Myosin structure and thyroidian control of myosin synthesis in urodelan amphibian skeletal muscle

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ABSTRACT Electrophoretic analysis in non-dissociating conditions reveals three types of myosin in adult urodelan amphibian skeletal muscles: 3 isoforms of fast myosin (FM), one isoform of intermediate myosin (IM) and one or two isoforms of slow myosin (SM). Each type is characterized by a specific heavy chain HCf (FM), HCi (IM) and HCs (SM), respectively. In all urodelan species, as in mammals, fast isomyosins associate HCf and the three fast light chains LC1f, LC2f, and LC3f. In most urodelan species the intermediate myosin contains LC1f and LC2f and can be considered as an homodimer of the alkali LC1f. However, in *Euproctus asper*, IM is characterized by the association of both slow and fast LC with HCi. Slow myosin is a hybrid molecule associating HCs with slow and fast LC. During metamorphosis, a myosin isoenzymic transition occurs consisting in the replacement of three larval myosins (LM) characterized by a specific heavy chain (HCl), by the adult isomyosins with lower electrophoretic mobilities. At the same time there is a change in the ATPase myofibrillar pattern, with the larval fiber types being replaced by adult fibers of types I, IIA and IIB. In the neotenic and perennibranchiate species, which do not undergo spontaneous metamorphosis, sexually mature larval animals present a change in the myosin isoenzymic profile, but no complete transition. The coexistence of larval and adult isomyosins and the persistance of transitional fibers of type IIC in the skeletal muscle are demonstrated. Experimental hypo- and hyperthyroidism indicate that thyroid hormone stimulates the regression of the larval isomyosins, possibly through indirect pathways. In contrast, the appearance and the persistence of the adult isomyosins seem to be independent of thyroid hormone. Thus, the control of the isoenzymic transition in the skeletal muscle of urodelan amphibians appears to imply indirect mechanisms, operating differently on each of the two phases of the complete transition.

KEY WORDS: *metamorphosis, skeletal muscle, myosin, thyroid hormone, urodelan amphibians*

Introduction

Urodelan amphibians represent a privileged model for studying the role of thyroid hormone in the regulation of some ontogenic events and differentiation processes. Development, which has been extensively investigated in various urodelan species, is slow enough as compared with anuran amphibians, and staging can be established with great precision by reference to both anatomical and metabolic changes. A unique advantage is the availability of species displaying different types of thyroidal status. Most species undergo spontaneous anatomical metamorphosis driven by a transient increase in serum thyroid hormone; certain hypothyroidian neotenic species or varieties display no external metamorphosis in natural conditions; finally in the perennibranchiate species anatomical metamorphosis never occurs even after experimental triiodothyronine treatments, due to low responsiveness to the thyroid hormone.

Myosin is a superfamily of proteins with a common basic structure, in which two heavy chains (HC) are associated with four light chains (LC). In birds and mammals, various kinds of myosin isoforms differing by their heavy chain and/or by their light chain constituents have been described in adults and during development

Abbreviations used in this paper: FM, fast myosin; FM_1, FM_2, FM_3, fast myosin type-1, type-2, type-3; HCF, fast heavy chain; HCi, intermediate heavy chain; HCl, larval heavy chain; HCs, slow heavy chain; IM, intermediate myosin; LM, larval myosin; LC, light chain; LC_1f, LC_2f, LC_3f, fast light chain type-1, type-2, type-3; LC_{1s}, LC_{2s}, LC_{1sa}, LC_{1sb}, light chain type-1, type-2, type-1a, type-1b; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid stimulating hormone.

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0214-6282/90

(Barton and Buckingham, 1985; Swynghedauw,1986; Stockdale and Miller, 1987; Weydert, 1988). It has been well established that innervation pattern (Hoh, 1975; Ishimura *et al.*, 1981; Gambke *et al.*, 1983) and thyroidian status (Butler-Browne *et al.*, 1984; Izumo *et al.*, 1986; d'Albis *et al.*, 1987; Russel *et al.*, 1988) play an active role in regulating myosin expression in the skeletal striated muscles of higher vertebrates.

In this review (Chanoine *et al.*, 1987, 1988; Chanoine and Gallien, 1989; Chanoine *et al.*, 1989, and recent unpublished results), the structural organization of adult skeletal striated muscle, as defined by the myofibrillar ATPase profile, is presented for different urodelan amphibian species differing according to their thyroidian status. The heavy chain and light chains constitution of the various myosin isoforms present in the adult skeletal muscle is characterized. Changes in the myofibrillar ATPase profiles and the myosin electrophoretic patterns in relation with structural modifications during the course of development are described. Finally the role of thyroid hormone on the expression of myosin isoenzymes during development and in adult urodelan amphibians is discussed on the basis of an experimental analysis of hypo- and hyperthyroidian animals.

