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EFFECT OF OXYGEN FREE RADICALS ON MYOSIN IN MUSCLE FIBRES
A DSC and EPR study

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Abstract

Differential scanning calorimetry (DSC) and electron paramagnetic resonance spectroscopy (EPR, both conventional and saturation transfer EPR) were used to study the motional dynamics and segmental flexibility of myosin in muscle fibres in the presence of free radical generating system.

Muscle fibre bundles isolated from psoas muscle of rabbit were spin-labelled with maleimide- and isothiocyanate-based probe molecules at the reactive sulfhydryl sites (Cys-707) of the motor domain. In the presence of hydroxyl free radicals the spectral intensity of the maleimide probe molecules decreased with time following a single exponential curve. MgADP and MgATP plus orthovanadate that produce flexibility changes in the multisubunit structure of myosin enhanced the reduction of the attached nitroxide molecules in free radical generating system. The analysis of the EPR spectra of spin-labelled and oriented fibres showed that the narrow distribution of spin labels changed in the presence of hydroxyl free radicals. Spectrum analysis by computer subtraction showed that short irradiation by UV light resulted in the enhancement of the ordered population at the expense of the disordered population. This suggests a transition of myosin heads from weak-binding state into strong-binding state.

DSC measurements performed on calf cardiac myosin resulted in two main transitions at 49.4 and 54.1°C, respectively. Addition of MgADP produced a decrease of the 49.4°C transition, whereas a shift towards higher temperature was detected at the 54.1°C transition. It shows that there is an inter-site communication between the domains of the myosin. Hydroxyl free radicals induced further shifts of the transition temperatures and affected the width of the heat absorption curves.

Keywords: DSC-measurement, oxygen free radicals, skeletal myosin, spin-labelling

Introduction

Current models of muscle contraction assume that force is generated by actin-myosin interaction coupled to the ATPase cycle. In the absence of nucleotides the head region of myosin (the catalytic domain) is rigidly attached to actin and forms a com-

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plex. In the presence of MgATP, the chemical energy liberated from ATP hydrolysis produces conformational changes in the actin-binding region of myosin. These structural changes produce internal motion of myosin head while bound to actin, and it makes the muscle to shorten [1–3]. The relationship between the energy transduction and the conformation of the intermediate states during the hydrolysis process is a crucial question of the muscle contraction.

Previous spectroscopic and thermodynamic measurements reported that proteolytic removal of the LC-2 light chain from cardiac myosin produced remarkable structural and dynamic changes in the myosin motor [4]. The dissociation of LC-2 light chains from myosin was also observed in cardial ischemic injury [5, 6]. During this process the generation of superoxide free radicals was recorded. In model experiments we showed that oxygen free radicals affected the local and global conformation of cardiac myosin [7]. It was experienced that oxygen free radicals modified the contractile proteins in time- and concentration-dependent manner. The essential –SH groups (Cys-707, Cys-697) locating close to the nucleotide binding site can be oxidized by the free radicals, and this alters the rate of the ATP hydrolysis and affinity of myosin for actin [8]. It was also published in an earlier paper that mild reactive oxidising agents are also able to generate free radicals in organic reactions and in –SH containing biological systems [9, 10].

Spectroscopic and DSC studies on skeletal myosin and muscle fibres showed that nucleotides, ADP or ATP plus orthovanadate induced significant increase of rotational mobility of the bound labels in the environment of the nucleotide binding sites, and shifts of the thermal transitions were observed in the DSC pattern after nucleotide binding to myosin [11–16]. In this study an attempt is made to observe data on skeletal myosin in muscle fibres in the presence of free radical generating system using EPR technique and paramagnetic probes which report upon molecular motions, and applying DSC which provides information about the structural stability. Using maleimide- and isothiocyanate-based paramagnetic probes attached to the thiol groups of myosin, the spectral intensity of probe molecules was significantly affected by hydroxyl free radicals. DSC measurements support the view that global conformational changes accompanied the effect of oxygen free radicals.

Materials and methods

Preparation and spin-labelling of muscle fibres

Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas muscle. Small stripes of muscle fibres (30–40 mm in length and 1 mm in diameter) were soaked in rigor solution (100 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 20 mM Tris. HCl, pH 7.0) plus 1% Triton X-100 for 1 h at 4°C and then transferred to rigor buffer plus 50% v/v glycerol for 1 h. The fibre bundles were stored overnight in 50% v/v glycerol and rigor buffer at 4°C. This procedure was repeated on the second day, then the fibre bundles were stored in 50% v/v glycerol plus rigor buffer in refrigerator at –18°C for 3 days to 1 month before use.

