Correlation of Enzymatic Properties and Conformation of Bovine Erythrocyte Myosin†

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ABSTRACT: Myosin was purified from bovine erythrocytes by chromatography on DEAE-cellulose, Sepharose CL-4B, hydroxylapatite, and DEAE-5PW. The yield was about 200 μg/L of packed cells. From SDS–polyacrylamide gels, the purity was estimated to be greater than 95%. The bovine erythrocyte myosin is composed of heavy chains of 200 kDa and light chains of 20 and 17 kDa, in a molar stoichiometry of 1. Myosin was also purified from human erythrocytes by the same method. The molecular weights of two light chains were 26K and 19.5K which confirmed the earlier reports [Fowler, V. M., Davis, J. Q., & Bennet, V. (1985) *J. Cell Biol. 100*, 47–55; Wong, A. J., Kiehart, D. P., & Pollard, T. D. (1985) *J. Biol. Chem. 260*, 46–49]. Phosphorylation by gizzard myosin light chain kinase, to a level of 1 mol of phosphate/mol of 20-kDa light chain, increased actin-activated ATPase, and the extent of activation was dependent on the $MgCl_2$ concentration. Both Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were dependent on KCl concentration and markedly decreased below 0.3 M KCl. Mg^{2+}-ATPase of phosphorylated myosin, while more resistant to decreasing ionic strength, was also decreased below 0.2 M KCl. These results are similar to those obtained with smooth muscle myosin and suggest that the 10S–6S transition occurs. In confirmation of this, gel filtration, viscosity, and electron microscopy (rotary shadowing) show that erythrocyte myosin forms extended and folded conformations in high and low salt, respectively. It is proposed that each conformation is characterized by distinct enzymatic properties. These results suggest that the conformational transition (10 S–6 S) or some part of the transition is important in determining the biological properties of erythrocyte myosin.

The peripheral proteins of the erythrocyte membrane form a cytoskeletal network or "cytoskeleton" which is thought to play an important role in controlling shape change as well as deformability (Gratzer, 1981; Schrier, 1985). The major constituents of the cytoskeleton are spectrin, ankyrin, actin, and band 4.1 (Gratzer, 1981; Schrier, 1985). Myosin (Fowler et al., 1985; Davis & Bennett, 1985; Matovick et al., 1985; Wong et al., 1985) and tropomyosin (Fowler & Bennett, 1984) also have been identified and characterized in human erythrocytes. The discovery of these proteins supports the earlier proposals that an actomyosin-based contractile system may be involved in the shape change of erythrocytes (Weed et al., 1969; Schrier et al., 1981; Sheetz et al., 1981).

Considerable evidence from a variety of experimental procedures indicates that phosphorylation of the 20-kDa myosin light chain is important in the regulation of the contractile apparatus in smooth muscle and nonmuscle cells and that phosphorylation of myosin is required for the expression of actin-activated ATPase activity (Adelstein & Eisenberg, 1980; Hartshorne, 1987). Recently, it has been shown (Suzuki et al., 1982; Trybus et al., 1982; Craig et al., 1983) that monomeric smooth muscle myosin forms two distinct conformations, "folded" (10 S) and "extended" (6 S). Each conformation is characterized by distinct enzymatic properties (Ikebe et al., 1983), and it was suggested that the effect of phosphorylation is to change conformation which would then be reflected by altered ATPase activity (Ikebe et al., 1983, 1984). This concept, termed the "shape–activity" hypothesis, has been developed by using only gizzard myosin and has not been challenged with myosins from other sources.

The objectives of this study using myosin isolated from bovine erythrocytes were to determine (1) if this myosin undergoes the 10S–6S transition [in previous studies with human erythrocyte myosin (Fowler et al., 1985; Wong et al., 1985), this was not determined] and (2) if the conformational change does occur to correlate enzymatic activity with conformation. Of these two points, the second is more interesting since it might be predicted from earlier work on nonmuscle myosin (Craig et al., 1983; Citi & Kendrick-Jones, 1986; Kendrick-Jones et al., 1987) that the 10S–6S transition would be observed with erythrocyte myosin. However, there have been no studies using nonmuscle myosin in which enzymatic properties are compared to myosin conformation.

Materials and Methods

Smooth muscle myosin (Ikebe & Hartshorne, 1985), myosin light chain kinase (MLCK)[1] (Walsh et al., 1983), and tro-

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[1] Abbreviations: MLCK, myosin light chain kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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pomyosin (Driska & Hartshorne, 1975) were prepared from frozen turkey gizzard. Calmodulin was isolated from bull testes (Walsh et al., 1983). F-Actin was prepared from rabbit skeletal muscle (Driska & Hartshorne, 1975). DEAE-Sephacel, Sepharose CL-4B, phenylmethanesulfonyl fluoride (PMSF), trypsin inhibitor (type II-O), cellulose (Sigmacell, type 50), and α-cellulose were purchased from Sigma Chemical Co., St. Louis, MO. Hydroxylapatite was from Bio-Rad Laboratories, Richmond, CA. [γ-^{32}P]ATP was obtained from ICN Radiochemicals, Irvine, CA.