Fast and intermediate myosin isoforms

In skeletal muscles of the adult rat, electrophoresis of native myosin in non-denaturing conditions typically demonstrated three isoenzymes of fast myosin (FM1, FM2, FM3), one isoenzyme of slow myosin (SM) with lower electrophoretic mobility, and one intermediate myosin isoform (IM) with an electrophoretic mobility between the slow and the three fast isomyosins.

The fast myosin isoforms and the intermediate isomyosin were shown to correspond respectively to myofibers of types IIB and IIA. In these two fiber types, two different myosin heavy chains, described as HC IIB and HC IIA, were characterized, together with the fast light chains. It was clearly established that in fast twitch muscles, the intermediate isomyosin is constituted by the association of HC IIA with the fast light chains (Billeter *et al.*, 1982; Fitzsimons and Hoh, 1983; Butler-Browne and Whalen, 1984; d'Albis *et al.*, 1986) .

In adult urodelan amphibians, we studied the dorsal skeletal muscle (*dorsalis trunci*), which appeared to be a fast type muscle, as shown by studying the myofibrillar ATPase profile. In the dorsal muscle of *Pleurodeles waltlii*, which is representative of the urodelans that metamorphose spontaneously, fibers of type IIA and type IIB represented respectively 67% and 20% of the total fibers (Table 1).

In this species, native myosin from the dorsal muscle was analyzed in non-dissociating conditions and revealed only three fast isomyosins with lower electrophoretic mobilities than the three fast isoforms of mammals (d'Albis *et al.*, 1985a). A study of the subunit composition of these three fast isomyosins demonstrated one specific heavy chain, namely HCf, and three fast light chains: LC1f, LC2f and LC3f with apparent molecular masses of 25 kDa, 20 kDa, and 16 kDa and isoelectric points of 5.45, 5.15, and 4.15, respectively (d'Albis *et al.*, 1985b). It should be noted that the phosphorylatable LC2f has a larger molecular mass in the urodelan amphibians than in mammals, and that the light chains of *P.waltlii* differ from the murine light chains by their isoelectric points (d'Albis *et al.*, 1985b). All fast isomyosins FM1, FM2 and FM3 are characterized by the same specific heavy chain homodimer HCf. As it has been generally accepted for mammalian muscles, we should consider that in the urodelan amphibian muscles, the fast isomyosin FM1 corresponding to the faster migrating electrophoretic band is an alkali light chain homodimer $(LC2f)_2$ $(LC3f)_2$, since FM2 is a heterodimer (LC1f $(LC2f)_2$ LC3f), and the slowest band FM3 again an homodimer $(LC1f)_2$ $(LC2f)_2$.

In spite of the high percentage of type IIA fibers in *P.waltlii* dorsal skeletal muscle, the intermediate isomyosin was not detected in our initial studies, which suggested a comigration of IM with the fast isoforms. However, in other urodelan species, *Triturus palmatus*, *Triturus alpestris*, and *Euproctus asper*, an intermediate myosin isoform with an electrophoretic mobilty between the slow and the fast isoenzymes was in fact demonstrated. In mammals, SDS-PAGE analysis in the presence of 25% glycerol (Carraro and Catani, 1983) showed this intermediate isomyosin to be characterized by a particular heavy chain (HCi), with different electrophoretic mobility than the slow (HCs) and the fast (HCf) heavy chains. It was interesting to note that in the urodelan amphibians, the light chain pattern of the intermediate myosin for a given muscle is species specific. In the dorsal skeletal muscle of *T. palmatus* and *T. alpestris* the intermediate myosin was shown to contain LC1f and LC2f, and can therefore be considered as a tetramer of the type $(LC1f)_2$ $(LC2f)_2$, thus containing a homodimer of the alkali LC1f as in FM3, according to d'Albis *et al.* (1986) for the intermediate myosin from murine masseter. In *E. asper*, intermediate myosin contained LC1f, LC2f, and also small amounts of the slow light chains LC1s and LC2s. The occurrence of such a hybrid molecule might account for particular physiological properties of *E. asper* dorsal muscle, in relation with specific locomotion modes and postures. By contrast, in mammals, it was reported (d'Albis *et al.*, 1986) that the light chain composition of intermediate myosin was specific for the muscle type but not for the species: the slow type soleus muscles of the rat and the mouse presented an intermediate myosin with mixed slow and fast LC content, and fast type muscles of the same species had an intermediate myosin with only fast LC content.

