Cooperativity in Scallop Myosin†

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ABSTRACT: Calcium binding and the calcium dependence of the actin-activated MgATPase activity were compared in scallop myofibrils, myosin, and heavy meromyosin (HMM). All these preparations bound two calciums per myosin molecule (or HMM) with identical stoichiometric constants: $K_1 = K_2 = 0.5–1.0 \times 10^7\ M^{-1}$ at pH 7.5, 25 °C. In the presence of adenosine tri- or diphosphate, the calcium binding curve shifted to higher calcium concentrations by about 0.2–0.3 pCa unit at half-saturation. Decreasing the pH from 7.5 to 7.0 decreased the calcium affinity by about tenfold. No evidence for cooperativity in calcium binding was obtained. Predictions for the calcium dependence of ATPase activity from the calcium binding curves depend on the way calcium activation is modeled. In an "independent-site" model where binding of calcium by a myosin head enhances the ATPase activity of that, the degree of normalized calcium binding and the calcium dependence of the normalized ATPase activity superimpose. In a "two-site" model where calcium binding by both myosin heads is required for ATPase activation, ATPase activity lags behind calcium binding. In a "one-site" model where calcium binding by one of the two myosin heads is sufficient for triggering the ATPase activity of the myosin molecule, ATPase activity rises faster than calcium binding. The ATPase activation of acto-HMM fits well a "two-site" model, indicating that two calciums must bind to each myosin molecule in order to switch the molecule on. Since the two calcium binding sites are on different myosin heads, the results also indicate an intramolecular cooperativity between the two myosin heads during calcium activation. The calcium dependence of the ATPase activity of myofibrils or actomyosin prepared from pure scallop myosin and pure rabbit actin showed a very sharp transition that could not be explained by any simple model of intramolecular cooperativity between the two calcium binding sites. Calcium dependence of tension development of skinned fiber bundles of the striated scallop adductor muscles showed a similarly sharp transition. Although activation initially followed predictions of the "two-site" model, activity rose sharply and crossed the calcium binding curve to follow the "one-site" model at higher calcium concentrations. As a possible explanation we propose that the shape of the activation curve in fiber bundles, myofibrils, and actomyosin is the result of an intermolecular cooperativity within the thick filaments. Such interaction would enable myosin molecules to switch on by binding of only one calcium ion once a certain number of myosin molecules (ca. 20%) had been initially activated by the binding of two calcium ions.

Scallop myosin is a regulatory myosin; in the presence of pure F-actin it acts as a calcium sensor and confers calcium sensitivity to the actin-activated ATPase activity in vitro (Kendrick-Jones et al., 1970; Szent-Györgyi, 1975) and to tension production in scallop fiber bundles (Simmons & Szent-Györgyi, 1978). The myosin-linked regulatory mechanism is in contrast to actin-linked regulation, which requires troponin and tropomyosin and is seen, for example, in vertebrate skeletal muscle (Ebashi et al., 1969; Lehman & Szent-Györgyi, 1975).

The calcium switch on scallop myosin comprises a calcium-specific site, one on each myosin head, which is a function of the scallop heavy chain and the regulatory light chain; when one or both regulatory light chains are removed, the molecule no longer confers calcium sensitivity to the actin-activated ATPase and is said to be desensitized (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976; Chantler & Szent-Györgyi, 1980). The main ligands involved in the formation of the calcium specific site appear to originate on the myosin heavy chain (Chantler & Szent-Györgyi, 1980). Nevertheless, this site is neither functional nor calcium specific unless the regulatory light chain is present.

Both regulatory light chains are required in an intact myosin molecule for the inhibition of ATPase activity, indicating a cooperativity between myosin heads (Chantler & Szent-Györgyi, 1980). However, these results are also explained if the loss of one regulatory light chain per myosin molecule causes the light chain free head to interfere with the function of the remaining regulatory light chain (Kendrick-Jones & Jakes, 1977; Chantler & Szent-Györgyi, 1980), and the cooperative effects may not be physiological. It was therefore of particular interest to check whether triggering of the actin-activated ATPase of intact myosin by calcium shows cooperative behavior. A comparison of pCa binding curves and pCa ATPase curves obtained under very similar conditions shows conclusively that two calciums must bind to each myosin molecule in order to switch the molecule on.

Materials and Methods

(*a*) *Materials.* *Aequipecten irradians* and *Placopecten magellanicus* were obtained from the Marine Biology Laboratory, Woods Hole, MA. The adductor muscles of these animals were suspended in an equal mixture of ethylene glycol and a solution containing 40 mM NaCl, 5.0 mM phosphate, pH 7.0, 1.0 mM $MgCl_2$, 0.1 mM EDTA,[1] 3.0 mM NaN_3, and 0.1% sulfadiazine to which 0.1 mM phenylmethanesulfonyl fluoride was freshly added; this was kept for 2 days at 0 °C and then stored at −20 °C prior to use.

(*b*) *Preparations.* Myofibrils were prepared exactly as described earlier (Chantler & Szent-Györgyi, 1980). Myosin

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HDTA, hexamethylenedinitrilotetracetic acid; $NaDodSO_4$, sodium dodecyl sulfate; HMM, heavy meromyosin; S-1, subfragment 1; S-2, subfragment 2.

