Activation of Skeletal Muscle Phosphorylase Kinase by Ca^{2+}. II. Identification of the Kinase Activating Factor as a Proteolytic Enzyme*

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ABSTRACT: The phosphorylase kinase activating factor (KAF) which is required for the irreversible activation of phosphorylase kinase by Ca^{2+} was purified 3000-fold from rabbit skeletal muscle and shown to possess proteolytic activity. Evidence was obtained that limited proteolysis constitutes the mechanism by which the factor brings about activation of the kinase. This consisted of showing that in the presence of Ca^{2+} the factor caused simultaneous enzyme activation and release of peptide material and that new NH_2-terminal amino acids arose during activation of the kinase by the factor. Furthermore, the extent of purification of KAF was found to be identical whether fractions were followed by peptide-releasing or kinase-activating activity measurements. The proteolytic nature of phosphorylase kinase activation by Ca^{2+} and KAF makes it unlikely that this mechanism constitutes a physiologically significant regulatory device.

Rabbit skeletal muscle phosphorylase kinase is activated by preincubation with ATP and Mg^{2+} (De Lange *et al.*, 1968), Ca^{2+} (Meyer *et al.*, 1964), and trypsin (Krebs *et al.*, 1964). In each instance, activation is characterized by a marked increase in the activity of the enzyme at pH 6.8 and a moderate increase in activity at pH 8.2. These changes have been shown to be due to an increased affinity of the activated forms for the substrate, phosphorylase *b* (Krebs *et al.*, 1964). Activation of the kinase by ATP and Mg^{2+} is accelerated by adenosine 3′, 5′-monophosphate and is accompanied by phosphorylation of seryl residues in the enzyme. Prior to the present study, however, the mechanism of phosphorylase kinase activation by Ca^{2+} had not been elucidated. It was known (Meyer *et al.*, 1964) that this type of action required the presence of a second protein, the kinase activating factor, or KAF,[1] but the question of whether KAF was an enzyme or functioned in some other manner remained open. In addition to information about this system in skeletal muscle, it was also known that phosphorylase kinase could be activated by preincubation by Ca^{2+} in the heart and that this effect was again mediated by a kinase activating factor (Hammermeister *et al.*, 1965; Drummond and Duncan, 1966).

Since phosphorylase kinase is activated by trypsin, the possibility was considered that KAF might be a calcium-requiring proteolytic enzyme, but attempts to demonstrate its proteolytic activity were unsuccessful (Meyer *et al.*, 1964). In the present study this question has been reopened and evidence has been obtained that the factor is in fact a proteolytic enzyme. The early attempts to show this were negative due to the great lability of KAF in the presence of Ca^{2+}. A method for purifying KAF to a point of near homogeneity is described.

Materials and Methods

Phosphorylase. Phosphorylase *b* was prepared as reported previously (Fischer and Krebs, 1958) using the modifications described by De Lange *et al.* (1968). Phosphorylase *a* and [^{32}P]phosphorylase *a* were made as described earlier (Fischer *et al.*, 1959).

Phosphorylase kinase was prepared in the nonactivated form by the method of Krebs *et al.* (1964) as improved and extended by De Lange *et al.* (1968). The final fraction, *i.e.*, the Sephadex G-200 fraction, was essentially free of KAF and showed only very slight activation when preincubated with Ca^{2+}. ^{32}P-labeled phosphorylase kinase was of the "maximally phosphorylated" type prepared by the method of Riley *et al.* (1968). The material was freed of [γ-^{32}P]ATP by precipitation at 1.2 M ammonium sulfate and passage through a Sephadex G-25 column equilibrated with 0.05 M glycerophosphate–0.002 M EDTA buffer (pH 7.0).

Heart muscle inhibitor of KAF was prepared from frozen rabbit hearts according to the method of Drummond and Duncan (1966) stopping after the ammonium sulfate step.

Other Materials. [γ-^{32}P]ATP was prepared by a method combining the procedures of Tanaka *et al.* (1959) and Jones (1962) as described by De Lange *et al.*

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: KAF, phosphorylase kinase activating factor; DNS, the 1-dimethylaminonaphthalene-5-sulfonyl group; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetate

(1968). Alumina Cγ was purchased from the Sigma Chemical Co.

Phosphorylase activity was measured in sodium maleate buffer at pH 6.5, using a glucose 1-phosphate concentration of 0.075 M, as described by Hedrick and Fischer (1965). One unit of phosphorylase *a* activity is defined as that quantity of enzyme which produces 1 μmole of inorganic phosphate/min in the absence of AMP, and a unit of phosphorylase *b* as that amount of enzyme giving an identical reaction rate in the presence of AMP.

