Journal of Thermal Analysis and Calorimetry, Vol. 65 (2001) 351–358

BINDING OF NUCLEOTIDES AT THE ACTIVE SITE MODULATES THE LOCAL AND GLOBAL CONFORMATION OF MYOSIN IN MUSCLE FIBRES

D. Lőrinczy[1*], *N. Hartvig*[2], *N. Farkas*[2] *and J. Belagyi*[2]

[1]Institute of Biophysics, Medical School, University of Pécs, H-7624 Pécs, Szigeti u. 12, Hungary
[2]Central Research Laboratory, Medical School, University of Pécs, H-7624 Pécs, Szigeti u. 12, Hungary

Abstract

Differential scanning calorimetry and electron paramagnetic resonance experiments were performed on glycerinated muscle fibres to study the effect of the binding of nucleotides (ADP and AMP·PNP) to myosin. The thermal unfolding of muscle fibres showed three discrete domain regions with thermal stabilities of 52.2, 58.8 and 67.8°C. AMP·PNP markedly affected the transitions, implying the strong interaction between AMP·PNP and catalytic domain, and partial dissociation of heads from actin. ADP produced only small changes in transition temperatures.

Spectrum deconvolution performed on isothiocyanate-labelled fibres in AMP·PNP-state resulted in two populations; 50% of labels was highly ordered with respect to fibre axis, whereas the other 50% of labels was randomly oriented. The conformation of the myosin heads which showed high degree of order were in the strongly binding ADP-state, the heads being attached to actin differ from those of heads in rigor. The results support the suggestion that the attached heads in strongly binding state to actin might have different local conformations.

Keywords: DSC, EPR, local and global conformation of myosin, nucleotide binding

Introduction

Recent studies on skeletal muscle fibres suggest that domain movements in the myosin head play a decisive role in the energy transduction process of the muscle contraction [1–3]. The main cleft in the 50-kDa segment of the subfragment-1 (S-1) opens when ATP binds to the active site, and during or after hydrolysis the specific formation of strong binding of myosin head for actin causes the closure of this cleft. As a consequence of this movement, the light chain-binding motif generates the power-stroke. This process is a multistep that can produce several conformational states of myosin. Extensive studies using different techniques indicated that the small nucleotide-induced conformational changes in the motor domain should be converted into

* Author for correspondence: Phone: (36) 72-536 260; Fax: (36) 72-536 261;
E-mail: denes.lorinczy@aok.pte.hu

1418–2874/2001/ $ 5.00

Akadémiai Kiadó, Budapest
Kluwer Academic Publishers, Dordrecht

larger movement. Spectroscopic probes – paramagnetic and fluorescence reporter molecules – widely used in muscle research to get information about orientations and rotational motion of myosin heads [4–6]. Paramagnetic probes provide direct method in which the rotation and orientation of specifically labelled proteins can be followed. DSC measurements in combination with spin label electron paramagnetic resonance (EPR) and saturation transfer (ST) EPR techniques allow us to characterize the rotational dynamics and internal flexibility of proteins, and compare these data with their structural stability derived from DSC data [7–9]. We report the effect of AMP·PNP (a non-hydrolysible ATP analogue) on the dynamics and structural stability of myosin head using different spin labels and DSC technique.

Materials and methods

Materials

Potassium chloride (KCl), magnesium chloride ($MgCl_2$), ethylene glycol-*bis*(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), histidine·HCl, glycerol, adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), 5'-adenylyl imidodiphosphate (AMP·PNP), 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MSL) and 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) were obtained from Sigma (Germany).

Fibre preparation

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, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 10 mM histidine·HCl, pH 7.0 at –18°C up to one month. Fibre bundles from glycerinated muscle were washed for 60 min in rigor buffer (80 mM potassium propionate (KPr), 5 mM $MgCl_2$, 1 mM EGTA in 25 mM Tris·HCl buffer, pH 7.0) 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 for actin that may correspond to the AM·ADP state. In experiments involving MgADP, the activity of adenylate kinase was inhibited by addition of 50 μM diadenosine pentaphosphate. The other analogue of intermediates in the ATPase pathway was formed by AMP·PNP, which binds stoichiometrically at the active site of myosin to form a stable complex [8]. The muscle fibres were stored in solution containing 80 mM KPr, 5 mM $MgCl_2$, 5 or 16 mM AMP·PNP in 10 mM histidine·HCl buffer, pH 7.0, for 15 min at 0°C, and then spectra were taken at ambient temperature (20–22°C).

