加熱および尿素変性速度からみた各種魚肉構造蛋白質の種特異性

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Species Specificity of Muscle Proteins of Fishes against Thermal and Urea Denaturation

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Thermal and urea labilities of actomyosin and myosin Ca^{2+}-ATPase from several fish species have been investigated at constant pH, KCl and tris-maleate buffer concentration.

A comparison of the first order denaturation rate constants (K_D) of the two proteins at 30° and 35°C revealed that both actomyosin and myosin of tilapia are most stable, followed by the skipjack, yellowfin and bigeye tunas, carp, star-spotted shark, yellowtail, flatfish and cod, in decreasing order of stability. But, both proteins from tilapia are remarkably unstable in comparison with those of rabbit.

The order of stability of Ca^{2+}-ATPase, as established from the K_D values for urea denaturation, is the same as the above excepting the star-spotted shark. The actomyosin Ca^{2+}-ATPase of this species is more resistant to thermal denaturation than those of carp and the tunas, whereas its urea lability is less than that of carp and comparable to those of the tunas.

The mechanism of thermal and urea denaturation and the molecular adaptation of both muscle proteins in fishes has been discussed.

CONNELL[1] suggested that a direct correlation may exist between the body temperature and the species specificity of myosin of vertebrates. Such a view was supported by other studies published in the following years[2-4], and also by our heat denaturation studies on the actomyosin and myosin of a large number of fish species[5-7].

It has also been pointed out that the interspecies order of stability of a specific protein remains unchanged irrespective of the denaturing factor[1-7]. Available literature further shows that lowly evolved animals usually possess less stable proteins compared to those placed at a higher level of organization. Thus, invertebrate or lower vertebrate proteins are highly unstable than those of mammals[8,9]. Yet exceptions seem to exist in certain respects. For instance, elasmobranchs considered lower in evolutionary merits than teleosts possess hemoglobins which are several times more urea-resistant than even human hemoglobin-A[9].

Since the urea level in the blood of elasmobranchs is several times higher than other vertebrates[10], there is reason enough to assume that their proteins may have undergone specific adaptation to sustain higher urea environment. We have, therefore, compared the lability of actomyosin Ca^{2+}-ATPase from several fish species, including an elasmo-

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branch; namely: tilapia (*Tilapia mossambica*), carp (*Cyprinus carpio*), yellowtail (*Seriola quinqueradiata*), flatfish (*Limanda angustirostris*), cod (*Gadus macrocephalus*) and three tunas, i.e. the skipjack (*Katsuwonus pelamis*), the yellowfin (*Thunnus albacares*) and the bigeye (*Thunnus obesus*). Due to its ready availability, the star-spotted shark (*Mustellus griseus*) was chosen from the elasmobranchs. The inactivation pattern of Ca^{2+}-ATPase of a few myosins was also included to show that basically the presence or absence of actin has no bearing upon the species specificity of the myosin molecule. In both cases, rabbit proteins were included as standard references.

The results show that the interspecies order of stability of Ca^{2+}ATPase remained unchanged, no matter whether the denaturing agent was heat or urea. However, the star-spotted shark deviated from this general trend as far as inactivation by urea is concerned. Therefore, urea-resistance appears to be one of the factors responsible for the species specific adaptation of myosin, at least in elasmobranchs.

Materials and Methods

All fish samples excepting tunas and tilapia were purchased in the fresh or frozen state at a local fish market. Muscles from tilapia and rabbit were obtained from live animals and immediately frozen at −20°C.

In the cases of rabbit, carp, yellowtail, flatfish and cod, both the frozen and unfrozen muscles of the animals kept for a few hours in ice following death, were utilized. But to ensure that tuna samples were not aged ones, slices of all three species were directly transported from the landing site in a deep frozen state. Actomyosin and myosin were prepared following the procedure of TAKASHI *et al.*[11,12] excepting rabbit actomyosin, which was extracted with WEBER-EDSALL solution of pH 9.6 and a composition previously described[7].

