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UV generated oxygen free radicals in cardiac myosin. DSC and EPR study

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Abstract

Differential scanning calorimetry (DSC) and electron paramagnetic resonance spectroscopy (EPR) were used to study the motional dynamics and segmental flexibility of cardiac myosin in the presence of free radical generating system.

Cardiac myosin isolated from calf heart muscle 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 attached to cardiac myosin decreased with time following a single exponential curve. MgADP and MgADP 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.

DSC measurements resulted in two main transitions at 49.4°C and 54.1°C, respectively. Addition of MgADP produced a decrease of 49.4°C transition, whereas a shift towards higher temperature was detected at 54.1°C transition. Hydroxyl free radicals induced further shifts of the transition temperatures and affected the width of the heat absorption curves. The total enthalpy of the UV irradiated myosin decreased significantly in different intermediate states of the ATP cycle.

Keywords: Cardiac myosin; Spin-labelling; DSC-measurement; Oxygen free radicals

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 is induced after myocardial ischaemic injury [2,3]. During this process the oxygen free radicals might interact with the active centre of myosin and/or the essential –SH groups altering the rate of the ATP hydrolysis and the affinity of myosin to actin. An exogenously administered, oxygen free radical-generating system has the capacity to cause cardiac dysfunction [4,5]. Myofibrillar creatine kinase, which is bound to myosin and localised at the M band of the sarcomere [6] has been demonstrated to be functionally coupled to myosin ATPase [7,8] and serves as an

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important intramyofibrillar ATP-regenerating system [9]. It was reported that inhibition of creatine kinase activity can result in dysfunction of the heart, especially at increased work loads [10]. Recently considerable data have been obtained about the effect of oxygen free radicals on sarcolemmal membrane [11–14], mitochondrial functions [15] and sarcoplasmic reticulum [16], but only a few data have been found about their effect on cardiac contractile protein [17,18]. Therefore experiments have been performed to investigate the effect of oxygen free radicals on calf cardiac myosin in the different intermediate states of the ATP hydrolysis cycle, namely in the nucleotide free state and in the MgADP state, by DSC and EPR techniques.

2. Materials and methods

2.1. Preparation of myosin

Calf heart myosin was prepared by the methods described by Shiverick et al. [19]. After washing of the tissue, myosin was extracted in buffer consisting of 0.6 M KCl, 20 mM imidazol, 1 mM DTT, 1 mM EDTA, pH 7.0. After centrifugation for 4 h at 0°C with 100 000 g, the crude myosin was purified using Sepharose 4B chromatography. The sample was precipitated with ice cold water, and thereafter the protein was collected by centrifugation and dissolved in 0.3 M KCl, 90 mM KH_2PO_4, 60 mM K_2HPO4, 2 mM $MgCl_2$ at pH 6.8. Protein concentration was determined by reading the absorption at 280 nm using absorption coefficient 0.55 mg ml^{-1} cm^{-1} for myosin.

2.2. UV irradiation of samples

The myosin solution was irradiated with a 200 W mercury lamp from a distance of 50 cm in a quartz sample cell. To avoid the warming of the samples a heat filter was used.

2.3. Calorimetric measurements

The thermal unfolding of muscle proteins in different states induced by ADP or ADP + V_i was monitored by a SETARAM Micro DSC-II calorimeter (SETARAM, France). All experiments were done between 0°C and 100°C with a scanning rate of 0.3°C/min. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μl sample volume on average. Rigor buffer was used as the reference sample. The sample and reference vessels were equilibrated with a precision of ±0.5 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 denaturated during each cycle. The repeated scan of denaturated sample was used as baseline reference which was subtracted from the original experiment. Calorimetric enthalpy was calculated from the area under the heat absorption curves using two points setting, SETARAM peak integration.

