the oil asphaltenes that we have examined so far yield squalene on mild pyrolysis, including those showing no sign of microbial degradation, the argument that this squalene is part of the original organic portion of the sediment appears to be strengthened. If so, then squalene must also be present in kerogens. The recent finding of squalene in Norwegian rocks supports this view (*12*). Since squalene is the biosynthetic precursor of plant steroids, it may also occur in trace amounts in coal.

All triterpenoids in nature are biosynthesized from squalene or from squalene-2-epoxide (*13*), and the chemically bound squalene found in asphaltene may be the squalene ether formed from squalene-2-epoxide. Experiments with added reagents known to promote carbon-oxygen bond cleavages in ethers, however, did not increase the squalene yields.

In conclusion, the widespread occurrence of squalene in petroleum asphaltene is a clear indication that squalene was a significant constituent of the biomass from which the oil formed and, as a new biological marker, will have practical application in oil correlations.

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Phosphorylation of Smooth Muscle Myosin: Evidence for Cooperativity Between the Myosin Heads

Abstract. *The relationship between the actin-activated adenosinetriphosphatase activity of smooth muscle myosin and the extent of myosin light chain phosphorylation is nonlinear. It is suggested that the phosphorylation of the two heads of smooth muscle myosin is an ordered process and that the two heads are influenced by cooperative interactions.*

The mechanism by which Ca^{2+} controls the activity of the contractile apparatus of smooth muscle is a controversial subject. The most popular theory is that the contractile activity is determined by the state of phosphorylation of two of the myosin light chains (*1*). Phosphorylation allows the activation by actin of the Mg^{2+}-adenosinetriphosphatase (Mg^{2+}-ATPase) activity of myosin and is thought to be a prerequisite for contraction; dephosphorylation of the myosin light chains eliminates the actin activation and therefore favors the relaxed state. The enzymes that are responsible for the phosphorylation and dephosphorylation have been characterized and are the myosin light chain kinase (*2*) and myosin light chain phosphatases (*3*), respectively. The alternative and minority viewpoint, held by Ebashi and co-workers (*4*), is that myosin phosphorylation is not significant and that regulation is achieved by a system based on the thin filaments, termed leiotonin. Whether these two mechanisms coexist within a single cell, are exclusive of each other, or are in some way complementary, is not known. The relative merits and contributions of each system must be established before the regulatory mechanism in smooth muscle is understood.

Our approach was to try to resolve the contribution made by one of the mechanisms above, namely phosphorylation. The relationship between the extent of myosin phosphorylation and the degree of activation by actin of the Mg^{2+}-ATPase activity has not previously been examined in a defined system, that is, one where each of the component proteins was separately purified and characterized. This is necessary to reduce the possible influence of unknown contaminant proteins. In particular, we wished to establish whether phosphorylation alone is sufficient for actin activation of ATPase activity and, if so, the relationship between the extent of phosphorylation and the level of ATPase activation.

The relationship between the extent of light chain phosphorylation and the activation of Mg^{2+}-ATPase activity by skeletal muscle actin is shown in Fig. 1. These data were collected by two procedures. In one method the time courses of myosin phosphorylation and adenosine triphosphate (ATP) hydrolysis were followed and at various times (4 to 10 minutes after the addition of ATP) the levels of phosphorylation were correlated to the ATPase rate, which was estimated from a tangent drawn to the relevant time point. In the other method myosin phosphorylation and ATPase activity were estimated from a single point at 10 minutes. The latter method is less accurate in that it could be affected by nonlinearity of either the phosphorylation or the ATPase reaction. However, use of the more convenient procedure is justified since both methods gave similar values, as shown in Fig. 1.

Figure 1 shows that the ATPase activity is not directly proportional to the degree of phosphorylation. Initially about 50 percent of the total sites are phosphorylated, and this results in only a slight activation of ATPase activity. At 50 percent phosphorylation the specific actin-activated ATPase activity is about 3 nmole of inorganic phosphate (P_i) liberated per minute per milligram of myosin. Subsequently, the remaining sites are phosphorylated and the ATPase activity is markedly enhanced. At saturating levels of phosphorylation the specific actin-activated ATPase activity is estimated to be about 28 nmole min^{-1} mg^{-1}. It appears, therefore, that significant activation of ATPase activity occurs only at relatively high levels of phosphorylation (> 50 percent), below which the ATPase activity does not show as marked a dependence on the degree of phosphorylation.

