Conformational Changes in Bovine Heart Myosin as Studied by EPR and DSC Techniques

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Abstract. Thermal behavior of intact and LC-2 deficient myosin obtained from bovine heart was studied using EPR and DSC techniques. The reactive thiol sites (Cys 704) of myosin was labelled with 4-maleimidopiperidine-nitroxyl, and the measurements were taken in X-band in the conventional and saturation transfer EPR time domains. DSC scans were made from 5° up to 60 °C with 0.25 °C/min scan rate. Bovine heart myosin was isolated by standard methods. The LC-2 deficient myosin was prepared by cleaving myosin with α-chymotrypsin (400:1 molar ratio) for 1.5 min at 25°. Our basic finding was a conformational change in LC-2 deficient myosin detected at 18 °C. It was not observed in intact myosin suggesting that the dissociation of the regulatory light chain resulted in a local structural change in the neighbourhood of the attached label in the 20 kD domain. The rotational correlation time of the label and the microwave saturation behavior of myosin at 25 °C exhibited no significant differences after removal of the LC-2 light chain. However, the mobility of the same label was significantly diminished in skeletal muscle. Studying the melting behavior of myosin, six endothermic peaks were detected at 19; 41.3; 43.3; 45.5; 48.5; and 54.3 °C (enthalpies: 708.4; 399; 773.8; 1089; 1612.8; and 3304.8 kJ/mol). They were assigned to the segment containing the essential thiols: HMM S-2, HMM S-1 (50kD and 20kD plus 27kD) and LMM. Removal of the LC-2 light chain was associated with the dissapearance of the 18° transition showing again a structural change in LC-2 deficient myosin which extended to a larger region.

Key words: EPR — DSC — Spin label — Melting behavior — Intact and LC-2 deficient heart muscle myosin

Introduction

Nowadays much attention is focused on the two main contractile proteins of the muscle — actin and myosin — in investigating the molecular dynamics of muscle contraction. From energetical and force generation points of view, both proteins play important role in the contraction and regulation. Cardiac muscle research has taken the leading role in this respect because of practical problems (alarming morbidity rates); the studies rely on data obtained for skeletal muscle ignoring the different physiological activities and structure of the two muscle types.

It is also known that the LC-2 chain can relatively easily be removed from the skeletal muscle myosin (Gersham and Dreisen 1970; Wagner and Giniger 1981), and that in the first approximation it has only little influence on the molecular dynamic behavior of the LC-2 deficient myosin head; however, it changes essentially the maximum velocity of muscle shortening (Moss et al. 1982, 1983) and can reduce the rotational motion of the myosin heads (Wells and Bagshaw 1983). Hoffmann and coworkers (personal communication) observed in hydrodynamic experiments (viscosity and sedimentation measurements) that — in contrast to skeletal muscle myosin — in intact myosin isolated from bovine and rat heart there is a small conformational change at 15 °C in addition to the known conformational changes of myosin at higher temperatures. In LC-2 deficient cardiac myosin this conformational transition has not been observed.

To shed more light into the internal structure of cardiac myosin and to study consequences of the regulatory light chain deficiency on the molecular dynamics, spin label and calorimetric measurements were performed. It could be concluded that the heart muscle myosin head region exhibits a greater flexibility than that of skeletal muscle myosin; the removal of the LC-2 light chain did not significantly influence the motional properties of the myosin head region. Both EPR and DSC measurements suggest that an interaction occurs between the myosin 20 k domain and the LC-2 light chain.

Materials and Methods

Preparation of cardiac myosin: Myosin from bovine heart was prepared essentially according to the method described by Shiverick et al. (1975). The protein was extracted from muscle tissue washed with 0.6 mol/l KCl, 1 mmol/l DTT, 1 mmol/l EDTA and 20 mmol/l imidazole buffer, pH 7.0. After centrifugation for 4 hours at 100,000 × *g*, the myosin was purified by chromatography on Sepharose-4B and precipitated by dilution with water.

Preparation of LC-2 deficient cardiac myosin: The cardiac myosin was digested with 400:1 (w/w) myosin: α-chromotrypsin at 25 °C for 1.5 minutes in 0.12 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l

DTT and 20 mmol/l phosphate buffer, pH 7.0. The fragments were purified by Sepharose-4B chromatography (Hoffmann and Siegert 1987; Hoffmann et al. 1987). The protein concentration was determined by the method of Lowry et al. (1951), as well as by measuring the absorption at 280 nm using extinction coefficients 0.55 mg ml^{-1} cm^{-1} for myosin, and 0.56 mg ml^{-1} cm^{-1} for LC-2 deficient myosin.

