Hydrogen Peroxide Generation in Ground Muscle Tissues

Stella Harel and Joseph Kanner*

Hydrogen peroxide (H_2O_2) and the superoxide radical (O_2^-) are normally present at low concentration as metabolites in aerobic cells. The generation of H_2O_2 in turkey muscle tissues was determined by using [^{14}C]formate oxidation to $^{14}CO_2$ by the peroxidative activity of catalase. The incubation of muscle tissues at 37 °C shows H_2O_2 generation of 14.0 nmol/g of fresh weight/30 min. In the presence of added catalase we found that muscle tissues generate almost 26.6 nmol of H_2O_2 under the same conditions. Muscle tissues aging at 4 °C increase the generation of H_2O_2. It seems that endogenous generation of H_2O_2 plays an important role in the formation of the primary pool of biological catalysts in muscle tissues.

INTRODUCTION

Hydrogen peroxide and the superoxide radical are normally present at low concentration as metabolites in aerobic cells (Ramasarma, 1982). The amount of superoxide radical, O_2^-, and hydrogen peroxide, H_2O_2, is maintained at approximately 10^{-12}–10^{-11} M and 10^{-9}–10^{-7} M, respectively, by superoxide anion dismutase and glutathion peroxidase (Oshino et al., 1973; Tyler, 1975).

Mitochondria (Loschen et al., 1971; Boveris et al., 1972), microsomes (Thurman et al., 1972; Hildebrandt and Roots, 1975), peroxisomes, (Boveris et al., 1972), and cytosolic enzymes (Chance et al., 1979) have all been recognized as effective H_2O_2 generators when fully supplemented by their substrates. Phagocytic leukocytes during phagocytosis generate large amounts of O_2^- and H_2O_2, which are released into the medium surrounding the phagocytes (Root et al., 1975).

Portwich and Aebi (1960) had estimated the H_2O_2-producing capacity of homogenates from different rat tissues. The generation of H_2O_2 was tested by using formate oxidation as an indicator reaction. It was found that liver homogenates produce 30–50 μmol of H_2O_2/g of dry weight/h. Decreasing activities were found: kidney > spleen > brain > muscles.

Hydrogen peroxide and the superoxide radical are cytotoxic to biological tissues, apparently due to the capacity of those intermediates of oxygen to generate other reactive oxygen species, such as the hydroxyl radical, which then initiate a radical chain reaction (Fridovich, 1983). This reaction can lead to extensive lipid and organic peroxide formation.

The aim of our study was to determine the generation of hydrogen peroxide in muscle tissues.

MATERIALS AND METHODS

Hydrogen peroxide, 30% for synthesis, was purchased from Merck-Schuchardt, Munich, West Germany; catalase 17600 U/mg, thymol-free and formate Na, were from Sigma Chemical Comp., St. Louis, MO; formate Na(^{14}C) with a specific activity of 4–5 mCi/mmol was obtained from New England Nuclear, Boston, MA; center well gross from Kontes, Vineland, NJ; Aqualuma scintillation liquid from Packard.

The amount of H_2O_2 was determined by using a method developed by Portwich and Aebi (1960) and modified by May and Haen (1979).

Fresh turkey muscle (dark or light, without the gross fat and connective tissues) was ground with a stainless steel food processor at 4 °C for 5 s.

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Ground muscle tissue (1 g) was added to a 50-mL Erlenmeyer flask in the presence of 425 U catalase, 0.05 M buffer acetate, pH 5.6, to a final volume of 4 mL.

The Erlenmeyer was stoppered with a rubber cap which held in the center a well gross contained a filter paper (Whatman M) soaked previously with 100 μL of KOH, 1 N.

Sodium formate, 1 mM (10.5 mL), which contained 0.5 μCi (^{14}C), was injected into the Erlenmeyer Flask just before the incubation in a shaking water bath. The reaction was stopped by the addition of 0.5 mL of acetic acid, 1 N.

The $^{14}CO_2$ collected by the KOH-soaked filter paper was transferred to a polyethylene scintillation vial and 10 mL of Aqualuma was added. The radioactivity was counted with 70% efficiency with a Kontron Liquid Scintillation Counter MR300.

RESULTS AND DISCUSSION

Hydrogen peroxide determination by the peroxidative activity of catalase is based on the presence of a specific electron donor compound to catalase compound I (Schonbaum and Chance, 1976). Formate is a substrate which donates two electrons and decomposes to CO_2 by the following reaction.

$$\text{catalase} + H_2O_2 \rightarrow \text{compound I} \quad (1)$$

$$\text{compound I} + HCOOH \xrightarrow{-2e} \text{catalase} + CO_2 \quad (2)$$

Formate oxidation by the peroxidative activity of catalase is high at low H_2O_2 concentration. At a high concentration of H_2O_2 the catalatic activity of catalase started to dominate and formate oxidation decreased.

Figure 1 shows that high levels of H_2O_2 are competitive to the interaction of compound I with formate.