Phosphorylase kinase activity was measured by a modification of the method previously described (Krebs *et al.*, 1964). The kinase reaction mixture and conditions were identical, except that (1) the phosphorylase *b* concentration was 8.7 mg of crystalline phosphorylase *b*/ml rather than being defined in terms of phosphorylase units and (2) the reaction was started by addition of phosphorylase kinase rather than ATP-Mg^{2+} solution. This last modification led to somewhat lower activities, but was more convenient, especially where the kinase assay was used as part of the KAF assay. A kinase unit was defined as that amount of enzyme converting 1 unit of phosphorylase *b* into phosphorylase *a* in 1 min at a specified pH. The kinase unit defined in this manner and used throughout this study is approximately equal to 2 units as defined previously (Krebs *et al.*, 1964).

KAF Activity. KAF fractions to be assayed were diluted in 0.05 M Tris-Cl–0.001 M EDTA–0.045 M 2-mercaptoethanol buffer (pH 7.5) containing 0.5 mg/ml of bovine serum albumin, and activated by preincubation for 1 hr at 30° prior to the activity test. To 0.2 ml of the diluted KAF was added 0.2 ml of nonactivated phosphorylase kinase solution in the same buffer at a concentration of 40,000 units/ml as assayed at pH 8.2. The reaction was started with 0.2 ml of 0.09 M Tris-Cl–0.03 M calcium acetate (pH 7.5) incubated for 5 min at 30°, and then stopped by a 1:30 dilution in cold neutral 0.015 M cysteine and assayed for phosphorylase kinase activity at pH 6.2. A control reaction without added KAF was run with each assay. The KAF unit[2] was defined as that amount of KAF which causes activation of phosphorylase kinase at a rate of 400 kinase units/min as measured at pH 6.2. The proportionality and precision of the assay is illustrated in Figure 1. In practice, dilutions of KAF were adjusted so that between 0.1 and 0.5 unit were used in the assay.

Detection of NH_2-Terminal Amino Acids in Phosphorylase Kinase. NH_2-terminal amino acids were determined by the method of Gray (1967), with modifications. Phosphorylase kinase fractions (5 mg in 4 ml) were dialyzed against two 60-ml portions of 0.3 M $NaHCO_3$–8 M before the addition of DNS-Cl. The precipitated DNS-kinase was washed by centrifugation with 50% acetone, acetone, and ether, then hydrolyzed 16 hr at 110°.

To separate most of the α-DNS-amino acids from the large amounts of DNS-OH, ε-DNS-lysine, *O*-DNS-tyrosine, and nonterminal amino acids, the dried hydrolysate was dissolved in 0.1 ml of 0.1 M sodium citrate (pH 3.4) and extracted twice with 2-ml portions of butyl acetate. The combined butyl acetate extracts were back-extracted twice with 0.1-ml portions of the citrate buffer, then dried. Since DNS-arginine and α-DNS-histidine are not extracted by the above procedure, their absence was confirmed by electrophoresis at pH 2.1 of a nonextracted portion of the hydrolysate.

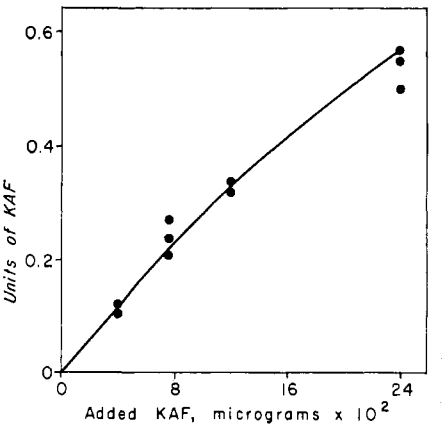

FIGURE 1: The proportionality of the KAF assay. Increasing amounts of partially purified KAF (the ammonium sulfate fraction) were assayed by the procedure described in the text. Triplicate analyses were performed at each dilution.

The extract of the hydrolysate was dissolved in acetone, then analyzed by two-dimensional thin-layer chromatography on silica gel G. The first solvent was $CHCl_3$–CH_3OH–HOAc (95:10:1), the second was *n*-$CH_3CH_2CH_2OH$–NH_4OH (80:20) (J. A. Black and G. H. Dixon, personal communication). Plates were dried 30 min at 110° between use of the two solvents.