Spin labelling: Myosin was labelled with either 4-maleimide TEMPO (NEM) or 4-iodoacetamide-TEMPO (IAA). The washed myosin was suspended in solution containing 0.5 mol/l KCl, 50 mmol/l Tris and 1 mmol/l EDTA, pH 8.0, and reacted on ice with 2—4 mol NEM for 60—90 minutes, or with 3.5 mol IAA over 1 mol myosin for 10—14 hours. The reaction was terminated by precipitation of myosin with ice cold water, the protein was collected by centrifugation and dissolved in 0.5 mol/l KCl, 25 mmol/l HEPES and 5 mmol/l $K_3Fe(CN)_6$ to reduce the EPR signal from the weakly immobilized labels, as suggested by Graceffa and Seidel (1980). Hexacynoferride was removed by exhaustive dialysis for 16 hours at 4 °C. The proteins were purified by centrifugation at 50,000 × *g* for 1 hour and used at a final concentration of 20—30 mg/ml (~ 50 μmol/l).

EPR experiments: The EPR measurements were performed with an ERS 220 X-band spectrometer (Center of Scientific Instruments, GDR). For the conventional EPR technique, 100 kHz field modulation (0.1—0.25 mT amplitude) and 2—20 mW microwave power were used. Second harmonic absorption out-of-phase (saturation transfer, ST) spectra were recorded with 50 kHz field modulation (0.5 mT amplitude) and detected out-of-phase at 100 kHz. The microwave power was 85 mW, which corresponds to an average microwave field amplitude of 0.025 mT in the central region of a flat Zeiss cell (GDR). The microwave magnetic field was determined according to Fajer and Marsh (1982) with peroxylamine disulphonate ion radicals in the same sample cell as the myosin samples.

EPR signals were recorded by a microcomputer system linked to the spectrometer. The spectra were recorded on a digital transient recorder and stored on magnetic media. Usually, 200 scans (scan time: 2.0 ms) were done and, in some cases, the signal to noise ratio was improved by the Fourier self-deconvolution procedure described by Kauppinen et al. (1981). The computer program written by dr. P. Gróf (1977) (Institute of Biophysics, University Medical School, Budapest) was employed.

Calculation of spectrum parameters: For the evaluation of the EPR scans the parameters used were: the ratio of the two low-field peak amplitudes h_1 and h_2, and the distance between the outermost hyperfine extrema (see Fig. 4). Rotational correlation times which characterize the rotational motion of the label were calculated using the value of the splitting constant of the sample and that of the same sample at the rigid limit approximation in the conventional EPR time scale according to Freed (1976):

$$\tau_2 = a/1 - 2A'_{zz}/2A_{zz}/^b,$$

where $a = 5.4 \times 10^{-10}$ s, $b = -1.36$ for Brownian diffusion, and $2A'_{zz}$ is the spilitting constant of the sample under investigation, $2A_{zz}$ is the splitting constant at rigid limit which could be obtained by extrapolation procedure ($T/\eta \to 0$, T is the temperature of the sample, η is the viscosity). The values of $2A'_{zz}$ were determined from the spectra of the samples at increasing viscosity, and extrapolated for $\eta \to \infty$ (myosin was dissolved in 10—40% sugar and/or 48—87% glycerol solution). The parameter $2A_{zz}$ can be determined by linear fitting of $2A'_{zz}$ data using the relationship (Freed 1976):

$$2A'_{zz} = f((T/\eta)^{-1/b}).$$

It should be noted that the hyperfine splitting constant measured for myosin in filamentous state can also be accepted as $2A_{zz}$.

