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Functional and structural differences in skeletal and cardiac myosins. A molecular dynamic approach

D. Lőrinczy[a,*], J. Belagyi[b]

[a]*Biophysical Institute, Medical University of Pécs, H-7643 Pécs, Szigeti út 12., Hungary*
[b]*Central Research Laboratory of Medical University of Pécs, H-7643 Pécs, Szigeti út 12., Hungary*

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Abstract

Conventional and saturation transfer electron paramagnetic resonance spectroscopy and differential scanning calorimetry were used to study the internal dynamics and stability of cardiac myosin.

Intact and LC 2-deficient myosin isolated from bovine heart were spin-labelled with maleimide and iodoacetamide probe molecules at the SH1 sites. It was found that the probe molecules rotate with an effective rotational correlation time of 42 ns, which is at least six times shorter than the rotational correlation time of the same label on skeletal myosin. Addition of MgADP induces intrinsic changes in the multisubunit structure of myosin, but it does not lead to changes of the overall rotational properties of the myosin head.

Temperature dependence of the EPR spectra of maleimide-labelled myosin shows continuous decrease of the spectral parameters (intensity ratio of the peak heights, hyperfine splitting) at increasing temperature. However, marked changes were obtained at about 16°C in LC 2-deficient myosin. DSC measurements also support the view that the removal of the LC 2 light chain produces change in the internal structure of cardiac myosin.

Keywords: Cardiac myosin; Spin-labelling; DSC-measurement; Order of myosin heads; Effect of nucleotides

1. Introduction

It is already known that the physiological and biophysical properties of cardiac muscle are different from skeletal muscle. The cardiac muscle myosin has greater susceptibility for alkaline denaturation and exhibits different amino acid composition [1,2] and/or amino acid sequence [3,4]. However, relatively few data were published on the molecular dynamical background of these different characteristics. In this study data will be presented for the interpretation of the different molecular behavior of cardiac- and skeletal muscle myosin using DSC technique and spectroscopic probes that report about global and local changes. It is known from earlier studies, that

(a) maleimide (MSL) and iodoacetamide (IASL) spin labels attach to the Cys 707 and Cys 697 amino acid residues which are localised in the 20 kDa domain of myosin subfragment-1(S1) [5–7];
(b) the LC 2 light chain can be removed from cardiac muscle myosin only by proteolytic digestion [1];

* Corresponding author. Tel.: +36-72-314017; fax: +36-72-314017.
E-mail address: microcal@apacs.pote.hu (D. Lőrinczy).

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(c) the greatest difference between the two kinds of myosin is thought to be in the 50 kDa domain that contains the actin binding site [8,9].

It was also shown by hydrodynamic measurements [10] that there are differences in the sedimentation of LC 2-deficient and intact cardiac muscle myosin. Cardiac myosin shows a conformational change at 15°C, that could not be detected after dissociation of the LC 2 light chain. It seems that the main functional difference is assigned to the interaction of the 50 kDa domain with LC 2 light chain.

Using IASL and MSL paramagnetic probes attached to the myosin SH1 group, the structure of the globular head portion of cardiac muscle myosin proved to be less rigid than that of the skeletal muscle. The IASL probe reflected the rotational motion of the entire globular subunit, and was a sensitive detector of local conformational changes in interaction with ADP. In the light of the recent data it seems to be disadvantageous that the paramagnetic probe molecules are localized in the largely conserved sequence of the 20 kDa domain that includes the SH-region, because during muscle activity the largest changes are expected between the motor and light chain domains [11]. The internal molecular arrangement of actin filaments is influenced by attachment of myosin to actin.

2. Materials and methods

2.1. Preparation of myosin

Bovine heart myosin was prepared by the method of Shiverick et al. [12]. After washing of the tissue, the protein 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 100000 × g, myosin was purified using Sepharose 4B chromatography.

