Kinetics of Oxidation of the Lipids and Proteins of Cod Sarcoplasmic Reticulum

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Lipid and protein oxidation in an NADH−Fe enzymic and an ascorbate−Fe nonenzymic system were determined simultaneously. The nonenzymic iron-reducing system gave rapid oxidation of lipid that leveled off at values much lower than those achieved in the enzymic system, which showed a continuous increase over the 1 or 2 h incubation times used. Protein sulfhydryl oxidation was more rapid in the nonenzymic system for total and accessible sulfhydryl groups, but the enzymic system oxidized the inaccessible sulfhydryl groups more rapidly. Both lipid and protein oxidations appeared to begin simultaneously. In the enzymic system, more lipid oxidation was achieved on a molar basis than oxidation of protein sulfhydryl groups, while in the nonenzymic system this was reversed. These data probably reflect the site specificity of the production of oxidizing elements in the two systems. The greater lipid oxidation in the enzymic system suggests that this may be the more important ferric iron-reducing system during storage of fish muscle.

Keywords: *Cod muscle; sarcoplasmic reticulum; oxidation of lipids and proteins; lipid oxidation; protein oxidation; membrane oxidation*

INTRODUCTION

It has been widely demonstrated that biological membrane components undergo oxidations both in vivo and in vitro that can modify their structure and function (Arstila et al., 1972; Astier et al., 1996). Much of the damage is due to reactive oxygen species (Burlando et al., 1997) that are generated during electron transport operations in most biological membranes (Ramasarma, 1982). Many endoplasmic and sarcoplasmic reticular systems have an NAD(P)H-dependent system for reducing ferric iron to the reactive ferrous iron (Lin and Hultin, 1976; Slabyj and Hultin, 1982; McDonald and Hultin, 1987; Han and Liston, 1989; Eun et al., 1992; Tampo and Yonaha, 1995). Kagan et al. (1985) ascribed the reduction to an NADPH−cytochrome P-450 reductase in the microsomal fraction from liver, while Tampo and Yonaha (1995) suggested that it was a heat-labile component that was probably different from cytochrome P-450. Han and Liston (1989) suggested that the active component in the microsomes of rainbow trout skeletal muscle was NADH−cytochrome b_5 reductase. Membrane preparations from homeothermic organisms are more active in the presence of NADPH than NADH (Lin and Hultin, 1976; Rashba-Step et al., 1993). The fish muscle systems that have been examined to date show that the iron-reducing system has a strong preference for NADH over NADPH (McDonald et al., 1979; Han and Liston, 1989; Hultin, 1992; Eun et al., 1992). Unpublished work in our laboratory indicates that fish liver microsomes have the same preference for NADH as do the membrane systems of fish muscle.

Other systems besides the NAD(P)H-driven ferric iron reductase can induce oxidations, usually measured in lipids, in membrane systems. The xanthine oxidase, xanthine system generates hydrogen peroxide and superoxide radicals, which in the presence of iron ions can produce the highly reactive hydroxyl radical (Srinivasan and Hultin, 1995). Low molecular weight iron chelates in the presence of a reducing substance such as ascorbate with or without hydrogen peroxide have also been used to generate reactive oxygen species (Halliwell and Gutteridge, 1989). Heme proteins can also catalyze this oxidation (Kanner et al., 1987).

In biological systems, protein oxidation may occur more rapidly than lipid oxidation (Davies and Goldberg, 1987; Srinivasan and Hultin, 1995). The protein is in the aqueous phase where many of the radicals are formed. Also, catalysts such as ferrous iron may bind to the protein, allowing radicals to be formed near sensitive protein side chains. Modification of the proteins of muscle sarcoplasmic reticulum may affect its ability to function. Using a mixed system of enzymic and nonenzymic catalysts of lipid peroxidation in vitro, it was demonstrated that the sarcoplasmic reticulum of winter flounder muscle lost its ability to take up calcium ions in the presence of ATP; concomitant with this loss of calcium-accumulating activity, there was a decrease in the Ca^{2+}, Mg^{2+}-ATPase protein and the formation of two high molecular weight polypeptides (Luo and Hultin, 1986a). The same high molecular weight polypeptides were present in higher concentrations in the sarcoplasmic reticulum of old fish as compared to young fish (Luo and Hultin, 1986b). Kukreja et al. (1988) generated oxygen radicals and found that a loss in Ca^{2+}-

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ATPase activity was accompanied by the formation of thiobarbituric acid-reactive substances (TBARS).

