Articles

Calcium Sensitivity of Vertebrate Skeletal Muscle Myosin†

Dallas L. Pulliam, Vitaly Sawyna, and Rhea J. C. Levine*

ABSTRACT: The calcium sensitivity of vertebrate skeletal muscle myosin has been investigated. Adenosinetriphosphatase (ATPase) activity was assayed in a reconstituted system composed of either purified rabbit myosin plus actin or myosin plus actin, tropomyosin, and troponin. The calcium sensitivity of actomyosin Mg-ATPase activity was found to be directly affected by the ionic strength of the assay medium. Actomyosin assayed at approximately physiological ionic strength (120 mM KCl) demonstrated calcium sensitivity which varied between 6 and 52%, depending on the myosin preparation and the age of the myosin. Mg-ATPase activity was increased when calcium was present in the assay medium at physiological ionic strength. Conversely, actomyosin Mg-ATPase activity assayed at a lower ionic strength (15 mM KCl) was inhibited by addition of calcium. Addition of tropomyosin and troponin to the assay increased the calcium sensitivity of the system at the physiological ionic strength still further (up to 99% calcium sensitivity) and conferred calcium sensitivity on the system at the lower ionic strength (>90% calcium sensitivity). A correlation also existed between myosin's calcium sensitivity and the phosphorylated state of light chain 2.

The interaction of actin and myosin in intact vertebrate skeletal muscle or in native reconstituted systems is dependent on the free calcium level as an "on–off" switch. Free calcium in the sarcoplasm or the assay medium, at a concentration of approximately 5×10^{-6} M, permits the cyclical attachment and detachment of myosin cross bridges of thick filaments with actin monomers of thin filaments and the resultant rapid release of adenosine diphosphate (ADP) and inorganic phosphate (P_i), the products of ATP hydrolysis, from myosin (Murray & Weber, 1973). Removal of free calcium to a concentration $\leq 10^{-7}$ M causes rapid relaxation; interaction between myosin cross-bridges and actin is inhibited, and ADP and P_i are no longer released from myosin at a rapid rate.

Ebashi & Endo (1968) have shown that in vertebrate striated muscles, the additional protein components of the thin filaments, tropomyosin and troponin (TMTN),[1] are responsible, at least in part, for the switching on of the activity of the contractile proteins when calcium is present. This TMTN "switch" has been demonstrated in reconstituted systems composed of myosin, actin, and TMTN at low ionic strength (Hartshorne & Mueller, 1967; Hitchcock et al., 1973) as well as in systems where soluble myosin fragments (HMM and S-1) are present together with thin filament proteins (Bremel et al., 1973). This type of control of contractile activity is termed thin filament linked, because of the location of the regulatory proteins, TMTN.

Thick filament linked or myosin-linked regulation is usually associated with invertebrate smooth and striated muscles (Lehman & Szent-Gyorgyi, 1975; Kendrick-Jones et al., 1973) and vertebrate smooth muscles (Chacko et al., 1977; Mrwa & Hartshorne, 1980). Most invertebrate striated muscles that display myosin-linked regulation also contain appreciable amounts of TMTN and thus exhibit a dual regulatory mechanism that is both myosin and thin filament linked (Lehman & Szent-Gyorgyi, 1975). In these muscles, as well as in vertebrate smooth muscle, free intracellular calcium levels have been shown to regulate contractility by affecting the phosphorylation of myosin light chain 2 (LC_2) via a calmodulin–protein kinase system (Mrwa & Hartshorne, 1980; Hoar et al., 1979; Sellers, 1981). Among invertebrates, only molluscan muscles exhibited purely myosin-linked regulation (Szent-Gyorgyi et al., 1973; Lehman et al., 1973; Chantler & Szent-Gyorgyi, 1980). In these tissues the myosin subunit responsible for calcium sensitivity is the EDTA light chain, which is easily removed from the protein, resulting in desensitization (Szent-Gyorgyi et al., 1973). The molluscan EDTA light chain is unique in that it confers calcium sensitivity on the myosin by binding calcium directly, rather than by a phosphorylating mechanism. The EDTA light chain of molluscan myosin is thought to be analogous to the DTNB light chain (Weeds, 1969) (LC_2) of vertebrate skeletal muscle myosin and the LC_2 of other myosins (Kendrick-Jones, 1974). Indeed, Kendrick-Jones (1974; Kendrick-Jones et al., 1976) and others (Sellers et al., 1980) have reported that addition of vertebrate striated muscle LC_2 to desensitized scallop myosin restored calcium sensitivity to the latter, if only 1 mol of EDTA light chain had been removed. This effect was rather puzzling, however, since vertebrate striated muscle myosin was not generally thought to be calcium sensitive itself. These results have been interpreted as reflecting cooperative behavior for the maintenance of the "off" state (Chantler et al., 1981).

The first indication that calcium sensitivity of myosin might occur in vertebrate striated muscle was Lehman's (1978) observation of 57% inhibition of ATPase hydrolysis, in the presence of EGTA, displayed by a vertebrate myofibrillar preparation from which the thin filament regulatory proteins, TMTN, had been extracted. But this phenomenon was evident only at 120 mM NaCl and not at the lower ionic strengths at which myosin-linked regulation can operate in invertebrate systems, and vertebrate ATPase assays are usually performed. Neither was Lehman (1978) able to observe calcium sensitivity in a reconstituted system containing just vertebrate skeletal

† From the Department of Anatomy, The Medical College of Pennsylvania/EPPI Division, Philadelphia, Pennsylvania 19129. *Received August 16, 1982; revised manuscript received January 3, 1983.* This work was supported by U.S. Public Health Service Grants BRSG RR-05418 and NS05615.

[1] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; AM, actomyosin; ATP, adenosine triphosphate; LC_2, light chain 2; TMTN, tropomyosin–troponin complex; EDTA, ethylenediaminetetraacetic acid; ATPase, adenosinetriphosphatase.

0006-2960/83/0422-2324$01.50/0

muscle myosin and actin, even at 120 mM NaCl.

More recently, Chin & Rowe (1982) reported that intact, native thick filaments, isolated from rabbit skeletal muscle, displayed calcium sensitivity at both low and "physiological" ionic strengths. In agreement with Lehman (1978), however, they found that synthetic thick filaments did not exhibit this property, under identical conditions. Chin & Rowe (1982) postulated that the calcium sensitivity they observed with native filaments was due to the fact that intact LC_2 was necessary for expression of this property. They suggested that exposure to high ionic strength, which is unavoidable during isolation of myosin, damages LC_2, resulting in loss of calcium sensitivity.

In the studies reported here, we will describe the calcium sensitivity that we have observed in a reconstituted system containing purified myosin and purified actin obtained from rabbit skeletal muscle.

