Armstrong, C. M. (1975) *Q. Rev. Biophys. 7*, 179–210.
Barchi, R. L., Cohen, S. A., & Murphy, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1306–1310.
Barrantes, F. J. (1975) *Biochem. Biophys. Res. Commun. 62*, 407–414.
Beneski, D. A., & Catterall, W. A. (1980 *Proc. Natl. Acad. Sci. U.S.A. 77*, 639–643.
Benzer, T., & Raftery, M. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3634–3637.
Bigelow, C. (1967) *J. Theor. Biol. 16*, 187–211.
Cahalan, M. (1980) in *The Cell Surface and Neuronal Function* (Cotman, C. W., Poste, G., & Nicolson, G. L., Eds.) pp 1–47, Elsevier/North-Holland, Amsterdam.
Ellisman, M. E., & Levinson, S. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6707–6711.
Ellisman, M. E., Agnew, W. S., Miller, J. A., & Levinson, S. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4461–4465.
Ferguson, K. A., & Wallace, A. L. C. (1961) *Nature (London) 190*, 629.
Gordon, A. H. (1975) *Electrophoresis of Proteins in Polyacrylamide and Starch Gels*, pp 190s–191s, North-Holland Publishing Co., Amsterdam.
Hartshorne, R. P., & Catterall, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4620–4624.
Horowitz, M. I. (1977) in *Glycoconjugates* (Horowitz, M. I., & Pigman, W., Eds.) Vol. I, pp 15–34, Academic Press, New York.
Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Cotman, C. W., Poste, G., & Nicolson, G. L., Eds.) pp 191–260, Elsevier/North-Holland, Amsterdam.
Keynes, R. D. (1975) in *The Nervous System* (Tower, P. B., Ed.) Vol. 1, pp 165–175, Raven Press, New York.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Levinson, S. R. (1975) *Philos. Trans. R. Soc. London, Ser. B 270*, 337–348.
Levinson, S. R., & Ellory, J. C. (1973) *Nature (London), New Biol. 245*, 122–123.
Levinson, S. R., Curatalo, C. J., Reed, J. K., & Raftery, M. A. (1979) *Anal. Biochem. 99*, 72–84.
Liu, Y. T., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842–2848.
Moore, A. C., Agnew, W. S., & Raftery, M. A. (1982) *Biochemistry 21*, 6212–6220.
Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 9*, 819–831.
Nozaki, Y., Schecter, N. M., Reynolds, J. A., & Tanford, C. (1976) *Biochemistry 15*, 3884–3890.
Pitt-Rivers, R., & Impiombato, F. S. (1968) *Biochem. J. 109*, 825–830.
Reinhold, U. (1972) *Methods Enzymol. 25*, 244.
Reynolds, J. A., & Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002–1007.
Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 369–372.
Sigworth, F. J., & Spalding, B. C. (1980) *Nature (London) 283*, 293–295.
Swank, R. T., & Munkries, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 412–418, Wiley, New York.
Udenfried, S., Stein, S., Bohlen, P., Daerman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–873.

Ordered Phosphorylation of the Two 20 000 Molecular Weight Light Chains of Smooth Muscle Myosin†

A. Persechini‡ and D. J. Hartshorne*

ABSTRACT: The time courses of phosphorylation of the M_r 20 000 light chains by purified myosin light chain kinase plus calmodulin were determined. In confirmation of an earlier report [Persechini, A., & Hartshorne, D. J. (1981) *Science (Washington, D.C.) 213*, 1383–1385], a steady-state kinetic analysis indicates that the phosphorylation occurs in an ordered manner; i.e., at a phosphorylation level of 0.5 mol of ^{32}P incorporated per mol of bound M_r 20 000 light chain, each myosin molecule would have one phosphorylated head. The kinetic parameters obtained for the phosphorylation of the more reactive myosin head are similar to those determined by using isolated light chains. It is suggested that the ordered, or sequential, phosphorylation, and the different reactivities of the two M_r 20 000 light chains, is the result of preexisting asymmetry of the myosin molecule. Similar patterns of myosin phosphorylation are obtained in both the absence and presence of skeletal muscle actin.

One of the theories which has been proposed to account for the activation of the contractile apparatus in smooth muscle is based on the phosphorylation of the myosin molecule. Phosphorylation of the M_r 20 000 light chains is thought to allow activation by actin of the Mg^{2+}-ATPase activity of myosin, whereas in the dephosphorylated state myosin is not activated by actin [see reviews by Adelstein & Eisenberg (1980) and Hartshorne & Mrwa (1982)]. Although this theory has received much support, it is not accepted universally [cf. Nonomura & Ebashi (1980)], and unequivocal evidence that myosin phosphorylation–dephosphorylation forms the dominant regulatory mechanism is still to be presented. Consequently, each phase of the phosphorylation scheme continues to be analyzed, and this includes the phosphorylation reaction which obviously is obligatory for subsequent phos-

† From the Muscle Biology Group, Departments of Biochemistry and Nutrition and Food Science, University of Arizona, Tucson, Arizona 85721. *Received April 8, 1982; revised manuscript received October 12, 1982.* This work was supported by Grant HL 23615 from the National Institutes of Health.

‡ Present address: Department of Biochemistry, The University of Leicester, Leicester, LE1 7RH, England.

