The Formation of Hydrogen Peroxide during the Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Cytochrome o from Vitreoscilla*

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SUMMARY

The formation of hydrogen peroxide during the oxidation of NADH by purified preparations of cytochrome o has been demonstrated by employing three independent methods: polarographic, colorimetric, and fluorometric. The first two methods were used to assay for the accumulation of hydrogen peroxide and showed that hydrogen peroxide did accumulate as a product, but only about 30% of the oxygen consumed or 15 to 20% of the NADH oxidized was recoverable as hydrogen peroxide. This lack of 1:1 stoichiometry was not due to residual catalase activity in these preparations which could be eliminated by freeze-thawing. Thus, hydrogen peroxide may not be the sole or primary product of the NADH-cytochrome o oxidase reaction. The fluorometric assay could be coupled directly to the NADH-cytochrome o oxidase reaction. The fluorometric assay could be coupled directly to the NADH-cytochrome o oxidase reaction in one medium, and this method showed that hydrogen peroxide was generated continuously from the beginning of the reaction in a 1:1 stoichiometry, hydrogen peroxide generated to NADH oxidized. This result suggests that hydrogen peroxide is an intermediate that can be trapped efficiently under the conditions of the fluorometric assay, whereas under the conditions of the first two assays most of the hydrogen peroxide generated undergoes further reaction.

Exogenously added FAD or FMN increased the percentage of hydrogen peroxide that accumulated in the NADH-cytochrome o oxidase reaction. Flavin is believed to act on the reductase side of cytochrome o so the increased percentage of hydrogen peroxide is not likely to result from the direct reaction of reduced flavin with oxygen.

The presence of cytochrome o in various bacteria has been reported by many authors but only in a few cases has the pigment, having the carbon monoxide absorption bands characteristic of cytochrome o, been shown to be a functional terminal oxidase (1-5). A heme protein is generally considered to be a terminal oxidase in respiration if it forms a light-reversible complex with carbon monoxide, and the action spectrum for the reversal of carbon monoxide-inhibited respiration has absorption maxima identical with the absorption maxima of the reduced carbon monoxide complex of the heme protein (6). The cytochrome o purified from Vitreoscilla meets these criteria (5).

In addition, since NADH-cytochrome o reductase is associated with cytochrome o preparations of all stages of purity, these preparations can catalyze the oxidation of NADH by molecular oxygen (7). During the aerobic oxidation of NADH an "oxygenated" form of cytochrome o is the predominant species detectable spectrophotometrically (7, 8). Like cytochrome oxidase, cytochrome o will react with hydrogen peroxide in the oxidized form and in both cases the spectrum of the complex is similar to that of the "oxygenated" form (8-10). Hydrogen peroxide was not detected as an intermediate during the reduction of oxygen to water by cytochrome oxidase (11). In contrast, the formation of hydrogen peroxide during the reaction of cytochrome o with oxygen has been demonstrated by three different methods in this report.

MATERIALS AND METHODS

The cytochrome o employed in these experiments was purified from Vitreoscilla cells as described previously (7). Catalase, present in the crude extract, is separated chromatographically from the cytochrome o during the purification procedure (7).

Hydrogen peroxide production was estimated polarographically with a YSI model 53 oxygen analyzer with a Heath ETW-2A recorder. Oxygen uptake was initiated by adding 10 μl of 0.03 M NADH to a 1-ml solution of cytochrome o in 0.1 M sodium phosphate buffer, pH 7.5, at 25°. After the reaction had proceeded to the extent desired, 1 μl of catalase (Sigma, beef liver, twice crystallized, 100,000 units/ml) was added, and the amount of oxygen released by this addition was employed to estimate the amount of hydrogen peroxide originally present using the relationship, H subscript 2 O subscript 2 → 1/2 O subscript 2 + H subscript 2 O. Thus, moles of hydrogen peroxide originally present were twice the moles of oxygen released by the addition of catalase, and hydrogen peroxide produced was expressed as percent of oxygen consumed.

