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ELECTRON PARAMAGNETIC RESONANCE AND NANOSECOND FLUORESCENCE DEPOLARIZATION STUDIES ON CREATINE-PHOSPHOKINASE INTERACTION WITH MYOSIN AND ITS FRAGMENTS

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Recent reports in the literature have indicated a physical association of creatine-phosphokinase (CPK) with the tail portion of the myosin molecule. The present paper describes further studies on the interaction of CPK with myosin and myosin fragments, using the techniques of electron paramagnetic resonance (EPR) and nanosecond fluorescence depolarization. From EPR work, spin-labeled CPK appears to interact with myosin, tail-less myosin (heavy meromyosin [HMM]), and myosin heads (subfragment-1 [S1]), the extent of interaction being proportional to the S1 content of myosin or its fragments. Spin-labeled CPK did not evidence interaction with the headless myosin "rods," with myosin tails (light meromyosin [LMM]), with S2 necks (which connect S1 to the rest of the myosin molecule), or with actin. When a fluorescent dye is directed to the essential ϵ-amino group of CPK, nanosecond fluorescence depolarization studies indicate a substantial interaction with myosin, HMM, and S1, but very little with F-actin. When the "fast-reacting" thiol of the S1 moiety or the "essential thiol" of CPK was labeled with either a fluorescent dye or a spin label, no interaction between CPK and myosin (or S1) was detected.

INTRODUCTION

The rapid regeneration of ATP,[1] from the ADP produced during muscular activity, is largely accomplished by creatine-phosphokinase (CPK) which normally is found in dimer form and catalyzes the rephosphorylation of ADP at the expense of phosphocreatine (PC). The relative abundance of CPK in muscle and its close cooperativity with muscle ATPases have led some investigators to look for a physical association between the dephosphorylating and rephosphorylating enzymes. In the past, various reports, largely based on enzyme kinetics studies, have indicated an interaction between CPK and myosin (e.g., 1–3) or heavy meromyosin (HMM) (4). More recently, Turner et al. (5) found CPK

[1] Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; PC, phosphocreatine; IAA, iodoacetamide; DNS, dansyl chloride = 1-dimethylaminonaphthalene-5-sulfochloride; 1, 5-IAEDANS, N-(2-iodoacetyl)-N′-(5-sulfo-1-naphthyl)ethylene diamine; CPK, creatinephosphokinase; HMM, heavy meromyosin; LMM, light meromyosin; S1, subfragment 1 of myosin; S2, subfragment 2 of myosin; EPR, electron paramagnetic resonance; spin labels (referred to here), N-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl) iodoacetamide or the corresponding maleimide; Tris, tris (hydroxymethyl) aminomethane.

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at the M line in sarcomeres. And Houk and Putnam (6), using steady state fluorescence depolarization measurements with a sulfhydryl-directed fluorescent dye on CPK, concluded that CPK interacts with the "rod" portion of myosin (myosin without its S1 heads) but not with subfragment 1 (S1). These observations are consistent with those of Turner et al. since only the rod portion of myosin appears at the M line. Houk and Putnam also noted that enzymatically inactive CPK, produced by exposure of CPK to a large excess of the dye or to dye followed by another sulfhydryl reagent (Houk, personal communication), shows no interaction with myosin. This suggested a close association between the "essential thiol" (the readily accessible thiol required for CPK enzymatic activity) and a myosin binding site on CPK.

METHODS

This report deals with efforts to study further the interaction of CPK with myosin and myosin fragments. Two techniques were used: electron paramagnetic resonance (EPR) and nanosecond fluorescence depolarization.

When a small paramagnetic compound ("spin label") is attached to a specific region of a protein molecule, changes in the EPR spectrum can reflect changes in the label mobility, due to whatever restrictions are imposed by its immediate environment. In the interpretation of EPR spectra, a change in the ratio of certain peak heights (peaks "1" and "2" in the muscle literature) indicates a change in the mobility of the bound spin label.

With the nanosecond fluorescence depolarization technique a fluorescent dye attached to a protein molecule absorbs a photon of plane polarized light from a rapidly flashing light source. The light emitted subsequent to any flash is observed by either of two photomultipliers which detect photons polarized, respectively, parallel and perpendicular to the exciting plane. This light becomes depolarized depending on the extent to which the molecule has randomized its orientation (due to rotational Brownian motion) during the time between absorption and emission of a photon. Data is collected for several tens of millions of photon emissions, and the accumulated intensities from each photomultiplier are recorded as a function of the time between a light flash and detection of the corresponding photon emission. The times required for the polarization ("anisotropy") and the total intensity to decay to 1/eth of their initial values are denoted as φ and τ, respectively. τ is responsive to the polarity of the immediate environment of the dye and therefore gives information somewhat analogous to that obtained from EPR measurements; φ depends on the tumbling time of the fluorescence-labeled molecule and is related to its size and shape.

