Affinity labelling of smooth-muscle myosin light-chain kinase with 5′-[*p*-(fluorosulphonyl)benzoyl]adenosine

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5′-{*p*-(Fluorosulphonyl)[^{14}C]benzoyl}adenosine (FSBA) was synthesized and used as a probe to study the ATP-binding site of smooth-muscle myosin light-chain kinase (MLCK). FSBA modified both free MLCK and calmodulin/MLCK complex, resulting in inactivation of the kinase activity. Nearly complete protection of the calmodulin/MLCK complex against FSBA modification was obtained by addition of excess ATP whereas MLCK activity alone was lost in a dose-dependent manner even in the presence of excess ATP. These results suggest that FSBA modified ATP-binding sites and ATP-independent sites, and the latter sites are protected by calmodulin binding. The results also suggest that the ATP-binding site is accessible to the nucleotide substrate regardless of calmodulin binding. The FSBA-labelled MLCK was completely proteolysed by α-chymotrypsin, and the ^{14}C-labelled peptides were isolated and sequenced. The sequence of the labelled peptide was Ala-Gly-X-Phe, where X is the labelled residue. The sequence was compared with the known MLCK sequence, and the labelled residue was identified as lysine-548, which is located downstream of the GXGXXG motif conserved among ATP-utilizing enzymes.

INTRODUCTION

In smooth muscle, contraction is initiated by Ca^{2+}-dependent phosphorylation of the regulatory light chain of myosin. Myosin light-chain kinase (MLCK) is the Ca^{2+}/calmodulin-dependent enzyme which catalyses the phosphorylation of myosin light chain, and is therefore the key regulatory protein in smooth-muscle contraction (Hartshorne, 1987; Sellers and Adelstein, 1987). The complete amino acid sequences of MLCK were determined from both skeletal muscle (Takio et al., 1986; Roush et al., 1988; Leachman et al., 1989; Herring et al., 1990a) and smooth muscle (Olson et al., 1990; Gallagher et al., 1991), and it is known that the molecules are quite distinct. For smooth-muscle MLCK, the sequence conserved among various protein kinases is found in the central portion of the molecule, suggesting it to be a catalytic domain. The regulatory domain resides at the C-terminal side of the catalytic core and consists of the auto-inhibitory region and calmodulin-binding region (Olson et al., 1990; Gallagher et al., 1991). The calmodulin-binding region was identified by isolating the calmodulin-binding peptide (Lukas et al., 1986) and later by truncation of the recombinant MLCK (Bagchi et al., 1989). The structure required for calmodulin binding was recently revealed by analysing the three-dimensional structure of the peptide–calmodulin complex by multi-dimensional n.m.r. spectroscopy (Ikura et al., 1992), and it was found that two hydrophobic residues, ten amino acid residues apart, play an essential role for the binding to calmodulin. On the other hand, it has been shown that the autoinhibitory region exists at the N-terminal side of the calmodulin-binding region. This was suggested by use of synthetic peptide analogues that modelled the N-terminal side of the calmodulin-binding region of MLCK. The peptides showed potent inhibitory activity against native MLCK (Kemp et al., 1987) as well as unregulated MLCK (Ikebe et al., 1987; Ikebe, 1990; Pearson et al., 1991). It was also demonstrated (Ikebe et al., 1987) that the proteolysis of smooth-muscle MLCK yields a 64 kDa inactive fragment which is further proteolysed to produce a 61 kDa active Ca^{2+}/calmodulin-independent kinase. The cleavage sites that produce the inactive fragment, i.e. 64 kDa fragment and the constitutively active MLCK, were identified to be on the N-terminal side of the calmodulin-binding region (Pearson et al., 1988; Ikebe et al., 1989).