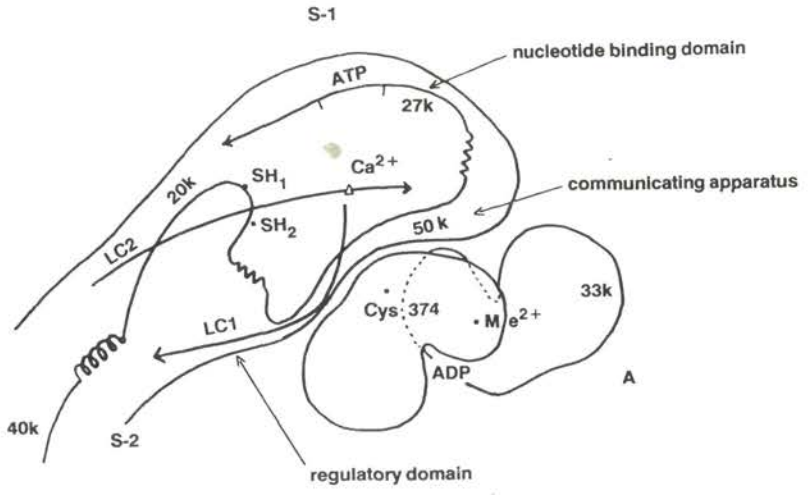

Fig. 1. Schematic diagram of the force generating unit of the muscle.

DSC measurements: Heat phenomena related to thermal transition were investigated in a DASM-4 (Privalov) scanning calorimeter. Denaturation proceeded at 0.25 K/min scan rate from 5 °C up to 60 °C. The thermograms were decomposed by the Fourier analysis (spectrum deconvolution) using the well known transitions of the skeletal muscle myosin as a reference.

Results and Discussion

Characterization of the labelled sites:

The method used to spin-label the SH-1 thiol site of cardiac myosin was essentially the same as described earlier by Thomas and colleagues (1975, 1980) and Manuck et al. (1986) for skeletal muscle myosin. Under the conditions used, the reaction of IAA and NEM with the SH-1 thiol groups has been reported to be quite selective, and even in myofibrils most of the paramagnetic probe molecules can be expected to attach to myosin SH-1 sites. The localization of the SH-1 site is shown in Fig. 1. The degree of labelling was 0.1—0.15 mol labels/mol protein for IAA label, and 0.5—0.75 mol label/mol protein for NEM label.

The EPR spectra of both NEM- and IAA-labelled cardiac myosin showed a superposition of spectra from strongly and weakly immobilized labels. The population of labels attached to weakly immobilizing sites varied a little from batch to batch, but it never exceeded 10% of the total EPR absorption. The

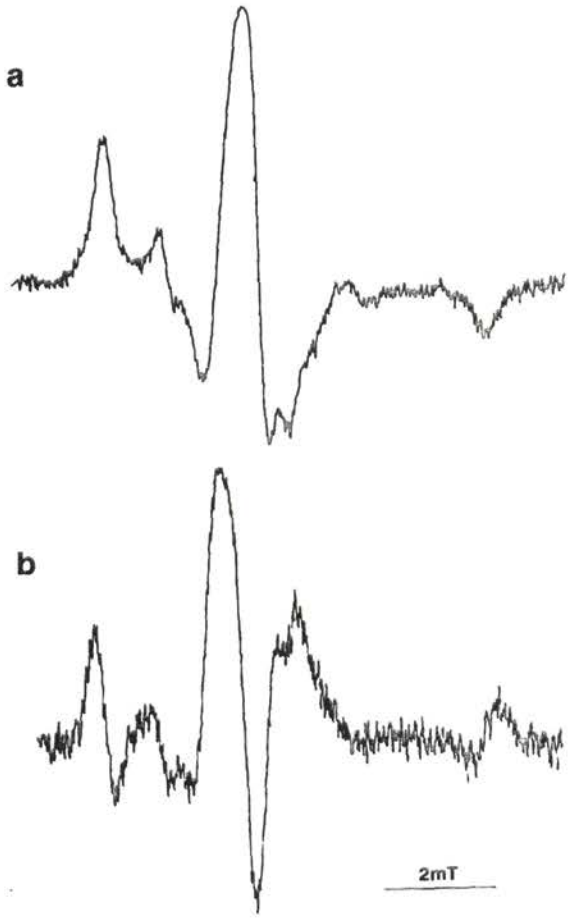

Fig. 2. Conventional (*a*) and ST (*b*) EPR spectra of IAA labelled heart myosin. Concentrations of myosin and bound label: 50 μmol/l and 25 μmol/l, respectively. Temperature 22 °C.