The loss of Ca^{2+}-ATPase activity as well as polymerization of the proteins indicates that proteins of the membranes are susceptible to oxidation. The loss of the ability to sequester calcium may have ramifications in post mortem storage. However, the oxidation of membrane lipids may have greater significance from the point of view of storage stability due to development of off-odors and flavors. It has been reported that the polar phospholipids of the membrane are more susceptible to oxidation than the neutral triacylglycerols (Hultin, 1994; Gandemer and Meynier, 1995; Gray et al., 1996). Membrane phospholipids have a higher content of the highly polyunsaturated fatty acids than do the neutral oils. Since the membrane phospholipids exist primarily in the form of a bilayer, there is a much larger surface area exposed per unit weight of lipid. It can be estimated that on equal wet weight basis the area of the phospholipids in contact with the aqueous phase is some 2 orders of magnitude greater than that of the neutral oils, which exist primarily as inter- and intracellular fat droplets. The membrane lipid fatty acids are also in close juxtaposition with the electron transport systems of the membranes with which they may directly interact. Alternatively, the production of reactive oxygen species by these electron transport systems near the site of membrane catalysts such as heme and nonheme iron proteins ensures that reactive radical species are produced near the highly oxidizable lipids. The proteins themselves may be attacked by these radicals. Protein radicals could react with susceptible lipids enhancing the rate of lipid oxidation. They could also serve to scavenge free radicals and thus have an antioxidant function with respect to the lipids.

The purpose of the work reported here was to determine the kinetics of both lipid and protein oxidation in the nonenzymic ascorbate−iron-driven system and the enzymic NADH−iron-driven system. The function of both systems is to reduce ferric iron to the reactive ferrous.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum. Cod fish (*Gadus morhua*) were obtained from day boats in Gloucester, MA, and were immediately iced, filleted, skinned, and minced. Sarcoplasmic reticulum was isolated from cod muscle using the method of Borhan et al. (1984) as modified by Decker et al. (1989). The final sarcoplasmic reticulum sediment was suspended in 0.12 M KCl and 5 mM histidine (pH 6.8) buffer at a protein concentration of 6.57 mg/mL, with a Potter homogenizer, and stored at −40 °C for no longer than one week.

Analytical Procedures. Moisture content was determined by drying to constant weight in an infrared oven. Lipid was extracted from the sarcoplasmic reticulum preparation or the muscle by the method of Lee et al. (1996) using a 1:2 (v/v) chloroform/methanol mixture. The protein concentration of the muscle was determined using the biuret method of Torten and Whitaker (1964). The protein concentration of the sarcoplasmic reticular suspension was determined using the bicinchoninic acid (BCA) method of Smith et al. (1985) using the assay reagent kit and a bovine serum albumin standard from Pierce Chemical Co. (Rockford, IL).

Oxidation of Sarcoplasmic Reticulum. The endogenous enzymic system (NADH−iron) contained a final concentration of 10 μM $FeCl_3$ (except where indicated differently), 100 μM ADP, 0.12 M KCl, 5 mM histidine buffer (pH 6.8), 100 μM NADH, and sarcoplasmic reticulum suspension (0.7 mg of membrane protein/mL). Final concentration in the assay volume was 5.5 mL. The nonenzymic system had a similar composition except that 100 μM ascorbate was substituted for the NADH in the enzymic system. Incubations were carried out in a shaking water bath at 6 °C in air in 25-mL Erlenmeyer flasks. Reactions were initiated by the addition of the sarcoplasmic reticulum suspension. All reagents were prepared fresh and used within 30 min. The assay medium was sampled at various times and assayed as described below.

Table 1. Composition of Cod Muscle and Sarcoplasmic Reticulum[a]

sample	yield (mg/100 g of muscle)	% water	% protein	% lipid
muscle		78.9 ± 0.6	18.5 ± 0.2	0.76 ± 0.03
sarcoplasmic reticulum	262.7 ± 0.4		59.0 ± 2.1	41.0 ± 2.0

[a] Protein and lipid percentages are on a dry weight basis.

Lipid Oxidation. TBARS were determined by the method of McDonald and Hultin (1987). Lipid hydroperoxides were determined according to the method of Shantha and Decker (1994) with modifications. Lipid extraction of the reaction mixture was performed with the method of Buege and Aust (1978). One milliliter of the aqueous suspension of the assay was mixed with 4 mL of a dichloromethane/methanol (2:1, v/v) solution in a test tube and mixed on a Vortex mixer. Fifty microliters of ammonium thiocyanate solution was then added and mixed on the vortex for 2−4 s. A solution of 50 μL of ferrous iron was added next and mixed on the vortex for 2−4 s. After the solution was incubated for 5 min in a dark place at room temperature, the absorbance at 500 nm was recorded against a blank. Cumene hydroperoxide (Sigma Chemical Co., St. Louis, MO) was used as a standard. All procedures were conducted within 10 min after the lipid was extracted.

