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Articles

Amino Acid Sequence of an Active Fragment of Rabbit Skeletal Muscle Myosin Light Chain Kinase†

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ABSTRACT: The amino acid sequence of a 368-residue segment at the carboxyl-terminus of rabbit skeletal muscle myosin light chain kinase (MLCK) has been determined. The sequence was derived primarily from analysis of two complementary sets of fragments obtained by cleavage at methionyl and arginyl bonds in S-carboxymethylated MLCK. The segment included a 360-residue fragment produced by limited tryptic digestion of MLCK. This fragment was both catalytically active and dependent on Ca^{2+}–calmodulin. Unique structural features of MLCK have been identified, and a likely calmodulin interaction site is suggested. Sequence comparisons of MLCK to other protein kinases indicate close structural relationships in spite of marked differences in physicochemical properties, enzymatic characteristics, and regulatory response among these enzymes.

Protein kinases perform important regulatory roles in response to both intracellular and extracellular signals [cf. reviews by Krebs & Beavo (1979), Flockhart & Corbin (1982), Klee & Vanaman (1982), Nishizuka (1984), and Stull et al. (1985)]. Specific protein kinases are thought to control a variety of cellular functions including glycogen metabolism, muscle contraction, and growth. The catalytic activities of the various protein kinases are regulated by agents as diverse as hormones, Ca^{2+}–calmodulin, cyclic nucleotides, growth factors, and diacylglycerol. Many protein kinases are key enzymes in cascade systems where they regulate, and are regulated by, other protein kinases. Thus, knowledge of the structure of these enzymes is important in understanding their function and their regulation.

Shoji et al. (1981, 1983) reported the amino acid sequence of the catalytic subunit of cAMP-dependent protein kinase, and subsequently Barker & Dayhoff (1982) found that the protein was homologous to an oncogene product ($pp60^{src}$).[1] The latter is also a protein kinase that phosphorylates tyrosine, rather than serine, residues of target proteins (Hunter & Sefton, 1980). Several other oncogene products and hormone

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[1] Abbreviations: cGK, cGMP-dependent protein kinase; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CM, *S*-carboxymethyl; HPLC, high-performance liquid chromatography; LBTI, lima bean trypsin inhibitor; MLCK, myosin light chain kinase; PbK, phosphorylase *b* kinase; $pp60^{src}$, transforming phosphoprotein of Rous sarcoma virus; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate.

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or mitogen receptors are also tyrosine protein kinases [cf. reviews by Sefton & Hunter (1984) and Pike & Krebs (1985)], thus providing a functional basis for the homologous relationship. Sequence analysis of cGMP-dependent protein kinase (cGK) from bovine lung (Takio et al., 1984a) and of the Ca^{2+}–calmodulin-dependent catalytic subunit of phosphorylase *b* kinase (PbK) from rabbit skeletal muscle (Reimann et al., 1984) further extended the homology and provided chemical evidence for the hypothesis that all protein kinases, including oncogene products and related hormone or mitogen receptors, have evolved from a common ancestral gene (Ullrich et al., 1984, 1985). The different molecular sizes and specificities within this family of enzymes appear to reflect an evolutionary history of gene duplication and acquisition of new function by gene splicing (Takio et al., 1984a).

Myosin light chain kinase, which was first described and purified to homogeneity from rabbit skeletal muscle by Perry and co-workers (Pires & Perry, 1977), catalyzes the phosphorylation of a specific class of myosin light chain known as the phosphorylatable or P-light chain (Frearson & Perry, 1975). Although the enzyme exists in species- and tissue-specific forms which differ markedly in their molecular weight, extractability, and antigenic determinants (Stull et al., 1985), the various isozymes share two important characteristics: (1) a high degree of substrate specificity, phosphorylating only the P-light chain at significant rates; (2) complete dependence of activity on Ca^{2+}–calmodulin. The role of myosin light chain phosphorylation in various tissues is an area of active investigation, but considerable evidence indicates that myosin phosphorylation is required for initiation of contraction in smooth muscle [reviewed by Kamm & Stull (1985)], whereas in skeletal muscle it is involved in modulating the amount of tension produced during contraction (Manning & Stull, 1982).

Skeletal muscle MLCK is one of the better characterized calmodulin-dependent enzymes, and its interactions with calmodulin have been studied by a number of equilibrium and kinetic techniques (Blumenthal & Stull, 1980; Johnson et al., 1981; Olwin et al., 1984; Stull et al., 1985). Nevertheless, there is very little information at the structural level concerning the molecular basis of this control mechanism. We have undertaken sequence analysis of rabbit skeletal muscle MLCK in order to provide insight into the molecular basis of the catalytic and regulatory mechanisms of the enzyme and to assess any structural relationship to other protein kinases.

We report herein the sequence of a 368-residue segment from the carboxyl-terminal half of MLCK. This segment includes a catalytically active, Ca^{2+}–calmodulin-regulated fragment (Tβ, 360 residues) which was produced by limited tryptic proteolysis of the whole protein.

Materials and Methods

MLCK was prepared from fresh rabbit back and hind limb skeletal muscle. All procedures were carried out at 4 °C. The tissue was minced in a chilled meat grinder and then homogenized for 1 min at high speed in a Waring blender in 2–3 volumes of buffer containing 4 mM NaEDTA, pH 7.0, 1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol. The homogenate was centrifuged for 20 min at 4800 rpm in a Beckman J-6 centrifuge. Supernatants were poured through cheesecloth and glass wool and then brought to pH 5.8 with 1 N acetic acid before being centrifuged for 40 min as described above. The resulting supernatants were poured through glass wool–cheesecloth, and the pH was adjusted to 7.0 with solid $NaHCO_3$. Magnesium acetate and MOPS buffer, pH 7.0, were added to final concentrations of 10 and 20 mM, respectively, and the conductivity was adjusted to 3.5 $m\Omega^{-1}$ with deionized water. The protein solution was stirred for 5–10 min before addition of DEAE-cellulose (Whatman DE-52, 200-mL packed volume per kilogram of starting tissue), preequilibrated in 20 mM MOPS, pH 7.0. After gently mixing for 40–60 min, the unadsorbed protein was removed by vacuum filtration on a sintered glass funnel. The DEAE-cellulose was washed on the funnel with 10 volumes of buffer containing 20 mM MOPS, pH 7.0, and 1 mM DTT, and with a conductivity adjusted to 3.5 $m\Omega^{-1}$ with NaCl. Protein was eluted from the DEAE-cellulose on the filter with several applications (1–2 volumes each) of buffer containing 20 mM MOPS, pH 7.0, 1 mM DTT, and 0.2 mM EDTA and with a conductivity adjusted to 7 $m\Omega^{-1}$ with NaCl. A total of 4.5–5 volumes of elution buffer were used. The eluates were pooled, and magnesium acetate, calcium chloride, and leupeptin were added to give final concentrations of 4 mM, 0.2 mM,and 5 mg/L, respectively. This solution was applied to a column containing calmodulin–Sepharose 4B [1 × 10 cm, with approximately 1 mg of calmodulin coupled per milliliter of resin prepared as described by Blumenthal & Stull (1980)] at the rate of 100–200 mL/h. The column was washed with 20 volumes of buffer containing 4 mM magnesium acetate, 0.2 mM calcium chloride, 10 mM MOPS, pH 7.0, 1 mM DTT, 0.2 M NaCl, and 1 mg/L leupeptin. Protein was eluted with buffer containing 2 mM EGTA, 10 mM MOPS, pH 7.0, 1 mM DTT, 0.2 M NaCl, and 1 mg/L leupeptin. Final purification was achieved by gel filtration chromatography using A 0.5m (Bio-Rad, 2.5 × 90 cm).