Spin-labelling of fibres was performed in relaxing medium (rigor solution plus 2 mM pyrophosphate at pH 7.0) either with 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (4-isothiocyanato-TEMPO, TCSL) or 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO, MSL). Thin bundle of fibres was reacted for 2 h with 2 moles of TCSL or with 2 moles of MSL per mole of myosin for 60–90 min over ice. After spin-labelling the fibre bundles were washed in great amount of rigor buffer plus 25 mM $K_3Fe(CN)_6$ for 16 h to remove the unreacted labels and to reduce labels bound to weakly immobilizing sites [17].

Free radical generation in samples

The spin-labelled muscle fibres were irradiated with a 200 W mercury lamp in the presence of 8 mM hydrogen peroxide from a distance of 50 cm in the same quartz sample cell used for EPR measurements. Hydrogen peroxide was added immediately before irradiation. To avoid the warming of the sample a heat filter was used. The time for irradiation was 90 s. Longer irradiation produced higher concentration of $\dot{O}H$ radicals that quickly reduced the MSL or TCSL labels bound to the essential thiol sites.

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). The microwave magnetic field intensity was determined with peroxylamine disulphonate ion radicals in the same sample cell as for the muscle samples following Fajer and Marsh [18].

DSC measurements

Thermal unfolding of calf myosin in the presence of hydroxyl free radical generating system and nucleotide (ADP) was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were done between 10 and 80°C with 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 ±0.5 mg. There was no need to perform any correction from the point of view of heat capacity between the sample and reference vessels.

Results and discussion

Characterization of the labelled sites

The method used to label the reactive thiol sites of myosin was essentially the same as described earlier by Thomas and colleagues [12] and Belágyi [15]. The degree of labelling was 0.15–0.25 mol label/mol protein for TCSL and 0.35–0.45 mol label/mol protein for MSL. The EPR spectra of both MSL- and TCSL-myosin in rigor state of the fibres showed strong dependence on orientation with respect to the fibre axis indicating that the *z*-axis of the magnetic tensor was oriented nearly perpendicular to the longer axis of the muscle fibres (Figs 1A and 1B). Rigorous analysis of spectra showed that TCSLs had two distinct orientations, the angles between the principal axis (*z*) of the spin label and the fibre axis were 56° and 75°, the fractions were 0.76 and 0.24, respectively [15]. From the EPR spectra we could conclude that in rigor the labels were immobilized on the time scale of the conventional EPR; in ST EPR time domain a small internal motion was detected even in rigor state; the effective rotational correlation time was about 100 μs calculated from the intensity ratio of the first two diagnostic peaks (L''/L=0.90±0.1, n=4; Fig. 1C).

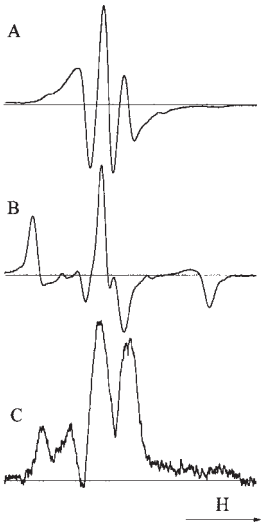

Fig. 1 Conventional EPR spectra of TCSL-muscle fibres in rigor. The TCSL-fibre bundles were oriented parallel (A) and perpendicular (B) to the laboratory magnetic field. (C) ST-EPR spectrum of fibres in perpendicular orientation. The field scan was 10 mT

Effect of UV irradiation on muscle fibres

UV irradiation in the presence of hydrogen peroxide generates hydroxyl free radicals in the buffer solution. The double integrals of the EPR spectra of MSL-fibres were compared with the double integral of a known concentration of spin label solution us-

ing the same sample cell and the same spectral parameters. Figure 2 shows the concentration of the maleimide spin labels attached to myosin as a function of time of UV irradiation. The semi-logarithmic plot of the spectral intensity gave a straight line evidencing that the interaction of the attached spin labels with the generated hydroxyl free radicals followed a pseudo first-order chemical reaction. The characteristic time of the reaction was 1.3 min.