2.4. EPR measurements

The isolated protein was labelled 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). Myosin was reacted for overnight with 2 mol of TCSL or with 2–4 mol of MSL per mol myosin for 60–90 min over ice. After reaction the samples were dialyzed overnight at 4°C against great excess of buffer solution. Before EPR measurements the protein was clarified by centrifugation at 50 000 g for 1 h and used at a final concentration of 20–100 μM. The EPR measurements were taken with an ESP 300 E (Bruker, Germany) *X*-band spectrometer. For the conventional EPR technique, 100 kHz field modulation (0.1–0.25 mT amplitude) and 2–20 mW microwave power were used. The double integrals of the spectra were normalized to unity for spectrum manipulation.

3. Results and discussion

Two conformations among a lot of intermediate 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, and H_2O_2 treated plus UV irradiated samples (Fig. 1a–c).

The thermal denaturation of rigor state shows a usual DSC scan with two big melting ranges in the

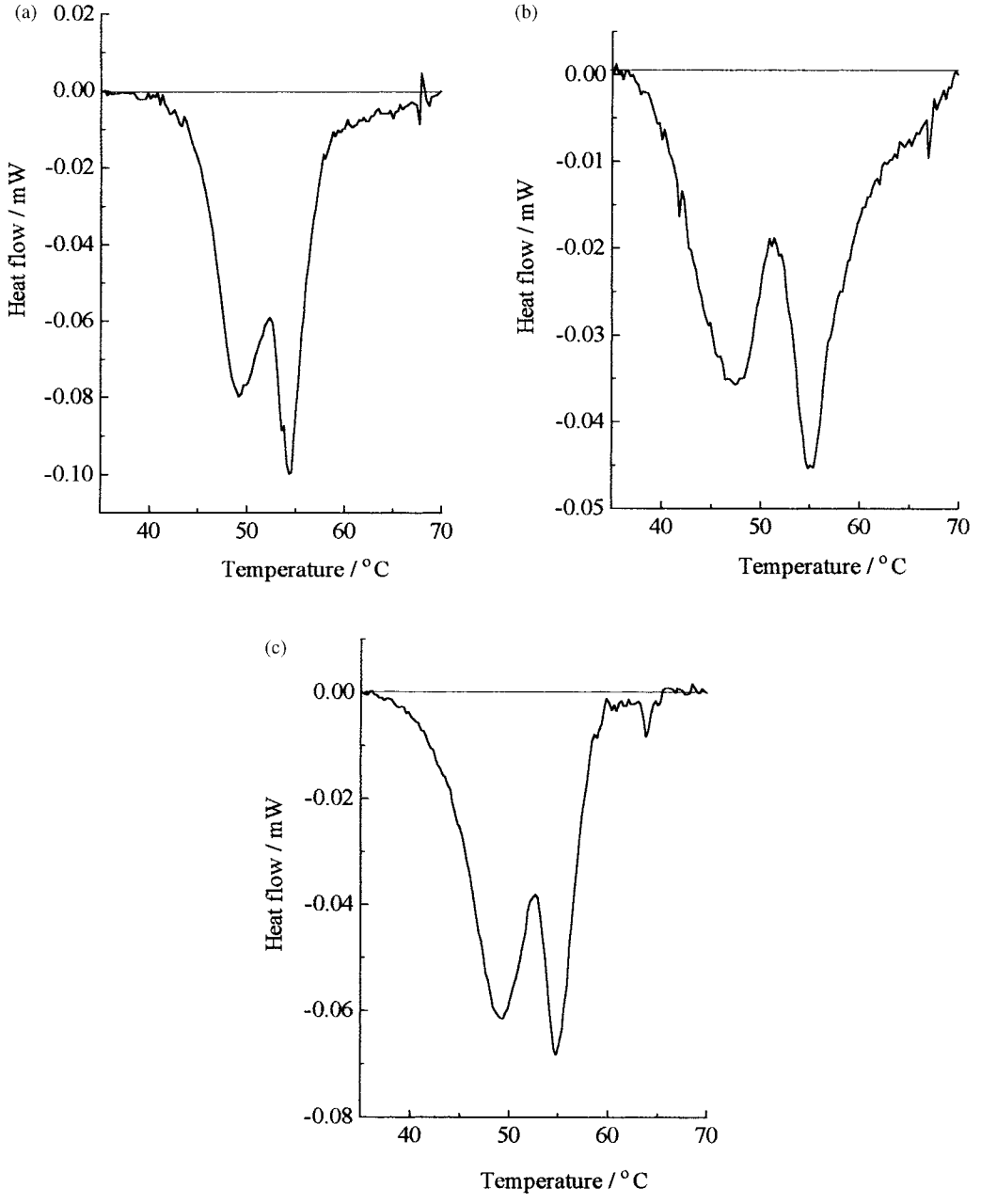

Fig. 1. (a) DSC curves of cardiac myosin (prepared from calf heart) in rigor; (b) in rigor treated with H_2O_2; (c) as well as in rigor treated with H_2O_2 after 4 min UV irradiation.

main transition (Fig. 1a). In present of hydrogen peroxide the separation of two main transition 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, Fig. 1b).

In the 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, Fig. 2b). After an UV irradiation of

