Mechanism of H_2O_2 Production in Porcine Thyroid Cells: Evidence for Intermediary Formation of Superoxide Anion by NADPH-Dependent H_2O_2-Generating Machinery†

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ABSTRACT: Hydrogen peroxide (H_2O_2), which is required for thyroid hormone synthesis, has been believed to be produced at the apical cell surface of thyroid follicular cells. However, we recently found that plasma membrane from porcine thyroid exclusively generated superoxide anion (O_2^-) by employing a novel method for simultaneous determination of H_2O_2 and O_2^- with diacetyldeuterioheme-substituted horseradish peroxidase (diacetyl-HRP) as the trapping reagent [Nakamura, Y., Ohtaki, S., Makino, R., Tanaka, T., & Ishimura, Y. (1989) *J. Biol. Chem. 264*, 4759–4761]. The present study describes the mechanism of H_2O_2 production as analyzed by this new method. Incubation of cultured porcine follicular cells with ionomycin, a Ca-ionophore, caused an increase in oxygen uptake of about 80%. During enhanced respiration, the cells released H_2O_2 in an amount equivalent to the amount of oxygen consumed as judged by the formation of compound II of diacetyl-HRP, and H_2O_2 adduct of the peroxidase. No formation of compound III of the peroxidase, an O_2^- adduct, was detected during burst respiration. Thus, the intact cells exclusively released H_2O_2 to the outside of the cells. On the other hand, when the cell fragments from follicular cells were incubated with NADPH or NADH in the presence of Ca^{2+}, the production of O_2^- was observed only during NADPH-dependent burst respiration, supporting our previous results that the plasma membrane exhibited NADPH-dependent O_2^--generating activity. O_2^- production by the plasma membrane was further confirmed by analyses of the effects of superoxide dismutase (SOD) and catalase on the reaction. These results suggested that H_2O_2 is secondarily produced through the dismutation of O_2^-. Indeed, high activity of SOD was observed in the cytosol and on the cytosolic side of the membrane of follicular cells. We propose that O_2^- is initially produced on the inside of the apical membrane and then released to the outside of the cell after being converted to H_2O_2 by the action of SOD.

Iodination of tyrosine residues in thyroglobulin and the subsequent coupling of the two iodotyrosine residues to form iodothyronine are key steps in the biosynthetic pathway of thyroid hormones (Serif & Kirkwood, 1958; Alexander, 1959; Lamas et al., 1972). Both reactions are catalyzed by thyroid peroxidase in the presence of H_2O_2 (Lamas et al., 1972; Nakamura et al., 1984). The major site of thyroglobulin iodination was found to be the apical cell surface in the follicle (Ekholm & Wollman, 1975; Ekholm, 1981); therefore, H_2O_2 was considered to be produced at the site. It was proved by cytochemical study that H_2O_2 generation occurred at the apical cell surface but not at the basal cell surface or in the intracellular space (Björkman & Ekholm, 1984).

Bénard and Brault (1970) found that H_2O_2 production in hog or calf thyroid slices was enhanced by thyroid-stimulating hormone (TSH),[1] although the TSH concentration used in the experiment was higher than the physiological concentration. H_2O_2 production is also evoked by Ca-ionophores (Björkman & Ekholm, 1984; Takasu et al., 1987), suggesting that TSH action is linked to an increase in intracellular concentration of Ca^{2+}. Thus, Ca^{2+} seemed an important factor in the regulation of H_2O_2 generation. The property of the enzyme(s) responsible for H_2O_2 production was analyzed by use of the particulate fraction and/or plasma membrane fraction from porcine thyroid (Dême et al., 1985; Nakamura et al., 1987; Dupuy et al., 1989). The results indicated that the H_2O_2-generating enzyme was a membrane-bound enzyme and required NADPH as an electron donor. Furthermore, H_2O_2-generating activity was found to be enhanced by a submicromolar Ca^{2+} concentration and an excess of ATP (Nakamura et al., 1987). Although it is generally accepted that H_2O_2 is produced at the cell surface by NADPH oxidase, the precise mechanism of the reaction has not yet been clarified. The possible formation of superoxide anion (O_2^-) by the enzyme has not been ruled out, since H_2O_2 production has been measured by indirect methods such as the scopoletin fluorescence assay in previous experiments. The ambiguity surrounding the primary oxygen metabolite of NADPH oxidase is due to a technical difficulty in determining O_2^- generated in crude preparations such as the plasma membrane fraction. To determine whether the plasma membrane produced O_2^- directly, we analyzed the NADPH oxidase reaction by the new assay method based on the stable adduct formation of diacetyl-HRP with O_2^- and H_2O_2. The result indicated that

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[1] Abbreviations: O_2^-, superoxide anion; SOD, superoxide dismutase; TSH, thyroid-stimulating hormone; DPBS, Dulbecco's phosphate-buffered saline; diacetyl-HRP, diacetyldeuterioheme-substituted HRP; HRP, horseradish peroxidase; DMEM, Dulbecco's-modified Eagle's medium; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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NADPH oxidase in the plasma membrane fraction from thyroid cells produced O_2^- as a primary metabolite of oxygen (Nakamura et al., 1989) and raised the question of whether O_2^- or H_2O_2 was the source of the oxidizing agent for hormone synthesis.

