Some Aspects of Fe^{2+}-Catalyzed Oxidation of Fish Sarcoplasmic Reticular Lipid

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The purpose of the work was to determine the role of ionic iron in lipid oxidation of isolated fish sarcoplasmic reticulum (SR) as a model for stored, ground fish muscle. Exogenous Fe^{3+} had only a minor role. Increasing concentrations of Fe^{2+} led to a greater extent of lipid oxidation, a more rapid maximal rate, but an increased lag time. It is suggested that the last occurs because Fe^{2+} functions as an antioxidant and reduces membrane antioxidant radicals to their active form. This conclusion is based on the observation that elimination of the antioxidative capacity of the membrane by storage or prior reaction eliminates the lag phase over a range of Fe^{2+} concentrations and Fe^{2+}/Fe^{3+} ratios. The rapid rate of Fe^{2+} oxidation in the presence of membrane compared to that in its absence suggests that the oxidation is driven by previously formed lipid peroxides reacting with Fe^{2+}.

INTRODUCTION

The quality deterioration that accompanies lipid oxidation is a problem with many food products, including those derived from muscle tissue. It is a vexing problem in fish due to the highly unsaturated nature of the fatty acids of fish muscle.

It is probable that the fatty acids of fish membrane lipids are a particular problem. The polar membrane lipids have a higher content of the highly polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), than do the triacylglycerols (Shewfelt, 1981). Fish muscle membranes have enzymic systems capable of transferring electrons from NADH to ferric iron to reduce it to ferrous iron (McDonald and Hultin, 1987; Luo, 1987), which may participate in the lipid oxidation reaction. Further, as in any heterogeneous system, the amount of lipid substrate available for oxidation by catalysts in the aqueous phase is proportional to the surface area exposed to the aqueous phase (Deems *et al.*, 1975). Since most all of the lipid in membrane exists as a bilayer, the surface area per unit weight of the lipid is much greater than that of the fat droplets which occur both intra- and intercellularly in fish muscle and contain primarily neutral lipids (Greene, 1913; Ke *et al.*, 1977; Howgate, 1979). Water-soluble lipid oxidation catalysts such as chelated non-heme iron, heme pigments, or ascorbate should also have a greater facility for interacting with the polar/charged surface presented by the polar lipids. For these reasons, it is believed that lipid oxidation of membrane lipids in stored fish muscle is more rapid than that occurring in the neutral lipids found in fat cells or dispersed as droplets inside or outside the muscle fibers.

Lipid oxidation in muscle membranes is a serious problem in minced tissue. Molecular oxygen is incorporated into the tissue at a much higher level than is found in intact post-mortem muscle, where oxygen penetration is generally limited to the top 1–4 mm (Lawrie, 1974). The loss of compartmentation in the muscle cell may also accelerate lipid oxidation by allowing pro-oxidants more ready access to sensitive sites. On the other hand, there is an opportunity to manipulate conditions in minced muscle tissue that cannot be easily done in intact tissue. For example, oxidizing or reducing agents could be added to maintain tissue iron in either the ferrous (2+) or ferric (3+) state.

In recent years it has been suggested that, in some systems and under some conditions, an optimal ratio of ferrous to ferric iron is required for maximal stimulation of lipid oxidation, perhaps through some type of a $Fe^{2+}/Fe^{3+}/O_2$ complex (Bucher *et al.*, 1983; Minotti *et al.*, 1987; Braughler *et al.*, 1987). This suggestion has more than a passing interest for prevention of lipid oxidation in stored minced fish muscle since it may be relatively easy to maintain the iron in either the 2+ or the 3+ state. If in fact an optimal ratio (suggested to be about 1) of Fe^{2+} to Fe^{3+} is required for lipid oxidation, maintaining the iron totally in either the 2+ or 3+ state should reduce lipid oxidation where non-heme iron is an important pro-oxidant. Since it is likely that the iron in heme is active in the oxidized state, maintaining both heme and non-heme iron in reduced form would inhibit lipid oxidation. On the other hand, it has been suggested that ionic iron is active in the ferrous state (Kanner *et al.*, 1987). If this hypothesis is correct, it would be difficult to inhibit both heme- and non-heme-iron-catalyzed lipid oxidation by manipulation of the redox potential of the system since heme iron would need to be maintained in the reduced state (Kanner *et al.*, 1987) and non-heme iron in the oxidized state.

