

Orientation Dependence and Motional Properties of Spin Labels in Cardiac and Skeletal Muscle Fibres

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Abstract. Orientation dependence and rotational motion of maleimide spin labels attached to the fast reacting thiol sites of myosin were studied in glycerinated cardiac and skeletal muscle fibres in rigor and in relaxing medium. The probe order in skeletal muscle was shown to be about one order of magnitude higher than that in cardiac muscle. In skeletal muscle in rigor the orientational order is static on the time scale of the saturation transfer electron paramagnetic resonance measurement (ST EPR, rotational correlation time of the label is greater than 1 ms), but in cardiac muscle fibres, a disorder was observed which was at least partly dynamical, the rotational correlation time being about 100 μ s. In relaxing solution the degree of order of probe molecules in both types of muscle was strongly reduced at and above the resting length. The disorder was at least partly dynamical on the ST EPR time scale, the apparent rotational correlation times being 200 μ s for skeletal muscle and 60 μ s for cardiac muscle, respectively. According to the results of ST EPR the rotational behavior of cross-bridges was identical in cardiac and skeletal muscle in relaxing medium.

Key words: Cardiac and skeletal muscle — Muscle contraction — Spin-labelling — Molecular dynamics

Introduction

It is known from earlier experiments that the mammalian heart muscle and vertebrate skeletal muscle have many structural similarities. It has been suggested that principal interactions between contractile proteins are the same in both muscles (Matsubara et al. 1977; Matsubara and Millman 1974; Stenger and Spiro 1961). It is also known that one of the low molecular weight subunits of myosin, the LC₂ light chain, which is localized in the neck region of the myosin rod (Winkelmann et al. 1984), can be partially removed from myosin by mild treatment with dithionitrobenzene (DTNB)-EDTA solution (Gazith et al. 1970;

Weeds and Lowey 1971). Partial removal of the LC₂ light chain modulates strongly the maximum velocity of shortening (Moss et al. 1983). However, in contrast to this finding, DTNB-EDTA treatment does not lead to the release of the LC₂ subunit from cardiac myosin (Sarkar et al. 1971; Malhotra et al. 1979).

The use of spectroscopic (fluorescence or paramagnetic) probe molecules has provided new informations about the spatial arrangement and the rate of motion of muscle proteins under various conditions (Eads et al. 1984; Thomas and Cook 1980; Mendelson and Wilson 1982). An analysis of EPR spectra of spin labelled skeletal and cardiac muscle fibres may provide information about differences between skeletal and heart muscle, and about the possible role of the LC₂ light chain in binding of cross-bridges to the thin filaments. By labelling the fast-reacting thiol sites on the head region of the myosin, we observed a disorder in cardiac muscle cell even in rigor in comparison with skeletal muscle fibres. The disorder was partly dynamical. In pyrophosphate relaxing solution the disorder increased significantly at and over the resting length of fibres in both muscles. The increased disorder could be explained with an altered motional state of cross-bridges in relaxation-inducing medium.

Materials and Methods

The cardiac muscle fibres were prepared from the papillary muscle of dog hearts, and the skeletal muscle fibres were isolated from rabbit psoas muscle. The glycerination and spin-labelling of the fast-reacting thiol sites after DTNB-EDTA treatment is described elsewhere (Belágyi et al. 1979; Belágyi and Gróf 1984). Briefly, the fibre bundles excised from the papillary muscle or the psoas muscle were kept for 30 min in solutions containing 0.1 mol/l KCl, 5 mmol/l MgCl₂, 4 mmol/l EGTA, 10 mmol/l histidine . HCl (pH 7.0 rigor buffer), or in rigor buffer plus 50% V/V glycerol to induce membrane destruction by osmotic shock. The fibres were then stored in 50% V/V glycerol and rigor buffer at -18 °C until use.