Slow myosin isoforms

A study of the myosin isoforms in the dorsal muscle of neotenic adult *Ambystoma mexicanum* suggested a comigration of slow and fast isomyosins, as also observed in different fishes (Karasinski and Kilarski, 1989; Martinez *et al.*, 1989). However, reelectrophoresis of the total myosin isoenzymes in SDS-PAGE failed to reveal any slow light chains. In the perennibranchiate *Proteus anguinus* small amounts of slow myosin from the dorsal muscle of adult animals were detected in pyrophosphate gels, but once again no slow light chains could be detected.

These observations were coherent with previous results obtained in the frog *Rana esculenta*. In the slow-tonic fibers of frog muscle, Pliszka *et al.* (1981) demonstrated in pyrophosphate gel a myosin isoform with lower electrophoretic mobility than the three fast isomyosins contained in fast-twitch fibers. While analysis of the proteolytic digestion pattern revealed differences in the heavy chain structure of the isomyosins from slow and fast fibers, SDS-PAGE failed to demonstrate light chains that would be specific of the myosin from slow tonic fibers.

The study of myosins from the dorsal muscle of adult *Tylototriton*

TABLE1

CHANGES IN THE ATPase MYOFIBRILLAR PATTERN DURING THE DEVELOPMENT OF *P.WALTLII* AND *A. MEXICANUM*. FIBER TYPES IN THE METAMORPHOSED ADULT *A. TIGRINUM*.

P. waltlii			*A. mexicanum*		
Stages	Fiber types		Fiber types		Stages/ages
53-54	Large (central)	Small (central or peripheral)	Large (central)	Small (central or peripheral)	54 4-6 months
55 a-b	Large (central) 30%	Small (central or peripheral) 70%	Large (central) 18%	Small (central or peripheral) 82%	7 months
55c	IIA 20%	IIC+IIB I 70% 10%	IIA IIC - 73%	IIB I 18% 9%	11-12 months
			IIA IIC 10% 60%	IIB I 16% 14%	Neotenic adult
Adult met.	IIA 67%	IIB I 20% 13%	IIA 57%	IIB I 26% 17%	*A. tigrinum* Adult met.

P.: *Pleurodeles*; A.: *Ambystoma*

verrucosus, another urodelan species that undergoes spontaneous metamorphosis, allowed a better understanding of the former results. In this case the isoenzymic pattern was characterized by the three fast myosin isoforms and a larger band with lower electrophoretic mobility. Analysis of the light chain pattern after reelectrophoresis of the total isomyosins and bidimensional electrophoresis, demonstrated the three fast light chains LC1f, LC2f and LC3f, and also three slow light chains LC1sa, LC1sb and LC2s. It has to be emphasized that only bidimensional gel electrophoresis allowed a clear separation of LC1sa, LC1sb and LC1f, since it happens that a comigration of these light chains occurs in one dimensional gel electrophoresis. In *T. verrucosus* the light chains presented apparent molecular masses of 25kDa for LC1s, 25 kDa for LC1f, 20 kDa for LC2f, 19 kDa for LC2s, and 16 kDa for LC3f.

An extensive study of myosins from the adult dorsal muscle of various other urodelan amphibian species, all submitted to spontaneous metamorphosis, revealed the existence of a well-defined slow myosin. This slow myosin was characterized, in pyrophosphate gel, by one *(Salamandra maculata T. palmatus and E. asper)* or two isoenzymes (*Cynops pyrrhogaster, T. alpestris*) with lower electrophoretic mobilities than the intermediate and the fast isoforms.

In these species, SDS-PAGE with 25% glycerol demonstrated a specific slow heavy chain (HCs) with an electrophoretic mobility distinct from HCi and HCf, which also corresponds to different results obtained in the human (Billeter *et al.*, 1981) or the rat (Carraro and Catani, 1983; Betto *et al.*, 1986; Bar and Pette, 1988). The slow myosin appeared to be a hybrid molecule, since the light chains associated to the HCs homodimer were fast light chains (LCf), always predominant, and slow light chains (LCs) in small amounts.

