Pederson, T., & Bhorjee, J. S. (1975) *Biochemistry 14*, 3238-3242.
Perle, M. A., & Newman, S. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4828-2830.
Razin, A., & Riggs, A. D. (1980) *Science (Washington, D.C.) 210*, 604-610.
Stalder, J., Groudine, M., Dodgson, J. B., Engel, J. D., & Weintraub, H. (1980a) *Cell (Cambridge, Mass.) 19*, 973-980.
Stalder, J., Larsen, A., Engel, J. D., Dolan, M., Groudine, M., & Weintraub, H. (1980b) *Cell (Cambridge, Mass.) 20*, 451-460.
Vertel, B. M., & Dorfman, A. (1978) *Dev. Biol. 62*, 1-12.
von der Mark, H., & von der Mark, K. (1977) *J. Cell Biol. 73*, 736-747.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 93*, 848-858.
Weintraub, H., Larsen, A., & Groudine, M. (1981) *Cell (Cambridge, Mass.) 24*, 333-444.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 631-635.
Weisbrod, S., Groudine, M., & Weintraub, H. (1980) *Cell (Cambridge, Mass.) 19*, 289-301.

Effects of pH, Ionic Strength, and Temperature on Activation by Calmodulin and Catalytic Activity of Myosin Light Chain Kinase†

Donald K. Blumenthal and James T. Stull*

ABSTRACT: The reversible association of Ca_4^{2+}-calmodulin with the inactive catalytic subunit of myosin light chain kinase results in the formation of the catalytically active holoenzyme complex [Blumenthal, D. K., & Stull, J. T. (1980) *Biochemistry 19*, 5608-5614]. The present study was undertaken in order to determine the effects of pH, temperature, and ionic strength on the processes of activation and catalysis. The catalytic activity of myosin light chain kinase, when fully activated by calmodulin, exhibited a broad pH optimum (>90% of maximal activity from pH 6.5 to pH 9.0), showed only a slight inhibition by moderate ionic strengths (<20% inhibition at $\mu = 0.22$), and displayed a marked temperature dependence ($Q_{10} \simeq 2$; $E_a = 10.4$ kcal mol^{-1}). Thermodynamic parameters calculated from Arrhenius plots indicate that the Gibb's energy barrier associated with the rate-limiting step of catalysis is primarily enthalpic. The process of kinase activation by calmodulin had a narrower pH optimum (pH 6.0-7.5) than did catalytic activity, was markedly inhibited by increasing ionic strength (>70% inhibition at $\mu = 0.22$), and exhibited nonlinear van't Hoff plots. Between 10 and 20 °C, activation was primarily entropically driven ($\Delta S° \simeq 40$ cal mol^{-1} deg^{-1}; $\Delta H° = -900$ cal mol^{-1}), but between 20 and 30 °C, enthalpic factors predominated in driving the activation process ($\Delta S° \simeq 10$ cal mol^{-1} deg^{-1}; $\Delta H° = -9980$ cal mol^{-1}). The apparent change in heat capacity (ΔC_p) accompanying activation was estimated to be -910 cal mol^{-1} deg^{-1}. On the basis of these data we propose that although hydrophobic interactions between calmodulin and the kinase are necessary for the activation of the enzyme, other types of interactions such as hydrogen bonding, ionic, and van der Waals interactions also make significant and probably obligatory contributions to the activation process.

Calmodulin is known to regulate a number of enzymes and cellular processes in a Ca^{2+}-dependent manner [for reviews, see Wolff & Brostrom (1979), Klee et al. (1980), Cheung (1980), Means & Dedman (1980), and Wang & Waisman (1979)]. Myosin light chain kinase is one of the several enzymes whose activity is completely dependent on the presence of Ca^{2+} and calmodulin. This enzyme is responsible for catalyzing the phosphorylation of a specific subunit of myosin, known as the phosphorylatable or P light chain (Frearson & Perry, 1975). The enzyme and its substrate are present in nonmuscle as well as muscle tissue. The phosphorylation reaction may play an important role in the regulation of contraction in smooth and skeletal muscles (Stull, 1980; Stull et al., 1980), as well as in modulating cellular function in certain nonmuscle tissues (Adelstein, 1978; Salisbury et al., 1980).

The details of the interaction of calmodulin with the many proteins it regulates are not well understood. Previous studies in our laboratory were concerned with determining the mechanism of activation of myosin light chain kinase (Blumenthal & Stull, 1980). The purpose of this investigation was to extend our previous studies and to determine the effects of pH, temperature, various salts, and ionic strength on the activation and catalytic activity of myosin light chain kinase. From analysis of these results it is possible to obtain information regarding the factors that play important roles in the regulation of myosin light chain kinase activity. Because calmodulin is highly conserved throughout eukaryotic evolution (Jamieson et al., 1980), the general features of the interaction of calmodulin with myosin light chain kinase may be applicable to other calmodulin-dependent processes.