Oxidation of Protein Sulfhydryl Groups. To determine total sulfhydryl groups, 0.1 mg of sarcoplasmic reticular protein per milliliter was suspended in 10 mM potassium phosphate (pH 7.4, freshly prepared) containing 2.5% sodium dodecyl sulfate (SDS, w/v). Sulfhydryl groups were reacted with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and the product was measured by absorbance at 412 nm (Viner et al., 1997). To determine accessible surface thiols, the sarcoplasmic protein was suspended in the potassium phosphate buffer without the SDS. Reference values containing only buffer and sample without the DTNB were subtracted from the sample readings. Inaccessible sulhydryl groups were defined as the difference between the total sulfhydryl groups and the accessible sulfhydryl groups (Soszynski and Bartosz, 1997). Reduced glutathione (GSH) (Sigma Chemical Co.) was used as a standard.

Electrophoresis of Oxidized Samples. Discontinuous SDS−polyacrylamide gel electrophoresis (SDS PAGE) in a 7.5% polyacrylamide gel was performed according to the method of Laemmli (1970) as previously described (Srinivasan and Hultin, 1995).

Statistical Methods. All experiments were performed in triplicate, and in each experiment at least two replicates were done. The results are expressed as the mean values and standard deviations of two replications from three independent determinations (SAS, 1988).

RESULTS

The average cod muscle sample used in this work contained 18.5% protein and 0.76% lipid (Table 1). The isolated sarcoplasmic reticulum contained 59.0% protein and 41.0% lipid on a dry weight basis. These results were similar to those of Borhan et al. (1984) who isolated sarcoplasmic reticulum from the winter flounder (*Pseudopleuronectes americanus*) and found 57% protein and 43% lipid. The yield of the current preparation from cod muscle was over twice as great as the yield

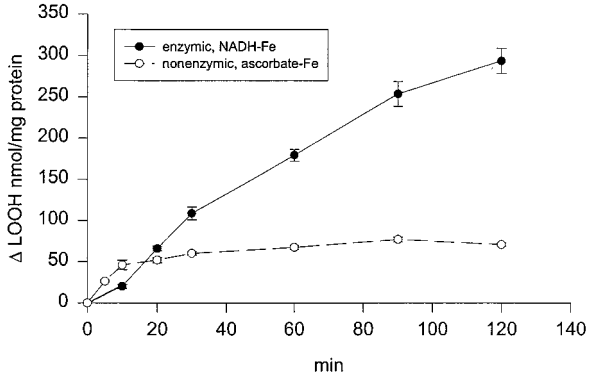

Figure 1. Effect of enzymic NADH−Fe and nonenzymic ascorbate−Fe systems on lipid hydroperoxide (LOOH) formation in cod sarcoplasmic reticulum. The NADH−Fe system contained 10 μM $FeCl_3$, 100 μM ADP, 0.12 M KCl, 5 mM histidine buffer, pH 6.8, 100 μM NADH, and 0.7 mg of protein/mL. The ascorbate−Fe system was the same except that 100 μM ascorbate was substituted for the NADH.

obtained in the earlier study with the flounder sarcoplasmic reticulum (i.e., 263 mg/100 g of muscle versus approximately 100 mg/100 g of muscle).

Lipid Oxidation. The pattern of build-up of lipid hydroperoxides in the enzymic and nonenzymic systems was different (Figure 1). In the nonenzymic ascorbate−iron system, there was a rapid early production of lipid peroxides. The rapid phase of lipid hydroperoxide build-up was over by 10 min, and there was little further increase in hydroperoxides after 30 min. On the other hand, although lipid peroxidation started more slowly in the enzymic NADH−iron system, almost linear production of lipid hydroperoxides occurred over the total reaction time. The total amount of lipid peroxides produced was over 4 times as great in the NADH−iron system as compared to the ascorbate−iron system.

Similar patterns of activity were observed when lipid oxidation was measured by TBARS. Kinetics of TBARS production in the NADH−iron and ascorbate−iron systems at three different iron concentrations are shown in Figure 2. Over a 60-min reaction period, there was a continuous increase in the enzymic NADH-driven system, while there was a leveling off of TBARS production in the nonenzymic system. The leveling off was most pronounced at the two higher iron levels, i.e., 50 and 100 μM. Apparently, the higher the iron concentration and the more rapid the oxidation, the more quickly the maximal value is reached. Since the 50 and 100 μM iron concentrations produced the same extent of lipid oxidation in the ascorbate−iron-driven system, it would appear that at these concentrations the iron is not the rate-limiting factor.

Oxidation of Protein Sulfhydryl Groups. The loss in the total sulfhydryl groups in the NADH−iron and ascorbate−iron systems is shown in Figure 3. There was a more rapid loss in the ascorbate−iron system in the initial stages of the reaction. The loss of the total sulfhydryl groups between the two systems was extensive and approximately the same after 60 min of incubation time. The more rapid loss of the total sulfhydryl content of the membrane proteins in the ascorbate−iron system is due entirely to those groups (accessible SH groups) that were exposed to the reagent in the absence of the denaturing agent SDS (Figure 4a). When the amount of the accessible sulfhydryl groups was subtracted from the total sulfhydryl groups of the membrane proteins, i.e., those determined in the presence of the denaturing SDS, a greater loss in these "inaccessible sulfhydryl groups" was observed in the NADH−iron system as compared to the nonenzymic ascorbate−iron system. Almost 60% of these inaccessible sulfhydryl groups were preserved during oxidation in the ascorbate−iron system (Figure 4b). In comparison, approximately two-thirds of these inaccessible sulfhydryl groups were lost when the oxidation was performed in the enzymic NADH−iron system after 2 h incubation.

