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Isolation and Properties of Platelet Myosin Light Chain Kinase†

James L. Daniel and Robert S. Adelstein*

ABSTRACT: A protein kinase which phosphorylates the 20 000-dalton light chain of platelet myosin has been isolated from human blood platelets and purified approximately 600-fold. Elution of a 7.5% polyacrylamide gel following electrophoresis of the partially purified enzyme yielded a single peak of kinase activity which could be aligned with a protein band on a stained gel. Assuming a globular shape, a native molecular weight of 83 000 (±10%) was determined by gel filtration on Bio-Gel P-200. The kinase requires Mg^{2+} for activity and is not sensitive to the removal of trace Ca^{2+}. The enzyme purified from human platelets phosphorylates the 20 000-dalton light chain of mouse fibroblast and chicken gizzard myosin, but does not phosphorylate human skeletal and cardiac myosin.

Human blood platelets contain actin and myosin which are similar in structure and function to the proteins found in smooth muscle cells (Adelstein and Conti, 1974). Platelet myosin is composed of two heavy chains (200 000 daltons) and two different light chains (20 000 and 15 000 daltons).

Previous work in this laboratory demonstrated that the 20 000-dalton platelet myosin light chain could be phosphorylated by incubating a crude extract of platelet actomyosin with γ-labeled $AT^{32}P$ (Adelstein et al., 1973). It was surmised that the platelet extract contained a kinase which phosphorylated the 20 000-dalton light chain. Moreover, phosphorylated platelet myosin differed from nonphosphorylated myosin in its ability to be activated by actin (Adelstein and Conti, 1975a).

Studying phosphorylation in rabbit skeletal muscle, Perrie et al. (1973) isolated a single chymotryptic peptide from the 18 500-dalton light chain of rabbit skeletal muscle myosin which contained the radioactive phosphorus. Recently, Pires et al. (1974) partially purified an enzyme from rabbit skeletal muscle responsible for this phosphorylation.

In this paper, we report the isolation and purification from

† From the Section on Molecular Cardiology, Cardiology Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received March 12, 1975.* A preliminary account of this work was given at the combined meetings of the Biophysical Society and the American Society of Biological Chemists, Minneapolis, Minnesota, June 1974 (Daniel, J., Conti, M. A., and Adelstein, R. S. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1579a).

platelets of the protein kinase responsible for phosphorylating the platelet myosin light chain. We also report on the properties of this kinase which differ from the kinase isolated by Pires et al. (1974) from rabbit skeletal muscle. Finally, we have investigated the substrate specificity of the platelet kinase with particular attention to myosins from various muscle and nonmuscle sources.

Methods

All steps, except as noted, were performed at 4 °C; deionized water was used throughout.

Preparation of Myosin. Platelet myosin was prepared by a variation of the method previously described (Adelstein et al., 1971). Washed and frozen human blood platelets were defrosted and mixed with two volumes of 0.5 M KCl, 15 mM Tris-HCl (pH 7.5), 1 mM EDTA,[1] 2.5 mM dithiothreitol (henceforth called buffer A) and 10 mM P ~ P. The cells were lysed by making the solution 3% with respect to *n*-butyl alcohol. After extraction for 30 min, the lysate was centrifuged for 30 min at 78 000*g*. The supernatant was diluted with 5 volumes of 1 mM EDTA and the pH adjusted to 6.4. This suspension was allowed to sit at 4 °C to permit complete precipitation (1–2 h). The cloudy suspension was centrifuged at 27 000*g* for 10 min. The pellet was dissolved in buffer A, made 10 mM with respect to $MgCl_2$ and ATP, and immediately fractionated with ammonium sulfate (to minimize phosphorylation of the platelet myosin by any associated kinase in the presence of the ATP). The ammonium sulfate fraction containing myosin (38–52%) was dialyzed in buffer A to remove the ammonium sulfate and ATP. Final purification was obtained by chromatography on Sepharose 4B (Adelstein and Conti, 1975b). Human cardiac, human skeletal, and canine cardiac myosin were prepared by a similar procedure.

Preparation of Platelet Myosin Light Chains. Platelet myosin was made 5 M with respect to guanidine hydrochloride and heavy chains were precipitated by adding 1 volume of water followed by 4 volumes of 99% ethanol using the procedure outlined by Perrie and Perry (1970). Heavy chains were removed by centrifugation at 27 000*g* for 10 min. The supernatant was lyophilized and then dialyzed in 25 mM Tris-HCl (pH 7.6) and 20 mM KCl. The resulting light chain fraction, which contained a mixture of both platelet myosin light chains, was found to be free of kinase activity which often contaminated undenatured myosin and was used for assay of kinase activity without further purification. As was the case for the native myosin, only the 20 000-dalton light chain (and not the 15 000-dalton light chain) was phosphorylated in this denatured light chain fraction (see Figure 1).

We would like to acknowledge the gifts of the following myosins: rabbit skeletal muscle from Dr. Evan Eisenberg; chicken gizzard from Drs. Steven Driska and David Hartshorne; and lobster myosin from Dr. Joel Regenstein.

Assay of ATPase Activity. The assay mixture contained 10 mM imidazole-HCl (pH 7.0), 2 mM ATP, 0.5 M KCl, and either 2 mM EDTA or 10 mM $CaCl_2$. Samples were incubated at 37 °C; aliquots were removed at zero time as well as two additional times to ascertain the linearity of phosphate release. Inorganic phosphate was measured by the method of Martin and Doty (1949).