Before spin-labelling, the fibres were washed in rigor solution to remove glycerol. DTNB and EDTA were subsequently added to the rigor buffer (in this case it did not contain EGTA) to a final concentration of 5 mmol/l, and the fibres were treated for 30 minutes. Then the fibres were washed in a rigor buffer for two hours and transferred to relaxing solution containing the maleimide spin label. (The spin label was a generous gift by K. Hideg, Central Laboratory, Medical University, Pécs). The concentration of the maleimide spin label was 2 mol label/mol myosin. The spin labelling lasted for two hours. Then, the fibres were washed again in rigor buffer for 16 hours to remove unreacted labels, followed by a treatment in rigor buffer plus 2-mercaptoethanol (in 500 fold molar excess over myosin) to reactivate the DTNB-blocked thiol sites in muscle fibres.

The conventional and saturation transfer EPR spectra of different muscle samples were taken with an ERS 220 type spectrometer (Centre of Scientific Instruments, GDR). The conventional EPR spectra of fibres were recorded in two orientations: the long axis of the fibres was oriented parallel and perpendicular to the laboratory magnetic field. The rotational correlation time for the attached probe molecules was obtained using the L'/L and C'/C spectral parameters of the ST EPR spectra and the calibration curves published by Thomas et al. (1976).

ATPase activity of the fibre bundles was measured under isotonic conditions. Bundles of about

10 mm in length and 0.1–0.15 mm in diameter were immersed in relaxing or activating solution (100 mmol/l KCl, 5 mmol/l MgCl₂, 5 mmol/l ATP, 10 mmol/l histidine. HCl with or without 0.1 mmol/l CaCl₂, pH 7.0). Aliquots of the respective solution were taken at times 0; 1; 2; 4; and 6 minutes and pipetted into the color reagent according to Lanzetta and coworkers (1979), and the optical absorbance was read at 660 nm. The rate of release of inorganic phosphate was obtained from a linear plot of absorbance against time.

The extent of reaction with DTNB was determined from the absorbance of liberated *p*-nitrothiophenolate ions at 412 nm after incubation of the fibres in rigor solution containing 2-mercaptoethanol, using a Hitachi type 124 spectrophotometer (Weeds and Lowey 1971).

The numbers of spin labels attached to myosin were obtained from the EPR spectra of denaturated muscle fibres by comparing them with spectra of known concentration of maleimide spin label in rigor buffer using the same flat cell for EPR measurements.

Results and Discussion

Characterization of labelled fibres. The mechanical properties of the spin-labelled and control muscle fibres were measured with a tension transducer (sensitivity: 0.6 μ N/mV) comparing the maximum tension and the rate of tension development during isometric contraction. The sarcomere length of the skeletal muscle was $2.3 \pm 0.1 \mu\text{m}$; in cardiac muscle fibres the force development was checked at a length, when the resting tension became just perceptible. This length should be very near to the normal length in situ under physiological conditions. A JEOL 100 C electron microscope was used to estimate indirectly the average sarcomere length in cardiac muscle fibres; it was $2.0 \pm 0.3 \mu\text{m}$.

After spin-labelling and treatment with 2-mercaptoethanol the tension produced in the presence of Ca²⁺-ATP was reduced in both muscle preparations. The decrease of ATP-induced contraction was 10–15 per cent in average in skeletal muscle, whereas in cardiac muscle, the drop of tension was significantly larger, the maximum tension being 54% that of the untreated fibres. Small changes in the maximum rate of tension and in the time to peak tension were also observed. An example of the muscle activity after spin-labelling is shown in Fig. 1.

In order to test further the properties of the muscle fibres, the ATPase activity of the muscle samples was measured in relaxing and activating solution. The ATPase activity of the spin-labelled psoas muscle fibres was $0.028 \pm 0.007 \mu\text{mol P}_i$ liberated/mg protein .min. In the presence of Ca²⁺ (0.1 mmol/l CaCl₂), the ratio of the ATPase activity of the spin-labelled and control fibres was 0.86 ± 0.15 .

