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CERIUM-MEDIATED FREE RADICALS IN CONTRACTILE PROTEINS
EPR and DSC study

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Abstract

The effect of free radicals obtained in hydroxyl and cerium(IV)-nitrilotriacetic acid free radical generating systems on contractile proteins (actin, myosin and their complexes in glycerinated muscle fibres) was studied using differential scanning calorimetry and spin trapping electron paramagnetic resonance technique. The analysis of spectra showed that selective attack of thiol groups – Cys-257 and Cys-374 residues of actin, and among others Cys-707 residue of myosin – and random attack of sidechains of the main proteins of muscle tissue produced structural and functional changes, which affected the ATP hydrolysis cycle and very likely the dynamics of actin. The melting curves obtained on protein systems support the view that global conformational changes accompany the local damage of free radicals.

Keywords: contractile proteins, DSC, hydroxyl and thiyl free radicals, spin-trapping EPR

Introduction

Previous biochemical researches revealed that myocardial ischemic injury affects the membrane integrity of the cells, and allows the oxidizing agents to enter the cells and to modulate the interaction of proteins [1–3]. In muscle fibres the oxygen free radicals are able to modify the catalytic centre of myosin. This process can cause impairment in the ATP hydrolysis cycle and force-generation of motile systems [4, 5]. The extension of modification depends on time, concentration and chemical structure of oxygen free radical species.

Reactions of hydroxyl free radicals generated by the Fenton-reaction with several proteins have been already investigated by trapping of the free radicals under different conditions [6]. The spin traps, in particular the widely used dimethyl-1-pyrroline-N-oxide (DMPO) and phenyl-N-tertier-butylnitrone (PBN) enabled the identification of the carbon-, oxygen- and sulphur-centred radicals in biological systems [7–9]. The backbone and sidechains of proteins and the components of the biological membranes undergo the random attack of free radicals [10]. However, some mild reactive oxidizing agents as cerium(IV) and chromium(VI) anions can generate

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thiyl free radicals in cellular systems containing cysteine [11, 12]. Cysteines might play an important role in biological systems; therefore the selective attack of thiol groups by oxidizing reactions could cause serious damage in the cell function.

Actin is one of the main components in the eukaryote cells, which plays a significant role in many cellular processes, like force-generation, maintenance of the shape of cells, cell-division cycle and transport processes. Actin is a highly conserved protein, varying little in its primary sequence from species to species. Actin is in a state of monomer (globular), G-actin, in a salt-free solvent and forms a helical polymer (filamentous actin), F-actin, with the addition of neutral salts. The globular form of actin can polymerise in the presence of Ca^{2+}- and Mg^{2+}-ions. In a given environmental condition, a polymerization equilibrium is established between G-actin and F-actin. The concentration of G-actin coexisting with F-actin in equilibrium depends on the salt concentration, pH, and temperature. The ability of actin monomers to form a filament is essential to the normal function in the cells; the filamentous form of actin is the principal component of the contractile or motile systems. On the other hand, the assembly and disassembly of actin is responsible for the cellular processes.

In this work we studied the effects of free radicals on specific sites of myosin and actin from rabbit skeletal muscle in solution and in supramolecular complexes using spin trapping EPR technique. The application of spin trapping supported the view that thiyl radicals can be generated in sulfhydryl-containing muscle protein by mild treatment. In addition, DSC measurements were performed to see whether the formation of free radicals on specific sites of myosin and actin could affect the global conformation of the proteins.

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′-triphosphate (ATP), nitrilotriacetic acid (NTA), phenyl-tert-butylnitrone (PBN) and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MSL), N-ethyl maleimide (NEM) were obtained from Sigma (Germany). Ammonium cerium(IV) nitrate was obtained from Merck (Germany). Lyophylized bovine hemoglobin and bovine serum albumin (BSA) were purchased by Sigma (Germany).