The mechanism by which the regulatory region controls the kinase activity is still obscure. Kemp et al. (1987) proposed that the autoinhibitory region serves as a pseudo-substrate inhibitor because of the similarity between the autoinhibitory region and the sequence of myosin light-chain N-terminus to the phosphorylation site. Although this is an attractive model to explain the regulatory mechanism, the replacement of basic amino acid residues in the autoinhibitory region with acidic residues, which is the key requirement of the pseudo-substrate inhibitor hypothesis, did not produce the constitutively active enzyme (Herring, 1991). We also observed that deletion of the Arg-Arg-Lys motif, essential for the pseudo-substrate inhibitor hypothesis, did not disrupt the inhibited form of smooth-muscle MLCK (K. Yano and M. Ikebe, unpublished work), suggesting that the inhibition does not arise from pseudo-substrate inhibition.

It is reasonable, however, to assume that the regulatory region of MLCK interacts with the catalytic domain in order to modulate the enzyme activity. In this respect, it is important to understand the structure of the active site and possible modulation of the active-site conformation due to interaction with the regulatory sites. In the present study, we employed 5′-[*p*-(fluorosulphonyl)benzoyl]adenosine (FSBA) as a probe to study the ATP-binding-site structure. FSBA is an ATP analogue which has been shown to be an effective affinity-labelling reagent which covalently modifies the nucleotide-binding site of enzymes (Zoller et al., 1981; Kamps et al., 1984; Russo et al., 1985). The compound consists of an adenosine moiety which binds to ATP-binding sites and a fluorobenzoyl moiety which reacts with amino acid residues at the fluorosulphonyl position (Figure 1). We found that FSBA specifically labelled a lysine residue which

Abbreviations used: MLCK, smooth-muscle myosin light-chain kinase; DTT, dithiothreitol; FSBA, 5′-[*p*-(fluorosulphonyl)benzoyl]adenosine.

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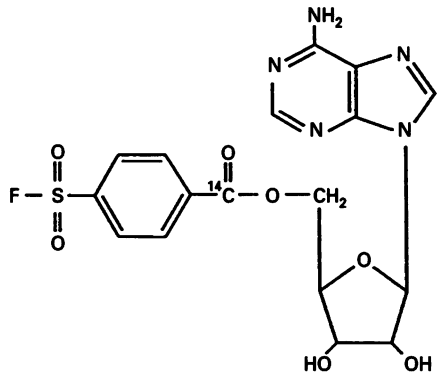

Figure 1 Structure of [^{14}C]FSBA

resulted in the loss of the kinase activity. The labelling was inhibited by the presence of Mg^{2+}-ATP, and occurred on the same lysine residue in the presence and absence of Ca^{2+}/calmodulin.

MATERIALS AND METHODS

Proteins were prepared by the following procedures: MLCK from frozen turkey gizzards (Ikebe et al., 1987); calmodulin from bull testes (Walsh et al., 1983); 20 kDa light chain from gizzard myosin (Hathaway and Haeberle, 1983; Ikebe et al., 1988). FSBA was purchased from Sigma. FSBA ^{14}C-labelled in the benzoyl moiety ([^{14}C]FSBA) (Figure 1) was synthesized as described by Colburn et al. (1987). The specific radioactivity was 1×10^3 c.p.m./nmol.

Assays of MLCK (0.04 or 0.03 μg/ml) activity were determined with the isolated 20000 Da light chain (0.25 or 0.2 mg/ml) in the presence of 35 or 5 μg/ml calmodulin, in 0.1 mM $CaCl_2$/1 mM $MgCl_2$/30 mM KCl/25 mM Tris/HCl (pH 7.5)/0.1 mM [γ-^{32}P]ATP at 25 °C. The extent of phosphorylation was estimated as described by Walsh et al. (1983).

MLCK (0.2 mg/ml) was treated with various concentrations of FSBA at 25 °C in 1 mM EGTA or $CaCl_2$, 1.68 mg/ml calmodulin, 10 mM $MgCl_2$, 0.1 M KCl, 10% glycerol, 5% dimethyl sulphoxide and 50 mM Tris/HCl, pH 7.5, with or without 10 mM ATP. At 30 min, 10 μl of reaction mixture was diluted into 1 ml of iced 20 mM dithiothreitol (DTT)/0.1 M KCl/25 mM Tris/HCl, pH 7.5, to stop the labelling reaction. DTT prevented the inactivation of MLCK by FSBA (results not shown). The activity of the labelled MLCK was determined as described above.