Materials and Methods

Chemicals were obtained from Sigma and were of analytical grade or better. [γ-^{32}P]ATP[1] was prepared by the method

† From the Department of Pharmacology and the Moss Heart Center, University of Texas Health Science Center at Dallas, Dallas, Texas 75235. *Received September 1, 1981.* This work was supported by grants from the National Institutes of Health (HL 23990) and the Muscular Dystrophy Association of America. D.K.B. was supported as a postdoctoral trainee (HL-07360).

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5'-triphosphate; NMR, nuclear magnetic resonance; SEM, standard error of the mean.

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of Walseth & Johnson (1979). Skeletal muscle myosin light chains and homogeneous myosin light chain kinase were prepared from fresh rabbit skeletal muscle as previously described (Blumenthal & Stull, 1980). Calmodulin was prepared from frozen bovine brain (Pel-Freez) by using fluphenazine–Sepharose affinity chromatography (Charbonneau & Cormier, 1979). Fluphenazine–Sepharose was prepared essentially as described by Kakiuchi et al. (1981). An extract enriched in calmodulin was prepared by using steps 1 and 4 of the procedure of Watterson et al. (1976). The precipitated pellet obtained in step 4 was resuspended in buffer containing 10 mM Tris, pH 8.0, and 50 mM NaCl and dialyzed against the same buffer overnight. $CaCl_2$ was added to a final concentration of 1 mM just prior to applying the solution to a column (2 × 20 cm) packed with fluphenazine–Sepharose and equilibrated with 10 mM Tris, pH 8.0, 50 mM NaCl, and 2 mM $CaCl_2$. The column was washed with 10 mM Tris, pH 8.0, 0.1 mM $CaCl_2$, and 50 mM NaCl until protein was no longer detected in the column effluent (approximately 10 volumes of buffer). Calmodulin was eluted with 10 mM Tris, pH 8.0, 50 mM NaCl, and 10 mM EGTA. Calmodulin purified by this procedure appeared homogeneous on polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and 1 mM EDTA.

Myosin Light Chain Kinase Assays. Enzyme activity was determined by rates of ^{32}P incorporation into skeletal muscle myosin light chains. Reactions were performed in 6 × 50 mm borosilicate glass tubes. All reaction mixtures (50-μL final volume) contained 95 μM skeletal muscle myosin P light chain, 100 μM calcium chloride, 10 mM magnesium acetate, 2 mM [γ-^{32}P]ATP (150–300 cpm/pmol), 15 mM 2-mercaptoethanol, and 0.1–0.3 nM rabbit skeletal muscle myosin light chain kinase. In all assays except those for the pH studies, the pH was buffered with 50 mM Mops, adjusted to pH 7.0 with NaOH. The pH buffer used for the pH studies contained 50 mM Hepps, 50 mM Mops, and 50 mM Mes adjusted to the appropriate pH with HCl or NaOH. Reaction mixtures were preincubated at assay temperature for at least 15 min. Reactions were initiated with [γ-^{32}P]ATP, and 20-μL aliquots of the reaction mixtures were removed after 5 and 15 min and spotted on 3MM filter paper squares. The squares were processed as previously described (Corbin & Reimann, 1975). Reaction mixtures containing all reaction components and 3 mM EGTA were used to determine background radioactivity. The amount of radioactivity associated with the EGTA reactions was typically <250 cpm. All assays were performed in duplicate.

A calmodulin concentration of 1 μM was used to obtain maximally activated rates (V_{max}), and 0.2–0.5 nM calmodulin was used to obtain subsaturating rates (v). The rate of phosphorylation catalyzed by maximally activated kinase under the reference conditions (30 °C; pH 7.0; μ = 0.02; no added KCl) was 20 μmol of ^{32}P incorporated min^{-1} (mg of enzyme)$^{-1}$ and is referred to as V_{max}^0. The ratio V_{max}^*/V_{max}^0 is the rate of phosphorylation catalyzed by maximally activated kinase under a specified set of experimental conditions (V_{max}^*) relative to V_{max}^0. Similarly, the rates of phosphorylation with subsaturating calmodulin concentrations are termed v^0 and v^*, indicating the rate under the reference and experimental conditions, respectively. The activation constant for calmodulin under the reference conditions (K_{CM}^0) relative to a specified set of experimental conditions (K_{CM}^*) was calculated by using the expression