Preparation of the biological samples

The experiments were carried out on differently prepared samples.

(i) Glycerol-extracted muscle fibre bundles were prepared from rabbit psoas muscle. Small stripes of muscle fibres were stored after osmotic shocks in 50% *v/v* glycerol, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 25 mM Tris·HCl, pH 7.0 at –18°C up to one month. Fibre bundles from glycerinated muscle were washed for 60 min in rigor buffer (80 mM potassium propionate (KPr), 5 mM $MgCl_2$, 1 mM

EGTA in 25 mM Tris·HCl buffer, pH 7.0) to remove glycerol, and then transferred to fresh buffer. This state models the rigor state of the muscle (AM, where M denotes myosin and A stands for actin). Spin-labelling of muscle fibres was performed as described earlier [13].

(ii) Actin was extracted from aceton-dried muscle powder at 0°C with 0.2 mM ATP, 0.1 mM $CaCl_2$ and 2 mM Tris·HCl buffer at pH 8.3 for 20 min. The extraction was followed by two polymerization-depolymerization cycles in the presence of 100 mM KCl and 0.5 mM $MgCl_2$ to remove the rest of the regulatory proteins. The concentration of actin was determined by measuring the optical absorption using a Genesys 7 type spectrophotometer (A(280 nm)=0.63 mg mL^{-1} cm^{-1}). G-actin was centrifuged with 100 000×g before EPR measurements in order to remove contamination of F-actin.

Preparation of samples for EPR measurements

(i) Generation of hydroxyl free radicals. OH radicals were generated by the metal ion-dependent breakdown of hydrogen peroxide, by the Fenton reaction. The composition of the reaction mixture was

85 μL buffer solution or test material,
1 μL $FeSO_4$ (100 mM stock solution),
1 μL ATP (100 mM stock solution),
5 μL PBN (1 M stock solution),
10 μL H_2O_2 (8.8 mM stock solution).

The components of the reaction mixture was subsequently added to the buffer solution, the last component was H_2O_2 and then spectra were taken within 3 min. The buffer solution was exchanged for the protein samples. The preparation of samples was essentially the same as described earlier [14, 15]. When muscle fibres were investigated in different states of the hydrolysis cycle, $FeSO_4$ was omitted from the buffer solution and the free radicals were generated by short UV irradiation using a 200 W HBO lamp (Zeiss, Germany). The irradiation lasted for 3 min from a distance of 50 cm.

(ii) Generation of thiyl free radicals. The Ce(IV)-nitrilotriacetic acid solution was prepared by adding one volume of 0.5 M $(NH_4)_2Ce(NO_3)_6$ solution to nine volume of 100 mM NTA, 0.5 M Tris·HCl, pH 8.25. The concentration of the stock solution of PBN was 1 M. The protein solution contained 30–40 μM actin, 12.5 mM $(NH_4)_2Ce(NO_3)_6$, 22.5 mM NTA and 200 mM PBN. Samples were prepared fresh, since, on standing, the radical concentration rapidly decreased ($T_{1/2}$=30 min).

DSC experiments

The thermal unfolding of muscle proteins in different states was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were done between 5 and 80°C. The heating rate was 0.3°C min^{-1}. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μL sample volume in average.

The concentration of actin varied between 36–65 μM. Preparation buffers were used as reference samples. The sample and reference vessels were equilibrated with a precision of ±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 curve. Calorimetric enthalpy was calculated from the area under the heat absorption curve using two-point setting, SETARAM peak integration. DSC diagrams were deconvoluted by the PeakFit 4.0 program from SPSS Corporation after data correction. In order to describe the single transitions Gaussian functions were used.

