Phosphorylation Reaction of Vertebrate Smooth Muscle Myosin: An Enzyme Kinetic Analysis†

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ABSTRACT: Phosphorylation of vertebrate smooth muscle myosin or its isolated 20 000-dalton light chains by myosin light-chain kinase (MLCK) was found to follow first-order kinetics not only at low ($[M] \ll K_m$) but also at high ($[M] \geq K_m$) substrate concentration. This observation can most simply be explained by a product inhibition for which the Michaelis constants (K_m) of the enzyme for the substrate (dephosphorylated myosin) and for the product (phosphorylated myosin) are approximately the same. For such a case, integration of the kinetic velocity equation gives an exponential formula similar to that of a true first-order reaction, the only difference being that its rate constant (k) depends additionally on the initial substrate concentration ($[M]_0$). The standard kinetic constants (k, K_m, V_{max}) have been calculated by using this pseudo-first-order relationship. Independent evidence for the validity of the derived kinetic relationship was obtained from binding studies with myosin and MLCK. These showed that MLCK binds to phosphorylated and dephosphorylated myosin with approximately equal affinity ($K_s = 30 \times 10^{-9}$ M). The possible applicability of the same kinetic relationship to other enzyme systems is discussed.

With a few exceptions [Ebisawa, 1983; for further references, see Ebashi et al. (1982) and Marston (1982)], it is now generally accepted that activation of vertebrate smooth muscle at the actomyosin level occurs as a result of a Ca-dependent phosphorylation of myosin [see reviews by Adelstein & Eisenberg (1980), Small & Sobieszek (1980), Hartshorne & Siemankowski (1981), and Perry (1983)]. The key regulatory enzyme involved in this molecular "switch", the myosin light-chain kinase (MLCK), has been purified and partially characterized (Dabrowska et al., 1978; Small & Sobieszek, 1980; Adelstein & Klee, 1981; Walsh et al., 1982; Uchiwa et al., 1982; Malencik et al., 1982). As its name implies, the myosin light-chain kinase specifically phosphorylates one of the myosin light chains, "P" light chain, which is common to vertebrate muscle and nonmuscle myosins. There are close similarities in the activation mechanisms of all MLCKs isolated (Blumenthal & Stull, 1980; Crouch et al., 1981; Hathaway & Adelstein, 1979; Sobieszek & Barylko, 1984) although, aside from smooth muscle, a regulatory role for light-chain phosphorylation has only been established in nonmuscle systems (Scordilis & Adelstein, 1977; Trotter & Adelstein, 1979; Scholey et al., 1981; Barylko & Sobieszek, 1983). Similar to some other regulatory enzymes (Klee et al., 1979; Cox et al., 1981; Malnoë et al., 1982; Burger et al., 1982), the activation of MLCK requires the presence of Ca^{2+} and calmodulin (Dabrowska et al., 1978; Adelstein & Klee, 1981; Sobieszek & Barylko, 1984). The involvement of an additional regulatory process has also been suggested from the finding that phosphorylation of the MLCK apoenzyme by the adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase reduces its affinity for calmodulin (Conti & Adelstein, 1981).

Even though some data on the properties of MLCK have been accumulated, the mechanism of the myosin phosphorylation reaction is far from being understood. This results in part from the fact that most of the studies have been carried out with whole light-chain preparations as a substrate rather than intact myosin (Walsh et al., 1979; Blumenthal & Stull, 1980; Adelstein & Klee, 1981). Thus, it is not clear whether the conclusions drawn from such studies can be applied to the in vivo situation. Not only is the native substrate (intact myosin) some 25 times larger but also the light chain itself must have a quite different configuration in vivo, when incorporated into the myosin molecule.

The present studies were carried out by using intact myosin in a filamentous form, which is the most native form of myosin, and the data were compared to those obtained with purified P light chain. A new interpretation of the mechanism of myosin phosphorylation has now been obtained by using these two systems. In contrast to the presently held view (Persechini & Hartshone, 1983; Sellers et al., 1983), it is shown that as a consequence of enzyme binding to the product, the myosin phosphorylation reaction is a first-order process. Phosphorylation then brings about a cooperative activation of smooth muscle actomyosin.

Materials and Methods

Actomyosin Extraction. Pig stomach or chicken and turkey gizzard muscles were used. Actomyosin and, subsequently, myosin were extracted with either normal or low MLCK content. Correspondingly, starting materials used were either myofibrils, prepared as described previously (Sobieszek & Bremel, 1975), or preextracted myofibrils from which a considerable amount of the kinase had been removed prior to actomyosin extraction. In the latter case, one or two washing cycles (Sobieszek & Bremel, 1975) were replaced by kinase extraction steps (Sobieszek & Small, 1977) followed by an additional washing in order to have approximately the same conditions for actomyosin extraction. The extraction of crude actomyosin was carried out exactly as described previously and actomyosin purified by divalent cation precipitation (Sobieszek & Small, 1976).

Purification of Myosin. To freshly extracted crude actomyosin (Sobieszek & Small, 1976) was added solid $MgCl_2$ up to 50 mM, and immediately after this had dissolved, the solution was saturated to 45% with ammonium sulfate added as a solid. The 45% precipitate, consisting mainly of actin-rich actomyosin, was then removed by centrifugation and the su-

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pernatant brought up to 60% saturation. The 60% pellet was collected by centrifugation and, as seen on sodium dodecyl sulfate (SDS) gels, consisted mainly of myosin and tropomyosin. This pellet was dissolved in buffer and clarified by 1–2 h of high-speed centrifugation (40000 rpm). Tropomyosin was then removed by dialysis of the mixture to 40 mM KCl and 10 mM imidazole, pH 6.8, under which conditions myosin is selectively precipitated. This resuspended myosin represented the first type of myosin used. Its MLCK content was relatively high, and it was therefore used only for progress curve experiments in which for each time point phosphorylation was initiated and terminated in a separate tube (see below).

Removal of MLCK from Myosin. The second type of myosin used had a low enough MLCK content to permit assays in which aliquots were withdrawn at each time point. This myosin was obtained by subjecting the first type of myosin to a gel filtration step on Sepharose 4B-CL. The myosin, at a concentration of 5–10 mg/mL, was dissolved and eluted with 0.6 M KCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 10 mM phosphate, pH 7.6, at a flow rate of not more than 3 mL cm^{-2} h^{-1}. The kinase activity of the fractions was assayed by using isolated P light chain as described previously [Sobieszek, 1977b; see also Sobieszek & Barylko (1985)]. The kinase-free myosin was collected and concentrated by dialysis against 20 mM KCl, pH 6.8, and imidazole buffer.

