Effect of oxygen free radicals on myosin in muscle fibres

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Abstract Experiments were performed on glycerol-extracted muscle fibres prepared from psoas muscle of rabbit in the presence of hydroxyl free radical generating system. Short irradiation of spin-labelled muscle fibres by UV light showed the interaction of probe molecules with oxygen free radicals. The intensity of the EPR signal from maleimide or isothiocyanate spin labels attached to the essential thiol groups decreased following irradiation. Oxygen free radicals affected the rate constant of the transition AM.ADP.V_i → AM.ADP in the ATP hydrolysis cycle. It was found that the essential –SH groups of myosin were involved in the oxidation of sulphydryls by Ce(IV). Ce(IV) complexed to nitrilotriacetic acid in the presence of spin trap produced long-lived free radicals located partly on SH-1 sulphydryls.

Key words: Myosin; Oxygen free radical; Spin-trapping

1. Introduction

According to previous spectroscopic and thermodynamic measurements the proteolytic removal of the LC2 light chain from cardiac myosin produces remarkable structural and dynamic changes in the myosin motor [1]. It is also known that dissociation of LC2 light chains is induced after myocardial ischaemic injury [2]. During this process the oxygen free radicals might interact with the active centre of myosin and/or the essential –SH groups (Cys-707, Cys-697) locating close to the nucleotide binding site, altering the rate of the ATP hydrolysis and affinity of myosin for actin. In relation to free radical reactions, it was demonstrated that superoxide dismutase treatment produced marked improvement in cardiac contractile function [3]. Modification of SH-1, the most reactive thiol, inactivates the K^+-EDTA ATPase while simultaneously activating the Ca^{2+}-ATPase activity [4,5]. The blocking of the essential thiols leads to change in the force development of the muscle concentration, and it might result in changes in the lifetime of the intermediate states. Mild reactive oxidising agents are able to generate thyil radicals in organic reactions and in –SH containing biological systems [6,7].

In this paper we demonstrate by EPR measurements on glycerol-extracted muscle fibres that the essential –SH groups are involved in the interaction of oxygen free radicals with myosin. In the presence of hydroxyl free radical generating system the intensity of the EPR signal arising from the spin labels bound to Cys-707 rapidly decreases. Moreover, oxygen free radicals increase the rate constant of the transition AM.ADP.V_i → AM.ADP in the ATP hydrolysis cycle. The use of Ce(IV), as a mild oxidising agent, provides evidence for selective attack on myosin in glycerinated muscle fibres at particular residues.

2. Materials and methods

2.1. Preparation of muscle fibre

Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas muscle. Small strips of muscle fibres (30–40 mm in length and 1 mm in diameter) were soaked in buffer solution (100 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 20 mM Tris-HCl, pH 7.0) plus 1% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride for 2 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 buffer at 4°C. This procedure was repeated on the second day, then the fibre bundles were stored in 50% v/v glycerol plus buffer in the refrigerator at −18°C for 3 days to 1 month before use.

2.2. Spin-labelling of muscle fibre

Spin-labelling of fibres was performed in relaxing medium (rigor solution plus 2 mM pyrophosphate at pH 7.0) with about 2 mol of 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) to 1 mol of myosin for 3 h at 0°C. In a few experiments 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MSL) was used. The composition of the rigor buffer was 80 mM K-propionate, 5 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris-HCl, pH 7.0. The spin labels were obtained from Sigma (Grünwalder, Germany). After spin-labelling the fibre bundles were washed in a large amount of rigor buffer for 16 h to remove the unreacted labels. In some cases 25 mM $K_3Fe(CN)_6$ was added to the rigor buffer to reduce labels bound to weakly immobilising sites [8]. The spin-labelled muscle fibres were stored in rigor solution for no longer than 24 h at 4°C before EPR measurements. The sarcomere lengths of the fibres were measured as reported earlier [9].

