Cytochrome Oxidase as an Indicator of Ice Storage and Frozen Storage

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The potential of cytochrome oxidase as an indicator of ice storage and frozen storage of fish was investigated. Optimal assay conditions for cytochrome oxidase in a crude homogenate from cod muscle were studied. Maximal cytochrome oxidase activity was found at pH 6.5−7.5 and an assay temperature of 30 °C. Maximal activation by Triton X-100 was obtained in a range of 0.62−1.25 mM Triton X-100. The specificity of the assay was high, as cytochrome oxidase was inhibited 98% by 33 μM of the specific inhibitor sodium azide. The coefficient of variation of cytochrome oxidase activity in different cods was 21%, and the coefficient of variation of different analyses on the same homogenate was 5%. It was shown that ice storage of muscle samples before they were frozen and thawed resulted in a major freezing-induced activation of cytochrome oxidase activity. The enzyme may therefore be used as an indicator of frozen fish to determine if the fish has been stored on ice before freezing. Cytochrome oxidase activity showed also potential as an indicator of frozen storage, as it was possible to distinguish between the frozen storage temperatures −9, −20, and −40 °C.

Keywords: *Cod muscle; cytochrome oxidase assay; frozen storage; ice storage; membrane-bound enzymes*

INTRODUCTION

Freezing and frozen storage are commonly used for preservation of fish. When the processes of freezing, frozen storage, and thawing of fish are done properly, the sensory properties of the frozen−thawed fish can be similar to those of fresh fish. However, proper treatment of fish after catch can sometimes be far from reality and, consequently, consumers often prefer fresh fish. Fresh fish has a limited shelf life and is susceptible to deterioration even when stored on ice (0 °C). In contrast, fish can be stored in a frozen state for several months without severe changes in quality. The limiting factor of frozen storage in lean fish species is denaturation of proteins, which results in a dry and firm texture of the fish muscle (*1*). In fatty fish species lipid oxidation will often be the limiting factor, resulting in a rancid flavor (*2*).

To determine whether a fish has been properly stored, it is necessary to develop a set of indicators that can differentiate between optimal and less optimal storage conditions. Numerous approaches have been taken to correlate physical and chemical changes in the fish muscle, such as protein solubility and oxidation, to quality changes in frozen fish (*3*). There is still a need, however, to find indicators that can give information on the time and temperature history of a frozen fish. Another problem is to distinguish between fresh and frozen fish (*4*). Enzyme activity has shown to be a useful indicator for this purpose, and most of the enzymes used are located in the lysosomes or in the mitochondrial matrix; as a result of membrane disruption they are released from the organelles after freezing and thawing.

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In this paper a new approach using membrane-bound enzymes as indicators of changes in fish muscle is presented. Membrane-bound enzymes, that is, enzymes which are located in the lipid bilayer of cellular membranes, are affected by changes in the lipid phase as well as in the aqueous phase in a muscle cell (*5*). Ice storage, freezing, and frozen storage are processes that result in changes in both of these phases. For example, membrane lipids are exposed to hydrolysis and oxidation, and the aqueous phase is changed by dehydration and changes in pH (*1*). These changes are known to affect the activity of membrane-bound enzymes (*6*). Hence, the activity of membrane-bound enzymes can be expected to be a good indicator of physical and chemical changes in muscle cells.

A previous study showed that the activity of cytochrome oxidase in a frozen−thawed homogenate of salmon muscle was highly affected by an ice storage period before freezing and freeze−thaw cycles (*7*). Cytochrome oxidase is embedded in the inner mitochondrial membrane, and like many other membrane-bound enzymes its activity depends strongly on the microenviroment of the enzyme (*8*). Specifically, it has been shown that cytochrome oxidase has a requirement of tightly bound cardiolipin to be fully active (*9*). Changes in the membrane during ice storage and frozen storage are likely to disturb the very sensitive interaction between cytochrome oxidase and the surrounding membrane lipids and proteins, resulting in a changed level of cytochrome oxidase activity. Therefore, this enzyme might be a good indicator of ice storage and frozen storage.