In the present study, we examined the oxygen metabolism of cultured thyroid follicular cells and their fragments by using this new assay method. The findings indicated that the cultured cells released H_2O_2 exclusively to the outside of cells during Ca^{2+}-stimulated respiration, while they produced O_2^- when lysed by freeze–thawing treatment. We also found that superoxide dismutase (SOD) activity was high both in the cytosol and on the cytosolic side of the plasma membrane as compared with that in neutrophils. The result was not compatible with the conventional idea that H_2O_2 is directly produced outside of cells but led to a new concept that O_2^- is produced and subsequently converted to H_2O_2 within the cells. The mechanism for the formation of active oxygen species in thyroid cells is different from the production of active oxygen species in neutrophils, in which O_2^- is secreted to the outside of cells (Makino et al., 1986a,b; Tanaka et al., 1988; Ueno et al., 1989).

Experimental Procedures

Preparations of Porcine Thyroid Cells and Neutrophils. Porcine thyroid cells were prepared as described by Sho and Kondo (1984) with slight modifications. Usually eight thyroids were minced and washed several times with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS). The minced tissue was treated with 150 mL of 0.1% collagenase (Wako Pure Chem., Tokyo, Japan) in Dulbecco's-modified Eagle's medium (DMEM) at 37 °C for 1 h and filtered through stainless steel mesh. The follicles suspended in DPBS were washed by centrifugation with DPBS several times. Then the follicles were treated with 20 mL of 0.1% trypsin and 0.2 mg/mL EDTA·4Na in DPBS at 37 °C for 1 h to disperse them. The treatment was stopped by adding 5 mL of 10% bovine serum albumin and 80 mL of cold DPBS. The isolated cells were collected and repeatedly washed by centrifugations with DMEM containing 100 mg of kanamycin/L. The cells [(1.5×10^6)/mL] were suspended in DMEM supplemented with 10% newborn calf serum (NBCS) and incubated at 37 °C in a 5% CO_2/95% air/water-saturated atmosphere for 20–26 h. The cells were used for experiments after being washed with DMEM and finally with DPBS.

Since the cells aggregated during incubation in the CO_2 incubator, the exact number of cells in the suspension could not be determined. The cell number in this study is the number counted before incubation. When necessary, a cell-free preparation of thyroid cells was prepared by freeze–thawing treatment as follows. Cells suspended in DPBS were frozen at −80 °C for 1 h and then lysed by addition of water at 0 °C. Cell fragments were separated from cytosolic components by centrifugation at 100000*g* for 30 min.

To analyze oxygen metabolism in the plasma membrane fraction, the membrane fraction prepared from porcine thyroid was further purified by the method described previously (Nakamura et al., 1987, 1989).

Porcine neutrophils were isolated and purified from porcine blood according to the method described previously (Iizuka et al., 1985). The cells were used to measure SOD activity on the same day that blood was drawn.

SOD activity in thyroid cells and neutrophils was measured after the following treatments. The cells were washed twice with DPBS, and sedimented cells were frozen for at least 1 h. Frozen cells were lysed by adding water, and cytosol and cell fragments were fractionated by centrifugation at 100000*g* for 30 min. After the cell fragments were washed twice, their SOD activity was measured.

Preparation of Porcine Superoxide Dismutase. Porcine erythrocytes were lysed by hypotonic treatment, and hemoglobin was removed by a heat treatment as described by Gärtner et al. (1984). The supernatant was applied to a DEAE-Sephacel column (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl buffer at pH 8.0. Porcine SOD was not adsorbed on the column. The passed-through fraction was applied to a CM-Cellulofine CH column (Seikagaku Kogyo, Tokyo, Japan) equilibrated with 50 mM sodium acetate buffer at pH 5.0, and the column was developed with use of a gradient of 0–0.5 M NaCl. The fractions with SOD activity were further purified by gel chromatography on Sephacryl S-200 (Pharmacia). The purity of the final SOD preparation was more than 95% as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Heme-Substituted Horseradish Peroxidase. Horseradish peroxidase was purchased from Toyobo (Osaka, Japan) and further purified by the method of Shannon et al. (1966). Isozyme B and C fractions were employed for the following heme substitution. The procedure for the preparation of the apoenzyme and its reconstitution with diacetyldeuterioheme were described earlier (Makino & Yamazaki, 1972).

Determinations. Simultaneous determination of O_2 consumption and generation of both O_2^- and H_2O_2 was carried out in a single cuvette set in a Shimadzu MPS-2000 double-beam spectrophotometer. In some experiments (Figure 4), absorbance change was measured on a Hitachi 557 dual-wavelength spectrophotometer. Oxygen consumption was measured with a Clark-type oxygen electrode (Diamond Electro-Tech Inc., Ann Arbor, MI) with a highly sensitive membrane (5776 membrane; YSI Inc., Yellow Spring, OH) under constant stirring. The electrode was mounted on the optical cuvette to permit the simultaneous measurement of oxygen consumption and the absorption spectrum. The formation of compounds II and III of diacetyl-HRP was measured as the difference in spectra against ferric peroxidase by use of extinction coefficients of 3.3 mM^{-1} cm^{-1} at 565 nm and 3.8 mM^{-1} cm^{-1} at 585 nm, respectively (Makino et al., 1986a,b).