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was prepared by the procedure of Focant & Huriaux (1976) as described in Chantler & Szent-Györgyi (1978); rabbit F-actin as described previously (Kendrick-Jones et al., 1970). Calcium-sensitive HMM was prepared as described by Craig et al. (1980) with the omission of $MgCl_2$ from the digestion mixture. The preparation showed a significant fragmentation of the heavy chains on $NaDodSO_4$–acrylamide gel electrophoresis, although on native gels low molecular weight components were not prevalent (E. M. Szentkiralyi, personal communication). Urea gels indicated that the light chains remained intact (Figure 4).

(*c*) *ATPase Assays.* Routine ATPase assays were performed as described earlier (Chantler & Szent-Györgyi, 1980) by following proton liberation in a pH-stat. pCa–ATPase curves were also performed in a pH-stat; however, the calcium level was adjusted incrementally using a Hamilton syringe. A routine curve was obtained as follows: a 0.1–0.3 mL aliquot of protein (myosin or myofibrils in 40 mM NaCl, 1.0 mM $MgCl_2$, 5.0 mM phosphate, and 0.1 mM EGTA, pH 7.0) was placed in ice for 5 min with the following additions—NaCl to 0.6 M; ATP to 2–5 mM; rabbit F-actin to a 10-fold molar excess over the myosin. This relatively clear solution would then be added dropwise with a Pasteur pipet to the pH-stat solution (final composition about 30 mM NaCl, 3.0 mM $MgCl_2$, 2.0 mM ATP, and 0.1 mM EGTA, pH 7.5, 25 °C; see individual experiments for precise details; the ionic strength varied by about 0.01 in experiments). Calcium was then added in 1–30-μL aliquots (20 mM $CaCl_2$ stock from a Hamilton syringe) and the rate obtained after each successive 2-min excursion of the chart paper. Several such experiments, with different volumes of calcium added at each point, were superimposed. A prerequisite to each experiment was that the actomyosin or myofibrils gave a straight line in the presence of 0.1 mM free calcium over a 15–20-min period. This control was always applied before embarking upon any pCa–ATPase curve. Contaminating calcium was estimated as 2 ± 1 μM; we thank Peter Barrett of Perkin-Elmer, Wellesley, MA, for these measurements which were made on a Perkin-Elmer 380 atomic absorption spectrophotometer.

ATPase determinations performed directly in the radioactive CaEGTA buffers which were used for calcium binding were made by the method of Taussky & Schorr (1952). Control experiments before each set of determinations showed that the actin-activated ATPase in each calcium buffer was linear for at least 4 min. Routinely, phosphate determination was made after 2 or 3 min in triplicates for each buffer.

(*d*) *Calcium Binding.* pCa binding curves on myosin and myofibrils, performed at low ionic strength, were executed by methods similar to those described previously (Kendrick-Jones et al., 1970). However, as several significant changes were made in this procedure, it is described in detail here.

CaEGTA buffers were made up from 50 mM CaEGTA and 100 mM EGTA stock solutions. The final total calcium concentration was always 0.1 mM and the EGTA concentration was varied so as to give the desired free calcium concentration. The ionic strength across a range of buffers varied from 0.040 to 0.047, although all but one buffer were in the 0.04–0.045 range. Sometimes HDTA was added to compensate for variations in EGTA concentrations in order to keep the ionic strength constant across the buffer range, usually at 0.045 (see individual experiments). ^{45}Ca was added to give a final count of around 20000 cpm/0.5 mL of buffer; 10 mM [^{3}H]glucose (20000 cpm/0.5 mL) was added to the solution as a volume marker. NaCl, imidazole, and $MgCl_2$ were also present in all CaEGTA buffers, the exact concentration depending upon the individual experiment. Binding experiments performed in the presence of ATP also contained 10 mM creatine phosphate and 0.25 mg mL^{-1} creatine phosphokinase as a backup system. All buffers were brought up to the desired pH (±0.02 pH unit) at the appropriate temperature just before use.

Proteins were thoroughly washed in a buffer containing NaCl, imidazole, and $MgCl_2$ (the same concentration as in the radioactive buffer) prior to resuspension in the radioactive CaEGTA buffer. For experiments performed in the absence of ATP, samples were incubated at the appropriate temperature 2 times for 10 min, the protein being spun down after each incubation. Only one 5-min incubation was performed for experiments in the presence of ATP. Each 10-mL aliquot was gently agitated during the incubations, using a separate Pasteur pipet for each aliquot. Duplicate samples at each pCa value were always made. Protein concentrations were 0.5–1.0 mg mL^{-1}. The final pellet was redissolved with 1.2 mL of 1.2 M NaCl, 10 mM phosphate, pH 7.0; from this solution two 0.5-mL aliquots were removed and added to 6 mL of scintillant (ACS, Amersham) and counted in a Beckman counter. Triplicate 10 or 20 μL samples were also removed for protein determinations. Each sample was counted twice. This pair of values and the pair of values from the sister aliquot were averaged; 60% by weight of the myofibril was considered to be myosin (Szent-Györgyi et al., 1973).

