FEBS Letters 472 (2000) 148–152 FEBS 23570

Conformational requirements for Ca^{2+}/calmodulin binding and activation of myosin light chain kinase

Roanna C. Padre, James T. Stull*

Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9040, USA

Received 23 March 2000

Edited by Shmuel Shaltiel

Abstract Myosin light chain kinase contains a regulatory segment consisting of an autoinhibitory region and a calmodulin-binding sequence that folds back on its catalytic core to inhibit kinase activity. It has been proposed that α-helix formation may be involved in displacement of the regulatory segment and activation of the kinase by Ca^{2+}/calmodulin. Proline mutations were introduced at putative non-interacting residues in the regulatory segment to disrupt helix formation. Substitution of proline residues immediately N-terminal of the Trp in the calmodulin-binding sequence had most significant effects on Ca^{2+}/calmodulin binding and activation. Formation of an α-helix in this region upon Ca^{2+}/calmodulin binding may be necessary for displacement of the regulatory segment allowing phosphorylation of myosin regulatory light chain.

Key words: Myosin light chain kinase; Calmodulin; Autoinhibition; Activation; Helix; Proline

1. Introduction

Myosin light chain kinase is a Ca^{2+}/calmodulin-dependent enzyme that catalyzes the phosphorylation of the Ser^{19} residue at the N-terminus of the regulatory light chain (RLC) of myosin II [1,2]. Phosphorylation of the RLC is essential for smooth muscle contraction [3], potentiation of skeletal and cardiac muscle contraction [4] and other cellular processes [5]. Myosin light chain kinase contains a catalytic core homologous to that of other protein kinases and a regulatory segment located C-terminal to the catalytic core. The regulatory segment consists of an autoinhibitory linker region that connects the calmodulin-binding sequence to the catalytic core. This portion of the kinase appears to fold back onto the catalytic site to inhibit kinase activity by intrasteric regulation. Ca^{2+}/calmodulin activates myosin light chain kinase presumably due to the specific displacement of the autoinhibitory region away from the catalytic cleft allowing RLC binding and phosphorylation.

The calmodulin-binding sequences on a variety of proteins consist of 16–25 amino acids and share very little homology in their primary structure, however many have the propensity to form a basic amphiphilic α-helix [6]. An α-helical wheel representation of the calmodulin-binding sequence shows a distribution of basic and polar residues on one side and hydrophobic residues on the opposite side of the helix. In aqueous solution, synthetic peptides containing calmodulin-binding sequences have very little structure. However, the peptides adopt an α-helical conformation upon binding Ca^{2+}/calmodulin or if the polarity of the solvent is decreased.

The structures of Ca^{2+}/calmodulin complexed with peptides containing the calmodulin-binding sequences from smooth and skeletal muscle myosin light chain kinase were determined by small-angle X-ray and neutron scattering [7], high-resolution NMR [8], and X-ray crystallography [9]. Ca^{2+}/calmodulin undergoes a conformational collapse with its two lobes wrapping around the peptide through the bending of the flexible central helix. The central helix unwinds bringing the two lobes closer to each other in a *cis* orientation. The collapse of the calmodulin structure around the target is accompanied by the peptide adopting a single α-helix in an antiparallel orientation. Trp^4 and Phe^{17} of the myosin light chain kinase peptide bind to the hydrophobic patches in the C- and N-terminal domains of calmodulin. However, small-angle scattering studies demonstrate that the binding of calmodulin to isolated peptide employs different determinants compared to calmodulin binding to the same sequence within the enzyme [10].

Prolines are known to destabilize an α-helix by introducing a kink in the helix [11], and are the least common residues within α-helices of crystallized proteins [12]. Substitution of proline residues at strategic positions in the autoinhibitory linker region and the calmodulin-binding sequence in myosin light chain kinase reveal the importance of α-helical structures in both autoinhibition and Ca^{2+}/calmodulin-binding activation.

