Correlation between biochemical properties and adaptive diversity of skeletal muscle myofibrils and myosin of some air-breathing teleosts

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Functional properties of myofibrils and relative stability of myosin of five teleosts Channa punctata, Clarias batrachus, Mastacembalus armatus, Labeo rohita and Catla catla adapted to different breathing modes were compared. Myofibrillar contractility and m-ATPase of air-breathing organ (ABO) possessing C. punctata and C. batrachus were low and least affected by pH in the range of 7.1-8.5. However, their myosin isoforms were relatively thermostable, more soluble at sub-neutral pH values, between 0.1 to 0.15 M KCl concentrations and less susceptible to α-chymotryptic digestion. In contrast, myofibrils and myosin of water-breather major carps L. rohita and C. catla were more contractile and susceptible to pH and salt concentrations. Thus, correlation between catalytic efficiency and relative stability of myofibrils and myosin of ABO-possessing teleosts was of reverse order and magnitude, as compared to water-bathers. Interestingly, myofibrils and myosin of the behavioral air-breather M. armatus showed intermediate properties. The specific levels of m-ATPase of all the five teleosts were in conformity with the levels of metabolic marker, the lactate dehydrogenase. The effect of chymotryptic cleavage of 94 and 173 kDa domains on ATPase, individuality of peptide maps of MyHC isomers and perturbation of phenylalanine residues by urea implicated hydrophobic residues in stabilizing myosin structure in these fish. The present study suggests two apparent evolutionary modifications of myofibrils and myosin in ABO-possessing teleosts: (i), 'down-regulation' of ATPase that explains sluggishness of such species and, (ii), more stable molecular structure to support stress of air-breathing modes of life.

Keywords: Air-breathing teleosts, Chymotryptic Peptide maps, Difference spectra, Hydrophobic interactions, m-ATPase, Muscle-type specificity, MyHC isoforms/isomers, SDS-PAGE, Structural plasticity

Temperature is widely regarded as evolutionary affector of the phenotypic plasticity of fish myosin1-5, which is the major contractile protein of myofibrils. A few reports on urea tolerance of actomyosin ATPase and myosin of elasmobranchs have also been published5,6. Urea has been reported to modify actin-myosin interaction in a way that is different from freshwater teleosts6. Exposure of thiols and conformational stability of myosin of two elasmobranchs in urea solutions has also been demonstrated5,6. However, reports about the influence of air-breathing on specificity of contractile proteins of fish muscle are lacking. Studies on LDH isozymes, however, suggest that air-breathing teleosts preferentially utilize anaerobic pathway to meet their energy needs during hypoxia7,8. The switchover to anaerobic metabolism indicates adaptability of glycolytic pathway in the muscle sarcoplasm. It is, therefore, conceivable that contractile proteins of air-breathing teleosts have undergone corresponding adaptive changes.

Myosin of air-breathing fishes should have ability to function at elevated temperatures during land excursions and survivability under mild desiccation and pH changes. Phenotypic changes in fish myosin result from the switchover to expression of alternative heavy chain (MyHC) isoforms9-11. The switchover may occur during gross anatomical changes such as development12-14 or during short duration acclimation to different temperatures13,15,16. ATP splitting (ATPase), actin-binding and thick filament formability, all three
essential activities of myosin are the functions of MyHC. While ATPase and actin-binding sites are located in twin globular heads of 400 kDa MyHC dimer, its α-helical rod portion determines solubility\textsuperscript{17,18}.

In the present study, an attempt has been made to investigate the extent to which air-breathing has modified myofibrillar contractility, m-ATPase, myosin ATPase and structural plasticity of myosin isomers. Since air-breathing was repeated several times during evolution among quite distant groups of fishes\textsuperscript{19}, we have selected teleosts which have air-breathing organs (ABO) of different types and origins. ABO-equipped species were: murrel, Channa punctata and the catfish Clarias batrachus, while Mastacembalus armatus, a behavioral air-breather has no ABO and instead uses skin as accessory respiratory organ\textsuperscript{19,20}. Major carps Labeo rohita and Catla catla were taken as water-breather controls.

