was found in the P face, together with numerous troughs which were not detectable in controls.

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Correlation of Enzymatic Properties and Conformation of Smooth Muscle Myosin†

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ABSTRACT: In the presence of adenosine 5′-triphosphate (ATP) and 1–10 mM $MgCl_2$, the relative viscosity (η_{rel}) of dephosphorylated gizzard myosin is reduced markedly over a range of KCl from 0.35 to 0.15 M. Sedimentation patterns show that the decrease in η_{rel} is due to the conversion of the 6S to 10S forms of myosin. Under similar conditions, η_{rel} of phosphorylated myosin is not altered, and at 0.2 M KCl, the 10S form is not observed. In 1 and 2 mM $MgCl_2$ and <0.2 M KCl, 10S can be formed from both phosphorylated myosin plus ATP and dephosphorylated myosin minus ATP. In the presence of ethylenediaminetetraacetic acid (EDTA), the decrease of η_{rel} and corresponding change in sedimentation pattern are independent of ATP and show only a dependence on KCl. Therefore, ATP and dephosphorylation are not obligatory for the 6S to 10S transition. In all instances, the 6S–10S transition of monomeric myosin is paralleled by an alteration of adenosine-5′-triphosphatase (ATPase) activity; i.e., the KCl dependence of the two processes is the same. Transition from 6S to 10S causes a decrease in Mg^{2+}- and Ca^{2+}-ATPase activity of myosin and an increase in K^+-EDTA-ATPase activity. The relationship between myosin shape and the ATP dependence of Mg^{2+}-ATPase activity also is consistent with this generalization. The phosphorylation dependence of the viscosity transition from 6S to 10S is not linear, and phosphorylation of both heads is required for the complete transition. In contrast, the ATP dependence of the transition is linear, and the binding of 2 mol of ATP/myosin is required for maximum effect. The idea is developed that the enzymatic properties of myosin are determined by its conformation, and thus, analogous changes in filamentous myosin may be important in the regulation of the activity of smooth muscle actomyosin.

The most popular theory to account for the regulation of the contractile apparatus of smooth muscle involves phosphorylation of the two M_r 20000 light chains of the myosin molecule. Several facets of this scheme have been analyzed, and considerable evidence in favor of the phosphorylation hypothesis has been accumulated [for reviews, see Adelstein & Eisenberg (1980) and Walsh & Hartshorne (1982)]. However, the role of phosphorylation in activating actomyosin adenosine-5′-

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triphosphatase (ATPase) activity is not universally accepted (Nonomura & Ebashi, 1980; Cole et al., 1982), and for this reason, it would be instructive to define the molecular changes that are induced in the myosin molecule as a consequence of phosphorylation. Although the molecular mechanism involving phosphorylation remains obscure, some interesting observations have been made. Suzuki et al. (1978) showed that filaments of dephosphorylated myosin in 0.15 M KCl were disassembled by adenosine 5′-triphosphate (ATP) and on ultracentrifugation showed a major component of 10 S. At higher ionic strengths, only a 6S component was detected in both the absence and presence of ATP. It was suggested (Suzuki et al., 1978) that the transition from 6S to 10S reflected the dimerization of myosin. Subsequently, Suzuki et al. (1982) showed that myosin dimers were not formed and the increase in sedimentation coefficient resulted from a large change in the radius of gyration. The dramatic change in hydrodynamic properties was visualized on electron microscopy with gizzard myosin by Onishi & Wakabayashi (1982), with vascular muscle myosin by Trybus et al. (1982), and with smooth muscle and thymus myosin by Craig et al. (1983). It was shown that the 10S component formed a looped structure in which the tail of the myosin molecule is bent back toward the head region whereas the 6S component remained in the extended, more asymmetric conformation. Trybus et al. (1982) also measured several physical parameters, e.g., Stokes radius and viscosity, and these results were consistent with a structure for the 10S component in which the myosin rod is bent. Mg-ATP favors the conversion of 6S to 10S (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983) but is not obligatory for the transition (Trybus et al., 1982), and the formation of the 10S component is enhanced also by dephosphorylation (Trybus et al., 1982; Craig et al., 1983).

In our laboratory, we have been interested in the effects of phosphorylation of smooth muscle myosin and have also observed that the viscosity of gizzard myosin, measured over a range of ionic strength, is dependent on several factors, including the state of myosin phosphorylation. In addition, it was found that the enzymatic properties of gizzard myosin differ under various solvent conditions and that the alteration of ATPase activity parallels the viscosity transition. The correlation of ATPase activity to a particular shape of myosin suggests that a conformational transition of myosin might be involved in the regulation of smooth muscle actomyosin. The studies leading to the formulation of this possibility are presented below.

Materials and Methods

Myosin was prepared from turkey gizzards by either the method of Persechini & Hartshorne (1983) or that of Ebashi (1976). Myosin obtained by either method behaved similarly. Cardiac myosin was a gift from Dr. S. Srivastava. Phosphate (^{32}P) incorporation into myosin was assayed as described previously (Mrwa & Hartshorne, 1980) and the level of prephosphorylation of myosin assessed by electrophoresis in alkaline urea gels (Perrie & Perry, 1970). Myosin was not used if prephosphorylation was detected.

ATPase activities in the millimolar range of ATP were determined at 25 °C as described by Ferenczi et al. (1978) by using [γ-^{32}P]ATP (New England Nuclear) under the following conditions: 1 and 10 mM $MgCl_2$, 30 mM Tris[1]-HCl (pH 7.5), 1 mM ATP; 7 mM $CaCl_2$, 30 mM Tris-HCl (pH 7.5), 1 mM ATP; 2 mM EDTA, 30 mM imidazole–HCl (pH 6.8), 1 mM ATP. Other conditions are given in the figure legends. For assays done in the micromolar range of ATP, the conditions were 70–100 units/mL pyruvate kinase (type II, Sigma Chemical Co.), 1 mM phosphoenolpyruvate (Sigma Chemical Co.), 10 mM $MgCl_2$, 1 mM EGTA, and 30 mM Tris-HCl (pH 7.5). Other conditions are given in the legends to Figures 7 and 8. The ATPase activities were determined by measuring the liberation of pyruvate (Reynard et al., 1961).