Since the fluorescent spots tend to fade with time, all plates were photographed. Long-wave ultraviolet light was provided by two 15-W black light tubes (GE F15T8.BLB), and exciting light was filtered from the film by a Wratten K2 filter. Polaroid type 42 film (ASA 200) was exposed 4–5 sec, or Polaroid type 55 P/N (ASA 50) was exposed for 30–40 sec, both at *f*/5.6.

Standard DNS-amino acids were prepared as described by Boulton and Bush (1964), except for α-DNS-histidine, which was prepared by acid hydrolysis of di-DNS-histidine, and ε-DNS-lysine, which was prepared by treating α-carbobenzoxylysine with DNS-Cl, then hydrolyzing in acid.

The release of peptides from various proteins catalyzed by KAF was assayed as the total ninhydrin-positive material present in trichloroacetic acid supernatants after alkaline hydrolysis according to the method of Hirs *et al.* (1956) as adapted by Fruchter and Crestfield (1965). After precipitation of proteins with 5% trichloroacetic acid, the supernatants were filtered through coarse sintered-glass funnels and extracted three times with two volumes of ether prior to hydrolysis.

Other Methods. Disc electrophoresis was carried out at approximately pH 7.5 as described by the method of Ornstein and Davis (1963) as modified by Davis *et al.* (1967). Protein in crude KAF fractions and in phosphorylase kinase preparations was determined by the

[2] This unit of KAF activity represents approximately 1000 × as much factor activity as the unit defined by Meyer *et al.* (1964).

TABLE I: Purification of KAF from Rabbit Skeletal Muscle.

Fraction	Vol (ml)	Protein (mg × 10^{-1})	KAF (units × 10^{-3})	Sp Act. (units/mg)
	Part A[a]			
Extract	5,100	12,900	238	1.1
pH 6.15 supernatant	5,100	11,000	259	2.4
pH 5.0 precipitate	190	190	119	63
	Part B[b]			
pH 5.0 precipitate	1,680	1,800	1,070	59
TEAE eluate	335	67	307	460
Alumina Cγ eluate	46	11	92	840
Ammonium sulfate precipitate	4	2.5	37	1,480
Sephadex G-200 eluate	45	0.9	49	5,400

[a] Fractions from a single preparation in which 2580 g of muscle were used. [b] Fractions from a KAF preparation in which combined pH 5.0 precipitates representing 14 kg of muscle were used.

biuret method of Weichselbaum (1946). In purer KAF fractions the spectrophotometric method of Warburg and Christian (1941) was used. Phosphorylase *b* concentration was determined using the $E_{278}^{1\%}$ 11.9 as determined by Applemen *et al.* (1963). ^{32}P was counted in the Model 3003 Packard Tri-Carb liquid scintillation spectrometer using a scintillant solution containing 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of 1,4-bis[2-(5-phenyloxazole)]benzene dissolved in 1 l. of dioxane.

Results

Purification of KAF. For the purification of KAF it was convenient to use a by-product of the phosphorylase kinase preparation, the pH 6.1–6.2 acid supernatant fraction (Krebs *et al.*, 1964) as the starting material. All steps were carried out at 0° unless indicated otherwise.

STEP 1. The acid supernatant fraction was adjusted to pH 4.9–5.0 with 1 N acetic acid and centrifuged 20 min at 5000*g*. The precipitate was homogenized in 70 ml of 0.1 M sodium glycerophosphate–0.004 M EDTA buffer (pH 8.2) per kg of muscle and the final pH of the suspension was adjusted to 7.0. This suspension was frozen and stored until sufficient material was available for the next step.

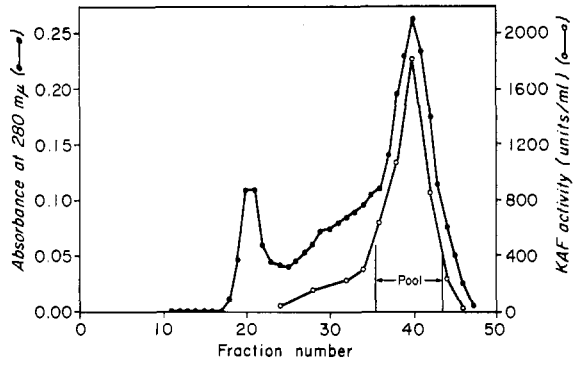

FIGURE 2: The purification of KAF on Sephadex G-200. An ammonium sulfate precipitate fraction from a typical preparation containing 37,000 units of KAF was applied to the column as described in the text. Fractions of 5.5 ml were collected and assayed for KAF.