EPR measurements

Conventional EPR spectra were taken with an ESP 300E (Bruker, Germany) X-band spectrometer. First harmonic, in-phase, absorption spectra were obtained using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1 or 0.2 mT at ambient temperature (20–22°C). For all spectra, sweep width was 10 mT; time constant 20.4 ms, conversion time 41 ms and 10 scans were accumulated. The samples were placed in a flat quartz cell (Scanlon Co., USA), and spectra were recorded immediately after mixing the components. The instrument was calibrated with potassium peroxylamine disulphonate which has an a_{iso}=1.302 mT in saturated bicarbonate solution. The spectra were normalized to the same number of unpaired electrons calculating the double integrals of the derived spectra, and the manipulations were performed on normalized EPR spectra. The concentration of free radicals in different samples was obtained by comparing the double integrals with that of a MSL solution of 50 μM concentration using the same sample cell and spectral parameters.

Results and discussion

EPR measurements

Reaction of –SH-containing compounds with Ce(IV)-ions in the presence of spin trap PBN results in the appearance of nitroxide free radicals. Ce(IV) complexed to NTA oxidizes sulfhydryl compounds *via* thiyl free radicals, which can be trapped by PBN [11]:

$$\text{protein–SH+Ce(IV)} \rightarrow \text{protein–S}^{\cdot}\text{+Ce(III)}$$

$$\text{protein–S}^{\cdot}\text{+PBN} \rightarrow \text{protein–S–PBN}^{\cdot}$$

The spectra were taken immediately after thiyl radical generation (Figs 1 and 2). In contrast to free cysteine, the PBN adduct molecules were strongly immobilized on actin. When F-actin was pretreated with 0.1 mM NEM for 10 min before addition of Ce(IV)-NTA, only a very low concentration of PBN spin adducts was detected. The hyperfine splittting constants obtained were about the same for both forms of actin

(G-actin: $2A_{zz}$=6.371±0.002 mT, F-actin: $2A_{zz}$=6.354±0.002 mT; SD, n=6), which implies that the mild oxidation by Ce(IV)-NTA could modify the protein structure in the neighbourhood of the thiol sites. The free radical concentration after Ce(IV)-NTA treatment was 0.42±0.06 (n=5) mole of free radical/mole of protein in G-actin, whereas 0.17±0.04 (n=4) mole of free radical/mole of actin was calculated in the case of F-actin.

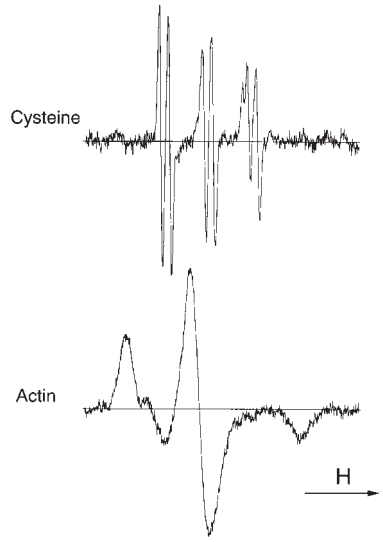

Fig. 1 EPR spectra of cysteine and monomer actin in Ce(IV)-NTA system. The concentration of actin was 52 μM, the field scan was 10 mT

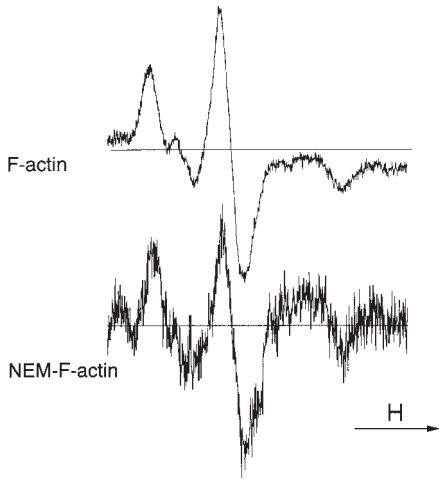

Fig. 2 EPR spectra of F-actin following treatment with Ce(IV)-NTA in the presence of PBN. N-ethyl-maleimide pretreatment blocks the Cys-374 residues in F-actin

