Goto, Y., & Aimoto, S. (1991) *J. Mol. Biol. 218*, 387-396.
Goto, Y., Calciano, L. J., & Fink, A. L. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573-577.
Goto, Y., Takahashi, N., & Fink, A. L. (1990b) *Biochemistry 29*, 3480-3488.
Goto, Y., Okamura, N., & Aimoto, S. (1991) *J. Biochem. (Tokyo) 109*, 746-750.
Hong, K., Yoshimura, T., & Papahadjopoulos, D. (1985) *FEBS Lett. 191*, 17-23.
Kaiser, E. T., & Kezdy, F. J. (1984) *Science 223*, 249-255.
Kono, K., Kimura, S., & Imanishi, Y. (1990) *Biochemistry 29*, 3631-3637.
Lau, S. Y. M., Taneja, A. K., & Hodges, R. S. (1984) *J. Biol. Chem. 259*, 13253-13261.
Lemire, B. D., Fankhauser, C., Baker, A., & Schatz, G. (1989) *J. Biol. Chem. 264*, 20206-20215.
Maezawa, S., & Yoshimura, T. (1990a) *Biochemistry 29*, 1813-1817.
Maezawa, S., & Yoshimura, T. (1990b) *Biochem. Biophys. Res. Commun. 173*, 134-140.
Maezawa, S., & Yoshimura, T. (1991) *Biochim. Biophys. Acta 1070*, 429-436.
Maezawa, S., Yoshimura, T., Hong, K., Düzgüneş, N., & Papahadjopoulos, D. (1989) *Biochemistry 28*, 1422-1428.
McLean, L. R., Hagaman, K. A., Owen, T. J., & Krstenansky, J. L. (1991) *Biochemistry 30*, 31-37.
Morgan, C., Williamson, H., Fuller, S., & Hudson, B. (1983) *Biochim. Biophys. Acta 732*, 668-674.
Morris, S., Sarkar, D. P., White, J. M., & Blumenthal, R. (1989) *J. Biol. Chem. 264*, 3972-3978.
Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987) *J. Biochem. (Tokyo) 102*, 957-962.
Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1988) *J. Biol. Chem. 263*, 4724-4730.
Parente, R. A., Nadasdi, L., Subbarao, N. K., & Szoka, F. C., Jr. (1990a) *Biochemistry 29*, 8713-8719.
Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1990b) *Biochemistry 29*, 8720-8728.
Pfanner, N., Rassow, J., Guiard, B., Sollner, T., Hartl, F.-U., & Neupert, W. (1990) *J. Biol. Chem. 265*, 16324-16329.
Stegmann, T., Doms, R. W., & Helenius, A. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 187-211.
Stegmann, T., White, J. M., & Helenius, A. (1990) *EMBO J. 9*, 4231-4241.
Stegmann, T., Delfino, J. M., Richards, F. M., & Helenius, A. (1991) *J. Biol. Chem. 266*, 18404-18410.
Suenaga, M., Lee, S., Park, N. G., Aoyagi, H., Kato, T., Umeda, A., & Amako, K. (1989) *Biochim. Biophys. Acta 981*, 143-150.
Szoka, F. C., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194-4198.
Szoka, F., Olson, F., Heath, T., Vail, W. J., Mayhew, E., & Papahadjopoulos, D. (1980) *Biochem. Biophys. Acta 601*, 559-571.
Takahashi, S. (1990) *Biochemistry 29*, 6257-6264.
Taqui Khan, M. M., & Martell, A. E. (1962) *J. Phys. Chem. 66*, 10-15.
White, J. M. (1990) *Annu. Rev. Physiol. 52*, 675-697.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.
Yoshimura, T., & Sone, S. (1987) *J. Biol. Chem. 262*, 4597-4601.
Yoshimura, T., Maezawa, S., & Hong, K. (1987) *J. Biochem. (Tokyo) 101*, 1265-1272.

Autophosphorylation of Skeletal Muscle Myosin Light Chain Kinase†

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Received February 11, 1992; Revised Manuscript Received April 14, 1992

ABSTRACT: Ca^{2+}/calmodulin-dependent myosin light chain kinase phosphorylates the regulatory light chain of myosin. Rabbit skeletal muscle myosin light chain kinase also catalyzes a Ca^{2+}/calmodulin-dependent autophosphorylation with a rapid rate of incorporation of 1 mol of ^{32}P/mol of kinase and a slower rate of incorporation up to 1.52 mol of ^{32}P/mol. Autophosphorylation was inhibited by a peptide substrate that has a low K_m value for myosin light chain kinase. Autophosphorylation at both rates was concentration-independent, indicating an intramolecular mechanism. There were no significant changes in catalytic properties toward light chain and MgATP substrates or in calmodulin activation properties upon autophosphorylation. After digestion with V8 protease, phosphopeptides were purified and sequenced. Two phosphorylation sites were identified, Ser 160 and Ser 234, with the former associated with the rapid rate of phosphorylation. Both sites are located amino terminal of the catalytic domain. These results indicate that the extended "tail" region of the enzyme can fold into the active site of the kinase.

Myosin light chain kinases catalyze the Ca^{2+}-dependent phosphorylation of myosin regulatory light chains. This phosphorylation results in potentiation of skeletal muscle contraction (Stull et al., 1986; Sweeney & Stull, 1990), whereas it is responsible for the initiation of smooth muscle contraction (Kamm & Stull, 1985; Hartshorne, 1987). Distinct forms of the kinase exist in smooth and skeletal muscle (Stull et al., 1986).

The complete amino acid sequence of the rabbit skeletal muscle myosin light chain kinase has been deduced by direct amino acid sequencing and cDNA cloning (Takio et al., 1986;

† The research described in this paper was supported in part by Grant HL06296 from the National Institutes of Health (to J.T.S.).
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0006-2960/92/0431-6126$03.00/0

Herring et al., 1990b). The 607-residue enzyme can be divided into four distinct domains: an amino-terminal "tail" of unknown function comprising almost half of the enzyme (Mayr & Heilmeyer, 1983); a central catalytic core that is homologous to other protein kinases (Hanks et al., 1988); a carboxyl-terminal calmodulin-binding, regulatory region (Blumenthal et al., 1985); and a myosin light chain binding site, which recently has been proposed to be located immediately amino terminal of the catalytic core (Herring et al., 1990a,b). A shape model of the kinase has been proposed in which the amino-terminal portion of the kinase (residues 1–255) forms an asymmetric, extension from the globular, carboxyl terminus (Mayr & Heilmeyer, 1983; Takio et al., 1986). The globular "head" structure contains the light chain binding site, catalytic core, and regulatory domains while the tail may form an extended polyproline II-like structure with two paired antiparallel segments.