What this type of relationship means at a molecular level is worthy of some speculation. If phosphorylation of one of the two myosin heads resulted in ATPase activation of either that head or both heads, then a linear relation between ATPase activity and phosphorylation would be observed. This situation does not apply to the experimental data. Thus it can be suggested that myosin with only one head phosphorylated is not activated significantly by actin. Assuming that both heads contain equivalent enzymatic sites, it appears that the nonphosphorylated head inhibits the activity of the phosphorylated head or that an

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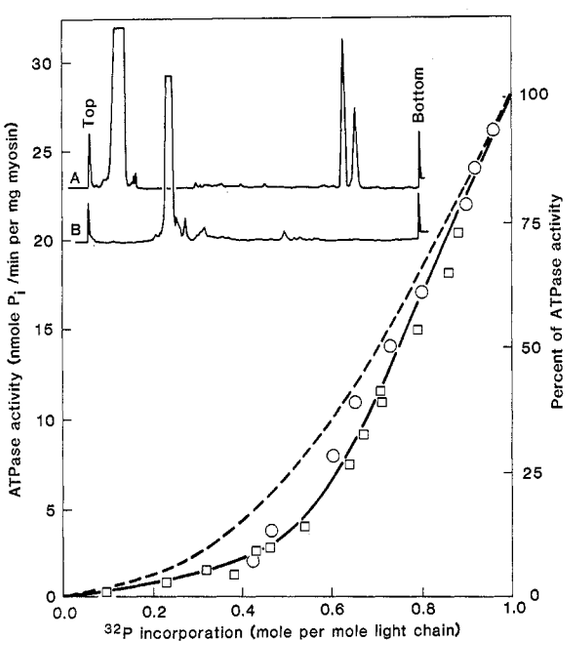
interaction between two phosphorylated heads is required for activity. Either hypothesis would account for the marked stimulation of ATPase activity as the second half of the available sites are phosphorylated. It is reasonable therefore to suggest that some cooperative interactions occur between the two myosin heads and are sensitive to the phosphorylation of the 20,000-dalton light chain. Cooperativity between the two myosin heads has also been found with scallop heavy meromyosin (*5*), and it was shown that both calcium sites (one on each head) on the myosin molecule needed to be filled before ATPase was activated. This is clearly analogous to the dependence of ATPase activity on phosphorylation reported here. It is interesting to note that the ATPase activity of smooth muscle heavy meromyosin subfragment 1 (a single head) is activated by actin, and phosphorylation is not required (*6*).

The next point to consider is whether the phosphorylation is random or is a sequential, or ordered, process. If the phosphorylation is random, the ATPase activity, due to the formation of myosin with both heads phosphorylated, should increase as the square of the fractional phosphorylation. For example, when half the total sites are phosphorylated, one-quarter of the myosin molecules would be active. The relation between ATPase activity and random phosphorylation is shown in Fig. 1, and although there are similarities between the theoretical and experimental curves, it appears that the latter does not fit the random model. The possibility of ordered phosphorylation should therefore be considered. If one of the myosin heads is phosphorylated more easily than the other, and only the doubly phosphorylated form of myosin is active, then the ATPase activity would be zero until 50 percent of the sites are phosphorylated and would then increase linearly as the second head is filled. The experimental observations do not conform to this situation. However, an ordered model is consistent with the data if it is assumed that myosin with one head phosphorylated has only a fraction of the activity (~ 10 percent) associated with the fully phosphorylated molecule.

Evidence supporting this hypothesis is shown in Fig. 2. The phosphorylation levels were determined at a fixed time (10 minutes) and at different concentrations of myosin light chain kinase. Phosphorylation of approximately half the total sites occurs with relative ease and the level of phosphorylation approaches a linear dependence on the kinase concentration. Subsequent phosphorylation, however, requires markedly increased levels of myosin light chain kinase. For example, a kinase concentration of approximately 1.5 μg/ml is required to achieve 50 percent phosphorylation, whereas more than ten times this amount is needed to reach the 80 percent level. This type of behavior is not found with isolated chicken gizzard myosin light chains (Fig. 2). To illustrate more dramatically the difference between the