In the case of pig stomach, crude tropomyosin-containing myosin was normally subjected to the same gel filtration step. This step enabled separation of intact myosin from myosin with its light chain clipped and at the same time removed the protease responsible for this degradation (see Figure 1F,G).

The third type of myosin, practically free of the kinase, was obtained in a similar way except that before the gel filtration step about 80% of the kinase was removed by batch treatment with hydroxylapatite. Thus, to myosin dissolved in 0.5 M KCl and 10 mM imidazole, pH 6.5, was added 0.3 volume of suspended hydroxylapatite (Bio-Rad, Bio-Gel HTP). After 15–20 min of incubation and brief centrifugation, the hydroxylapatite-free supernatant was diluted 2 times with the "Sepharose 4B-CL" buffer and chromatographed as above.

Myosin Phosphorylation Assays. The radioactive ATP solution was made by 10–15-fold dilution of [γ-^{32}P]ATP (~3000 mCi/mmol, New England Nuclear) with cold ATP (Sigma, St. Louis, MO) at a concentration of 10–20 mM. It was stored at −70 °C, and its exposure to assay temperature (25 °C) was limited only to the assay time in order to minimize losses in specific activity.

The phosphorylation reaction was initiated by addition of an appropriate amount of the above [γ-^{32}P]ATP solution to make 1.5–2.5 mM final ATP concentration. Two phosphorylation assays were used. When phosphorylation was sufficiently slow and myosin concentration not higher than 15 mg/mL, aliquots were withdrawn at given time points from a single incubation mixture, applied to 2 × 4 cm pieces of Whatman 3MM chromatographic paper, and immediately dropped into 5% trichloroacetic acid (Cl_3CCOOH). The paper pieces were washed with several changes of Cl_3CCOOH, then rinsed in ethanol, and counted in water. It was found to be very convenient to count without scintillant, since for Cherenkov radiation, counting efficiency was only 2-fold less and the background counts were very low.

Progress curves were also obtained from a number of separate identical incubations, stopped at the time points indicated by addition of solid urea to make 8.5 M final concentration. Results were comparable to the Cl_3CCOOH-stopped reaction, indicating that the reaction was stopped by urea within approximately 1 s. After solubilization, aliquots were again applied to Whatman 3MM paper pieces, dropped into 5% Cl_3CCOOH, and processed as described above. When needed, a small amount (20–50 μL) of urea-containing assay mixture was kept and examined on urea/glycerol gel electrophoresis (P. Jertschin and A. Sobieszek, unpublished results).

Other Procedures. Smooth muscle myosin light-chain kinase, from pig stomach or gizzard (chicken or turkey) muscles, was purified as described in detail previously (Sobieszek & Barylko, 1985). Corresponding calmodulins were obtained as a byproduct of kinase purification. Following diethylaminoethyl (DEAE) chromatography (Sobieszek & Barylko, 1985), calmodulin-containing fractions were concentrated by addition of solid ammonium sulfate to 80% saturation and subjected to further gel filtration and ion-exchange purification steps.

Myosin light chains were isolated from purified myosin or actomyosin essentially according to Perrie & Perry (1970) with improvements relating to a larger scale purification (Sobieszek & Barylko, 1985). Protein concentration was measured by the biuret method. SDS gel electrophoresis was carried out according to Laemmli (1970) but using a 3% stacking gel and an 8–20% linear gradient in the separating gel.

Results

Myosin-Associated MLCK. Myosin isolated by the rapid method described carries much more associated kinase activity than is commonly observed in other preparations (Sellers et al., 1981, 1983; Persechini & Hartshorne, 1983; Ikebe et al., 1982; Cole et al., 1983; Kendrick-Jones et al., 1983), where activity is possibly reduced as a result of aging. Consequently, the development of a method to free enzyme from substrate was one of the major initial problems.

Following preextractions for the kinase, freshly isolated myosin is electrophoretically homogeneous (Figure 1A,C), but it is nevertheless fully phosphorylated on addition of Ca^{2+} within 15–30 s. Aging of the preparation naturally reduces the kinase activity. Since, however, enzyme kinetics require substrate free from enzyme, it was necessary to further purify the myosin chromatographically.

Application of myosin on a gel filtration column at high ionic strength, at slightly alkaline pH and with divalent cations removed, separates the kinase relatively well (Figure 2). Nevertheless, this relatively troublesome purification step does not produce myosin that is fully free of the kinase, its phosphorylation being completed without added MLCK within 4–12 min. Reconstitution of the purified kinase and myosin returns the phosphorylation rate to the initial levels (Figure 2, insert). A second application of this myosin on the same column entailing a further 3 days resulted in the removal of most but still not all of the kinase (detectable with 15–30-min incubation time) and led to a deterioration of the myosin. The difficulty associated with the removal of the kinase indicates that its affinity for myosin is very high and that the binding is of the equilibrium type.

As indicated under Materials and Methods, the most effective way of removing kinase was to use another method involving a rapid initial batch treatment with hydroxylapatite followed by a single gel filtration step. In the experiments described, myosin purified by one of the three different methods was used, according to the requirements of the experiment.

Interestingly, the kinase bound to myosin was associated with stoichiometric amounts of calmodulin. Its addition to any of the three types of myosin preparations did not increase