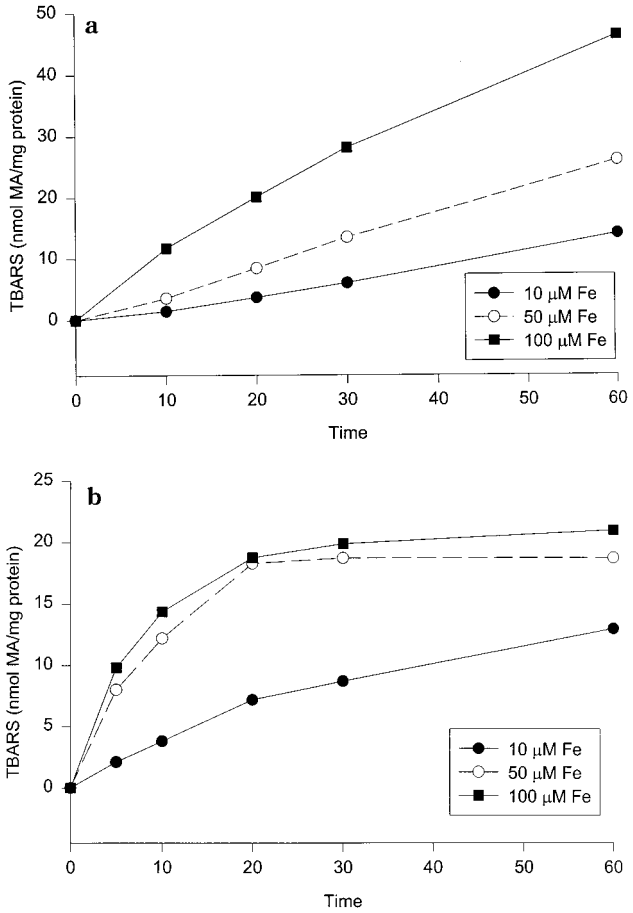

Figure 2. Effect of iron concentration on TBARS production in cod sarcoplasmic reticulum catalyzed by an enzymic system of NADH−Fe (a) or a nonenzymic system of ascorbate−Fe (b). Other conditions were as described in Figure 1.

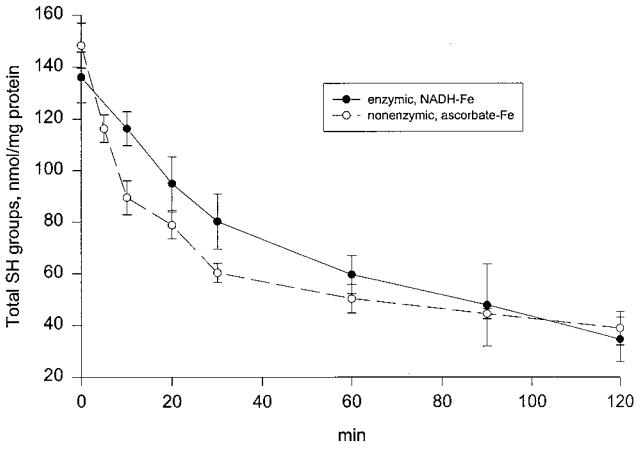

Figure 3. Loss of total sulfhydryl groups in the proteins of cod sarcoplasmic reticulum catalyzed by an enzymic system of NADH−Fe or a nonenzymic system of ascorbate−Fe. Other conditions were as described in Figure 1.

