Identification of 5,5'-Dithio-bis-2-nitrobenzoic Acid (DTNB) and Alkali Light Chains of Piscine Myosin

Shugo WATABE,* Yoshihiro OCHIAI,* and Kanehisa HASHIMOTO*

(Received October 24, 1981)

Myosins were prepared from the ordinary muscles of 14 species of fish, and analyzed for light chain subunits by SDS-gel electrophoresis. DTNB light chain and two alkali light chains A_1 and A_2 , in the order of increasing mobility, were dissociated from the myosin of each fish. Molecular weights of the myosin subunits were determined to be in the ranges 17,500–21,000 for DTNB light chains, 25,000–30,000 for A_1 , and 13,500–22,500 for A_2 light chains, respectively.

In each scombroid fish tested, the molecular weight of A_2 was clearly larger than that of DTNB light chain; the opposite result was found in several other fishes including carp and pufferfish. In the group of fishes such as sardine and horse mackerel, both light chains showed the same molecular weight. These results suggested that there is some correlation between the molecular weight of A_2 and the locomotory activity of fish.

Quantitative analysis showed that piscine myosins contained approximately two moles each of DTNB and of alkali light chains per mole. However, the ratios of A_1/A_2 differed widely, from 0.1 (grunt) to 2.6 (requiem shark).

Myosin, a fibrous protein whose molecular weight is approximately 480,000 is composed of four light chains of molecular weight of about 20,000 and two heavy chains of that of about 200,000.¹⁻⁵ The myosins from rabbit fast muscle and other sources have three kinds of light chain designated LC_1 , LC_2 , and LC_3 in the order of decreasing molecular weight.⁶⁻¹⁰ One of them can easily be dissociated when myosin is treated with a sulfhydryl reagent, 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB), and is referred to as DTNB light chain.^{2,4,11} The DTNB light chains from various sources have so far been demonstrated to correspond to LC_2 .^{2,4,7-9,11} The dissociation of DTNB light chain does not significantly change the ATPase activity of myosin.^{2,12} Two other light chains LC_1 and LC_3 can be separated from myosin by alkaline treatments, and are called alkali light chains A_1 and A_2 , respectively.^{8,13,14} As their amino acid sequences indicate, these two chains are encoded by homologous genes,¹⁵ and are essential to myosin ATPase.⁵

The piscine myosin is very similar to the mammalian myosin in physicochemical properties such as amino acid profile, sedimentation coefficient, and intrinsic viscosity, suggesting that both myosins have essentially the same molecular structure.¹⁸⁻²⁴

However, the stability of fish myosin generally is much lower than that of mammalian myosin when measured using ATPase activity or aggregation rate as a parameter.^{18,24-28} In addition, the stability of fish myosin is remarkably species-specific: *e.g.*, tilapia and carp myosins are about 30 and 10 times, respectively, more stable than mackerel myosin at 30°C, when examined on ATPase activity.^{24,27} In this connection, it has been demonstrated by sodium dodecyl sulfate (SDS)-gel electrophoresis that the ordinary muscle myosins from various fishes contain three types of light chains, and that the electrophoretic patterns are mostly species-specific.²⁸⁻³²

These findings led us to suppose that myosin light chains of fish are closely related to the stability and physiological functions of the myosin. Studies along those lines are, however, quite poor: FOCANT *et al.*³³⁻³⁸ isolated three types of light chains from the myosin of carp, trout, and pike, and showed that the molecular weight of DTNB light chain is between those of both other chains as in the mammalian myosin. No other papers are available.

We attempted to purify myosins from the ordinary muscles of 14 species of fish, mostly marine, and to analyze their light chain patterns. Results showed that A_2 light chain has the molecular

* Lab. of Marine Biochem., Fac. of Agric., The Univ. of Tokyo, Bunkyo, Tokyo 113 (渡部終五・落合芳博・橋本周久: 東京大学農学部水産化学研究室).

weight larger than or equal to DTNB light chain, in migratory fishes such as bigeye tuna and skipjack.