For the determination of the FSBA-binding site, 2.5 mg of MLCK (0.5 mg/ml) was labelled with [^{14}C]FSBA (1 mM) at 25 °C for 20 min in 0.1 M KCl/10% glycerol/5% dimethyl sulphoxide/50 mM Tris/HCl, pH 7.5. The reaction was also carried out in the presence of MgATP (10 mM $MgCl_2$ and 10 mM ATP) to evaluate the specificity of the FSBA labelling. To examine the effects of Ca^{2+}/calmodulin binding on the FSBA labelling, 0.2 mM $CaCl_2$ and 0.32 mg/ml calmodulin were added to the reaction mixture. The reaction was stopped by 2-fold dilution into iced 0.1 M DTT/0.1 M KCl/25 mM Tris/HCl, pH 7.5. After addition of guanidine/HCl to 6 M, the solution was applied to a Sephadex G-25 column (1.5 cm × 48 cm) equilibrated with 10 mM sodium phosphate, pH 6.0, containing 6 M guanidine/HCl to remove [^{14}C]FSBA that had not reacted. Protein-containing fractions were pooled and dialysed against 10 mM NH_4HCO_3.

The [^{14}C]FSBA-labelled protein was digested by α-chymotrypsin (1:440, w/w) at 25 °C for 16 h. The digest was loaded on a TSK DEAE 5 PW column (7.5 mm × 750 mm) (Tosoh, Tokyo, Japan) attached to a Perkin–Elmer h.p.l.c. system which was equilibrated with 0.01 M NH_4HCO_3. The peptides were eluted with a linear NH_4HCO_3 gradient. The fractions (0.6 ml/tube) were collected at a flow rate of 0.6 ml/min. A portion (120 μl of α-chymotrypsin-treated MLCK reacted with FSBA in the presence of MgATP, or 60 μl of α-chymotrypsin-treated MLCK reacted with FSBA in the absence of MgATP) of each fraction was mixed with 5 ml of scintillation cocktail, and the radioactivity of each fraction was measured in a Beckman LS 3801 scintillation counter. The radioactive fractions were pooled, freeze-dried and applied to a Spheri-5 RP-18 (Brownlee Labs) C_{18} reverse-phase column attached to the h.p.l.c. system. The peptides were eluted with a linear CH_3CN gradient in 0.1% trifluoroacetic acid. Fractions (0.8 ml/tube) were collected at a flow rate of 0.8 ml/min, and a portion (300 μl) of each fraction was taken to measure the radioactivity as described above. Amino acid sequences of the radioactive peptides were determined on an Applied Biosystem 477A protein sequencer.

Protein concentrations were determined by the method of Bradford (1976) or by spectrophotomeric measurements for MLCK ($A^{1\%}_{278} = 11.4$) (Adelstein and Klee, 1981), calmodulin ($A^{1\%}_{277} = 1.9$) (Watterson et al., 1976), and the 20 kDa light chain ($A^{1\%}_{277} = 3.37$) (Hathaway and Haeberle, 1983).

RESULTS AND DISCUSSION

MLCK was modified by FSBA in the presence and absence of Ca^{2+}. As shown in Figure 2, MLCK was inactivated by FSBA in the absence of ATP in a dose-dependent manner in both the presence and the absence of Ca^{2+}; however, the inactivation of MLCK occurred at a lower concentration of FSBA in the absence of Ca^{2+} (IC_{50} in the presence and absence of Ca^{2+} was 0.47 mM and 0.33 mM respectively). The inactivation of MLCK by FSBA was almost completely blocked by addition of excess ATP in the presence of Ca^{2+}. On the other hand, the MLCK activity was gradually inhibited by FSBA in the absence of Ca^{2+} even though the modification was carried out in the presence of excess ATP (IC_{50} 1.37 mM). The results suggest that MLCK was inactivated by FSBA in the absence of Ca^{2+}/calmodulin due to the modification of the ATP-binding site and of sites which are not directly related to the ATP-binding site, with the former site showing a higher affinity for FSBA than the latter sites.

