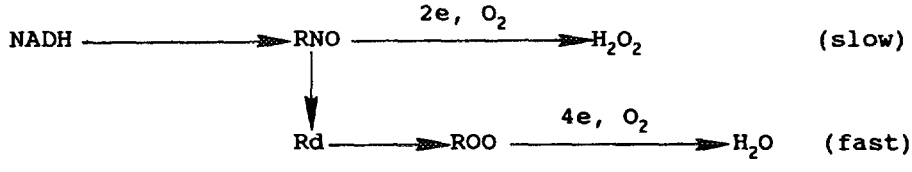


Fig. 1. The NADH oxidation rate with different components of the electron transfer chain that couples NADH oxidation to O_2 as a terminal electron acceptor and the effect of cyanide, azide and CO on the NADH oxidation rate. (——): the reaction mixture contains 0.06 μmoles NADH and 10 pmoles (RNO) in a total volume of 1 ml; (---): the reaction mixture contains 0.06 μmoles NADH, 10 pmoles RNO, and 3 nmoles D. gigas Rd in a total volume of 1 ml; (·-·-·): the reaction mixture contains 0.06 μmoles NADH, 10 pmoles RNO, 3 nmoles D. gigas Rd, and 0.35 nmoles ROO in a total volume of 1 ml. The NADH oxidation rates are the same when cyanide or azide (1μM) is added into the reaction mixture. After the same reaction mixture is incubated with CO, the NADH oxidation rate is decreased (····).

reaction mixture only containing RNO. Rd was found to be necessary for the coupling of RNO to ROO. Experiments with a Clark electrode established that the stoichiometry NADH oxidized/O_2 reduced was 2:1 in the presence of RNO, Rd, and ROO, thus showing the full reduction of oxygen to water. Also, hydrogen peroxide, abundant when oxygen is reduced by RNO alone, was not found in the complete system. These results allow us to propose the following electron transfer chain:



Cyanide and azide up to 1 mM concentrations were not inhibitors of that reaction, in contrast, CO inhibited oxygen reduction (fig. 1).

Characterization of ROO: the native molecular weight of ROO was estimated to be 86 kDa by FPLC-gel filtration, with 43 kDa for each monomer from SDS-PAGE. After extraction of TCA, the flavin component was identified as FAD by paper chromatography. Quantification by spectrophotometry was consistent with the presence of two flavin moieties per monomer of the native protein. Plasma emission data indicated the presence of 1.4±0.3 Fe per monomer of ROO. No other metals were found. The oxidized and dithionite-reduced spectra of ROO are shown on fig. 2; the presence of α and ß bands at 551 nm and 522 nm, respectively, is reminiscent of a hemochrome-type of linkage similar to the one found in cytochrome c; this is also apparently supported by our observation that TCA only extracted the flavins but not the heme, suggesting its covalent linkage to the protein. However, two unique features are found: the presence of an additional peak at 585 nm that does not seem to be sensitive to the redox state of the protein and unlike proto- or meso-heme containing cytochromes, the Soret band is not shifted toward larger wavelengths after reduction of the protein, instead, it remains at the same position: 416 nm. Consistent with its inhibitory effect on O_2 reduction, CO induces large spectral changes (fig. 2) with, in particular, a shift of the Soret band to 413 and the appearance of a shoulder at 405 nm and the disappearance of α and ß bands, again, the 585 nm peak is not affected. After a long period of incubation with CO, the protein precipitated and unlike what was found after TCA treatment, both flavin and heme were solubilized showing that the latter was not covalently bound to the protein.

The pyridine hemochrome of the ferrous hemoprotein is shown on fig. 3: the Soret peak is now found at 409 nm and the α and ß peaks are at 548 nm and 518 nm, respectively. The secondary peak now at 589 nm still remains insensitive to the redox state. These spectral properties are not entirely consistent with the ones of known hemes: in particular, the 589 nm peak, being insensitive to the redox state of the heme, does not appear to be related to the heme-iron axial ligands and could reveal a different heme-ring structure. Clearly, the determination of the chemical structure of this heme deserves further studies.