In the present study we investigate the potential of cytochrome oxidase (EC 1.9.3.1) as an indicator of an ice storage period before freezing and as an indicator of frozen storage conditions. To measure the effect of

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changes in the membrane on cytochrome oxidase due to ice storage and frozen storage, the activity will be measured in a crude homogenate to avoid the effects of a fractionation procedure to interfere with the results. Cytochrome oxidase activity has been measured in crude homogenates from heart and red muscle from several fish species (*10*) but not in white muscle from cod as used in this study. In the following an assay of cytochrome oxidase activity in crude homogenate from cod muscle is described. To establish well-defined and stable assay conditions, important aspects such as pH, temperature, detergent, and substrate concentration were studied to obtain optimal assay conditions of the enzyme. A high specific activity of the enzyme will increase the possibility of identifying significant changes in activity as a result of changes in the membrane induced by ice storage and frozen storage.

MATERIALS AND METHODS

Materials. Just caught (JC) cod (*Gadus morhua*) with body weights of ~3 kg were obtained from Öresund, Denmark. All chemicals used in this study were of analytical grade. Cytochrome *c* (Cyt *c*) from bovine heart was obtained from Sigma.

Homogenate Preparation. Muscle samples were taken from the area between the dorsal fin and the lateral line. Each homogenate was made from 3.0 g of muscle, which was cut up into small pieces in 24 mL of cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. This suspension was homogenized by 10 up-and-down strokes in a Potter-Elvehjem apparatus at 750 rpm using a Teflon pestle. The temperature was kept below 4 °C, and the homogenization procedure did not exceed 5 min.

Ice Storage and Freezing. Homogenates were prepared from nine different JC and gutted cods and analyzed immediately for cytochrome oxidase activity. Muscle samples (4−6 g) from the JC cods were vacuum-packed and frozen at −30 °C for 12 h. Cods were then stored on ice for 3 days, and muscle samples from ice-stored cod were vacuum-packed and frozen at −30 °C. After freezing, muscle samples were thawed (1 h at 0 °C) and cytochrome oxidase activity was measured.

Frozen Storage. Nine JC cods were gutted and stored on ice for 18 h. Muscle samples (4−6 g) were taken from each cod, vacuum-packed, and frozen in a blast freezer (−40 °C) for 15 h. Fish samples were then distributed at −9, −20, and −40 °C (all samples from one fish at the same temperature). Muscle samples were thawed at 0 °C for 1 h before homogenate preparation and measurement of enzyme activity.

Cytochrome Oxidase Assay. The method of determination of cytochrome oxidase activity was based on assays for fish tissues (*11*, *12*). The activity of cytochrome oxidase was measured by following the decrease in reduced cytochrome *c* spectrophotometrically at 550 nm. One milliliter of homogenate was incubated with 1 mL of detergent solution consisting of 1.24 mM Triton X-100, 1 mM EDTA, and 1 mM $NaHCO_3$. After incubation, samples were centrifuged at 1000*g* (10 min, 4 °C) and the supernatant was used for measurement of cytochrome oxidase activity.

Reduced Cyt *c* solution (41 μM, pH 7.0) was prepared just before the analysis of enzyme activity: Cyt *c* was dissolved in a 30 mM potassium phosphate buffer containing 1 mM EDTA and reduced with dithionite (0.2 mg of dithionite in 1 mL of buffer). The absorbance of the reduced Cyt *c* solution should be 0.7 ± 0.05 read against a reference cuvette containing completely oxidized Cyt *c*. One hundred microliters of supernatant was added to 1 mL of reduced Cyt *c* solution in a cuvette to start the reaction, and the absorbance (*A*) was recorded every 20 s in 4 min. Finally, a few crystals of potassium ferricyanide were added to completely oxidize the Cyt *c* and the absorbance (A_0) was registrated.

A linear relationship between logarithmic ($A - A_0$) and reaction time was obtained, and enzyme activity was calculated by multiplying a pseudo-first-order constant (*k*) by the total concentration of Cyt *c*. The activities of cytochrome oxidase are reported as micromoles of Cyt *c* per minute per gram of muscle.

