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The Two Terminal Oxidases of the Aerobic Respiratory Chain of Escherichia coli Each Yield Water and Not Peroxide as a Final Product

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The aerobic respiratory chain of Escherichia coli contains two terminal oxidases, the cytochrome d complex and the cytochrome o complex. Each of these enzymes catalyzes the oxidation of ubiquinol-8 within the cytoplasmic membrane and reduces molecular oxygen. The purpose of this work is to experimentally verify that each of the terminal oxidases yields water as a product with no significant amount of hydrogen peroxide. This was accomplished by preparing membranes which were washed so as to eliminate membrane-associated catalase and peroxidase activities. The NADH oxidase activity of the membrane-bound respiratory chain was measured by monitoring the rates of both NADH and oxygen utilization. This was performed using membranes from strains in which either cytochrome o or cytochrome d were absent. Results using each strain showed two NADH utilized per oxygen, indicating a four-electron reduction of oxygen to water.

The aerobic respiratory chain of Escherichia coli contains two terminal oxidases (1). The cytochrome o complex and the cytochrome d complex each catalyze the two electron oxidation of ubiquinol-8 within the bacterial cytoplasmic membrane. Electrons are transferred in each case to molecular oxygen, and this electron transfer results in the generation of a transmembrane potential across the cytoplasmic membrane. The two oxidases are redundant, at least under normal laboratory aerobic growth conditions. Mutants have been isolated which lack either the cytochrome o or cytochrome d complex and these mutants grow as well as does the wild-type strain (2,3). Both enzymes have been purified to

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homogeneity (4-7) and each can function as a coupling site *in vitro* in reconstituted systems (8,9). The genes encoding each multi-subunit complex have been mapped, cloned, and fully sequenced to yield the primary structure of each subunit (10; Chepuri, Lemieux, Au and Gennis unpublished). A combination of biophysical methods in conjunction with molecular genetics approaches are being used to elucidate the structure and catalytic mechanisms of these enzymes.

It has been assumed, but never demonstrated, that each of these enzymes catalyzes the four-electron reduction of molecular oxygen to water without yielding hydrogen peroxide as its final product. Since this is a rather critical point in understanding how these enzymes function, and since there are other systems in which hydrogen peroxide is released as an intermediate in energy conserving electron transport chains (11,12), the experiments described in this paper were performed.

Two problems have presented difficulties. First, the substrate ubiquinol-1 which is used to assay each of the oxidases, will chemically reduce hydrogen peroxide to water. Hence, one cannot simply examine the purified enzymes to see how many molecules of ubiquinol-1 are oxidized to reduce a molecule of oxygen. Even if the product of each oxidase were peroxide, this would not accumulate but would be directly reduced to water by ubiquinol. Hence, two molecules of ubiquinol will be oxidized per oxygen molecule regardless of the product of the enzyme, as has been observed (7).

A second problem complicates efforts to examine the products of the intact respiratory chain *in situ* in isolated membranes. By examining membranes, one can use NADH as a reductant, and NADH will not react directly with hydrogen peroxide at a significant rate. However, isolated membranes from *E. coli* have been shown to contain catalase activity due to a membrane-associated heme protein (13). Hence, peroxide will not accumulate but will disproportionate to water and molecular oxygen.

In this study, a simple washing of the isolated membranes was found to strip off the membrane-associated hydroperoxidase(s) leaving the respiratory components intact. Respiration was examined from NADH to oxygen, and it has been confirmed that each oxidase yields water and not hydrogen peroxide as a final product.

Materials and Methods

Materials

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO): nicotinamide adenine dinucleotide (reduced), Tween-20, N-lauroyl sarcosine (Sarkosyl), bovine catalase, horse hemoglobin, horse radish peroxidase, superoxide dismutase, ethylenediaminetetraacetate, nitroblue tetrazolium. From Difco (Detroit, MI) the following were purchased: beef extract, peptone and yeast extract. BCA assay were purchased from Pierce Chemical Company (Rockford, IL). Bio-Rad (Richmond, CA) was the source for sodium dodecyl sulfate, Coomassie Brilliant Blue R-250, and all electrophoresis reagents. Kodak Laboratory Chemicals (Rochester, NY) supplied sodium dithionite and dithioerythritol. Ultrex hydrogen peroxide was purchased from Baker Chemical Company (Phillipsburg, NJ). Sodium hydroborate was purchased from Ventron Alfa Division (Danvers, MA). Potassium ferricyanide and pyridine were obtained from Fisher Scientific Company (Pittsburg, PA).

Strain and Growth Conditions

E. coli RG145 (*cyd*$^-$) (14) was grown aerobically into early logarithmic phase in 200 liters at 37°C. The following salts were sterilized in the fermentor: 700g of sodium chloride, 736g of potassium phosphate, dibasic and 264g of potassium phosphate, monobasic. The following solutions were autoclaved separately then added to the fermentor: 300g beef extract, 300g yeast extract, 1000g peptone, 200g dextrose, 2g vitamin B_1, 2g nicotinic acid and 10g ampicillin. Approximately 600g of cells were harvested from 200 liters of media.

E. coli MR43L(F152) was grown aerobically into early stationary phase at 37°C in 200 liters of the following medium: 612g of potassium phosphate, 384g of ammonium sulfate, 800g potassium hydroxide, 24g magnesium sulfate, 3g calcium chloride, 290g casamino acids, 1.3 liters sodium lactate, and 1g ferrous sulfate.

