Raman Spectroscopic Study of Changes in Fish Actomyosin during Setting

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Actomyosins (AMs) isolated from tilapia, lemon sole, ling cod, and rock fish were heated at 40 °C, and structural changes in AMs were investigated using Raman spectroscopy to elucidate low-temperature gelling phenomenon, that is, "setting", of surimi. The following conformational transitions were observed in lemon sole, ling cod, and rock fish gels during setting: a slow unfolding of α-helix and exposure of hydrophobic amino acid residues occurring in long-time incubation at 40 °C, thereby forming hydrophobic interactions among AM molecules. In addition, the most frequent conformation in disulfide bonds was gauche–gauche–trans (g–g–t) form in the set gel. On the other hand, tilapia AM did not form a gel with heating at 40 °C, its α-helical structure in the myosin being far more stable than that of the other species. The heat resistance of the tight α-helix may prevent the gelation of tilapia AM. It is, therefore, likely that the unfolding of the α-helix of myosin is a prerequisite for gelation of AM during setting.

Keywords: *α-Helix; hydrophobic interaction; disulfide linkages; gel; setting; surimi*

INTRODUCTION

Rheological changes from sol to gel are referred to as setting when salted surimi, a fish meat paste, is maintained at a low temperature around 40 °C or at refrigerated temperature. Setting is an important process for surimi-making due to enhancement of the gel strength of kamaboko. It has been hypothesized that setting is induced by hydrophobic and sulfhydryl–disulfide exchange reactions of actomyosin (AM) molecules in fish meat (Itoh et al., 1979; Niwa et al., 1981, 1983). Taguchi et al. (1978) reported that the interaction between myosin and actin is related to the setting. Seki et al. (1990) suggested a deep involvement of transglutaminase (TGase) in the muscle for the setting of surimi. A decrease of the myosin heavy chain (MHC) was observed along with the production of cross-linked MHC during setting, probably owing to the action of TGase (Numakura et al., 1985). However, setting occurred even without TGase (Nowsad et al., 1994). Setting properties of fish meat are dependent on species (Shimizu et al., 1981, 1983). Recently, Ogawa et al. (1995) reported that an unfolding of α-helix in myosin molecules would initiate setting. Because setting involves complex reactions by forming intra- and inter-molecular interactions of myosins, further clarification of the mechanism of setting phenomenon is necessary.

Laser Raman spectroscopy is useful in studies of proteins in aqueous solution and does not require chemical modifications of proteins, especially at high protein concentrations. Bands from amide I, amide III, and C–C stretching modes can be measured to investigate the changes in the peptide backbone conformation. Information on the environment of the aromatic side chains of tyrosine (Tyr) and tryptophan (Trp) as well as the conformation of the disulfide bond can be also obtained from the Raman spectra. During thermally induced gelation, proteins undergo structural changes, for example, exposure of functional groups followed by protein–protein interactions. Thus, conformational studies of thermal gelation have been conducted for many globular proteins using Raman spectroscopy (Chen et al., 1973; Li-Chan and Nakai, 1991; Nonaka et al., 1993).

Recently, Bouraoui et al. (1997) and Careche and Li-Chan (1997) demonstrated that such spectroscopic studies may allow assessment of the structural changes in fibrous proteins, such as gel formation of surimi-based products. The objective of this study was to investigate the mechanism of conformational changes of fish AMs during setting using laser Raman spectroscopy.

MATERIALS AND METHODS

Materials. Four species of fish, tilapia (*Tilapia nilotica*), lemon sole (*Microstomus kitt*), ling cod (*Ophiodon elongatus*), and rock fish (*Sebastes maliger*), were used as samples immediately after post-mortem in this study. All chemicals used were of analytical grade.

Preparation of Fish AM. AM was separated from the dorsal muscle according to the method of Sano et al. (1986) after modifications as follows. Fish muscle (100 g) was homogenized in 500 mL of 50 mM NaCl/20 mM sodium

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phosphate buffer (pH 6.8) containing 0.05 mM phenylmethane-sulfonyl fluoride (PMSF) in a Waring blender, and the macerate was centrifuged at 10000g for 5 min. These procedures of homogenization and centrifugation were repeated once more. The resultant residue was homogenized in 1 L of 0.6 M NaCl/20 mM sodium phosphate buffer (pH 6.8) in the Waring blender, and the suspension was centrifuged at 10000g for 5 min. The supernatant was filtered through triple-layer cheesecloth. The supernatant was diluted with 6 L of 20 mM sodium phosphate buffer (pH 6.8) with constant agitating. The precipitate was collected by centrifugation at 10000g for 10 min and then suspended in 500 mL of 50 mM NaCl/20 mM sodium phosphate buffer (pH 6.8). The washed precipitate was collected by centrifugation at 10000g for 10 min. All procedures were carried out at 4 °C.

Setting Conditions and Gelling Properties. Gelling properties of AM were measured as gel strength (N·mm). The purified AM was concentrated by centrifuging at 30000g for 60 min. The AM was ground with NaCl adjusted to the final concentration of 2.5% (w/w). After the AM paste thus prepared was used to fill a 2.0 mL plastic microcentrifuge tubes (10 mm diameter), the tubes were centrifuged at 5000g for 1 s to deaerate. For setting, the samples were heated at 40 °C for 30 min in a thermostated water bath. After the heated samples were cooled in an ice–water bath for 60 min, the bottoms of the tubes were cut, and the gel formed inside was squeezed out. The released cylindrical gels were cut to the length of 8 mm, except 12 mm for ling cod.

Gel strength was measured by the puncture test using a TA-XT2 texture analyzer (Stable Micro Systems, Surrey, England) with a cylindrical plunger (2 mm in diameter). The plunger resting on the cylindrical gel sample moved at a speed of 3 cm/min. The breaking force (N) and the indentation (mm) were taken, and the product of these values (N·mm) was defined as the gel strength.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). AM sample (20 mg) was mixed with 0.4 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 10% SDS (w/v), 8 M urea, and 2% β-mercaptoethanol and heated at 90 °C for 5 min followed by mechanical shaking overnight at room temperature. After centrifugation at 15000g for 10 min, the supernatant was applied for SDS–PAGE. The electrophoresis was carried out according to the method of Laemmli (1970) using 10% acrylamide separating gel. The gel was stained with 0.025% Coomassie brilliant blue R-250.

Protein concentration was determined according to the modified biuret method of Umemoto (1966).

