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EFFECT OF NUCLEOTIDES ON THERMAL TRANSITIONS OF MYOSIN

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Abstract

The internal dynamics and the thermal stability of myosin in rabbit psoas muscle fibres in different intermediate states of the ATP hydrolysis cycle were studied by differential scanning calorimetry (DSC) and electron paramagnetic resonance (EPR) spectroscopy. Three overlapping endotherms were detected in rigor, in strongly binding and weakly binding state of myosin to actin. The transition at 58.4°C can be assigned to the nucleotide-binding domain. The transition at highest temperature represents the unfolding of the actin and the contributions arising from the actin–myosin interaction. The transition of 54°C reflects the interaction between the subunits of myosin. Nucleotide binding induced shifts of the melting temperatures and produced variations in the calorimetric enthalpy changes. The changes of the EPR parameters indicated local rearrangements of the internal structure in myosin heads.

Keywords: conformation of myosin, DSC, EPR, spin labelling

Introduction

As most of the manifestations of biological motility, the muscle contraction is also based on the cyclic interaction of myosin heads with actin. The energy source is the myosin-catalysed ATP hydrolysis, its rate is enhanced by the presence of actin [1]. In the presence of CaATP the energy released from hydrolysis produces conformational changes in myosin [2–4] and/or in actin [5] which can be manifested as an internal motion of myosin head while bound to actin. The force generation involves structural rearrangements of myosin, therefore internal motions and flexibility changes of the main proteins of muscle could be an integral part of the contractile process. The shortening of striated muscle is performed by cyclic interaction of myosin (M) with ATP and actin (A), and at least six intermediates are proposed for actomyosin ATPase in solution [6, 7]. The AM·ADP and AM·ADP·P_i complexes play important roles in the powerstroke of cross bridge cycle, therefore the main

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goal of the recent efforts is the investigation of energetics of the actomyosin. ATPase in the different intermediates of the contractile cycle. Recently, elegant studies were done on myosin subfragment complexes [8, 9].

We have extended the experiments to study the fibre system prepared from psoas muscle of rabbit in rigor, strongly binding and weakly binding states of myosin to actin where the inorganic phosphate (P_i) was substituted by the phosphate analogue orthovanadate [10]. The better understanding of the interrelation between the chemical events observed in protein solutions and the mechanical activities on cellular level requires experiments on supramolecular complexes where stabilizing forces may modulate the hydrolysis process.

Materials and methods

Fibre preparation

Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas 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) at −18°C up to one month. In DSC experiments the fibre bundles 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. 5 mM MgADP was added to the rigor solution to simulate the strong-binding state of myosin to actin which may correspond to the AM·ADP state. The other analogue of intermediates in the ATPase pathway is formed by ADP (5 mM) and orthovanadate (5 mM), which together bind stoichiometrically at the active site of myosin to form a stable complex $AM^+ADP{\cdot}V_i$. This complex is believed to be analogue of the steady-state intermediate $AM^{**}ADP{\cdot}P_i$ (M^+ and M^{**} denote different conformations of myosin) [10].

Calorimetric measurements

The thermal unfolding of myosin in different states was monitored by a Setaram Micro DSC-II calorimeter (Setaram, France). All experiments were done between 0 and 100°C with a scanning rate of 0.3°C min^{-1}. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 µl sample volume in average. Rigor buffer was used as reference. The sample and reference vessels were equilibrated with a precision of ±0.5 mg. There was no need to do any correction from the point of view of heat capacity between the sample and reference vessels. The samples were irreversibly denaturated during each cycle.

The repeated scan of a denaturated sample was used as baseline reference which was subtracted from the original DSC scan. The deconvolution was performed with Jandel PeakFit 4.0 program. Gaussian peak functions were used to approximate the unfolding of the structural units of myosin. Calorimetric enthalpy change was calculated from the area under the heat absorption curves using two points setting Setaram peak integration.