In all protein preparations, the final concentrations of KCl and tris-maleate buffer of pH 7.0 were 0.6 M and 20 mM, respectively. The concentration of protein was determined with the Biuret reagent standardized by micro-Kjeldahl. The concentrations of various protein preparations were adjusted to give a final concentration of 5–6 mg/m*l* upon addition of urea solutions as described below. The procedures of thermal inactivation have already been described in detail[5,6].

Urea inactivation experiments were conducted at 25°C, and at definite intervals tubes were transferred to ice and immediately diluted with 7.5 m*l* of cold distilled water. A comparison of the Ca^{2+}-ATPase activity of actomyosin or myosin diluted in this way and stored under ice for a few hours showed no differences when compared with the samples analysed immediately after dilution.

ATPase assaying was performed in a reaction mixture which contained 60 mM KCl, 25 mM tris-maleate buffer of pH 7.0, 5 mM $CaCl_2$ and 1 mM ATP. The incubation tem-

perature was 25°C for actomyosin and 20°C for the myosin Ca^{2+}-ATPase assay.

At a given molarity of urea, the first order inactivation rate constants (K_D') were calculated from the slopes of straight lines of logarithmic plots of relative ATPase activity versus time, essentially as described by YASUI *et al.*[13]. The equation used was: $K_D = (\ln C_0 - \ln C_t)\ 1/t$, where C_0 and C_t are the ATPase activities before and after inactivation by urea for a given incubation time t. For convenience' sake K_D' values have been used taking $K_D' = K_D/2.303$.

Results and Discussion

Inactivation by heat First order inactivation rate constants of Ca^{2+}-ATPase of actomyosins from several fish species along with the rabbit at 30° and 35°C are given in Table 1. Depending upon the species, the time required for complete inactivation of this protein varied widely from 30 min to 6 hr.

Table 1. The apparent rate constants of inactivation of actomyosin ATPase of fish species and rabbit at 30° and 35°C in 0.6 M KCl of pH 7.0.

Actomyosin was incubated at 30° and 35°C in 0.6 M KCl containing 20 mM tris-maleate buffer of pH 7.0. Protein concentration was adjusted within the chosen range of 5–6 mg/ml.

Activity was measured at pH 7.0 in 60 mM KCl, 25 mM tris-maleate buffer, 5 mM $CaCl_2$, and 1 mM ATP at 25°C.

The values in parenthesis show the number of determinations.

	Inactivation rate constant K_D' (sec^{-1})	
	30°C	35°C
Rabbit		0.3×10^{-5}
Tilapia		1.6×10^{-5}
Skipjack tuna		$4.2-7.3\times10^{-5}$ (5)
Yellowfin tuna		$6.9-10.7\times10^{-5}$ (8)
Bigeye tuna		$7.2-9.3\times10^{-5}$ (4)
Carp		9.7×10^{-5}
Star-spotted shark	$3.3-4.7\times10^{-5}$ (2)	19.3×10^{-5} (2)
Yellowtail	7.7×10^{-5}	20.0×10^{-5} (2)
Flat fish (Magarei)	17.3×10^{-5}	73.3×10^{-5}
Cod	30.0×10^{-5}	

At 30°C, actomyosin Ca^{2+}-ATPase activity of rabbit, tilapia, skipjack tuna, yellowfin tuna and bigeye tuna did not decline significantly even after six hours. Hence K_D' values were calculated only for the shark, yellowtail, flatfish and cod. Results show that cod actomyosin is about 1.5 times as unstable as that of flatfish, which is in turn much less stable than those of yellowtail and shark.

At 35°C the actomyosin ATPase of tilapia is most stable. Its K_D' is less than half of the skipjack tuna's. The K_D' of the latter species is only 1/12th of flatfish. There is no marked variation among the K_D' values of skipjack ($K_D' = 4.2-7.3\times10^{-2}\ sec^{-1}$), yellowfin

($K_D'=6.9$–10.7×10^{-5} sec^{-1}) and bigeye tuna ($K_D'=7.2$–9.3×10^{-5} sec^{-1}) actomyosin ATPases. Cod actomyosin was too unstable at this temperature to give any value.