Circular Dichroism (CD). The purified AM was diluted to a protein concentration of 0.2 mg mL^{-1} with 0.6 M NaCl/20 mM sodium phosphate buffer (pH 6.8). CD spectra were taken with a J-500A spectropolarimeter (JASCO, Tokyo, Japan). A 1 mm path length quartz cell in a water-jacketed cell holder was used to maintain a constant temperature by circulating water at 40 °C. The instrument was calibrated using d-10-camphorsulfonic acid, $[\theta]_{290.5} = 7800$ (deg·cm²·dmol^{-1}). Molar ellipticities of AM were determined with an assumption of a mean residue weight of 115 (g·mol^{-1}).

Raman Spectroscopy. The purified AM sample was sedimented to 8–12% protein concentration by centrifugation at 30000g for 60 min and was ground with NaCl adjusted to the final concentration of 2.5% (w/w). After the sample was packed into hematocrit capillary tubing (Nichiden-Rika Glass Co., Tokyo, Japan), both ends of the tubing were heat-sealed. The sealed dispersions were then heated in a water bath held at 40 °C for 1, 5, 10, 20, and 30 min. After heating, the samples were kept in an ice–water bath until measurement of the spectra.

Raman spectra were recorded on a model NR-1100 laser Raman spectrophotometer (JASCO) with excitation emitted from the 488-nm line of a Spectra-Physics model 168B argon ion laser (Spectra-Physics, Mountain View, CA). The Raman scattering of the samples in a transverse/transverse arrangement (capillary held horizontally and incident laser beam perpendicular to the capillary axis) was measured. The conditions used were as follows: incident laser power, 200 mW; slit
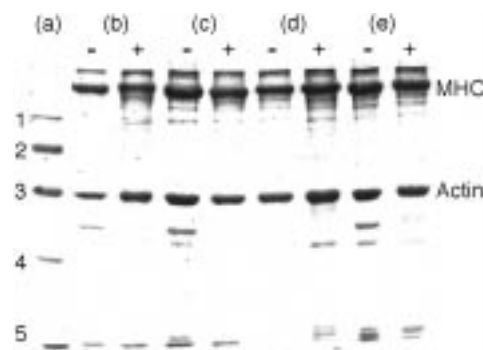

Figure 1. SDS–PAGE of AM using 10% acrylamide gel: (a) molecular weight markers; (b) tilapia; (c) lemon sole; (d) ling cod; (e) rock fish. + and − indicate AM samples with and without heating at 40 °C for 30 min, respectively. The molecular weight markers used were (1) phosphorylase b (94000), (2) bovine serum albumin (67000), (3) ovalbumin (43000), (4) carbonic anhydrase (30000), and (5) soybean trypsin inhibitor (20100).

height, 4 mm; spectral resolution, 5.0 cm^{-1} at 19000 cm^{-1}; sampling speed, 120 cm^{-1}·min^{-1} with data collected at every cm^{-1}. The sample mounted in the spectrometer was thermostated at 4 ± 0.5 °C using a JASCO temperature controller model RT-IC. Five scans were averaged for the regions 450–1800 and 2450–3350 cm^{-1}. The Raman spectra of the corresponding solvents were also taken. All computations on the recorded spectra were performed using Grams/386 (Galactic Industries Corp., Salem, NH) on an IBM-compatible computer. All spectra were corrected for solvent background by subtracting of the spectrum of the buffer.

The intensity around 1660 cm^{-1}, arising from the HOH-bending vibration, of the buffer spectrum was by far weaker than that around 1660 cm^{-1} of the sample spectra. This process allowed the analysis of the amide I band without deuteration to remove the superimposing H$_2$O band. The criterion for the exact amount of water subtraction was satisfied as a flat baseline in the region 1750–1800 cm^{-1} (Barrett et al., 1978). The HOH-bending vibration exists in this region, and there is no competition from any of the protein bands, including the amide I. Proper solvent subtraction, therefore, should provide a flat baseline in the region. When the relative Raman intensities were calculated, spectra of samples required correction for a background convex, which resulted from sample luminescence. Background correction was done according to the procedure of Lippert et al. (1976): the background is usually a straight line between minima in the spectra, except in the region of solvent bands. Raman intensities were standardized to the Raman band near 1450 cm^{-1} due to methylene bending mode, which is believed to be invariable during conformational changes of proteins after baseline correction. All analyses were repeated three times, and the results were reported as the average of these replicates.

RESULTS

Electrophoresis. Figure 1 shows the SDS–PAGE patterns of the AM samples used in this study. Each fish species has strong MHC and actin bands, suggesting that the samples are typical band patterns of AM. Heating at 40 °C for 30 min did not cause degradation of MHC, which may lead to the destruction of gel. These 40 °C set gels were translucent in appearance, different from white and opaque gels when heated to 90 °C.

Gel Strength. The result is shown in Table 1. AMs of lemon sole, ling cod, and rock fish underwent sol-to-gel transition, whereas that of tilapia did not, as reported by Shimizu et al. (1981). Lemon sole AM and ling cod AM formed strong gels (gel strengths of 21.46 and 32.89 N·mm, respectively) with appreciable inden-

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Table 1. Gel Strength of Fish AM Heated at 40 °C for 30 min[a]

species	gel strength/N·mm
tilapia	NFG[b]
lemon sole	21.46 ± 2.31 (n = 13)
ling cod	32.89 ± 8.63[c] (n = 9)
rock fish	3.84 ± 1.22 (n = 4)

[a] Protein concentrations for tilapia, lemon sole, ling cod, and rock fish are 11.9, 10.0, 10.0, and 8.4%, respectively. [b] NFG, not form gel. [c] The lengths of cylindrical gel samples were 12 mm.
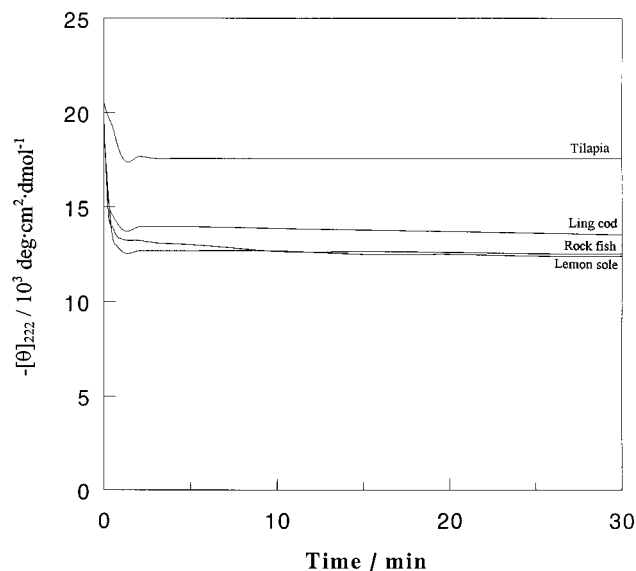

Figure 2. Changes in $[\theta]_{222}$ of AM during heating at 40 °C.

tation, which was characteristic of set gels of AM (Liu et al., 1997), whereas rock fish AM became a weaker gel with a gel strength value of 3.84 N·mm.