The above procedures could not be used for HMM as it is soluble even at low ionic strength. A rapid equilibrium dialysis technique was used (Englander & Crowe, 1965), as this overcame the problem of ATP hydrolysis during the course of the experiment. CaEGTA buffers were prepared similar to those above except that the total calcium concentration amounted to 0.05 mM and they lacked tritiated glucose. For each determination, 1.0 mL of HMM ($\sim$10 mg mL^{-1}) in 15 mM NaCl, 15 mM imidazole, 3.0 mM $MgCl_2$, and 2.0 mM ATP, pH 7.5, was placed in a Spectrapor 2 dialysis sac and held between the two extremes of the rapid dialysis apparatus (Englander & Crowe, 1965). The sac was immersed in a cylinder containing 450 mL of the radioactive CaEGTA buffer (including 15 mM NaCl, 15 mM imidazole, 3.0 mM $MgCl_2$, and 2.0 mM ATP, pH 7.5) and was stirred vigorously on magnetic stirrer for 30–35 min at 22 °C. After this time the contents of the sac and aliquots of the dialysate were prepared for scintillation counting and protein determination. Because the HMM could have removed only a minute fraction of the total calcium, a duplicate sample could be started immediately in the same buffer. Control experiments showed that while calcium in the absence of EGTA reached equilibrium after only 10 min, 20 min was required for calcium to reach equilibrium in the presence of EGTA. A control experiment with [^{32}P]ATP showed that ATP also reached equilibrium during this time; this means that the pool of ATP outside the dialysis sac was available to the HMM within the dialysis sac at all times during the experiment.

HMM was prepared from Aequipecten muscles; the pCa–ATPase data of *Aequipecten* and *Placopecten* myofibrils superpose under identical conditions.

(*e*) *Calculations.* Free calcium and magnesium concentrations were calculated on a Wang 500 programmable calculator by using an iterative procedure (Storer & Cornish-Bowden, 1976) as described earlier in Chantler & Szent-Györgyi, (1980). The following logarithms of binding constants were used (Sillen & Martell, 1971): EGTA/H^+, 9.53 (25 °C), 9.95 (0–5 °C); HEGTA/H^+, 8.88 (25 °C), 9.20 (0–5 °C); H_2EGTA/H^+, 2.68; H_3EGTA/H^+, 2.00; EGTA/Ca^{2+},

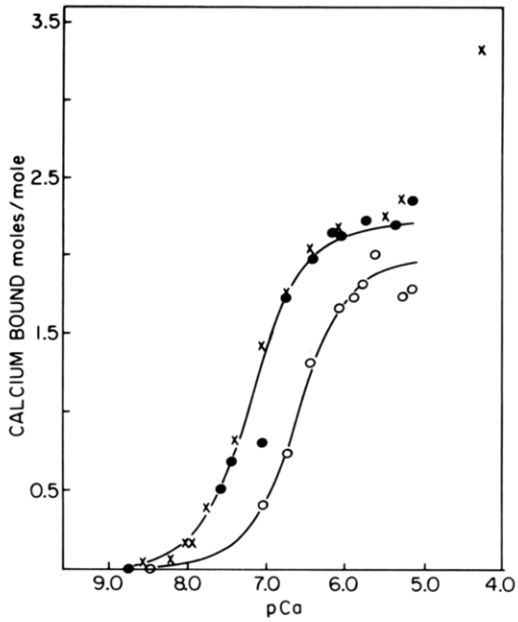

FIGURE 1: Calcium binding by *Placopecten* myofibrils. (×) 1 mM $MgCl_2$, 40 mM NaCl, and 10 mM imidazole, pH 7.5, 0 °C. (●) Same conditions but at 25 °C. The results are fitted by the line drawn with $K_1 = K_2 = 1.5 \times 10^7$ M^{-1}. (○) 10 mM $MgCl_2$, 40 mM NaCl, and 10 mM imidazole, pH 7.5, 25 °C. The line is drawn by using $K_1 = K_2 = 4 \times 10^6$ M^{-1}.

10.97 (25 °C), 11.53 (0–5 °C); HEGTA/Ca^{2+}, 5.30; EGTA/Mg^{2+}, 5.20 (25 °C), 4.83 (0–5 °C); HEGTA/Mg^{2+}, 3.40; ATP/H^+, 6.54 (25 °C), 6.54 (0–5 °C); HATP/H^+ 4.06 (25 °C), 4.29 (0–5 °C); ATP/Ca^{2+} 3.90 (25 °C), 4.10 (0–5 °C); ATP/Mg^{2+} 4.22 (25 °C), 3.97 (0–5 °C); creatine phosphate/Ca^{2+}, 1.1; creatine phosphate/Mg^{2+}, 1.60.

pCa binding and ATPase curves were fitted by using two stoichiometric constants (Klotz & Hunston, 1979) and the best fit determined by eye on a trial and error basis. When the maximum amount of calcium bound per mole of myosin is normalized to 1.0 after such a curve-fitting procedure, then the predicted ATPase values at each point can be established directly. pCa–ATPase curves were normalized from 0 to 1.0 so as to be directly comparable with the pCa binding curve under the same conditions.

(f) Other Procedures. Polyacrylamide disc gel electrophoresis in the presence of urea was performed as described previously (Kendrick-Jones et al., 1976). $NaDodSO_4$ slab gel electrophoresis was performed according to Matsudaira & Burgess (1978).

Protein concentrations were determined either by the Folin–Lowry procedure (Lowry et al., 1951) with bovine serum albumin as a standard or by the biuret method (Gornall et al., 1949) with an optical density of 0.07 cm^{-1} $(mg/mL)^{-1}$ at 550 nm.