The peroxidase-o-dianisidine colorimetric assay used for the estimation of hydrogen peroxide was essentially that described in the Worthington Biochemical Corp. Enzymes Manual. This assay was sensitive to approximately 5 μm hydrogen peroxide. The peroxidase used for both this assay and the fluorometric assay described below was horseradish peroxidase, Sigma type II. The oxidation of NADH, at initial concentrations of 30 to 450 μm, by cytochrome o was followed spectrophotometrically at 340 nm, and aliquots were removed at appropriate times to assay for hydrogen peroxide accumulation. Catalase activity of cytochrome o preparations was assayed spectrophotometrically at 240 nm using the method of Beers and
The estimation of hydrogen peroxide fluorometrically was with the procedure of Keston and Brandt (13), which can be used for hydrogen peroxide concentrations as low as $10^{-11}$ M. The assay medium contained 2 μM 2',7'-dichlorofluorescin diacetate, activated by hydrolysis as described (13), peroxidase, 2 μg/ml, ZnSO$_4$, iH$_2$O, 0.04 mg/ml, 0.02 M sodium phosphate, pH 7.1, cytochrome o, 0.1 to 0.8 μg heme, and 10 μM NADH. The reaction was started by the addition of the NADH or cytochrome o to 3 ml of the assay medium and the increase in fluorescence at 525 nm was followed with an Aminco-Bowman spectrophotofluorometer using an excitation wavelength of 503 nm. The rate of NADH oxidation was determined independently at 340 nm with a Zeiss DMR 21 spectrophotometer.

The standard curves for the colorimetric and fluorometric assays were obtained using fresh dilutions of 30% hydrogen peroxide (Merck Superoxol). The actual concentration of this concentrated solution was determined by measuring the $A_{260}$ of an appropriate dilution and using the molar extinction coefficient of 43.6 cm$^{-1}$.

RESULTS

The polarographic technique provided the first direct evidence for the accumulation of hydrogen peroxide in the NADH-cytochrome o oxidase reaction (Fig. 1). For several different cytochrome o preparations tested at concentrations of 2.5 to 15 μg heme, the moles of hydrogen peroxide accumulated per 100 ml of oxygen consumed ranged from 12 to 50 and averaged about 27. Both the low ratio of hydrogen peroxide accumulated to oxygen consumed and the variability of the data could have been due to residual catalase activity known to be present in purified preparations of cytochrome o (7). This residual catalase activity could be eliminated by repeated freezing-thawing of cytochrome o solutions, but preparations so treated showed no significant difference in the relative amount of hydrogen peroxide detected. Exogenously added catalase, on the other hand, eliminated hydrogen peroxide accumulation in these experiments (Table I).

When limited amounts of NADH were completely oxidized by solutions of cytochrome o the ratio of NADH oxidized to oxygen consumed averaged about 1.4, consistent with roughly 50% of the product being hydrogen peroxide, assuming water and hydrogen peroxide are the only products and no degradation of accumulated hydrogen peroxide. This ratio would be 2.0 if water were the sole product and 1.0 if hydrogen peroxide were the sole product.

The addition of FMN or FAD, both of which increased the rate of NADH oxidation by cytochrome o (14), resulted in an increase in the relative amount of hydrogen peroxide detected (Table I). Flavin is believed to stimulate the NADH oxidase activity of cytochrome o preparations by acting on the reductase side of the cytochrome because it stimulates the rate of formation of the oxygenated form of cytochrome o from NADH and the oxidized cytochrome o (14). When a solution of cytochrome o with exogenously added flavin was bubbled with carbon monoxide to replace approximately 50% of the oxygen with CO, the oxygen uptake was inhibited completely, further evidence that the electrons from NADH to oxygen were flowing through cytochrome o even in the presence of flavin.

Fig. 1 (left). Polarographic assay for hydrogen peroxide accumulation during the oxidation of NADH by purified preparations of cytochrome o. The reaction solution was 1.0 ml of 0.1 M sodium phosphate, pH 7.5, containing 14 μM cytochrome o heme at a temperature of 25°C. The solution for the bottom tracing contained, in addition, 20 μM FMN. The reaction was initiated with 10 μl of 0.05 M NADH and 1 μl of catalase (100,000 units/ml) was added to determine hydrogen peroxide accumulation as described in the text.

Fig. 2 (center). Colorimetric-peroxidase assay for hydrogen peroxide accumulation during the oxidation of NADH by purified preparations of cytochrome o. The reaction solution contained 5 μM cytochrome o and 450 μM NADH in 0.02 M sodium phosphate, pH 7.5, at 21°C. At appropriate times after the addition of the NADH, aliquots were removed and assayed using the peroxidase-o-dianisidine procedure described under "Materials and Methods."