CD Profile. CD spectra were measured to monitor structural changes of fish AMs during heating at 40 °C. Changes in $-[\theta]_{222}$ of the AM solution (0.02%) are shown in Figure 2. For all species, values of $-[\theta]_{222}$, indicating α-helical content, decreased considerably within 1 min, but only slightly thereafter. The decreases in the α-helical content were different among the species. Upon heating at 40 °C for 30 min, α-helical content decreased by 35, 30, and 37% in the lemon sole, ling cod, and rock fish systems, respectively, whereas that of tilapia decreased by only 14%. This indicates that AM of tilapia is more stable against heating than those of lemon sole, ling cod, and rock fish.

Raman Spectra at 450−1800 cm⁻¹. Figures 3−6 show Raman spectra (450−1800 cm⁻¹) of 8−12% fish AMs heated at 40 °C for 0 (a), 5 (b), and 30 min (c). Table 2 shows the assignment of major bands to amino acid side chains and peptide backbones. As seen in Figures 3a, 4a, 5a, and 6a, unheated AMs exhibit relatively a flat spectrum in 1750−1800 cm⁻¹ region, after subtraction of the corresponding solvent, indicating that the solvent subtraction is allowed without damaging accuracy (Barrett et al., 1978). Superimposed on each Raman spectrum was a broad luminescence of unknown origin, despite use of purified AMs.