Viscosity was measured at 25 °C in Cannon-Ubbelohde viscometers with water flow times of approximately 25 s. Myosin concentration was 2 mg/mL; other conditions are given in the figure legends. The viscosity data are expressed as η_{rel} (viscosity of protein solution/viscosity of solvent). When phosphorylated myosin was used, the level of phosphorylation was determined before and after viscosity measurements, and data were taken only if these levels were identical.

Sedimentation velocity experiments were carried out in the Beckman Model E analytical ultracentrifuge at 22 °C with schlieren optics. Conditions used were as follows: 2 mg/mL myosin under various solvent conditions (given in figure legends) in 12 mm light path cells with quartz plain and 1° positive wedge windows; An-H rotor, final speed at 60000 rpm; bar angle 65°. During the rotor speed-up, the schlieren patterns were monitored for the presence of very rapidly sedimenting aggregates that would be sedimented before final speed was reached. These were not observed.

Gel filtration was carried out with columns (1.8 × 42 cm) of Sephacryl S-300 (Sigma Chemical Co.) at a flow rate of 25 mL/h. Solvent conditions are given in the legend to Figure 5. Other preparative and analytical procedures were as described by Walsh et al. (1982).

Results

The KCl dependence of the relative viscosity (η_{rel}) of phosphorylated gizzard myosin in 10 mM $MgCl_2$ is shown in Figure 1. Phosphorylated myosin (approximately 2 mol of P incorporated/mol of myosin) in the presence of 1 mM ATP shows no change in viscosity over the range of KCl concentration of 0.2–0.5 M. In contrast, η_{rel} of dephosphorylated myosin in the presence of 1 mM ATP is progressively reduced at KCl concentrations of less than 0.35 M. At this concentration of $MgCl_2$, the viscosity transition requires the presence of ATP, and as shown in Figure 1 in the absence of ATP, η_{rel} of dephosphorylated myosin is not reduced at lower KCl concentrations.

The sedimentation patterns of dephosphorylated myosin plus ATP at 0.15 and 0.35 M KCl are shown in the inset of Figure 1. At both concentrations of KCl, only one component is detected, and clearly, the species present at 0.15 M KCl sediments more rapidly than that at 0.35 M KCl. (The absence of rapidly sedimenting polymers was verified by monitoring the schlieren patterns at lower centrifugal forces.) The similarity of these sedimentation patterns to those reported by others (Suzuki et al., 1978; Trybus et al., 1982) suggests that in the presence of ATP dephosphorylated gizzard myosin at 0.15 and 0.35 M KCl sediments as a 10S and 6S component, respectively. The effect of phosphorylation at 0.2 M KCl and the presence of 1 mM ATP is also illustrated by sedimentation patterns shown in Figure 1. Phosphorylated myosin (2.0 mol of P/mol of myosin) sediments more slowly than the dephosphorylated form, and the two sedimenting species approximate the 6S and 10S components, respectively. In each case, only one sedimenting species is detected. A comparison of the viscosity and ultracentrifuge data suggests that the conformational changes detected by viscosity reflect the 6S

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

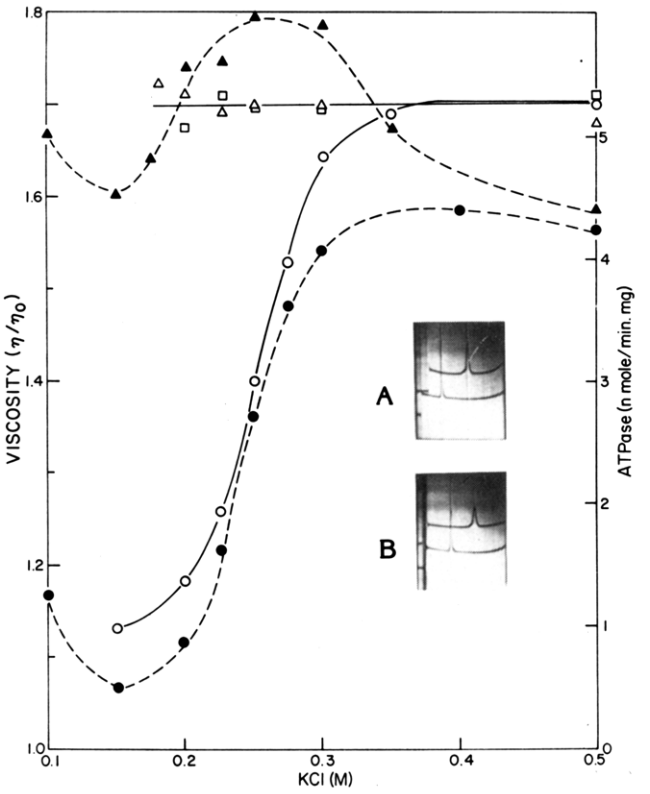

FIGURE 1: KCl dependence of Mg^{2+}-ATPase activity, viscosity, and sedimentation patterns of phosphorylated and dephosphorylated gizzard myosin at 10 mM $MgCl_2$. Myosin was phosphorylated to approximately 2 mol of P/mol of myosin by incubating for 10 min at 25 °C in 1 mM ATP, 10 mM $MgCl_2$, 0.2 M KCl, 30 mM Tris-HCl (pH 7.5), $\sim 1 \times 10^{-4}$ M $CaCl_2$, 10 μg/mL gizzard myosin light chain kinase, and 5 μg/mL calmodulin. Viscosity data: phosphorylated myosin, 1 mM ATP, and 10 mM $MgCl_2$ (△); dephosphorylated myosin, 1 mM ATP, and 10 mM $MgCl_2$ (○); dephosphorylated myosin minus ATP and 10 mM $MgCl_2$ (□). ATPase activity under identical conditions shown for phosphorylated (▲) and dephosphorylated (●) myosin. Sedimentation patterns in 1 mM ATP and 10 mM $MgCl_2$ shown in inset for (A) dephosphorylated myosin in 0.15 (upper) and 0.35 M KCl (lower) and (B) dephosphorylated myosin in 0.2 M KCl (upper) and phosphorylated myosin in 0.2 M KCl (lower). Patterns are shown at 40 min after reaching speed of 60000 rpm. Other conditions are as given under Materials and Methods.