Smooth muscle myosin light chain kinase is phosphorylated by a number of different protein kinases that regulate its activation by Ca^{2+}/calmodulin (Kamm & Stull, 1989; Stull et al., 1990). Rabbit skeletal muscle myosin light chain kinase is phosphorylated by cyclic AMP-dependent protein kinase at a slow rate with no change in the Ca^{2+}/calmodulin activation properties (Edelman & Krebs, 1982). Geuss et al. (1985) reported that rabbit skeletal muscle myosin light chain kinase was phosphorylated in the presence of Ca^{2+}/calmodulin and MgATP. However, no data were presented on the properties of this autophosphorylation reaction. Considering the potential importance of phosphorylation, including autophosphorylation, for affecting the biochemical properties of kinases (Edelman et al., 1987), we decided to examine autophosphorylation of rabbit skeletal muscle myosin light chain kinase. Results from these studies provide interesting structural information about the tail region of the kinase.

Experimental Procedures

Chemicals and Reagents. [γ-^{32}P]ATP was obtained from ICN. Trypsin, α-chymotrypsin, and *Staphylococcus aureus* V8 protease were purchased from Boehringer Mannheim, and IDA–Sepharose 6B was from Pharmacia. All other reagents were of analytical grade.

Purification of Proteins and Peptides. Rabbit skeletal muscle myosin light chain kinase was prepared as described previously (Herring et al., 1990b). Rabbit skeletal muscle myosin light chains were purified according to Blumenthal and Stull (1980). Calmodulin was purified from bovine testes (Bowman et al., 1992). Synthetic peptides of skeletal muscle myosin light chain, LC17 (PKKAKRRAAEGSSNVFS) and LC17A6A7 (PKKAKAAAAEGSSNVFS), were synthesized as described previously (Michnoff et al., 1986).

Autophosphorylation of Myosin Light Chain Kinase. Myosin light chain kinase was autophosphorylated at 30 °C in 50 mM MOPS,[1] 1 mM dithiothreitol, 10 mM magnesium acetate, 3 μM myosin light chain kinase, 4 μM calmodulin, 0.15 mM $CaCl_2$, or 2 mM EGTA[1] at pH 7.0. The reaction was initiated by the addition of [γ-^{32}P]ATP to a final concentration of 1 mM. The reaction was terminated by spotting an aliquot of the reaction mixture onto Whatman P81 phosphocellulose paper, which was then immersed in 75 mM phosphoric acid and washed as previously described (Roskoski, 1983). Radioactivity bound to the phosphocellulose papers was measured by liquid scintillation spectrometry.

Phosphoamino Acid Analysis. Myosin light chain kinase (10 μg) was autophosphorylated in the presence of Ca^{2+}/calmodulin for 50 min as described above and subjected to SDS–PAGE[1] (10% polyacrylamide). After staining and destaining, the gel slice containing myosin light chain kinase was excised and incubated with trypsin (20 μg) in 1 mL of 50 mM ammonium bicarbonate (pH 8.4) for 37 °C for 20 h. The solution was removed and lyophilized. More than 90% of the initial radioactivity in the gel slice was recovered. The sample was mixed with phosphoserine, phosphothreonine, and phosphotyrosine standards, and partial acid hydrolysis for phosphoamino acid analysis was performed (Cooper et al., 1983). The mixture was dried in a Speed Vac (Savant, Hicksville, NY), dissolved in 6 N HCl, and subjected to acid hydrolysis under reduced pressure at 110 °C for 2 h. Hydrolysates were dried in a Speed Vac, dissolved in H_2O, and subjected to thin-layer electrophoresis on cellulose plates (20 × 20 cm), Pyronin G (0.5%), [^{32}P]phosphate, and the three standard phosphoamino acids were also spotted. Electrophoresis was performed at 1000 V at 4 °C for 40 min at pH 3.5 in pyridine/glacial acetic acid/H_2O, 1/10/189 (v/v). After electrophoresis, the radioactivity was located by autoradiography with Kodak Omat film at −70 °C, and the phosphoamino acid standards were visualized by ninhydrin staining.

Peptide Mapping of Autophosphorylated Myosin Light Chain Kinase. Following autophosphorylation of myosin light chain kinase for 50 min, the reaction mixture was cooled on ice for 5 min, and 20 mM NaCl, 8% glycerol, 100 mM Tris (pH 8.0), 2 mM EGTA, and chymotrypsin (total protein/chymotrypsin, 20/1 w/w) were added. At the indicated time, aliquots (10 μg of myosin light chain kinase) were added to the SDS–PAGE sample buffer (see below) containing 1 mg/mL TPCK[1] and 7 mM PMSF[1] and immediately placed in boiling water for 4 min. After SDS–PAGE (12% polyacrylamide) and protein staining, the gel was dried for autoradiography. Chymotryptic peptides were identified by amino-terminal amino acid sequence analysis following electrophoretic transfer to PVDF[1] membranes (Millipore Corp., Bedford, MA) (Matsudaria, 1987).

For CNBr digestion, 10 μg of autophosphorylated myosin light chain kinase was precipitated with 10% trichloroacetic acid, followed by a couple of cycles of washing with 10% trichloroacetic acid. The protein pellet was dissolved in 100 μL of 70% formic acid and two small crystals of CNBr were added. Following incubation at room temperature overnight (approximately 16 h), the digestion was terminated by the addition of 1 mL of H_2O and lyophilization. The lyophilized sample was dissolved in sample buffer and subjected to SDS–PAGE (20% polyacrylamide). The gel was dried for autoradiography.

Purification of Autophosphorylated Peptides. After autophosphorylation of 150 μg of myosin light chain kinase, 100% trichloroacetic acid was added to the reaction mixture to a final concentration of 10%. The sample was cooled on ice for 10 min, followed by centrifugation. The pellet was dissolved in 300 μL of 8 M urea and dialyzed extensively against 50 mM ammonium bicarbonate (pH 7.8), 2 mM dithiothreitol, 0.3 mM EDTA,[1] and 0.02% NaN_3. After dialysis, 10 μL of 1 mg/mL V8 protease (in 100 mM Tris-HCl, pH 6.8) was added and the reaction mixture incubated for 4 h at 37 °C. Another 10 μL of solution containing V8 protease was added

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); EDTA, ethylenediaminetetraacetic acid; IDA, iminodiacetic acid.

and the reaction mixture incubated overnight. The digest was lyophilized, dissolved in 0.1 M acetic acid (pH 2.9), and applied to a 1.5-mL column (1-cm diameter) of IDA–Sepharose[1] charged with Fe^{3+} as described by Andersson and Porath (1986). The column was washed sequentially with more than 7 column volumes of the following buffers: 0.1 M acetic acid, 0.1 M sodium acetate (pH 5.0), 0.1 M ammonium acetate (pH 5.7), 0.1 M ammonium acetate (pH 8.8), and 0.2 M EDTA (pH 7.5). An aliquot of each eluate was added to Cytoscint (ICN) to measure radioactivity. The eluate obtained at pH 8.8 containing most radioactivity was lyophilized and then dissolved in 10% eluent B (84% acetonitrile–0.1% trifluoroacetic acid). The material was applied to a reverse-phase column (LKB-Pharmacia Spherisorb ODS-2, 5 μm, 4 × 250 mm) equilibrated with 90% eluent A (0.1% trifluoroacetic acid in H_2O) and 10% eluent B. Peptides were eluted with a linear gradient of 10–70% eluent B over 100 min at 0.8 mL/min. Absorbance was measured at 214 nm, and the radioactivity was monitored by liquid scintillation spectrometry. Nearly 80% of the total radioactivity applied to the column was recovered.