An important polymorphism appeared in the light chains composition of slow myosin. In *S. maculata*, reelectrophoresis in SDS-PAGE of the slow myosin isoform, first separated in non dissociating conditions, showed LC1f, LC2f and LC1s. Moreover, analysis in bidimensional gel electrophoresis demonstrated the existence of two LC1s, LC1sa and LC1sb, differing by their isoelectric point. In the other species slow myosin contained LC1f, LC2f, one single form of LC1s, and an associated LC2s. The occurrence of two slow myosin isoenzymes in *C. pyrrhogaster* and *T. alpestris* remains unclear, since they appeared to possess the same HCs and presented no detectable differences in LC composition and stochiometry. A similar observation was noted by Karasinski and Kilarski (1989) in the fish *Rutilus rutilus*.

In all those urodelan species, molecular masses of the light chains, as deduced from standard markers run in parallel, were 27 kDa for LC1s and 22kDa for LC2s.

Analysis of the myofibrillar ATPase profile in the dorsal muscle of adult *P. waltlii* showed 13% of type I fibers (Table1). Although it had been shown In mammals that fibers of type I contained slow myosin, the initial analysis in pyrophosphate gel did not allow the demonstration of slow (as well as intermediate) myosin in the dorsal muscle of *P. waltlii*. Yet, modifications of the basic electrophoretic conditions have recently allowed in *P. waltlii*, as in the other species, the clear separation of one slow (SM), one intermediate (IM) and three fast isomyosins (FM1, FM2, FM3) (Fig.1), characterized by specific heavy chains, respectively HCs, HCi and HCf (unpublished data).

Changes in myosin isoforms during ontogenesis

In *P. waltlii*, analysis of myosin isoforms during the course of ontogenesis revealed a complete myosin isoenzymic transition. From stage 42 to stage 54, which corresponds to larval life (Gallien and Durocher, 1957), three electrophoretic bands representing larval myosin isoenzymes were characterized in pyrophosphate gel. At stage 55, during anatomical metamorphosis, new bands of lower electrophoretic mobility were detected, corresponding to the fast isomyosins and to heterogeneous transitional myosins having a mobility intermediate between the larval and the fast isomyosins. As many as nine bands were observed at the late stage 55c (ending

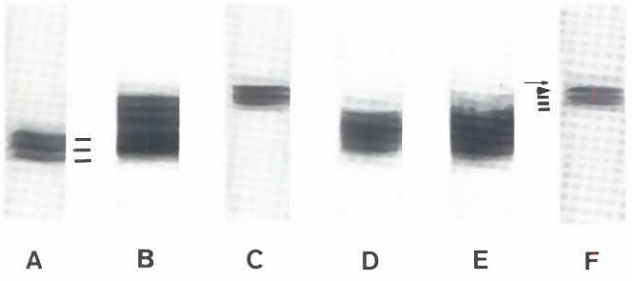

Fig. 1. Electrophoresis in non-dissociating conditions of myosins from the dorsal muscle of *P. waltlii* and *A. mexicanum*. (**A**) *Hypophysectomized* P.waltlii *aged 6 months. Bars indicate the larval myosin isoforms.* (**B**) *Hypophysectomized* P.waltlii *aged 3 years.* (**C and F**) *Metamorphosed adult* P.waltlii *(→ Slow myosin isoform; ⇢ Intermediate myosin isoform; _ Fast myosin isoforms).* (**D**) *Neotenic* A. mexicanum *aged 9 months (control).* (**E**) *Hypophysectomized* A. mexicanum *aged 9 months.*

of external metamorphosis). After metamorphosis was complete (stage 56), isomyosins of intermediate electrophoretic mobility disappeared first, followed by the three larval myosin isoforms. Finally, in the sexually mature adults, only the three fast isomyosins having the lowest electrophoretic mobility were detected in the conditions of the initial technique.

In the species *Ambystoma mexicanum* and *Ambystoma tigrinum* the same nomenclature established for *P.waltlii* by Gallien and Durocher (1957) was used for staging, in order to make comparison easier.

In the neotenic species *A. mexicanum*, electrophoresis of native myosin extracted from the dorsal muscle at stages 42 to 54 (animals aged 4 months) demonstrated three larval isomyosins having electrophoretical mobilities similar to the three *P.waltlii* larval isomyosins. Other isoenzymes of lower mobility corresponding to heterogeneous transitional myosins and fast myosins appeared gradually in the course of subsequent development, but the larval isoforms did not disappear and were still observed in neotenic animals aged 6 years and more. So, there was no complete myosin isoenzymic transition in this species.