A determination of the role of iron in oxidation of fish muscle membrane lipid could thus be useful in devising a procedure to minimize lipid oxidation in stored fish muscle, especially if it was minced. The sarcoplasmic reticulum (SR) was chosen because it is the major membrane in light or ordinary fish muscle tissue and presents a simpler system to evaluate than the other major fish muscle membrane system, mitochondria. The effect of ferrous and ferric iron and their combinations was evaluated on isolated cod SR that was not protected from

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oxidation during preparation to make it similar to SR in minced post-mortem fish muscle.

MATERIALS AND METHODS

Materials. Atlantic cod (*Gadus morhua*) was obtained from fish distributors in Gloucester, MA. Adenosine 5'-diphosphate (ADP) and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bathophenanthroline was purchased from Sigma Chemical Co. (St. Louis, MO). Coomassie Blue protein assay reagent was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Preparation of Sarcoplasmic Reticulum. Sarcoplasmic reticulum (SR) was isolated from the muscle of Atlantic cod (*G. morhua*) using the method of Borhan *et al.* (1984) except that the minced muscle was homogenized three times at 30-s intervals, the pH of the homogenate was adjusted to 7.3, the ratio of supernatant/20% sucrose/45% sucrose used for the discontinuous sucrose gradient was 50:9:3, and the gradient was centrifuged using a Type 35 rotor at 33 000 rpm (130000*g*) in a Beckman L5-65B Ultracentrifuge for 60 min at 6 °C. One hundred parts per million of streptomycin was added to SR preparations used for aging studies. The protein concentration of the SR preparation was determined according to the method of Bradford using a commercial Coomassie Blue G-250 protein binding agent from Pierce (Bradford, 1976; Pierce Chemical Co., 1989).

Lipid Oxidation Measured by Thiobarbituric Acid-Reactive Substances (TBARS). Standard assays for lipid oxidation were performed by first mixing equimolar iron and ADP followed by 5 mM histidine, pH 6.8, and 0.01 mg of SR protein/mL. Freshly prepared ferrous or ferric iron or combinations of both iron species were used in the experiments. The reaction mixture was maintained at 6 °C for all of the lipid oxidation assays throughout this study.

The extent of lipid oxidation was measured by TBARS using the method described by McDonald and Hultin (1987). TBARS is expressed as nanomoles of malondialdehyde per milligram of SR protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 532 nm.

Continuous Measurement of Lipid Oxidation. Lipid oxidation was initiated by adding an aliquot of the mixture containing equimolar $FeCl_2$ and ADP and 5 mM histidine into a cuvette to which SR had been previously added. The final SR concentration was either 0.01 or 0.1 mg of protein/mL. The reaction medium was scanned against a reference at 274 nm for specified time periods in a Hitachi U-3110 spectrophotometer equipped with a temperature control device. The reference cuvette contained the same mixture without $FeCl_2$.

To determine that the changes observed at 274 nm were due to changes in the lipid fraction, SR was oxidized in a reaction medium containing 30 μM $FeCl_2$, 30 μM ADP, 5 mM histidine, pH 6.8, and 3 mg of SR protein/mL for 20 min. The lipid of the oxidized SR was extracted following the method of Bligh and Dyer (1959) as modified by Palmer (1971) except that 40 mL of the SR medium was substituted for 50 g of muscle. The solvent was removed by evaporation under vacuum at room temperature and the lipid resolubilized in methylene chloride. The spectrum of the lipid was obtained in a Hitachi U-3110 spectrophotometer.

Determination of Lipid Hydroperoxides. Lipid hydroperoxides in the SR were determined by the iodometric assay described by Buege and Aust (1978). The peroxide value was calculated using a molar extinction coefficient of 1.73×10^4 M^{-1} cm^{-1}.

Determination of Ferrous Iron Oxidation. The oxidation of ferrous iron in the presence of SR was assayed according to the method of Aust *et al.* (1990). At specified time intervals, aliquots of 2.8 mL of the reaction mixture containing $FeCl_2$, ADP, histidine, and SR were mixed with 0.2 mL of acetate buffer and 3 mL of bathophenanthroline (batho) in 95% ethyl alcohol. The Fe^{2+}–batho was extracted into 2 mL of chloroform. The samples were centrifuged at 500*g* for 5 min. The chloroform layer was transferred to a 5-mL volumetric flask, and the volume was made up with 95% ethyl alcohol. The absorbance was read at 533 nm (Lee and Clydesdale, 1979). Standard curves were constructed with 5, 10, and 20 μM $FeCl_2$ for each trial. For easy comparison with oxidation of lipid, Fe^{2+} concentrations from assays were subtracted from the initial Fe^{2+} concentration to obtain the amount of Fe^{3+} in the assay mixtures.