Kinase Assay. Kinase activity was measured by the amount of radioactive phosphate transferred from γ-labeled $AT^{32}P$ to 0.15 mg of isolated light chain fraction. The assay volume of 0.2 ml contained 25 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.15 mM ATP, and approximately 5–15 μCi of $AT^{32}P$. The reaction was measured at 23 °C for varying time intervals (0–24 h) with 30 min being used most often. The reaction was stopped by adding an aliquot (0.025–0.050 ml) of the reaction mixture to 1 ml of 10% Cl_3CCOOH, 2% pyrophosphate. These samples were then heated at 90 °C for 20 min and cooled on ice for 5 min. Each sample was applied to a Millipore filter Type HA (25 mm) on a Millipore 3025 sampling manifold. The samples were washed with 5% Cl_3CCOOH, 1% pyrophosphate to remove any nonprotein-bound radioactivity. The filters were placed in Aquasol (New England Nuclear) and after being well dispersed were counted in a Packard Tricarb liquid scintillation spectrometer.

Protein Determinations. Protein concentrations were determined by the method of Lowry et al. (1951) after precipitation in 10% Cl_3CCOOH; bovine serum albumin was used as a standard.

Polyacrylamide Gel Electrophoresis. Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was performed by the method of Fairbanks et al. (1971). Either 7.5 or 10% acrylamide gels were run in the presence of 40 mM Tris-acetate (pH 7.4), 2 mM EDTA, 1% sodium dodecyl sulfate.

In order to determine the location of radioactively labeled polypeptides on the gels, two methods were used. Gels were crushed with a Savant autogel divider and the resulting fractions were counted in Aquasol. For more exact location of ^{32}P, bands of protein stained with Coomassie brilliant blue were cut from the gels and crushed with a glass rod. These bands were incubated with a 9:1 mixture of Nuclear Chicago NCS tissue solubilizer and water at 50 °C for 2 h. This mixture was suspended in 0.04% 2,5-diphenyloxazole and toluene and counted in the liquid scintillation spectrometer.

In order to preserve enzymatic activity of the platelet kinase 7.5% polyacrylamide gels were electrophoresed (2.0 mA per tube) in the absence of sodium dodecyl sulfate and in the presence of 2.5 mM dithiothreitol, using the same buffer and gel system employed by Fairbanks et al. (1971). The gels were pre-electrophoresed overnight to remove the ammonium persulfate before the proteins were applied. The kinase was eluted from the gels by incubation of gel slices (2.5 mm) overnight in individual tubes containing 0.1 ml of the following buffer: 20 mM KCl, 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2.0 mM dithiothreitol (henceforth called buffer B). The following day each gel slice was assayed for kinase activity by adding 0.15 mg of platelet myosin light chains, $MgCl_2$, ATP, and $AT^{32}P$ to each incubation tube and bringing the total volume to 0.2 ml (i.e., excluding the gel volume; for details of kinase assay, see above).

The following procedure was used for elution of material from 7.5% polyacrylamide gels prior to electrophoresis of the material on sodium dodecyl sulfate–polyacrylamide gels. A group of eight identical gels were simultaneously sliced with a razor blade at 2.5-mm intervals. Each set of eight gel slices was allowed to elute for 36 h into deionized water. The eluate was then removed from the slices, lyophilized, solubilized in 10 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate and electrophoresed on 7.5% sodium dodecyl sulfate–polyacrylamide gels as described above.

Results

Purification of Platelet Myosin Light Chain Kinase. Isolation of the protein kinase responsible for platelet myosin

[1] Abbreviations are as follows: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, cyclic adenosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; Tris, tris(hydroxymethyl)aminomethane.

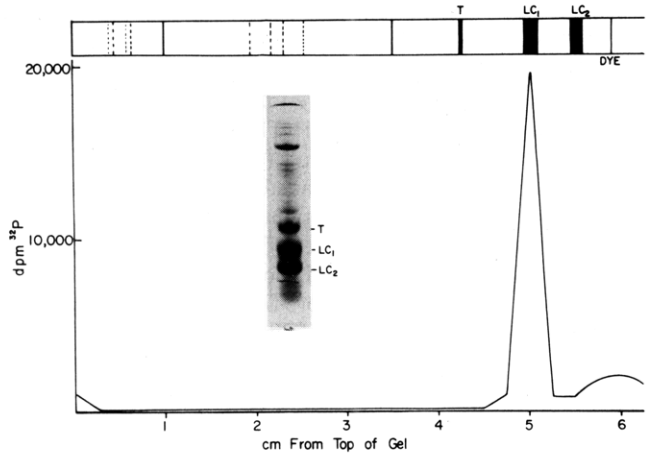

FIGURE 1: A 1% sodium dodecyl sulfate–7.5% polyacrylamide gel of the platelet myosin light chain fraction. The graph indicates the location and amount of radioactivity found in the gel when it was sliced at 2.5-mm intervals. The photograph in the center shows the actual gel which was sliced. A drawing of this gel is provided above for purpose of alignment. Tropomyosin is designated by the letter T; LC_1 and LC_2 indicate the 20 000 and 15 000 dalton light chains of platelet myosin, respectively.

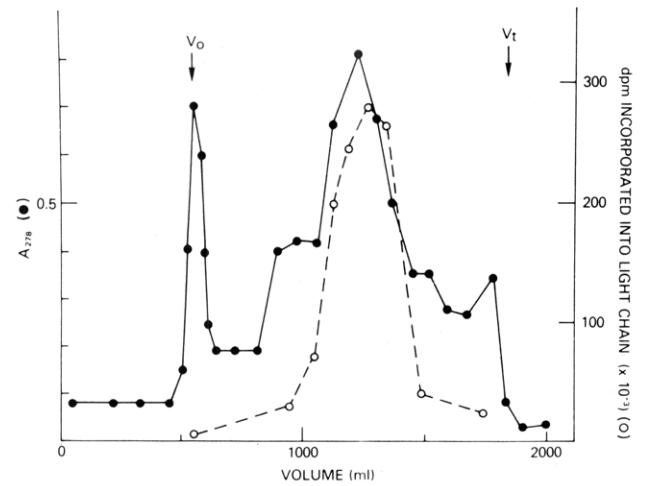

FIGURE 2: Gel filtration of the 30–55% ammonium sulfate fraction on Sepharose 6B. Eight hundred and fifty milligrams of protein was applied in 40 ml to a 5.0 × 90 cm column of Sepharose 6B equilibrated with buffer B. Fractions were collected every 30 min at a flow rate of 30 ml per h. Protein concentration was monitored by the absorbance at 278 nm. Fractions eluting between 1150 and 1360 ml were pooled for the DEAE-Sephadex A-25 column.