In the related species *A. tigrinum*, two different lines were studied and displayed two different situations. In one variety, adult individuals were neotenic as in *A. mexicanum*, and reached sexual maturity in the form of giant larvae. In the other line metamorphosis occurred spontaneously at the end of stage 54. Myosin changes in the neotenic adult *A. tigrinum* were similar to *A. mexicanum*, i.e. the larval and adult isoforms coexisted continuously; in contrast a full myosin isoenzymic transition was observed in the dorsal muscle of metamorphosed adult *A. tigrinum* as in *P. waltlii*.

Finally, in the perennibranchiate *Proteus anguinus*, there was also no complete transition, as in *A. mexicanum*. Electrophoretic analysis in non-dissociating conditions, of the myosin extracted from the dorsal muscle of *P. anguinus* individuals aged 3 months demonstrated three distinct bands of larval-type isomyosins. Other bands of lower electrophoretic mobility, corresponding to adult isomyosins, appeared at the end of the larval phase in animals aged 6 to 9 months. The adult isoforms gradually increased in intensity during the course of development, which is very slow in this species (Vandel and Durand, 1970), but the three larval isomyosins were continuously detected, and were still present in sexually mature animals aged 16 years.

Changes in the myosin subunits (HC and LC) during ontogenesis

In *P. waltlii*, myosin heavy chains from the dorsal skeletal muscle were analyzed by electrophoresis in 5% polyacrylamide separation slab gel, in the presence of 0.1% SDS and 25% glycerol, at different stages of the development. It was demonstrated that changes in the myosin isoenzymic pattern implied modifications in the heavy chain content. The three larval isomyosins (stage 54) were characterized by a specific heavy chain (HCl), which differed from HCf by its electrophoretic mobility.

In *A. mexicanum*, analysis of the heavy chains in denaturing conditions and the study of the proteolytic digestion pattern revealed the presence of one single larval heavy chain in individuals aged three months (stage 54), and the coexistence of two distinct heavy chains, one larval and one adult, in the neotenic sexually mature individuals.

In the metamorphosed sexually mature *A. tigrinum* individuals, electrophoresis in dissociating conditions revealed only one band, corresponding to a single adult myosin heavy chain. In contrast, two bands were observed in the neotenic adult individuals, thus demonstrating the coexistence of both larval and adult heavy chains.

In the three species that have been investigated, analysis of the light chain pattern under denaturing conditions in one-dimensional gels revealed the existence of the three fast light chains LC1f, LC2f, and LC3f, which showed no modification during the course of development.

These observation thus demonstrated that during ontogenic development, the modifications in myosin isoenzymic patterns are clearly accompanied by a change in the heavy chains, while no change in the global light chain composition can be detected.

Changes in the ATPase myofibrillar pattern during ontogenesis

In parallel with the complete *(P. waltlii)* or incomplete *(A. mexicanum, P. anguinus)* myosin isoenzymic transition, changes were also shown to occur in the ATPase myofibrillar profile of the dorsal skeletal muscle.

In *P. waltlii*, at the larval stages 53 and 54, a study of the ATPase activity revealed one single type of fiber, classified as larval fiber type. However, among the larval fibers, two distinct populations were identified by their size and localization. The peripheral part of the dorsal muscle consisted in small fibers only, whereas in the central part both small and large fibers were present, thus giving the dorsal muscle of *P. waltlii* a typical mosaic aspect. At stage 55a (beginning of anatomical metamorphosis) the two fiber types still displayed the same spatial repartition, and small fibers were shown to represent 70% of the total. Gradually during metamorphosis, transitional fibers of type IIC, and adult fibers of types I, IIA and IIB could be demonstrated. After the total completion of anatomical metamorphosis, only the fiber types I (13%), IIA (67%) and IIB (20%) remained. A very similar pattern (type I 17%, type IIA 57%, type IIB 26%) was observed in the dorsal muscle of adult metamorphosed *A. tigrinum* (Table 1).

