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Role of basic residues in the phosphorylation of synthetic peptides by myosin light chain kinase

(smooth muscle/calmodulin)

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ABSTRACT **The substrate specificity of the chicken gizzard myosin light chain kinase has been studied by using a series of synthetic peptide analogs of the NH_2-terminal sequence of the chicken gizzard myosin light chain (M_r = 20,000). An 18-residue synthetic peptide, Arg-Pro-Gln-Arg-Ala-Lys-Ala-Lys-Thr-Thr-Lys-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2, corresponding to the sequence reported by Maita *et al.* [Maita, T., Chen, J. I. & Matsuda, G. (1981) *Eur. J. Biochem.* 117, 417–424], was phosphorylated with a 22-fold higher K_m and a V_{max} that was decreased to 1% of the native protein substrate. This peptide was also an inferior substrate when compared with an 18-residue synthetic peptide with an alternative sequence, Lys-Ala-Lys-Thr-Thr-Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2, which was phosphorylated with an apparent K_m of 6.9 μM, comparable to the native protein substrate of 8.6 μM, and a V_{max} of 3.9 μmol·min^{-1}·mg^{-1}, 11% of that for the protein substrate. The kinetics of phosphorylation of shortened peptides corresponding to both sequences, together with peptides with appropriate substitutions, indicated that basic residues were the primary determinants of specificity for the smooth muscle myosin light chain kinase. In the latter peptide sequence, lysine residues 11 and 12 and the arginine at position 13 had a major influence on the kinetics of peptide phosphorylation.**

The myosin light chain kinase in skeletal muscle was reported by Pires *et al.* in 1974 (1) and various forms of this enzyme are now recognized to be widely distributed in other muscle and nonmuscle tissues (2). The role of this enzyme is presently best understood in the control of smooth muscle contraction (2, 3). Nevertheless, it may have important physiological functions in other cell types, including platelets (4), macrophages (5), and pancreatic islets (6).

The myosin light chain kinase exhibits strong specificity for its natural substrate and fails to phosphorylate exogenous proteins (2). Moreover, the enzyme has a preference for the myosin light chains isolated from the same tissue type (7, 8). For instance, the skeletal muscle enzyme does not phosphorylate smooth muscle light chains and vice versa.

Previously, we reported (9) that the myosin light chain kinase phosphorylated a synthetic 17-residue peptide corresponding to the local amino acid sequence around the phosphorylation site in the chicken gizzard myosin light chain (J. Kendrick-Jones, personal communication; see ref. 10). The complete amino acid sequence of the chicken gizzard myosin light chain has now been reported by Maita *et al.* (11) and this contained a number of differences to the sequence previously synthesized by us. Therefore, we have synthesized peptide analogs corresponding to the sequence reported by Maita *et al.* (11) as well as a further revised sequence (J. Kendrick-Jones, personal communication) containing a different assignment of tryptic peptides. In this paper we report the comparison of the kinetics of phosphorylation of synthetic peptides corresponding to all three sequences and related analogs, which demonstrate that specific residues have a strong influence on the kinetics of peptide phosphorylation by the chicken gizzard myosin light chain kinase.

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MATERIALS AND METHODS

All materials were reagent grade unless otherwise indicated. [γ-^{32}P]ATP was obtained from Amersham.

Peptide Synthesis and Purification. The synthetic peptides were synthesized as the COOH-terminal amide form by the Merrifield solid-phase synthesis procedure (12). Amino acid derivatives protected with the *t*-butyloxycarbonyl group in the α-amino position and benzhydrylamine resin were obtained from the Protein Research Foundation (Osaka, Japan). The peptides were assembled on a Beckman 990 synthesizer. The completed peptides were simultaneously deprotected and cleaved from the resin in anhydrous HF (13) and purified by ion-exchange chromatography (14). Peptide purity was assessed by amino acid analysis and high-voltage electrophoresis at pH 1.9 and 6.4 as described (14). All peptides electrophoresed as single spots and had the expected amino acid compositions. The 23-residue peptide, K-MLC$_{1-23}$, chromatographed as a single symmetrical peak on Sephadex G-25 superfine in 5.5 M acetic acid.

