The MgATP-Binding Site on Chicken Gizzard Myosin Light Chain Kinase Remains Open and Functionally Competent during the Calmodulin-Dependent Activation-Inactivation Cycle of the Enzyme†

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ABSTRACT: An ATP-like affinity labeling reagent, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), was used to probe the MgATP-binding site of smooth muscle myosin light chain kinase from chicken gizzard (smMLCK) and its calmodulin (CaM) complex. Native smMLCK has an absolute requirement for the binding of the calcium complex of CaM for expression of its catalytic activity. FSBA reacted with smMLCK–CaM and with the CaM-free, inactive enzyme as well. Both reactions were dependent on time and FSBA concentration. Reaction was accompanied by the incorporation of covalently bound [^{14}C]FSBA into smMLCK protein at a molar ratio of approximately 1:1 in each case. *p*-(Fluorosulfonyl)benzoic acid, an analogue of FSBA lacking the adenosine targeting group, did not react at a significant rate with either form of smMLCK. Reaction of CaM-free and CaM-bound smMLCK with FSBA displayed saturation kinetics. The first-order rate constants for the conversion of the reversible, noncovalent enzyme–FSBA complex to form the irreversibly inhibited, covalently modified enzyme were similar for both smMLCK and smMLCK–CaM, 0.15 and 0.07 min^{-1}, respectively. The concentrations of FSBA yielding the half-maximal rate of inactivation, K_I, were essentially identical—0.65 and 0.64 mM, respectively—for smMLCK and smMLCK–CaM. MgATP, but not MgGTP or a substrate peptide, potently inhibited reaction with FSBA. Inhibition by MgATP was competitive. The measured inhibitory constant for MgATP was essentially the same—33 versus 34 μM—for both smMLCK and smMLCK–CaM. It therefore is concluded that the MgATP-binding site on smMLCK remains accessible and recognizable as such when the enzyme becomes inactivated upon dissociation of CaM.

The myosin light chain kinases (MLCKs)[1] are a family of tissue-specific enzymes that phosphorylate the regulatory light chain of myosin [reviewed in Stull et al. (1986)]. In smooth muscle, this event is an obligate step in the initiation of contraction, while in striated muscle it serves a modulatory role [reviewed in Sellers and Adelstein (1987) and Stull et al. (1985)]. Distinct forms of the enzyme are expressed in smooth, cardiac, and skeletal muscle tissue as well as in nonmuscle tissues. Although these various forms show marked differences with respect to molecular size, antigenicity, and susceptibility to and the subsequent effects of phosphorylation by the cAMP-dependent protein kinase, they share several important properties in common. These include an absolute dependence on the binding of the calcium complex of calmodulin (CaM) for expression of their protein kinase activity. This requirement allows cells to stringently regulate MLCK activity through fluctuations in the level of the intracellular second messenger calcium.

The MLCKs have the distinction of being the first CaM-regulated enzymes whose CaM-binding domains were identified and sequenced [for rabbit skeletal muscle MLCK, see Blumenthal et al. (1985) and Edelman et al. (1985); for chicken gizzard MLCK, see Lukas et al. (1986)]. The discovery of these CaM-binding domains has led to intensive efforts to determine the molecular mechanisms by which CaM activates this target enzyme. Models for this process fall into two distinct categories, the conformational-shift type and the autoinhibitory type [reviewed in Kennelly et al. (1989) and Soderling (1990)]. Conformational-shift models propose that the critical event that takes place upon CaM binding is a conformational rearrangement of the active site of MLCK from a nonfunctional to a catalytically competent configuration. Attempts to detect the occurrence and to determine the nature of any conformational changes that take place in the MLCKs upon CaM binding by spectroscopic (Johnson et al., 1981; Malencik et al., 1982), physicochemical (Adelstein & Klee, 1981; Mayr & Heilmeyer, 1983), and proteolytic means (Walsh, 1985) indicate that some small degree of change in overall enzyme conformation does occur upon activation. However, it has not proved possible to discern whether or not this reflects the occurrence of a regio-specific active-site conformational event of the type predicted by conformational-shift models.