EPR spectra of myosin labelled with IAA spin label in the conventional (*a*) and ST time domain (*b*) are illustrated in Fig. 2. The spectra were characterized by the distance of the outermost hyperfine extrema, $2A'_{zz}$ and the rotational correlation time of the labels. The results for cardiac myosin are summarized in Table I. The rigid limit value for $2A'_{zz}$ at $\eta \to \infty$ was obtained by a least squares fit procedure, and a value of 6.674 mT was obtained for the NEM label. This value was significantly higher than the values of $2A'_{zz}$ measured for myosin minifilaments ($2A'_{zz} = 6.484$ mT) or myosin precipitate ($2A'_{zz} = 6.510$ mT). Since the

Table I. Spectral parameters and rotational correlation times of spin labels bound to the fast reacting thiol sites in cardiac and skeletal muscle myosins

	Sample	Conventional EPR		Saturation transfer EPR			Notes	
		$2A'_{zz}$(mT)	$\tau_2(\mu s)$	C''/C	(L''/L)	$\tau_3(\mu s)$		
Cardiac	myosin (NEM)	6.402 ± 0.02	0.042	-0.93 ± 0.25		0.2	$n = 11$	$n = 5$
	LC-2-myosin (NEM)	6.396 ± 0.03	0.041	-1.17		0.1	$n = 4$	$n = 2$
	myosin (IAA)	6.901 ± 0.02	0.50				$n = 8$	
	myosin (NEM)	6.484 ± 0.03	0.070	-0.40		2	minifilaments	
	rigid limit	6.510 ± 0.03	0.084	-0.03 ± 0.03		7	precipitates $n = 4$	
		6.674					sugar plot	
	myosin (IAA)							
	rigid limit	6.944 ± 0.03			(0.93)	150	$n = 3$	
Skeletal	myosin (NEM)	6.803 ± 0.02	0.26					
	myosin (IAA)	6.930 ± 0.03	0.27					
	myosin (NEM)	6.875 ± 0.02						
	rigid limit							
	muscle fibres	6.935 ± 0.02			(1.22 ± 0.06)	1000	rigor	$n = 10$
	myosin (IAA)							
	rigid limit	7.003 ± 0.03						

labels were strongly immobilized at $\eta \to \infty$ but not in side by side aggregates of myosin molecules, the former value was used as the rigid limit. Using $2A_{//} = 6.674$ mT for the NEM-label, the rotational correlation time of the label was 42 ns at room temperature and at 1 cP (Table I). Addition of glycerol to myosin precipitate resulted in a sudden increase of hyperfine splitting, suggesting a strong effect of glycerol on the rotational mobility of the myosin head region, or at least on the neighbourhood of the bound label. This suggests that

(i) the attached labels show significant rotational mobility even in minifilament form and in aggregates of cardiac myosin molecules;

(ii) there is a substantial difference in the rotational motional properties between cardiac and skeletal myosin: the rotational correlation time calculated for cardiac myosin was twice shorter than that for skeletal muscle myosin, suggesting that the labels were located on SH-1 sites of myosin (Thomas et al. 1975).

It should be noted that no significant changes of the rotational correlation time were observed for myosin and LC-2-deficient myosin; hence, the removal of the LC-2 chain from cardiac myosin had no influence on the rotational motion of the attached NEM label.

It is known from earlier measurements and hydrodynamic properties of myosin that the rate of reorientation of the long axis of isolated S1 is 1.63×10^{-7} s, and 2.53×10^{-7} s for myosin; thus, the maleimide probe molecules do not attach rigidly to skeletal myosin (Thomas et al. 1975). The probe rotated relative to the head region of S-1. Our results allow the same conclusion to be drawn for cardiac muscle myosin.

From the ST EPR spectra of myosin filaments the following spectral parameters L''/L and C'/C were calculated: $C'/C = -0.041$ and $L''/L = 0.24$. They correspond to rotational correlation times of 6 μs and 2 μs, respectively. These values indicate that the self-organization of myosin into filaments was accompanied by a strong immobilization of labels in the head region of the myosin molecules. Hence, a significant portion of mobility originated from the segmental flexibility of the protein.

Comparing the motion of the probe molecules in skeletal and cardiac myosin, a substantial reduction in the mobility of the maleimide probe molecule attached to skeletal myosin can be stated. The characteristic hyperfine constant was $2A'_{//} = 6.803 \pm 0.025$ mT. Using $2A_{//} = 6.875$ mT as rigid limit (the hyperfine splitting constant for maleimide labelled myosin at $\eta \to \infty$), the rotational correlation time was 0.26 μs according to Goldman and coworkers (1972). In contrast, the mobility of the cardiac myosin labelled with maleimide spin label under identical conditions could be characterized by $\tau_2 = 42$ ns, i.e. the maleimide label attached to cardiac myosin rotates about 5 times faster than the same label bound to SH-1 of skeletal myosin.