phosphorylation required the choice of a suitable substrate. Platelet myosin proved unsuitable since, as isolated, it often contained a small amount of kinase activity prior to Sepharose chromatography. Therefore, a platelet light chain fraction was prepared from guanidine hydrochloride denatured platelet myosin following the procedure used by Perrie and Perry (1970) for preparing light chains from skeletal muscle. Figure 1 shows that this fraction retained both receptor activity and specificity in that the 20 000-dalton light chain (LC_1) was the only protein phosphorylated (the low, broad peak of radioactivity at the bottom of the gel (6 cm from the top) may be due to phosphorylated proteolytic fragments of the 20 000-dalton light chains since it was found to increase at a slow rate with prolonged storage). Other components of the fraction (e.g., platelet tropomyosin (T) and the 15 000-dalton platelet myosin light chain (LC_2)) were not phosphorylated. Since the 20 000-dalton light chain is specifically phosphorylated (see below) and since no kinase activity was found in this fraction, the fraction was considered to be a suitable substrate for the isolation of the kinase.

TABLE I: Summary of the Purification Procedures.

	Protein (mg)	Total Act.[a]	% Act. Recovered	Spec Act.[b]
Extract	1700	995		0.59
$(NH_4)_2SO_4$ 30–55%	846	840	84	0.99
Sepharose 6B	169	648	67	3.83
DEAE-Sephadex A25	9.7	249	29	25.7
Hydroxylapatite	0.30	104	12	346

[a] Nanomoles of ^{32}P incorporated into standard amount (0.15 mg) of platelet myosin light chain fraction in 30 min corrected up to the total volume of each fraction. [b] Total activity/mg protein.

Kinase Extraction. Washed, frozen platelet concentrates (1-day old) were taken from stocks on hand and thawed (25-g wet weight). The platelets were extracted with 2 volumes of 40 mM potassium phosphate (pH 6.2), 2 mM dithiothreitol, and 1 mM EDTA for 30 min at 4 °C. Butanol was added to a concentration of 3% to assure cell lysis. This suspension was sedimented at 30 000*g* for 20 min. The pellets were reextracted twice more with 1 volume of the same buffer. The three supernatants were combined and dialyzed in buffer B (see Table I).

Ammonium Sulfate Fractionation. The dialyzed extract was made 30% with respect to ammonium sulfate by adding an appropriate volume of saturated ammonium sulfate. The pH was maintained between 7 and 8. The precipitate was allowed to form at 4 °C for 1 h and then sedimented at 28 000*g* for 15 min. Ammonium sulfate was then added to the supernatant to 55% saturation. The precipitate was again collected by centrifugation at 28 000*g*. Both 0–30% ammonium sulfate fraction and 30–55% fraction precipitates were suspended in and then dialyzed against buffer B. Assay of both ammonium sulfate fractions for kinase activity showed the 0–30% fraction contained less than 10% of the total activity; hence, the 0–30% fraction was discarded.

Gel Filtration on Sepharose 6B. The 30–55% ammonium sulfate fraction (20–40 ml) was applied to a 5.0 × 90 cm column of Sepharose 6B (Pharmacia Fine Chemicals Inc.) which had been equilibrated with buffer B (Figure 2).

A single peak of kinase activity (as measured by incorporation of ^{32}P into platelet myosin light chain fraction) eluted from the column at an elution volume expected for proteins of molecular weight in the range of 100 000 daltons. The peak fractions were combined for application to an ion-exchange column.

Chromatography on DEAE-Sephadex A-25. The fraction (200 ml) obtained from the Sepharose 6B column was applied to a 2.5 × 95 cm column of DEAE-Sephadex A-25 (Pharmacia Fine Chemical Inc.) which had been equilibrated with buffer B (Figure 3). Protein kinase activity was eluted with a gradient of KCl generated by a reservoir containing 1 l. of 25 mM Tris-HCl (pH 7.6), 0.4 M KCl, 2 mM dithiothreitol, and 1 mM EDTA and a mixing chamber containing 1 l. of buffer B. As illustrated in Figure 3, a single major peak of kinase activity (measured with platelet myosin light chain as the substrate) was eluted between 0.14 and 0.18 M KCl. An initial peak of kinase activity was found associated with the protein which

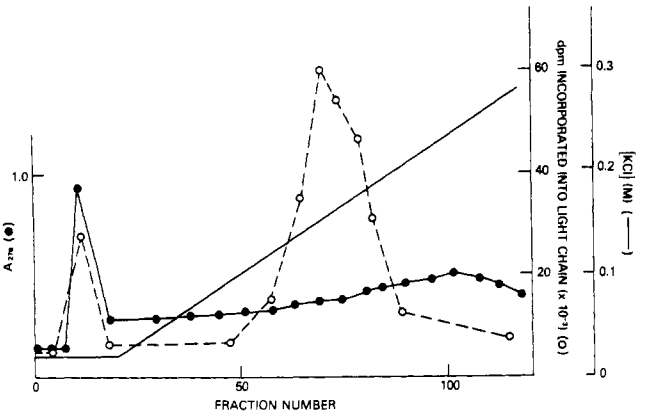

FIGURE 3: DEAE-Sephadex A-25 chromatography of the pooled fraction from Sepharose 6B. One hundred and seventy milligrams of protein was applied in 200 ml to a 2.5 × 95 cm column of DEAE-Sephadex A-25 equilibrated with buffer B. The column was eluted with a KCl gradient as described under Results at a flow rate of 30 ml per h. Fractions were collected at 30-min intervals. Kinase activity was assayed using platelet myosin light chain fraction (see Methods) as substrate. Protein concentration was monitored as in Figure 1. Fractions 65–80 (about 250 ml) were pooled and concentrated using a Diaflo UM-10 membrane to 20 ml. This material was dialyzed vs. 2 mM potassium phosphate (pH 7.0), 2 mM dithiothreitol.