Denaturation studies on myosin Ca^{2+}-ATPase of many fish species have been hampered by its very unstable nature. In the present study as well, reliable K_D' values could only be obtained for a few species given in Table 2. Their order of comparative stabilities is the same as in the presence of actin (Table 1).

Table 2. The apparent rate constants of inactivation of myosin ATPase from fish species and rabbit at 30° and 35°C in 0.6 M KCl of pH 7.0.

The conditions of incubation and ATPase measurements were the same as in the legend of Table 1.

	Inactivation rate constant K_D' (sec^{-1})	
	30°C	35°C
Rabbit		1.22×10^{-5}
Tilapia	6.7–7.3×10^{-5} (2)	3.57×10^{-5}
Skipjack tuna	12.2×10^{-5}	
Yellowfin tuna	15.8×10^{-5} (2)	
Carp	20.0×10^{-5}	
Yellowtail	65.6×10^{-5}	

At 35°C, only tilapia myosin Ca^{2+}-ATPase gave reasonable K_D' value, being about one third that for the rabbit. At 30°C, tilapia myosin ATPase is approximately twice as stable as that of skipjack tuna and ten times that of yellowtail.

Inactivation by Urea K_D' values of urea denaturation of actomyosin Ca^{2+}-ATPase of rabbit and fish species are given in Table 3.

Table 3. The apparent rate constants of inactivation of actomyosin ATPase of fish species and rabbit in 0.5 and 1.0 M urea solutions, containing 0.6 M KCl of pH 7.0.

Actomyosin was incubated in urea solutions of 0.5 and 1.0 M which were 0.6 M in KCl of pH 7.0 at 25°C. Protein concentration was adjusted to give a definite final concentration within the chosen range of 5–6 mg/ml.

The conditions of ATPase measurements were the same as in the legend of Table 1.

	Inactivation rate constant K_D' (sec^{-1})	
	Urea concentration (M)	
	0.5	1.0
Rabbit		0.3×10^{-5}
Tilapia		5.6×10^{-5}
Skipjack tuna		11.7×10^{-5}
Yellowfin tuna		16.3–24.4×10^{-5} (5)
Bigeye tuna	2.8×10^{-5}	16.7–23.0×10^{-5} (8)
Carp	2.9–3.3×10^{-5} (3)	39.2–48.3×10^{-5} (3)
Star-spotted shark		18.6–24.9×10^{-5} (3)
Yellowtail	14.9×10^{-5}	21.1–45.0×10^{-5} (3)
Flat fish (Magarei)	21.1–27.8×10^{-5} (4)	
Cod	47.5–52.0×10^{-5} (2)	

Apparently K_D' values of actomyosin ATPase of yellowfin and bigeye tunas are within the same range, if denatured with 1.0 M urea. Tilapia actomyosin still showed the highest stability than other fish species, though it is much less stable than that of rabbit. Bigeye tuna actomyosin ATPase is as stable as that of carp in 0.5 molar urea, but its unstability compared to the latter species became apparent when denatured with 1.0 M urea.

Table 4. The apparent rate constants of inactivation of myosin ATPase of fish species and rabbit in urea solutions of 0.5 and 1.0 M, containing 0.6 M KCl of pH 7.0. The conditions of incubation and ATPase measurements were the same as in the legend of Table 3.

	Inactivation rate constant K_D' (sec^{-1})	
	Urea concentration (M)	
	0.5	1.0
Rabbit		5.8×10^{-5}
Tilapia	$7.5\text{–}14.0\times10^{-5}$ (2)	$28.5\text{–}33.3\times10^{-5}$ (3)
Skipjack tuna	15.8×10^{-5}	
Yellowfin tuna	$21.3\text{–}23.3\times10^{-5}$ (3)	58.3×10^{-5}
Yellowtail	49.2×10^{-5}	

Table 4 shows the corresponding K_D' values of myosin Ca^{2+}-ATPase inactivation by urea. It shows tilapia myosin ATPase to be about 1.5 times as stable as that of skipjack tuna, 2 times that of yellowfin tuna, and over 4 times that of yellowtail, in 0.5 M urea. At this concentration, rabbit myosin ATPase was vertually unaffected. At 1.0 M, however, a relative order based on K_D' comparison can be drawn, which reveals tilapia myosin Ca^{2+}-ATPase to be as stable as that of yellowfin tuna appears to be at 0.5 molar urea. The K_D' of rabbit myosin ATPase is over 6 times less than that of tilapia.

