Urea-resistibility of shark myosin

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SUMMARY: Elasmobranch fish except for some freshwater rays contain urea, one of the most typical protein denaturants, in the range from 0.2 to 0.6 M to exert resistancy against high osmolarity of seawater. However, proteins in urea-containing elasmobranch can maintain their physiological functions even in the presence of urea as proteins of teleost in the absence of urea. The objective of this study was to analyze the actin-activated Mg²⁺-ATPase activity, α-helical structure, surface hydrophobicity, effects of trimethylamine-N-oxide on the urea-resistibility of requiem shark Triakis scyllia myosin at various urea concentrations and its deduced amino acid sequence for better understanding of the mechanism underlying urea-resistibility of requiem shark myosin.

The results obtained suggest that regulatory mechanisms to compensate for undesirable effects of urea at high concentrations are related to developing protein structures resistant to urea at least in the case of above analyses in marine elasmobranchs.

KEYWORDS: shark, urea-resistibility, myosin, myofibrillar protein

INTRODUCTION

Unlike teleosts, elasmobranch fish except for some freshwater rays contain one of the most typical protein denaturants, urea, in the range from 0.2 to 0.6 M in the muscle. It is well known that urea interferes with hydrogen bonds of biological macromolecules as an effective protein denaturant, affects hydrophobic interaction in proteins, and destabilizes the structure of water bound to proteins. Thus, urea damages the structure and function of most proteins including various enzymes, collagen, and myofibrillar proteins. However, marine elasmobranch have evolved regulatory mechanisms to compensate for such undesirable effects of urea at high concentrations in two ways. One strategy is to accumulate unusually high levels of methylamine compounds such as betaine and trimethylamine oxide (TMAO), in order to establish urea-methylamine counteraction. The other is to develop protein structures resistant to urea denaturation. This paper deals with the mechanism(s) underlying urea-resistibility of requiem shark myofibrils.

Urea-resistibility of myofibrillar proteins from requiem shark

The urea resistibility of myosin, reconstituted actomyosin and myofibril of requiem shark Triakis scyllia were investigated. Myosin, reconstituted actomyosin and myofibril were prepared from the requiem shark, and their urea-resistibilities were measured at 25°C, taking Ca²⁺-ATPase activity as a parameter. The relative urea-resistibility of requiem shark myosin was somewhat less than that of carp (Cyprinus carpio) myosin. In contrast, reconstituted actomyosin and myofibril were remarkably activated in the presence of urea (Fig.1). These results demonstrated the existence of urea-resistibility of this shark myofibrillar proteins. Then denaturation rate constants (Kₚ) of myofibrillar proteins from requiem shark and carp were measured in the absence and presence of urea. As shown in Fig.2, in the absence of urea, both the reconstituted actomyosin and myofibril showed about 10 times as high a resistibility as myosin, the magnitude which was clearly less than that with the carp, 18-20 times.

In 1.4M urea, on the other hand, the reconstituted actomyosin and myofibril exhibited a higher resistibility than myosin. The magnitude was 4-6 times with requiem shark, in contrast to less than...
Denaturation rate constants (×10⁻⁵ s⁻¹)

Fig. 2 Denaturation rate constants (Kd, as expressed in ×10⁻⁵ s⁻¹) of myosin, reconstituted actomyosin (AM) and myofibril (MF) from requiem shark and carp in 0 M and 1.4 M urea. 2 times with carp, suggesting a species-specificity of requiem shark myofibrillar proteins. These results led us to hypothesize that actin-myosin interaction of requiem shark may be different from that of carp.

Effects of urea on kinetic constants of actin-activated Mg²⁺-ATPase of requiem shark myosin

The objective of this study was to measure actin-activated Mg²⁺-ATPase activity of requiem shark myosin at various urea concentrations and to compare kinetic constants to those of carp to test the above assumption on the mechanism underlying urea-resistibility of requiem shark myofibrils. The influence of urea on actin-activated myosin Mg²⁺-ATPase of requiem shark was examined and compared to that of carp. As shown in Fig. 3, no changes in the maximum turnover rate, Vmax, was observed for shark myosin up to 0.3 M urea which is an approximate physiological concentration in marine elasmobranch tissues. The rate for carp myosin decreased by 70% at this urea concentration. The affinity of myosin to actin also remained unchanged up to about 0.7 M urea for shark in marked contrast to carp myosin which decreased by 30%, even in 0.1 M urea. Since it has been reported that urea-resistibility of requiem shark myosin Ca²⁺-ATPase in the absence of actin is comparable to that of carp, a high resistibility against urea in actin-activated Mg²⁺-ATPase of shark myosin is partly accounted for by its stable interaction with actin.

Effects of urea on surface hydrophobicity of requiem shark myosin

In the previous section, the urea resistibility of marine elasmobranch myofibrillar proteins against inactivation of ATPase activity has been argued. The objective of this section was to measure the surface hydrophobicity of requiem shark myosin as well as those of its subfragment-1 (S1) and rod at various urea concentrations, and further to compare the results obtained with those of carp counterparts for better understanding of mechanisms involved in structural changes of the myosin molecule by urea.

As shown in Fig. 4, the requiem shark myosin showed almost the same urea concentration dependency of the fluorescence intensity change as that of its rod, whereas the corresponding pattern of carp myosin was similar to that of its S1. These results indicate that shark myosin is more resistant to urea treatments than carp myosin, and that the region connecting S1 and rod is most responsible for such differences between requiem shark and carp myosins.