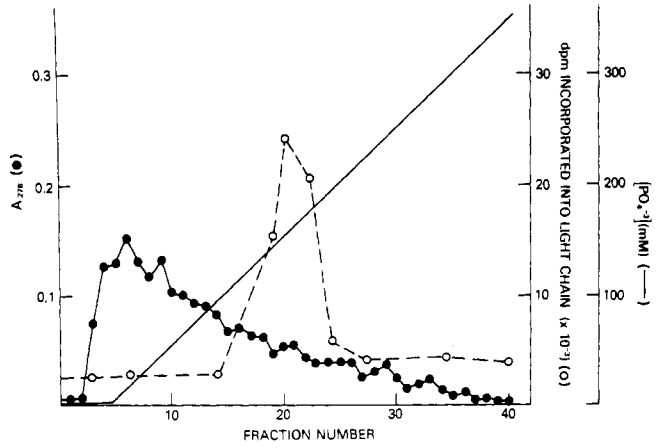

FIGURE 4: Hydroxylapatite chromatography of platelet kinase. The fraction (9.5 mg) from the DEAE-Sephadex A-25 column was applied to a 2.0 × 15 cm column of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories). The column was eluted at a flow rate of 20 ml/h and 8.0-ml fractions were collected. A linear phosphate gradient was used for elution. Kinase activity was measured using the light chain fraction as a substrate. Protein concentration was monitored by measurement of the absorbance at 278 nm. The peak fractions (20–24) were pooled and concentrated using a Diaflo UM-10 membrane.

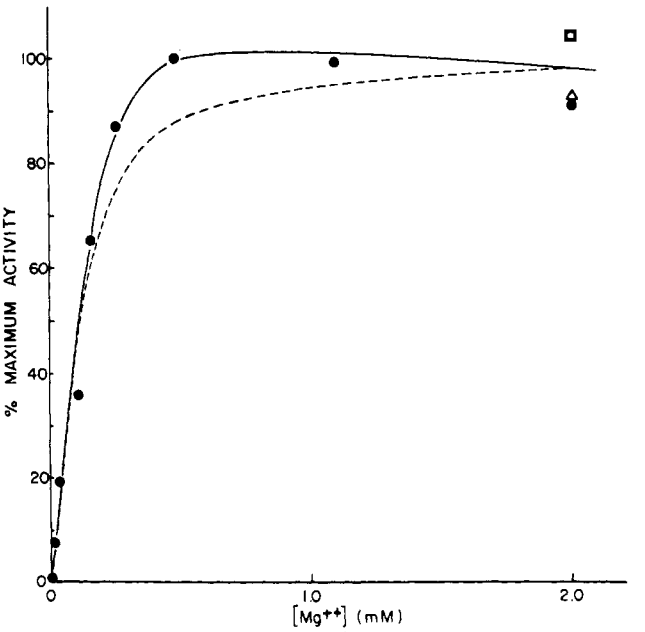

FIGURE 5: The effect of increasing Mg^{2+} concentration on platelet kinase activity. Conditions: 40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, and 1 mM EDTA, 0.15 mM ATP. The concentration of Mg^{2+} was changed by adding varying proportions of two solutions, one containing magnesium acetate and EDTA and the other containing only EDTA, thus allowing the EDTA concentration to remain constant. A binding constant of 9.4×10^5 M^{-1} (at pH 7.6) of EDTA for magnesium was used to calculate magnesium concentration. This constant was calculated using the appropriate stability constants and equations (from eq 22, Chaberek and Martell, 1959). The triangle indicates the effect of the addition of 0.25 mM $CaCl_2$; the square shows the effect of the addition of 1 mM EGTA to the assay system which contained 2 mM Mg^{2+} and no EDTA. The dashed line indicates the theoretical curve which would be obtained if MgATP were acting as substrate. This curve was calculated using a binding constant of 2×10^4 M^{-1} for MgATP (Nanninga, 1961).

eluted at the beginning of the KCl gradient. Rechromatography of this peak material on a similar DEAE-Sephadex column resulted in a peak of kinase activity eluting at about 0.16 M KCl. This indicated that the initial peak of activity was due to the same kinase found in the major peak (Figure 3) which failed to bind to the column during the initial chromatography. Fractions from the major peak were combined and concentrated by ultrafiltration using an Amicon Diaflo UM-10 membrane (from 300 to 20 ml) and dialyzed in 2 mM potassium phosphate (pH 7.0), 2 mM dithiothreitol.

Chromatography on Hydroxylapatite. The protein kinase fraction from the DEAE-Sephadex A-25 column was applied to a (1.5 × 20 cm) column of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) that had been previously equilibrated with 2 mM potassium phosphate (pH 7.0), and 2 mM dithiothreitol. The column was eluted with a linear phosphate gradient generated by a reservoir containing 250 ml of 0.4 M potassium phosphate (pH 7.0) and 2 mM dithiothreitol and a mixing chamber containing 250 ml of 2 mM potassium phosphate (pH 7.0) and 2 mM dithiothreitol. A single peak of kinase activity was obtained which eluted at a phosphate concentration of about 0.16 M (Figure 4). The peak fractions were pooled and concentrated to a final volume of 1 ml with ultrafiltration followed by vacuum dialysis (using buffer B as dialysis buffer).

A summary of the purification procedure is given in Table I. The enzyme was purified about 600-fold by the procedure outlined. About 12% of the initial activity remained after the final step. Starting with about 25 g of packed platelets, the hydroxylapatite fraction yielded approximately 300 μg of protein.