These results indicate that modification of the ATP-sensitive site is unaffected by Ca^{2+}/calmodulin binding to the enzyme, whereas modification at the ATP-insensitive sites, which also resulted in the loss of MLCK activity, is protected by Ca^{2+}/calmodulin binding. The IC_{50} value in the absence of Ca^{2+} and ATP (0.33 mM) was comparable with that (0.35 mM) calculated from the IC_{50} for the ATP-binding site (estimated from the IC_{50} in the presence of Ca^{2+}/calmodulin, i.e. 0.47 mM) and for ATP-insensitive sites (IC_{50} in the presence of excess ATP, i.e. 1.37 mM). This suggests that the ATP-binding site is open to the substrate, regardless of calmodulin binding. Similar results have been obtained for skeletal-muscle MLCK (Colburn et al., 1987) and recently for smooth-muscle MLCK (Kennelly et al., 1992).

Protection against the inactivation of MLCK by FSBA was examined in the presence of various nucleotides (Table 1). MLCK was better protected against inactivation in the presence of

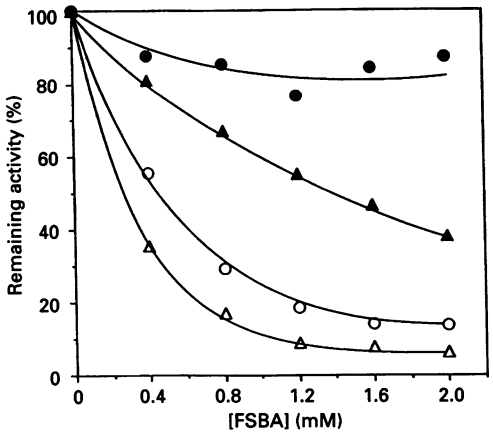

Figure 2 FSBA concentration-dependence of MLCK activity

MLCK was incubated with 0–2 mM FSBA at 25 °C for 30 min in 1 mM EGTA (△, ▲) or $CaCl_2$ (○, ●), 1.68 mg/ml calmodulin, 10 mM $MgCl_2$, 0.1 M KCl, 10% glycerol, 5% dimethyl sulphoxide and 50 mM Tris/HCl, pH 7.5, with (▲, ●) or without (△, ○) 10 mM ATP. Remaining activity was determined as described in the Materials and methods section.

Table 1 Protection by various nucleotides against the inactivation of MLCK by FSBA

MLCK (0.2 mg/ml) was incubated with 0.6 mM FSBA at 25 °C in 0.32 mg/ml calmodulin, 6 mM $MgCl_2$, 0.1 M KCl, 10% glycerol, 5% dimethyl sulphoxide and 50 mM Tris/HCl, pH 7.5. The remaining activity at 30 min was measured as described in the Materials and methods section. The 100% value was taken as the activity of MLCK under the above conditions incubated in the absence of FSBA. Nucleotides were added at 6 mM, and EGTA and $CaCl_2$ at 1 mM.

	Remaining activity (%)	
Nucleotide	EGTA/calmodulin	Ca^{2+}/calmodulin
None	23.0	26.8
ATP	69.8	87.0
ADP	61.5	97.1
CTP	47.8	55.7
GTP	31.3	45.0
ITP	40.7	57.1
UTP	36.7	57.5
8-Azido-ATP	52.7	63.5