Materials and Methods

Myosin. Most of the purified myosin we used was prepared according to a modification of the procedures of Perry (1955) and Offer et al. (1972). New Zealand White rabbits were killed with an intravenous injection of Nembutal, and 100 g of muscle was removed from the back and hind legs. The muscle was minced in a meat grinder and crude myosin was extracted with 3 volumes of extracting solution while stirring in an ice water bath for 15 min. The extracting medium contained 0.3 M KCl, 75 mM K_2HPO_4, 75 mM KH_2PO_4, 0.2 mM DTT, and 0.5 mM ATP, pH 6.6. After centrifugation for 20 min at 2000*g*, the supernatant was filtered through Pyrex wool (Corning Glass, Corning, NY). The precipitate was saved at −20 °C to be used for preparation of actin and the tropomyosin–troponin complex (TMTN). The myosin in the supernatant was precipitated with 9 volumes of cold deionized water and centrifuged at 16000*g* for 15 min. The precipitate was washed twice with a 1:10 dilution of extracting solution and was then redissolved to give a final concentration of 0.5 M KCl, 32.5 mM K_2HPO_4, 17.5 mM KH_2PO_4, 0.2 mM DTT, and 1 mM EDTA, pH 7.0. Protein concentration was determined by the biuret technique (Gornall et al., 1949), and the myosin concentration was adjusted to 10 mg/mL. Removal of actin, actomyosin, and F protein was performed by ammonium sulfate fractionation according to Offer et al. (1972). Up until this time the myosin was in a medium containing phosphate buffer. The final precipitate was redissolved in 0.3 M KCl, 10 mM Tris–maleate, 0.2 mM DTT, 2 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.0, at a concentration of approximately 5 mg/mL. Ammonium sulfate was removed by dialysis of the myosin solution for a minimum of 4 h vs. 30 volumes of the same medium which was changed after 2 h. An aliquot of the myosin solution was removed to assay ATPase activity. This myosin was dialyzed vs. 30 volumes of a solution containing either 120 mM KCl or 15 mM KCl plus 10 mM Tris–maleate, 0.2 mM DTT, and 2 mM $MgCl_2$, pH 7.0, which was changed after 2 h. The remaining myosin was either stored at 4 °C or was diluted 50% with glycerol and was stored at −20 °C.

Several myosin preparations which had been purified according to the method of Weber (1956) were also used for assays. A total of 12 different myosin preparations were tested.

Actin. Acetone dried muscle powder was prepared from the precipitated residue remaining after the first step in the myosin extraction procedure. Actin was prepared from the acetone powder according to the Eisenberg & Kielley (1974) modification of Spudich & Watt's (1971) procedure. Actin was extracted from 10 g of acetone powder at 0 °C for 30 min with 200 mL of their buffer A at pH 8.0. We found that it was essential to maintain the extracting buffer at 0 °C to eliminate contamination of the actin preparation with other thin filament proteins. To accomplish this, a sealed ice–water bath was used to lower the temperature of the extracting solution. The G-actin obtained according to Eisenberg & Kielley (1974) was clarified by centrifugation at 80000*g*, then lyophilized in their buffer A, and stored at −20 °C. Prior to use the actin was reconstituted with ion-free water, brought to 50 mM KCl, and allowed to polymerize for 90 min.

Preparation of Tropomyosin and Troponin Complex. Tropomyosin and troponin complex (TMTN) was prepared from the residue remaining after myosin and actin extraction, according to the procedures of Spudich & Watt (1971) and Hartshorne & Mueller (1969). TMTN was stored at −20 °C in the mercaptoethanol buffer, pH 8.3, of Spudich & Watt (1971).

Myosin Light Chain Kinase. Myosin light chain kinase was prepared from both fresh myosin and lyophilized myosin according to the procedures of Pires et al. (1974) and Pemrick (1980). Phosphorylation of LC_2 was carried out on both fresh and stored myosins according to Pemrick (1980).

ATPase Activity Determination. New Kimble 13 × 100 mm borosilicate glass test tubes were always used for ATPase determination. All other glassware used was washed with phosphate-free detergent and thoroughly rinsed.

ATPase and all chemicals were weighed out in a desiccated environment. Sigma grade ATP (Sigma Chemical Co., St. Louis, MO) was dissolved at a concentration of 50 mM, pH 6.9, in deionized water, and 5-mL aliquots were kept frozen at −20 °C prior to use.

ATPase activity was assayed in a shaking water bath in 2 mL of a medium containing either 120 mM KCl or 15 mM KCl. The medium also contained 10 mM Tris–maleate, 2 mM $MgCl_2$, and varying concentrations of $CaCl_2$, pH 7.0. The reaction medium was prepared with 0.5 mL of a 1 mg/mL suspension of myosin. To this was added 0.5 mL of actin at 0.33 mg/mL. When TMTN was present, 0.5 mL of actin at 0.33 mg/mL combined with TMTN was added. Calcium buffered with EGTA was added to the desired concentration, and the reaction medium was brought to 2 mL with the myosin dialysate. After equilibration to a temperature of 25 °C, the reaction was initiated by the addition of 80 μL of 50 mM Sigma grade ATP, pH 6.9. ATPase activity was terminated 5 min later by the addition of 0.2 mL of the reaction medium to 1 mL of 10% trichloroacetic acid and 2% ascorbic acid. Inorganic phosphate concentration was determined by the method of Baginski et al. (1967).

Standards which were run with each assay included buffer with added ATP at the beginning and the end of the assay, protein standards in buffer with no ATP, buffer with no ATP or protein added, and inorganic phosphate standards with concentrations ranging from 4 to 400 nM P_i/mL. Additionally, each of the above standards was run with the addition of varying calcium and magnesium concentrations to determine if these ions had any effect on ATP hydrolysis and/or inorganic phosphate determination. The same buffer against which the myosin had last dialyzed was used to make up all standards and to dilute all protein samples.

Inorganic Phosphate Determination. The amount of inorganic phosphate (P_i) liberated was calculated as nM P_i (mg of myosin)$^{-1}$ min^{-1} using a computer program which compensated for Cl_3CCOOH hydrolysis of ATP (based on a calculated linear regression), subtracted absorption due to protein, and subtracted background P_i contained in the buffer.