Properties of the Light Chain Kinase. Since kinases are often found associated with myosin (e.g., myokinase), it was essential to show that this kinase preparation did not contain platelet myosin. This was found to be the case by assaying the kinase fraction after chromatography on DEAE-Sephadex and finding no detectable myosin ATPase activity. Electrophoresis of this fraction on sodium dodecyl sulfate–polyacrylamide gels confirmed this observation by showing no evidence of the 200 000-dalton heavy chain of myosin.

The purified kinase is dependent on Mg^{2+} ions for activity as demonstrated in Figure 5. Ca^{2+}, in the absence of Mg^{2+}, does not activate the enzyme. A close correlation with a calculated curve of MgATP concentration suggests that MgATP

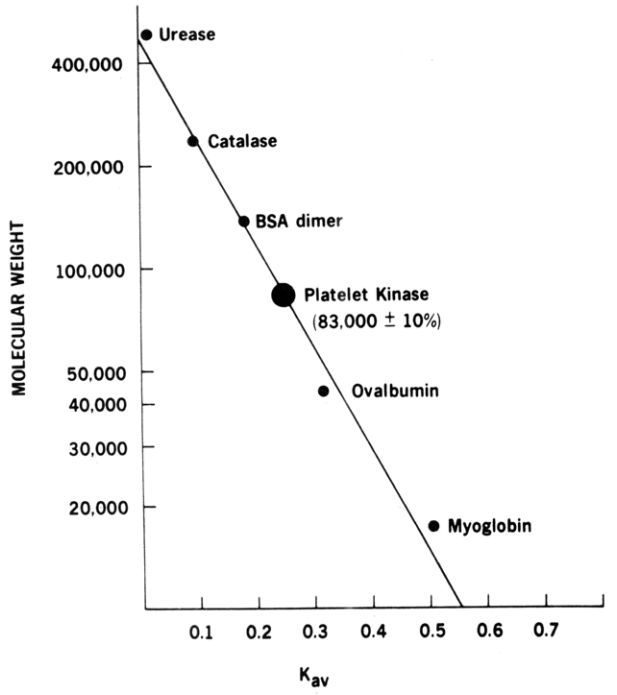

FIGURE 6: The native molecular weight of platelet light chain kinase. Samples of each standard (2 ml) were applied to a 2.0 × 48 cm column of Bio-Gel P-200. The elution volume was determined by measurement of absorbance at 278 nm. The same volume of hydroxylapatite kinase fraction was applied and elution volume determined by assaying for kinase activity using platelet myosin light chains as a substrate.

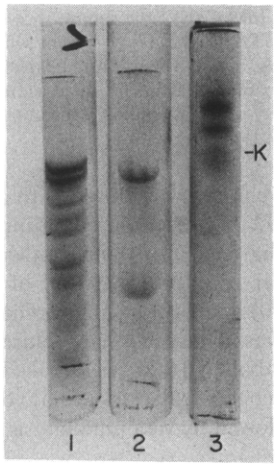

FIGURE 7: (Number 1) A 1% sodium dodecyl sulfate–7.5% polyacrylamide gel of the kinase obtained from hydroxylapatite; (number 2) a 1% sodium dodecyl sulfate–7.5% polyacrylamide gel of the material obtained from elution of eight 7.5% polyacrylamide gels (without sodium dodecyl sulfate) which comigrated with the peak of kinase activity; (number 3) a 7.5% polyacrylamide gel (without sodium dodecyl sulfate) of the same kinase shown in number 1 (note that gels 1 and 2 were approximately 7-cm long and contained sodium dodecyl sulfate; gel 3 was about 9 cm and contained no sodium dodecyl sulfate). The letter K indicates the band which had the same R_f as the kinase activity.

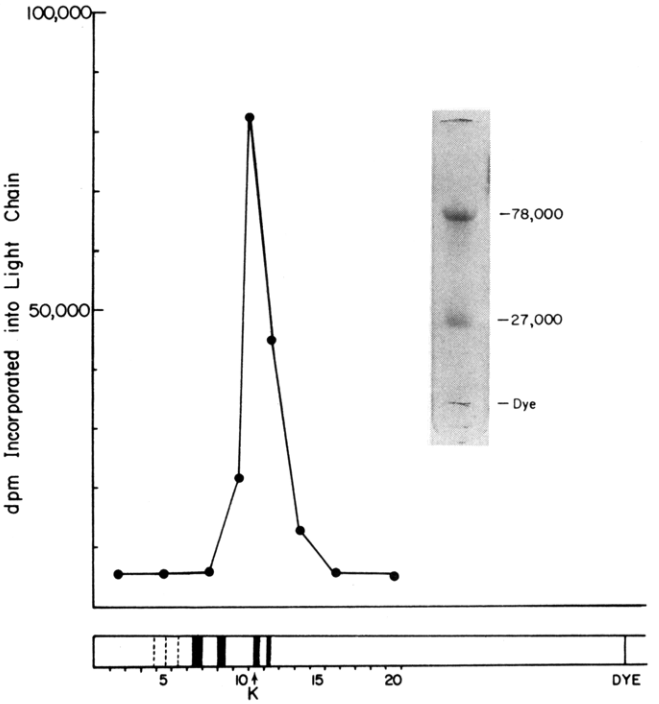

FIGURE 8: Elution of kinase activity from a polyacrylamide gel. A sample of the hydroxylapatite fraction was electrophoresed on a 7.5% polyacrylamide–0.2% bisacrylamide gel, and protein was eluted as described under Methods. Kinase activity was measured using 0.15 mg of platelet myosin light chain fraction as substrate. The fractions from the gel are plotted on the abscissa. Below the figure is a diagram of a similar gel which was stained with Coomassie brilliant blue. The letter K indicates the band which aligned with the kinase peak. A picture of this gel is shown in Figure 7 (number 3).

is the substrate for the enzyme. The magnesium-dependent kinase activity was not sensitive to removal of trace Ca^{2+} by EGTA, as illustrated in the figure.