The ATPase activity (Ca-Mg activity) of the control cardiac muscle fibre was higher than that of the psoas muscle fibres by a factor of 1.27, and it became reduced to about 80% of the original activity after spin-labelling.

Spectrophotometric measurements provided evidence that the number of

-SH groups which reacted with DTNB was 5.8 ± 0.4 mol DTNB/mol myosin ($n = 15$, skeletal muscle) and 7.8 ± 0.4 mol DTNB/mol myosin ($n = 8$, cardiac muscle), assuming that DTNB molecules reacted only with myosin. In contrast, the number of spin-labelled sulfhydryl sites was much lower, 0.17 ± 0.08 mol spin label/mol myosin head (skeletal muscle), and 0.25 ± 0.1 mol spin label/mol myosin head (cardiac muscle), respectively.

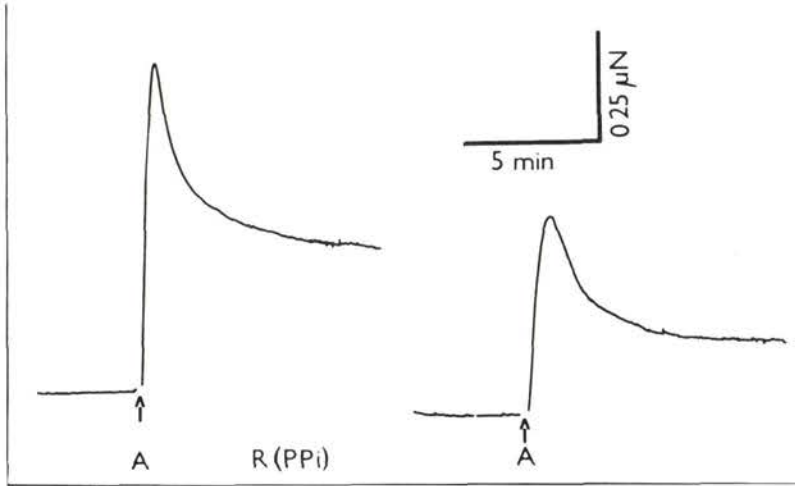


Fig. 1. Mechanical activity of spin-labelled cardiac muscle fibres; *left*: control fibres, *right*: spin-labelled fibres. The length of the bundles was 3.5 mm, their mass was about 0.2 mg. A — activating solution, R(PPi) — pyrophosphate-relaxing solution.

An analysis of myosin extracted from spin-labelled psoas muscle fibres showed that the labels were attached to strongly immobilizing sites (Fig. 2). The characteristic hyperfine splitting constant was $2A_{zz} = 6.824$ mT. Using $2A_{zz} = 6.889$ mT as rigid limit (the hyperfine splitting constant for precipitated myosin) the rotational correlation time was $0.3 \mu\text{s}$ according to Goldman and coworkers (1972). From the ST EPR spectra ($L''/L = 0.7$ and $C'/C = -1.3$) we calculated $\tau_2 = 0.3$ and $0.1 \mu\text{s}$. Both values agree quite well with the earlier

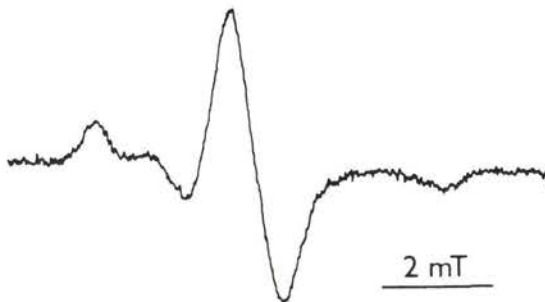


Fig. 2. A conventional EPR spectrum of myosin. Myosin was extracted from spin-labelled skeletal muscle fibres and purified before measurement. The degree of labelling of the muscle fibres was 0.52 mol label/mol myosin.

measurements on myosin (Thomas et al. 1975). This also supports the assumption that the labels in glycerinated muscle fibres reacted predominantly with the fast-reacting thiol sites of myosin.