2. Materials and methods

2.1. Oligonucleotide-directed mutagenesis

A 1300 base pair 5′ *Bam*HI-*Xba*I-3′ cDNA fragment, coding for the C-terminal half of rabbit smooth muscle myosin light chain kinase without the telokin Ig-like motif (amino acids 587–1003) was subcloned into M13mp18 vector and was used to make single-stranded DNA template for oligonucleotide-directed mutagenesis. To produce the M968P, A983P, and A986P mutants (Fig. 1) with desired substitutions at particular amino acid residues, oligonucleotides (M968P, 5′-GTACTTCTTCGGGCGGTCCTTGG-3′; A983P, 5′-GCTCTCACAGGATTGCCCGT-3′; and A986P, 5′-GTCTTCCAATGGGTCTCACAGC-3′) were designed as the non-coding strand of smooth muscle myosin light chain kinase. The oligonucleotides were phosphorylated by 0.4 units/μl T4 polynucleotide kinase (Life Technologies, Inc.) at 37°C for 30 min followed by heat inactivation at 70°C for 15 min prior to use in mutagenesis. Oligonucleotide-directed

*Corresponding author. Fax: (1)-214-648-2974.
E-mail: jstull@mednet.swmed.edu

Abbreviations: RLC, regulatory light chain; Ig-like motif, immunoglobulin-like motif; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid; α-MLCK, anti-myosin light chain kinase monoclonal antibody; α-CaM, anti-calmodulin monoclonal antibody

PII: S0014-5793(00)01434-4

mutagenesis was performed using Sculptor mutagenesis kit (Amersham Pharmacia Biotech). DNA sequencing was performed to verify the desired nucleotide substitutions for each mutant cDNA [13]. The mutated regions were subcloned into pat21/pCMV$_5$B containing the nucleotide sequence for the C-terminal half of the kinase.

2.2. Expression of wild-type and mutant smooth muscle myosin light chain kinases

COS-7 African green monkey kidney cells (obtained from ATCC, CRL-1651) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 ug/ml streptomycin at 37°C with 5% CO_2. The myosin light chain kinase proline mutants in pCMV$_5$ expression vector were transfected into COS-7 cells using Fugene™-6 transfection reagent (Roche Molecular Biochemicals) as described by the manufacturer. The cells were collected and lysed on ice in a buffer (lysis buffer) containing 20 mM MOPS at pH 7.5, 1% Nonidet P-40, 0.5 mM EGTA, 50 mM magnesium chloride, 10% glycerol, 10 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, 0.25 mM benzamidine, 120 μg/ml *N*α-*p*-tosyl-L-lysine chloromethyl ketone, and 100 μg/ml phenylmethylsulfonyl fluoride. The lysates were centrifuged at 7000×*g* for 2 min at 4°C to remove the insoluble fraction. Aliquots of the COS-7 cell lysates were frozen in liquid nitrogen and stored at −80°C. The quantity of the kinases in COS-7 cell lysates was determined by immunoblotting with various amounts of rabbit smooth muscle myosin light chain kinase purified from Sf9 cells [14] as standard and probing with anti-myosin light chain kinase monoclonal antibody which recognizes the N-terminus of the kinase.

2.3. Myosin light chain kinase assays

To determine the Ca^{2+}/calmodulin-dependent activity of wild-type and mutant smooth muscle myosin light chain kinases, ^{32}P incorporation into myosin RLC was measured [15]. Myosin RLC was purified from turkey gizzards [16]. Calmodulin was purified from bovine testes [17] and from *Escherichia coli* SMH 174 (DE3) expressing bovine calmodulin [18] as previously described. Maximal kinase activity was determined in reactions having 50 mM MOPS at pH 7.0, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.3 mM $CaCl_2$, 1 mM [γ-^{32}P]ATP (200–300 cpm/pmol, purchased from ICN), 1 μM calmodulin, 26.5 μM RLC, and diluted lysates of myosin light chain kinase. Lysates were freshly diluted in 25 mM MOPS at pH 7.0, 5 mM magnesium acetate, and 1 mM dithiotreitol and added to reaction mixtures. Final concentrations of myosin light chain kinase used showed linear phosphorylation rates with respect to time and enzyme concentration. K_m and V_{max} values were obtained from Lineweaver-Burke plots after measuring the rates of ^{32}P incorporation under varying myosin RLC concentrations. Ca^{2+}/calmodulin-independent activity of the lysates was measured in the presence of 4 mM EGTA in the absence of exogenous Ca^{2+} and calmodulin. Mock transfected lysates diluted 1:10 exhibited $<5\%$ of the total kinase activity, with no detectable activity observed in the presence of 4 mM EGTA.

