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MYOSIN SWITCHES IN SKELETAL MUSCLE DEVELOPMENT OF AN URODELAN AMPHIBIAN, PLEURODELES WALTLII. COMPARISON WITH A MAMMALIAN, MUS MUSCULUS[1]

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The isomyosins from dorsal axial muscle, which appear successively through metamorphosis of P.waltlii, are shown to be composed of identical fast-type light chains but of distinct heavy subunits. We observe that this modification goes with a change in ATPase activity as also in the case of mouse. Metamorphosis in amphibian as well as birth in mammalian are thus both accompanied by the synthesis of new myosins of higher catalytic efficiency.

Contractile properties of adult muscles are gradually acquired during late prenatal and early postnatal development. A biochemical basis for this ancient observation has been obtained by recent electrophoretic and immunocytochemical studies which have demonstrated that fetal, neonatal, and adult myosins appear successively in skeletal muscles of birds and mammalians [1-9]. Hatching and birth represent major physiological events which are thus accompanied by the synthesis of new types of myosins.

Metamorphosis in amphibians involves the passage from an aquatic to a terrestrial mode of life and constitutes similarly a model of the organism adaptation to a new vital situation. We have shown in previous studies [10,11] that fast skeletal muscles of adult amphibians, both the frog Rana esculenta and the urodelan Pleurodeles waltlii, contain three isomyosins. We have also analyzed the myosin patterns of the dorsal muscle of P.waltlii at various stages of its development and observed for the first time that, as in the other vertebrates, there is a succession of myosins forms which differ by their electrophoretic properties in the native state [11].

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We present in this paper the characterization of these isoforms.We show that larval, metamorphosis, and adult myosins of the dorsal axial muscle of P.waltlii are all composed of a combination of the same fast-type light chains, but that they differ by both their heavy subunits and their ATPase activities. Similar results are obtained with neonatal and adult myosins from a mammalian so far not investigated, Mus musculus, for which a recombinant plasmid demonstrating the transition from fetal to adult myosin heavy chain $_{m}RNA_{s}$ has been recently cloned [12].

EXPERIMENTAL

Animals and muscles. Dorsal axial muscles from P.waltlii, at selected stages of development defined from the staging series by [13], originated from the breeding of C.Gallien. Dorsal and lingual muscles from mice of various ages and from 18 day embryos of muscular dysgenesis mutants were gifts of C. Gallien and of F.Rieger.

Myosin extracts. Rapid preparation of crude myosin was performed in ice. The muscles were cut in small pieces and washed with either 4 vol. of 600 mM KCl, 40 mM $NaHCO_3$, 10 mM Na_2CO_3, 1 mM $MgCl_2$, 10 mM $Na_4P_2O_7$ pH 8.8 (buffer A) in the case of P.waltlii, or with 5 vol. of 20 mM NaCl, 5 mM sodium phosphate and 1 mM EGTA pH 6.5 in the case of M.musculus. Myosin was then extracted with either 4 vol. of A or with 3 vol. of 100 mM $Na_4P_2O_7$, 5 mM EGTA and 1 mM dithiothreitol pH 8.5 ; after 20 mn of gentle agitation, the mixtures were centrifuged at 10 000g and the myosin containing supernatants diluted twice with glycerol for conservation at -20°C.

Electrophoretic analysis of native myosin. Non-dissociating conditions were used but for a few modifications as previously described [14,15]. Running buffer was 20 mM $Na_4P_2O_7$ (pH 8.5), 10 % glycerol, 0.01 % 2-mercaptoethanol, 2 mM $MgCl_2$ and 1 mM ATP. Cylindrical gels (6x0.5cm) were 4 % in polyacrylamide ; about 1 µg myosin per band was loaded. Electrophoresis was carried out at 5 mA per tube for 20 to 22 hours at 2°C.

ATPase activity measurements. Myosin ATPase activity was measured on the gels in the conditions just described, but for replacing the $Na_4P_2O_7$ buffer by 30 mM $K_4P_2O_7$. After a short run of $4^{h}30$, the buffer in the upper tank was changed for ATP for an other $1^{h}30$. The gels were then incubated at 37°C with ATP and $CaCl_2$ in the ATPase assay medium [14] and the kinetics of the amount of phosphate released was obtained by measuring at successive times the absorbance of the white band of calcium phosphate formed.

Analysis of myosin light chains by isoelectric focusing. Myosin isoenzymes were first separated by electrophoresis in non-dissociating conditions. Several bands coming from 4 to 8 gels were sliced after light staining or without staining by comparison with a control and then loaded on tubes. The gels (9x0.27cm) contained 2% ampholines pH 4-6.5. Isoelectric focusing was run for 16 000 V.h. according to [16], and gels stained according to [17].