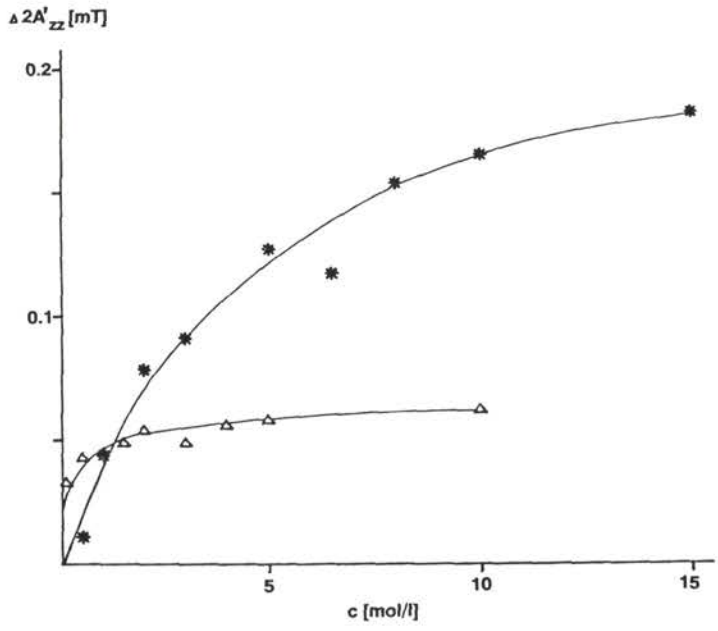

Fig. 3. Effect of MgADP on the hyperfine splitting of NEM- and IAA-labelled heart myosin. Myosin concentration 20 μmol/l, temperature: 22 °C. Symbols: Asterisks: IAA labelled myosin (0.15 mol label/mol protein); Triangles: NEM labelled myosin (0.7 mol label/mol protein). Buffer composition: 0.5 mol/l KCl, 25 mmol/l HEPES, 1 mmol/l EDTA, pH = 7.0.

Interaction of spin labelled myosin with ADP

As has been shown previously, the binding of ADP to myosin results in a significant decrease in the proportion of strongly immobilized label (Seidel et al. 1970). The data from spectral changes could be fitted by curves predicted for either one or two independent ADP binding sites and an association constant in the range of 10—12.4×10^2 mol (Stone 1970). Also it has been shown that the changes in the ratio of the heights of the two first peaks depended on whether the myosin was skeletal or cardiac myosin: it was significantly lower for cardiac myosin suggesting different local conformational changes in the two myosin classes (Stone et al. 1981).

Experiments with cardiac myosin labelled with SH-directed spin labels showed that the substrate-dependent conformational changes depended strongly on the probe molecules; both iodoacetamide and maleimide labelled myosins exhibited a decrease in their hyperfine splitting constants after addition of ADP to the buffer. The increased mobility of the attached labels varied with the ADP

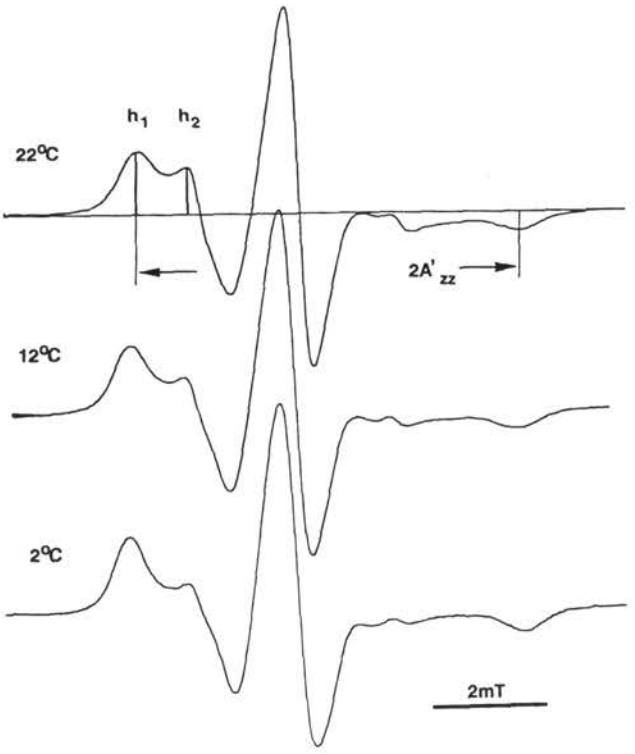

Fig. 4. Spectrum parameters and temperature dependence of EPR spectra of NEM-labelled heart myosin.

concentration, reaching a saturation level, but the conformational change induced by ADP was significantiy higher for IAA labelled myosin.