In contrast to conformational-shift models, autoinhibitory models—first proposed by Kemp and co-workers (Kemp et al., 1987)—suggest that no significant changes occur to the conformation of the active site of MLCK during the CaM binding/activation process. Rather, it is proposed that over-

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[1] Abbreviations: MLCK, myosin light chain kinase; smMLCK, smooth muscle myosin light chain kinase; skMLCK, skeletal muscle myosin light chain kinase; CaM, calmodulin; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; FSB, *p*-(fluorosulfonyl)benzoic acid; CaM kinase II, multifunctional calmodulin-dependent protein kinase type II; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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lapping with or closely adjacent to the enzyme's CaM-binding domain is a substratelike, also called a pseudosubstrate, domain homologous to the phosphorylation sites on myosin P-light chains. In the absence of CaM, this domain binds to the myosin light chain substrate-binding site within an essentially conformationally-constant active site. Here it acts as a self-inhibitor or autoinhibitor of catalysis. Binding of CaM activates the enzyme by removing the inhibitor. This is suggested to result either through association of CaM with amino acids that form the binding determinants needed for autoinhibitor recognition by the active site or via a highly localized, CaM-induced conformational transformation of the autoinhibitor region that renders it unrecognizible or inaccessible to the enzyme's active site. The central evidence for this model is the observation that synthetic peptides modeled after the CaM-binding regions of smooth and skeletal muscle MLCK act as inhibitors of their respective MLCK isozymes that are competitive with respect to myosin light chain or peptide substrates (Kemp et al., 1987; Kennelly et al., 1987). Further, the sites of proteolytic cleavage that lead to the CaM-independent activation of smMLCK (Pearson et al., 1988; Ikebe et al., 1989) and skMLCK (Edelman et al., 1985) in vitro by partial proteolysis appear to map precisely to those areas suggested by peptide inhibition studies to form the autoinhibitory region. Similar observations have been made regarding the location of inhibitory domains in smMLCK (Ito et al., 1991) or a nonmuscle MLCK (Shoemaker et al., 1990) identified using site-directed mutagenesis.

Although autoinhibitory models for the activation of MLCK have enjoyed fairly widespread popularity, with similar models being suggested for the regulation of several other CaM-dependent enzymes such as calcineurin (Hashimoto et al., 1990), plasma membrane Ca^{2+} pump (Vorherr et al., 1990), and CaM kinase II (Colbran et al., 1988), the evidence for the regulation of the MLCKs in this manner remains largely inferential. Recently it has been observed that the alteration of skMLCK by either deleting the entire regulatory region by site-directed mutagenesis (Herring et al., 1990) or mutating the basic amino acid residues deemed essential for autoinhibition (Herring, 1991) failed to produce constitutively active enzyme forms as predicted by autoinhibitory models. A mutant form of calmodulin, with three amino acid substitutions in domain I, recently has been reported to bind smMLCK without stimulating its catalytic activity (VanBerkum & Means, 1991). Such results indicate that the picture painted by autoinhibitory models must be modified to include currently unknown additional—presumably conformational—elements or that the basic premise of this model is completely lacking in validity for the MLCKs.

In order to gain further insight into the events that take place when CaM binds to and activates smMLCK from chicken gizzard—especially those affecting the enzyme's active site—we have probed the MgATP–substrate-binding site of the enzyme in both its CaM-free and CaM-bound states with the ATP-like affinity labeling reagent 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (FSBA) [reviewed in Colman et al. (1977)]. This reagent has been used to covalently label the nucleotide-binding sites of a number of proteins, including several protein kinases: the cAMP-dependent protein kinase (Zoller et al., 1981), the cGMP-dependent protein kinase (Hashimoto et al., 1982), $pp60^{src}$ (Kamps et al., 1984), and the EGF receptor kinase (Russo et al., 1985). In addition, Saitoh et al. (1987) reported that the CaM-bound, active form of smMLCK reacted with this compound with a consequent loss of catalytic activity, indicating that FSBA might prove a suitable probe of the MgATP-binding site of this protein kinase as well. Specifically, we wished to determine (a) whether the MgATP-binding site that must be present when the enzyme is rendered active by CaM—a site that therefore should bind to and react with FSBA—remains accessible and recognizable as such in the CaM-free state as evidenced by reactivity with this affinity labeling reagent and (b) whether this site undergoes a change, conformational or otherwise, of sufficient magnitude during the CaM activation process to alter its reactivity with or affinity for FSBA.