Orientalional order of probe molecules. Both the striated muscle and the cardiac muscle fibres are highly structurally organized. It may thus be expected that EPR spectra of paramagnetic probe molecules will reflect the distribution of the fast-reacting thiol sites (myosin heads) along the thick filaments. The conventional EPR spectra of muscle fibre bundles are shown in Figs. 3 and 4. The maleimide spin labels attached to the fast-reacting thiols of the myosin head region in relaxing solution after DTNB-EDTA treatment exhibited a strong orientational order with respect to the fibre axis. In rigor state of the fibre bundles, the principal axes of the magnetic tensors were uniformly oriented relative to the fibre axes; the mean value of the angle between the fibre axis (k) and the z principal axis for the spin label was 84° (Belágyi and Gróf 1984). The hyperfine splitting constant was $2A_{zz} = 6.968 \pm 0.03$ mT for cardiac muscle fibres and $2A_{zz} = 7.066 \pm 0.03$ mT for fibre bundles of psoas muscles. (In minced sample, where the distribution of labels (cross-bridges) was isotropic, the hyperfine splitting constant was 6.935 mT). Both values are very close to the

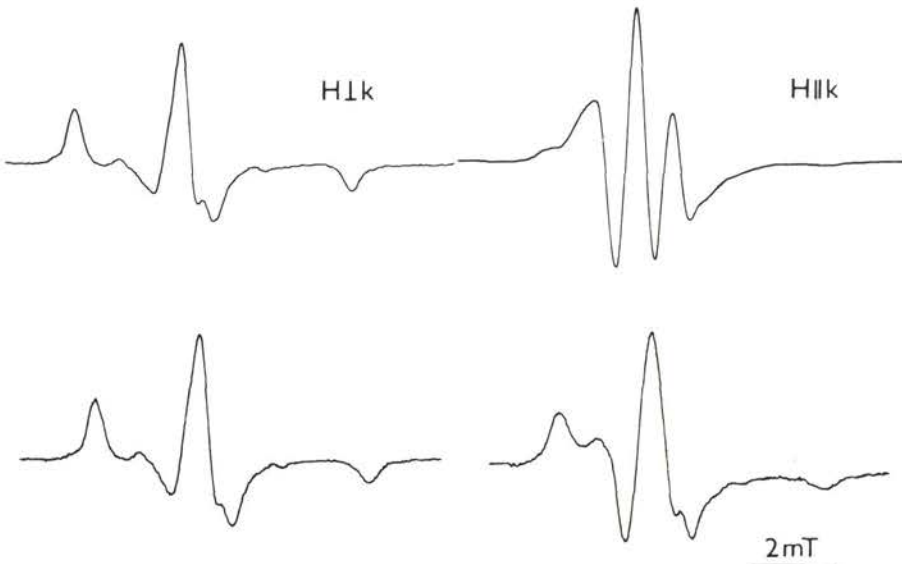


Fig. 3. Conventional EPR spectra of skeletal muscle fibres. The glycerinated muscle fibres were labelled with maleimide spin label in pyrophosphate-relaxing solution after DTNB-EDTA treatment. The molar ratio of spin label to myosin was 0.27. The fibre axis was oriented perpendicularly or in parallel to the magnetic field applied as indicated. The fibres were in rigor at resting sarcomere length. *Bottom:* EPR spectra of muscle fibres at resting length in pyrophosphate-relaxing solution.

rigid limit evidencing that in rigor the rotational motion of probe molecules is highly restricted on the time scale of the conventional EPR technique. This corresponds to the generally accepted fact that most (if not all) myosin cross-bridges are rigidly attached to the thin filaments in rigor (Ready et al. 1965).