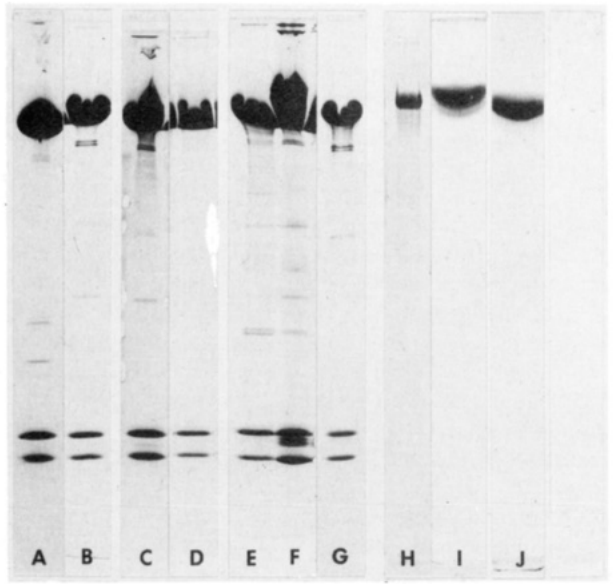

FIGURE 1: Representative SDS gels of myosin and myosin light-chain kinase preparations used. Note that gels A–G were run as described under Materials and Methods while gels H–J were run as in Sobieszek & Bremel (1975). (A) Chicken gizzard myosin after ammonium sulfate fractionation and tropomyosin removal. The two dense lower bands correspond to myosin light chains of 20000 and 17000 daltons, respectively. The two faint bands correspond to traces of tropomyosin. (B) Chicken gizzard myosin as in (A) after hydroxylapatite treatment followed by a gel filtration step on Sepharose 4B-CL. (C and D) Turkey gizzard myosin after the same purification steps as for (A) and (B), respectively. (E) Pig stomach crude myosin directly after ammonium sulfate fractionation. The doublet around the middle of the gel corresponds to tropomyosin. Pig stomach muscle tropomyosin migrates differently than that of gizzard (see gel A). (F) Same as (E) but after dialysis and sedimentation for tropomyosin removal. Two bands have appeared from slight proteolytic degradation of the regulatory light chain. (G) Pig stomach myosin as in (F) after a gel filtration step on Sepharose 4B-CL. The slightly degraded myosin is separated. (H and I) Myosin light-chain kinase preparations from pig stomach and chicken gizzard muscle, respectively.

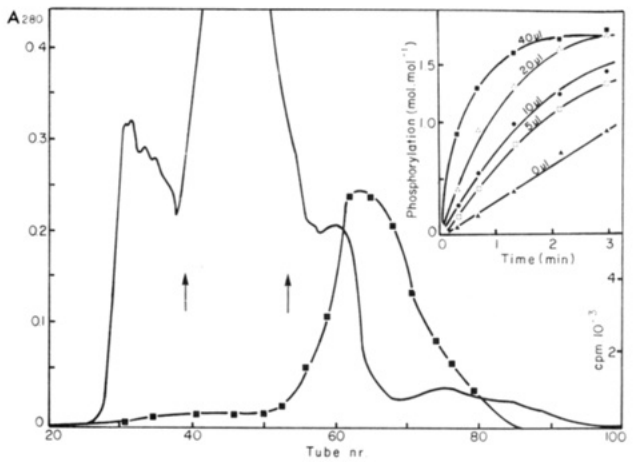

FIGURE 2: Separation of MLCK from turkey gizzard myosin on a Sepharose 4B-CL column. The elution profile detected at 280 nm is shown as a solid line while MLCK activity, detected as described under Materials and Methods, is shown by linked solid squares. The myosin obtained from the main peak (arrows) could be fully phosphorylated by addition of purified MLCK (insert), the rate of incorporation increasing with increasing amounts of kinase. However, the myosin itself had significant MLCK content as is shown by the lowest line (solid triangles; –0 µL) in the insert.

the existing phosphorylation rate, while addition of purified MLCK apoenzyme reduced the rate as would be expected for an unproductive enzyme/substrate complex. In the case of column-purified myosin, addition of very low concentrations

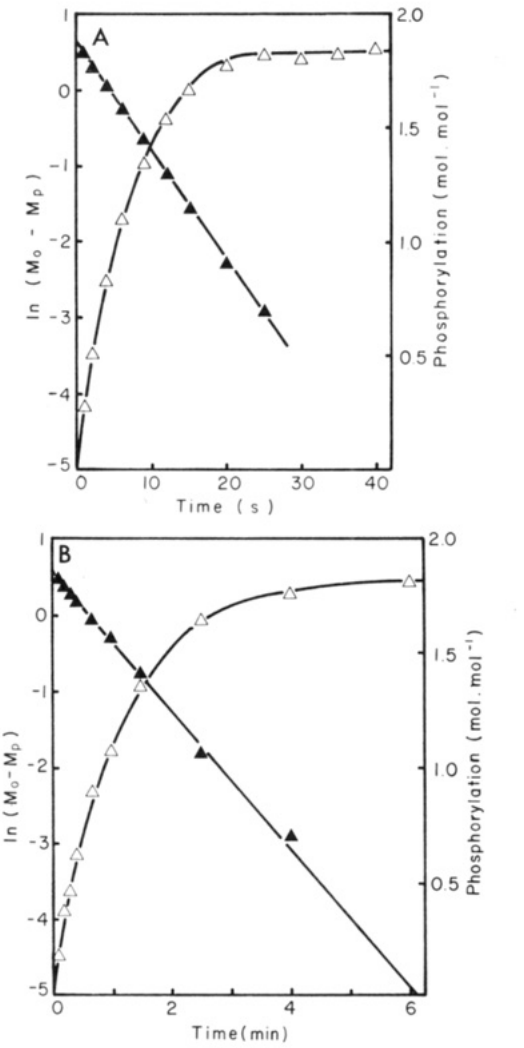

FIGURE 3: Phosphorylation progress curves for pig stomach myosin before (A) and after (B) the gel filtration step on Sepharose 4B-CL (see text). Myofibrils from which the myosin in (B) was obtained were preextracted for MLCK removal (see Materials and Methods). The myosin concentrations in (A) and (B) were 26 and 23 µM, respectively.

of the kinase apoenzyme did not produce inhibition but rather a small increase in phosphorylation rate. This indicates that this myosin retained calmodulin at levels over and above the levels of the residual kinase. Binding of calmodulin and the kinase apoenzyme to myosin was independent of Ca^{2+} concentration. The high affinity of this binding was most clearly observed when diluted myosin solutions at 0.3 M KCl were applied on trifluoperazine (TFP) and calmodulin affinity columns arranged in tandem. In both cases, small, probably stoichiometric amounts of myosin were bound to the columns. This myosin was eluted by an ethylene glycol bis(β-aminoethyl ester)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) containing solution together with calmodulin and the kinase, respectively, from the TFP and calmodulin columns.

Myosin Phosphorylation. The phosphorylation reaction is most conveniently followed by incubation of myosin with [γ-^{32}P]ATP and assaying the product (phosphorylated myosin). In assays of this type, the easiest way of obtaining elementary kinetic parameters is to use an integrated form of the velocity equation. For this reason, the time course of myosin phosphorylation for many different myosin preparations has been monitored.

