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INTERNAL FLEXIBILITY OF CARDIAC MYOSINS

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Abstract

Conventional and saturation transfer electron paramagnetic resonance spectroscopy (EPR and ST EPR) and differential scanning calorimetry (DSC) were used to study the motional dynamics and segmental flexibility of cardiac myosins.

Cardiac myosins isolated from bovine and human heart muscle were spin-labelled with isothiocyanate- or maleimide-based probe molecules at the reactive sulfhydryl sites (Cys-697 and Cys-707) of the motor domain. The maleimide probe molecules attached to human cardiac myosin rotated with an effective rotational correlation time of 33 ns which was at least eight times shorter than the rotational correlation time of the same label on skeletal myosin (260 ns). In the presence of MgADP and MgADP plus orthovanadate, flexibility changes in the multisubunit structure of myosins were detected, but this did not lead to changes of the overall rotational property of the myosin heads. Significant difference in the internal flexibility was detected on myosin samples isolated from ischemic tissue, the rotational correlation time decreased to 25 ns.

DSC measurements supported the view that addition of nucleotides produced additional loosening in the multisubunit structure of cardiac myosin. It is postulated that there is an intersite communication between the nucleotide binding domain and the 20 kDa subunit where the reactive thiol sites are located.

Keywords: cardiac myosin, DSC, flexibility of myosin heads, spin-labelling

Introduction

Current models of muscle contraction assume that force is generated by myosin-actin interaction coupled to the ATPase cycle. The head portion of myosin molecule (subfragment one, S 1) is rigidly attached to actin and forms a complex in the absence of nucleotides, in rigor state. In the presence of MgATP, the chemical energy liberated from ATP hydrolysis produces conformational changes in the head region of myosin. The structural changes might produce rotation of myosin head while bound to actin, and it makes the muscle to shorten [1, 2]. This mechanism of the energy transduction is a crucial question of the muscle contraction.

The biochemical characteristics of cardiac muscle is different from skeletal muscle [3–5]. In an effort to identify the extent of myocardial infarction and test

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the efficiency of appropriate therapies, it was found that during myocardial infarction the intracellular acidosis or the activation of proteolytic enzymes led to the release of the structurally bound myosin light chains [6]. Cell death, due to irreversible disruption of sarcolemma, results in the accessibility of myosin heavy chain to exogenous agents, the fragments of contractile proteins were found to circulate for long-term period in serum. The level of cardiac myosin LC 2 light chain in serum correlates closely with the infarct size [7]. An increased myocardial activity of xanthine oxidase generating superoxide free radicals has been recorded in ischemia and was assumed to be the main source of oxygen radicals [8]. Oxygen free radicals are able to modify the contractile proteins in time- and concentration-dependent manner. This interaction leads to the loss of protein sulfhydryl groups, and thereby the enzyme activity is affected.

It is also known from hydrodynamic measurements [9] that there were differences in the sedimentation of LC-2 deficient and intact cardiac muscle myosins; intact cardiac myosin showed a conformational change at 18°C which could not be detected after removal of the LC-2 light chain. The main functional difference was assigned to the 50 kDa domain and/or to the interaction of heavy chain with the LC-2 light chain. Biochemical and spectroscopic studies show that nucleotides and nucleotides plus orthovanadate induce significant increase of rotational mobility of the bound labels in the environment of the nucleotide binding sites, therefore changes are expected in the DSC pattern after nucleotide binding to myosin.

In this study an attempt is made to observe data about the different molecular behaviour of cardiac- and skeletal muscle myosins using EPR technique and paramagnetic probes which report upon molecular motions, and applying DSC which provides information about the structural stability. Using maleimide-, iodoacetamide- and isothiocyanate-based paramagnetic probes attached to the thiol groups of myosin, the globular head portion of cardiac muscle myosin containing the catalytic and transmission domains proved to be less rigid than that of the skeletal muscle. Maleimide probes attached to myosin isolated from ischemic tissue reflected reduced rotational motion. Segmental flexibility of cardiac myosin was influenced by attachment of actin to myosin.

