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NUCLEOTIDES INDUCED CHANGES IN SKELETAL MUSCLE MYOSIN BY DSC, TMDSC AND EPR

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Abstract

Electron paramagnetic resonance (EPR, ST-EPR) and differential scanning calorimetry (DSC) were used in conventional and temperature modulated mode to study internal motions and energetics of myosin in skeletal muscle fibres in different states of the actomyosin ATPase cycle. Psoas muscle fibres from rabbit were spin-labelled with an isothiocyanate-based probe molecule at the reactive sulfhydryl site (Cys-707) of the catalytic domain of myosin. In the presence of nucleotides (ATP, ADP, AMP·PNP) and ATP or ADP plus orthovanadate, the conventional EPR spectra showed changes in the ordering of the probe molecules in fibres. In MgADP state a new distribution appeared; ATP plus orthovanadate increased the orientational disorder of myosin heads, a random population of spin labels was superimposed on the ADP-like spectrum.

In the complex DSC pattern, higher transition referred to the head region of myosin. The enthalpy of the thermal unfolding depended on the nucleotides, the conversion from a strongly attached state of myosin to actin to a weakly binding state was accompanied with an increase of the transition temperature which was due to the change of the affinity of nucleotide binding to myosin. This was more pronounced in TMDSC mode, indicating that the strong-binding state and rigor state differ energetically from each other. The different transition temperatures indicated alterations in the internal microstructure of myosin head region The monoton decreasing TMDSC heat capacities show that C_p of biological samples should not be temperature independent.

Keywords: DSC, EPR, nucleotides, temperature modulated calorimetry, skeletal muscle contraction

Introduction

Muscle is a chemo-mechanical machine that converts chemical energy into work and heat. The energy source is the myosin-catalysed ATP hydrolysis; the rate is enhanced

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in the presence of actin [1, 2]. In the presence of MgATP the energy released from hydrolysis produces conformational changes in myosin [3] and/or in actin [4, 5], which can be manifested as an internal rotation of myosin head while bounds to actin. The force generation is performed by cyclic interaction of myosin with ATP and actin, and at least six intermediates are proposed for actomyosin ATPase in solution [6] . The better understanding of the interrelation between the chemical events observed in protein solutions and the mechanical activity on cellular level requires experiments on supramolecular complexes where stabilizing forces may modulate the hydrolysis process. We have extended the experiments to study the fibre system prepared from psoas muscle of rabbit in rigor, strongly binding and weakly binding states of myosin to actin where P_i was substituted by the phosphate analogue orthovanadate [7, 8].

Studies using different techniques indicated that the nucleotide-binding pocket did not experience large conformational changes during the hydrolysis cycle [8–10]. However, the small nucleotide-induced conformational changes in the motor domain should be converted into larger movement. Data agree that while the structure of the motor domain remains similar to rigor, the regulatory domain swings about a point in the distal end of the motor domain [11]. The changes in the 50 kDa domain might effect the segment of the 20 kDa domain that contains the essential sulfhydryl groups.

Spectroscopic probes widely used in biological systems to get information about orientation and rotational motion of proteins. In muscle fibre studies, the maleimide-based nitroxides (MSL) are usually attached to the reactive sulfhydryl sites (Cys-707, Cys-697) of myosin heads, and two different orientations are tested: the longer axis of the fibres is oriented parallel and perpendicular to the magnetic field [12]. The different labels have different chemical structure and different attaching linkage, therefore, it is reasonable to use different labels to understand the molecular motion of the head region of myosin in the presence of nucleotides.

In this report, we studied the effect of MgADP and MgATP-orthovanadate on the dynamics and orientation of myosin head using isothiocyanate spin label and EPR spectroscopy. In order to find correlation between local and global structural changes in the intermediate states of the ATPase cycle, the spectroscopic technique was combined with conventional and temperature modulated DSC measurements that report domain stability and interactions.

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 orthovanadate (Na_3VO_4), 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) were obtained from Sigma (Germany).