Raman spectra of fish AMs revealed a relatively strong S−S stretching vibration around 525 cm⁻¹ attributable to a gauche−gauche−trans (g−g−t) conformation of the disulfide bond (Tu, 1982). This indicates that the g−g−t form is a regular conformation of the disulfide bond in fish AM. Each spectrum revealed a distinct peak at 1004−1006 cm⁻¹ attributable to C−C ring stretch of phenylalanine (Phe). In the 1220−1320 cm⁻¹ region, an amide III mode was observed with overlapping of a C−H bend mode. An outstanding peak
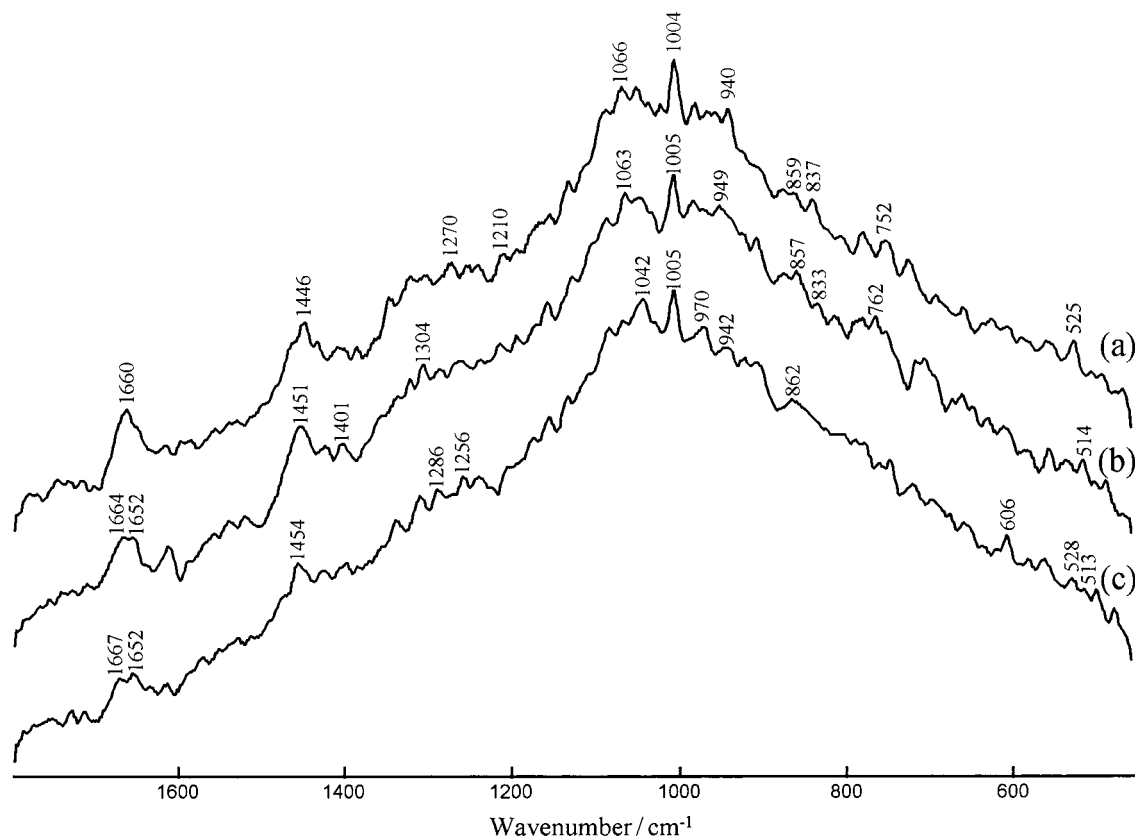
Figure 3. Raman spectra (450−1800 cm⁻¹) of tilapia AM (a) unheated, (b) heated at 40 °C for 5 min, and (c) heated at 40 °C for 30 min. Protein concentration was 11.9%.
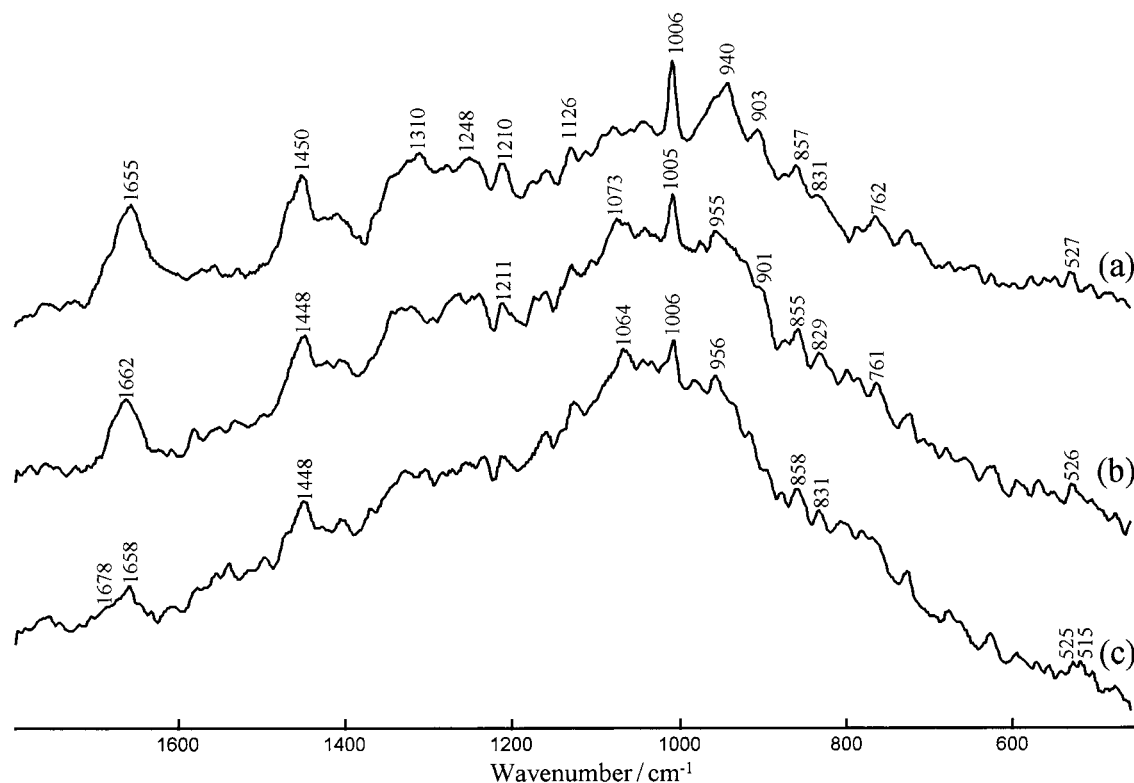
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Figure 4. Raman spectra (450−1800 cm⁻¹) of lemon sole AM (a) unheated, (b) heated at 40 °C for 5 min, and (c) heated at 40 °C for 30 min. Protein concentration was 10.0%.
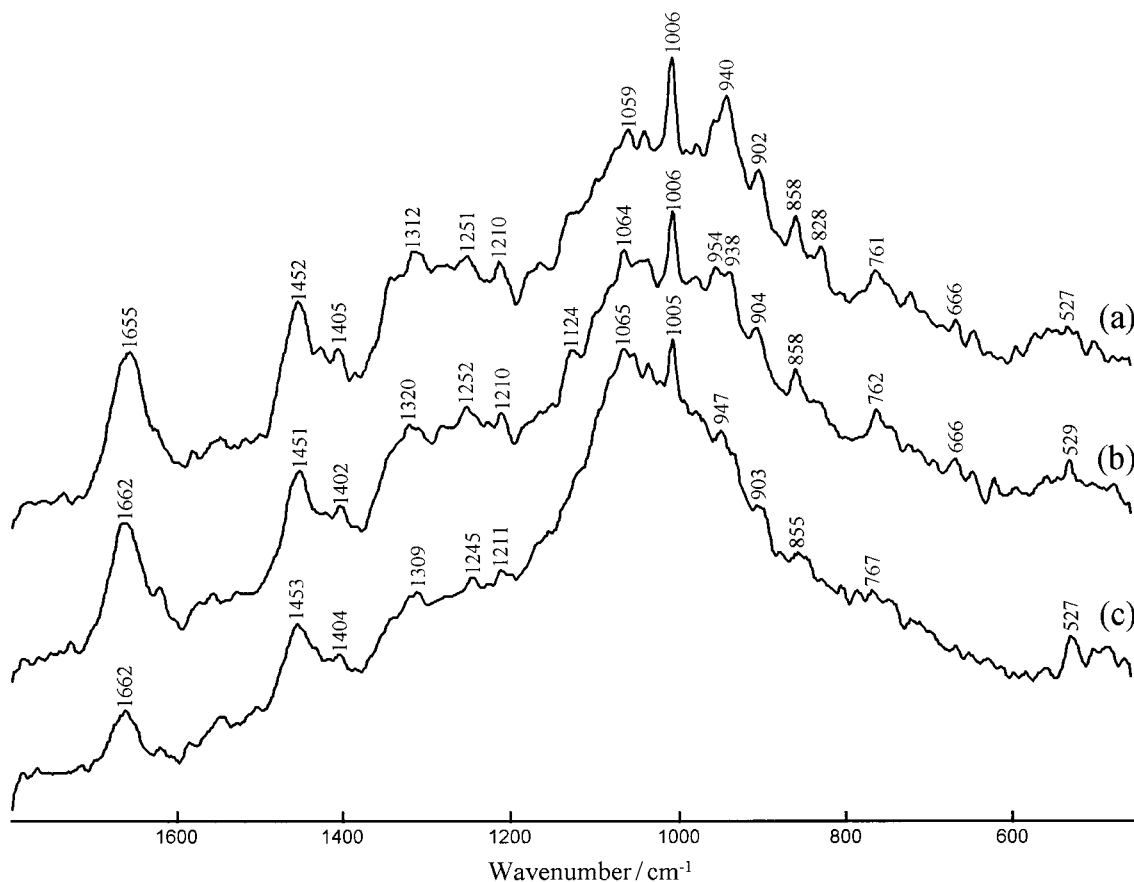

Figure 5. Raman spectra (450−1800 cm⁻¹) of ling cod AM (a) unheated, (b) heated at 40 °C for 5 min, and (c) heated at 40 °C for 30 min. Protein concentration was 7.9%.

