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VANADATE(V_i) AND ADP INDUCED DOMAIN MOTIONS IN MYOSIN HEAD BY DSC AND EPR

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Abstract

Thermal stability and internal dynamics of myosin head in psoas muscle fibres of rabbit in the intermediate state AM.ADP.P_i – mimicked by AM.ADP.V_i – of the ATP hydrolysis cycle was studied by differential scanning calorimetry and spin label electron paramagnetic resonance spectroscopy.

Three overlapping endotherms were detected in rigor, in strongly binding ADP and weakly binding AM.ADP.V_i state of myosin to actin. The transition at 54.0°C can be assigned to the 50 k actin-binding domain. The transition at highest temperature (67.3°C) represents the unfolding of actin and the contributions arising from the nucleotide-myosin head interaction. The transition at 58.4°C reflects the melting of the large rod part of myosin. Nucleotide binding (ADP, ATP plus orthovanadate) induced shifts of the melting temperatures and produced changes in the calorimetric enthalpies. The changes of the EPR parameters indicated local rearrangements of the internal structure in myosin heads in agreement with DSC findings.

Keywords: ATP hydrolysis, conformation of myosin, DSC, EPR, nucleotide-myosin interaction, orthovanadate

Introduction

The muscle contraction is based on the cyclic interaction of myosin heads with actin. The energy source is the ATP hydrolysis catalysed by myosin; the rate of hydrolysis is significantly enhanced by the presence of actin [1]. In the presence of CaATP the energy released from hydrolysis produces conformational change in myosin [2–4] and in actin [5], which can be manifested as an internal motion of myosin head while bounds 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 with ATP and actin, and at least six intermediates are proposed for actomyosin ATPase in solution [6–9].

$$\mathrm{AM+ATP} \leftrightarrow \mathrm{A+M{\cdot}ATP} \leftrightarrow \mathrm{A+M^{*}{\cdot}ATP} \leftrightarrow \mathrm{AM^{**}.ADP{\cdot}P_i} \leftrightarrow$$
$$\mathrm{AM^{*}.ADP{\cdot}P_i+AM^{*}.ADP+P_i} \leftrightarrow \mathrm{AM+ADP+P_i}$$

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where *M* denotes myosin, *A* stands for actin and the asterisks (*) and (**) identify intermediate conformations. The AM·ADP and AM·ADP·P_i complexes play important roles in the powerstroke of cross bridge cycle; therefore the main 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 and actomyosin complexes [9–12].

We have combined the DSC experiments with the electron paramagnetic resonance (EPR) technique to study the local and global behavior of muscle proteins in fiber system 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 [13]. It is believed that the specific binding of ATP and other nucleotides to myosin head is coupled with local conformational change in the environment of the binding site. These local changes can enforce further reaction steps, which are accompanied with global conformational changes. Therefore, a better understanding of the interrelation between the hydrolysis events observed in protein solutions and the mechanical activities on cellular level requires experiments on supramolecular complexes where the interaction of the ordered protein structure may generate stabilizing forces that modulate the hydrolysis process.

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) and 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) were obtained from Sigma (Germany).

Preparation of muscle fibres

Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas muscle. Small stripes of muscle fibres (30–35 mm in length and 0.5 mm in diameter) were stored after osmotic shocks in 50% v/v glycerol and rigor solution (80 mM potassium propionate (KPr), 5 mM $MgCl_2$, 1 mM EGTA in 25 mM Tris.HCl buffer, 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. 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. 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 is formed by ATP (5 mM) and orthovanadate (5 mM), which together bind stoichiometrically at the active site of myosin to form a stable complex $AM^{+}ADP.V_i$. This complex is believed to be analogue of the steady-state intermediate $AM^{**}ADP.P_i$ (M^{+} and M^{**} denote different conformations of myosin) [13].

DSC 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 ±0.1 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 irreversible denatured during each cycle.

The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC scan. The deconvolution was performed with SPSS PeakFit 4.0 program. Gaussian peak functions were used to approximate the unfolding of the structural units of muscle proteins. Calorimetric enthalpy was calculated from the area under the heat absorption curves using two points setting SETARAM peak integration.

Spin-labelling of myosin

Spin-labelling of myosin in 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 of 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 (0.1–0.25 mT amplitude) and 2–20 mW microwave power were used. Second harmonic absorption, 90° out-of-phase (saturation transfer EPR, ST EPR) 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 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 normalised to unity for spectrum manipulation.