Materials and Methods

Materials. Purchased materials include magnesium chloride, calcium chloride, Tris, Triton X-100, NaCl, DTT, EGTA, and DMF (Fisher Scientific Co., Pittsburgh, PA); HEPES (Research Organics, Inc., Cleveland, OH); FSBA, ATP, GTP, and BSA (Sigma Chemical Co., St. Louis, MO); FSB (Aldrich Chemical Co., Milwaukee, WI); [*adenine*-8-^{14}C]FSBA (DuPont/New England Nuclear, Boston, MA); and Coomassie protein assay reagent and a standardized solution of BSA (Pierce, Rockford, IL). The peptide substrate for MLCK was the generous gift of Dr. Edwin G. Krebs, University of Washington, Seattle, WA. All other materials are from previously listed sources (Kennelly et al., 1987, 1990).

Proteins. Calmodulin was purified from bovine testes as described by Charbonneau et al. (1983). Chicken gizzard myosin light chain kinase was extracted from washed myofibrils prepared from fresh gizzards by a modification of the procedure of Sobieszek and Bremel (1985) and then purified to a single band of ≥98% electrophoretic homogeneity on SDS–polyacrylamide gels (Laemmli, 1970) by ion-exchange, calmodulin affinity, and gel filtration chromatography essentially as described by Takio et al. (1985) for the purification of skMLCK from rabbit muscle. smMLCK protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (Pierce) as standard. smMLCK molarity was determined from the protein concentration data using a molecular weight of 107 534 (Olson et al., 1990). CaM was estimated from its UV absorbance maximum at 277 nm, using an extinction coefficient of 3300 M^{-1} cm^{-1} (Klee, 1977).

Conditions for Reaction with FSBA. The reaction between skMLCK or skMLCK–CaM and FSBA was carried out at 25 °C in a volume of 80 μL containing 50 mM Tris (pH 7.5), 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 0.16–0.32 mg/mL smMLCK, 10% (v/v) DMF, and the indicated amounts of FSBA. For reaction with the smMLCK–CaM complex, CaM was added to a concentration of 10 μM. Reaction was initiated by adding FSBA in DMF and mixing vigorously on a Vortex mixer. The FSBA stock solution was made fresh immediately prior to use. FSBA concentration was determined from its UV absorbance maxima at 259 nm following 500-fold dilution of an aliquot into 95% EtOH using an extinction coefficient of 15 800 M^{-1} cm^{-1} (Colman et al., 1977). At timed intervals, the reaction was stopped by removing 10-μL aliquots of the reaction mixture and diluting them into 500 μL of ice-cold 10 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.2 mM EGTA, 0.5 mg/mL BSA, 0.1 M NaCl, and 0.1% (v/v) Triton X-100 (dilution buffer). The combination of dilution of the reactants and rapid reaction of the remaining FSBA with DTT (Colman et al., 1977) halts further reaction. smMLCK activity was then determined by assaying aliquots of this mixture by conventional means using a synthetic peptide, KKRAARATSNVFA, as substrate (Kemp & Pearson, 1985). The rate of reaction was expressed as the apparent first-order rate constant, k_{app}, as defined by the equation $\ln (E/E_0) = -k_{app}t$ where E is defined as the amount of enzyme activity remaining at any time t and

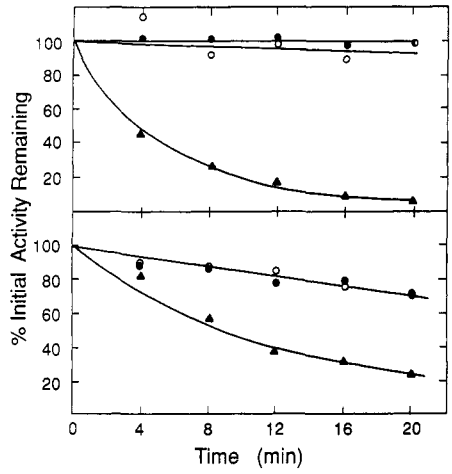

FIGURE 1: Inactivation of smMLCK or smMLCK–CaM by FSBA. smMLCK (top) or smMLCK–CaM (bottom) was incubated with either no inactivating reagent (○), 1.5 mM FSB (●), or 1.5 mM FSBA (▲) under standard conditions as described under Materials and Methods. At the indicated times, aliquots of the reaction mixture were then removed and assayed for enzyme activity.

E_0 is defined as the amount of enzyme activity present at $t = 0$. All results were corrected with reaction mixtures to which DMF without FSBA was added. Experiments testing FSB as an inactivator utilized identical procedures, with the exception that FSB was substituted for FSBA. The FSB was quantitated by weight instead of absorbance.