Results

Calcium Binding. Intact Placopecten myofibrils bind approximately 2 mol of calcium per mol of myosin (Figure 1). The experimental points from pCa 9.0 to 5.5 are fitted well by using two identical stoichiometric constants ($K_1 = K_2 = 1.5 \times 10^7$ M^{-1} in 1 mM $MgCl_2$, pH 7.5). At pCa values below 5.5 additional lower affinity sites begin to bind calcium. Affinity for calcium is the same at 0 °C as at 25 °C (Figure 1). Calcium binding is not influenced by small changes in ionic strength, and the same results were obtained when the ionic strength was held constant with HDTA as in the experiment shown (Figure 1), where the ionic strength was allowed to vary with EGTA concentration between 0.043 and 0.047. Other

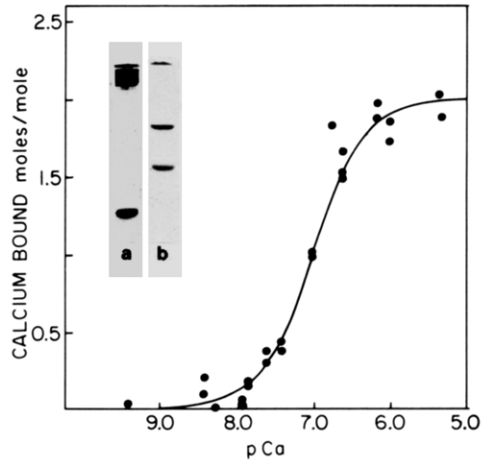

FIGURE 2: Calcium binding by *Placopecten* myosin. (●) 1 mM $MgCl_2$, 40 mM NaCl, and 10 mM imidazole, pH 7.5, 25 °C. The results are fitted by the line drawn by use of $K_1 = K_2 = 10^7$ M^{-1}. Inset: (a) myosin, 7.5% $NaDodSO_4$–polyacrylamide gel electrophoresis; (b) myosin, 10% urea–gel electrophoresis.

workers have also found that calcium binding by rabbit myosin in unaffected by limited changes in ionic strength or temperature (Morimoto & Harrington, 1974; Holroyde et al., 1979). A tenfold increase in magnesium concentration decreases the apparent affinity of *Placopecten* myofibrils for calcium (Figure 1; $K_1 = K_2 = 4.0 \times 10^6$ M^{-1}), although ionic strength effects have not been rigorously excluded. Decreasing the pH from 7.50 to 7.00 decreases the calcium affinity of scallop myofibrils by about an order of magnitude (data not shown). The calcium binding curve for myosin, under similar conditions, is almost superimposable on the myofibril curve (Figure 2, $K_1 = K_2 = 1.0 \times 10^7$ M^{-1}).

In order to relate pCa binding and pCa–ATPase curves under identical conditions, the effect of MgATP on calcium binding has to be evaluated. Difficulties arise if one tries to complete a calcium binding curve with scallop myofibrils in the presence of ATP: as the actin-activated rate is high at higher free calcium concentrations ($\geq$1.00 μmol min^{-1} mg^{-1}), it is difficult to prevent hydrolysis within the time of the experiment, even with a backup system. This problem is exacerbated by the pelleting step in the calcium binding methodology for insoluble proteins because high local protein concentrations may exhaust local ATP supplies. In addition, these conditions in the absence of calcium parallel those for separation of actin and myosin between the pellet and supernatant (Szent-Györgyi et al., 1971). Myosin was used, therefore, in the presence of a creatine phosphokinase–creatine phosphate backup system in order to obtain a pCa binding curve in the presence of ATP. This ensured that ATP was still present after the 15–20-min interval required for all manipulations. Magnesium was always present over nucleotides to keep $[Mg^{2+}]_{free}$ about 1 mM. It is tacitly assumed that actin is not a requirement for initiation of calcium-dependent changes on myosin (see Kendrick-Jones et al., 1976; Chantler & Szent-Györgyi, 1978). In the presence of ATP (or ADP), the calcium binding curve shifts to higher calcium by about 0.2–0.3 pCa unit at half-saturation, and the data can be best fitted with stoichiometric affinity constants, $K_1 = K_2 = 5 \times 10^6$ M^{-1} (Figure 3). A similar shift in the presence of ATP is also seen at pH 7.0 (data not shown). Creatine phosphate and creatine phosphokinase in the absence of adenine nucleotides had no effect on calcium binding.

Since HMM is soluble even at low ionic strength, calcium binding studies in the presence of ATP require a rapid dialysis technique (Englander & Crowe, 1965). Using this method,

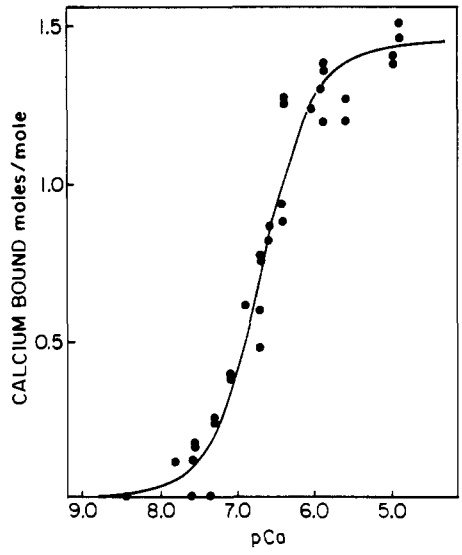

FIGURE 3: Calcium binding by *Placopecten* myosin in the presence of ATP: 3 mM $MgCl_2$, 2 mM ATP, 5 mM NaCl, 15 mM imidazole, pH 7.5, 10 mM creatine phosphate, and 0.25 mg mL^{-1} creatine phosphokinase. The results are fitted by the line drawn by use of $K_1 = K_2 = 5 \times 10^6$ M^{-1}.