Materials and Methods

Acrylamide, ATP, bis-acrylamide, coomassie brilliant blue R-250, DEAE-cellulose, PMSF, TLCK-treated α-chymotrypsin and Tris (hydroxymethyl) aminomethane were from Sigma Chemicals, USA and 2-mercaptoethanol was from CDH Chemicals, India. Buffers and other salts used in the experiments were of analytical grade purchased from Merck, CDH or Qualigens, India.

Collection of fish

Fish murrel Channa punctata, Mastacembalus armatus, the catfish Clarias batrachus and major carps Labeo rohita and Catla catla were collected from upper Ganges canal at Nanau, 20 km from Aligarh during November, when water temperature at the site was 12-15°C. Fish were transported to laboratory under oxygen in polythene bags partly filled with water. ABO-bearing species could be transported without this arrangement. Fish were acclimated for 24 h in separate tanks of 60 L capacity in the water of 15±2°C with continuous aeration by bubbling. The density was 10 each for C. punctata and C. batrachus, 6 for M. armatus and 4 each for the major carps.

Preparation of myofibrils or natural actomyosin (NAM) and measurement of contractility

Fish were stunned by a severe blow to the head and white muscle from anterior myotomes was carefully removed, avoiding contamination of dark muscle. Muscle pieces were immediately transferred to polythene bags, which were dipped in ice bath to stop glycolysis and delay rigor mortis. Myofibrils were prepared as described earlier\textsuperscript{23}.

Natural actomyosin (NAM) from myofibrils was prepared by extracting in 0.45 M KCl containing 25 mM phosphate buffer (pH 7.0), followed by precipitation with 10 vol of distilled water\textsuperscript{24}Precipitation cycle was repeated three times and resulting precipitate dissolved in 0.6 M KCl or NaCl containing Tris-HCl buffer, pH 7.5. The contractility was measured by the procedure described previously\textsuperscript{23} with the modifications that pH was a variable. It was estimated in 0.1 M KCl and 40 mM buffer at a specific pH and was defined as (Pv0−PvATP)/Pv0×100, where Pv0 was the initial packed volume of myofibrils and PvATP after adding 1 mM Mg-ATP. Mfs were packed by centrifugation at 1000 rpm and 20°C for 10 min.

Lactate dehydrogenase (LDH) assay

First wash saved during preparation of myofibrils was dialyzed against three changes of 50 mM Tris-HCl. Precipitated material was removed as pellet, following centrifugation at 10 K rpm and 4°C. Supernatant was used for the assay of LDH activity. Essentially, the protocol of Bergmeyer\textsuperscript{24} was followed, while l-lactate was used as the substrate. Assays were made in 60 mM phosphate buffer of pH 7.2, 1 mM NAD or 0.2 mM NADH at 20°C against 50 mM substrates.

Preparation of myosin and MyHC isomers

Myosin extraction was performed according to the protocol\textsuperscript{24}, with the modification that prior to extraction myofibrils were washed twice with 5 mM MgCl\textsubscript{2}-ATP. The final pellet was suspended in phosphate buffer (pH 8.0) to give a final concentration of 0.05 M. This was followed by the addition of MgCl\textsubscript{2} and ATP to the final concentrations of 0.1 M and 0.005 M, respectively. The method gave a fairly good yield (up to 250 mg/50 g muscle), with only minor contamination of actin, which could be removed by treating with DEAE-cellulose. Inclusion of 0.001 M PMSF at each extraction step prevented the frequent cleavage of MyHC due to intrinsic proteases.

MyHC isomers from myosin were prepared essentially as described previously\textsuperscript{25}. Briefly, myosin was gently stirred in 2.4 M LiCl overnight. Tri-potassium citrate was slowly added with continuous and gentle stirring to a final concentration of 0.8 M.
The thick and sticky precipitate, thus formed was collected by centrifugation. After extensive dialysis against 0.5 \( N \) \( \text{NaCl} \) in Tris-HCl (\( pH \) 7.5), followed by centrifugation at 10 K rpm, the supernatant was taken as MyHC preparation. It was screened by SDS-PAGE and checked for \( \text{Ca}^{2+} \)-ATPase activity.

