

Comparative study of myosins in solutions and supramolecular complexes. Effect of nucleotides.

D. Lőrinczy^{a,*}, J. Belagyi^{1,b}

^a Biophysical Institute, University Medical School, 12 Szigeti Str., H-7643 Pécs, Hungary

^b Central Research Laboratory, University Medical School, 12 Szigeti Str., H-7643 Pécs, Hungary

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Abstract

Electron paramagnetic resonance (EPR) and differential scanning calorimetric (DSC) measurements were performed to study the motional dynamics and structural stability of myosins prepared from skeletal muscle of rabbit and cardiac myosin of bovine heart in different intermediate states of the ATP pathway in solution and supramolecular complexes. ADP, the nonhydrolyzable ATP analogue 5'-adenylyl imidodiphosphate (AMP.PNP) and ADP plus orthovanadate (V_i) were used in different muscle model systems, as chemically skinned muscle fibres, myofibrils prepared from chemically skinned fibres and myosin solutions to simulate the pre-power (AMP.PNP-state, ADP. V_i -state) and post-power (rigor state, ADP-state) stroke conformations of myosin heads.

Both DSC and EPR measurements support the view that the myosin heads following attachment to actin undergo a sequence of conformational states that differ both dynamically and energetically from each other. The differences in the melting temperatures and rotational mobility at the different intermediates clearly indicate significant alterations in the internal microstructure of myosin head region induced by binding of nucleotides.

Keywords: DSC; EPR; Myosin conformations; Myosin unfolding

1. Introduction

Current models of muscle contraction assume that force is generated by myosin–actin interaction fuelled by ATP during the ATPase cycle. The head of myosin molecule (subfragment one, S 1) is rigidly attached to actin and forms a supramolecular complex in the absence of nucleotides, in rigor. In the presence of MgATP, the chemical energy liberated from ATP hydrolysis produces conformational changes in myo-

sin. The structural change might induce an internal rotation of myosin head while bound to actin, and it causes the muscle to shorten [1–3]. This mechanism of the energy transduction is a crucial question of the muscle contraction.

The internal motion of myosin heads generated by the chemical energy of the hydrolysis of ATP requires the cyclic interaction of myosin with ATP and actin, and for actomyosin ATPase the presently accepted mechanism in model systems suggests at least six intermediates [4]. The dynamical state and orientation of heads with respect to the fibre axis can be determined by using spectroscopic techniques [5,6]. The use of different labels attaching to the same site can

*Corresponding author. Tel.: 003672-314017; fax: +(36 72) 314 017; e-mail: microcal@apacs.pote.hu.

¹Fax: +(36 72) 315 864; e-mail: belagyi@main.pote.hu.

give different information about the internal flexibility of the proteins. In earlier papers it was reported that an isothiocyanate-based spin label reflected domain orientations in the presence of MgADP, whereas the maleimide-based spin label exhibited only the orientation of the entire head [7,8]. The combination of spectroscopic and thermodynamic methods (e.g. DSC), gives good chance to analyse not only the local structural and dynamical changes of well-defined domains and/or subunits in supramolecular systems, but the global conformation and stability of myosin driving the force generation fuelled by ATP [9].

The aim of the present paper was to investigate the myosin head conformations, the orientational distribution and structural stability in the presence of nucleotides as ADP, the nonhydrolyzable ATP analogue 5'-adenylyl imidodiphosphate AMP.PNP and nucleotides plus orthovanadate in solution and supramolecular complexes. In order to obtain data about internal motions of myosin heads and interaction of functional domains in myosin correlating actomyosin ATPase, the spin-labelling technique and the differential scanning calorimetry (DSC) were used. The results show that the isothiocyanate probe molecules (TCSL) which exhibited in rigor narrow orientational distribution with respect to the longer axis of the fibres, reflected large changes in the fractions of the populations in the presence of MgAMP.PNP and MgADP plus orthovanadate, whereas small change was measured for the maleimide spin label (MSL) in the same state indicating that MSL was not able to sense domain rotation or only local conformational changes were induced by nucleotides in the myosin head, but no large-scale head rotation. The results also showed that in the presence of MgADP + V_i an increase of the probe mobility was detected in the ST EPR time domain.