STEP 2. The pH 5.0 precipitate fraction from 14 kg of muscle was thawed, diluted to 140 ml/kg of muscle with cold distilled water, and centrifuged 2 hr at 78,000*g*. The supernatant was divided into two equal parts, each of which was pumped through a 2.5 × 45 cm column of TEAE-cellulose equilibrated with 0.05 M sodium glycerophosphate–0.002 M EDTA (pH 7.0) at a flow rate of 300–400 ml/hr. After washing with 1 l. of 0.1 M sodium glycerophosphate–0.002 M EDTA (pH 7.0), the KAF was eluted using 0.3 M sodium glycerophosphate–0.002 M EDTA (pH 7.0). Fractions of 25 ml were collected and assayed for KAF. Those containing at least 100 units/mg of protein were pooled.

STEP 3. The combined eluates from the two TEAE columns were dialyzed overnight against 30 volumes of neutral 0.002 M EDTA, concentrated two- to threefold by ultrafiltration, and then dialyzed overnight against two 1-l. portions of 0.01 M sodium glycerophosphate–0.002 M EDTA (pH 6.5). The fraction was then diluted to 4 mg/ml with the pH 6.5 buffer. Three successive portions of Alumina Cγ, each at 0.3 mg of solids/mg of protein, were added and the mixture was homogenized by hand. The suspension was stirred for 15 min and centrifuged 5 min at 12,000*g* after each addition of gel. Each portion of gel was then homogenized carefully with 15 ml of 0.05 M sodium glycerophosphate–0.002 M EDTA (pH 7.0) and stirred and centrifuged as above. Although the eluate from the first portion of gel contained a considerable amount of KAF, those from the second and third portions possessed a higher specific activity and usually only these were combined and used for further purification.

STEP 4. The Alumina Cγ product was adjusted to 1.8 M ammonium sulfate by the addition of 0.90 volume of 3.75 M ammonium sulfate, stirred at 0° for 20–30 min,

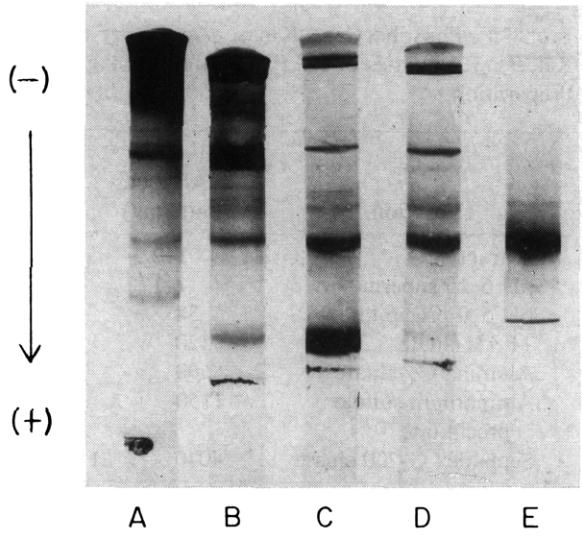

FIGURE 3: Disc electrophoresis of fractions in the KAF preparation. From the left the fractions are: (A) pH 5.0 precipitate, (B) TEAE eluate, (C) Alumina Cγ eluate, (D) ammonium sulfate precipitate, and (E) Sephadex G-200 eluate. The arrow indicates the direction of migration of the protein components.

and centrifuged 5 min at 25,000*g*. The precipitate was dissolved in 4 ml of 0.05 M sodium glycerophosphate–0.002 M EDTA–0.5 M NaCl (pH 7.0) and applied immediately to a 2.5 × 80 cm column of Sephadex G-200 equilibrated with the same buffer. The elution rate was 6–12 ml/hr. The fractions with the highest KAF activity were pooled and dialyzed overnight against 1 l. of 0.05 M sodium glycerophosphate–0.002 M EDTA (pH 7.0). An elution diagram is shown in Figure 2.

A summary of an individual KAF preparation is given in Table I. Data on those steps which are part of the phosphorylase kinase preparation are also given. It should be noted that the very large loss of activity shown in the Alumina Cγ step was not seen ordinarily; a yield of 60–80% was more common. Unless indicated otherwise the experiments reported in this paper were all carried out with the most highly purified fraction of KAF.