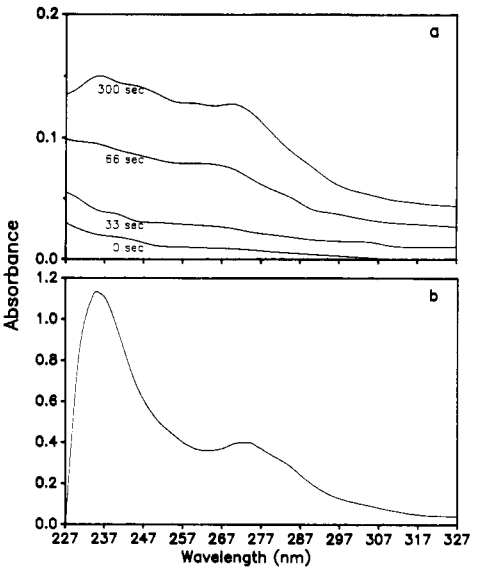

Figure 1. (a) Differential scanning for Fe^{+2}-induced lipid oxidation in fish sarcoplasmic reticulum at different time intervals on the same sample. The reaction medium containing 10 μM Fe^{2+} and ADP, 5 mM Pipes, pH 6.8, and 0.01 mg of SR protein/mL was scanned against a reference in which Fe^{3+} was substituted for Fe^{2+}. The scanning speed was 800 nm/min. (b) Spectrum of isolated SR lipid.

Determination of Membrane-Bound Iron. Reaction medium containing 20 μM $FeCl_2$, 20 μM ADP, 5 mM histidine, pH 6.8, and 0.01 or 0.1 mg of SR protein/mL was incubated for 20 min at 6 °C followed by centrifugation at 130000*g* for 60 min. The resulting pellet was washed in deionized distilled water and centrifuged again. The final iron-enriched SR was resuspended in histidine buffer and the protein concentration determined. Membrane-bound iron was determined in a Perkin-Elmer 3030B atomic absorption spectrophotometer equipped with a HGA-400 graphite furnace (Osinchak *et al.*, 1992). The suspension of membrane-bound iron was directly injected onto a L'vov platform in a pyrolytically coated graphite tube. Pyrolysis and atomization took place in that graphite tube. Pyrolysis temperature was 1400 °C with a 10-s ramp time and a 20-s hold. Atomization was at 2400 °C with no ramp time and a 5-s hold.

Tocopherol Determination. Tocopherol content in SR lipids was determined by high-performance liquid chromatography (HPLC) with an ultraviolet absorbance detector (Carpenter, 1979). Membrane lipids were extracted from either fresh or aged SR by the modified method of Bligh and Dyer (1959) as mentioned in the previous section. The lipid was separated from solvent by evaporation under vacuum and resolubilized in 1.5% isopropyl alcohol in hexane. The separation system contained a 4.6 mm × 250 mm Microsorb silica column with 5 μm particle size and 100-Å pore size from Rainin Instrument Co., a Du Pont 870 HPLC pump module, a Waters 484 absorbance detector, and a HP integrator. The mobile phase was 1.5% isopropyl alcohol in hexane at a flow rate of 1 mL/min. A 20-μL injection loop was used.

RESULTS

Since a continuous measurement of lipid oxidation would be useful in following the changes induced by iron, the possibility of following lipid oxidation by change in absorption due to conjugated dienes or trienes was evaluated. Difference spectra with time of peroxidizing fish SR are shown in Figure 1a. To determine that the increase in absorption was due to a change in conjugated diene or conjugated triene content, the lipid was extracted and absorption maxima were obtained (Figure 1b). Absorption maxima were found at 274 and 234 nm, values

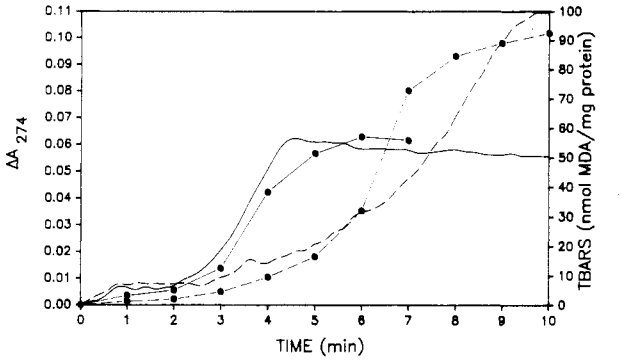

Figure 2. Fe^{2+}-induced lipid oxidation measured as TBARS (●—● 10 μM Fe^{2+}, ●- - -● 20 μM Fe^{2+}) and absorbance at 274 nm (— 10 μM Fe^{2+}, - - - 20 μM Fe^{2+}). Reaction conditions were as described in Figure 1a except 5 mM histidine was used instead of Pipes.