Stoichiometry of FSBA Incorporation into smMLCK and smMLCK–CaM. smMLCK, 0.95 mg/mL final, was incubated under standard conditions in a volume of 400 μL with 0.4 mM [^{14}C]FSBA labeled at a specific activity of (20–30) × 10^3 cpm/nmol. When inactivation of smMLCK–CaM was desired, CaM (20 μM final) was added, and the FSBA concentration was increased to 0.6 mM. The reaction was initiated by adding 40 μL of a 10×-concentrated stock solution of [^{14}C]FSBA in DMF and mixing thoroughly. The [^{14}C]-FSBA stock solution was prepared by drying an aliquot of radioactive FSBA in 95% ethanol under a stream of dry air and then immediately dissolving it in a solution of nonradioactive FSBA in DMF and mixing thoroughly. The concentration was determined by measuring the OD_{259} of the mixture as described above. At various times following the start of the reaction, two aliquots were removed from this mixture. The smaller of the two, 5 μL, was diluted into 1.0 mL of ice-cold dilution buffer and later assayed for remaining MLCK activity as described previously. The second aliquot, 55 μL in volume, was analyzed for the incorporation of [^{14}C]FSBA into enzyme protein as described by Colburn et al. (1987).

Results

FSBA reacted with both smMLCK and smMLCK–CaM in a time-dependent manner, as evidenced by the loss of assayable smMLCK activity (Figure 1). *p*-(Fluorosulfonyl)-benzoic acid, which contains the reactive fluorosulfonyl group of FSBA but lacks the adenosine targeting moiety, did not react at an appreciable rate with either the free enzyme or its CaM complex (Figure 1). The reaction obeyed pseudo-first-order kinetics (data not shown). The rate of reaction—as expressed by the pseudo-first-order rate constant $k_{apparent}$ (k_{app})—was dependent upon FSBA concentration and was observed to saturate with increasing levels of FSBA (Figure 2).

Kinetic parameters for the reactions-K_I, the dissociation constant for the reversible, noncovalent FSBA–enzyme com-

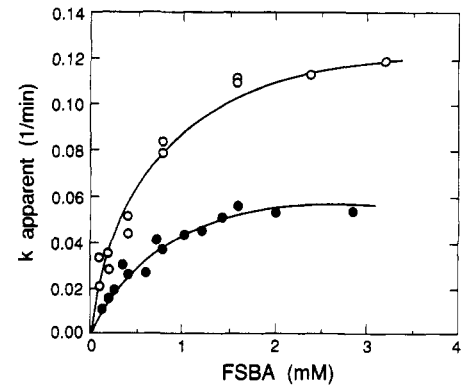

FIGURE 2: FSBA inactivation of smMLCK or smMLCK–CaM: dependence of reaction rate on FSBA concentration. smMLCK or smMLCK–CaM was reacted with the indicated concentrations of FSBA under standard conditions as described under Materials and Methods. Shown is the apparent first-order rate constant for inactivation of smMLCK (○) or smMLCK–CaM (●), k_{app}, as a function of FSBA concentration. All values for k_{app} were corrected for the apparent rate of inactivation observed in control incubations lacking FSBA.