Materials and Methods

Materials

Thirteen marine and one fresh water fish were used: Mackerel *Pneumatophorus japonicus*, skipjack *Katsuwonus pelamis*, bigeye tuna *Thunnus obesus*, round herring *Etrumeus micropus*, sardine *Sardinops melanosticta*, horse mackerel *Trachurus japonicus*, bigeye scad *Thachurus crumenophthalmus*, threadfish *Alectis ciliaris*, cutlassfish *Trichiurus lepturus*, pufferfish *Fugu pardale*, grunt *Parapristipoma trilineatum*, filefish *Navodon modestus*, carp *Cyprinus carpio*, and requiem shark *Triakis scyllia*. Rabbit was also used for comparison. With the sardine, horse mackerel, requiem shark and carp, live specimens were used. Other fishes were frozen with dry ice immediately after capture and kept at -40°C until used. Piscine ordinary and rabbit fast muscles were carefully excised from the dorsal part, avoiding any dark or slow muscle.

Preparation of Myosin Light Chains

The following procedures were performed at $0-4^{\circ}\text{C}$ unless otherwise stated. Myosins were prepared by the method of STAFFORD *et al.*,³⁶⁾ except that the ammonium sulfate fractionation was carried out between 40–50% instead of 40–70% saturation. The whole, DTNB and alkali light chains were prepared from each myosin as described below.

Whole light chains: To a portion of the myosin solution thus obtained was added an equal volume of 0.05 M Tris-HCl (pH 8.0) containing 8 M urea, 0.01 M EDTA, and 0.01 M dithiothreitol according to LOWEY and HOLT.³⁷⁾ The mixture was stirred at room temperature for 30 min, added 10 volumes of ice-cold water, and centrifuged at $5,000 \times g$ for 15 min. The resulting supernatant was used as whole light chain fraction.

DTNB light chain: To another portion of the myosin solution was added an equal volume of 0.01 M Tris-HCl (pH 8.5) containing 0.5 M KCl, 0.02 M DTNB, and 0.02 M EDTA according to FOCANT and HURIAUX.³³⁾ The mixture was allowed to stand at 0°C for 10 min, added 10 volumes of ice-cold water, and centrifuged. The supernatant obtained was used as DTNB light chain fraction.

Alkali light chains: The precipitate obtained

at the last step in DTNB light chain separation was used for preparing alkali light chains by the method of GAETJENS *et al.*³⁵⁾ The precipitate was allowed to stand at 0°C for 10 min in 0.1 M glycine-KOH (pH 11.1) containing 2 M LiCl and 1 mM dithiothreitol. To the solution was added a neutralized 2.5 M potassium citrate to a final concentration of 0.8 M. The mixture was left for another 10 min, and centrifuged to obtain alkali light chain fraction.

Analytical Methods

SDS-gel electrophoresis was performed according to WEBER and OSBORN,³⁸⁾ using 10% polyacrylamide gels containing 0.1% SDS. Slab-gel electrophoresis was carried out by the method of PERRIE and PERRY,³⁹⁾ using 10% polyacrylamide gels containing 8 M urea. After electrophoresis, gels were stained with Coomassie brilliant blue.

Molecular weights of the light chains were measured using bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome c (Schwartz/Mann) as standard protein markers.

The myosins prepared as above were electrophoresed to estimate molar ratios of DTNB and both alkali light chains. After the run, gels were stained and scanned at 550 nm with a Gelman ACD-18 densitometer. Molar ratios of respective light chains were calculated using their molecular weights, on the assumptions that their staining intensities per unit weight match each other, and that each myosin contains four light chains.

Results and Discussion

The piscine myosins used contained some actin, tropomyosin, and troponin, as seen in SDS-gel electropherograms of their whole light chain fraction (pattern 2 in Fig. 1). However, simultaneous analyses of the DTNB and alkali light chain fractions (patterns 1 and 3 in Fig. 1) made it possible to differentiate respective light chains. The results obtained are schematically shown in Fig. 2. Three light chains were observed in most piscine myosins, but only two in those of round herring, sardine, bigeye scad, and horse mackerel. In these four fishes, DTNB and A_2 light chains were expected to possess practically the same mobility under the electrophoretic conditions adopted. When the whole light chain fraction of horse mackerel was electrophoresed in the presence of 8 M urea, three bands appeared, one of which was DTNB light chain with an intermediate mobility (Fig. 3). This was also

Fig. 1. SDS-gel electrophoretic patterns of DTNB (1), whole (2), and alkali (3) light chain fractions from piscine ordinary muscle myosins (10% gels).

Fig. 2. Schematic diagram of SDS-gel electrophoretic patterns of light chains from piscine ordinary muscle myosins. Grey, black, and dotted bands represent A_1 , A_2 , and DTNB light chains, respectively.