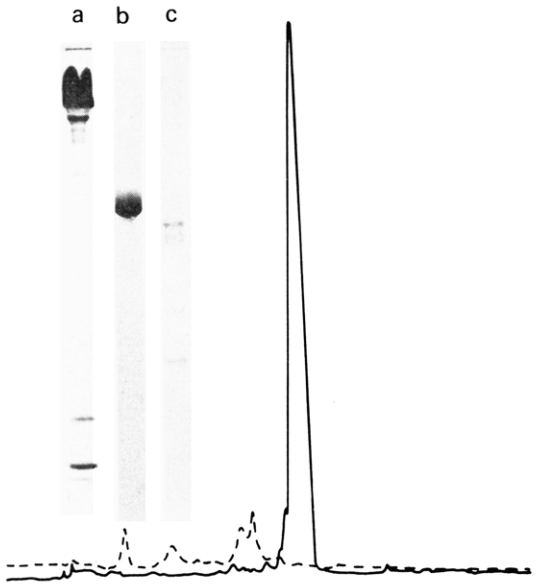

FIGURE 1: Representative gels of the proteins used, with scans of thin filament proteins. Myosin (a) was run on 10% $NaDodSO_4$–polyacrylamide gels. Myosin (40 μg) had bands corresponding to heavy chains, C protein, and light chains. G-Actin [(b) 30 μg] was run on 12.5% $NaDodSO_4$–polyacrylamide gels and gave a single band. TMTN [(c) 5 μg] was run on 12.5% $NaDodSO_4$–polyacrylamide gels and gave bands corresponding to tropomyosin and troponin subunits. Spectrophotometric scans were done of actin (30 μg; solid line) and TMTN (1 μg; dashed line).

The value thus obtained was used to calculate the amount of inorganic phosphate which was liberated by hydrolysis of ATP based on the P_i standards which were run in each assay.

Electrophoresis Techniques. All proteins were tested for purity by electrophoresis on 1.5 mM $NaDodSO_4$–polyacrylamide slab gels according to the procedure of Laemmli (1970). Proteins were electrophoresed at 30 mA/gel through a 5% stacking gel and then a 10.0% or 12.5% separating gel. The staining procedures were those of Fairbanks et al. (1971). The last optional staining step which intensifies staining of minor components was consistently utilized.

Urea slab gel procedures were those of Perrie et al. (1973). Urea (6 M) gels were 12% polyacrylamide and did not utilize a stacking gel. Gels were preelectrophoresed for 45 min at 10 mA/gel and after loading at 10 mA/gel for 3 h.

The purity of all protein preparations and determination of percentage of LC_2 phosphorylation were assessed by spectrophotometry. Stained gels were scanned at 579 nm on an ISCO UA5 absorbance monitor with an ISCO 1310 gel scanner attachment linked to a Kipp & Zonen BD12 chart recorder.

Determination of Free Calcium Concentration (pCa_f'). Free calcium concentrations were calculated by using the apparent stability constants of Fabiato & Fabiato (1978). Values obtained agreed closely (difference $<10^{-9}$) with those obtained when a different method was used (Fabiato & Fabiato, 1979).

Results

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis of myosin, actin, and TMTN (Figure 1), respectively, demonstrated that these proteins were pure. Myosin (Figure 1a) gels exhibited bands corresponding to the heavy chains of myosin, C protein, and three light chains. Actin (Figure 1b) gels had only one band corresponding to G actin, and TMTN (Figure 1c) gels contained bands corresponding to tropomyosin and troponin subunits. We were able to identify bands when as little as 1 μg of TMTN was added to gels. All myosin and actin preparations used in the following experiments have identical patterns on gels. When actin gels were scanned, the pattern was as indicated in Figure 1 (solid line). Without changing the sensitivity of the spectrophotometer, a scan of the gel containing 1 μg of TMTN gave the pattern indicated in Figure 1 (dashed line). It can be seen that the peaks corresponding to TMTN do not appear on the scan of the actin-containing gel. These results allowed us to be confident that no contaminating thin filament regulatory proteins were present in preparations used for actomyosin ATPase assays.

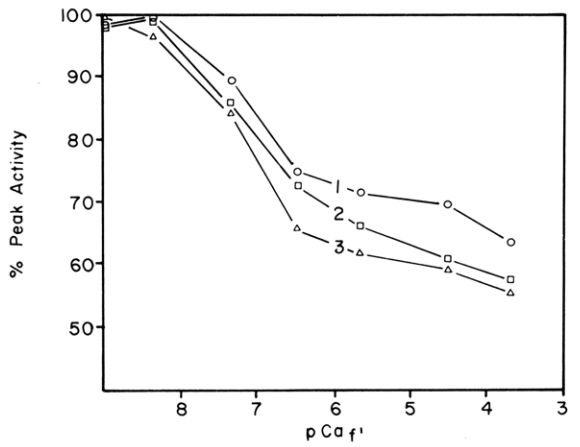

FIGURE 2: Percent peak actomyosin ATPase activities (Table I) of three different, representative, myosin preparations, assayed at 15 mM KCl, are plotted vs. pCa_f'.

Myosin ATPase Activity. The calcium-dependent ATPase activity of purified myosin was determined in the assay medium described previously with either 15 mM KCl or 120 mM KCl. Myosin ATPase activity was determined at a myosin concentration of 0.25 mg/mL. With magnesium present at 2 mM, myosin ATPase activity was suppressed at all calcium concentrations which were tested and never exceeded 7 nM P_i (mg of myosin)$^{-1}$ min^{-1} (0.03 P_i s^{-1} site^{-1}).

Actomyosin ATPase Activity. The magnesium-dependent actomyosin ATPase activity was determined for a reconstituted system containing rabbit myosin and rabbit actin. Fully reconstituted and polymerized actin was added to the assay medium to a final concentration of 0.084 mg/mL. Freshly prepared myosin was added to a concentration of 0.25 mg/mL. This gave an actin to myosin molecular ratio in the assay medium of approximately 4:1. Conditions for the assay are described under Materials and Methods and were identical in all cases except for the KCl concentration used. We observed essentially identical results with myosins prepared either according to Perry (1955) and Offer et al. (1972) or according to Weber (1956).

Actomyosin ATPase Activity at 15 mM KCl. The results obtained when the assay medium contained 15 mM KCl were significantly different from those obtained by using the same system with the KCl concentration raised to 120 mM. The percent peak ATPase activity is plotted vs. pCa_f' in Figure 2 for actomyosin assayed at a KCl concentration of 15 mM. The peak activities and calcium sensitivities of several different myosin preparations are listed in Table I. When the actomyosin ATPase activities of all preparations were assayed in 15 mM KCl, calcium was found to be inhibitory with increasing free calcium concentration.

