PhD Thesis

Research of muscle proteins applied by luminescence spectroscopy

Bódis Emőke

Programme	Biochemistry and molecular biology
Head of the Programme	Dr. Sümegi Balázs
Subprogramme	B-130: Resarch of functional dynamics of proteins applying biophysical methods
Head of the Subprogramme	Dr. Somogyi Béla

University of Pécs, Faculty of Medicine
Department of Biophysics

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Introduction

One of the biggest cell of the organisation is the striated cell, its volume is filled in by two types of filamental system: (i) the thin myofilament, containing mainly actin and the (ii) thick myofilament, containing myosin. These filaments are regularly arranged, their paralell build-up creats the base of the muscle contraction, the sarkomer. At muscle contracion the two types of filements move pass each other causing the shortening of the sarkomer and the whole muscle cell (muscle fibre). The energy which is needed for the power generation is arrised from ATP hydrolysis.

Myosin is a motor protein, which is able to convert the chemical energy from ATP hydrolysis into mechanical work. This is also an actin-activated Mg^{2+}-ATPase enzyme, which can function in the absence of actin too. It is a hexameryc polipepdide containing two, ~220 kDa heavy chains and two pairs of ~20 kDa light chains. The N-terminal of the polipeptide, approximately 850 amino acides form the myosin head („cross-bridge"), while the rest of the protein has the function of stability and takes part in the forming of the thick filaments. The globular head contains the ATP-binding cleft and the actin-binding regions.

The smallest portion of the molecule, which doesn't loose its functional activity, is the subfragment 1 (S1). Applying S1 digested from myosin by α-chymotrypsin in *in vitro* circumtenses is practical, because it is soluble in a solution with low salt concentration in contrast with intact myosin. The heavy chain of S1 can be digested by trypsin in 3 fragments: (i) 20 kDa, (ii) 50 kDa and (iii) 25 kDa.

During one cycle of ATP hydrolysis the nucleotide-binding cleft of myosin undergoes a chain of conformational changes, which can affect the whole molecule. In the absence of nucleotide the cleft is open, then after the ATP association the cleft will be closed, and this closed state will remain after the hidolysis at ADP.P_i binding as well. At inorganic phosphate (P_i) and ADP release the cleft will have again an open position, now it is able to bind the next ATP molecule. The investigation of ATP and ADP.P_i states by spectroscopic methods are difficult, because they have

extremly short lifetimes. Solving the problem, it is able to apply non-hydolysable nucleotide analogues, they can mimic the given nucleotide states. It is widely used to mimic the ATP state by applying ADP.BeF_X, while the ADP.P_i state by applying ADP.AlF_4^- or ADP.V_i

Actin is the unit of the cytoskeletal microfilament and also the thin filaments of the muscle tissue. It has two appearing form both *in vivo* and *in vitro*: monomer and polimer. Monomer has a globular structure, it contains bivalent cation (Ca^{2+}, Mg^{2+}), and nucleotide (ADP, ATP) binding sites in the pocket inside the molecule. The X-ray christallographic 3D modell of monomer actin was born in 1990, the filament actin was preper by modelling based on the monomeric form. The monomer can be divided into two domains, the smaller domain contains two subdomains: I and II, the bigger domain contains also two subdomains: III and IV.

There are 4 tryptophans in a monomer, all of them are located in the subdomain I, which region comprises the amino acides binding to myosin. The huge advantage of tryptophan is that it can function as a fluorophor or cromophore in spectroscopic experiments. G. B. Strambini and S. S. Lehrer investigated the conformational and dynamic differences between the monomer and filament forms applying tryptophan phosphorescence spectroscopy. According to their results the environments of tryptophans are highly heterogen, the conclusion is given that the local environments have different polarity and local flexibility. Compering the spectroscopic features of the monomer and the filament forms, they found that subdomain I hardly changes during the polimerisation.

Aims

In the focus of our research the intramolecular flexibility of myosin is as well as the relation of actin-myosin complex characterising eiter from the myosin side or from the actin side.

1.) Our aim was to investigate the intramolecular flexibility as a dynamic parameter between enzimatically active regions: (i) actin-binding region, (ii) ATP-binding cleft and (iii) highest reactivity cysteine (SH1, Cys^{707}). Furthermore we pland to follow, how this parameter changes during one cycle of ATP hydrolysis, where ATP, ADP.Pi, ADP and nucleotide-free states can be distinguished. We applied fluorescence spectroscopic methods: resonance energy transfer (FRET), lifetime and steady-state anisotropy measurements.