Concentration of myofibrils or myosin was determined by biuret method\(^{26} \). Native myosin was also read directly at 280/260 nm using the formula: Protein concentration (mg/ml) = \( A_{280} \times 1.5 - A_{260} \times 0.75 \).

**ATPase assay**

ATPase was assayed at 20\(^\circ\)C and the liberated Pi was colorimetrically determined\(^{26} \). Assays were made at 20\(^\circ\)C, 0.05 \( M \) \( \text{KCl} \), 1 \( mM \) \( \text{MgCl}_2/\text{CaCl}_2 \), 1 \( mM \) ATP and 0.3 mg/ml protein concentration. Buffers were 25 mM Tris-maleate for \( pH \) 6.0 to 7.0 and Tris-HCl for \( pH \) 7.5 to 8.5.

**SDS-PAGE and molecular weight estimation**

SDS-PAGE of Laemmli\(^{27} \) was employed to screen the NAM, myosin or MyHC preparations. Gels contained 10\% acrylamide (A to C ratio 30:0.8), 1\% SDS and 10\% glycerol. Dimensions of the gel slabs were 100 \( \times \) 150 \( \times \) 1 mm. Lower gels were made in 0.375 \( M \) Tris-HCl (\( pH \), 8.6) and upper gel (3\%) in 0.125 \( M \) Tris-HCl (\( pH \), 6.8). Runs were made in Tris-glycine of \( pH \) 8.3 (25 mM and 0.25 \( M \), respectively). Routine protein staining was performed with Coomassie brilliant blue R-250. Electrophoretic patterns were documented by digital imaging. Molecular weights (\( M_w \)) of individual polypeptides were estimated by GelPro software (Cybernetics, USA). Chicken NAM was used as the molecular weight marker.

**Purification of individual isoforms by preparative SDS-PAGE**

Individual preparations of myosin from white muscle of selected fish species were screened by the preparative SDS-PAGE. MyHC bands were visualized following 2 h washing in distilled water and then placing the gels in cold. Gels were then washed with distilled water and placed on ice over a polythene sheet to visualize MyHC-SDS complex as white bands. The bands were cut out and treated as described earlier\(^{28} \). Individual gel pieces with MyHC were crushed and squeezed by smooth glass rods in dialysis tubing in the presence of 1x running buffer containing SDS. Electro-elution was carried out for 3 h and the contents were centrifuged at 10,000 g for 15 min. The supernatant containing a specific MyHC isoform was saved and used for peptide mapping.

**Electrophoresis of MyHC isoforms**

MyHC isoforms were resolved by SDS-PAGE essentially according to previously described protocol\(^{26} \), with the modification that gels were 10\% in glycerol. Vertical slab gels (100 \( \times \) 150 \( \times \) 1 mm) containing 8\% acrylamide concentration (A:C= 200:1) in the separating and 4\% in stacking gel (A:C = 20:1) were used. With the exception of lower-gel buffer that was 0.75 \( M \) and \( pH \) of 9.3, other solutions and buffers were the same as in Laemmli’s protocol\(^{27} \). Scion imaging software programme was used for densitometry of individual MyHC isoform.

For urea-PAGE, molarity of Tris-HCl buffer in gel and Tris-glycine in running buffer was the same as in Laemmli’s\(^{27} \) protocol, except that SDS was replaced by 5 \( M \) urea (final concentration) and no upper gel was cast.

**\( pH \) Dependence of solubility**

Solubility of different myosins as a function of variations in \( pH \) values and salt concentrations was estimated as follows. Myosin solution (2.5 mg/ml) was incubated with the buffers of variable salt concentrations and desired \( pH \) for 3 h at ice temperature. Buffers of different \( pH \) values were the same as used for ATPase activity.