MLCK activity was determined by the incorporation of ^{32}P into myosin light chains as described by Edelman & Krebs (1982).

Rabbit skeletal muscle myosin light chains were prepared as described by Blumenthal & Stull (1980). α-Chymotrypsin and TPCK–trypsin were obtained from Worthington, lima bean trypsin inhibitor (LBTI) was from Millipore, *Staphylococcus aureus* V8 protease was from Miles, and Endoproteinase Lys-C was from Boehringer Mannheim. Rat mast cell protease II was a generous gift of Dr. N. Katunuma, Tokushima University. Radiolabeled iodo[^{14}C]acetic acid, iodo[^{3}H]acetic acid, and [^{14}C]methyl iodide were products of New England Nuclear/du Pont.

Limited proteolysis with trypsin was carried out as follows: MLCK (0.4 mg/mL) was treated with TPCK–trypsin (1/1000 w/w) at 22 °C for 20 min in 5 mM MOPS (pH 6.5) containing 5 mM DTT and 0.1 M NaCl. Digestion was terminated by the addition of LBTI to 4 μg/mL, and the mixture was applied to a Sephacryl S-200 (or S-300) column. Limited proteolysis with chymotrypsin was performed under the same conditions except that the concentration of MLCK was 0.86 mg/mL and the enzyme/substrate ratio was 1/500 (w/w).

Intact MLCK and fragments of limited proteolysis were S-carboxymethylated as described by Takio et al. (1983). ^{14}C-Methylation of methionyl residues in MLCK followed the procedures of Link & Stark (1968). [^{3}H]CM-MLCK (200 nmol) was treated with 25 μmol (250 μCi) of [^{14}C]CH_3I in 1 mL of 6 M guanidine hydrochloride, 0.1 M KNO_3, and 0.1 M acetate (pH 4) at room temperature for 20 h in the dark. The protein was separated from the mixture by extensive dialysis.

The citraconylation procedure of Habeeb & Atassi (1970) was used to limit tryptic cleavage or to unfold and solubilize the protein before glutamyl bond cleavage. ^{14}C-Methylated [^{3}H]CM-MLCK (100 nmol) was treated with 200 μL of citraconic anhydride in 1 mL of 8 M guanidine hydrochloride in a pH stat at pH 8.5 and then extensively dialyzed against

0.1 M NH_4HCO_3 at pH 8.8. For the cleavage of arginyl bonds, citraconylated, ^{14}C-methylated [^3H]CM-MLCK was incubated with TPCK–trypsin at 37 °C at a substrate to enzyme weight ratio of 100 for 1 h. The digest was applied either to a Sephadex G50 column in 0.1 M NH_4HCO_3, pH 8.5, after addition of LBTI (LBTI to trypsin weight ratio of 1.5) or to a tandem series of molecular-exclusion HPLC columns (in 6 M guanidine hydrochloride and 10 mM phosphate, pH 6.0) after decitraconylation (9% formic acid, 37 °C, 2 h). For digestion with *S. aureus* V8 protease, citraconylated ^{14}C-methylated [^3H]CM-MLCK was further dialyzed against 0.1 M NH_4HCO_3, pH 8.0, for 2 h prior to the digestion (37 °C, substrate to enzyme weight ratio of 50, 4 h). The digest was fractionated on a series of two TSK G2000SW columns (in 6 M guanidine hydrochloride and 10 mM phosphate, pH 6.0) and decitraconylated by treatment with 0.1 volume of 90% formic acid (2 h, 37 °C) before further fractionation by reversed-phase HPLC. Although decitraconylation takes place slowly at pH 6.0 (Habeeb & Atassi, 1970), during the size-exclusion chromatography, no effect of the decitraconylation on the separation was evident. Methionine-containing peptides were evident from the ^{14}C radiolabel.

Cleavage at methionyl residues was achieved in 70% formic acid as described by Gross (1967). Enzymatic subdigestions of isolated fragments were performed at 37 °C by using substrate to enzyme weight ratios of 20–100 for 2–6 h in 0.1 M NH_4HCO_3 (pH 8.0) except for *S. aureus* V8 protease (50 mM phosphate buffer, pH 7.8).

Peptides were separated by size-exclusion or by reversed-phase HPLC. For the primary separation, TSK-G2000SW and G3000SW columns (LKB Instruments, Inc.), connected in series, were eluted at 0.4 mL/min with 10 mM phosphate buffer (pH 6.0) containing 6 M guanidine hydrochloride. Pooled fractions were subsequently applied to a reversed-phase HPLC column, either Ultrapore RPSC (Beckman) or SynChropak RP-P (SynChrom), and eluted with an acetonitrile gradient in aqueous trifluoroacetic acid (Mahoney & Hermodson, 1980) for further fractionation and for the removal of salts. Amino acid analyses, sequenator analyses (spinning cup and gas phase), and identifications of phenylthiohydantoins followed procedures described previously by Takio et al. (1983, 1984b). Molecular weights were estimated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate according to Laemmli (1970) unless stated otherwise.

Homologous sequences were sought and aligned on a VAX/VMS computer using the programs described by Dayhoff et al. (1983). Optimal alignment of related proteins was established by using the ALIGN program as before (Takio et al., 1984a), and alignment scores were expressed in units of standard deviation.

RESULTS

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate suggested that the molecular weight of rabbit skeletal muscle MLCK is between 81 000 and 91 000, the value depending upon the system used. Sedimentation equilibrium data indicated a molecular weight of 70 300–75 000 (Mayr & Heilmeyer, 1983; Crouch et al., 1981). Thus, the polypeptide chain length appeared to be in the range of 600–800 residues, and anomalous behavior during gel electrophoresis was indicated. Several attempts to determine its amino-terminal sequence failed, indicating that the amino-terminus is blocked.