Chemical Modification and Sequencing of Autophosphorylated Peptides. Amino acid and sequence analyses of peptides were performed on an Applied Biosystems Inc. (Foster City, CA) Model 475A sequencer equipped with a Model 120A PTH amino acid analyzer. Because the standard manufacturer's programming and chemicals resulted in the rapid dissociation of peptide-bound phosphate from phosphoserine under the strongly alkaline conditions of the Edman coupling reaction, phosphoserine was identified by converting it to *S*-ethylcysteine for detection as a PTH amino acid with distinctive chromatographic properties. The modification was performed prior to sequencing by β-elimination and subsequent addition of ethanethiol as described by Meyer et al. (1986). PTH-*S*-ethylcysteine was identified under reverse-phase chromatographic conditions similar to those recommended by the manufacturer and utilized by Meyer et al. However, these standard conditions were changed by employing an A solvent containing 10% (v/v) tetrahydrofuran in water instead of the normal 3.5% in order to decrease the retention time of PTH-*S*-ethylcysteine relative to dimethylphenylthiourea, a major side product of the Edman degradation which obscured the PTH-*S*-ethylcysteine peak under standard chromatographic conditions. Because this change also altered the retention times of several other PTH amino acids, residues from phosphopeptides were subjected to chromatographic analysis under both the conventional and modified conditions to confirm all identifications.

Miscellaneous. SDS–PAGE was performed as described by Laemmli (1970). Samples were prepared by incubation with electrophoresis sample buffer for 4 min in boiling water. Final concentrations were 50 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 5% sucrose, 0.01% bromphenol blue, 1% SDS, and 2 mM EGTA. Myosin and myosin light chain kinase concentrations were determined by the dye-binding method of Bradford (1976) with bovine γ-globulin as standard. Calmodulin concentration was determined by UV absorbance at 277 nm and an extinction coefficient of 3300 M^{-1} cm^{-1} (Klee, 1977). The activity of myosin light chain kinase was measured by ^{32}P incorporation into the rabbit skeletal muscle myosin regulatory light chain as described previously (Blumenthal & Stull, 1980).

Results

Time Course for Phosphorylation of Myosin Light Chain Kinase. Purified rabbit skeletal muscle myosin light chain kinase was incubated in the presence of [γ-^{32}P]ATP. There was a time-dependent ^{32}P incorporation that was significantly greater in the presence of Ca^{2+}/calmodulin than in the presence of EGTA (Figure 1). In the presence of Ca^{2+}/calmodulin the stoichiometry obtained by 50 min was 1.52 mol of ^{32}P incorporated/mol of myosin light chain kinase. Incubation up to 2 h did not significantly increase this extent of phosphorylation. If calmodulin was not added to the reaction mixture, the phosphorylation was not significantly greater than that obtained in the presence of EGTA (data not shown). These results show that the phosphorylation is Ca^{2+}/calmodulin-dependent and at least two sites are phosphorylated.

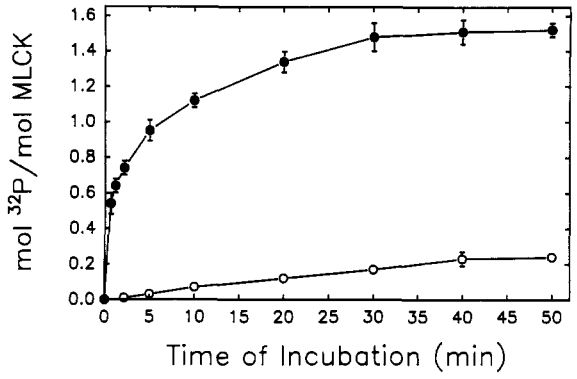

FIGURE 1: Time course of autophosphorylation of skeletal muscle myosin light chain kinase. Autophosphorylation of the kinase (MLCK) was performed in the presence of Ca^{2+}/calmodulin (solid circles) or EGTA (open circles) as described under Experimental Procedures.

The rate of phosphorylation in the presence of Ca^{2+}/calmodulin was more rapid within the first 5 min with nearly 1 mol of ^{32}P incorporated/mol of myosin light chain kinase (Figure 1). Subsequently, the reaction was much slower. These data suggest that two sites may be phosphorylated at different rates.

Effect of Myosin Light Chain Kinase Concentration on the Rates of Phosphorylation. Autophosphorylation of protein kinases can occur by intermolecular or intramolecular mechanisms. To distinguish between these two possibilities, the dependency of the rate of phosphorylation was examined as a function of myosin light chain kinase concentration. Autophosphorylation was measured after 2 min of incubation over a 50-fold range of kinase concentrations which temporally represents the faster phase (Figure 2A). Under these conditions the rate of phosphorylation was independent of kinase concentration.

After the enzyme was phosphorylated for 5 min under standard conditions, the reaction mixture was diluted with the reaction buffer without myosin light chain kinase to vary the kinase concentration 12-fold and then incubated for another 17 min. The rate of phosphorylation between 5 and 22 min represents primarily the slower phase. As can be seen in Figure 2B, this rate of phosphorylation was also independent of myosin light chain kinase concentration. Thus, in both cases, autophosphorylation appears to occur by an intramolecular mechanism.

