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DSC AND EPR STUDY ON AMP.PNP, BEF_x AND AlF_4 CONTAINING MYOSIN NUCLEOTIDE COMPLEXES

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Abstract

Differential scanning calorimetry and electron paramagnetic resonance experiments were performed on glycerinated skeletal muscle fibres to study the effect of the binding of nucleotides and nucleotide analogues to myosin. The thermal unfolding of muscle fibres in rigor showed three discrete domain regions with thermal stability of 52.2, 58.8 and 67.8°C. AMP.PNP and ATP plus AlF_3 or BeF_2 affected markedly the transitions, which implies the strong interaction between AMP.PNP or nucleotide analogues and catalytic domain of myosin, and a partial dissociation of heads from actin. ADP.BeF_x and ADP.AlF_4^- states model the transition states of the ATP hydrolysis cycle which precede the powerstroke of the muscle fibres.

Spectrum deconvolution 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 myosin heads which showed high degree of order were in the strongly binding ADP-state. The spectra in ADP.AlF_4^- - and ADP.BeF_x state reflected random orientation of labels with increased rotational mobility in comparison with rigor. The results suggest that myosin in muscle fibres in ADP.BeF_x state exists in two forms.

Keywords: aluminium and beryllium fluoride, ATP hydrolysis, conformation of myosin, DSC, EPR, nucleotide-myosin interaction

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 domain of the myosin head – called subfragment-1 (S-1) – opens when ATP binds to the active site, and during hydrolysis the specific formation of strong binding of myosin head for actin causes the closure of this cleft. As a consequence of this conformational change, the light chain-binding domain amplifies the force of the contraction. This process is a multistep process that can produce several conformational states of myosin. Extensive studies using different techniques indicated that the nucleotide-induced conformational changes in the motor domain should be converted into larger movement in the actomyosin system [4].

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Spectroscopic probes – paramagnetic and fluorescence reporter molecules – widely used in muscle research to get information about orientation and rotational motion of myosin heads [5–7]. Spin label EPR technique in combination with DSC measurements allow us to characterize the rotational dynamics and internal flexibility of proteins, and compare these data with their structural stability derived from DSC data [8–10]. We report the effect of the non-hydrolysible ATP analogue AMP.PNP and ATP plus AlF_3 or BeF_2 on the dynamics and structural stability of myosin heads in muscle fibres using DSC and spin label EPR technique. AMP.PNP state mimics the ATP state, whereas ADP.BeF_x and ADP.AlF_4^- states model the transition state (ADP.P_i) of the ATP hydrolysis cycle which precedes the powerstroke of the muscle fibres [11–13]. Analysis of transition temperatures by DSC showed that beryllium and aluminium fluorides, similar to vanadate affected strongly the thermal stability of myosin.

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-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl spin label (TCSL), aluminium chloride, beryllium sulphate and sodium fluoride 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 was added of 5 mM concentration to the rigor solution to simulate the strongly binding state of myosin for actin that may correspond to the AM.ADP state. For experiments with the ATP analogue AMP.PNP 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). The nucleotide analogues were formed by ATP (5 mM) and beryllium fluoride or aluminium fluoride which stoichiometrically bind to myosin to form a stable complex. Beryllium fluoride and aluminium fluoride were prepared from 10 mM NaF and 3 mM $AlCl_3$ and $BeSO_4$ immediately before experiments.

DSC measurements

Thermal unfolding was 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 ±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 denatured sample was used as baseline reference that 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 TCSL to one mole myosin for 20 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 [14]. After spin-labelling the fibre bundles were washed in great amount of rigor buffer plus 5 mM dithiotreitol 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. In the central region of the flat cell a few segments of the muscle fibres (6–7 mm long) were mounted parallel to each other. 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 magnetic 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 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

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 interactions in the highly ordered fibre system increases the transition temperatures and the in-

teraction enthalpies in comparison with myosin and actin in solutions. The thermal unfolding of muscle proteins in the absence of nucleotides could be characterized by three discrete domain regions with different thermal stability (T_m=52.2, 58.8 and 67.8°C), one further transition was derived [15] by deconvolution at 63.0°C (Fig. 1). 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 [15] that the first two transitions were only little affected by the binding of nucleotides (Fig. 2). Earlier experiments using tryptic digestion gave evidence that the most labile part of myosin was the 50-kDa segment (actin-binding domain) 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 [16]. The binding

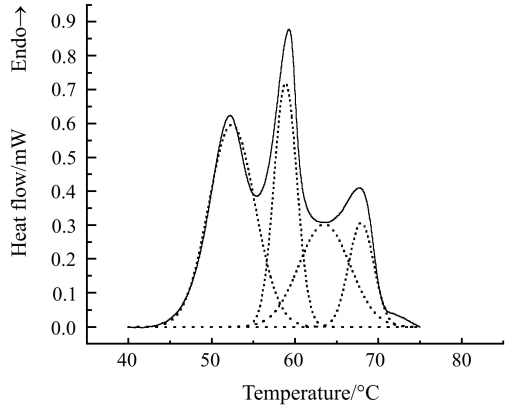

Fig. 1 Melting curve of muscle fibres in rigor. Deconvolution procedure resulted in four transitions which can be assigned to different protein components of the muscle fibres. The third transition at 63°C can be assigned to actin and actin-binding proteins

