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Thermal Denaturation and Aggregation Properties of Atlantic Salmon Myofibrils and Myosin from White and Red Muscles

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Thermal denaturation and aggregation abilities of salmon myofibrils and myosin were studied measuring turbidity, intrinsic fluorescence, 8-anilino-1-naphtalene sulfonic acid binding, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linking. The thermal behaviors of protein preparation from white and red muscles were compared, and the relationship with thermal gelation properties is discussed. The low gelation ability of salmon muscle proteins was related to a limited extent of protein denaturation and aggregation upon heating. These properties seemed to be carried by myosin molecules as a similar behavior was observed for both myofibrils and myosin preparations. The higher thermal stability observed for red muscle proteins with higher transition temperatures in rheological profiles was related to a shift to higher temperature in denaturation and aggregation processes. The extent of denaturation and aggregation was very similar for both muscle types as was the final rigidity of the gels formed.

KEYWORDS: Salmon; muscle; myofibrils; myosin; muscle type; thermal gelation; *Salmo salar*

INTRODUCTION

The architecture of teleost (finfish) fish is unique in the animal kingdom with the contractile units being compartmentalized into short segments called myotomes, separated one from another by soft connective tissue. The muscle fibers are relatively short (2−5 mm) and organized in a parallel fashion, spanning the shortest dimension of the myotome. The red muscle fibers in fish are generally localized superficially, particularly abundant along the lateral line, in a thin subcutaneous layer, whereas the bulk of the fillet interior is comprised of fast-twitch (white) fibers. Pelagic (fatty) species require the ability to swim long distances in search of food and therefore contain a higher proportion of red muscle as compared to demersal (lean, white-fleshed) species.

The thermal gelation ability of fish myofibrillar proteins has been well documented (*1*−*4*). This functional property was shown to occur in a three-step process: (i) dissociation of the contractile protein structure in the presence of salt, (ii) unfolding of protein molecules due to heating, and (iii) aggregation of unfolded protein domains via hydrogen and disulfide bonds, electrostatic and hydrophobic interactions, to form a three-dimensional network (*2*, *5*).

This thermal gelation process has been extensively investigated on crude myofibrils, and molecular mechanisms underlying this property have been investigated using a variety of complementary physicochemical methods including rheology, ultrastructural characterization, and measurements of changes in protein hydrophobicity. Large differences were thus observed between species. Chan et al. (*6*) demonstrated relationships between thermal gelation and denaturation/aggregation processes of fish myosin and myofibrillar proteins. The thermal aggregation appeared to be influenced by the surface hydrophobicity of the unfolded domains and the temperature at which these domains unraveled.

Differences in thermal gelation properties of muscle protein related to muscle type origin (red vs white) have also been reported. Although largely investigated in mammals (*7*−*10*) and birds (*11*−*13*), relatively little information has been published on fish (*3*). In salmonids, myofibrils extracted from red and white muscles exhibit different thermal gelation behaviors (*3*, *14*−*16*). White muscle myofibrils formed gels at lower temperatures than red, but no differences in gel characteristics were observed after heating to 80 °C (*15*). Moreover, white muscle myofibrils are apparently more sensitive to the physicochemical environment (pH and ionic strength) than red muscle. For instance, white muscle myofibrils exhibit a high gelling capacity only at low pH values (pH 5.6) or low ionic strength (≤0.3 M KCl) (*16*).

The physical behavior related to muscle type suggests that differences must exist between the thermal stabilities and the aggregation phenomena for red and white muscle components. Red slow-twitch muscle proteins are generally more heat-stable

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than white fast-twitch muscle proteins. This has been demonstrated by comparisons using differential scanning calorimetry or by measurements of thermal stability of ATPase activity in mammals (*17*) and fish (*18*). Rheological measurements have shown that the transition temperature is 5 °C higher for red muscle proteins than for white muscle proteins (*9*, *15*, *19*). Changes in the surface hydrophobicity of heated red and white rabbit myosin differ, suggesting a potentially greater degree of hydrophobic interaction in white muscle than in red muscle during gel formation (*20*).

Furthermore, myosin was shown to be the major protein involved in thermal gelation (*21*−*25*) in several different species although other muscle proteins can influence the thermal gelation process. Purified myosin formed more rigid gels after heating than those from actomyosin or myofibrils (*19*, *26*). The thermal gelation of purified myosin also depends on muscle type (*19*). This has not been extensively studied, especially in fish, although the biochemical characteristics of fast and slow myosins are very specific. For instance, the temperature dependencies of myosin ATPase of these two muscle types are different (*18*) but the consequence for the thermal gelation process is not known. Differences in thermal gelation behavior between myofibrils from these two muscle types were suspected to be related to distinct denaturation or aggregation characteristics of myosin (*15*), but no data are available to corroborate this hypothesis.

The gelation behavior of demersal (white-fleshed) and pelagic (dark-fleshed) species is different as reported by Chan et al. (*6*), and these differences arise from differential rates of unfolding of the myosin heavy chain (MHC), exposing hydrophobic groups in response to the thermal energy input. Dark-fleshed myosins appear to unfold more slowly upon heating. Accordingly, Chan and Gill (*27*) and Gill et al. (*28*) demonstrated that by slowing down the gelation process, using a low-temperature setting strategy, the thermal gelation of herring muscle could be significantly improved.

The present study was undertaken with salmon muscle as a model system for pelagic species. The aim was to see if denaturation and/or aggregation properties of myofibrils and myosin from white and red muscles could explain their different thermal gelation properties using measurements of turbidity, intrinsic fluorescence (IF), exposure of hydrophobic amino acids, protein−protein interactions, and rheological properties.

MATERIALS AND METHODS

Fish Samples. Atlantic salmon (*Salmo salar*), mean body weight 2 kg, were reared in seawater facilities of the "Aquatron" at Dalhousie University (Halifax, Canada). Fish were anesthetized with eugenol, stunned by a blow on the head, bled by gill arch section immediately after death, and transported on ice to the laboratory. All steps from slaughter were carried out at 4 °C. Fish were eviscerated, and muscles were prepared within a few hours after death. After the skin was removed, the red muscle tissue was carefully dissected along the median line of each side and traces of white muscle were carefully removed. Deep lateral dorsal white muscle was excised from the left fillet. The muscles were minced to prevent any effect due to the heterogeneity between the front and the caudal part, and the protein preparation was performed 24 h postmortem (*16*).