In the dorsal muscle of young *A. mexicanum* individuals (animals

aged less than 4 months), fibers were of the larval type, with two distinct populations, as already observed in *P. waltlii*, However, the mosaic aspect was less obvious in *A. mexicanum* since the small fibers were essentially peripheral and appeared scarcely in the central region among the larger fibers. In animals aged 7 months, the large and the small fibers represented respectively 18% and 82% of the total fibers. Three fiber types could be identified in animals aged 8 months. Part of the small fibers, essentially located in peripheral position under the skin and around the ribs in the latero-ventral part of the muscle, were of the type I, while another population of small fibers located in a more central position was of the type IIB. The other fibers, whatever their size, were identified as type IIC. In animals aged 11 months the respective proportions were 9% type I, 18% type IIB, and 73% type IIC; no fibers of the type IIA were differentiated at this time, but appeared later in animals aged 12 months. Finally in neotenic adults aged 3 years, four fiber types were characterized: type I (14%), type IIA (10%), type IIB (16%) and type IIC (60%) (Table 1).

In the perennibranchiate *P. anguinus*, the situation appeared to be not so different from *A. mexicanum*. In animals aged 3 months the muscular fibers presented a uniform larval type. In juvenile individuals aged 4 years, three fiber types were demonstrated: type I (24%), type IIB (17%) and type IIC (59%). No fibers of the type IIA were detected at this time, but appeared later on, in animals aged 6 years. In sexually mature *P. anguinus* aged 16 years, four fiber types were also identified: type I (15%), type IIA (4%), type IIB (29%) and type IIC (52%) (Table 2). It should be noted that in this species, the fibers reported as type IIC represented in fact a heterogeneous population and required a more precise classification. Fibers of type IIC" located in the peripheral part of the muscle and displaying a stronger staining intensity were distinguished from fibers of type IIC' located in the central part of the muscle, and were weaker in coloration.

The persistence of a great number of type IIC fibers in the skeletal dorsal muscle of neotenic adults of the species *A. mexicanum* and *P. anguinus* represents an interesting situation as compared with *P. waltlii* and *A. tigrinum,* in which no type IIC fibers are detected in the dorsal muscle of metamorphosed adults. This observation will be connected with the fact that the myosin isoenzymic transition is incomplete in the adults of the two first species. In fact different studies (Billeter *et al.*, 1980; Pierobon-Bormioli *et al.*, 1981) have also emphasized the heterogeneity of type IIC fibers in mammalian species, suggesting that they might represent "transitional" fibers. Fibers of type IIC might then be made up of various mixtures of larval and adult myosin isoforms.

TABLE 2

CHANGES IN THE ATPase MYOFIBRILLAR PATTERN DURING THE DEVELOPMENT OF *PROTEUS ANGUINUS*

Stages	Fiber types			
3 months	100% larval types			
4 years	IIA -	IIB 17%	IIC 59%	I 24%
6 years	IIA 1%	IIB 25%	IIC 54%	I 20%
16 years	IIA 4%	IIB 29%	IIC 52%	I 15%

Thyroidal status and myosin isoenzymic transition

The anatomical metamorphosis that occurs spontaneously in most urodelan amphibian species corresponds to a dramatic developmental crisis that has been directly correlated with thyroid hormone activity (Etkin, 1968; Hourdry, 1980). Three types of thyroidal status were defined in the urodelan amphibians. In most species the anatomical metamorphosis that accompanies an increase in the circulating thyroid hormone level occurs spontaneously. Indeed, a rise in circulating hormone levels during anatomical metamorphosis was reported for various urodelan species (Eagelson and McKeown, 1978; Larras-Regard *et al.*, 1981). In *P.waltlii* it appeared that thyroxine (T4) was not detectable before stage 54, and was first demonstrated in the serum of larvae at late stage 54. From then on, a regular rise in the circulating T4 level was observed at stage 55 during the course of the external metamorphosis. Soon after metamorphosis was completed (stage 56) the amount of T4 decreased in the serum, and thyroxine was undetectable in the serum of the adult animals.

In some neotenic species or varieties, individuals do not undergo anatomical metamorphosis in natural conditions, and reach sexual maturity as giant larvae. However, when treated with triiodothyronine (T3) they complete a full metamorphosis. Several studies on *A. mexicanum* demonstrated that failure to undergo external metamorphosis might be attributed to the young larvae's inability to attain a sufficient level of thyroidian activity (Blount, 1950; Prahlad and Delanney, 1965; Prahlad, 1968; Ituriza, 1971; Norris and Platt, 1973). In fact we have shown that thyroxine could not be demonstrated in the serum of individuals aged 3 months; was only detected in very small amounts in animals aged 4 to 9 months, and fell again to undetectable levels in the serum of the neotenic adults.