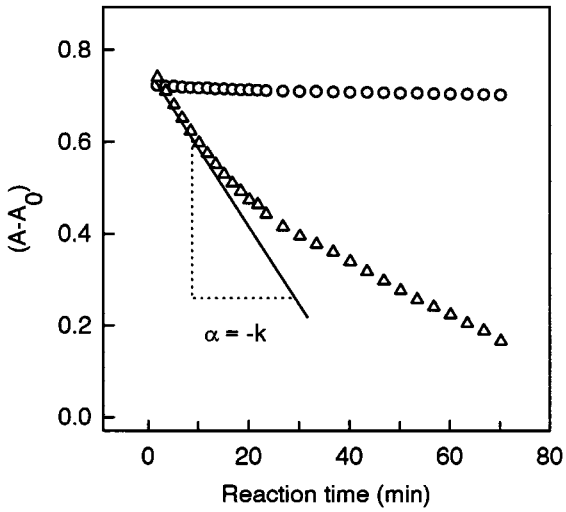

Figure 1. Assay of cytochrome oxidase activity in a homogenate incubated with (○) and without (△) sodium azide. Oxidation of Cyt *c* was followed as a decrease in reduced Cyt *c* concentration measured at 550 nm. *A* is the absorbance at a given time, and A_0 is the absorbance of completely oxidized Cyt *c*. $A - A_0$ was plotted on a logarithmic scale against reaction time. *k* is a first-order constant obtained from the slope (α) of the initial linear part of reaction.

Statistical Analysis. Student's *t* test (one-sided) was used to determine statistically significant differences between mean values. *p* values of <0.01 were considered to be significant.

RESULTS

Although cytochrome oxidase activity has been measured in fish tissue (*11*, *12*), studies on optimal assay conditions for this enzyme are insufficient. Therefore, experiments were performed to establish optimal assay conditions for cytochrome oxidase activity in cod muscle homogenate.

Assay and Specificity. The reaction product of cytochrome oxidase activity, oxidized Cyt *c*, is a competitive inhibitor of the enzyme. The constant for half-inhibition, K_i, of cytochrome oxidase by oxidized Cyt *c* (3 μM) is equal to the constant of half-activation, K_m, for the enzyme by its substrate, reduced Cyt *c* (*13*). Determination of enzyme activity therefore had to be based on initial reaction rates. The result of a typical assay for cytochrome oxidase activity in a cod muscle homogenate is shown in Figure 1. During the first 10 min of reaction a linear relationship between $\ln(A - A_0)$ and reaction time was obtained, and the slope of the line (α) was used to calculate the initial rate of Cyt *c* oxidation. After 10 min of reaction, the rate of oxidation of Cyt *c* decreased, probably due to a low concentration of substrate and/or inhibition by oxidized Cyt *c*.

NaN_3 is a specific inhibitor of cytochrome oxidase (*14*), and the specificity of the assay was investigated by inclusion of NaN_3 in the assay before the reaction was initiated by the addition of homogenate. Figure 1 also shows an assay for cytochrome oxidase activity with 33 μM NaN_3 present in the assay, resulting in an inhibition of 98% of the enzyme activity. Increasing the concentration of NaN_3 >10 times in the assay (370 μM) did not further inhibit cytochrome oxidase.

Linearity of the assay was confirmed when cod muscle homogenate was added to the assay in a range of 0−200

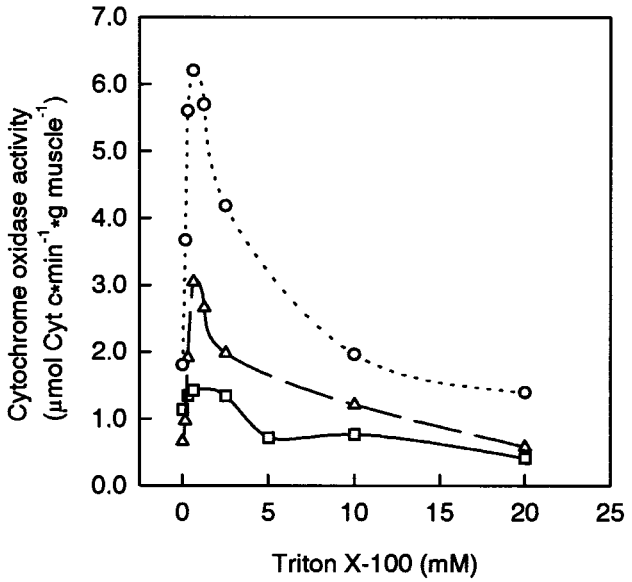

Figure 2. Effect of Triton X-100 on cytochrome oxidase activity. Homogenates prepared from a just caught cod (○), an ice-stored (0 °C for 3 days) cod (△), and a frozen stored (−20 °C for 3 months) cod (□) were incubated with increasing concentrations of Triton X-100. Each datapoint corresponds to a duplicate determination of enzyme activity.