It soon became apparent that one important characteristic of these progress curves was their exponential form (Figures

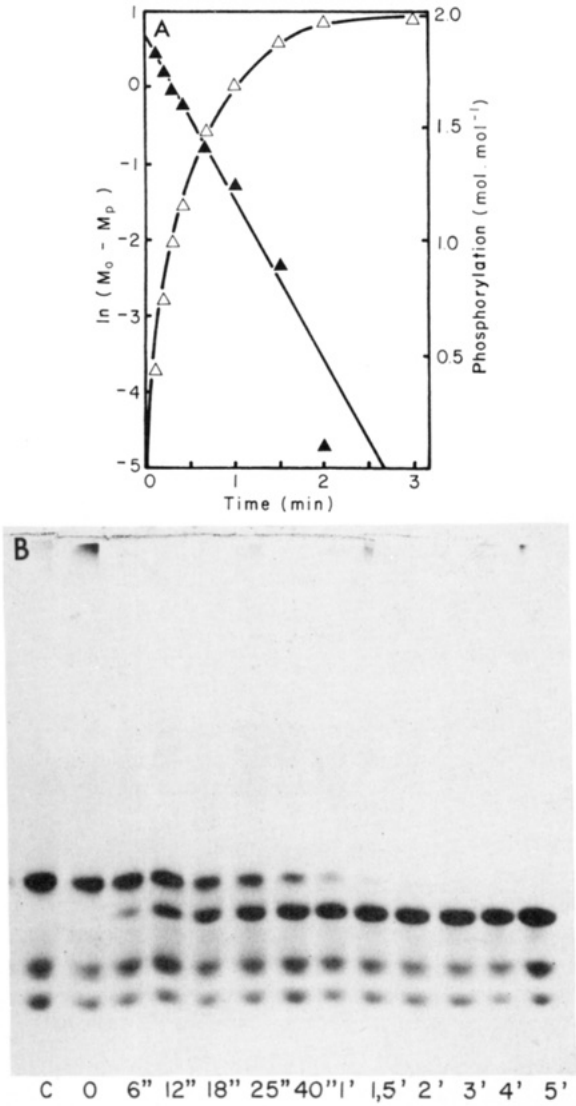

FIGURE 4: Phosphorylation progress curve for chicken gizzard myosin (A) of the same type as in Figure 3B. Panel B shows urea/glycerol gels of the myosins corresponding to the successive time points in (A). Note a gradual transfer of density of the upper band (dephosphorylated light chain) to the lower one (phosphorylated light chain). The two lower bands correspond to two types of 17 000-dalton light chains present in all our smooth muscle myosin preparations. "C" (control) is the same as "O" except at a somewhat higher loading.

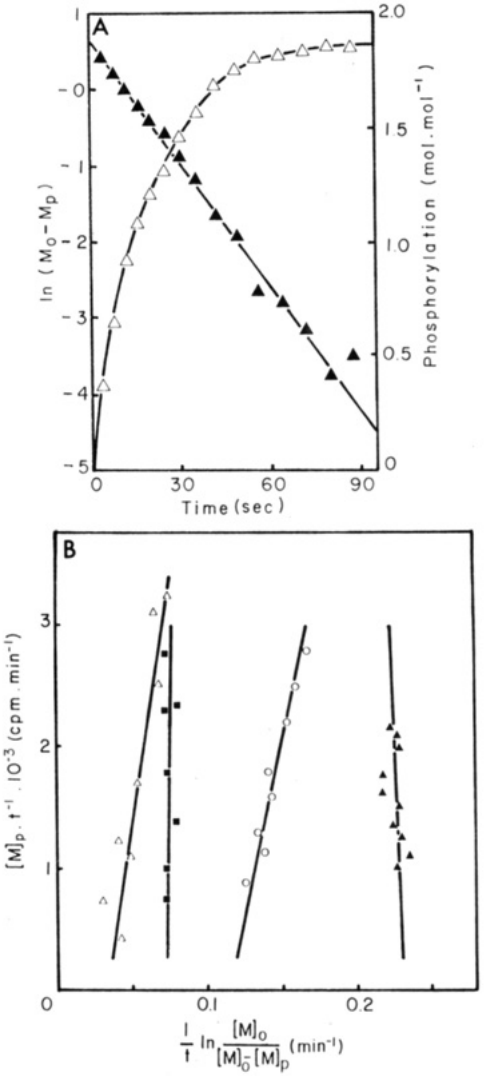

FIGURE 5: (A) Phosphorylation progress curve for chicken gizzard myosin at 150 μM, the highest concentration assayed. The myosin is of the same type as in Figure 3B. (B) Representative plots of the integrated Henri–Michaelis–Menten equation for phosphorylation of myosin from turkey (■) and chicken (▲) gizzard as well as from pig stomach (△ and ○) muscles. All myosin concentrations were in the range 20–40 μM.

3–5). Thus, plots of the logarithm of remaining substrate concentration (dephosphorylated myosin) vs. time were linear (Figures 3–5). Since the measured parameter is product, i.e., phosphorylated myosin, the experimental points fit a straight line most exactly at the shortest times and become progressively more scattered as the phosphorylation level gets closer to the maximal level. It is therefore important to estimate the maximum level of incorporation from the entire progress curve, and not just from the last few points. This level was estimated from an exponential best fit for a given set of experimental points and compared to the value calculated from the myosin concentration and specific activity of [γ-^{32}P]ATP.

Normally, the incorporation obtained was between 1.2 and 2.8 mol of P_i per mol of myosin. For fresh preparations, this always corresponded to full phosphorylation of myosin as judged from urea/glycerol gel electrophoresis (P. Jertschin and A. Sobieszek, unpublished results). Figure 4 shows an example of such a phosphorylation course. Estimations of nominal levels of phosphorylation were often unrealistic if calculated from ^{32}P incorporation. This discrepancy between the urea gels and the radioactivity assay is attributed to uncertainty in the specific activity of [γ-^{32}P]ATP, which decomposes at room temperature about 4% per h.

