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Protein structure and function

EPR and DSC study of glycerol-extracted muscle fibres in intermediate states of ATP hydrolysis

PhD Thesis

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Introduction

One of the most- significant properties of the living systems is the motion, which is one of the emphasized fields of the biology for a long time. In the world we can see different types of the motion, where the most important rule is connected with specially developed protein system.

The best-known motion type of the advanced living systems is the muscle contraction. Muscle is a "chemo-mechanical machine" that converts chemical energy into mechanical work and heat during contraction. The energy source is ATP, its hydrolysis is driven by myosin, and the rate is enhanced in the presence of actin. The products liberated from ATP hydrolysis produce conformational changes in myosin and very likely in actin. The structural change might induce rotation of myosin head while bound to actin, and it causes the muscle to shorten. The force generation involves some structural rearrangement of myosin and consequently, internal motion and flexibility of the major components of muscle may be an integral part of the contractile process in the actomyosin system. The sliding motion in striated muscle requires the cyclic interaction of myosin (M) with ATP and actin (A), and for actomyosin ATPase the presently accepted mechanism in model system Bagshaw and Trentham's:

$$M+ATP \leftrightarrow M^*.ATP \leftrightarrow M^{**}.ADP.Pi \leftrightarrow M^*.ADP+Pi \leftrightarrow M+ADP+Pi$$

The aim of our study was to investigate the energetic of the actomyosin ATPase in the presumed different intermediates of the contractile cycle using differential scanning calorimetry (DSC) and electron paramagnetic resonance (EPR) technique. We have extended the experiments to study myofibrils prepared from chemically skinned (glycerinated) m.psoas fibres in rigor (AM), in the presence of nucleotides (ATP, ADP, AMP.PNP) as well as Pi analogues {orthovanadate (Vi), AlF_4^- or BeF_3^-}. Rigor and ADP state model the strongly binding state of myosin to actin, whereas the non-hydrolyzable ATP analogue AMP.PNP, ATP plus Pi analogues mimic weakly binding states of myosin to actin.

The aim of study

We have examined by electron paramagnetic resonance spectroscopy (EPR) and differential scanning calorimetry (DSC) the complexes of myosin with actin in fibre system in the absence of nucleotides and in the intermediate state of ATP hydrolysis in muscle fibres. In our experiments we also studied the effect of environmental parameters during muscle contraction.

1. During muscle contraction the most important structural changes are expected in the myosin head. One of the possible dynamic assays is the spin label technique. Myosin in fibers was spin-labelled with an isothiocyanate based spin label (ITC) and maleimid spin label (MSL) as well. It is believed that the labels bind to the fast reacting thiol sites in the catalytic domain of myosin, especially to the side chain cystein 707. Spectroscopic probes provide direct information about the orientation of myosin heads, during the ATP hydrolysis of the muscle contraction; in the *strong binding* state (rigor and ADP) and in the *weak binding* state.

2. In line with EPR measurements we examined the muscle contraction by differential scanning calorimetry (DSC). The powerful DSC technique allows the derivation of heat changes as a function of temperature. From the deconvolution of the thermal unfolding patterns it is possible to characterize the structural domains of the motor protein. In this work we tried to approach the temperature-induced unfolding processes in different intermediate state of ATP hydrolysis in striated muscle fibres. In recent literature we didn’t find related data by DSC about the ATP-hydrolysis cycle in muscle fibres.

Materials and methods

EPR spectroscopy

The EPR is one of the spectroscopy methods which examine the electromagnetic interaction in different systems. It is required that the system should posses an unpaired electron. Usually the biological system cannot be examined directly by EPR, because the system hasn't unpaired electron or electrons, therefore we used the spin label technique. Spectroscopic probes are widely used in muscle research to obtain information about orientations and rotational motion of myosin heads. Paramagnetic probes provide a direct method in which the rotation and orientation of specifically labelled proteins can be followed. In muscle fiber studies, the probe molecules, especially the maleimide-based nitroxides (MSL) and the isothiocyanate-based spin label (TCSL) are usually attached to the reactive sulfhydryl sites. The main problems limiting the interpretation of the spectroscopic measurements are in connection with the relative orientation of the spin labels; in addition, the attached label can cause local modifications. The different labels have different chemical structures, and therefore they have a slightly different orientation with respect to the longer axis of S1. The change of the orientation of the entire S1 or a change in the internal structure of the S1 during experiment is reported differently by the different labels. Therefore, it is reasonable to use different labels to understand the molecular motion of S1 in the presence of nucleotides. We observed that the isothiocyanate-based spin label is more sensitive to the domain orientation in myosin head than the widely used maleimide spin label. Very likely, this label has a particular orientation that reflects smaller changes in the orientation of the myosin heads and has a more flexible linkage, and therefore senses the internal rearrangement of the segments.