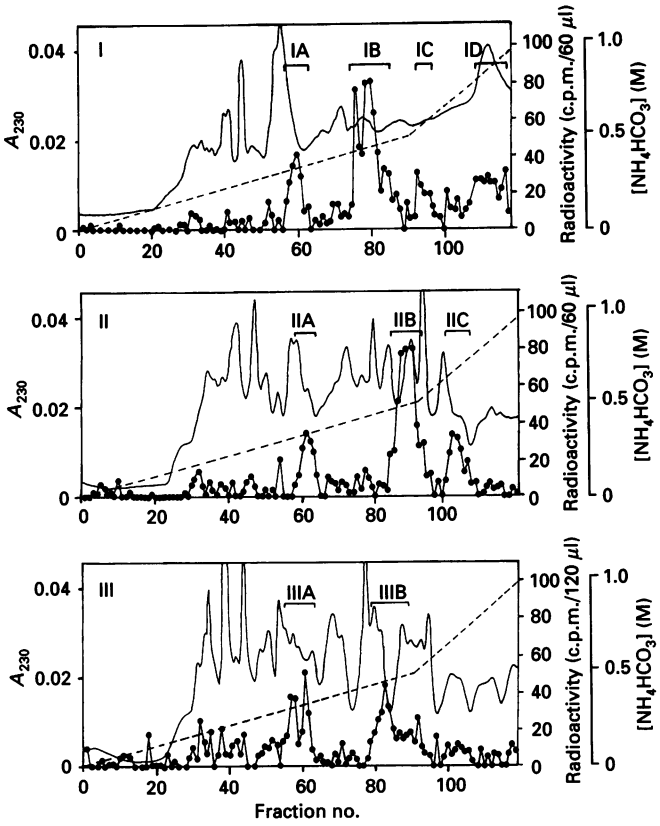

Figure 3 DEAE ion-exchange h.p.l.c. of α-chymotryptic digests of [^{14}C]FSBA-labelled MLCK

MLCK was reacted with [^{14}C]FSBA in the absence of ATP, Ca^{2+} and calmodulin (I), in the absence of ATP and in the presence of Ca^{2+} and calmodulin (II), or in the presence of ATP and in the absence of Ca^{2+} and calmodulin (III). Other conditions were indicated in the Materials and methods section. α-Chymotryptic digestion and chromatography were done as described in the Materials and methods section. Fractions (600 μl) were collected and monitored for A_{230} (——) and for radioactivity (●); ----, NH_4HCO_3 gradient. The radioactive peaks were collected as indicated.

Ca^{2+}/calmodulin (Table 1), and this is because MLCK was also inactivated by FSBA due to the modification at ATP-insensitive sites (Figure 2) in the absence of Ca^{2+}. MLCK was well protected by both ADP and ATP against inactivation, suggesting that ADP also binds well to MLCK. This was consistent with the results that the K_m for ADP in the reverse reaction is comparable with the K_m for ATP in the forward reaction (Ikebe and Hartshorne, 1986). 8-Azido-ATP, in which the bulky N_3 group is introduced at the position 8 of the adenine ring (and therefore the conformation in respect to the N–C bond between the purine moiety and the ribose moiety is *syn*, due to this substitution; the conformation of ATP is *trans*), protected against inactivation with less potency. This may be due to the decreased affinity of 8-azido-ATP to MLCK due to the introduction of a bulky group on the adenine ring. GTP, ITP, CTP and UTP, which do not serve as substrates for MLCK, also protected against inactivation to some extent, and among them GTP was the least potent. The results suggest that these nucleotides, although they do not function as substrates, can bind to MLCK at the ATP-binding site. The results of Table 1 also suggest that the structure of the purine ring at positions 1–6 is more important in determining the nucleotide binding, since the pyrimidine nucleotides (CTP, UTP) also showed protection potency comparable with that of purine nucleotides (ITP, GTP).