Fibre preparation

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 and buffer solution (80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris·HCl, pH 7.0, rigor buffer) at –18°C up to one month. Fibre bundles of glycerinated muscle 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 (AM, where M denotes myosin and A stands for actin). 5 mM MgADP was added to the rigor solution to simulate the strongly binding state of myosin for actin which 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 was formed by ADP or ATP plus orthovanadate (abbreviated as V_i) which together bind stoichiometrically at the active site of myosin to form a stable complex AM·ADP·V_i. The muscle fibres were stored in solution containing 80 mM K-propionate, 5 mM $MgCl_2$, 5 mM ADP or 5 mM MgATP plus 5 mM V_i, 1 mM EGTA in 25 mM Tris·HCl buffer, pH 7.0, for 15 min at 0°C before measurements.

Spin-labelling of fibres was performed in relaxing medium (rigor solution plus 2 mM pyrophosphate at pH 6.5) with about two moles of 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl (TCSL) to one mole 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 7.5) for 1 h to achieve selective labelling of the reactive thiols. After spin-labelling the fibre bundles were washed in great amount of rigor buffer plus 5 mM DDT for 30 min at 0°C, pH 7.0 to remove the unreacted labels and restore the preblocked thiol groups.

EPR measurements

Conventional EPR spectra were taken with an ESP 300E (Bruker, Germany) spectrometer. First harmonic in-phase absorption spectra were obtained using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1–0.2 mT. Second harmonic 90° out-of-phase absorption spectra were recorded with 63 mW microwave power and 50 kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out-of-phase. The microwave power corresponds to an average microwave field amplitude of 0.025 mT in the center region of the standard tissue cell of Zeiss (Carl Zeiss, Germany), and was obtained using the standard protocol [13]. In this region of the tissue cell, five small segments (5–6 mm long) of the muscle fibres (total wet mass about 15–25 mg) were mounted parallel to each other. The spectra were recorded in two positions at temperature of 22±1°C, where the long axis of the fibres was oriented parallel or perpendicular to the static field. The manipulations were performed on normalized EPR spectra by digital subtraction.

Calorimetric measurements

The thermal unfolding of myosin in fibres 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 sample. 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 denaturated during each cycle. The modulated DSC measurements were performed by series with temperature jump of 0.5°C under 900 s followed by 900 s isotherms.

Evaluation of DSC scans

The repeated scan of denaturated sample was used as baseline reference which was subtracted from the original DSC scan. After SETARAM ASCII conversion the deconvolution was performed with PeakFit 4.0 program from SPSS Corporation. Gaussian peak functions were used to approximate the unfolding of the structural units of myosin. Calorimetric enthalpy was calculated from the area under the heat absorption curves using two points setting SETARAM peak integration. Fast Fourier transformation developed in Schick's laboratory was applied to evaluate the modulated scans.

Results

DSC measurements

The unfolding of proteins in muscle fibres by thermal excitation is a complex process and depends on the state of the actomyosin complex. The experimental data suggest at least four transitions in the temperature range examined. The minor transition at T_{m1}= 18°C is not shown in the figures; it might be attributed to the interaction of LC-2 light chain with the long α-helical part of the myosin head [14]. This transition could be evaluated as the sign of an interdomain communication.

The starting point of the evaluation was that the main transition between 45 and 70°C is the superposition of endotherms that are believed to correlate mainly with T_ms of the larger domains of myosin and a smaller contribution from thin filaments. On isolated myosin three endotherms were reported, therefore in the deconvolution procedure three transitions were assumed.

In the absence of nucleotides (Fig. 1, bottom) three transitions could be decomposed from the DSC scan with T_{m1}=54.05°C, T_{m2}=58.36°C and T_{m3}=62.29°C melting temperatures. In the presence of MgADP (Fig. 1, middle) there is an increase in the peak of transition 53.5°C which could be the energetic consequence of the internal rearrangement of myosin structure [11, 15].