DSC measurements

Thermal unfolding was monitored by a Setaram Micro DSC-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 ±0.1 mg. There was no need to perform any correction from the point of view of heat capacity between the sample and reference vessels.

Evaluation of DSC scans

The repeated scan of denaturated sample was used as baseline reference which was subtracted from the original DSC scan. Calorimetric enthalpy was calculated from the area under the heat absorption curves using two points setting Setaram peak integration.

Spin-labelling of muscle fibres

Spin-labelling of fibres was performed in relaxing medium (rigor solution plus 2 mM pyrophosphate at pH 6.5) with about two moles of MSL or TCSL to one mole myosin for 10 (MSL) or 20 (TCSL) min at 0°C. Before spin-labelling the fibres were incubated in low ionic strength buffer (1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTNB and 20 mM MOPS, pH 7.0) for 1 h to achieve selective labelling of the reactive thiols [10]. After spin-labelling the fibre bundles were washed in great amount of rigor buffer plus 5 mM dithiotretiol for 30 min at 0°C, pH 7.0 to remove the unreacted labels and restore the preblocked thiol groups.

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 (0.2 mT amplitude) and 20 mW microwave power were used. Second harmonic absorption, 90^0 out-of-phase spectra were recorded with 50 kHz field modulation 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 [11, 12]. In this region of the tissue cell, few segments of the muscle fibres (6–7 mm long) were mounted parallel to each other. The spectra were recorded in two positions at temperature of 22±1°C, where the longer axis of the fibres was oriented parallel and perpendicular to the laboratory field. The spectra were normalised to the same number of unpaired electrons calculating the double integral of the derived spectra. We assumed that the spectra from MSL- or TCSL-fibres in different states could be composed of a linear combination of spectra, the manipulations were performed on normalised EPR spectra by digital subtraction.

Results and discussion

EPR measurements

Myosin in fibres is usually spin-labelled with a maleimide- or an isothiocyanate based spin labels which are believed to bind to the fast reacting thiol site (Cys-707) in

the catalytic domain of myosin as supported by ATPase measurements [13, 14]. According to EPR measurements the labels in fibres were strongly immobilized on the microsecond time scale, the labels rotated with an effective rotational correlation time of 1 s (MSL) and 100 μs (TCSL) respectively, calculated from ST EPR spectra in the absence of nucleotides. In rigor – in the absence of nucleotides – the myosin heads had only one mode of binding to actin filaments. The attached MSL probes showed a narrow Gaussian distribution with respect to the longer axis of the fibres with a mean angle of 82^0 and an angular spread of 6^0 [4]. Using TCSL probes, the EPR spectra also reported high dependence on orientation, but in comparison to MSL-fibres with different mean angle and angular spread ($\vartheta=75^0$, $\sigma=16^0$; [15]). Addition of MgADP to buffer solution did not affect the orientational order of spin labels in MSL-fibres, that is, myosin heads in rigor and myosin heads with bound ADP exhibit the same orientational order. However, the incubation of TCSL-fibres in rigor buffer containing MgADP resulted in significant changes of the orientation dependence (Fig. 1). It is probably due to the fact that TCSL molecules possess more flexible attaching linkage than MSL probes, as can be derived from the ST-EPR spectra of the labelled fibres. This supports the view that TCSL probes can reflect internal structural changes in the catalytic domain of myosin induced by nucleotides.