2.3. Spin-trapping of PBN in muscle fibres

The fibre bundles were preincubated in rigor buffer for 15 min and then immersed in the same solution containing 5 mM Ce(IV)-nitrilotriacetic acid plus 30 mM PBN. The Ce(IV) solution was prepared by adding 1 volume of 50 mM $(NH_4)_2Ce(NO_3)_6$ to 9 volumes of 10 mM nitrilotriacetic acid, 50 mM Tris at pH 7.0 with vigorous stirring. After 10 min incubation the fibre bundles were immediately transferred to EPR flat cells.

2.4. UV irradiation of muscle fibres

The spin-labelled muscle fibres were irradiate with a 200 W mercury lamp from a distance of 50 cm in the same quartz sample cell used for EPR measurements. To avoid warming of the muscle sample a heat filter was used. The time for irradiation was 90 s. Longer irradiation produced a higher concentration of $^{\bullet}OH$ radicals that quickly reduced the TCSL labels bound to the cross-bridges, and the newly generated EPR spectrum suppressed the spectrum from spin labels. In some cases 4-isothiocyanato-2,2,6,6-tetramethylpiperidine (ITC) was used instead of TCSL, to study the effect of oxygen free radicals on muscle fibres. When the irradiation was performed on ITC-labelled muscle fibres, an EPR spectrum was detected arising from strongly immobilised free radicals (Fig. 1). This suggests that the illumination led to nitroxide formation.

2.5. ATPase activity

The K^+-EDTA ATPase activity of myosin in muscle fibres was measured by determining the rate of release of inorganic phosphate as described earlier [9]. The activity was measured in solution containing 600 mM KCl, 5 mM EDTA, 5 mM ATP and 50 mM TES, pH

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7.0. From the solutions aliquots were taken at times $t = 1$, 2, 4, 6 and 8 min and pipetted into the colour reagent according to Lanzetta and co-workers [10] and the optical absorbance was read at 660 nm using a Hitachi 124 spectrophotometer. The rate of release of the inorganic phosphate was obtained from the linear plot of absorbance against time. The activity of control fibres was 0.0816 μM P_i/mg myosin/min, whereas the Ce(IV)-PBN-treated fibres exhibited a reduced ATPase activity: 0.0454 μM P_i/mg myosin/min.

2.6. EPR measurements

Conventional and ST EPR spectra were taken with ESP 300E (Bruker, Germany) spectrometer. First harmonic, in-phase, absorption spectra were obtained using 20 mW microwave power and 100 kHz field modulation with an amplitude of 0.1–0.2 mT. Second harmonic, 90° out-of-phase, absorption spectra were recorded with 63 mW and 50 kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out-of-phase. The 63 mW microwave power corresponds to an average microwave field amplitude of 0.025 mT in the central region of the standard tissue cell of Zeiss (Carl Zeiss, Germany), and the values were obtained using the standard protocol of Fajer and Marsh [11]. In this region of the tissue cell, small segments of the muscle fibres (6–7 mm long) were mounted parallel to each other. The spectra were recorded in two positions at a temperature of 22 ± 1°C, where the longer axis of the fibres was oriented parallel and perpendicular to the laboratory field. The number of spin labels bound to myosin was determined from the EPR spectra of muscle fibres by comparing the double integrals of the spectra with known concentration of TCSL in aqueous solution in the same sample cells; it was 0.28 ± 0.07 ($n = 12$). The double integral of the spectra was normalised to unity for spectrum manipulation.

2.7. Analysis of spectra

In the MgADP.V_i state of the fibres and after UV irradiation, the superposition of two or more spectral components was recorded, and the fractions of the populations depended on the state of the muscle fibres. In order to determine whether the spectra from TCSL-labelled fibres in different states could be composed of a linear combination of spectra, manipulations were performed on normalised EPR spectra by digital subtraction. In the case of the AM^+.ADP.V_i state, the spectrum of fibres detected in the AM.ADP state (Fig. 2B) was subtracted from the EPR spectrum of fibres obtained after MgADP+V_i)-induced conformational changes (Fig. 3B). During this procedure the fraction of the ADP spectrum was varied until phase inversion appeared.