The EPR spectrum of muscle fibres labelled with the maleimide spin label exhibited a non-isotropic angular dependence of the labels with respect to the fibre axis. From a comparison of the characteristic maxima it has been possible to estimate the orientational order in the two muscle types according to Hubbell and McConnell (1969). The first maximum (I_1) of the first derivative spectrum arises from labels where the $2p\pi$ molecular orbital of the unpaired electron (the z axis of the nitroxide coordinate system) is lying approximately parallel to the laboratory magnetic field (H), whereas the second maximum (I_2) arise from those labels that have an orientation of their z -axis being approximately perpendicular to the static magnetic field.

In $H \perp k$ orientation the ratio of the second to the first maximum (I_2/I_1) was 0.120 ± 0.06 ($n = 9$), and it did not differ significantly from that measured in homogenized samples. It follows that the distribution of spin labels (cross-bridges) nearly corresponds to a two-dimensional random distribution in a plane perpendicular to the long axis of the fibres. However, in $H \parallel k$ orientation of the fibre axis, the ratios were 0.547 for cardiac muscle and 3.861 for skeletal muscle fibres. The ratio for cardiac muscle was never greater than 0.90 even in the best preparations.

The ratio defined by $(I_2/I_1)_{\parallel}/(I_2/I_1)_{\perp}$ could be used as a measure of the orientational order. It reached 29 for skeletal muscle, but is was only 4.2 for cardiac muscle fibres. The probability that the principal axis z of the label is positioned perpendicular to the fibre axis is about 30 times greater than that of a parallel orientation of the principal axis z , in skeletal muscle. This means that in cardiac muscle the orientational order is lower by a factor of about 7. This is partly due to different cellular organization of cardiac muscle fibres.

In some cases, the conventional EPR spectrum for cardiac muscle fibres reflected two populations of labels, the smaller population (5–7% of the total absorption) rotating with a rotational correlation time of about 1 ns. It seems likely that this latter population were third class thiols whose blockage does not influence the myosin ATPase activity in cardiac muscle. They react as rapidly as the fast-reacting thiol sites termed S_1 (Pfister et al. 1975; Reisler et al. 1974).

In relaxing solution at and over the resting length, considerable changes were observed in the spectra in the $H \parallel k$ orientation. By contrast, no or very little alteration was obtained in $H \perp k$ orientation. The ratio $(I_2/I_1)_{\parallel}$ decreased from 3.861 to 1.256 ± 0.207 ($n = 8$) in skeletal muscle samples, whereas in cardiac muscle fibres this ratio dropped to 0.350 (Fig. 4). The ordering of the probe molecules decreased from 29 to 9.66 in psoas muscle fibres, and from 4.2 to 2.7

in cardiac muscle fibres at resting length. In both cases, the orientational order of cross-bridges (probe molecules) approached random distribution. However, conventional EPR measurements cannot differentiate between myosin heads with disorganized distribution in relaxed state and those reorienting themselves slowly enough on the time scale of measurements.

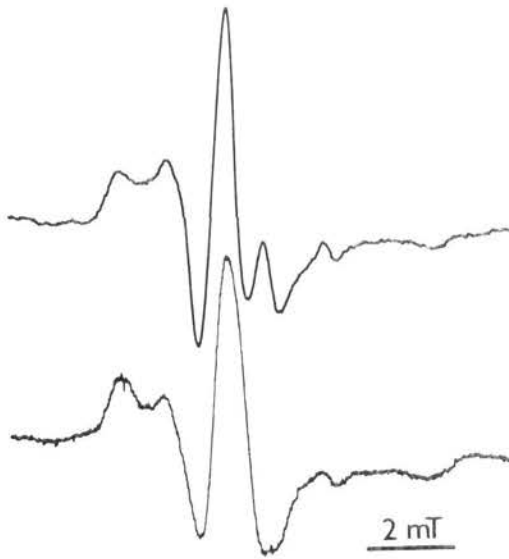


Fig. 4. Conventional EPR spectra of cardiac muscle fibres. The fibre axis was oriented in parallel to the magnetic field applied. The cardiac muscle fibres were labelled with maleimide spin label in pyrophosphate-relaxing solution after DTNB-EDTA treatment. The molar ratio of spin label to myosin was 0.23. *Top*: rigor state; *bottom*: after 30 minutes incubation in pyrophosphate-relaxing solution.