The peak ATPase activity of this system occurred at either no added calcium or at a pCa_f' of 8.4. The lowest activity was always seen at 1 mM added calcium. The three curves (1–3) in Figure 2 represent three different myosin preparations which

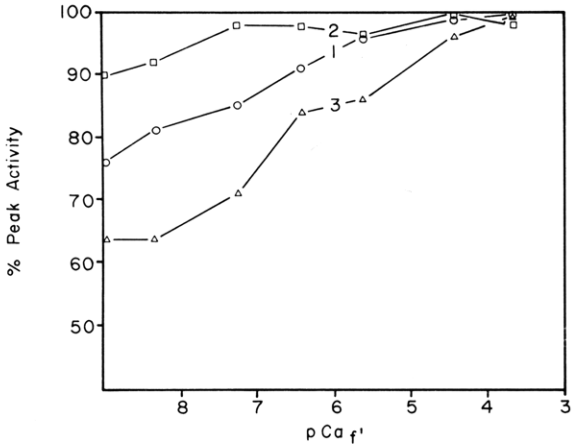

FIGURE 3: Percent peak actomyosin ATPase activities (Table I), of the same three representative myosin and actin preparations as illustrated in Figure 2, assayed at 120 mM KCl, are plotted vs. pCa_f'.

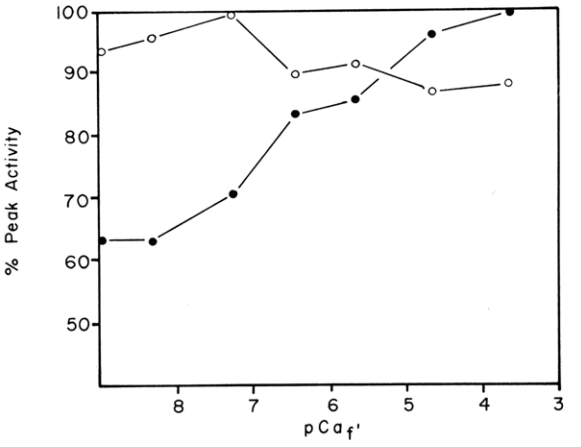

FIGURE 4: Percent peak actomyosin ATPase activities of a representative myosin preparation (Table II) assayed at 120 mM KCl before (●) and after (○) storage at 4 °C for 48 h are plotted vs. pCa_f'.

are representative of the ranges of calcium sensitivities we observed in the 12 preparations tested. Additionally, curve 1 represents a different actin preparation than curves 2 and 3, which were assayed with the same actin.

Actomyosin ATPase Activity at 120 mM KCl. When the same proteins were run in the identical manner as above, except for KCl concentration, the response of the system to added calcium was reversed. In Figure 3, percent of peak activity is plotted vs. pCa_f'. Peak activity and calcium sensitivity of this preparation are listed in Table I. Curve 1 of Figure 3 represents the results obtained by using the same actin and myosin preparations at the same concentrations as curve 1 of Figure 2. The same is true of curves 2 and 3. In all three cases peak actomyosin ATPase activity occurred when the pCa_f' was 4.42 or greater. The total amount of calcium sensitivity we observed varied depending on the myosin preparation and ranged between 10% and 52% under optimal conditions (Table I).

It is possible, although highly unlikely, that TMTN was a contaminant of the actin we prepared. According to our gel patterns, described previously, TMTN could only amount to 3% (1 part in 30) of each actin preparation without being detected on gels. If we were to assume that TMTN were a contaminant of actin in a ratio of 30:1, however, then 84 μg/mL of actin would also contain 2.8 μg/mL of contaminating TMTN. It seems unlikely that 2.8 μg/mL of contaminating TMTN would produce the 37% calcium sensitivity observed (in the absence of added TMTN) when the same actin assayed with myosin at 15 mM KCl gave no calcium sensitivity (Figure 2, curve 3).

The calcium sensitivity of the actomyosin ATPase activity also appeared to be inversely related to time. This was demonstrated when an aliquot of the myosin preparation that exhibited 37% calcium sensitivity when fresh was stored at 4 °C for 48 h and then was reassayed for actomyosin ATPase activity by using fresh actin, under identical conditions. Figure 4 shows the ATPase activity of this myosin both before and after storage. The peak activity and calcium sensitivity of this preparation are listed in Table I. After storage, this myosin demonstrated only 6% calcium sensitivity.

A series of experiments was performed to compare the phosphorylated state of LC_2 with the time-dependent decrease in calcium sensitivity described above. Electrophoresis of myosin on urea gels (Figure 5) with subsequent spectrophotometric scanning provided information regarding the percent of LC_2 which was present in both phosphorylated and unphosphorylated states. These data are presented in Table II.

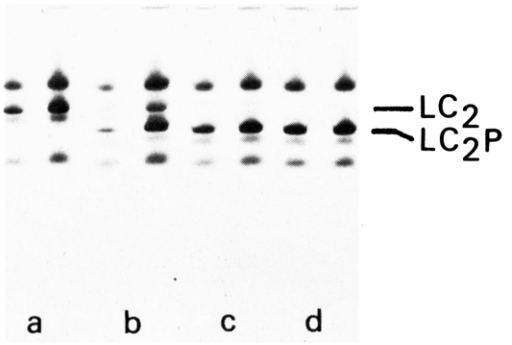

FIGURE 5: In vitro phosphorylation of LC_2 of a representative myosin preparation. Myosin electrophoresed on 6 M urea–13% polyacrylamide gels shows good separation between the phosphorylated (LC_2P) and unphosphorylated (LC_2) populations of LC_2. The left-hand member of each pair of wells (a–d) was loaded with 10 μg of rabbit myosin and the right-hand member of each pair of wells with 15 μg of rabbit myosin. Myosins run in well pairs a, b, c, and d were incubated for 0, 10, 30, and 60 min, respectively, with rabbit myosin light chain kinase, as described under Materials and Methods.

The actomyosin ATPase activity of this myosin at 120 mM KCl was determined both with and without calcium at time intervals from 0.5 to 28 days after myosin extraction. Purified myosin was stored at 4 °C in 120 mM KCl assay buffer (Materials and Methods) with 0.2 mM DTT. Actin was freshly reconstituted from the same lyophilized actin prior to each assay. Aliquots of myosin were taken prior to ATPase assay and, in some cases, immediately after assay, for urea gel electrophoretic determination of LC_2 phosphate content. There was no significant difference ($p < 0.001$) between the degree of phosphorylation of LC_2 before or after assay. Thus, LC_2 did not undergo phosphorylation during incubation with Ca^{2+} and ATP for the 5 min of assay. The percent phosphorylation of LC_2 is listed in Table II. Additionally one aliquot of myosin (Table II, f) was partially (21%) rephosphorylated by incubation with myosin light chain kinase.