in the amide I region was centered in the 1651−1660 cm⁻¹ region, corresponding to α-helical structure: 1660 cm⁻¹ for tilapia, 1655 cm⁻¹ for lemon sole and ling cod, and 1651 cm⁻¹ for rock fish.
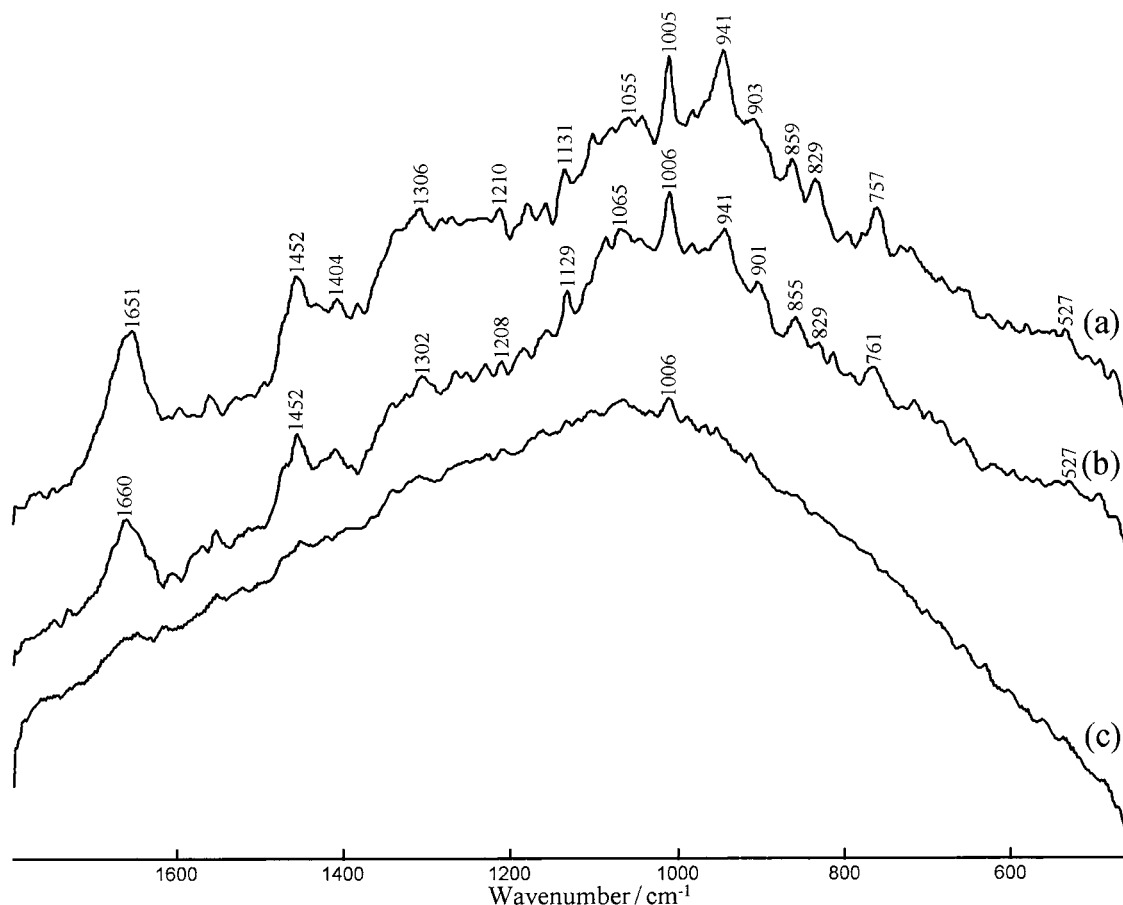
Figure 6. Raman spectra (450−1800 cm^{-1}) of rock fish AM (a) unheated, (b) heated at 40 °C for 5 min, and (c) heated at 40 °C for 30 min. Protein concentration was 8.4%.

Table 2. Tentative Assignment of Some Major Bands in the Raman Spectra of Fish AM

peak/cm^{-1} (±4 cm^{-1})	tentative assignment
525	S−S stretching vibration (gauche−gauche−trans)
760	Trp
830, 860	Tyr
900	C−C residue stretch
940	C−C residue stretch; CH$_3$ symmetric stretch
1005	C−C ring stretch (Phe)
1060	Backbone C−C, C−N stretch
1210	Tyr and Phe modes
1244	amide III (β + random coil)
1304	amide III (α-helix); C$_\alpha$H bend, CH$_2$ twist
1340	Trp; CH bend
1404	ionized side-chain carboxyl groups
1450	CH$_3$ (asymmetric), CH$_2$, CH bend
1650−1670	amide I regions
2855	CH$_2$ symmetric stretch
2880	CH$_2$ asymmetric stretch
2938	CH$_3$ symmetric stretch, CH$_2$ asymmetric stretch

Tilapia AM shows a decrease in the 525-cm^{-1} band intensity and an increase in the intensity of the band at 510 cm^{-1}, which may imply the formation of a gauche−gauche−gauche (g−g−g) conformation of the disulfide bond during heating at 40 °C (Figure 3). The heating resulted in a decreased intensity of the 940-cm^{-1} band arising from C−C residue stretch and CH$_3$ symmetric stretch. The 1660-cm^{-1} peak in the amide I region was shifted to 1652 and 1667 cm^{-1} attributable to disordered structure. The intensity ratio of doublet bands at 860 and 830 cm^{-1}, $R = I_{860}/I_{830}$, can monitor the microenvironment of the Tyr side chain (Tu, 1982;

Van Dael et al., 1987). The value increased to 1.5 ± 0.2 from 0.7 ± 0.1 with heating at 40 °C for 30 min. This shows that tyrosyl residues located around the hydrophobic environment moved to the polar environment.

Lemon Sole AM. In the spectra of lemon sole, the band intensity at 903 cm^{-1} attributable to C−C stretch vibration was weakened by heating at 40 °C, whereas the 940-cm^{-1} band was shifted to 956 cm^{-1} (Figure 4). The amide I band at 1655 cm^{-1} was markedly diminished, as clearly seen by comparison with the 1450-cm^{-1} peak considered to be independent of conformational changes. Raman intensity in the region 1060−1100 cm^{-1}, which contains conformationally sensitive skeletal vibrations (mostly C−N and C−C stretch modes) (Carew et al., 1975), increased upon heating. The R value (1.4 ± 0.1) decreased by 0.2 after heating at 40 °C for 30 min, indicating that tyrosyl residues were slightly buried in the nonpolar environments.