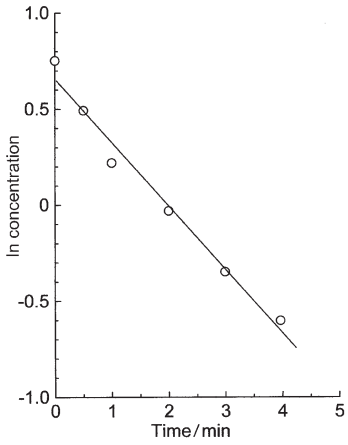

Fig. 2 Semi-logarithmic plot of spectral intensity of spin-labelled muscle fibres on the time of irradiation by UV light. Muscle fibres were spin-labelled with MSL. The samples were irradiated immediately before EPR measurements with a 200 W high-pressure mercury lamp from a distance of 50 cm

Since the reactive sulfhydryl sites are near to the nucleotide binding pocket in the crystal structure of myosin [19], in the presence of nucleotides changes are expected in the environment of the probe molecules. In solution containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ADP, 1 mM EGTA, 100 μM P1,P5-di(adenosine-5')pentaphosphate (Ap_5A), in 10 mM histidine. HCl buffer, pH 7.0, the conventional EPR spectra of fibres labelled with TCSL showed large changes in the probe distributions. In contrast to this observation the low-angle X-ray diffraction studies reported that the angle of attachment for the myosin head in the ternary actin-myosin-ADP complex was the same as in rigor muscle [20]. This supports the view that the TCSL reports internal changes in the environment of the labelled sites without significant global reorientation of the heads.

The addition of orthovanadate ions with ATP led to a drop in tension, therefore it was proposed that the myosin heads with MgADP.V_i are in weakly binding state to actin. The lineshape of the EPR spectrum (bottom spectrum in Fig. 3) supports the suggestion that the myosin heads are in loosely bound state or the heads are already dissociated from the actin filaments, because almost no orientation dependence could be detected.

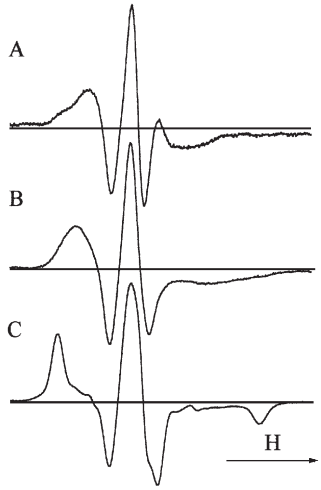

Fig. 3 Conventional EPR spectra of TCSL-fibres in parallel orientation to the laboratory magnetic field in the presence of MgADP and MgATP+V_i.
Symbols: (A) rigor state; (B) AM.ADP state; (C) AM^+.ADP.V_i state. The fibres were kept in rigor buffer plus 4.0 mM MgADP and 4.0 mM MgATP plus 2.0 mM $NaVO_3$ for 5 min at 0°C before UV irradiation

The spectral intensity of the MSL- or TCSL-myosin changed immediately after addition of 8 mM H_2O_2. It indicated that hydroxyl free radicals were already generated in the buffer solution containing nucleotide and H_2O_2. After UV irradiation the change of the spectral intensity of the samples was more pronounced (Table 1). In some cases, when the time of irradiation was increased, a singlet superimposed on the spin label signal was also detected. Under specific conditions in muscle fibres we estimated the portion of the –SH groups participating in the reaction with hydroxyl free radicals [9]. The comparison of the spectral intensity of the protein sample with the rate of the ATP hydrolysis showed that about 40% of the essential thiols reacted with the hydroxyl free radicals. The decrease of the spectral intensity was accompanied by a change of the lineshape of the EPR spectrum in parallel orientation. The change was more pronounced in the case of ADP.V_i state. Spectral analysis by digital subtraction showed that there was a significant increase –6–14 % of the total absorption – in the portion of the highly ordered population of spin labels observed in rigor at the expence of the disordered population detected in ADP.V_i state. This supports the view that the hydroxyl free radicals interact not only with the spin labels located on the essential thiol site of the protein, but affect the intermediate states of the ATP hydrolysis cycle. It was reported earlier on skeletal myosin that irradiation by UV light modified the rate of ATP hydrolysis, and accelerated the dissociation of ADP and V_i [21]. The dissociation of the products leads to the rapid increase of the rigor population of myosin heads.

Table 1 Concentration of attached TCSL in myosin in the presence of oxygen free radicals

Protein state	Concentration of label/ ($\cdot 10^8$)	Treatment
Rigor	8.523	
ADP	2.077	8 mM H_2O_2
ADP	0.521	8 mM H_2O_2+5 min UV irr.
ADP	1.145	8 mM H_2O_2
ADP	0.512	8 mM H_2O_2+5 min UV irr.
ADP	2.304	8 mM H_2O_2
ADP	1.426	8 mM H_2O_2+3 min UV irr.