Electrophoretic analysis of myosin heavy chains in the presence of SDS. This was performed in 5 % polyacrylamide separation slab gels, in the presence of 0.1 % SDS [18] and of 25 % glycerol, according to [7]. Loads were of about 2 to 10 ng myosin per band. Electrophoresis was carried out at 20 mA per slab for 5 hours. Gels were stained with silver nitrate [19].

Analysis of the proteolytic digestion patterns of the heavy chains. This was done according to [20] as previously published [21].

RESULTS and DISCUSSION

The various myosins which appear sequentially in development of P.waltlii (Fig.1) may be ranged into three categories, the larval, the metamorphosis, and the adult types [11]. This compares well to what is observed in the other classes of vertebrates, such as the mammalians. In rat, for instance, one encounters successively embryonic, neonatal and adult isomyosins [3]. Similar patterns are obtained for fast muscles of the mouse, both the lingual [11] and the dorsal (Fig.1) muscles. It appears however that development in mouse is more precocious than in rat, as adult myosins appear a few days earlier and as 18 day embryos do not display the fetal but already the neonatal forms. The same three neonatal isomyosins are found to be present in muscular dysgenesis 18 day embryos which undergo a neuromuscular lethal mutation (results not shown).

The quite distinct electrophoretic mobilities displayed by the native myosins at successive steps of development of M.musculus and even more of P.waltlii may come from structural differences in either the large, or the small, or both subunits which compose the molecules. We present in figure 2 the light chain contents of these myosins. Neonatal and adult murine myosins are composed of a combination of the same fast-type light chains, two alkali ones LC_{1F} and LC_{3F} of isoelectric points pI respectively 5.5 and 4.85 and one one phosphorylable form LC_{2F} of pI equal to 5.2. We observe that larval, metamorphosis, and adult myosins of P.waltlii are also all composed of identical fast-type light chains ; these

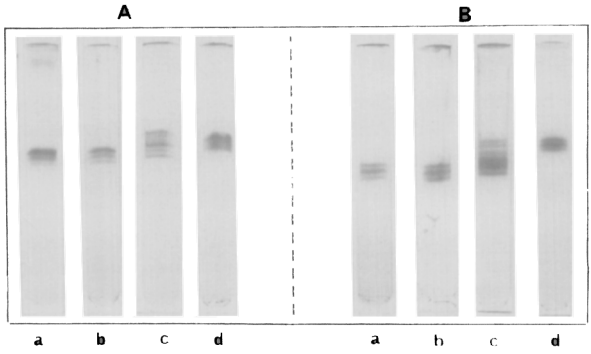

Figure 1. Myosin isoenzymes of fast-type muscles through development of a mammalian (A) and of an amphibian (B). Electrophoretic analysis in non-dissociating conditions.

A. Dorsal muscle myosins from M.musculus of selected ages, displaying successively neonatal (a and b : 3 days prenatal and 1 day postnatal), a mixture of neonatal and adult (c : 11 days), and adult (d) myosins.

B. Dorsal muscle myosins from P.waltlii, at selected stages of development [13], displaying larval (a and b : stages 42 and 54), a mixture of larval, metamorphosis, and adult (c : stage 55_C), and adult (d) myosins.

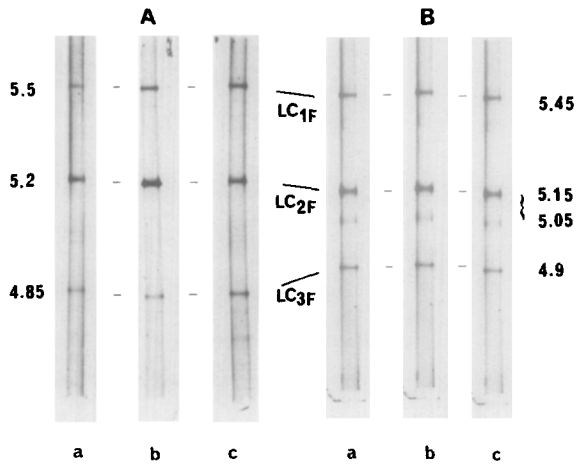

Figure 2. Light chain analysis by isoelectric focusing of myosins first separated by electrophoresis in non-dissociating conditions.

A. **M. musculus** a. neonatal myosins from 1 day mouse
b. mixture of neonatal and adult myosins from 11 days mouse
c. adult myosins.