Limited Proteolysis of MLCK. Preliminary experiments with trypsin, chymotrypsin, *S. aureus* V8 protease, mast cell protease II, and thermolysin suggested the presence of a stable fragment of molecular weight approximately 40 000 which is

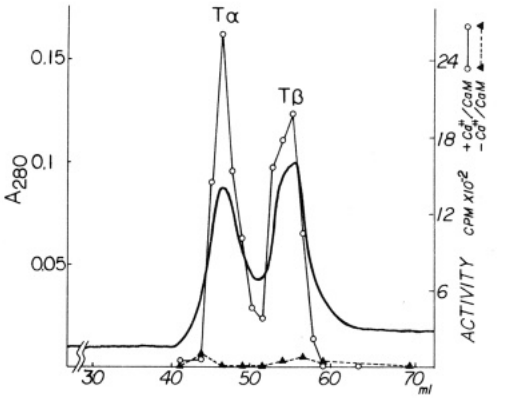

FIGURE 1: Separation of products (Tα and Tβ) of limited tryptic digestion of MLCK (see Materials and Methods) on Sephacryl S-200 (1.0 × 105 cm) in digestion buffer containing 1 mM EDTA. The flow rate was 4 mL/h. Enzyme activity is expressed as ^{32}P incorporated per minute in the presence of either calcium–calmodulin or EGTA (2 mM).

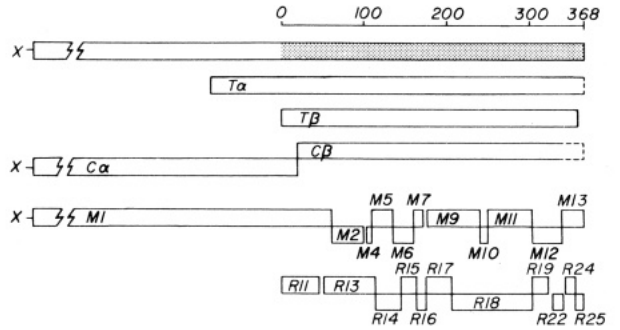

FIGURE 2: Relative alignment of major fragments isolated from MLCK. The upper segment represents the whole protein, blocked at the amino-terminus. The amino acid sequence of the carboxyl-terminal shaded portion is presented herein; that of the amino-terminal segment is not yet determined. Dashed portions of enclosures of fragments from limited proteolysis by trypsin (Tα, Tβ) and chymotrypsin (Cα, Cβ) indicate that the precise termini were not established. Prefixes M and R denote products of cleavage at methionyl and arginyl residues, respectively.

relatively resistant to these proteolytic enzymes and which, in most cases, retained both kinase activity and Ca^{2+}–calmodulin dependence. For example, very mild treatment with trypsin degraded MLCK to an intermediate fragment of ~60 000 daltons (Tα) and a more stable fragment of ~40 000 daltons (Tβ). Figure 1 shows the separation of these two fragments on a Sephacryl S-200 column and the dependence of their catalytic activities on Ca^{2+}–calmodulin. Amino-terminal analysis yielded the sequences RGSPA and GIEFQ in fragments Tα and Tβ, respectively. Detailed analyses in this report provide the complete amino acid sequence of the active fragment Tβ.

Similarly, chromatography on Sephacryl S-300 separated two fragments of limited chymotryptic hydrolysis, an ~37 000-dalton fragment, Cβ, which retained Ca^{2+}–calmodulin-dependent activity, and a ~42 000-dalton fragment (Cα), which lacked any activity (data not shown). The amino-terminus of the larger fragment was blocked, indicating that it might be derived from the amino-terminus of the whole protein (Figure 2), whereas the 37 000-dalton fragment had the amino-terminal sequence XLPAR, shown later to be CLPAR (Figure 3). Cysteinyl residues were not identified in Cβ because this fragment was not S-alkylated. Amino acid compositions of the tryptic and chymotryptic fragments Tβ and Cβ were similar to each other (Table I), but that of Cα was characterized by an unusually high proportion of Pro, Glx,

Table I: Amino Acid Compositions[a] of Tryptic Peptides of Citraconylated MLCK and of Other Selected Fragments

Peptide		R11	R12	R13	R14	R15	R16	R17	R18[b]	R19[c]	R22[d]	R24[d]	R25	T_β[e]	C_β[e]	C_α[f]	M1
Residues No.[g]		1-46	47-51	52-116	117-146	147-164	165-177	178-207	208-304	305-325	330-343	345-357	358-368	1-360	21-ND[h]	N-term to 20	N-term to 61
Asp/Asn	(D/N)	3.0(3)		4.1(5)	1.3(1)	2.1(3)	1.1(1)	3.8(4)	14.3(17)	2.3(2)	0.4(0)	1.9(2)		40.0(39)	35.8	26.5	23.7
Thr	(T)			4.1(5)	1.1(1)	1.4(2)		2.2(2)	5.2(6)					16.7(16)	15.1	18.9	16.5
Ser	(S)	1.2(1)		4.0(5)					7.0(8)	1.2(1)	0.8(1)	0.9(1)	2.4(3)	24.3(17)	15.6	26.7	29.7
Glu/Gln	(E/Q)	7.5(8)	1.1(1)	7.3(8)	6.4(7)	1.6(2)	1.1(1)	1.6(1)	11.3(11)	2.5(2)	1.2(1)			50.2(42)	39.7	60.9	72.5
Pro	(P)	9.7(10)		1.5(1)	1.0(1)			1.1(1)	3.9(4)	0.9(1)				20.4(18)	17.2	51.5	56.5
Gly	(G)	2.2(2)		5.9(6)	2.2(2)		1.1(1)	2.3(2)	6.3(6)				1.2(1)	27.6(19)	20.3	45.7	45.1
Ala	(A)	4.1(4)		4.6(4)	2.0(2)			1.2(1)	3.7(3)	5.0(5)	0.7(0)	3.0(3)	1.1(1)	26.3(22)	24.9	59.8	61.7
CMCys	(C)	1.6(3)		0.9(2)			0.7(1)	0.7(1)		0.9(1)				8.5(9)	---	ND	5.4
Val	(V)	2.1(2)	0.7(1)	4.9(5)	1.1(1)	3.1(4)		2.9(3)	5.9(8)	0.7(0)		1.1(1)		21.9(25)	23.4	16.5	14.6
Met	(M)			3.0(4)	0.7(1)	0.9(1)	0.8(2)		2.1(2)	1.0(1)	0.8(1)		0.8(1)	10.5(12)	10.7	1.9	
Ile	(I)	1.9(2)	0.7(1)	1.8(2)	3.2(4)	0.6(1)	1.7(2)	2.3(3)	2.4(3)		0.9(1)	0.9(1)	0.9(1)	16.5(20)	16.8	5.8	5.8
Leu	(L)	3.0(3)	1.0(1)	5.0(5)	4.0(4)	1.3(1)	1.0(1)	6.0(6)	10.0(10)	4.3(3)	4.0(4)		1.0(1)	38.0(38)	38	15.4	17.4
Tyr	(Y)				1.4(2)	0.8(1)			3.0(4)	0.5(0)	0.8(1)			7.4(8)	6.7	2.7	
Phe	(F)	2.8(3)		1.6(2)	1.7(2)	1.0(1)	1.1(1)	1.1(1)	5.0(6)	0.5(0)		1.1(1)	1.1(1)	17.7(18)	16.3	4.5	9.0
Trp[h]	(W)								(2)	(1)		(1)		(4)			
His	(H)	1.0(1)		0.8(1)	1.0(1)	0.8(1)	0.9(1)	1.8(2)		1.1(1)				8.8(8)	7.9	2.8	3.7
Lys	(K)	0.5(0)		8.5(9)			1.1(1)	1.9(2)	4.5(5)	2.9(2)	3.6(4)	1.9(2)	1.9(2)	24.7(27)	24.3	29.0	26.5
Arg	(R)	3.6(4)	1.0(1)	0.8(1)	1.1(1)	0.9(1)	1.0(1)	1.1(1)	1.8(2)	1.4(1)	1.3(1)	1.5(1)		16.9(18)	16.1	6.6	11.9
Total res.		46	5	65	30	18	13	30	97	21	14	13	11	360	ND[h]	ND[h]	ND[h]
Yield (%)		45	55	45	61	50	60	57	50	39	70	55	60	40	32		
N-terminus														GIEFQ-	XLPAR-	blocked	blocked
Mr[i]														40,000	37,000	42,000	ND[h]

