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Activation mechanism of rabbit skeletal muscle myosin light chain kinase

5′-*p*-Fluorosulfonylbenzoyl adenosine as a probe of the MgATP-binding site of the calmodulin-bound and calmodulin-free enzyme

Peter J. Kennelly[1], Joel C. Colburn[2]*, James Lorenzen[3], Arthur M. Edelman[3]**, James T. Stull[2] and Edwin G. Krebs[3]

[1] *Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0308 (U.S.A.),* [2] *Department of Physiology and Moss Heart Center, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235 (U.S.A.)* and [3] *Howard Hughes Medical Institute and Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)*

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5′-*p*-Fluorosulfonylbenzoyl adenosine (FSBA), an ATP-like affinity labelling reagent, reacted with rabbit skeletal muscle myosin light chain kinase (skMLCK) and its calmodulin complex in a site-specific manner. Reaction was dependent upon the presence of the adenosine moiety of FSBA, saturated with increasing FSBA, was inhibited by MgATP, and was accompanied by stoichiometric incorporation of [^{14}C]FSBA. The kinetic constants describing the reaction were similar for skMLCK and its calmodulin complex: $k_3 = -0.040$ min^{-1} and -0.038 min^{-1}, and $K_i = 0.18$ mM and 0.40 mM, respectively. It is concluded that the MgATP-binding site on skMLCK remains accessible at all times and maintains a near constant conformation.

Autoinhibition; Pseudosubstrate; Myosin light chain kinase; Calmodulin; 5′-*p*-Fluorosulfonylbenzoyl adenosine

1. INTRODUCTION

The myosin light chain kinases (MLCK) possess an absolute requirement for the binding of the calcium-bound form of calmodulin (CaM) for expression of catalytic activity (reviewed in [1]). The discovery of the location and primary structure of the CaM-binding domain of rabbit skeletal muscle MLCK (skMLCK) [2,3] sparked intense efforts to elucidate the mechanism by which it and other enzymes are activated by CaM. Much of this effort has focused on the possibility, first suggested by Kemp and coworkers [4], that the region immediately adjacent to or overlapping with the CaM-binding domain might serve as an endogenous pseudosubstrate or autoinhibitor of catalysis by binding to the protein/peptide substrate binding pocket within the active site of the enzyme. The binding of CaM would remove the auoinhibitor from the active site allowing catalysis to proceed (reviewed in 5–7]). The principal evidence for autoinhibition sprang from the observation that peptides modelled after the CaM-binding domains of chicken gizzard MLCK and skMLCK acted as competitive, CaM-mediated inhibitors of catalysis with respect to protein or peptide substrates [4,8]. With skMLCK it was also observed that the binding model peptides to the active site counteracted the effects of CaM binding upon the thermal stability properties of the enzyme [9]. Zot and Puett [10] reported that the complex produced by the covalent crosslinking of CaM to skMLCK retained significant catalytic activity in the absence of Ca^{2+}, indicating that attachment of CaM at the proper location was sufficient to partially turn on catalysis. Shoemaker et al. [11] demonstrated that inversion of the sequence of the CaM-binding domain of a nonmuscle MLCK, which leaves the adjacent putative autoinhibitory domain undisturbed, produced a fully functional, CaM-regulated enzyme.

Recently it has been proposed that the behavior of skMLCK and several other CaM-regulated models

Correspondence address: P.J. Kennelly, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308, USA. Fax: (1) (703) 231-9070.

* *Present address:* Applied Biosystems Inc., 3745 N. First St., San Jose, CA 95134, USA.

** *Present address:* Dept. of Pharmacology and Therapeutics, State University of New York at Buffalo, Buffalo, NY 14214, USA.

Abbrevations: CaM, calmodulin; MLCK, myosin light chain kinase; skMLCK, rabbit skeletal muscle MLCK; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2′-ethane sulfonic acid; MOPS, 3-(*N*-morpholino) propane sulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DMF, *N,N*-dimethyl formamide; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; FSB, *p*-fluorosulfonyl benzoic acid

could be explained by a 'flip-flop model' in which MLCK possess a CaM-like domain to which the CaM-binding domain associates in the absence of CaM [12]. This binding event is thought to induce a conformational shift affecting the active site that renders it catalytically non-functional. CaM-binding would activate the enzyme by disrupting the association of the CaM-binding domain with the internal CaM-like domain and allowing the active site to relax into its catalytically-competent conformation.