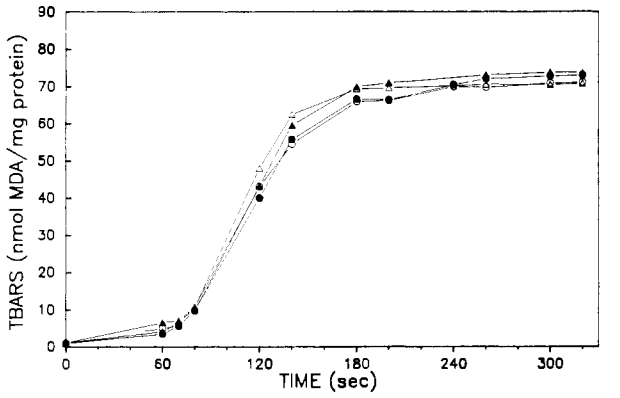

Figure 3. Lipid oxidation catalyzed by 10 μM Fe^{2+} plus 0–20 μM Fe^{3+}. The incubation media contained 10 μM Fe^{2+}, Fe^{3+} (○ 0 μM; ●, 5 μM; △, 10 μM; ▲, 20 μM), ADP (equimolar to total Fe), 5 mM histidine, pH 6.8, and 0.01 mg of SR protein/mL.

typical of conjugated trienes and dienes, respectively (Kates, 1972; Brown, 1980). For continuous assay of lipid oxidation, absorbance at 274 nm was chosen since histidine at the concentration used strongly absorbed in the 234-nm region. The absorbance of histidine at 5 mM was so high that insufficient energy could be transmitted through the suspension. For that reason the reaction in Figure 1a had 5 mM Pipes as buffer rather than the histidine used in the rest of the studies. In addition, histidine is a free-radical acceptor and may undergo oxidation. Oxidation of histidine could interfere with the measurement of conjugated diene formation at 234 nm. For these reasons absorbance at 274 nm was used to monitor lipid oxidation continuously.

A comparison was made between lipid oxidation measured by change in absorbance at 274 nm with a determination of TBARS at 10 and 20 μM ferrous iron concentrations (Figure 2). The kinetic patterns of oxidation of the two measurements follow similar time courses, indicating that ΔA_{274} is an acceptable substitute for TBARS as a measure of lipid oxidation in the sarcoplasmic reticulum. It is possible that components other than conjugated trienes contribute to ΔA_{274}, and results must be evaluated with this in mind. Formation of TBARS may also involve components other than those derived from lipid oxidation (Janero and Burghardt, 1988).

The rate of oxidation of SR lipids as measured by TBARS in the presence of 10 μM ferrous iron was not affected by ferric iron added over the concentration range 0–20 μM (Figure 3). This is in contrast to what was observed when ferric iron was held constant at 10 μM and ferrous iron was added over the concentration range 0–40 μM (Figure 4). The rate of oxidation of SR lipid in the presence of 10 μM ferric iron with no ferrous iron was negligible over the time period of these experiments.

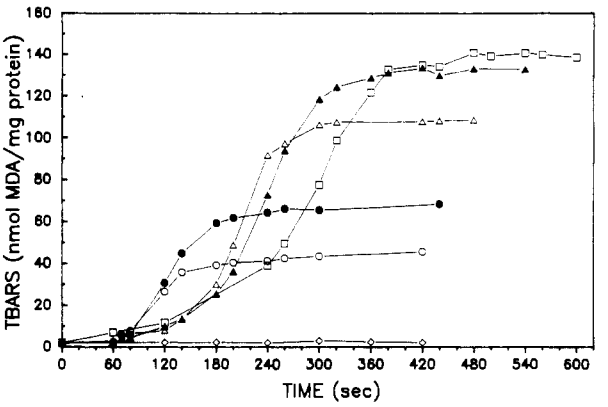

Figure 4. Lipid oxidation catalyzed by 10 μM Fe^{3+} plus 0–40 μM Fe^{2+}. The incubation media contained 10 μM Fe^{3+}, Fe^{2+} (◇, 0 μM, ○, 5 μM, ●, 10 μM, △, 20 μM, ▲, 30 μM, □, 40 μM), ADP (equimolar to total Fe), 5 mM histidine, pH 6.8, and 0.01 mg of SR protein/mL.