2.2. Preparation of LC 2-deficient myosin

Myosin was cleaved with α-chymotrypsin (400 : 1) for 1.5 min at 25°C in buffer containing 0.12 M NaCl, 20 mM phosphate, 1 mM EDTA, 1 mM DTT, at pH 7.0. The product was purified by column chromatography on Sepharose 4B. Protein concentrations were determined either by reading the absorption at 280 nm using absorption coefficients 0.55 mg/ml/cm for myosin and 0.56 mg/ml/cm for LC2-deficient myosin.

2.3. Spin-labelling

The isolated proteins were labelled either with 4-maleimido-TEMPO (MSL) or with 4-iodoacetamido-TEMPO (IASL). Myosin suspended in 0.5 M KCl, 50 mM TRIS, 1 mM EDTA, pH 8.0 was reacted for 60–90 min with 2 mol of MSL or with 3 mol of IASL per mole of myosin for 10–12 h on ice. The reaction was terminated by precipitation of myosin with ice cold water, and thereafter the protein was collected by centrifugation and solved in 0.5 M KCl, 25 mM HEPES, 1 mM EDTA at pH 7.0. In some cases myosin was treated with 5 mM $K_3Fe(CN)_6$ to remove the weakly immobilized labels [13]. The protein was clarified by centrifugation at 50000 × g for 1 h and used at final concentration of 20 mg/ml.

2.4. EPR experiments

The EPR measurements were taken with an ESP 300 E X-band spectrometer (Bruker, Germany). 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 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 (Germany). The microwave magnetic field was determined with peroxylamine disulphate ion radicals in the same sample cell as for the myosin samples following Fajer and Marsh [14].

2.5. DSC measurements

Thermal unfolding of muscle proteins was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were done between 5 and 60°C with 0.3°C/min scan rate. Conventional Hastelloy batch vessels were used during the thermal transition experiments with 850 μl sample volume on average. The buffer solution 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 perform any correction from the point of view of heat capacity between the sample and reference vessels.

3. Results and discussion

3.1. Characterization of the labelled sites

The method used to label the SH1 thiol site of cardiac myosin was essentially the same as described earlier by Thomas and colleagues [15,5] and Belagyi et al. [16] for skeletal muscle myosin. The degree of labelling was 0.1–0.15 mol label/mol protein for IASL and 0.5–0.75 mol label/mol protein for MSL. The EPR spectra of cardiac MSL-myosin are shown in Fig. 1. The conventional spectrum exhibited a small amount of weakly immobilized labels, but its population never exceeded 10% of the total EPR absorption. The conventional EPR spectra were characterized by the distance between the outermost hyperfine extreme $2A'_{ZZ}$, whereas the L''/L parameter was calculated from ST-EPR spectra (Fig. 1, bottom). For determination of the rotational correlation time the method of Goldman and coworkers [17] was used. The EPR spectra were recorded with increasing concentration of glycerol or

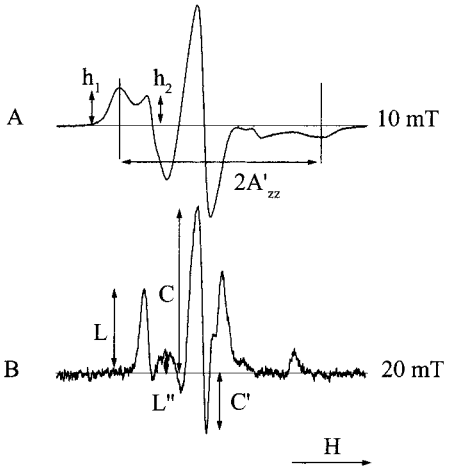

Fig. 1. EPR spectra of myosin: (A) conventional spectrum of myosin in solution; (B) ST-EPR spectrum of myosin in minifilament form. Myosin was labelled with MSL. The hyperfine splitting constant $2A'_{ZZ}$, the L''/L and C'/C ratios are characteristic for the rotational motion of the attached spin labels.