Fig. 3 (right). Fluorometric assay for hydrogen peroxide formation during the oxidation of NADH by purified preparations of cytochrome o. The circles are the amount of hydrogen peroxide formed as determined fluorometrically at 525 nm, and the solid lines are the amount of NADH oxidized as determined spectrophotometrically at 340 nm. Lower curve and solid circles are for cytochrome o at 0.1 μg heme. Upper curve and open circles are for cytochrome o at 0.2 μg heme. The closed squares are the controls (minus NADH or cytochrome o). Average of two experiments. For concentration of other reagents and other details see "Materials and Methods."
The assay was in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.5, at 25°. Cytochrome o was 14 &amMP; with respect to heme. The reaction was initiated by the addition of 10 &mu;M of 0.08 M NADH. After approximately 50 to 70 mmol of oxygen were consumed, 1 &mu;M of catalase (100,000 units/ml) was added and the amount of oxygen released was used to estimate the accumulated hydrogen peroxide as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Hydrogen peroxide formed as per cent of oxygen consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24</td>
</tr>
<tr>
<td>FMN, 20 μM</td>
<td>39</td>
</tr>
<tr>
<td>FAD, 20 μM</td>
<td>29</td>
</tr>
<tr>
<td>Catalase, 5 units/ml</td>
<td>0</td>
</tr>
</tbody>
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For kinetic studies of hydrogen peroxide formation and more precise data, another method for the assay of hydrogen peroxide was tested, namely, the standard colorimetric assay, in which peroxidase is used to catalyze the oxidation of a dye (usually o-dianisidine) by hydrogen peroxide. Both the low pH and the high concentration of o-dianisidine used in this assay inhibited the oxidation of NADH by cytochrome o so it was not possible to assay for hydrogen peroxide formation in the NADH-cytochrome o oxidase reaction by direct coupling in one medium. Consequently, aliquots were removed at appropriate times from the NADH oxidase medium and added to the peroxide assay medium. The results showed no evidence for hydrogen peroxide formation during the early part of the reaction, indeed not for the first 30 min (Fig. 2). Results obtained with the polargraphic technique, on the other hand, did show hydrogen peroxide formation clearly occurring within 5 to 10 min, although more precise data at earlier reaction times was not feasibly obtainable. At 60 min and after the peroxidase-colorimetric assay gave qualitatively the same results as the polargraphic assay including an increased amount of hydrogen peroxide formed in the presence of added FMN or FAD.

Since peroxidase is known to oxidize NADH (15) the residual NADH present in the assay medium could have interfered with the colorimetric assay. This was tested and confirmed; NADH did interfere with the colorimetric assay, apparently being a better substrate for the enzyme than o-dianisidine. The possibility of interference by residual NADH in this assay for hydrogen peroxide was eliminated by having limited amounts of NADH be completely oxidized by solutions of cytochrome o at several concentrations within various time periods less than 30 min. These solutions, containing no residual NADH, were then assayed for hydrogen peroxide accumulation, but again no peroxide accumulation was detected. The failure of the colorimetric assay to detect hydrogen peroxide accumulation during the first 30 min of reaction cannot be ascribed solely to interference by residual NADH.

Two observations provided indirect evidence for the formation of hydrogen peroxide early in the NADH-cytochrome o oxidase reaction. First, catalase inhibited the rate of oxygen uptake of the reaction when followed polarographically (see Fig. 1), but had no effect on the rate of NADH oxidation followed spectrophotometrically at 340 nm. Second, peroxidase stimulated the rate of NADH oxidation, followed spectrophotometrically, exactly 2-fold when added simultaneously with NADH to the cytochrome o solution. When added several minutes after the NADH the initial stimulation was even greater, but the rate then returned to the 2-fold stimulated rate. Since peroxidase can catalyze the oxidation of NADH by hydrogen peroxide (15) these observations are consistent with the early and continued production of hydrogen peroxide in the NADH-cytochrome o oxidase reaction.