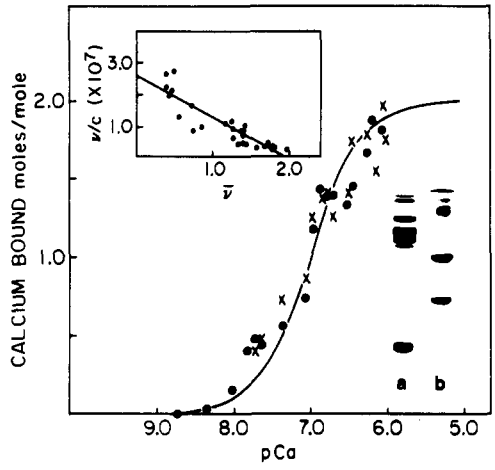

FIGURE 4: Calcium binding by *Aequipecten* heavy meromyosin: 3 mM $MgCl_2$, 2 mM ATP, 15 mM NaCl, and 15 mM imidazole, pH 7.5, at 25 °C. The results are fitted by the line drawn by use of $K_1 = K_2 = 10^7$ M^{-1}. Different symbols represent measurements with two different preparations. Inset: Scatchard plot; (a) HMM, 7.5% $NaDodSO_4$–acrylamide gel electrophoresis; (b) HMM, 10% urea–acrylamide gel electrophoresis.

we were able to attain equilibrium within 20 min in ^{45}CaEGTA at 25 °C without substantial hydrolysis of the total ATP available. The results can be fitted with stoichiometric affinity constants $K_1 = K_2 = 1 \times 10^7$ M^{-1} (Figure 4). Myofibrils, myosin, and HMM bind 2 mol of calcium/mol of myosin (or HMM) in a similar manner, and no cooperativity in calcium binding is seen in the presence or in the absence of ATP. The results indicate that presence of actin, tropomyosin, or the filamentous state of myosin does not influence calcium binding by myosin.

Models Relating Calcium Binding to pCa of ATPase. Calcium binding by scallop myosin switches on the actin-activated MgATPase (Szent-Györgyi et al., 1973; Kendrick-Jones et al., 1976). As the specific calcium sites are on the myosin molecule, a definite relationship must exist between binding and ATPase activation. Three models may be envisaged. In the first model, two calcium ions must bind per molecule of myosin in order to switch the actin-activated ATPase on ("two-site" model). In the second model, one

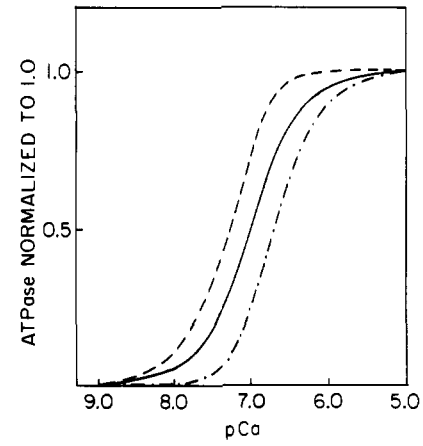

FIGURE 5: Models relating calcium binding to ATPase activity. (—) Normalized calcium binding ($K_1 = K_2 = 10^7$ M^{-1}) and "independent site" model ATPase curve; (- - -) 1 mol of calcium/mol of myosin is sufficient to switch on the ATPase; (·····) 2 mol of calcium/mol of myosin is required to switch on the ATPase.

calcium ion must bind per molecule in order to elevate the ATPase of the entire molecule ("one-site" model). The third model requires that an enhancement of the actin-activated ATPase per head of myosin occurs when one calcium ion binds per head ("independent site" model). These models are illustrated in Figure 5. The solid line in this figure represents near-random calcium binding in myosin, with $K_1 = K_2 = 10^7$ M^{-1}. If one normalizes the calcium binding in the plateau region (pCa of 5.50 at pH 7.50) to 1.0, then one may predict the pCa–ATPase curve according to the models by simple probability. Thus, if the degree of calcium binding is X (where $0 \leq X \leq 1.0$), then the predicted ATPase at that point (also normalized between 0 and 1.0) would be X^2 according to the "two-site" model, $X^2 + 2X(1 - X)$ according to the "one-site" model, or simply, X for the "independent-site" model. As seen in Figure 5, if two calcium ions are required to switch on a myosin molecule, then the predicted ATPase always falls below the binding curve, has a steeper slope than the binding curve, and falls away maximally from the binding curve in the "take-off" region of calcium concentrations. If only one calcium is required to switch on the myosin molecule, then the predicted ATPase curve is always above the binding curve, has a steeper slope than the binding curve, and deviates maximally from it at calcium concentrations around the approach to the plateau. If one calcium bound per site switches on that site, then the ATPase curve follows the binding curve precisely.

pCa of ATPase. The actin-activated MgATPase of scallop HMM preparations is usually enhanced 4- to 5-fold by calcium. The calcium dependence of this activation (normalized to 1.0) is related to calcium binding of the same HMM preparations in Figure 6. The curves are the theoretically predicted ATPase activities calculated for "one-site", "two-site", and "independent-site" models from the stoichiometric binding constants of $K_1 = K_2 = 10^7$ M^{-1} (Figure 6). ATPase activation clearly lags behind calcium binding, and its rise is steeper. The points follow rather closely the curve predicted for the "two-site" model for activation.