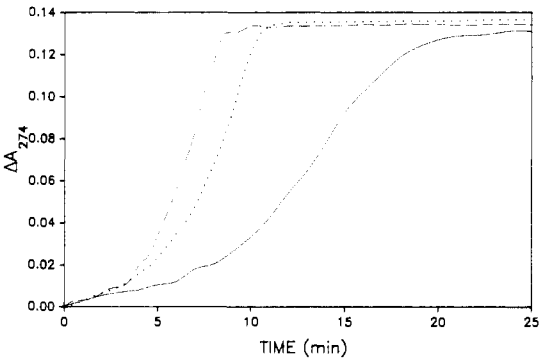

Figure 5. Fe^{2+}-induced lipid oxidation of SR prepared from fresh fish muscle and aged for 1 day (—), SR prepared from fresh fish muscle and aged for 5 days (· · ·), and SR prepared from fish muscle aged for 6 days post-mortem (- - -). Reaction conditions were as described in Figure 2.

The time course of the reaction follows typical lipid oxidation kinetics with a lag phase, an accelerated phase, and a third phase of greatly reduced increase in TBARS. The extent of oxidation where this slow phase was reached was related to the amount of ferrous iron that was added to the assay (correlation coefficient = 0.997 over the range of ferrous iron 5–20 μM; three experiments). Increases in the total amount of oxidation as measured by TBARS were not as great at the higher concentrations of ferrous iron as they were at concentrations up to 20 μM. There was an increase in lag time at the higher concentrations of ferrous iron, although the differences appeared to be greatest between two groups, those of ferrous iron concentrations of 10 μM and less and those of 20 μM and greater ($P < 0.05$, paired t-test).

When fish muscle was aged post-mortem before preparation of the SR or when SR prepared from fresh fish was allowed to age *in vitro* at 6 °C, the lag time of lipid oxidation decreased as measured by ΔA_{274} (Figure 5). It is possible that freshly prepared SR of recently killed fish contained a component which contributed to the lag phase but which was destroyed by post-mortem storage of the fish or the SR. To test for possible loss of this "antioxidant" during exposure to ferrous iron which leads to lipid oxidation, SR was incubated with 1 μM ferrous iron until well past the lag phase of the reaction. At that point additional ferrous iron was added in concentrations ranging from 1 to 40 μM.

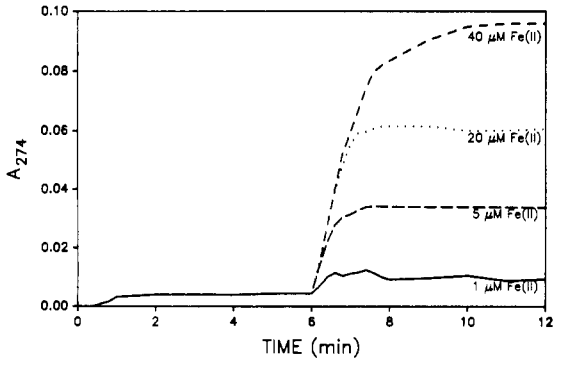

Figure 6. Lipid oxidation on readdition of Fe^{2+}. The first reaction medium contained 1 μM Fe^{2+} and ADP, 5 mM histidine, pH 6.8, and 0.01 mg of SR protein/mL. At the end of the reaction (6 min), additional Fe^{2+} and ADP were added to the reaction media to obtain the desired concentrations of Fe^{2+} and maintain ADP equimolar. Additional histidine was added to maintain a concentration of 5 mM.

Table I. Effect of Storage Time on Tocopherol Content of SR[a]

storage time, days	tocopherol, ng/mg of protein
1	1940
9	<2

[a] The SR preparation was stored at 6 °C in 5 mM histidine buffer, pH 6.8.

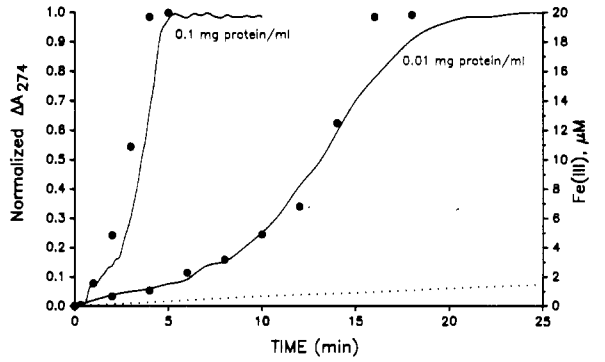

Figure 7. Lipid oxidation (—) and simultaneous Fe^{2+} oxidation (●). The value of 1.0 of normalized absorbance at 274 nm represents absorbances of 0.191 and 0.132 for the sample containing 0.1 and 0.01 mg of SR protein/mL, respectively. The dotted (· · ·) line is the autoxidation of 20 μM Fe^{2+} in the presence of equimolar ADP and 5 mM histidine.