$$(V_{max}^0/v^0 - 1)/(V_{max}^*/v^* - 1) = K_{CM}^0/K_{CM}^*$$

The value of K_{CM}^0/K_{CM}^* was only calculated when both v^*/V_{max}^* and v^0/V_{max}^0 had observed values ≤ 0.7. The equation used assumes that activation follows simple hyperbolic kinetics, which we previously demonstrated in the case of myosin light chain kinase activation by calmodulin (Blumenthal & Stull, 1980). The equation also implicitly assumes that the Ca_4^{2+}·calmodulin concentration in the assays containing subsaturating concentrations of calmodulin is not significantly different between the reference condition and the particular experimental condition. In order to ensure that the latter assumption was valid, we employed a $CaCl_2$ concentration of 100 μM in all assays. Increasing the concentration of $CaCl_2$ to 300 μM did not significantly affect the results obtained with varying temperature or ionic strength, indicating that the concentration of Ca_4^{2+}·calmodulin did not vary significantly in the experiments involving ionic strength and temperature. The value of K_{CM}^0, the concentration of calmodulin required for half-maximal activation of the enzyme under the reference conditions, was 0.3 nM.

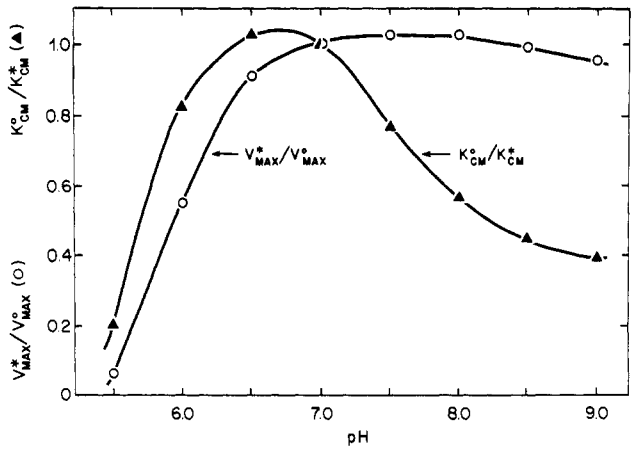

FIGURE 1: Effects of pH on activation and catalytic activity of myosin light chain kinase. Data are plotted relative to values observed at pH 7.0, 30 °C, and no added salt. The indicated values were calculated as described under Materials and Methods.

Results

Effects of pH. The effects of pH on the activation (K_{CM}^0/K_{CM}^*) and catalytic rate (V_{max}^*/V_{max}^0) of myosin light chain kinase are plotted in Figure 1. The catalytic activity of maximally activated enzyme was low at pH 5.5 but increased with increasing pH until about pH 6.5. The optimum catalytic activity was observed from pH 6.5 to pH 9.0. In contrast, activation of myosin light chain kinase by calmodulin showed a much narrower pH optimum, from about pH 6.0 to pH 7.5. Activation was markedly inhibited below pH 6.0 and moderately inhibited from pH 7.5 to pH 9.0.

Effects of Temperature. Figure 2 illustrates the effect of temperature on the catalytic rate of myosin light chain kinase, at various concentrations of KCl. The data are plotted in an Arrhenius format. The plots obtained at each KCl concentration are linear from 10 to 30 °C. The rates obtained at 35 °C are much lower than expected (i.e., the points at 35 °C fall well below the projected line), suggesting that the enzyme is unstable above 30 °C. In support of this, phosphate incorporation at 35 °C was not linear with respect to time, whereas linear rates of phosphorylation were observed at all lower temperatures. The instability of the enzyme at 35 °C appeared to be increased with increasing concentrations of KCl. In contrast, at temperatures between 10 and 30 °C, KCl concentrations up to 100 mM did not significantly change the catalytic rate from that seen with no added salt. Adding 200 mM KCl to the reaction mixture decreased the catalytic rate

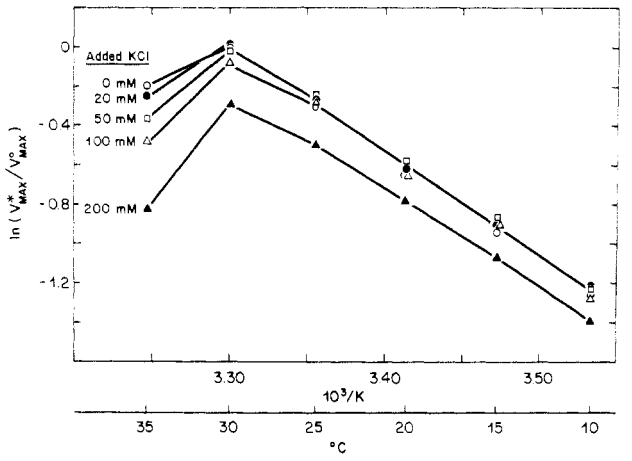

FIGURE 2: Effects of temperature on catalytic activity of myosin light chain kinase at various concentrations of KCl. The logarithm of the ratio of the catalytic rate under the indicated conditions (V_{max}*) relative to the rate observed at 30 °C, pH 7.0, and no added KCl (V_{max}^0) is plotted as a function of the reciprocal of the absolute temperature. Thermodynamic parameters calculated from the slopes are given in the text.