The evidence for both autoinhibitor and conformational-shift models remains inferential in nature. In order to gain further insight into the events that take place when CaM binds to and activates rabbit skMLCK – especially those affecting catalysis, we have probed the MgATP substrate binding site of the enzyme using the ATP-like affinity labelling reagent 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) [13]. Specifically, we wished to determine (i) whether the MgATP binding site that must be present when the enzyme is activated by CaM remains accessible and recognizable in the absence of CaM, and (ii) whether this site undergoes a conformational or other change of sufficient magnitude upon CaM binding so as to alter its affinity for or reactivity with FSBA. Portions of this work have been reported previously in preliminary form [14].

2. MATERIALS AND METHODS

2.1. *Materials*

Purchased materials included DMF (Fisher Scientific, Pittsburg, PA); HEPES (Research Organics Inc., Cleveland, OH); FSBA (Sigma Chemical Co., St. Louis, MO); *p*-fluorosulfonyl benzoic acid (Aldrich Chemical Co., Milwaukee, WI); and [adenine-8-^{14}C]FSBA (New England Nuclear). Peptides were synthesized by the chemical synthesis facility of the Howard Hughes Medical Institute in Seattle, Washington and purified and analyzed as previously described [8]. All other materials were obtained from previously listed sources [8,9].

2.2. *Enzymes and other proteins*

skMLCK was purified from the back and hind limb skeletal muscle tissue of New Zealand white rabbits [15]. CaM was purified from bovine testes [16]. skMLCK concentration was determined by the method of Bradford [17] using bovine serum albumin as standard and a molecular mass of 65 000 Da [18] to calculate molarity. CaM was estimated from its UV absorbance at 277 nm [19].

2.3. *Reaction conditions for affinity labelling*

The reaction between skMLCK and FSBA was carried out at 25°C in a volume of 60 μl of 50 mM HEPES buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 M NaCl, 5% (v/v) DMF, and the concentrations of FSBA indicated in the individual figure legends. The concentration of skMLCK was 0.1 mg/ml (1.5 μM). For reactions involving the skMLCK·CaM complex, 5 μM CaM was also included. Mg^{2+} and NaCl were included in order to stabilize the skMLCK·CaM complex [9]. FSBA was added as a freshly made 20-fold concentrated stock solution in DMF. FSBA concentrations were determined by measuring the UV absorbance at 259 nm using an extinction coefficient of 15800 $M^{-1}\cdot cm^{-1}$ [13]. Reaction was initiated by adding FSBA in DMF and mixing vigorously. At timed intervals the reaction was stopped by removing 4 μl aliquots of the reaction mixture and diluting them 500-fold into 10 mM MOPS, pH 7.0, containing 1 mM DTT, 0.2 mM EGTA, 0.1 M NaCl, 0.5 mg/ml BSA, and 0.1% Triton X-100. The proportion of skMLCK molecules undergoing irreversible modification by FSBA was determined by measuring the MLCK activity remaining [8] using a peptide substrate (KKRAARATSNVFA; [20]). All results were corrected with mixtures containing DMF without FSBA. Experiments with *p*-fluorosulfonyl benzoic acid (FSB) utilized identical procedures, with the exception that the FSB was quantitated by weight.

2.4. *Stoichiometry of FSBA incorporation into skMLCK*

The stoichiometry of incorporation of covalently bound FSBA into skMLCK was determined as described by Colburn et al. [14] using [adenine-8-^{14}C]FSBA.

3. RESULTS AND DISCUSSION

FSBA binds to and reacts with both skMLCK and its CaM complex, rendering the enzyme catalytically inactive through the covalent modification of the enzyme's MgATP binding site. This conclusion is based on several criteria. First, inactivation required the presence of the adenosine moiety of FSBA (Fig. 1). FSB, which contains the reactive fluorosulfonyl group of FSBA but lacks the adenosine targeting moiety, produced little or

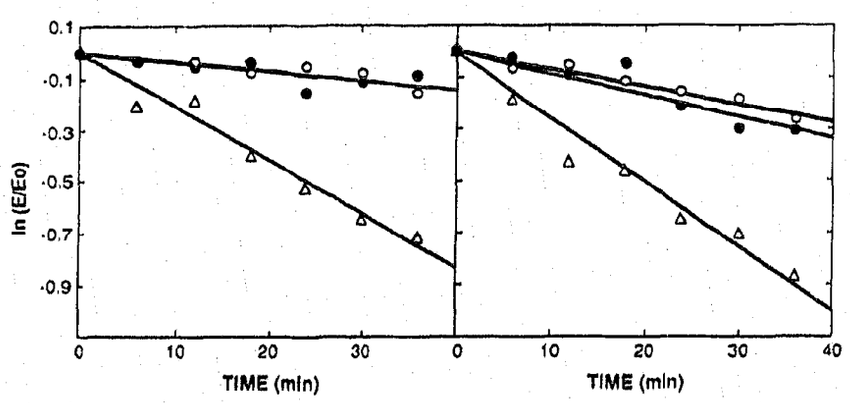

Fig. 1. Inactivation of skMLCK or skMLCK-CaM by FSBA. skMLCK (top) or skMLCK·CaM (bottom) was incubated in the presence of no inactivating agent (○), 0.6 mM FSB (●), or 0.6 mM FSBA (△) and aliquots of the reaction mixture removed and assayed for activity at the indicated times as described in Materials and Methods. Shown is the ln of the fraction of initial enzyme activity remaining [$\ln(E/E_o)$] versus time of incubation.