Examination of Table II, a–e, indicates that a direct correlation exists between calcium sensitivity and percent phosphorylation of LC_2. With increasing storage time there is a progressive loss of phosphate from LC_2 and a concomitant decrease in the observed calcium sensitivity. Indeed, the correlation coefficient (0.96) for the five data pairs obtained was highly significant ($p < 0.01$). This could implicate phosphorylation of LC_2 as a necessary condition for expression of myosin calcium sensitivity. Restoration of lost calcium

Table I: Actomyosin ATPase Activity and Calcium Sensitivity[a]

A. Activities of Three Representative Myosin Preparations[b]

proteins	[KCl] (mM)	peak ATPase act.[c]	Ca^{2+} sensitivity (%)	% SD (n = 24)	ATPase act. at zero Ca^{2+}
(1) myosin 8, actin 7 (curve 1)	15	271.3 ± 15.3	−36	<7	172.1 ± 4.4
	120	81.6 ± 3.7	27	<5	62.4 ± 1.3
(2) myosin 10, actin 1 (curve 2)	15	315.0 ± 13.5	−46	<5	181.6 ± 8.5
	120	87.5 ± 2.5	10	<4	78.4 ± 3.1
(3) myosin 11, actin 1 (curve 3)	15	226.7 ± 14.9	−47	<7	122.4 ± 2.4
	120	66.7 ± 3.3	37	<10	42.1 ± 4.3
(4) 48-h-old myosin 11, actin 1	120	102.0 ± 3.8	6	<8	96.3 ± 6.6

B. Activities of Two Different Myosin Preparations[d]

proteins	[KCl] (mM)	peak ATPase act.[c]	Ca^{2+} sensitivity (%)	% SD (n = 24)	ATPase act. at zero Ca^{2+}
(1) myosin 14, actin 1, TNTM 6	50	230.0 ± 13.5	90	<6	32.8 ± 1.6
(2) myosin 15, actin 3, TNTM 6	120	83.2 ± 2.7	99	<8	0.1 ± 0.6

C. Various Degrees of Calcium Sensitivity[e]

proteins	Ca^{2+} sensitivity (%) at 120 mM KCl	other conditions
(1) myosin 7, actin 1	(a) 52	
	(b) 0	stored for 48 h at −20 °C
(2) myosin 8, actin 7	(a) 27	
	(b) 23	aged 5 min
	(c) 22	aged 30 min
(3) myosin 12, actin 1	(a) 17	
	(b) 0	stored for 20 h at −20 °C
(4) myosin 13, actin 3	(a) 33	
	(b) 19	stored for 48 h at −20 °C
(5) myosin 14, actin 3	(a) 25	
(6) myosin 15, actin 3	(a) 32	
(7) myosin 16,[f] actin 3	(a) 90[g]	
	(b) 33[h]	stored for 7 days at −20 °C

[a] Calcium sensitivity was calculated by the equation Ca^{2+} sensitivity = 100 [1 − (ATPase activity at zero added Ca^{2+})/(peak ATPase activity)]. A negative calcium sensitivity indicates that the addition of calcium had an inhibitory effect on ATPase activity. The standard deviations for each assay were less than the percentage indicated in the last column; e.g., if the SD <10%, a value of 50 nM P_i (mg of myosin)$^{-1}$ min^{-1} would have a SD <5 nM P_i (mg of myosin)$^{-1}$ min^{-1}. [b] The peak ATPase activities of the three representative myosin preparations, chosen for the range of calcium sensitivities they exhibited, in actomyosin ATPase assay at both low and physiological ionic strengths, with and without added calcium. The effect of storage (aging) on the calcium sensitivity at physiological ionic strength of one of these preparations is shown in (4). [c] nM P_i (mg of myosin)$^{-1}$ min^{-1}. [d] The peak ATPase activities of two different myosin preparations, in the presence of troponin and tropomyosin, assayed for actomyosin ATPase at moderately low (50 mM KCl) and physiological ionic strengths. [e] Various degrees of calcium sensitivity exhibited by several different myosin preparations, other than those graphed in Figures 2 and 3 (except for indicating the effect of storage); conditions are indicated in the right-handed column. [f] Myosin preparation according to Offer et al. (1972). [g] Very low activity. [h] Activity was increased over (a).

sensitivity by rephosphorylation of LC_2 would have added considerable strength to this hypothesis. To investigate this, myosin which was 0% phosphorylated was rephosphorylated to 21% (Table II, f). This rephosphorylated myosin, however, did not exhibit calcium sensitivity. The possibility remains, however, that additional, irreversible light chain degradation also occurs with aging and that this abolishes myosin calcium sensitivity.

Table II: Age Dependence of Myosin Calcium Sensitivity[a]

age[a]	ATPase act. with Ca^{2+}	ATPase act. with EGTA	Ca^{2+} sensitivity (%)	LC_2 phosphorylation (%)
a	50.2 ± 3.4	31.1 ± 4.5	38	15
b	74.6 ± 2.4	53.8 ± 3.4	28	13
c	113.8 ± 2.3	97.9 ± 3.4	14	10
d	129.5 ± 1.8	127.0 ± 2.5	2	0
e	175.0 ± 9.5	177.9 ± 0.8	−2	0
f	144.1 ± 7.4	133.6 ± 2.5	8	21

[a] Effect of aging and LC_2 phosphorylation on myosin calcium sensitivity. The actomyosin ATPase activity [nM P_i (mg of myosin)$^{-1}$ min^{-1}] ± standard deviation both with Ca^{2+} and without Ca^{2+} (with EGTA) is listed for one representative myosin preparation. ATPase activity was assayed when myosin was fresh (a), 48 h old (b), 7 days old (c), 14 days old (d), 28 days old (e), and 28 days old after incubation with myosin light chain kinase (f). For each assay actin was freshly reconstituted from the same lyophilized powder. Calcium sensitivity was calculated as described in footnote *a* of Table I. Determination of percent of LC_2 phosphorylation is described under Materials and Methods.

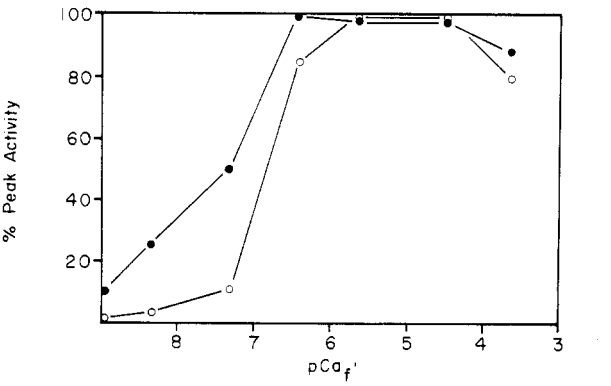

FIGURE 6: Effect of thin filament regulatory proteins on AM ATPase activity at low and physiological ionic strengths. The percent peak actomyosin ATPase activity (Table I) in the presence of TMTN is plotted vs. pCa_f'. Activity was assayed at 50 mM KCl (●) and 120 mM KCl (○).