Bovine erythrocytes were isolated from freshly collected blood, anticoagulated with acid–citrate–dextrose containing 2 mM NaN_3 (Bennett, 1983). Human whole blood was obtained from the blood bank at Case Western Reserve University Hospital. Platelets and white blood cells were removed by a modification of the method of Beutler et al. (1976) as follows: 5 L of anticoagulated bovine blood was centrifuged for 10 min at 400g at room temperature. The supernatant and buffy coat were removed, and 1 volume of 0.15 M NaCl and 10 mM Tris-HCl (pH 7.5) was added. The mixture was passed through a cellulose column (5.5 × 30 cm; Sigmacell:α-cellulose ratio = 1.1) to removed contaminating platelets and white blood cells. The erythrocytes were washed 3 times with 10 volumes of 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM NaN_3 by centrifugation for 15 min at 300g at 4 °C. About 1.5 L of packed erythrocytes was obtained. Lysis was accomplished by adding 10 volumes of 10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 20 μg/mL PMSF, 10 μg/mL trypsin inhibitor, and 0.1 mM dithiothreitol with stirring on ice. Sodium pyrophosphate (pH 7.5) was then added to a final concentration of 20 mM. After 30 min, the ghosts were pelleted by centrifugation at 10000g for 30 min. The hemolysate (~14 L) was absorbed to 500 mL of DEAE-Sephacel equilibrated with 20 mM sodium pyrophosphate, 10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM NaN_3, and 2 mM dithiothreitol (buffer A) for 2 h with stirring on ice. The DEAE was collected on a Büchner funnel, washed with 2 L of buffer A, poured into a column (5.5 × 20 cm), washed with 1 volume of buffer A, and eluted with a 0–0.4 M NaCl linear gradient (2 × 1 L) in buffer A at 50 mL/h. Twelve-milliliter fractions were collected. The eluted proteins were monitored by K^+-EDTA-ATPase and Ca^{2+}-ATPase activities. Fractions containing ATPase activity were pooled (~450 mL); 1.5 volumes of cold saturated ammonium sulfate (pH 7.5) containing 5 mM dithiothreitol, 0.2 mM EDTA, 20 μg/mL PMSF, 25 μg/mL trypsin inhibitor, and 1 mM NaN_3 were then added. After 25 min with continuous stirring on ice, the sample was centrifuged for 30 min at 10000g. The precipitate was suspended in 10 mL of 0.5 M KCl, 1 mM EGTA, 10 mM Tris-HCl (pH 7.5), and 2 mM dithiothreitol (buffer B) plus 10 μg/mL trypsin inhibitor and dialyzed against buffer B. The solution was clarified by centrifugation at 100000g for 30 min, and the supernatant was applied to a Sepharose CL-4B column (2.5 × 90 cm) equilibrated with buffer B. Five-milliliter fractions were collected at a flow rate of 30 mL/h. Fractions containing ATPase activity were pooled (~110 mL) and applied to a hydroxylapatite column (1 × 20 cm) equilibrated with buffer B. The column was washed with 2 volumes of buffer B and eluted with a linear gradient of 0–0.5 M potassium phosphate (pH 7.5), 2 × 100 mL, at 12 mL/h. Fractions containing ATPase activity were pooled (~20 mL) and dialyzed against 20 mM sodium pyrophosphate, 10 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 2 mM dithiothreitol. The samples were clarified by filtration (0.45 μm, Millipore), applied to a TSK-DEAE-5PW column (7.5 × 75 mm) equilibrated with the dialysis buffer, and eluted with a linear gradient of 0–0.35 M NaCl, 2 × 15 mL, using a Perkin-Elmer series 4 HPLC system. Fractions (0.4 mL) containing ATPase activity were pooled (~2 mL) and dialyzed against 0.5 M KCl, 30 mM Tris-HCl (pH 7.5), and 5 mM dithiothreitol and stored on ice.

Electrophoresis was carried out on 7.5–20% or 10–23% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ using the discontinuous system of Laemmli (1970). The gels were stained in 0.06% Coomassie Brilliant Blue R 250 (Sigma) and scanned by a GS 300 scanning densitometer (Hoefer Scientific Instruments) attached to an LCI-100 laboratory computing integrator (Perkin-Elmer). Molecular weights were estimated by using the following standards: skeletal muscle myosin heavy chain 205 000), β-galactosidase (116 000), phosphorylase b (97 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), smooth muscle myosin light chains (20 000 and 17 000), and lysozyme (14 300).

The extent of phosphorylation was measured as described by Walsh et al. (1983). ATPase activities were determined at 25 °C as described previously (Ikebe & Hartshorne, 1985b). Assay conditions are given in the figure legends.

Viscosity was measured at 23 °C in Cannon-Ubbelohode viscometers with a water flow time of approximately 54 s. Conditions are given in the figure legends. The viscosity data are expressed as η_{rel} (viscosity of protein/viscosity of solvent).

Urea gel electrophoresis was performed as described earlier (Perrie & Perry, 1970). The gels were fixed in 30% methanol and 7% glutaraldehyde and subjected to the silver stain procedure of Eschenbruch and Burk (1982).

The TSK 4000 SW column was attached to a Perkin-Elmer series 4 HPLC system (see Figure 6). Rotary shadowing of purified erythrocyte myosin was carried out essentially as described by Craig et al. (1983). Myosin (30–100 μg/mL), of 0.1 M NaCl (or 0.3 M ammonium acetate), 0.3–1 mM ATP, 0.3–1 mM $MgCl_2$, 1 mM Tris-HCl (pH 7.5), and 50% (v/v) glycerol was sprayed onto mica and rotary shadowed at an angle of 6°, and the specimens were examined in a JEOL 100CX electron microscope operated at 80 kV.

Protein concentrations were estimated by the method of Bradford (1976) or by the biuret reaction (Gornall et al., 1949) using bovine serum albumin (Bio-Rad) as a standard. Gizzard smooth muscle myosin was used as a standard to determine the concentration of erythrocyte myosin.

Results

Purification and Characterization of Erythrocyte Myosin. Bovine erythrocyte myosin was purified by using the above procedures. Each stage of the purification was monitored, and the results are shown in Table I. Ca^{2+}-ATPase activity was measured since this activity is not inhibited with actin and therefore gives more realistic estimates of the extent of purification. Figure 1A shows the SDS–polyacrylamide gel patterns obtained at each stage of the procedure. Chromatography on DEAE-Sephacel was effective in that it removed hemoglobin and also concentrated the myosin from a large volume of hemolysate. The myosin eluted between 0.2 and 0.3 M NaCl. On the subsequent Sepharose CL-4B column, myosin was eluted slightly after the void volume (data not shown). Actin and various low molecular weight proteins were contaminants at this stage. In the hydroxylapatite step, actin and myosin were eluted separately (the former at approximately 0.2 M phosphate and the latter at 0.3 M phosphate). Finally, the HPLC ion-exchange column removed other contaminants, principally at protein of M_r ~100 000 (see lane

Table I: Purification of Erythrocyte Myosin[a]

	volume (mL)	concn (mg/mL)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	purification (x-fold)
lysate[b]	14500	50.0	906	0.0014	
DEAE-Sephacel	400	2.5	594	0.594	424
60% $(NH_4)_2SO_4$	32	14.0	372	0.830	593
Sepharose CL-4B	83	0.3	149	5.960	4257
hydroxylapatite	22	0.059	77	59.231	42308
HPLC	3.4	0.081	66	240.0	171429

[a] Ca^{2+}-ATPase was used to estimate the myosin content. Assay condition: 0.5 M KCl, 50 mM Tris-HCl (pH 7.5), and 10 mM $CaCl_2$. [b] Lysate sample was dialyzed against 0.5 M KCl, 10 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol before assay for Ca^{2+}-ATPase to remove Mg^{2+}.