[a] Residues per peptide by amino acid analysis (6N HCl 110°C, 20h). Values of less than 0.3 are not reported. Numbers in parentheses indicate those found in the sequence (Fig. 3). Ratios are calculated to the integral values underlined. One letter amino acid abbreviations are indicated in parentheses.
[b] R18 was contaminated with about 0.3 equivalents of the peptide lacking the amino-terminal Arg.
[c] R19 was contaminated with ~0.14 equivalents of R15 and ~0.06 equivalents of R22 (as judged by sequence analysis).
[d] R22 and R24 were accompanied by 0.2 and 0.3 equivalents respectively of the corresponding peptides with amino terminal arginyl residues.
[e] Results of single analyses of fragments of limited proteolysis. In each case minor contamination with the amino-terminal segment (high content of Glx, Pro, Gly, Ala, Lys) distorts the composition.
[f] Fragment C_α was not well separated from residual MLCK during chromatography on Sephacryl S-300.
[g] Numbered as in Fig. 3, and representing only the carboxyl-terminal segment of MLCK.
[h] Not determined.
[i] As determined by $NaDodSO_4$/PAGE.

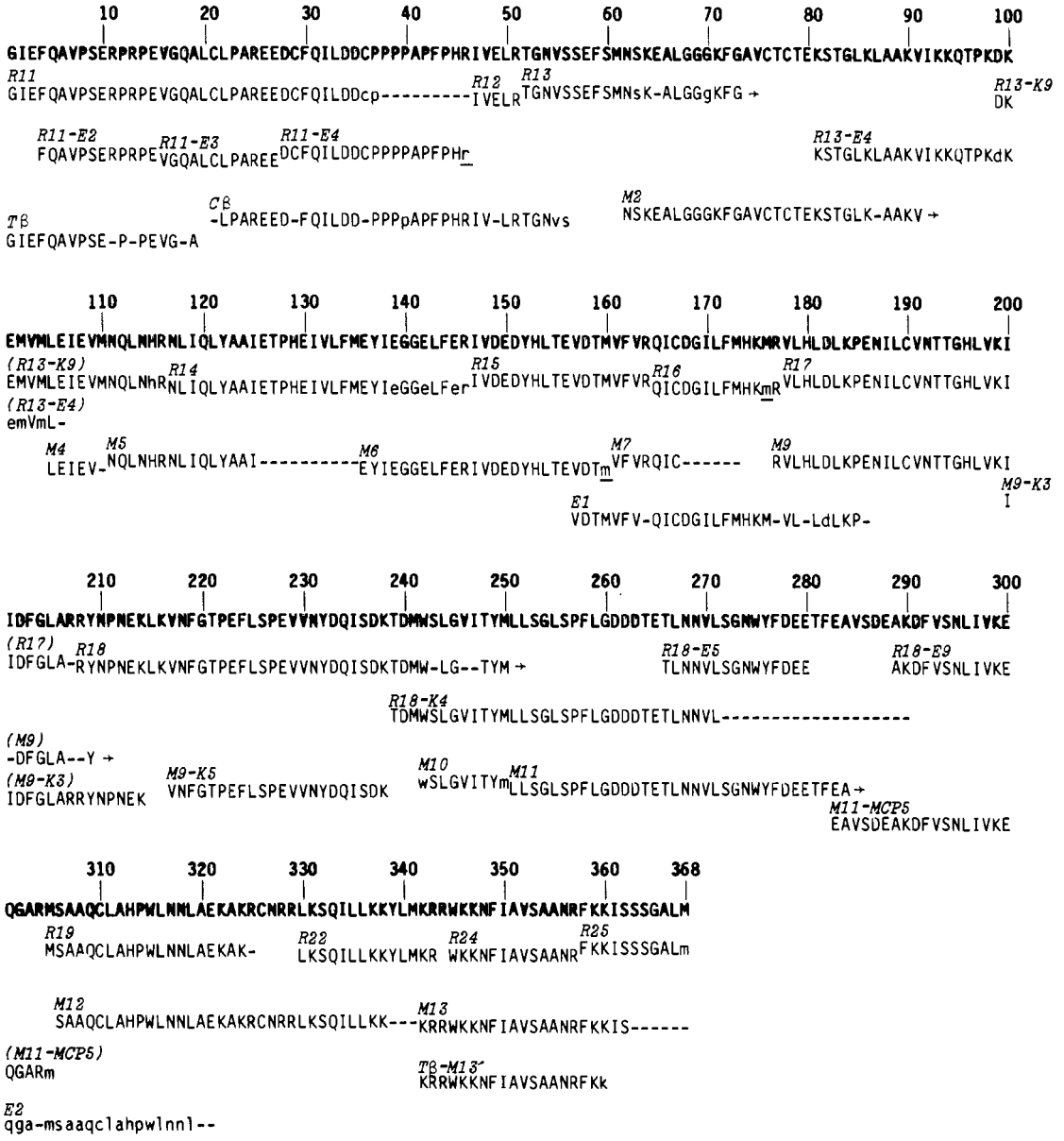

FIGURE 3: Detailed summary of the proof of sequence of the carboxyl-terminal 368 residues of MLCK. The proven sequences of specific peptides (named in italics) are given below the summary sequence (bold type). Prefixes R, M, T, and C are identified in Figure 2. Subpeptides are identified by hyphenated suffixes, where E, K, and MCP denote subdigestion at glutamyl bonds (*S. aureus* protease), at lysyl bonds (endoproteinase Lys-C), or by mast cell protease II, respectively. Peptide sequences written in upper case letters were proven by Edman degradation; lower case letters indicate tentative identifications, and those underlined are deduced from amino acid compositions. Those not identified are shown by dashes or by an arrow for a long unidentified sequence. Residue numbers 1–368 start at the amino-terminus of the ~40-kDa tryptic fragment Tβ (approximately the middle of intact MLCK) and continue to the carboxyl-terminus of MLCK.

Ala, and Gly and a low content of hydrophobic residues.