Effect of Peptide Substrates on the Autophosphorylation of Myosin Light Chain Kinase. The effect of two synthetic peptide substrates on the rate of autophosphorylation of myosin light chain kinase was examined. The two peptides, LC17 and CL17A6A7, were chosen because of the marked differences in their K_m values (2.8 and 162 μM, respectively) (Michnoff et al., 1986). The ^{32}P incorporation into myosin light chain kinase was measured after separation of the kinase from the peptide and [γ-^{32}P]ATP by SDS–PAGE. LC17 (100 μM)

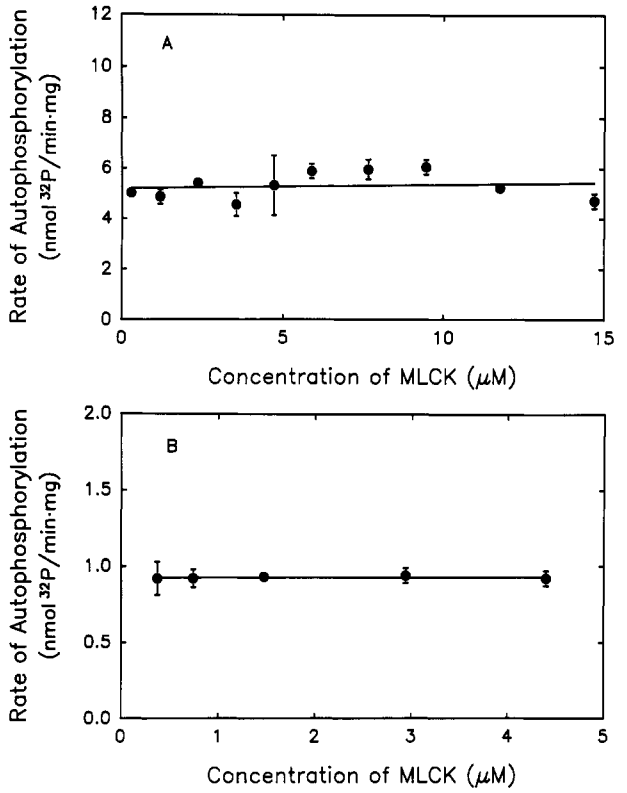

FIGURE 2: Effect of myosin light chain kinase concentration on the rates of autophosphorylation. (A) Autophosphorylation was performed at 15 μM calmodulin and the indicated concentrations of myosin light chain kinase (MLCK) at 30 °C for 2 min. (B) Autophosphorylation was performed at 6 μM calmodulin and 4.4 μM myosin light chain kinase for 5 min. Then the mixtures were immediately diluted to the indicated concentrations with the same buffer mixture for autophosphorylation except without myosin light chain kinase and calmodulin and incubated at 30 °C for a total of 22 min. Best-fit lines were determined by least-squares linear regression analysis.

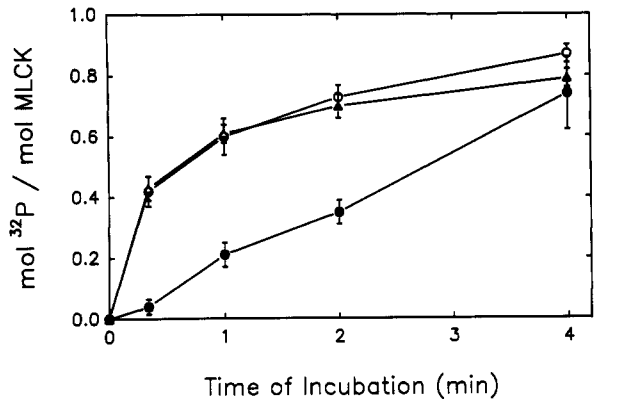

FIGURE 3: Effect of synthetic peptide substrates on autophosphorylation of myosin light chain kinase. Autophosphorylation was performed as described under Experimental Procedures except that the concentration of calmodulin was 1 μM and that of myosin light chain kinase (MLCK) was 0.1 μM. The reaction mixtures contained 100 μM peptide LC17 (solid circles) or LC17A6A7 (triangles) or no peptide (open circles). At the indicated times, SDS–PAGE sample buffer was added, and the mixture was immediately heated in boiling water for 4 min followed by SDS–PAGE (10% polyacrylamide). After staining and destaining, myosin light chain kinase was excised, and the radioactivity was measured by liquid scintillation spectrometry.

depressed the initial rate of the autophosphorylation reaction, while LC17A6A7 at a similar concentration had no significant influence on the reaction (Figure 3). Thus the synthetic peptide substrate with a high affinity for myosin light chain kinase inhibited autophosphorylation.

Table I: Kinetic Parameters of Autophosphorylated and Nonphosphorylated Myosin Light Chain Kinase[a]

	autophosphorylated		nonphosphorylated	
	$K_{0.5}$[b]	V_{max}[c]	$K_{0.5}$[b]	V_{max}[c]
calmodulin	2.8 ± 0.2	17.4 ± 0.6	3.0 ± 0.2	17.0 ± 1.0
light chain	2.9 ± 1.0	22.6 ± 0.0	2.6 ± 0.1	22.0 ± 1.0
myosin	3.8 ± 0.2	9.6 ± 0.4	4.0 ± 0.4	8.6 ± 0.6
ATP	135 ± 11	21.6 ± 2.6	101 ± 2	17.0 ± 1.0

[a] Myosin light chain kinase was autophosphorylated in the presence of Ca^{2+} and calmodulin for 40 min as described under Experimental Procedures. Following autophosphorylation, the reaction mixture was directly diluted 1000-fold with 20 mM MOPS, 1 mg/mL BSA, 1 mM dithiothreitol, and 1 mM EGTA at pH 7.0 and assayed for kinase activity for 30 s. Nonphosphorylated kinase was treated the same as above except without ATP at the autophosphorylation step. With varying concentrations of calmodulin, light chain, and ATP, the reaction mixtures of the kinase assay contain 0.3 nM myosin light chain kinase. With varying concentrations of myosin, the mixture contains 2 nM myosin light chain kinase, 50% glycerol, and 250 mM KCl. With varying concentrations of calmodulin and ATP, the initial rates of ^{32}P incorporation into myosin light chain were measured. Kinetic parameters were derived from double-reciprocal plots. All values represent the mean ± SEM for at least three experiments. [b] The values for calmodulin represent the concentration (nM) of calmodulin required for half-maximal activation. The values for light chain, myosin, and ATP represent K_m values (μM). [c] The V_{max} values are in μmol of ^{32}P incorporated min^{-1} (mg of enzyme)$^{-1}$.

Biochemical Effects of Myosin Light Chain Kinase Autophosphorylation. We examined the effect of autophosphorylation on myosin light chain kinase activity at varying concentrations of calmodulin, myosin light chain, myosin, and ATP, respectively, to obtain the kinetic parameters listed in Table I. There were no apparent differences in K_m and V_{max} values for the substrates before and after autophosphorylation. There were also no differences in the calmodulin activation properties. A time was chosen (30 s) so that the kinetic parameters could be determined with little autophosphorylation during the kinase assays themselves. Thus, autophosphorylation of myosin light chain kinase has no demonstrable effect on the catalytic or activation properties of the kinase.

We also examined the effect of autophosphorylation on the heat stability of the kinase. Myosin light chain kinase was heated to 45 °C for different periods of time, and kinase activity was measured. The nonphosphorylated and autophosphorylated kinases lost enzyme activity at the same rate (data not shown). Thus, autophosphorylation does not appear to affect the heat stability of the kinase.