Effect of urea on α-helical structure of requiem shark myosin

Myosin and its fragments, rod and S1, were prepared from requiem shark and examined for the effects of urea on their α-helical structure by monitoring with circular dichroism (CD) using corresponding proteins prepared from carp as references. As shown in Fig. 5, all of the protein preparations from requiem shark
showed decreased α-helical contents with increasing urea concentrations\(^5\)). However, rod was most stable against urea treatment. When compared with above results for requiem shark, proteins prepared from carp were less stable in terms of α-helical structure at least up to 2 M urea examined in the present study, irrespective of myosin and its fragments. Furthermore, α-helical structure of myosin, rod and S1 from requiem shark were completely refolded when urea was removed from the sample solutions (Fig. 6). On the other hand, the refolding of α-helix in carp proteins after urea treatment was not perfectly accomplished, especially in the case of myosin and S1. Taking together, it is concluded that α-helical structure of requiem shark myosin is highly resistant to urea denaturation.

The effects of urea on transition temperature of requiem shark myosin and its rod

Differential scanning calorimetry (DSC) was employed for studying the unfolding of myosin rods from requiem shark and carp, induced by urea, in the presence of various concentration of urea. The deconvolution analysis gave three endotherms having three transition temperatures (Tm) from 30°C to 50°C. The prominent endotherms of both myosin rod were decreased almost linearly with the increasing urea concentration. The decreasing rate of Tm value of carp was larger than that of shark, showing the presence of urea-resistibility of shark myosin rod.

The effects of TMAO on the urea-resistibility of requiem shark myofibrils were investigated, taking Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase activities as a parameter\(^6\)). As shown in Fig. 7 and 8, both activities were hardly changed or activated up to 0.6 M urea. On the other hand, the two activities were both decreased to less than 50% in the presence of TMAO up to 0.5 M. When measured at a 2:1 molar ratio of urea and TMAO, Ca\(^{2+}\)- and Mg\(^{2+}\)-ATPase activities were similar to those in the presence of TMAO alone, indicating that TMAO declined urea-resistibility of myofibrils. Myosin, the most abundant protein in myofibrils, from requiem shark exhibited the effects of urea and TMAO on its Ca\(^{2+}\)-ATPase activity primarily similar to those of myofibrils. However, Ca\(^{2+}\)-ATPase activities in the coexistence of urea and TMAO for actomyosin reconstituted from requiem shark myosin and chicken F-actin were in the
average of those independently measured in the presence of either urea or TMAO alone.

![Graph](image)

**Fig. 8** Effects of urea and TMAO on myofibrillar Mg²⁺-ATPase activity of requiem shark and carp. The activity was measured at various concentrations of urea (●), TMAO (▲) and in a mixture (■) of urea and TMAO at a 2:1 molar ratio of requiem shark (a) and carp (b)².

Carp reconstituted actomyosin, and myosin, which were used as teleost references, showed their tendency in the effects of urea and TMAO on Ca²⁺-ATPase activities similar to those of requiem shark counterparts. These results indicate that the regulatory mechanisms to compensate for the undesirable effects of urea at high concentrations are related to developing protein structures that are resistant to urea, at least in the case of myofibrillar ATPases in marine elasmobranch fish.

**Cloning and sequencing of cDNA for requiem shark myosin and its subunit fragments**

The object of this section was to isolate cDNA clones encoding myosin heavy chain and three light chains from requiem shark in order to demonstrate whether there is a domain structure for urea-resistibility of these proteins. The cDNA libraries were constructed from fast skeletal muscle of requiem shark and were screened for myosin alkali light chains, DTNB light chains and myosin heavy chain, using an antiserum raised against requiem shark myosin A1 light chain and heavy chain. The amino acid sequence of three types of A1, A2 and DTNB light chains were deduced from cDNA nucleotide sequences, predicting 193, 150 and 168 amino acid residues respectively. A1 light chain contained the so-called difference peptide in the N-terminal regions, where Ala, Pro and Lys are abundant. According to database searches on Gen Bank or DDBJ, the amino acid sequence showed the homology of 62-67%, 65-67% and 77-82% to 9 fish species respectively. The heavy chain was analyzed up to 1728 amino acid residue. The analysis are now in progress to elucidate the mechanism of urea-resistibility of this shark.

**DISCUSSION**

The proteins in urea-containing elasmobranch can maintain their physiological functions even in the presence of urea as proteins of teleost in the absence of urea. Then, two strategies have been proposed to explain how these elasmobranch compensate for such undesirable effects of high concentrations of urea. One is to accumulate unusually high levels of methylamine compounds, in order to establish urea-methylamine counteraction. However, this mechanism is not always observed. No counteraction has been reported for some proteins. Furthermore, the counteraction is exerted differently on the altered protein functions even for the same protein. Bovine liver catalase showed urea-methylamine counteraction on its thermal stabilities, but not on the activity⁷. The other strategy is to develop protein structures resistant to urea. Proteins from requiem shark can maintain their physiological functions even in their physiological concentrations of urea without methylamines. Our results support the latter mechanisms, but more information of the structure of requiem shark myosin required for more understanding of urea-resistibility of elasmobranch.

**REFERENCES**


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