General Strategy of Sequence Analysis. Two primary sets of overlapping fragments were generated by cleavage at methionyl residues and at arginyl residues (Figure 2). These provided data to determine the sequence of the 360-residue, active fragment Tβ and to align that sequence with the carboxyl-terminus of MLCK. Details of the proof are summarized in Figure 3. The residue numbering system starts with the amino-terminus of Tβ, approximately in the middle of the MLCK molecule (Figure 2). Products of arginyl cleavage are arbitrarily numbered R11, R12, etc., from the amino-terminus of Tβ; those between the amino-terminus of MLCK (not shown) and that of Tβ are numbered Ra, Rb, etc. Methionyl cleavage products (M1–M13) are numbered from the amino-terminus to the carboxy-terminus of MLCK (Figure 2). Greek letters identify products of limited proteolysis. Details of the amino-terminal sequence of MLCK, including most of the fragments M1 and Cα and all of the arginyl bond cleavage fragments Ra, Rb and Rc (derived from the unsequenced segment amino-terminal to fragment Tβ), will be presented elsewhere.

Cleavage at Methionyl Bonds. Eight milligrams of [^{14}C]-CM-MLCK (100 nmol) was treated with cyanogen bromide, and the mixture was separated into nine fractions on TSK G2000SW columns, as illustrated in Figure 4. Fraction 1 contained only fragment M1, and it was simply dialyzed to remove salts. All other fractions were applied to reversed-phase HPLC for further fractionation and desalting. Two additional small peptides, M3 (Val-Hse) and M8 (His-Lys-Hse), were isolated from a separate cyanogen bromide digest of [^{14}C]CM-MLCK, using a Sephadex G50 column (data not shown). Of the 13 primary cleavage products, only the largest, M1, was blocked at the amino-terminus, indicating that it represented the amino-terminus of the whole protein (Figure 2). This was consistent with its amino acid composition, which resembled that of the amino-terminal chymotryptic fragment

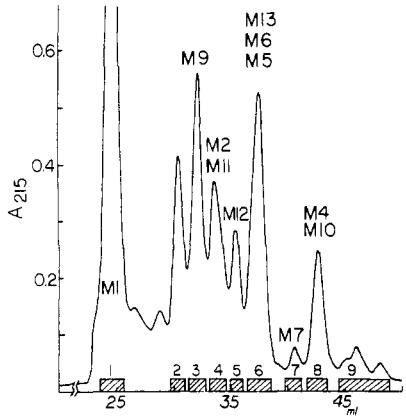

FIGURE 4: Primary separation of cyanogen bromide fragments of [^{14}C]CM-MLCK (1 mg) on a tandem series of two TSK G2000SW columns (each 7.5 × 600 mm) eluted with 10 mM phosphate (pH 6.0) containing 6 M guanidine hydrochloride at 0.4 mL/min. Hatched bars indicate pooled fractions. Symbols M1–M13 identify the locations of fragments. The order of the symbols (top to bottom) indicates the order of elution during subsequent fractionation on an Ultrapore RPSC column. Pooled fraction 2 contained a peptide overlapping M11 and M12. Pooled fraction 9 contained a mixture of M3 (Val-Hse) and M8 (His-Lys-Hse).

Cα in its high content of Pro, Glx, Ala, and Gly and its low content of hydrophobic amino acid residues (Table I).

Cleavage at Arginyl Bonds. Figure 5 shows the primary separation by size in an HPLC system of the tryptic digest of citraconylated ^{14}C-methylated [^{3}H]CM-MLCK. Each of the pooled fractions was applied to reversed-phase HPLC columns for further separation and for desalting. Pools C and G contained essentially pure R11 and R12, respectively. Amino acid compositions of peptides R11–R25 are listed in Table I. Peptide Ra is blocked, and it shared with Rb an amino acid composition characteristic of the amino-terminal region. Rc had the same amino-terminal sequence as that of fragment Tα (Figure 2), also indicating that it was derived from the amino-terminal half of MLCK. In confirmation, these three fragments (Ra, Rb, and Rc) could also be isolated from M1 after arginyl bond cleavage, as could R11, R12, and a peptide corresponding to the amino-terminal 10 residues of R13 (Figure 3).

Sequence Analysis. Since the 40-kD fragment Tβ retained both catalytic activity and calmodulin dependence (Figure 1), the present study is focused on that segment of MLCK and its relationship to the carboxyl-terminus of the whole protein. Most of the sequence of this domain was determined by analyses of the two complementary sets of peptides derived by cleavage at methionyl and arginyl bonds (Figure 3).

The amino-terminal sequences of R11 and Tβ were identical, and the sequence of R11 overlapped fragment Cβ (Figure 3). Hence, the sites of limited proteolysis by the two enzymes are within 20 residues of each other and in the approximate middle of the MLCK molecule (Figure 2). Partial sequence analysis of fragment Cβ provided overlaps linking R11–R12–R13. Subdigestion of R11 and R13 with *S. aureus* V8 protease or endoproteinase Lys-C yielded peptides that, together with M2, aligned residues 1–116. Peptides M5–M7 overlapped R13–R16 and extended the sequence to Arg-177. As mentioned earlier, peptides corresponding to R11, R12, and a fragment of R13 had been recovered from a tryptic digest of citraconylated M1. Thus, residues 1–61 must represent the carboxyl-terminus of the much larger fragment M1.

The remainder of the proof of sequence of the 368-residue segment is most easily discussed in two portions. A middle segment (residues 178–305) was defined by overlapping sequences of R17, R18, and M9–M11. Subdigestions of M9 with endoproteinase Lys-C and of M11 with mast cell protease yielded some of the information. Peptide R18 was primarily the product of cleavage of an Arg–Arg bond at residues 207–208 but contaminated with about 0.3 equiv of the same peptide lacking the amino-terminal Arg. The overlap at residues 282–285 was only two residues long. Otherwise, the proof of sequence of this middle segment was rigorous. In the carboxyl-terminal segment (residues 305–368), peptides R19–R25 were overlapped by M12 and M13. As above with R18, both R22 and R24 were found as mixtures with and without amino-terminal arginine from Arg–Arg sequences. In these cases, the forms lacking the amino-terminal Arg predominated (Table I). Of the series of arginyl cleavage products, only R25 lacked arginine entirely, placing it at the carboxyl-terminus of MLCK.

The three segments of the 368-residue domain were linked together by using two peptides isolated after *S. aureus* protease digestion of citraconylated, ^{14}C-methylated [^{3}H]CM-MLCK. Peptide E1 (residues 157–186) overlapped R16 and R17, linking the amino-terminal segment to the middle segment and providing a continuous sequence from residues 1 to 305. Peptide E2 was contaminated with an equimolar amount of a fragment observed in an analogous digest of M1 (data not shown). The amino-terminal sequence of the contaminant (GQAAARRGSPAFLHSPSCP) was subtracted from the results of analysis of the mixture to derive the sequence of E2 (residues 301–321). This completed the linkage of the middle segment to the carboxyl-terminal segment. The contaminant was apparently derived from the region surrounding the amino-terminus of Tα (Figure 2), which had the amino-terminal sequence RGSPA. The juxtaposition of M11 and M12 was confirmed by analysis of an overlapping peptide (pooled fraction 2, Figure 4) with a composition equivalent to the sum of M11 and M12, and with the amino-terminal sequence of M11.