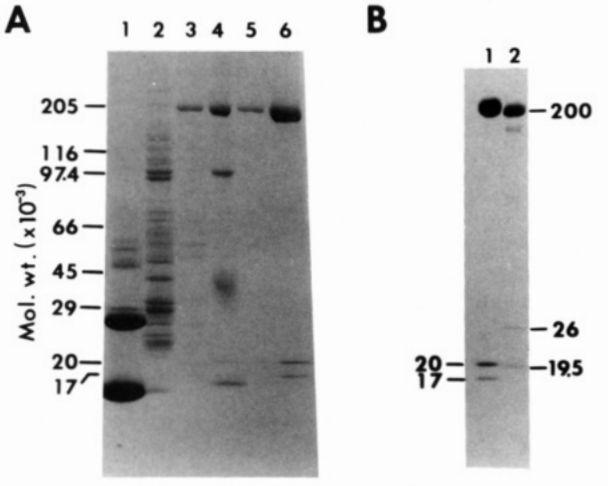

FIGURE 1: Purification of myosin from bovine and human erythrocytes. (A) 7.5–20% polyacrylamide linear gradient SDS gel of samples of hemolysate (17 μL) (lane 1), the DEAE pool (35 μL) (lane 2), the Sepharose CL-4B pool (100 μL) (lane 3), the hydroxyapatite pool (100 μL) (lane 4), and the purified myosin from bovine erythrocytes (4 μg/mL and 20 μg/mL) (lanes 5 and 6, respectively). (B) 10–25% polyacrylamide linear gradient SDS gels. Gizzard smooth muscle myosin (25 μg) (lane 1) and purified human erythrocyte myosin (15 μg) (lane 2).

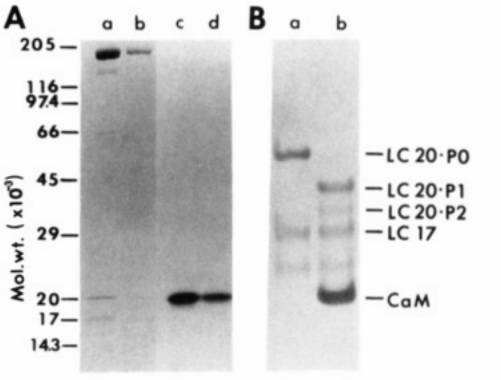

FIGURE 2: Electrophoresis of myosin from purified bovine erythrocytes and gizzard smooth muscle on a 10–23% polyacrylamide linear gradient SDS gel (A) and urea gel (B). (A) Myosin was phosphorylated by incubation with 1 μg/mL calmodulin, 1 μg/mL MLCK, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.125 M KCl, and 30 mM Tris-HCl (pH 7.5) for 20 min. Coomassie Blue staining: (a) myosin from gizzard smooth muscle (12 μg); (b) erythrocyte myosin (5 μg); (c) and (d) are the corresponding autoradiograms; (c) gizzard smooth muscle; (d) erythrocyte. (B) Myosin was phosphorylated by incubation with 15 μg/mL calmodulin, 4 μg/mL MLCK, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.125 M KCl, and 30 mM Tris-HCl (pH 7.5) for 20 min. The reaction was stopped by the addition of urea to 9 M. (a) Dephosphorylated erythrocyte myosin; (b) phosphorylated erythrocyte myosin. LC20-P0, dephosphorylated 20-kDa light chain. LC20-P1, single-phosphorylated 20-kDa light chain. LC20-P2, double-phosphorylated 20-kDa light chain. LC17, 17-kDa light chain. CaM, calmodulin.

4 in Figure 1A), although this protein was occasionally negligible. The yield was approximately 200 μg from 1 L of packed erythrocytes.

The purity of the final product was estimated (by densitometry) to be greater than 95%. The bovine erythrocyte myosin is composed of heavy chains of approximately 200 kDa and two light chains of 20 and 17 kDa. From the densitometric scans, the relative proportions of each subunit were calculated to 1.12:1.0:0.91 for the heavy chain, 20-kDa, and 17-kDa light chains, respectively. The light-chain composition is indistinguishable from gizzard smooth muscle myosins (see Figure 1B and Figure 2A) or bovine platelet myosin (data not shown). The molecular weights of two light chains from bovine erythrocytes were different from those from human erythrocytes (Fowler et al., 1985; Wong et al., 1985).

To determine whether the difference in light-chain molecular weights between bovine erythrocytes and human erythrocytes reflected the difference in species, we purified human erythrocyte myosin by our procedures (see Materials and Methods). The yield was about 270 μg from 1 L of packed erythrocytes. The human erythrocyte myosin is composed of heavy chains of approximately 200 kDa and two light chains of 26 and 19.5 kDa (Figure 1B). These values are the same as those reported previously (Wong et al., 1985). The human erythrocyte myosin was more easily degradated during purification compared to bovine erythrocyte myosin, as judged by immunoblotting using polyclonal antibodies against bovine platelet myosin (data not shown). The major degradation fragment was a protein of M_r ~170 000 as reported by Wong et al. (1985). There was a faint band of 17 kDa when 20–30 μg of myosin was applied (see Discussion).

In the presence of Ca^{2+}, calmodulin, and gizzard MLCK, only the 20-kDa light chain was phosphorylated (Figure 2A, autoradiogram). No heavy-chain phosphorylation was detected. Light-chain phosphorylation was assessed also by urea gel electrophoresis (Figure 2B). The purified myosin was obtained in the dephosphorylated form, and following incubation with MLCK, Ca^{2+}, and calmodulin, the mobility change for the major phosphorylated subunit was consistent with a phosphorylation level of 1 mol of P/mol of light chain. A more rapidly migrating doubly phosphorylated light chain (Ikebe & Hartshorne, 1985a) was also observed occasionally as a minor component (Figure 2B).

The time course of phosphorylation of bovine erythrocyte myosin by gizzard MLCK is shown in Figure 3. Up to 1.9 mol of P/mol of myosin was incorporated under these conditions. These data fit a single exponential, suggesting random phosphorylation. Random phosphorylation was also reported with thymus myosin (Wagner et al., 1985).