μL of homogenate. When the total concentration of Cyt *c* in the assay was varied from 1 to 80 μM, saturating levels of Cyt *c* were obtained above 30 μM. Therefore, maximal activity of cytochrome oxidase was obtained using 37 μM Cyt *c* in the assay.

pH and Temperature. Cytochrome oxidase activity was measured in two different cod muscle homogenates in the pH interval 5.5−8.0, and maximal activities were found in the pH area 6.5−7.5. The temperature dependence of cytochrome oxidase was measured in a cod muscle homogenate in the temperature interval between 2 and 34 °C. Cytochrome oxidase activity increased with increasing temperatures until 28−29 °C, at which maximal activity was observed. Raising the temperature to 34 °C caused a decrease in activity, probably due to heat denaturation of the enzyme. On this basis an assay temperature of 25 °C seems to be appropriate.

Triton X-100 Activation. Homogenates were incubated with Triton X-100 to make the outer mitochondria membrane permeable to external Cyt *c*. Addition of detergent may also prevent formation of membrane vesicles and aggregation of membrane proteins. The concentration of Triton X-100 during incubation which results in maximal cytochrome oxidase activity was studied in a homogenate prepared from a JC fish, a homogenate stored on ice for 3 days, and a homogenate stored at −20 °C for 3 months (Figure 2). In all three homogenates maximal cytochrome oxidase activity was obtained at a Triton X-100 concentration in the range of 0.62−1.25 mM. In homogenate prepared from JC cod, enzyme activity increased by 240%, and in ice-stored homogenate activity increased by 360% when the Triton X-100 concentration was raised from 0 mM to the concentration at which maximal activity was obtained. In frozen homogenate the increase in activity was only 25%. When homogenates were incubated at Triton X-100 concentrations >1.25 mM, cytochrome oxidase activity decreased, and at 20 mM Triton X-100 the activity was reduced to the same level as in the absence of Triton X-100. The decrease in cytochrome oxidase activity was presumably due to changes in the lipid microenvironment of the enzyme.

Table 1. Biological and Analytical Variability in Cytochrome Oxidase Activity

variation between	CV (%)
individuals[a]	20.6 (n = 18)
homogenates[b]	7.6 (n = 5)
Cyt *c* solutions[c]	4.1 (n = 3)
analyses[d]	5.1 (n = 4)

[a] Eighteen JC cod. [b] Five different homogenates from the same cod. [c] Three different solutions of Cyt *c*. [d] Four measurements on the same homogenate.

Activity in the Supernatant Fraction. After incubation with Triton X-100, the assay procedure included a centrifugation step to remove visible particles of fish muscle that otherwise could interfere with the spectrofotometric measurements. Although the centrifugation was mild (1000*g*, 10 min), it was investigated whether a part of the cytochrome oxidase activity was removed from the supernatant by centrifugation. Supernatant activity was compared with total activity in different homogenates from the same fish. The total activity was measured as cytochrome oxidase activity as described under Materials and Methods, omitting the centrifugation step in the assay procedure and with manual mixing in the reaction cuvette to obtain a constant influence of visible particles on the absorbance. Cytochrome oxidase activities (supernatant and total) were measured in a muscle homogenate from JC cod, ice-stored cod, cod that was frozen, and cod that was frozen after ice storage. The range of supernatant cytochrome oxidase activity in these homogenates was 3.5−6.0 μmol·min^{-1}·g of muscle^{-1}. Nearly all (93−98%) of the cytochrome oxidase activity was found in the supernatant fraction, and different combinations of ice storage and freezing did not have any effect on the distribution of cytochrome oxidase activity after centrifugation.