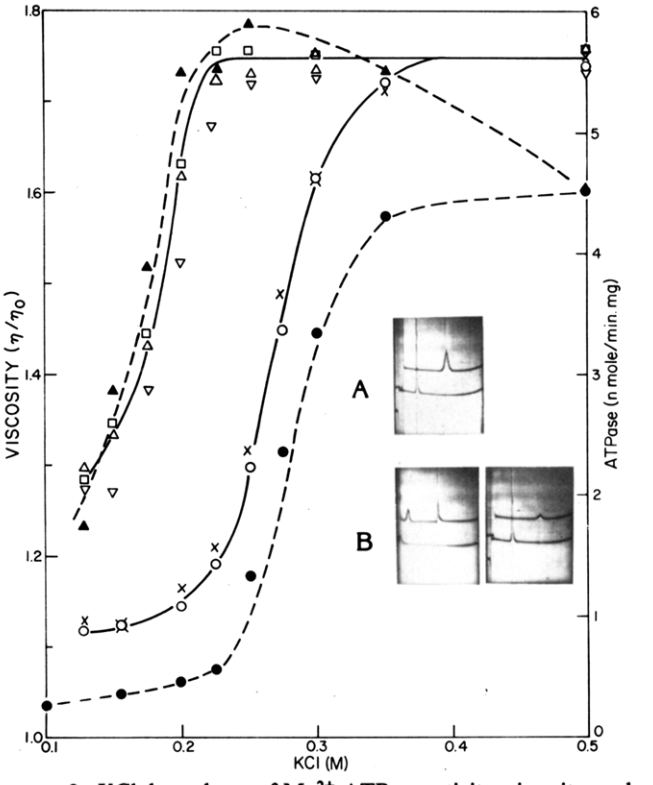

FIGURE 2: KCl dependence of Mg^{2+}-ATPase activity, viscosity, and sedimentation patterns of phosphorylated and dephosphorylated gizzard myosin at 1 and 2 mM $MgCl_2$. Phosphorylation of myosin as in Figure 1. Viscosity data: dephosphorylated myosin, 1 mM ATP, and 1 (○) or 2 mM $MgCl_2$ (×); dephosphorylated myosin minus ATP and 1 mM $MgCl_2$ (□); phosphorylated myosin, 1 mM ATP, and 1 (△) or 2 mM $MgCl_2$ (▽). ATPase activity in 1 mM $MgCl_2$ shown for phosphorylated (▼) and dephosphorylated (●) myosin. Sedimentation patterns in 1 mM ATP and 1 mM $MgCl_2$ shown in inset for (A) dephosphorylated myosin in 0.15 (upper) and 0.35 M KCl (lower) and (B) phosphorylated myosin in 0.125 (upper) and 0.25 M KCl (lower). Patterns are shown at 32 min (A) and 1 and 36 min (B) after reaching speed of 60000 rpm. Other conditions are as given under Materials and Methods.

to 10S transition. Further, it is likely that when the transition is complete, at either side of the transition zone, either the 6S or 10S component forms the major species. A study of the species present within the transition zone, i.e., at KCl concentrations between 0.2 and 0.35 M KCl, is in progress and will be presented later. However, preliminary evidence has suggested that within this zone a single sedimenting species is always present, which, depending on the solvent conditions, sediments at s values intermediate between the 6S and 10S components.

The dramatic change in the conformation of dephosphorylated myosin as a function of ionic strength is reflected also by an alteration in the Mg^{2+}-ATPase activity, as shown in Figure 1. Myosin at relatively high KCl concentrations (0.4 and 0.5 M) is in the extended form, i.e., 6S, and has a higher Mg^{2+}-ATPase activity than at lower concentrations (<0.2 M) where it exists in the folded conformation. The reduction of the Mg^{2+}-ATPase activity and of η_{rel} with decreasing KCl concentrations clearly exhibits a similar KCl dependence, and this suggests that the 6S to 10S transition induces an alteration of the enzymatic properties of myosin. In contrast, phosphorylated myosin does not show the marked conformational changes described for dephosphorylated myosin over a similar range of KCl concentrations, and correspondingly, the Mg^{2+}-ATPase activity of phosphorylated myosin is not markedly altered. A slight activation of Mg^{2+}-ATPase activity is observed over the range of 0.5–0.25 M KCl, but the marked reduction of ATPase activity associated with the formation of 10S is not found with phosphorylated myosin. Suzuki et al. (1978) have previously reported that the 10S and 6S forms of chicken gizzard myosin have different Mg^{2+}-ATPase activities.

The KCl dependence of the conformational change and Mg^{2+}-ATPase activity of gizzard myosin at lower total $MgCl_2$ concentrations (1 and 2 mM) are shown in Figure 2. Several features of these curves are similar to those reported in the previous figure. The viscosity of dephosphorylated myosin in the presence of ATP is reduced markedly at KCl concentrations below 0.35 M at both concentrations of $MgCl_2$, and the viscosity decrease is paralleled by a reduction of Mg^{2+}-ATPase activity. At 0.15 and 0.35 M KCl in the presence of ATP (1 mM), the sedimentation patterns of dephosphorylated myosin indicate a single sedimenting component consistent with the 10S and 6S species, respectively. There are several additional points, however, that can be made from the data expressed in Figure 2. The viscosity of phosphorylated myosin (2.0 mol of P/mol of myosin) in the presence of ATP, while more resistant to decreasing ionic strength than dephosphorylated myosin, is reduced at KCl concentrations of less than 0.2 M. Again, the reduction of viscosity is paralleled by a decrease in Mg^{2+}-ATPase activity (Figure 2). Sedimentation patterns of phosphorylated myosin in the presence of ATP at 0.125 and