0006-2960/83/0422-0470$01.50/0

phorylation-induced events. However, most of the kinetic parameters for this reaction have been determined by using isolated light chains rather than intact myosin. The dissimilarity of these two substrates was pointed out by Mrwa & Hartshorne (1980), and it was shown that the time course of phosphorylation of myosin was not linear in contrast to an approximately linear rate for the isolated light chains. Subsequently, this discrepancy was explained by Persechini & Hartshorne (1981), who presented evidence to indicate that the phosphorylation of each of the two heads of myosin was not equivalent. It was suggested that the phosphorylation followed an ordered process in that the phosphorylation of the first myosin head was achieved much more readily than the phosphorylation of the second head. Our explanation for this effect was that the two myosin heads were subject to cooperative interactions. The ATPase activity also exhibited evidence of cooperativity, and it appeared that both heads of the molecule must be phosphorylated in order to achieve activation by actin (Persechini & Hartshorne, 1981).

In order to confirm that the phosphorylation of myosin does indeed follow a sequential pathway, it was necessary to examine the reaction in more detail, and to do this, we chose to analyze time courses of phosphorylation. These data, presented herein, confirm that phosphorylation is an ordered process. In addition to establishing this point, we were also concerned with clarifying the reason for the nonequivalent reaction of the two heads. There are two basic possibilities, which would both generate an ordered process. The first, and simplest possibility, is that initially the two heads are equivalent but that the phosphorylation of the first head hinders the phosphorylation of the second head; i.e., the sequential reaction is based on negative cooperative interactions between the two myosin heads. The second possibility is that the sequential phosphorylation of the two myosin light chains is due to a preexisting asymmetry of the myosin molecule resulting in different reactivities of two heads. The interpretation of the data presented in this report, in contrast to our earlier suggestion (Persechini & Hartshorne, 1981), favors the latter as the more likely reason for the ordered phosphorylation process.

Materials and Methods

Proteins were prepared by using the following procedures, with myosin light chain kinase from frozen turkey gizzards (Walsh et al., 1980), calmodulin from frozen bull testes (Autric et al., 1980), and skeletal muscle actin from rabbits (Driska & Hartshorne, 1975). Myosin was prepared as follows: frozen turkey gizzard mince was suspended in 3 volumes of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] (pH 7.6), 50 mM KCl, 2 mM EGTA, 15 mM $MgCl_2$, 0.2 mM dithiothreitol, and 3% (v/v) Triton X-100 (buffer A), homogenized by using either a Polytron (setting 11, 5 s, probe PT 20 ST) or a Waring blender (20 s), and centrifuged at 1500g for 5 min. The supernatant was discarded and the pellet subjected to another three cycles of homogenization and centrifugation with buffer A. The Triton X-100 was washed out of the muscle debris by repeating the homogenization–centrifugation sequence 3 times with buffer A minus Triton X-100. Extraction of myosin was achieved by suspending the pellet with the aid of the Polytron in 2 volumes (based on original muscle weight) of 40 mM imidazole (pH 7.1–7.2), 5 mM ATP, 4 mM EDTA, 2 mM EGTA, and 0.5 mM dithiothreitol. The supernatant following centrifugation at 14000g for 20 min was filtered through glass wool and the pellet discarded. $MgCl_2$ was added slowly, by using a peristaltic pump, to the supernatant to a final concentration of 150 mM, and ATP was added to 2.5 mM. The mixture was left for 10 min and centrifuged at 1500g for 10 min. The supernatant was filtered through glass wool and centrifuged overnight at 50000g. The resulting supernatant was diluted with 10 volumes of cold H_2O, and the precipitated myosin was collected by centrifugation at 11000g for 15 min. The pellet was suspended in approximately 1 volume of H_2O, using a Potter homogenizer to form a homogeneous opaque suspension. The following were then added in order: EGTA to 1.5 mM, sodium phosphate (pH 7.6) to 10 mM, $MgCl_2$ to 0.2 M, ATP to 5 mM. For the latter two reagents, a peristaltic pump was used. After 10 min, the mixture was centrifuged at 160000g for 3 h. The supernatant was diluted with 10 volumes of cold H_2O and the myosin collected by centrifugation at 11000g for 15 min. The pellet was dissolved in the minimum volume of 0.3 M KCl, 10 mM Tris-HCl (pH 7.6), and 0.2 mM dithiothreitol and dialyzed against this solvent. The final myosin solution was clarified, if necessary, by centrifugation at 25000g for 40 min. The average yield of myosin was 2.8 mg/g wet weight of gizzard. The purity of the myosin preparations was assessed as described previously (Persechini & Hartshorne, 1981), and preparations were not used if the purity was less than 95%. An additional indicator of the homogeneity of the myosin preparations was provided by the finding that 1.0 mol of ^{32}P could be incorporated (at high myosin light chain kinase concentrations) per mol of 20000-dalton light chain. The frozen turkey gizzards were obtained from Deaktor Meat Packing Co., Phoenix, AZ.

Proteins were monitored by electrophoresis on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ by using the discontinuous buffer system of Laemmli (1970). The level of prephosphorylation of myosin was checked by electrophoresis in alkaline urea gels (Perrie & Perry, 1970). Myosin was not used if prephosphorylation was detected.

Protein concentrations were determined by the biuret method (Itzhaki & Gill, 1964) or by dye binding as described by Spector (1978). The concentration of the M_r 20000 myosin light chain was calculated by assuming 4.26 nmol of light chain per mg of myosin.

Phosphorylation assays were carried out at 25 °C in 25 mM Tris-HCl (pH 7.45), 4 mM $MgCl_2$, approximately 50 μM $CaCl_2$, and 1 mM [γ-^{32}P]ATP (about 3000 cpm/nmol; New England Nuclear). All assays were initiated by the addition of ATP following a 10-min preincubation period at 25 °C. Other conditions are given in the figure legends. Aliquots (0.5 mL) were removed after various time intervals and assayed for acid-stable ^{32}P as described by Mrwa & Hartshorne (1980).