Protein Purification. The purification of myosin light chains (15), calmodulin (16), and the myosin light chain kinase (8) were by published procedures modified as described (9).

Myosin Light Chain Kinase Assay. Myosin light chain kinase was assayed in a volume of 0.08 ml of 40 mM Hepes buffer, pH 7.0/5 mM magnesium acetate/0.5 mM [γ-^{32}P]ATP (100–2,000 cpm/pmol)/0.55 mM $CaCl_2$/5 μg of calmodulin/1 mg of bovine serum albumin per ml/0.1% (wt/vol) Tween 80 and myosin light chains or peptide as indicated. The enzyme was diluted in 25 mM Tris·HCl/1 mM dithiothreitol buffer, pH 7.5, containing 0.1% (wt/vol) Tween 80 to prevent loss of enzyme through binding to plastic and to ensure an accurate measure of the enzyme specific activity (17). Incubations were carried out at 30°C and aliquots (0.03 ml) were taken at 3 and 6 min. The aliquots were applied to phosphocellulose ion-exchange paper and were washed as described (14). The phosphorylated forms of the shortest peptides, containing two or less basic residues, were separated from [γ-^{32}P]ATP by using the ion-exchange column procedure (14).

RESULTS

The sequences of the parent peptides used in this study are given in Table 1. The 18-residue synthetic peptide (M-MLC$_{6-23}$) corresponding to residues 6–23 with serine at position 23 of the amino acid sequence of the chicken gizzard myosin light chain reported by Maita *et al.* (11) was a relatively poor

Table 1. Effect of amino acid sequence on kinetic constants

Peptide	Sequence	Apparent K_m, μM	V_{max}, μmol·min^{-1}·mg^{-1}	K_m/V_{max}
Myosin light chains		8.6 ± 1.5	36.2 ± 1.6	0.2
K-MLC$_{6-23}$	K-A-K-T-T-K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	6.9 ± 0.6	3.9 ± 0.2	1.8
M-MLC$_{6-23}$	R-P-Q-R-A-K-A-K-T-T-K-A-T-*S*-N-V-F-S-NH$_2$	193 ± 15	0.39 ± 0.02	495
MLC$_{1-17}$*	S-S-K-T-T-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	90 ± 4	2.05 ± 0.03	44

See text for peptide and protein phosphorylation procedures. Kinetic constants (mean ± SEM) were estimated by fitting the data to the Michaelis–Menton equation by using the method of least squares. Phosphorylated residues are italicized. A one-letter notation for amino acid sequences is used (18).
*Data from ref. 9.

substrate for the myosin light chain kinase, with an apparent K_m of 193 μM and a V_{max} of 0.39 μmol·min^{-1}·mg^{-1}. Compared with the chicken gizzard myosin light chains, this 18-residue peptide (M-MLC$_{6-23}$) had a 22-fold higher K_m and a V_{max} that was decreased by 99% (Table 1). This synthetic peptide also had inferior kinetics compared with the 17-residue synthetic peptide substrate (MLC$_{1-17}$) previously synthesized in this laboratory (9).

We have tested synthetic peptide analogs of the chicken gizzard myosin light chain with a different assignment of tryptic peptides than that reported by Maita *et al.* (11) (J. Kendrick-Jones, personal communication). The 18-residue synthetic peptide, K-MLC$_{6-23}$, with this revised sequence was an excellent substrate, having a 10-fold higher V_{max} and a K_m that was decreased to 3.6% of the corresponding 18-residue peptide M-MLC$_{6-23}$ (Table 1). The apparent K_m of the 18-residue synthetic peptide with the revised sequence was comparable to that of the myosin light chains. These results indicate that the amino acid sequence of the 18-residue peptide has a very strong effect on the kinetics of peptide phosphorylation.

Effect of Chain Length. The finding that the apparent K_m of peptide K-MLC$_{6-23}$ (Table 1) was comparable to the natural substrate suggested that this peptide may contain structural features important for recognition by the enzyme. To examine this further, we prepared a series of synthetic peptides varying in length, based on both amino acid sequences (Table 2) and tested them as substrates for the myosin light chain kinase.