Materials and methods

Preparation of myosin

Bovine and human heart myosin was prepared by the methods described by Shiverick [10] and Léger and coworkers [11]. After washing of the tissue, myosin was extracted in buffer consisting of 0.6 *M* KCl, 20 *mM* imidazol, 1 *mM* dithiothreitol (DTT), 1 *mM* EDTA, *pH* 7.0. After centrifugation for 4 h at 0°C with 100 000×g, the crude myosin was purified using Sepharose 4B chromatog-

raphy. The sample was precipitated with ice cold water, and thereafter the protein was collected by centrifugation and dissolved in 0.3 *M* KCl, 90 *mM* KH_2PO_4, 60 *mM* K_2HPO_4, 2 *mM* $MgCl_2$ at *pH* 6.8. Protein concentrations were determined either by the method of Lowry [12] or by reading the absorption at 280 nm using absorption coefficients 0.55 mg $ml^{-1}cm^{-1}$ for myosin.

Preparation of muscle fibre

Glycerol-extracted muscle fibre bundles were prepared from dog cardiac muscle. Small stripes of muscle fibres (20–25 mm in length and 1 mm in diameter) were stored after osmotic shocks in 50% *V/V* glycerol and rigor solution (100 *mM* KCl, 5*mM* $MgCl_2$, 1 *mM* EGTA, 10 *mM* histidine. HCl, *pH* 7.0) in refrigerator at –18°C for 3 days to 1 month before use.

Spin-labelling of proteins and muscle fibres

The isolated proteins were labelled either with 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (4-isothiocyanato-TEMPO, TCSL) or 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO, MSL). Few samples were labelled with 4-iodoacetamido-2,2,6,6-tetramethylpiperidinooxyl (4-iodoacetamido-TEMPO, IASL). Myosin was reacted for overnight with 2 moles of TCSL or with 2–4 mol of MSL per mole of myosin for 60–90 min over ice. After reaction the samples were dialyzed overnight at 4°C against great amount of buffer solution. In some cases myosin was treated with 5 *mM* $K_3(Fe(CN)_6)$ to reduce the signal intensity arising from weakly immobilized labels [13]. The protein was clarified by centrifugation at 50 000×g for 1 h and used at a final concentration of 20–100 μM .

Spin-labelling of muscle fibres was performed in relaxing medium (rigor solution plus 2 *mM* pyrophosphate at *pH* 7.0) with about one mole of MSL to one mole myosin for 30 min at 0°C. After spin-labelling the fibre bundles were washed in great amount of rigor solution plus 25 *mM* $K_3Fe(CN)_6$ for 16 h to remove the unreacted labels and to reduce labels bound to weakly immobilizing sites [13]. The spin-labelled muscle fibres were stored in rigor solution no longer than over 24 h at 4°C before EPR measurements. The sarcomere length of the fibres were measured by laser diffraction using a He–Ne gas laser and was 2.25±0.15 μm in rigor solution.

EPR measurements

The EPR measurements were taken with an ESP 300 E (Bruker, Germany) X-band spectrometer. For conventional EPR technique 100 kHz field modulation (0.1–0.25 mT amplitude) and 2–20 mW microwave power were used. Second harmonic absorption, 90° out-of-phase spectra were recorded with 50 kHz field modulation (0.5 mT amplitude) and detection at 100 kHz out-of-phase. The microwave power was 63 mW which corresponds to an average

microwave field amplitude of 0.025 mT in the central region of the flat cell of Zeiss (Jena, Germany). The microwave magnetic field intensity was determined with peroxylamine disulphonate ion radicals in the same sample cell as for the myosin samples following Fajer and Marsh [14].

Signals due to EPR absorption were detected by the Bruker microcomputer system interfaced to the EPR spectrometer and the spectra were stored on diskettes. The standard WIN EPR software (Bruker, Germany) was used for evaluation. The double integrals of the spectra were normalized to unity for spectrum manipulation.

DSC measurements

Thermal unfolding of cardiac myosin in the present of different nucleotides (ADP, ADP+orthovanadate(V_i)) were monitored by a SETARAM Micro DSC II calorimeter. All experiments were done between 10 and 80°C with a 0.3°C min^{-1} scan rate.