Molecular Weight Determination of the Native and Denatured Kinase. Figure 6 shows the elution characteristics of the kinase and a number of proteins of known molecular weight on a column of Bio-Gel P-200. The molecular weight of each protein is plotted against its relative mobility (K_{av}). All standard proteins were selected on the basis of their reported elution pattern from Sephadex G-200 which correlated with their molecular weights (Andrews, 1965). The K_{av} of the kinase was consistent with a molecular weight for the native enzyme of 83 000 ± 10% assuming a globular shape for the enzyme.

Since the kinase peak eluted from the hydroxylapatite column was not a pure protein (as demonstrated by a sodium dodecyl sulfate–polyacrylamide gel of this fraction; Figure 7, number 1), the following experiment was performed in order to obtain information about the subunit composition of the kinase. Approximately 25 μg of the hydroxylapatite kinase was loaded on each of ten polyacrylamide gels (see Methods for polyacrylamide gels in the absence of sodium dodecyl sulfate). After electrophoresis, these gels were divided into three groups. One gel was stained to allow visualization of the protein bands. This gel is shown in Figure 7, number 3. Another gel was cut into 2.5-mm sections and eluted overnight into buffer B for assay of kinase activity the next day (see Methods). The other eight gels were also cut into 2.5-mm slices and were eluted overnight into water. The resulting eluates were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels (all bands on the original sodium dodecyl sulfate–polyacrylamide gel of the hydroxylapatite peak were accounted for using this method).

A summary of this experiment is shown in Figure 8. A drawing representing the stained gel from Figure 7, number 3, is shown on the abscissa in order to demonstrate the relationship between the gel sections used for enzymatic analysis and the protein electrophoresis pattern. The results of the assay for kinase activity are given on the graph above. A single peak of activity was found. This peak comigrated with a protein band seen on the original stained gel. The sodium dodecyl sulfate–polyacrylamide gel of the material obtained from this

TABLE II: Substrates of Platelet Myosin Light Chain Kinase.

Substrate	Receptor[a] Act. (%)	Inhibitor Act.
Nonmuscle myosins		
Platelet	100	
Platelet light chain (denatured)	8–12	
Mouse fibroblast	8–12	
Muscle Myosins		
Chicken gizzard	15–20	
Lobster	15–20	
Human skeletal	0	+[b]
Rabbit skeletal	0	+[b]
Human cardiac	0	N.D.[c]
Canine cardiac	0	N.D.[c]

[a] Relative to platelet myosin. [b] Present. [c] Not determined.

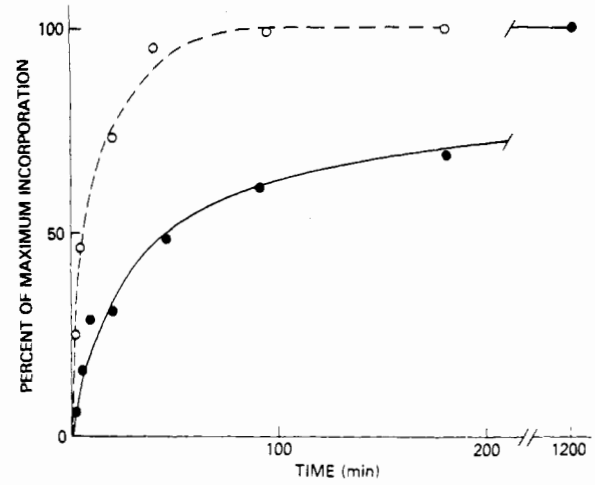

FIGURE 9: The time course for phosphorylation of intact platelet myosin and for isolated platelet myosin light chain. Either platelet myosin (0.19 mg) or platelet myosin light chains (0.02 mg of 20 000-dalton light chain) was incubated with hydroxylapatite kinase fraction (5 μg) at 25 °C in 0.4 ml of 0.3 M KCl, 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM dithiothreitol, and 0.15 mM ATP (containing 20 μCi of γ-labeled $AT^{32}P$). Aliquots (25 μl) were taken at the indicated times and incorporation of ^{32}P was determined as described in the Methods. In each case, approximately the same amount of phosphate was incorporated per mol of 20 000-dalton light chain when the reaction was complete (about 0.75 mol of phosphate per mol of light chain).

region of the gel is shown to the right (see also Figure 7, number 2). The staining pattern obtained is much less complex than that of the original sodium dodecyl sulfate–polyacrylamide gel of the hydroxylapatite column fraction. Only two protein bands were found: one with a molecular weight of 78 000 daltons and another with a molecular weight of 27 000 daltons. Since the native molecular weight obtained from gel filtration was 83 000 ± 10%, the 78 000-dalton band is thought to be the kinase.

The Stoichiometry for the Phosphorylation of Intact Platelet Myosin and Isolated Platelet Myosin Light Chain. A time course for the labeling of platelet myosin and platelet myosin light chain fraction is shown in Figure 9. The two experiments were performed under identical conditions and nearly identical ratios of kinase to substrate (mole:mole). As shown, the reaction with myosin as a substrate is approximately six times more rapid. In each case, when the reaction was completed almost stoichiometric amounts of labeling were found. Approximately 0.75 mol of phosphate was transferred to each mol of 20 000-dalton light chain whether the light chain was a constituent of native myosin or had been separated from the myosin heavy chain by denaturation.

The finding that both native myosin and isolated light chain are phosphorylated to the same extent indicated that they both might be phosphorylated at the same site. The following experiment was performed in order to more firmly establish this point. A sample of myosin was divided into two aliquots. To one aliquot the protein kinase and nonradioactive ATP were added and phosphorylation was allowed to proceed until completion as indicated by the time course in Figure 9. A light chain fraction was prepared from each aliquot (phosphorylated and nonphosphorylated) and the ability of each sample to be phosphorylated was measured using γ-labeled $AT^{32}P$ and the kinase. In two different experiments, only 4 and 20% of the pre-labeled light chain were phosphorylated relative to the control light chains (those light chains prepared from the aliquot of myosin which had not seen ATP). Thus, light chains prepared from phosphorylated myosin are not phosphorylated to a significant extent following denaturation, indicating that both native and denatured light chains are phosphorylated at the same site.