Calmodulin activation properties of mutant smooth muscle myosin light chain kinase in COS-7 cell lysates were examined by performing Ca^{2+} activation assays as previously described [19]. To assess the relative K_{CaM} values of mutant kinases in COS-7 cell lysates, the kinase activities were measured at 1 μM CaM with varying free Ca^{2+} concentrations from 200 nM to 100 μM with a Ca^{2+}/EGTA buffer system [20]. The added calmodulin was in great excess of the endogenous calmodulin contributed by the COS-7 cell lysate which provides controlled conditions to determine the relative concentrations of Ca^{2+}/calmodulin required for kinase activation. Therefore, the free Ca^{2+} concentration determines the concentration of Ca^{2+}/calmodulin. To establish the quantitative changes in the calmodulin activation properties (K_{CaM}) of mutant myosin light chain kinases, the ratio of activities at Ca^{2+} concentrations that resulted in less than maximal activity to the maximal activity measured at 100 μM Ca^{2+} was determined as previously described [21–23]. The ratio of activity at a specific Ca^{2+} concentration that is less than required for maximal activity decreases as the K_{CaM} value for a mutant myosin light chain kinase increases relative to the wild-type enzyme. In the same manner, if the ratio of activity increases, the K_{CaM} value decreases. In this analysis, it is assumed that the Ca^{2+}-binding properties of calmodulin are not changed and that a single Ca^{2+} complex is needed to activate the kinase. The ratio of activities is used to calculate the fold difference in K_{CaM} relative to wild-type smooth muscle myosin light chain kinase with an average K_{CaM} value of 1 nM [1,21–23].

2.4. Immunoprecipitation of wild-type and mutant smooth muscle myosin light chain kinase

COS-7 cell lysates were diluted in lysis buffer containing 1 μM calmodulin, 1 mg/ml bovine serum albumin, 0.5 mM EGTA or 10 mM Ca^{2+}. The diluted lysates were incubated with polyclonal antibodies raised against the full-length kinase followed by the addition of protein A-Sepharose (Amersham Pharmacia Biotech). The amount of polyclonal antibody used was sufficient to precipitate all of the proline myosin light chain kinase mutant as determined by titrating increasing quantities of the antibody with a fixed amount of the target protein. The immune complexes were collected by centrifugation, washed with lysis buffer, boiled in 5×Laemmli sample buffer containing 0.5 mM EGTA, and then subjected to SDS–PAGE. Immunoprecipitates were analyzed by Western blotting using anti-myosin light chain kinase and anti-calmodulin monoclonal antibodies (Upstate Biotechnology, Inc.).

3. Results

3.1. Kinetic properties of smooth muscle myosin light chain kinase proline mutants

Residues Met^{968}, Ala^{983}, or Ala^{986} in the regulatory segment of smooth muscle myosin light chain kinase show no evidence of direct interaction with the catalytic core or calmodulin thus mutagenesis of these residues should not affect autoinhibition and Ca^{2+}/calmodulin activation of the kinase by altering the conformation of the regulatory segment. In addition, the Garnier-Osguthorpe-Robson (GOR) secondary structure prediction method version IV [24] predicts the α-helix as the predominant secondary structure in the regulatory segment of myosin light chain kinase and that individual substitution of residues Met^{968}, Ala^{983}, or Ala^{986} to proline (Fig. 1) would disrupt the putative α-helical structure at the N- or C-terminal of the regulatory segment (Fig. 2). Thus, proline substitution mutations of these residues were performed to disrupt α-helical formation. The proline substitution of residue Met^{968} within the autoinhibitory linker region showed a modest change in $[Ca^{2+}]_{0.5}$ with a K_{CaM} value of 0.4 nM compared to 1 nM for wild-type kinase (Table 1). In addition, Ca^{2+}/calmodulin-independent kinase activity (10% of total activity)

