Study of the State of F-Actin in Denervated Muscle Fibre by Polarised UV Fluorescence Microscopy

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Abstract. In order to establish the effect of denervation on the structure of F-actin, polarisation of fluorescence of tryptophan residues of F-actin was determined in intact and denervated ghost muscle fibres (freed from myosin, troponin and tropomyosin) under various conditions. $P_{\parallel}$, the polarisation parameter obtained by excitation of the fluorescence of tryptophan residues of F-actin by light polarised parallel to the long axis of the fibre, was found to be lower for denervated fibres than for the intact ones, i.e., $P_{\parallel}$ (denervated) $< P_{\parallel}$ (intact). On the contrary, the parameter $P_{\perp}$ obtained by excitation of the fluorescence of tryptophan residues of F-actin by light polarised perpendicular to the long axis of the fibre was greater for denervated fibres than for intact fibres, i.e., $P_{\perp}$ (denervated) $> P_{\perp}$ (intact). Moreover, solution containing adenosine triphosphate (ATP) caused an increase in $P_{\perp}$ and a decrease in $P_{\parallel}$ of F-actin in intact fibres, and at the same time a decrease in $P_{\perp}$ and an increase in $P_{\parallel}$ of F-actin in denervated fibres. The binding of the heavy meromyosin (HMM) and the myosin subfragment-I (S-I) to F-actin to intact as well as to denervated fibres decreases $P_{\perp}$ and increases $P_{\parallel}$ though to different degrees. It is supposed that denervation impairs the structure of actin in the regions in which actin-actin and actin-myosin interactions take place.

Key words: Conformational changes of F-actin — Tryptophan fluorescence — Effect of denervation on F-actin

Introduction

There is sufficient ground to assume that denervation alters the structure and specific properties of F-actin. Thus it has been shown that actin from denervated muscles is not readily polymerized nor does it actively combine with heavy meromyosin (Kalamkarova et al. 1976; Moskalenko and Strankfeld 1980); it also differs in its aminoacid composition (Ibragimov et al. 1981).

It has been shown in recent years that structural changes in contractile proteins of muscle fibres can be studied with the aid of the highly sensitive polarised UV (ultraviolet) fluorescence microscopy (Aronson and Morales 1969; Borovikov et al. 1971; Dos Remedios et al. 1972; Borovikov et al. 1974). This method has been used to reveal local conformational changes of F-actin induced by ATP (Borovikov et al. 1978), by fibre stretching (Borovikov et al. 1981) and by S–I and HMM binding (Borovikov et al. 1982 a, b). Furthermore, polarised UV fluorescence microscopy is a promising method for studying the state of contractile proteins in the process of the appearance and spreading of destruction (Borovikov et al. 1979; Borovikov 1980).

In the present work the above method has been used to investigate the structural changes of F-actin in muscle after denervation.

Material and Methods

Preparation of single ghost muscle fibres. White skeletal muscles (extensor digitorum longus, gastrocnemius, biceps femoris, vastus lateralis) of rabbit hind legs were denervated as described previously (Kalamkarova et al. 1976). Denervated muscles (after 14, 30, 50 days) and intact muscles of normal rabbits were taken from no less than 5 animals. In some of the experiments muscles were similarly taken from the undenervated legs (conditionally normal muscles).

Glycerinization was carried out according to Szent-Györgyi (1949). The glycerol was washed out with a solution containing (in mmol.l^{-1}): 100 KCl, 1 $MgCl_2$, 67 phosphate buffer, pH 7.0 (solution 1). Single fibres were isolated from the glycerinated muscles and placed in a flow chamber for 30—40 minutes to extract myosin, troponin and tropomyosin with a solution containing (in mmol.l^{-1}): 800 KCl, 5 $MgCl_2$, 5 ATP, 4 ethyleneglycol bis (2-aminoethyl)-N, N′-tetraacetic acid (EGTA), 67 phosphate buffer, pH 7.0.

Ghost fibres were tested in standard and "relaxing" solutions which differed from solution 1 by 4 mmol.l^{-1} EGTA or 5 mmol.l^{-1} ATP and 4 mmol.l^{-1} EGTA, respectively. In some experiments acrylamide (concentration — 0.15 mol.l^{-1}) was used to quenching the tryptophan fluorescence. Acrylamide had no effect on the contraction of glycerinated muscle fibres.