The longest peptide tested (K-MLC$_{1-23}$) was a 23-residue peptide corresponding to the alternative amino acid sequence (J. Kendrick-Jones, personal communication). This peptide was readily phosphorylated by the myosin light chain kinase, with an apparent K_m of 2.7 μM, 33% of that of the natural substrate (Fig. 1*B*; Table 2). This peptide (K-MLC$_{1-23}$) was phosphorylated to the extent of 0.97 mol of [^{32}P]phosphate per mol of peptide (Fig. 1*A*). On high-voltage paper electrophoresis, the phosphorylated product migrated as a single spot as assessed by ninhydrin staining and autoradiography (results not shown).

The capacity of the shorter peptides, corresponding to both amino acid sequences, to act as substrates for the myosin light chain kinase varied over a wide range, with a 10^5-fold change in the K_m/V_{max} ratio when going from the 23-residue peptide to the 8-residue peptide. The change in kinetic parameters for the K-MLC peptides occurred largely when going from the 13-residue peptide to the 11-residue peptide K-MLC$_{13-23}$ (Table 2). A corresponding, but less dramatic, change was observed for the M-MLC series of peptides when going from the 15-residue peptide to the 11-residue peptide (Table 2). The change in kinetics of phosphorylation of the M-MLC series correlated with the loss of three basic residues, arginine-9, lysine-11, and lysine-13. In the light of the differences in the kinetics of phosphorylation of the 18-residue peptides K-MLC$_{6-23}$ and M-MLC$_{6-23}$ (see above), the results with the shorter peptide analogs suggested that specific residues rather than chain length may have an important influence on the kinetics of peptide phosphorylation.

Effect of Specific Residues. The change in the apparent K_m observed in reducing the peptide chain length of the K-MLC sequence from 13 to 11 residues suggested that the three basic residues, lysine-11, lysine-12, and arginine-13, may be important. To distinguish between the contributions of these residues and peptide chain length, we prepared synthetic peptide analogs with alanine substituted for each of these basic residues.

There was a substantial increase in the apparent K_m when alanine was substituted for lysine-11, lysine-12, and arginine-13. The V_{max} was only slightly influenced by these substitutions (Table 3). In the case of the peptide containing alanine at position 13, the COOH-terminal serine was also replaced by alanine. Further support for the contribution of the basic residues is apparent from a comparison of peptides 5–7 with peptide 1 in Table 3. Substitution of threonine at position 11 had a similar effect on the kinetics of phosphorylation as did substitution with

Table 2. Kinetic constants for synthetic peptide substrates

Peptide	Sequence	Apparent K_m, μM	V_{max}, μmol·min^{-1}·mg^{-1}	K_m/V_{max}
K-MLC$_{1-23}$	S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	2.7 ± 0.1	3.0 ± 0.1	0.9
K-MLC$_{6-23}$	K-A-K-T-T-K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	6.9 ± 0.6	3.9 ± 0.2	1.8
K-MLC$_{8-23}$	K-T-T-K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	9.1 ± 0.9	3.4 ± 0.2	2.7
K-MLC$_{11-23}$	K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	20 ± 2	2.5 ± 0.1	8
K-MLC$_{12-23}$	K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	470 ± 23	0.82 ± 0.03	5.7×10^2
K-MLC$_{13-23}$	R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	3,400 ± 200	0.32 ± 0.01	1.06×10^4
K-MLC$_{16-23}$	R-A-T-*S*-N-V-F-S-NH$_2$	13,000 ± 5,000	0.13 ± 0.03	1.0×10^5
M-MLC$_{6-23}$	R-P-Q-R-A-K-A-K-T-T-K-A-T-*S*-N-V-F-S-NH$_2$	193 ± 15	0.39 ± 0.02	495
M-MLC$_{9-23}$	R-A-K-A-K-T-T-K-A-T-*S*-N-V-F-S-NH$_2$	207 ± 28	0.56 ± 0.04	3.70×10^2
M-MLC$_{11-23}$	K-A-K-T-T-K-A-T-*S*-N-V-F-S-NH$_2$	1,350 ± 160	0.11 ± 0.01	1.23×10^4
M-MLC$_{13-23}$	K-T-T-K-A-T-*S*-N-V-F-S-NH$_2$	6,900 ± 1,200	0.022 ± 0.002	3.14×10^5
M-MLC$_{16-23}$	K-A-T-*S*-N-V-F-S-NH$_2$	14,800 ± 8,000	0.036 ± 0.013	4.11×10^5

See legend to Table 1. Phosphorylatable residues are italicized.