Linear plots of the type shown in Figures 3 and 4 indicate that the reaction is first order with respect to myosin. From the enzyme kinetic point of view, this implies that the substrate concentration ([M]) is much lower than the Michaelis constant (K_m); [M] $\ll K_m$. Thus, it would appear that in order to see the enzyme/substrate effects the myosin concentration has to be increased. The curves shown in Figures 3 and 4 were obtained by using 15–25 mg/mL of myosin, some 5–10-fold more than commonly employed in other studies (Persechini & Hartshorne, 1983; Sellers et al., 1983). Using the urea procedure (see Materials and Methods), we could assay myosin (in filamentous form) at concentrations as high as 150 μM (75 mg/mL), and the shape of the progress curves was unchanged (Figure 5A).

Phosphorylation of Isolated Light Chain. The phosphorylatable light chain can be separated from myosin under denaturing conditions (Perrie & Perry, 1970) and when renatured represents a very convenient substrate for MLCK. Naturally, myosin does not survive the denaturation, but as

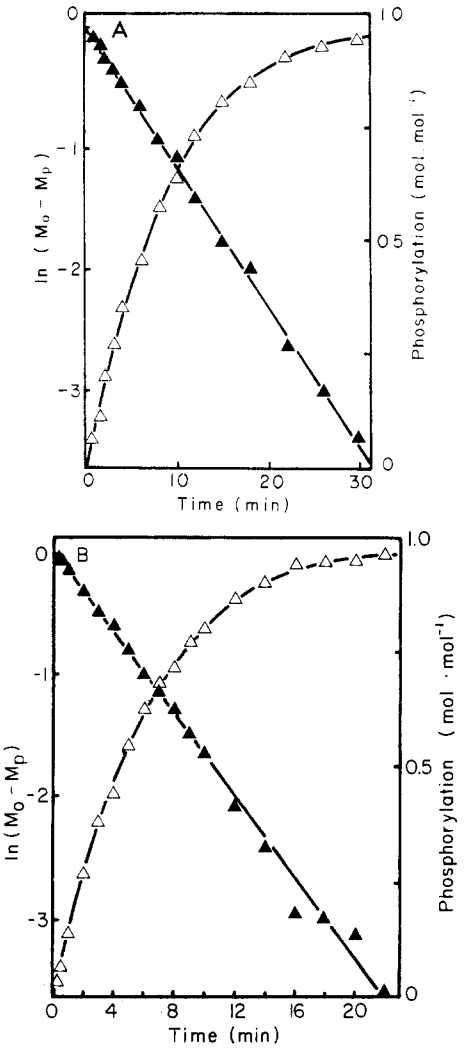

FIGURE 6: Phosphorylation progress curves for purified P light chain. The light-chain concentrations were 300 and 200 μM for (A) and (B), respectively, and the amount of kinase added was chosen to give maximal incorporation after 20–30 min of incubation. The ATP concentration in (B) was equal to the light-chain concentration (200 μM), in order to facilitate second-order behavior (see text).

shown by Adelstein & Klee (1981), the light chain so obtained is contaminated by calmodulin.

For most types of phosphorylation assay, the presence of calmodulin is either desired or irrelevant. Only in the case of a calmodulin-type assay, utilizing MLCK, must calmodulin be removed from the light chains used. This separation is easily achieved by a single ion-exchange purification step (Adelstein & Klee, 1981). It is clear from the present studies that this strong association of calmodulin with the light chain is direct. It does not result from the binding of MLCK to myosin. The binding is not affected by calcium concentration and may be hydrophobic in nature.

Significantly, the phosphorylation time course of isolated light chain, in the presence of purified MLCK, follows the same pattern as for intact myosin (Figure 6A). It was possible in this case to extend the range of substrate concentrations used up to 1 mM. Since, in contrast to the intact myosin, isolated light chain itself does not hydrolyze ATP, it was possible to examine the phosphorylation reaction under conditions in which any second-order behavior would readily be observed (Gutfreund, 1972), i.e., when initial ATP and light-chain concentrations are equal. Significantly, plots of ln [M] vs. time were linear (Figure 6B) while plots of $[M]^{-1}$ vs. time were nonlinear, again confirming first-order behavior.

Kinetic Analysis. As mentioned above, a convenient way of analyzing progress curves is to use an integrated form of the velocity equation. By plotting product concentration over time ($[M^P]/t$) vs. t^{-1} ln $[[M]_0/([M]_0 - [M^P])]$, it should be possible to obtain K_m and V_{max} values even from a single progress curve. Plots of this type are shown in Figure 5B and as can be seen are vertical or almost so. This indicates that the system under study exhibits a strong product inhibition effect. Exactly vertical lines are obtained when the apparent affinities of enzyme for its substrate and its product are the same, a case not analyzed in detail in the literature.

For a single substrate/single product reaction exhibiting product inhibition, the differential form of the velocity equation can be written as

$$v = -\frac{d[M]}{dt} = -\frac{V_{max}[M]}{K_m + K_m[M]_0/K_m^P + (1 - K_m/K_m^P)[M]}$$

Integration of this equation is relatively simple and provides the following formula:

$$V_{max}t = \left(K_m + \frac{K_m[M]_0}{K_m^P}\right) \ln \frac{[M]_0}{[M]} + \left(1 - \frac{K_m}{K_m^P}\right)([M]_0 - [M])$$

An important case of this formula is when the Michaelis constants for phosphorylated and dephosphorylated myosin are the same ($K_m = K_m^P$). It then reduces to

$$V_{max}t = (K_m + [M]_0) \ln \frac{[M]_0}{[M]}$$

or

$$[M] = [M]_0 \exp\left\{-\frac{V_{max}}{K_m + [M]_0}t\right\}$$

Thus, in this special case, the reaction is essentially first order. That is, at any given myosin concentration, the plot of ln [M] vs. time is linear with respect to the apparent first-order rate constant given as

$$k_{app} = \frac{V_{max}}{K_m + [M]_0}$$

Since this constant is itself a function of the initial myosin concentration, $[M]_0$, the reaction could be referred to as pseudo first order. However, it is not a pseudo-first-order reaction in the common sense, where a change in the reactant concentration results in a change in reaction order. Its simple dependence on $[M]_0$ is very useful since, in contrast to the presently held view (Orsi & Tipton, 1979), it enables us to estimate the elementary kinetic constants.