But the most striking feature of the inactivation of actomyosin Ca^{2+}-ATPase is the deviation shown by star-spotted shark protein from this general trend. As shown in Table 3 its K_D' value in 1.0 M urea is in proximity of the values of yellowfin and bigeye tunas, and about half of the values for carp and yellowtail actomyosins. The difference between the Ca^{2+}-ATPase of actomyosins from carp and star-spotted shark can further be seen in Fig. 1.

From K_D' values presented in Tables 1 and 2, it is evident that in stability Ca^{2+}-ATPases of actomyosin or myosin of even tilapia do not match those of the rabbit. However, rabbit actomyosin Ca^{2+}-ATPase in itself has been found to be much more heat or urea labile than that of the whale[7].

Interspecies variations are much more widely expressed by Ca^{2+}-ATPase of actomyosin than by that of myosin. For example, K_D' of tilapia actomyosin ATPase is about 5 times as large as that of the rabbit, whereas the ratio of K_D' values of their myosins is less than three. That the Ca^{2+}-ATPase of actomyosin complex is generally several times as stable as that of myosin suggests that actin renders myosin more stable against thermal

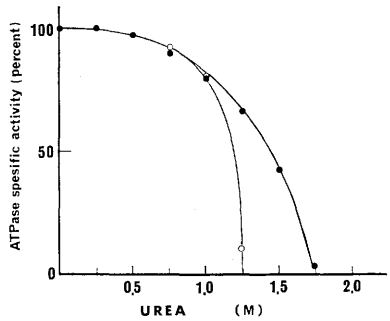

Fig. 1. Effect of different concentrations of urea on actomyosin ATPase of star-spotted shark and carp muscle.

Activities are expressed as the percentages of the initial activity of individual preparations in the absence of urea.

Calculated amounts of solid urea was added to the protein solution at 25°C, and dissolved by shaking in the reaction tube. After 15 minutes other constituents of the reaction mixture to determine ATPase activity were added, which also inhibit the inactivation.

—●—, star-spotted shark, —○—, carp.

inactivation: e.g., K_D' of yellowtail myosin is 9 times as much as that of actomyosin ATPase at 30°C (Tables 1 and 2); or at 35°C rabbit myosin ATPase inactivates at about 20 times faster rate than its actomyosin (Tables 1 and 2). Therefore, actomyosin can safely be chosen for understanding the myosin of a particular species, where removal of actin leaves it highly vulnerable to inactivation.

Using the Ca^{2+}-ATPase of actomyosin as a criterion, we have already shown that flatfishes appear to comprise the group of highly unstable myofibrillar proteins[7], but the present study indicates that cod proteins are most labile as far as thermal and urea denaturation is concerned. Though, most of the pioneering work on fish muscle proteins was carried out on cod and flatfishes[1], one of the conclusions drawn—that myosin adaptability seems closely related with the body temperature of fish—has served as a satisfactory explanation for a number of studies appearing in the following years.

As a matter of fact, the body temperature of fish usually falls within the temperature range of its habitat, and this could closely be related with the fact that myosin or actomyosin of fishes inhabiting the waters of the same temperature are of comparable stabilities. But notwithstanding this generalization, an extraordinary stability has been shown by the proteins of tuna species[5-7]. The temperature range of their habitat is not so widely different than that of other fishes, but they markedly differ from other fish species in having a very high body temperature[14], which has been demonstrated to be up to 15°C above that of the surroundings. Thus, the exceptional stability of their myofibrillar proteins correlates well with the higher body temperature.