SOD activity in the supernatant and membrane fractions was assayed by the system using cytochrome *c*, xanthine, and xanthine oxidase under the conditions described by Crapo et al. (1978). One unit of SOD is defined as that amount of enzyme that inhibits the cytochrome *c* reduction rate by 50% (Crapo et al., 1978). SOD activity was detected on an agarose gel electrophoretic film (Universal Electrophoresis Film; Ciba Corning Diagnostics, Palo Alto, CA) as described by Flohé and Ötting (1984).

Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Enzymes and Chemicals. Erythrocyte catalase was purified from porcine erythrocytes according to the method of Takeda and Samejima (1977). Acetylated cytochrome *c* was prepared according to the method of Kakinuma and Minakami (1978). Crystalline bovine liver catalase was purchased from Boehringer Mannheim GmbH. The enzyme was further purified by an AcA 34 gel filtration column (IBF Biotechnics, France) and concentrated by ultrafiltration (Amicon, Danvers, MA). Bovine erythrocyte SOD was purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin was the product of Calbiochem (La Jolla, CA). All other chemicals were of the highest grade commercially available.

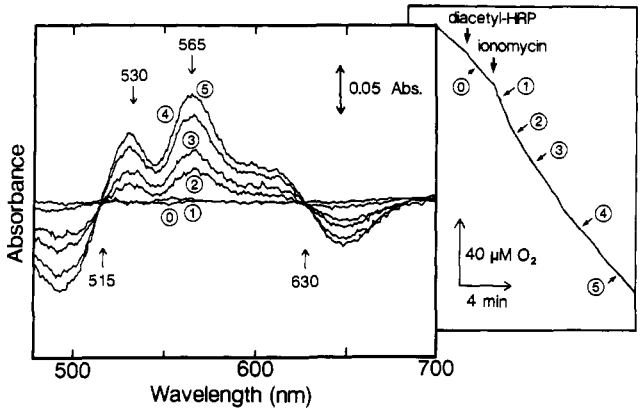

FIGURE 1: H_2O_2 generation from thyroid follicular cells as measured by diacetyl-HRP. Changes in the spectrum of diacetyl-HRP and O_2 consumption (inset) were recorded simultaneously during incubation of intact thyroid cells with the Ca-ionophore ionomycin. The reaction medium contained 3×10^7 cells/mL, 93 μM diacetyl-HRP, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM glucose in DPBS at 28 °C. The reaction was started by the addition of 0.5 μM ionomycin, and spectra 1–5 were recorded at the times indicated by arrows in the inset.

RESULTS

Detection of Hydrogen Peroxide and Superoxide Anion from Intact Cells and Lysed Cells. Figure 1 shows the spectral changes in diacetyldeuterioheme-substituted horseradish peroxidase during enhanced respiration of the cultured thyroid cells brought about by the addition of the Ca-ionophore ionomycin. As shown in the inset, the cells consumed O_2 at a rate of about 15.3 μM/min; this rate was not affected by the addition of diacetyl-HRP. With the addition of 0.5 μM ionomycin, the rate increased markedly to 24.6 from 15.3 μM/min. The spectrum of diacetyl-HRP changed to that of another species with the enhanced O_2 consumption. The conversion gave a set of isosbestic points at 515 and 630 nm, and the resultant species had absorption maxima of 530 and 565 nm. The spectral characteristics of this species agreed with those of compound II of diacetyl-HRP, an adduct of the peroxidase with H_2O_2 (Makino & Yamazaki, 1972; Makino et al., 1986a,b). During the conversion, no formation of compound III, an O_2^- adduct of the peroxidase, was detected. The rate of compound II formation calculated from the increase in absorbance at 565 nm was 9.8 μM/min. This value approximates the oxygen consumption rate achieved by the addition of ionomycin (9.3 μM/min), indicating that the O_2 consumed is converted stoichiometrically to H_2O_2. These results indicate that intact follicular cells release H_2O_2 extracellularly, but not O_2^-. This appears to be inconsistent with the previous findings obtained for the thyroid plasma membrane, in which O_2^- is an initial oxygen metabolite (Nakamura et al., 1989). The discrepancy implies that H_2O_2 is formed secondarily from O_2^-.

To substantiate this hypothesis, oxygen metabolism was examined under the condition in which intact follicular cells were lysed by freeze–thawing treatment. Under this condition, diacetyl-HRP, as an indicator for both H_2O_2 and O_2^-, should be freely accessible to the plasma membrane from both outside and inside the cell. The primary cultured thyroid cells were disrupted by freeze–thawing, and the fragmented cells were separated from the cytosolic components by ultracentrifugation (see Experimental Procedures). The resultant pellet of lysed cells is hereafter referred to as the cell fragment. NADPH-dependent oxygen metabolism of the cell fragments was examined by diacetyl-HRP at 25 °C (Figure 2). The rate of O_2 consumption was much slower than that observed in the intact cells, indicating that the endogenous substrate for