Variability. To use cytochrome oxidase as an indicator of ice storage and/or frozen storage, it is important to know the level and variability of cytochrome oxidase activity in cod as well as the reproducibility of the assay. Mean activity and standard deviation in 18 JC cods from the same catch was 2.4 ± 0.5 μmol·min^{-1}·g of muscle^{-1}, corresponding to a coefficient of variation (CV) of 21%. The CV on activities measured in five different homogenates prepared from the same fish was 8%, and when activities were measured in the same homogenate, the CV was 5%. Measuring the activity in the same homogenate using three different solutions of reduced Cyt *c* resulted in a CV of 4%. The results are summarized in Table 1. The variability due to different Cyt *c* solutions seems to be negligible, as this CV was less than the CV of the analysis as a whole.

Ice Storage and Freezing. JC cods and cods ice-stored for 3 days were frozen and thawed as described under Materials and Methods to see if 3 days of ice storage had an effect on cytochrome oxidase activity after freezing and thawing (Figure 3). Cytochrome oxidase activity increased significantly in both JC (unpaired *t* test, $p < 0.009$) and ice-stored fish (paired *t* test, $p < 0.00003$) after freezing (−30 °C) and thawing of muscle samples. However, the freezing-induced increase in enzyme activity in ice stored cod was 97%, while in JC cod it was only 24%. This results in a remarkably high activity in frozen samples, which were ice stored before freezing (Figure 3). By a further

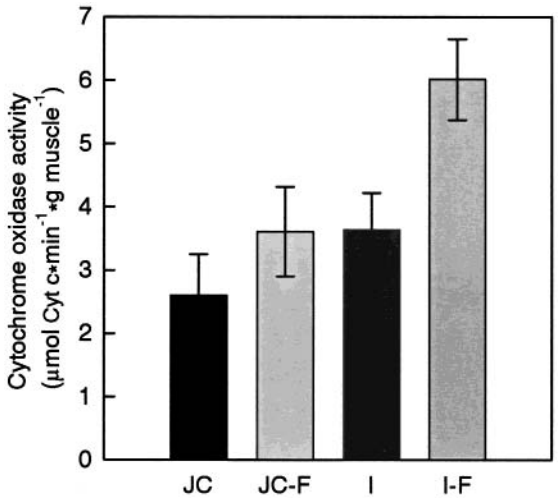

Figure 3. Effect of ice storage and freezing on cytochrome oxidase activity in cod muscle. Cytochrome oxidase activity was measured in muscle samples from nine different cods (n = 9) just after catch (JC), in cod muscle samples frozen just after catch (JC-F, n = 6), in muscle samples from cods that were stored on ice for 3 days (I, n = 6), and in cod muscle samples frozen after 3 days of ice storage (n = 6) (I-F). Columns represent mean values; standard deviations are indicated by error bars.

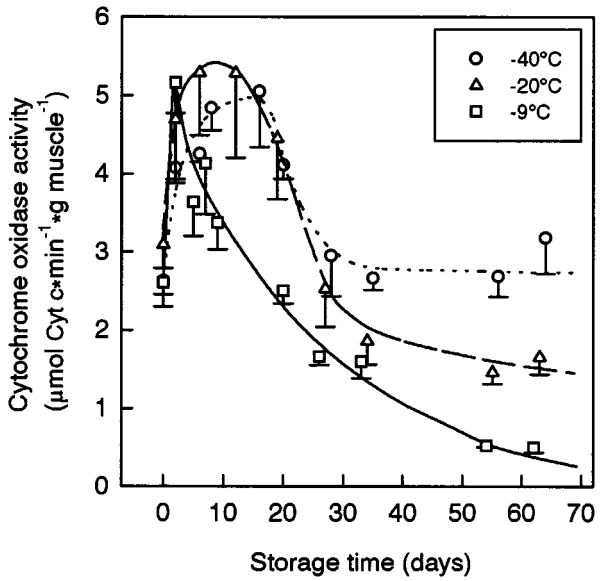

Figure 4. Stability of cytochrome oxidase at different subzero temperatures. At each temperature the activity of cytochrome oxidase was measured in muscle samples from three cods. Results are presented as mean values; standard deviations are indicated by error bars. A storage time of 0 days corresponds to cytochrome oxidase activity in cods stored for 18 h on ice after catch.

investigation of the freezing-induced activation it was found (results not shown) that when muscle samples from ice-stored cods (n = 3) were frozen at −80 °C instead of −30 °C, no significant activation by freezing was observed (unpaired t test, p = 0.08). Furthermore, no freezing-induced increase in activity was observed when homogenates (n = 3 homogenates from ice-stored cods) were frozen at −30 °C instead of freezing of muscle samples.