Ling Cod AM. The unheated ling cod AM exhibits a spectrum similar to that of lemon sole AM (Figure 5). An increase in the intensity of 527-cm^{-1} peak is striking. Heating at 40 °C resulted in a decrease in the intensity at 940 cm^{-1} accompanied by a shift to higher frequency. The peaks in the 1050−1100-cm^{-1} region are sharpened upon heating, similar to that of lemon sole. Besides, the amide I band centered at 1655 cm^{-1} shows a shift to 1662 cm^{-1} with a concomitant decrease in the 1655-cm^{-1} band intensity. The R value increased to 1.6 ± 0.4 from 1.0 ± 0.1 with heating at 40 °C for 30 min, suggesting that Tyr residues are exposed to a more polar environment.
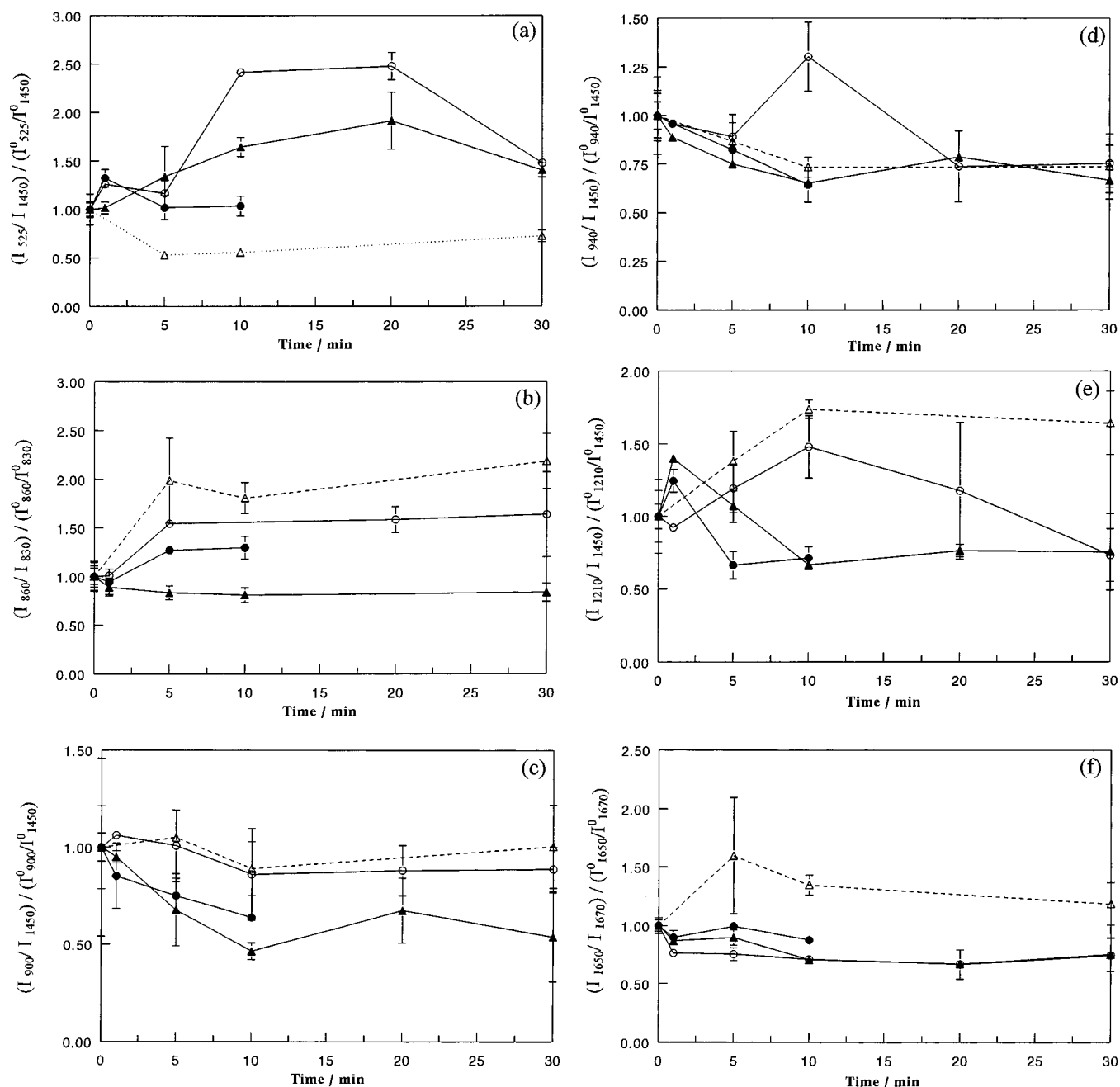
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Figure 7. Changes in relative intensity of main Raman bands during heating at 40 °C: (a) 525 cm^{-1}; (b) 860 cm^{-1}/830 cm^{-1} ratio; (c) 900 cm^{-1}; (d) 940 cm^{-1}; (e) 1210 cm^{-1}; (f) 1650 cm^{-1}/1670 cm^{-1} ratio; (△) tilapia AM; (▲) lemon sole AM; (○) ling cod AM; (●) rock fish AM. Intensities of each spectrum were normalized to the 1450-cm^{-1} band, and normalized intensities were expressed as relative values to the intensity of the sample without heating (time = 0). The error bars represent standard deviations of triplicate experiments.

Rock Fish AM. A prominent peak at 940 cm^{-1} appears in the Raman spectrum of unheated AM of rock fish, which is weakened by heating for 5 min (Figure 6). The 1651-cm^{-1} peak shifted to 1660 cm^{-1} with heating at 40 °C for 5 min. Rock fish AM had a strong luminescence during the incubation time, thereby making Raman peaks undetectable after the AM samples were heated for >20 min. The *R* value of rock fish increased from 1.1 ± 0.2 to 1.4 ± 0.1 upon heating at 40 °C for 5 min.

Overall Observation. The changes in the Raman intensity are summarized in Figure 7; these changes have been standardized as quotients against the Raman band near 1450 cm^{-1} due to methylene bending mode. Each panel (a–f) of the figure represents the relative value to unheated control.

In Figure 7a, the 525-cm^{-1} band of tilapia AM reduced intensity by half after heating at 40 °C for 30 min. Meanwhile, the intensity increased by 50% in lemon sole and ling cod, whereas that of rock fish did not change within 10 min.