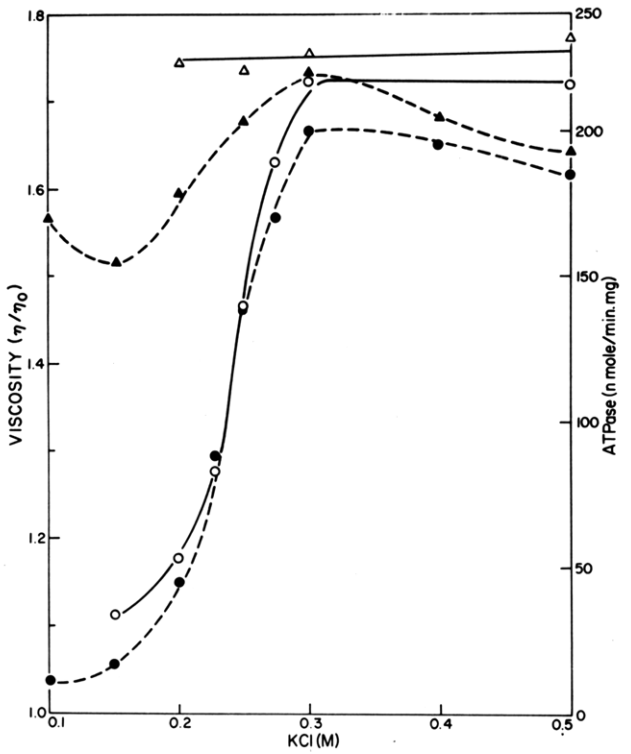

FIGURE 3: KCl dependence of Ca^{2+}-ATPase activity and viscosity for phosphorylated and dephosphorylated myosin at 7 mM $CaCl_2$. Myosin was phosphorylated as in Figure 1 and dialyzed vs. 0.5 M KCl, 30 mM Tris-HCl (pH 7.5), and 0.2 mM dithiothreitol. Viscosity data: dephosphorylated myosin and 1 mM ATP (○); phosphorylated myosin and 1 mM ATP (△). ATPase activity shown for dephosphorylated (●) and phosphorylated myosin (▲). Other conditions are as given under Materials and Methods.

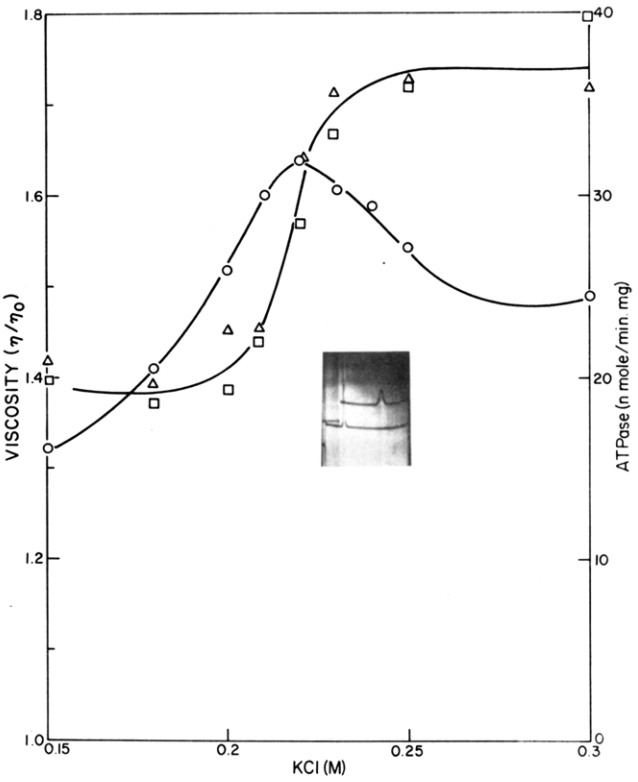

FIGURE 4: KCl dependence of K^+EDTA-ATPase activity, viscosity, and sedimentation patterns of dephosphorylated myosin in 2 mM EDTA. Viscosity data: myosin plus (△) and minus 1 mM ATP (□). ATPase activity (○). Sedimentation patterns in 1 mM ATP shown in inset for 0.2 (upper) and 0.5 M KCl (lower). Patterns are shown at 26 min after reaching speed of 60 000 rpm. Other conditions are as given under Materials and Methods.

0.25 M KCl are shown in the inset of Figure 2. At the lower ionic strength, two sedimenting components are present, representing the 10S species and myosin polymer. At the higher ionic strengh, only the 6S component is observed. The points to emphasize are that phosphorylated myosin can exist in the 10S conformation and whether or not it does so is dictated by the solvent conditions (e.g., Mg^{2+} and KCl concentrations) and, secondly, that the 10S conformation of phosphorylated myosin shows a reduced Mg^{2+}-ATPase activity, i.e., similar to the 10S conformation of dephosphorylated myosin.

The data in Figure 2 illustrate also that ATP is not essential for the 6S–10S transition. The viscosity of dephosphorylated myosin in 1 mM $MgCl_2$ and in the absence of ATP is reduced markedly at concentrations of KCl of less than 0.2 M, suggesting the formation of the 10S component. Sedimentation patterns (not shown) are similar to those for phosphorylated myosin (Figure 2) and indicate the presence of polymer plus the 10S species at 0.125 M KCl and only the 6S species at 0.25 M KCl. Even though ATP is not essential for the 6S–10S transition, it is clear that in the presence of ATP the transition zone is shifted to higher ionic strength and is therefore more readily visualized. Dephosphorylated bovine cardiac myosin, assayed in 1 mM $MgCl_2$, 1 mM ATP, and 0.2–0.5 M KCl, does not show the viscosity transition seen with gizzard myosin and maintains a high viscosity consistent with the extended 6S species.

Figure 3 shows that the conformational change reflected by viscosity measurements occurs in the presence of Ca^{2+}-ATP. The viscosity of phosphorylated myosin (2 mol of P/mol of myosin) remains constant between 0.2 and 0.5 M KCl, whereas the η_{rel} of dephosphorylated myosin is reduced markedly below 0.3 M KCl. Again, the enzymatic activity of myosin is reflected by the conformation of myosin. For dephosphorylated myosin, the reduction of Ca^{2+}-ATPase activity shows a similar KCl dependence to the viscosity change, but for the phosphorylated myosin, the effect of KCl on the Ca^{2+}-ATPase activity is not as dramatic.