Electrophoretic evaluation of the membrane proteins

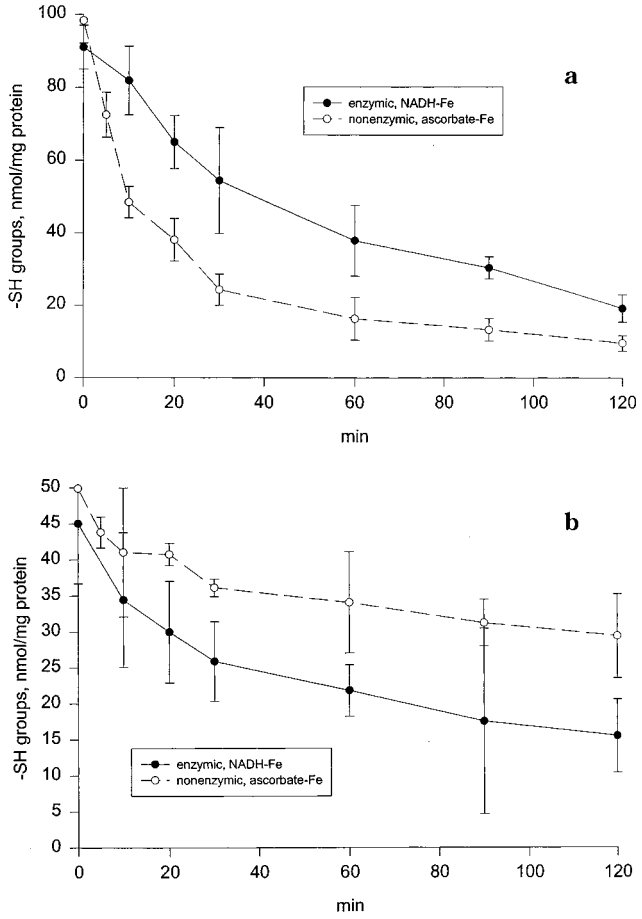

Figure 4. Loss of accessible (a) and inaccessible (b) sulfhydryl groups in the proteins of cod sarcoplasmic reticulum catalyzed by an enzymic system of NADH−Fe or a nonenzymic system of ascorbate−Fe. Other conditions were as described in Figure 1.

after different times of oxidation in the presence (Figure 5a) and absence (Figure 5b) of dithiothreitol (DDT) were performed. The results shown here were obtained with the NADH−iron system; similar results were observed with the ascorbate−iron system (Soyer and Hultin, 1999). When the electrophoresis was carried out in the presence of DTT, changes occurred slowly. A decrease in the major Ca^{2+}-ATPase band at 105 kDa was noticeable after 60 min. At about the same time, there was a decrease in the band just below the 205 kDa marker polypeptide. A similar loss was observed with two high molecular weight bands present in relatively low concentrations (arrowheads). However, when the electrophoresis was carried out in the absence of the DDT, changes were observed much sooner. After a 20-min reaction time, much of the major Ca^{2+}-ATPase was lost and a smudging of the bands occurred. A similar effect was seen for the high molecular weight doublet (arrowhead, Figure 5b), which had changed little when the proteins were examined in the presence of DTT, and the band just below the 205 kDa marker polypeptide. These results indicate that a major effect of the protein oxidation was the formation of disulfide bonds between the polypeptides. The spreading of the Ca^{2+}-ATPase band could have been due to interaction of various low molecular weight peptides caused by breaking of peptide bonds or by interactions with breakdown products from lipid oxidation.

Comparison of Lipid and Protein Oxidation Rates. A comparison of the rate of loss of sulfhydryl

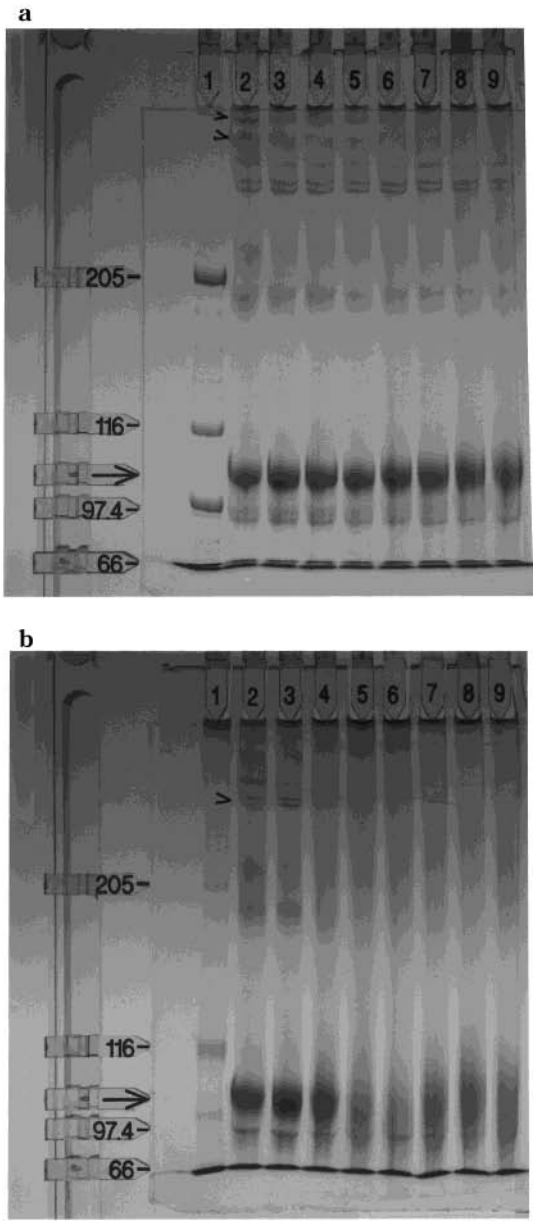

Figure 5. SDS−PAGE of cod sarcoplasmic reticulum proteins in the presence (a) or absence (b) of dithiothreitol (DTT) after exposure of the membrane to the enzymic NADH−Fe oxidizing system. Conditions for oxidation were as described in Figure 1. To each lane was added 10.5 μg of protein. Lane 1, molecular weight markers. Lane 2, membrane proteins. Lanes 3−9, oxidized membrane proteins after 0 (lane 3), 10 (lane 4), 20 (lane 5), 30 (lane 6), 60 (lane 7), 90 (lane 8), or 120 (lane 9) min.