Muscle fibres were spin-labelled with two moles of TCSL to 1 mol of myosin for 180 min at 0°C in 80 mM Kpropionate, 5 mM $MgCl_2$, 1 mM EGTA and phosphate buffer, pH 6.5. Unreacted labels were removed by dialysis overnight against the same buffer solution at 4°C. The free radical generation was performed by addition of 5 mM MgADP and 8 mM H_2O_2 immediately before EPR measurement

DSC measurements

Two conformations among a lot of intermedier states of muscle contraction were investigated: the rigor and the MgADP or strong-binding state of calf cardiac myosin. In each experiment three subsets were used, the 'native', H_2O_2 treated (these states are not presented on figures), and H_2O_2 treated plus UV irradiated samples.

The thermal denaturation of rigor state shows a usual DSC scan with two big melting ranges in the main transition (Fig. 4). In the present of hydrogen peroxide the

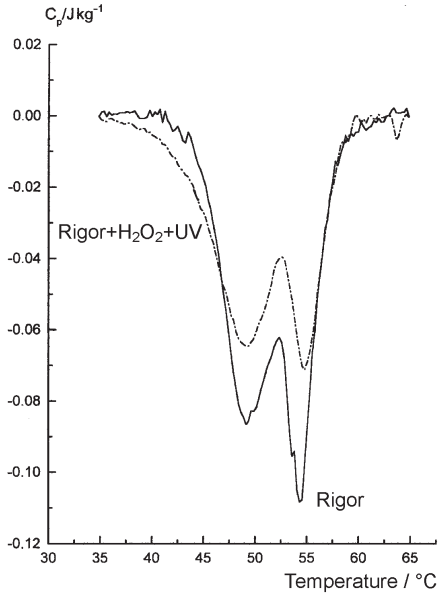

Fig. 4 DSC profiles of cardiac myosin in nucleotide-free-states. The heat flow in mW units is plotted *vs.* temperature (endothermic deflection is directed downwards). The UV irradiation lasted for 90 s

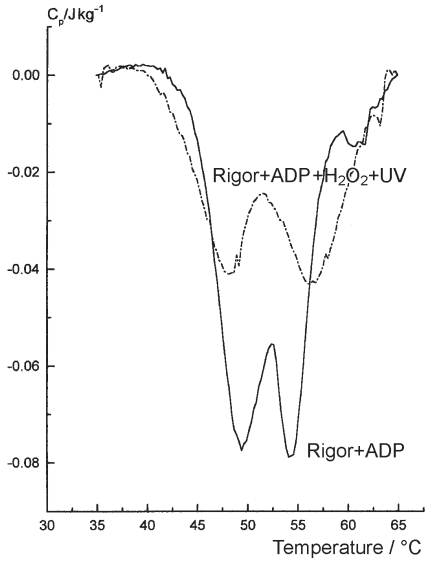

Fig. 5 Melting behaviour of bovine cardiac myosin in the presence of 4 mM MgADP. Note the increase of separation between the two main transitions and the changes after UV irradiation

separation of two main transitions is more pronounced including a temperature shift of catalytic domain to a lower (46.9°C) and for the rod part to the higher temperature (55.1°C).

In strong-binding state the effect of hydrogen peroxide is more effective, the catalytic domain becomes less stabile (T_{m1}=45.5°C) while the rod part shows an increased thermal stability (T_{m2}=56.3°C). After an UV irradiation of treated samples in rigor (Fig. 4) we can observe only a broadening of the melting temperature range practically with the same transition temperatures while in MgADP state (Fig. 5) the catalytic domain will be less (47.9°C) and the rod part of myosin earns more (56.1°C) thermal stability. In both states there is significant decrease in the total enthalpy change.

The experiments support the view that both local and global conformational changes play an important role in the interaction of oxygen free radicals with motor proteins which lead to protein damage. The photomodification produced by UV irradiation on myosin could influence the internal flexibility and rotational state of the myosin heads, and could also transfer a portion of heads into rigor state. Moreover, it cannot be excluded that the free radicals generated by the irradiation can also influence the power of the myosin motor as well. It seems further work is needed to explain in details the relations among dynamics of myosin heads in fibres and the effect of free radicals.

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