Spin-labelling of myosin

The muscle fibres were labelled with 4 maleimido 2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO, MSL) for 90 min with 2 moles of MSL over ice. After reaction the samples were washed overnight at 4°C *vs.* great amount of buffer solution. In some cases the fibres were treated with 25 mM $K_3Fe(CN)_6$ to reduce the signal itensity arising from weakly immobilized labels [11].

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). Signals due to EPR absorption were detected by the Bruker microcomputer system interfaced to the EPR spectrometer. The standard WIN EPR program (Bruker, Germany) and programs written in our laboratory were used for evaluation of EPR spectra. The double integrals of the spectra were normalized to unity for spectrum manipulation.

Results and discussion

The unfolding of proteins in muscle fibres by thermal excitation is a complex process and depends on the state of actomyosin complex. The experimental data sug-

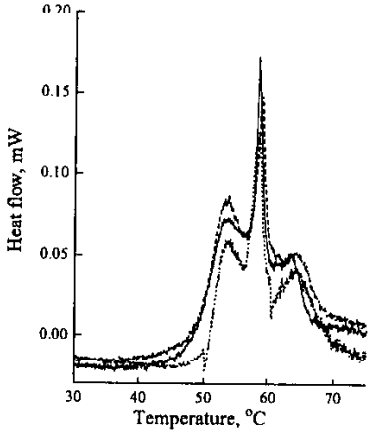

Fig. 1 DSC pattern of myosin in muscle fibres in different intermediate states. The heat flow in mW units is plotted against temperature (endothermic deflection is directed upwards). Symbols: solid line: rigor state; dashed line: ADP-state; dotted line: ADP·V_i - state

gest at least four transitions in the temperature range examined. The minor transition at T_{m1}=18°C is not shown in the Figures; it might be attributed to the interaction of LC-2 light chain with the long α-helical part of the myosin head [12]. The starting point of the evaluation was that the main transition between 50 and 60°C was the superposition of endotherms that are believed to correlate mainly with T_ms of the larger domains of myosin and a smaller contribution from thin filaments. On isolated myosin three endotherms were reported, therefore in the deconvolution procedure three transitions with Gaussian peak function were assumed.

Table 1 DSC parameters of melting of contractile proteins in fibre system

Muscle state	Transition temperature/°C		
	T_{m1}	T_{m2}	T_{m3}
Rigor	54.05	58.36	62.29
ADP	53.49	58.61	63.64
ADP·V_i	53.98	58.08	63.78
	Excess enthalpy/J g^{-1}		
	ΔH_1	ΔH_2	ΔH_3
Rigor	54.08	18.06	27.85
ADP	44.51	29.96	25.51
ADP·V_i	40.94	25.14	33.91

Glycerinated muscle fibres isolated from psoas muscle of rabbit were measured in different intermediate states of the ATP hydrolysis cycle. The states were rigor state, strong-binding (ADP) state and weak-binding (ADP·V_i) state

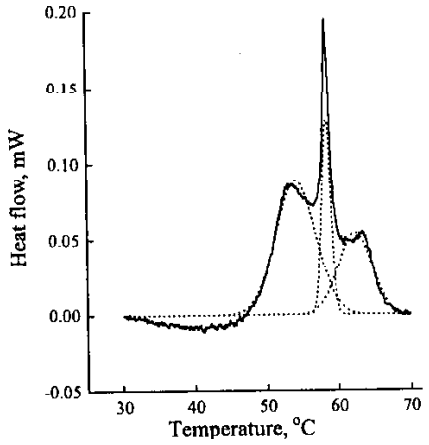

Fig. 2 Deconvolution of the main transition of myosin in fibre system in rigor state (no nucleotide is present). The deconvolution procedure was performed using Jandel PeakFit software. During the procedure Gaussian peak functions were assumed

Denaturation behaviour of the fibre bundles in different intermediate states can be seen in Fig. 1 and Table 1. From the DSC scans three transitions could be decomposed with T_{m1}=54.0°C, T_{m2}=58.4°C and T_{m3}=62.3°C melting temperatures (Fig. 2). We suggest the unfolding around 54°C describes the interdomain interaction of myosin [12, 13], while the transition around 58.4°C on the experimental scan may be related to the stabilizing energy between catalytic domain and nucleotide. The last transition (T_{m3}=62.3°C) may describe the unfolding of actin and the interaction between actin and the actin-binding domain of myosin. Little information can be obtained from the experiments about the melting of the myosin rod [9, 14, 15].