Data Analysis. Linear fits were obtained by least-squares analysis (nonweighted). Means and sample standard deviations were calculated by using standard formulas. Polynomials were generated by polynomial regression by using orthogonal polynomials. This process was carried out by computer with a commercially available program [Biomedical Computer Programs (P series); BMDP5R] as described by Dixon & Brown (1979).

Kinetic Approach

The overall approach was to obtain time courses of myosin phosphorylation from which relevant data were extracted by using two methods of analysis.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

First, data may be fit to an integrated form of the Michaelis–Menten equation [see Orsi & Tipton (1979)]:

$$\ln (S_r/S_i) = -(V_m/K_m)t \quad (1)$$

This form is valid only when K_m exceeds the effective substrate concentration, and the reaction is essentially first order with respect to substrate, i.e.

$$V = (V_m/K_m)S_r \quad (2)$$

[S_r = effective substrate remaining at time t; S_i = effective initial substrate concentration (t = 0)]. The fact that time course data taken at five different myosin concentrations fit the same curve verifies that rates had a first-order dependence on substrate concentration (see Figures 1 and 4).

Second, time course data may be fit by a polynomial expression of the form

$$P = a + bt + ct^2 + dt^3 \quad (3)$$

which may be differentiated to an expression of the form

$$V = dP/dt = b + 2ct + 3dt^2 \quad (4)$$

where P = moles of ^{32}P per mole of light chain. From eq 3 and 4, it is possible to calculate values for the velocity of the phosphorylation reaction (V) and the concentration of substrate remaining at any value of t.

An ordered phosphorylation model requires that segments of the reaction, above and below a level of 0.5 mol of ^{32}P/mol of light chain, are each kinetically homogeneous. Given this requirement, it can then be proposed that the ordered phosphorylation is generated either by negative cooperativity between a phosphorylated and a nonphosphorylated myosin head or by preexisting asymmetry of the myosin molecule (see Discussion for a more detailed consideration). Our data are consistent with the assumption that preexisting asymmetry produces an ordered phosphorylation. According to such a model, at t = 0 the total initial substrate concentration (S_0) consists effectively of two pools of substrate, S_I and S_{II}, each of which equals $S_0/2$. Each pool reacts separately, S_I from 0 to 0.5 mol of ^{32}P/mol of light chain and S_{II} from 0.5 to 1.0 mol of ^{32}P/mol of light chain. During the first phase of the reaction, the total substrate concentration at any time (S_t) is the sum of substrate from both reactant pools. The effective concentration can be obtained by correcting for the substrate in the second, as yet unreacted, pool.

$$S_D = S_t - S_{II} \quad (5)$$

S_D = the effective substrate concentration, S_t = the total substrate concentration at time t, and S_{II} is simply $S_0/2$. During reaction of the second head, S_t includes substrate from only the second pool, and no correction is necessary; therefore, S_t equals the effective substrate concentration.

We need only consider the concentration of myosin light chain since the concentration of the other substrate, ATP, is essentially constant over the time course examined ([ATP] = 0.9–1.0 mM).

Results

First Head. The myosin used in the following experiments was not prephosphorylated as determined by alkaline urea gel electrophoresis. Therefore, at t = 0, $S_I = S_0/2 = S_D$. Once the reaction is initiated phosphorylation causes an increasing deviation between S_I and S_D, and for any specified time point, S_D must be calculated. Phosphorylation time courses were determined at five myosin concentrations and were superimposable if expressed as moles of ^{32}P per mole of M_r 20000 light chain vs. time. This suggests that over this concentration range the reaction was first order with respect to substrate concentration. Therefore, data plotted according to eq 1 should produce a straight line. There are, however, two ways in which this can be done. First, if the total substrate concentration and the total initial substrate concentration are used, then ln S_t/S_0 is plotted against time. Contrary to the simplest expectation, this plot is curved (Figure 1A), suggesting that a first-order process as a function of total substrate concentration is not a realistic description of myosin phosphorylation. However, if phosphorylation is ordered, then a correction, as described under Kinetic Approach, must be applied for the substrate which cannot react during the first segment of phosphorylation (i.e., phosphorylation of the first head). This represents the second method of presenting the data, and as shown in Figure 1A, a plot of ln S_D/S_I vs. time is linear. A relationship of this type supports the idea that the phosphorylation of myosin is ordered. Under the conditions normally used for the phosphorylation assays, myosin is in an aggregated form, and the possibility was considered that the sequential phosphorylation is induced as a result of myosin aggregation rather than being a property of the individual myosin molecule. To test this hypothesis, we carried out phosphorylation time courses under conditions where myosin is soluble, i.e., in 0.3 M KCl. Values of S_t and S_D were calculated and plotted as before, as shown in Figure 1B. Similar to the results obtained at lower salt concentrations, a first-order dependence only on S_D is demonstrated. For this reason, it is unlikely that the ordered phosphorylation process, at least with respect to the phosphorylation of the first myosin head, is generated as a direct consequence of myosin–myosin interactions.