Results and discussion

Thermal stability of contractile proteins in fibre system

Previous X-ray crystallographic studies of myosin established that myosin is a multisubunit protein consisting of distinct domains that can produce coupled movements induced by the binding of nucleotides to myosin [15]. The architecture of the molecule requires changes of the internal flexibility and conformational changes when the chemical energy of ATP is converted directly into mechanical movement. The conformational changes are associated with alteration of the structural stability of the protein that can be studied by DSC in the different intermediate states of the biochemical cycle.

The unfolding of proteins in muscle fibres by thermal excitation is a complex process and depends on the state and the domain structure of the motor proteins, in rigor four transitions have been observed under the three main peaks (Fig. 1 [16]). In earlier experiments on myosin solution three larger transitions were detected at 46, 52.5 and 57°C temperatures in high ionic strength buffer at pH 7.0 [17]. The low temperature transition of T_m=19°C observed first and reported by us on cardiac myosin was omitted, it might be attributed to the interaction of LC-2 light chain with the C-terminal domain of the myosin head [18]. The starting point of the analysis was that the main transition between 50 and 60°C can be separated into nearly independent thermal transitions according to the domain structure of myosin which is main protein of the striated muscle, the other proteins, actin, tropomyosin and troponin have only minor contribution to the total transition enthalpy.

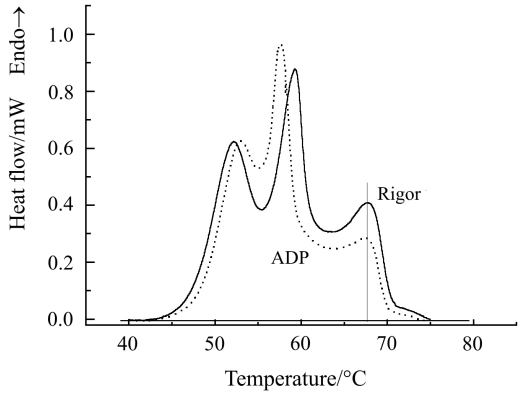

Fig. 1 DSC melting profiles of glycerinated muscle fiber bundles in rigor and ADP state. Only the main transitions between 40–70°C are shown

The lowest transition at 54.0°C was only little affected by the binding of nucleotides. It was shown by tryptic digestion that the most labile part of myosin was the 50 k segment or a part of it, therefore it is believed that the first thermal transition can be assigned to the 50 k domain even in muscle fibres [10, 19]. This domain plays an essential role in actin-myosin interaction and actin-myosin binding [2]. The middle

part of the decomposed DSC scan at 58.4°C may refer to the rod part of myosin, which seems to undergo subtle changes in different states [20–23]. The third component of the main transition at 67.3°C is in relation with the nucleotide-myosin interaction [16], and this temperature range overlaps with the thermal transitions of actin and actin-binding proteins [24].

The binding of ADP to myosin induced only a little change in the DSC pattern indicating that ADP alone produced a small local conformational change in the myosin heads in agreement with the EPR results (Fig. 1). The changes in the DSC pattern may reflect the increased affinity of myosin to ADP [3, 25].

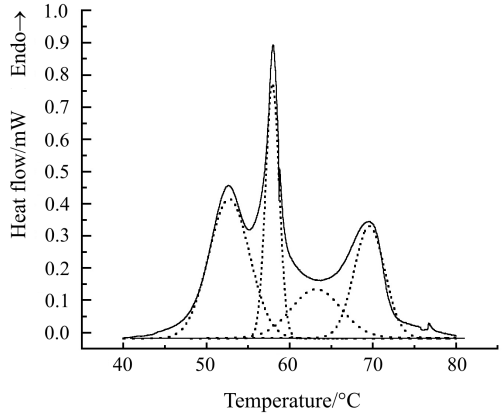

Fig. 2 Melting pattern of muscle fibre in ADP.V_i state. The Gaussian curves were obtained by a deconvolution procedure. The contributions from actin and actin-binding proteins were neglected

In the presence of 5 mM MgADP plus 5 mM orthovanadate the separation of the high temperature transition is also pronounced (Fig. 2). 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. In weakly binding state of myosin to actin the myosin heads are loosely attached to actin, and the fibres do not produce tension. The difference in the melting temperatures clearly indicates differences in the conformation of myosin head region. This latter finding is in good agreement with the model from Rayment *et al*. [2], because in rigor the narrow cleft between the upper and lower domains of the 50 k segment is in a closed conformation, while in AM.ADP.V_i state it opens.