Preparation of proteins. Myosin was extracted from rabbit white skeletal muscles as described by Holtzer and Lowey (1959). HMM was obtained by digesting myosin with trypsin according to Szent-Györgyi (1953). The ATPase activity of HMM in a solution containing (in mmol.l^{-1}): 5 $CaCl_2$, 1 ATP, 50 Tris-HCl buffer, pH 7.5 at 25°C was 0.98 μmol P_i/min/mg.

The subfragment-1 was obtained by digesting myosin with papain in the presence of 2 mmol.l^{-1} EGTA (Morgossian and Lowey 1978). The ATPase activity of S–1 in a solution containing (in mmol.l^{-1}) 5 $MgCl_2$, 1 ATP, 50 Tris-HCl buffer, pH 7.5 at 25°C was 0.04 μmol P_i/min/mg.

The concentration of HMM and S–1 was determined by means of a spectrophotometer, using the extinction coefficient $E_{280}^{1\%} = 6.0$ cm^{-1} for HMM and $E_{280}^{1\%} = 8.1$ cm^{-1} for S–1 (Morgossian and Lowey 1978). The concentration was 2—5 mg/ml for HMM and 1—2.5 mg/ml for S–1.

Binding of HMM and S–1 to F-actin of ghost muscle fibre. The process of HMM or S–1 binding to F-actin was accomplished by incubating a single ghost fibre over 1—1.5 hours in a solution containing 2—5 mg/ml HMM or 1—2.5 mg/ml S–1, 30 mmol.l^{-1} Tris-HCl buffer, pH 7.0. Unbound protein was washed away from the fibre with the standard solution.

SDS-polyacrylamide gel electrophoresis. The protein composition of ghost and of glycerinated fibres was determined by the method used by Davis (1964), modified for maximum separation of contractile proteins (Sheludko 1975). Ghost and glycerinated fibres were put in a homogenizer and dissolved in a solubilizating solution (12% glycerol, 2% SDS, 5% 2-mercaptoethanol, 50 mmol.l^{-1} Tris-HCl buffer, pH 6.8) for 5 minutes at 100°C, the specimens were then cooled to 0°C and applied to a gel. The separating gel contained 12% acrylamide, the concentrating gel 2.5% acrylamide.

Measurement of polarisation of the tryptophan fluorescence of ghost fibres. Measurements were made by a microfluorimeter (Ioffe et al. 1974). The tryptophan fluorescence of the fibre was excited at 303 ± 4 nm. The tryptophan fluorescence was recorded at wavelengths ranging from 320 to 380 nm. Four components of polarized fluorescence were determined, namely, ${}_{\parallel}I_{\parallel}$, ${}_{\parallel}I_{\perp}$, ${}_{\perp}I_{\parallel}$, ${}_{\perp}I_{\perp}$, where the subscripts $\parallel$ and $\perp$ denote the direction of the polarisation of fluorescence parallel and perpendicular to the fibre axis; the left symbol refers to the exciting light, the right symbol denotes the direction of the polarisation of the fluorescence light. The degree of the polarisation of the fluorescence was determined on orienting the fibre parallel ($P_{\parallel}$) and perpendicular ($P_{\perp}$) to the plane of the exciting light on the basis of the following ratio:

$$P_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\parallel}I_{\perp})/({}_{\parallel}I_{\parallel} + {}_{\parallel}I_{\perp})$$
$$P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel})/({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel})$$

Intensity of the tryptophan fluorescence of the fibre (I_{av}) was estimated (in %) on the basis of the following relationship:

$$I_{av} = ({}_{\parallel}I_{\parallel}/2 + {}_{\perp}I_{\perp}/2) \times 100\,\%$$

The changes in P and L_{av} were recorded with an accuracy to the nearest 0.001 (rel. units) and 1 %, respectively.