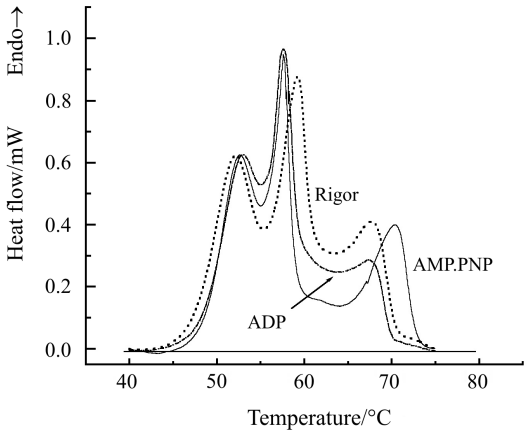

Fig. 2 DSC patterns of muscle fibres in rigor, ADP and AMP.PNP state. Most significant change can be observed at the highest temperature transition. Symbols: rigor (dotted line), ADP state (dashed line), AMP.PNP state (solid line)

of ADP to myosin induced only little change in the DSC pattern, indicating that ADP alone produced small local conformational change in the myosin heads [17].

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 this interaction appears very likely in the last transition, which is shifted from 67.8 to 70.1°C. The third transition at 63.0°C can be assigned to myosin heads bound to actin.

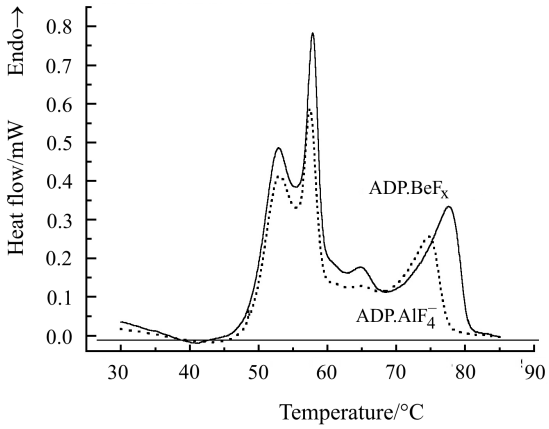

Fig. 3 Melting profiles of muscle fibres in ADP.AlF_4^- and ADP.BeF_x states. Note the large shift of the last transition in comparison with rigor

In the presence of ATP plus BeF_x or AlF_4^-, the high temperature transition was shifted from 67.7°C to T_m=74.6°C and T_m=77.4°C, respectively (Fig. 3). Biochemical experiments reported dissociation of heads from actin in ADP.AlF_4^- and ADP.BeF_x states [20]. From our experiments on muscle fibres we could confirm that the last transition characterized the interaction of the nucleotide binding domain with nucleotides or nucleotide analogues. X-ray diffraction studies reported that ADP.BeF_x in complex with myosin mimicked the ATP bound state, whereas ADP.AlF_4^- complex was the analogue of the metastabile ADP.P_i state after ATP hydrolysis [21, 22]. According to biochemical experiments the complexes of ADP with phosphate analogues are long-lived complexes, which might explain the larger shift of temperature for the complex of myosin with ADP and BeF_3 or AlF_4^-, in comparison with AMP.PNP. DSC measurements on subfragment-1 with ADP and beryllium fluoride also revealed significant shift of T_m in comparison with nucleotide free subfragment-1 [22].

EPR measurements

Myosin in fibres is usually spin-labelled with a maleimide (MSL)- or an isothiocyanate based spin labels which are believed to bind to the fast reacting thiol site (Cys-707) in the catalytic (nucleotide-binding) domain of myosin as supported by ATPase measurements [23, 24]. According to EPR measurements the labels in fibres were strongly immo-

bilized 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 saturation transfer (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 probes show a narrow Gaussian distribution with respect to the longer axis of the fibres [5, 25]. Incubation of TCSL-fibres in rigor buffer containing MgADP resulted in significant change of the orientation dependence (Fig. 4). This supports the view that TCSL probes can reflect internal structural changes in the catalytic domain of myosin induced by nucleotides.