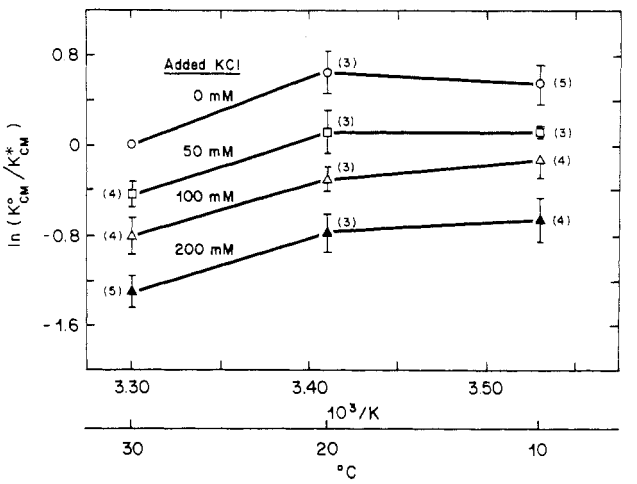

FIGURE 3: Effects of temperature on the activation of myosin light chain kinase by calmodulin at various concentrations of KCl. The ratio of the activation constant for calmodulin at 30 °C, pH 7.0, and no added KCl relative to the activation constant under the indicated experimental condition (K_{CM}^0/K_{CM}*) was calculated as described under Materials and Methods. The logarithm of this value is plotted as a function of the reciprocal of the absolute temperature. The indicated points represent the average of several separate experiments (the number of experiments is indicated in parentheses beside each point); the error bars indicate the variability between experiments (±SEM). Thermodynamic parameters calculated from the plots are presented in the text.

at each temperature, but the plot had a slope that was essentially identical with that obtained at lower KCl concentrations. The activation energy (E_a) for the rate-limiting step of catalysis was calculated from the Arrhenius plot (Figure 2) with the relation $E_a = -(\text{slope})R$, where R is the gas constant (1.99 cal mol^{-1} deg^{-1}). The average value of E_a obtained from the several plots was 10.4 kcal mol^{-1}. Other thermodynamic parameters associated with the energy barrier of catalysis were calculated (for 30 °C) with the following equations: $\Delta H^\ddagger = E_a - RT$, $\Delta G^\ddagger = RT\,[\ln (kT/h) - \ln V_{max}^0]$, and $\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T$, where k represents Boltzmann's constant, h is Planck's constant, and T is 303 K. The values calculated were $\Delta H^\ddagger$ = 9.9 kcal mol^{-1}, $\Delta G^\ddagger$ = 15.7 kcal mol^{-1}, and $\Delta S^\ddagger$ = −19.2 cal mol^{-1} deg^{-1}.

van't Hoff plots of myosin light chain kinase activation by calmodulin at various concentrations of KCl are shown in Figure 3. Activation occurred more readily at 10 and 20 °C than at 30 °C, and although KCl inhibited activation, similar changes in K_{CM} were observed with changing temperature at each concentration of KCl examined. Thermodynamic parameters were calculated from the slopes of the plots in Figure 3 with the following relations: slope = $\Delta H°/R$, $\Delta G° = RT \ln K_{CM}$, $\Delta S° = (\Delta H° - \Delta G°)/T$, and $\Delta(\Delta H°)/\Delta T = \Delta C_p$. The average standard enthalpy changes ($\Delta H°$) calculated were −900 (±2000) cal mol^{-1} for the plots between 10 and 20 °C and −9980 (±1100) cal mol^{-1} for the 20–30 °C range. Thus, between 10 and 20 °C activation was primarily entropically driven ($\Delta S° \simeq 40$ cal deg^{-1}), whereas the activation process was driven by enthalpic factors between 20 and 30 °C ($\Delta S° \simeq 10$ cal deg^{-1}). The nonlinearity of the van't Hoff plots indicates that activation by calmodulin was associated with a large apparent decrease in the molar heat capacity (ΔC_p = −910 cal mol^{-1} deg^{-1}).