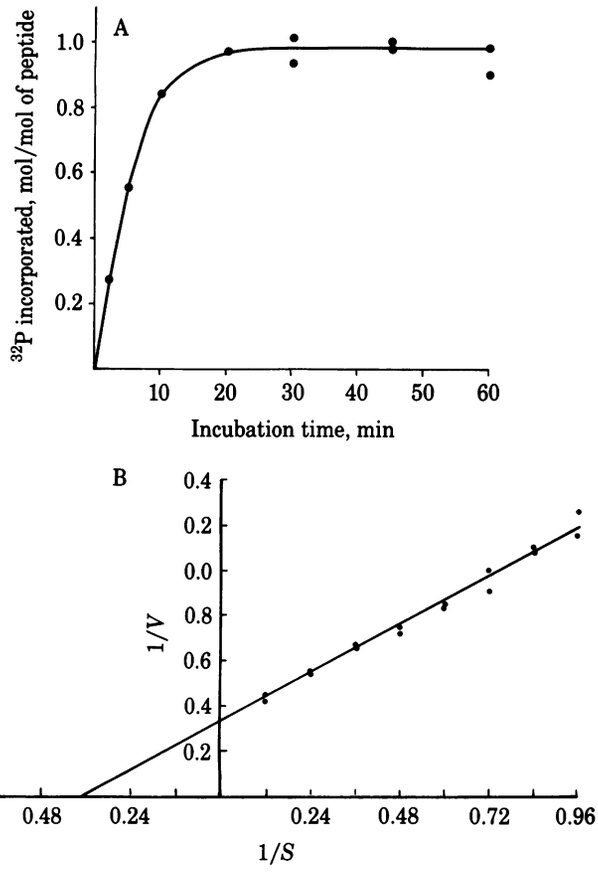

FIG. 1. (*A*) Time course of 23-residue synthetic peptide phosphorylation. Peptide [K-MLC$_{1-23}$, S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-S-NH$_2$ (0.48 mM)] was incubated with myosin light chain kinase (20.3 μg/ml) in a reaction mixture (0.44 ml) having the composition described in the text. Aliquots (20 μl) were withdrawn at intervals and the ^{32}P incorporation was determined by liquid scintillation spectroscopy after separation of the synthetic peptide from [γ-^{32}P]ATP. The specific activity of the [γ-^{32}P]ATP was corrected for the presence of [^{32}P]P$_i$ (13%) and the presence of [^{32}P]ADP (5%), as assessed by anion-exchange chromatography on a Partisil-10 SAX column (Whatman). (*B*) Double-reciprocal plot of synthetic peptide phosphorylation as a function of substrate (peptide) concentration. ^{32}P incorporation into the synthetic peptide (K-MLC$_{1-23}$) was measured, and aliquots (30 μl) were withdrawn at intervals to verify that the rates of phosphorylation were linear with respect to time for all concentrations of peptide tested. Kinetic constants were determined as described in the legend to Table 1.

alanine. Peptide 5 had an apparent K_m of 90 μM, intermediate between that of peptide 1 and 2. This result indicated that shifting lysine-11 two residues towards the NH$_2$ terminus had a deleterious effect on the kinetics of phosphorylation but not as marked as substituting with alanine (peptide 6, Table 3). It is clear from these results that lysine-11, lysine-12, and arginine-13 have a strong influence on the kinetics of phosphorylation. The length of the peptide appears to be a less significant factor, at least for the synthetic peptides studied here.