Urea destroys the native configuration of proteins by disrupting hydrogen bonds, producing major cleavages in the inter and intramolecular hydrophobic bonds. The

extent of the damage thus caused is so severe that even if the protein molecule is renatured its configuration is far from being native[15]. In the case of myosin the changes in helical contents are spontaneous[1], though there exists a possibility that at least helical contents may be restored by renaturation, in either cases. In spite of these differences in the mechanisms of inactivation by heat and urea, the relative order of stabilities of actomyosin or myosin is the same through all the teleosts and mammals yet studied. CONNELL[1] and the authors[7] have shown that the critical urea concentration beyond which the rate of denaturation is sharply increased differs from species to species. This view is further supported by the present findings.

Another point brought up by this study is the importance of protein concentration in urea denaturation studies. Protein concentrations of 5–6 mg/m*l* were considered appropriate because of rather insignificant variations of K_D' at these concentrations (Table 5).

Table 5. The apparent rate constants of inactivation of actomyosin ATPase from fish species and rabbit in 1.0 M urea solution containing 0.6 M KCl of pH 7.0 at various protein concentrations.

The conditions of incubation and ATPase measurements were the same as in the legend of Table 3.

Actomyosin	Protein (mg/m*l*)	Urea (M)	K_D' (sec^{-1})
Star-spotted shark	6.17	1.0	21.7×10^{-5}
	5.55	1.0	18.6×10^{-5}
Bigeye tuna	3.75	1.0	20.0–20.6×10^{-5}
	4.16	1.0	20.0–21.1×10^{-5}
	5.08	1.0	20.0×10^{-5}
Yellowfin tuna	4.42	1.0	16.3–17.5×10^{-5}
	6.00	1.0	17.2×10^{-5}

Since SH groups have been reported to participate in the aggregation of myosin during frozen storage of muscle[17], some experiments were performed to elucidate if such a possible aggregation influences the denaturation rate of protein by urea. Results showed no differences between the denaturation rate of Ca^{2+}-ATPase of fresh bigeye tuna actomyosin and that stored in ice for two weeks. It was further confirmed by causing more severe denaturation by heat. K_D' value of freshly prepared actomyosin of this species having Ca^{2+}-ATPase specific activity of 0.240 μmoles/min/mg protein was 20.0×10^{-5} sec^{-1} while denatured with 1.0 M urea. When the specific activity was brought down to 0.171 μmoles/min/mg by heat denaturation at 35°C for 30 minutes, a value of 17.7×10^{-5} sec^{-1} was obtained. Similarly respective values for the freshly prepared (specific activity=0.368 μmoles/min/mg) and heat denatured myosin (specific activity= 0.177 μmoles/min/mg) of tilapia were 30.8×10^{-5} sec^{-1} and 33.3×10^{-5} sec^{-1}. In these cases, the myosin was incubated at 35°C for 6 minutes. Likewise, insignificant differences

were observed in K_D' values of actomyosins obtained from fresh and frozen muscles of carp and rabbit.

As in the heat denaturation, interspecies differences of K_D' values during urea denaturation of actomyosin Ca^{2+}-ATPase were wider than those of myosin. For instance, in 1.0 M urea K_D' of tilapia actomyosin ATPase was 19 times as much as that of rabbit, while myosin ATPase of the former only 5 times as much as the latter. As a rule, the actomyosin complex was more stable than myosin during urea inactivation as well.

In contrast to the teleosts, the star-spotted shark was rather unique in the pattern of urea inactivation of actomyosin ATPase as described above, thus raising a possibility that more than one factors must have been involved in the molecular evolution of myosin. It is well known that the blood and other tissue of cartilagenous fishes maintain urea concentrations up to 0.45 M to balance the osmotic pressure against environmental salinity. In the muscle of star-spotted shark, a concentration of 1740 mg/100 g tissue has been reported very recently[16], whereas its actomyosin showed resistance to urea concentration as high as 1.5 M. The critical urea concentration required to drastically damage the star-spotted shark actomyosin ATPase is obviously high compared to that of teleosts showing comparable or even higher thermal stabilities. During thermal inactivation, for instance, shark actomyosin is quite unstable than those of carp and three tuna species, but during inactivation by urea shark enzyme proved to be more stable than or comparable to those of carp and tunas.