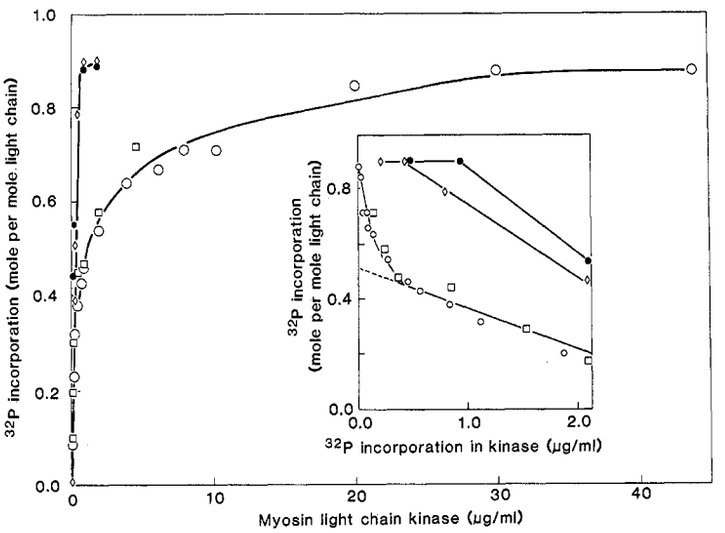


Fig. 1 (left). Relationship between actin-activated ATPase activity and phosphorylation of gizzard myosin. (○) Points taken from the time courses of phosphorylation and ATPase activity; (□) single 10-minute time point assays. A theoretical curve based on a random phosphorylation is shown by the dashed line. The ATPase activity and phosphorylation levels were determined at 25°C with different concentrations of myosin light chain kinase (0 to 43 μg/ml) and constant calmodulin (11 μg/ml). The ATPase activity of myosin alone (~ 2 nmole min^{-1} mg^{-1}) was subtracted from each point. Assay conditions: 4 m*M* $MgCl_2$; 60 m*M* KCl; 25 m*M* Tris-HCl (*p*H 7.5); 1 m*M* [γ-^{32}P]ATP (~ 3000 count min^{-1} $nmole^{-1}$ for phosphorylation assays and 1000 count min^{-1} $nmole^{-1}$ for ATPase assays); gizzard myosin, 0.44 mg/ml; and skeletal muscle actin, 0.25 mg/ml. Phosphate incorporation (*8*) and ATPase activity (*9*) were determined as described previously. Chicken gizzard myosin was purified as described previously (*10*) with a modification to include three initial washing steps with 3 percent Triton X-100, 15 m*M* $MgCl_2$, 50 m*M* KCl, and 10 m*M* Tris-HCl (*p*H 7.5). Scans of gradient sodium dodecyl sulfate (SDS)–polyacrylamide gels (*11*) of myosin (A) and myosin light chain kinase (B) are also shown. From densitometry the myosin was estimated to be > 96 percent pure and the myosin light chain kinase > 92 percent pure. Major contaminants in the kinase preparation were fragments derived by proteolysis of the parent 105,000-dalton apoenzyme. No myosin light chain kinase or phosphatase activity was evident in the myosin preparation. The latter was measured by allowing the phosphorylation of myosin to proceed under the assay conditions and then inactivating the kinase by addition of EGTA to 1 m*M* and following any subsequent dephosphorylation over a 10-minute period. The level of phosphorylation in the isolated myosin was estimated by electrophoresis on alkaline urea gels (*12*) and preparations were used only if it was not detectable. This ensured that the incorporation of ^{32}P-labeled phosphate accurately reflected the extent of total phosphorylation. Other proteins prepared were: skeletal muscle actin (*13*), chicken gizzard myosin light chain kinase (*14*), and bull testes calmodulin (*15*) with a final purification on a fluphenazine affinity column (*16*). Fig. 2 (right). Phosphorylation of intact myosin and partially purified myosin light chains at different concentrations of myosin light chain kinase. Conditions as in Fig. 1. Phosphorylation of myosin at (□) 0.1 mg/ml and (○) 0.44 mg/ml. Phosphorylation of isolated light chains at (●) 0.01 mg/ml and (◇) 0.02 mg/ml. The concentration of unphosphorylated 20,000-dalton light chain in the partially purified light chain preparation was determined by densitometry of gradient SDS-polyacrylamide gels (*11*) and alkaline urea gels (*12*).

phosphorylation sites of whole myosin and those of the isolated light chains, the data are presented in an alternative form in the inset of Fig. 2. It could be inferred from Fig. 2 that the phosphorylation sites of whole myosin fall into two classes, the first 50 percent being phosphorylated relatively easily and the second 50 percent phosphorylated with more difficulty. When the light chains are removed from the myosin molecule, only one class of sites is apparent. This behavior can be explained by assuming cooperative interaction of the myosin heads, and it is suggested that phosphorylation of the first head hinders that of the second head. This would generate a sequential or ordered phosphorylation process.