Phosphorylation Site. All the phosphorylated peptides were subjected to partial acid hydrolysis to assess whether serine or threonine was being phosphorylated. In the case of the rapidly phosphorylated longer peptides, serine was the principal site of phosphorylation, with little phosphothreonine present (Table 4). In contrast, phosphothreonine was most abundant for the shortest phosphopeptides with only 8 or 11 residues. The results suggest that peptide length had a greater influence on the selectivity between serine and threonine than the amino acid sequence (Table 4). The phosphorylation of threonine was calmodulin dependent (results not shown). When the 23-residue peptide (K-MLC$_{1-23}$) was phosphorylated in the presence of high levels of enzyme equivalent to those used to phosphorylate the poorer short peptide substrates, the ratio of phosphothreonine to phosphoserine increased from 0.08 to only 0.13, indicating that a contaminating enzyme was probably not responsible for the threonine phosphorylation of the short peptides. It was not possible to competitively inhibit peptide threonine phosphorylation with myosin light chains because the differences in kinetics resulted in the light chains becoming stoichiometrically phosphorylated before threonine phosphorylation could be measured.

The site(s) of phosphorylation in each peptide was studied. In all but three cases, the ^{32}P-phosphorylated peptides were found to be alkali labile (>97% released in 20 min in 0.1 M NaOH at 100°C) and stable in acid (>94% remaining after 20 min in 0.1 M HCl at 100°C). The phosphorylated peptides K-MLC$_{16-23}$, M-MLC$_{13-23}$, and M-MLC$_{16-23}$ were alkali labile to a lesser extent (84–78%) and acid stable (95–99%) under these conditions. These results are consistent with [^{32}P]phosphate being esterified to serine or threonine residues in phosphorylated peptides.

The site(s) of phosphorylation was further investigated by proteolytic digestion and high-voltage paper electrophoresis, as described (9). When the phosphorylated peptides were sequentially digested with trypsin and chymotrypsin, the ^{32}P radioactivity was associated with a peptide carrying a net negative charge at pH 3.5, with a mobility of 0.13 relative to phosphoserine. In the case of the 8-residue peptides K-MLC$_{16-23}$ and M-MLC$_{16-23}$, which contained a single basic residue at their NH$_2$ termini, trypsin did not alter the peptides' electrophoretic mobility. The yield of ^{32}P-phosphorylated peptide after both enzymic digestion and paper electrophoresis averaged 70% over the range of peptides. Autoradiography indicated a single major ^{32}P-phosphorylated species at each stage of the analysis. Amino acid analysis of the tryptic/chymotryptic ^{32}P-labeled phosphopeptides after acid hydrolysis revealed stoichiometric amounts of Asp, Thr, Ser, and Ala and lesser amounts of Val

Table 3. Effect of specific substitutions on peptide phosphorylation

Peptide	Sequence	Apparent K_m, μM	V_{max}, μmol·min^{-1}·mg^{-1}	K_m/V_{max}
1	K-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	20 ± 2	2.5 ± 0.1	8
2	A-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	463 ± 13.6	2.10 ± 0.03	220
3	K-A-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	445 ± 59	1.64 ± 0.09	271
4	K-K-A-P-Q-R-A-T-*S*-N-V-F-A-NH$_2$	951 ± 94	2.7 ± 0.14	352
5	S-S-K-T-T-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$*	90 ± 4	2.05 ± 0.03	44
6	S-S-A-T-T-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	570 ± 55	1.19 ± 0.06	479
7	T-T-K-R-P-Q-R-A-T-*S*-N-V-F-S-NH$_2$	1,257 ± 64	1.07 ± 0.03	1,175

See legend to Table 1. Amino acid substitutions are shown in **boldfaced** type and phosphorylated residues are italicized.
* Data from ref. 9.