The skeletal actin has five –SH groups (Cys-10, 217, 257, 285, 374) with different accessibilities [16]. According to Liu *et al.* [17] the most reactive thiol group was Cys-257 in monomer form, using a maleimide-based fluorescence thiol reagent. In polymerized form, it seems the most reactive group is Cys-374, as reported by other authors using different thiol-directed reporter molecules [18–21]. These results suggest that mostly the reactive Cys-257 thiol groups are involved in the reaction with G-actin, and only a smaller fraction of Cys-374 thiol groups contributes to the PBN signal. Polymerization of actin buries the exposed Cys-257 groups (and the other three less reactive thiol sites), and therefore only the thiol sites of the Cys-374 residues participate in the reaction.

The amount of strongly immobilized spin labels on the thiol sites, calculated by the double integration of the EPR spectra, can be overestimated by the non-specific binding properties of PBN spin adduct to the proteins. Proteins, actin and myosin have hydrophobic areas, which can bind small molecules. Serum albumin has many hydrophobic areas, and it is known that it really binds to small molecules as steroid hormones, fatty acids that are only slightly soluble in the blood serum [22]. The binding of PBN radical adducts to hydrophobic areas can contribute to the apparent spin concentration, assuming that this fraction has similar rotational correlation time. In order to test the possibility of the non-specific binding of PBN, experiments were performed on BSA in hydroxyl free radical generating system. The EPR spectrum in BSA-PBN solution was superposition of two spectral components, one large fraction of weakly immobilized PBN spin adducts and a second smaller fraction of strongly immobilized PBN spin adducts (Fig. 3). The appearance of strongly immobilized nitroxide free radicals might show the non-specific binding of PBN spin adduct to protein, and/or the formation of thiyl radicals on albumin after secondary reactions, which were trapped by PBN. The hyperfine splitting of this fraction was 6.520±0.035 mT, which is a reasonable value for large protein as BSA. MSL attached

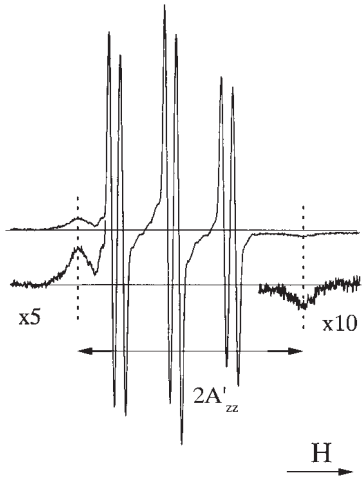

Fig. 3 EPR spectrum of BSA in hydroxyl free radical generating system in the presence of PBN

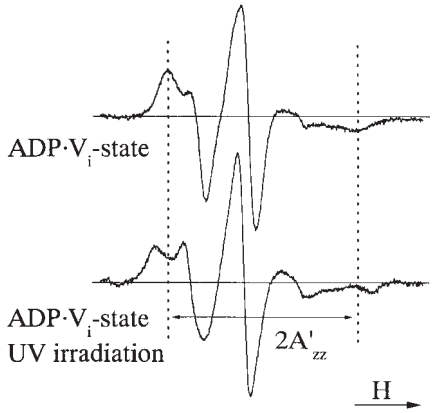

Fig. 4 EPR spectra of MSL-muscle fibres in ADP·V_i state before and after free radical generation

to the single sulfhydryl group of BSA resulted in a value of 6.501±0.035 mT. The former experimental finding showed that seemingly albumin molecules were able to bind PBN spin adduct molecules produced in hydroxyl free radical generating system. Two interpretations are possible. The PBN spin adduct molecules can bind to the hydrophobic areas of the proteins, and the non-covalent bindings appear as the fraction of strongly immobilized labels detected by the spectrometer. An alternative explanation might be that thiyl free radicals are also generated in sulfhydryl-containing proteins by secondary reactions even in hydroxyl free radical generating systems, as reported recently on hemoglobin by Mason and coworkers [23]. These thiyl free radicals are trapped by PBN on the surface of the protein. The appearance of both radical formations cannot be excluded.