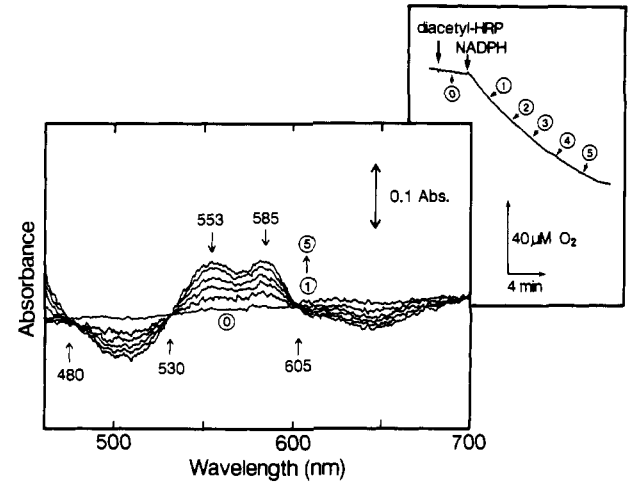

FIGURE 2: O_2^- generation from cell-free preparation of thyroid follicular cells. Changes in the spectrum of diacetyl-HRP and oxygen consumption (inset) were recorded simultaneously during incubation of the cell-free preparation (cell fragment) with NADPH in the presence of Ca^{2+}. The reaction mixture (0.74 mL) contained cell fragments prepared from 6×10^7 cells (approximately 2 mg of protein), 115 μM diacetyl-HRP, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 90 mM KCl, 0.25 M sucrose, 1 mM EGTA, and 20 mM MOPS at pH 7.0 and 25 °C. The reaction was started by the addition of 106 μM of NADPH and recorded as described in Figure 1.

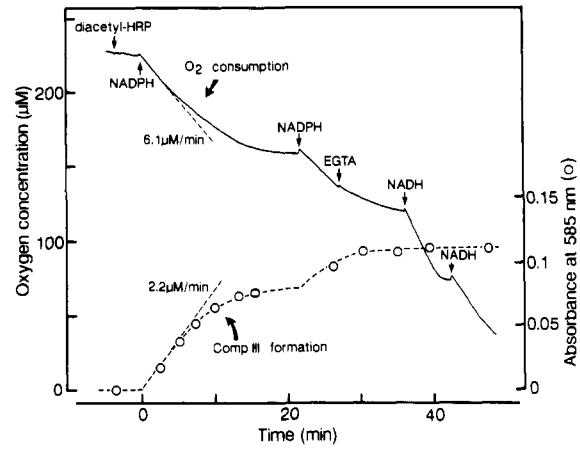

FIGURE 3: Effects of EGTA and NADPH on oxygen consumption and generation of O_2^- by cell fragment. The amount of compound III (O) accumulated during respiration was determined from an increase in absorbance at 585 nm and plotted with the time course of the simultaneous measurement of O_2 consumption. Other conditions were the same as those in Figure 2. The additions indicated by arrows (left to right) were 115 μM diacetyl-HRP, 106 and 146 μM NADPH, 5 mM of EGTA, and 106 μM of NADH twice.

respiration was deficient in the fraction. The addition of NADPH to the mixture caused rapid O_2 consumption with the formation of a new spectral species of diacetyl-HRP. The spectral species was produced through a set of isosbestic points at 480, 530, and 605 nm, and the resultant species exhibited absorption maxima at 553 and 585 nm in the difference spectrum. These spectral characteristics were exactly the same as those of compound III. Essentially the same result was also obtained at 30 °C. These results, together with the previous finding (Nakamura et al., 1989), indicate that the oxidase in the membrane fraction exclusively produces O_2^-.

In Figure 3, the time course for compound III formation is depicted with the oxygen consumption trace. The effects of NADH and EGTA upon O_2^- formation were also examined in the experiment. The formation of compound III initiated by the addition of NADPH increased linearly with time and reached a plateau when O_2 consumption terminated. The

initial rates for O_2 consumption and O_2^- production were 6.1 and 2.2 μM/min, respectively. This means that 36% of the O_2 consumed is converted to O_2^-. A residual part of O_2 might be consumed by other membrane-bound NADPH-dependent redox enzymes (Dupuy et al., 1989), although the exact reasons remain unclear. The second addition of NADPH restarted both reactions. The subsequent addition of EGTA terminated compound III formation and decreased O_2 consumption. This confirms the earlier observation that NADPH oxidase requires Ca^{2+} for O_2^- formation (Nakamura et al., 1987, 1989). The addition of NADH induced rapid O_2 consumption, but the formation of compound III was not observed. The NADH-supported rapid O_2 consumption could be ascribed to mitochondrial respiration, since the cell fragment preparation was found to contain mitochondrial fractions. These results indicate that O_2^- formation by the cell fragment is highly specific to NADPH and that the contribution of mitochondria to O_2^- formation is nil.

Reexamination of O_2^- Production of the Plasma Membrane Fraction. Dupuy et al. (1989, 1990) recently reported that the membrane fraction from thyroid produced only H_2O_2. In response to this controversial result, we reexamined the oxygen metabolism of the membrane fraction purified from thyroid. When the NADPH-dependent burst respiration of several membrane fractions was examined by the diacetyl-HRP method, only the formation of compound III was observed, as reported previously (Nakamura et al., 1989) (data not shown). Simultaneous analyses of oxygen consumption and compound III formation indicated that more than 85% of the oxygen consumed was converted to O_2^-. We also examined the spectral change of diacetyl-HRP at the various concentrations (20–150 μM) of the peroxidase. In this experiment, thyroid membrane fraction with O_2^--generating activity of 5.5 μM/min was used. At the concentration over 30 of diacetyl-HRP, the product of the peroxidase was compound III and the rate of compound III formation was constant irrespective of the concentration of diacetyl-HRP employed. At less than 20 μM, compound III was formed in the initial stage of the reaction (within 4 min) without showing an appreciable decrease in the formation rate. After the lapse of 4 min, a small amount of compound II was accumulated. The result, however, does not mean the direct formation of H_2O_2, since spontaneous dismutation of O_2^- to H_2O_2 is considered to proceed at a rate comparable to that of the reaction between diacetyl-HRP and O_2^- in the late stage of the reaction.