Results

Polarisation of fluorescence of the tryptophan residues of glycerinated muscle fibres is characterized by higher values of $P_{\parallel}$ than of $P_{\perp}$ (Aronson and Morales 1969; Borovikov et al. 1971) and by the ratio $P_{\perp}/P_{\parallel} < 1$ (see Table 1). As extraction of myosin, troponin and tropomyosin from the fibre proceeds, $P_{\parallel}$ decreases and $P_{\perp}$ increases; then the value of $P_{\perp}$ becomes higher than that of $P_{\parallel}$ and the ratio $P_{\perp}/P_{\parallel} > 1$ (see Table 1). Maximum values for this ratio are reached when the intensity of the tryptophan fluorescence of the fibre is reduced by 60% (Borovikov et al. 1982a). In such fibres (both intact and denervated) myosin, troponin and tropomyosin are practically absent and only actin is left (see Fig. 1). In ghost fibres treated with a solution of low ionic strength (Kirillina et al. 1979), $P_{\perp} = P_{\parallel}$ and the ratio $P_{\perp}/P_{\parallel} = 1$. Consequently, polarisation anisotropy in ghost muscle fibres is closely related to F-actin of thin filaments.

The polarisation parameters $P_{\perp}$ and $P_{\parallel}$ and the ratio $P_{\perp}/P_{\parallel}$ are noticeably different for intact and denervated fibres (see Table 1). $P_{\perp}$ (denervated) $> P_{\perp}$ (intact) and $P_{\parallel}$ (intact) $> P_{\parallel}$ (denervated). Hence $P_{\perp}/P_{\parallel}$ (denervated) $> P_{\perp}/P_{\parallel}$ (intact). In other words, polarisation anisotropy of the tryptophan fluorescence of denervated ghost fibres is greater than that of intact fibres, whereas $P_{\perp}$ (dener-

Table 1. Polarization of tryptophan fluorescence of intact and denervated muscle fibres of rabbit

Muscle Fibres	$P_\perp$	$P_\parallel$	$P_\perp/P_\parallel$
	Glycerinated Fibres		
Intact	0.138 ± 0.003	0.375 ± 0.005	0.368 ± 0.010
Denervated (50 days)	0.167 ± 0.006	0.341 ± 0.008	0.500 ± 0.015
Conditionally normal (50 days)	0.170 ± 0.012	0.352 ± 0.015	0.483 ± 0.050
	Ghost Fibres		
Intact	0.222 ± 0.002	0.200 ± 0.001	1.110 ± 0.020
Denervated (14 days)	0.228 ± 0.002	0.189 ± 0.002	1.210 ± 0.025
Denervated (30 days)	0.230 ± 0.003	0.180 ± 0.002	1.280 ± 0.030
Denervated (50 days)	0.232 ± 0.003	0.176 ± 0.003	1.320 ± 0.040
Conditionally normal (50 days)	0.230 ± 0.005	0.180 ± 0.006	1.280 ± 0.070

Measurements were carried out in a standard solution containing (in mmol.l^{-1}): 100 KCl, 1 $MgCl_2$, 4 EGTA, 67 phosphate buffer pH 7.0. No less than 15 fibres taken from 5 rabbits were investigated in each experiment.

vated) $\simeq P_\perp$ (conditionally intact) and $P_\parallel$ (denervated) $\simeq P_\parallel$ (conditionally intact).

The polarisation characteristics and fluorescence intensity of intact and denervated ghost fibres are noticeably affected by the ATP solution (see Fig. 2). In intact fibres $P_\perp$ increases and $P_\parallel$ decreases, i.e., anisotropy of the fluorescence polarisation fibres $P_\perp$ decreases and $P_\parallel$ increases, i.e. the anisotropy decreases ($P_\perp/P_\parallel$ decreases) and these differences are the greater the longer the process of atrophy (see Fig. 2, 3). As for the intensity of the tryptophan fluorescence of both intact and denervated fibres it increases under the influence of the ATP solution by $10 \pm 5\%$.

These differences disappear when fluorescence is quenched by acrylamide (see Fig. 2, 3). In the presence of acrylamide both intact and denervated fibres respond to ATP by a decrease in anisotropy of the fluorescence. Under these conditions the intensity of the fibre fluorescence is decreased in the standard solution by $30 \pm 5\%$ and in ATP solution by $10 \pm 5\%$. It follows that in the presence of ATP the fluorescence intensity increases by 15—20%.