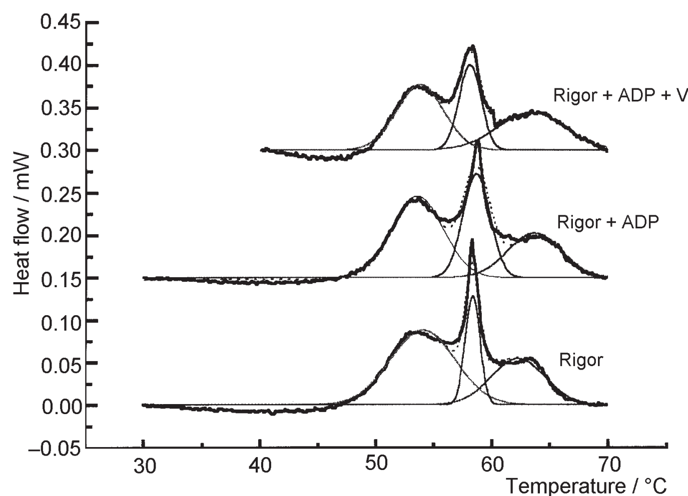

Fig. 1 DSC patterns of muscle fibre system in rigor (no nucleotide is present, bottom; AM·ADP or strong binding state, middle; AM·ADP·V_i or weak binding state, upper). Symbols: raw line – experimental DSC scan without filtering; dotted line – sum of the deconvoluted curves. The deconvolution procedure was performed using PeakFit 4.0 program from SPSS Corporation. In order to describe the single transitions Gaussian functions were used

Table 1 DSC parameters of melting of contractile proteins in fibre system. Glycerinated muscle fibres isolated from psoas muscle of rabbit were measured in different intermediate state of the ATP hydrolysis cycle. The states rigor state, strong-binding state (ADP) and weak-binding state (ADP·V_i)

Transition temperature			
Muscle state	T_{m1}/°C	T_{m2}/°C	T_{m3}/°C
Rigor	54.05	58.36	62.29
ADP	53.49	58.61	63.64
ADP·V_i	53.98	58.08	63.78
Excess enthalpy			
Muscle state	H_1/J g^{-1}	H_2/J g^{-1}	H_3/J g^{-1}
Rigor	54.08	18.06	27.85
ADP	44.51	29.96	25.51
ADP·V_i	40.94	25.14	33.91

When 5 mM MgADP was added in the presence of 5 mM V_i (Fig. 1, upper) the separation of the first two peaks is less pronounced. It is obvious that addition of nucleotides produces conformational changes in the multisubunit structure of myosin. The enthalpy of the thermal unfolding (Table 1) depends on the nucleotides, the conversion from a strongly attached state of myosin for actin to a weakly binding state is

accompanied with an increase of the transition enthalpy of the third melting peak which might relate to the change of the affinity of nucleotide binding to myosin.

EPR results: effects of ADP and ADP+V_i

TCSL-fibres indicated that the binding of ADP induced a large change in the mean orientation of the segment containing the label. Spectrum analysis showed (Fig. 2) that the mean orientation of the *z*-axis of the TCSL changed from 76 to 56° with respect to the fibre axis after addition of ADP, and this change was accompanied with the increase of the angular spread. The hyperfine splitting constant ($2A'_{zz}$) in parallel orientation was 3.265±0.025 mT (*n*=4) in rigor and 4.871±0.025 mT (*n*=4) in ADP state, respectively. However, the ST-EPR spectra showed (Fig. 3) only small differences between rigor and ADP-state evidencing that the global conformation of actin-myosin complex remained the same, the parameters characterising the motional state of the labels were L''/L=0.75±0.1 (*n*=5) in rigor state, and L''/L=0.72±0.3 (*n*=4) in ADP state.