In the next experiments, the effect of SOD and/or catalase on O_2^- formation was examined to confirm O_2^- formation. As shown in the inset of Figure 4A, adding diacetyl-HRP and SOD did not evoke oxygen consumption. The subsequent addition of NADPH, however, induced rapid oxygen consumption. When the absorption spectral changes in diacetyl-HRP were recorded at the times indicated in the inset during burst respiration, compound II with absorption maxima at 532 and 566 nm appeared (Figure 4A). This indicated that the O_2^- produced was completely converted to H_2O_2 by the action of SOD. To examine the relationship between the oxygen consumed and compound II formation, the increase in absorbance at 566 nm caused by compound II formation was measured simultaneously with O_2 consumption (Figure 4B). The initial rates calculated from the traces were 37 and 33 μM/min for O_2 consumption and compound II formation, respectively, indicating that at least 90% of the consumed oxygen was converted to H_2O_2 in the presence of SOD.

The effect of catalase on burst respiration was examined in detail. The membrane fraction used in the experiment

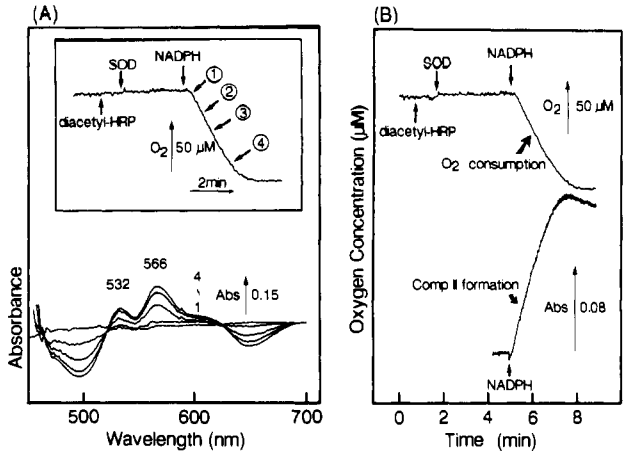

FIGURE 4: Effect of SOD on oxygen metabolism of a purified plasma membrane fraction. The reaction mixture contained a plasma membrane fraction (1.1 mg/mL as protein) and 5 mM ATP. In panel A, changes in the spectrum of diacetyl-HRP (180 μM) and oxygen uptake (inset) induced by the addition of 0.13 mM NADPH were recorded simultaneously in the presence of SOD (4.5 μM). Spectra 1–4 were recorded at the times indicated by arrows in the inset. The spectra were recorded on a dual-wavelength spectrophotometer in which the reference wavelength was fixed at 700 nm. In panel B, formation of compound II was followed by measuring the change in absorbance at 566 nm minus that at 625 nm (isosbestic point) on a dual-wavelength spectrophotometer. The concentrations of diacetyl-HRP, SOD, and NADPH were the same as those in panel A. Other experimental conditions were the same as those in Figure 2.

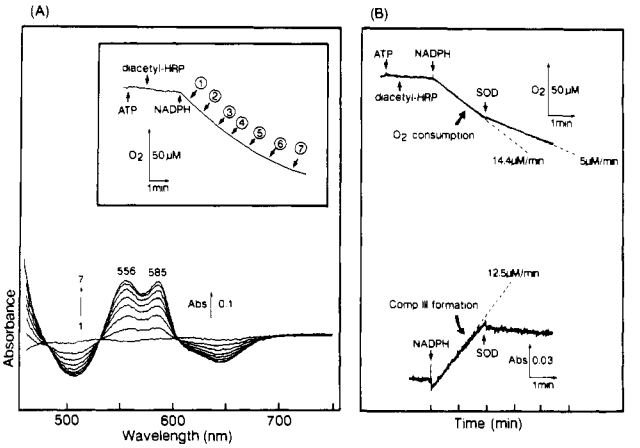

FIGURE 5: Effect of erythrocyte catalase on oxygen metabolism of purified plasma membrane fraction. In panel A, the reaction medium contained 33 μM erythrocyte catalase and the absorption due to catalase was initially memorized and recorded as base line. The additions indicated by arrows were ATP (3.7 mM), diacetyl-HRP (105 μM), and NADPH (105 μM). The spectral changes in diacetyl-HRP were recorded at the times indicated by arrows in the oxygen consumption trace (inset). In panel B, the absorbance increase at 585 nm due to compound III formation and oxygen consumption were simultaneously measured. The reaction mixture contained 33 μM erythrocyte catalase, and the reaction was started by the addition of NADPH (105 μM). ATP (3.7 mM), diacetyl-HRP (105 μM), and SOD (9.5 μM) were added at the times indicated by arrows. Other experimental conditions were the same as those in Figure 2. The concentration of catalase was expressed as heme concentration.