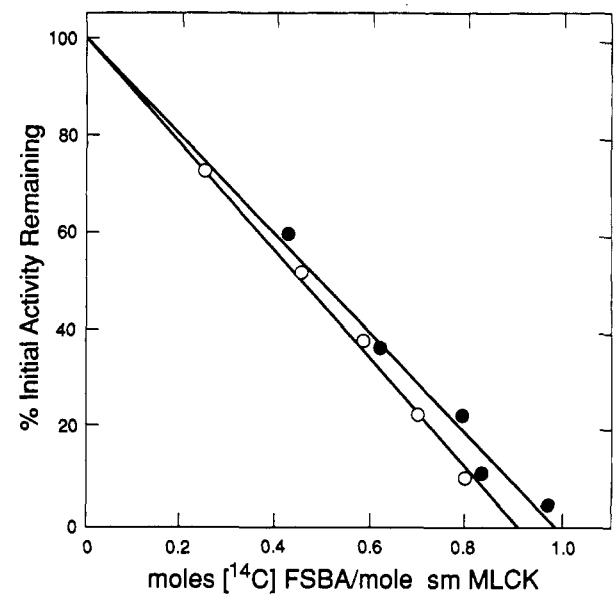

FIGURE 3: Stoichiometry of [^{14}C]FSBA incorporation into smMLCK or smMLCK–CaM. smMLCK (○) or smMLCK–CaM (●) was reacted with [*adenine*-8-^{14}C]FSBA and analyzed for activity and incorporation of radioactive FSBA into enzyme protein as described under Materials and Methods. Shown is a plot of the percentage of initial assayable smMLCK activity remaining versus the quantity of FSBA incorporated into each mole of smMLCK or smMLCK–CaM present. The stoichiometry of FSBA incorporated when all of the smMLCK (–CaM) had become inactivated was 0.9 mol/mol for smMLCK and 1.0 mol/mol for smMLCK–CaM, respectively, as determined by linear regression analysis by the computer program Cricket Graph with the point (0.0 mol/mol, 100% activity) weighted 15-fold over all other points.

plex, and k_3, the rate constant for the reaction of the noncovalent complex to the form covalently modified, inactive enzyme (Kitz & Wilson, 1962)—were determined using a Hanes–Woolf plot. The rate constant k_3 differed somewhat between smMLCK and smMLCK–CaM, being 0.15 min^{-1} for the former and 0.07 min^{-1} for the latter. The affinities of smMLCK and its CaM complex for FSBA, as described by K_I, were essentially identical at 0.65 and 0.64 mM, respectively. Inactivation was accompanied by the covalent incorporation of FSBA into smMLCK protein as shown by the incorporation of radioactivity from [^{14}C]FSBA into the enzyme. Using radiolabeled FSBA, it was observed (Figure 3) that the in-

Table I: Effect of Potential Ligands on FSBA Inactivation of smMLCK and smMLCK–CaM[a]

enzyme form	expt	additions			rate of inactivation (% control)
		MgATP	MgGTP	peptide	
smMLCK	1	–	–	–	100
		+	–	–	0
		–	+	–	75
		–	–	+	98
smMLCK–CaM	2	–	–	–	100
		+	–	–	6
		–	+	–	71
smMLCK–CaM	3	–	–	–	100
		+	–	–	3
		–	–	+	94

[a] smMLCK or smMLCK–CaM was reacted with 0.4 mM FSBA as described under Materials and Methods except that, where indicated, the following compounds were present at the indicated final concentrations: MgATP, 10 mM; MgGTP, 10 mM; MLCK substrate peptide (KKRAARATSNVFA), 0.2 mM.

corporation of FSBA into smMLCK or smMLCK–CaM was proportional to the quantity of enzyme activity lost, with an estimated stoichiometry of 0.9 or 1.0 mol of FSBA incorporated per mole of smMLCK or smMLCK–CaM inactivated, respectively.

To further ascertain whether the inactivation of smMLCK and smMLCK–CaM by FSBA resulted from the interaction of this reagent with the enzyme's MgATP-binding site, the effect of added MgATP on this process was examined. As can be seen in Table I, MgATP blocked inactivation of both smMLCK and smMLCK–CaM by FSBA. MgGTP, a purine nucleotide triphosphate that does not function as a phosphoryl donor substrate for smMLCK, offered little protection against catalytic inactivation even at a concentration that was at least 100 times higher than the enzyme's K_m for MgATP (Hartshorne & Mrwa, 1980; Foster et al., 1986). The addition of saturating concentrations of a substrate peptide, KKRAARATSNVF (Kemp & Pearson, 1985), had no effect on the reaction. Further examination revealed that MgATP acted as a competitive inhibitor with respect to FSBA when either smMLCK or smMLCK–CaM was the target of the affinity labeling reagent (data not shown). Secondary plots describing the inhibition were linear, indicating that the mechanism was simple competitive inhibition, with the K_i for MgATP protection estimated to be 33 μM for smMLCK and 34 μM for the smMLCK–CaM complex.

Discussion

One of the key features that distinguishes conformational-shift models for the activation of the MLCKs by CaM from autoinhibitor models concerns the status of the enzyme active site during the activation–inactivation process. The former class of models predicts that a conformational change of sufficient magnitude to convert the active site from a totally inert to a catalytically competent state takes place upon CaM binding. The latter predicts that the active site is a passive spectator to the binding and removal of a pseudosubstrate domain from the protein substrate-binding site. The former implies that as part of the active-site region, the other substrate-binding-site, that for MgATP, should be subject to CaM-induced conformational changes, while the latter predicts that the MgATP-binding site remains open and in its functionally competent configuration at all times.