EPR measurements

Myosin in muscle fibres was spin-labelled with an isothiocyanate based spin label which is believed to bind to the fast reacting thiol site (Cys 707) in the catalytic domain of myosin [26, 27]. The ST EPR measurements on TCSL-fibres in rigor and also in ADP state showed that the labels were strongly immobilized on the millisecond time domain

(Fig. 3). The spectral parameter L″/L was greater than 0.8 (0.820±0.064, n=5) which evidences that the rotational correlation time of the label is about 60–80 μs. We could not find significant difference between rigor and ADP state. This suggests – in agreement with previous data – that there is only a small difference in the rotational mobility of the powerstroke state and rigor (AM.ADP and AM states) of the muscle mashine. This statement is supported by conventional EPR data as well.

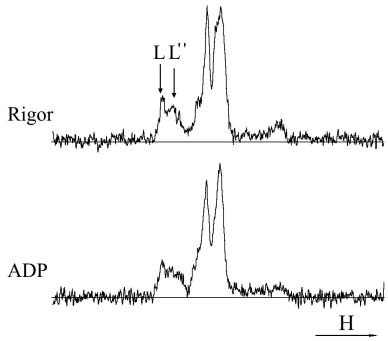

Fig. 3 Saturation transfer EPR spectra of TCSL-muscle fibre in rigor and ADP state. The laboratory magnetic field was oriented perpendicular to the long axis of the fiber bundle. The field scan was 20 mT. The diagnostic L and L″ parameters are shown

Spectroscopic probes provided direct information about the orientation of myosin heads; in rigor the myosin heads had only one mode of binding. In oriented fibre system, the mean angle of the Gaussian distribution of attached label was found to be ϑ=82° and the angular spread was σ=6° [28, 29]. The fibres were spin-labeled with a maleimide-based spin label. Using TCSL probes, the EPR spectra also reported a high dependence of orientation with different mean angle and angular spread (ϑ=75°, σ=16°, Fig. 4) [26].

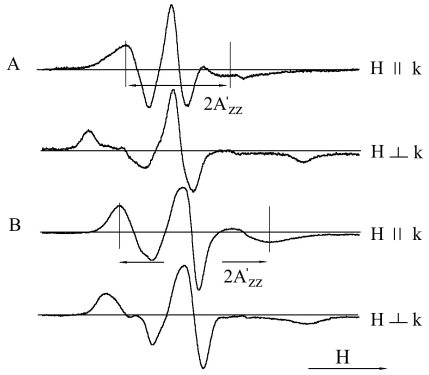

Fig. 4 Conventional EPR spectra of oriented fiber bundles labelled with TCSL in rigor (spectrum A) and ADP state (spectrum B). The long axis of the fibre was oriented parallel and perpendicular to the laboratory magnetic field. The field scan was 10 mT

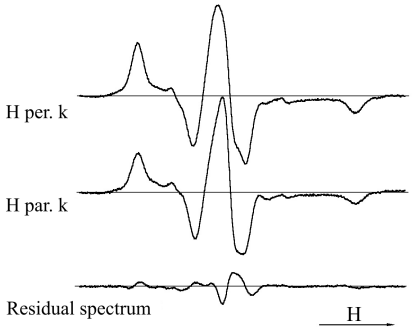

Fig. 5 Conventional EPR spectra of oriented fiber bundles labelled with TCSL in ADP.V_i state. The long axis of the fibre was oriented parallel and perpendicular to the laboratory magnetic field. The residual spectrum indicates that the orientation dependence is almost disappeared. The field scan was 10 mT

In the presence of nucleotides (ADP or ATP plus orthovanadate), the conventional EPR spectra showed changes in the ordering of the probe molecules in fibres (Figs 4 and 5). Addition of 5 mM MgADP resulted in a change in the mean angle of the distribution of spin labels, it decreased from 75 to 56° and the angular spread increased by four degree, but the orientation order remained preserved. In contrast, 5 mM MgATP plus 5 mM orthovanadate to rigor buffer produced an orientation disorder of myosin heads, only one spectral component could be detected which was charateristic to random population of spin labels. It shows the dissociation of myosin heads from actin or a weak interaction between M^+ADP.V_i and actin.

Conclusions

The constrain in muscle fibre system generated by the particular filament organisation and the unique protein-protein interaction produces an increase of the rigidity of the supramolecular structure, and this stabilises the system. The structure formation alters the thermodynamic and the motional dynamic properties of the actomyosin system. The consequence of that is a remarkable shift of the thermal transitions to higher temperatures detected at 39, 47 and 51°C in solution on isolated myosin and myosin fragments. However, the basic melting pattern of myosin in fibre system is preserved. EPR data support the view that ADP binding induces only a local conformational change in the environment of the nucleotide binding domain of myosin without significant change of the rotational mobility, but according to DSC data this produces an alteration in melting temperature and transition enthalpy. This is evidence that particular regions of myosin are subjected to global conformational change after nucleotide binding.