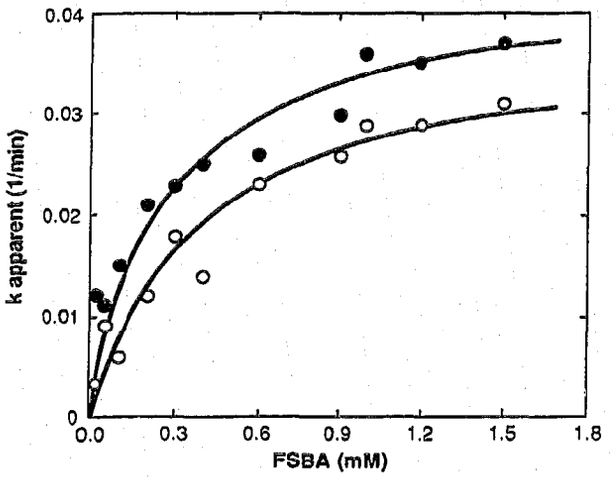

Fig. 2. Dependence of rate of inactivation on FSBA concentration. skMLCK (○) or skMLCK·CaM (●) was incubated with the indicated concentrations of FSBA under standard conditions as described in Materials and Methods. Shown plotted is the apparent first-order rate constant for inactivation, k_{app}, as a function of FSBA concentration.

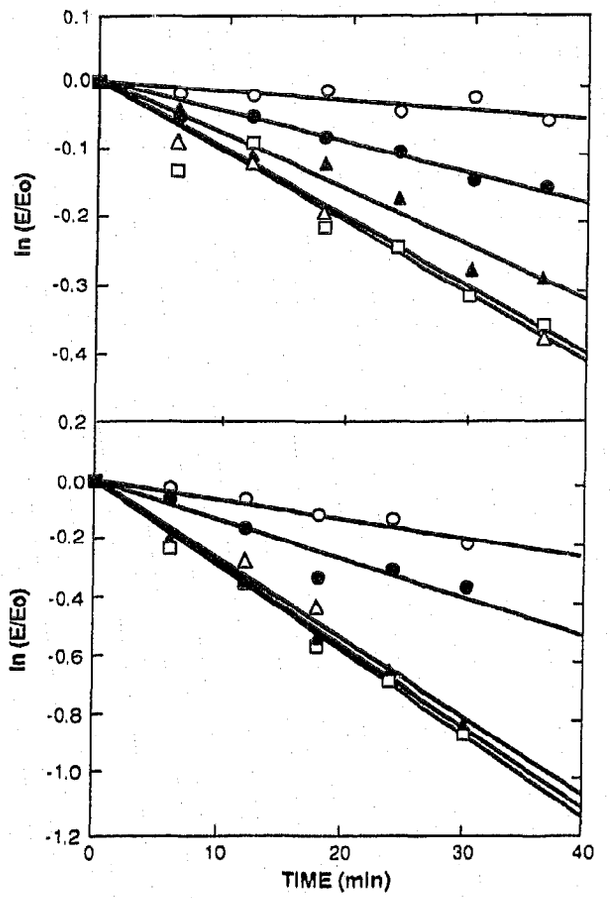

Fig. 3. Inhibition of FSBA inactivation by MgATP. skMLCK [top] or skMLCK·CaM [bottom] was incubated with 0.6 or 0.4 mM FSBA, respectively, under standard conditions with the following modifications. Included in each reaction mixture were either no additional compounds (□), 10 mM MgATP (●), 10 mM MgGTP (△) or 0.2 mM substrate peptide - KKRAARATSNVFA (▲). The open circles (○) represent the average of control values obtained for reaction mixtures containing each of the above-listed compounds, but no FSBA. Average values were presented for the sake of clarity.