groups and the formation of lipid hydroperoxides as a function of time is shown for the NADH−iron enzymic system in Figure 6 and the ascorbate−iron nonenzymic system in Figure 7. The purpose of these graphs is to show the relative rates of protein and lipid oxidations. The loss of sulfhydryl groups was some 16 times greater than the formation of protein carbonyl groups in the NADH−iron system, and it was over 30 times greater than found in the ascorbate−iron system (Soyer and Hultin, 1999). Thus, it seems reasonable to expect that the loss of sulfhydryl groups represents a large portion of the total change occurring in side groups of the membrane proteins. The lipid hydroperoxides were chosen as a measure of the oxidation rate of the lipids since they are among the first reaction products of the

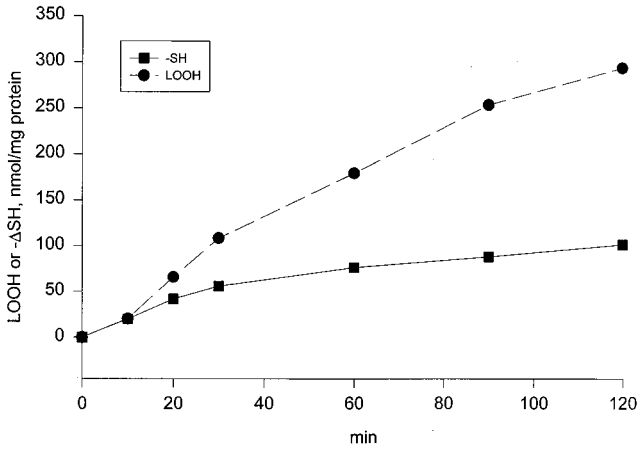

Figure 6. Comparison of lipid (as LOOH) and protein (as loss of −SH) oxidation as a function of time in the enzymic NADH−Fe system.

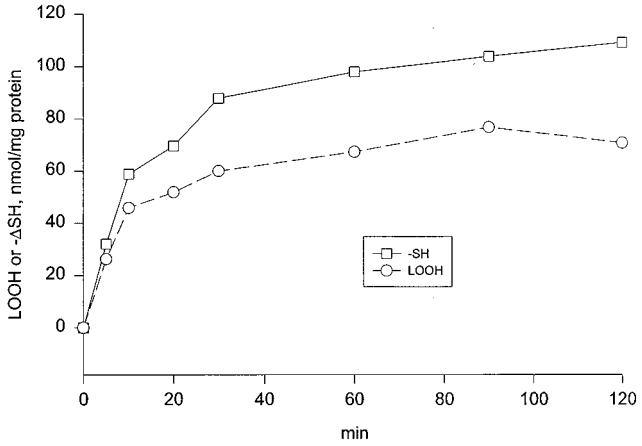

Figure 7. Comparison of lipid (as LOOH) and protein (as loss of −SH) oxidation as a function of time in the nonenzymic ascorbate−Fe system.

oxidation sequence. The initial rates of lipid and protein oxidation in the NADH−iron system were the same, but lipid oxidation from 20 min on became more substantial than the rate of loss of the sulfhydryl groups. In the ascorbate−iron system, the initial rate of loss of sulfhydryl groups was somewhat faster than the formation of lipid hydroperoxides, and this relatively faster rate continued through at least the first 60 min. Thus, in the enzymic NADH−iron system there was greater lipid oxidation relative to loss of sulfhydryls, while in the nonenzymic ascorbate−iron system the situation was reversed.

DISCUSSION

The kinetics of lipid oxidation was vastly different in the nonenzymic ascorbate−Fe and the enzymic NADH−Fe systems. The rapid phase of lipid oxidation was over after 10 min in the nonenzymic system, and there was relatively little increase thereafter. Lipid hydroperoxides were chosen as the measurement of lipid peroxidation since they occur early in the reaction sequence, and it is most likely that most of the oxidizing lipid forms these compounds. Lipid hydroperoxides may break down rapidly under appropriate conditions, such as the presence of ferrous iron. However, since the production of TBA-reactive substances (TBARS) follows the same kinetic course (Figure 2), it seems that lipid hydroperoxide breakdown is not the main cause for the leveling off of this reaction.