A correlation (−0.92) also exists between the peak ATPase activity observed and the percent phosphorylation of LC_2. As the phosphate content of LC_2 decreases, the AM ATPase activity increases. As noted above, however, this relationship holds true only as long as the rephosphorylated myosin is not considered. Rephosphorylation did decrease activity (Table II, f) below that of the same myosin which was unphosphorylated (Table II, e). However, this level was still greater than that of phosphorylated myosin (Table II, d). This result may again reflect light chain degradation with aging.

Actomyosin ATPase Activity in the Presence of TMTN. TMTN-regulated actomyosin ATPase activity was assayed at two different KCl concentrations, 50 mM KCl and 120 mM KCl. All other conditions of assay were identical (Materials and Methods). Actin was premixed with TMTN complex prior to its addition to the assay medium. The calcium sensitivity of TMTN-regulated actomyosin was ≥90% at 50 mM KCl and 99% at 120 mM KCl (Figure 6 and Table I).

Discussion

Until the experiments reported here were performed, all of our ATPase assays using reconstituted systems containing

either vertebrate or invertebrate (*Limulus*) striated muscle myosin were run routinely at the ionic strengths commonly used for such assays (15–50 mM KCl) and yielded results in agreement with those in the literature: vertebrate myosin was not calcium sensitive, while *Limulus* myosin was. However, when we simultaneously assayed the effect of calcium on the ATPase activity of reconstituted vertebrate actomyosin at both 120 mM and 15 mM KCl with the identical purified proteins, we observed myosin-linked calcium sensitivity at the physiological but not at the lower ionic strength. Indeed, addition of calcium was actually inhibitory in the latter case. This inhibition of actomyosin ATPase activity by calcium when myosin was assayed at 15 mM KCl cannot be explained at this time. Such an effect of calcium, however, has also been observed by others (Bremel, 1972; Bremel & Weber, 1971). In the presence of TMTN, however, our system exhibited calcium sensitivities of 90% or better at both 120 mM KCl and 15 mM KCl, over the range of calcium concentrations tested. We have not yet collected enough data to establish whether the calcium sensitivity of our actomyosin at 120 mM KCl is dependent on a change in V_{max} or K_m. Preliminary results on myosin assayed at 120 mM KCl at two different actin concentrations, in the presence of EGTA or Ca^{2+}, suggest that V_{max} is affected. Additional work on this question is in progress.

That the ionic strength (KCl concentration) of the assay medium alone is responsible for the expression of vertebrate striated muscle myosin calcium sensitivity in our hands is indicated by our adherence to identical conditions of assay in all cases (save for KCl concentration), our use of the identical proteins for assay at both ionic strengths, and our observation (V. Sawyna et al., unpublished result) that a reconstituted system containing purified *Limulus* myosin and pure rabbit actin demonstrates an increase in calcium sensitivity at 120 mM KCl over that seen at 15 mM KCl.

That myosin is implicated as the protein responsible for the calcium sensitivity we observed at 120 mM KCl is apparent, since each preparation of actin, always used within 12 h of reconstitution and polymerization, was assayed with more than one myosin preparation. In one case, by use of the same actin, one myosin preparation demonstrated as little as 10%, while a different myosin preparation showed as much as 37%, calcium sensitivity. Furthermore, when myosin was stored, calcium sensitivity was significantly decreased. This shows not only that actin is not responsible for this behavior but also that aging appears to abolish the calcium sensitivity of vertebrate striated muscle myosin.

The fact that an increase in age of the myosin decreased the calcium sensitivity at first suggested to us that oxidation of myosin thiol groups might have occurred with time. Such a loss in calcium sensitivity in reconstituted preparations was reported by both Daniel & Hartshorne (1972) and Goodno et al. (1978) when the thiol groups were in an oxidized state. However, TMTN was present in the systems examined by these investigators, while the loss of calcium sensitivity that we observed occurred in the absence of these regulatory proteins. We also found that myosin preparations which demonstrated a decrease in calcium sensitivity with aging showed a concomitant increase in ATPase activity. This finding is inconsistent with the expected effects resulting from the oxidation of thiol groups (Pinset-Harstrom & Whalen, 1979; Srivastava & Wikman-Coffelt, 1980). Because of these data, and the fact that we always stored myosin in the presence of 0.2 mM DTT, we believe that we can rule out the oxidation of myosin thiol groups as a cause of the loss of calcium sensitivity in our aged myosin preparations.

The loss of myosin calcium sensitivity with aging together with the increase in actomyosin ATPase activity that we observed, however, does fit well with several independent observations of others and serves to implicate LC_2 as the moiety most likely responsible for these effects. First, the breakdown of this light chain with aging (Pinset-Harstrom & Whalen, 1979) supports our observation on the loss of calcium sensitivity under similar conditions. Second, the ability of LC_2 to restore calcium sensitivity to desensitized scallop myosin (Kendrick-Jones, 1974; Kendrick-Jones et al., 1976) might possibly be explained as a real property of this myosin subunit as well as a cooperative effect of the two light chains. Finally, it has been reported that loss of LC_2 does not lower actomyosin ATPase activity (Weeds, 1969), which agrees with the increase in ATPase activity we observed under conditions such as aging, which Pinset-Harstrom & Whalen (1979) have found responsible for LC_2 breakdown.

An attractive hypothesis to explain myosin calcium sensitivity is that the expression of this property may be related to the degree to which LC_2 is phosphorylated. While phosphorylation of LC_2 per se has not been found to affect regulation of striated muscle as it does in smooth muscle (Chacko et al., 1977; Kerrick et al., 1980), some investigations (Stull et al., 1979; Barany et al., 1980; Pemrick, 1980; Cooke et al., 1981, 1982) suggest that phosphorylation of skeletal muscle LC_2 may modulate actin–myosin interaction.

Our data on phosphorylation could be interpreted as evidence that the phosphorylated state of LC_2 influences both the AM ATPase activity and the calcium sensitivity. In our hands, phosphorylation of LC_2 affected AM ATPase activity in a negative manner, in agreement with the findings of Cooke et al. (1981, 1982) but at variance with those of Pemrick (1980). Indeed, Pemrick observed as much as 181% increase in ATPase activity following phosphorylation of LC_2.