3. Results and discussion

3.1. Effect of MgADP and orthovanadate

In contrast to MSL fibres that report global motion, the TCSL fibres indicated that the binding of MgADP induced a large change in the mean orientation of the labels. It can be demonstrated by the significantly different conventional EPR spectra at parallel orientation of fibres in rigor and ADP state and by the different distribution density functions (Fig. 2A,B)

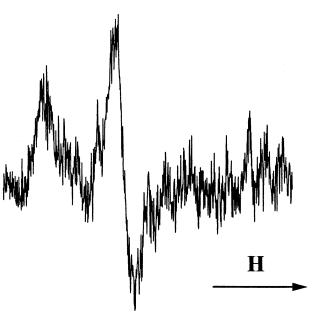

Fig. 1. EPR spectrum of ITC-labelled muscle fibres after short UV irradiation. The fibres were irradiated for 5 min in rigor buffer containing 8 mM H_2O_2, and immediately afterwards the sample was measured. The field scan was 10 mT.

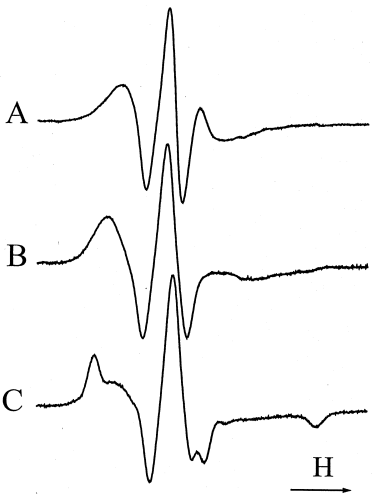

Fig. 2. EPR spectra of TCSL fibres. Symbols: A: rigor state; B: MgADP state; C: MgADP.V_i state. The fibres were oriented parallel to the laboratory magnetic field. The field scan was 10 mT.

[9]. MgADP+orthovanadate increased the orientation disorder of myosin heads, and a random population of spin labels was superimposed on the ADP-like spectrum evidencing conformational and motional changes in the internal structure of the myosin heads (Fig. 2C) [12]. Deconvolution of spectra yielded fractions for a 0.34:0.66 mixture of highly oriented and disoriented labels. The results on fibres in the AM^+.ADP.V_i state showed that the fraction of the disoriented population depended on whether MgADP of MgATP was used. When the fibres were incubated in rigor buffer plus MgATP and V_i for 15 min, no highly oriented fraction was observed implying the dissociation of heads from actin, or the non-specific binding of cross-bridges on actin.

3.2. Effect of UV irradiation on muscle fibres in the $AM^+.ADP.V_i$ state

Short irradiation (30 s) of control muscle fibres in the quartz sample cell used for EPR observations generated hydroxyl free radicals in the presence of H_2O_2 [13]. The EPR signal recorded had no hyperfine structure, a broad singlet was observed (Fig. 3, top spectrum), its spectral intensity increased with the time of irradiation. Longer irradiation of spin-labelled fibres produced a significant decrease of the spectral intensity of the TCSL signal, therefore as a compromise 90 s was chosen for irradiation. Using MSL fibres and increasing the duration of the irradiation, the double integral of the spectra decreased with a time constant of about 2 min. During this study the differences in irradiation between the different states of the muscle fibres were not followed. So far, we have no evidence that $^{\bullet}$OH radicals influence differently the intermediate states of the myosin motor. The $^{\bullet}$OH free radicals interacted very quickly with myosin and with the spin labels located on it, resulting in the reduction of the bound TCSL labels.