In this study, we have observed that smMLCK–CaM reacted with the ATP-like affinity labeling reagent FSBA in a time- and concentration-dependent manner. The reaction was judged to take place directly at the MgATP-binding site since (a) it was dependent upon the presence of the adenosine moiety

Table II: Comparison of Kinetic Constants for the Reaction between FSBA and MLCKs and Their CaM Complexes with Those Reported for Other Protein Kinases

protein kinase	k_3 (min^{-1})	K_I (mM)	ref
smMLCK	0.15	0.65	this paper
smMLCK–CaM	0.07	0.64	this paper
skMLCK	0.04	0.40	Kennelly et al. (1991)
skMLCK–CaM	0.04	0.18	Kennelly et al. (1991)
CaM kinase II + CaM	0.086	1.5	King et al. (1988)
cAMP-dependent PK[a] (C-subunit)	0.054	0.15	Hixson & Krebs (1979)
cGMP-dependent PK	0.129	1.05	Hixson & Krebs (1981)
phosphorylase kinase	[b]	0.8	King & Carlson (1982)
casein kinase II	0.043	0.88	Hathaway et al. (1981)

[a] PK = protein kinase. [b] Not reported.

of FSBA, (b) it displayed saturation kinetics, (c) it was accompanied by the stoichiometric incorporation of FSBA into smMLCK protein, (d) it was effectively inhibited by MgATP, but not MgGTP (a purine nucleotide that is not a substrate for the enzyme) or a substrate peptide, and (e) inhibition by MgATP was competitive. Moreover, sequence analysis of cDNA clones revealed that smMLCK contains only a single nucleotide-binding domain, that required for substrate (MgATP) binding (Olson et al., 1990). The kinetic parameters for this reaction were quite comparable to those observed for the reaction of FSBA with other protein kinases (Table II). The K_i for MgATP, 34 μM, compared quite favorably with the reported K_m values for smMLCK–CaM of 30–70 μM (Hartshorne & Mrwa, 1980; Foster et al., 1986).

When smMLCK alone, which is completely inactive in the absence of CaM, was incubated with FSBA, it also reacted with this reagent, as judged by both the consequent loss of assayable enzyme activity and the covalent incorporation of FSBA into enzyme protein, in an apparently site-specific manner. As with smMLCK–CaM, reaction took place at the enzyme's MgATP-binding site as judged by the same criteria that were used to assess the site-specificity of the reaction with FSBA–CaM. These observations indicate that the MgATP-binding site of smMLCK remains open and accessible, as well as recognizable, to ligands such as FSBA and MgATP even when the enzyme is rendered catalytically inert upon its dissociation from calmodulin. This conclusion is further reinforced by the earlier observation of Malencik and co-workers (Malencik et al., 1982; Malencik & Anderson, 1986) that the binding of the fluorescent dye 9-anthroylcholine to turkey gizzard smMLCK and its CaM complex could be competed by MgATP. Further, experiments with MLCK from rabbit skeletal muscle also suggest that its MgATP-binding site remains open and functional, since it too reacts with FSBA in the CaM-free as well as the CaM-bound state (Colburn et al., 1987; Kennelly et al., 1991).

Kinetic analyses indicate that smMLCK binds FSBA or MgATP with the same affinity in the presence or absence of CaM. The rate constant for the reaction of the noncovalent enzyme–FSBA complex to form the covalently modified enzyme, k_3, is only about 2-fold higher for the CaM-free enzyme as for the CaM-bound form, a relatively minor shift. These observations suggest that the conformation of the MgATP-binding site varies little between the active and inactive states of smMLCK. Such behavior is quite consistent with the basic tenets of autoinhibitory models for MLCK activation, although it does rule out variants of this model in which the autoinhibitory domain is suggested to block both the myosin light chain and the MgATP substrate-binding sites on the protein (Ikebe, 1990). It also is in marked contrast to that of CaM kinase II, a CaM-stimulated protein kinase hypothesized to

be regulated by the binding of an autoinhibitor domain to its MgATP-binding site, rather than to its protein-binding site as has been suggested for MLCK (Colbran et al., 1988).

Further work is required to ultimately resolve the question of how CaM activates smMLCK. However, whatever models are put forward in the future to describe the activation–inactivation process, these results suggest that they must include the presence of an accessible and functionally competent MgATP-binding site in the CaM-free enzyme.

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Registry No. FSBA, 57454-44-1; MgATP, 1476-84-2; MLCK, 51845-53-5.