2. Experimental

Preparation of myosin. Bovine heart myosin was prepared by the methods described by Shiverick [10] and Bouvagnet and coworkers [11]. After washing the tissue, myosin was extracted in buffer consisting of 0.6 M KCl, 20 mM imidazole, 1 mM dithiothreitol (DTT), 1 mM EDTA, pH 7.0. After centrifugation for 4 h at 0°C with $100\,000 \times g$, the crude myosin

was purified by repeated precipitation-dissolution cycle.

Preparation of muscle fibres. Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas muscle. Small stripes of muscle fibres were stored after osmotic shocks in 50% v/v glycerol and rigor solution (100 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 10 mM histidine.HCl, pH 7.0) at -18°C up to one month. Fibre bundles of glycerinated muscle were washed for 60 min in rigor buffer to remove glycerol, and then transferred to fresh buffer. This state models the rigor state of the muscle (AM, where M denotes myosin and A stands for actin). MgADP of 5 mM concentration was added to the rigor solution to simulate the strongly binding state of myosin which may correspond to the AM.ADP state. The other analogue of intermediates in the ATPase pathway is formed by AMP.PNP (16 mM) and ADP (5 mM) plus orthovanadate (2 mM, abbreviated V_i) which together bind stoichiometrically at the active site of myosin to form a stable complex, $AM^+.ADP.V_i$ (M^+ represents a myosin isomer). This complex is believed to be analogue of the steady-state intermediate $AM^{**}.ADP.P_i$ [12].

Spin-labelling. The isolated protein was labelled with 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl (4-maleimido-TEMPO, MSL). Myosin suspended in 0.5 M KCl, 50 mM TRIS, 1 mM EDTA at pH 8.0 was reacted with 2 to 2.5 mol of MSL per 1 mol of myosin for 60 to 90 min. The reaction was terminated by precipitation of myosin with ice cold water, and thereafter the protein was collected by centrifugation and dissolved in 0.5 M KCl, 25 mM HEPES, 1 mM EDTA at pH 7.0. In some cases myosin was treated with 5 mM K₃(Fe(CN)₆) to reduce the signal intensity arising from weakly immobilized labels [13]. The protein was clarified by centrifugation at $50\,000 \times g$ for 1 h and used at a final concentration of 10 to 20 μ M.

Spin-labelling of the fibres was performed in rigor solution (100 mM KCl, 5 mM MgCl₂, 1 mM EGTA, pH 7.0) plus 2 mM pyrophosphate or 2 mM ADP with about 1 mol of MSL to 1 mol myosin for 60 min or with about 2 mol of 4-isothiocyanato-2,2,6,6-tetramethylpiperidinoxyl (TCSL) to 1 mol myosin for 3 h at 0°C. Myosin has two reactive cysteine residues (Cys-707 and Cys-697) that can be labelled specifically with MSL or TCSL. The spin labels were

obtained from SIGMA (Grunwalder, Germany). After spin-labelling the fibre bundles were washed in a great amount of rigor buffer 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 as reported earlier [8].

The number of spin labels bound to myosin was determined from the EPR spectra of muscle fibres by comparing the double integrals of the spectra with known concentration of MSL in aqueous solution in the same sample cell.

EPR measurements. The EPR measurements were taken with ESP 300 E (Bruker, Germany) X-band spectrometers. For conventional EPR technique 100 kHz field modulation (0.1 to 0.25 mT amplitude) and 2 to 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 (Germany). The microwave magnetic field intensity was determined with peroxyamine disulphate 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 microcomputer system interfaced to the spectrometer. For evaluation of the spectra the standard WIN EPR software was used. The double integrals of the spectra were normalized to unity for spectrum manipulation.

DSC measurements. The thermal unfolding of muscle proteins in different states induced by AMP.PNP and ADP plus V_i were monitored by a SETARAM Micro DSC-II calorimeter. All experiments were done between 5 and 80°C. The heating rate was 0.3°C/min. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μ l sample volume in average. Rigor buffer was used as reference sample. The sample and reference vessels were equilibrated with a precision of ± 1 mg. There was no need to do any correction from the point of view of heat capacity between the sample and reference vessels.

3. 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. The maximum tension of the labelled fibres was in an average about 10 to 15% smaller than the maximum tension produced by the untreated fibres. This agrees with the earlier observations [15].