Reaction of the cysteine residues of myosin with Ce(IV)-NTA in the absence of nucleotides produced PBN spin adducts molecules, which were strongly immobilized on myosin [11, 13]. In glycerinated muscle fibres ATPase activity measurements supported that at about half of the thiyl radicals were generated on the essential thiols of myosin (Cys 697, Cys 707). The rotational correlation time of the attached PBN spin adduct molecules was estimated in the saturation transfer EPR time domain. The characteristic spectral parameter, L''/L was about one – similar value as in rigor – which corresponded to an effective rotational correlation time of 100 μs. In rigor, in the absence of nucleotides the myosin molecules are rigidly attached to the actin filaments, almost no rotational motion of the head portion of myosin can be expected.

An interesting result was obtained in the presence of ATP and orthovanadate (V_i). It is known that incubation of fibres in buffer solution containing the components of the rigor buffer plus 4 mM ATP and 4 mM orthovanadate mimics the ADP·V_i-state of the ATP hydrolysis cycle that precedes the working stroke of the muscle. The myosin·ADP·V_i intermediate forms a weak binding to actin, which is accompanied with increased rotational motion of spin labels, attached to Cys-707 residues. This residue is located in the neighbourhood of the nucleotide binding site, the apparent rotational correla-

tion time of the attached label was 0.18 μs. In hydroxyl free radical generating system a significant mobility change could be detected, evidencing that the free radical attack enhanced the dissociation of the hydrolysis products ADP and V_i from myosin. The dissociation is followed with the development of a tight binding of myosin to actin completing the hydrolysis cycle. The resulting EPR spectrum exhibited an increased hyperfine splitting $2A'_{zz}$ characteristic of the rigor state; the increase indicates the formation of the strong actin-myosin complex. In former experiments UV irradiation above 300 nm of the head portion of myosin in ADP·V_i-state in buffer solution resulted in photooxidation of a serine residue in the 23-kDa segment of the myosin heavy chain [24, 25]. The photooxidation induced the release of both ADP and V_i. Very likely the same process was responsible for the increase of immobilization of the actomyosin complex in the highly organized muscle structure.

DSC measurements

Earlier experiments on globular and filamentous actin by DSC showed that the main thermal transitions were at 47.3 and 51°C for G-actin, and much higher transitions were detected for F-actin, they were at 59.7, 60.6 and 61.3°C [26]. The monomer actin consists of structural domains, which are separated by a cleft, and the bound nucleotide (ATP for G-actin and ADP for F-actin) is localized in the cleft. The larger and smaller domains can move as two units relative to one another. The transitions obtained as a result of the deconvolution could be assigned to these structural domains. During polymerization the monomer-monomer interaction enhances the thermal stability, and this leads to an increase of melting enthalpy and transition temperatures.

The free radical reactions produce significant alterations in the DSC pattern of F-actin. The narrow peak broadens drastically and is shifted by about 5°C to lower

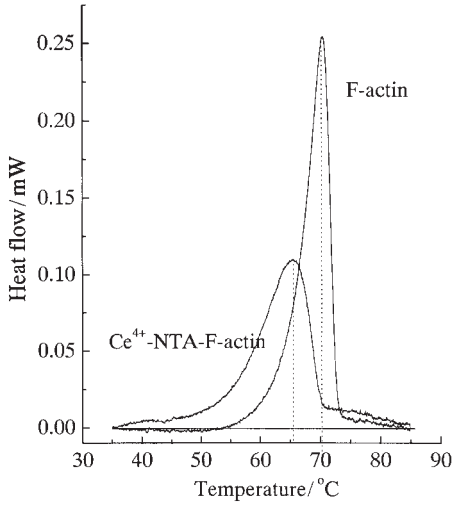

Fig. 5 DSC patterns of F-actin before and after treatment with Ce(IV)-NTA. The protein concentration was 50 μM