Table 4. Distribution of [^{32}P]phosphate in phosphoserine and phosphothreonine after partial acid hydrolysis

Synthetic peptide	Length, no. of residues	Ratio*
K-MLC$_{1-23}$	23	0.08
K-MLC$_{6-23}$	18	0.08
M-MLC$_{6-23}$	18	0.09
K-MLC$_{8-23}$	16	0.02
M-MLC$_{9-23}$	15	0.17
K-MLC$_{11-23}$	13	0.03
M-MLC$_{11-23}$	13	0.11
K-MLC$_{12-23}$	12	0.07
K-MLC$_{13-23}$	11	0.51
M-MLC$_{13-23}$	11	1.91
K-MLC$_{16-23}$	8	2.44
M-MLC$_{16-23}$	8	3.30

See text for peptide phosphorylation procedures. The phosphorylated peptides were subjected to partial acid hydrolysis (5.7 M HCl, 110°C, 2 hr) and high-voltage paper electrophoresis at pH 1.9. The position of ^{32}P-phosphorylated amino acids was located by autoradiography and quantitated by liquid scintillation spectroscopy. Peptide sequences were as shown in Table 2.

* Phosphothreonine-to-phosphoserine ratio.

and Phe (results not shown). The amino acid composition was consistent with that expected from the peptide $\overset{17}{\text{Ala}}$-$\overset{18}{\text{Thr}}$-$\overset{19}{\text{Ser}}$-$\overset{20}{\text{Asn}}$-$\overset{21}{\text{Val}}$-$\overset{22}{\text{Phe}}$. These results together with those obtained from studying the partial acid hydrolysates indicate that serine-19 is the major phosphorylation site for the peptides containing little phosphothreonine. The shortest peptides K-MLC$_{16-23}$, M-MLC$_{16-23}$, and M-MLC$_{13-23}$ were phosphorylated on either threonine-18 or serine-19. In the case of K-MLC$_{13-23}$, there was evidence of a minor phosphorylated species (7% of total radioactivity) that migrated with a position expected of diphosphorylated material. Because the peptides phosphorylated on threonine residues were very poor substrates (Table 2), it was not practical to phosphorylate them stoichiometrically, and the possibility of minor amounts being multiphosphorylated was not investigated further.

DISCUSSION

In this investigation we have studied the capacity of the chicken gizzard smooth muscle myosin light chain kinase to phosphorylate synthetic peptides corresponding to three amino acid sequences. Previously, we reported (9) the phosphorylation of a synthetic 17-residue peptide (MLC$_{1-17}$, as listed below) by the myosin light chain kinase. This peptide corresponded to an early version of the chicken gizzard myosin light chain sequence (J. Kendrick-Jones, personal communication) (see ref. 10) and was phosphorylated with an apparent K_m of 90 μM (9). Subsequently, Maita *et al.* (11) reported the complete amino acid sequence of the chicken gizzard myosin light chain (M_r = 20,000) with an extended NH_2-terminal sequence containing a different order of basic residues. We have synthesized an analog of this sequence, M-MLC$_{6-23}$ (see below), with serine in place of alanine at position 23, similar to the MLC$_{1-17}$ peptide. This 18-residue peptide, M-MLC$_{6-23}$, was phosphorylated with a K_m of 193 μM, higher than that of the 17-residue peptide, MLC$_{1-17}$ reported previously (9). A revised version of the chicken gizzard myosin light chain sequence (J. Kendrick-Jones, personal communication) was also tested. The NH_2-terminal region of this sequence (K-MLC$_{1-23}$, as listed below) is similar to that reported by Maita *et al.* (11) but differs in the assignment of tryptic peptides. The 18-residue peptide analog of this sequence, K-MLC$_{6-23}$, was phosphorylated with a low K_m of 6.9 μM, which is comparable to the chicken gizzard myosin light chains.

MLC$_{1-17}$

S-S-K-T-T-K-R-P-Q-R-A-T-S-N-V-F-S

M-MLC$_{1-23}$

S-S-K-R-K-R-P-Q-R-A-K-A-K-T-T-K-A-T-S-N-V-F-S

K-MLC$_{1-23}$

S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-S

The observation that the synthetic peptide (M-MLC$_{6-23}$) corresponding to the sequence reported by Maita *et al.* (11) was a relatively poor substrate does not necessarily imply any error in the sequence reported by Maita *et al.* (11). Nevertheless, because J. Kendrick-Jones (personal communication) has suggested an alternative sequence that is an excellent substrate for the enzyme, the local phosphorylation site sequence of the chicken gizzard myosin light chain must be investigated further.