Students’ \( t \)-test was applied to check the significant rise in solubility values (\( P<0.05 \)). The values were the average of duplicate determinations of three different samples.

**Kinetics of thermal inactivation of \( \text{Ca}^{2+} \)-ATPase**

Myosin (1.0-1.5 mg) in 0.5 \( M \) \( \text{KCl} \) containing 25 mM Tris-HCl of \( pH \) 7.5 was incubated at 45\(^\circ\)C for predetermined time intervals and the inactivation was stopped by freezing the tubes to crushed ice. Aliquots were also saved separately in the crushed ice for turbidity measurement and read at 340 nm on GENESYS-10 UV-Visible Spectrophotometer. Rate constants of inactivation (\( k_o \)) were calculated by the formula:\( = \left( \ln C_a - \ln C_t \right)/t \), where \( C_a \) and \( C_t \) were the ATPase activities, before and after incubation time \( t \) (in sec).

**Limited proteolytic cleavage and changes in ATPase**

It was monitored, by digesting white myotomal muscle NAM of each species with TLCK-treated \( \alpha \)-chymotrypsin at 20\(^\circ\)C and a ratio of 50:1. At different time intervals, 1 ml aliquots were pipetted out and 0.5 mM PMSF (final concentration) was added to stop the proteolysis. Part of aliquots was used for \( \text{Ca}^{2+} \)-ATPase assay\(^{25} \) or SDS-PAGE analysis\(^{27} \).
Peptide mapping

MyHCs isolated from white myotomal muscle were further purified electrophoretically. Initially, SDS-PAGE in 10% gels was performed. After washing with distilled water, gels were placed on ice over a polythene sheet. MyHC-SDS complex could be visualized as white bands, which were cut out and treated as described earlier. TLCK-treated α-chymotrypsin solution at a substrate to enzyme ratio = 25:1 was added to each well. Electrophoresis was initiated, but interrupted after 30 min and “within-gel digestion” of MyHC was allowed to proceed for 1 h. Electrophoresis was then resumed and continued till the BPB line reached the end of the gel. Protein bands were visualized by silver staining.

UV-difference (UV-DS) spectroscopy

For spectral analysis, solutions of 2 mg myosin/ml (dissolved in 0.5 M KCl in 20 mM Tris-maleate of pH, 7.0) were mixed with equal volume of 2x urea solution of a specific strength. All urea solutions contained the myosin dissolution buffer (0.5 M KCl in 20 mM Tris-maleate of pH 7.0). UV-DS were recorded after 2 h incubation at room temperature on UV-5704 SS spectrophotometer (ECL, India).

Results

Concordance between pH dependence of contractility and LDH level of muscle

Fig. 1 displays two important characteristics of myofibrillar proteins: (i), at pH values of 7.0 to 8.0, contractility of C. punctata and C. batrachus myofibrils was lower than of carps, and (ii), concordance of specific myosin ATPase (Fig. 1B) with specific LDH activities of muscle of corresponding species (Fig. 1C). LDH is an established glycolytic marker of muscle sarcoplasm. Profiles of m-ATPase are not shown here, since for each species they were identical to contractility. Interestingly, contractility of M. armatus myofibrils and LDH levels were intermediate to ABO-possessing species and the carps.

Species-specificity of MyHC isoforms

MyHC prepared from white trunk muscle according to Lied and Decken had no ATPase activity. Also, no appreciable inter-species differences were observed in the stacking of MyHC in routine SDS-PAGE (Fig. 2A). MyHC preparations further purified by preparative SDS-PAGE were homogenous in urea gels (Fig. 2B). Highly purified MyHCs of white muscle of each of the five teleosts stacked as single (monomorphic) bands in SDS-PAGE system of (Fig. 2C). The densitograms (Fig. 2D) supported the inter-species variability in electrophoretic stacking of MyHC isomers displayed in Fig. 2C.