The fractionation of KAF was greatly facilitated by the use of disc electrophoresis. Often the efficacy of a particular step was indicated by its usefulness in removing a particular impurity rather than by a major increase in specific activity of KAF. This is illustrated in Figure 3 which shows the electrophoretic pattern of different fractions in the preparation. It can be seen, for example, that the ammonium sulfate step was especially effective in removing a fast-migrating impurity even though this procedure resulted in only a modest increase in KAF activity. The final product showed a major band comprising 60–80% of the total protein as judged from densitometric analysis of the pattern. KAF activity was found to be associated only with this band as determined by extracting and assaying slices from a duplicate gel (Figure 7). The molecular weight of KAF, as determined on Sephadex G-200 using aldolase and bovine serum albumin as markers (Andrews, 1965), was estimated to be about 110,000.

The Release of Ninhydrin-Positive Material during Activation of Phosphorylase Kinase by KAF. Earlier experiments designed to test the hypothesis that KAF is a proteolytic enzyme were negative (Meyer *et al.*, 1964). With the availability of more highly purified preparations of the factor this question was reopened and evidence consistent with this idea was obtained. Figure 4 shows the result of an experiment in which simultaneous measurements were made of phosphorylase kinase activation and the formation of trichloroacetic acid soluble ninhydrin-positive material during treatment of the enzyme with KAF and Ca^{2+}. The experiment shows that there was a KAF-dependent release of peptide material during this reaction and fair agreement was obtained between the extent of activation and the amount of such material formed.

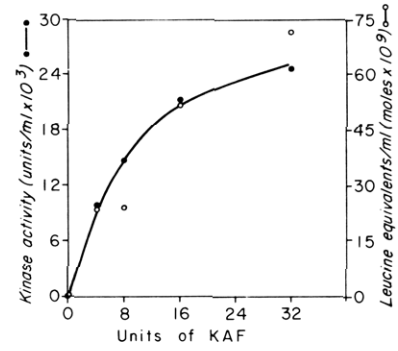

FIGURE 4: The release of peptides from phosphorylase kinase during activation by KAF and Ca^{2+}. Reaction mixtures of 1.5 ml were identical in composition with those used in the standard assay for KAF except that the concentration of phosphorylase kinase was higher: 4.2 mg/ml or 80,000 pH 8.2 units/ml. Aliquots were removed for measurement of kinase activity at pH 6.2 and the release of trichloroacetic acid soluble, ninhydrin-positive material after base hydrolysis as explained in Methods. Controls without added KAF showed no increases in kinase activity or acid-soluble peptides.

The Release of ^{32}P Peptides from ^{32}P-Labeled Phosphorylase Kinase by KAF. It was found that ^{32}P-labeled phosphorylase kinase, obtained by preincubating the enzyme with [^{32}P]ATP and Mg^{2+} (De Lange *et al.*, 1968), served as a more sensitive and useful substrate than nonactivated phosphorylase kinase in studying the release of peptides catalyzed by KAF. Accordingly, this form of the enzyme was employed in experiments designed to gain further information on the identity of the apparent proteolytic activity of KAF and the activity involved in kinase activation. Figure 5 shows that KAF catalyzes the release of acid-soluble ^{32}P. The radioactive material released was not $^{32}P_i$ as shown by the fact that it was not extractable into isobutyl alcohol–benzene as a phosphomolybdate complex (Walters and Cooper, 1965) nor did it migrate like P_i on paper electrophoresis. Since the ^{32}P in ^{32}P-labeled phosphorylase kinase is known to be present in serine (or threonine) residues (Riley *et al.*, 1968) it can be assumed that this material consisted of phosphopeptides.

The leveling off of the reactions in Figure 5 with varying amounts of KAF was reminiscent of the effect which had been seen in the activation of phosphorylase kinase by KAF (Meyer *et al.*, 1964; Drummond and Duncan,

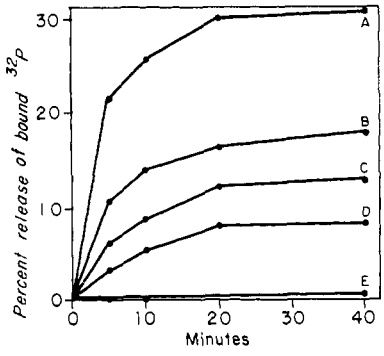

FIGURE 5: Release of ^{32}P from ^{32}P-labeled phosphorylase kinase by KAF. Reaction mixtures at 30° were identical in composition with those used in the KAF assay procedure (see Methods) except that ^{32}P-labeled activated phosphorylase kinase was used as substrate instead of nonactivated phosphorylase kinase. The KAF concentrations were 8 units/ml in A, 2 units/ml in B, 1 unit/ml in C, 0.5 unit/ml in D, and no KAF in E. The kinase contained 1.6 moles of bound $^{32}P/10^5$ g of protein and was present at a concentration of 0.2 mg/ml in the reaction mixtures. At the specified times 0.8-ml aliquots were added to 0.1 ml of 1% bovine serum albumin and the proteins precipitated at 5% trichloroacetic acid. The samples were centrifuged and the supernatants were counted.