Interaction of spin-labelled myosin with ADP. The binding of ADP to skeletal myosin resulted in a significant decrease in the proportion of the strongly immobilized label [16]. Our results on cardiac myosins showed that the increase of the mobility induced by MgADP varied with the concentration of nucleotide and it attained to a saturating level. Calculations gave evidence that cardiac myosins bound two moles of MgADP per 1 mol of myosin [17].

It had previously been shown on glycerol-extracted muscle fibres that the addition of MgADP to maleimide spin labelled fibres in rigor buffer did not result in significant axial rotation of the cross-bridges [7], but in the case of an isothiocyanate-based spin label, the nucleotide binding produced a 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 [8]. It can be concluded that MgADP produced an intrinsic change in the multi-subunit structure of the myosin head region, but this did not lead to the changes of the global rotational properties of the myosin heads.

Effect of AMP.PNP and ADP plus orthovanadate on rotational mobility. In an effort to understand the intermediates of the myosin ATPase in muscle fibres, we have begun with the binding of the nonhydrolyzable ATP analogue AMP.PNP to cross-bridges in glycerinated muscle fibres. The muscle fibres were stored in solution containing 100 mM KCl, 5 mM $MgCl_2$, 16 mM AMP.PNP, 1 mM EGTA, in 10 mM histidine. HCl buffer, pH 7.0, for 15 min at 0°C, and then spectra were taken at room temperature. The AM.ADP. V_i -state was induced by addition of 5 mM ADP to the rigor solution in the presence of 2.0 mM NaV_3O_5 and 100 μ M P^1 , P^5 -di (adenosine-5') penta-phosphate. In the presence of AMP.PNP and ADP plus orthovanadate, the conventional EPR spectra

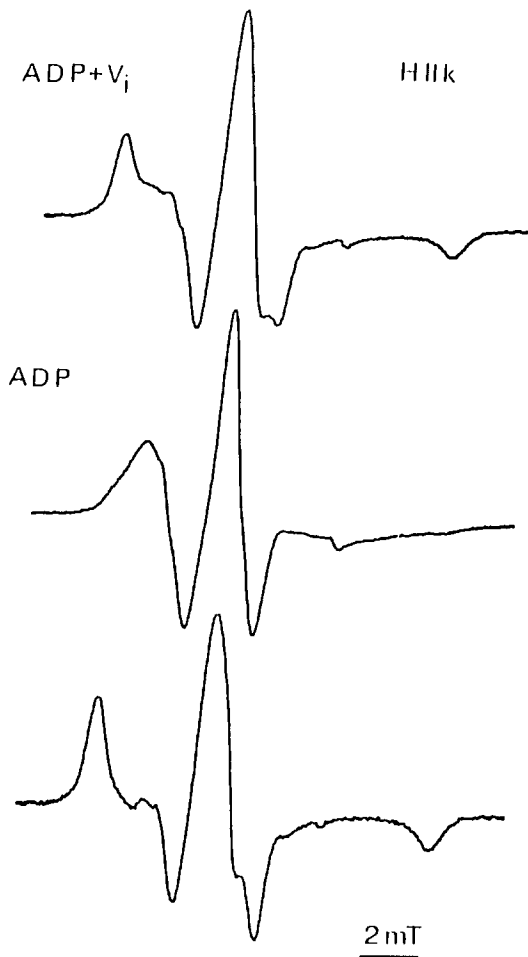


Fig. 1. Conventional EPR spectra of glycerol-extracted muscle fibres labelled with isothiocyanate spin label. The first derivative of the energy absorption is recorded as a function of the magnetic field. The magnetic field increases from left to right, and the sweep width is 10 mT. The double integrals of the spectra were normalized to unity. Fibre axis was oriented parallel to the laboratory magnetic field. The fibres were incubated in rigor buffer plus 4 mM MgADP, 100 μ M A_p5A , and plus 2 mM Na_3VO_5 for 15 min at 0°C before spectra were taken. Bottom: difference spectrum.

showed large changes in the ordering of the probe molecules in fibres, and a new distribution appeared. (ADP + orthovanadate) and AMP.PNP increased the orientational disorder of myosin heads, and a random population of spin labels was superimposed on the ADP-like spectrum giving evidence of conformational and motional changes in the internal structure of the myosin heads (Fig. 1 and Fig. 2). The fractions of