The pCa–ATPase curve obtained with *Placopecten* myosin in the presence of a 10 molar excess of rabbit F-actin under nearly identical conditions with those of Figure 3 is seen in Figure 7. The normalized curve is superimposable on the one obtained for *Placopecten* or *Aequipecten* myofibrils (data not shown). The data are compared to the predicted ATPase. The best fit to the binding data of Figure 3 has been drawn and has also been used to calculate the expected ATPase for all

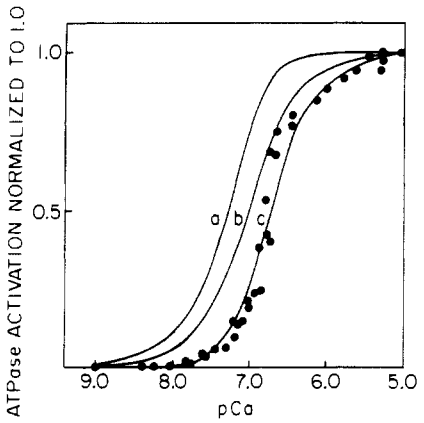

FIGURE 6: Calcium dependence of the ATPase activity of *Aequipecten* heavy meromyosin: 3 mM $MgCl_2$, 2 mM ATP, and 30 mM NaCl, pH 7.5, at 25 °C, 0.14 mg mL^{-1} HMM, 0.1 mM EGTA, and 0.35 mg mL^{-1} rabbit actin in 10-mL final volume. Sequential addition of $CaCl_2$. Specific activity: 0.24 μmol min^{-1} mg^{-1} (+Ca) and 0.05 μmol min^{-1} mg^{-1} (–Ca). Specific activity of *Aequipecten* myosin under the same conditions: 0.55 μmol min^{-1} mg^{-1}. V_{max} of HMM from double-reciprocal plots in 3 mM $MgCl_2$, 2 mM ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, and 5 mM NaCl: 1.0 μmol min^{-1} mg^{-1}. (Curve a) One calcium ion per myosin is sufficient for activation. (Curve b) Calcium binding, fitted to $K_1 = K_2 = 10^7$ M^{-1} and "independent-site" ATPase curve. (Curve c) Two calcium ions are required for activation.

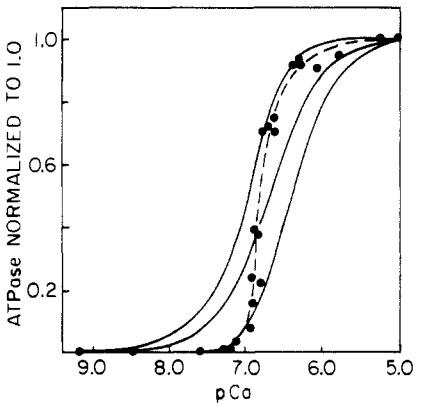

FIGURE 7: Calcium dependence of the ATPase activity of Placopecten myosin. (---) Normalized ATPase activity in 3 mM $MgCl_2$, 2 mM ATP, 20 mM NaCl, and 0.1 mM EGTA at pH 7.5, 25 °C; 10 mol of pure rabbit actin per mol of myosin. Sequential addition of $CaCl_2$. pCa values were corrected for 2 μM calcium contamination. The solid line in the center is best fit to calcium binding with $K_1 = K_2 = 5 \times 10^6$ M^{-1} (from Figure 4). Lower line: expected ATPase if two calcium ions per myosin are required for activations. Upper line: expected ATPase if one calcium per myosin is sufficient for activation. Specific activity 1.36 μmol min^{-1} mg^{-1} (+Ca) and 0.02 μmol min^{-1} mg^{-1} (–Ca).

three models. The ATPase data fit the theoretical curve for the model requiring two calcium ions very well at the low free calcium end of the curve and through the first part of the rise. This region is the most critical one for the model (see above). However, at higher free calcium concentrations the data deviate from the expected line and even cross over the binding data such that the ATPase diverges greatly from and is above the binding curve in the region of the approach to the plateau. Such a curve is not predicted by any simple model.

The pCa dependence of tension of skinned fiber bundles of *Placopecten* also shows a very sharp transition (R. M. Simmons and A. G. Szent-Györgyi, unpublished experiments). The tension data obtained at pH 7.0 agree closely with ATPase data obtained with myofibrils under similar conditions with a midpoint at pCa values of about 5.7 for both tension and ATPase (Figure 8). The higher calcium requirement corresponds to the lowered affinity for calcium at this pH. The