KCl concentration and pH dependence of solubility profiles

Between ionic strength of 0.1 to 0.15 M (Fig. 3A), myosin of ABO-possessing teleosts was 20-25% more soluble than the homologues from major carps. The maximum solubility at 0.2 M KCl was a common property of all myosin isoforms. But, myosin of ABO-possessing teleosts was distinct in displaying 10% higher solubility (P<0.05) between pH values of 6.5 and 7.1 (Fig. 3B).
Effect of thermal incubation

Rate constants of inactivation $k_D$ calculated from the first order plots of inactivation of myosin $\text{Ca}^{2+}$-ATPase (Fig. 4A) were $5.74 \times 10^{-4}$ s$^{-1}$ and $5.85 \times 10^{-4}$ s$^{-1}$ for $C. batrachus$ and $C. punctata$, respectively. The $k_D$ values for myosin $\text{Ca}^{2+}$-ATPase of $L. rohita$, $C. catla$ and $M. armatus$ were $20.18 \times 10^{-4}$ s$^{-1}$, $19.93 \times 10^{-4}$ s$^{-1}$ and $10.7 \times 10^{-4}$ s$^{-1}$, respectively. Clear species variations were displayed by the turbidity profiles also (Fig. 4B); turbidity reached maxima at 20 min of incubation at 45°C. Absorbance of carp myosin at this peak time of denaturation was more than twice as high as those of $C. batrachus$ and $C. punctata$. Turbidity profiles of myosin of $M. armatus$ were in

between those of the myosin of major carps and of ABO-possessing teleosts.

**a-Chymotryptic cleavage of MyHC and its correlation with ATPase**

Fig. 5 compares proteolytic susceptibility at NAM to chymotrypsin ratio of 50:1. SDS-PAGE profiles demonstrated that it was essentially the cleavage of 200 kDa MyHC into low $M_c$ polypeptides (Fig. 5A-D). Myofibrillar proteins of $C. punctata$ and $C.$
**batrachus** were most stable. Following 10 min of digestion, a fraction of undigested MyHC isomers was retained as a thin band of 200 kDa in SDS-PAGE profiles of these species (Fig. 5A-B). Fast degradation of *L. rohita* and *C. catla* MyHC was concomitant with the gradual increase in intensity of 173 kDa HMM-HC and 94 kDa S1-HC bands (Fig. 5C-D). As apparent from thickening of actin band, degradation products co-stack with actin. Susceptibility of Ca\(^{2+}\)-ATPase of these species was reflected by two main characteristics (Fig. 5E-F). Either the magnitude of activation was low as in carps (130-140%) or the rapid decline followed as in *M. armatus* and *L. rohita*.

**Fig. 5**—SDS-PAGE profiles showing changes in sub-molecular composition of polypeptides due to α-chymotryptic digestion of NAMs at the substrate to protease ratio of 50:1 for 30 min. The panels are (A): *C. punctata*; (B): *C. batrachus*; (C): *L. rohita*; and (D): *C. catla*. Profiles of *M. armatus* (not included here) were quite similar to those of major carps. Undigested chicken NAM is used as marker. Changes in Ca\(^{2+}\)-ATPase activity during course of digestion: (E): NAMs of two ABO-possessing teleosts and *M. armatus* (F): NAMs of the carps. Experimental details are given under 'Materials and Methods'.

In contrast, Ca\(^{2+}\)-ATPase activation of 160-170% occurred in NAM of *C. punctata* and *C. batrachus* (Fig. 4E). In these species, activity was maintained at 100%, even after 80 min of digestion.

**Sub-molecular diversity of peptide maps of MyHC isoforms**

Substructural basis of differences between MyHC isoforms of white skeletal muscle were compared by peptide mapping in 15% gels, following digestion with TLCK-treated α-chymotrypsin\(^{28}\) (Fig. 6). The maps of MyHC of *C. punctata*, *C. batrachus* and *M. armatus* consisted of 44, 35 and 32 polypeptides respectively and 41 each for *L. rohita* and *C. catla*.