When the fibres were incubated in rigor buffer plus MgATP and V_i for 15 min, no orientation dependence was detected showing the dissociation of all heads from actin, or the non-specific binding of cross-bridges on actin. However, after addition of MgADP and V_i for 15 min the dissociation of myosin heads was not complete. Un-

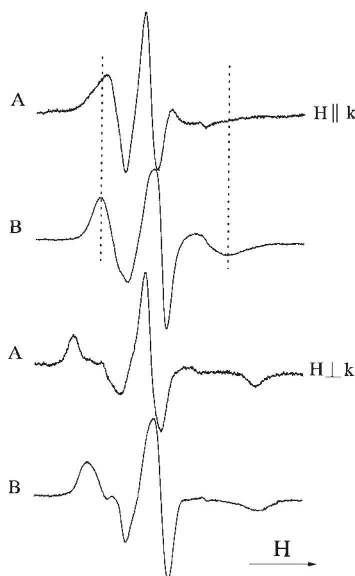

Fig. 2 Conventional EPR spectra of TCSL-fibres in rigor (A) and ADP-state (B). The spectra were taken in parallel and perpendicular orientations. The fibres were kept in buffer solution (Materials and methods) during spectrum accumulation. MgADP was added in 5 mM concentration and the incubation of fibres lasted for 15 min before EPR measurements. The field scan was 10 mT. The hyperfine splitting constant ($2A'_{zz}$) is larger in MgADP state which is the sign of the change in orientational order

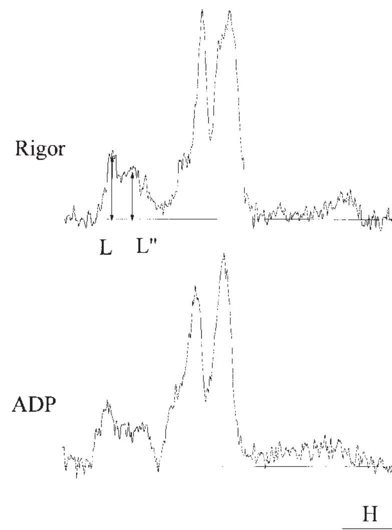

Fig. 3 Saturation transfer EPR spectra of TCSL-fibres in rigor (A) and ADP-state (B). The spectra were taken in perpendicular orientation. MgADP was added in 5 mM concentration and the incubation of fibres lasted for 15 min before EPR measurements. The position of the low-field diagnostic peaks *L*, *L″* are shown on the spectrum. The field scan was 10 mT

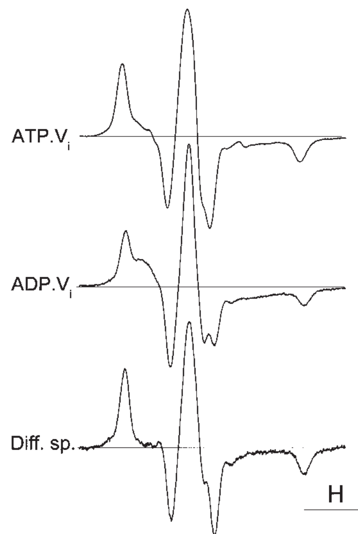

Fig. 4 Conventional EPR spectra of TCSL-fibres in ADP·V_i-state. The spectra were taken in parallel orientations. MgADP or MgATP and V_i was added in 5 mM concentration and the incubation of fibres lasted for 15 min before EPR measurements. A – MgATP and V_i; B – MgADP and V_i; C – residual spectrum. The spectrum obtained in MgADP state (Fig. 3) was subtracted from spectrum B. The field scan was 10 mT

der conditions used in our experiments (Fig. 4) about 30–35% of the cross bridges remained in ADP state, whereas the larger population (65–70%) seemed to be in dissociated state as calculated by spectrum deconvolution.

TMDSC measurements

The total C_p (Fig. 5) in each case of simulated muscle contraction showed practically the same result as the conventional DSC scans (plotted in gray). The very surprising new finding was that the modulated C_p (plotted in black) instead of sharp transitions revealed continuous decrease in the function of temperature in the main melting range at the modulation frequency and amplitude used in our experiment. This support the proposal of Chun [16, 17] that during the unfolding of proteins in the calculation of different thermodynamic functions C_p cannot be handled as constant, temperature independent variable.