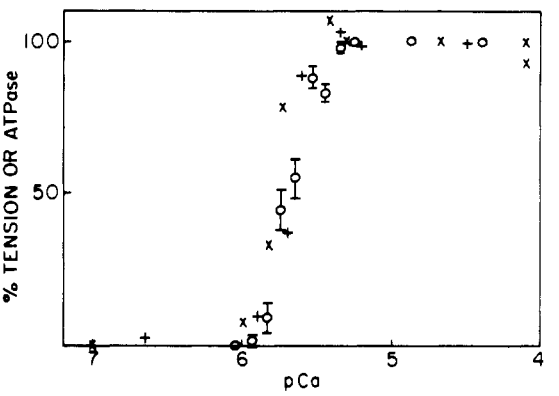

FIGURE 8: Calcium dependence of ATPase activity of *Placopecten* myofibrils and of tension development by fiber bundles. (×) ATPase, 3 mM $MgCl_2$, 2 mM ATP, 30 mM NaCl, and 0.1 mM EGTA at pH 7.0 with sequential addition of $CaCl_2$ measured in a pH-stat. (+) ATPase, 3 mM $MgCl_2$, 2 mM ATP, 30 mM NaCl, 15 mM imidazole, pH 7.0, and 1 mM CaEGTA–EGTA mixtures to obtain appropriate free calcium levels. Phosphate measured in individual samples by the method of Taussky & Schorr (1952). (○) Tension measurements. Conditions as in Simmons & Szent-Györgyi (1980). Data taken from R. M. Simmons and A. G. Szent-Györgyi (unpublished experiments).

similar shape and position of the tension and ATPase data indicate that the steep calcium dependence of ATPase activity in myofibrils and myosin is not an experimental artifact. The ATPase results obtained from proton release in the pH-stat or from phosphate determinations using CaEGTA buffers are in good agreement.

Discussion

Each molecule of scallop HMM must bind two calcium ions before the ATPase of the molecule is enhanced in the presence of actin. Since the two calcium binding sites are located on different myosin heads, the results directly show that a cooperativity exists between the two heads of scallop myosin during the calcium switch. Since HMM retains both of its regulatory light chains, this cooperativity cannot be derived from interactions between light chain free and light chain containing myosin heads (Kendrick-Jones & Jakes, 1977; Bagshaw, 1980). The results support a previous suggestion based on the negatively cooperative reuptake of regulatory light chains by EDTA-treated myofibrils that two calcium ions may be required to switch on the myosin molecule (Chantler & Szent-Györgyi, 1980). It may be noted that the pCa binding and pCa–ATPase data of scallop myofibrils, from which regulatory light chains had been removed, are fitted by the same curves [K = 6.0 at pH 7.5 in the presence of 1 mM $MgCl_2$; Figure 5 in Chantler & Szent-Györgyi (1980)]. This indicates that calcium binding to these heavy chain remnants of calcium-specific sites (Chantler & Szent-Györgyi, 1980) is such that each calcium alters the ATPase per site; i.e., cooperativity between the two halves of the bipartite myosin molecule as seen by pCa-binding/pCa–ATPase relationships is abolished when regulatory light chains are removed. This observation does not support Bagshaw's (1980) "clumping" hypothesis in which he proposed that head-to-head interaction is the result of the "stickiness" the heads acquire by the removal of regulatory light chains.

The calcium activation curve of scallop myosin (complexed with rabbit actin), myofibrils, or fiber bundles is complex. Although the initial portion of the activation curve is the one expected if two calcium ions switch on one myosin molecule, the remainder of the activation is completed at a very narrow range of calcium concentrations that cannot be explained by a simple "two-site" model.

A possible explanation for the shape of the ATPase curve could be the existence of cooperative activity within the thick filament, i.e., intermolecular cooperativity. Calcium binding is still randomized, as shown by calcium binding experiments that were carried out at low ionic strength where the myosin exists as thick filaments. Such thick filament cooperativity could operate as follows: once a certain number of myosin molecules (~20%) within the filament have been switched on (two calcium ions being required to activate each myosin molecule as seen by the initial rise of the pCa–ATPase curve), some form of intermolecular communication occurs within the filament that enables molecules to be switched on by binding only one ion of calcium. The possibility that adjacent molecules may switch on in the absence of calcium binding may be ruled out, for one would then predict 100% ATPase activity at, even prior to, 50% calcium binding. The similarity of the half-maximum points of the ATPase activity and calcium binding of myofibrils and actomyosin would be the consequence that calcium activation switches from the "two-site" to the "one-site" model. Such a hypothesis readily explains the lack of intermolecular effects with HMM since HMM does not assemble into filaments. This cooperativity does not require tropomyosin since the same sharp transition was obtained with actomyosin, myofibrils, and fiber bundles.

Thus the scallop myosin molecule exhibits four forms of cooperativity. In double-headed myosins, in contrast to single-headed fragments, the two regulatory light chains bind with different affinities in a negatively cooperative manner (Stafford et al., 1979; Chantler & Szent-Györgyi, 1980). Although the sensitivity of pure hybrid myofibrils (containing only foreign regulatory light chains) depends on the source of foreign regulatory light chains, mixed hybrids (one foreign and one scallop regulatory light chain) are always calcium sensitive (Sellers et al., 1980; Simmons & Szent-Györgyi, 1980), suggesting a cooperativity for the maintenance of the off state. The third form of cooperativity is that the two heads must cooperate during the switching on mechanism, as was suggested earlier (Chantler & Szent-Györgyi, 1980) and demonstrated directly in this paper; two calcium ions are required to switch the molecule on. All three forms of cooperativity may be described as intramolecular cooperativity and may be separate manifestations of an underlying mechanism for head–head interaction. This interaction seems to involve the S-2 region of the myosin molecule (Stafford et al., 1979). Evidence has also been presented in this paper for a fourth form of cooperativity which may be termed intermolecular. Thus the ATPase of scallop actomyosin or myofibrils may be activated by a considerably smaller increase in calcium than theoretically predicted provided the myosin is present as thick filaments.