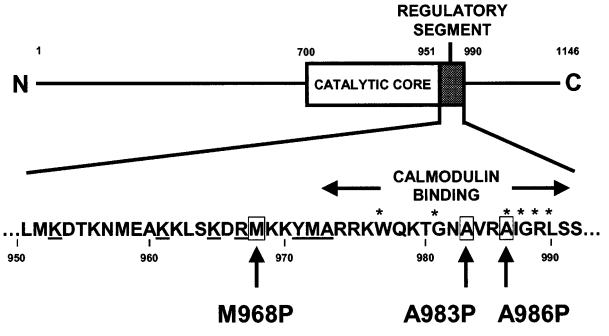

Fig. 1. Proline substitution mutants of rabbit smooth muscle myosin light chain kinase. The regulatory segment of smooth muscle myosin light chain kinase contains the calmodulin-binding sequence (Ala^{973} to Ser^{992}) and the linker region, which connects the catalytic core to the calmodulin-binding sequence. Underlined residues are those amino acids that contribute to autoinhibition [35–38]. The asterisks identify residues essential for high-affinity calmodulin binding [39]. The proline substitutions at Met^{968}, Ala^{983}, and Ala^{986} are shown below the sequence of the regulatory segment.

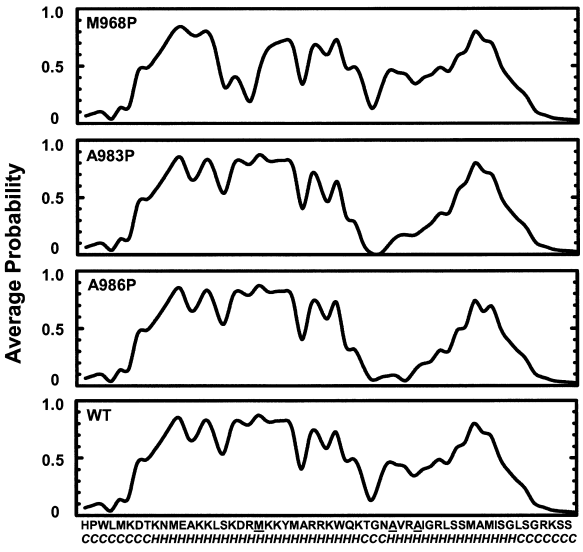

Fig. 2. Secondary structure predictions in the rabbit smooth muscle myosin light chain kinase regulatory segment. The Garnier-Osguthorpe-Robson (GOR) secondary structure prediction method version IV [24] was used to determine the α-helical content of residues 948–1005 for wild-type and mutant M968P, A983P, and A986P kinases. The amino acid sequence of the wild-type kinase residues 948–1005 is given at the bottom with the corresponding secondary structure prediction (*H*, helix; *C*, coil) and the sites of proline mutations are underlined.

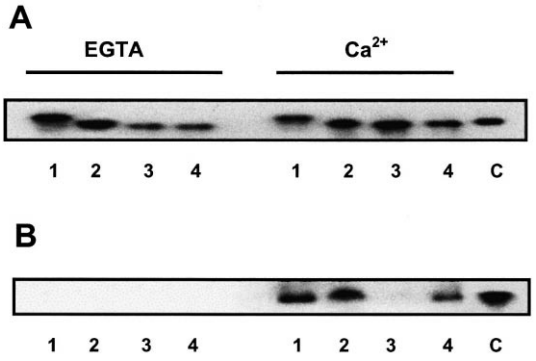

Fig. 3. Binding of calmodulin to myosin light chain kinases containing proline mutations of residues within the regulatory segment. COS-7 cell lysates expressing wild-type and mutant kinases were immunoprecipitated with anti-myosin light chain kinase (α-MLCK) antibody in the presence of calmodulin and Ca^{2+} or EGTA. Immunoprecipitates were analyzed by Western blotting using anti-myosin light chain kinase (α-MLCK; panel A) and anti-calmodulin (α-CaM; panel B) antibodies following SDS–PAGE. Lane 1, wild-type; lane 2, M968P; lane 3, A983P; lane 4, A986P; lane C, purified kinase or calmodulin control.

was observed with the M968P mutant (data not shown). The mutant A983P in the calmodulin-binding sequence resulted in a dramatic increase in the Ca^{2+} required for half-maximal activation ($\gg 10$ μM) with greater than a 20-fold increase in the K_{CaM} values. The A986P mutant required significantly more Ca^{2+} for half-maximal activation (3.8 μM) with only a 5-fold increase in the K_{CaM} value relative to wild-type kinase. There were modest changes in the K_m and V_{max} values of the M968P mutant. The K_m value for light chain could not be measured for the A983P mutant; the specific activity of the kinase was 2.4 vs. 18 μmol ^{32}P/min/mg for the wild-type kinase (Table 1).