temperature evidencing that the monomer-monomer interaction and/or the filament-filament association are affected (Fig. 5). This result correlates with the EPR experiments, the hyperfine splitting, which is characteristic of the rotational mobility, exhibited similar values in both forms of actin. The oxidation of the Cys-374 residues by Ce(IV)-NTA in F-actin results in conformational changes in the environment of the subdomain-1, which has consequence in the global structure of the protein. The significant broadening of the melting curve is probably due to the fact that the effect of the radical reactions modified the compact structure of F-actin, and this led to a decreased cooperativity between the protomers. The weakened interaction appears as a reduced transition enthalpy.

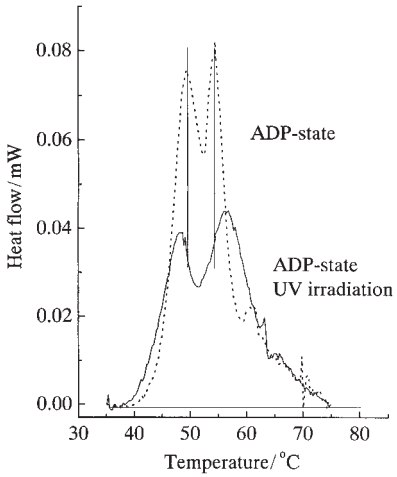

Fig. 6 Melting curves of ADP-myosin before and after hydroxyl free radical generation

Similar broadening effect was observed on myosin in the presence of nucleotides (Fig. 6). The starting point of the evaluation was that the main transitions between 45 and 60°C were the superposition of endotherms. They were believed to correlate mainly with T_ms of the larger domains of myosin: the head portion of myosin, which contains the nucleotide binding site, the actin binding site (50 kDa segment) and the long tail with the two light chains, and the rod-like parts. On isolated myosin two large endotherms were detected – characteristic of the head and rod portion of myosin – but in the deconvolution procedure usually three or four transitions with Gaussian peak function were assumed to resolve the internal domains of the head [27, 28]. It was shown that the most labile part of myosin was the 50-kDa segment or a part of it, therefore it is believed that the first thermal transition can be assigned to the 50-kDa domain [29, 30]. This transition might involve the unfolding of the neck region of myosin [31], and the long rod part of the myosin as well [32, 33]. In the presence of nucleotides the largest changes were measured at higher temperature transitions.

Denaturation behavior of myosin in the absence and presence of hydroxyl free radicals in ADP-state can be seen in Fig. 6. From the DSC scan two transitions could be decomposed with T_{m1}=50.1°C and T_{m2}=54.4°C melting temperatures. We suggest the unfolding around 50.1°C describes the transitions of the rod portion and the actin binding domain, while the transition around 54.4°C on the experimental scan may be related to the catalytic domain with the bound nucleotide. In the presence of free radicals there are broadenings in the peak of transitions (44.5 and 55.2°C) together with a significant decrease of the enthalpy, which could be the energetic consequence of the internal change of myosin structure evoked by the random attack of free radicals.

Conclusions

Selective attack by mild oxidation at particular amino acid residues on contractile proteins provided evidence, that the radical damage led to conformational changes, which affected the dynamic and interaction of actin and myosin.

Specifically on actin, the formation of free radicals depends on the form of actin, and the subsequent reactions are able to modify the cooperative interaction between the protomers. In muscle fibres, a large fraction of the essential sulfhydryl sites is involved in the radical attack, which affects the ATPase activity of myosin.

Random attack of hydroxyl free radicals on myosin in solution and in supramolecular complex with actin modifies the hydrolysis cycle, the free radical attack is accompanied with the enhanced release of the hydrolysis product in weakly binding state of myosin for actin.

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