Location of the Autophosphorylation Sites. Purified myosin light chain kinase that had been autophosphorylated to 1.5 mol of ^{32}P incorporated/mol of kinase was subjected to partial acid hydrolysis and high-voltage electrophoresis. Autoradiographs of the electrophoretograms showed ^{32}P that migrated with phosphoserine, not phosphothreonine or phosphotyrosine (data not shown).

To determine the location of the autophosphorylation sites, myosin light chain kinase phosphorylated to 1.5 mol of ^{32}P/mol of kinase was partially digested by chymotrypsin as described under Experimental Procedures and subjectd to SDS–PAGE. Figure 4A shows the protein stain of the chymotryptic digest of the autophosphorylated myosin light chain kinase. The enzyme (M_r = 89 000 by SDS–PAGE) was rapidly degraded to a major fragment of 35 kDa which was resistant to further proteolysis. The protein bands were transferred to PVDF membrane after SDS–PAGE, and the 35-kDa band was subjected to amino-terminal sequence analysis as described below. The sequence obtained, 256 CLPA 259, is consistent with the identification of this fragment in the carboxyl-terminal

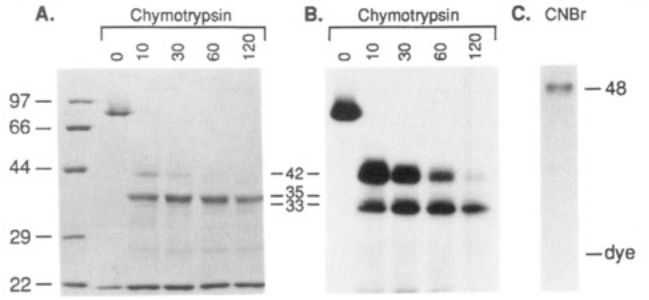

FIGURE 4: Digestion of autophosphorylated myosin light chain kinase. After limited chymotryptic digestion, autophosphorylated myosin light chain kinase was separated by SDS–PAGE (12% polyacrylamide), and the gel was subjected to autoradiography. Panel A: Stained gel. The lane on the left contains molecular mass markers with the numbers indicating kilodaltons. For 0 min, 4 μg of autophosphorylated myosin light chain kinase was applied. In the following lanes 10 μg of autophosphorylated myosin light chain kinase was applied after incubation with chymotrypsin on ice for 10, 30, 60, and 120 min, respectively. Panel B: Autoradiogram of the gel in (A). Panel C: Autoradiogram of autophosphorylated myosin light chain kinase after CNBr digestion and SDS–PAGE (20% polyacrylamide) as described under Experimental Procedures. The molecular mass was determined by comparison with a stained gel containing M_r standards (not shown).

head containing the catalytic and regulatory domains (Edelman et al., 1985). Fragments having molecular masses of 42 and 33 kDa, respectively, were poorly stained. Figure 4B shows an autoradiogram of the same gel. Following degradation, radioactivity was distributed into the 42- and 33-kDa fragments, while no radioactivity was found with the 35-kDa fragment.

After CNBr digestion the autophosphorylated kinase was subjected to SDS–PAGE and autoradiography (Figure 4C). There was only one radioactive band with a molecular mass of 48 kDa, which is consistent with its identification as the M1 fragment (residues 2–296) reported by Takio et al. (1985). These results, obtained with chymotryptic and CNBr digestion, show that the autophosphorylation sites are located in the amino-terminal tail of the enzyme, not the carboxyl-terminal head which contains the catalytic and regulatory domains.

Purification and Sequences of ^{32}P-Labeled Peptides Obtained from Autophosphorylated Kinase. Autophosphorylated myosin light chain kinase was digested by V8 protease, dried, and dissolved in 0.1 M acetic acid. The digest was applied a Fe^{3+}–IDA–Sepharose column (Figure 5). Most of the nonphosphorylated peptides were eluted from the column by stepwise washing with the acidic pH buffers as described under Experimental Procedures (pH 2.9, 5.0, and 5.7), whereas phosphopeptides were eluted at pH 8.8. Approximately 89% of the total radioactivity applied to the column was recovered at this step. This fraction was applied to reverse-phase HPLC (Figure 6). Five radioactive peaks were eluted from the HPLC column with 80% recovery of the applied radioactivity.

The most prominent peaks in chromatograms of radioactivity derived from myosin light chain kinase that had been subjected to phosphorylation for 50 min (peaks A, D, and E, Figure 6) were subjected to amino-terminal sequence analysis. Sequencing was performed after treatment of the peptides to convert phosphoserine to the more readily detected amino acid *S*-ethylcysteine as described under Experimental Procedures. The sequencing results are shown in Table II. Peak A contained two peptides from myosin light chain kinase, one spanning the sequence Lys 219 to Glu 238 and the other extending from Lys 170. The 16th residue in the former peptide was identified as *S*-ethylcysteine, indicating that Ser 234 was phosphorylated. Combined peaks D and E contained three peptides. One was from calmodulin and began at Ala

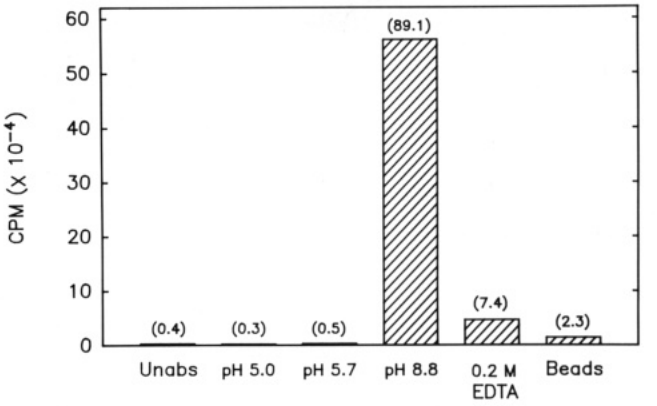

FIGURE 5: Fe^{3+}–IDA–Sepharose chromatography of the V8 protease digest of autophosphorylated myosin light chain kinase. After digestion of autophosphorylated myosin light chain kinase by V8 proteases, the peptides were applied to a 1.5-mL column of Fe^{3+}–IDA–Sepharose as described under Experimental Procedures. Unabs is the unabsorbed and 0.1 M acetic acid (pH 2.9) wash fraction; pH 5.0, pH 5.7, pH 8.8, and 0.2 M EDTA represent the fractions eluted from the column with 0.1 M sodium acetate, pH 5.0; 0.1 M ammonium acetate, pH 5.7, 0.1 M ammonium acetate, pH 8.8; and 0.2 M EDTA, pH 7.5, respectively. Beads represent the radioactivity remaining on the resin after elution. Numbers in parentheses represent the percentage of total radioactivity applied to the column.