Rotational motion of myosin heads. The rate of motion of spin-labelled protein domains was determined from both conventional and saturation transfer EPR spectra. It was shown earlier that in partially oriented systems, like the muscle sample, the orientational order of spin labels can be estimated in $H \parallel k$ orientation using conventional EPR technique, whereas the $H \perp k$ orientation was promising for the calculation of the rotational correlation times on the time scale of the ST EPR technique (Belágyi and Gróf 1984). In rigor buffer at resting length of the fibre bundles, the L''/L spectral parameter was greater than 1.20 for skeletal muscle ($L''/L = 1.22 \pm 0.06$, $n = 10$), this indicated no or very little rotational motion in the submillisecond time range ($\tau_2 > 1$ ms). This supports the view about the rigid attachment of almost all myosin heads to the thin filaments (Lowell and Harrington 1981).

In contrast, measurement of the rotational correlation time gave $\tau_2 = 120 \pm 40 \mu\text{s}$ ($n = 4$) for cardiac muscle; this suggested some residual motion also during the rigor state (Fig. 5). The shorter rotational correlation time of labels in cardiac muscle can be interpreted by the supposition that in rigor, a fraction of cross-bridges was in detached state and the cross-bridges rotated

randomly within a restricted area. Another possibility is that all cross-bridges are in attached state in rigor buffer, but the cardiac myosin has a different supramolecular organization, the LC₂ light chain somehow influencing the spatial arrangement and dynamical properties of cardiac myosin resulting in little flexibility, even in the actomyosin complex.

In relaxing solution (in the presence of ATP or pyrophosphate plus the components of the rigor buffer) at and over the resting length, small but significant changes in the spectral parameters L''/L and C'/C were observed. The changes indicated that at least a sub-population of the labelled sites has an increased rotational mobility (Fig. 6).

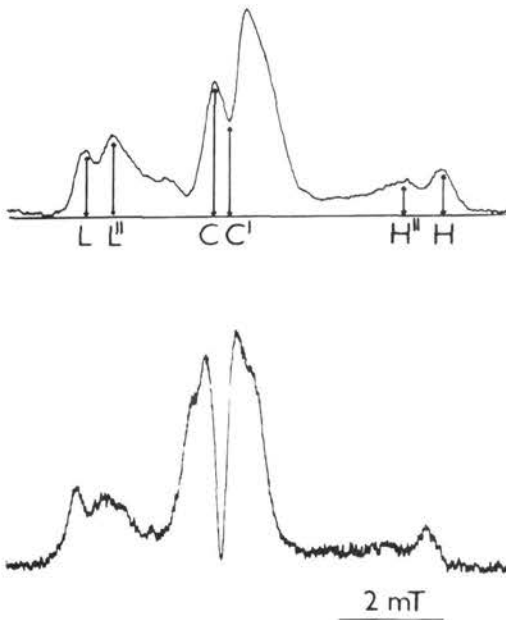


Fig. 5. ST EPR spectra of skeletal and cardiac (bottom) muscle fibres. The fibre axis was oriented perpendicularly to the laboratory magnetic field. The average microwave field amplitude during measurements was 0.025 mT in the sample cell. The spectral parameters used to evaluate the measurements are indicated.

For skeletal muscle, the apparent rotational correlation time decreased by about a factor of two, τ_2 was 200 μs ($L''/L = 1.03 \pm 0.03$, $n = 8$); corresponding values for cardiac muscle were 60 μs ($L''/L = 0.76 \pm 0.1$) and 20 μs ($C'/C = 0.21$). The difference in the rotational correlation times estimated from spectral parameters L''/L and C'/C may be due to anisotropic motion of the labels attached.