The carboxyl-terminus of fragment Tβ is apparently eight (or nine) residues short of the carboxyl-terminus of MLCK. This conclusion was based on analysis of a CNBr digest of Tβ which, besides yielding M2–M12 as from whole MLCK, produced a shorter version of M13 (Tβ-M13′ in Figure 3) and a fragment of M1 (residues 1–61). Hence, it appears that the active and calmodulin-sensitive fragment Tβ comprises residues 1–360 (or 359) of Figure 3. The uncertainty arises from the content in Tβ-M13′ of 4.3 residues of Lys after acid hydrolysis.

Discussion

Proof of the structure of an active fragment within the carboxyl-terminal half of rabbit skeletal muscle myosin light chain kinase involved two primary sets of overlapping peptides derived by cleavage at either arginyl or methionyl residues. In general, the two sets proved to be quite complementary, although it was necessary to subdigest some of the larger peptides and to generate two specific overlapping peptides by digestion of the whole enzyme at glutamyl residues. In addition, two fragments generated by limited proteolysis of the whole enzyme (Tβ and Cβ) provided important data in the terminal regions (Figure 3).

The sequence presented in this report comprises the carboxyl-terminal 368 residues of MLCK. This segment includes the 41 006-dalton fragment Tβ (numbered residues 1–360 in this report) and the eight residues linking it to the carboxyl-terminus of the whole protein. This fragment retains not only catalytic activity toward myosin light chains but also calmodulin dependence, indicating the functional significance of this segment even in the absence of the amino-terminal

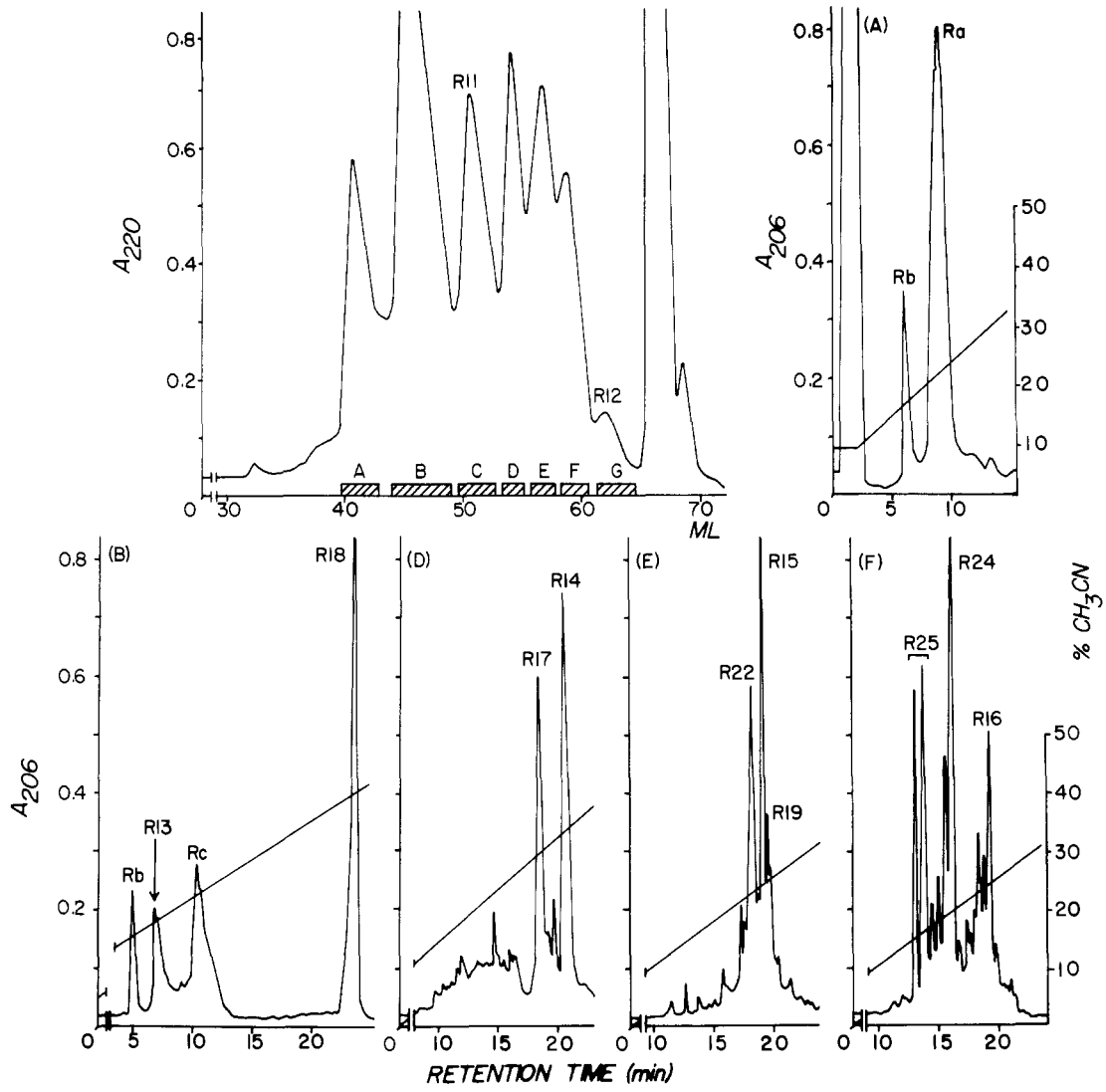

FIGURE 5: Separation of tryptic peptides of 2 mg of citraconylated, ^{14}C-methylated [^{3}H]CM-MLCK. Symbols R11–R25 (and Ra, Rb, and Rc) identify the location of fragments. Upper left: Primary separation at 0.4 mL/min on a tandem arrangement of three TSK columsn (G3000SW, G2000SW, and G2000SW, each 7.5 × 600 mm) in 10 mM phosphate (pH 6.0) containing 6 M guanidine hydrochloride. Hatched bars indicate pooled fractions A–G. (Panels A–F) Further separation of pooled fractions on a reversed-phase Ultrapore RPSC column (A–D) or a SynChropak RP-P column (E and F).

segment of the intact molecule, the function of which remains unknown.

The proof of sequence relies largely on replicate identifications of phenylthiohydantoins rather than upon amino acid compositions, particularly when suboptimal amounts of peptides were hydrolyzed. Ninety percent of the residues were identified in more than one peptide, but the remainder were examined only once (e.g., residues 74–79, 92–98, 126–135, etc.; cf. Figure 3). In most cases, independent identifications were made in two complementary HPLC systems.