In the present case, instantaneous velocity is given by

$$v = \frac{V_{max}[M]}{K_m + [M]_0} = k_{app}[M]$$

Thus, it is enough to measure an apparent first-order rate constant for a series of initial myosin concentrations. Since the amount of enzyme added has to be constant, the myosin used must be free of kinase. A replot of k_{app}^{-1} vs. $[M]_0$ will give the reciprocal of the true first-order rate constant (k^{-1}) as the vertical axis intercept and the negative value of the Michaelis constant ($-K_m$) as the horizontal axis intercept. A series of logarithmic plots obtained from progress curves are shown in Figure 7A. The highest concentration is about 50 μM (25 mg/mL) with a corresponding k_{app} = 0.9 min.$^{-1}$ The

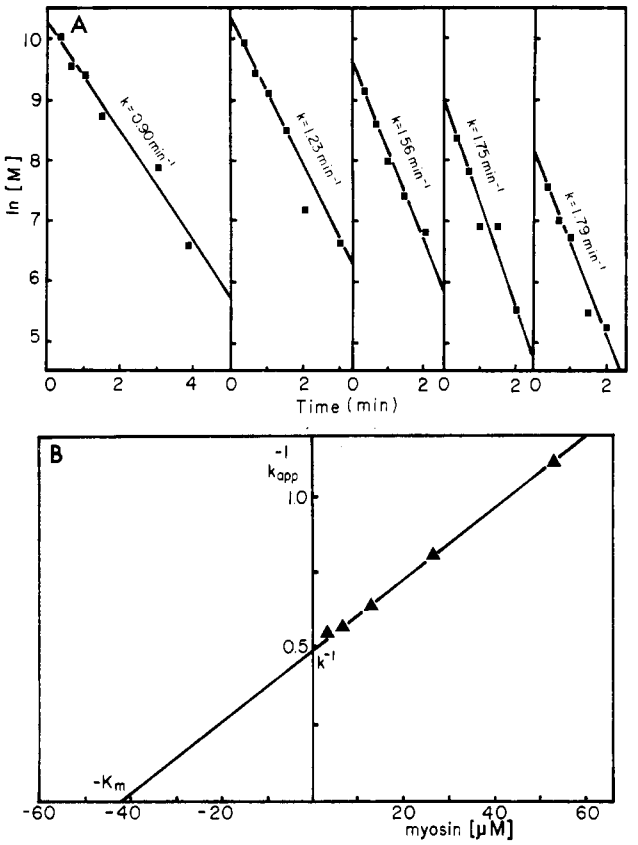

FIGURE 7: Evaluation of kinetic constants for phosphorylation of chicken gizzard myosin by turkey gizzard MLCK. From a series of progress curves obtained at fixed MLCK and variable myosin concentrations, corresponding apparent first-order rate constants were estimated (A). The reciprocals of these rate constants were replotted in (B) as a function of the initial myosin concentration. K_m (the Michaelis constant) and k (the true first-order rate constant) were obtained, respectively from the x and y intercepts. V_{max} can then be calculated from the relationship $k = V_{max}/K_m$.

Table I: Kinetic Constants of Myosin Light-Chain Kinases

substrate	enzyme	V_{max} (μmol L^{-1} min^{-1})	K_m[d] (μM)	k (min^{-1})	K_m[e] (μM)
PS[a] myosin	PS MLCK	10		0.70	15
PS myosin	ChG MLCK	29		1.32	22
PS P light chain	ChG MLCK	78	171	2.86	165
PS P light chain	PS MLCK	100	167	2.63	171
ChG[b] myosin	TG[c] MLCK	77		2.03	42
ChG P light chain	ChG MLCK	250		3.30	75

[a] PS, pig stomach. [b] ChG, chicken gizzard. [c] TG, turkey gizzard. [d] K_m value estimated from the Lineweaver–Burk plot by using the Lee & Wilson (1971) approximation for the initial substrate concentration. [e] K_m value estimated by using the pseudo-first-order assumption described here.

K_m value obtained in this case is 42 μM (Figure 7B), corresponding to the value obtained here for the chicken gizzard myosin/kinase system. The Michaelis constants for other combinations of pig stomach or chicken gizzard MLCK and myosins or isolated light chains are given in Table I. In the case of the light chains, the Michaelis constants were also determined by the initial rate method using the Lee & Wilson (1971) approximation for the initial substrate concentration. As expected, K_m values obtained by the two methods were the same. Such a comparison is not possible in the case of the intact myosin/kinase system in view of the limited solubility of myosin.

Binding of Kinase to Myosin. The only assumption made in the above section was that the Michaelis constants for the

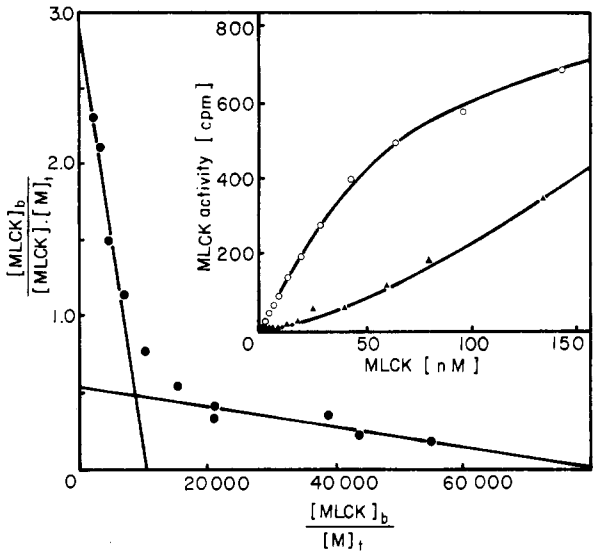

FIGURE 8: Scatchard plot of binding of turkey gizzard MLCK to chicken gizzard dephosphorylated myosin. The insert shows the kinase activity in the pellets (O) and the supernatants (▲) after sedimentation. Conditions were as for the phosphorylation assays except that ATP was not present and the pH was 6.75. The myosin concentration was 35 μM.

binding of kinase by phosphorylated and dephosphorylated myosin under steady-state conditions are similar (i.e., when the phosphorylation reaction is in progress). To determine the validity of this assumption, three types of equilibrium binding experiments were carried out. In the first type, dephosphorylated kinase-free filamentous myosin was incubated with increasing amounts of kinase. After centrifugation, the concentrations of kinase in the pellets and supernatants were assayed, using isolated light chains as substrate. As shown in Figure 8, two affinities of kinase binding are detectable when myosin is used in the form of filaments. The high-affinity site has a K_s^{app} of 30 nM and the lower affinity site a K_s^{app} of 1.3 μM.