Protein Preparation. Myofibrils were prepared according to the method of Olson et al. (*29*) with minor changes. White and red muscle tissue (≈20 g) were homogenized with a Polytron model PT 10/35 (Brinkmann Instruments, Westbury, NY) at 10000 rpm for 30 s in 10 volumes of a 2 °C isolation medium (20 mM potassium phosphate buffer, 100 mM KCl, and 1 mM EDTA, pH 7.0). The homogenate was centrifuged at 1000*g* for 15 min at 2 °C. The pellet was resuspended in 5 volumes of isolation medium and centrifuged again using the same conditions. The pellet was resuspended in 5 volumes of isolation medium, and the suspension was passed through a strainer, to remove connective tissue and debris. Five more volumes of isolation medium was added to further facilitate passage of the myofibrils through the strainer and centrifuged again using the same conditions. Myofibrils were then washed three times by suspending them in 5 volumes of isolation medium and centrifuging at 1000*g* for 15 min at 2 °C. Finally, the myofibrils were stored in 1 M KCl, 50 mM phosphate buffer (pH 7.0), and 50% (v/v) glycerol at −30 °C.

Myosin from white and red muscles was extracted using the method described by Martone et al. (*30*) except that the first muscle-washing step was replaced by the myofibril preparation procedure described above. The pellet of myofibrils was resuspended in 5 volumes of extraction buffer [0.45 M KCl, 1 mM DDT, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-maleate, pH 6.8] and was kept for 1 h in ice. ATP solution (5 mM final concentration) was added to the mixture at t_0 and t_{30} min. The suspension was centrifuged at 10000*g* for 15 min at 2 °C. The supernatant was passed throuh gauze to remove insoluble debris, then diluted with 25 volumes of 1 mM $KHCO_3$, and kept for 15 min in ice. Precipitated myosin was recovered by centrifugation (12000*g* for 10 min at 2 °C). The pellet was resuspended by 5 volumes of a solution C (0.5 M KCl, 1 mM DDT, and 20 mM Tris, pH 7.5) and kept for 10 min in ice, and then, 3 volumes of 1 mM$KHCO_3$ and Mg^{2+} (10 mM final concentration) was added. The mixture was kept at 4 °C overnight and then centrifuged at 22000*g* for 15 min at 2 °C. The pellet was finally resuspended in 5 volumes of solution C. The extracted myosin was stored in 1 M KCl, 50 mM phosphate buffer (pH 7.0), and 50% (v/v) glycerol at −30 °C.

The composition of purified myofibrils and myosin extracts was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to Laemmli (*31*) using an LKB model 2001 Vertical Electrophoresis Unit equipped with an LKB model 2297 Macrodrive 5 constant power supply.

Protein Content Determination. For rheological measurements, the protein content was determined by the Biuret method (*32*) with bovine serum albumin (BSA) as a standard. For other experiments, protein determinations were performed by the Bradford (*33*) method using BSA as a standard.

Turbidity Measurements. The turbidity was measured with a Hewlett-Packard model 8453 spectrophotometer equipped with a model 89090A temperature controller (Agilent Technologies, Mississauga, ON, Canada). Myofibrils or myosin samples (0.2 g/L) were suspended in deaerated assay buffer (0.6 M NaCl and 50 mM imidazole buffer, pH 6.5), placed in covered glass cuvettes (1 cm path length), and heated continuously in the spectrophotometer at a rate of 1 °C/min from 10 to 70 °C, under agitation, and the change in absorbance at 350 nm (ΔA_{350}) was measured continuously as heating proceeded.

Initial heating trials were made with solution containing 1 g/L of protein, but for red muscle preparations, large aggregates were formed upon heating and precipitated, precluding taking accurate turbidity measurements even with agitation. Subsequently, all turbidity measurement experiments were done at protein concentrations of 0.2 g/L.

IF Measurements. The fluorescence measurements were performed with a Perkin-Elmer LS50 Luminescence Spectrometer (Perkin-Elmer Ltd., Boston, MA) equipped with a data processor and a thermocontrolled cell. Myofibrils or myosin samples (0.2 g/L) were suspended in deaerated assay buffer (0.6 M NaCl and 50 mM imidazole buffer, pH 6.5), and 3.5 mL was placed in a covered cuvette and held at constant temperature for 10 min within a 10−70 °C temperature range. The fluorescence intensity was recorded continuously using excitation and emission wavelengths of 290 and 339 nm, respectively. Both excitation and emission slit widths were set at 2.5 nm (*6*). At each temperature tested, the fluorescence intensity decreased with time to reach a plateau within the 5 first minutes. The value obtained after 10 min (FI 10 min) was considered to be characteristic of each sample at a determined temperature.

8-Anilino-1-naphthalene Sulfonic Acid (ANS) Binding Experiments. Four milliliter samples of contractile proteins, in 0.6 M NaCl and 50 mM imidazole buffer, pH 6.5, were held at constant temperature for 10 min within a 10−70 °C temperature range. Then, a 20 μL aliquot of 8 mM ANS was added, and the samples were mixed and held at

room temperature for 1 h in dark before reading. The fluorescence intensities were recorded using excitation and emission wavelengths of 390 and 470 nm, respectively. The excitation and emission slits were 2.5 and 5.0 nm, respectively. Surface hydrophobicity, S_0 (*34*, *34*), was determined as the initial slope of the plot of fluorescence intensity against protein concentration (0.05−0.25 mg/mL) at each temperature.

1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC) Experiment. The EDC cross-linking approach described by Chan et al. (*35*) was used with some modifications to study the noncovalent cross-linking of myofibrils and myosin from white and red muscles after heating within a 10−70 °C temperature range. A 1 mL aliquot of protein solution containing 2.5 g/L in deaerated assay buffer (0.6 M NaCl and 50 mM imidazole buffer, pH 6.5) was heated for 10 min within a 10−70 °C temperature range. After heating, 10 μL of 454.5 mM EDC (Sigma #39391, St. Louis, MO) dissolved in assay buffer was added to the fish protein solution to obtain a final concentration of 4.5 mM and the mixture was further incubated at the selected temperature for 10 more min. EDC cross-linking was stopped by adding 1 mL of a quenching solution (5% 2-mercaptoethanol, 2.5% SDS, and 8 M urea in 0.1 M Tris/glycine, pH 8.8). An internal electrophoretic standard, 0.5 mL of catalase, 2.04 g/L in the quenching solution, was added to all of the samples, which were subsequently heated at 95 °C for 30 min to ensure complete saturation with SDS. Samples were dialyzed overnight against quenching buffer without urea and electrophoresed on 10% acrylamide gels according to Laemmli (*31*).

Viscoelastic Measurements. Heat-induced gelation of proteins was studied using a controlled-stress rheometer (Bohlin CS rheometer, Malvern Instruments, Malvern, Worcestershire, United Kingdom) in the oscillatory mode (0.1 Hz). Protein samples (15 g/L) in deaerated assay buffer (0.6 M NaCl and 50 mM imidazole buffer, pH 6.5) were heated between the two parallel plates of the rheometer (diameter = 4 cm, gap = 0.1 cm) from 20 to 80 °C at 1 °C/min. Rheological analyses were carried out with a maximum deformation of 3%. Viscoelastic parameters, i.e., storage modulus (G'), loss modulus (G''), and phase angle [δ = Arctan (G''/G')], were recorded continuously. Sample dehydration during heating was prevented by the use of paraffin oil.