1966) and had been interpreted as indicating that a possible stoichiometric relationship existed between KAF and the kinase. An explanation for this behavior was found in the fact that KAF exhibits marked instability in the presence of Ca^{2+} (Meyer *et al.*, 1964). In the present study (not illustrated) it was demonstrated that the time required for complete loss of KAF activity during preincubation of the factor with Ca^{2+} corresponded almost exactly with the time at which phosphorylase kinase activation or the ^{32}P-releasing reactions leveled off. This type of experiment was carried out at several KAF concentrations. Furthermore, it was demonstrated in experiments similar to those of Figure 5 that readdition of substrate at 20 min resulted in no new burst of activity.

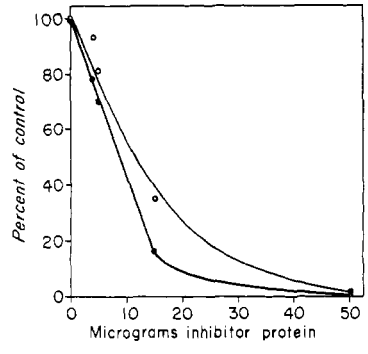

FIGURE 6: Inhibition of "^{32}P-releasing activity" (○—○) and phosphorylase kinase activating activity (●—●) of KAF by a protein inhibitor for heart muscle. The reaction mixtures for measuring the release of ^{32}P had a final volume of 0.6 ml and were essentially identical in composition with those of the KAF assay except that ^{32}P-labeled phosphorylase kinase, 0.12 mg containing 2.2 moles of $^{32}P/10^5$ g, was used instead of nonactivated phosphorylase kinase. KAF (0.4 unit) was used. Incubation was for a period of 5 min at 30°.

TABLE II: Phosphorylase Kinase Activating and ^{32}P-Releasing Activities of KAF at Different Stages of the Preparation.[a]

Fraction	KAF Act. (units/mg)	^{32}P-Releasing Act.
Extract	1.2	103
pH 6.15 supernatant	1.1	111
pH 5.0 precipitate	34	104
TEAE eluate	520	79
Alumina Cγ eluate	560	102
Ammonium sulfate precipitate	1150	135
Sephadex G-200 eluate	4040	100

[a] A typical preparation of KAF was assayed for specific KAF activity using nonactivated phosphorylase kinase as the substrate and ^{32}P-releasing activity using ^{32}P-labeled phosphorylase kinase as the substrate. The method employed for the latter assay is described in the legend of Figure 6 and is reported for each fraction as 100 × (rate of release of ^{32}P/KAF unit)/(rate of release of ^{32}P/KAF unit in the G-200 eluate).

The extent of ^{32}P release in 5 min under the conditions of the experiment of Figure 5 was used as a measure of "^{32}P-releasing activity" of KAF. It was found that this activity could be inhibited by the heart muscle KAF inhibitor[8] described by Drummond and Duncan (1966) and that the extent of inhibition with varying amounts of the inhibitor more or less paralleled inhibition of phosphorylase kinase activation (Figure 6).

Parallel Purification of ^{32}P-Releasing and Phosphorylase Kinase Activating Activities of KAF. To aid in determining that the same protein was responsible for the phosphorylase kinase activation reaction and the release of ^{32}P peptides from the labeled kinase, various fractions from the KAF preparation were assayed for both activities. The result of this experiment is shown in Table II, and it is apparent that the degree of purification of both activities is parallel throughout the procedure. The final fraction was examined by disc gel electrophoresis using duplicate gels. One gel was stained for protein and the other was cut into sections which were eluted with buffer and assayed for each type of activity. Figure 7 shows that the protein stain, which was most intense in the region of section 39, coincided closely with the point of maximum KAF activity as well as the ^{32}P-releasing activity.

The Appearance of NH_2-Terminal Amino Acids in Phosphorylase Kinase during Activation by KAF. Using the fluorodinitrobenzene method for estimating and identifying the NH_2-terminal amino acids in nonactivated phosphorylase kinase, De Lange (1965) found small but

[8] The purified inhibitor fraction was also found to act as an inhibitor of trypsin.