The above results showed that FSBA modified residues important for MLCK activity, and we therefore attempted to identify the FSBA-labelled residues. To accomplish this, we synthesized FSBA in which ^{14}C is incorporated in the benzoyl moiety (Colburn et al., 1987), since the commercially available radioactive FSBA (New England Nuclear) contains ^{14}C in the adenine ring, which would be lost from the peptide by hydrolysis at the ester bond under the acidic conditions used for purification of the affinity-labelled peptides. The affinity labelling of smooth-muscle MLCK with ^{14}C-labelled FSBA was performed under the following three conditions: (I) in the absence of ATP and Ca^{2+}/calmodulin, (II) in the absence of ATP but in the presence of Ca^{2+}/calmodulin, (III) in the presence of 10 mM ATP but in the absence of Ca^{2+}/calmodulin. The labelling of MLCK with ^{14}C-labelled FSBA was done as described in the Materials and

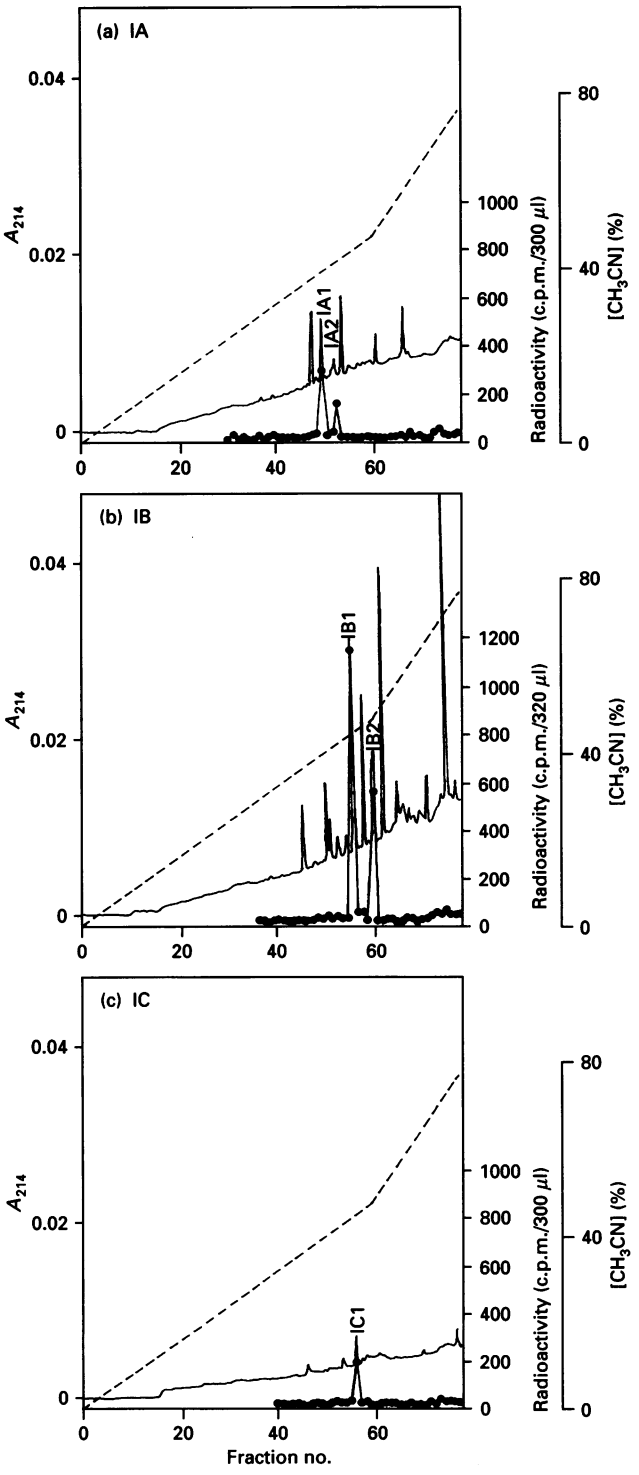

Figure 4 Separations of α-chymotryptic radioactive peptides by reverse-phase h.p.l.c.

The fractions from Figure 3 were purified by reverse-phase h.p.l.c. as described in the Materials and methods section. Fractions (800 μl) were collected and monitored for A_{214} (——) and for radioactivity (●); – – – –, CH_3CN gradient. The radioactive peaks were numbered as indicated.

methods section, and the labelled MLCK was digested with α-chymotrypsin, and then the digests were subjected to DEAE 5 PW anion-exchange chromatography (Figure 3). Four radio-

Table 2 Amino acid sequences of ^{14}C-labelled peptides

X, Unidentified amino acid.