The weakest points in the proof of structure are in the area of residues 283, 305, and 342. The overlaps at residues 282–285 and 341–344 were only dipeptides, and that around Met-305 relied on analysis of an impure peptide, E2. Although the latter analysis lacked rigor, the alignment was confirmed by analysis of a product of incomplete methionyl cleavage corresponding to linked segments M11 and M12. The occurrence of methionine at the carboxyl-terminal position (residue 368) precluded the usual identification of the carboxyl-terminal cyanogen bromide fragment by its lack of homoserine. Since trypsin has been reported to cleave methionyl bonds occasionally (e.g., in calmodulin; Sasagawa et al., 1982), one should be cautious in concluding that the only tryptic peptide lacking arginine was the carboxyl-terminal fragment. In the present case, the same peptides were found even when the methionyl residues were S-^{14}C-methylated prior to tryptic digestion, a procedure which should alter susceptibility to tryptic digestion. Thus, these data support the conclusion that Met-368 is the true carboxyl-terminus of MLCK. The tryptic fragment Tβ was eight residues shorter at the carboxyl-terminus, and this will be discussed later in connection with the putative calmodulin binding site in MLCK.

Limited Proteolysis of MLCK. There have been several reports in the last 10 years that both skeletal and smooth muscle MLCK are susceptible to limited proteolysis. In several of these cases, proteolytic cleavage activated the rabbit skeletal muscle molecule, rendering it unresponsive to calcium–calmodulin [e.g., see Tanaka (1980) and Srivastava & Hartshorne (1983)]. In other reports, limited proteolysis generated shortened forms of the molecule (M_r ~36000) which retained dependence upon calcium–calmodulin [e.g., see Mayr & Heilmeyer (1983)]. These various studies involved different digestion conditions, but in each case the active fragment was much smaller than the starting material. The precise size of intact rabbit skeletal MLCK has been a matter of controversy with reports ranging from 70300 to 91000 daltons [reviewed

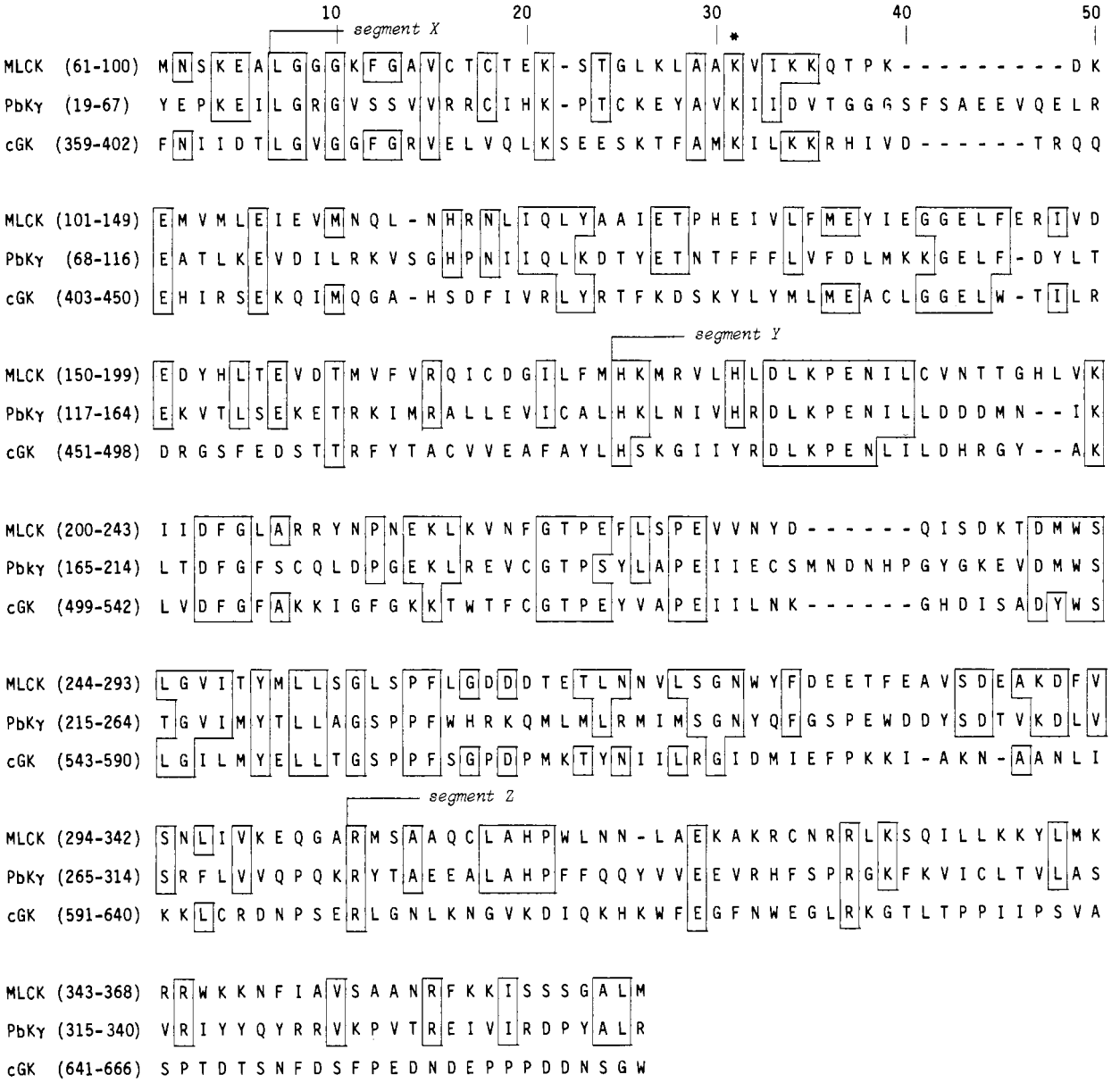

FIGURE 6: Alignment of a segment of MLCK (residues 61–368 in Figure 3) with the corresponding segments of cGK and the γ subunit of PbK. Residues identical with MLCK are enclosed in boxes. Lysine residue 389 in cGK (*) is at the ATP binding site (Hashimoto et al., 1982). The sequences of PbK_γ and cGK are from Reimann et al. (1984) and Takio et al. (1984a), respectively. Little similarity in sequence is observed in segments on the amino-terminal side of those illustrated; hence, they were omitted from the figure. The carboxyl-termini of cGK and PbK are residues 670 and 386, respectively (not shown). The locations of segments X, Y, and Z (Figure 7) are identified.

by Stull et al., (1985)]. The detailed hydrodynamic study of Mayr and Heilmeyer indicates 70 300 daltons, but larger values were estimated by gel electrophoresis. Their studies of frictional coefficients led to a model of MLCK with a highly asymmetric "tail" fragment (M_r 33 000) and a compact globular "head" fragment (M_r 36 500) containing both the catalytic center and the calmodulin binding domain. The present data suggest that the asymmetric tail must be on the amino-terminal side of the catalytic and regulatory head. In these terms, the complete amino acid sequence of their fragment is described herein, although it appears somewhat larger than their estimate of 36 500 daltons by $NaDodSO_4$–polyacrylamide gel electrophoresis. However, our conditions of tryptic digestion were not identical with those of Mayr and Heilmeyer. Our fragment Tβ (cf. Figure 2) lacked eight amino acids from the carboxyl-terminus of MLCK, giving a molecular weight (calculated) of 41 006. We observed a larger intermediate (Tα in Figures 1 and 2) which also possessed catalytic and calmodulin-dependent activities. The placement of Tα and of the chymotryptic fragment Cα within the structure of MLCK will be presented elsewhere with the complete amino-terminal sequence of MLCK.