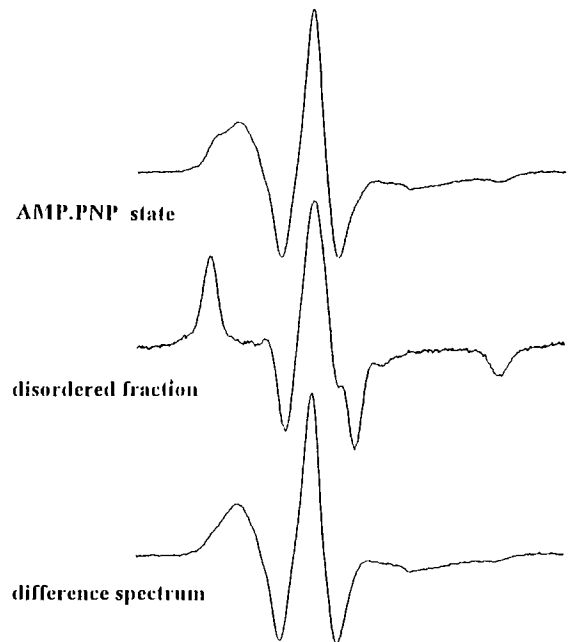


Fig. 2. Conventional EPR spectra of glycerinated muscle fibres labelled with TCSL. Fibre axis was oriented parallel to the laboratory magnetic field. The fibres were incubated in rigor buffer plus 16 mM AMP.PNP for 15 min at 0°C before EPR measurement. Upper spectrum: after addition of MgAMP.PNP, middle spectrum: ($AM^+.ADP.V_i$ - $AM.ADP$) spectrum (difference spectrum in Fig. 1); bottom: residual spectrum.

ordered populations depended on the nucleotide. Saturation transfer EPR measurements reported increased rotational mobility of spin labels in the presence of ADP plus orthovanadate.

The comparison of EPR spectra obtained on myofibrils in AMP.PNP- and $ADP.V_i$ -states showed significant decrease of $2A_{ZZ}$ (the distance between the outermost hyperfine extrema in mT) which is an evidence of the increased rotational mobility. The hyperfine splitting constant ($2A_{ZZ}$) of the difference spectrum is 6.740 ± 0.02 mT in $MgADP.V_i$ -state. This value is significantly smaller than the value obtained on homogenized rigor fibres ($2A_{ZZ} = 6.780$ mT). The calculation results in a rotational correlation time of 0.64 μ s. This suggests that a fraction of the myosin heads in $AM^+.ADP.V_i$ -state is detached or the binding of ADP and V_i produces segmental mobility in the environment of the labelled sites. Addition of AMP.PNP is associated with disordering of heads which is probably due to detachment of heads. The

hyperfine splitting of the resonance in AMP.PNP-state on homogenized samples was 6.667 ± 0.03 mT. The apparent rotational correlation times ($\tau_r = 0.14$ μ s) correspond to rapidly rotating detached myosin heads or to the motion of large segments of the protein structure.

Orientational order of spin labels in presence of MgADP and V_i . In $AM^+.ADP.V_i$ -state of muscle fibres, large changes were detected in the conventional EPR spectra of TCSL-fibres (Fig. 1). It indicates that the binding of substrates to the catalytic domain of the myosin head influenced significantly the orientation of the segment that held the probe molecules.

It was reported earlier that in ADP-state little changes were detected in the azimuthal and torsional angles of the cross-bridges using multiple probes attached to myosin [8]. This statement is in accordance with the low-angle x-ray diffraction studies that the angle of attachment for the myosin head in the ternary actin-myosin-ADP complex was the same as in rigor muscle [18]. This supports the view that the TCSL reports slow internal changes without significant head reorientation. The addition of orthovanadate ions with ADP led to a drop in tension, therefore, it was proposed that the myosin heads with MgADP and V_i are in weakly binding state. The lineshape of the EPR spectrum (upper spectrum in Fig. 1) suggests that the spectrum is in fact a superposition of two spectra; one component may arise from myosin heads which are in strongly binding state, the other component might correspond to a state with little restricted rotational motion (isotropic distribution, bottom spectrum in Fig. 1). The probability for the labels being in the rotational state 1 is about 0.34, and about 0.66 in state 2. These values were calculated from the double integrals of the composite spectra. In $AM^+.ADP.V_i$ -state, the orientational order of labels was significantly reduced, this strongly suggests that the rotational state 2 reflected either the disorder of a fraction of heads or the internal motion of a larger segment in the cross-bridge.