In the presence of EDTA, i.e., absence of Mg^{2+} and Ca^{2+}, the viscosity transition of dephosphorylated myosin, occurring over the range of KCl concentrations from 0.1 to 0.25 M, is not dependent on the presence of ATP as shown in Figure 4. Sedimentation patterns, taken in the presence of ATP and 0.2 and 0.25 M KCl, show in each case a single species with sedimentation characteristics similar to those of the 10S and 6S forms, respectively (inset of Figure 4). Over the transition zone of KCl concentrations, the K^+EDTA-ATPase activity of dephosphorylated myosin also is altered. A possible explanation for the bell-shaped curve is as follows: at higher concentrations of KCl (>0.3 M), the K^+EDTA-ATPase activity is dependent on the KCl concentration and shows a marked inhibition with reducing KCl levels. If the progressive reduction of activity is extrapolated, then the activity at 0.2 M KCl would be predicted to be approximately 50% of the activity at 0.3 M KCl. Clearly, this is not the case. The range of KCl concentrations over which the ATPase activity shows an apparent activation is also that range over which the change in myosin conformation occurs (Figure 4). It is therefore suggested that the increase in ATPase activity is due to the formation of the 10S conformation, which has a higher specific K^+EDTA-ATPase activity than does the 6S form. Opposing the increase in activity due to the formation of 10S is a decrease in activity due to the decreasing KCl concentrations. The sum of these opposing trends is the bell-shaped curve as shown in Figure 4.

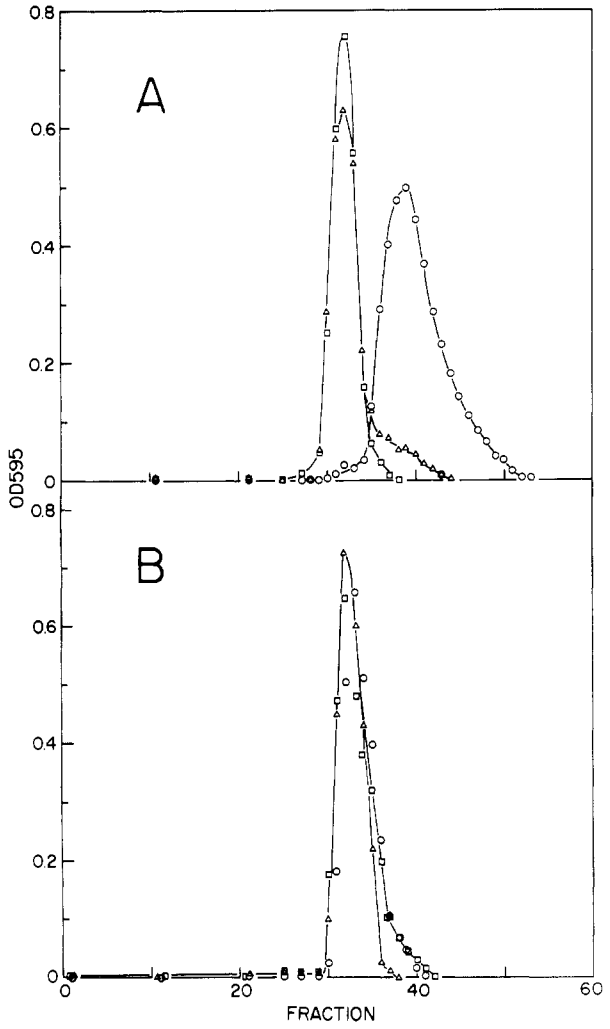

FIGURE 5: Elution patterns of phosphorylated and dephosphorylated gizzard myosin from Sephacryl S-300. Conditions are (A) 0.2 M KCl, 10 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5) and (B) 0.3 M KCl, 10 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5). Other conditions: (A) phosphorylated myosin plus 1 mM ATP (△); dephosphorylated myosin plus (○) and minus 1 mM ATP (□); (B) phosphorylated myosin plus 1 mM ATP (△); dephosphorylated myosin plus (○) and minus 1 mM ATP (□). Protein concentration was measured as described by Spector (1978). Other conditions are as given under Materials and Methods.

It was shown by Trybus et al. (1982) that the 10S and 6S species of myosin could be separated by gel filtration. This is confirmed by data presented in Figure 5. The more asymmetric conformation of myosin, i.e., the 6S form, elutes prior to the 10S form. The elution profiles of phosphorylated and dephosphorylated myosin in the presence and absence of ATP and at different KCl concentrations are shown in Figure 5. The assignment of the elution positions to either 6S or 10S conformations is consistent with the viscosity data presented previously.

The phosphorylation dependence of the viscosity change is shown in Figure 6. Phosphorylation reactions were carried out at either 75 or 200 mM KCl, and viscosity was measured at 200 mM KCl, 10 mM $MgCl_2$, and 1 mM ATP (other conditions are given in the legend to Figure 6). The relationship between η_{rel} and the extent of myosin phosphorylation is not linear. Phosphorylation of 50% of the total sites causes only approximately 25% of the total viscosity change, and phosphorylation of the remaining sites is required to complete the viscosity transition. A correlation of the sedimentation patterns with the extent of myosin phosphorylation is being

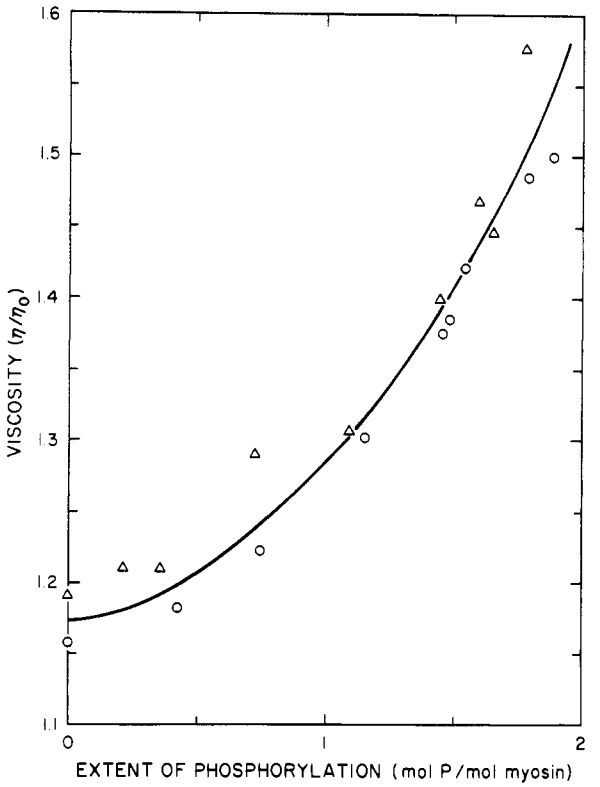

FIGURE 6: Relationship between extent of myosin phosphorylation and viscosity. Varying extents of phosphorylation were obtained by varying kinase concentration and time of incubation; reactions were stopped by addition of EGTA (1 mM). Phosphorylation was carried out in either 75 mM (△) or 0.2 M KCl (○). Other conditions are as in Figure 1. Conditions for viscometry: 0.2 M KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 30 mM Tris-HCl (pH 7.5).