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Aminonaphthalenesulfonamides, A New Class of Modifiable Fluorescent Detecting Groups and Their Use in Substrates for Serine Protease Enzymes†

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ABSTRACT: A series of new compounds, 6-amino-1-naphthalenesulfonamides (ANSN), were used as fluorescent detecting groups for substrates of amidases. These compounds have a high quantum fluorescent yield, and the sulfonyl moiety permits a large range of chemical modification. Fifteen ANSN substrates with the structure (N^{α}-Z)Arg-ANSNR_1R_2 were synthesized and evaluated for their reactivity with 8 proteases involved in blood coagulation and fibrinolysis. Thrombin, activated protein C, and urokinase rapidly hydrolyzed substrates with monosubstituted sulfonamide moieties (R_1 = H). The maximum rate of substrate hydrolysis for acrylic substituents was observed when R_2 = C_4H_9 (*n*-butyl homologue). The hydrolysis rates for substrates with branched substituents were slower than their linear analogues. Monosubstituted (N^{α}-Z)Arg-ANSNR_1R_2 possessing cyclohexyl or benzyl groups in the sulfonamide moiety were hydrolyzed by these three enzymes at rates similar to that of the *n*-butyl homologue (except the cyclohexyl compound for u-PA). Factor Xa rapidly hydrolyzed substrates with short alkyl chains, especially when R_1 = R_2 = CH_3 or C_2H_5. Lys-plasmin and rt-PA demonstrated low activity with these compounds, and the best results were accomplished for monosubstituted compounds when R_2 = benzyl (for both enzymes). Factor VIIa and factor IXaβ exhibited no activity with these substrates. A series of 14 peptidyl ANSN substrates were synthesized, and their reactivity for the same 8 enzymes was evaluated. Thrombin, factor Xa, APC, and Lys-plasmin hydrolyzed all of the substrates investigated. Urokinase, rt-PA, and factor IXaβ exhibited reactivity with a more limited group of substrates, and factor VIIa hydrolyzed only one compound (MesD-LGR-ANSN$(C_2H_5)_2$). The substrate ZGGRR-ANSNH(cyclo-C_6H_{11}) showed considerable specificity for APC in comparison with other enzymes (k_{cat}/K_M = 19 300 M^{-1} s^{-1} for APC, 1560 for factor IIa, and 180 for factor Xa). This kinetic advantage in substrate hydrolysis was utilized to evaluate the activation of protein C by thrombin in a continuous assay format. Substrate (D-LPR-ANSNHC_3H_7) was used to evaluate factor IX activation by the factor VIIa/tissue factor enzymatic complex in a discontinuous assay. A comparison between the commercially available substrate chromozyme TH (*p*-nitroanilide) and the ANSN substrate with the same peptide sequence (TosGPR) demonstrated that aminonaphthalenesulfonamide increased the specificity (k_{cat}/K_M) of substrate hydrolysis by thrombin more than 30 times, with respect to factor Xa substrate hydrolysis.

The proteases involved in coagulation and fibrinolysis are trypsin-like serine proteases which preferentially hydrolyze peptide, ester, or amide bonds in which a basic amino acid provides the carbonyl group of the scissile bond. The specificity of enzyme/substrate interactions is a complex function of a variety of structural factors which include binding domains in the protease for specific amino acid side chains located on both the amino (P_1–P_n) and carboxyl (P_1'–P_n') side of the targeted lysine or arginine residue in the substrate protein.[1] The idea that short peptide substrates can be designed which incorporate enough information to discriminate among different proteases relies on the concept that each active site is comprised of a unique series of side chain binding "pockets".

A number of studies evaluating the utility of synthetic substrates for many of the proteases involved in blood coagulation and fibrinolysis have been reported (Cho et al., 1984; McRay et al., 1981; Lottenberg et al., 1981). Generally, these studies have evaluated the reactivity of substrates containing 4-nitroaniline and amides of 7-amino-4-methylcoumarins for this class of serine proteases. This work has provided a large amount of information regarding contributions of the P_1–P_3 amino acids in an attempt to optimize enzymatic substrate specificity for a particular protease. However, a design strategy employing structural variations of the detecting group

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[1] The nomenclature used to identify individual amino acid residues of the substrate (P_2, P_1, P_1', etc.) and the subsites of the enzyme (S_2, S_1, S_1', etc.) is that of Schechter and Berger (1967).

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