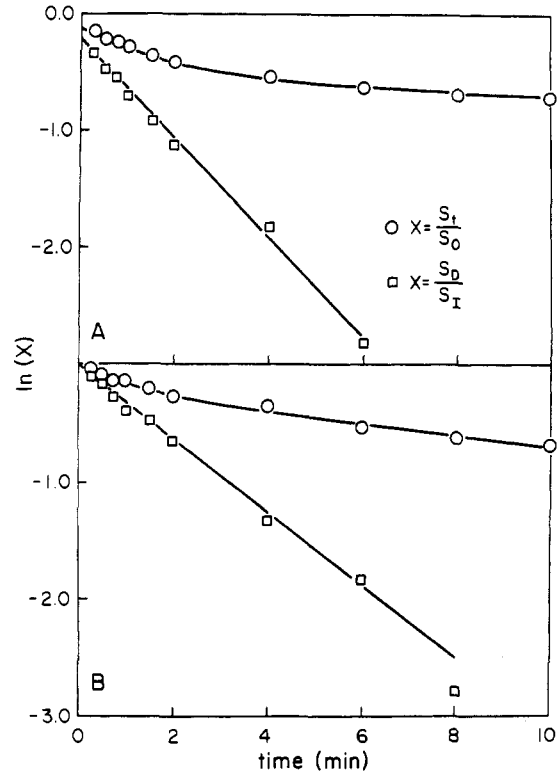

FIGURE 1: (A) Plot of natural logarithm of the fraction of the effective initial substrate remaining vs. time. The fractional substrate concentration at the indicated times was calculated in two ways (see text) to give S_t/S_0 or S_D/S_I. Each data plot is the mean of values taken from five time coures (S_0 = 0.84, 1.68, 2.52, 3.36, and 4.2 μM bound M_r 20000 light chain). The sample standard deviation was ≤14%. For assay conditions, see Materials and Methods. Other conditions: myosin light chain kinase, 3.3 nM; calmodulin, 10 nM; KCl, 60 mM. (B) Same for (A) except that KCl concentration was 300 mM and calmodulin concentration was 65 nM.

The phosphorylation reaction at low ionic strength frequently showed a nonzero intercept in the first-order plots as

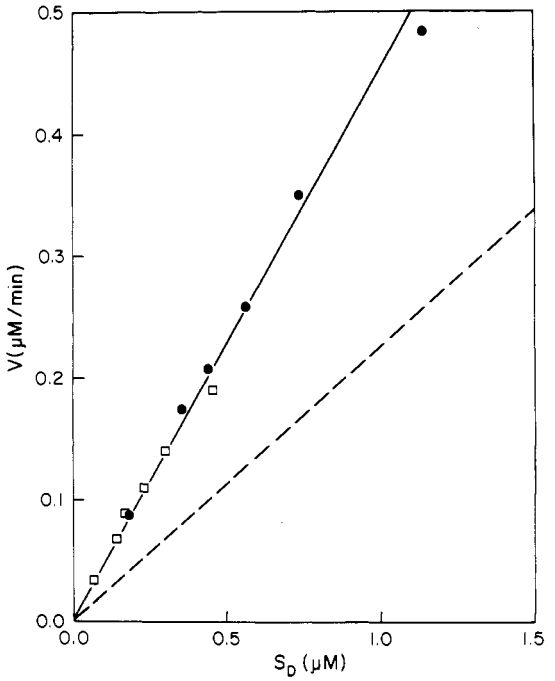

FIGURE 2: Plot of velocity vs. corrected substrate concentration (S_D). Values were derived from a polynomial expression, generated by using the data points in Figure 1A, and replotted as moles of ^{32}P per mole of M_r 20000 light chain vs. time (polynomial not shown). S_0 = 4.2 (●) or 1.68 (□) μM bound M_r 20000 light chain. The dashed line indicates a plot of velocity vs. S_D', i.e., the results expected for the negative cooperativity model.

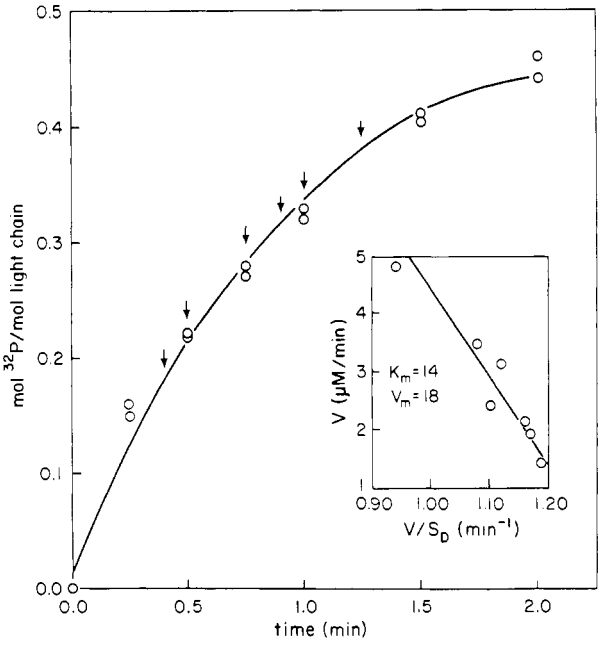

FIGURE 3: Phosphorylation time course for a reaction of the first half of the total M_r 20000 light chain population. The solid line is described by a third-order polynomial. S_0 = 10 μM bound M_r 20000 light chain; myosin light chain kinase, 10 nM; calmodulin, 30 nM; KCl, 60 mM. The relationship between velocity and S_D is shown in the inset as an Eadie–Hofstee plot. Velocity and corresponding values of S_D were calculated from the polynomial at the times indicated by the arrows. The initial values (t = 0) of velocity and S_D were determined and also included.

seen in Figure 1A. The deviation from linearity was variable and was due to an initial rapid phosphorylation of the myosin light chains. The size of this rapidly reacting pool usually was equivalent to between 5 and 10% of the total light chain sites. An explanation for this phenomenon is not available, but since gizzard myosin is quite labile, these anomalous sites could be generated by a conformational change of myosin, possibly as a result of limited proteolysis. Since the magnitude of this effect was quite small and its occurrence did not alter the interpretation of our results, it was not investigated further. Why this deviation from linearity is not found in the phosphorylation reactions carried out at higher ionic strength (Figure 1B) is not known.