Effects of Ionic Strength and Various Chloride Salts. The effects of increasing ionic strength on the rate of catalysis of maximally activated myosin light chain kinase are shown in Figure 4. Ionic strengths up to 0.22 had minimal effects on the activity of the enzyme at temperatures between 10 and 30 °C. The apparent instability of the enzyme at 35 °C was, however, increased with increasing ionic strength. At temperatures between 10 and 30 °C the effects of ionic strength did not vary with temperature, and, conversely, the temperature dependence of the catalytic rate (Figure 2) was unaffected

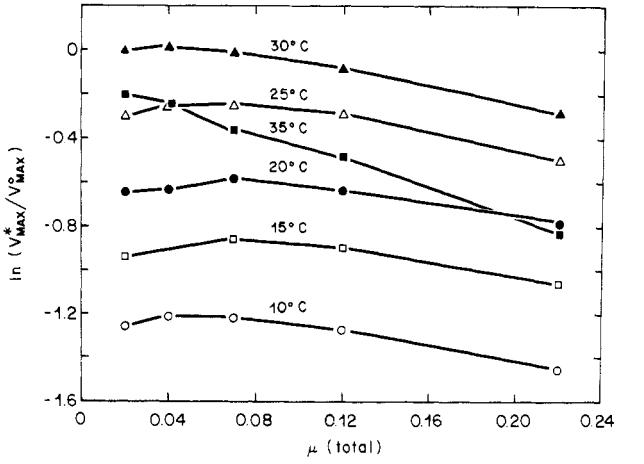

FIGURE 4: Effect of ionic strength on catalytic activity of myosin light chain kinase at various temperatures. The data shown are identical with those in Figure 2 but are plotted here as a function of total ionic strength in the assay. The ionic strength of an assay mixture containing no added KCl was determined by comparing the conductivity of such an assay mixture to that of a known concentration of KCl.

by ionic strengths up to 0.22. Approximately half of the inhibition of catalytic activity at μ = 0.22 can be attributed to an increase in the K_m value for myosin P light chain substrate (data not shown). The value of the K_m increased from 10 μM at μ = 0.02 to 14 and 27 μM at μ = 0.12 and 0.22, respectively. The data in Figure 4 were not corrected for this effect. The effect that various chloride salts (0.2 M) had on the catalytic activity of the enzyme is shown in Figure 6. NH_4^+, K^+, Na^+, and Li^+ are arranged in order of decreasing ionic radius. KCl and NaCl had the least effect on catalytic activity, followed by NH_4Cl and LiCl. Guanidinium chloride, a potent chaotropic agent, was the most effective chloride salt in inhibiting enzyme activity.

The effect of ionic strength on the activation of the enzyme by calmodulin is shown in Figure 5. Activation was inhibited more than 70% on going from μ = 0.02 to μ = 0.22. This effect did not appear to vary significantly with temperature

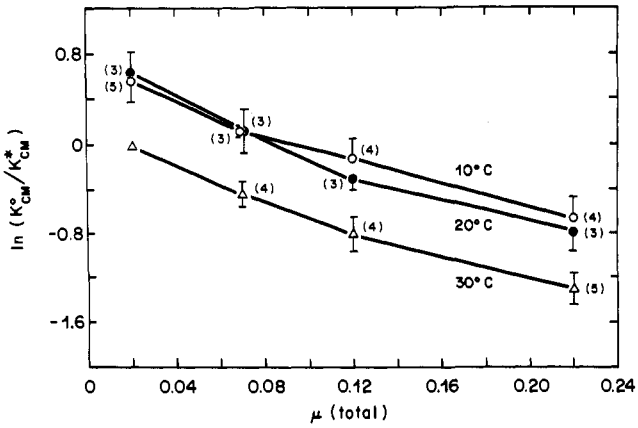

FIGURE 5: Effect of ionic strength on activation of myosin light chain kinase at various temperatures. The data shown are identical with those in Figure 3 but are plotted as a function of total ionic strength.

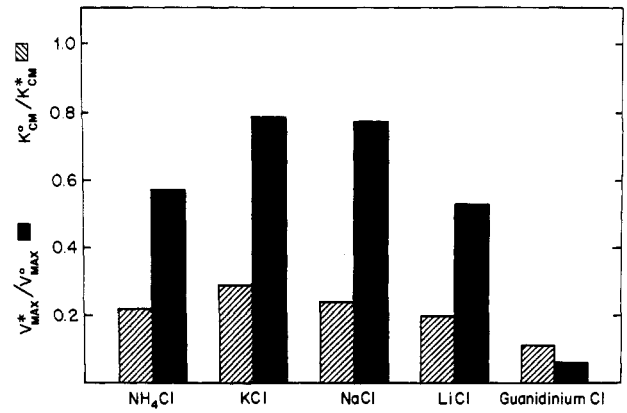

FIGURE 6: Effect of various chloride salts on catalytic activity and activation by calmodulin of myosin light chain kinase. The values indicated were calculated as described under Materials and Methods. The values obtained with each of the indicated chloride salts (0.2 M) were compared to the value obtained with no added salt.

between 10 and 30 °C. Conversely, ionic strength did not appear to affect the temperature dependence of activation. The inhibition of activation by various chloride salts (0.2 M) followed the same order of effectiveness as the inhibition of catalytic activity (Figure 6). Although guanidinium chloride was the most effective salt in attenuating activation, the relative effect (compared to KCl) on activation was much less than the effect on catalytic activity (Figure 6).