If a labeled protein binds to a second protein, the environment of the label may be altered sufficiently to cause a change in the EPR spectrum of a spin label or in the excited lifetime, τ, of a labeling fluorescent dye. Changes in the rotational properties of a labeled protein following interaction with a second protein can be reflected as a change in φ.

RESULTS

EPR Spectra

Exposure of CPK to a paramagnetic derivative of IAA, in the absence of any protection of the CPK essential thiol, resulted in no detectable interaction with myosin. With

the same spin label directed to the fast-reacting thiol on either myosin or S1, there was also no evidence of interaction with CPK. When the essential thiols of CPK are protected (7) through a reaction mixture containing Mg^{++}, Cl^{-}, creatine, and ADP, and spin labeling is accomplished with a paramagnetic derivative of maleimide, the CPK retains virtually all of its enzymatic activity and gives evidence of interacting with myosin, S1, and HMM. Furthermore, when the concentrations of these proteins or fragments are expressed in terms of their content of S1 heads, and the change in EPR peak ratio is plotted against this S1 head concentration, a single linear relationship fits the data for skeletal myosin, cardiac myosin, HMM, and S1. This linear relationship holds for all the concentrations tested, ranging up to a ratio of [S1 heads] – [CPK dimer] = 8:1 in the case of S1 itself and a ratio of about 3:1 in the case of HMM heads. By contrast, F-actin at a concentration of 4.5 monomers per CPK dimer produced no significant change in peak ratio for CPK bearing the maleimide spin label. Rods or S2, the short neck that joins each S1 to the rest of the myosin molecule, in a 1:1 concentration ratio with labeled CPK, or LMM in a 1.7:1 ratio, also caused no change. A concentration ratio of 1:1 is somewhat smaller than that (1.6:1) used by Houk and Putnam (6) in their experiments with rods, but the absolute concentrations of proteins in the spin label experiments are several-fold higher and would therefore favor interaction on the basis of mass action. In any case, it is clear that failure of a second protein to perturb the spin label on CPK does not necessarily mean a lack of interaction between the two proteins. On the other hand, a definite change in peak height ratio, proportional to the S1 head concentration of the second protein, strongly suggests interaction of CPK with the S1 moiety. Since the response does not saturate even at an [S1] – [CPK dimer] ratio of 8:1, the interaction may not be a strong one. The observed EPR changes brought about by myosin heads are somewhat stabilized at higher ionic strengths but show no dependence on Ca^{++}, Mg^{++}, substrates, or products of the enzymatic reactions governed by either enzyme.

Fluorescence Experiments

In initial experiments with nanosecond fluorescence depolarization, the thiol-directed dye, N-(2-iodoacetyl)-N′-(5-sulfo-1-naphthyl)ethylene diamine (1, 5-IAEDANS), employed previously (6, 8) was used to label either CPK or S1. With S1 this dye goes preferentially to the "fast-reacting thiol"; with CPK it probably goes mainly to the "essential thiol" (9). In either case, the dye concentration was only one-third to one-half the protein monomer concentration, and the enzymatic activity of the dye-labeled protein was greater than half the control value. When S1 was labeled with 1, 5-IAEDANS, addition of CPK (dimer), at 1- to 2-fold the S1 concentration, gave no evidence of interaction between the two proteins. Most experiments were carried out in the presence of 0.2–0.4 mM MgATP, 6–12 mM PC, 0.16–0.17 M KCl with 28 mM Tris buffer at pH 8.0–8.3 at 25°C. This approximated ionic conditions under which earlier evidence of interaction between myosin and CPK had been reported (3). However, a higher ATPase concentration in the present experiments would be expected to lower the steady state levels of ATP and deplete the PC supply more rapidly. Data are normally gathered for at least half an hour in the nanosecond fluorescence depolarization studies. In control experiments with labeled S1 in the presence of the usual reaction mixture, but in the absence of the ATP-regenerating CPK, no change in either φ or τ values was found over an extended time

interval up to 3 hr at 25°C.