In the second type of experiment, the binding of the kinase to phosphorylated and dephosphorylated myosin was compared. Native myosin/kinase complexes were sedimented in a 5–30% sucrose gradient. In 60 mM KCl, 1 mM MgATP, and 10 mM imidazole, pH 6.75, both phosphorylated and dephosphorylated myosin filaments sedimented to the bottom of the gradient. However, examination of the pellets by urea/glycerol gel electrophoresis revealed that during the sedimentation an average of 50% of the myosin became dephosphorylated.

As shown in Figure 9A,B, resuspended pellets obtained from dephosphorylated and semiphosphorylated myosins have the same kinase activities. The upper curves represent kinase activities assayed by using isolated light chain, while the lower correspond to incorporation into myosin itself.

As expected for an equilibrium type of binding, the unbound kinase activity is present at the top of the gradient and exponentially decreases toward the bottom (Figure 9C,D). Figure 9C,D shows additionally that there is a peak of kinase activity around the middle of the gradient and that this peak sedimented a little faster in the presence of Ca^{2+}. SDS gel electrophoresis revealed that this activity was associated with small amounts of myosin in low-polymer form.

In the third type of binding experiment, the affinity of the kinase for fully phosphorylated and dephosphorylated myosin was compared. The experiments were carried out under the same conditions as for the sucrose gradients. In this case, 0.5 mM EGTA or 0.1 mM $CaCl_2$ was added to a mixture of

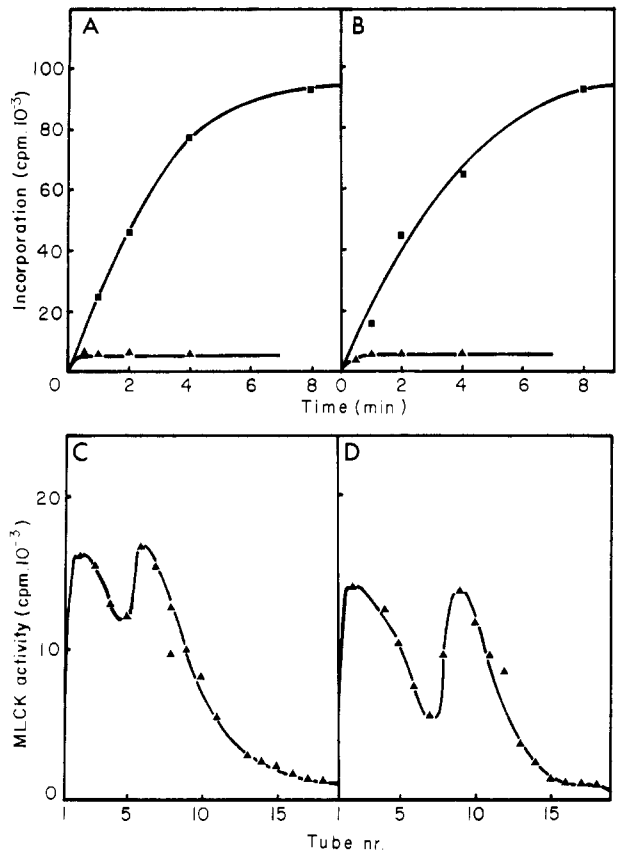

FIGURE 9: Sucrose gradient sedimentation of phosphorylated (B, D) and dephosphorylated (A, C) chicken gizzard myosin. Panels A and B show the MLCK content of the myosin pellets as measured by ^{32}P incorporation into added P light chain (■) or myosin itself (▲). Panels C and D show the MLCK activity of the sucrose gradient fractions. Tube 1 corresponds to the top (5% sucrose) of the gradient while tube 20 to the bottom (30% sucrose) of the gradient. Conditions as for phosphorylation assays except that in the case of dephosphorylated myosin 0.5 mM EGTA and not 0.1 mM $CaCl_2$ was present. ATP concentration was 0.5 mM in the gradients and 1.0 mM in the sample.

kinase and myosin filaments. The kinase/myosin ratio was chosen so that after 2–3 min of incubation with 1 mM ATP, myosin became fully phosphorylated in the presence of Ca, while for the EGTA-containing tubes it remained dephosphorylated. After the incubation, myosin filaments were immediately sedimented to the bottom of the tubes in a high-speed table centrifuge.

Analysis of the supernatant and the pellets for kinase content showed that there was half as much kinase sedimenting with phosphorylated myosin as compared to dephosphorylated myosin. Correspondingly, there was practically no kinase activity in the supernatant of the dephosphorylated myosin while the other supernatant had significant levels of kinase activity. The kinase activity ratio of the pellets was very close to 2; thus, the binding constant of the kinase for phosphorylated myosin is on the order of 0.1 μM.

All three types of binding experiments thus indicate that the kinase was bound to myosin with very high affinity essentially independent of its state of phosphorylation, but the amounts of binding were far below stoichiometric levels.

Discussion

Since the smooth muscle myosins used in recent studies by others (Sellers et al., 1981; Malencik et al., 1982; Persechini & Hartshorne, 1983; Cole et al., 1983) were isolated by procedures similar to those developed in our earlier work (Sobieszek & Bremel, 1975; Sobieszek & Small, 1976; Sobieszek, 1977a,b), we may conclude from the present report that they all contained significant levels of MLCK. It is apparent that the kinase is present in all relatively fresh smooth muscle myosin preparations, tightly bound to its substrate. Even myosin preparations such as those shown in Figure 1A, that appear homogeneous after SDS gel electrophoresis, may have enough MLCK to become fully phosphorylated within 10–30 s after addition of Ca^{2+} and ATP. The removal of MLCK from myosin constituted the primary problem in the present studies and could be achieved only after two purification steps, each resulting in a substantial loss of myosin. This problem could have been overlooked in other studies on myosin phosphorylation in which MLCK usually had to be added to myosin in order to obtain phosphorylation rates 10–50-fold slower than those reported here (Persechini & Hartshorne, 1983; Sellers et al., 1983).

The levels of MLCK present in the two smooth muscle myosins used in this study were variable. It was often possible to obtain pig stomach myosin practically free of active MLCK after a single gel filtration step. This, however, may reflect higher levels of proteolytic activity present in pig stomach preparations since analogous preparations of gizzard myosin remained contaminated by kinase.