Conventional Hastelloy batch vessels were used during the thermal transition experiments with 850 µl sample volume in average. Rigor buffer was used as reference sample. The sample and reference vessels were equilibrated with a precision of ±1.0 mg. There was no need to perform any correction from the point of view of heat capacity between the sample and reference vessels.

Results and discussion

Characterization of labelled muscle fibres

The fibre bundles could produce tension in activating solution (rigor buffer plus 0.1 *mM* $CaCl_2$ and 5 *mM* ATP) after spin labelling (Fig. 1). The maximum tension of the labelled fibres was in an average about 10–15% smaller than the maximum tension produced by the untreated fibres. This agrees with the earlier observations [15]. From the extent of the reduction of the K^+-EDTA ATPase activity and from the number of spin labels bound to myosin we estimated that about 90% of spin labels were located on the reactive sulfhydryl sites. The Ca^{2+}-ATPase of the extracted myosin showed no remarkable change after spin-labelling; it supports the view that TCSL binds to the SH_2 sulfhydryl site of myosin [16, 17].

Characterization of the labelled sites

The method used to label the reactive thiol sites of cardiac myosins was essentially the same as described earlier by Thomas and colleagues [18] and Belagyi [19] for skeletal muscle myosin. The degree of labelling was 0.15–0.25 mol label/mol protein for TCSL and 0.35–0.45 mol label/mol protein for MSL.

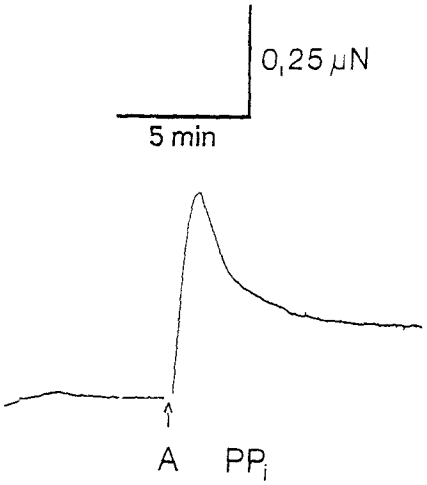

Fig. 1 Mechanical response of spin-labelled glycerinated cardiac muscle fibre bundles. Fibre bundle was isolated from dog heart, glycerinated as described in Materials and Methods and spin-labelled with MSL in relaxing medium. The mechanical response was initiated by addition of 5 *mM* ATP and 0.1 *mM* $CaCl_2$ to the rigor buffer. Arrow shows the time of addition of the activation solution. Inorganic phosphate (PP_i) was used to induce relaxation

The EPR spectra of both MSL- and TCSL cardiac myosins showed the superposition of spectra from strongly and weakly immobilized labels (Figs 2 and 3). The fractions of labels attached to weakly immobilizing sites varied a little from batch to batch, but never exceeded 10% of the total EPR absorption. The conventional EPR spectra were characterized by the distance between the outermost hyperfine extrema $2A_{zz}'$ and the rotational correlation time of the labels (Table 1). For determination of the rotational correlation time the method of Goldman *et al.* [20] was used. The rigid limit value for $2A_{zz}'$ at $\eta \rightarrow \infty$ was obtained by a least square fit procedure, and was (6.782±0.03) mT for MSL. In contrast, $2A_{zz}$ =6.875 mT was obtained for maleimide labelled skeletal myosin at $\eta \rightarrow \infty$ and 6.800±0.025 mT at 1 cP and 20°C. The rotational correlation times of MSL myosins at room temperature and 1 cP were 33 ns (human cardiac myosin) and 260 ns (skeletal myosin), respectively. The results suggest that there is a substantial difference between the internal mobility of cardiac and skeletal myosins; the effective rotational correlation time for cardiac myosin was 8 times shorter than that of for skeletal muscle myosin assuming that the labels were located on the same sites of myosin. At low ionic strengths, where the human cardiac myosin was in filament form, the hyperfine splitting constant increased significantly and the value attained to $2A_{zz}'$ =6.556±0.03 mT at room temperature (τ_2 =55 ns). The comparison of $2A_{zz}'$ values indicate that the self-