Fig. 3. Slab-gel electrophoretic patterns of DTNB (1) and whole (2) light chain fractions from horse mackerel ordinary muscle myosin (10% gel in the presence of 8 M urea).

true for three other fishes. It follows that A_2 and DTNB light chains of these four fishes have the same molecular weight, though both light chains differ from each other in net charge.

The molecular weights of the light chains determined by SDS-gel electrophoresis are shown in Table 1. The molecular weights of DTNB and A_1 light chains fall in fairly narrow ranges of

17,500–21,000 and 25,000–30,000, respectively. Another alkali light chain A_2 , elicited the widest range of 13,500 (requiem shark)–22,500 (skipjack).

The molecular weight of A_2 was larger than that of DTNB light chain in mackerel, skipjack, and bigeye tuna, whereas all the known DTNB light chains fall between the two alkali light chains.^{2,4,7-9,11,33-35} Therefore, it is not suitable to designate myosin light chains as LC_1 , LC_2 , and LC_3 in the order of decreasing molecular weight in these scombroid fishes. They should be designated DTNB, A_1 and A_2 light chains, based on their inherent properties. The LC-designation system is also unsuitable to the fishes whose DTNB and A_2 light chains overlap with each other as described above.

All fishes possessing A_2 which is larger than or equal to DTNB light chain in molecular weight, are migratory ones, indicating that there may exist some relationship between the molecular weight of A_2 and the locomotory activity of fish.

Finally attempts were made to examine the light chain composition of the myosins. As summarized in Table 2, the piscine myosins contained about two moles of DTNB light chain per mole as has already been reported for rabbit fast

Table 1. Molecular weights of light chains from piscine ordinary muscle myosins, as determined by SDS-gel electrophoresis

	Light chains	Molecular weight		Light chains	Molecular weight
Mackerel	A_1	29,000	Cutlassfish	A_1	27,000
	A_2	21,500		DTNB	19,000
	DTNB	19,500		A_2	16,000
Skipjack	A_1	30,000	Pufferfish	A_1	28,000
	A_2	22,500		DTNB	19,000
	DTNB	20,000		A_2	16,000
Bigeye tuna	A_1	27,500	Grunt	A_1	28,500
	A_2	21,000		DTNB	19,500
	DTNB	19,000		A_2	16,000
Round herring	A_1	28,500	Filefish	A_1	28,000
	A_2 , DTNB	21,000		DTNB	19,000
Sardine	A_1	28,000		A_2	16,500
	A_2 , DTNB	21,000	Carp	A_1	27,500
				DTNB	18,000
Bigeye scad	A_1	28,000		A_2	16,500
	A_2 , DTNB	20,000	Requiem shark	A_1	25,500
				DTNB	17,500
Horse mackerel	A_1	26,000		A_2	13,500
	A_2 , DTNB	18,000	Rabbit	A_1	25,000
	A_1	26,000		DTNB	18,500
Threadfish	DTNB	18,000		A_2	14,000
	A_2	15,000			

Table 2. Light chain compositions of piscine ordinary muscle myosins

	Moles/mole of myosin			A_1/A_2
	DTNB	A_1	A_2	
Mackerel	2.4	0.5	1.1	0.4
Skipjack	2.2	0.6	1.2	0.5
Bigeye tuna	2.4	0.9	0.7	1.3
Threadfish	2.5	0.6	0.9	0.6
Cutlassfish	1.9	0.6	1.5	0.4
Grunt	2.0	0.1	1.9	0.1
Pufferfish	2.2	0.6	1.2	0.5
Filefish	2.4	0.7	0.9	0.8
Carp	2.3	0.8	0.9	0.9
Requiem shark	2.2	1.3	0.5	2.6
Rabbit	2.0	1.4	0.6	2.3

muscle myosin.^{20, 40, 41)} The remaining two moles were accounted for by A_1 and A_2 . However, their relative ratios in myosin molecule fluctuated very widely among the fishes tested. The molar ratios of A_1 and A_2 were in the range from 0.1 (grunt) to 2.6 (requiem shark). It seems interesting from the viewpoint of comparative biochemistry that the light chains of requiem shark and rabbit myosins resembled each other not only in their molecular weights but also in their molar ratios. Further studies will be needed to elucidate possible correlations between the piscine myosin light chain pattern and the degree of evolution.

Isolation and further characterization of myosin light chains from several fishes are now in progress.

Acknowledgements

The authors express their gratitude to Dr. Y. HIROSAKI and Mr. K. SEZAKI, the Enoshima Aquarium for a kind help in supplying requiem shark. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science and Culture.

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