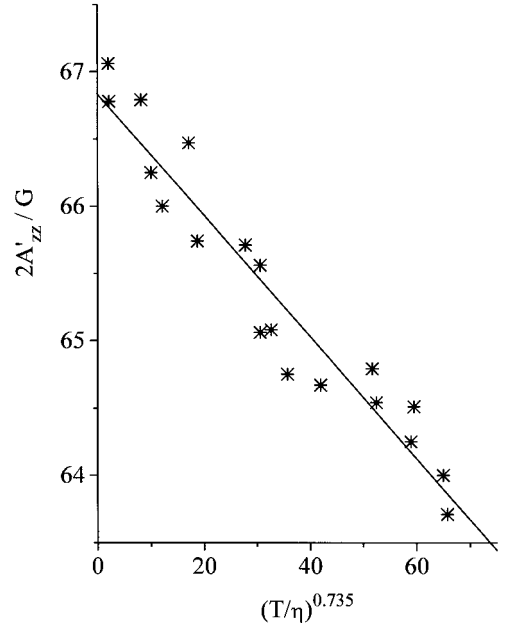

Fig. 2. Variation of the $2A'_{ZZ}$ value for myosin with $(T/\eta)^{0.735}$. For details see text. T and η are the temperature on Kelvin scale (K) and viscosity of the solution in mPa s, respectively.

sugar (Fig. 2). The rigid limit value for $2A'_{ZZ}$ at $\eta \rightarrow \infty$ was obtained by a least square fit procedure, and was 6.674 mT. This value was significantly larger than the $2A'_{ZZ}$ value which was measured for myosin in minifilament form ($2A'_{ZZ} = 6.484$ mT) or for myosin precipitated by centrifugation ($2A'_{ZZ} = 6.510$ mT). Since the labels were strongly immobilized at $\eta \rightarrow \infty$, but not in the side- by- side aggregate of myosin molecules, the former value (6.674 mT) was used as rigid limit. In contrast, $2A_{zz} = 6.875$ mT was obtained for maleimide labelled skeletal myosin at $\eta \rightarrow \infty$ and 6.80 ± 0.025 mT at 1 mPa s. The rotational correlation times of the MSL were 42 ns and 260 ns, respectively, at room temperature and at 1 mPa s. Addition of glycerol to the myosin precipitate resulted in a sudden increase of hyperfine splitting, which indicated the strong effect of glycerol on the rotational mobility of the cardiac myosin head region or at least in the neighbourhood of the bound label. This suggests that

(a) significant rotational mobility of the attached labels existed even in minifilament form and in the aggregate of myosin molecules;

(b) there is a substantial difference in the rotational motional properties of cardiac and skeletal myosin; the calculated rotational correlation time for cardiac myosin was six times shorter than that of for skeletal muscle myosin assuming that the labels were located on SH1 sites of myosin.

It should be noted that we did not observe significant changes of the rotational correlation time of myosin and LC 2-deficient myosin evidencing that the removal of the LC 2 chain from cardiac myosin did not influence the rotational motion of the attached MSL label. This supports the view that there is a flexible coupling between the catalytic and LC domain. At low ionic strengths, where myosin was in filament form, the hyperfine splitting constant increased significantly and the value attained to $2A'_{ZZ} = 6.484 \pm 0.03$ mT at room temperature ($\tau_2 = 70$ ns). From the ST EPR spectra of myosin filaments the spectral parameters L''/L and C'/C were were calculated to be 0.24 and -0.041, which correspond to rotational correlation times of 6 and 2 μs, respectively. These values indicate that the self-organization of myosin into filaments is accompanied by a strong immobilization of labels in the head region of the myosin molecules. It suggests that a significant portion of the mobility originates from the segmental flexibility of the protein.

The low-field spectral parameter in the ST EPR spectrum of glycerinated muscle fibres had a value of $L''/L = 1.22 \pm 0.06$ ($n = 10$) for skeletal muscle, whereas $L''/L = 0.83 \pm 0.1$ ($n = 4$) was calculated for cardiac muscle fibres in rigor [18]. The values of τ_2 showed that the rate of the reorientation of the label in cardiac muscle fibres was faster approximately with one order of magnitude in rigor, evidencing some internal flexibility of cross-bridges even in their attached state.