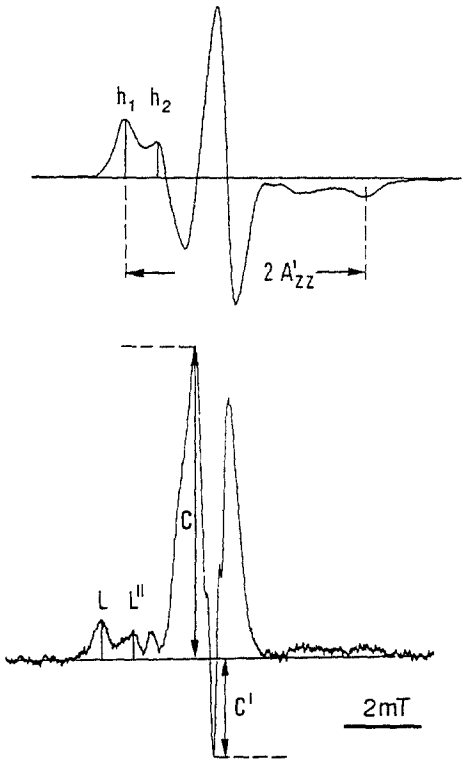

Fig. 2 Conventional (upper) and ST EPR spectrum (bottom) of human cardiac myosin labelled with MSL. The spectra were recorded at 20°C. The concentration of myosin was 20 μM. The spectral parameters (the low-field peaks h_1 and h_2, the hyperfine splitting constant $2A_{zz}'$ and the diagnostic peaks of the ST EPR spectrum L, L'', C and C') used to characterize the EPR spectra are also shown

organization of myosin into filaments was accompanied with a strong immobilization of the labels in the head region of the myosin. It suggests that a significant portion of the mobility originates from the segmental flexibility of the protein.

From ST EPR spectra of myosin filaments we calculated the spectral parameters L''/L and C'/C, they were

$$L''/L = 0.19 \pm 0.1 \ (n=4) \text{ and}$$

$$C'/C = -0.34 \pm 0.15 \ (n=4)$$

which correspond to rotational correlation time of 2 μs, respectively (Fig. 2). Addition of actin to myosin strongly reduced the rotational mobility of the label bound to myosin (Fig. 3). The low-field spectral parameter, L''/L, in the ST EPR spectrum of glycerinated skeletal muscle fibres took up a value of

$$L''/L = 1.22\pm0.06\ (n=10), \text{ whereas}$$

$$L''/L = 0.83\pm0.1\ (n=4)$$

was obtained for cardiac muscle fibres in rigor [21]. The values of τ_2 (> 1 ms and about 70 μs, respectively) showed that the rate of the reorientation of the label in cardiac muscle fibres was faster approximately with one order of magnitude in rigor, evidencing some internal flexibility of cross-bridges even in their attached state. Preliminary experiments also showed that the diagnostic peak ratios (*L''/L, C'/C)* depended on the state of the cardiac myosins (Table 2).

In contrast to MSL fibres, the measurements on TCSL-labelled fibres indicated the motion of a larger segment or domain in the head region of skeletal myosin as revealed by ST EPR technique, the spectral parameter *L''/L* was (0.85±0.1), much smaller than 1.2 that is usually detected on rigor MSL fibres. The ordering of isothiocyanate-based spin labels attached to the fast-reacting thiol site is nearly static on the millisecond time scale in rigor; but the spectra of spin-labelled fibres exhibited two narrow distributions with mean angles of 75° and 56° as published recently [19].