Preparation of Membranes

Cells were harvested by centrifugation and washed once with 10 mM Tris-HCl, pH 8.0. Washed cells (50 grams) were resuspended in 250 ml of 10 mM Tris-HCl, pH 8.0, containing 5 mM magnesium sulfate, 2 mg pancreatic DNase (type I) and 1 mM PMSF, freshly prepared in ethanol. Antifoam A was used to prevent foaming of the buffer while resuspending the cells. The suspension was passed once through a French pressure cell (SLM-Aminco, Urbana, IL) at 20,000 psi and unbroken cells were removed by centrifugation at 10,000g for 10 minutes, 4°C. The supernatant was then centrifuged at 150,000g for 1 hour to sediment the membrane fraction. Approximately 1g of membranes were prepared from 50g of cells.

The pelleted membranes were suspended to 10 mg/ml in 50 mM potassium phosphate, pH 7.0, and sonicated for 10 minutes at 25% of maximum power with 50% cycle (Heat Systems Sonifier W-375, Branson Sonic Power

Co., N.Y.). The membranes were pelleted at 100,000g for 1 hour at 5°C. This cycle of sonication and pelleting was repeated twice. Membranes were resuspended in potassium phosphate buffer, pH 7.5, and analyzed for catalytic activities.

Determination of Catalase, Peroxidase and Superoxide Dismutase Activities

The washed membranes were assayed for catalase, peroxidase and superoxide dismutase activities. To measure catalase activity, the decomposition of hydrogen peroxide was observed spectrophotometrically at 240 nm at 37°C (15). To a 3-ml cuvette containing 50 mM potassium phosphate, pH 7.5, hydrogen peroxide was added to a final concentration of 42 mM. To initiate the reaction, washed membranes from strains RG145 or MR43L(F152) were added. Catalase activity was also measured in an oxygen electrode chamber containing 3.5 mM hydrogen peroxide in phosphate buffer, pH 7.0, at 37°C. Washed membranes were added to initiate the reaction.

To measure peroxidase activity, the oxidation of o-dianisidine was observed spectrophotometrically at 460 nm, at 37°C (15). A 3-ml cuvette contained 0.024 mM o-dianisidine and 42 mM hydrogen peroxide. The reaction was initiated by the addition of washed RG145 or MR43L(F152) membranes. Horse radish peroxidase was used as a control.

To measure superoxide dismutase activity, the reduction of nitroblue tetrazolium (NBT) was observed spectrophotometrically at 460 nm at 37°C. To initiate the reaction, washed membranes were added to the reaction mixture in the cuvette. Superoxide dismutase was used as a control in the presence of EDTA and riboflavin (16).

Measurement of the Rate of Oxygen Consumption

The rate at which the oxidases in the NADH:O_2 oxidoreductase chain consumed oxygen was measured with a YSI model oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). All measurements were made at 37°C. A buffer of 50 mM potassium phosphate, pH 7.5, was used. The oxygen concentration at 37°C was assumed to be 250 µM for all of the buffers. NADH was added to the reaction vessel at a concentration of 2 mM.

Results and Discussion

Two strains were used for these studies. RG145 contains cytochrome o as its only terminal oxidase and MR43L(F152) contains predominantly cytochrome d as its respiratory oxidase. RG145 is a mutant in which cytochrome d is not expressed and which contains a plasmid resulting in the overproduction of cytochrome o (14). MR43L(F152) is a wild-type strain that overproduces cytochrome d and, under the growth conditions used, ie. harvesting late in stationary phase of growth, cytochrome o is present to only a very small extent (4). Membranes from these strains contained respectively 1.3 and 2.0 nmol heme b per mg membrane protein.

After sonicating and pelleting the membranes several times as described, the membranes were devoid of catalase activity as well as any superoxide dismutase or o-dianisidine peroxidase activities. Furthermore, the addition of either catalase (88 units) horseradish peroxidase (24 pmol) or superoxide dismutase (1400 units) had no effect on the rate of oxygen utilization in the presence of NADH (2mM). The rates of oxygen utilization by the membranes were 613 and 223 nmol O_2/min/mg for the membranes containing cytochrome o or cytochrome d, respectively. The rates of NADH oxidation was measured spectroscopically using the same conditions and membrane preparations, yielding 1205 and 451 nmol NADH oxidized/min/mg respectively. The data show no evidence for the accumulation of either hydrogen peroxide or superoxide.

Table I summarizes the data on NADH and oxygen consumption rates for membranes from both MR43L(F152) and RG145. When measured under identical conditions, the ratio of NADH oxidation to oxygen consumption is 1.93 for the membranes containing cytochrome o and 2.02 for the membranes containing cytochrome d.

These data verify that each of these E. coli enzymes reduces molecular oxygen to water without the release of hydrogen peroxide. The experiments do not rule out a tightly bound peroxy intermediate in the

Table I

Determination of the Ratio of NADH Oxidized:Oxygen Consumed

Strain	Predominant or Only Oxidase present	Units NADH[a]	Units O_2[b]	ratio NADH:O_2
RG145	cytochrome o	1205	613	1.93
MR43L(F152)	cytochrome d	451	223	2.02

[a]nmoles NADH oxidized/min/mg
[b]nmoles oxygen consumed/min/mg

catalytic mechanisms of these oxidases, and there is some evidence to support such an intermediate for cytochrome d (17). Further studies are required to clarify how these enzymes activate molecular oxygen and stabilize bound intermediates formed prior to the formation of water.

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