The incubation of ground muscle tissues led to the generation of 14.0 nmol of H_2O_2/g of fresh wt/30 min. The oxidation of formate in this homogenate seemed to be activated by endogenic catalase. In the presence of added catalase, muscle homogenate generated almost 26.6 nmol of H_2O_2/g of fresh wt/30 min, in a 4-mL reaction mixture. This amount is equal to 5.3 μM H_2O_2 in the reaction mixture. The generation of H_2O_2 for the first minute was 0.3 μM. As the reaction mixture contained 4 mL of aqueous solution in the presence of 1 g of muscle tissues, the rise in H_2O_2 concentration in situ of 1 g of muscle tissues was almost 5-fold higher, about 1.5 μM H_2O_2. From Figure 1, it is possible to see that catalase (425 U) reacts with formate until 3–4 μM H_2O_2 at a stoichiometric rate of 1:1. We found also that in a model system glucose oxidase, which generated 1 μM H_2O_2/min, could activate metmyoglobin for a significant membranal lipid peroxidation (Harel and Kanner 1985). Azide (1 mM) in-

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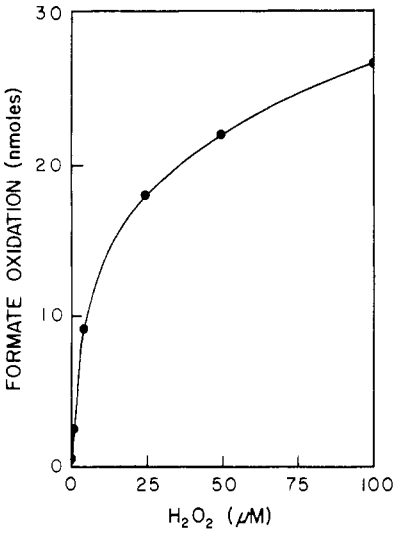

Figure 1. Formate peroxidation by catalase as affected by H_2O_2 concentration. Catalase (425 U) in the presence of formate Na 1 mM contained 0.5 μCi (^{14}C) in buffer acetate, 0.05 M, pH 5.6, and was incubated at 37 °C. The reaction was initiated by various concentrations of H_2O_2 and stopped after 1 min by the addition of 0.5 mL of acetic acid 1 N. $^{14}CO_2$ was collected and determined as described in Materials and Methods.

Table I. Production of H_2O_2 by Ground Muscle Tissues Incubated at 37 °C, pH 5.6

treatment	production of H_2O_2 nmol/g of fresh wt/30 min
ground dark muscle	14.0
ground dark muscle plus catalase (425 U)	26.6
ground dark muscle plus sodium azide (1 mM)	3.8
ground dark muscle plus catalase plus azide	3.8

hibited almost 85% of the formate oxidation (Table I). The significant increase in formate oxidation by the addition of catalase indicates clearly that this oxidation is activated by the presence of H_2O_2.

Hyrogen peroxide accumulated in dark muscle homogenate at a rate of 1.5 nmol/min/g of fresh wt (for the first minute) at 37 °C and pH 6.5. The generation of H_2O_2 decreased signicantly after 30 min of incubation. Similar results were obtained by the incubation of light muscle tissue under the same conditions.

The incubation of dark muscle homogenate at 60 °C increased the rate of H_2O_2 generation. Azide inhibition of formate oxidation at high temperature showed that the oxidation of formate is produced by catalase and not affected by the heating itself (Figure 2).

Increasing the incubation temperature to 100 °C permitted the accumulation of 24 nmol/g of fresh wt of H_2O_2 for the first 10 min, but after this time catalase was completely inhibited (results not shown) and this also inhibited formate oxidation. Heating of [^{14}C]formate does not elaborate $^{14}CO_2$.

Muscle tissues were aging at 4 °C for a period of 5 days, and the generation of H_2O_2 was determined. The results in Figure 3 show an increase in H_2O_2 production of almost 2.3-fold during a storage of 5 days.

The formation of hydrogen peroxide by muscle tissues could be generated by the activity of a few enzymes located in mitochondria, peroxisomes as well as by cytosolic enzymes (Loschen et al., 1971; Boveris et al., 1972; Chance et al., 1979). However, the increased production of H_2O_2 at high temperature indicated that in muscle tissues a large

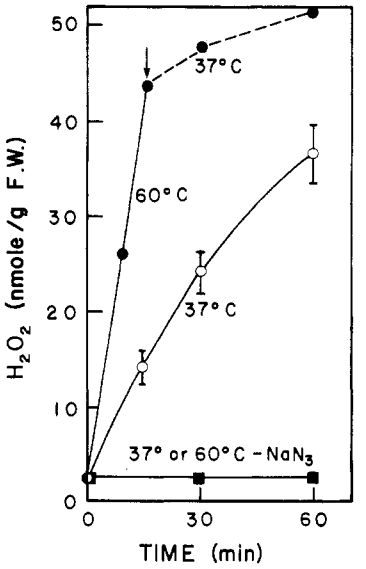

Figure 2. Generation of H_2O_2 by dark muscle incubated at 37 and 60 °C in the presence of added catalase (425 U): ↓, cooled to 37 °C and catalase added once more; ■, in the presence of added catalase and sodium azide (1 mM).