In the presence of MgADP (Fig. 1) there is a broadening in the peak of transition 54°C together with a significant increase of the enthalpy at 58.4°C which could be the energetic consequence of the internal rearrangement of myosin structure in strongly binding state and increased affinity of myosin to ADP [3, 16, 17]. In the presence of 5 mM MgADP plus 5 mM V_i the separation of the high-temperature transition is also pronounced (Fig. 1). The enthalpy of the thermal unfolding depends on the nucleotides, the conversion from a strongly attached state of myosin for actin (rigor state and ADP-state) to a weakly binding state (ADP·V_i-state) is accompanied with increase of the transition temperature and enthalpy which are due to the change of the increased affinity of nucleotide binding to myosin.

It is known that the thermal effects are related to structural and mechanical events. Recent data suggest that MgADP induces an internal change in the multisubunit structure of S-1 which are served by spectroscopic [4, 16], electrical birefringence [18] and DSC techniques [8, 9, 19]. The EPR measurements on MSL-fibres in rigor and in ADP-state showed that the labels were strongly immobilized on the millisecond time domain as revealed by the saturation transfer EPR data (Fig. 3).

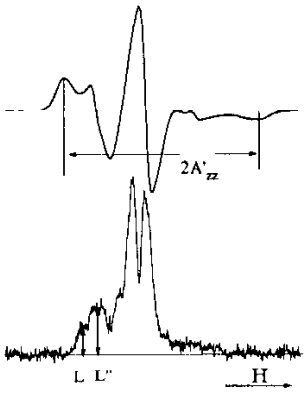

Fig. 3 Conventional (upper) and ST EPR spectrum (bottom) of myosin labelled with MSL in fibre system at room temperature. The spectral parameters (the hyperfine splitting constant $2A'_{zz}$ and the diagnostic peaks of the ST EPR spectrum L, L'') used to characterize the EPR spectra are also shown. The scan width was 10 mT (conventional spectrum) and 20 mT (ST EPR spectrum), respectively

The spectral parameter L''/L was greater than 1.2 evidencing that the rotational correlation time of the label is larger than 1 ms. In contrast, the addition of MgADP and orthovanadate to the rigor buffer induced large change in the mobility of the attached labels (Fig. 4). The decrease of the hyperfine splitting is the sign of the increase of the rotational mobility in the environment of the labelled site. The EPR data support the view that local conformational changes are also induced by nucleotides and orthovanadate.

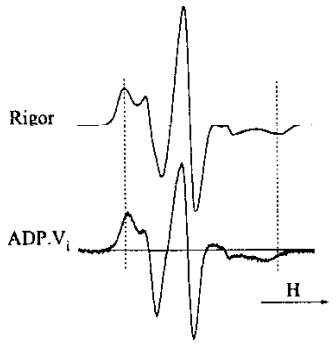

Fig. 4 Effect of nucleotide on MSL-myosin in the presence of MgADP and orthovanadate. The sample was incubated for 10 min in buffer solution before EPR measurement. Nucleotide and orthovanadate induced significant decrease of the hyperfine splitting in fibre system

We can conclude that our data are in a good agreement with experiments performed on protein solutions [20] and on myosin subfragment [8], and they seem to be consistent with the model recently proposed by Rayment and coworkers [2]. The constrain generated by filament association and protein–protein interaction increases the rigidity of the supramolecular structure, and this stabilizes the system. The structure formation alters the dynamical and energetical behaviour of contractile proteins, the consequence of that is the shift of the melting points 39, 47 and 51°C, measured in solution of intact myosin, to higher temperatures in rigor (Table 1). This is an evidence that particular regions of myosin are subjected to stabilizing forces leading to alteration of the transition temperatures.

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