Orientational order of spin labels in presence of MgAMP.PNP. The EPR spectrum at H||k orientation exhibited significant changes in comparison to rigor spectra. The $AM.AMP.PNP$ -state can be interpreted as a complex state: 55% of the total absorption arises from labels which are in strong-binding state, and the state can be characterized with high degree of order,

whereas 45% spectral component arises from labels which are randomly oriented. The state of labels with high degree of order seems to be the same as in $AM.ADP$ state. Fig. 2 shows the result of the digital subtraction; the residual spectrum (bottom spectrum) was obtained by subtracting the difference spectrum ($MgAMP.PNP$ state-random population, bottom spectrum of Fig. 1) from the spectrum detected in the presence of MgADP (upper spectrum of Fig. 1.). As regards the distinction of the populations, the result agrees quite well with the observations of Fajer [7], but recent measurements gave EPR evidence as well that the cross-bridges which exhibited high degree of static order, have a state differing from rigor state. Using MSL-fibres and AMP.PNP (Fig. 3), the digital subtraction resulted about the same fractions (44% oriented labels, upper spectrum of Fig. 3 and 56% randomly oriented labels, Fig. 3), but the EPR spectrum of oriented labels resembled to a spectrum characteristic to rigor state.

Fajer et al. [19] found that AMP.PNP (in the presence of Mg) induced dissociation of heads from actin, resulting single-headed cross-bridges, but no change in the orientation of heads remained attached to actin was detected. Our results on AMP.PNP fibres suggests that about half of the heads represents the disordered population with reduced rate of rotational motion as reported by Fajer and coworkers, but our results gave EPR evidence as well that the cross-bridges which exhibited high degree of static order are in strongly binding MgADP state, the head being attached to actin differs from that of rigor. The binding of AMP.PNP might induce change in the orientation of the segment that holds the label by rotation resulting in another conformationally stable state.

DSC measurements. To gain further insight into the structural properties of myosins, DSC measurements were performed on intact and LC-2 deficient cardiac myosins in the temperature range of 5 to 60°C. It is known that myosin is a multi-subunit protein consisting of domains, therefore a rather complex thermogram comprising at least three endothermic transitions are expected, corresponding to the thermal transition of the myosin rod with α -helical structure, and to those of the structural domains in the head region of myosin reported recently by Rayment and coworkers [20]. The measurements performed on skeletal myosin showed that the highest transition temperature could