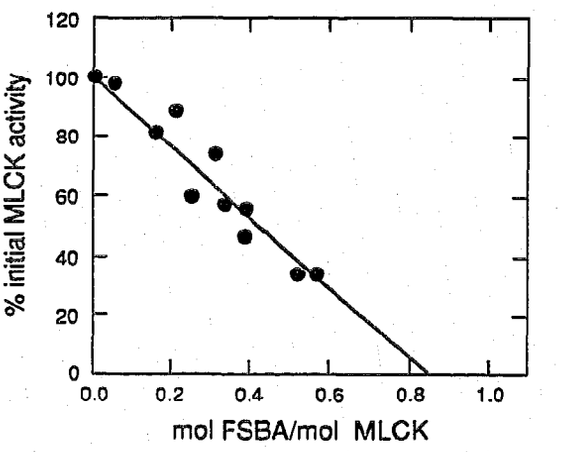

Fig. 4. Stoichiometry of [^{14}C]FSBA incorporation into skMLCK. Samples of skMLCK were incubated with [adenine-8-^{14}C]FSBA, 0.8 mM, for varying periods of time, then analyzed for remaining enzyme activity and incorporation of [^{14}C]radioactivity as described in Materials and Methods. Shown plotted is the proportion of enzyme activity remaining versus the mol of [^{14}C]FSBA incorporated per mol of skMLCK. The line represents a least-squares fit of the data.

no inactivation of either form of the enzyme (Fig. 1). Second, the rate of reaction - as measured by the apparent first order rate constant k_{app} as related by the equation $\ln(E/E_o) = -k_{app} \times t$ where E is the quantity of activity measured at time t and E_o is the amount of activity measured at $t=0$ - saturated with increasing concentrations of FSBA (Fig. 2). Third, MgATP, but not MgGTP or a peptide substrate, inhibited the reaction (Fig. 3). Lastly, inactivation of skMLCK was accompanied by the nearly stoichiometric incorporation, 0.84 mol/mol, of covalently-bound ^{14}C-labelled FSBA into skMLCK (Fig. 4). The kinetic parameters for each reaction were determined using a Hanes-Woolf plot

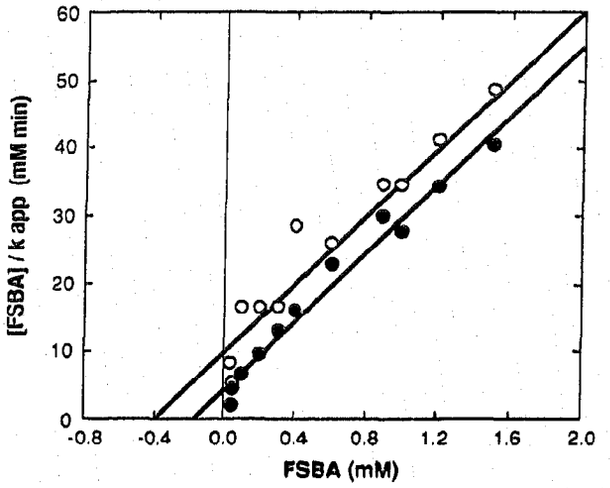

Fig. 5. Analysis of the kinetic parameters for the FSBA inactivation of skMLCK or skMLCK·CaM. Shown is a Hanes-Woolf plot [S/V versus S] of the data in Fig. 2 describing the dependence of the rate of inactivation of skMLCK (○) or skMLCK·CaM (●) on the concentration of FSBA. The lines shown represent least-squares fits of the data.

($FSBA/k_{app} = FSBA/k_3 + K_i/k_3$ [21]) as shown in Fig. 4. The rate constant for the conversion of the reversible enzyme·FSBA complex to the covalent enzyme–FSBA complex, k_3, is essentially identical whether skMLCK or skMLCK·CaM is the target species (0.038 min^{-1} and 0.040 min^{-1}, respectively). The equilibrium dissociation constants of the non-covalent skMLCK·FSBA and skMLCK·CaM·FSBA complexes differed by about two-fold (K_i = 0.40 mM and 0.18 mM, respectively), with skMLCK·CaM displaying the greater affinity.

The reactivity of skMLCK and its CaM complex with FSBA indicates that the MgATP binding site on the enzyme remains accessible, and recognizable as such, even when the enzyme is in the catalytically inactive, CaM-free state. This behavior contrasts with that of the multifunctional CaM-dependent protein kinase type II, for which it has been proposed that activity is modulated through the blockage of the MgATP-binding site [22]. The shift in K_i, the dissociation constant for FSBA, of some two-fold upon the transit of skMLCK between the CaM-bound and CaM-free states indicates that this site was subject to some degree of change, perhaps conformational in nature, during this process. However, the relatively small shift in K_i observed, coupled with the virtually unchanged value of k_3, implies that the degree of alteration is relatively small. Such behavior is consistent with the basic tenets of autoinhibitor models for the regulation of skMLCK. The possibility still remains that another quite different mechanism might more accurately describe the process of CaM activation. However, any such model must accommodate the observed maintenance of the accessibility and recognizability of the MgATP-binding site during the activation-inactivation cycle.

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