Discussion

Catalysis. Although the emphasis of the present investigation was directed toward elucidation of the factors important in the interaction of calmodulin with myosin light chain kinase, data were also obtained regarding the effects of the various experimental variables on the enzyme's catalytic rate. So that possible effects on catalytic rate due to changes in substrate K_m values could be minimized, the concentrations of myosin P light chain (95 μM) and ATP (2 mM) employed herein were in 10-fold excess of their respective K_m values determined under the reference conditions. Similarly, the concentration of calmodulin used to fully activate the enzyme (1 μM) was 3000-fold higher than the value of K_{CM}^0.

The enzyme exhibits optimal activity at pH values above pH 6.5. Below this, enzyme activity is markedly attenuated. The pH profile shown in Figure 1 is qualitatively similar to the profiles previously reported for myosin light chain kinases from rabbit skeletal (Pires & Perry, 1977), bovine cardiac (Walsh et al., 1979), and chicken gizzard smooth muscle (Mrwa & Hartshorne, 1980). The marked inhibition observed above pH 8.0 in the report by Pires & Perry (1977) could have resulted from the fact that calmodulin may not have been saturating in those experiments (Nairn & Perry, 1979) or because a different pH buffering system was used.

The effects of temperature on fully activated myosin light chain kinase at various added concentrations of KCl indicate that catalysis is markedly temperature dependent. Linear Arrhenius plots were observed between 10 and 30 °C ($Q_{10} \simeq 2$). It was found that KCl concentrations up to 0.1 M had no effect on the slope of the Arrhenius plot between 10 and 30 °C, but increasing KCl concentration appeared to increase the instability of the enzyme at temperatures above this. As might be expected in the case of a rate involving covalent modification, the Gibb's energy change associated with the energy barrier of the rate-limiting step of the phosphorylation reaction is primarily due to enthalpic contributions at 30 °C. The results obtained with rabbit skeletal muscle myosin light chain kinase are in excellent agreement with data regarding the temperature dependence of the avian gizzard kinase (Mrwa & Hartshorne, 1980).

Moderate ionic strength (μ = 0.02–0.22) has little effect on the catalytic activity of myosin light chain kinase, in agreement with previous reports (Pires & Perry, 1977; Walsh et al., 1979; Mrwa & Hartshorne, 1980). A small amount of salt (μ = 0.05–0.10) appears to be necessary for optimal catalytic efficiency (Figure 4), probably because charged groups that hinder maximal catalytic rate are shielded. As noted above, increasing ionic strength appears to increase the instability of the enzyme at temperatures above 30 °C. The order of effectiveness of various chloride salts in inhibiting catalysis ($K^+ \simeq Na^+ < NH_4^+ < Li^+$) follows the so-called Hofmeister or lyotropic series (von Hippel & Wong, 1964; von Hippel & Schleich, 1969). Guanidinium chloride was the most effective inhibitor of enzyme activity of the several chloride salts tested and was the only chloride salt that inhibited catalytic activity of the enzyme more than it inhibited calmodulin activation of the enzyme.

Activation by Calmodulin. Optimal activation of myosin light chain kinase by calmodulin was observed between pH 6.0 and pH 7.5. Because this covers the range of pH values thought to exist intracellularly, these data indicate that modest changes in intracellular pH would not be expected to significantly affect the activation of myosin light chain kinase. Many investigators routinely assay myosin light chain kinase activity at pH values above 7.5 (Pires & Perry, 1977; Nairn & Perry, 1979; Walsh et al., 1979; Mrwa & Hartshorne, 1980) and, thus, are underestimating the ability of calmodulin to activate the enzyme. Indeed, the value of K_{CM}^0 determined in this study was at least 2- or 3-fold lower than that of these other studies.

van't Hoff plots of enzyme activation by calmodulin are nonlinear, indicating that the enthalpy change associated wth the activation process is temperature dependent. Thus, there appears to be a significant change in the heat capacity associated with the activation process. Between 10 and 20 °C the activation process is entropically driven, with little or no apparent contribution to the free energy change contributed by enthalpy changes. In contrast, between 20 and 30 °C most of the free energy change of the activation process is contributed by enthalpic factors, with an apparently small contribution by entropic factors. A number of protein–protein and protein–ligand interactions (Edelhoch & Osborne, 1976; Ross & Subramanian, 1981; Beaudette & Langerman, 1980) exhibit thermodynamic profiles that are very similar to that

displayed by calmodulin activation of myosin light chain kinase.