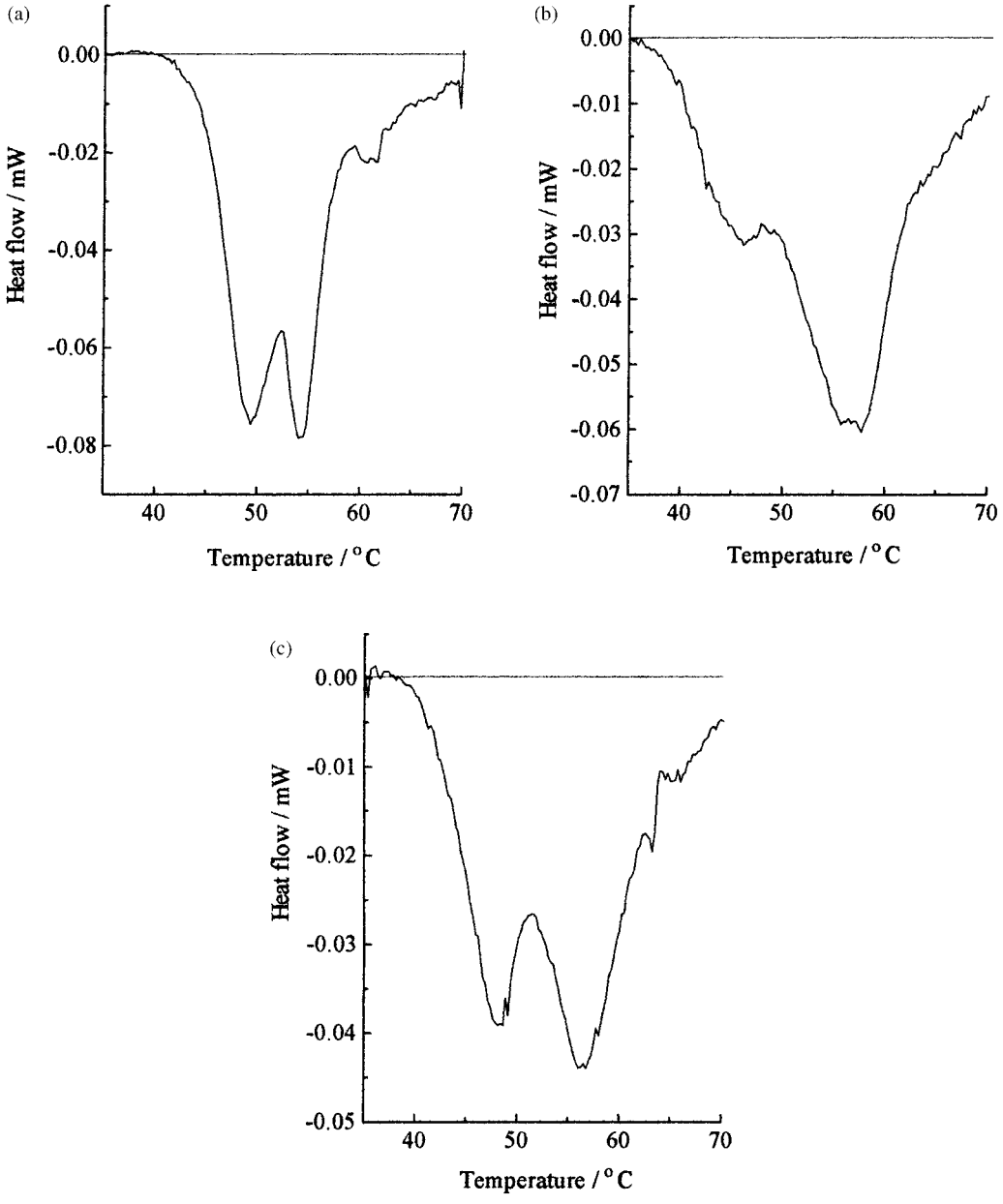

Fig. 2. (a) Thermal denaturation curves of calf cardiac myosin in strong-binding state (MgADP); (b) nucleotide binding state treated with H_2O_2; (c) the (b) state after 4 min UV irradiation.

treated samples in rigor we can observe only a broadening of the melting temperature range practically with the same transition temperatures while in MgADP state the catalytic domain will be less (47.9°C) and the rod part of myosin has more (56.1°C) thermal stability. In both state there is significant decrease in the total enthalpy change.

After UV irradiation the main profile of the thermal transition did not change basically, no new transition was detected. It indicates that the hydroxyl free radi-

cals interacted with the protein did not induce large structural changes in the internal structure of the cardiac myosin. However, the remarkable shifts of the transition temperatures and the changes of the form of the heat absorption curves show that the interaction of the free radicals with the side chains of the protein resulted in the altered affinity of myosin to nucleotides and in the altered flexibility of the internal structure of the protein (Figs. 1 and 2). The modifications can contribute to the change of the ATPase activity and to the increased probability of the LC2 light chain dissociation observed in cardiac muscle after ischaemic injury. It should be noted that after addition of 8 mM H_2O_2, shifts and enthalpy changes were already observed in the heat absorption curves. UV