It has been known from earlier experiments that maleimide spin labels get rigidly attached to myosin heads even in the presence of nucleotides; hence, motions detected under different conditions reflect those of the cross-bridges, or of a large domain of them (Seidel et al. 1970). The conclusions derived from

conventional EPR spectra agree with the earlier results in that a well-defined orientation of cross-bridges exists, and that both myosin heads interact simultaneously with the actin filaments under rigor conditions (Belágyi et al. 1979; Thomas and Cook 1980; Yanagida 1981).

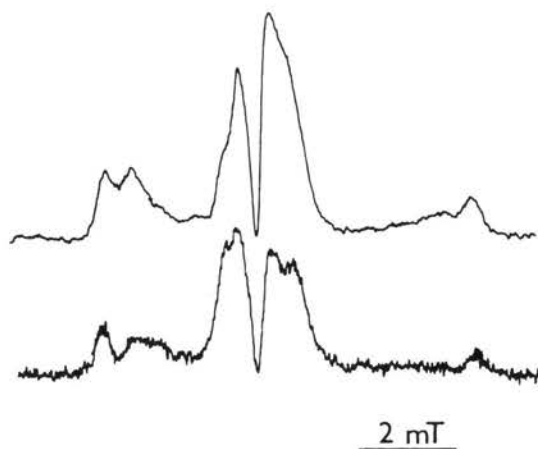


Fig. 6. ST EPR spectra of skeletal and cardiac (bottom) fibres in pyrophosphate-relaxing solution. The fibre axis was oriented perpendicularly to the laboratory magnetic field. The fibres were treated for 30 minutes at 0°C prior to EPR measurements.

The disorganization of myosin heads occurring in the presence of $MgPP_i$ is at least partly dynamical, since ST EPR spectra in $H \perp k$ orientation indicated a significant reduction of the rotational correlation times. However, $MgPP_i$ is also known not to induce complete dissociation of cross-bridges from actin (pseudorelaxed state or intermediate state between rigor and relaxation). Thus, in the simplest case, the EPR spectrum reflects two states of cross-bridges: (i) rigor-like motion and orientation, (ii) motional state for detached myosin heads. Recently, Ishiwata and coworkers (1986) have noted that the fraction of myosin heads bound to the thin filaments in myofibrils can be calculated using a suitable calibration curve. Supposing that the same calibration curve is valid for oriented muscle fibres, the application of the procedure allowed to calculate the fraction of the detached myosin heads. This assumption is supported by ST EPR measurements: in rigor, the spectral parameter L''/L was 1.22, and in relaxed state induced by ATP, $L''/L = 0.5$. Both values agree quite well with those obtained on myofibrils. From ST EPR spectra, $f^* = 0.60$ — 0.65 for skeletal muscle fibres,

* f is the fraction of the heads attached to the thin filaments in the given state.

and the corresponding value f for cardiac muscle fibres was 0.35–0.40; i.e. at resting length in pyrophosphate-relaxing solution as seen by ST EPR spectrometer, 35–40% of the cross-bridges rotated quite freely, whereas 60–65% of the cross-bridges remained bound to the thin filament in oriented skeletal muscle fibres.

A greater mobility of spin labels was observed in cardiac muscle fibres in rigor state. For the sake of comparison, the average mobility of probe molecules in relaxing medium was referred to this motional state. The ratio of $f_{\text{relax}}/f_{\text{rigor}}$ for cross-bridges was the same as in skeletal muscle; this supports the conclusion that there are no significant differences in dynamical processes in relaxing medium between cardiac and skeletal muscle fibres.

It seems reasonable to suppose that under conditions employed in this study, there is no substantial difference between cardiac and skeletal muscle in fast-reacting thiols labelled with maleimide probe molecules. Based on ST EPR measurements it can thus be concluded that cross-bridges in cardiac and skeletal muscle have similar general rotational properties. Nevertheless, it is likely that a more detailed analysis of the conformational and motional states will eventually lead to differentiation between the two muscles in accordance to their different functions.

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