Activation of the enzyme by ice storage and freezing of muscle samples at −30 °C was observed in all examined cod muscle samples, and therefore the observed standard deviations are due to differences in cytochrome oxidase activity between individual cods, as well as differences in the extent of activation upon ice storage and freezing.

Frozen Storage. The stability of cytochrome oxidase during frozen storage was studied when cod muscle samples were frozen at −40 °C and stored at −9, −20, and −40 °C (Figure 4). At all temperatures an initial increase in cytochrome oxidase activity was observed as a result of freezing and during the first period of frozen storage. In muscle samples stored at −9 °C there was a maximal increase in enzyme activity (98%) after 2 days of frozen storage. Muscle samples stored at −20 °C showed a maximal increase in activity (71%) between 6 and 12 days of frozen storage, and the muscle samples stored at −40 °C showed a maximal increase in activity (92%) between 8 and 16 days of frozen storage. After maximal activation of cytochrome oxidase in muscle samples stored at −9 °C, the enzyme activity decreased rapidly to almost no activity after 60 days. In muscle samples stored at −20 and −40 °C cytochrome oxidase activity decreased rapidly to a lower level, but after 30−40 days of frozen storage the rate of cytochrome oxidase inactivation was decreased. After 60 days, cytochrome oxidase activity in muscle samples stored at −20 °C had decreased to a level of ∼1.8 μmol·min^{-1}·g of muscle^{-1}, whereas the activity in muscle samples stored at −40 °C had decreased to ∼2.8 μmol·min^{-1}·g of muscle^{-1}.

DISCUSSION

A simple method for the measurement of cytochrome oxidase activity was used for the study of some important aspects of the assay and the enzyme in crude homogenate from cod muscle. An almost complete inhibition of cytochrome oxidase by NaN_3 suggests that the assay is highly specific for cytochrome oxidase. NaN_3 inhibition is specific for the cytochrome oxidase except for the inhibitory effect on catalase (*15*). Catalase catalyzes the breakdown of hydrogen peroxide, and this enzyme is not expected to be able to contribute to the oxidation of Cyt *c*. The activity not inhibited by NaN_3 could be due to an incomplete inhibition of the enzyme when the activity in a supernatant fraction from a crude homogenate was measured. A complete inhibition of cytochrome oxidase by 1 mM NaN_3 has been reported for the isolated enzyme from the photosynthetic bacterium *Rhodopseudomonas palustris* (*16*).

A pH value of 7.0 is used in assays for cytochrome oxidase in fish (*11, 12*), but no studies on optimal pH have been reported on fish tissue. The pH optimum found in this study (6.5−7.0) is close to the pH optimum for the isolated enzyme from rat liver (6.0−6.3) (*17*). Differences in pH optima may be due to different ionic strengths in the assay media. According to Nicholls and Chance (*14*) the optimal pH value decreases when the concentration of phosphate buffer is increased.

Cytochrome oxidase activity in fish tissues has been measured in a wide range of assay temperatures, 15−37 °C (*10*−*12*), but studies on the optimal assay temperature of the enzyme have to the authors' knowledge not been performed. The optimal temperature of 28−29 °C of cytochrome oxidase found in this study was close to the optimal assay temperature of 30 °C for sarcoplasmic reticulum Ca^{2+}-ATPase in cod muscle (*18*).

A higher activation of cytochrome oxidase by Triton X-100 in ice-stored homogenate (360%) than in homogenate prepared just after catch (240%) may be due to solubilization of protein aggregates formed during the

ice storage period. The minor activation of cytochrome oxidase by Triton X-100 in frozen homogenate suggests that freezing and frozen storage disrupt the mitochondria membrane. A disrupted membrane may result in easier diffusion of substrate to the enzyme, and the effect of Triton X-100 is greatly reduced.

The effect of detergent on mitochondrial membranes can give information about the integrity of the membrane. Rustin et al. (*13*) suggested that measurement of cytochrome oxidase activity in the absence and in the presence of a detergent could be used as a method to estimate the intactness of the outer mitochondria membrane.