Analogous proteolytic abolition of regulator dependence has been observed in other systems, for example, in the response of cyclic nucleotide dependent kinases to limited proteolysis (Inoue et al., 1976; Lincoln et al., 1978; Monken & Gill, 1980; Takio et al., 1983) and in the loss of the calcium–calmodulin dependence of several enzymes including phosphodiesterase, calcineurin, phosphorylase kinase, and Ca-ATPase [cf. review by Manalan & Klee (1984)]. In each case, the loss of a segment necessary for regulation was coincident with the conversion of the inactive protein to its active state. A detailed study of the limited proteolysis of MLCK by chymotrypsin is presented separately by Edelman et al. (1985), who contrast fragments of slightly different size, one of which lacks and two of which retain calmodulin dependence. In that study, comparison of the fragments provides insight into the location within MLCK of a segment crucial for calmodulin interaction.

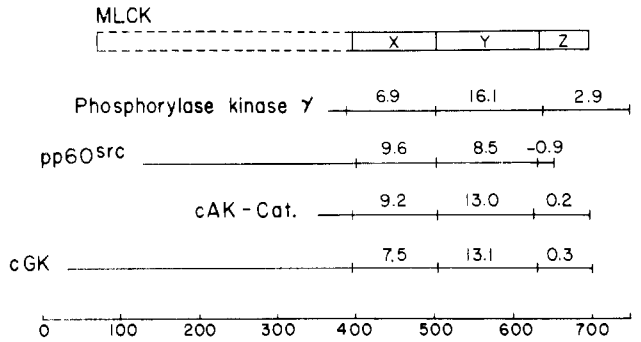

FIGURE 7: Three somewhat arbitrarily chosen segments of MLCK (X, Y, and Z in the top segmented bar) are aligned and compared with corresponding segments of homologous proteins, as previously described by Takio et al. (1984a). Each protein is indicated as a segmented line, above each segment of which is its alignment score with the vertically aligned segment of MLCK. Residues included in the boxed segments of MLCK are indicated by the following residue numbers from Figure 3: X = 67–174, Y = 175–304, and Z = 305–368. In the other proteins, the corresponding segments begin (or end) at the following residue numbers: PbKγ, 24, 140, 275, (386); pp60^{src}, 272, 377, 506, (526); catalytic subunit of cAMP-dependent protein kinase (cAK-Cat.), 48, 157, 280, (350); catalytic domain of cGMP-dependent protein kinase (cGK), 364, 474, 601, (670).

Search for Homologous Proteins. The sequence of the 368-residue segment of MLCK was arbitrarily divided into 45-residue segments and compared with the sequences in the November (1984) edition of the data bank of the Protein Identification Resource (2898 proteins, supplemented with locally generated sequences) by using the SEARCH program of Dayhoff et al. (1983). The group of proteins that scored highest by this analysis were protein kinases, including cAMP-dependent and cGMP-dependent kinases, the γ-subunit of phosphorylase kinase, the epidermal growth factor receptor, and the pp60^{src} family. The specific alignment of MLCK with phosphorylase kinase and with cGMP-dependent protein kinase is illustrated in Figure 6, and similar alignments were observed for the other members of this family of proteins. The statistical significance of these alignments is indicated in Figure 7, wherein arbitrarily chosen segments X and Y display great similarity to the corresponding segments of four other kinases and the carboxyl-terminal segment Z differs from all the other proteins except the γ chain of phosphorylase kinase. This discontinuity in the pattern of homology is also evident in Figure 6, where MLCK, phosphorylase kinase, and cGMP-dependent protein kinase appear to be homologous through glycine-273 of MLCK. After that point, cGMP-dependent protein kinase bears no structural resemblance to MLCK, whereas phosphorylase kinase continues to show weak similarity. However, the alignment score of segment Z (2.9 in Figure 7) is too low to provide a compelling argument that the carboxyl-terminal segments of MLCK and of phosphorylase kinase are related structurally or evolutionarily.

Other studies do indicate that the carboxyl-terminal region of MLCK is involved in the regulation of the catalytic activity by calcium–calmodulin. The lack of homology of the corresponding region of the γ subunit of phosphorylase kinase with that of cAMP-dependent protein kinase had led to such speculation about phosphorylase kinase (Reimann et al., 1984). Proof is now derived in two studies that are presented separately (Blumenthal et al., 1985; Edelman et al., 1985) and related to the sequence in the present paper. In one of these studies (Blumenthal et al., 1985), it is shown that the carboxyl-terminal 27-residue cyanogen bromide fragment, M13 (Figure 3), inhibits calcium–calmodulin activation of MLCK (K_I = 1 nM). Since fragment Tβ in the present work is also calmodulin dependent but lacks residues 361–368, residues 342–360 (KRRWKKNFIAVSAANRFKK) must contain information crucial for calmodulin interaction. In a separate but related study, Edelman et al. (1985) show that limited chymotryptic digestion of MLCK yields not only the calmodulin-dependent fragment Cβ but also a catalytically active, but calmodulin-*insensitive* fragment ("Cβ35") that lacks all but the eight amino-terminal residues of segment M13. Thus, both of these studies, when interpreted in terms of the sequence in Figure 3, do provide evidence of the site of interaction of MLCK with calmodulin.

The homologous relationship of MLCK with the other protein kinases also leads to a prediction of the site of ATP binding at the active center of the enzyme. Lys-31 in MLCK (Figure 6) corresponds to Lys-389 in the sequence AMKIL of cGMP-dependent protein kinase, which has been identified at the ATP-binding site by Hashimoto et al. (1982). Corresponding lysines in homologous sequences were identified in cAMP-dependent protein kinase by Zoller & Taylor (1979) and in pp60^{src} by Kamps et al. (1984). Thus, lysine in the sequence AAKVI of MLCK may provide the ATP-binding site corresponding to AMKIL in the nucleotide-dependent protein kinases and to AIKTL in the viral tyrosine protein kinases.

The proof of sequence of the amino-terminal half of MLCK is not yet completed. Source fragments for this study are suggested by the alignments in Figure 2, where fragments Tα, Cα, and M1 provide obvious starting points. Even at this stage, it is evident that MLCK must have a chimeric nature like cGMP-dependent protein kinase and other members of the protein kinase family (Takio et al., 1984a). The composition of fragment M1 (Table I) is sufficient by itself to exclude a homologous relationship with the amino-terminal half of cGMP-dependent protein kinase. Segment M1 is enriched in proline, glutamic acid, glycine, and alanine, residues that may play a role in the highly asymmetric nature of this segment of the molecule. Further studies of this amino-terminal segment should both establish its sequence and identify its role in the enzyme.

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