IA1*	Ala-Gly-X-Phe
IA2*	Ala-Gly-X-Phe
IB1*	Ala-Gly-X-Phe
IB2*	Ala-Gly-X-Phe
ICl*	Ala-Gly-X-Phe
IIB2†	Ala-Gly-X-Phe

* Peptide obtained from MLCK labelled in the absence of Ca^{2+}/calmodulin.
† Peptides obtained from MLCK labelled in the presence of Ca^{2+}/calmodulin.

active peaks were observed. Among them, peak ID was found to be large peptide fragments of MLCK which were not completely digested by α-chymotrypsin, as judged by SDS/PAGE. Therefore, peak ID was not further analysed. The other three radioactive peaks were observed in all three conditions. The radioactive fractions were pooled, and subjected to C_{18} reverse-phase chromatography (Figure 4).

From fraction A from the labelling conditions I, two radioactive peaks, IA1 and IA2, were obtained (Figure 4a). The two radioactive peaks, IB1 and IB2, were also obtained from fraction B from the labelling condition I (Figure 4b). From fraction C, a single radioactive peak was obtained (Figure 4c). These purified radio-labelled peptides were subjected to sequence analysis. The sequences of all five peptides were identified as Ala-Gly-X-Phe (Table 2), in which X is the FSBA-labelled residue, although the elution positions of these peptides were distinct. The reason for the difference in the elution position for these five fractions during chromatography is unclear, but possibly it may arise from additional reactions involving the benzoyl group after hydrolysis during isolation of the peptide, arising as a consequence of the difference in the bound moieties of FSBA to the amino acid residue. To support this, it was found that the elution positions of amino acid phenylthiohydantoins of the labelled residues (X) in the peptides were distinct from one another (results not shown). The radioactive peptides which were labelled with FSBA in condition II were also purified, and five radioactive peptides were obtained. The elution positions of these peptides were nearly identical with those of the peptides labelled in condition I (results not shown), suggesting that the peptides obtained in labelling condition II are the same as those obtained in labelling condition I (Figure 4). Actually the sequence of peptide IIB2 was found to be Ala-Gly-X-Phe (Table 2). When MLCK was labelled with FSBA in the presence of excess ATP, the amount of radioisotope incorporated in the corresponding labelled peptides, i.e. IIIA1, IIIB1 and IIIB2, was markedly decreased (only one-fifth to one-tenth of radioisotope was incorporated into the peptides) (results not shown). Peaks A2 and C1 were not detected in this condition. These results clearly show that the labelling of the X residue in the peptide Ala-Gly-X-Phe is ATP-sensitive. Although ATP-sensitive labelling of MLCK by FSBA was abolished by adding excess ATP, MLCK activity was decreased slowly even in the presence of ATP (Figure 2). However, we could not find any particular radiolabelled peptides derived from ATP-insensitive labelling (results not shown). It has been shown (Togashi and Reisler, 1982) that FSBA modifies cysteine residues to form thiosulphonate, which is followed by displacement of FSBA by another cysteine residue to yield a disulphide. This process may be responsible for the ATP-insensitive inactivation of MLCK by FSBA. The location of the labelled peptide was