No lag phase was observed upon this second addition of ferrous iron at any concentration (Figure 6). After the second addition of the ferrous iron, the extent of oxidation was again related to the amount of iron that was added. When isolated SR was stored at 6 °C in histidine buffer, pH 6.8, no detectible tocopherol was found in the membrane preparation after 9 days (Table I).

Rates of oxidation of SR lipid were determined at low (0.01 mg of SR protein/mL, the standard assay) and high (0.1 mg/mL) SR protein concentration. Oxidation of ferrous iron was also measured under these conditions (Figure 7). Oxidation of lipid is expressed as ΔA_{274} and shown as a continuous line, while the amount of ferric iron is shown as solid circles. The initial concentration of ferrous iron was 20 μM at both concentrations of SR protein. The rate of autoxidation of ferrous iron under the same conditions but without the SR is included (dotted line). Rapid oxidation of ferrous to ferric iron depends on the presence of the membrane. The extent of lipid oxidation observed was about 50% higher in the assay containing the higher concentration of SR protein. The accelerated phase of oxidation was considerably greater

Table II. Effect of SR Concentration on Adsorption of Iron[a]

mg of SR protein/mL	nmol of Fe/mg of SR protein
0.01	636 (32%)
0.1	114 (57%)

[a] SR was incubated with 20 μM $FeCl_2$, 20 μM ADP, and 5 mM histidine, pH 6.8, for 20 min. Percentages in parentheses indicate the amount of the total iron added that was bound. The original SR contained 0.6 nmol of iron/mg of SR protein.

and the lag phase was considerably shorter in the sample that had the higher protein concentration. Ferrous iron oxidation rather closely followed absorbance changes at 274 nm at the low concentration of SR protein, whereas it slightly preceded ΔA_{274} at the higher concentration of SR. In Figure 7 the relative oxidative activities with respect to absorbance changes at 274 nm are adjusted to fit on the same scale so that their relationship to ferrous iron oxidation could be readily seen for the two samples. Since iron content was not changed when the SR protein content was increased 10-fold, iron oxidation cannot be compared on the same scale as ΔA_{274} for both samples without normalization of the values.

The iron content of the sarcoplasmic reticulum was measured after the assays described in Figure 7 (Table II). Fifty-seven percent of the total iron added was bound to the membrane at the concentration of 0.1 mg of SR protein/mL, whereas 32% of the total iron was bound at a protein concentration of 0.01 mg of SR protein/mL. The content of bound iron, however, calculated on a milligram of protein basis, was some 5.5-fold greater at the lower SR protein concentration.

DISCUSSION

Lipid oxidation of fish SR was greatly dependent on the concentration of ferrous iron with respect to lag phase, extent of oxidation after the rapid phase of lipid oxidation was completed, and, to a lesser degree, the maximal oxidation rate (Figure 3). This effect was observed in the presence or absence of ferric iron. Although the maximal rate of lipid oxidation and the total oxidation increased with increasing ferrous iron concentrations, surprisingly the lag phase also increased with higher concentrations of ferrous iron.

If an initial oxidation with a low concentration of iron was allowed to go to completion, no lag phase was observed when a second dose of Fe^{2+} was added. A similar result had been reported by Zollner *et al.* (1990). The response of the system to the second addition of Fe^{2+} was immediate, and the time to reach the maximal rate did not depend on the total amount of Fe^{2+} added. Thus, it did not depend on the ratio of Fe^{2+}/Fe^{3+} in the system. As was observed when the ferrous iron concentration was varied, both the maximal rate of oxidation and the extent of the oxidation increased with increasing Fe^{2+} concentrations.

It appears to be likely that in the cod muscle sarcoplasmic reticulum system studied, the effect of various ferrous iron concentrations on the lag phase of lipid oxidation relates to some change in the membrane rather than to the Fe^{2+}/Fe^{3+} ratio. Evidence for this was the observation that when the fish muscle tissue from which the SR was prepared was aged post-mortem or when the isolated SR itself was stored, the lag phase observed at any given concentration of Fe^{2+} decreased relative to that observed with freshly prepared SR obtained from fresh muscle tissue. There are at least two processes that could contribute to the change in the SR membrane that could account for these observations. The first is a decrease in

the antioxidative potential of the membrane. A loss of α-tocopherol was observed in this study when the SR membrane was aged for 9 days. It is possible that this component may have decreased soon enough post-mortem to have contributed to the decrease in lag phase. Takenaka *et al.* (1991) found that a decrease in arachidonic acid and an increase in TBARS formation were suppressed in membrane lipids until the tocopherol content in the membrane fell below a critical level. Changes in sulfhydryl groups could also contribute to an antioxidative effect (Takenaka *et al.*, 1991).