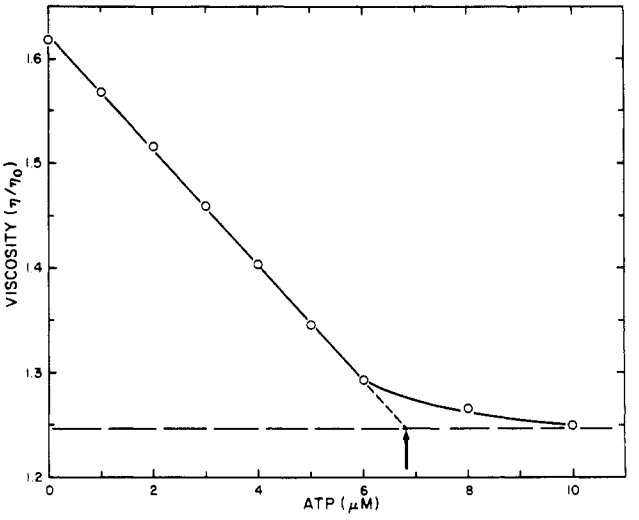

FIGURE 7: Relationship between ATP concentration and viscosity of dephosphorylated gizzard myosin. Myosin was exhaustively dialyzed against 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM dithiothreitol to remove bound nucleotide. Conditions for viscometry: 3.4 μM myosin, 0.2 mg/mL pyruvate kinase, 1 mM phosphoenolpyruvate, 200 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 30 mM Tris-HCl (pH 7.5). Arrow designates 2 mol of ATP added per mol of myosin.

carried out and will be presented later.

The ATP dependence of the viscosity change is shown in Figure 7. Under the conditions chosen, i.e., 0.2 M KCl and 10 mM $MgCl_2$, myosin exists predominantly as the 6S form in the absence of ATP (see Figure 1). As ATP is added, the reduction in η_{rel} is linear, and the change is complete at 2 mol of ATP/mol of myosin. (The concentration of myosin heads

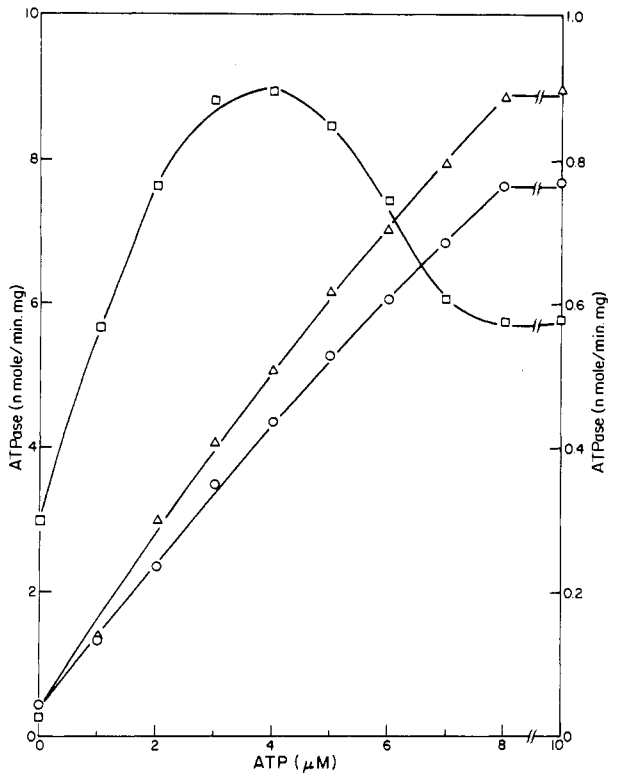

FIGURE 8: Relationship between ATP concentration and Mg^{2+}-ATPase activity of phosphorylated and dephosphorylated gizzard myosin. Myosin was dialyzed as in Figure 7. Myosin concentration was 4 μM. ATPase activities in 10 mM $MgCl_2$ shown for phosphorylated myosin and 0.2 M KCl (△) and dephosphorylated myosin in 0.3 (○) and 0.2 M KCl (□). Other assay conditions are given in Figure 7 and under Materials and Methods. The non-zero intercept at zero added ATP indicates slight residual nucleotide in the myosin preparation. Note the lower ATPase activity (right axis) for dephosphorylated myosin in 0.2 M KCl (□).

is indicated by the arrow in Figure 7.) No further change in viscosity occurs on increasing the ATP concentration to 1 mM. It is likely that the binding of ATP to one of the myosin heads induces a partial conformational change and that the complete transition occurs only when both active sites of myosin are occupied. [K_{app} for ATP and gizzard myosin is less than 1 μM for both phosphorylated and dephosphorylated myosin (Ikebe et al., 1982b), and therefore, the ATP to myosin stoichiometries would approximate those represented by the total concentration of each component.] These experiments were carried out in the presence of an ATP-regenerating system (see legend to Figure 7). Under conditions where the ATP supply is not maintained, the reversibility of the viscosity change may be demonstrated (data not shown). On the addition of ATP (20 μM) to gizzard myosin (4.3 μM) in 0.2 M KCl, the viscosity decreased and remained constant for approximately 4 min. When the level of ATP was reduced to substoichiometric values, the viscosity increased and returned to that obtained in the absence of ATP. From these results it is also evident that ADP cannot elicit the viscosity transition.