In this report, we studied the effect of nucleotide and their analogues on the dynamics and orientation of myosin head using 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) and N-(1-oxyl-2,2,6,6-tetrametyl-4-piperidinyl) maleimide (MSL) by EPR spectroscopy.

EPR measurements were recorded with an ESP 300E (Bruker) spectrometer. First harmonic in-phase absorption spectra were obtained using 20 mW microwave power and 100

kHz field modulation with amplitude of 0.1–0.2 mT. Second harmonic 90° out-of-phase absorption spectra were recorded with 63 mW microwave power and 50-kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out-of-phase.

Differential scanning calorimetry

In order to find correlation between the local conformational changes obtained by EPR spectroscopy and the global structural changes that might be expected in the intermediate states of the ATPase cycle, the spectroscopic measurements were combined with DSC measurements that report domain stability and interactions. The measurements support the view that many of the local conformational changes produced by nucleotide binding are accompanied with global conformational changes.

The only direct and sensitive method to follow this change is the differential scanning calorimetry (DSC). The sample cell contains the given macromolecular system together with its buffer, the reference cell is filled with buffer and both of them have a very strict mass equilibrium to set a relatively equal total heat capacity. During a controlled heating-cooling cycle without any molecular rearrangement in the sample cell, there is no temperature difference between the two cells. Any molecular or spatial reorganization needs extra energy, therefore a temperature delay will appear in the sample cell compared to the reference one.To sustain the same temperature, it needs extra energy which equals to the transition energy. This appears in the heat flow-temperature (or time) diagram as an endotherm response (in the case of a heat flow calorimeter).

The thermal unfolding of myosin in fibers was monitored by a SETARAM Micro DSC-II calorimeter in the University of Pécs, Institute of Biophysics. All experiments were carried out between 5 and 80°C. Conventional Hastelloy batch vessels were used during the denaturation experiments with an average 850-µL sample volume. Rigor buffer was used as a reference sample. The sample and reference vessels were equilibrated with a precision of ± 0.1 mg. It was not necessary to correct for heat capacity between the sample and reference vessels. The samples were irreversibly denatured during each cycle.

Results and discussion

We have examined by EPR the complexes of myosin with actin in fibre system in absence of nucleotides and the intermediate state of ATP hydrolysis, by means of spin probes. From EPR spectrum of the attached MSL label we could conclude to the global orientation of myosin head, and in the case of TCSL label to the local changes as well.

a) From the results of conventional EPR spectra measured in glycerol-extracted muscle fibres it could be concluded that both labels (MSL, TCSL) had very high orientation dependence with respect to fibre long axis. Both labels were located on the reactive cys 707. There was only a difference in the centre of orientation and in the angular disorder. This difference can be explained by the different quality of spin labels, because their chemical structure and the mode of connection are different. From the line shape of spectrums we concluded, that the spin labels were high immobilized in rigor with an effective rotational correlation time of about 100-300 μs.

b) ST EPR measurements suggested that the connection of the two main proteins actin and myosin are rigid, in rigor all of the myosin molecules were attached to actin. The connection is highly stereospecific and rigid, so the binding can release only one way.

c) In the presence of ADP, the EPR spectrum reflects the AM.ADP strong binding intermediate state. In the case of MSL spin label comparing the EPR spectra of the rigor and ADP state, no change in the orientation of myosin head was detected. In contrast to MSL label, in the case of TCSL the EPR spectra of fibres were significantly different.

d) In our work we used MSL and TCSL spin label to analyse the weak binding of myosin to actin in different states of ATP hydrolysis cycle. To mimic the ADP.P_i state the fibres were incubated in rigor buffer containing ATP and orthovanadate, this keeps the long dissociation of myosin from actin. In the case both MSL and TCSL spin labels we observed highly significant difference in the angle of distribution of labels (myosin heads) as compared

with EPR spectra of rigor and ADP states. Both MSL and TCSL spin labels exhibited random distribution of labels. We found difference when ATP was used in place of ADP in the presence of V_i. In the case of ATP exclusively the random population was appeared, while using ADP we detected a fraction of the oriented population as well characterizing the ADP state. In proves that application of ADP does not produce complete dissociation of myosin from actin.