Immediately after UV irradiation a composite EPR spectrum was recorded (Fig. 3B). As a first step of the spectrum analysis, the spectrum obtained after UV irradiation of control fibres was subtracted from the complex spectrum. The contribution of the $^{\bullet}$OH free radicals to the total absorption

was about 15–20%. In the second step, the spectrum recorded in the presence of MgADP and orthovanadate (Fig. 2C) was subtracted from the same spectrum obtained after UV irradiation. The residual spectrum (Fig. 3C) resembles the spectrum obtained in rigor (bottom spectrum in Figs. 3 and 2A). The concentration of this fraction varied between 5 and 11% of the total absorption. More precise determination of this fraction was not possible because of the large dispersion in the estimation of the spectral intensity produced by UV irradiation. The results provide strong evidence that UV irradiation induced a process that enhanced the dissociation of ADP and V_i from the myosin heads, and forced the transition of cross-bridges from weakly binding state into rigor. It was shown in an earlier paper that irradiation with UV light modified covalently the stable MgADP-V_i-myosin subfragment 1 complex and induced the release of trapped MgADP and V_i [14]. Our experiment shows that the photomodification of myosin by UV light might take place even in a more complex system as well.

3.3. Oxidation of myosin by Ce(IV)

The mild oxidation of myosin by Ce(IV) in muscle fibres in the presence of PBN resulted in a strongly immobilised nitroxide EPR spectrum (Fig. 4A); the hyperfine splitting constant $2A'_{zz}$ was equal to 6.391 ± 0.016 mT ($n = 6$). ST EPR measurements revealed that the rotational correlation time is in the millisecond time range (Fig. 4B). The ratio of the first two diagnostic peaks L″/L is near to 1 ($L''/L = 0.918 \pm 0.017$; $n = 4$); e.g. the PBN spin adduct is very rigidly attached to the oxidised thiol group. The apparent rotational correlation time of the bound spin adduct is 170 μs taken from calibration curves [15]. In earlier papers [6,7] it has been suggested that the reaction

$$\text{myosin} - \text{SH} + \text{Ce(IV)} \rightarrow \text{Ce(III)} + \text{myosin} - {}^{\bullet}\text{S}$$

$$\text{myosin} - {}^{\bullet}\text{S} + \text{PBN} \rightarrow \text{myosin} - \text{S} - \text{PB}^{\bullet}\text{N}$$

took place after addition of Ce(IV)-nitrilotriacetic acid to myosin solution.

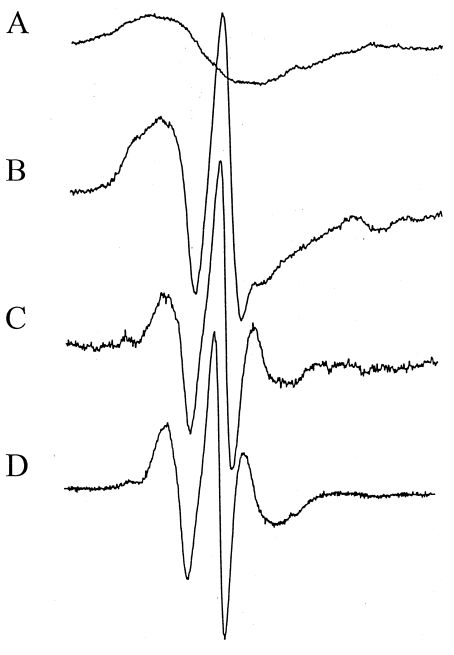

Fig. 3. EPR spectra of TCSL fibres after UV irradiation. Symbols: A: control fibres in MgADP.V_i state after 90 s irradiation; B: TCSL fibres in MgADP.V_i state after 90 s irradiation; C: rigor-like residual EPR spectrum after spectrum subtraction. The spectrum from Fig. 2C was subtracted from that of 3B; D: fibres in rigor state. The muscle fibres were oriented parallel to the laboratory magnetic field. The scan width was 10 mT.