consumed O_2 at a rate of 15.5 μM/min and produced O_2^- at a rate of 13.5 μM/min in the presence of diacetyl-HRP when NADPH was added (data not shown). When the reaction was induced in the presence of an excess amount of erythrocyte catalase, diacetyl-HRP was converted to compound III with a rate of O_2 consumption of 14.5 μM/min (Figure 5A). In addition, compound III formation was observed when liver catalase instead of erythrocyte catalase was included in the medium at a final concentration of 24 μM. When the time

Table I: SOD Activity in Thyroid Cells and Neutrophils[a]

	units/10^6 cells	units/mg of protein
thyroid cells		
cytosol (n = 6)	0.59 ± 0.10	15.1 ± 1.8
cell fragment (n = 5)	0.26 ± 0.11	6.7 ± 1.6
neutrophils		
cytosol (n = 8)	0.035 ± 0.009	1.23 ± 0.31
cell fragment (n = 8)	<0.005	<0.1

[a] The cell pellet frozen for at least 1 h was thawed and then lysed by the addition of water. The lysate was centrifuged at 100000g for 30 min to separate cytosol and cell fragment fractions. Then, the SOD activity of both fractions was assayed. Values are the mean ± SE for n preparations.

courses of oxygen consumption and compound III formation were measured under the same experimental conditions (Figure 5B), the initial rates were calculated to be 14.4 and 12.5 μM/min for O_2 consumption and compound III formation, respectively, indicating that oxygen metabolism was essentially unaffected by catalase. The subsequent addition of an excess amount of SOD during burst respiration promptly terminated compound III formation and decreased the O_2 consumption rate. In a separate experiment, it was confirmed that no compound II formation was detected after the addition of SOD. This finding meant that the H_2O_2 generated by the action of exogenous SOD was decomposed to $^1/_2$ mol each of H_2O and O_2 by an excess amount of catalase without being trapped in diacetyl-HRP. These results provide definitive evidence that the NADPH oxidase in the thyroid plasma membrane does not produce H_2O_2 directly during burst respiration. The mechanism of compound III formation of diacetyl-HRP is further discussed later.

We attempted to detect O_2^- by a classical method using cytochrome c reduction. However, we failed to detect SOD-inhibitable cytochrome c reduction because of a significantly high background of cytochrome c reductase activity in the membrane fraction (Nakamura et al., 1987; Dupuy et al., 1989). The difficulty in detecting O_2^- was overcome by use of acetylated cytochrome c as an O_2^- trapping reagent, which lacked the reactivity for reductase without a loss of the reducibility for O_2^- (Kakinuma & Minakami, 1978). By using acetylated cytochrome c, we confirmed the O_2^- formation during burst respiration of thyroid membrane fraction. The amount of O_2^- calculated from SOD-inhibitable acetylated cytochrome c reduction, however, was approximately 30% of the amount calculated from the diacetyl-HRP method. The reason for the discrepancy is unknown at present.

Superoxide Dismutase Activity and Its Localization in Thyroid Cells. The results described above suggested that O_2^- produced in the intact thyroid cells was efficiently dismutated to H_2O_2 prior to reaction with diacetyl-HRP. For this reason, the activities and localization of SOD in thyroid cells were examined. The results, which are summarized in Table I, were compared with those in neutrophils. Cytosolic SOD activity per 10^6 thyroid cells was 17 times greater than that in neutrophils. When activity was expressed as specific activity (units/mg of protein), cytosolic SOD activity in the thyroid cell was 12-fold higher than that in neutrophils. Furthermore, the activity of membrane-bound SOD in the thyroid cell fragment (6.7 units/mg of protein) was significantly higher than that in neutrophils.

The activity of SOD on the surface of thyroid cells was assessed by the inhibitory action of intact thyroid cells on cytochrome c reduction in DPBS at pH 7.4 and 37 °C. The experimental condition for the measurements was slightly different from that described in the original literature (Crapo et al., 1978), and the sensitivity was about one-quarter of that under the conditions originally reported. The addition of 3 × 10^6 cells did not cause a significant inhibition of cytochrome c reduction. This indicated that the amount of SOD on the cell surface is negligible and that most of the SOD assessed as membrane-bound SOD is localized on the cytosolic side of the plasma membrane.

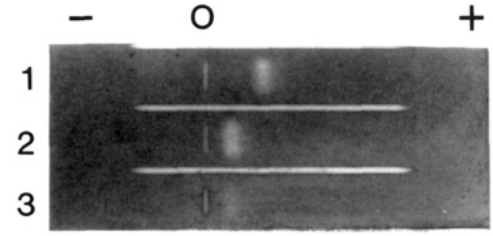

FIGURE 6: Electrophoretic analysis of SOD in the cytosol of thyroid cells. The cytosolic proteins from thyroid cells were separated on agarose gel, and SOD activity was detected by Nitro Blue tetrazolium. SOD activity was visualized as a colorless band in the background of a blue formazan. The samples applied at the origin (O) were 0.1 μg of bovine erythrocyte SOD (lane 1), 0.1 μg of porcine erythrocyte SOD (lane 2), and 2 μL of 4 mg/mL cytosolic protein from thyroid cells (lane 3).