It is not clear whether any homology in the mechanisms of heat or urea inactivation of Ca^{2+}-ATPase has lead to the similarity of interspecies order of stability, excepting shark. The only evidence available is that SH groups take part in both. BUTTKUS[17,18] has shown that disulphide bonding is responsible for the molecular aggregation of myosin during frozen storage, contrary to earlier findings[19,20]. Involvement of SH groups during thermal inactivation may be taken into account because of their participation in more drastic thermal changes like cooking[21].

There is no report on the mechanism of urea denaturation of muscle proteins, but in some other proteins the involvement of disulphide bonds in the formation of gels at very high molar concentrations of urea has been suggested[20]. Whether the same mechanism is responsible for the changes at lower urea concentrations also, where the SS bonding presumably may be in initiation, has not been elucidated so far. But changes in titrable SH groups of fish myosin at quite lower concentrations of urea have been reported by CONNELL[1]. Thus there exists a possibility that the two mechanisms may share some common steps, but they can not be altogether the same because of the reverse order of shark actomyosin ATPase inactivation.

Exceptional resistance of shark actomyosin against urea denaturation seems to provide another support to the view that the molecular adaptation of myosin is the function

of multiple factors.

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References

1) J. J. CONNELL: *Biochem. J.*, **80**, 503–509 (1961).
2) I. M. MACKIE: *Biochim. Biophys. Acta*, **115**, 160–172 (1966).
3) G. HAMOIR, H. A. MCKENZIE, and M. B. SMITH: *Ibid.*, **74**, 374–385 (1963).
4) C. S. CHUNG, E. G. RICHARDS, and H. S. OLCOTT: *Biochemistry*, **6**, 3154–3161 (1967).
5) K. ARAI and M. FUKUDA: This Bull., **39**, 625–631 (1973).
6) K. ARAI, K. KAWAMURA, and T. HAYASHI: *Ibid.*, **39**, 1077–1085 (1973).
7) A. HASNAIN, K. ARAI, and T. SAITO: *Ibid.*, **39**, 1195–1200 (1973).
8) M. BARANY and K. BARANY: *Biochem. Z.*, **345**, 37–56 (1966).
9) J. BONAVENTURA, C. BONAVENTURA, and B. SULLIVAN: *Science*, **184**, 57–59 (1974).
10) C. L. PROSSER and F. A. BROWN Jr.: in "Comparative Animal Physiology" (ed. by same authors), Saunders, Philadelphia (1961), p. 30.
11) R. TAKASHI, K. ARAI, and T. SAITO: This Bull., **36**, 169–172 (1970).
12) R. TAKASHI, K. ARAI, and T. SAITO: *Ibid.*, **36**, 165–168 .1970).
13) T. YASUI, H. KAWAKAMI, and F. MORITA: *Agr. Biol. Chem.*, **32**, 225–233 (1968).
14) F. G. CAREY and J. M. TEAL: *Proc. Natn. Acad. Sci.*, **56**, 1464–1469 (1966).
15) R. A. FEENEY and R. G. ALLISON: in "Evolutionary Biochemistry of Proteins" (ed. by same authors), Wiley-Interscience, N.Y. (1969) p. 4–5.
16) M. SUYAMA and H. SUZUKI: This Bull., **41**, 787–790 (1975).
17) H. BUTTKUS: *J. Food Sci.*, **35**, 558–562 (1970).
18) H. BUTTKUS: *Ibid.*, **39**, 484–489 (1974).
19) R. TAKASHI: This Bull., **39**, 197–205 (1973).
20) J. J. CONNELL: *J. Sci. Fd. Agric.*, **11**, 245–249 (1960).
21) R. HAMM and K. HOFFMANN: *Nature*, **207**, 1269–1271 (1965).