In the presence of the enzymic NADH−Fe system, there was a short lag phase, quickly followed by a steady production (not quite linear) of peroxides over the 2-h incubation period. The net effect was that there was approximately a 4-fold greater production of peroxides produced by the enzymic NADH−Fe system over the 2-h incubation period than by the nonenzymic system. The production of TBARS in the enzymic system was almost linear. Since the amount of TBARS produced in the NADH-driven system was at least equal to that produced by the nonenzymic system, the higher values of the peroxides in the enzymic NADH system as compared to the nonenzymic system could not have been due to reduced conversion of peroxides to breakdown products.

A lag phase was noted in the enzymic NADH system at 10 min, while there was no indication of a lag in the oxidation of the nonenzymic ascorbate−Fe system with a first measurement at 5 min. It would seem that the initial reaction of the nonenzymic ascorbate−Fe system is faster than that of the enzymic system. It is not easy to explain this. Previous results in a system in which ferrous iron was added directly to the reaction medium without a reducing agent showed a lag phase and strongly suggested that this lag phase was due to the presence of antioxidants in the membrane (Huang et al., 1993). The greater initial oxidative activity of the ascorbate−ferric iron system as compared to ferrous iron may be related to the production of an activated ascorbate product that accelerates the reaction. The semidehydroascorbate seems a logical contender for this product (eq 1; Halliwell and Gutteridge, 1989; Buettner, 1993). In any case, the limitation of lipid hydroperoxides

$$\text{ADP}-\text{Fe}^{3+} + \text{ascorbate}^{-} \rightarrow \text{ADP}-\text{Fe}^{2+} + \text{semidehydroascorbate}^{\bullet -} \quad (1)$$

produced in the nonenzymic ascorbate−Fe system was not due to lack of substrate. Considerably more lipid hydroperoxides were produced in the enzymic NADH−Fe system, which had the same amount of lipid substrate available to it.

The greater extent of peroxide formation observed in the enzymic NADH−Fe system would indicate that this catalytic system might be more important in determining quality attributes of stored fish muscle than the nonenzymic ascorbate−Fe system. One of the factors that could have contributed to the much greater lipid oxidation observed in the enzymic system is that this enzymic ferric iron-reducing system can operate very efficiently with respect to NADH availability. Although comparable data for the cod muscle sarcoplasmic reticular system are not available, it has been determined that the K_M for NADH of the sarcoplasmic reticular enzymic system of the winter flounder (*P. americanus*) in the presence of 0.1 μM ADP is approximately 1 μM (McDonald and Hultin, 1987). It has been demonstrated that in red hake muscle (*Urophycis chuss*) the level of NADH never dropped below 7 μmol/kg of tissue for fillets after 14 days of storage at 0 °C (Phillippy and Hultin, 1993). Even in minced red hake muscle tissue stored under the same conditions, there was 4 μmol of NADH/kg of tissue remaining after the same time. Thus, there would be little expectation that the activity of the enzyme would drop off much with storage of the

muscle tissue if the cod membrane system had similar kinetic characteristics to that of the winter flounder system and if the cod retained NADH anywhere near as well as the red hake.

Another explanation relates to the possibility of a site-specific oxidation in the case of the enzymic NADH−Fe system. The enzyme that reduces iron is part of the sarcoplasmic reticulum membrane. In the assay system, a certain percentage of the iron binds to the membrane (Huang and Hultin, 1992). At the concentration of iron used in this study, a majority of the iron would be bound to the membrane. If the reduction of the ferric iron to ferrous iron takes place on or in the membrane, free radicals produced by the ferrous iron would be close to the membrane lipids and/or proteins. Thus, the enzymic ferric iron-reducing system is not only highly efficient because of its low K_M for NADH, but it is located close to the lipid component of the membrane. It is interesting that in the earlier study it was determined that there was no difference between soluble iron and membrane-bound iron in their ability to catalyze lipid oxidation, in that case as measured by TBARS (Huang and Hultin, 1992). Another interesting facet of the role of iron is that both in the earlier study and in this study, using the enzymic NADH−Fe system and TBARS as the measure of oxidation, there was almost a linear progression in oxidation rates with increasing iron concentration. This is in contrast to the nonenzymic system which showed no greater activity at 100 μM $FeCl_3$ than it did at 50 μM $FeCl_3$. This contrast, along with the greater activity from the enzymic system, implies that the iron is a more efficient catalyst when its redox cycle involves the membrane enzyme.