ATPase Activities of Bovine Erythrocyte Myosin. ATPase activities were determined under a variety of experimental conditions. In general, these activities were similar to those displayed by gizzard myosin. The pH profile of Ca^{2+}-ATPase

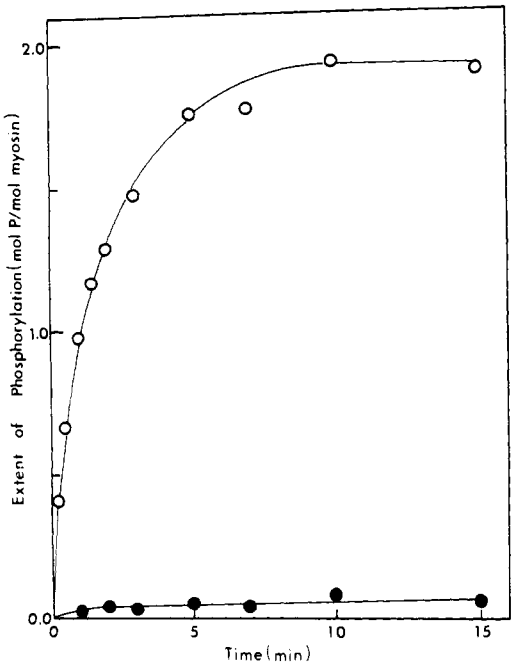

FIGURE 3: Time course of phosphorylation of erythrocyte myosin by MLCK. Conditions: erythrocyte myosin (20 μg/mL) was incubated at 25 °C with 2 μg/mL MLCK from turkey gizzard, 15 μg/mL calmodulin, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), 0.125 M KCl, 100 μM [γ-^{32}P]ATP, and either 0.1 mM $CaCl_2$ (○) or 1 mM EGTA (●).

(assayed at high ionic strength) showed two optima at pHs of approximately 6 and 10. For the K^+-EDTA-ATPase, a single relatively broad maximum was observed between pHs 8.6 and 9.8. For both activities, an increase in pH above 10 resulted in a marked inhibition. The pH dependencies of Ca^{2+}- and K^+-EDTA-ATPases are similar to those observed with both gizzard myosin (Onishi et al., 1978) and skeletal myosin (Mommaerts & Green, 1954). As with skeletal and smooth muscle myosins, the K^+-EDTA-ATPase activity of erythrocyte myosin was inhibited by skeletal muscle F-actin. At an equal weight ratio of actin to myosin, the K^+-EDTA-ATPase activity was inhibited over 90% (data not shown). This is similar to smooth muscle myosin but not to skeletal myosin whose K^+-EDTA-ATPase activity is inhibited only by 50% (Krisanda & Murphy, 1980). At pH 7.5 and 0.5 M KCl, the Ca^{2+}- and K^+-EDTA-ATPase activities were similar, i.e., approximately 200 nmol/(min·mg) (1.6 s^{-1}).

Figure 4 shows the typical results for the $MgCl_2$ dependence of actin-activated ATPase activity of phosphorylated myosin in the presence and absence of gizzard tropomyosin. For the dephosphorylated myosin, the extent of actin activation was negligible, and the Mg^{2+}-ATPase activity was similar in the presence and absence of actin. Phosphorylation increased the actin-activated ATPase, and the extent of activation was dependent on the $MgCl_2$ concentration. Maximum activity was observed only at relatively high $MgCl_2$ concentrations, i.e., above 6 mM $MgCl_2$ (total). The actin-activated ATPase activity of smooth muscle myosin shows a similar Mg^{2+} dependence (Ikebe et al., 1984). However, the effects of tropomyosin on the actin-activated ATPase activities of gizzard and erythrocyte myosins are different. With phosphorylated smooth muscle myosin, tropomyosin causes an increase of actin-activated ATPase activity (Chacko & Rosenfeld, 1982; Ikebe et al., 1984, 1985b). In contrast, this effect was not observed with the phosphorylated erythrocyte myosin (1.8 mol of P/mol of myosin) over the entire range of $MgCl_2$ concentrations (Figure 4). The ATPase activity in 6 mM $MgCl_2$ in

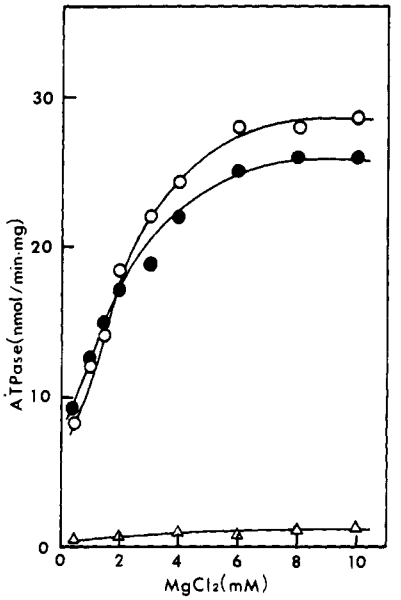

FIGURE 4: Mg^{2+} concentration dependence of actin-activated ATPase of phosphorylated erythrocyte myosin in the presence or absence of tropomyosin. The $MgCl_2$ concentration is the total concentration. Assay conditions: 0.1 mg/mL erythrocyte myosin, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.1 mM ATP, 0.3 mg/mL skeletal muscle actin, and 0.1 mM $CaCl_2$ in the presence (●) or absence (○) of gizzard tropomyosin (0.06 mg/mL). Myosin was phosphorylated by incubation in the assay solvent conditions with 15 μg/mL calmodulin and 4 μg/mL MLCK for 15 min at 25 °C (○, ●). MLCK was omitted from the control assay (△). The level of phosphorylation was 1.8 mol of P/mol of myosin. ATPase assay was started by the addition of actin (○, △) or actin plus tropomyosin (●).

the presence of gizzard tropomyosin was 90–120% of that in the absence of tropomyosin (from four separate experiments).

The KCl dependencies of Ca^{2+}- and K^+-EDTA-ATPase activities of dephosphorylated erythrocyte myosin are shown in Figure 5A. The Ca^{2+}-ATPase activity shows a sharp increase between 0.05 and 0.25 M KCl and a plateau at higher KCl concentrations. This was also observed with human erythrocyte myosin (data not shown). With gizzard myosin, the change in ATPase activity reflected the 6S to 10S transition (Ikebe et al., 1983). The K^+-EDTA-ATPase activity showed a gradual activation on increasing the KCl concentration (Figure 5A), and this is similar for skeletal and smooth muscle myosins (Ikebe et al., 1983).

The KCl dependence of Mg^{2+}-ATPase activities of phosphorylated and dephosphorylated erythrocyte myosin (in the absence of actin) is shown in Figure 5B. For the dephosphorylated myosin, the activity decreased at KCl concentrations below 0.3 M, but for the phosphorylated myosin, a decrease in activity was observed only below 0.2 M KCl. Similar results were obtained with gizzard myosin (Ikebe et al., 1983) and were interpreted on the basis of the 6S–10S transition (see Discussion). However, it should be noted that the Ca^{2+}- and Mg^{2+}-ATPase activities of dephosphorylated erythrocyte myosin in 0.2 M KCl are more than twice those in 0.1 M KCl. In contrast, these activities with gizzard myosin are essentially the same (Ikebe et al., 1983). These differences suggest that the dephosphorylated erythrocyte myosin in 0.2 M KCl is partially in the 6S state.