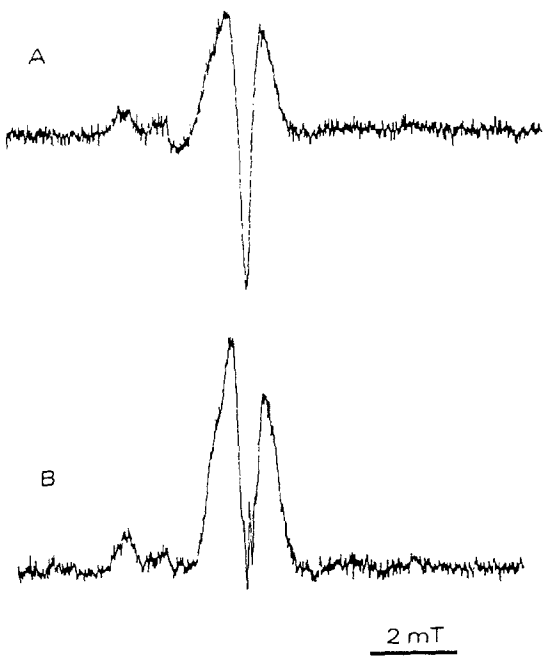

Fig. 3 ST EPR spectra of bovine cardiac myosin before (A) and after (B) of addition of skeletal muscle actin. The molar ratio of actin to myosin was 5 to 1. Spectra were taken at 20°C. The concentration of myosin was 20 μM and was labelled with MSL

Table 1 Segmental flexibility in spin-labelled cardiac myosin. Cardiac myosins isolated from normal and ischemic human heart muscle were spin-labelled with a iodoacetamide- and maleimide-based spin label to block the reactive - SH1 group in the motor domain of myosin. 1.0 mol spin label/mol protein was reacted for 24 h at 0°C. Unreacted labels were removed by overnight dialysis at 4°C. The protein concentration was 33 μM. The table shows the hyperfine splitting constants $2A'_{zz}$ measured in mT in different states. The spectra were taken at 20°C

Maleimide label	Normal	Ischemic
Solution	6.456*	6.385
Filaments	6.514	6.459
Precipitate	6.558	6.529
Iodoacetamide label		
Solution	6.850	6.832
Filaments	6.855	6.841
Precipitate	6.973	6.873
(atrium)	–	6.703

* error of determinations: ±0.025 mT

Table 2 Domain motion of spin-labelled cardiac myosin. Cardiac myosins isolated from normal and ischemic human heart muscle were spin-labelled with a maleimide-based spin label to block the reactive -SH1 and -SH2 groups of the motor domain of myosin. 0.75 mol spin label/mol protein was reacted for 1 h at 0°C. Unreacted labels were removed by overnight dialysis at 4°C. The protein concentration was 20 μM. The table shows the ratio of the diagnostic peaks of the ST EPR spectra. The spectra were taken at 20°C

Normal		Ischemic	
L''/L	C'/C	L''/L	C'/C
0.184	–0.34	not measurable	–0.62

Interaction of spin labelled myosin with ADP

The binding of ATP or ADP to IASL skeletal myosin resulted in a significant decrease in the proportion of the strongly immobilized label. The experimental data could be fitted by curves predicted for either one or two independent ADP binding sites and association constant in the range of 10–12.4 $\times 10^2$ M^{-1}[22]. Since the reactive sulfhydryl sites are near to the nucleotide binding pocket in the crystal structure of myosin [23], changes are expected in the environment of the probe molecules. Our results on cardiac myosins showed that the conformational changes obtained after MgADP binding depended strongly on the probe molecules [24].

Experiments performed on glycerol-extracted muscle fibres showed that the addition of MgADP to maleimide spin labelled fibres in rigor buffer did not result in significant axial rotation of the cross-bridges [25], but in the case of an

isothiocyanate-based spin label, the nucleotide binding produced remarkable change of the distribution of the attached labels with respect to the longer axis of the filaments, but no measurable change of the rate of rotational motion was observed in the ST EPR time domain after addition of MgADP [19]. We came to the conclusion that MgADP produced an intrinsic change in the multisubunit structure of the myosin head region, but this did not lead to the changes of the global rotational properties of the myosin heads. This finding corroborates with the X-ray diffraction studies that the pattern was identical with that of rigor when ADP was bound to myosin [26]. However, after addition of V_i a marked decrease in the L''/L spectral parameter was detected ($L''/L=0.70\pm0.1$) evidencing the alteration of the cross-bridge state. Unfortunately, the absence of two spectral components in homogenized fibres did not allow the separation of spectra, we suggest that the decrease of the rotational correlation time can be due to the component arising from the disordered fraction detected in the presence of MgADP $+V_i$. Spectrum subtraction showed a marked decrease in the rotational mobility of the attached labels; the L''/L spectral parameter was ($L''/L=0.58\pm0.1$) which corresponds to an apparent rotational correlation time of 20 ns.