In Fig. 3 the changes in $2A'_{zz}$ are plotted against ADP concentration. In the presence of ADP the mobility of spin labels slightly increased and the difference in the hyperfine splitting constants (designated $\Delta 2A'_{zz} = 2A'_{zz}$ in the absence of ADP and $-2A'_{zz}$ in the presence of ADP) reached a maximum at the saturating amount of ADP.

Iodoacetamide probes are known to exhibit a greater flexibility of the attaching links than do maleimide molecules. Hence, the myosin (NEM)-ADP complex can be regarded as behaving like a rigid moiety on the time scale of the conventional EPR, whereas the IAA probe molecule is a sensitive monitor of the nucleotide binding accompanied by a local conformational change. Experiments performed with glycerol-extracted muscle fibres showed that the addition

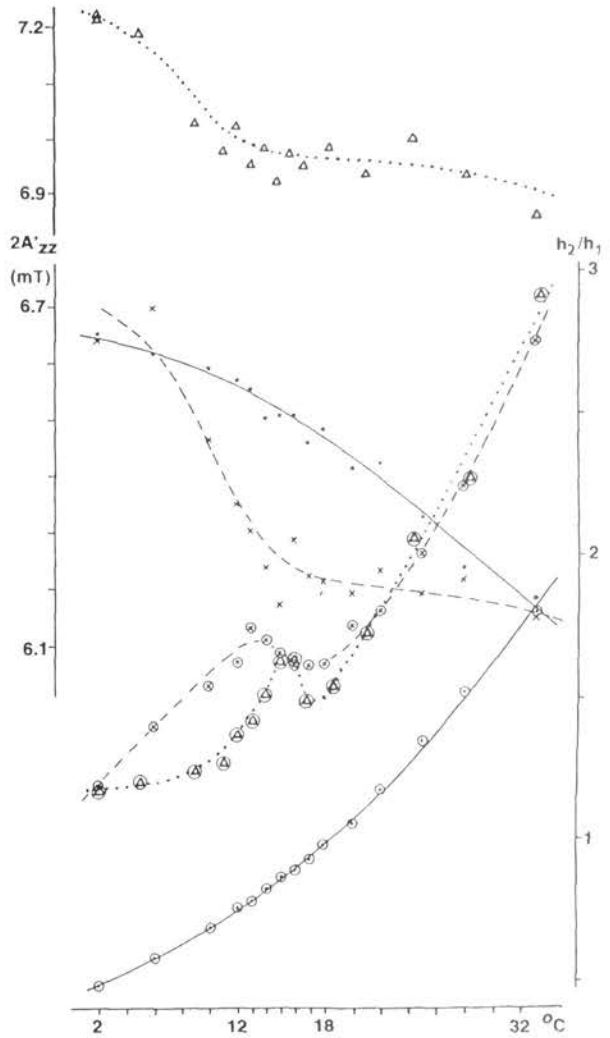

Fig. 5. Plot of hyperfine splitting of IAA-labelled intact (△) and NEM-labelled intact (●) and LC-2 deficient (×) heart myosin against temperature. The proper circles refer to h_2/h_1.

of MgADP to maleimide spin labelled fibres in rigor buffer did not resulted in a significant axial rotation of the crossbridges (Thomas et al. 1985).

In contrast, significant alteration of the probe distribution was observed in the ternary actin-myosin -ADP complex in fibres, when IAA spin labels were located on the SH-1 thiol sites in myosin (Belágyi 1988). On the basis of these and other results it could be concluded that MgADP induced an intrinsic change in the multisubunit structure of the myosin head region, which nevertheless did not change the overall rotational properties of the myosin head.