Conformation of Erythrocyte Myosin. Since the availability of erythrocyte myosin was limited, the techniques that could be used to monitor the myosin conformation were also restricted. The three techniques that were applied were gel filtration, viscosity, and electron microscopy. It was previously shown that the 10S and 6S species of myosin could be separated by gel filtration (Trybus et al., 1982; Ikebe et al., 1983)

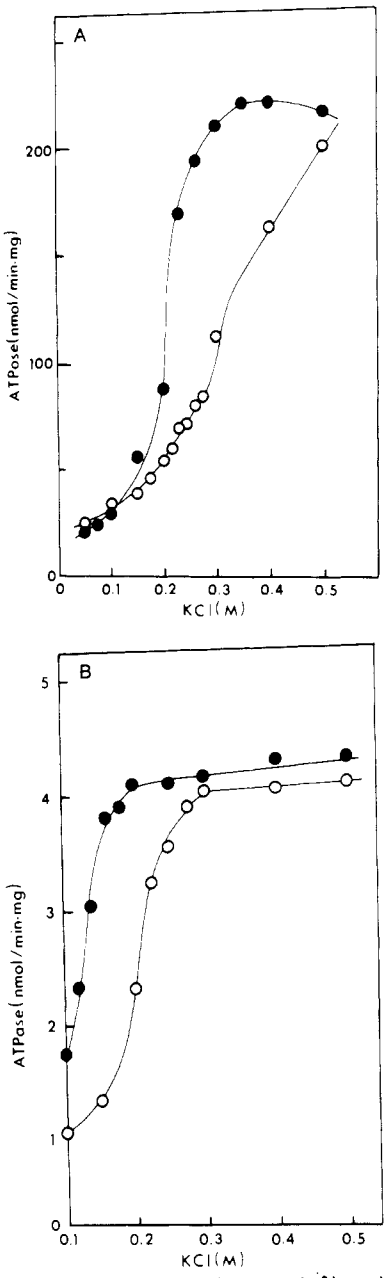

FIGURE 5: KCl concentration dependence of Ca^{2+}-, K^+-EDTA-, and Mg^{2+}-ATPase activities. (A) Ca^{2+}-ATPase (●) and K^+-EDTA-ATPase (○). Assay conditions: variable KCl concentrations, 50 mM Tris-HCl (pH 7.5), and 10 mM $CaCl_2$ or 2 mM EDTA. (B) Mg^{2+}-ATPase. ATPase activities in 1 mM $MgCl_2$ are shown for phosphorylated (●) and dephosphorylated (○) myosin. Myosin was phosphorylated to approximately 1.8 mol of P/mol of myosin by incubation for 15 min at 25 °C in 0.1 mM ATP, 1 mM $MgCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 15 μg/mL calmodulin, and 4 μg/mL gizzard myosin light chain kinase.

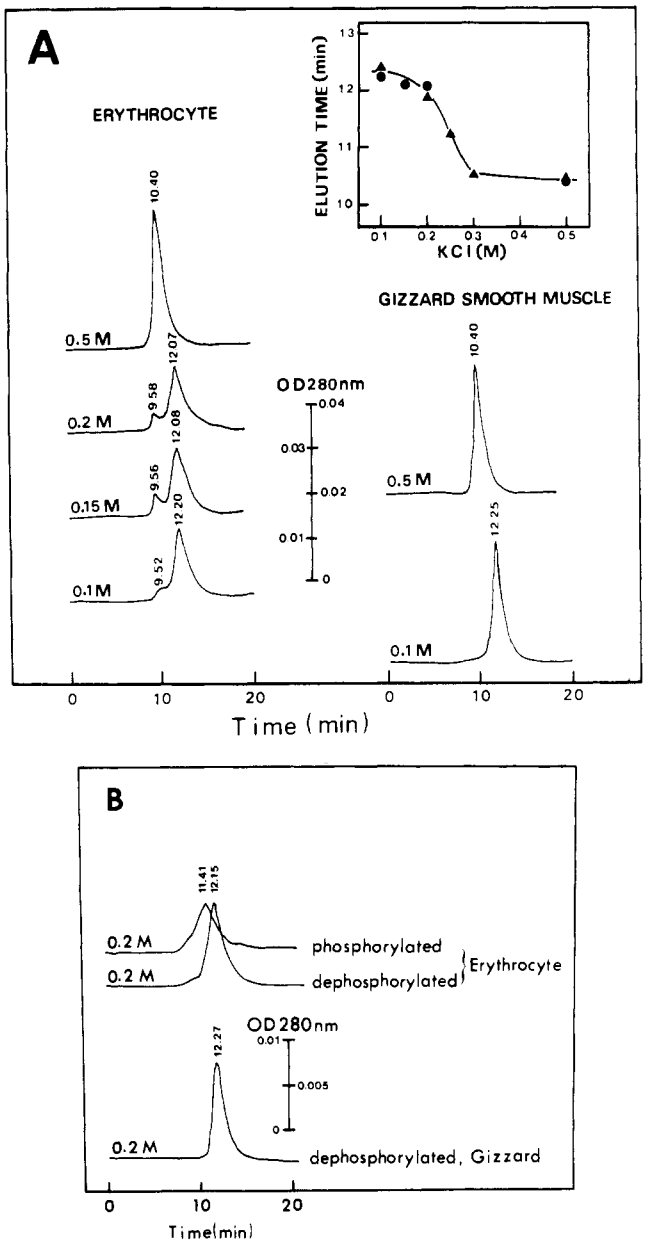

FIGURE 6: Elution patterns of phosphorylated and dephosphorylated erythrocyte myosin from the TSK 4000 SW column. (A) Myosin was dialyzed against 1 mM $MgCl_2$, 0.5 mM ATP, 30 mM Tris-HCl (pH 7.5), and KCl (0.1–0.5 M) as indicated. Erythrocyte myosin applied was 105 μg (0.1 M KCl), 70 μg (0.15 and 0.2 M KCl), or 112 μg (0.5 M KCl). Gizzard myosin was 100 μg in both 0.1 and 0.5 M KCl. (B) Myosin was dialyzed against 1 mM $MgCl_2$, 1 mM ATP, 30 mM Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM dithiothreitol. Phosphorylated myosin was obtained by incubation of myosin in 0.2 M KCl with 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 15 μg/mL calmodulin, and 2 μg/mL MLCK for 20 min. A 500-μL sample was applied to a TSK 4000 SW column (30 × 0.75 cm) attached to a Perkin-Elmer series 4 HPLC system at a flow rate of 0.6 mL/min. Myosin applied was 20 μg (erythrocyte) or 25 μg (gizzard). The bed volume determined with dithiothreitol was 22.15 min. The void volume determined with blue dextran was 9.67 min. The inset shows the KCl dependence of elution time of dephosphorylated myosins from two different preparations (●, ▲).

and the viscosity measurement offered a convenient and rapid means of following the conformational change of smooth muscle myosin (Ikebe et al., 1983).