ATPase activity of actomyosin and tension development of skinned fibers of vertebrate skeletal muscles also have a considerably sharper calcium dependence than calcium binding (Weber et al., 1964; Ebashi & Endo, 1968; Endo, 1972; Hellam & Podolsky, 1969; Julian, 1971), although the transition zone is not quite as narrow as with scallop. As pointed out by Murray et al. (1975) and by Tawada & Tawada (1975), the sharp rise of contraction cannot be accounted for by the interactions of the four calcium binding sites of troponin; in any case it would seem that only two sites on troponin bind calcium under physiological conditions (Potter & Gergely, 1975). Tawada & Tawada (1975) suggested that intermolecular cooperation of tropomyosin molecules that join end to end in the groove of the actin filament may account for the differences between the pCa dependence of calcium binding and contraction. They observed that in the presence of a nonpolymerizable tropomyosin (modified by carboxypeptidase treatment) the calcium dependence of the superprecipitation of actomyosin broadened significantly. Hydrodynamic evidence for cooperativity within rabbit thick filaments has been reported (Morimoto & Harrington, 1974). Harrington (1971) has also presented a model of how adjacent molecules in the thick filament might interact and suggested that the interaction involves the S-2 region of the molecule. Intermolecular cooperativity within the thick filaments of vertebrate muscles, however, is unlikely to be responsible for the sharpness of regulatory functions since soluble rabbit S-1 preparations complexed with regulated actin have a similarly sharp calcium-dependent ATPase as do insoluble actomyosins (Murray et al., 1975; J. M. Murray and A. Weber, personal communication). The simplest interpretation thus restricts cooperative modifications of the calcium dependence of myosin control to interactions within the thick filaments and similar modifications of actin control to the thin filaments.

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Chromatographic Resolution of Insulin Receptor from Insulin-Sensitive D-Glucose Transporter of Adipocyte Plasma Membranes†

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ABSTRACT: The chromatographic properties of the affinity-labeled adipocyte insulin receptor and the insulin-sensitive hexose transporter were evaluated in order to test the hypothesis that the transporter might be composed of receptor polypeptides. [^{125}I]Insulin was linked specifically and covalently to the high-affinity insulin receptor in dimethylmaleic anhydride extracted rat adipocyte plasma membranes by using the cross-linking reagent disuccinimidyl suberate [Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375-3381]. The membranes were solubilized with sodium cholate and resolved into three peaks of protein following hydroxylapatite chromatography. The first or void volume peak contained 10-20% of the protein, no receptor-bound [^{125}I]insulin, and no cytochalasin B sensitive D-glucose transport activity when reconstituted into phospholipid vesicles. The second major peak contained about half of the protein and as much as 87% of the affinity-labeled receptor but no hexose transport activity. The third peak contained 30-40% of the protein, 13-20% of the receptor-bound [^{125}I]insulin, and all of the recoverable D-glucose transport activity. Similar results were obtained by using diethylaminoethylcellulose (DEAE-cellulose) anion-exchange chromatography. More than 90% of the recovered receptor-linked [^{125}I]insulin eluted from this column at concentrations of NaCl below 1 M. These fractions contained no recoverable hexose transport activity. All of the hexose transporter recovered from the DEAE-cellulose column was eluted at higher NaCl concentrations. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the column fractions that contained all of the recovered D-glucose transporter from hydroxylapatite or DEAE-cellulose chromatography showed after autoradiography only traces of the affinity-labeled insulin receptor. These data indicate that the insulin receptor and D-glucose transporter represent distinct polypeptides in the fat cell membrane and imply that a transduction event must mediate hexose transport activation by insulin.

Although significant advances have been made in understanding how hormones interact with receptor proteins and what changes in cell function are brought about as a result of hormone–receptor interactions, little is known about the spatial relationship of membrane-bound hormone receptors and their effector systems. Two of the hormone receptor–effector systems for which such information is available are the acetylcholine and β-adrenergic receptor–effector systems. Huganir et al. (1979) have recently reported that the acetylcholine-dependent Na^+ channel appears to be part of the same protein complex as the acetylcholine receptor. The complex which can be isolated by using a choline carboxymethyl affinity column (Huganir et al., 1979) or α-neurotoxin affinity column (Heidmann & Changeux, 1978) is composed of four tightly associated but nondisulfide-linked peptides. These four peptides can be dissociated only following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Huganir et al., 1979; Heidmann & Changeux, 1978). When this purified acetylcholine receptor complex was reconstituted into phospholipid vesicles, carbamoylcholine-dependent, neurotoxin-sensitive Na^+ transport was observed (Huganir et al., 1979). In contrast to this observation is the finding that the β-adrenergic receptor is structurally distinct from its effector protein, adenylate cyclase. Sahyoun et al. (1977) separated erythrocyte ghost fragments enriched in β-adrenergic receptor from fragments enriched in adenylate cyclase by using discontinuous sucrose gradients. Vauquelin et al. (1977) found that the β-adrenergic receptor was retained by an alprenolol–agarose affinity column while adenylate cyclase activity

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