3.2. Calmodulin-binding properties of proline mutants of smooth muscle myosin light chain kinase

The ability of calmodulin to induce helicity in the target sequence may be required for calmodulin binding. To show binding of calmodulin to the proline mutants, immunoprecipitation was performed using anti-myosin light chain kinase monoclonal antibody. COS-7 cell lysates expressing wild-type and mutant kinases were subjected to immunoprecipitation with an anti-myosin light chain kinase monoclonal antibody in the presence of Ca^{2+} or EGTA. Both wild-type and mutant kinases were immunoprecipitated (Fig. 3A). In the presence of Ca^{2+}, calmodulin co-immunoprecipitated with wild-type, M968P, and A986P mutant kinases. However, calmodulin was not present in immunoprecipitates of A983P mutant kinase. Calmodulin did not co-immunoprecipitate with either the wild-type or mutant kinases in the presence of EGTA (Fig. 3B).

4. Discussion

Alanine scanning mutagenesis within the calmodulin-binding sequence of smooth muscle myosin light chain kinase revealed that calmodulin binding by the enzyme is determined by an interplay of specific hydrophobic and electrostatic interactions [25]. Accordingly, we selected neutral alanine residues within the calmodulin-binding sequence and substituted them with proline residues that would disrupt helix formation. The proline substitution mutants A983P and A986P in the calmodulin-binding sequence were less easily activated by Ca^{2+}/calmodulin (increased $[Ca^{2+}]_{0.5}$ and K_{CaM} values relative to wild-type) most likely due to the disruption of helix formation induced by calmodulin binding.

Mutagenesis of residue Ala^{983} not only resulted in a dramatic increase in the K_{CaM} value but also inhibited high-affin-

Table 1
Kinetic properties of myosin light chain kinase containing proline mutations within the regulatory segment

Kinase	$[Ca^{2+}]_{0.5}$ (μM)	K_{CaM} (nM)	K_mLC (μM)	V_{max} (μmol^{32}P/min/mg)
Wild-type	0.6 ± 0.03	1.0	14 ± 1.4	15 ± 0.9
M968P	0.3 ± 0.02	0.4 ± 0.03	6.7 ± 0.5	7.9 ± 0.5
A983P	$\gg 10$	$\gg 20$	n.d.	(2.4 ± 0.3)
A986P	3.8 ± 0.69	5.1 ± 0.26	11 ± 0.8	15 ± 0.6

Mutagenesis, expression in COS-7 cells, quantitation of expressed kinase in COS-7 cell extracts, and measurement of kinetic properties of rabbit smooth muscle myosin light chain kinases were performed as described in Section 2. Values are means ± standard errors for at least three experiments with lysates from transfections representing at least two independent mutations. $[Ca^{2+}]_{0.5}$ values represent the Ca^{2+} concentration required for half-maximal activation at 1 μM calmodulin. The relative K_{CaM} values were calculated for at least three Ca^{2+} concentrations. V_{max} and K_m values were determined from Lineweaver-Burke plots. K_mLC refers to K_m values for the RLC; n.d., values not determined. Specific activity value of A983P enclosed in parentheses vs. 18 ± 0.8 μmol ^{32}P/min/mg for wild-type kinase.

ity Ca^{2+}/calmodulin binding to the kinase. The results with Ala^{983} were most interesting since this residue is close to the tryptophan residue at the N-terminus of the calmodulin-binding sequence that has been shown by Bayley and coworkers [26] to be important for binding to the calmodulin C domain, which then induces a high degree of helicity in a model peptide. The ability to induce helicity in the target sequence may be connected with the ability to activate the enzyme. The C domain of calmodulin is generally more effective than the N domain in maintaining helicity of the peptide; likewise, the separated C domain of calmodulin activates myosin light chain kinase to 65%, whereas the N domain hardly activates the enzyme. Based on the relative effects of proline mutations in Ala^{983} and Ala^{986}, the region immediately C-terminal of the tryptophan in the calmodulin-binding sequence may be most important in forming an α-helix with the C domain of calmodulin leading to activation.