The means by which LC_2 phosphorylation may affect calcium sensitivity is not readily apparent. Our data suggest that the regulatory effect of phosphorylation may not be directly mediated by a calcium-dependent kinase system, since we found that the percent phosphorylation of LC_2 was the same in both preassay and postassay and in both the presence and absence of calcium. It may be that phosphorylation affects the calcium binding properties of LC_2. Studies on rabbit S-1, myosin, and glycerinated fibers by Bagshaw & Reed (1977) and Bagshaw & Kendrick-Jones (1979) suggested that the calcium binding affinity of LC_2 is too low for this peptide to function as a regulatory switch under the conditions of their experiments. It has been shown, however, that the affinity of LC_2 for calcium is affected by environmental conditions such as the presence of myosin heavy chains, ionic strength, and pH (Wikman-Coffelt, 1980; Wikman-Coffelt et al., 1979; Mrakovcic et al., 1979).

Thus, several parameters may govern the expression of myosin's calcium sensitivity. This property may be partly dependent upon the nature of the "ionic sphere" surrounding the myosin aggregates. This environmental factor clearly is modified by increasing the ionic strength of the medium to near-physiological levels. Perhaps the addition of the TMTN complex, which possesses charged groups, to the system is sufficient to modify the ionic environment surrounding the myosin in a similar fashion, even at a low, less physiological, ionic strength. Further support for the idea that the effect of calcium on myosin aggregates may be related to the ionic strength of the environment is provided by Morimoto & Harrington's (1974) observation of a calcium-induced decrease

in the viscosity and increase in the sedimentation coefficient of a suspension of native thick filaments in a medium of 0.12 ionic strength. It would be interesting to know if these changes would also occur at a lower ionic strength. Although we did not make measurements, it was subjectively apparent to us that the turbidity of our purified myosin suspension at 120 mM KCl was less than that at 15 mM KCl, at the same protein concentration.

In the light of our results, as well as those of others, we conclude that both the degree of phosphorylation and the ionic strength of the environment may affect the calcium binding characteristics of LC_2. The fact that rephosphorylation of our aged myosin failed to restore the lost calcium sensitivity does not rule out this interpretation. Since Pinset-Harstrom & Whalen (1979) have demonstrated that LC_2 undergoes a time-dependent proteolysis, it is possible that LC_2 may be present in our aged preparations in a damaged state, which, although capable of being phosphorylated, is incapable of affecting the myosin calcium sensitivity.

The relationship of myosin calcium sensitivity, such as we described, to the activity of the muscle itself is not known. Srivastava et al. (1980) have shown that tension development by actomyosin threads was identical whether the myosin present in their reconstituted system was intact or LC_2 deficient. These studies, however, were performed at 50 mM KCl, an ionic strength at which myosin calcium sensitivity may not be expressed. In fact, it is clear that the behavior of myosin in actomyosin ATPase assays carried out under more nearly physiological conditions such as intact molecules in an aggregated state and at a more physiological ionic strength differs both from that exhibited by its more soluble subfragments (HMM and S-1) and from that observed under assay conditions designed to optimize enzymatic activity (low ionic strength). We would argue that in order to understand the fine features of regulation of actomyosin ATPase activity as it occurs in vivo, it would be advantageous to study these phenomena under conditions which resemble the physiological situation.

Acknowledgments

We thank Kathy McGlynn for technical assistance and glassware handling and Dolores Wells and Cheronne Holt for secretarial help.

Registry No. ATPase, 9000-83-3; calcium, 7440-70-2.

References

Baginski, E. S., Foa, P. O., & Zak, B. (1967) *Clin. Chim. Acta 15*, 155–158.

Bagshaw, C. R., & Reed, G. H. (1971) *FEBS Lett. 31*, 386–390.

Bagshaw, C. R., & Kendrick-Jones, J. (1979) *J. Mol. Biol. 130*, 317–336.

Barany, K., Barany, M., Gillis, J. M., & Kushmerick, M. J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1547–1551.

Bremel, R. D. (1972) Ph.D. Thesis, St. Louis University, St. Louis, MO.

Bremel, R. D., & Weber, A. (1971) *Biophys. J. 11*, 237a.

Bremel, R. D., Murray, J. M., & Weber, A. (1973) *Cold Spring Harbor Symp. Quant. Biol. 37*, 267–275.

Chacko, S., Conti, A. M., & Adelstein, R. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 129–133.

Chantler, P. D., & Szent-Gyorgyi, A. G. (1980) *J. Mol. Biol. 138*, 473–492.

Chantler, P. D., Sellers, J. R., & Szent-Gyorgi, A. G. (1981) *Biochemistry 20*, 210–216.

Chin, T. F., & Rowe, A. J. (1982) *J. Muscle Res. Cell Motil. 3*, 118.

Cooke, R., Franks, V., Ritz-Gold, C. J., Toste, T., Blumenthal, D. K., & Stull, J. T. (1981) *Biophys. J. 33*, 235a.

Cooke, R., Franks, K., & Stull, J. T. (1982) *FEBS Lett. 144*, 33–37.

Daniel, J. L., & Hartshorne, D. J. (1972) *Biochim. Biophys. Acta 278*, 567–576.

Ebashi, S., & Endo, M. (1968) *Prog. Biophys. Mol. Biol. 18*, 123–183.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Fabiato, A., & Fabiato, F. (1978) *J. Gen. Physiol. 72*, 667–699.

Fabiato, A., & Fabiato, F. (1979) *J. Physiol. (Paris) 75*, 463–505.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Goodno, C. C., Wall, C. M., & Perry, S. V. (1978) *Biochem. J. 175*, 813–821.

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

Hartshorne, D. J., & Mueller, H. (1969) *Biochim. Biophys. Acta 175*, 301–319.

Hitchcock, S. E., Huxley, H. E., & Szent-Gyorgyi, A. G. (1973) *J. Mol. Biol. 80*, 825–836.

Hoar, P. E., Kerrick, W. G. L., & Cassidy, P. S. (1979) *Science (Washington, D.C.) 204*, 503–506.

Kendrick-Jones, J. (1974) *Nature (London) 249*, 631–634.

Kendrick-Jones, J., Szentkiralyi, E. M., & Szent-Gyorgyi, A. G. (1973) *Cold Spring Harbor Symp. Quant. Biol. 37*, 47–53.

Kendrick-Jones, J., Szentkiralyi, E. M., & Szent-Gyorgyi, A. G. (1976) *J. Mol. Biol. 104*, 747–775.

Kerrick, W. G. L., Hoar, P. E., & Cassidy, P. S. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1558–1563.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lehman, W. (1978) *Nature (London) 274*, 80–82.

Lehman, W., & Szent-Gyorgyi, A. G. (1975) *J. Gen. Physiol. 66*, 1–30.

Lehman, W., Kendrick-Jones, J., & Szent-Gyorgyi, A. G. (1973) *Cold Spring Harbor Symp. Quant. Biol. 37*, 319–330.

Morimoto, K., & Harrington, W. F. (1974) *J. Mol. Biol. 88*, 693–709.