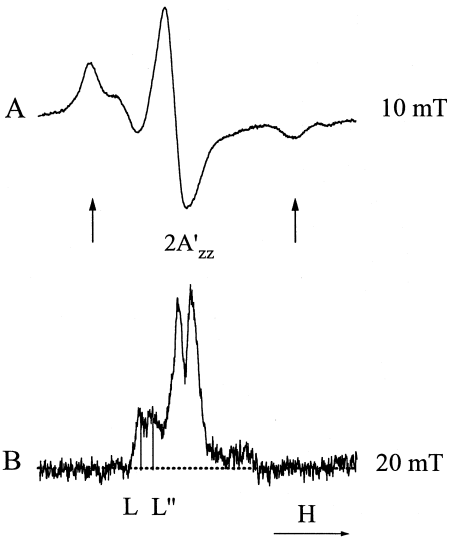

Fig. 4. A: Conventional EPR spectrum of muscle fibres after Ce(IV)-nitrilotriacetic acid treatment in the presence of 30 mM PBN. Fibres were incubated for 10 min and immediately measured. The field scan was 10 mT. B: ST EPR spectrum of the same sample. The fibres were oriented perpendicular to the laboratory magnetic field. The field scan was 20 mT.

The spectrum of Ce(IV)-PBN-treated muscle fibres exhibited no signal arising from weakly immobilised PBN spin adduct, implying that the nitroxide radicals were strongly bound to the protein structure. The average concentration of free radicals trapped by myosin was 0.28 mol PBN/mol myosin after 10 min incubation. It should be noted that efforts to obtain a spectrum that is characteristic of oriented nitroxide groups were not successful. Ordering of probe molecules in MSL and TCSL fibres in rigor and in the ADP state has already been reported [9,16]. The absence of orientation dependence might have two reasons: (i) not only the SH-1 groups are responsible for the observed EPR spectra, or (ii) the PBN nitroxide radical might have different orientations with respect to the longer axis of the muscle fibre after binding to the oxidised SH-1 thiol group. Graceffa [7] has shown in his experiments that the K^+-EDTA ATPase activity of myosin isolated from rabbit muscle did not change significantly after Ce(IV) oxidation and PBN spin-trapping. This would mean that the thiyl radicals involved in the oxidation process by Ce(IV) differ partly from the essential thiols. However, in muscle fibres the Ce(IV) plus PBN treatment decreased the K^+-EDTA ATPase activity of myosin that accompanied the modification of the essential thiols [17]. The fractional inhibition of the ATPase activity was 0.54, larger than would be expected on the basis of PBN free radical concentration. One explanation for that might be the short lifetime of PBN radicals located on the essential thiols. In a series of experiments the double integral of the PBN signal was recorded as a func-

tion of time. Under conditions used in our study the PBN signal followed a single exponential decay with a time constant of 13.5 min. The first possibility is supported by the data obtained on muscle fibres which were pretreated with *N*-ethylmaleimide or iodoacetamide before Ce(IV) plus PBN treatment. The muscle fibres were incubated in rigor buffer containing 1 mM *N*-ethylmaleimide for 1 h at 0°C. The incubation with iodoacetamide lasted for 5 h. The concentration (double integral) of the PBN signal decreased significantly; the mean value of 0.95 ± 0.05 μM free radical/mg wet muscle ($n = 6$) was reduced to 41% of the control value after treatment with *N*-ethylmaleimide, and 52% of the control value was obtained after iodoacetamide treatment. These values agree quite well with the value of the fractional inhibition of the ATPase activity, which suggests that at least half of the PBN molecules are located on the SH-1 sites.

Earlier experiments reported that the essential thiols are highly reactive even in the fibre system [18], the other thiol groups of myosin are buried in the organised structure of the muscle. Therefore, it is possible that only the essential thiols are accessible to Ce(IV). It cannot be excluded that Ce(IV)-nitrilotriacetic acid perturbs the local structure of myosin heads around the segment containing the essential thiols, and this affects the ordering of probe molecules in the head region of myosin and the ATPase activity of myosin independently of the state of the SH-1 groups. On the other hand, the perturbation can induce the loosening of the structure and other –SH groups are accessible to Ce(IV) as well.

In summary, the present study suggests a linkage between the function of the myosin motor and the particular effect of free radicals and oxidation on myosin. We might speculate that a suitable reducing agent which helps to keep the essential –SH groups of myosin in the intact state could contribute to the maintenance of the proper function in heart muscle.

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