The activation of cytochrome oxidase by freezing and thawing of muscle samples from both JC cod and cod that had been stored on ice (Figure 3) may be due to changes in the membranes during freezing and thawing. Nilsson and Ekstrand (*19*) used the volume of centrifuged tissue fluid (CTF) and release of lysosomal enzymes as an indirect measure of membrane permeability. After freezing and thawing of rainbow trout, they observed an increased lysosomal enzyme activity in CTF, indicating a breakdown of the cellular membranes during freezing. It is well documented that the interaction of cytochrome oxidase with membrane lipids is essential for activity (*8*), and it has also been reported that cytochrome *c* exists in different conformations depending on the physical state of the membrane (*20*). A possible theory for the freezing-induced activation of cytochrome oxidase is therefore that the observed activation is due to changes in the interaction between cytochrome oxidase, Cyt *c*, and the surrounding membrane lipids.

According to this theory, the absence of a freezing-induced activation in muscle samples frozen at −80 °C (Figure 3) could be explained by a less pronounced membrane damage at low freezing temperatures (fast freezing). Furthermore, a decreased rate of membrane changes at low storage temperature (−40 °C) could explain a delay in frozen storage induced activation (Figure 4). The absence of a freezing effect on cytochrome oxidase activity when the cod muscle sample was homogenized before freezing indicates that the mechanism of freezing activation is sensitive to changes in the chemical and physical environment of the membrane-bound enzyme. In a homogenate the muscle cells are disintegrated and enzymes and other compounds are released from intracellular organelles. All of this may have dramatic effects on membrane structures in the cell, destroying sensitive interactions between membrane proteins and lipids responsible for the freezing-induced activation of cytochrome oxidase.

The amplification of the freezing-induced activation by an ice storage period before freezing is an interesting result. A breakdown of muscle cells during 3 days of ice storage (0 °C) is inevitable, and it is likely to have an effect on membrane-bound enzymes as well as other enzymes. The reason for the enlargement of the freezing-induced activation of cytochrome oxidase could be conformational changes of cytochrome oxidase, an increased enzyme−lipid interaction, or stabilization of the enzyme by free fatty acid and lysophospholipids formed during ice storage by enzymatic cleavage. The mechanisms of cytochrome oxidase activation by ice storage and by freezing cannot be resolved without further experiments, but on the basis of the present results it can be suggested that interactions between cytochrome oxidase and the surrounding membrane lipids is an important factor. Although the exact mechanisms are unknown, the results (Figure 3) clearly demonstrate that the extent of freezing-induced activation of cytochrome oxidase may be used to determine whether a frozen fish has been ice stored or not. Cytochrome oxidase activities >5 μmol·min^{-1}·g of muscle^{-1} in a frozen fish indicate that a frozen fish has been ice stored before freezing.

Despite individual variations in cytochrome oxidase activity in different fish, it seems that it is possible to use cytochrome oxidase as an indicator of frozen storage temperatures (Figure 4). Different rates of inactivation resulting in different levels of enzyme activity make it possible to differentiate between frozen storage temperatures (−9, −20, and −40 °C) after 30 days of frozen storage. This result could be explained by a decrease in deteriorative processes in the muscle tissue at low storage temperatures. Nilsson and Ekstrand (*19*) found that storage temperature had a marked effect on muscle membranes. They found a higher lysosomal enzyme activity in CTF in fish stored at −18 °C compared to −40 °C after 3 months of frozen storage, indicating increased membrane damage at higher storage temperatures. In the present study it was shown that the activity of cytochrome oxidase was highly influenced by physical and chemical changes in the environment of the enzyme. It seems that the sensitive interactions between cytochrome oxidase and the surrounding membrane lipids in the intact fish muscle make cytochrome oxidase a potential indicator of frozen storage temperature and an ice storage period before freezing.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; CTF, centrifuged tissue fluid; Cyt *c*, cytochrome *c*; JC, just caught; CV, coefficient of variation; JC-F, just caught and frozen; I, ice stored; I-F, ice stored and frozen; SUP, supernatant.