Statistical Analysis. When the measurements done at different temperatures were independent, a two-way analysis of variance (ANOVA) was used to test the effects of muscle type and heating temperatures. The significance of differences ($p < 0.05$) between means was tested using a Newman−Keuls test.

For experiments with linear heating (turbidity and rheological measurements), the effect of muscle type was tested with an ANOVA in which dependent variables were figures measured at 20, 30, 40, 50, 60, and 70 °C, considered as repeated measurements due to temperature factor.

RESULTS

Turbidity Measurements. Some studies demonstrated that an increase in absorbance of myofibrillar protein solutions was related to protein aggregation (*6*, *35*, *36*). Moreover, increases in turbidity of heated fish (cod and herring) myosin were suggested to be a consequence of either more and/or larger myosin aggregates (*28*). For myofibrils from both muscle types (**Figure 1A**), a small decrease in ΔA_{350} was observed at temperatures below 30 °C, which then increased with subsequent heating as the myofibrillar proteins aggregated. This increase started at 30 °C for white muscle myofibrils and reached an apparent maximum at 50 °C; then, a decrease in ΔA_{350} was observed at heating temperatures between 50 and 70 °C. The increase in ΔA_{350} for red muscle myofibrils began at 37 °C, increasing linearly to about 45 °C, and then, a plateau was reached in the 45−60 °C range. Subsequent heating of the red muscle myofibrils above 60 °C resulted in large increases in ΔA_{350}. The aggregation capacity for white myofibrils was higher than that for red in the 40−50 °C range ($p < 0.05$) but lower after heating to 70 °C.

Turbidity values for myosin preparations were generally higher than for myofibrils from both muscle types although both

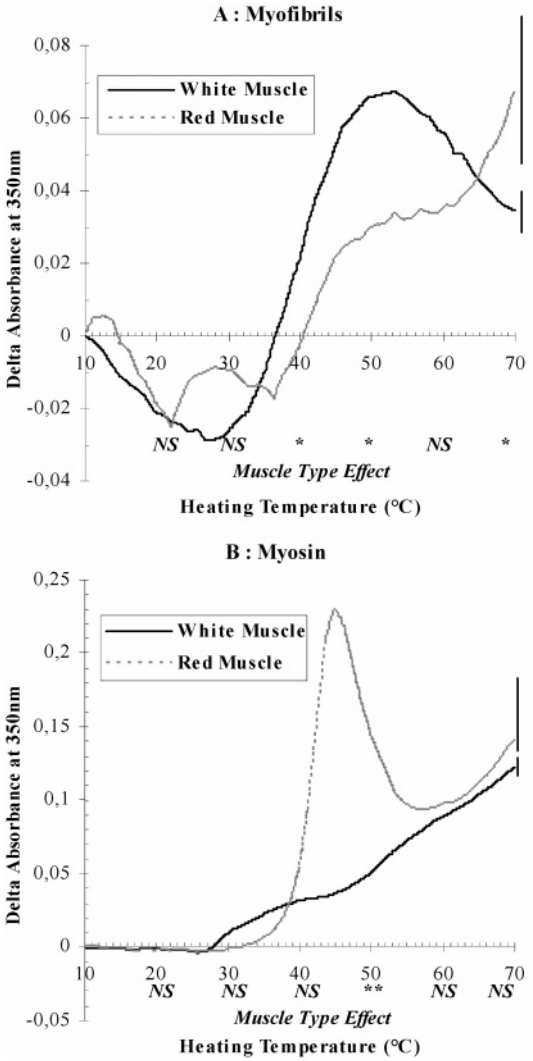

Figure 1. Turbidity of salmon white and red muscles myofibrils (**A**) and myosin (**B**), 0.2 g/L in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5, with a heating rate of 1 °C/min. The vertical bar at the end of each curve indicates the mean standard error. Analysis of variance was done from figures measured at 20, 30, 40, 50, 60, and 70 °C considering each figure as repeated measurement related to temperature; NS, not significant; ∗, $p < 0.05$, ∗∗, $p < 0.01$, and ∗∗∗, $p < 0.001$, $n \geq 5$.

	myofibrils	myosin
muscle type effect	NS	NS
temperature effect	∗∗∗	∗∗∗
MT × temperature effect	∗∗∗	∗

sets of experiments (heated myosin and myofibrils) were carried out with the same protein concentration (0.2 g/L). For myosin preparations (**Figure 1B**), turbidity started to increase from 27 and 34 °C for white and red muscle samples, respectively. For white muscle myosin, turbidity increased almost linearly up to 70 °C, whereas for red muscle myosin, a sharp increase in ΔA_{350} was initially observed from 38 °C, with a peak at 45 °C, followed by a rapid decrease up to 55 °C. This peak was followed by a steady increase between 60 and 70 °C. Despite the obvious differences in behavior between the red and white myosins, there was a great deal of variability among replicate samples and only at 50 °C ($p < 0.01$) were the turbidities higher for red muscle myosin than for white.

Fluorescence Measurements. The major contribution to the IF of proteins comes from aromatic amino acid residues (mainly

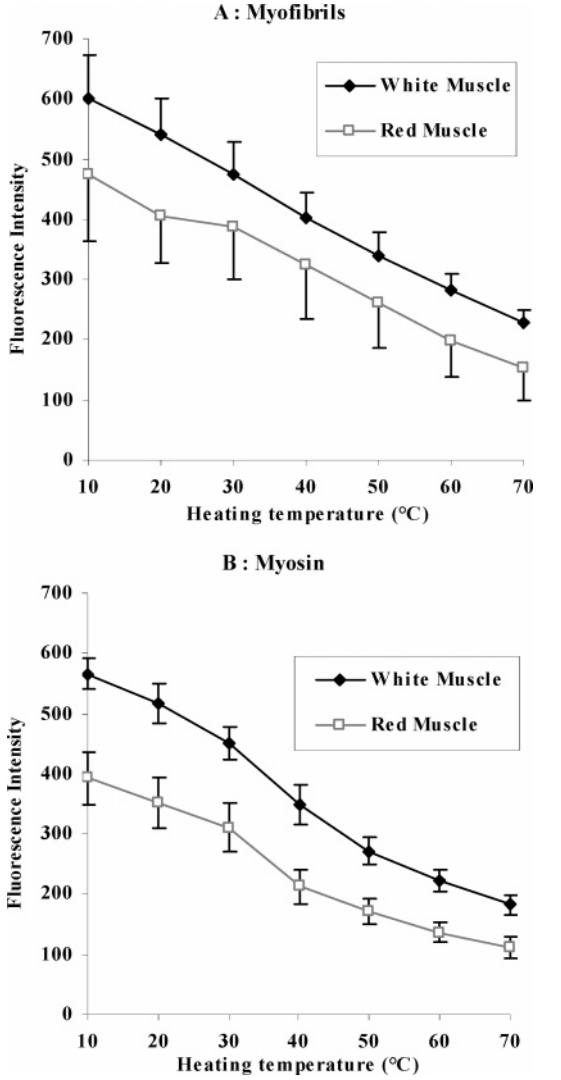

Figure 2. IF intensity of salmon white and red muscles myofibrils (**A**) and myosin (**B**), 0.2 g/L in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5, after a 10 min thermal treatment (mean of three experiments ± standard deviation).