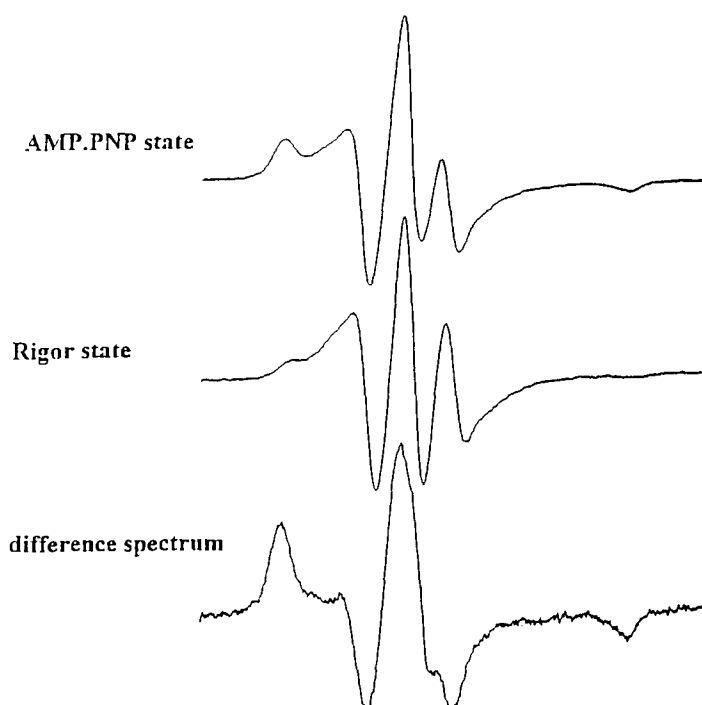


Fig. 3. Conventional EPR spectra of glycerinated muscle fibres labelled with MSL. Fibre axis was oriented parallel to the laboratory magnetic field. The fibres were incubated in rigor buffer plus 16 mM AMP.PNP for 15 min at 0°C before EPR measurement. Upper spectrum: after addition of MgAMP.PNP; middle spectrum: spectrum in rigor; bottom: difference spectrum.

be assigned to the unfolding of the coiled-coil α -helix rod portion of the protein moiety [17].

The complex heat capacity profile of intact myosin showed three major transitions with $T_m = 17.5$, 45 and 54.5°C, as transition temperatures. Removal of the LC-2 light chain was accompanied with the disappearance of the 17.5°C transition. The total enthalpy of the transitions was 7265 kJ/mol, similar results were obtained on skeletal myosin [17]. 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 627, 681, 1158, 1258 and 3172 kJ/mol. An accurate analysis by deconvolution allowed the identification of six transitions with total enthalpy of 7875 kJ/mol calculated from the part areas of the melting profile. The contribution of the LC-2 light chain was 631 kJ/mol.

For the interpretation of the lowest endothermic transition we have two possibilities: (i) melting of the LC-2 light chain of the myosin; (ii) more likely it

could be related to the structural property of the 20 kDa segment. This region is stabilized by specific ionic interactions between the LC-2 light chain and the 20 kDa segment of the myosin, the stabilization energy might contribute to the transition enthalpy. This assumption correlates with the EPR spectral changes at 17°C. The removal of the LC-2 light chain increases the instability of the 20 kDa domain which is accompanied with an increase of the flexibility and the enhancement of the rate of internal motion. We can only speculate that the flexibility changes observed here are of importance to the transmission of signals from regulatory domain to the catalytic domain in thick filament-regulated myosins allowing the switching of the myosin motor. The mechanism by which the conformational changes are directly induced in the large myosin head remains to be established.

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 hypothesized intermediate states of the ATPase cycle.

Bovine cardiac myosin in rigor buffer during a thermal denaturation showed an endothermic transition with two main meltings at $T_{m1} = 49.1^\circ\text{C}$ and 54.5°C , respectively. The total change of enthalpy normalized to the protein mass was $-16.49 \pm 1.38 \text{ J/g}$. Addition of 5 mM MgADP to the protein (strong-binding state of myosin to actin), the two transitions were clearly separated ($T_{m1} = 48.3^\circ\text{C}$ and $T_{m2} = 56.2^\circ\text{C}$). The total enthalpy change decreased, $\Delta H = -13.75 \pm 1.2 \text{ J/g}$.

Decrease of the total enthalpy by MgADP might be explained by internal loosening in the domain structure of the myosin head region following the nucleotide binding. This suggestion is supported by EPR measurements evidencing the redistribution of spin labels after MgADP addition [8]. 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 ($\Delta H = -9.01 \pm 0.98 \text{ J/g}$) with an increased melting

points $T_{m1} = 55.2^\circ\text{C}$ and $T_{m2} = 65.2^\circ\text{C}$. According to both EPR and DSC measurements, the segmental 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 catalytic domain is able to reduce or cancel the intersite communication among the different structural domains [21].

Denaturation behaviour of homogenized myofibrils in rigor can be seen in Fig. 4. From the DSC scan at least three main transitions can be derived. The low temperature transition ($T_m = 19^\circ\text{C}$), which was firstly detected and described by us [17] might be attributed to the interaction of the LC-2 light chain with the 20 kDa domain of the myosin head. The transition characterizes the interaction stabilizing the regulatory domain. The unfolding around $T_m = 52^\circ\text{C}$ refers to myosin head, whereas transition $T_m = 58^\circ\text{C}$ is very