Discussion

The structure of actomyosin complex is well-known in the absence of ATP [3], but the structural changes which are followed during tension development is less understood. It was proposed that this process involves the transition of the myosin head from a 'weak' to a 'strong' binding state to actin [2, 18–23]. The constrain generated by the filament association and protein-protein interaction increases the rigidity in the supramolecular structure, and this stabilizes the system. The structure formation alters the dynamical and energetical behaviour of contractile proteins, the consequence of that is the shift of the melting points 39, 47 and 51°C, measured in solution on myosin, to higher temperatures in rigor (Fig. 1). This is an evidence that particular regions of myosin are subjected to stabilizing forces leading to alteration of the transition temperatures (Table 1).

Earlier studies on myosin suggested that the endotherms were correlated with T_ms of the principal domains of the myosin molecule, and concluded that the head region (S-1) has a distinct transition around T_m=52.5°C in 0.1 M KCl (pH 7.0), whereas the long tail (LMM) showed two endothermic transitions (T_m=45°C, T_m=56°C) at high salt concentration [23]. Precise DSC measurements allowed the deconvolution of the melting curve on S-1 and three transitions were obtained [24]. The unfolding around 47°C may refer to the catalytic domain in S-1 [14, 25] which is the most labile part of S-1, while the transitions at higher temperature could be assigned to the unfolding of the long α-helical part of the myosin head [1, 26, 27]. Nucleotides (ADP, AMP·PNP) and nucleotides plus orthovanadate affected differently the thermal transitions of the different domains [24, 28]. The melting of actin appeared at 63°C [29]. The results cited above suggest that a clear and unique assignment of the thermal transitions to the components of muscle fibres is not easily possible. The only way to obtain information about the structural domains of myosin and their thermal properties is the simulation of the different intermediate states of the ATP hydrolysis under controlled conditions. As a starting point, we assumed that at first approximation the

contributions from LMM and actin filaments are the same in the intermediate states, independently of the state of myosin heads.

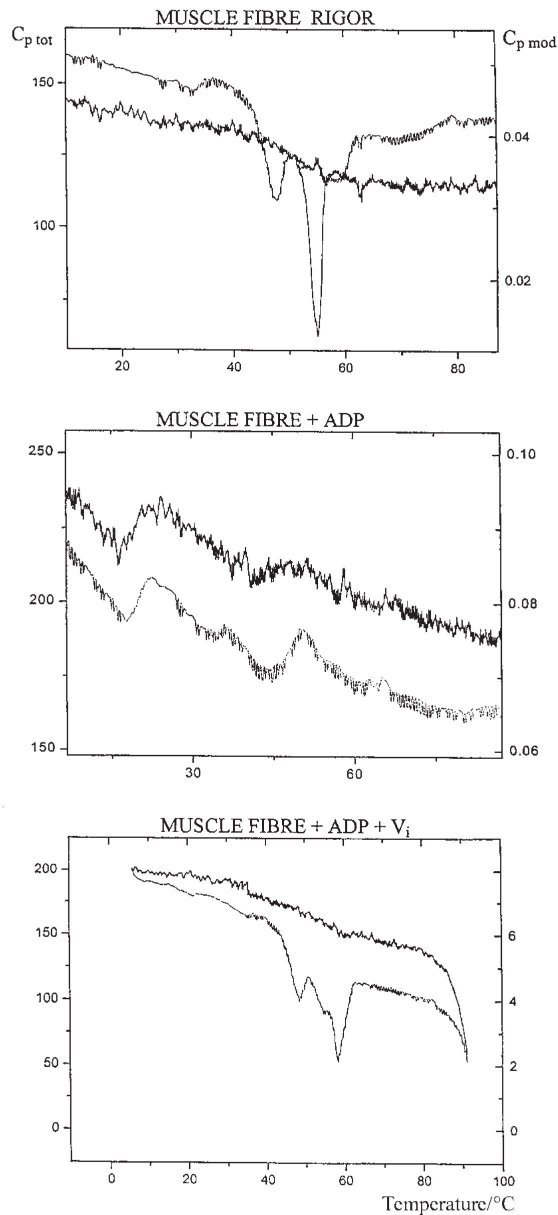

Fig. 5 TMDSC scans of different state of muscle contraction in rabbit psoas fibres. Left axis C_{ptotal}, right axis $C_{pmodulated}$. Conventional DSC scans plotted in grey, modulated curves in black

In the presence of 5 mM MgADP a significant decrease in the enthalpy of the transition 54 – it is shifted to 53.49°C as well – was calculated which might be an energetic consequence of the internal rearrangement of myosin structure (Fig. 1). The binding of ADP can induce loosening of the association between the myosin domains and/or a decreased interaction between the two myosin heads [24, 30]. When 5 mM MgADP plus 5 mM V_i were added to the muscle sample, the first two transitions were less separated and pronounced (Fig. 1).