It is believed that acrylamide quenches the emission of a tryptophan residue at colliding or at forming a complex with it, the quenching effect on tryptophan found on the surface is much greater than the quenching effect on tryptophan residues in the deeper regions of the protein (see Burshtein 1977). From this it could be

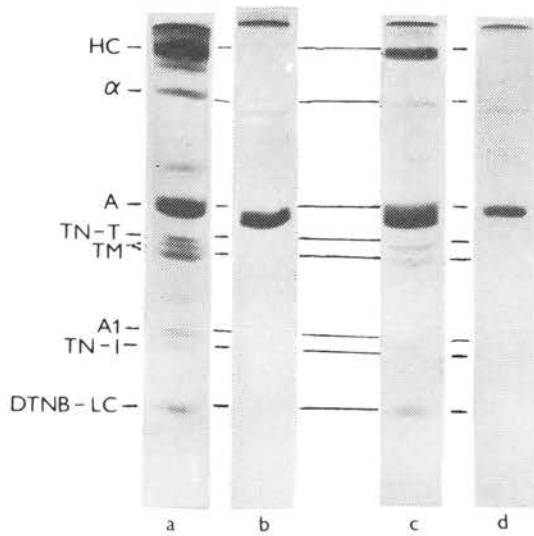

Fig. 1. SDS-polyacrylamide gel electrophoresis of myofibrills preparations. a) intact glycerinated fibres; b) intact ghost fibres; c) denervated (50 days) glycerinated fibres; d) denervated (50 days) ghost fibres. Symbols: HC — heavy myosin chains; A — actin; A1, DTNB-LC — light myosin chains; α — actinin; TM-tropomyosin; TN — troponin.

supposed that the tryptophan residues located in the regions readily accessible to acrylamide are responsible for the different response to ATP of intact and denervated muscle fibres.

The polarisation parameters of intact as well as of denervated ghost fibres change when HMM and S–1 are bound to F-actin (see Fig. 4). In these experiments $P_\perp$ decreases while $P_\parallel$ increases, the only difference being that the changes in polarisation parameters are appreciably less at later stages of the denervation atrophy. The changes in the parameters are accompanied by an increase in the intensity of the tryptophan fluorescence caused by the appearance of tryptophan residues, HMM or S–1 in the ghost fibres (Yanagida and Oosawa 1978). In the present work, the increase in I_{av} was identical for both intact and denervated fibres at HMM or S–1 binding and equalled to $75 \pm 5\%$ and $80 \pm 5\%$, respectively.

Previously Yanagida and Oosawa (1978) showed that on binding HMM or S–1 to actin, the increase in the tryptophan fluorescence of ghost fibres by 75—80% corresponds to the molar ratio of HMM or S–1 to actin equal to 0.3 and 0.6, respectively. As the test conditions in our investigations and in those of Yanagida and Oosawa were similar, we can conclude that in our case the molar ratios HMM/actin and S–1/actin are the same for denervated and intact fibres and are also equal to 0.3 and 0.6, respectively. Hence, it follows that the different levels

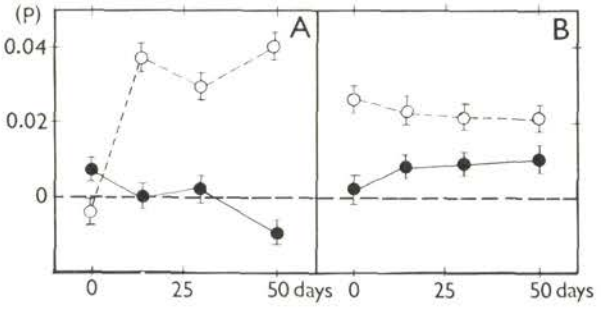

Fig. 2. Dependence of changes in tryptophan fluorescence of ghost muscle fibres induced by ATP in the absence (A) and presence (B) of acrylamide on duration of the denervation (abscissa). Ordinate: polarised fluorescence changes (ΔP) in arbitrary units. ●–● — $\Delta P_{\perp}$; ○–○ — $\Delta P_{\parallel}$. Each point represents the average value for 15 fibres. The concentration of acrylamide was 0.15 mol.l^{-1}.

in the polarisation changes (see Fig. 4) are not associated with differences in the quantitative ratios of myosin fragments to actin in intact and denervated fibres.