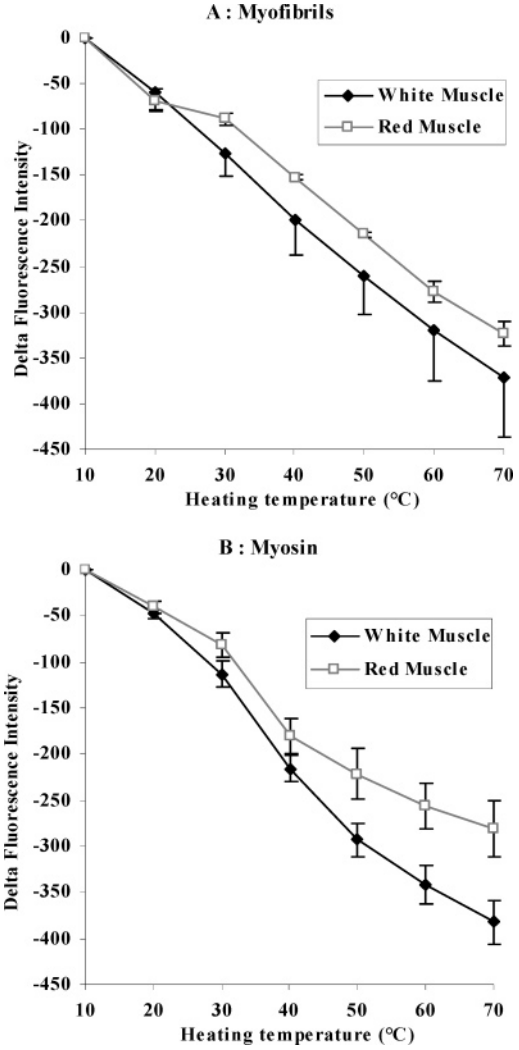

Figure 3. Change in IF intensity of salmon white and red muscles myofibrils (**A**) and myosin (**B**), 0.2 g/L in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5, after a 10 min thermal treatment (mean of three experiments ± standard deviation).

tryptophan), and the location of these aromatic amino acid residues in proteins affects the fluorescence energy (*37*). IF (**Figure 2**) decreased steadily as heating temperatures increased for both the heated myofibril and the myosin suspensions, suggesting a progressive exposure of aromatic amino acid residues as they moved from a moderately hydrophobic to a polar environment. The patterns of loss of IF were approximately parallel although the white myofibrils had higher IF readings ($p < 0.05$) at 20, 60, and 70 °C (**Figure 2A**). The heated white muscle myosin solutions had significantly ($p < 0.01$) higher IF values than the heated red muscle myosin at all heating temperatures (**Figure 2B**). The IF curves for salmon red muscle myofibrils and both red and white muscle myosins were distinctly sigmoidal in shape, suggesting that the protein changes (unfolding and subsequent aggregation) occur in a stepwise manner where the inflection points occur at distinct transition temperatures. One major transition was evident at about 30 °C.

To compare IF changes more directly, mean IF data were expressed as ΔIF relative to the 10 °C readings [i.e., IF (given temperature) − IF (10 °C)] and were plotted in **Figure 3**. This parameter showed clearly that the salmon white muscle myofibrils were more sensitive to heating. These differences were more evident for purified red and white myosins particularly when exposed to higher temperatures of denaturation (**Figure 3B**).

ANS Binding Studies. The ANS surface hydrophobicity, S_0 values, was higher for white muscle myofibrils and myosin than for red muscle ones whatever the temperature considered (**Figure 4**, $p < 0.001$). For both muscle types, S_0 increased with increasing temperature from 30 °C for myofibril preparations and from 20 °C for myosin samples, suggesting the progressive exposure of aromatic hydrophobic amino acid residues during the heating process. The effect of heating temperature was not significant ($p > 0.05$) for white muscle myofibrils probably because of the high variability observed with these samples, but a significant effect of heating temperature was obtained for red muscle myofibrils ($p < 0.05$). For myosin preparations, the effect of heating temperature on S_0 values was significant for both muscle types ($p < 0.05$).

When considering the relative change in ANS hydrophobicity due to heating temperature (**Figure 5**), where $\Delta S_0 = [S_0$ (given temperature) $- S_0$ (10 °C)], red muscle myosin exhibited a higher increase in S_0 value after heating at temperatures ≥50 °C (**Figure 5B**, $p < 0.05$), although no significant heating effect was observed for myofibrils. The changes in ANS S_0 values due to heating were higher for myosin preparations than for myofibrils.

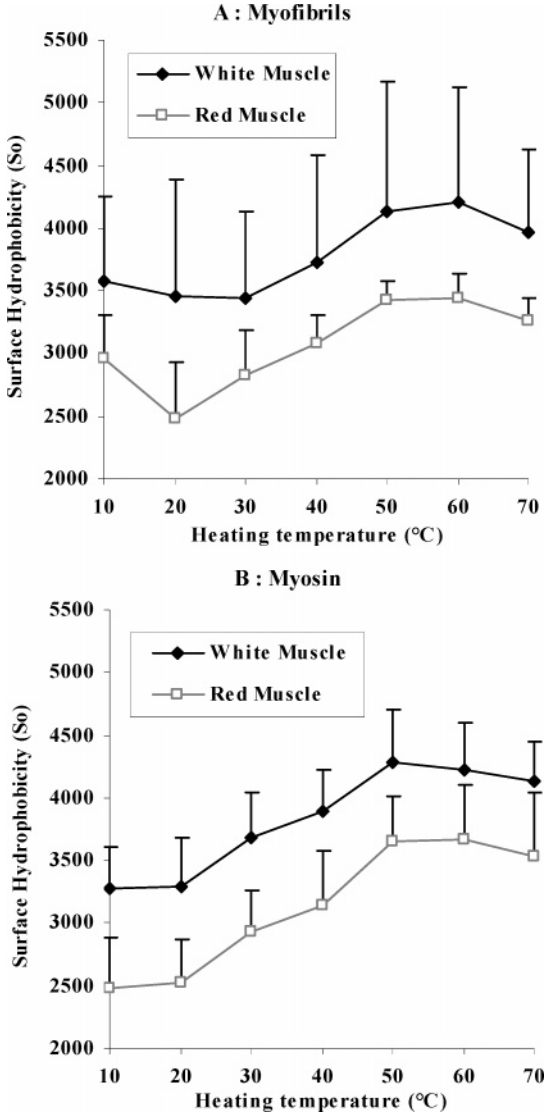

Figure 4. ANS surface hydrophobicity (S_0) of salmon white and red muscles myofibrils (**A**) and myosin (**B**) in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5, after a 10 min thermal treatment (mean of three experiments ± standard deviation).