Finally, in the perennibranchiate species, anatomical metamorphosis has never been described; apparently it never occurs even after triiodothyronine treatments. The indications were that perennibranchiate urodelan amphibians retain most of their larval external characters mainly because their tissues have not developed a sufficient responsiveness to thyroid hormone (Schreiber, 1937, 1939). In fact it was demonstrated that the neotenic status in the perennibranchiate species could hardly be due to a deficiency of the thyroid, which has been shown to be active (Swingle, 1922; Vialli, 1931).

Effects of experimental hyperthyroidism

In *P. waltlii*, larvae were treated with T3 when reaching stage 54. In experimental animals the anatomical metamorphosis was fully completed within 3 weeks, when controls had just reached stage 55a (beginning of metamorphosis). The myosin isoenzymic transition was accelerated by the treatment, and the proportions of transitional and adult isomyosins among the experimentally metamorphosed animals were greatly increased as compared with controls. However, no significant differences in myofibrillar profiles were observed in experimental metamorphosed individuals versus controls still in the larval condition.

TABLE 3

MYOSIN ISOFORMS AND THEIR SUBUNITS IN URODELAN AMPHIBIAN SKELETAL MUSCLE

	Myosin isoforms	Myosin heavy chains	Myosin light chains
Larvae	3 LM	HCl	LC1f, LC2f, LC3f
	3 FM	HCf	LC1f, LC2f, LC3f
Metamorphosed adults	1 IM	HCi	LC1f, LC2f, (LC1s, LC2s)
	SM1	HCs	LC1f, LC2f, LC1s/LC2s
	SM2		

In *A. mexicanum* , all the animals aged from 2 months to 6 years treated with T3 presented a full external metamorphosis within 3 weeks. At that time both the myofibrillar ATPase profile and myosin isoenzymic distribution were similar in experimentally metamorphosed animals and in the controls. Individuals aged 7 months that had been metamorphosed for 5 weeks displayed little difference with controls in respect to the myofibrillar ATPase profile. In contrast, the myosin isoenzymic distributions differed clearly in the two populations: in the experimentally metamorphosed *A. mexicanum* a large enhancement of the adult myosin isoforms and the regression of the isomyosins of intermediate mobility were observed. Experimental animals from the same group were analyzed when aged 18 months (one year after experimental metamorphosis). Their myosin isoenzymic pattern revealed adult isomyosins only, the transitional and the larval isoforms were no longer detected. Thus the myosin isoenzymic transition was fully complete in these animals. It should be noted that individuals that had been treated when aged one year or more presented little differences with their controls, both in the myofibrilar ATPase profile and the myosin isoenzymic pattern. Moreover they hardly survived more than 6 months in the metamorphosed form.

In *P. anguinus* , animals aged 6 months, 1 year and 4 years were treated with T3 for continuous periods of 3 months and 7 months. After 3 months no differences were detected between experimental animals and their controls. No modifications were recorded in the experimental individuals, whatever the age, either in external aspect, myofibrillar ATPase profile or myosin isoenzymes electrophoretic distribution. Animals that had been continuously treated for 7 months presented a cornification of the tegument, as already described by Durand (1971). The myosin isoenzymic pattern differed in the animals that had been treated, as compared with controls. Experimental individuals aged 6 months or 1 year at the beginning of the T3 treatment demonstrated a slight enhancement in the staining of the adult myosin isoforms. In animals that were aged 4 years at the beginning of the T3 treatment, the enhancement of the adult isomyosins was accompanied by a clear regression of some larval isomyosins. The myofibrillar profile was also modified in these animals, since there was a significant increase in the proportion of the fibers of type IIB, and a regression in the percentage of the type I and IIC fibers. However, in no case could a change be detected in the light chain composition or their relative proportion in the T3 treated animals.

Effects of experimental hypothyroidism

Since thyroid stimulating hormone (TSH) produced by the hypophysis controls thyroid activity, precocious hypophysectomy was used to obtain hypothyroidian individuals. A chronic treatment with thiourea added to the water of breeding tanks was also performed to induce hypothyroidism.