For a simple first-order Michaelis–Menten process, a plot of reaction velocity (V) vs. S_D should also be linear (see eq 2), and this is verified in Figure 2. (Values of V and S_D were derived from a polynomial expression fit to the data shown in Figure 1A). The slope of the plot gives a value for V_m/K_m of 0.45 min^{-1}, which agrees well with the value of 0.40 min^{-1} obtained from Figure 1A. A comparison of these two values of V_m/K_m allows a choice to be made regarding the two possible models responsible for the ordered phosphorylation process. Similar values from the two types of plot are obtained only if the model based on preexisting asymmetry is used (see Discussion).

In order to obtain more accurate values of K_m and V_m, we carried out time course experiments at higher protein concentrations, which were close to the practical limit. These data were then fit by a polynomial (Figure 3, solid line). At the times indicated by the arrows, and at t = 0, values of S_D and V were calculated, and an Eadie–Hofstee plot of these data is shown in the inset of Figure 3. Values of K_m and V_m were calculated and were 14 μM and 18 μM/min, respectively. A double-reciprocal plot of the same data (not shown) gave a K_m of 17 μM and a V_m of 21 μM/min. It is essential that the corrected substrate concentration (S_D) be used in these plots. Use of the uncorrected substrate concentration (S_t) results in negative, and therefore meaningless, values for K_m and V_m. This was also found to be true for an analysis of the phosphorylation reaction at high salt (data not shown).

Values of k_{cat} and k_{cat}/K_m were calculated from the above data, assuming a molecular weight of 130000 for the myosin light chain kinase, and were 1800–2100 min^{-1} and 110–150 min^{-1} μM^{-1}, respectively. The value of k_{cat}/K_m agrees well with those obtained from Figure 1A, i.e., 122 min^{-1} μM^{-1}, and from Figure 2, i.e., 136 min^{-1} μM^{-1}.

The data presented so far are consistent with the supposition that the phosphorylation of myosin follows an ordered sequence, generated by a preexisting nonequivalence of the two myosin heads. It is interesting that the kinetic parameters obtained from the analysis of the phosphorylation of the first head are similar to those obtained with isolated light chains [see Hartshorne (1982) and Walsh & Hartshorne (1982)].

Second Head. Since the phosphorylation of the second head should occur only when all of the first reactant pool is exhausted (S_D = 0), the kinetics of phosphorylation would be expected to depend simply on S_t; i.e., only one type of substrate should be present. However, it was found that the analysis of the data for the second head was more complicated than that for the first head, and a simple kinetic scheme does not entirely fit the observed phosphorylation profiles.

Phosphorylation data from time courses at five myosin concentrations were fit to a polynomial (Figure 4), and from this curve, values of V and S_t were calculated at the indicated time points. A plot of ln S_t/S_{II} vs. time is shown in Figure 5, and this indicates that at least two first-order processes are involved. One process describes the phosphorylation between 0.5 and 0.7 mol of ^{32}P/mol of light chain, and the second process accounts for the phosphorylation of the remaining sites. In the inset of Figure 5, double-reciprocal plots of V vs. S_t are

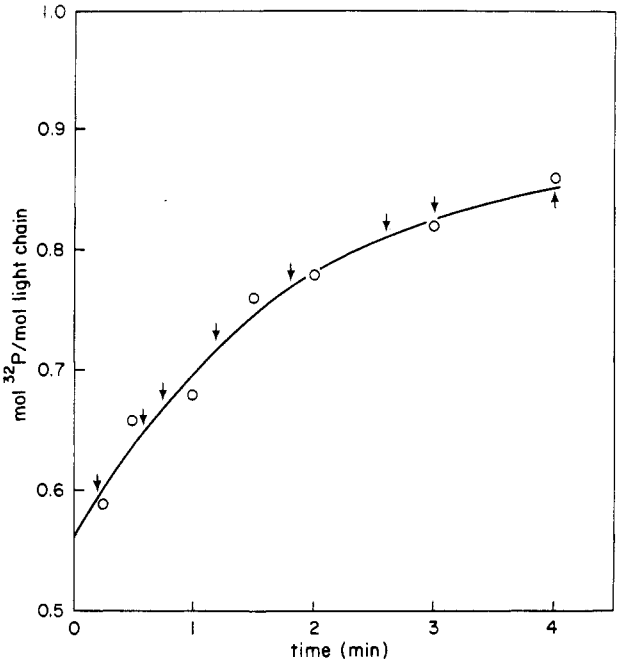

FIGURE 4: Phosphorylation time course for a reaction of the second half of the total M_r 20000 light chain population. The solid line is described by a third-order polynomial. Each data point is the mean of values taken from five phosphorylation time courses (S_0 = 0.84, 1.68, 2.52, 3.36, and 4.2 μM bound M_r 20000 light chain). The sample standard deviation was ≤5%. Myosin light chain kinase, 116 nM; calmodulin, 350 nM; KCl, 60 mM. Values of V and S_t were calculated at the time points indicated by the arrows, and included in Figure 5.