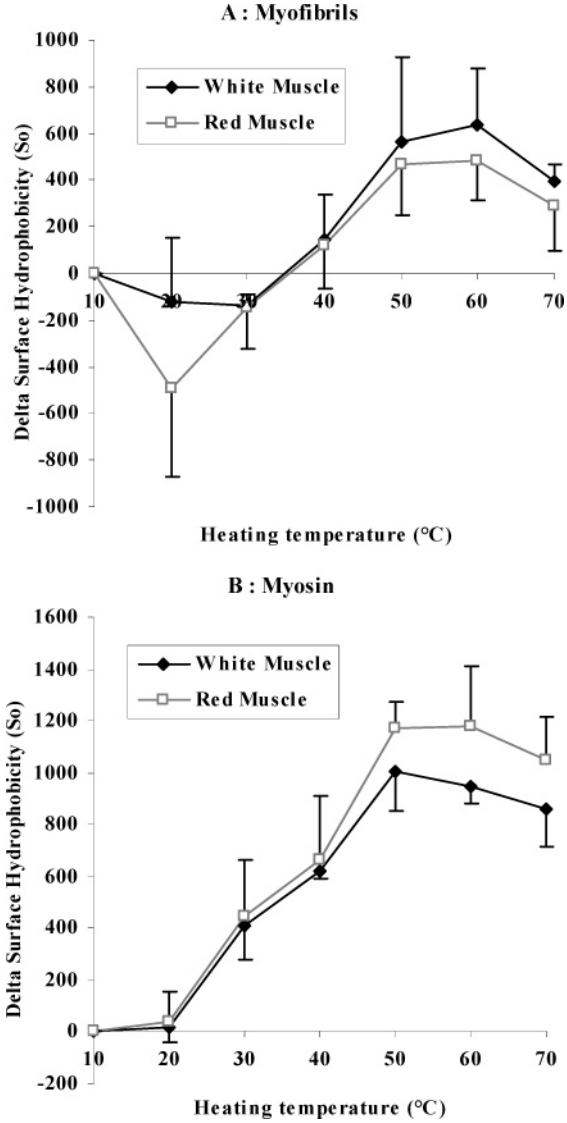

Figure 5. Change due to heating in ANS surface hydrophobicity (S_0) of salmon white and red muscles myofibrils (**A**) and myosin (**B**) solutions in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5 (mean of three experiments + standard deviation).

EDC Experiment. Heating myofibrillar protein samples with EDC as a "zero-length" covalent cross-linker is a means to electrophoretically examine the noncovalent interactions in heated muscle proteins (*25*) since EDC cross-linking can only take place between proteins that are already in intimate contact (2−5 Å) and will not cross-link noninteracting proteins. Heating led to the progressive noncovalent cross-linking and disappearance of the MHC (MW, ∼200 kDa). The disappearance of these bands was concomitant with the development of polymerized material at the top of the gel for samples heated in the 20−50 °C temperature range. In addition, a decrease in the actin band was evident for samples heated above 40 °C (**Figure 6A**,**B**). The electrophoretic patterns suggest the formation of high molecular mass aggregates at intermediate and higher temperatures. All of the myofibrillar proteins disappeared from the gels, suggesting the formation of polymers that were too large to enter the 10% acrylamide. Note that the internal standard, catalase, added after protein polymerization, remained unaffected. Also, as expected, samples not receiving the EDC treatment (C10 and C70) were unaffected by heating, indicating that the changes were due only to noncovalent interactions.

Similar results were obtained with myosin samples (**Figure 6C**,**D**). The band corresponding to the MHC was not visible after heating at temperatures ≥40 °C, and a band of high molecular mass aggregates disappeared when the heating temperature was above 50 °C. A band of high molecular mass aggregates was present even for control without EDC, suggesting a spontaneous covalent polymerization of myofibrillar proteins with storage for this muscle type.

Thermal Gelation Experiments. For myofibril samples (**Figure 7A**), the main differences in rheological behavior between muscle type were the ability of white muscle myofibrils to form a gel at 30−40 °C with a maximum G' at 35 °C. The local thermal transition for red muscle myofibrils was less pronounced and was about 2 °C higher, thus demonstrating a slightly higher thermal stability. After heating to 80 °C, there was no significant difference in storage modulus (G') values between muscle types but red muscle myofibrils formed more elastic gels (lower phase angle, δ, values) than white muscle ones ($p < 0.01$). For myosin preparations (**Figure 7B**), no gel formation was observed at low temperature, whatever the muscle type, and differences in thermal stability between muscle types