Figure 7b shows changes in the intensity ratio of the doublet at 860 and 830 cm^{-1} associated with the environment of the Tyr side chain. For tilapia, ling cod, and rock fish, the relative intensity increased during the first 5 min of heating, but increased only slightly after 5 min. This may suggest that Tyr side chains of AM were exposed on the surface earlier during heating. Notably, the *R* (I_{860}/I_{830}) value of tilapia at 30 min was greater than double that of the unheated AM. In contrast, the *R* value of lemon sole was slightly decreased in the first 5 min. The
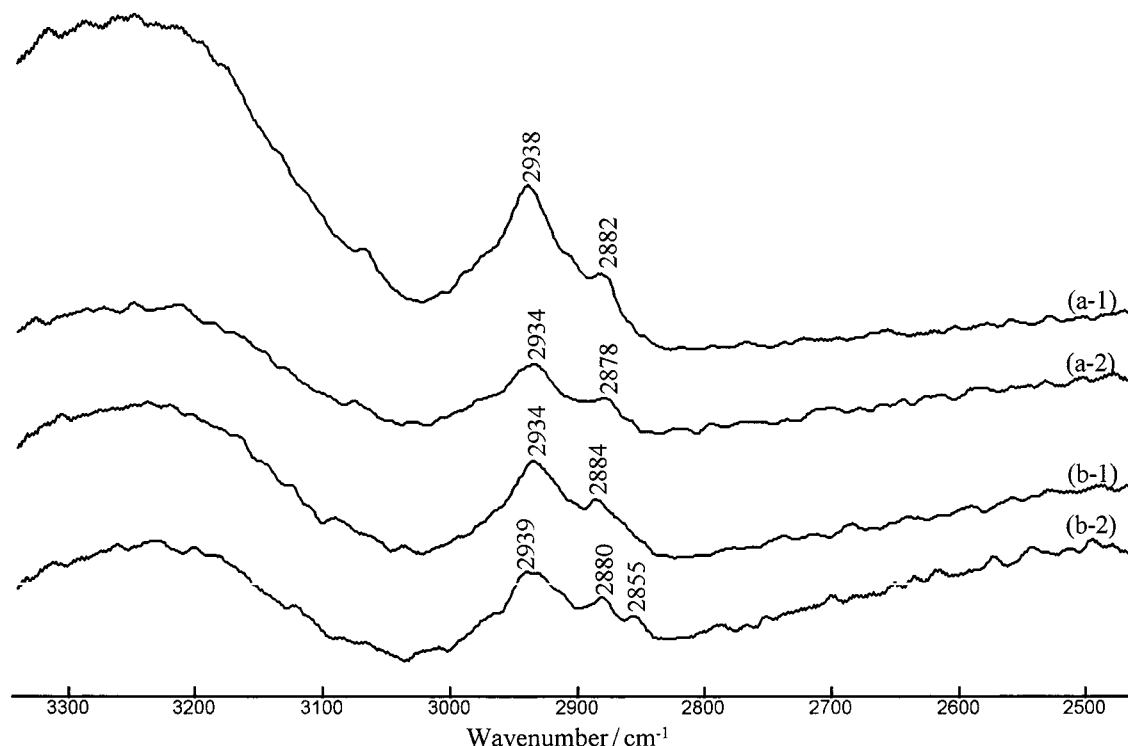
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Figure 8. Raman spectra (2450−3350 cm^{-1}) of AM from (a) lemon sole and (b) tilapia, without subtraction of the corresponding solvent spectrum: (a-1, b-1) without heating; (a-2, b-2) with heating at 40 °C for 30 min. Protein concentrations were the same as in Figures 3 and 4.

structural meaning of these contradictory phenomena is unknown.

A substantial decrease in the intensity at 900 cm^{-1} is observed during the first 10 min in lemon sole and rock fish (Figure 7c). The intensity decreased by 10% in ling cod, but only little in tilapia.

In all species, the band at 940 cm^{-1} was gradually reduced, except for 10-min-heated ling cod, reaching 25−30% below the ratio at 0 time after 30 min heating (Figure 7d). Carew et al. (1983) have indicated that, in myosin molecule, the 940 cm^{-1} mode may reflect conformational changes in the S-1 or S-2 regions, whereas the intensity at 902 cm^{-1} reflects changes only in the myosin tail. On the basis of their findings, it is possible that the degrees of conformational changes in the myosin tail are different between species. However, the extents of changes in the other portions of myosin may be similar among species.

Changes in the intensity of the 1210-cm^{-1} peak attributable to Tyr and Phe modes are indicated in Figure 7e. An increase in 1210-cm^{-1} band was observed in tilapia AM. For lemon sole and ling cod, the 1210-cm^{-1} band intensity after heating for 30 min was reduced by 70%, suggesting that the buried Tyr and Phe residues were exposed (Li-Chan and Nakai, 1991).

Changes in the I_{1650}/I_{1670} ratio revealed a significant discrepancy between tilapia and other species (Figure 7e). The ratio for tilapia increased by 18% after heating for 30 min, whereas those for the other fish were lowered during the first 10 min to 71−82% of that of unheated control. The ratio of the band heights at 1650 cm^{-1}/1670 cm^{-1} in the amide I region reflects changes in the proportion of α-helix in the same protein molecule. However, it raises a question whether the decrease in the α-helical content is due to an increase in the content of β-structure or random coil (Barrett et al., 1978). Therefore, it is a speculation that AMs from

lemon sole, ling cod, and rock fish undergo alteration of α-helix to β-structure and/or random structure during setting process. The increase in the I_{1650}/I_{1670} ratio found in tilapia may not mean formation of α-helix, because the lack of increase in the I_{900} and I_{940} bands is also attributable to α-helical conformation. Our CD results support this interpretation. Explanation of the increase in the I_{1650}/I_{1670} ratio is difficult, with only possible explanation that the remaining structure forms some unknown structures.

Raman Spectroscopy at 2450−3350 cm^{-1}. Raman spectra in the wavenumber range of 2450−3350 cm^{-1} were compared before and after heating at 40 °C (Figure 8). In this frequency region, there exists no appropriate peak to standardize the spectrum. This fact made it difficult to quantitatively compare the spectra. At any rate, the spectra would provide some qualitative information on the environment of aliphatic residues in proteins (Verma and Wallach, 1977; Arteaga, 1994). Unheated tilapia AM showed a distinct peak at 2884 cm^{-1} attributable to the CH_2 asymmetric stretch, along with a discernible shoulder at 2934 cm^{-1} arising from the CH_3 symmetric and/or CH_2 asymmetric stretch. After heat treatment for 30 min, these two peaks shifted slightly and a small shoulder appeared at 2855 cm^{-1} due to CH_2 symmetric stretch. However, the spectra as a whole were quite similar before and after heating at 40 °C for 30 min. The spectrum of unheated lemon sole AM shows a distinct peak at 2938 cm^{-1} with a shoulder at 2882 cm^{-1}, and the peaks shifted slightly toward lower frequencies of 2934 and 2878 cm^{-1} upon heat treatment. The main peaks in the spectra of ling cod and rock fish did not shift after 40 °C heating for 30 min (Table 3), despite the shifts in the shoulder. In all species, no peaks are seen in the 2500−2600 cm^{-1} region indicating S−H vibration. This suggests that

Table 3. Changes in Peaks Assigned to Aliphatic Residues in 2800–3000 cm⁻¹ by 40 °C Heating

sample		wavenumber/cm⁻¹
tilapia	no heating	2934, 2884 (sh)
	40 °C, 30 min	2939, 2880 (sh), 2855 (sh)
lemon sole	no heating	2938, 2882 (sh)
	40 °C, 30 min	2934, 2878 (sh)
ling cod	no heating	2938, 2881 (sh)
	40 °C, 30 min	2938, 2884 (sh)
rock fish	no heating	2934, 2905 (sh), 2882 (sh)
	40 °C, 5 min	2934, 2910 (sh), 2876 (sh)
	40 °C, 30 min	not determined[a]

[a] The peaks cannot be determined because of strong luminescence. (sh) means shoulder peak.

total sulfhydryl (SH) content in AM is undetectable in the form of S–H vibration.

DISCUSSION

To investigate the gelling mechanism of fish muscle in "setting", the Raman spectroscopy was applied to AMs isolated from four species of fish. Tilapia was the species in which AM did not form a gel with heating at 40 °C for 30 min. Changes induced by setting of AM sol are summarized in Table 4.