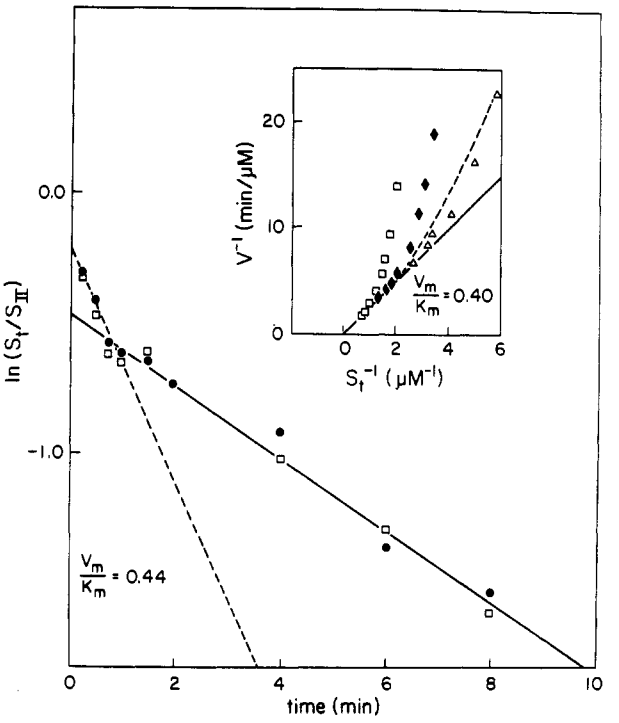

FIGURE 5: Plot of ln S_t/S_{II} vs. time. S_0 = 8 (□) or 10 (●) μM bound M_r 20000 light chain. The dashed line describes the phosphorylation up to about 0.70 mol of ^{32}P/mol of M_r 20000 light chain. The solid line describes the phosphorylation of the remaining sites, up to ~0.90 mol of ^{32}P/mol. The inset shows a double-reciprocal plot of values for velocity and S_t, calculated from the polynomial in Figure 4. Arrows (in Figure 4) indicate points in the time course where data were taken. S_0 = 0.84 (△), 1.68 (♦), and 3.36 (□) μM bound M_r 20000 light chain.

shown. The values obtained at different substrate concentrations only fit a single linear double-reciprocal plot at phosphorylation levels below 0.7 mol/mol of light chain. Subsequent phosphorylation follows more complex kinetics, possibly involving some form of product inhibition. However, if this occurs, the inhibition is neither simple competitive nor simple noncompetitive since plots of V^{-1} against S_t^{-1} at constant product concentration (i.e., constant inhibitor concentration) are curved. This is illustrated as the dashed line in the inset of Figure 5.

If it is accepted that phosphorylation between 0.5 and 0.7 mol of ^{32}P/mol of light chain follows simple Michaelis–Menten kinetics, then a value for V_m/K_m of 0.39 min^{-1}, or a k_{cat}/K_m of 3.4 min^{-1} μM^{-1}, can be estimated from Figure 5. These values are only approximate and are given merely to emphasize the differences between the phosphorylation of the first and of the second head of myosin.

Effect of Actin. If the patterns of phosphorylation described above are to be of physiological significance, then the effect of actin must also be considered. This is addressed in Figure 6. Phosphorylation of myosin at a fixed time point and at varying concentrations of myosin light chain kinase was measured in the presence (at a 6-fold molar excess over myosin) and absence of skeletal muscle actin. No significant difference is apparent. The time course of phosphorylation in the presence and absence of actin, shown in the inset of Figure 6, also did not differ significantly. Similar results suggesting that actin does not influence phosphorylation were also obtained by using isolated light chains as the substrate (results not shown).

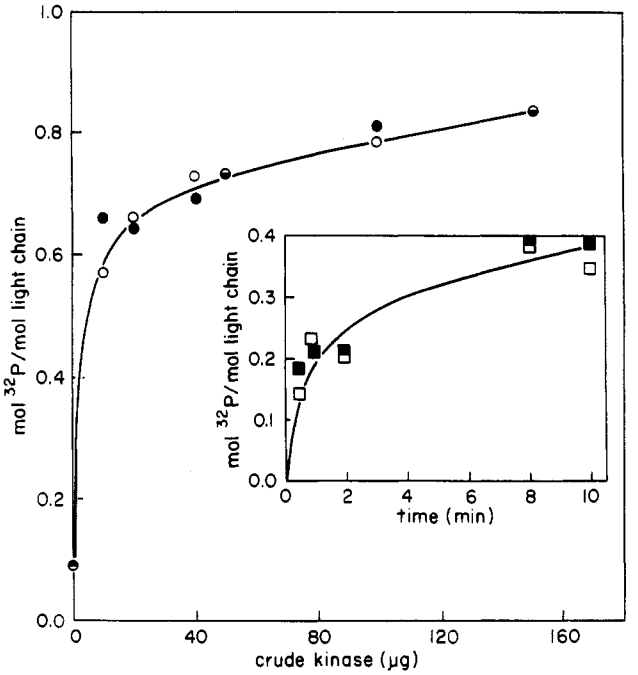

FIGURE 6: Plot of myosin phosphorylation at various concentrations of crude myosin light chain kinase in the absence (●) and presence (○) of skeletal muscle actin (6 μM). Data were taken at a fixed time point (10 min). Myosin concentration was 1 μM. The inset shows a time course of phosphorylation in the absence (■) and presence (□) of skeletal muscle actin (6 μM). Myosin, 0.42 μM; myosin light chain kinase, 3.5 nM; calmodulin, 11 nM; KCl, 60 mM.