The two-dimensional NMR structure of the autoinhibitory linker region and part of the calmodulin-binding sequence of smooth muscle myosin light chain kinase shows that an amino acid corresponding to Met^{968} is located at the start of an α-helical segment extending to the Trp residue in the calmodulin-binding sequence [27]. In addition, the crystal structures of Ca^{2+}/calmodulin-dependent protein kinase I, twitchin kinase and titin kinase demonstrate that the region in their regulatory segments equivalent to the linker region in myosin light chain kinase also forms an α-helix [28–31]. Furthermore, results obtained upon substitution of Cys^{289} to a proline in the linker sequence preceding the calmodulin-binding sequence in Ca^{2+}/calmodulin-dependent protein kinase II suggests the presence of an α-helical structure important for Ca^{2+}/calmodulin activation [32]. Amino acid Met^{968} in the linker region of myosin light chain kinase corresponds to Cys^{289} in Ca^{2+}/calmodulin-dependent protein kinase II. Mutagenesis of residue Met^{968} made the kinase more easily activated by Ca^{2+}/calmodulin, however the magnitude of the effect was small. In addition, there was enhanced Ca^{2+}/calmodulin-independent kinase activity (10% of total activity) while maximal activity was decreased. We conclude that secondary structure within the linker sequence may be important for the proper orientation of residues within the autoinhibitory linker region to interact with the catalytic core.

Alternatively, proline in a polypeptide chain has less conformational freedom than other amino acids, as the pyrrolidine ring of proline imposes rigid constraints on the $N–C_\alpha$ rotation and restricts the available conformational space of the preceding residue of proline [33]. In regions devoid of α-helical structures proline may not function as a helix breaker but may provide molecular rigidity at turns or in random regions by tightening the surrounding conformation as a result of reducing the backbone flexibility [34]. However based on the following observations that (1) α-helical segments are present in synthetic peptide containing the autoinhibitory linker region of myosin light chain kinase; (2) an increase in the helicity of the calmodulin-binding sequence of myosin light chain kinase is accompanied by the collapse of calmodulin about its target peptide; and (3) calmodulin forms a collapsed structure about the intact kinase, it is most likely that introducing an α-helix breaker within the linker region and the calmodulin-binding sequence of myosin light chain kinase disrupts autoinhibition and prevents the collapse of calmodulin about its target sequence resulting in the lost of high-affinity binding of calmodulin to the kinase.

In summary, substitutions of proline residues at strategic positions reveal that the region C-terminal of the tryptophan in the N-terminus calmodulin-binding sequence is important in calmodulin binding and activation. The ability of calmodulin to induce helicity in its binding sequence when it forms a collapsed structure may be connected to its ability to activate myosin light chain kinase. Finally, the presence of an α-helical structure within the linker sequence may help to orient residues to interact with the catalytic core during intrasteric regulation of the kinase.

Acknowledgements: Support provided in part by the Bashour Research Fund and NIH grants HL06296 and HL29043.