All fish AMs contain α-helical structure in the sol state in 2.5% salt solutions. The decline of the 900-cm⁻¹, 940-cm⁻¹, and amide I band intensities shows that the α-helices of lemon sole, ling cod, and rock fish AMs are dissociated to some extent by heat treatment at 40 °C. A decrease in the intensity at 900 cm⁻¹ seemed to reflect changes only in the myosin tail (Carew et al., 1983) in lemon sole, ling cod, and rock fish, thereby forming gel upon heating at 40 °C. This result suggests that interaction between myosin tails with high helical content would create gel elasticity (Sano et al., 1990a,b). In contrast, α-helix in tilapia AM was far less sensitive to heating at 40 °C than those of the other species. This high stability of tilapia AM is consistent with the report of Park and Lanier (1989) that tilapia myosin and actin seldom undergo structural changes at temperatures below 40 °C. Tilapia AM, the α-helix of which was not affected by heating at 40 °C, did not form a gel, whereas three other species with significant unfolding of α-helix caused sol–gel transition. These results strongly support the dissociation of α-helix closely associated with the low-temperature gelling phenomenon, that is, setting, of AM. The unfolding of AM in a sol state at high protein concentrations occurred gradually during the first 5–10 min, which was different from the quicker unfolding of AM in solution. This slow unfolding may require a long incubation time to form gel in the setting process. Such dissociation of α-helix of fish AM may occur in myosin, because α-helicity of AM belongs to myosin (Ogawa et al., 1996).

Raman spectra of AM revealed the characteristic Tyr bands at 830 and 860 cm⁻¹ and the phenolic ring band

at 1210 cm⁻¹ along with the Phe residues. Without heating, the Tyr residues of tilapia AM are totally buried ($R = 0.7$) compared with the other fish species. In contrast, those of lemon sole are exposed ($R = 1.4$), whereas those of ling cod and rock fish AMs are moderately exposed ($R = 1.0$ and 1.1, respectively). This situation may mean that the protein molecules of tilapia are more rigid and compact than those of other fish. For tilapia, ling cod, and rock fish, 40 °C heating of AM increased the R value ratio, suggesting that the tyrosyl residues are more exposed to the molecular surface. Despite an insignificant decrease in α-helical structure, tilapia AM appears to expose the Tyr residues to the surface. In contrast, the Tyr residues of lemon sole AM become less exposed to the surface, probably because of intermolecular interactions during gel network formation. Similar changes are reported in the gelation of α-lactalbumin (Nonaka et al., 1993).

According to changes in the 1210-cm⁻¹ band, it was found that the microenvironment of two aromatic residues of Tyr and Phe had changed after 40 °C heating. The Tyr and Phe residues of tilapia AM were more extensively buried, whereas those of the other species were exposed to a greater extent. Differences in the microenvironmental changes between tilapia and the other species may be related to the gelation at 40 °C. This would suggest that the exposure of the aromatic residues occurs during the "setting" process at 40 °C. In tilapia and lemon sole, the behaviors of Tyr and Phe residues are different from that of Tyr alone as represented by the R values. This may be explained by the differences in the R values and bands at 1210 cm⁻¹.

Setting of AM is caused by myosin, not by actin (Shimizu et al., 1983). Besides, actin shows no detectable change at the temperatures around 40 °C (Wu et al., 1985; Torigai and Konno, 1997). These results suggest that the spectral changes regarding the Tyr and Phe residues are restricted to myosin. According to the primary structure information of myosin (Suzuyama et al., 1980; Hirayama and Watabe, 1997; Hirayama et al., 1997), ~90% of total Tyr residues and ~80% of total Phe residues contribute to S-1. Thus, the changes in the Tyr and Phe bands appearing in the Raman spectra are mainly derived from S-1 portion.

Careche and Li-Chan (1997) have reported that a shift to higher wavenumbers in the C–H stretching band at 2933 cm⁻¹ is observed after frozen denaturation of cod myosin, and it stems from enhanced exposure of the aliphatic residues to an aqueous environment. Thus, the observed peak shift in the 2855–2940 cm⁻¹ region suggests that the environment of aliphatic side chains may have been changed upon heating, although it is not confirmed whether the changes can be interpreted as enhanced exposure. In particular, tilapia AM is believed to undergo remarkable environmental changes as well

Table 4. Summary of Changes Induced by 40 °C "Setting" of AM Sol

	gelation	g–g–t (S–S bond)	total α-helix	α-helix (S-1 or S-2)	α-helix (myosin tail)	exposure of buried Tyr and Phe	surface hydrophobisity of aliphatic residues
tilapia	−	⇊	→	⇊	→	⇊ (⇈ for Tyr alone)	++
lemon sole	+++	⇈	⇊⇊	⇊	⇊	↑ (↓ for Tyr alone)	+
ling cod	+++	⇈	⇊⇊	⇊	⇊	↑	+
rock fish	++	→[a]	⇊⇊[a]	⇊[a]	⇊[a]	↑[a]	+[a]

[a] The changes were determined on the basis of spectral data up to 10 min. −, no occurrence; +, occurrence with slight degree; ++, occurrence with moderate degree; +++, occurrence with consequential degree; ↓, slight decrease; ⇊, moderate decrease; ⇊⇊, consequential decrease; →, no consequential changes; ↑, slight increase; ⇈, moderate increase; ↑↑↑, consequential increase.

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as changes in aromatic residues, without affecting α-helical structures.

Although a myosin molecule freshly prepared had no less than 42 free SH groups (Buttkus, 1970), SH signals were not detected owing to the low sensitivity of the S−H vibration mode. The Raman spectra showed the relatively strong stretching vibration of disulfide bonds with the g−g−t conformation. In many naturally occurring proteins with disulfide bonds, the g−g−g form is the most preferred conformation (Tu, 1982). Thus, AMs having the main conformation of disulfide linkages in g−g−t form are different from the many other proteins. Sols of lemon sole and ling cod AMs heated at 40 °C have increased g−g−t form. Itoh et al. (1980) suggested that the intermolecular S−S linkages of AM were formed during heating at 40 °C. Accordingly, increases in the g−g−t conformation of disulfide bonds observed in lemon sole and ling cod must have resulted from S−S linkages formed during heating at 40 °C or transformation from g−g−g and/or t−g−t. In tilapia AM, the loss of the g−g−t form could be derived from the g−g−g form, along with simultaneous environmental changes of hydrophobic side chains.

In conclusion, gel formation of fish AMs in setting requires g−g−t conformation of disulfide bonds as well as a slow unfolding of α-helix.

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