Recent data suggest that MgADP induces an internal change in the multisubunit structure of S-1 which were served by spectroscopic [15, 31] and electrical birefringence [32] techniques. In the presence of MgADP the myosin heads remain attached to actin, as could be seen from the ST-EPR spectra, the spectral parameters L''/L were the same in rigor and in ADP state in the limits of the experimental error. In contrast, large difference in the static order of the attached labels was detected in the conventional EPR spectra, evidencing that the binding of MgADP allowed the rotation of the segment that holds the label from one conformationally stable state into another one, and this is accompanied by a subtle rotation of the entire head [12, 31, 33–35].

Very likely, the myosin head has only one stereospecific orientation with that binds strongly to actin, but with different internal structure depending on bound nucleotide. It was already reported that nucleotide binding influenced the region containing the essential sulfhydryls [34, 36, 37].

It is obvious from our data that the strongly binding state and rigor state differ energetically from each other (Table 1). Saturation of myosin with MgADP leads to stabilization of the head regions [24], which is reflected in the increased excess enthalpy H_2 at T_{m2}=58.6°C. In contrast to this enthalpy change, there is a decrease of excess enthalpy H_1 at T_{m1}=53.5°C. Very probably, the binding of ADP results in a decreased interaction between the two myosin heads. This interpretation implies a small global conformational change of the myosin head following ADP binding. The active site pocket in ADP state has nearly the same closed conformation as in rigor, and this results in a strong interaction between myosin and actin. The increase of excess enthalpy H_2 observed in strongly and weakly binding states could be a sign of the ‘stretched state’ of the myosin heads during force generation. It seems the middle part of the decomposed DSC scan (Table 1) may refer to the nucleotide binding domain which undergoes small conformational change in different states as seen in EPR spectra. However, the transition temperature exhibits only a subtle change, evidencing that the global structure in rigor and in strong-binding state does not differ significantly.

The third component of the main transition might refer partly to the rod part of myosin [38, 39] and the melting of actin [40]; but its broadening and small shifting towards higher temperature and yielding a greater transition enthalpy in weakly binding state (AM·ADP·V_i state) could be the sign that the environment of the nucleotide pocket becomes more packed in the presence of orthovanadate. It was shown by fluorescence measurements that the distance between εADP and SH1 is shorter in S-1. εADP·V_i complex, but this distance is unperturbed in acto·S-1·εADP complex [41]. Comparison with various nucleotide bound S-1 complexes indicates that the shape of

S-1 in S1·ADP and S1·ADP·P_i states significantly differs from the shape of S-1 in nucleotide free states [42].

It seems our interpretation is in fairly agreement with the experimental data obtained on protein solutions [28] and on S-1 subfragment [43], and it seems to be consistent with the model recently proposed by Rayment *et al.* [3]. The local changes in the myosin head induced by nucleotide binding (revealed by EPR spectroscopy) appeareded in the thermal stability of the simulated intermedier states as a global change.

We have performed the first modulated experiments in proteins as we know from the literature. We have expected these data to provide information on the fine internal domain structural rearrangement during the different state of muscle contraction. C_{ptotal} gave practically the same result as the conventional DSC except for ADP state which should be carefully reinvestigated, but in $C_{pmodulated}$ we could not observed any sharp transition. It showed a stepwise change: before and after the melting region it was nearly constant and between 45 and 70°C it decreased monotonically (Fig. 5). This support those proposals that C_p of biological macromolecules (proteins) should be temperature dependent [16, 17] in contrast to the recent opinions. To clarify this problem we should perform experiments in the function of modulation frequency and amplitude, too.

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