Alternatively, it might be argued that the nonlinearity of the phosphorylation reaction is due to progressive reduction of available substrate. This possibility cannot be eliminated since the Michaelis constant, K_m, for the myosin and myosin light chain kinase is not known under our assay conditions. However, we think this explanation is unlikely because a similar phosphorylation profile is obtained at two different myosin concentrations (0.4 and 1.9 μM with respect to the 20,000-dalton light chain) and because phosphorylation of the isolated light chains (at 0.5 and 1.0 μM) showed a markedly different dependence on kinase concentration.

These results imply that the two heads of the smooth muscle myosin molecule do not act independently. This cooperative behavior of smooth muscle myosin has apparently not been noted previously, possibly because of the use of more complex protein systems and different assay conditions (*7*) and because the prephosphorylation levels of myosin are often not taken into account.

In these studies we used the simplest assay system that was available with our current level of expertise. Although this approach has the advantage that it allowed a less ambiguous interpretation of the effects of phosphorylation than was possible previously, it has the disadvantage that the system does not fully represent the native situation. The regulatory mechanism must be more complex than our experiments indicate, and the cooperative behavior of myosin remains to be investigated when additional components, such as tropomyosin, are added.

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A Conjugate of α-Amanitin and Monoclonal Immunoglobulin G to Thy 1.2 Antigen Is Selectively Toxic to T Lymphoma Cells

Abstract. *A covalent conjugate of an α-amanitin azo derivative and a monoclonal immunoglobulin G to the Thy 1.2 antigen on murine T lymphocytes was synthesized. The conjugate was 375- to 750-fold more inhibitory to murine T lymphoma S49.1 cells than the unconjugated derivative. At 0.7 × 10^{-7} to 1.5 × 10^{-7}M and at 4 × 10^{-7}M amanitin equivalents, the conjugate inhibited protein synthesis in S49.1 cells by 50 percent and 80 to 96 percent, respectively. At these concentrations, mutant Thy 1–deficient S49 cells and other murine lymphomas lacking Thy 1 altogether or carrying Thy 1.1 antigens were unaffected. This result demonstrates the potential for targeting amanitin to specific cell types.*

Conjugates of toxins and proteins have been constructed by coupling diphtheria toxin, ricin, the toxin A chains, gelonin, and amanitin to antibodies, hormones, or concanavalin A; and the targeting potentials of the conjugates have been evaluated with mammalian cells (*1–6*). Conjugates in which a monoclonal antibody directed against a colorectal carcinoma are linked either to a diphtheria A chain or to a ricin A chain are cell-specific and inhibit protein synthesis by the carcinoma cells at a median effective dose (ED_{50}) of $10^{-9}M$ (*5*). An ED_{50} of 10^{-9} to $10^{-11}M$ has been demonstrated with an epidermal growth factor coupled to a ricin A chain (*2*), with a monoclonal antibody to Thy 1.2, a differentiation antigen on murine T cells, coupled to a ricin A chain (*1*), and with a monoclonal antibody to Thy 1.2 coupled to ricin (*6*). However, in several studies, the conjugates of ricin, diphtheria toxin, or the toxin A chains with antibodies or hormones were of relatively low potency or completely nontoxic, even when the receptor-binding activity was retained (*1*, *3*). Although it is not known to what extent this decreased toxicity of the conjugates is due to the lysosomal degradation of the toxins before they reach intracellular targets, such degradation may be significant in inactivating many of the chimeric toxins.

α-Amanitin, a potent and relatively specific inhibitor of RNA polymerase II, is a bicyclic octapeptide (*7*, *8*). Although lactoperoxidase inactivates α-amanitin in vitro (*9*), the relative stability of amanitin observed in studies of whole animals (*10*) indicates that it is not readily affected by lysosomal inactivation. Conjugates of amanitin with monoclonal antibodies of varying specificities would therefore be useful in comparisons with conjugates of diphtheria toxin, ricin, or their respective A chains for the study of cellular uptake and processing of the conjugates. We now report that a conjugate of an α-amanitin azo derivative and a monoclonal immunoglobulin G (IgG) to Thy 1.2 (anti-Thy 1.2) is a selective toxic agent directed by the antibody to a murine T lymphoma S49.1. The conjugate is 133 to 277 times more toxic against S49.1 cells than native α-amanitin and 375 to

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