2.) Lys^{553} amino acid of S1 is located close to the actin-binding surface and can be labelled selectively with extrinsic fluorophore (FHS). We required to characterise the environment of the fluorophore by fluorescence quenching measurements in the presence and absence of actin and/or ADP.

3.) In addition our aim was to characterise the changes in actin upon actin-S1 complex formation. We applied steady-state and time-resolved phosphorescence methodes, where tryptophans functioned as chromophores. This method allows to detect the conformational and dynamic changes of the local environment of the probes upon the complex formation. Modelling of myosin we used tryptophan-free motor domain (MD/W-), which enzimatic activity and ability of actin-binding are close to the native S1.

Methods

The steady-state fluorescence measurements were carried out on Perkin Elmer LS50B spectrofluorometer. To determine the transfer efficiency (E) of the Förster-type resonance energy transfer (FRET) we measured the intensity of the donor in the presence (F_{DA}) and in the absence (F_D) of the acceptor:

$$E = 1 - (F_{DA} / F_D) \qquad (1)$$

The distance (R) between the donor and acceptor can be calculated in the knowledge of transfer efficiency (E) and Förster-type critical distance (R_o):

$$E = R_o^6 / (R_o^6 + R^6) \quad (2)$$

The Förster-type critical distance is that of the distance between the donor and acceptor, which would be at 50% transfer efficiency. The definition of the normalised transfer efficiency (f ') is the following:

$$f' = E / F_{DA} \quad (3)$$

The temperature dependence of f ' provides information about the flexibility of the protein matrix between the two labelled points. The higher slope of f ', the more felxible is the protein matrix between the donor and acceptor.

Steady-state anisotropy measurements on S1 were carried out in nucleotide-free state between 6-26^{o}C at 8 temperature values. The results were analised by the Perrin-equation:

$$1 / r = 1 / r_0 (1 + (k T / V \eta) \tau) \quad (4)$$

where r is the steady-state anisotropy, r_0 is the limited anisotropy, k is the Boltzman constant, T is the absolute temperature, V is the volume of the sphere-shape rotating unit, η is the viscosity and τ is the lifetime of the fluorophore.

Fluorescence lifetime measurements were made on ISS K2 multifrequenz phasefluorometer. The average lifetime were calculated as following:

$$\tau_{átl} = \Sigma \tau_n^2 \alpha_n / \Sigma \tau_n \alpha_n \quad (5)$$

where τ_n is n^{th} lifetime component, α_n is the belonging amplitude.

For the determination of the efficiency of fluorescence quenching corrected steady-state fluorescence spectra were taken up and also the lifetime values in the presence of different concentration of quencher were measured. The results were analised with the help of Stern-Volmer equation:

$$F_0 / F = \tau_0 / \tau = 1 + K_{SV}[Q] \quad (6)$$

where F_0 and τ_0 are the intensity and lifetime in the absence of quencher, F and τ are the intensity and lifetime in the presence of quencher, K_{SV} is the Stern-Volmer constant .

$$K_{SV} = k_+ \tau_0 \quad (7)$$

where k_+ is the bimolecular quenching constant, its value gives information about the exposition of the fluorophore, in case of charged quencher about the distribution of charges of the protein matrix.

The phosphorescence measurements were carried out on a home-made phosphorometer in G.B. Strambini's laboratory (CNR, Pisa, Italy). The steady-state spectra of actin and/or myosin motor domain (MD/W-) were taken up at 140 K, where the glycerol concentration of the solvent was 60%, in this case the matrix has a so-called glass state („glass matrix"). The tryptophans of actin were the cromophores of the spectroscopic measurements. By the analysis of the spectra we investigated the relaxation between the triplet excited state and the lowest vibronic level of the singlet ground state ($\lambda_{0,0}$). The peaks of the spectra as a function of the wavelengh characterises the solvent exposition of the chromophore.

The phosphorescence lifetime decays were taken at 0.5 and 20 ^{o}C. The decays were analised by the fitting of exponential functions applying Global software (Global Unlimited, LFD, University of Illinois). In all cases the addition of 3 exponential functions gave a better fitt, than the addition of 2 exponential functions. Based on an empirical study the longer lifetime refers to a less flexible microenvironment of the cromophore, where the quenching processes are less effective.