Discussion

Our observations are not consistent with the simplest possible model in which reaction of the entire light chain substrate pool can be described by a single Michaelis–Menten process. As an alternative, we have suggested that phosphorylation of smooth muscle myosin is an ordered process, and this can be generated as a result of two possibilities. One is that the two

heads are initially equivalent and that the reduced reactivity of a second head results from phosphorylation of the first (i.e., negative cooperativity); a second possibility is that myosin is asymmetric with respect to its heads with the result that one head is preferentially phosphorylated. These two models are very similar from a kinetic point of view. In each case, the kinetics of light chain phosphorylation would consist of two distinct segments, which would correspond to phosphorylation of the two myosin heads. Therefore, the first reaction segment would be expected to depend not on the total remaining substrate (S_t) but on the effective substrate concentration (S_D) which would include only the more reactive first head. However, the value obtained for S_D depends upon which of the two ordered models the calculations are based. The effective substrate concentration (S_D') predicted by using the model based on negative cooperativity is defined as

$$S_D' = 2S_t - S_0 \quad (6)$$

It can be seen that initially $S_D' = S_t = S_0$ and that as the reaction progresses S_D' and S_t become further apart. On the other hand, a model based on preexisting asymmetry and initial substrate inequality requires that the effective substrate concentration (S_D) be defined

$$S_D = S_t - S_0/2 \quad (7)$$

It can be seen that while initially $S_t = S_0$, S_D is only half this value ($S_0/2$ or S_I). It is also apparent that at any time during the reaction of the first head

$$S_D = S_D'/2 \quad (8)$$

This difference between the two models can be used to determine which is the correct description of myosin phosphorylation. The analysis is simplified if fairly low substrate concentrations are used so that the reaction is essentially first order.

First-order Michaelis–Menten processes can be described by either of two linear equations. One is a logarithmic plot (eq 1); the other is a reduction of the familiar Michaelis–Menten equation (eq 2). Data plotted according to eq 1 clearly produce a straight line if we assume that an ordered model, based on a preexisting asymmetry, applies (see Figure 1). However, use of the alternative model, based on negative cooperativity, will produce exactly the same line, since at any level of phosphorylation the ratio of S_r to S_i reduces to the same expression $[(2S_t - S_0)/S_0]$ for either possibility. This is because there is a factor of 2 difference not only in S_r (S_D or S_D') but also in S_i (S_I or S_0). Data plotted according to eq 2, however, produce different curves for the two models (see Figure 2) since the slope (V_m/K_m) depends directly on S_r. The rationale that was followed was that the correct model should produce an agreement in the values of V_m/K_m obtained from data plotted according to eq 1 or eq 2. If data are plotted according to eq 2 and a model based on preexisting asymmetry is assumed (Figure 2, solid line), then a value for V_m/K_m of 0.45 min^{-1} is obtained, and this compares favorably with a value of 0.40 min^{-1} derived from Figure 1. If a model based on negative cooperativity is assumed, then a plot of S_D' against V would be as indicated by the dashed line in Figure 2. The value of V_m/K_m calculated from the slope of this line would obviously not be consistent with the value obtained from Figure 1. From this evidence, it can be argued that an ordered model based on preexisting asymmetry is an adequate description of myosin phosphorylation.

While the reaction of the first light chain can be described in terms of a simple ordered model, the phosphorylation of the second site follows more complex kinetics. The reason for the apparent complexity is not understood. If it is assumed that the phosphorylation of myosin represents a composite of two first order reactions, one for each head, then with the parameters calculated from our data (k_{cat}/K_m for the first head is approximately 40-fold higher than that for the second head) one would expect at longer time intervals that the slow phase of phosphorylation would begin to contribute to the total extent of phosphorylation. This would be evident by a downward concavity in the linear plots of Figure 1.[2] That this does not occur indicates either that the kinetic parameters derived for the second head are not reliable or that the phosphorylation of each site is exclusive, i.e., not a composit of two first-order reactions. With regard to the latter, it is possible that the initial difference in reactivity of the two heads is enhanced by phosphorylation of the first site, and, for example, could generate an increased K_m for the second site. These possibilities are not resolved and since they are pertinent to the mechanism underlying sequential phosphorylation should be studied further. Despite our lack of success in describing the entire reaction, we feel that the analysis of the phosphorylation of the more reactive light chain is the more important component and is adequate to identify an ordered phosphorylation reaction.

In an earlier paper (Persechini & Hartshorne, 1981), we suggested that the different reactivities of the two heads resulted from negative cooperative interactions. Our present preference is that cooperative interactions between the two heads probably exist prior to phosphorylation and these dictate an ordered phosphorylation sequence. Whether or not there is additional negative cooperativity between the phosphorylated and nonphosphorylated heads cannot be answered at this time.

The concept of preexisting asymmetry, if accepted, can alter the way in which we conventionally think about the functioning of the two myosin heads. The idea is not new, and for a more detailed discussion, the review of Schaub & Watterson (1981) is recommended. What induces the asymmetry? A number of factors could be involved, including structural constraints or cooperative interactions between the myosin heads to alter nucleotide or cation binding. It is unlikely that the asymmetry is induced as a result of binding to actin since the phosphorylation profiles of myosin are similar in the presence and absence of actin. But with respect to the phosphorylation reaction it appears that the asymmetry inhibits the phosphorylation of the second head, rather than enhancing the reactivity of the first site. This prediction is based on the observation that isolated light chains follow phosphorylation kinetics that are much closer to the first attached site than to the second. Kinetic parameters obtained for the phosphorylation of isolated light chains by using myosin light chain kinases isolated from a variety of muscle sources range from 5 to 50 μM for K_m and from 290 to 3900 min^{-1} for k_{cat} (Hartshorne, 1982; Walsh & Hartshorne, 1982).

Based on the assumption of ordered phosphorylation, it is to be expected that the myosin in which only one site is phosphorylated would be more prevalent than fully phosphorylated myosin. It is certainly our experience that unless precautions are taken to eliminate myosin light chain kinase activity during the isolation of myosin the product is frequently phosphorylated at one site. This point emphasizes the need to determine preexisting levels of phosphorylation before attempting any kinetic analyses. It is probably also an important consideration when preparing proteolytic subfragments of myosin since it is thought that light chain phosphorylation influences the proteolysis of the heavy chain (Okamoto &

[2] We thank the reviewers for pointing this out to us.