In Figure 6A are shown the elution profiles of gizzard and erythrocyte myosin applied to a TSK 4000 SW column at different ionic strengths. For the gizzard (control) myosin, the conformations at 0.5 M KCl and 0.1 M KCl are the 6 S and 10 S, respectively (Ikebe et al., 1983). The elution positions were 10.4 min for the 6 S and 12.25 min for the 10 S. By comparison, it appears that the erythrocyte myosin in 0.5 M KCl exists in the 6S state and that at 0.1 M KCl the 10S conformation is formed. It is suggested that the minor components in 0.1–0.2 M KCl eluting before the 6S peak represent myosin oligomers since they appear close to the void volume. The elution time of erythrocyte myosin increased markedly with decreasing KCl concentration from 0.3 to 0.2 M (Figure 6A, inset). This suggests that the conformational change (6 S to 10 S) actually occurs in erythrocyte myosin in the above KCl concentration range. The effect of phosphorylation on the elution position of erythrocyte myosin is shown in Figure 6B. The dephosphorylated erythrocyte myosin in 0.2 M KCl

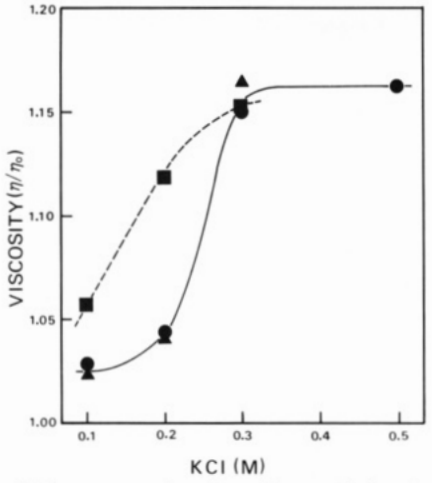

FIGURE 7: KCl concentration dependence of viscosity. Myosin preparation was started with 3–3.5 L of packed erythrocytes, and the HPLC step was omitted. Myosin fractions after the hydroxylapatite column were concentrated by sucrose and dialyzed in 0.35 M KCl, 30 mM Tris-HCl (pH 7.5), and 2 mM dithiothreitol. Purity was greater than 90%. Phosphorylated myosin was obtained by incubation of myosin in 0.2 M KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 15 μg/mL calmodulin, and 2 μg/mL MLCK for 25 min. The level of phosphorylation was 1.8 mol of P/mol of myosin. Myosin concentration was adjusted to 0.5 mg/mL in variable KCl concentration, 30 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP, and 2 mM dithiothreitol. Dephosphorylated myosins from two different preparations (●, ▲); phosphorylated myosin (■).

eluted a little faster (12.15 min) than dephosphorylated gizzard myosin in 0.2 M KCl (12.27 min, i.e., consistent with the 10S conformation). This small shift to the 6S conformation is in parallel with the small increase in Ca^{2+}- and Mg^{2+}-ATPase activities in 0.2 M KCl (Figure 5). The elution position of phosphorylated (1.9 mol of P/mol of myosin) erythrocyte myosin is earlier, at 11.41 min, and this suggests that under these conditions phosphorylation induces a partial transition to the 6S state (6S myosin elutes at 10.4 min).

Viscosity measurements were also used to monitor myosin conformation as a function of KCl concentration (Figure 7). The viscosity of dephosphorylated myosin decreased markedly at KCl concentrations less than 0.3 M. For the phosphorylated myosin (1.8 mol of P/mol of myosin), a decrease in viscosity also occurred with decreasing ionic strength, but the viscosity decrease is shifted to lower ionic strength compared to dephosphorylated myosin. By comparison with similar studies on smooth muscle myosin, these data are interpreted to indicate that erythrocyte myosin exists in the 6S conformation at high ionic strength and forms the 10S conformation at lower ionic strength. The effect of phosphorylation is to shift the transition zone to lower ionic strength.

The conformation of erythrocyte myosin was also studied by electron microscopy of rotary-shadowed specimens. At high salt (0.3–1.0 M ammonium acetate), almost all molecules showed the typical myosin structure of two heads at one end of an extended tail (i.e., 6 S, Figure 8A). In contrast, at a lower salt concentration (i.e., 0.1 M NaCl), most myosins favored the folded form (i.e., 10 S) in which the tail of myosin bent back toward the neck region of the myosin heads (Figure 6B). These two conformations are similar to those observed with smooth muscle myosin (Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983) and nonmuscle myosin: thymus (Craig et al., 1983; Kendrick-Jones et al., 1987) and brush border cell (Citi & Kendrick-Jones, 1986; Kendrick-Jones et al., 1987). From the results of gel filtration and viscosity, it was suggested that phosphorylation at a lower salt induces a partial transition to the 6S myosin. Some support

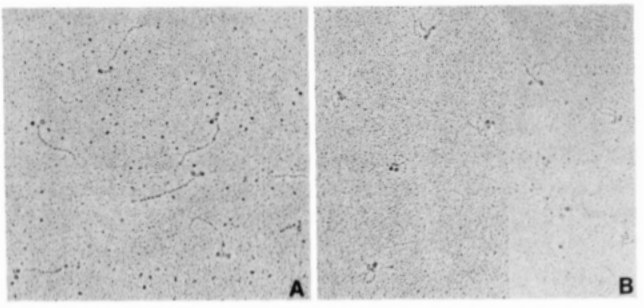

FIGURE 8: Rotary-shadowed erythrocyte myosin molecules. (A) Specimen sprayed in 0.3 M ammonium acetate, 0.3 mM ATP, 0.3 mM $MgCl_2$, 1 mM Tris-HCl (pH 7.5), and 50% (v/v) glycerol. (B) Specimen sprayed in 0.1 M NaCl, 0.3 mM ATP, 0.3 mM $MgCl_2$, 1 mM Tris-HCl (pH 7.5), and 50% (v/v) glycerol. Magnification: 55000×.

for this was observed by electron microscopy. Phosphorylated myosin at low salt (0.1 M NaCl) was examined by electron microscopy, and fewer molecules of phosphorylated myosin formed a folded conformation although folded molecules were still observed (data not shown).