**Conformational variations of myosins in urea solutions**

Typical conformational changes at the lowest (0.5 M) and highest molarity (4.0 M) of urea are shown in Fig. 6A and B, respectively. With the exception of *L. rohita*, each myosin showed a distinct peak at ~265 and troughs at 259, 263 and 270 nm (Fig. 7A-B). The wavelengths were essentially the perturbation range of phenylalanine residues (Fig. 7A-B). As buried phenylalanine residues got exposed, due to increasing concentrations of urea, slight blue shift in spectra also occurred, accompanied by the appearance of new peaks. The ∆A values of deepest trough at 268-270 nm were used to compare the conformational differences as the function of molarity of urea (Fig. 7C). It was obvious that ∆A values for myosins of two ABO-possessing teleosts were substantially lower than those of *M. armatus* and the major carp myosin isoforms.
Discussion

Partitioning into aerial and aquatic modes of breathing is characteristic of ABO-possessing teleosts *Clarias batrachus* and *Channa punctata* \(^{19,20}\). As a result, these species are capable of surviving outside water and bypass water scarcity by aestivating in mud. The sustenance outside water, however, depends on the type of ABO. Air-breathing organs of *C. batrachus* and *C. punctata* differ in anatomy, organization, evolutionary origin and complexity \(^{20}\). *M. armatus* lacks any specialized ABO, but behaves as an air-breather. Gaseous exchange takes place through skin, which has a rich subcutaneous blood supply \(^{19}\). The two carps respire by aquatic mode only. Thus, the investigated teleosts had different life styles and modes of breathing. Biochemical evidence presented here discriminate these fish broadly as air- and water-breathers and also at species level.

For each species, plots of myofibrillar contractility and m-ATPase were identical (Fig. 1A) and hence independent of breathing mode. However, magnitude of these properties was related with the mode of breathing. ABO-possessing teleosts had distinctly lower contractility and m-ATPase than the water-breathing carps. The equivalence of these functions with specific Mg\(^{2+}\)-ATPase of myosin suggests that catalytic properties of myosin are of prime importance for teleosts of either mode of life. The agreement between specific m-ATPase of each species with the activity levels of LDH (Fig. 1C) points to an integrated evolution of glycolytic metabolism and muscle motility.

MyHC, the core functional polypeptide assembly displayed inter-species variations in electrophoretic mobility of the isomers, suggesting structural differences (Fig. 2). MyHC isomers of each species were monomorphic, indicating that white muscle was composed of single type of muscle fibers \(^{11}\). Therefore, the observed biochemical differences in myosin of air- or water-breathing species cannot be attributed to complexity of muscle fiber types. Mosaic muscles composed of more than one type of muscle fibers display the presence of several MyHC isomers \(^{9,10}\). Occurrence of monomorphic MyHC is consistent with histochemical evidence on *C. punctata* and *M. punctalus*, a sister species of *M. armatus* \(^{30}\).

Ionic strength and pH dependence of solubility of native myosin also revealed species differences (Fig. 3A-B). The solubility of stable myosin of ABO-possessing teleosts between 0.1-0.15 M KCl was higher, than that of unstable isomers of *L. rohita* and *C. catla*, which obviously should be attributed to air-breathing linked stability. Two arguments supported this inference. First, due to less denaturation below pH 7.0 (Fig. 4B) myosin isomers of ABO-possessing teleosts should remain in a soluble state around 0.1 to 0.15 M (Fig. 3A). Second, solubility profiles of myosin of even behavioral air-breather *M. armatus* resembled closely with those of ABO-possessing teleost isomers.