B. **P.waltlii** a. larval myosins from stage 54
b. metamorphosis myosins from stage 55c
c. adult myosins.

The numbers indicate the isoelectric points of the various light chains, the two alkali types LC_{1F} and LC_{3F}, and the phosphorylable one LC_{2F}.

differ from the murine ones by their isoelectric points, LC_{1F} and LC_{2F} being somewhat more acidic, with pI equal to 5.45 and 5.15, and LC_{3F} somewhat more basic with a pI of 4.9. In the case of amphibian myosins, about 20 % of the LC_{2F} is under the phosphorylated form with a pI of 5.05. As in birds or in mammalians [1,5,22], one may reasonably suppose that the three myosins in each category, the larval, the metamorphosis, and the adult ones, are alkali light chain homodimers and heterodimer. The difference in electrophoretic mobility between the isoforms within one class originates in the small subunits ; on the other hand, homologous myosins, that is LC_{1F} or LC_{3F} homodimers or the heterodimer, of two different categories, such as the larval and the adult ones, must differ by their heavy chains.

Differences in the large subunits are demonstrated by electrophoretic analysis both of the whole proteins and of their proteolytic peptide maps. Fig.3 shows that the heavy chains of larval myosins (b) migrate in SDS slightly ahead the adult ones (a), and this is accompanied by distinct peptide maps (Fig.4). Different patterns of neonatal and adult heavy chains of murine myosins are also presented in Fig.4.

The basic ATPase activity of myosin is generally recognized to be linked to the nature of its heavy chains, the light chains playing a role in stabilizing the conformation of

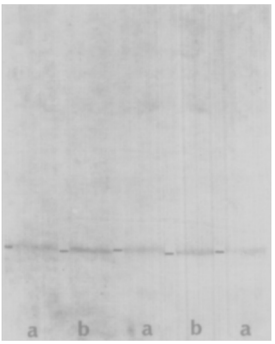

Figure 3. Electrophoresis in the presence of SDS and glycerol of the heavy chains of adult (a) and larval (b) myosins of P.waltlii.

the whole molecule and also possibly in the interaction with actin. As we have just described, myosins of both P.waltlii and M.musculus display the same light chains but different heavy chains through development. The ATPase activity of the various classes of myosins has been measured on gels after separation in non-dissociating conditions. This determination was not simple in the case of the amphibian myosins, as they are known to be fragile and less active than the mammalian ones [23,24]. Two modifications to the original technique [14] were therefore found essential, replacing the electrophoresis sodium pyrophosphate buffer by potassium pyrophosphate and allowing the run to last altogether not more than 6 hours at 2°C. In these conditions of assay,no alkaline inactivation was

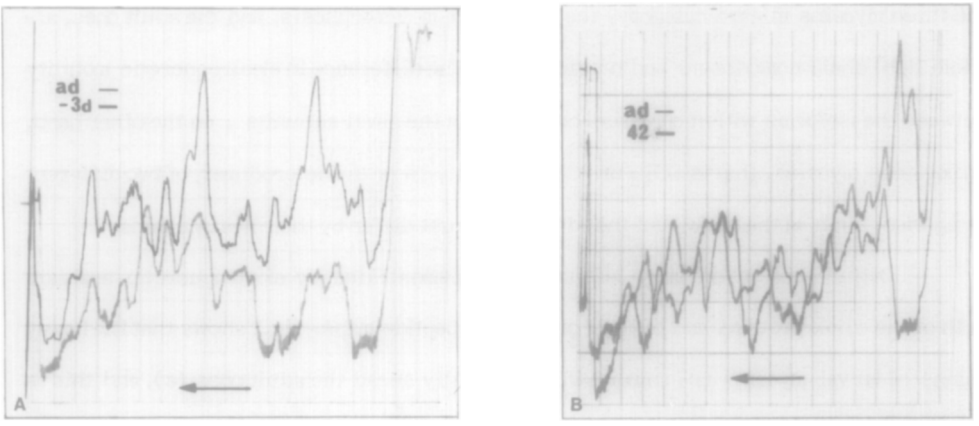

Figure 4. Superimposed densitometric scans of the proteolytic digestion patterns of heavy chains isolated from myosin isoenzymes prealably separated by gel electrophoresis in non-dissociating conditions.

A. **M.musculus** : neonatal (3 days) and adult myosins.
B. **P.waltlii** : larval (stage 42) and adult myosins.

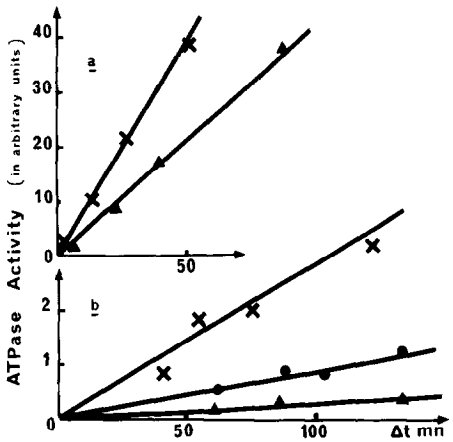

Figure 5. Ca^{2+}-activated ATPase activity of myosin on non-dissociating gels. The activity is expressed by the ratio of the calcium phosphate band at successive intervals of time over the protein band stained after the assay with Coomassie blue.