irradiation induced further changes in the DSC patterns, but the total DSC profile approximated the nucleotide-free DSC profile. It indicates that not only the local conformation, but the global conformation of the cardiac myosin reflected the enhanced dissociation of the hydrolysis products from the nucleotide binding site, similarly to EPR measurements.

3.1. Characterization of the labelled sites

The method used to label the reactive thiol sites of cardiac myosin was essentially the same as described earlier by Thomas and co-workers [20] and Belagyi [21] for skeletal muscle myosin. 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 cardiac myosins showed the superposition of spectra from strongly and weakly immobilized labels (Fig. 3). This showed that the labels were located on a specific site of the protein, very likely on Cys-707. The conventional EPR spectra were characterized by the distance between the outermost hyperfine extrema $2A'_{zz}$. The hyperfine splitting for myosin was 6.456 ± 0.03 mT ($n = 4$) at room temperature.

3.2. Interaction of spin labelled myosin with oxygen free radicals

UV irradiation in the presence of hydrogen peroxide generates hydroxyl free radicals in buffer solution. Fig. 4 shows the concentration of MSL spin labels

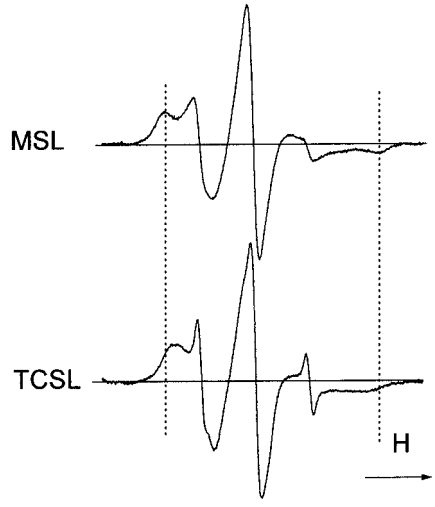

Fig. 3. The EPR spectra of MSL and TCSL labelled cardiac myosins. The hyperfine splitting of MSL-myosin is larger than that of TCSL-myosin evidencing that the MSL labels are more rigidly attached to the reactive thiol site –SH1.