Cytosolic SOD activity in thyroid cells was inhibited to less than 10% of the original value by the addition of 1 mM KCN, suggesting that SOD in the cytosol was Cu,Zn-SOD. This was confirmed by activity staining on gel electrophoresis (Figure 6). When cytosolic protein was separated by agarose gel electrophoresis and SOD activity was stained by the reaction with Nitro Blue tetrazolium, SOD activity was detected as a single band. Its mobility was exactly the same as that of SOD purified from porcine erythrocytes but was significantly different from that for bovine SOD.

Discussion

So-called reactive oxygen species such as O_2^- and H_2O_2 have been shown to participate in the bactericidal actions of neutrophils (Rossi, 1986). In this instance, O_2^- is formed by an NADPH oxidase system localized in the plasma membrane and is released to the outside of the membrane (Makino et al., 1986a,b). We have recently demonstrated that NADPH oxidase in the plasma membrane fraction from porcine thyroid catalyzes the massive production of O_2^- and have suggested that an enzyme system similar to the NADPH oxidase system in neutrophils is responsible for the production of H_2O_2 in the thyroid (Nakamura et al., 1989). In the present study, however, the liberation of H_2O_2 but not O_2^- was demonstrated with intact thyroid cells stimulated with a Ca-ionophore, ionomycin. SOD activity in thyroid cells was found to be much greater than that in neutrophils, and cell fragments of thyroid cells without cytosol generated only O_2^- when reacted with NADPH. These results indicate that O_2^- is an intermediate in H_2O_2 formation and suggest that SOD plays an important role as an H_2O_2 supplier in thyroid hormone synthesis. On the basis of these findings, we postulate a mechanism for H_2O_2 production as illustrated in Figure 7. In the model, O_2^- is initially produced inside the plasma membrane and converted to H_2O_2 by the action of SOD. Then, H_2O_2 is released to the outside of cells by traversing the plasma membrane and reacted with thyroid peroxidase to synthesize thyroid hormone.

Thyroid peroxidase catalyzes two reactions necessary for hormone biosynthesis, that is, the iodination of tyrosine residues and the coupling of two iodotyrosine residues. By reacting with H_2O_2, thyroid peroxidase converts to compound I. This compound is a reaction intermediate for the iodination reaction and is reduced to compound II in the coupling reaction in

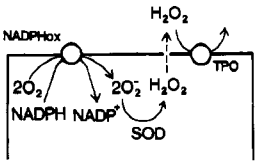

FIGURE 7: Hypothetical scheme for H_2O_2 production from O_2^-. NADPHox and TPO on the plasma membrane (solid line) denote NADPH oxidase and thyroid peroxidase, respectively.

which iodotyrosine is oxidized to the radical species (Nakamura et al., 1984). Through the reaction with O_2^-, on the other hand, compound III may be formed. Compound III is a labile state for thyroid peroxidase and therefore may not participate in hormone synthesis (Dr. Masao Nakamura, Hokkaido University, personal communication). Thus, O_2^- is a useless and harmful oxidant for thyroid peroxidase but an intermediate in H_2O_2 formation. The clinical significance of SOD in the thyroid has been suggested by Sugawara et al. (1988). They found a deficiency of cytosolic SOD in the tissues of patients with endemic goiter in Brazil and suggested that decreased enzyme activity causes degeneration of the tissue, presumably due to prolonged exposure to oxygen-free radicals. In addition to their suggestion for a protective role, we believe that SOD plays an essential role in hormone biosynthesis as the supplier of H_2O_2.

The present results indicated that O_2^- is a primary oxygen metabolite in the burst respiration of both thyroid follicular cells and their fragments. However, the recent reports by Dupuy et al. (1989, 1990) described that the thyroid plasma membrane fraction directly produces H_2O_2, not O_2^-. This conclusion was drawn on the basis of observation that compound III formation of diacetyl-HRP was inhibited by catalase. In their scheme, NADPH is first oxidized to NADPH$^\cdot$ by the diacetyl-HRP–H_2O_2 system, and then the resulting NADPH$^\cdot$ reacts with O_2 to yield O_2^-: NADPH$^\cdot$ + O_2 → NADP$^+$ + O_2^-. Thus, the reaction is triggered by H_2O_2 and is autocatalytic. The reaction was well characterized by Yokota and Yamazaki (1977) as a peroxidase–oxidase reaction. We examined the diacetyl-HRP-catalyzed oxidase reaction of NADPH in the presence of H_2O_2. The addition of NADPH caused no spectral change in diacetyl-HRP, and a successive addition of H_2O_2 evoked rapid formation of compound II. Thus, the diacetyl-HRP-catalyzed oxidase reaction was extremely slow, being different from native peroxidase. The reason is ascribable to the marked decrease in the reactivity of compound II of diacetyl-HRP for hydrogen donors (Makino & Yamazaki, 1972; Makino et al., 1986a,b).