A buildup of lipid hydroperoxides could also shorten the lag phase. The extent of oxidation that occurred during the rapid rate of reaction was roughly linearly related to the amount of ferrous iron up to a concentration of about 20 μM. In addition, at both 10 and 20 μM the loss of measurable ferrous iron occurred at the point where the rapid phase of lipid oxidation ceased. Thus, it seems to be likely that Fe^{2+} played a critical role in the oxidation process. However, the rate of autoxidation of Fe^{2+} was slow compared to its oxidation in the presence of the SR (Figure 7). Clearly, some component in the membrane must have contributed to this rapid oxidation of Fe^{2+}. It is likely that lipid hydroperoxides were the components responsible for the rapid ferrous iron oxidation; the reaction of ferrous iron with lipid hydroperoxides is known to be very rapid (Halliwell and Gutteridge, 1989). The low rate of lipid oxidation that occurs after the rapid phase concludes could be the result of chain reaction processes or be dependent on a slow reduction of ferric to ferrous iron by reducing substances in the membrane such as sulfhydryl groups.

Typically, freshly prepared SR from fresh cod muscle had a lipid hydroperoxide content of approximately 60 nmol/mg of membrane protein. Since this is only a small fraction of the amount of ferrous iron oxidized, e.g., 3% of the moles of iron oxidized when 20 μM Fe^{2+} was used, the role of the lipid hydroperoxide must be to stimulate lipid oxidation by reaction with Fe^{2+}. Lipid hydroperoxides could be formed in the living animal, during the post-mortem handling period, or during the preparation of the SR membrane itself. Although the autoxidation of Fe^{2+} by molecular oxygen was slow compared to the reaction in the presence of SR (Figure 7), it was not negligible. After 2.5 h, approximately 30% of a 20 μM solution of Fe^{2+} was oxidized, and 50% was oxidized after 5 h. Ferrous iron oxidation by molecular oxygen could produce superoxide; the latter could dismutate to hydrogen peroxide which would be converted to hydroxyl radical by Fe^{2+}-catalyzed Fenton reaction. The hydroxyl radical could, in turn, initiate oxidation of membrane lipids, producing lipid hydroperoxides. Thus, ferrous iron contact with molecular oxygen and the membrane lipids in the living fish or during preparation of the membrane could result in production of lipid hydroperoxides. Recently, it has been suggested that chelated iron could initiate oxidation of linoleic acid emulsified with Tween 20 via perferryl ions or chelator–iron–oxygen complexes (Yin *et al.*, 1992).

Increasing concentrations of ferrous iron increased the lag phase of membrane lipid oxidation (Figure 4). This indicates that the ferrous iron is acting as an antioxidant in the system. It is possible that Fe^{2+} is doing this by reducing antioxidant free radicals to their active form

$$R^{\cdot} + TOH \rightarrow RH + TO^{\cdot}$$

$$Fe^{2+} + H^{+} + TO^{\cdot} \leftrightarrow Fe^{3+} + TOH$$

where R· represents any radical and TOH represents the antioxidants of the membrane; tocopherol is probably the principal one. The reaction involving iron and tocopherol is probably reversible (Fukuzawa, 1987) and is dependent on the relative concentrations of antioxidant and ferrous and ferric ions. At high concentrations of ferrous iron, reduction of the antioxidant free radical, TO·, is favored. As long as ferrous iron was present and had access to the tocopheryl radical, this radical could be reduced and could scavenge other radicals, such as those formed from unsaturated fatty acids in the membrane. This continuous cycling of tocopherol between reduced and free-radical forms could continue until the ferrous iron was completely oxidized to the ferric iron. The tocopheryl radical would then be oxidized to its quinone form on interaction with another free radical.

$$R^{\cdot} + TO^{\cdot} + H^{+} \rightarrow T{=}O + RH$$

Alternatively, the ferrous iron could reduce lipid radicals such as LOO· (Schaich, 1992). The anion produced would form lipid hydroperoxide, which would be subject to breakdown by other ferrous ions, hastening the oxidative process.