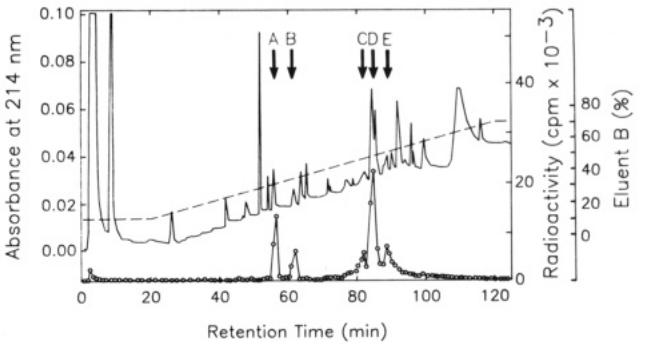

FIGURE 6: Separation of phosphopeptides eluted from the Fe^{3+}–IDA–Sepharose column by HPLC. The column was equilibrated with 10% eluent B (84% acetonitrile–0.1% trifluoroacetic acid) and developed with a gradient of 10–70% eluent B (dashed line) as described under Experimental Procedures. Absorbance was measured at 214 nm (solid line) while radioactivity was monitored by liquid scintillation spectrometry (open circles).

88. The other two were from myosin light chain kinase and displayed overlapping sequences spanning Gly 144 to Glu 169 or Ala 148 to Glu 169. Edman degradation of the peptide mixture yielded *S*-ethylcysteine residues in cycles 13 and 17, indicating that Ser 160 in myosin light chain kinase was phosphorylated. Both peptides contained Ser 152, Ser 158, and Ser 167 in unmodified form, confirming that the phosphoserine modification reaction was behaving specifically. Unphosphorylated serine was not detected at either position 160 or position 234. In the case of Ser 160, this is expected because phosphorylation was stoichiometric. In the case of Ser 234, however, where the stoichiometry was less than 1, the absence of unmodified serine indicates either that endogenous phosphorylation had occurred or that the quantity of unmodified PTH-Ser was below the limits of detectability. Detection of PTH-Ser is relatively insensitive compared to other residues because various reactions occur that degrade the side chain of this amino acid during the Edman chemistry. All of the phosphopeptides identified in this study arose by cleavage on the carboxyl-terminal side of glutamic acid residues as expected for V8 protease, but the component peak D that began at Ala 148 was the product of an atypical

Table II: Amino Acid Sequence Analysis of Phosphopeptide Peaks A, D, and E Isolated by HPLC[a]

position	peak A				peaks D and E					
1	Lys m219/m170	*14*			Ala m148/c88	*395*;	Gly m144	*90*		
2	Ala m220	*54*;	Leu m171	*30*	Arg m149	*60*;	Gln m145	*78*;	Phe c89	*277*
3	Pro m221/m172	*57*			Arg m150/c90	*152*;	Ala m146	*99*		
4	Gly m222	*46*;	Ala m173	*25*	Gly m151	*124*;	Ala m147	*165*;	Val c91	*264*
5	Gln m223/m174	*69*			Ser m152	*46*;	Ala m148	*141*;	Phe c92	*283*
6	Ala m224	*40*;	Lys m175	*6*	Pro m153	*84*;	Arg m149	*61*;	Asp c93	*162*
7	Asp m225	*35*;	Pro m176	*9*	Ala m154	*117*;	Arg m150	*90*;	Lys c94	*79*
8	Gln m226	*50*;	Leu m177	*13*	Phe m155	*94*;	Gly m151	*48*;	Asp c95	*127*
9	Ala m227	*34*;	Ser m178	undetected	Leu m156	*99*;	Ser m152	*24*;	Gly c96	*145*
10	Lys m228	*8*;	Glu m179	undetected	His m157	*14*;	Pro m153	*37*;	Asn c97	*21*
11	Val m229	*27*			Ser m158	*26*;	Ala m154	*47*;	Gly c98	*16*
12	Gln m230	*25*			Pro m159	*43*;	Phe m155	*33*;	Tyr c99	undetected
13	Gly m231	*16*			etCys m160	*31*;	Leu m156	*36*		
14	Asp m232	*16*			Cys m161	–;	His m157	*6*		
15	Thr m233	*12*			Pro m162	*34*;	Ser m158	*14*		
16	etCys m234	*15*			Ala m163	*37*;	Pro m159	*31*		
17	Arg m235	*12*			Ile m164	*27*;	etCys m160	*10*		
18	Gly m236	*11*			Ile m165	*36*;	Cys m161	–		
19	Ile m237	*11*			Ala m166	*30*;	Pro m162	*16*		
20	Glu m238	*6*			Ser m167	*8*;	Ala m163	*25*		
21					Thr m168	*6*;	Ile m164	*5*		
22					Glu m169	*4*;	Ile m165	*7*		
23							Ala m166	*3*		
24							Ser m167	*2*		
25							Thr m168	*2*		
26							Glu m169	*1*		

[a] Peak A contained two peptides from myosin light chain kinase (m); peaks D and E contained three peptides, two from myosin light chain kinase and one from calmodulin (c). Residues are assigned to the various peptides in these mixtures and labeled according to the positions they occupy in myosin light chain kinase (m) or calmodulin (c) on the basis of the known sequences of these peptides. Yields of PTH amino acids in picomoles are shown in italics and are derived from analyses performed under conditions that had been modified for identification of PTH-*S*-ethylcysteine, a derivative of phosphoserine (see text). Cysteine residues were identified by the presence of derivatives for which quantitation standards are unavailable. *S*-Ethylcysteine residues (etCys) were quantitated by comparison with signals derived from a standard phosphorylated peptide, kemptide.

cleavage carboxyl terminal to an alanine residue. The less prominent phosphopeptide peak, C, contained a peptide beginning at Gly 144 (data not shown). In a separate experiment, peak B contained a major peptide beginning at Lys 219 and containing Ser 234.

Ratio of ^{32}P Incorporated into the Two Sites. Myosin light chain kinase was autophosphorylated in the presence of Ca^{2+} for 1 and 50 min or in the presence of EGTA for 50 min, respectively. The autophosphorylated enzyme was precipitated by trichloroacetic acid, dialyzed, and digested by V8 protease as described under Experimental Procedures. The resultant digest was directly applied to HPLC without the Fe^{3+}–IDA–Sepharose chromatography, and the radioactivity was measured in the peaks to establish the relative rates of phosphorylation of Ser 160 and Ser 234. The retention times of each radioactive peak were the same as the radioactive peaks obtained after Fe^{3+}–IDA–Sepharose chromatography shown in Figure 6 (data not shown).