3.2. Interaction of spin labelled myosin with ADP

The binding of ATP or ADP to myosin results in a significant decrease in the proportion of the strongly immobilized label [19,20]. It was reported that the changes in the ratio of the first two peak height depended on whether the myosin was skeletal or cardiac myosin, the ratio at cardiac myosin was significantly lower indicating different local conformational changes in the two classes of myosins [9].

Our results for cardiac myosin showed that the conformational changes obtained after ADP binding depended strongly on the probe molecules. The increase in the mobility induced by ADP varied with its concentration and attained to a saturating level. The conformational change induced by ADP was significantly higher for IASL-myosin.

It is known that IASL exhibits a larger flexibility of the attaching linkage in comparison with MSL. Therefore, we can state that MSL-myosin-ADP complex behaves like a rigid moiety on the time scale of the conventional EPR, whereas the IASL is a sensitive monitor of the nucleotide binding, and it is accompanied by a local conformational change. Experiments performed on glycerol-extracted muscle fibres showed that the addition of MgADP to MSL-fibres did not result in significant axial rotation of the cross-bridges [16]. In contrast, significant alteration of the orientational distribution was observed in the ternary actin-myosin-ADP complex in fibres, when IASL was located on the SH1 thiol sites of myosin. It was concluded that MgADP produces an intrinsic change in the multisubunit structure of the myosin head region, but this does not lead to the change of the overall rotational properties of the myosin head.

3.3. Orientational dependence of EPR spectra in macroscopically aligned actin filament system

It is known from earlier reports that MSL or IASL attached to SH1 sites of myosin in glycerinated muscle fibres in rigor exhibited large degree of order of spin labels with respect to the fibre axis [5]. Similar orientation dependence was detected when MSL-HMM penetrated into the fibres and bound to actin [21]. The results indicated the particular attachment of myosin heads to actin in the absence of ligands like ATP, ADP or PPi. It is expected that in macroscopically aligned actin filament system, an orientation dependence of spin labels can be measured when spin labelled myosin or its fragments are allowed to bind to the system.

Our experiments on MSL-myosin bound to actin showed that $2A'_{ZZ}$ depended on the orientation of the aligned actin filaments with respect to the laboratory magnetic filed. The measurements were performed in

two orientations, $H \| k$ and $H \perp k$, where k is the filament long axis. The orientational anisotropy was small, but differed significantly from 0. The orientational anisotropy was directly obtained from the difference of hyperfine splittings $A_{\|} - A_{\perp}$, normalized to that value of single crystal $(A_{zz} - 1/2(A_{xx} + A_{yy}))$, where all the labels are perfectly oriented. The small effect induced by cardiac myosin can be explained by the larger internal flexibility of the cardiac myosin, and that the binding of myosin to actin reduces the orientational order of aligned F-actin filaments [22].

3.4. Temperature dependence of spectral parameters

The results for intact myosin and LC 2-deficient myosin labelled either with IASL or MSL are presented in Fig. 3. The hyperfine splitting constant ($2A'_{ZZ}$) and the ratio of the low-field peak heights (h_2/h_1) were used to characterize the temperature induced changes.