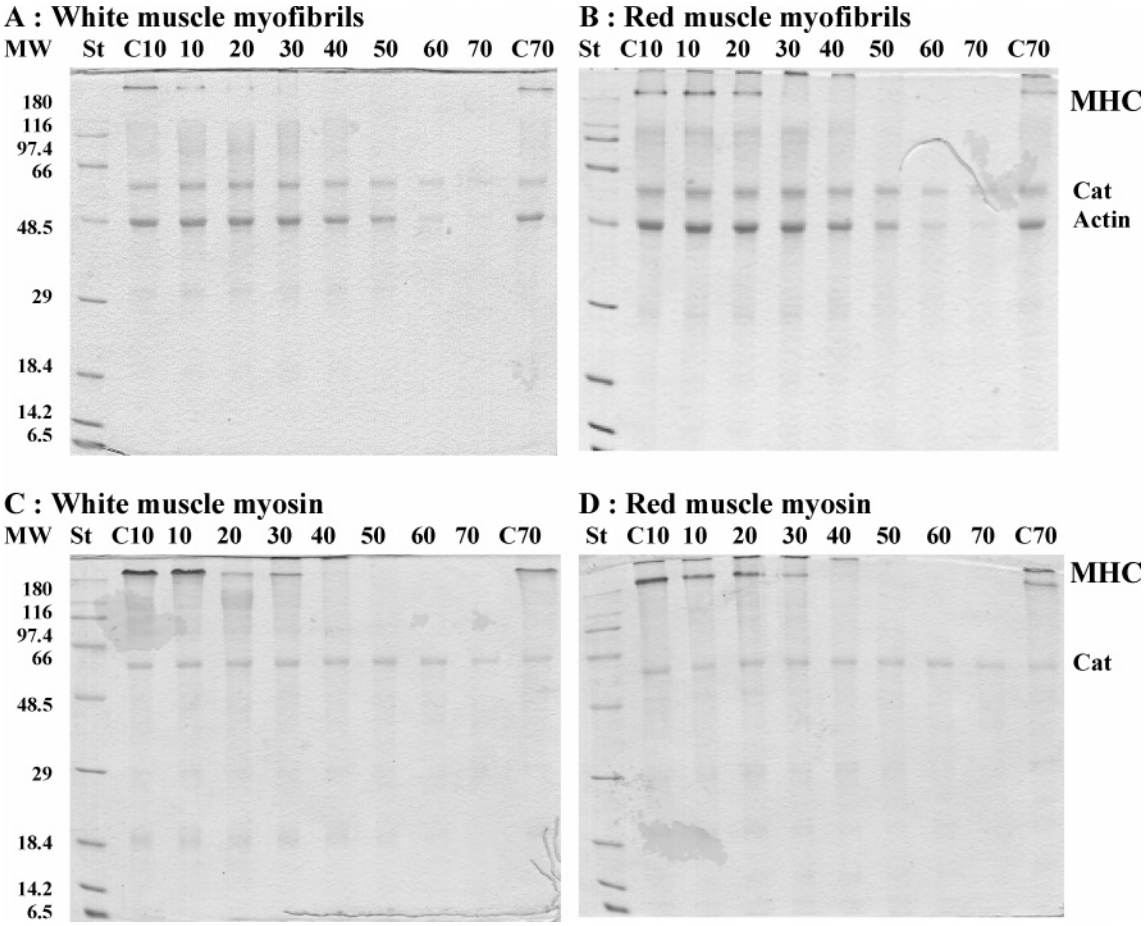

Figure 6. SDS-PAGE on 10% acrylamide gel of salmon white and red muscles myofibrils (**A** and **B**) and myosin (**C** and **D**) samples from EDC experiment. MW, molecular mass in kDa; St, molecular mass standard; numbers 10–70 correspond to heating temperatures for each sample; C10 and C70 are control without EDC incubated at 10 and 70 °C, respectively; Cat, internal electrophoresis standard catalase; and Act, actine.

were much less evident. Indeed, no overall significant effect of muscle type was measured even if G' values tended to be higher for white muscle myosin at low temperature ($p = 0.059$ at 30 °C) and δ values were lower for red muscle samples at higher temperature ($p < 0.05$ at 70 °C). Rheological behavior of myosin preparations differed from myofibrils ones as no important thermal transition at low temperature was observed. However, no significant difference in rheological parameters was observed after heating to 80 °C between myofibrils and myosin samples.

DISCUSSION

Effect of Heating on Denaturation and Aggregation of Salmon Muscle Proteins. The aim of this study was to clarify whether the denaturation and aggregation properties of salmon muscle protein could explain some specificity of thermal gelation of salmonids myofibrils. Thermal gelation experiments showed that salmon muscle proteins had some complex rheological profiles. The profiles observed here for salmon myofibrils were similar to those obtained with brown trout muscle proteins (*14*, *15*), except that with brown trout white muscle myofibrils, a single peak in rheological profile was observed at 36 °C (*15*) whereas two transitions were observed in the present study: a shoulder at 29 °C and a peak at 35 °C. Salmon red muscle myofibrils showed a localized maximum in G' at 36 °C followed by a dip and then steady increase in rigidity from 43 to 80 °C. No similar low-temperature setting was observed with brown trout proteins (*15*). This low-temperature setting phenomenon at low temperature ("suwari" in Japanese) is a result of the initial formation of a three-dimensional protein network immobilizing a large proportion of the free water. One explanation for the differences observed between the trout and salmon data may be the different pH (6.0 vs 6.5, respectively) used in these studies, as it was shown that higher pH values favored protein interaction and gelation at low temperature (*16*). It is also interesting to note that there was no evidence for the gel-softening phenomenon in the 60 °C range ("modori" in Japanese) sometimes evident due to the presence of heat-stable alkaline proteases in certain fish species. The rheological data shown in **Figure 7** suggest that initial gel formation is primarily due to the interaction of white muscle proteins and tends to suggest that at least for salmon, the other (nonmyosin) components play a key role in initial setting since no low-temperature setting was observed for the purified myosin samples.

Thermal denaturation of myofibrils and myosin was shown to be a progressive phenomenon. Aromatic amino acid exposure, as measured by IF, was almost linear. Such a progressive process was previously obtained for cod, herring, and silver hake myosin (*6*) and suggest the continuous exposure of aromatic amino acids to an exterior polar environment. On the contrary, carp actomyosin demonstrated that IF changes over a narrow (30−50 °C) temperature range. It has been suggested that for carp, this may be due to a greater concentration of aromatic amino acid residues in the head portion of myosin, and their exposure due to the unfolding of a non-α-helical portion of the molecule (*38*). An increase in surface hydrophobicity (ANS binding) due to heating also revealed a progressive exposure of aromatic hydrophobic amino acid residues during the heating process but was limited to a more narrow range of temperature (20−50 °C).

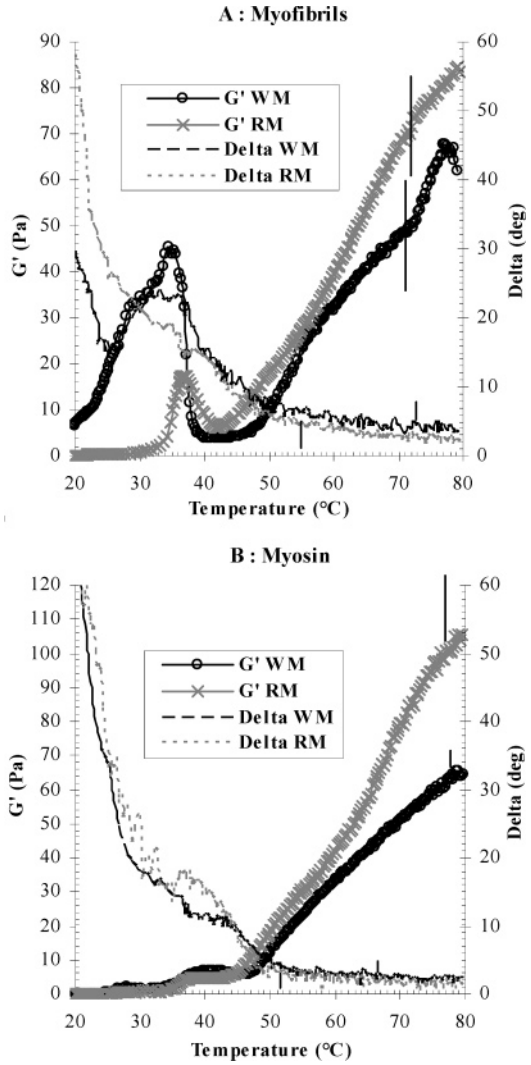

Figure 7. Thermal gelation profile of salmon white (WM) and red (RM) muscles myofibrils (**A**) and myosin (**B**) 15 g/L in 50 mM imidazole buffer and 0.6 M NaCl, pH 6.5, with a heating rate of 1 °C/min ($n \geq 3$). The vertical bar on each curve indicates the mean standard error. Analysis of variance was done from figures measured at 20, 30, 40, 50, 60, 70, and 80 °C considering each figure as a repeated measurement related to temperature:

	myofibrils		myosin	
	G'	δ	G'	δ
muscle type effect	NS[a]	NS	NS	NS
temperature effect	***[b]	***	***	***
muscle type × temperature	NS	NS	NS	$p = 0.095$

[a] NS, not significant. [b] ***, $p < 0.001$, $n \geq 3$.

Similar observations were previously reported for chicken breast and leg salt-soluble proteins (*39*), actomyosin from carp and flying fish (*40*), and myosin from tilapia (*41*, *42*) and rabbit (*20*). Such a phenomenon suggests a rapid unfolding of proteins within this temperature range (*39*). Moreover, a plateau or even a slight decrease in ANS hydrophobicity at temperatures >50 °C has also been reported for tilapia myosin (*41*). The decrease in ANS fluorescence intensity at high temperature was apparently not due to thermal quenching effects (*41*) but perhaps to a decrease in accessibility of hydrophobic groups probably due to their involvement in aggregation (*34*). However, such a phenomenon was not observed in IF measurements for which the decrease was linear up to 80 °C.

Thermal aggregation of salmon myofibrils and myosin, measured by turbidimetry, appeared to be a discontinuous phenomenon with very different shapes of curves depending on the muscle type or the kind of preparation. Turbidity increased above a given temperature threshold and then stabilized or decreased at high temperature. It is interesting to notice that the threshold temperature measured with turbidity was different from the temperature observed for the onset of thermal gelation, as measured rheologically. That is, the rheological data in **Figure 7** suggest a "typical" suwari that begins at about 22 °C for white myofibrils. Turbidity data for white myofibrils do not begin to rise until 30 °C, and these apparent differences between different types of measurements may be due to differences in protein concentrations for the two tests (rheological tests carried out at 15 g/L; turbidity tests at 0.2 g/L). Use of higher concentrations for turbidity tests on salmon protein was precluded since higher concentrations resulted in protein precipitation without heating. Comparison of the present results with previously published data is also difficult as most of the experiments have been carried out under different conditions, protein concentrations, and with various fish species. Moreover, with croaker actomyosin, Liu et al. showed that the change in turbidity due to heating is greatly influenced by protein concentration and the temperature at which turbidity started to increase is also concentration-dependent (*43*). Indeed, even with very low protein concentrations, decreases in A_{350} were observed for white muscle myofibrils between 10 and 28 °C and at temperatures >55 °C and for red muscle myosin solutions at intermediate temperatures (45−55 °C range). Such unexpected decreases in turbidity have also been observed for whiting myosin after a maximum value obtained around 50 °C (*4*). The decreases in absorbance may be due to the precipitation of very big aggregates at various stages in the heating regime. Although it is tempting to implicate the possible presence of modori-forming proteases, there was no rheological evidence for this phenomenon in salmon. Except for white muscle myosin, the increase of turbidity was limited to a narrow temperature range. White muscle myofibrils showed an increase in turbidity only in the 30−50 °C temperature range. A similar observation, but in a different temperature range, was already observed with arrowtooth flounder myosin, which aggregated between 23 and 40 °C (*44*). For red muscle myofibrils and myosin, a narrow range of temperature concerned by a turbidity increase was also observed but in two steps in the 37−45 and 65−70 °C ranges for both myofibrils and myosin. This biphasic increase in aggregation process has already been observed with myosin from black marlin (*18*) and chicken breast muscle (*45*) and was shown to be related, for chicken myosin, to the presence of myosin light chains as a continuous increase in turbidity was measured with MHC alone (*45*).

EDC experiments showed that the MHC was the first and the principal myofibrillar protein cross-linked during a heat treatment, which is consistent with results obtained with myofibrils and myosin from cod, silver hake, and herring (*35*). These authors showed that the cross-linking ability and the size of the MHC polymers formed were species-dependent, probably due to the inherent differences of MHC from different species (*35*). In that study, it was shown that the cod MHC polymerized during heat treatment at 50 °C prior to the involvement of actin or any of the other myofibrillar proteins. Electrophoretic data also demonstrated that actin and other low molecular mass components interacted preferentially with aggregated myosin

rather than self-associating to form high molecular mass polymers (*25*). In the present study, the formation of high molecular mass compounds was observed only at intermediate temperature probably because heating at higher temperature led to very high molecular mass compounds, which did not enter the stacking gel. Some trials were done to analyze the size of the polymers formed with open-pored agarose−polyacrylamide composite gels, but no bands were detected with this method, suggesting the formation of very high molecular mass compounds with salmon muscle proteins.

As compared with other fish species, isolated proteins from salmonids muscle formed weak gels after heating (*3*, *14*−*16*) and this is confirmed in this study. Salmonids are pelagics and as such are considered to have poor gelling ability for the production of surimi-based products (*46*−*48*). This has been related to the low activity of endogenous transglutaminase, an enzyme implicated in covalent cross-link between MHC. Moreover, salmon contains less calcium (necessary for transglutaminase activity) and more anserin (a powerful inhibitor of transglutamisase) (*47*−*49*). Another explanation could be that salmonid muscle proteins have a limited exposure of hydrophobic amino acids due to heating and therefore a low aggregation capacity. Indeed, the extent of denaturation and aggregation phenomena measured in this study appeared to be low as compared with other studies on mammals (*20*) and even with some other fish species (*6*, *35*, *38*). In some case, but not all, the difference could be due to different heating methods; the linear heating rate applied in some study (*6*, *20*) allows a progressive and more complete unfolding of protein molecules than the constant heating temperature method that we applied for IF and ANS biding experiments. Limited interaction ability could be related to characteristic of the primary sequence of myosin as it was shown in chum salmon that MHC had several residues substituted to glycine especially in the HMM region (*50*). This small neutral amino acid could participate in the instability of the molecule and does not favor the formation of interaction. So, to fully understand the molecular mechanisms underlying the specific thermal gelation behavior of salmonids muscle proteins, it would be necessary to get the complete sequence of red and white muscle myosins and to study all of the kind of interactions involved in the formation of a thermal gel. Indeed, the importance of the effect of pH and ionic strength revealed that electrostatic interactions are determinant in thermal gelation at low temperature but also for the final rigidity of the gel after heating, especially for white muscle myofibrils. So, further studies are needed including the thermal unfolding of myosin and subfragments by circular dichroism to better understand if the primary amino acids sequence of white muscle myosin could explain the low-gelling ability of salmonid muscle preparations.