After autophosphorylation for 1 min in the presence of Ca^{2+}, the ^{32}P incorporated into Ser 160 accounted for 98% of the total ^{32}P incorporation (Table III). After incubation for 50 min, the ^{32}P incorporated into Ser 160 accounted for 70% of the total radioactivity. Nearly 1 mol of ^{32}P was incorporated into Ser 160, while 0.5 mol of ^{32}P was incorporated into Ser 234. In the presence of EGTA, the rates of autophosphorylation at both sites were significantly slower.

Table III: Ratio of ^{32}P Incorporated into Ser 160 and Ser 234 of Rabbit Skeletal Muscle MLCK[a]

incubation	mol of ^{32}P/ mol of MLCK	% of cpm[b]	
		Ser 160	Ser 234
+Ca^{2+}, 1 min	0.62	97.8	2.2
+Ca^{2+}, 50 min	1.52	70.0	30.0
+EGTA, 50 min	0.24	98.0	2.0

[a] Myosin light chain kinase was autophosphorylated as described under Experimental Procedures for 1 or 50 min. Specific sites of phosphorylation were identified and quantitated after digestion of myosin light chain kinase and isolation of phosphopeptides by HPLC (Figure 6) as described under Results. [b] Total cpm of Ser 160 and Ser 234 was designated as 100%.

Discussion

Rabbit skeletal muscle myosin light chain kinase phosphorylates itself. The autophosphorylation reaction depends upon Ca^{2+}/calmodulin, which is consistent with the activation necessary for phosphorylation of its other substrate, myosin light chain. However, the rates of phosphorylation are significantly different. The V_{max} value obtained for myosin light chain is 20 μmol of ^{32}P incorporated min^{-1} (mg of kinase)$^{-1}$ whereas the value for the maximal rate of autophosphorylation is 5 nmol of ^{32}P incorporated min^{-1} mg^{-1}. A synthetic peptide substrate with a high affinity but low V_{max} value for the kinase inhibits phosphorylation of myosin light chain kinase. Furthermore, ^{32}P is incorporated into the kinase to an appreciable stoichiometry of 1.52 mol of ^{32}P/mol of myosin light chain kinase.

Autophosphorylation is independent of kinase concentration, indicating an intramolecular mechanism. It has recently been reported that smooth muscle myosin light chain kinase could exist in oligomeric forms at high enzyme concentrations (14 μM or greater) (Sobieszek, 1991), which raises the possibility that skeletal muscle myosin light chain kinase could also form oligomers. However, the skeletal muscle kinase has different biochemical properties compared to those of the smooth muscle enzyme, including molecular size, mechanism of regulation by other protein kinases, reaction mechanism, and binding to contractile proteins (Stull et al., 1986). These differences are based on unique structural properties of skeletal versus smooth muscle kinases (Gallagher et al., 1991; Leachman et al., 1992). The molecular mass of rabbit skeletal muscle myosin light chain kinase determined under nondenaturing conditions by

gel filtration and sedimentation equilibrium centrifugation is between 70 and 80 kDa, similar to the values obtained by SDS–PAGE (Pires & Perry, 1977; Yazawa & Yagi, 1978; Blumenthal & Stull, 1980; Mayr & Heilmeyer, 1983). These results suggest that the skeletal muscle kinase exists as a monomer in the native state. With sedimentation equilibrium and sedimentation velocity studies, Mayr and Heilmeyer (1983) found that more than 90% of the protein exists in a nonaggregated state at kinase concentrations ranging from 3 to 23 μM. The rate of autophosphorylation was not changed when the kinase concentration was varied from 0.29 and 14.7 μM. These results indicate an intramolecular autophosphorylation.

The biochemical function of the autophosphorylation reaction is not clear. It had no significant influence on the kinetic properties of myosin light chain phosphorylation or calmodulin activation. The addition of the purified tail fragment to the head fragment of rabbit skeletal muscle myosin light chain kinase increased the catalytic activity of the isolated head fragment to values closer to those of the native kinase (Mayr & Heilmeyer, 1983). Geuss et al. (1985) reported that a phosphorylated myosin light chain kinase was inhibited more strongly than the nonphosphorylated kinase by phosphorylated light chain. Since myosin light chain kinase is phosphorylated in the tail region (see below), these results suggest that the amino-terminal portion of the kinase may play a structural role in influencing the activity of the catalytic core. Other unidentified functions for the autophosphorylations cannot be ruled out. Physiologically, myosin light chain kinase is probably not readily phosphorylated in skeletal muscle. The time required for autophosphorylation is substantially slower than for muscle contractions (milliseconds). Additionally, the high light chain substrate concentration (200–350 μM) would be expected to inhibit competitively the autophosphorylation of myosin light chain kinase.

Autophosphorylation of rabbit skeletal muscle myosin light chain kinase occurs only in the amino-terminal tail region. This conclusion is supported by results obtained with chymotrypsin and CNBr digestion where no evidence was found for ^{32}P incorporation into the head region containing the light chain binding site, catalytic core, and calmodulin binding-autoinhibitory domain. These results are in contrast to observations made with some other protein kinases. For example, a threonine residue in the middle of the catalytic core of the catalytic subunit of the cyclic AMP-dependent protein kinase is autophosphorylated (Taylor et al., 1990). Because it cannot be readily dephosphorylated, it has not been possible to study the functional importance of this phosphorylation. Additionally, Ca^{2+}/calmodulin-dependent protein kinase II phosphorylates itself in the regulatory region, resulting in changes in its calmodulin activation properties (Soderling, 1990).

Sequence analysis of purified peptides from autophosphorylated myosin light chain kinase demonstrates ^{32}P incorporation into Ser 160 and Ser 234. These residues are not close to the catalytic core which starts at about residue 300 (Hanks et al., 1988; Knighton et al., 1991a,b). Previous work demonstrated that three regions containing basic residues on the amino-terminal side of the phosphorylatable serine were important substrate determinants in synthetic peptide substrates (Michnoff et al., 1986). Region I contained an arginine that was three residues on the amino-terminal side of the phosphorylatable serine; region II contained three basic residues that were 6–8 residues amino terminal of the phosphorylatable serine; and region III contained two basic residues that were 10–11 residues amino terminal of the phosphorylatable serine. Examination of the sequences around the autophosphorylation sites reveals three basic residues at the 3rd (trimethylhistidine), 10th (arginine), and 11th (arginine) positions amino terminal of autophosphorylated Ser 160 (Takio et al., 1986; Meyer & Mayr, 1987; Herring et al., 1990b). These basic residues in the kinase correspond to regions I and III in the light chain substrate. The second site of autophosphorylation (Ser 234) has no obvious structural similarities with the phosphorylation consensus sequence for myosin light chain kinase. The apparent difference in the phosphorylation rates for these two sites might be related to the differences in the sequences on the amino-terminal side of the two autophosphorylated serine residues. Avian and mammalian smooth muscle myosin light chain kinases do not have phosphorylatable residues at similar positions (Olson et al., 1990; Gallagher et al., 1991), which may account for the very slow rates of autophosphorylation (Foyt & Means, 1985; Stull et al., 1990).