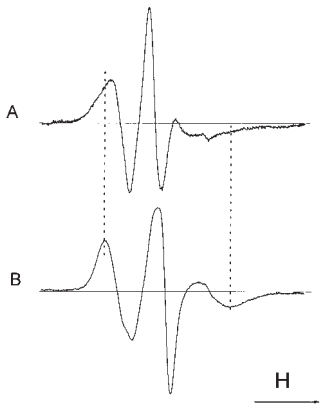

Fig. 1 EPR spectra of muscle fibres in rigor (A) and ADP-state (B). The long axis of fibres was oriented parallel to the laboratory magnetic field. The field scan is 10 mT

Measurements on MSL-fibres in the presence of AMP·PNP showed that about half of the myosin heads dissociated from actin [16]. This fraction had dynamic disorder, whereas the other population had the same spectral feature as in rigor. In our experiments the muscle fibres from rabbit were spin-labelled with isothiocyanatebased probe molecules at Cys-707 of myosin. Spectrum deconvolution resulted in two populations; about 50% of labels belonged to the ordered fraction, and 50% of labels was randomly oriented. Our results on AMP·PNP-fibres, using TCSL suggest that about half of the heads represents a disordered population with reduced rate of rotational motion, but the myosin heads which exhibited high degree of order were in the

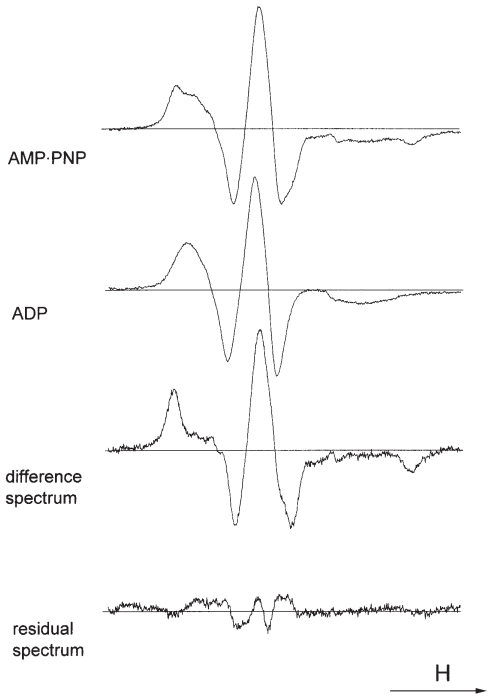

Fig. 2 EPR spectra of TCSL-fibres in different states of the ATP hydrolysis cycle. Difference spectrum: subtraction of the ADP spectrum from the AMP·PNP spectrum. Bottom: the residual spectrum, the difference of the ADP spectrum and the ADP-like spectrum derived from the AMP·PNP spectrum. The field scan is 10 mT

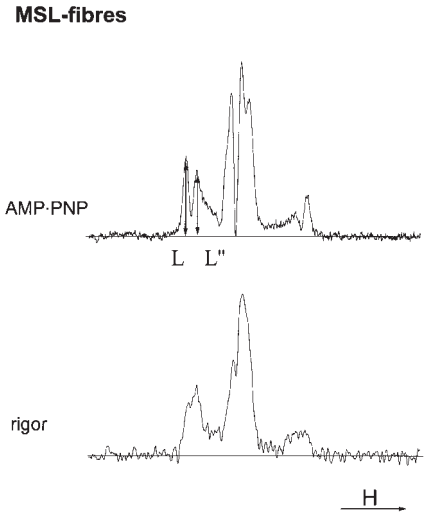

Fig. 3 ST- EPR spectra of MSL-fibres in rigor and AMP·PNP-state. The field scan is 20 mT

strongly binding ADP-state, the heads being attached to actin differ from those of heads in rigor (Fig. 2). Subtracting the ADP spectrum from the AMP·PNP spectrum, the difference spectrum was characteristic of random population of spin labels (third spectrum in Fig. 2). When the spectrum of TCSL-fibres in ADP·V_i-state (weakly binding state of myosin for actin) or the difference spectrum in Fig. 2 was subtracted from the AMP·PNP spectrum, the resulting spectrum was similar to an ADP spectrum. The weakly binding state was performed by adding 5 mM MgATP plus 5 mM orthovanadate to rigor buffer before EPR measurements. The residual spectrum obtained after subtraction of the ADP spectrum from the 'ADP-like' spectrum can be seen on the bottom of Fig. 2. AMP·PNP increased the orientational disorder of myosin heads, a random population of spin labels was superimposed on the ADP-like spectrum evidencing conformational and motional changes in the internal structure of myosin heads. ST EPR measurements reported increased rotational mobility of spin labels in the presence of AMP·PNP, the population of heads belonging to the disordered fraction either dissociated from actin filaments or exhibited binding property differing from rigor (Fig. 3; [16]).