Differences between Myosin and Myofibrils (Table 1). Turbidity curves of myofibrils and myosin samples were very different as were the thermal gelation profiles. Rheological profiles of myosin samples gave no peak at low temperature, and similar results were also observed with carp (*36*) and rabbit (*20*). The presence of actomyosin filaments in the myofibril samples could be responsible for the first sharp increase in gel rigidity; our result seemed to confirm this. As myosin was shown to be the main actor of muscle protein gelation (*21*−*25*), myosin solution behavior upon heating would allow one to better understand the behavior of the complex myofibrils system. In our work, myofibrils and myosin preparations exhibited quite similar denaturation and aggregation behaviors, which is consistent with their similar gelling ability. Myosin solutions demonstrated a higher aggregation ability (measured by turbidimetry) and a slightly higher thermal increase in ANS surface hydrophobicity, as compared to myofibrillar solutions. This little difference did not lead to a significantly higher gelation capacity of myosin solution. This similar gel rigidity obtained for myofibrils and myosin samples was in contradiction with results obtained on mammals species for which three- and six-fold more rigid gels were obtained between myofibrils and purified myosin from beef and rabbit, respectively (*19*, *26*). Two hypotheses may explain such an observation: (i) Salmonids were shown to have poor gelation capacity (*46*), and this could be inherent to low gelation capacity of myosin in relation with its specific amino acid sequence (*50*); (ii) pH conditions used in this study were demonstrated to be far from optimal values obtained for salmonids muscle proteins (*16*), and this might not allow the expression of correct thermal gelation of myosin. Moreover, a previous study with salmonids muscle protein showed that, surprisingly, the extraction of myofibrillar proteins, as compared with raw myofibrils, did not improve the gelation ability of trout protein, suggesting that salmonids myofibrils were not very sensitive to the presence of contaminants (*14*). The present work compared for the first time the thermal behavior of myofibrils and myosin preparation in a salmonids, and further studies will be needed to confirm if the similar behaviors of these preparations are due to intrinsic properties of proteins molecule or are very dependent on the physicochemical environment.

Table 1. Summary of the Effects of the Muscle Type (White Muscle or Red Muscle) and the Type of Preparation (Myofibrils or Myosin) Obtained with All of the Methods Tested[a]

	white vs. red muscle		myofibrils vs. myosin sample	
method	myofibrils	myosin	white muscle	red muscle
turbidity	thermal stability: RM > WM		global extent of aggregation: myosin > Myof	
	global extent of aggregation: RM ≥ WM		curve shape ≠	
IF	thermal stability: RM > WM			
	absolute value: WM > RM		absolute value: Myof > myosin	
	decrease due to heating (>30°C): WM ≈ RM	decrease due to heating (>30°C): WM > RM	similar decrease due to heating	decrease due to heating (>40°C): Myof > myosin
ANS	absolute value: WM ≈ RM	absolute value: WM ≥ RM	absolute value: Myof ≈ myosin	
	increase due to heating (>30°C): WM ≈ RM	increase due to heating (>40°C): RM > WM	increase due to heating: myosin > Myof	
EDC	similar		similar	
rheology	thermal stability: RM > WM		no big thermal transition at low temperature for myosin	
	gel rigidity after heating: WM ≈ RM		gel rigidity after heating: Myof ≈ myosin	

[a] RM, red muscle; WM, white muscle; and Myof, myofibrils.

Effect of Muscle Type (Table 1). Our results confirmed a higher thermal stability for salmonids myofibrils from red muscle as compared with white muscle ones and showed a similar result for myosin. This appeared with, for proteins from red muscle, a higher threshold temperature for the onset of aggregation, measured by turbidity, and a shift in IF curves toward higher temperature. So, white muscle proteins denatured and aggregated at a lower temperature, which is consistent with their ability to form gels at lower temperature and with lower transition temperatures in rheological profiles. These results are consistent with most of the published data comparing proteins from different muscle types (*9*, *15*, *17*−*19*, *39*, *51*).

Moreover, myofibrils and myosin from red and white muscles exhibited some differences in their thermal behavior. For thermal gelation, the shape of the rheological profiles varied depending

on muscle type but a similar rigidity was obtained after heating to 80 °C for both red and white muscles myofibrils and myosin, which was in agreement with results obtained with brown trout myofibrils (*14*−*16*). The differences in aggregation profiles that we observed between muscle types seem to be classically observed (*17*, *39*, *51*, *52*) but depend on the physicochemical environment (*17*). The total extent of aggregation after heating to 70 °C was higher for red muscle myofibrils than for white muscle ones, but no significant difference was observed between white and red muscle myosins. This higher aggregation capacity of proteins from red muscle was also observed with poultry SSP and myosin (*39*, *51*), but this parameter does not appear determinant in thermal gelation ability, as a higher gelling ability is generally observed for white muscle proteins. The study of denaturation also showed some differences between muscle types. Significant differences in absolute values of IF and surface hydrophobicity were measured between muscles types, presumably due to differences in amino acid composition of myosin isoforms. Moreover, decreases of IF values were slightly higher for both white muscle preparations as compared to the red ones. On the contrary, an increase in ANS surface hydrophobicity due to heating tended to be higher for red muscle proteins, which is in contradiction with results obtained with rabbit myosins (*20*). So, the small difference measured in thermal unfolding, by IF, for white muscle myosin might be counterbalanced by the higher thermal increase in surface hydrophobicity for red muscle myosin, and finally, no difference in thermal gelation ability was observed between myosins from red and white muscles.

In conclusion, the present work showed that the low gelation ability of salmon muscle proteins was related to a limited extent of protein denaturation upon heating, associated with a limited extent of aggregation. These specific thermal behaviors seemed to be carried by myosin molecules, as a similar behavior was observed for both myofibrils and myosin preparations. Comparison of preparations from red and white muscles, studied presently for the first time, showed that higher transition temperatures measured in rheological profiles for red muscle proteins were related to a shift to higher temperature in denaturation and aggregation processes for proteins from this muscle type. Red and white muscle myofibrils and myosin demonstrated similar gel rigidity after heating, which was coherent with similar aggregation properties. Denaturation experiments showed distinct behavior of proteins from the two muscle types. White muscle myosin showed a higher extent of aromatic amino acids exposition but a lower increase in surface hydrophobicity during heating process. The balance of these two opposite tendency might explain the similar gelling ability between proteins from the two muscle types.

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