Mrakovcic, A., Oda, S., & Reisler, E. (1979) *Biochemistry 18*, 5960–5965.

Mrwa, U., & Hartshorne, D. J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1564–1568.

Murray, J., & Weber, A. (1973) *Physiol. Rev. 53*, 612–673.

Offer, G., Moos, C., & Starr, R. (1972) *J. Mol. Biol. 74*, 653–676.

Pemrick, S. M. (1980) *J. Biol. Chem. 255*, 8836–8841.

Perrie, W. T., Smillie, L. B., & Perry, S. V. (1973) *Biochem. J. 135*, 151–164.

Perry, S. V. (1955) *Methods Enzymol. 2*, 582.

Pinset-Harstrom, I., & Whalen, R. G. (1979) *J. Mol. Biol. 134*, 189–197.

Pires, E., Perry, S. V., & Thomas, M. A. W. (1974) *FEBS Lett. 41*, 292–296.

Sellers, J. R. (1981) *J. Biol. Chem. 256*, 9274–9278.

Sellers, J. R., Chantler, P. D., & Szent-Gyorgyi, A. G. (1980) *J. Mol. Biol. 144*, 223–245.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Srivastava, S., & Wikman-Coffelt, J. (1980) *Biochem. Biophys. Res. Commun. 92*, 1383–1388.
Srivastava, S., Cooke, R., & Wikman-Coffelt, J. (1980) *Biochem. Biophys. Res. Commun. 92*, 1–7.
Stull, J. T., Manning, D. R., High, C. W., & Blumenthal, D. K. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1552–1557.
Szent-Gyorgyi, A. G., Szentkiralyi, E. M., & Kendrick-Jones, J. (1973) *J. Mol. Biol. 74*, 179–203.
Weber, A. (1956) *Biochim. Biophys. Acta 19*, 345–351.
Weeds, A. G. (1969) *Nature (London) 223*, 1362–1364.
Wikman-Coffelt, J. (1980) *Biochem. J. 185*, 265–268.
Wikman-Coffelt, J., Srivastava, S., & Mason, D. T. (1979) *Biochimie 61*, 1309–1314.

Evolutionary Aspects of Accuracy of Phenylalanyl-tRNA Synthetase. A Comparative Study with Enzymes from *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and Turkey Liver Using Phenylalanine Analogues†

Hans-Joachim Gabius, Friedrich von der Haar,‡ and Friedrich Cramer*

ABSTRACT: The phenylalanyl-tRNA synthetases from *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and turkey liver activate a number of phenylalanine analogues (tyrosine, leucine, methionine, *p*-fluorophenylalanine, β-phenylserine, β-thien-2-ylalanine, 2-amino-4-methylhex-4-enoic acid, mimosine, *N*-benzyl-L- or *N*-benzyl-D-phenylalanine, and ochratoxin A), as demonstrated by K_m and k_{cat} of the ATP/PP_i pyrophosphate exchange. Upon complexation with tRNA, the enzyme–$tRNA^{Phe}$ complexes show a significantly increased initial discrimination of these amino acid analogues expressed in higher K_m and lower k_{cat} values, as determined by aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2). The overall accuracy is further enhanced by a second discrimination, a proofreading step. The strategies employed by the enzymes with respect to accuracy differ. Better initial discrimination in the aminoacylation and less elaborated proofreading for the *E. coli* enzyme can be compared to a more efficient proofreading by other synthetases. In this way the comparatively poor initial amino acid recognition in the case of the *S. cerevisiae* and *N. crassa* enzymes is balanced. The extent of initial discrimination is therefore inversely coupled to the hydrolytic capacity of the proofreading. A striking difference can be noted for the proofreading mechanisms. Whereas the enzymes from *E. coli*, *S. cerevisiae*, and *N. crassa* follow the pathway of posttransfer proofreading, namely, enzymatic hydrolysis of the misaminoacylated tRNA, the turkey liver enzyme uses tRNA-dependent pretransfer proofreading in the case of natural amino acids. In spite of the same subunit structure and similar molecular weight, the phenylalanyl-tRNA synthetases from a prokaryotic and lower and higher eukaryotic organisms show obvious mechanistic differences in their strategy to achieve the necessary fidelity.

Aminoacyl-tRNA synthetases esterify an amino acid with its cognate tRNA with an error rate of smaller than 10^{-4} (Loftfield & Vanderjagt, 1972). In fact, a specificity of smaller than 10^{-5} is observed as a result of a proofreading step subsequent to initial binding and activation of the amino acid (Cramer et al., 1979; von der Haar et al., 1981). For the proofreading capacity of the aminoacyl-tRNA synthetases, different mechanistic interpretations are given in the literature. It was suggested by Hopfield that specificity is enhanced by kinetic proofreading via the preferential dissociation of the wrong aminoacyladenylate (Hopfield, 1974; Hopfield et al., 1976). This pathway has been questioned by von der Haar (1977), Fersht (1977b), and Igloi et al. (1978). von der Haar and Cramer have suggested chemical proofreading as a corrective step after transfer of the amino acid to the tRNA, which leads to emzymatic hydrolysis of the ester linkage between the tRNA and the wrong amino acid (von der Haar & Cramer, 1975, 1976). A similar scheme for the discrimination between cognate and noncognate substrates depending on the relative rates of synthesis and hydrolysis was developed by Fersht, who introduced the double-sieve model (Fersht, 1977a; Tsui & Fersht, 1981). Generalization of such mechanistic descriptions must be done with caution, since synthetases from specific organisms are necessarily used for these investigations. Discrepancies in the mechanistic interpretations are possible because it is not sufficiently well established that the proofreading mechanisms of specific synthetases from different organisms are identical. An indication of different behavior is also provided by the example of different extent of activation of some nonprotein amino acids that are produced only in certain plants [e.g., Lea & Norris (1977)]. It should also be noted that there are differences in the catalytic mechanisms of other enzymes with respect to evolution, for example, the fatty acid synthetase (Lynen, 1980).

In this paper, the interaction of 11 phenylalanine analogues with the phenylalanyl-tRNA synthetase from *Escherichia coli*, *Saccharomyces cerevisiae* (yeast), *Neurospora crassa*, and turkey liver was studied by analysis of ATP/PP_i pyrophosphate exchange, aminoacylation of yeast $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A(3′NH_2), and AMP production during aminoacylation of $tRNA^{Phe}$-C-C-N. From the studies, a comprehensive picture emerges of how the phenylalanyl-tRNA

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany. *Received November 9, 1982.*
‡ Present address: Braun-Melsungen A.G., D-3508 Melsungen, Federal Republic of Germany.

0006-2960/83/0422-2331$01.50/0