A novel feature of the present data is the range of myosin concentrations used in the measurements of phosphorylation rates. Myosin concentrations of about 5 μM have been commonly used in kinetic studies (Persechini & Hartshorne, 1983; Sellers et al., 1983). With myosin in filamentous form and the assay method described, it was possible to follow the progress of phosphorylation at myosin concentrations up to about 150 μM which corresponds to 0.3 mM phosphorylatable sites. This enabled for the first time an estimate of the Michaelis constant for the kinase-intact myosin system. Its value of 20–40 μM is about 5–10-fold higher than previous estimates made by using impure isolated light chains (Adelstein & Klee, 1981), when the concentration of phosphorylatable sites is compared. In contrast, in the present study, the K_m values for purified isolated light chains were 2–4-fold higher than those obtained for myosin. Thus, the isolated light chain is a more convenient but not a better substrate for the kinase than is intact myosin. As might be expected, the former values compare more realistically with estimates of the intracellular myosin concentration based on the myosin concentration in smooth muscle (Cohen & Murphy, 1978).

Under the above conditions, substrate concentration was comparable to K_m, yet the kinase/myosin system nonetheless displayed first-order kinetics. This suggests product inhibition; that is, the K_m's for substrate and product are approximately the same. Since the kinase concentration was extremely low, a steady-state approach could be applied to the data analysis. In this case, the Michaelis and binding constants are not necessarily the same, even for a single substrate/single product reaction such as that considered here. Thus, the kinetic results do not necessarily indicate identical affinities of phosphorylated and dephosphorylated myosins for kinase.

Our sucrose gradient experiments indicate that the amounts of kinase bound to dephosphorylated and to one-head-phosphorylated myosins are the same. However, fully phosphorylated myosin binds approximately half as much kinase. Such a small difference in binding is not significant, but it implies that only after the second myosin head becomes phosphorylated does myosin adopt a new configuration in which product release is facilitated. Conceivably, further acceleration of product release might take place when actin is present in the system. It has already been demonstrated

that the calmodulin/MLCK complex binds to F-actin, and a flip-flop mechanism for this system has been suggested (Sobue et al., 1982). Consistent with this idea, we find that the binding of calmodulin and MLCK to myosin is an independent and Ca^{2+}-insensitive process, most likely of a hydrophobic nature. It is possible that the binding of the kinase to F-actin, under activating conditions (Ca^{2+} present), is needed for an effective product release and for the "transport" of the enzyme along the myosin filament.

The independent binding of the MLCK apoenzyme and calmodulin to myosin implies that the mechanism of activation for this system is different from that of phosphodiesterase (Klee et al., 1979; Cox et al., 1981). In the latter system, it is the apoenzyme that is activated by binding of Ca-saturated calmodulin. From the present data and from the observation that myosin, as well as its isolated P light chain, forms unproductive substrate/enzyme complexes, it is clear that Ca^{2+} alone activates a preexisting complex. It is possible that some conformational change of the myosin/kinase/calmodulin complex arising from ATP binding to myosin is also necessary for such Ca activation. On the basis of the present evidence, however, one cannot distinguish whether Ca^{2+} binding alone is sufficient to induce this conformational change or whether the presence of ATP is also necessary. It may be noted, however, that ATP binding to dephosphorylated myosin can induce a major conformational change in the molecule (Suzuki et al., 1982; Kendrick-Jones et al., 1983; Ikebe et al., 1983).

Pseudo-first-order reactions carry no restriction on substrate concentration and can be temporally characterized by only one or two parameters. The pseudo-first-order approach employed here enabled the limitations imposed by myosin solubility to be overcome. It seems likely that this approach could usefully be applied to any enzyme reaction which shows, or can be manipulated to show, pseudo-first-order kinetics, that is, any reaction in which there is no contribution of a quadratic term in the integrated form of the rate equation. Exceptions would be true first-order reactions, which are easily recognized because the rate constant is independent of the initial substrate concentration.

For the myosin/kinase system, the fact that the phosphorylation reaction has two substrates and two products does not invalidate the single substrate approximation, since one of the substrates (ATP) is present at saturating concentration while the corresponding product (ADP) only inhibits at about 100× higher concentrations than those present during the assays. The fact that it is possible to describe myosin phosphorylation by a single logarithmic term may indicate that the reaction has a ping-pong-type (Segel, 1975) mechanism. This conclusion could be tested by product inhibition studies, but these are only possible for isolated P light chain and not for intact myosin.

During the course of these studies, there appeared two reports on the type of progress curves that are observed for myosin phosphorylation (Persechini & Hartshorne, 1983; Sellers et al., 1983). From their data on sequential phosphorylation of the two myosin heads (Persechini & Hartshorne, 1981), Persechini & Hartshorne (1983) treated phosphorylation of the first and the second head completely separately. Such an approach may only be possible for an ideal sequential system in which phosphorylation of at least 90% of the population of first heads is completed before any significant phosphorylation of the second head can take place. It is evident that this is not true for the kinase/myosin systems studied here. Indeed, Persechini and Hartshorne found instead a poor fit of their data to the sequential model which they support.

In another report, Sellers et al. observed single exponential progress curves for phosphorylation of smooth muscle heavy meromyosin or monomeric myosin (Sellers et al., 1983). However, their curves for myosin in the filamentous form could only be fitted with two exponential terms. This they interpreted in terms of two populations of myosin heads within the filament differing in their configurations and accessibilities to the kinase. Our data covering a much wider range of myosin filament concentrations are consistent with the existence of a single population of myosin heads in the range 0–95% of phosphorylation. The discrepancy may be due to different ways of estimating the initial myosin concentration, $[M]_0$. It was often seen in our study that a progress curve with apparently two logarithmic terms could be "condensed" into one, when $[M]_0$ was determined as described above.

The observation of a single population of myosin heads with respect to the phosphorylation rate does not necessarily mean that phosphorylation is random since the nature of the methods does not allow a distinction between the phosphorylation rates except when the differences are relatively great (10-fold or more). In conclusion, the deeper understanding of the phosphorylation process provided by these studies should now allow a closer investigation of the interdependence of the phosphorylation and the actin activation of myosin. Such studies, now in progress, already indicate that phosphorylation is random and that the activation is not simple but has to be considered in terms of a cascade regulatory system [see Chock & Stadtman (1980)] where phosphorylation and dephosphorylation "loops" are equally important.

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