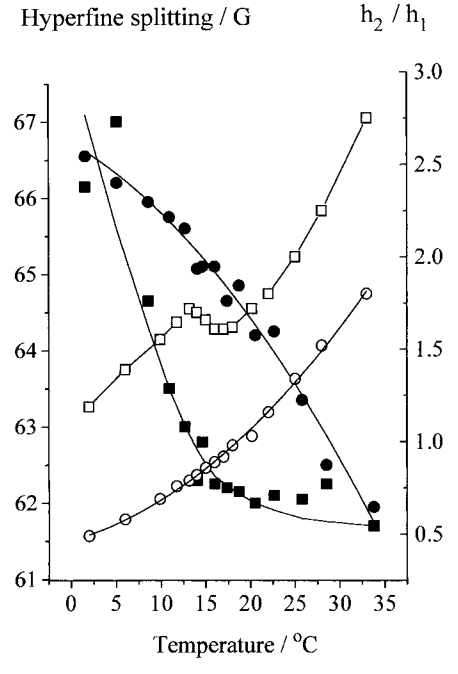

Fig. 3. Temperature dependence of EPR spectral parameters. Cardiac myosin was labelled MSL. Full symbols show the variation of the hyperfine splitting with temperature (● intact myosin, ■ LC 2-deficient myosin), whereas open symbols (○, □) represent the peak height ratio (h_2/h_1) at increasing temperature.

For MSL-myosin, a continuous decrease of $2A'_{ZZ}$ together with a similarly continuous increase in the peak height ratio at increasing temperature was detected as evidence for the slow loosening of the protein structure due to continuous heat absorption. In contrast, a sudden change of h_2/h_1 was detected at around 16°C on LC 2-deficient myosin, and at the same temperature there was a larger alteration in the shape of the curve for $2A'_{ZZ}$. A similar effect of temperature on the protein conformation in the neighbourhood of the attaching site of the labels was measured on IASL-myosin.

The measurements on MSL-myosin exhibited only continuous alteration of the spectral parameters at increasing temperature. It is known that MSL has larger rigidity of the attaching linkage in comparison to IASL. Therefore, the two probe molecules have different sensitivity towards the conformational change of myosin. The measurements in the ST EPR time range support the view that no sudden change in the submillisecond rotational motion of the motor domain exists (Fig. 4). The results confirm that only the segmental motion of the IASL-myosin head was detected under the melting temperature.

Removal of the LC 2 light chain influenced greatly the motional dynamics of MSL-myosin. Significant changes were obtained in spectral parameters of $2A'_{ZZ}$ and h_2/h_1 at about 16°C. This finding supports the

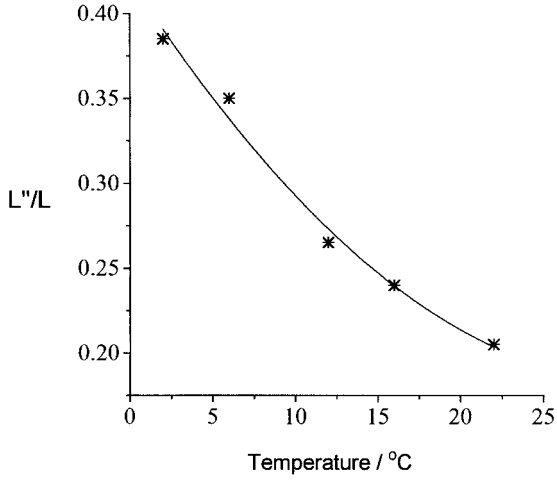

Fig. 4. Plot of saturation transfer spectral parameter ($L''L$) against reciprocal temperature. Spectra were taken on LC 2 deficient MSL-myosin.

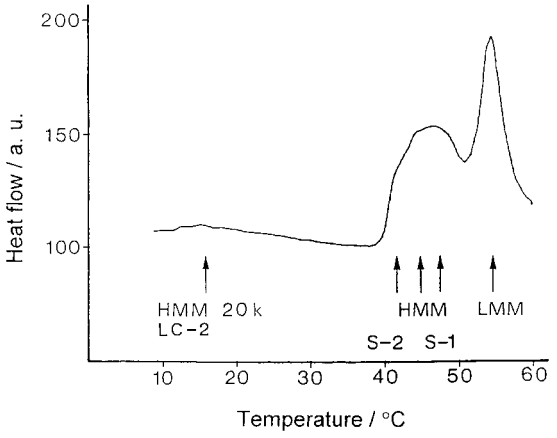

Fig. 5. DSC scan of intact heart myosin. Arrows show the transition temperatures of the subunit of the myosin. The heat flow is given in arbitrary units.

conclusion that the predominant change induced by temperature under the melting point was a local conformational change in the neighbourhood of the thiol site. Earlier experiments showed that the segment containing the essential thiols, SH1 and SH2 is highly flexible [23]. Very likely, the LC 2 light chain modulates the flexibility of the 20 kDa segment of cardiac myosin that holds the label, and its removal affects the local environment of the probe molecules.