The loss of total sulfhydryl groups in the membrane proteins in both the enzymic and the nonenzymic iron-reducing systems showed some similarity to the production of lipid hydroperoxides. The initial rate of loss was faster in the nonenzymic system than it was in the enzymic system. This was especially pronounced in the early stages of the reaction. For example, after a 10-min reaction time, 15% of the total sulfhydryl groups had been lost in the enzymic system as compared to 40% in the nonenzymic system. With time, however, the difference in total production decreased until they were essentially the same after 90 min. This, however, may simply be a reflection that there was limited substrate for the reactions. It could be questioned as to whether the loss of sulfhydryl groups is a good measure of protein oxidation. There are of course many side groups of proteins that are susceptible to oxidation by free radicals (Stadtman, 1993). Production of carbonyls in this system was 15−30 times less on a molar basis than the loss of sulfhydryl groups (Soyer and Hultin, 1999). While loss of sulfhydryl groups may not be as definitive a measure of protein oxidation as the production of lipid hydroperoxides is of lipid oxidation, it seems reasonable to suggest that it probably is a reflection of the general susceptibility of the membrane proteins to oxidation. Other changes in proteins that would cause either cross-linking (polymerization) or fragmentation were not readily observed in the first 60 min of reaction time (Figure 5a). A comparison of the SDS−PAGE gels that were prepared in the absence of dithiothreitol show a very different story. Significant changes were observed as early as 10 min, and by 30 min, there was an extensive redistribution of polypeptides as a function of size. Some of this change involves polymerization since there is a production of some higher molecular weight materials that are spread out over the upper part of the gels. This is consistent with the oxidation of sulfhydryl groups to form disulfides, whose formation can be reversed in the presence of the dithiothreitol.

Not only were the sulfhydryl groups lost less rapidly when the oxidation was catalyzed by the NADH−Fe enzymic system, but there was a difference between the enzymic and the nonenzymic systems in the rate and the extent of accessible and inaccessible sulfhydryl oxidation. The enzymic system was much more efficient at oxidizing the inaccessible sulfhydryls than was the nonenzymic. Presumably, the sulfhydryl groups that were not accessible to the reagent are those which are not generally exposed to components in the aqueous phase. Total sulfhydryl groups are determined by completely denaturing the proteins with SDS. The inaccessible sulfhydryls, therefore, are most likely those that are buried away from the surface of the membrane. This would suggest that there is a different spatial location of the causative agent(s) of oxidation in the NADH−Fe enzymic system as compared to the ascorbate−Fe nonenzymic system. It would indicate that it is easier for these reactive oxidative components to reach the interior of the membrane when supplied by the enzymic NADH−Fe system. This may also be another reason lipid oxidation is so extensive in the enzymic system, i.e., the reactive oxidizing components are produced near the unsaturated fatty acids that are in the interior of the membrane.

The difference in the localization of the oxidizing reactions is also illustrated by looking at the relative production of lipid peroxides and the loss of sulfhydryl groups in each of the two oxidizing systems separately. In the ascorbate−Fe nonenzymic system, more sulfhydryl groups are lost than lipid peroxide formed. In the case of the enzymic NADH−Fe system, it is just the opposite, with many more lipid hydroperoxide groups formed than sulfhydryl groups lost. Although it appears that there are some sulfhydryl groups that are buried, one would expect that a high percentage of the sulfhydryl groups would occur on the surface. The unsaturated hydrocarbon tails of the fatty acids would be buried in the interior. Thus, the preference for the enzyme system for lipid oxidation over protein oxidation relative to what happens in the nonenzymic system is also indicative that the oxidizing compounds are produced either in the interior of the membrane or in an area where their effectiveness can be transferred more readily to the interior.

The oxidations that occur in a system such as the cod sarcoplasmic reticular membrane could be expected to be very complex. In addition to the initiation reactions that could directly affect both the lipids and the proteins, free radicals produced in one of the fractions could initiate oxidations in the other, e.g., lipid-free radicals could initiate protein oxidations and vice versa. One of the purposes of this study was to determine the relative rate of oxidations of the protein and lipid fractions and to assess which fraction was more susceptible to oxidation. It is impossible to distinguish from the data presented here which, if either, of the fractions oxidize first. Because of the nature of the two catalytic systems, it is not unreasonable to expect that there was perhaps more lipid oxidation in the enzymic system and more protein oxidation in the nonenymic system in the early stages. However, oxidation of only protein or only

lipid initially would seem to be unlikely. At this point, we can probably not suggest any more than that the oxidation of both fractions proceeds more or less simultaneously and begins almost immediately, there being only an indication of a slight lag phase in lipid oxidation in the NADH−Fe enzymic system. When the oxidation of the lipids of the cod sarcoplasmic reticulum were carried out in a model system of washed, minced cod muscle catalyzed by a free radical-generating system of ferric iron, hypoxanthine, and xanthine oxidase, there was evidence that oxidation of the muscle proteins occurred before oxidation of the membrane lipids (Srinivasan and Hultin, 1995). The membrane proteins were not evaluated separately in that study.

The results of this study may have practical applications in quality deterioration of stored fish muscle. It is unlikely that the oxidation of membrane proteins has a severe impact on storage life quality. Their primary role may be as competition for free radicals for the membrane lipids. The oxidation of membrane lipids is thought to be one of the major sources of quality deterioration in muscle food products. Thus, one might expect that the enzymically catalyzed system in the presence of NADH may be one of the major sources of quality deterioration due to lipid oxidation in stored fish muscle.

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