DSC measurements

The results of the melting process of myosin and actin in the supramolecular structure of muscle fibres cannot be interpreted easily. It is expected that the formation of new interaction in the highly ordered fibre system increases the transition temperatures and the interaction enthalpies in comparison to myosin and actin solutions. The thermal unfolding of muscle proteins in the absence of nucleotides could be characterized by three discrete domain regions with different thermal stabilities (T_m=52.2, 58.8 and

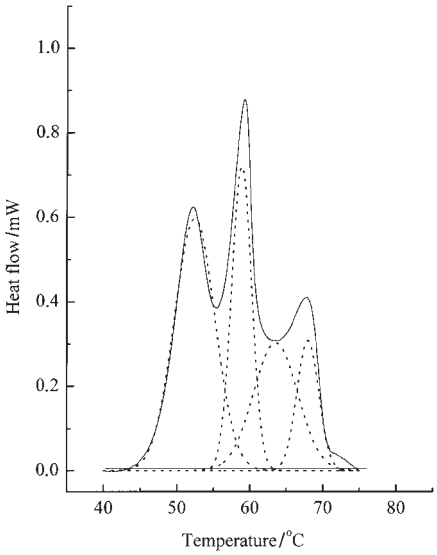

Fig. 4 Melting curve of muscle fibres in rigor

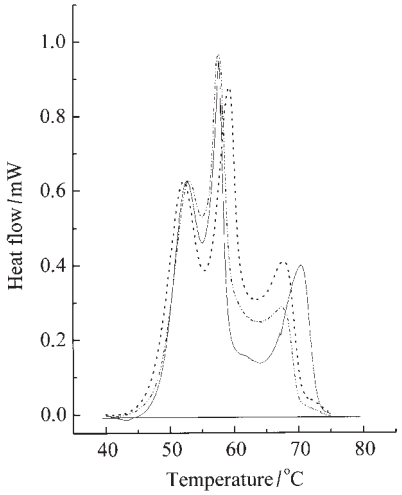

Fig. 5 DSC patterns of muscle fibres in rigor, ADP- and AMP·PNP-states. Symbols: rigor (dotted line), ADP-state (dashed line), AMP·PNP-state (solid line)

67.8°C), one further transition was derived by deconvolution at 63.0°C (Fig. 4). Deconvolution was performed using PeakFit 4.0 from SPSS Corporation, and assumed that the single transitions follow Gaussian processes. The comparison of the melting curves in the presence of nucleotides, ADP or AMP·PNP showed that the first two transitions were only little affected by the binding of nucleotides (Fig. 5). Earlier experiments using tryptic digestion gave evidence that the most labile part of myosin was the 50-kDa segment or a part of it, therefore it is believed that the first thermal transition can be assigned to the 50-kDa domain even in muscle fibres [17]. The binding of ADP to myosin induced only a little change in the DSC pattern, indicating that ADP alone produced small local conformational changes in the myosin heads in agreement with the EPR results.

The experiments on isolated myosin heads (S-1) showed the appearance of a new transition at higher temperature in the presence of AMP·PNP, which was attributed to the nucleotide interaction with the domains of the myosin head [18, 19]. The increased thermal stability of the dissociated globular heads induced by the interaction between AMP·PNP and the nucleotide-binding domain in muscle fibres appears very likely in the last transition, which is shifted from 67.8 to 70.1°C. The contribution of the third transition at 63.0°C to the total melting enthalpy reduced to its half, whereas the enthalpy of the last transition markedly increased. It suggests that the third transition can be assigned to myosin heads bound to actin. The dissociation of myosin heads from actin (or the transition from strongly binding state into weakly binding state of myosin to actin) induced by AMP·PNP resulted in a significant decrease of their contributions to the total enthalpy of the denaturation process.

In summary, DSC and EPR results suggest that in the presence of ADP and AMP·PNP the attached heads have the same global orientation as in rigor, but the internal structure undergoes local conformational changes.

This work was supported by research grants from the National Research Foundation (OTKA T 030248, CO-123 for J. B. and CO-272 for D. L.), Ministry of Education (FKFP 0387/2000), Hungary.

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