The myosin light chain kinase phosphorylated all three peptide sequences on the same serine corresponding to serine-19 in the Maita *et al.* (11) sequence. This residue is also phosphorylated in the intact protein. Thus, in respect to the phosphorylation site specificity, all three synthetic peptides reflect the properties of the intact myosin light chain. We have assessed the contribution of specific residues to the kinetics of peptide phosphorylation by investigating a series of peptides. The longest peptide tested, K-MLC$_{1-23}$, corresponding to the revised sequence of J. Kendrick-Jones (personal communication), had an apparent K_m of 3 μM, less than that for the myosin light chains. The 23-residue peptide corresponding to the sequence of Maita *et al.* (11) was not synthesized, so it is not clear what effect extending this peptide sequence beyond 18 residues would have had on the kinetics of phosphorylation.

Shortening the peptide length corresponding to both sequences caused a large increase in the apparent K_m—in the case of the revised Kendrick-Jones sequence, much of this increase occurred in the region of the three basic residues, lysine-11, lysine-12, and arginine-13. Similar, although less dramatic, results were obtained with the shortened version of the sequence corresponding to that reported by Maita *et al.* (11). In this case, deletion of arginine-6 had little effect, whereas deletion of arginine-9, lysine-11, and lysine-13 had pronounced effects on the kinetics of phosphorylation. Therefore, the conclusion that basic residues are important—in particular, two lysines and one arginine—is supported by the results obtained with both amino acid sequences and does not reflect some unique properties of a specific peptide sequence. Additional support for this concept was obtained by preparing analog peptides containing substitutions in place of these basic residues. Replacement of any of these residues in the revised Kendrick-Jones sequence by alanine caused a large increase in the apparent K_m. Moreover, substitution with threonine in place of lysine-11 or separation of lysine-11 and lysine-12 by threonine residues caused a corresponding increase in the apparent K_m. Thus, the results obtained with the shortened peptides of both sequences together with specific substitution experiments clearly demonstrate that the kinetics of peptide phosphorylation for chicken gizzard myosin light chain kinase are dependent on nearby basic residues.

The finding that threonine-18 was phosphorylated to a significant extent in the shorter peptides of both sequences (7 or 11 residues) was unexpected. The kinetics of threonine-18 phosphorylation were extremely poor and the results described

herein do not rule out the possibility of phosphorylation at this site by a contaminating protein kinase. Nevertheless, the complete calmodulin dependence of threonine-18 phosphorylation is consistent with the myosin light chain kinase being responsible. Limited phoshorylation of threonine has also been observed by using the myosin light chains as substrate (10), so that threonine phosphorylation may not be restricted to peptide substrates. Nevertheless, the enzyme source and purification used by Noiman (10) was similar to that used in the present study and therefore leaves open the question of a contaminating enzyme.

The critical lysine residues (positions 11 and 12) in the 23-residue peptide substrate are separated by 6 residues from the site of phosphorylation. This is in contrast to the cAMP-dependent protein kinase, in which the important basic residues tend to be less distant from the phosphorylation site (19).

In the case of the corresponding skeletal and cardiac myosin light chains, basic residues are also present in the proximity of the phosphorylation sites (20). In the light of the results reported herein, it would seem most likely that some of these residues will be important specificity determinants for the corresponding myosin light chain kinases of these tissues. However, it is not yet clear to what extent the particular arrangement of the basic residues or other factors are responsible for the tissue-specific substrate specificity properties of the myosin light chain kinase.

The myosin light chain kinase can now be added to a growing list of protein kinases that have been shown to require basic residues as substrate specificity determinants. These include phosphorylase *b* kinase (21), the cAMP-dependent protein kinase (22, 23), histone H4 kinase (24), and the cGMP-dependent protein kinase (25). Although these enzymes have a requirement for basic residues, they nevertheless exhibit important differences in their capacity to phosphorylate model synthetic substrates (22, 24).

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