A general conceptual model that is capable of explaining the thermodynamic behavior of a variety of protein–protein and protein–small molecule interactions in terms of various inter- and intramolecular forces has recently been proposed by Ross & Subramanian (1981). The formation of a protein–protein or protein–ligand complex is proposed to occur in two steps, the first step stabilized by hydrophobic interactions and the second step stabilized by short-range interactions such as electrostatic (ionic) interactions, protonations, hydrogen bonding, and van der Waals interactions. Hydrophobic and ionic interactions contribute to the stability of the complex via positive entropy changes, whereas hydrogen bonding, protonations, and van der Waals interactions stabilize the complex by negative changes in enthalpy. Evidence from this investigation, as well as earlier studies from other laboratories, indicates that this model may be useful in understanding the interaction of calmodulin with other proteins and with small molecules.

The binding of Ca^{2+} to calmodulin is known to result in the exposure of a hydrophobic domain, which is thought to serve as the interface for Ca^{2+}-dependent interactions of calmodulin with other proteins, such as Ca^{2+}-sensitive cyclic nucleotide phosphodiesterase and troponin I (LaPorte et al., 1980; Tanaka & Hidaka, 1980). Various hydrophobic ligands such as the phenothiazine antipsychotics are also presumed to bind to this site and thereby antagonize calmodulin–protein interactions (LaPorte et al., 1980; Weiss & Levin, 1978; Tanaka & Hidaka, 1980). Thermodynamic data from this study indicate that the activation of myosin light chain kinase by calmodulin is entropy driven under certain conditions (i.e., 10–20 °C) and are thus consistent with the concept that hydrophobic interactions play an important role in calmodulin–protein interactions.

However, the thermodynamic profile of calmodulin activation of myosin light chain kinase indicates that short-range interactions including van der Waals interactions, protonations, and/or hydrogen bond formation are also factors of some importance in stabilizing the catalytically active calmodulin–myosin light chain kinase complex. These factors are all characterized by negative enthalpy changes [references cited by Ross & Subramanian (1981)]. Since activation of myosin light chain kinase is enthalpy driven between 20 and 30 °C, one or more of these factors appear to be contributing to the stability of the active calmodulin–enzyme complex. These factors are also characterized by large decreases in entropy (Ross & Subramanian, 1981). Presumably, these unfavorable entropy changes are offset by the positive entropy changes resulting from hydrophobic and ionic interactions.

The marked inhibition of calmodulin activation of myosin light chain kinase by increasing ionic strength indicates that electrostatic interactions are important in stabilizing active calmodulin–protein interactions. Indeed, if hydrophobic interactions were the sole force stabilizing calmodulin–protein interactions, then increasing ionic strength would be expected to facilitate calmodulin–protein interactions. There is evidence from recent proton NMR spectral studies that suggests that ionic interactions are involved in the Ca^{2+}-dependent association of calmodulin with troponin I (Evans & Levine, 1980) and that the specific amino acid residues that participate in these interactions are immediately adjacent to one of the hydrophobic binding sites on calmodulin (Klevit et al., 1981). On the basis of these data, along with data from photoaffinity cross-linking studies, which suggest that troponin I, myosin light chain kinase, and Ca^{2+}-sensitive cyclic nucleotide phosphodiesterase share a common calmodulin-binding domain (Andreasen et al., 1981), it is reasonable to expect that ionic interactions may be a general feature of many calmodulin–protein interactions.

Because the various short-range interactions can only come into play when the distance between interacting molecules is small, it would be expected that the primary, secondary, and tertiary protein structure of both calmodulin and myosin light chain kinase must not vary significantly from some optimal configuration in order that proper alignment of interacting amino acid residues might occur. Nairn et al. (1980) have concluded that the whole calmodulin molecule is necessary for activation of myosin light chain kinase, based on studies with proteolytic fragments of calmodulin. The idea that the integrity of calmodulin is required for activation is consistent with the concept that short-range interactions play an essential role in the activation process. Indeed, the chloride salts of NH_4^+, Li^+, and guanidinium [salts that are known to alter macromolecular secondary structure (von Hippel & Wong, 1964; von Hippel & Schleich, 1969)] may be inhibiting activation by disrupting or preventing the secondary and/or tertiary structure of either calmodulin, the enzyme, or both proteins, which is optimal for activation. Finally, the specificity of calmodulin-dependent enzymes such as myosin light chain kinase may be due to short-range interactions since hydrophobic interactions are relatively unspecific (Lesk & Chothia, 1980). Even though troponin C's from both skeletal and cardiac muscle show a high degree of sequence homology with calmodulin, they are not able to activate myosin light chain kinase (Walsh et al., 1980; Nairn et al., 1980). Thus, the calmodulin-specific activation of myosin light chain kinase and the highly conserved primary sequence of calmodulin might both be attributable to the obligatory contributions made by short-range interactions to calmodulin-dependent processes such as myosin light chain kinase activation.