Sekine, 1981a,b; Ritz-Gold et al., 1980; Pemrick et al., 1980).

There is very little published data with which our results can be compared. Most of the previous studies on the phosphorylation of smooth muscle myosin have been concerned with the effects of phosphorylation on actomyosin ATPase activity rather than with the kinetics of the phosphorylation reaction itself. An exception to this is the recent work of Ikebe et al. (1982), who suggested, in agreement with our results, that the phosphorylation of gizzard myosin is an ordered process but that the phosphorylation of heavy meromyosin is random. The latter point was made also by Sellers & Adelstein (1982). Using various smooth muscle fiber preparations, it is well established that the initiation of tension is associated with an increase in myosin phosphorylation. However, the phosphorylation levels of myosin in resting and contracting muscles are so variable [cf. Aksoy et al. (1982), Bárány & Bárány (1981), Butler & Siegman (1982), Cassidy et al. (1981), and Silver & Stull (1982)] that it is not possible at this time to define the kinetics of phosphorylation in these fiber preparations.

The possibility that the observed phosphorylation profiles are directed by some factor other than an ordered mechanism should also be considered. An alternative explanation is that two separate populations of myosin molecules exist with different phosphorylation kinetics. However, there is no evidence for the existence of two classes of phosphorylation sites on the isolated light chains, and the M_r 20 000 light chain appears to be chemically homogeneous as evidenced from its primary structure reported recently by Maita et al. (1981). From our earlier results (Persechini & Hartshorne, 1981), it was suggested that both heads of the myosin molecule must be phosphorylated in order to allow actin-activated ATPase activity. Should myosin isozymes exist, they must be present in a 1:1 ratio, and considering this with the ATPase data, it would mean that the more reactive isozyme has no ATPase activity, whereas the less reactive isozyme possesses all of the activity. Obviously this situation is not likely, and the possibility that the phosphorylation reaction is directed by different myosin isozymes is not a reasonable alternative to the ordered phosphorylation mechanism.

Acknowledgments

We thank Dr. M. P. Walsh for purified myosin light chain kinase and calmodulin, S. Hinkins for technical assistance, and M. Celi for clerical help.

Registry No. Myosin light chain kinase, 51845-53-5.

References

Adelstein, R. S., & Eisenberg, E. (1980) *Annu. Rev. Biochem. 49*, 921–956.

Aksoy, M. O., Murphy, R. A., & Kamm, K. E. (1982) *Am. J. Physiol. 242*, C109–C116.

Autric, F., Ferraz, C., Kilhoffer, M.-C., Cavadore, J.-C., & Demaille, J. G. (1980) *Biochim. Biophys. Acta 631*, 139–147.

Bárány, M., & Bárány, K. (1981) *Am. J. Physiol. 241*, H117–H128.

Butler, T. M., & Siegman, M. J. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 204–208.

Cassidy, P. S., Kerrick, W. G. L., Hoar, P. E., & Malencik, D. A. (1981) *Pfluegers Arch. 392*, 115–120.

Dixon, W. J., & Brown, M. B. (1979) in *Biomedical Computer Programs* (P-series), pp 444–452, University of California Press, Los Angeles, CA.

Driska, S., & Hartshorne, D. J. (1975) *Arch. Biochem. Biophys. 167*, 203–212.

Hartshorne, D. J. (1982) in *Vascular Smooth Muscle: Metabolic, Ionic, and Contractile Mechanisms* (Crass, M. F., III, & Barnes, C. D., Eds.) pp 135–161, Academic Press, New York.

Hartshorne, D. J., & Mrwa, U. (1982) *Blood Vessels 19*, 1–18.

Ikebe, M., Ogihara, S., & Tonomura, Y. (1982) *J. Biochem. (Tokyo) 91*, 1809–1812.

Itzhaki, R. F., & Gill, D. M. (1964) *Anal. Biochem. 9*, 401–410.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Maita, T., Chen, J.-I., & Matsuda, G. (1981) *Eur. J. Biochem. 117*, 417–424.

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

Nonomura, Y., & Ebashi, S. (1980) *Biomed. Res. 1*, 1–14.

Okamoto, Y., & Sekine, T. (1981a) *J. Biochem. (Tokyo) 89*, 697–700.

Okamoto, Y., & Sekine, T. (1981b) *J. Biochem. (Tokyo) 90*, 833–842.

Orsi, B. A., & Tipton, K. F. (1979) *Methods Enzymol. 63*, 159–183.

Pemrick, S. M., Schneiderman, S., & Stracher, A. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2043.

Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31–38.

Persechini, A., & Hartshorne, D. J. (1981) *Science (Washington, D.C.) 213*, 1383–1385.

Ritz-Gold, C. J., Cooke, R., Blumenthal, D. K., & Stull, J. T. (1980) *Biochem. Biophys. Res. Commun. 93*, 209–214.

Schaub, M. C., & Watterson, J. G. (1981) *Biochimie 63*, 291–299.

Sellers, J. R., & Adelstein, R. S. (1982) *Biophys. J. 37*, 262a.

Silver, P. J., & Stull, J. T. (1982) *J. Biol. Chem. 257*, 6145–6150.

Spector, T. (1978) *Anal. Biochem. 86*, 142–146.

Walsh, M. P., & Hartshorne, D. J. (1982) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 3, Academic Press, New York (in press).

Walsh, M. P., Cavadore, J.-C., Vallet, B., & Demaille, J. G. (1980) *Can. J. Biochem. 58*, 299–308.