Temperature dependence of EPR spectrum parameters

Temperature induced changes in the EPR spectra of heart muscle myosin labelled with NEM are shown in Fig. 4. The temperature dependence of spectrum parameter $2A'_{zz}$ in the temperature interval of 2—32 °C is illustrated in Fig. 5. The spectrum parameters did not change upon heating or cooling, therefore all data were included in the same diagram. Monotonic changes of the hyperfine splitting constant were obtained for intact myosin (Fig. 5, full circles) labelled with NEM; the parameter $2A'_{zz}$ decreased which corresponds to the slow loosening of the protein structure due to the absorbed heat energy. LC-2 deficient myosin (Fig. 5, crosses) shows a clear-cut change of the hyperfine splitting constant at around 15 °C; this might refer to a conformational change. Interesting by a similar pattern was observed for intact myosin labelled with IAA probe molecules (Fig. 5, triangles).

This result, which seems contradictory at first sight, can be interpreted quite well on the basis of different attaching of the label molecules to myosin Cys 704 thiol groups. The maleimide label is well known to bind more rigidly to the SH-groups of proteins very likely (it is stabilized by H-bonds), the librational motion of the probe is smaller than that of the iodoacetamide label, so the rotational motion of the maleimide probe molecule reflects almost entirely the global motion of a large protein segment in the myosin head region.

In contrast, due to its more flexible attachment the IAA label is sensitive also to the internal motion. Considering that the ST-EPR measurements exhibited no sudden change in the spectrum parameters of myosin labelled with NEM in the given temperature range, a local conformational change at 15 °C can be suggested to occur as detected by the IAA label. Very likely, dissociation of LC-2 light chains modulates the flexibility of the 20 kDa segment, and this influences the rigidity of the attachment of NEM label. The reduced flexibility allows rotational motion of the segment that holds the label, and a local conformational change is detected on increasing temperature. The present state of experiments allows no reasonable explanation as to the possible physiological importance of this conformational change.

Thermal transitions of heart muscle myosin

Intact myosin shows a slow thermal transition at around $Tm_1 = 18°$ with $H_1 = 708.44$ kJ/mol enthalpy according to the DSC scan (Fig. 6); this was not detectable with LC-2 deficient myosin. Fourier analysis of the thermograms yielded the following components: peaks with transition temperatures $Tm_2 = 41.3°$ and $Tm_5 = 48.5$ °C and with enthalpies $H_2 = 399.02$ kJ/mol and $H_5 = 1612.8$ kJ/mol originate probably from the HMM component of the pro-

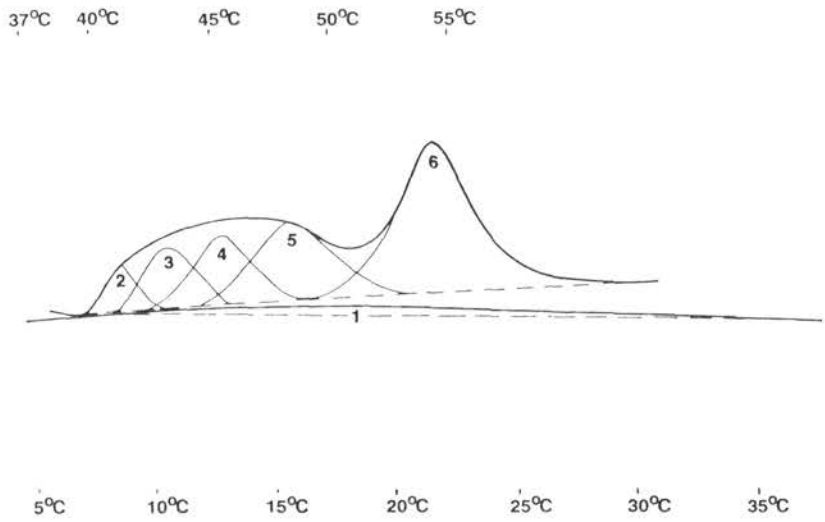

Fig. 6. Fourier deconvolution of DSC scan of intact cardiac myosin. The upper scale pertains to the high temperature range (37—60 °C), the lower one to the low temperature range (5—35 °C).

tein. The lower transition temperature component very likely is due to the phase transition of the S-2 domain. The above transitions at 41 °C and 48 °C were found for HMM prepared from rabbit skeletal muscle myosin by chymotryptic digestion (Shriver et al. 1988), the higher temperature transition might concern the S-1 domain. Its topology is illustrated in Fig. 1.