Discussion

Using the procedure described above, we purified bovine erythrocyte myosin to approximately 95% homogeneity. This is apparently of a higher purity than the preparation described by Fowler et al. (1985). In the report by Wong et al. (1985), it was stated that none of the individual contaminants represented more than 5% of the protein; however, several contaminants are visible in the SDS profile. The low yield of our preparation as compared with other reports (Fowler et al., 1985; Wong et al., 1985) may be due to the difference in preparation procedures and the number of steps involved. After the Sepharose CL-4B chromatography, 5–10 mg of myosin (purity was 80–85% by densitometric scanning) was obtained from 1 L of packed cells. This yield is comparable with those of other reports (Fowler et al., 1985; Wong et al., 1985). One of our intentions for the procedure was to prepare dephosphorylated myosin (Figure 2B), and it was observed that the use of Mg^{2+}-ATP (particularly in the absence of EGTA) resulted in a partially phosphorylated myosin [see the method of Wong et al. (1985)].

The myosin isolated previously (Fowler et al., 1985; Wong et al., 1985) from human erythrocytes was different from our preparation with respect to the light-chain composition. The human erythrocyte myosin had light chains of 25 (Fowler et al., 1985) to 26 (Wong et al., 1985) kDa and 19.5 kDa, whereas the bovine erythrocyte myosin possessed light chains of 20 and 17 kDa. Since the human erythrocyte myosin purified by our own method also had light chains of 26 and 19.5 kDa, it is concluded at present that the difference in molecular weights of two light chains is derived not from preparative artifacts but reflects the difference in source species.

Since platelet myosin has light chains of 20 and 17 kDa, an obvious concern was that the putative bovine erythrocyte myosin was in fact derived from platelets. This is unlikely for the following reasons. Most of the platelets were removed from the packed erythrocytes by the cellulose column step [88% of the contaminating platelets was removed by this method (Beutler et al., 1976)], and more were removed in the subsequent three wash steps. It was found by light microscopy that the number of platelets in the erythrocyte preparation was less than 0.1% of the total cell number. Assuming that the volume of the bovine platelet (10 μm^3) is one-ninth of the volume of the erythrocyte (90 μm^3) and that 1 mg of myosin

was obtained from 12 mL of packed platelets,[2] the amount of platelet myosin in the final preparation of erythrocyte myosin would be less than 4%. The SDS gel profile of human erythrocyte myosin had a faint band of 17 kDa when 20–30 μg of myosin was applied. Assuming that all of this band is derived from the light chain of contaminating platelet myosin, we estimate by densitometric scanning that 4–5% of the total myosin is derived from platelets. This value is consistent with the value calculated by the yield as mentioned above.

In general, the ATPase properties of the bovine erythrocyte myosin were similar to those of smooth muscle myosin. These included the pH–activity profiles and the marked inhibition of K^+-EDTA-ATPase activity by actin. In addition the actin-activated ATPase activity of erythrocyte myosin is increased by phosphorylation, and the extent of activation is dependent on the $MgCl_2$ concentration (Figure 4). Again, this is similar to smooth muscle myosin. It is suggested also (Figure 2B) that the 20-kDa light chain of erythrocyte myosin can be phosphorylated at two residues [in gizzard myosin, serine-19 and threonine-18 of the 20-kDa light chain are phosphorylated by MLCK (Ikebe et al., 1986)] although the influence of double phosphorylation on the biological properties of erythrocyte myosin remains to be elucidated. There is one interesting point of difference between gizzard and erythrocyte myosin, and that is the effect of tropomyosin on the actin-activated ATPase activity of phosphorylated myosin. With the smooth muscle system, a marked activation is observed (Chako & Rosenfeld, 1982; Ikebe et al., 1984; Ikebe & Hartshorne, 1985b), and this was not apparent with erythrocyte myosin. These relatively small effects of tropomyosin on actin-activated Mg^{2+}-ATPase activity (especially with fully phosphorylated myosin; 1.8–2 mol of P/mol of myosin) were also seen in myosin from other nonmuscle cells, i.e., brush border cells (Citi & Kendrick-Jones, 1986) or thymus (Scholey et al., 1982; Wagner et al., 1986). Whether or not the lack of activation is dependent on the level of myosin phosphorylation and is characteristic of nonmuscle myosins must be determined. It has been suggested that gizzard myosin interacts directly with tropomyosin (Merkel et al., 1986) and this interaction could be modified or missing with the erythrocyte myosin.

The above results show that the bovine erythrocyte myosin undergoes the 10S–6S transition and in general is similar to smooth muscle myosin. The procedures that were used to demonstrate the transition were, by necessity, those appropriate for a restricted supply of myosin. However, the direct visualization of each conformation by electron microscopy offers compelling evidence for the existence of the two states, and the gel filtration data (Figure 6) and viscosity results (Figure 7) show ionic strength dependence of the transition. Since thymus myosin (Craig et al., 1983; Kendrick-Jones et al., 1987) and brush border myosin (Citi & Kendrick-Jones, 1986; Kendrick-Jones et al., 1987) have been shown previously to undergo the 10S–6S transition, our results on another nonmuscle myosin were not unexpected. However, a unique and more interesting contribution is the proposed correlation of enzymatic activity with the conformation of erythrocyte myosin. This has been demonstrated before only with gizzard myosin (Ikebe et al., 1983), and the hypothesis was developed that conformational changes within the 10S–6S transition form part of the in vivo regulatory mechanism of smooth muscle. It is obviously important to determine if this hypothesis can be extended to nonmuscle systems or is restricted to smooth muscle. Our present results suggest that the "shape–activity" hypothesis might be a general mechanism applicable to those systems that are regulated by myosin phosphorylation. The evidence in support of this is based on the ATPase properties of the erythrocyte myosin compared to the viscosity results that reflect the conformational changes. For the $MgCl_2$ dependence of the actin-activated ATPase activity, it was suggested previously (Ikebe et al., 1984) with gizzard myosin that the level of activity was inversely proportional to the concentration of 10S myosin. The Ca^{2+}-ATPase activity of erythrocyte myosin shows a sharp decline below 0.3 M KCl, i.e., where the folded conformation begins to form. At 0.1 M KCl, most of the erythrocyte myosin is 10 S, and the ATPase level is low (Figure 5A). For the Mg^{2+}-ATPase activity, a similar pattern is observed, where formation of the 10S myosin results in a decrease of Mg^{2+}-ATPase activity (Figure 5B). The influence of phosphorylation of erythrocyte myosin is also similar to the smooth muscle situation. For the Mg^{2+}-ATPase activity, phosphorylation of bovine erythrocyte myosin alters the KCl dependence, and this is interpreted as a stabilization of the 6S conformation at an ionic strength of less than 0.3 M. The gel filtration data (Figure 6B) as well as viscosity data (Figure 7) are consistent with this and indicate that phosphorylation induces a partial transformation to the 6S state in 0.2 M KCl, although Mg^{2+}-ATPase activity of phosphorylated myosin in 0.2 M KCl reached almost the maximum level which is consistent with the 6S state. One possible explanation for the discrepancy between gel filtration (or viscosity) and the level of Mg^{2+}-ATPase activity in 0.2 M KCl is that some subtle conformational change in the head–neck region which may responsible for dictating the ATPase activity, as suggested in gizzard myosin (Ikebe & Hartshorne, 1984, 1985b), has fully occurred under these conditions, while the tail portion is still partially folded.