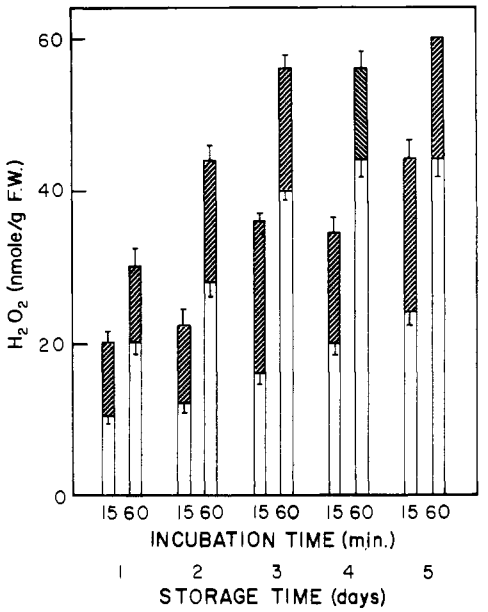

Figure 3. The effect of storage time on H_2O_2 generation by incubated dark muscle at 37 °C: □, without added catalase; ▨, with added catalase.

part of H_2O_2 is generated by a non-enzymatic reaction. A non-enzymatic reaction which could generate H_2O_2 in muscle tissues is the oxidation of oxymyoglobin and oxyhemoglobin. It was reported by several researchers that the oxidation of oxymyoglobin and oxyhemoglobin led to the production of superoxide radical and H_2O_2 (Mirsa and Fridovich, 1972; Satoh and Shikama, 1981; Wallace et al., 1982). High temperature was found to increase the autoxidation of oxymyoglobin and oxyhemoglobin (Brown and Mebine, 1969; Wallace et al., 1982) and the formation of metmyoglobin in muscle foods (Brown and Dolev, 1963; Snyder and Skrdlant, 1966; Giddings, 1977).

The cytotoxic activity of H_2O_2 in biological tissues is due especially to the capacity of this compound to be reduced to the hydroxyl radical (Walling, 1975; Kong and Davison, 1980; Rowley and Halliwell, 1983). This very active free radical could initiate oxidation of lipids and other biomolecules (Willson, 1979). Hydroxyl radicals also oxidize formate, however we had increased formate oxidation by

about 2-fold with the addition of exogenous catalase. These results indicate that most of the formate oxidation is via the H_2O_2/catalase complex (compound I) and not by HO· radicals.

Most recently, we found that H_2O_2 activates metmyoglobin, probably to a porphyrin cation radical which initiates lipid peroxidation (Harel and Kanner, 1985). The oxidation of unsaturated fatty acids in muscle foods is one of the major causes of quality deterioration. It seems possible that endogenous generation of H_2O_2 could play an important role in the formation of the primary pool of radicals which initiate lipid peroxidation in muscle tissues.

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Muscle Membranal Lipid Peroxidation Initiated by H_2O_2-Activated Metmyoglobin

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Metmyoglobin (MetMb), H_2O_2-activated by glucose-oxidase/glucose system, initiated membranal lipid peroxidation. No such peroxidation occurred in the presence of the glucose oxidase system, H_2O_2, or MetMb alone. Heated MetMb maintained its capacity to be activated by H_2O_2. The accumulation of thiobarbituric reactive substances (TBA-RS) and oxygen absorption showed a higher rate of lipid peroxidation by H_2O_2-activated MetMb in microsomes separated from turkey than from chicken muscle tissues. Membranal lipid peroxidation initiated by activated MetMb was inhibited by low concentrations of either ascorbyl palmitate, α-tocopherol, or butylated hydroxytoluene (BHT). Inhibition was also observed by very low concentrations of ascorbic acid in the presence of EDTA. Only very high concentration of EDTA (1–10 mM) inhibited significantly membranal lipid peroxidation by activated metmyoglobin.

INTRODUCTION

The problem of lipid peroxidation in food systems is of significant importance in maintaining a stable food supply.

With modern techniques of food formulation and the production of structured food products, the problem of lipid peroxidation has increased in importance (Pearson et al., 1983). The use of mechanically deboned muscle tissues in the production of many of the restructured food products offers an entirely new approach to the development of food products. However, minced tissue is more readily subject to lipid peroxidation changes, either due to the disruption of the normal structure of the cells, or to the incorporation of more oxygen in the tissues.

Oxygen is vital for many purposes in biological systems and to the process of lipid peroxidation. The use of oxygen by biological matter is by controlled reactions. However, following food processing, such reactions can no longer be controlled. The electronic structure of oxygen, which reflects the Pauli principle, has two unpaired electrons at the energy level of π antibonding in triplet state $^3\Delta g$. The reaction of oxygen, therefore, is spin forbidden, with ground-state molecules of singlet multiplicity, such as polyunsaturated fatty acids (PUFA) or other molecules, in the same state. This barrier does not apply to reactions with atoms or molecules containing unpaired electrons, such as transition metals and free radicals. With regard

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