The endothermic peaks with transition temperatures $Tm_3 = 43.3\,°C$, $Tm_4 = 45.5\,°C$ and $Tm_6 = 54.3\,°C$ ($H_3 = 773.76\,kJmol^{-1}$, $H_4 = 1089\,kJmol^{-1}$ and $H_6 = 3304.8\,kJmol^{-1}$) can be attributed to the LMM fragments (Bertazzon et al. 1988). The same transitions were measured for rabbit skeletal muscle myosin at 44.7; 46.6; 54.8 and 55.1 °C respectively (Privalov 1982). The sum of enthalpies measured (Table II) are in very good agreement with the values calculated by Fourier analysis.

DSC measurements reflect the physico-chemical property (structural stability) of a given functional and/or structural domain, whereas spin-label measurements probe the dynamics of the microenvironment of the label; hence, the EPR and DSC measurements which are partly different at first sight are not contradictory. They provide different mutually complementary information.

The monotonic temperature dependence of the hyperfine splitting constant (and of the correlation time) is the consequence of increasing internal fluctuations. One possible explanation for the structural change occurring at about

Table II. Transition temperatures and enthalpies of intact cardiac myosin fragments

Number of transitions with increasing temperature	1	2	3	4	5	6
Transition temperatures T_i (°C)	18	41.3	43.3	45.5	48.5	54.3
Transition enthalphy H_i (kJmol^{-1})	704.44	399.02	773.76	1089	1612.8	3304.8

(Intact and LC-2 deficient myosins of cardiac muscle in 500 mmol/l KCl, 25 mmol/l HEPES and 1 mmol/l EDTA pH = 7.0, were measured in differential scanning calorimeter (DASM-4, Privalov). Myosin concentration 20 μmol/l, scan rate 0.25 K/min.)
Sum of enthalpies: 8080.5 kJ mol^{-1} (measured); 7888.3 kK mol^{-1} (deconvoluted).

15 °C might be a temperature-induced alteration of the packing, or interaction between the segments containing SH-1 (Cys 704) and SH-2 (Cys 694) groups and the 27 kDa domain containing the nucleotide binding site (see Fig. 1). This assumption is supported by experiments in which cross-linking between the 20 kDa domain containing SH-1 and the 27 kDa domain with bound ATP could be attained (Lu et al. 1986). Removal of LC-2 seems to induce structural loosening of the microenvironment.

Intact myosin isolated from heart muscle shows another conformational change at low temperatures in addition to the well-known phase changes observed for skeletal muscle myosin. This can be attributed to different structural construction manifested also in different physiological action. ST EPR experiments showed that the paramagnetic probe attached to Cys 704 residue had a rotational motion in the submillisecond time range even in heart muscle fibers in the rigor state (Belágyi and Röth 1987). The lack of conformational change in LC-2 deficient myosin may refer to some interaction among the heads, resulting in a rigidization of the structure. This kind of interaction was already reported for LC-2 deficient myosin of smooth muscle (Wells and Bagshaw 1983). Another possibility would be a conformational change of the LC-2 light chain itself; however, no experimental evidence is available to support this possibility. The most plausible explanation would be a domain-domain interaction between the LC-2 light chain and the 20 kDa subunit, resulting in a structural instability of the 20 kDa segment. The nature of the structural instability in a protein system was analyzed by Privalov (1982) and Potekhin and Privalov (1982). The sign of a change arising from structural instability is an endothermic transition at low temperature.

In conclusion, the monotonic change in the hyperfine splitting constant ($2A'_{//}$ decrease) for intact myosin labelled with NEM corresponds to the slow loosening of the protein structure caused by the absorbed heat energy. A marked change of parameter $2A'_{//}$ is observed at around 18 °C for LC-2 deficient myosin which may refer to a local conformational change in the 20 kDa domain.

According to DSC experiments, a conformational change occurs in intact cardiac myosin at low temperature (18 °C) in addition to the well-known phase changes of skeletal muscle myosin; the additional change might be attributed to different interaction between the LC-2 light chain and the 20 kDa domain of the myosin head.

DSC measurements reflect the physico-chemical properties of a given functional or structural domain of myosin, whereas spin label measurements record the microdynamics of the segment containing the Cys-704 amino acid residue; consequently, there is no contradiction between the results of EPR and DSC measurements which provide different and mutually complementary information on the structural stability of myosin.

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