We carefully and repeatedly analyzed the oxygen metabolite during burst respiration of the plasma membrane fraction by the diacetyl-HRP method. Under our experimental conditions in which "an excess amount of diacetyl-HRP" was used to trap all the O_2^- or H_2O_2 generated, we observed only the formation of compound III and failed to detect compound II, an H_2O_2 adduct of the peroxidase. The formation of H_2O_2 was observed only when an excess amount of SOD was included in the medium. If compound III of diacetyl-HRP had been formed by the peroxidase–oxidase reaction during NADPH-induced burst respiration, the formation of compound III should be suppressed by the addition of an excess amount of catalase. However, neither compound III formation nor O_2 consumption in burst respiration was catalase-inhibitable under our experimental conditions. Furthermore, the possibility that H_2O_2 is utilized for the diacetyl-HRP-catalyzed peroxidase reaction even in the presence of an excess amount of catalase was ruled out for the following reason: H_2O_2 generated by exogenously added SOD was efficiently decomposed by catalase without being trapped in diacetyl-HRP, as shown in Figure 5. These close studies confirmed our previous result that the plasma membrane fraction produces O_2^- as a primary oxygen metabolite in NADPH-dependent burst respiration (Nakamura et al., 1989). The reasons for the conflicting results are unclear at present, but their use of less than 10 μM of diacetyl-HRP for the detection of O_2^- might have caused the difference. Our conclusion for O_2^- formation in thyroid cells was recently confirmed by a different experimental method. By using a spin-trapping technique, Verma et al. (1990) observed the formation of O_2^- in follicular cells from mouse thyroid when an SOD inhibitor was included in the reaction medium. They also suggested that the H_2O_2 generated was a secondary product of the dismutation of O_2^-.

Registry No. SOD, 9054-89-1; NADPH, 53-57-6; H_2O_2, 7722-84-1; O_2, 11062-77-4; Ca, 7440-70-2.

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Investigation of Laser-Induced Long-Lived States of Photolyzed MbCO†

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ABSTRACT: We present evidence from resonance Raman and absorption measurements that the extended exposure of MbCO to CW laser light at low temperatures alters the CO rebinding kinetics and leads to a significantly increased population of very long lived states of photolyzed MbCO. This optical "pumping" process is observed for samples frozen in both aqueous buffer and glycerol/buffer and exhibits power law behavior with a very weak temperature dependence. A comparison of the nonexponential rebinding kinetics of CO molecules from the pumped states with the rebinding observed in flash photolysis experiments suggests that the pumped states are distinct geminate states, not observed in flash photolysis experiments. Thus, a four-state model, with two geminate states, is implicated for MbCO. Pumped states may represent "separated geminate pair" states with the CO molecule still in the heme pocket or possibly trapped within a cavity on its way through the protein matrix, consistent with molecular dynamics simulations. The possibility of significant deoxyheme relaxation from a less domed to a more domed configuration, as a result of the multiple photolysis events associated with the pumping process, is also explored. However, the small changes observed in the Soret band line shape and position subsequent to pumping at $T < 180$ K tend to rule out this explanation for the pumping process. Since the yield for creating a pumped state is small (e.g., $<10^{-7}$ for $T > 100$ K), pumping can be observed only after extended illumination and is absent in flash photolysis measurements, even after multiple flashes. At higher temperatures ($T > 180$ K), the escape of the CO molecule to the solvent is observed. Our data are consistent with a "phase transition" of the protein that is coupled to the surrounding matrix. The protein fluctuations are quenched below ~185 K for a solvent composed of 70% glycerol and below ~260 K for aqueous buffer. We also present the first large amplitude measurements of CO rebinding from the protein exterior, observed below 200 K after freezing the sample under laser illumination.

MbCO at low temperatures has been extensively studied by use of the flash photolysis technique (Austin et al., 1975; Beece et al., 1980). These studies have revealed a nonexponential nature of the geminate rebinding process at low temperatures and require a distribution of exponential rebinding rates in order to be explained. Two possible scenarios for the observed power law rebinding are (1) all Mb molecules are identical and each has multiple sites with different rebinding rates (homogeneous ensemble) and (2) each molecule at low temperatures is frozen in a slightly different conformation than the other molecules (quenched disorder), leading to a distribution of rebinding rates throughout the ensemble (inhomogeneous ensemble). One experiment that can distinguish between these two possibilities involves photodissociating flashes that are repeated at time intervals short enough so that only partial rebinding occurs between two flashes. If all the molecules are identical and have a variety of sites for the photolyzed CO molecule to go to, repetitive flashes would lead to an increasing number of molecules with CO "trapped" at sites with smaller rebinding rates. Each flash will, thus, alter the distribution of rates and after each flash, slower rebinding would be observed. If, on the other hand, the ensemble of molecules is inhomogeneous, repetitive flashes will not alter the distribution of rates and, after each flash, the same nonexponential relaxation function will be observed. Since the multiple-flash experiment (Austin et al., 1975) in MbCO revealed no change in the rebinding after repeated pulses, it was concluded that inhomogeneity of the ensemble of Mb molecules was the source of the nonexponential rebinding behavior at low temperatures. The inhomogeneous distribution of protein conformations has been supported by several other measurements (Frauenfelder et al., 1979; Champion & Sievers, 1980; Parak et al., 1982; Hartmann et al., 1982) and model calculations (Case & Karplus, 1979) on Mb and Hb.

However, EXAFS experiments (Powers et al., 1984; Chance et al., 1986) showed that, after extended continuous illumination of a MbCO sample with white light, some pumping to long-lived states of photolyzed molecules occurs. Subsequent flash photolysis experiments (Ansari et al., 1987), performed

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