A. **M.musculus** myosins. ▲ neonatal × adult
B. **P.waltlii** myosins. ▲ larval ● metamorphosis × adult

observed and straight lines for the hydrolysis of ATP were obtained (Fig.5). Adult P.waltlii myosins were found to be about 10 times more active than larval ones ; they were on the other hand some 25 times less active than the adult murine ones, which are in turn 2 times more active than the neonatal ones.

An increase in ATPase activity of mammalian and avian myosin during development had been observed since a long time [25]. Difficulties inherent to the purification of myosin at the perinatal period had however shed some doubt on the validity of such result and a recent paper [8] reports that embryonic and adult myosins from chicken pectoralis fast muscle show the same level of ATPase activity. In the two cases we present in this paper, the ATPase assay does not involve a long preparation of myosin ; the purification is obtained by electrophoresis of a crude extract, and no degradation can be observed. We can thus conclude that, both in mouse and in P.waltlii, the physiological changes of birth and metamorphosis are accompanied by the synthesis of myosins of higher ATPase activity, which may account for the new contractile properties of the adult muscles.

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REFERENCES

1. **Hoh, J.F.Y. and Yeoh,G.P.S.** (1979) Nature (London) 280, 321-323.
2. **Rushbrook, J.I. and Stracher, A.**(1979)Proc. Natl. Acad. Sci. U.S.A.76, 4331-4334.
3. **Whalen, R.G., Sell,S.M., Butler-Browne,G.S.,Schwartz,K., Bouveret,P. and Pinset-Härström,I.** (1981) Nature (London) 292, 805-809.
4. **Syrovy, I.** (1981) Int. J. Biochem. 13, 237-24
5. **Takano-Ohmuro,H., Obinata, T., Masaki,T. and Mikawa,T.** (1982 J. Biochem. (Tokyo) 91,1305-1311.
6. **Bandman,E., Matsuda,R. and Strohman,R.C.** (1982)Dev. Biol. 93,508-518.
7. **Carraro,U. and Catani,C.** (1983)Biochem. Biophys. Res. Commun. 116, 793-802.
8. **Lowey,S., Benfield,P.A., LeBlanc,D.D. and Waller,G.S.** (1983)J. Muscle Res. Cell Motility 4, 695-716.
9. **Winkelmann,D.A., Lowey,S. and Press,J.L.** (1983).Cell 34, 295-306.
10. **Pliszka,B., Strzelecka-Golaszewska,H., Pantaloni,C. and d'Albis,A.** (1981) Eur. J. Cell. Biol. 25, 144-149.
11. **d'Albis,A., Janmot,C., Weinman,J, and Gallien ,C.L.** (1984) in Recent Advances on Contractile Proteins(Alia,E.E., Arena,N. and Russo, M.A. eds.) Praeger Scientific, New-York, in press.
12. **Weydert,A., Daubas,P., Caravatti,M., Minty,A., Bugaisky, Cohen,A. Robert,B. and Buckingham,M.** (1983) J.Biol. Chem. 258, 13867-13874.
13. **Gallien,L. and Durocher, M.** (1957)Bull. Biol. France et Belgique 19, 97-114.
14. **Hoh,J.F.Y., Mc Grath,P.A. and White, R.I.** (1976) Biochem. J. 157, 87-95.
15. **d'Albis,A., Pantaloni,C., and Béchet, J-J.** (1979)Eur. J. Biochem. 99, 261-272.
16. **O'Farrell,P.H.** (1975)J. Biol. Chem. 250, 4007-4021.
17. **Righetti,P.G. and Drysdale,J.W.** (1974)J. Chromatogr. 98, 271-321.
18. **Laemmli,U.K. and Favre,M.** (1973)J. Mol. Biol. 80, 575-599.
19. **Morrissey,J.H.** (1981) Anal. Bioch. 117, 307-310.
20. **Cleveland,D.W., Fischer,S.G., Kirschner,M.W. and Laemmli,U.K.** (1977)J. Biol. Chem. 252, 1102-1106.
21. **d'Albis,A., Pantaloni,C., and Béchet, J-J.** (1979) FEBS Lett. 106, 81-84.
22. **Hoh,J.F.Y.** (1978) FEBS Lett. 90, 297-300.
23. **Ferenczi,M.A., Homsher,E., Trentham,D.R. and Weeds,A.G.** (1978)Biochem. J. 171, 155-163.
24. **Pliszka,B., Szpacenko,A., Strzelecka-Golaszewska,H.** (1979) Int. J. Biochem. 10, 343-359.
25. **Trayer,I.P. and Perry,S.V.** (1966)Biochem. Z. 345, 87-100.