Since the CPK dimer is somewhat smaller than S1 and considerably smaller than myosin, labeled CPK might be expected to provide a more sensitive fluorescent probe for interaction with the myosin family of protein fragments. Although 1, 5-IAEDANS-CPK retained more than half of its enzymatic activity and although conditions approximating those of Houk and Putnam (6) were included in the experimental series, neither myosin nor S1 elicited a change in the φ or τ values of the labeled CPK at 25°C.

The fluorescent dye dansyl chloride (DNS) can be directed largely to ϵ-amino groups when thiols are masked before exposure of protein to the dye (10). The DNS-CPK retained about 45% of its enzymatic activity after thiols were unmasked, indicating that about half of the essential ϵ-amino groups had reacted with the dye. DNS, compared with 1, 5-IAEDANS, presents some disadvantages as a fluorescent label: it has a somewhat shorter excited lifetime, its intensity decay curve is not as well approximated by a single exponential, and the polarization curve is less smooth. Despite these difficulties, it seems clear that the presence of myosin, HMM, or S1 brings about changes in the polarization curves corresponding to significant increases (over 2-fold) in the DNS-CPK φ value. Small increases in the τ value are also observed. Evaluation of φ and τ were generally made over the time interval between 9 and 20 nsec after the peak intensity of fluorescence. Over a range of S1 concentrations (0–17 μM) up to several times the total CPK concentration (1.0–1.5 μM, about half of which is DNS labeled), an increase in φ value parallels an increase in S1 concentration. (Increases in τ values tended to level off with increasing S1 concentrations and were, in any case, very small.) The range of myosin or HMM concentrations was more limited but gave φ values similar to those for the corresponding S1 concentrations. Since concentrations of myosin or S1 which alter the DNS-CPK response did not affect 1, 5-IAEDANS-CPK, the effects of these proteins on DNS-CPK cannot be readily atrributed to changes (e.g., in viscosity) which might be expected to occur to a similar extent in each label system. Qualitatively, the effect of S1, myosin, and HMM on the DNS-CPK φ value parallels the effect of these moieties on the spin-labeled CPK.

In preliminary studies with F-actin and DNS-CPK, a small increase in φ value was observed in the presence of actin. But, compared with S1, a much higher F-actin (monomer) concentration was required to achieve the same increase. S1 (9 μM) and one or two times this concentration in F-actin form a highly viscous (unpourable) gel that remains nearly clear for at least 1 hr (constant OD at 500 nm) in the presence of the CPK ATP-regenerating system. The DNS-CPK φ value in this S1-actin system is not much larger than that for the same concentration of S1 in the absence of actin. Thus, a large increase in the macroviscosity and the presumed immobilization of a large part of the S1 seem to have little effect on the CPK rotational properties as reflected in the φ value. Other myosin fragments have not yet been tested for possible interaction with DNS-CPK.

DISCUSSION

At present, there are unresolved differences among the various experimental approaches to the physical interaction between CPK and myosin or myosin fragments. Considerably higher protein concentrations are used in the EPR experiments; longer data collection times are required for nanosecond fluorescence depolarization measurements;

different labels affect the proteins in different ways. In both fluorescence depolarization and EPR experiments, it would be possible for interaction between a labeled and an unlabeled protein to elude detection. In particular, the failure of rods or LMM to register an effect on spin-labeled CPK does not necessarily stand in disagreement with other observations (5, 6). The cause of the discrepancies in the results between the steady state (6) and the nanosecond fluorescence depolarization experiments with 1, 5-IAEDANS-CPK is not clear. However, EPR and both fluorescence depolarization studies demonstrate direct interaction between myosin and CPK. There is also some indication that thiols may be involved (either directly or indirectly) in the interaction of CPK with myosin or its fragments. When thiols are protected, EPR and nanosecond fluorescence depolarization measurements appear to reflect interaction of CPK with S1 and HMM as well as myosin. If, in each case, the S1 moiety furnishes a common site for interaction with DNS-CPK, the similarities in the resulting φ values might be interpretable in terms of CPK loosely bound to S1 (and making little distinction as to whether the S1 is free in solution, a part of HMM, a part of myosin, or perhaps a part of an S1-actin gel). In view of the foregoing results, it seems interesting to reconsider the role that CPK may play in the contractile cycle and to consider the possibility that on each S1 arm of myosin there is a binding site for CPK.

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