In *P. waltlii* , individuals were hypophysectomized at embryonic stage 22, or at larval stage 45. The experimental animals did not undergo external metamorphosis; they remained in the larval condition while controls were totally metamorphosed, and presented no detectable level of T4 in the serum. The myosin content of hypothyroidian animals aged 105, 120 and 130 days was initially analyzed in pyrophosphate gels. The experimental animals aged 105 days presented only the three larval isomyosins; their controls attained stage 55b-55c and presented as many as 9 bands, including the three adult isoforms of lower electrophoretic mobility. In animals aged 120 and 130 days, controls were totally metamorphosed and revealed the three adult fast myosin isoforms which represented as much as 65% of the total myosin, while the hypophysectomized individuals still showed only the three larval isomyosins. However, further experiments demonstrated that hypothyroidism in fact does not prevent the production of adult isomyosins. Hypophysectomized animals could be maintained as giant larvae for respectively 6 months, 12 months and 14 months. Electrophoretic analysis of the myosin from the dorsal muscle of these experimental individuals showed some weakly stained transitional and adult bands to appear. Finally, hypothyroidian giant larvae were shown to present a clear coexistence of the larval and the adult isoforms three years after they had been hypophysectomized (Fig. 1).

In *A. mexicanum*, young individuals (stage 45) were submitted to a continuous 6-month thiourea treatment. No significant difference was detected between experimental animals and their controls. The adult isomyosins appeared at the same time, and the ATPase profile evolved in the same way. Similar results were obtained with animals that had been hypophysectomized at stage 45 (Fig.1).

Discussion

Our observations in urodelan amphibians, and different experimental results obtained in rodents (Whalen *et al.*, 1985; Butler-Browne *et al.*, 1987; d'Albis *et al.*, 1987) are in favor of a major role for thyroid hormone, acting on the fast skeletal muscle to promote the transition from the neonate (or larval) to the adult myosin, by regulating the expression of the heavy chains.

In the urodelan amphibians, during the course of ontogenesis,

TABLE 4

TERMINAL DIFFERENTIATION IN *P. WALTLII* SKELETAL MUSCLE

Larval stages	Metamorphosis / Thyroid hormone	Metamorphosed adult
3LM		3FM, IM, SM
HCl		HCf, HCi, HCs
LC1f, LC2f, LC3f		LC1f, LC2f, LC3f
Larval fibers		Fiber types I, IIB, IIA

changes in the myosin isoenzymic pattern were thus accompanied by sequential modifications of the heavy chains only, while no changes were detected in the light chain composition (Tables 3 and 4). Such a sequential appearance of distinct heavy chains during muscular differentiation was also reported for birds and mammals (Whalen *et al.*, 1981; Stockdale and Miller, 1987). It was shown that heavy chains are encoded by different genes (Weydert *et al.*, 1985), whose expression might be regulated by thyroid hormone (Izumo *et al.*, 1986; Russel *et al.*, 1988).

In both rodents and urodelan amphibian models the terminal differentiation of the skeletal muscle is characterized, at the level of the myosin isoenzymic profile, by two major events: i) the appearance of the adult myosin isoforms, and ii) the disappearance of the neonate (or larval) myosin isoforms.

- *Appearance of adult myosin isoforms*: in the urodelan amphibians, whatever their thyroidal status, adult myosin isoforms always appear between the third and the eighth month, depending on the species. Experimental hypothyroidism may slow down, but does not prevent the appearance of transitional and/or adult myosin isoforms in *P. waltlii* and *A. mexicanum*. In these two species a T3 treatment results in a rapid increase of the transitional and adult isoforms. In *P. anguinus* a slight enhancement of the adult isomyosins is also demonstrated in individuals that have been treated as long as 7 months with T3.

- *Disappearance of larval myosin isoforms*: in *P.waltlii* , a rapid regression and disappearance of the larval myosin isoforms is related with the rise in the level of circulating thyroid hormone during spontaneous metamorphosis. A complete disappearance of the larval myosin isoforms may be obtained in *A. mexicanum* following T3 treatment. This regression appears to be a long-term process, initiated by T3, but independent of the continuous presence of thyroid hormone. It occurs only in young individuals displaying a high metabolic activity. A significant regression of some larval isomyosins is also demonstrated in *P. anguinus* individuals aged at least 4 years, that have been submitted to a long-term T3 treatment.

These accumulated results indicate clearly that thyroid hormone is in fact involved in the control of the myosin isoenzymic modifications that occur in the skeletal muscle at the time of metamorphosis in the urodelan amphibians. However, this control appears to imply indirect mechanisms, operating differently in each of the two phases of a complete myosin transition; it may be long delayed and related to the age (i.e. the metabolic conditions). The appearance, and persistence, of adult isomyosins seems to be independent of thyroid hormone, although a rise in the circulating T3 may rapidly increase their production. The regression of the larval isomyosins appears to be stimulated by thyroid hormone but, apparently, through indirect pathways.

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