The addition of MgADP and orthovanadate to the rigor buffer results in an intermediate state mimicking the AM.ADP.P_i state. The large change of the mobility and the orientational order of the attached labels are the sign of a remarkable structural rearrangement. On the basis of the DSC data we can suggest that the most significant change appears in the interaction between the nucleotide binding domain and the

ADP.V_i ligand. The result of this interaction leads to dissociation of myosin heads from actin and/or to the formation of weak binding of myosin to actin.

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References

1 R. W. Lymn and E. W. Taylor, Biochemistry, 10 (1971) 4617.
2 I. Rayment, H. M. Holden, M. Whittaker, C. B. John, M. Lorenz, K. C. Holmes and R. A. Milligan, Science, 261 (1993) 58.
3 I. Rayment and H. M. Holden, TIBS, 19 (1994) 129.
4 J. Belagyi and D. Lőrinczy, Biochem. Biophys. Res. Commun., 219 (1996) 936.
5 A. Orlova and E. H. Engelman, J. Mol. Biol., 232 (1993) 334.
6 L. A. Stein, R. P. Schwarz, P. Boon Chock and E. Eisenberg, Biochemistry, 18 (1979) 3895.
7 E. Eisenberg and L. E. Greene, Ann. Rev. Physiol., 42 (1980) 293.
8 M. Nyitrai, A. G. Szentgyörgyi and M. A. Geeves, Biochem. J., 365 (2002) 19.
9 A. Malnasi-Csizmadia, D. S. Pearson, M. Kovacs, R. J. Wooley, M. A. Geeves and C. R. Bagshaw, Biochemistry, 40 (2001) 12727.
10 D. I. Levitsky, V. L. Shnyrov, N. V. Khvorov, A. E. Bukatina, N. S. A. E. Vedenkina, E. A. Permyakov, O. P. Nikolaeva and B. F. Poglazov, Eur. J. Biochem., 209 (1992) 829.
11 A. A. Bobkov and D. I. Levitsky, Biochemistry, 34 (1995) 9708.
12 E. Kim, E. Bobkova, Gy. Hegyi, A. Muhlrad and E. Reisler, Biochemistry, 41 (2002) 86.
13 C. C. Goodno, Proc. Natl. Acad. Sci. USA, 76 (1979) 2620.
14 L. Zhao, J. Gollub and R. Cooke, Biochemistry, 35 (1996) 10158.
15 S. Highsmith and D. Eden, Biochemistry, 29 (1990) 4087.
16 D. Lőrinczy and J. Belagyi, Eur. J. Biochem., 268 (2001) 5970.
17 K. Samejima, M. Ishioroshi and T. Yasui, Agric. Biol. Chem., 47 (1983) 2373.
18 D. Lőrinczi, U. Hoffmann, L. Pótó, J. Belagyi and P. Laggner, Gen. Physiol. Biophys., 9 (1990) 589.
19 A. Setton and A. Muhlrad, Arch. Biochem. Biophys., 235 (1984) 411.
20 D. Lőrinczy and J. Belagyi, Biochem. Biophys. Res. Commun., 217 (1995) 592.
21 L. King and S. Lehrer, Biochemistry, 28 (1989) 3489.
22 A. Bertazzon, G. H. Tian and T. Y. Tsong, Biophys. J., (1988) 236a.
23 M. Zolkiewski, M. J. Redowicz, E. D. Korn and A. Ginsburg, Arch. Biochem. Biophys., 318 (1995) 207.
24 D. Lőrinczy and J. Belagyi, Thermochim. Acta, 259 (1995) 153.
25 A. J. Fisher, C. A. Smith, J. Thoden, R. Smith, K. Sutoh, H. M. Holden and I. Rayment, Biophys. J., 68 (1995) 19s.
26 J. Belagyi, I. Frey and L. Pótó, Eur. J. Biochem., 224 (1994) 215.
27 N. Hartvig, D. Lőrinczy, N. Farkas and J. Belagyi, Eur. J. Biochem., 269 (2002) 2168.
28 D. D. Thomas and R. Cooke, Biophys. J., 32 (1980) 891.
29 P. G. Fajer, Proc. Natl. Acad. Sci. USA, 91 (1994) 937.