LITERATURE CITED

(1) Mackie, I. M. The effects of freezing on flesh proteins. *Food Rev. Int.* **1993**, *9*, 575−610.
(2) Hultin, H. O. Biochemical deterioration of fish muscle. In *Quality Assurance in the Fish Industry*; Huss, H. H., Jakobsen, M., Liston, J., Eds.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1992; pp 125−138.
(3) Gould, E.; Peters, J. A. *On Testing the Freshness of Frozen Fish*; Grosvenor Press: London, U.K., 1971.
(4) Rehbein, H. Physical and biochemical methods for the differentiation between fresh and frozen thawed fish or fillets. *Ital. J. Food Sci.* **1992**, *4* (2), 75−86.
(5) Haltia, T.; Freire, E. Forces and factors that contribute to the structural stability of membrane proteins. *Biochim. Biophys. Acta* **1995**, *1228* (1), 1−27.
(6) Stanley, D. W. Biological membrane deterioration and associated quality loss in food tissues. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 487−553.
(7) Godiksen, H.; Jessen, F. The effect of ice storage and freeze/thaw cycles on Ca^{2+}-ATPase and cytochrome oxidase activity in salmon (*Salmo salar*). *Ann. Soc. Sci. Færoensis* **2000**, 151−159.
(8) Freedman, R. B. Membrane-bound enzymes. In *Membrane Structure*; Finean, J. B., Michell, R. H., Eds.; Elsevier: Amsterdam, The Netherlands, 1981; pp 259−299.

(9) Paradies, G.; Ruggiero, F. M.; Petrosillo, G.; Quagliariello, E. Enhanced cytochrome oxidase activity and modification of lipids in heart mitochondria from hypothyroid rats. *Biochim. Biophys. Acta* **1994**, *1225*, 165−170.
(10) West, J. L.; Bailey, J. R.; Almeida-Val, V. M. F.; Val, A. L.; Sidell, B. D.; Driedzic, W. R. Activity levels of enzymes of energy metabolism in heart and red muscle are higher in north-temperate-zone than in Amazonian teleosts. *Can. J. Zool.* **1999**, *77*, 690−696.
(11) Sidell, B. D.; Driedzic, W. R.; Stowe, D. B.; Johnston, I. A. Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. *Physiol. Zool.* **1987**, *60* (2), 221−232.
(12) Barbagli, C.; Crescenzi, S. Influence of freezing and thawing on the release of cytochrome oxidase from chicken's liver and from beef and trout muscle. *J. Food Sci.* **1981**, *46*, 491−493.
(13) Rustin, P.; Chretien, D.; Bourgeron, T.; Gérard, B.; Rötig, A.; Saudubray, J. M.; Munnich, A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin. Chim. Acta* **1994**, *228*, 35−51.
(14) Nicholls, P.; Chance, B. Cytochrome *c* oxidase. In *Molecular Mechanisms of Oxygen Activation*; Hayashi, O., Ed.; Academic Press: New York, 1974; pp 479−534.
(15) Bennett, M. C.; Mlady, G. W.; Kwon, Y. H.; Rose, G. M. Chronic in vivo sodium azide infusion induces selective and stable inhibition of cytochrome *c* oxidase. *J. Neurochem.* **1996**, *66* (6), 2606−2611.
(16) King, M. T.; Drews, G. Isolation and partial characterization of the cytochrome oxidase from *Rhodopseudomonas palustris*. *Eur. J. Biochem.* **1976**, *68*, 5−12.
(17) Höchli, L.; Hackenbrock, C. R. Cytochrome *c* oxidase from rat liver mitochondria: purification and characterization. *Biochemistry* **1978**, *17*, 3712−3719.
(18) Godiksen, H.; Jessen, F. Sarcoplasmic reticulum Ca^{2+}-ATPase activity in cod (*Gadus morhua*) muscle measured in crude homogenates. *J. Food Biochem.* **2000**, in press.
(19) Nilsson, K.; Ekstrand, B. The effect of ice storage and various freezing treatments on enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*). *Z. Lebensm. Unters. Forsch.* **1993**, *197*, 3−7.
(20) Kim, U.; Kim, Y. S.; Han, S. Modulation of cytochrome *c*-membrane interaction by the physical state of the membrane and the redox state of cytochrome *c*. *Bull. Korean Chem. Soc.* **2000**, *21* (4), 412−418.

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