The Mg^{2+}-ATPase activity of myosin as a function of ATP concentration is shown in Figure 8. The Mg^{2+}-ATPase activity of phosphorylated myosin (1.8 mol of P/mol of myosin) in 0.2 M KCl and 10 mM $MgCl_2$ increases linearly on the addition of ATP and reaches a maximum at approximately 2 mol of ATP added per mol of myosin. Similar results are obtained for dephosphorylated myosin in 0.3 M KCl and 10 mM $MgCl_2$. However, for dephosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$, the results are quite different. Addition of ATP increases the Mg^{2+}-ATPase activity, but this is optimal at approximately 1 mol of added ATP/mol of myosin. Addition of further ATP causes a decrease in activity, which reaches a constant value at about 2 mol of added ATP/mol of myosin. Note also the difference in the levels of ATPase activity observed in each case. For phosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$ and dephosphorylated myosin in 0.3 M KCl and 10 mM $MgCl_2$, the specific activity is about 8 nmol min^{-1} mg^{-1}. For dephosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$, the maximum activity is about 0.9 nmol min^{-1} mg^{-1} and the specific activity (at saturating concentrations of ATP) about 0.6 nmol min^{-1} mg^{-1}. The significant difference between these two distinct patterns of ATP dependence is that, in the two examples exhibiting a linear relationship, the myosin conformation remains at all levels of ATP in the 6S conformation, whereas, for dephosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$, the conformation in the absence of ATP begins at that of 6S but is progressively altered (see Figure 7) to that of 10S on saturation with ATP.

Discussion

The viscosity measurements described above offer a very convenient and rapid means of following the conformational change of smooth muscle myosin, previously described by other investigators (Suzuki et al., 1982; Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). The point of including the sedimentation patterns was to get an assessment of the species present under various ionic conditions and different levels of phosphorylation. With the exception of experiments performed at relatively low concentrations of KCl (0.125 M, see Figure 2) where myosin polymers are formed, the conditions show only one sedimenting component. At either side of the transition zone, the components approximate either 6S or 10S, and within the transition zone, intermediate boundaries are observed. The fact that the viscosity transitions are shown by the sedimentation velocity experiments to be essentially complete and do not exhibit marked heterogeneity under various ionic conditions is also consistent with the results obtained by gel filtration. These data provide some confidence in the acceptance of the viscosity measurements (with the exception of those carried out at low ionic strength) as a reflection of the 6S–10S transition that occurs with smooth muscle myosin.

The conditions under which the transition occurs are varied, and the influence of ionic strength is the most consistent. Under any conditions, decreasing ionic strength favors the formation of the folded myosin conformation. In general, the effect of the other important variables, e.g., phosphorylation and ATP and Mg^{2+} concentration, is to shift the zone of ionic strength over which the transition occurs. For example, dephosphorylation and the presence of ATP shift the viscosity transition to higher ionic strength (see Figure 2), and in the absence of Mg^{2+}, the transition occurs at lower KCl concentrations (see Figure 4). The points to emphasize are that neither phosphorylation–dephosphorylation nor the presence of ATP is obligatory for the transition. Phosphorylated myosin in the presence of ATP can exist as 10S, and dephosphorylated myosin in the absence of ATP can also form the folded conformation. In either case, whether or not the 10S conformation is formed depends upon the other solvent conditions.

However, under specified solvent conditions, the 6S–10S transition is affected by both the level of myosin phosphorylation and the presence or absence of ATP. The conditions chosen to measure the phosphorylation dependence and ATP dependence of the viscosity transition were 0.2 M KCl and 10 mM $MgCl_2$. In this solvent, phosphorylation favors the

10S to 6S transition, and the addition of ATP favors the 6S to 10S transition. This transition shows a linear dependence on ATP concentration and is complete when ATP is bound to both myosin heads. In contrast, the phosphorylation dependence is not linear, and the more marked conformational change occurs when the extent of phosphorylation exceeds 50% of the available sites. It has been proposed that the phosphorylation of gizzard myosin is sequential (Persechini & Hartshorne, 1981, 1983), and thus at 50% phosphorylation, myosin would exist predominantly as the singly phosphorylated species. From the data presented in Figure 6, it is doubtful that the phosphorylation of one of the myosin heads can cause the transition from 10S to 6S, and it is likely that the complete transition requires phosphorylation of both sites. It is not known if the slight increase in viscosity that is observed at 50% phosphorylation is due to the formation of an intermediate conformation, or is the result of an alteration in the 10S–6S equilibrium.

A point that we have tried to emphasize is the finding that the 10S and 6S conformations are characterized by different enzymatic properties. Suzuki et al. (1978) showed originally that the Mg^{2+}-ATPase activity of gizzard myosin was different for the two conformations, and this we have confirmed. In addition, we have shown above that the KCl-dependent transition in viscosity is reflected closely by an alteration of ATPase activity; i.e., the K^+ dependence of viscosity and ATPase activity are the same. This is shown for the Mg^{2+}-, Ca^{2+}-, and K^+EDTA-ATPase activities. The ATP dependence of Mg^{2+}-ATPase activity (Figure 8) is also consistent with these results. Dephosphorylated myosin in 0.3 M KCl and 10 mM $MgCl_2$ and phosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$ remain in the 6S conformation on the addition of ATP. The ATPase activity increases linearly as the active sites are saturated, and maximum activity is obtained at the expected ATP to myosin stoichiometry of 2:1. In contrast, the addition of ATP to dephosphorylated myosin in 0.2 M KCl and 10 mM $MgCl_2$ elicits a change in conformation. The conformation in the absence of ATP is 6S and, when saturated with ATP, is 10S (as shown in Figure 7). The higher ATPase activity that is observed at one ATP/myosin could be due to the formation of an intermediate conformation, possibly a partially folded form of myosin. This intermediate state, however, has a considerably lower ATPase activity ($\sim$0.9 nmol min^{-1} mg^{-1}) than the equivalent 6S form at this level of ATP ($\sim$5 nmol min^{-1} mg^{-1}). It is interesting that the approximately 10-fold inhibition of Mg^{2+}-ATPase activity characterizing the conversion of 6S to 10S is largely complete at the intermediate state of the transition.