The recent model proposed by Ross & Subramanian (1981) to explain the thermodynamic behavior of protein–protein interactions may be useful in conceptualizing the sequence of events that result in the activation of myosin light chain kinase by calmodulin. The first step of the activation process would involve binding of calmodulin to the enzyme and would be driven by hydrophobic interactions. These hydrophobic interactions would be Ca^{2+} dependent, since the hydrophobic domain at the site of interaction on calmodulin is only exposed when Ca^{2+} is bound to the protein. As a consequence of hydrophobic interactions between calmodulin and the enzyme, short-range interactions such as hydrogen bonding, ionic, and van der Waals interactions would become possible due to the juxtapositioning of appropriate amino acid residues. Although these short-range interactions probably cannot maintain the integrity of the calmodulin-enzyme complex in the absence of the hydrophobic interactions (i.e., in the absence of Ca^{2+}), they are probably responsible for strengthening the stability of the complex, conferring calmodulin specificity to the interaction, and inducing structural changes in the enzyme, which result in activation. Although this sequence of interactions is consistent with the thermodynamic data presented here, the molecular details of the interaction between calmodulin and its various binding proteins must await data from a variety of other techniques.

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References

Adelstein, R. S. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, 27–30.

Andreasen, T. J., Keller, C. H., LaPorte, D. C., Edelman, A. M., & Storm, D. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2782–2785.

Beaudette, N. V., & Langerman, N. (1980) *CRC Crit. Rev. Biochem. 9*, 145–170.

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

Charbonneau, H., & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun. 90*, 1039–1047.

Cheung, W. Y. (1980) *Science (Washington, D.C.) 207*, 19–27.

Corbin, J. D., & Reimann, E. M. (1975) *Methods Enzymol. 38*, 287–290.

Edelhoch, H., & Osborne, J. C. (1976) *Adv. Protein Chem. 30*, 183–250.

Evans, J. S., & Levine, B. A. (1980) *J. Inorg. Biochem. 12*, 227–239.

Frearson, N., & Perry, S. V. (1975) *Biochem. J. 151*, 99–107.

Jamieson, G. A., Bronson, D. D., Schachat, F. H., & Vanaman, T. C. (1980) *Ann. N.Y. Acad. Sci. 356*, 1–13.

Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakon, M., & Kosaki, G. (1981) *FEBS Lett. 126*, 203–207.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489–515.

Klevit, R. E., Levine, B. A., & Williams, R. J. P. (1981) *FEBS Lett. 123*, 25–29.

LaPorte, D. C., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814–3819.

Lesk, A. M., & Chothia, C. (1980) *Biophys. J. 30*, 35–47.

Means, A. R., & Dedman, J. R. (1980) *Nature (London) 285*, 73–77.

Mrwa, U., & Hartshorne, D. J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1564–1568.

Nairn, A. C., & Perry, S. V. (1979) *Biochem. J. 179*, 89–97.

Nairn, A. C., Grand, R. J. A., Wall, C. M., & Perry, S. V. (1980) *Ann. N.Y. Acad. Sci. 356*, 413–414.

Pires, E. M. V., & Perry, S. V. (1977) *Biochem. J. 179*, 89–97.

Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.

Salisbury, J. L., Condeelis, J. S., & Satir, P. (1980) *J. Cell Biol. 87*, 132–141.

Stull, J. T. (1980) *Adv. Cyclic Nucleotide Res. 13*, 39–93.

Stull, J. T., Blumenthal, D. K., & Cooke, R. (1980) *Biochem. Pharmacol. 29*, 2537–2543.

Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078–11080.

von Hippel, P. H., & Wong, K. Y. (1964) *Science (Washington, D.C.) 145*, 577.

von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 417–574, Marcel Dekker, New York.

Walseth, T. F., & Johnson, R. A. (1979) *Biochim. Biophys. Acta 526*, 11–31.

Walsh, M. P., Vallet, B., Autric, F., & Demaille, J. G. (1979) *J. Biol. Chem. 254*, 12136–12144.

Walsh, M. P., Vallet, B., Cavadore, J.-C., & Demaille, J. G. (1980) *J. Biol. Chem. 255*, 335–337.

Wang, J. H., & Waisman, D. M. (1979) *Curr. Top. Cell. Regul. 15*, 47–107.

Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501–4513.

Weiss, B., & Levin, R. M. (1978) *Adv. Cyclic Nucleotide Res. 9*, 285–304.

Wolff, D. J., & Brostrom, C. O. (1979) *Adv. Cyclic Nucleotide Res. 11*, 27–88.