TABLE III: The Hydrolysis of Various Proteins by KAF and Trypsin.[a]

Substrate	Leucine Equiv Released by KAF (μmoles)	Leucine Equiv Released by Trypsin (μmoles)
Phosphorylase *b*	0.08	0.12
Phosphorylase *a*	0.08	0.08
Casein	2.2	1.4
Hemoglobin	0.38	0.48
Bovine serum albumin	1.7	0.83
Phosvitin	0.14	0.81

[a] The protein substrates were denatured by boiling a 0.4% solution made up in 0.05 M sodium glycerophosphate buffer (pH 7.5) for 10 min; with phosphorylases *b* and *a* and hemoglobin the resulting suspensions were homogenized. Reaction mixtures with KAF contained 0.05 M sodium glycerophosphate buffer, 0.015 M mercaptoethanol, 0.01 M calcium acetate, 0.8 mg of protein substrate, and 65 units (0.01 mg) of KAF. The final volume was 0.7 ml. Reaction mixtures with trypsin were identical except that no mercaptoethanol or calcium acetate were included, and trypsin (2.5 μg) was used instead of KAF. Incubations were for 10 min at 30°. Reactions were stopped with an equal volume of 10% trichloroacetic acid and ninhydrin reactions after base hydrolysis were carried out as described in Methods.

reproducible amounts of threonine (0.14 mole/10^5 g), serine (0.10 mole/10^5 g), aspartic acid (0.06 mole/10^5 g), and glutamic acid (trace). In the present study these same four NH_2-terminal amino acids were also readily detected in all samples of the nonactivated kinase by the dansyl chloride procedure. In addition variable traces of glycine, alanine, methionine, leucine, and isoleucine were also seen in some preparations. Incubation of the kinase with Ca^{2+} alone or KAF alone did not change the pattern of NH_2-terminal amino acids, but in the presence of both components definite increases occurred in leucine, isoleucine, glutamic acid, serine, alanine, and glycine; in addition, NH_2-terminal lysine appeared. No changes were noted in the amount of NH_2-terminal threonine, aspartic acid, or methionine. These studies were of a qualitative or at best a semiquantitative nature with the relative amounts of the different NH_2-terminal amino acids being estimated simply by determining the extent of dilution that could be carried out before a given fluorescent spot disappeared.

The Relative Activities of KAF, Trypsin, and Chymotrypsin in the Activation of Phosphorylase Kinase. It was of interest to compare the relative specific activity of purified KAF with that of other proteolytic enzymes in their activation of phosphorylase kinase. As shown in Figure 8, trypsin and chymotrypsin were approximately equal in their activating ability, while KAF had about

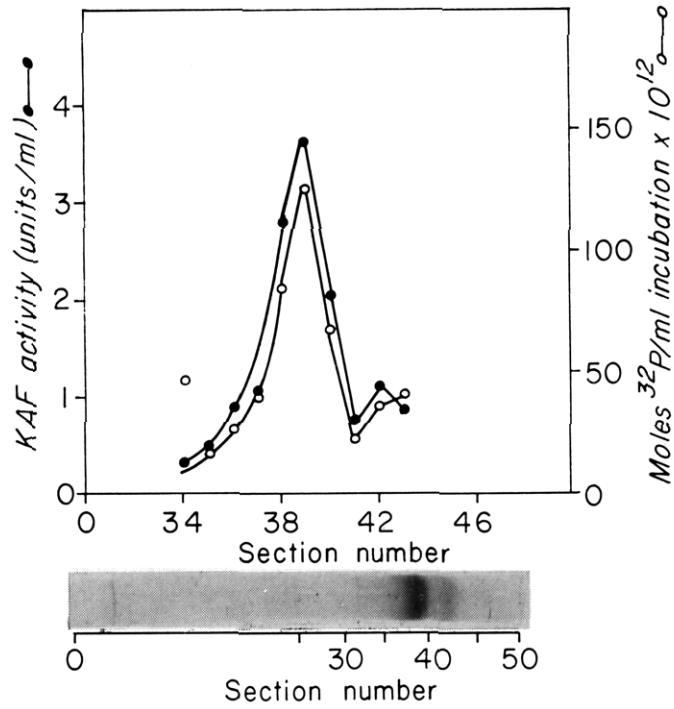

FIGURE 7: The migration of protein, kinase-activating activity, and ^{32}P-releasing activity of purified KAF on disc gel electrophoresis. Duplicate gels were run. One of these was stained for protein. The other gel was sliced and the sections were eluted and assayed for KAF activity and for ^{32}P-releasing activity using the system described in the legend of Figure 6.

one-third of their activity on a weight basis. In view of the fact that the molecular weight of KAF is probably several times greater than that of trypsin or chymotrypsin, it would appear that on a molar basis the three enzymes are approximately equal in their kinase-activating ability. It should be noted that phosphorylase kinase activation as a result of limited proteolysis constitutes an extremely sensitive test for low concentrations of proteolytic activity.