It should be noted that the nature of the changes of $P_{\perp}$, $P_{\parallel}$ and $P_{\perp}/P_{\parallel}$ induced by ATP solution and by HMM or S–1 binding in conditionally normal fibres does not differ from that of the changes induced in denervated fibres. It can be implied that fibres taken from the contralateral leg (that was not operated upon) should not be used for the control test.

It has previously been shown that changes in polarisation characteristics should not be attributed to measurement errors due to scattering of light, birefringence or to other errors of measurement (Aronson and Morales 1969; Dos

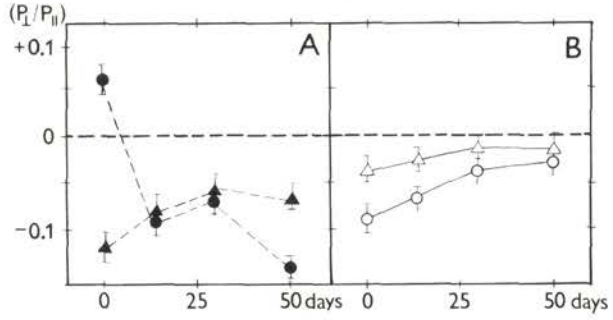

Fig. 3. Dependence of changes in tryptophan fluorescence anisotropy $\Delta(P_{\perp}/P_{\parallel})$ of rabbit ghost muscle fibres induced by ATP (A) by HMM and S–1 binding (B) on duration of denervation atrophy (abscissa). Ordinate: fluorescence anisotropy changes (in arbitrary units). ●–● — in the absence of acrylamide; ▲–▲ — in the presence of acrylamide; ○–○ — HMM; △–△ — S–1. The test conditions are the same as in Fig. 2.

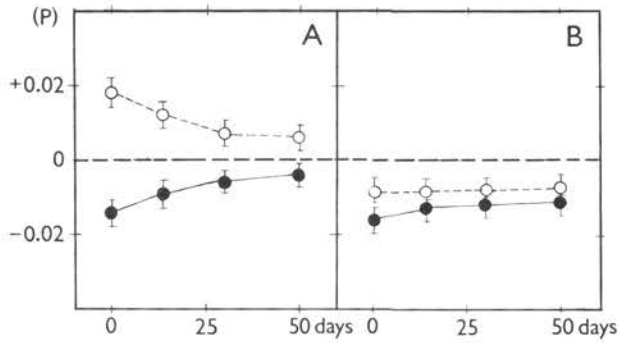

Fig. 4. Dependence of changes in tryptophan fluorescence polarisation of rabbit ghost muscle fibres, induced by binding HMM (A) and S–1 (B) on duration of denervation atrophy (abscissa). Symbols and test conditions are the same as in Fig. 2.

Remedios et al. 1972; Chernogriadskaya et al. 1978). On the basis of this data it can be assumed that the changes in $P_\perp$, $P_\parallel$ and $P_\perp/P_\parallel$ recorded in this investigation reflect changes in the emission of tryptophan residues of ghost muscle fibres.

Discussion

It has been shown that in cross-striated muscle fibres the tryptophan residues of myosin are predominantly arranged along the long axis of the fibre, while the tryptophan residues of F-actin mainly perpendicular to it (Yanagida et al. 1974; Borovikov et al. 1972, 1974). On the other hand the tryptophan residues of the other contractile proteins are in fact arranged isotropically and their contribution to the total emission of the fibre is considerably lower than that of myosin and actin (Yanagida et al. 1974; Chernogriadskaya et al. 1978). Since anisotropic orientation of tryptophan residues of myosin is higher than that of F-actin, for glycerinated muscle fibres $P_\parallel > P_\perp$ (see Table). After selective extraction of myosin, troponin and tropomyosin, tryptophan residues of F-actin remain in the ghost fibre; the absorption and emission oscillators are mainly oriented perpendicular to the long axis of the fibre (Yanagida and Oosawa 1978; Borovikov et al. 1978; Borovikov and Chernogriadskaya 1979); $P_\perp$ becomes therefore greater than $P_\parallel$ (see Table). It is apparent that anisotropy of the polarisation of fluorescence of ghost muscle fibres depends on the optical properties of tryptophan residues of F-actin and is defined by the spatial parameters of the actin helix.