Results and Conclusions

1.) Research of the intramolecular flexibility of myosin S1

We made three different FRET (donor-acceptor) pairs inside S1: Ser^{181} (ANN, donor) - Lys^{553} (FHS, acceptor), Ser^{181} (ANN, donor) - Cys^{707} (IAF, acceptor), Cys^{707} (IAEDANS, donor) - Lys^{553} (FHS, acceptor). We measured the transfer efficiency between the donor-acceptor pairs and calculated the f' parameter az a function of the temperature. We investigated four different nucleotide states: nucleotide-free, ADP,

ATP, ADP.P_i. The last two states were mimiced by nucleotide analogues (ADP.BeF_X, ADP.AlF_4^- és ADP.V_i).

According to our results the intramolecular flexibility of S1 is heterogen, and this heterogenity is independent on the quality of the bound nucleotides. The actin-binding region (50 kDa lower domain) is a highly rigid area of the protein, the conformational changes during the ATP hidrolysis in the 4 nm far nucleotid-bindng cleft don't influence the rigidity of the actin-binding domain. The 50 kDa upper domain seemed highly flexible, which flexibility remains during the hydolysis cycle. We suppose, this remarkable flexibility is needed for the quick phosphate binding, conformationl change and release. The great flexibility and dynamic movement gives opportunity for the quick enzymatic processes coupled with significant conformational changes. Altough the S1 is dynamically heterogen , in general it is true, that the closed state is more rigid, while the open state is more flexible. The reason can be, that the more rigid conformation provides a more stable mechanical base for the transfer of infomation from the nucleotide binding cleft to the lever arm. It is widely thought, that the ADP.BeF_x molecule mimics the ATP state, the ADP.AlF_4^- and ADP.V_i molecules mimic the ADP.P_i state, however our results could find differences between the three nucleotide analogue states. According to our results closed state is produced by ADP.V_i, while the two other analogues generate an equilibrium between the open-closed transition.

2.) Characterisation of the actin-binding region of S1 by fluorescence quenching experiments

We modified Lys^{553} amino acid of S1 by FHS extrinsic fluorophore. The quenching experiments were carried out by applying $CoCl_2$ as a quencher. According to our results $CoCl_2$ quenched the fluorescence of FHS in the presence and in the absence of actin, both in nucleotide-free and ADP states. By the Stern-Volmer plots the mechanism of quenching process is static. The binding of actin to S1 doesn't change the efficiency of quenching, therefore we think, that the effect of actin doesn't cause such a change, which could be revealed by applying positive quenchers. Nevertheless the efficiency of quenching depends on the quality of bound nucleotide: in case of

ADP state the efficiency decreses by 40% realtive to the nucleotide-free states. Our conclusion compering to the results of MacLean laboratory is, that in ADP state the spatial exposition changes (not the distribution of charges).

3.) Investigation of actin upon myosin binding

We examined the steady-state spectra of actin tryptophans at 140 K and the lifetime decays at room temperature. According to our results both the spectra and lifetime decays are heterogen, refering to the heterogen microenvironment of tryptophans. By the results we assigned the spectroscopic parameters to the proper tryptophans.

We investigated, how myosin binding can influence the local environment of tryptophans of actin. S1 has 6 tryptophans, therefore the spectra cannot be devided into components, because of the too many signal-superposition. Among the lifetime components a new component was appeared, which is not characteristic either actin, or S1. Modelling the binding of S1 to actin, we applied a tryptophan-free construction: MD/W-. This motor domain hepls us to divide the spectroscopic features of actin-myosin complex and myosin itself. According to our results, the bound motor domain changes mainly the microenvironment of Trp356 amino acid, although this amino acid is not in direct connection with the actin-myosin binding surface. On the actin surface localised Trp79 was also sensitive to the motor domain binding, but the experiments show only a slightly conformation change. It directly proves, that this amino acid doesn't take part in myosin binding. Interestingly, the rigidity of the deeply burried Trp340 amino acid is increasing, although it is „far" from the binding surface, the effect of myosin binding can only indirectly influence the local region around Trp340.

We examined the effect of tropomyosin on actin and/or MD/W-. We could not found any changes in spectra or lifetime decays. It means, that this regulating protein has either no effect on actin and/or MD/W- or its effect cannot detect based on phosphorescence spectroscopy methods.

Publications

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