e) To examine further the weak binding state of myosin to actin we measured ADP.AlF_4 and ADP.BeF_x states of muscle fibres. From EPR results we could conclude that the EPR spectrum of ADP.BeF_4 was distinct from the spectra of ADP.AlF_4 and ADP.V_i. In ADP.BeF_x state we identified two conformations in proportion of 60-40%. The larger population was referred to M**ADP.P_i weak binding state, while the smaller was assigned to M*ATP state. Our assumptions were confirmed with spectrum manipulation and with further EPR experiments with γ-ATP. On the basis of our EPR results we suggest that ADP.BeF_x state and the M*.(γ–ATP) state probably represent the same intermediate state, while fibres in ADP.AlF_4 state cannot be distinguished from the ADP.V_i or ADP.P_i state .

f) To mimic fibres in the ATP state we used AMP.PNP. The EPR spectra exhibited superposition of two spectra. One of the spectra was characteristic to a random population of labels that was experienced in ADP.P_i state, while the other one showed an oriented population of labels, similar to that state obtained in strong binding state of myosin to actin. Using MSL spin label, the ordered state looks like as rigor state. However, using TCSL label, this population represented the ADP state. This supports the view that in the presence of AMP.PNP a dynamic equilibrium exists between the strong and weak binding state, or the two heads of myosin behaves differently, due to their different stereo positions in the fibre structure. The ordered fraction was approximately 50% of the total spin label concentration.

In order to study the global conformational changes during ATP hydrolysis DSC technique was used. DSC is not structure examination method, therefore the results of DSC can give only information about the thermodynamic behaviour of the actomyosin complex, thus from the thermograms we can draw the conclusions only carefully, however the DSC is a very good complementary of the EPR results.

In strongly and weakly actin binding states the thermograms could be decomposed into three separate transitions in the main transition temperature range. Considering the muscle structure a fourth heat transition was also assumed which was assigned to actin. Deconvolution into four components was performed by using PeakFit 4.0 software from SPSS Corporation. For analysis of the single thermal transitions Gaussian functions were assumed. The comparison between the DSC patterns different states of the fibre bundles suggested that the second and third Gaussian curves represented the myosin rod and the actin moiety. The third and second transition curves were subsequently subtracted from the main transition curve. The deconvolution resulted in four transitions, the first three transition temperature were almost independent of the intermediate state of the muscle, the last transition temperature was shifted to higher temperature, when the buffer solution was manipulated to mimic the intermediate states of ATP hydrolysis.

The comparison of the main transitions for myosin and actin in solution and in fibres system shows significant increase of T_m, which is due to the interaction between actin and myosin in the filament structure. The stereospecific binding of the two myosin heads to actin induces a stabilisation at the head-to-tail junction and rod part of the myosin molecules in the thick filaments that leads to decrease of cooperativity.

The comparison between the DSC patterns in weakly binding states of myosin to actin, as in AM.ADP.P_i or in AM.ADP.V_i state, showed that the transition temperature at the second and third transitions (actin binding domain and myosin rod) varied only slightly, whereas the last one (the fourth transition) shifted markedly to higher temperature depending on the ternary complex. The last transition can be assigned to the nucleotide binding domain of myosin or to actin filaments.

a) Between rigor and ADP states differences could be detected only in the presence of MOPS buffer, since the pH stability was constant during the denaturation process. Incubation of fibres in ADP containing buffer solution resulted in a significant effect

of the thermal stability of the fibres, the transition temperature of the fourth transition rose with about 3°C. It's very likely; that binding of ADP produces flexibility- and/or orientation changes in the head portion of myosin head, which appears as a change in the altered thermal transition of the rigor and ADP states.

b) Significant difference was found between the strongly and weakly binding states of myosin to actin when ADP was trapped by V_i on myosin head. The binding of complex induced remarkable stabilization in the globular part of myosin, which is reflected in a 2.0–6.0°C shift of the transition temperature to higher temperature. Neglecting the small changes observed in the linewidth of transitions at half-height for myosin rod and actin filaments, we can conclude that the global conformational changes mostly occur in the globular portion of myosin heads.

c) We established that the binding of AM.ADP.V_i, AMP.PNP, ATP and BeF_x or AlF_4 to myosin affected differently the conformation and energetic state of myosin; all effects were significantly different from that of the strong binding state. The different transition temperatures and increased calorimetric enthalpies indicated significant differences in the inner structural stability of nucleotide-myosin head complex together with the assumed structural change of F-actin. The temperature increase of the fourth transition is followed in the order of the nucleotide analogues: V_i, AMP.PNP, AlF_4 and BeF_x. However, it cannot be excluded, that above mentioned weak binding states of ATP hydrolysis does not simulate well the ADP.P_i state. Recent experimental results support the suggestion that the nucleotide analogue-F-actin interaction would produce the dominant change observed in the DSC pattern.

Significant contributions to Thesis

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