3.5. DSC measurements

DSC measurements were performed on intact and LC 2-deficient myosin in the temperature range of 5–60°C. Myosin is a multisubunit protein consisting of several domains. Therefore, a rather complex thermogram comprising at least four endothermic transitions is expected, corresponding to the thermal denaturation of the myosin rod with α-helical structure and the structural domains of myosin head [7,24,25].

The DSC profile of the intact myosin is shown in Fig. 5. Three major transitions were detected with $T_m = 17$, 45 and 54.5°C as transition temperatures. Removal of the LC 2 light chain was accompanied by the disappearance of the 17°C transition. The total enthalpy of the transitions was 7276.7 kJ/mol. Studying the melting profile of myosin we could identify five endothermic peaks (six peaks by deconvolution analysis) at peak maximum of 17, 41.5, 45, 48 and 54.5°C. The calculated enthalpies were 628, 682.5, 1160, 1260 and 3127.5 kJ/mol. The thermodynamic data and our suggestion for the assignment of the transitions to the substructure of myosin are given in Table 1.

According to the recent model of myosin, it is believed that it comprises structural units that fold up independently into a stable domain structure [7,26]. The measurements performed on skeletal myosin showed that the highest transition temperature could be assigned to the unfolding of the coiled-coil α-helix rod portion of the protein moiety [25]. This finding and further data found for myosin head strongly suggest that the endothermic peak at 54.5°C indicated the melting of the rod part.

By examining the effect of mild heat treatment on the ATPase and proteolytic sensitivity of myosin S 1, it was concluded that the 50 kDa segment of the myosin head was preferentially unfolded during heat treatment at 35°C, while the 21 and 27 kDa fragments

Table 1
Transition temperatures and enthalpies of myosin unfolding[a]

Structural subunit	Transition temperature (°C)	Transition enthalpy (kJ/mol)
HMM 20 kDa or LC 2 light chain	17.0	628[b]
HMM S-2	41.5	682
HMM 50 kDa domain	45.0	1578
HMM 25 + 20 kDa domains	48.0	1260
LMM	54.5	3127.5
		total enthalpy: 7277 kJ/mol

[a]Intact and LC 2-deficient cardiac myosin was measured in buffer solution containing 500 mM KCl., 25 mM HEPES and 1 mM EDTA, pH 7.0. The concentration of the samples was 20 μM. Measurement was made with DASZM-4 Privalov-type and Micro DSC-II (SETARAM) differential scanning calorimeters with a scan rate of 0.3°C/min.

[b]The transition at 17°C could not be detected on LC 2-deficient myosin.

remained unchanged [27]. It was shown by limited tryptic digestion that in term of thermal stability the head region of myosin comprised of two domains, the unstable 50 kDa domain which unfolded independently of the remainder structure, and the more stable 20 and 25 kDa segments of the heavy chain [28]. Recent measurement on the thermal unfolding of skeletal myosin gave evidence that the first transition of the profile 41°C was very likely due to the thermal transition of the S 2 region [29]. Considering all the experimental data it seems to be not unreasonable to assign the thermal transitions obtained in our measurements to the following structural subunits: HMM S 2 at $T_m = 41.5$°C, HMM 50 kDa at $T_m = 45$°C, HMM $20 + 25$ kDa at $T_m = 48$°C and LMM at $T_m = 54.5$°C. The higher T_m values calculated for the domains of HMM in our experiments can be explained by the differences of the conditions of the measurements (sensitivity of the DSC apparatus, type of buffer solution, protein concentration).