The lag phase was reduced and the maximal rate of ΔA_{274} was increased when the ferrous iron concentration was kept constant but the content of sarcoplasmic reticulum in the assay was increased (Figure 7). Both of these phenomena could be the result of the greater amount of lipid hydroperoxides associated with the increased SR. Lipid free radicals would be produced initially at a faster rate due to the higher concentration of lipid hydroperoxides causing a more rapid increase in ΔA_{274}. The lipid free radicals would produce more tocopheryl free radicals. Both the initial production of lipid free radicals from lipid hydroperoxides and the reduction of tocopheryl free radicals consume ferrous iron, shortening the time during which ferrous iron can function as an antioxidant; this would shorten the lag phase.

Yamamoto and Niki (1988) demonstrated that α-tocopherol incorporated into artificial liposomes rapidly reduced ferric to ferrous iron. They also observed that the α-tocopherol in intact erythrocyte membranes did not reduce ferric iron in the aqueous phase. Stoyanovsky *et al.* (1989) studied the interaction of ferrous iron with α-tocopherol incorporated into phospholipid liposomes. They showed that the presence of tocopherol increased the amount of iron associated with the liposomes, and they suggested that a tocopherol–iron complex was formed. Preferential binding of the ferrous iron to tocopherol or to membrane proteins located near tocopherol would greatly favor bound iron serving as antioxidant for reducing tocopheryl radicals. It is possible that the interaction of membrane antioxidant and ferrous iron under our reaction conditions occurs preferentially with bound iron. It has been demonstrated previously that increasing concentrations of iron in a suspension of cod muscle SR caused more iron to be bound to the membrane fraction (Huang and Hultin, 1992). In this paper, we demonstrated that the total amount of iron bound to the membrane at the end of an oxidative assay is greater when a higher concentration of SR protein is examined (Table II). Iron bound at or near the membrane lipid hydroperoxides and antioxidants such as tocopherol could facilitate bound iron serving as a decomposer of lipid hydroperoxides and an antioxidant for reducing tocopheryl or lipid radicals.

The reactivity of iron depends on the components to which it is chelated. The amount of low molecular weight iron in fish muscle tissue is about 10% of the total soluble

iron (Decker and Hultin, 1990). Nucleotides, amino acids, and organic acids are all potential chelators for iron in this tissue. ATP has been suggested to be a major binder of iron in biological systems (Weaver *et al.*, 1990). In postmortem fish muscle ATP is degraded rapidly through a series of nucleotides (Mulchandani *et al.*, 1990). ADP would be a major nucleotide present in the first few hours after the death of the animal. Free histidine is present in high concentrations in some fish muscles, e.g., up to 100 mM in some tuna species and 50 mM in mackerel (Ikeda, 1980). This, along with its highly stimulatory activity in the enzyme-catalyzed system, has led us to use this amino acid as a chelator as well as a buffer for membrane lipid oxidation studies.

In this study we used a mixture of ADP and histidine, which on the basis of kinetic results, has been suggested to form a 1:1:1 complex with ferric iron (Erickson *et al.*, 1990; Erickson and Hultin, 1992). This complex is highly stimulatory toward the enzymic lipid oxidation system of fish SR whereby ferric iron is reduced by NADH. In the enzymic system, stimulation by ADP is maximal at equimolar concentrations to the iron, while histidine continuously stimulates oxidation to a concentration of at least 100 mM. At the concentrations used in the experiments reported here, equimolar ADP and 5 mM histidine would have a slight inhibitory effect on nonenzymic lipid oxidation (Erickson and Hultin, 1992).

Identification of the actual chelator compounds in postmortem muscle tissue will be an important step in evaluating the role of iron *in situ* in lipid oxidation processes. Unfortunately, this has not yet been accomplished.

Our process of preparing fish muscle sarcoplasmic reticulum is not unlike the conditions to which the membrane would be subjected in the production of food products made from minced fish muscle tissue. During grinding, oxygen is incorporated into the tissue, cellular structural barriers are destroyed, and pro-oxidants come into close contact with unsaturated fatty acid molecules. The highly unsaturated nature of the fatty acids of fish muscle sarcoplasmic reticulum makes them particularly sensitive to oxidative reactions. This work has suggested that ferrous iron can act as a pro-oxidant by decomposing lipid hydroperoxides and as an antioxidant by increasing the lag period, probably by ferrous iron reduction of antioxidant and lipid free radicals. The concentrations of ferrous iron that showed this ability to serve as an antioxidant were high compared to those found in fish muscle.

It might be expected that decomposition of peroxides is the major effect of ferrous iron in post-mortem fish muscle *in situ*, and it would be prudent to maintain iron in the ferric form if possible. In our system, neither Fe^{3+} nor the Fe^{2+}/Fe^{3+} ratio played a significant role in lipid oxidation.

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