The Effect of Cyclic Nucleotides. We have not been able to demonstrate any effect of cAMP on platelet myosin light chain kinase. In these experiments, the nucleotide was tested at varying pH (pH 6.5–8) and at different concentrations (10^{-6} to 10^{-4} M), including a large excess to be certain that enzymatic cleavage by phosphodiesterase was not masking the effect. In order to discern if a regulatory cyclic nucleotide binding subunit might have been lost during the purification, the kinase was tested with cAMP at all stages of the preparation with negative results. Furthermore, a protein which is known to bind cAMP and inhibit cAMP dependent kinases (Gilman, 1970) did not inhibit the platelet myosin light chain kinase. The dibutyryl derivative of cAMP had no effect on kinase activity. Another cyclic nucleotide, cGMP, also had no effect on the enzymatic activity of the kinase.

Substrates of the Kinase. A list of proteins assayed as substrates of platelet myosin light chain kinase is shown in Table II. The table gives an estimate of the receptor activity for each protein relative to platelet myosin in 0.3 M KCl (based on the number of moles of substrate phosphorylated in 30 min). In each case, substrates were assayed by the Millipore filter method outlined above which was used to demonstrate substrate activity. The results of these assays were used for the data shown in Table II. In order to confirm these results, radioactively labeled bands were located on sodium dodecyl sulfate–polyacrylamide gels. In each of the myosins which incorporated ^{32}P, only the 20 000-dalton light chain was phosphorylated by the kinase. This had already been demonstrated for platelet myosin (Adelstein et al., 1973). We have extended this finding to show that the guanidine denatured 20 000-dalton platelet light chain retained acceptor activity. Lobster, chicken gizzard, and mouse fibroblast myosins (all undenatured) were substrates of the kinase but with reduced affinity relative to platelet myosin. Rabbit skeletal muscle, human skeletal muscle, human cardiac, and canine cardiac myosins did not serve as substrates; moreover, the two skeletal muscle myosins inhibited the phosphorylation of platelet myosin light chain.

Discussion

A kinase which phosphorylates the 20 000-dalton light chain of platelet myosin has been partially purified from human

blood platelets. The procedure outlined above has produced about a 600-fold purification of the kinase (Table I). The procedure was facilitated by the use of a denatured light chain fraction in which only the 20 000-dalton light chain acted as a substrate (Figure 1). This step not only provided a specific substrate for the kinase but was necessary since native myosin was often associated with the kinase. Specificity was also indicated by the finding that denatured light chains made from previously phosphorylated native myosin could not be phosphorylated to a significant extent. As indicated in Figure 9, native myosin was phosphorylated at a more rapid rate than denatured light chain.

The kinase was judged to be impure due to the presence of several protein bands following electrophoresis of the hydroxylapatite fraction on sodium dodecyl sulfate and ordinary polyacrylamide gels. However, elution and assay of kinase activity from ordinary (non-sodium dodecyl sulfate) polyacrylamide gels have allowed alignment of the kinase activity with a single band found on a similar gel stained with Coomassie blue (Figure 8). Material eluted from the same position on like gels showed only two bands when electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel. One of the two bands migrated with a molecular weight of 78 000 daltons which corresponds closely to the native molecular weight obtained from gel filtration. This indicates that the kinase is comprised of a single polypeptide chain of about 80 000 daltons. Although other possibilities cannot be disregarded e.g., a trimer of 27 000 daltons or a dimer of 78 000 and 27 000, both these possibilities seem less likely. The 27 000-dalton peptide may well be a contaminant since the kinase migrates very close to another protein which does not possess any kinase activity (see Figure 7, gel 3). The presence of a single peak of kinase activity at each stage of the preparation and on elution from polyacrylamide gels indicates that phosphorylation of platelet myosin light chain is probably due to single species of kinase.

The kinase, isolated from platelets, is similar in some of its properties to the enzyme isolated from rabbit skeletal muscle by Pires et al. (1974); Perry et al. (1975). Corresponding to the observations of Pires et al. (1974), we have not found an effect of cAMP on our kinase at any stage of the preparation. The native molecular weight of the two enzymes is not inconsistent with this observation since most cAMP-regulated enzymes are larger (Krebs, 1972; Rubin et al., 1972). This is also consistent with the observation that the kinase contains only one polypeptide chain. Further support for the lack of regulation by cAMP is indicated by the failure of the specific protein kinase inhibitor to inhibit the platelet myosin light chain kinase.

The platelet and rabbit skeletal muscle kinases appear to have different specificities for divalent cations and for substrates. While both enzymes are activated by Mg^{2+} ion, the platelet kinase, unlike the skeletal muscle kinase, *does not* require low levels of Ca^{2+} for full activation (see Figure 5). The independence of the platelet enzyme of Ca^{2+} and the dependence of the skeletal muscle kinase on Ca^{2+} may indicate a fundamental difference between the regulation of muscle and cellular contractile systems.

While the skeletal muscle enzyme isolated by Pires et al. (1974) phosphorylates mammalian skeletal muscle and cardiac myosin, these proteins are not substrates for the platelet kinase. Indeed, mammalian skeletal muscle myosins not only failed to be phosphorylated by the kinase, but they inhibited the enzyme's ability to phosphorylate platelet myosin light chain. This suggests structural similarities between the platelet and skeletal muscle myosin light chains which allow binding of the kinase to the skeletal muscle myosin without phosphorylation of the skeletal muscle myosin light chain. Studies on the primary structure of the phosphorylated site of platelet myosin light chain will allow for comparison with that of the skeletal muscle myosin light chain.