For the interpretation of the lowest endothermic transition there are two possibilities; (a) a small conformational change in the LC 2 light chain itself; (b) more likely it could be related to the interaction of the long α-helical region of myosin with the LC 2 light chain. This region is stabilized by the interaction of the LC 2 light chain and the 20 kDa segment of the myosin, and this appears as small endothermic peak in the thermogram. The structural perturbation might correlate with the EPR observation on the conformational transition at 16°C.

Acknowledgements

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References

[1] M. Tada, G. Bailin, K. Barany, Biochemistry 8 (1969) 4842.
[2] W.M. Kuehl, R. S Adelstein, Biochem. Biophys. Res. Commun. 39 (1970) 956.
[3] G. Huszár, M. Elzinga, J. Biol. Chem. 247 (1972) 745.
[4] N. Bálint, F.A. Sréter, J. Gergely, Arch. Biochem. Biophys. 168 (1975) 557.
[5] D.D. Thomas, R. Cooke, J. Biophys 32 (1980) 891.
[6] M.S. Crowder, R. Cooke, J. Musc. Res. Cell Motil. 5 (1984) 131.
[7] I. Rayment, W.R. Rypniewski, K. Schmidt-Bäse, R. Smith, D.R. Tomchick, M.M. Benning, D.A. Winkelmann, G. Wesenberg, H.M. Holden, Science 261 (1993) 50.
[8] D.B. Stone, Biochemistry 12 (1973) 3672.
[9] D.B. Stone, R.A. Mendelson, J. Botts, P.H. Cheung, Circ. Res. 49 (1981) 677.
[10] U. Hoffmann, J. Belagyi, Biol. Chem. Hoppe-Seyler 372 (1991) 679.
[11] O. Roopnarine, A.G. Szent-Györgyi, D.D. Thomas, Biochemistry 37 (1998) 14428.
[12] K.T. Shiverick, L.L. Thomas, N.R. Alpert, Biochim. Biophys. Acta. 393 (1975) 124.
[13] P. Graceffa, J.C. Seidel, Biochemistry 19 (1980) 33.
[14] P. Fajer, D. Marsh, J. Mag. Res. 49 (1982) 212.
[15] D.D. Thomas, J.C. Seidel, J. Gergely, J.S. Hyde, J. Supramol. Struct. 3 (1975) 376.
[16] J. Belagyi, I. Frey, L. Pótó, Eur. J. Biochem. 24 (1994) 215.
[17] S.A. Goldman, G.V. Bruno, C.F. Polnaszek, J.F. Freed, J. Chem. Phys. 56 (1972) 716.
[18] J. Belagyi, E. Rőth, Gen. Physiol. Biophys. 6 (1987) 571.
[19] J.C. Seidel, M. Chopek, J. Gergely, Biochemistry 9 (1970) 3265.
[20] D. Lőrinczy, J. Belagyi, Thermochim. Acta 296 (1997) 161.
[21] J. Belagyi, G. Pallai, P. Gróf, K. Trombitás, Studia Biophysica 73 (1978) 159.
[22] Gy. Hegyi, L. Szilágyi, J. Belagyi, Eur. J. Biochem. 175 (1988) 271.
[23] E.E. Huston, J.C. Grammer, R.C. Yount, Biochemistry 27 (1988) 8945.
[24] A. Bertazzon, G.H. Tian, T.Y. Tsong, Biophys. J. 53 (1988) 236a.
[25] P.L. Privalov, Adv. Protein Chem. 35 (1982) 1.
[26] D. Mornet, R. Bertrand, P. Pantel, E. Audemard, R. Kassab, Biochemistry 20 (1981) 2110.
[27] A. Setton, A. Mühlrad, Arch. Biochem. Biophys. 235 (1984) 411.
[28] M. Burke, S. Zaager, J. Bliss, Biochemistry 26 (1987) 1492.
[29] J.W. Shriver, I. Zegar, U. Kamath, Biophys. J. 53 (1988) 236a.