attached to myosin as a function of time of UV irradiation. The semi-logaritmic plot of the spectral intensity gave a straight line evidencing that the interaction of the attached spin labels with the gen-

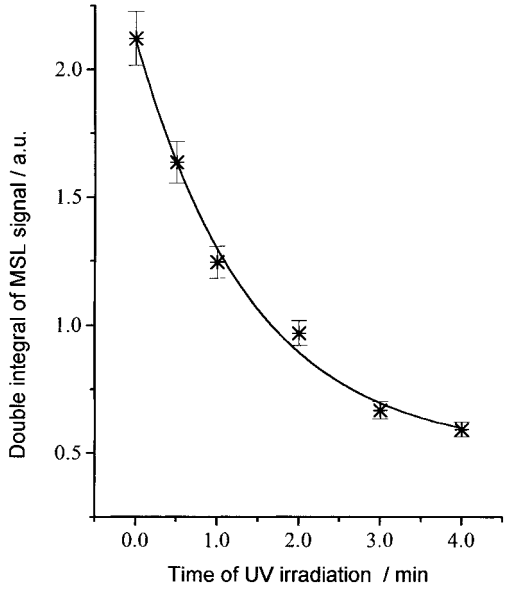

Fig. 4. The concentration of MSL spin labels attached to myosin as the function of time of UV irradiation.

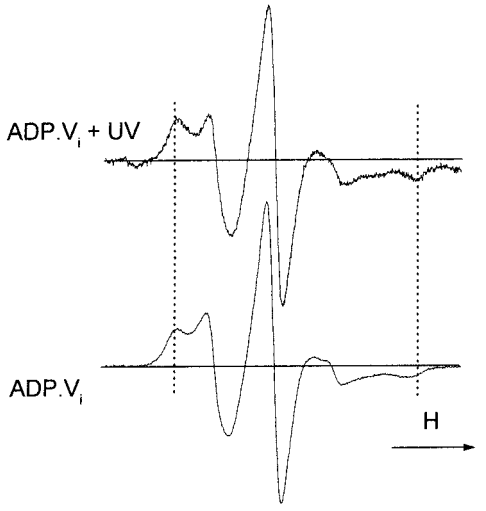

Fig. 5. Effect of oxygen free radicals on TCSL-labelled myosin in the presence of MgADP and orthovanadate. The sample was irradiated for 90 s after addition of 8 mM hydrogen peroxide to generate hydroxyl free radicals.

erated hydroxyl free radicals followed a pseudo first order chemical reaction. The characteristic time of the reaction was 1.3 min.

It is known that the binding of nucleotides, ADP or ADP plus orthovanadate (V_i) to myosin resulted in a significant increase in the mobility of the strongly immobilized labels. Since the reactive sulfhydryl sites are near to the nucleotide binding pocket in the crystal structure of myosin [22], changes are expected in the environment of the probe molecules. Experiments performed on cardiac myosin showed that the addition of 4 mM MgADP and orthovanadate to TCSL myosin affected strongly the mobility of the attached spin labels [23]. The change in the rotational mobility of spin labels was much larger when MgADP and orthovanadate was added to cardiac myosin (Fig. 5). The hyperfine splitting constant changed from 6.456 to 6.199 mT in ADP + V_i state of myosin.

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. In some cases, when the time of irradiation was increased, a singlet superimposed on the spin label signal was also detected. Independent of the nucleotide, ADP or ADP + V_i, the spectral intensity decreased (Fig. 5). The decrease of the spectral intensity was accompanied by the increase of the hyperfine splitting. The increase was about 0.1 mT in the case of MSL-myosin in ADP + V_i state. This shows 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 for skeletal myosin that irradiation by UV light modified the rate of ATP hydrolysis, and accelerated the dissociation of ADP and V_i [24]. In nucleotide-free state greater hyperfine splitting is expected. Under specific conditions in muscle fibres we estimated the portion of the –SH groups participating in the reaction with hydroxyl free radicals [18]. 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 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 that leads to protein damage. It can be suggested that suitable chemicals that suppress and/or quench the generation of free radicals in biological systems can reduce the ischaemic injury.

Acknowledgements

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