Another characteristic of ROO deserves some comments: NADH, in the presence of RNO and Rd, does not appear to fully reduce ROO

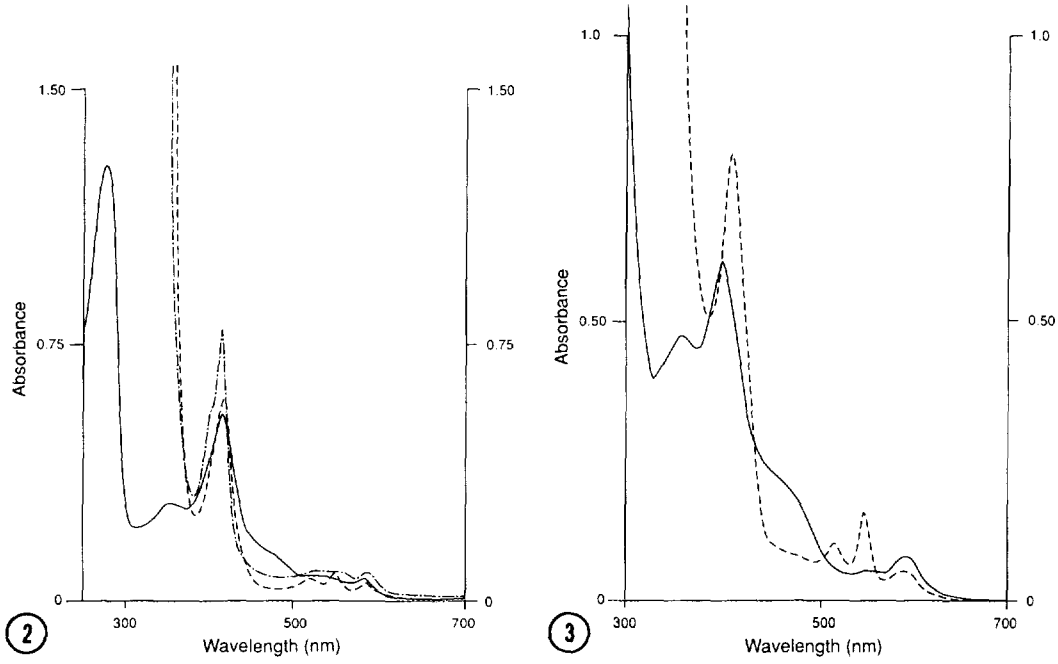


Fig. 2. The spectra of ROO in oxidized, reduced states and after incubation with CO. (——): 3.5 μM oxidized ROO in 10 mM potassium phosphate buffer (pH 7.6); (---): 3.5 μM ROO reduced by dithionite; (·-·-·): 3.5 μM reduced ROO incubated with CO.

Fig. 3. The spectra of pyridine hemochrome of ROO. The reaction mixture contains 20% pyridine, 0.2 mmoles NaOH, 4.6 nmoles ROO in a total volume of 1 ml. (——): the oxidized spectrum; (---): after reduction by dithionite.

heme moiety since the only absorption change is centered around 450 nm, corresponding to flavin reduction (spectra not shown), and the α and β absorption peaks, observed after the addition of dithionite, are not seen. This probably indicates that the fully reduced protein is not the catalytically competent form of the enzyme.

DISCUSSION

The present work demonstrates the presence of an authentic terminal reductase (formally a rubredoxin:oxygen oxidoreductase, a new entry in E.C.) in Desulfovibrio species, which is capable of reducing oxygen to water. It also provides, for the first time, a physiological role of rubredoxin in these organisms.

It has been shown, several years ago, that some strains of sulfate-reducing bacteria, including D. gigas, contain both catalase and SOD activities. These two proteins have been fully characterized (11). At that time, the presence of these proteins,

otherwise characteristic of aerobic life, was interpreted as evidence for a defense mechanism protecting these strictly anaerobic bacteria from accidental contact with air. This work, by showing that oxygen can indeed be a terminal electron acceptor through a specific reductase, provides a full and more classical interpretation for the presence of SOD and catalase in some Desulfovibrio species.

ROO is most probably not directly related to the cytochrome (quinol)-oxygen oxidoreductases which are hemo-proteins but contain copper, a metal not found in the D. gigas enzyme. Also, cyanide and azide, two popular inhibitors of the copper-containing enzymes, have little or no effect on the new protein.

The relatively small molecular weight of this protein which contrasts with the complexity of its redox components makes it an excellent tool for further studies of its mechanism of action. This, together with the determination of the chemical nature of its heme moiety and its possible link to sulfur and hydrogen metabolisms, is currently under investigation in our laboratories.

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