DSC measurements

According to the recent model of myosin, it is believed that it comprises structural units that fold up independently into a stable domain structure

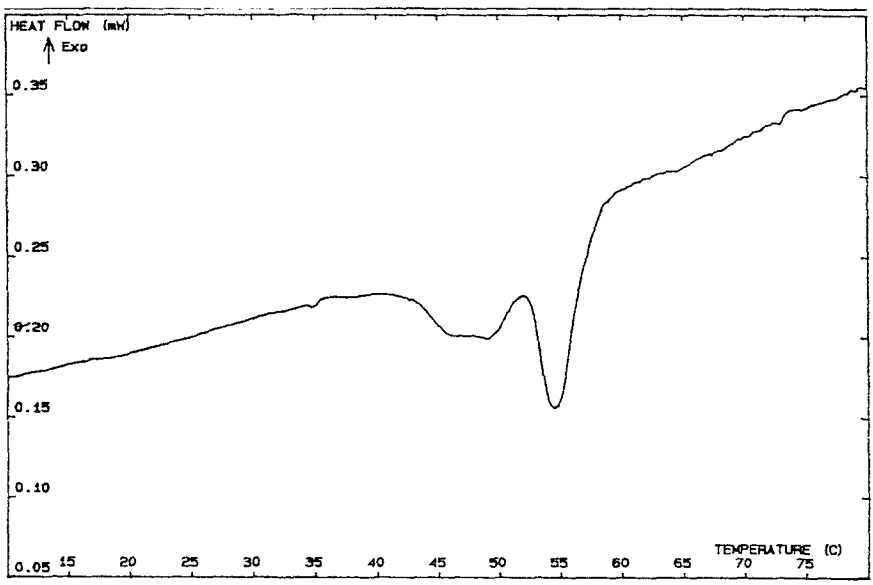

Fig. 4 DSC profile of intact cardiac myosin in the absence of nucleotides. The heating current (I) in mW units is plotted against temperature (endothermic deflection is directed downwards)

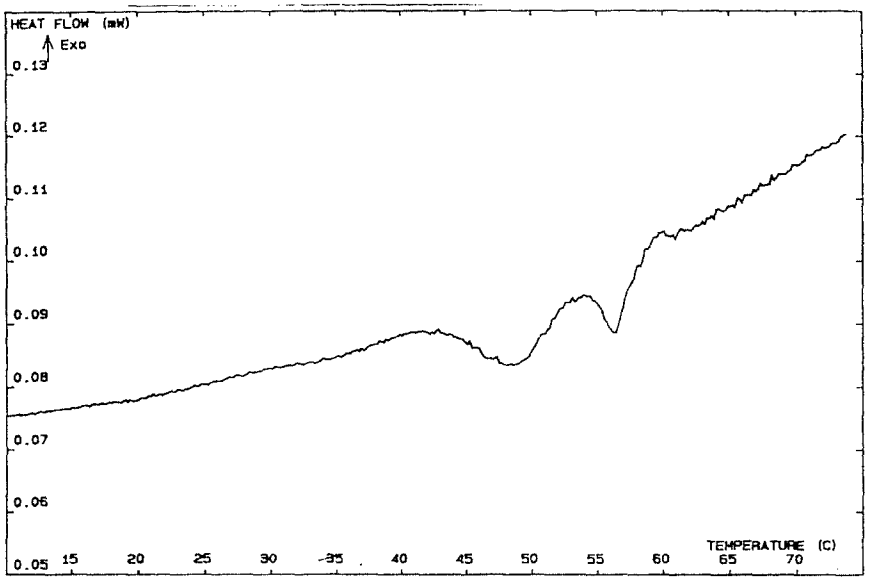

Fig. 5 Thermal transition of bovine cardiac myosin in the presence of 5 *mM* MgADP (endothermic deflection is directed downwards)