The Hydrolysis of Protein other than Phosphorylase Kinase Catalyzed by KAF. Various heat-denatured proteins were tested as potential substrates for the proteolytic action of KAF. Parallel experiments were carried out using trypsin. The results are shown in Table III.

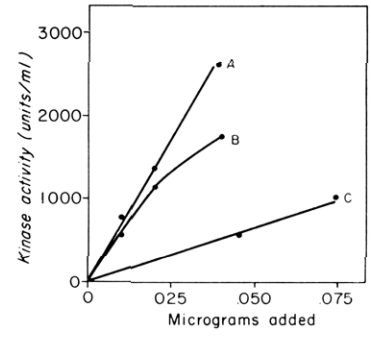

FIGURE 8: Comparison of the effectiveness of KAF, trypsin, and chymotrypsin in the activation of phosphorylase kinase. The conditions for phosphorylase kinase activation were those employed in the standard KAF activity test. A = chymotrypsin, B = trypsin, and C = purified KAF.

Control experiments demonstrated that the release of peptides from the various protein substrates was Ca^{2+} dependent in each instance.

Discussion

When the work presented in this paper was initiated, it was known that incubation of phosphorylase kinase with Ca^{2+} led to activation of the kinase and that a protein factor (KAF) was required for this activation. Studies of the kinetics of the activation reaction had shown increasing rates of activation with increasing amounts of KAF, but the final levels of activation also increased with increasing KAF (Meyer *et al.*, 1964; Drummond and Duncan, 1966). For this reason it was not possible to decide whether the action of KAF was stoichiometric or catalytic. No information was available as to the mechanism of action of KAF, although it had been shown (Krebs *et al.*, 1964) that phosphorylase kinase activated by KAF showed no gross changes in its sedimentation characteristics. The present studies indicate that KAF is a proteolytic enzyme. This was evidenced by the KAF-catalyzed release of ninhydrin-positive material from nonactivated phosphorylase kinase as well as from other protein substrates. In addition, KAF caused the ready release of ^{32}P-labeled peptides from ^{32}P-labeled activated phosphorylase kinase. KAF was purified 3000-fold to a state of near homogeneity with the extent of purification being the same whether calculated on the basis of phosphorylase kinase activation assays or the release of ^{32}P-labeled peptides. The final material gave one major band on disc electrophoresis, and this band contained both activating and proteolytic activities. Treatment of phosphorylase kinase with KAF and Ca^{2+} resulted in the appearance of new and increased NH_2 terminals in the kinase. The proteolytic manifestations of KAF were inhibited by the heat-stable protein inhibitor of KAF described by Drummond and Duncan (1966).

The finding that KAF is a proteolytic enzyme casts great doubt as to its physiological role in the activation of phosphorylase kinase. Any continually functioning mechanism for activation of the kinase would be expected to be reversible, and it would appear very unlikely that reversal of peptide-bond hydrolysis could occur as part of a control mechanism of a type involving phosphorylase kinase. Particularly under conditions in which the actual release of peptides occurs during activation of the kinase by KAF, it is difficult to conceive of any repair process that might be operating. The complete destruction of activated kinase molecules and the resynthesis of nonactivated kinase would also seem like an extremely remote possibility.

The probability that KAF has no physiological role in controlling phosphorylase kinase activity should not be construed to mean that Ca^{2+} cannot be involved in regulating the activity of this enzyme. Meyer *et al.* (1964) noted that apart from the role of Ca^{2+} as a metal required for the action of KAF, Ca^{2+} also appears to be essential for the activity of phosphorylase kinase. Thus with either activated or nonactivated phosphorylase kinase, the introduction of EGTA into the phosphorylase *b* to *a* reaction mixture caused marked inhibition of the reaction which was relieved most effectively by addition of Ca^{2+}. Recently Ozawa *et al.* (1967) calculated that as little as 1×10^{-6} M Ca^{2+} is required for maximal stimulation of phosphorylase kinase by this mechanism. The hypothesis advanced by Meyer *et al.* (1964) that Ca^{2+} may play a role in the coupling of muscle contraction to glycogenolysis through its effect on phosphorylase kinase remains very much alive.

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