There was also a suggestion of the effect of phosphorylation from rotary shadowing results which showed that more molecules of phosphorylated myosin formed an extended conformation, although folded molecules of the phosphorylated myosin were still observed at the ionic strength (0.1 M NaCl) at which the electron microscopic experiments were performed [this salt level may have been too low for phosphorylation to convert the majority of folded molecules to the extended state, as is observed under certain conditions (Craig et al., 1983; Trybus & Lowey, 1984)]. The extent of transition from 10 S to 6 S as judged from rotary shadowing is not inconsistent with the viscosity results (Figure 7) and also consistent with the results in Figure 5B where the Mg^{2+}-ATPase activity of phosphorylated myosin in 0.1 M KCl increased only twice that of dephosphorylated myosin. This phosphorylation-dependent transition between folded and extended conformations has been described for other nonmuscle myosins, i.e., thymus (Craig et al., 1983; Kendrick-Jones et al., 1987) and brush border myosin (Citi & Kendrick-Jones, 1986; Kendrick-Jones et al., 1987).

In general, and based on limited experimental data, the human erythrocyte myosin is similar to the bovine erythrocyte myosin and thus presumably undergoes the 10S–6S transition.

In summary, the above results show that bovine erythrocyte myosin can exist in two conformations, namely, the folded (10S) form and the extended (6S) form. The enzymatic characteristics associated with each conformation support the hypothesis, developed with gizzard myosin, that the conformational transition (10 S–6 S) or some part of the transition is important in determining biological properties of myosin and

[2] This yield was obtained with several preparations of bovine platelet myosin using the same procedures as in the text.

could therefore be involved in the in vivo regulatory mechanisms.

Registry No. ATPase, 9000-83-3.

References

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

Bennett, V. (1983) *Methods Enzymol. 96*, 313–324.

Beutler, E., West, C., & Blume, K.-G. (1976) *J. Lab. Clin. Med. 88*, 328–333.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chacko, S., & Rosenfeld, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 292–296.

Citi, S., & Kendrick-Jones, J. (1986) *J. Mol. Biol. 188*, 369–382.

Craig, R., Smith, R., & Kendrick-Jones, J. (1983) *Nature (London) 302*, 436–439.

Davis, J. Q., & Bennett, V. (1984) *J. Biol. Chem. 259*, 14850–14856.

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

Echenburgch, M., & Burk, R. R. (1982) *Anal. Biochem. 125*, 96–99.

Fowler, V. M., & Bennett, V. (1984) *J. Biol. Chem. 259*, 5978–5989.

Fowler, V. M., Davis, J. Q., & Bennett, V. (1985) *J. Cell Biol. 100*, 47–55.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Gratzer, W. B. (1981) *Biochem. J. 198*, 1–8.

Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) Vol. I, 2nd ed., pp 423–482, Raven Press, New York.

Ikebe, M., & Hartshorne, D. J. (1985a) *J. Biol. Chem. 260*, 13146–13153.

Ikebe, M., & Hartshorne, D. J. (1985b) *Biochemistry 24*, 2380–2387.

Ikebe, M., Hinkins, S., & Hartshorne, D. J. (1983) *Biochemistry 22*, 4580–4587.

Ikebe, M., Barsotti, R. J., Hinkins, S., & Hartshorne, D. J. (1984) *Biochemistry 23*, 5062–5068.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1986) *J. Biol. Chem. 261*, 36–39.

Kendrick-Jones, J., Smith, R. C., Craig, R., & Citi, S. (1987) *J. Mol. Biol. 198*, 241–252.

Krisanda, J. M., & Murphy, R. A. (1980) *J. Biol. Chem. 255*, 10771–10776.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Matovick, L. M., Groschel-Stewart, U., & Schrier, S. L. (1986) *Blood 67*, 1668–1674.

Merkel, L., Ikebe, M., & Hartshorne, D. J. (1986) *Biophys. J. 49*, 65a.

Mommaerts, W. F. M., & Green, I. (1954) *J. Biol. Chem. 208*, 833–843.

Onishi, H., & Wakabayashi, T. (1982) *J. Biochem. (Tokyo) 92*, 871–879.

Onishi, H., Yamada, Y., Ikebe, M., Suzuki, H., & Watanabe, S. (1978) *J. Biochem. (Tokyo) 83*, 129–135.

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

Scholey, J. M., Smith, R. C., Drenckhahn, D., Groeschel-Stewart, U., & Kendrick-Jones, J. (1982) *J. Biol. Chem. 257*, 7737–7745.

Schrier, S. L. (1985) *Clin. Haematol. 14*, 1–12.

Schrier, S. L., Hardy, B., Junga, I., & Ma, L. (1981) *Blood 58*, 958–962.

Sheetz, M. P., Painter, R. G., & Singer, S. J. (1976) *Biochemistry 15*, 4486–4492.

Suzuki, H., Kamata, T., Onishi, H., & Watanabe, S. (1982) *J. Biochem. (Tokyo) 91*, 1669–1705.

Trybus, K. M., & Lowey, S. (1984) *J. Biol. Chem. 259*, 8564–8571.

Trybus, K. M., Huiatt, T. W., & Lowey, S. (1982) *Proc. Natl. Acad.* Sci. *U.S.A. 79*, 6151–6155.

Wagner, P. D., & George, J. N. (1986) *Biochemistry 25*, 913–918.

Wagner, P. D., Vu, N. D., & George, J. N. (1985) *J. Biol. Chem. 260*, 8084–8089.

Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.

Weed, R. I., LacCelle, P. L., & Merrill, E. W. (1969) *J. Clin. Invest. 48*, 795–809.

Wong, A. J., Kiehart, D. P., & Pollard, T. D. (1985) *J. Biol. Chem. 260*, 46–49.