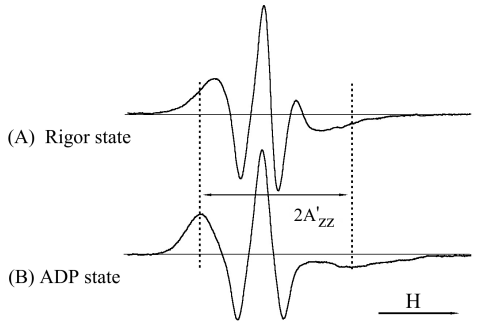

Fig. 4 EPR spectra of muscle fibres in rigor and ADP state. The long axis of fibres was oriented parallel to the laboratory magnetic field. The change of the hyperfine splitting constant ($2A'_{zz}$) shows the different static order of spin labels in rigor and ADP-state. The field scan is 10 mT

In the presence of AMP.PNP the spectrum deconvolution resulted in two populations; about 50% of labels belonged to the ordered fraction, and 50% of labels was randomly oriented [26, 27]. AMP.PNP increased the orientation disorder of myosin heads, a random population of spin labels was superimposed on the ordered fraction evidencing 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. The myosin heads that exhibited high degree of order were in the strongly binding ADP-state, the heads being attached to actin differ from those of heads in rigor (Fig. 5). Subtracting an ADP.V_i spectrum from the AMP.PNP spectrum, the difference spectrum was characteristic of ordered population of spin labels (ADP-like spectrum, third spectrum in Fig. 5).

Almost no orientation dependence was detected in the presence of ATP and beryllium or aluminium fluoride (Fig. 6). The hyperfine splitting constants of the conventional EPR spectra were different; 6.661±0.04 mT (ADP.BeF_x complex) and 6.712±0.03 mT (ADP.AlF_4^- complex), respectively. The myosin heads represented disordered populations with reduced rate of rotational motion, characterising of dissociated myosin heads or non-specific binding of myosin heads to actin. The spectrum of ADP.BeF_x complex seemed to be the superposition of an ADP.V_i-like spectrum and the spectrum of a protein moiety which rotated with an effective rotational

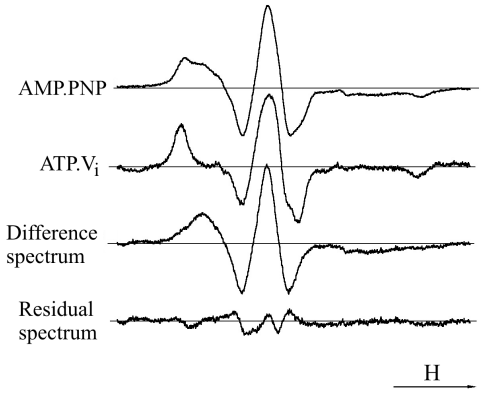

Fig. 5 EPR spectra of TCSL-fibres in different states of the ATP hydrolysis cycle. Digital subtraction of an ADP.V_i spectrum (randomly oriented spin labels) from the AMP.PNP spectrum resulted in a spectrum (difference spectrum), which is characteristic of ADP spectrum. Bottom: the residual spectrum, the difference of the ADP spectrum and the ADP-like spectrum derived from the AMP.PNP spectrum. The field scan is 10 mT

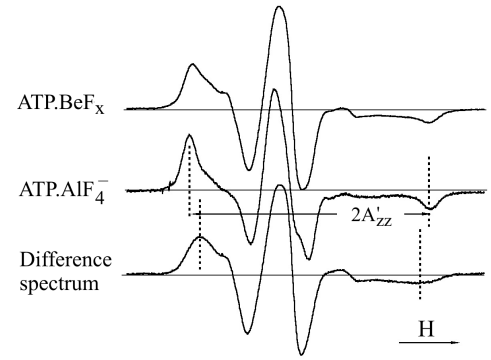

Fig. 6 EPR spectra of TCSL-fibres in ADP.AlF_4^- and ADP.BeF_x states. Digital subtraction of the ADP.AlF_4^- spectrum from the ADP.BeF_x spectrum resulted in a spectrum with larger rotational mobility of about 15 nsec. This spectrum might mimick the M.ATP state

correlation time of 12–15 ns [28]. Digital subtraction of either an ADP.V_i spectrum or an ADP.AlF_4^- spectrum from the ADP.BeF_x spectrum resulted in two fractions. This result suggests that under experimental conditions two conformers existed in ADP.BeF_x state, mimicking the M*.ATP and M**.ADP.P_i states.

Conclusions

The DSC and EPR results suggest that in the presence of ADP and AMP.PNP the attached (oriented) heads have the same global orientation as in rigor, but the internal structure undergoes local conformational changes. Our data in skeletal muscle fibres on BeF_x and AlF_4^- binding support the view that the conformation and the energetic

of the ADP.BeF_x and ADP.AlF_4^- states differ from each other and represent different transition states. The differences in the melting temperatures and the changes in the EPR spectra indicate significant alterations in the internal structure of myosin head region and the nucleotide-myosin head interaction. The results of ADP.BeF_x state suggest that this state is heterogeneous, the superposition of the intermediate states M*.ATP and AM**.ADP.P_i. In contrast, ADP.AlF_4^- state cannot be distinguished from the ADP.P_i (AM.ADP.V_i) state.

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