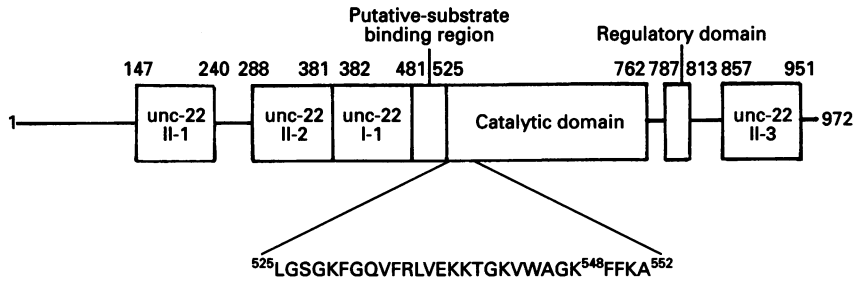

Figure 5 Location of [^{14}C]FSBA-labelled lysine residue of MLCK

The numbering of amino acid residues and domain structure of MLCK were referenced with the amino acid sequence data reduced from a full-length cDNA (Olson et al., 1990). The putative substrate-binding site was referenced with reports of skeletal-muscle MLCK by Herring et al. (1990a,b). K^{548} was labelled with [^{14}C]FSBA. unc-22 I and II, *C. elegans* unc-22 gene product consensus motifs I and II.

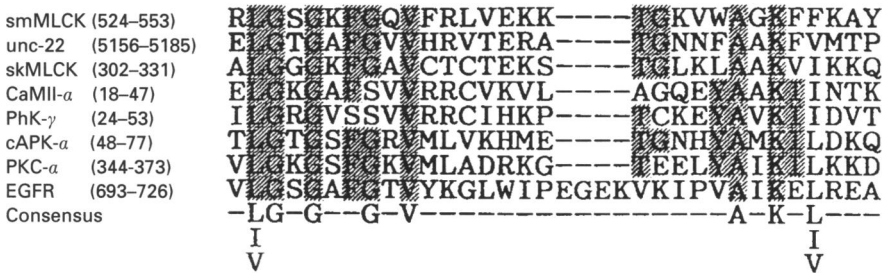

Figure 6 Alignment of the amino acid sequence of ATP-binding site from smooth-muscle MLCK and those of other protein kinases

The ATP-binding site of smooth-muscle MLCK (smMLCK) (Olson et al., 1990) was compared with the following sequences of the ATP-binding site of protein kinases: unc-22 (Benian et al., 1989); skeletal-muscle MLCK (skMLCK) (Herring et al., 1990a); Ca^{2+}/calmodulin-dependent protein kinase type II (CaMII-α) (Lin et al., 1987); phosphorylase kinase γ subunit (PhK-γ) (Reimann et al., 1984); cyclic-AMP-dependent protein kinase catalytic subunit, α-form (cAPK-α) (Shoji et al., 1983); protein kinase C, α form (PKC-α) (Parker et al., 1986), and epidermal-growth-factor receptor (EGFR) (Ullrich et al., 1984). For each position, residues found in at least four copies are shaded. Numbers in parentheses indicate positions in amino acid sequences of the protein kinases. Alignment and consensus sequence were referenced with a review by Hanks et al. (1988).

assigned according to the complete amino acid sequence (Olson et al., 1990), and it was found that the residue labelled with FSBA was lysine-548 (Figure 5). This is located downstream of the GXGXXG motif which is conserved among ATP-utilizing enzymes and is thought to be a part of the ATP-binding pocket (Figure 5). The acidic-residue-rich sequence, corresponding to the putative substrate (light chain)-binding region proposed by Herring et al. (1990a,b) in skeletal-muscle MLCK, is positioned upstream of this lysine residue. This lysine residue is conserved among various protein kinases (Figure 6), and it was previously shown that FSBA labelled this consensus lysine residue of cyclic-AMP-dependent protein kinase (Zoller et al., 1981), V-*src* (Kamps et al., 1984) and epidermal-growth-factor receptor kinase (Russo et al., 1985).

Recently, the three-dimensional structure of the catalytic subunit of cyclic-AMP-dependent protein kinase was determined by X-ray crystallography (Knighton et al., 1991). Since all known protein kinases share a conserved catalytic core homologous to the catalytic subunit of cyclic-AMP-dependent protein kinase, the catalytic core of MLCK was modelled according to the structure of the cyclic-AMP-dependent protein kinase (Knighton et al., 1992). According to the model, lysine-548 is on β-sheet 3 in the small lobe which serves as the binding site for MgATP.

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