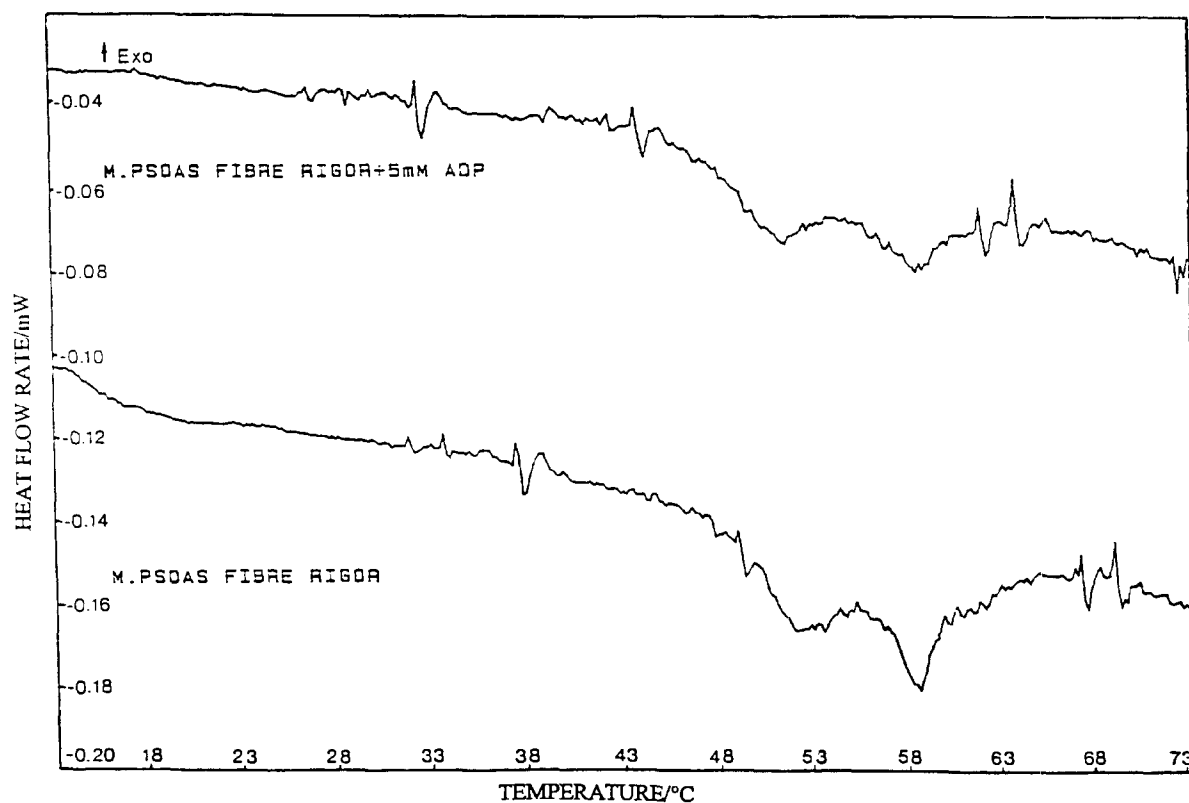


Fig. 4. DSC scans of *m. psoas* fibres in rigor (lower curve) and after 5 mM ADP treatment (upper curve). Endothermic deflections are directed downwards.

likely the superposition of two transitions, one of them is due to the conformational change of actin, the second one describes the unfolding of the rod part of myosin. This latter peak has a relatively narrow width at half height of T_m which could be the sign of a more rigid conformation. The rod has a negligible role in the force-generation, very likely it is involved only in the stabilization of myosin, therefore, its contribution to the transition enthalpy should be practically the same at different stages of the contraction. After addition of ADP, the low temperature transition was shifted towards to the higher values, and there is a definite decrease in melting profile for actin which could be an energetic consequence of the internal rearrangement of myosin structure in strongly binding state. Similarly, in the presence of 5 mM ADP and 5 mM V_i , a further shift of the low temperature transition was observed, but the separation between the peaks was less pronounced. The total enthalpy change of the strongly and weakly binding states did not differ significantly from each other, but in rigor the enthalpy change was about 20% smaller than in other cases.

Our data strongly support the idea that the strongly binding state and rigor state differ both dynamically and energetically from each other. The differences in the melting temperatures and rotational mobility at the different intermediates clearly indicate significant alterations in the internal microstructure of myosin head region induced by binding of nucleotides.

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