An attractive possibility raised by this discussion is that if the two conformations of myosin possess different biological properties, then the 6S–10S transition could be involved in the regulation of the activity of smooth muscle actomyosin. Specifically, the hypothesis is that phosphorylation of myosin converts the "inactive" 10S species to an "active" 6S form. The activity referred to being the ability of actin to activate the Mg^{2+}-ATPase activity of myosin. At the moment, a direct confirmation of this hypothesis, e.g., by measuring the actin activation of the two forms of myosin, is not available, and supportive evidence relies on less direct correlations. This is summarized as follows: (1) Three types of ATPase activities (Mg^{2+}, Ca^{2+}, K^+ EDTA) have been measured, and in each case, the activity of the 10S and 6S forms differ. (2) The phosphorylation dependence of the viscosity change (Figure 6) and of actin-activated ATPase activity (Persechini & Hartshorne, 1981; Ikebe et al., 1982a) is similar, and it is suggested that for a complete conformational change and for full activation of ATPase activity, myosin must be phosphorylated on both heads. (3) The Mg^{2+} dependence of actomyosin ATPase activity may be correlated to the Mg^{2+} dependence of the viscosity transition. It is established that in vitro smooth muscle actomyosin requires relatively high concentrations of free Mg^{2+} (in the millimolar range) in order to express full ATPase activity (Hartshorne & Gorecka, 1980). At low Mg^{2+} concentrations, phosphorylated myosin is partly in the 10S form (at lower KCl concentrations, Figure 2) and also at low free Mg^{2+} levels the actomyosin ATPase activity is low. It is suggested that an increase in Mg^{2+} concentrations favors the formation of 6S phosphorylated myosin and also causes an increase in actomyosin ATPase (this effect will be reported in more detail later). Myosin light chain kinase does not require relatively high concentrations of free Mg^{2+} for maximum activity (Hartshorne et al., 1980), and therefore, the earlier examples of the Mg^{2+} dependence of actomyosin ATPase activity were not due to different levels of myosin phosphorylation. An inhibition of Mg^{2+}-ATPase at low Mg^{2+} levels is not found with skeletal muscle actomyosin (Hartshorne & Gorecka, 1980).

Thus, it is suggested that the determinant of ATPase activity is the conformation of myosin and that the activity of a given shape is independent of phosphorylation. The point to emphasize is that the shape transformation is usually effected by phosphorylation, and by this indirect route, phosphorylation may regulate Mg^{2+}-ATPase activity of actomyosin. But, conceivably other factors, e.g., oxidation and high $MgCl_2$ concentrations, could also induce a shape change and thereby activate ATPase activity.

The relationship of the 10S to 6S forms to the regulation of actin-activated ATPase activity remains to be established, but since myosin filaments exist in both contracted and relaxed smooth muscle (Somlyo et al., 1981), it is unlikely that the transition observed with monomeric myosin reflects exactly the in vivo situation. It is possible that similar effects occur in myosin filaments, but these could be confined to inter- rather than intramolecular interactions.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5.

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Chain Length Dependent Modification of Lipid Organization by Low Levels of 25-Hydroxycholesterol and 25-Hydroxycholecalciferol. A Laser Raman Study†

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ABSTRACT: We have used Raman spectroscopy to investigate the thermal transitions of multibilayered liposomes composed of lecithins, i.e., dilauroyllecithin, dimyristoyllecithin, dipalmitoyllecithin, distearoyllecithin, or egg lecithin, plus 5-cholesten-3,25-diol (25-hydroxycholesterol), 25-hydroxycholecalciferol, and vitamin D_3. We recorded the CH-stretching (2800–3000-cm^{-1}) regions of the Raman spectra at various temperatures and employed plots of temperature vs. the intensity of the 2880- or 2930-cm^{-1} bands relative to that of the 2850-cm^{-1} feature, i.e., the ratios I_{2880}/I_{2850} and I_{2930}/I_{2850}, to estimate thermal transitions. These plots show multiple discontinuities, each of which may be ascribed to a state change of a separate phase with distinctive proportions of lecithins and cholesterol derivatives. Low concentrations of 25-hydroxycholesterol and 25-OH-D_3 (≥0.2 mol %) abolish the pretransition and split the main transitions of dilauroyllecithin (4 °C) and dimyristoyllecithin (23 °C) into two. The midpoint of the new small transition centers at about 3–4 °C lower than those of the respective main transitions of dilauroyllecithin and dimyristoyllecithin. A further increase in the molar ratio of 25-hydroxycholesterol and 25-hydroxycholecalciferol decreases the amplitudes of the new and the main transitions (dilauroyl- and dimyristoyllecithin). The transitions of dipalmitoyllecithin and distearoyllecithin at 2 mol % concentrations of either sterol remain unaffected. There was no splitting in the main transition of either dipalmitoyllecithin or distearoyllecithin in the presence of these sterols. The perturbing effect of the 25-hydroxysterols follows the order dilauroyllecithin > dimyristoyllecithin > dipalmitoyllecithin > distearoyllecithin. Our data suggest that 25-hydroxysterols disorganize phospholipids of 12–14-carbon acyl-chain length more than those with 16–18-carbon chains. As assessed by changes in the intensities of the 1653-cm^{-1} C=C stretching band, interaction with phospholipids also involves the aliphatic double bonds of 25-hydroxycholecalciferol.

Most cells can derive cholesterol from two sources, namely de novo biosynthesis from acetyl coenzyme A (Sabine, 1977) or receptor-mediated uptake and liposomal degradation of plasma low-density lipoprotein (Brown & Goldstein, 1979). The control mechanisms for cellular cholesterol homeostasis are not perfectly known. Endogenous cholesterol biosynthesis is regulated by a feedback mechanism that controls the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterogenesis, located in reticulum endoplasmic membranes (Rodwell et al., 1976). As shown by Kandutsch & Chen (1975), certain oxidation products of cholesterol, rather than cholesterol itself, regulate sterol biosynthesis at the HMG-CoA reductase level. The list of the oxygenated sterols that have this capacity continues to increase (Schroepfer et al., 1977, 1980; Shakespeare & Wilton, 1980) and includes several cholesterol precursors (Gibbons et al., 1980) and some oxygenated derivatives of vitamin D_3 (Philippot et al., 1976).

The several sterically dissimilar sterols, all more polar than cholesterol, suppress HMG-CoA reductase activity suggests the possibility that this endoplasmic reticulum enzyme is regulated by the state of its membrane lipid environment, as is also indicated by Arrhenius plot discontinuities of HMG-CoA reductase (Mitropoulos & Venkatesan, 1977, Sabine & James, 1976; Philippot & Wallach, 1979), signifying cooperative changes of phase within the enzyme or its microenvironment (Raison, 1973).

In order to elucidate the mechanism of suppression of HMG-CoA reductase activity by these oxygenated sterols, we have used Raman spectroscopy to study the interactions be-

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