It has been reported that a monoclonal antibody, 14a, inhibits rabbit skeletal muscle myosin light chain kinase activity competitively with respect to the light chain substrate (Nunnally et al., 1987). The epitope for this antibody was located between residues 165 and 173 (Herring, et al., 1990b), which is near the first autophosphorylation site. The inhibition of kinase activity by the antibody is probably due to steric inhibition because of its close proximity to Ser 160 which is near the active site.

The phosphorylation of two residues in a single polypeptide chain by an intramolecular mechanism requires significant protein flexibility. Each phosphorylatable serine must lie sufficiently close to the active site in the folded protein so that it can make contact with the essential catalytic amino acid. The proximity of these regions to the catalytic site and conformational constraints on the site probably target particular residues for modification. In this respect, it is worthwhile to note that while Ser 160 and Ser 234 were phosphorylated, nearby residues, including Ser 152, Ser 158, and Thr 233, were not. Because the tail regions of smooth muscle myosin light chain kinases are also thought to extend from a globular catalytic head region, the results obtained on autophosphorylation of the rabbit skeletal muscle myosin light chain kinase imply that a general structural property of this region is flexibility.

In summary, two autophosphorylation sites of rabbit skeletal muscle myosin light chain kinase have been identified at Ser 160 and Ser 234. The intramolecular autophosphorylation of the enzyme has intriguing implications for both the folding structure and the function of the amino-terminal tail of myosin light chain kinase.

Acknowledgments

We express our appreciation to Li-Chu Hsu for help in the purification of calmodulin and myosin light chain kinase. We acknowledge helpful discussion with B. Paul Herring and Patricia J. Gallagher and preparation of the manuscript by Phyllis Foley.

Registry No. ATP, 56-65-5; myosin light chain kinase, 51845-53-5; phosphoserine, 407-41-0.

References

Andersson, L., & Porath, J. (1986) *Anal. Biochem. 154*, 250–254.

Blumenthal, D. K., & Stull, J. T. (1980) *Biochemistry 19*, 5608–5614.

Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., & Krebs, E. G. (1985) *Proc.*

Natl. Acad. Sci. U.S.A. 82, 3187–3191.
Bowman, B. F., Peterson, J. A., & Stull, J. T. (1992) *J. Biol. Chem. 267*, 5346–5354.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Cooper, J. A., Sefton, B. M., & Hunter, T. (1983) *Methods Enzymol. 99*, 387–402.
Edelman, A. M., & Krebs, E. G. (1982) *FEBS Lett. 138*, 293–298.
Edelman, A. M., Takio, K., Blumenthal, D. K., Hansen, R. S., Walsh, K. A., Titani, K., & Krebs, E. G. (1985) *J. Biol. Chem. 260*, 11275–11285.
Edelman, A. M., Blumenthal, D. K., & Krebs, E. G. (1987) *Annu. Rev. Biochem. 56*, 567–613.
Foyt, H. L., & Means, A. R. (1985) *J. Cyclic Nucleotide Protein Phosphorylation Res. 10*, 143–156.
Gallagher, P. J., Herring, B. P., Griffin, S. A., & Stull, J. T. (1991) *J. Biol. Chem. 266*, 23936–23944.
Geuss, U., Mayr, G. W., & Heilmeyer, L. M. G., Jr. (1985) *Eur. J. Biochem. 153*, 327–334.
Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42–52.
Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) pp 423–482, Raven Press, New York.
Herring, B. P., Stull, J. T., & Gallagher, P. J. (1990a) *J. Biol. Chem. 265*, 1724–1730.
Herring, B. P., Fitzsimons, D. P., Stull, J. T., & Gallagher, P. J. (1990b) *J. Biol. Chem. 265*, 16588–16591.
Kamm, K. E., & Stull, J. T. (1985) *Am. J. Physiol. 249*, C238–C247.
Kamm, K. E., & Stull, J. T. (1989) *Annu. Rev. Physiol. 51*, 299–313.
Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.
Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991a) *Science 253*, 407–414.
Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991b) *Science 253*, 414–420.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Leachman, S. A., Gallagher, P. J., Herring, B. P., McPhaul, M. J., & Stull, J. T. (1992) *J. Biol. Chem. 267*, 2930–4938.
Matsudaria, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Mayr, G. W., & Heilmeyer, L. M. G., Jr. (1983) *Biochemistry 22*, 4316–4326.
Meyer, H. E., & Mayr, G. W. (1987) *Biol. Chem. Hoppe-Seyler 368*, 1607–1611.
Meyer, H. E., Hoffman-Posorske, E., Korte, H., & Heilmeyer, L. M. G., Jr. (1986) *FEBS Lett. 204*, 61–66.
Michnoff, C. H., Kemp, B. E., & Stull, J. T. (1986) *J. Biol. Chem. 261*, 8320–8326.
Nunnally, M. H., Hsu, L. C., Mumby, M. C., & Stull, J. T. (1987) *J. Biol. Chem. 262*, 3833–3838.
Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., & Means, A. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2284–2288.
Pires, E. M. V., & Perry, S. V. (1977) *Biochem. J. 167*, 137–146.
Roskoski, R. (1983) *Methods Enzymol. 99*, 3–6.
Sobieszek, A. (1991) *J. Mol. Biol. 220*, 947–957.
Soderling, T. R. (1990) *J. Biol. Chem. 265*, 1823–1826.
Stull, J. T., Nunnally, M. H., & Michnoff, C. H. (1986) in *The Enzymes* (Krebs, E. G., & Boyer, P. D., Eds.) pp 113–166, Academic Press, Orlando, FL.
Stull, J. T., Bowman, B. F., Gallagher, P. J., Herring, B. P., Hsu, L. C., Kamm, K. E., Kubota, Y., Leachman, S. A., Sweeney, H. L., & Tansey, M. G. (1990) *Prog. Clin. Biol. Res. 327*, 107–126.
Sweeney, H. L., & Stull, J. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 414–418.
Takio, K., Blumenthal, D. K., Edelman, A. M., Walsh, K. A., Krebs, E. G., & Titani, K. (1985) *Biochemistry 24*, 6028–6037.
Takio, K., Blumenthal, D. K., Walsh, K. A., Titani, K., & Krebs, E. G. (1986) *Biochemistry 25*, 8049–8057.
Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990) *Annu. Rev. Biochem. 59*, 971–1005.
Yazawa, M., & Yagi, K. (1978) *J. Biochem. 84*, 1259–1265.