References

[1] Stull, J.T., Nunnally, M.H. and Michnoff, C.H. (1986) in: The Enzymes (Krebs, E.G. and Boyer, P.P., Eds.), Calmodulin-Dependent Protein Kinases, pp. 113–116, Academic Press, New York.
[2] Gallagher, P.J., Herring, B.P. and Stull, J.T. (1997) J. Muscle Res. Cell Motil. 18, 1–16.
[3] Stull, J.T., Krueger, J.K., Kamm, K.E., Gao, Z.-H., Zhi, G. and Padre, R. (1996) in: Biochemistry of Smooth Muscle Contraction (Barany, M., Ed.), Myosin Light Chain Kinase, pp. 119–130, Academic Press, New York.
[4] Sweeney, H.L., Bowman, B.F. and Stull, J.T. (1993) Am. J. Physiol. 264, C1085–C1095.
[5] Stull, J.T., Lin, P.-J., Krueger, J.K., Trewhella, J. and Zhi, G. (1998) Acta Physiol. Scand. 164, 471–482.
[6] O'Neil, K.T. and DeGrado, W.F. (1990) Trends Biochem. Sci. 15, 59–64.
[7] Heidorn, D.B., Seeger, P.A., Rokop, S.E., Blumenthal, D.K., Means, A.R., Crespi, H. and Trewhella, J. (1989) Biochemistry 28, 6757–6764.
[8] Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. (1992) Science 256, 632–638.
[9] Meador, W.E., Means, A.R. and Quiocho, F.A. (1992) Science 257, 1251–1255.
[10] Krueger, J.K., Bishop, N.A., Blumenthal, D.K., Zhi, G., Beckingham, K., Stull, J.T. and Trewhella, J. (1998) Biochemistry 37, 17810–17817.
[11] Piela, L., Nemethy, G. and Scheraga, H.A. (1987) Biopolymers 26, 1587–1600.
[12] Richardson, J.S. and Richardson, D.C. (1988) Science 240, 1648–1652.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Lin, P.-J., Luby-Phelps, K. and Stull, J.T. (1997) J. Biol. Chem. 272, 7412–7420.
[15] Blumenthal, D.K. and Stull, J.T. (1980) Biochemistry 19, 5608–5614.
[16] Hathaway, D.R. and Haeberle, J.R. (1983) Anal. Biochem. 135, 37–43.
[17] Bowman, B.F., Peterson, J.A. and Stull, J.T. (1992) J. Biol. Chem. 267, 5346–5354.
[18] Zhi, G., Abdullah, S.M. and Stull, J.T. (1998) J. Biol. Chem. 273, 8951–8957.
[19] Herring, B.P. (1991) J. Biol. Chem. 266, 11838–11841.
[20] Potter, J.D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628–4633.
[21] Fitzsimons, D.P., Herring, B.P., Stull, J.T. and Gallagher, P.J. (1992) J. Biol. Chem. 267, 23903–23909.
[22] Miller, J.R., Silver, P.J. and Stull, J.T. (1983) Mol. Pharmacol. 24, 235–242.
[23] Stull, J.T., Hsu, L.C., Tansey, M.G. and Kamm, K.E. (1990) J. Biol. Chem. 265, 16683–16690.
[24] Garnier, J., Gibrat, J.F. and Robson, B. (1996) Methods Enzymol. 266, 540–553.

[25] Bagchi, I.C., Huang, Q.H. and Means, A.R. (1992) J. Biol. Chem. 267, 3024–3029.
[26] Barth, A., Martin, S.R. and Bayley, P.M. (1998) J. Biol. Chem. 273, 2174–2183.
[27] Barden, J.A., Sehgal, P. and Kemp, B.E. (1996) Biochim. Biophys. Acta 1292, 106–112.
[28] Hu, S.H., Parker, M.W., Lei, J.Y., Wilce, M.C., Benian, G.M. and Kemp, B.E. (1994) Nature 369, 581–584.
[29] Goldberg, J., Nairn, A.C. and Kuriyan, J. (1996) Cell 84, 875–887.
[30] Kobe, B., Heierhorst, J., Feil, S.C., Parker, M.W., Benian, G.M., Weiss, K.R. and Kemp, B.E. (1996) EMBO J. 15, 6810–6821.
[31] Mayans, O., van der Ven, P.F., Wilm, M., Mues, A., Young, P., Furst, D.O., Wilmanns, M. and Gautel, M. (1998) Nature 395, 863–869.
[32] Mukherji, S., Brickey, D.A. and Soderling, T.R. (1994) J. Biol. Chem. 269, 20733–20738.
[33] Schimmel, P.R. and Flory, P.J. (1968) J. Mol. Biol. 34, 105–120.
[34] Zhu, G.P., Xu, C., Teng, M.K., Tao, L.M., Zhu, X.Y., Wu, C.J., Hang, J., Niu, L.W. and Wang, Y.Z. (1999) Protein Eng. 12, 635–638.
[35] Shoemaker, M.O., Lau, W., Shattuck, R.L., Kwiatkowski, A.P., Matrisian, P.E., Guerra-Santos, L., Wilson, E., Lukas, T.J., Van Eldik, L.J. and Watterson, D.M. (1990) J. Cell Biol. 111, 1107–1125.
[36] Yano, K., Araki, Y., Hales, S.J., Tanaka, M. and Ikebe, M. (1993) Biochemistry 32, 12054–12061.
[37] Krueger, J.K., Padre, R.C. and Stull, J.T. (1995) J. Biol. Chem. 270, 16848–16853.
[38] Fitzsimons, D.P., Herring, B.P., Stull, J.T. and Gallagher, P.J. (1992) J. Biol. Chem. 267, 23903–23909.
[39] Bagchi, I.C., Huang, Q.H. and Means, A.R. (1992) J. Biol. Chem. 267, 3024–3029.