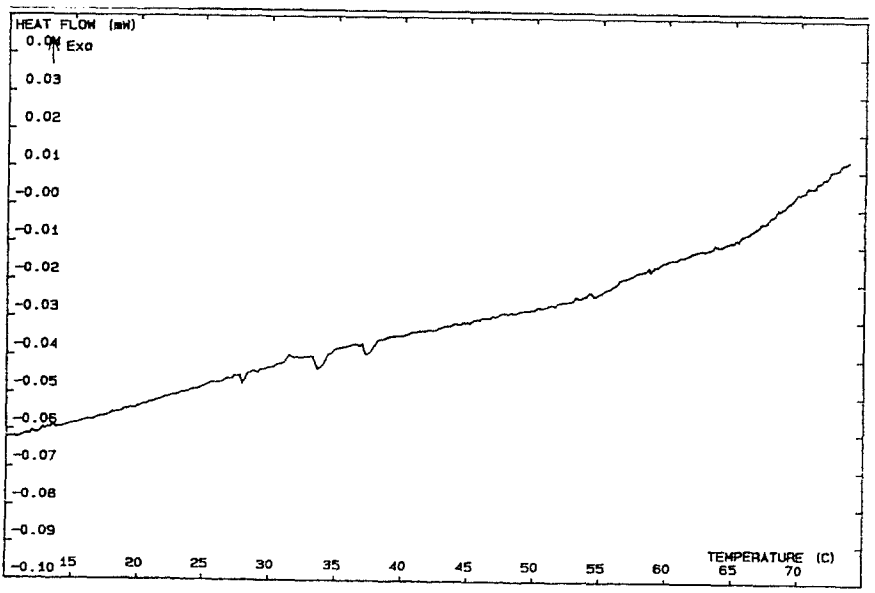

Fig. 6 Melting behaviour of bovine cardiac myosin in the presence of 5 *mM* MgADP and orthovanadate (V_i). Note the increase of separation between the two main transitions

[5, 23, 27]. The measurements performed on skeletal myosin showed that the highest transition temperature could be assigned to the unfolding of the coiled-

coil α-helix rod portion of the protein moiety [28]. More recent experiments provided evidence that the melting profile of myosin rod could be resolved into five or six quasi-two state transitions. Studying the melting profile of cardiac myosin we could identify five endothermic peaks at peak maximum of 17.5, 41.5, 45, 48 and 54.5°C, the transition enthalpies were 150 kcal mol^{-1}, 163 kcal mol^{-1}, 277 kcal mol^{-1}, 301 kcal mol^{-1} and 747 kcal mol^{-1} [24].

In order to simulate the possible states of the contraction cycle we have focused our attention to the rigor, strongly and weakly binding states which are hypothetized intermediate states of the ATPase cycle (Figs 4, 5 and 6). Bovine cardiac myosin in rigor buffer during a thermal denaturation showed an endothermic transition with two main meltings at T_{m1} =49.1°C and 54.5°C. The total enthalpy change normalized for the protein mass was −16.49±1.38 J g^{-1}. Addition of 5 mM MgADP to the system (strong-binding state of myosin to actin) the two transitions were clearly separated by lower T_{m1} =48.3°C and higher T_{m2} =56.2°C. The total enthalpy change decreased to −13.75±1.2 J g^{-1}. In the presence of 5 *mM* MgADP plus 5 *mM* orthovanadate (weak-binding state of myosin to actin), we have observed a remarkable decrease in the total enthalpy of transitions (ΔH=−9.01 ±0.98 J g^{-1}) with an increased melting points T_{m1} =55.2°C and T_{m2} =65.2°C.

Addition of MgADP caused a decrease of the total enthalpy which could be explained by an internal loosening in the domain structure of the myosin head region following the nucleotide binding. This is supported by EPR measurements as well evidencing the redistribution of spin labels after MgADP binding. According to both EPR and DSC measurements, the greater internal flexibility is enhanced by addition of orthovanadate which appeared as an increase of the rotational mobility and a drastic total enthalpy change of the thermal transitions. It can be assumed that the binding of orthovanadate to the motor domain is able to reduce or cancel the intersite communication among the different structural domains. We can only speculate that the flexibility changes observed here are of importance to the transmission of signals from motor domain to actin allowing the switching or rotation of the myosin motor on actin. The mechanism by which the conformational changes are directly induced in the large myosin head remains to be established.

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