More direct evidence for this postulate was obtained from the fluorometric assay, which also used peroxidase to catalyze the oxidation of a dye, 2',7'-dichlorofluorescin diacetate, by hydrogen peroxide (13). The oxidized dye is fluorescent with an emission maximum at 525 nm. The assay, which is sensitive to hydrogen peroxide concentrations as low as 10^-11 M, is performed at neutral pH with relative low concentrations of reagents, and it was possible to assay directly for hydrogen peroxide formation by adding cytochrome o and NADH to the assay medium. The assay medium did not inhibit the oxidation of NADH by cytochrome o nor was NADH oxidized by the assay medium in the absence of cytochrome o. This experiment showed that hydrogen peroxide was detectable early in the reaction (within 1 min) and was generated continuously during the oxidation of NADH by cytochrome o in a stoichiometric ratio of 1:1 hydrogen peroxide formed to NADH oxidized (Fig. 3).

**DISCUSSION**

The results of three independent methods showed the formation of hydrogen peroxide during the oxidation of NADH by purified cytochrome o preparations. Although both the polargraphic and peroxidase-colorimetric assays showed net hydrogen peroxide accumulation, at least later in the reaction, it cannot be concluded that hydrogen peroxide is the only product. The accumulated hydrogen peroxide as determined by these methods was approximately 30% of the oxygen consumed or approximately 15 to 20% of the NADH oxidized even in preparations devoid of detectable catalase activity (Figs 1 and 2, Table 1). The fluorometric assay, on the other hand, showed the generation of 1 mol of hydrogen peroxide per mol of NADH oxidized continuously during the reaction (Fig. 3). These apparently discrepant results can be explained on the basis of the different experimental conditions used for each assay method, even though the colorimetric and fluorometric assays for hydrogen peroxide are both based on the same principle, peroxidase is used to catalyze the oxidation of a dye by the hydrogen peroxide. The dyes used in the two assays were different, but, more important, the concentration of reagents was lower in the fluorometric assay, and it could be performed at neutral pH. These differences enabled the NADH-cytochrome o oxidase reaction to be coupled directly to the fluorometric assay in one medium, and under these conditions hydrogen peroxide apparently can be trapped efficiently. In the colorimetric assay it was necessary to run the NADH-cytochrome o oxidase reaction independently in a separate medium and remove aliquots to the hydrogen peroxide assay medium, eliminating the possibility of trapping hydrogen peroxide as an intermediate in the reaction and allowing only accumulated hydrogen peroxide to be determined. The results of this assay showed no hydrogen peroxide accumulation early in the reaction, but later, hydrogen peroxide appeared as an increasing per cent of the product (Fig. 2), whereas the results of the fluorometric assay showed the constant formation of one hydrogen peroxide per NADH oxidized from the beginning of the reaction (Fig. 3).
These apparently different results are explainable if the hydrogen peroxide generated in the reaction is an intermediate, some or all of which, depending on the conditions, normally undergoes further reduction, but which is trapped efficiently under the conditions used in the fluorometric assay. The polarographic assay, like the colorimetric assay, would only determine accumulated hydrogen peroxide.

Details of the role of flavin in the NADH-cytochrome o oxidase reaction are still under investigation in this laboratory. There is now evidence that the NADH-cytochrome o reductase activity of purified cytochrome o preparations is associated with the presence of a contaminating flavoprotein, although this flavoprotein has not yet been separated from the cytochrome o (14). Exogenously added flavin is believed to act on the reducing side of cytochrome o, serving as a mobile carrier between the flavoprotein and the cytochrome (14). The evidence is against a direct reaction of reduced flavin with oxygen with the resultant formation of hydrogen peroxide, for example, oxygen uptake is inhibited completely when carbon monoxide replaces part of the oxygen in a solution containing cytochrome o, flavin, and NADH. Thus, the increased accumulation of hydrogen peroxide observed in the presence of flavin could be due to a change in the reaction mechanism, an increased rate of peroxide formation, or a decreased rate of peroxide removal in the cytochrome o-oxygen reaction itself.

There is no evidence for hydrogen peroxide as an intermediate during the reaction of reduced cytochrome oxidase with oxygen (11). To this author’s knowledge, however, this has not been tested with the sensitive fluorometric assay used in these experiments so successfully. Considering the observed limitations of the standard colorimetric assay it would be worthwhile to retest the cytochrome oxidase-oxygen reaction for a peroxide intermediate using the fluorometric assay.

REFERENCES
The formation of hydrogen peroxide during the oxidation of reduced nicotinamide adenine dinucleotide by cytochrome o from Vitreoscilla.

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