It is known that the great majority of tryptophan residues of F-actin are localized within the protomers (Elzinga and Collins 1973) and are not affected by the solution because they are surrounded by a hydrophobic microenvironment (Vedenkina et al. 1973). Taking this data into consideration and on the basis of the

concept that the changes in fluorescence of tryptophan residues can indicate structural changes in the protein macromolecule (Konev 1965; Chernitsky et al. 1972; Burshtein 1977; Chernogriadskaya et al. 1978) it can be assumed that the changes in fluorescence polarisation induced by ATP in intact and denervated fibres occur as a result of conformational changes in F-actin. It should be noted that conformational changes of the proteins in denervated and intact fibres differ at least in some respects, since one and the same agent initiates dissimilar changes in fluorescence anisotropy. This in turn indicates that the structure of F-actin in intact and denervated fibres can be different.

It is of interest to discuss possible localization of changes in the structure of F-actin in the process of denervation atrophy.

Earlier, by using acrylamide and NO_3^- to quenching fluorescence in polarisation studies it became possible to assume that under the influence of ATP local structural changes occur in F-actin of intact fibres which reduce the intramollecular mobility of the polypeptide chain of this protein (Borovikov et al. 1978; Borovikov and Chernogriadskaya 1979). These structural changes of actin are accompanied by an increase in fluorescence intensity and a decrease in polarisation anisotropy in thin filaments. In this work similar changes were observed in thin filaments of denervated fibres. Apparently F-actin of intact and denervated fibres responds similarly to ATP — by a decrease in the intramollecular mobility of the peptide chain of the protomer. Most probably, the structural changes take place near the surface of the protein, since quenching of emission of tryptophan residues localized in such regions of the protomer removes the differences in response to ATP in intact and denervated fibres (see Fig. 2, 3).

It is known that out of 4—5 tryptophan residues of actin one of them, Trp–74, is located close to the surface of the protomer; this aminoacid residue is found in the immediate vicinity to the region of the polypeptide involved in the polymerization of actin (Elzinga and Collins 1973). Perhaps the change in the response of F-actin to ATP and the inhibition of polymerization of denervated actin (Moskalenko and Strankfeld 1980) are both caused by structural changes of the protein in the region where Trp–74 is located.

The results of this work imply the possibility of the appearance of structural changes of F-actin in the parts of the polypeptide chains of actin protomers that bind the heads of myosin molecules, since after denervation a noticeable weakening in the ability of F-actin to respond by a decrease in anisotropy of fluorescence of F-actin to HMM and S–1 binding has been observed (see Fig. 4). The structural changes of actin in the myosin-binding part of the polypeptide chain agree with the data on the reduced ability of denervated actin to combine with HMM in vitro (Moskalenko and Strankfeld 1980).

The conclusion that the structure of F-actin changes in the process of denervation is also supported by data showing different values of anisotropy for

tryptophan fluorescence of denervated and intact ghost fibres (see Table). The greater absolute values of anisotropy of fluorescence of denervated fibres as compared with those of intact fibres show that the thin filaments are not seriously impaired by the denervation. The structural changes which take place in actin are accompanied by an increase in anisotropy of fluorescence of the ghost fibre. The increase in anisotropy of fluorescence of thin filaments after denervation could be explained by changes in the arrangement of tryptophan residues in actin protomers, by changes in orientation of protomers in F-actin, or by a decreased flexibility of thin filaments.

On the basis of the given data it is not easy to indicate exactly at what organizational level of the protein macromolecule the structural changes of F-actin occur. However, it seems unlikely that in the process of denervation atrophy it is the quarternary structure of this protein alone that changes. This point of view is supported by data showing that changes in the state of the hydrogen bonds of myofibrill proteins of a muscle fibre induced by changes in the ion strength and pH of the solution, do not change practically anisotropy of the tryptophan fluorescence of the fibre (Aronson and Morales 1969). On the contrary, changes at deeper levels of organization of contractile proteins induced by functional changes of the fibre are accompanied by appreciable changes in anisotropy of the tryptophan fluorescence (Aronson and Morales 1969; Dos Remedios 1972; Borovikov et al. 1972, 1974).

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