Myosins from nonmuscle cells (human blood platelets, Adelstein et al. 1973, and mouse fibroblasts, unpublished results in this laboratory), smooth muscle cells (chicken gizzard, Kendrick-Jones, 1973; Leger and Focant, 1973), and lobster (Dr. J. Regenstein, personal communication) resemble each other in containing a light chain of 20 000 daltons which migrates in 8 M urea–polyacrylamide gel electrophoresis with a more negative charge than the light chains of skeletal muscle and cardiac muscle myosin. These structural similarities are also manifested in the ability of the 20 000-dalton light chain of platelet, fibroblast, chicken gizzard, and lobster myosin to serve as a substrate for platelet myosin light chain kinase (see Table II).

Recent results from this laboratory (Adelstein and Conti, 1975a) have indicated a potentially important physiological role for the kinase. Phosphorylation of the 20 000-dalton platelet myosin light chain results in a five-to-eightfold increase in the actin-activated myosin ATPase activity measured at low ionic strength. Dephosphorylation, using alkaline phosphatase (*E. coli*), results in a decrease in the actin-activated ATPase activity. Hence, platelet myosin light chain kinase appears to control the interaction of actin and myosin in human blood platelets. The findings reported here on the ability of this enzyme to phosphorylate the 20 000-dalton light chain of fibroblast and chicken gizzard myosin suggest that the function of phosphorylation may not be confined to platelets.

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Acetylcholine Receptor Oligomers from Electroplax of *Torpedo* Species†

Raymond E. Gibson, Richard D. O'Brien,* Stuart J. Edelstein,‡ and William R. Thompson§

ABSTRACT: Sedimentation in sucrose gradients of α-bungarotoxin-labeled crude and pure acetylcholine receptor preparations from *Torpedo californica* showed two major oligomers. The molecular weights, corrected for the bound Triton X-100 by comparing sedimentation in H_2O and in D_2O, were 330 000 for the heavy (H) oligomer and 190 000 for the light (L) oligomer. Lesser peaks were found in preparations of *T. marmorata* and purified preparations of *T. californica* with molecular weights of 500 000 (HH) and 80 000 (LL). These molecular weights are based upon the assumption of globularity, and may require adjustment if the assumption is wrong. The H and L peaks have similar drug sensitivities, but at pH 10 the L peak was stable whereas the H peak dissociated to components sedimenting as LL. Treatments with *p*-chloromercuribenzoate, which blocks acetylcholine binding partially without affecting α-bungarotoxin binding, had no effect upon the pattern of sedimentation. This and other evidence suggested that the heterogeneity of oligomers was unrelated to the heterogeneity of site affinities for acetylcholine and nicotinic drugs.

The great specificity of α-bungarotoxin for acetylcholine receptor (AChR)[1] was demonstrated by the physiological studies of Chang and Lee (1963). Subsequently, radioactive α-bungarotoxin has been shown to react irreversibly with both particulate and soluble receptors from *Torpedo marmorata* (Miledi et al., 1971); preparations solubilized by 1% Triton X-100 showed, on sucrose gradients, one major peak and a smaller, more rapidly sedimenting peak of one-fifth the height of the major peak. Subsequently, Raftery et al. (1972) fractionated similar material from *T. californica* on Sepharose 6B, and then showed that different fractions contained differing amounts of a rapidly and slowly sedimenting peak. The major binding component sedimented at 9.0 S and appeared to predominate at high toxin concentrations; at lower toxin concentrations, a minor component of 13.7 S was present. They pointed to the possibility that the two classes of binding site might correspond to one form reversibly associated with, and another irreversibly bound to, bungarotoxin; and also pointed to a possible parallel with the high and low affinity sites for acetylcholine binding which have been reported (Eldefrawi et al., 1971; O'Brien and Gibson, 1974).

In the present study, we have used various conditions to demonstrate a series of differently sedimenting forms of receptor–α-bungarotoxin complex, and have interpreted them in terms of a series of oligomers. We show that the oligomeric heterogeneity bears no relation to the two kinds of acetylcholine binding site.

Materials and Methods

Sucrose gradients were prepared over the range of 5–20% sucrose in a Ringer solution containing the indicated percentage of Triton X-100, and the following millimolar concentrations of salts: NaCl, 115; KCl, 4.6; $CaCl_2$, 0.65; $MgSO_4$, 1.15; and Na_2HPO_4, 15.7. The gradients were 12 ml in volume, and were made with a Beckman gradient former. The receptor preparation was incubated for 15 min at 0 °C with the indicated radioactive toxin at a ratio ("toxin–receptor ratio") of 1 except as indicated; the ratio is that of the calculated number of moles of toxin to the moles of acetylcholine binding sites in the receptor preparation, measured by equilibrium dialysis against acetylcholine (O'Brien and Gibson, 1974).

The receptor preparations were as follows. "Stored" *Torpedo marmorata* electroplax was collected in Naples, Italy, in 1968 and the heavy membrane fraction was prepared there, lyophilized, and stored at −25 °C (O'Brien et al., 1970). A concentration of 10 mg or 40 mg/ml, as indicated (corresponding to 0.73 or 2.92 g of original electroplax per ml), was prepared in the above Ringer with 1% Triton X-100, and the supernatant after centrifuging at 100 000*g* for 90 min was prepared as previously described (O'Brien and Gibson, 1974).

† From the Section of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853. *Received September 30, 1975.* This work was supported in part by Grants NS 09144 and GM 53317 from the National Institutes of Health.

‡ Alfred P. Sloan Research Fellow, Section of Biochemistry, Molecular and Cell Biology, Cornell University.

§ Present address: Department of Chemistry, The University of Texas at Dallas, Dallas, Tex. 75230.

[1] The following abbreviations are used: ACh, acetylcholine; AChR, acetylcholine receptor; *M*, molecular weight; pD, −log [D_3O^+]; ClHgBzO, *p*-chloromercuribenzoate.