Thermal incubation revealed functional as well as structural differences of the myosin isomers. Thermal inactivation rate constants (k\(_D\)) of myosin ATPase were about four times as low as those of the major
carps. Thus, characteristics of ABO-possessing teleosts myosin were low physiological (Mg\(^{2+}\)) ATPase and substantially thermostable Ca\(^{2+}\)-ATPase (Fig. 4A). The \(k_D\) of ATPase inactivation for Cyprinus carpio was reported to differ by a magnitude of 10 for a temperature difference of 20°C. However, all of the teleosts, in the present study, inhabited the water of 12-15°C, hence the differences in thermostability were not due to acclimation to different temperatures. The other parameter, turbidity also supported higher thermostability of ABO-possessing teleosts. Turbidity is a solubility related change and an indicator of ability of inter-molecular aggregation. In case of fish myosin, aggregation occurs by the interaction of rod\(^{31}\), rather than the head-to-head reaction typical of mammalian myosin\(^{32}\). Thermal denaturation was carried out at a pH 7.5, where according to pH dependence of the solubility, each of the native myosin isoform was totally soluble (Fig. 4B). Therefore, differences in turbidity profiles were due to inter-species differences in aggregation behavior\(^{18}\), which in turn, reflect structural differences of myosin isomers.

To compare susceptibility of ATPase domains, chymotryptic cleavage of MyHC by SDS-PAGE and ATPase assay were carried out simultaneously. Cleavage of two domains of 173 kDa HMM-HC and 94 kDa S1 was parallel to decline in Ca\(^{2+}\)-ATPase (Fig. 5). Since, we used TLCK-treated chymotrypsin, it was free of trypsin contamination that would generate HMM, due to different cleavage specificity. MyHC of ABO-possessing teleosts was least susceptible, since release as well as subsequent cleavage of HMM-HC (Heavy Meromyosin Heavy Chain) and S1 (subfragment-1) proceeded slowly. Moreover, in these two species, a fraction of undigested MyHC persisted in SDS-PAGE profiles (Fig. 5A-B; lanes 2-3) up to 10 min of proteolysis. A compact molecular structure or the absence of one or more critical sites might account to the decreased susceptibility. Either of the possibilities supports differences in primary structure of MyHC isoforms. Primary sequence of Cyprinus carpio myosin provides an example of critical location of a specific residue. Just a single lysine at position 301 in 50 kDa domain (accession nos GenBank U8992 and U32574) makes its MyHC highly susceptible to tryptic cleavage\(^{24}\). Chymotrypsin cleaves a polypeptide at carboxyl groups contributed by phenylalanine, tryptophan and tyrosine residues. Therefore, most probably some of these hydrophobic residues had a critical location in vicinity or within catalytic domains of susceptible MyHC isomers.

More extensive variations in hydrophobic residues were indicated by the peptide maps (Fig. 6). The number of countable polypeptides in the maps varied between 32-41, pointing to deletion/addition of a number of specific sites. During evolution, a specific protease cleavage site could be eliminated or added by alteration in the reading frame of the gene due to deletion or addition of a nucleotide. Hence, the observed sub-molecular variations of MyHC isoforms reflected their different evolutionary histories.

Few data are available on spectral comparisons of fish myosin. Mirror carp myosin is known to acquire random structure at rather low concentration (4.0 M) of urea, while tyrosine residues of elasmobranch myosin are not exposed at even 6.5 M urea\(^{5}\). In the present study, no perturbation of tyrosine or tryptophan was observed up to 4.0 M urea, for any of the myosins. The exposure of buried phenylalanine residues, however, occurred, which was also accompanied by a blue shift as the molarity of urea increased (Fig. 7A and B). The increase in ΔA at 268-270 nm (Fig. 7C) displayed wide variations in the magnitude of perturbation by urea. As obvious, myosin isomers of ABO-possessing teleosts were identifiable as one stable class against that of the aquatic breathers. The chymotryptic cleavage and UV-DS data suggested that hydrophobic interactions substantially contributed to structural stabilization of investigated myosin isoforms.

This is the first report that demonstrates modification of ATPase, contractility and thermostability of fish myofibrillar proteins, in context to breathing modes. The data highlight two main adaptive characteristics of myosin isoforms of ABO-possessing teleosts: (i) a ‘down-regulation’ of catalytic activity to make muscle sluggish; and (ii) the exceptional stability of myosin isomers to sustain stressful conditions. In contrast, during low temperature acclimation, catalytic efficiency (Mg\(^{2+}\)-ATPase) of teleost myosin was enhanced while thermostability decreased\(^{43,33,34}\).

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