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Studies on the Subunit Structure of Trypsin-Activated Phosphorylase Kinase†

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ABSTRACT: Trypsin-activated phosphorylase kinase with a sedimentation constant of approximately 22 S can be dissociated to lower molecular weight species. In the presence of ATP at 5° a 13S component which has a molecular weight of approximately 350,000 is slowly formed. Rewarming the enzyme to 20° does not reverse the dissociation process. At lower enzyme concentrations, 9S and 6S species are formed as identified by sucrose gradient centrifugation. These subfragments of the enzyme are active in the assay, suggesting that the large molecular weight of phosphorylase kinase is not essential for catalysis of the phosphorylase *b* to *a* reaction. Analysis of the 6S fraction by gel electrophoresis in sodium dodecyl sulfate shows that subunits A and B which are present in native phosphorylase kinase are absent in this fraction. Subunit C, however, is still present. Cold denaturation of trypsin-activated kinase occurs and is accentuated in the presence of ATP. No effect of cold is seen with native nonactivated phosphorylase kinase or enzyme activated by phosphorylation. These forms of phosphorylase kinase are not dissociated by ATP.

In a previous report (Hayakawa *et al.*, 1973b), it was shown that phosphorylase kinase (ATP–phosphorylase phosphotransferase, EC 2.7.138) can be dissociated into three separable subunits by treatment with sodium dodecyl sulfate. Since no enzymic activity could be restored following this treatment, less stringent dissociating conditions were sought so that the relationships which these subunits have to the regulatory and catalytic function of phosphoylase kinase could be examined.

Nonactivated phosphorylase kinase, phosphorylase kinase that had been phosphorylated and activated by protein kinase, and trypsin-activated phosphorylase kinase were used in a comparative study. Phosphorylase kinase obtained by treatment with trypsin is catalytically active (Krebs *et al.*, 1964; Huston and Krebs, 1968) and sediments in the ultrcentrifuge with a sedimentation coefficient similar to that of nonactivated phosphorylase kinase (Krebs *et al.*, 1964) suggesting that only a limited attack of the enzyme by trypsin has occurred. The results reported herein show that trypsin-treated kinase dissociates upon dilution or by incubation with ATP to give active subfragments. The conditions found to dissociate trypsin-treated kinase to its subunits were ineffective with nonactivated and protein kinase-activated phosphorylase kinase.

Experimental Section

Materials. Phosphorylase *b*, nonactivated and protein kinase-activated phosphorylase kinase, and [γ-^{32}P]ATP were

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prepared as described in the preceding papers (Hayakawa *et al.*, 1973a,b). Trypsin-activated phosphorylase kinase was prepared by incubation of the nonactivated enzyme (1–4 mg/ml) with trypsin for 5 min at pH 6.8 at a weight ratio of kinase:trypsin of 1000:1. A sixfold excess of soybean trypsin inhibitor on a weight to weight basis was added to stop the reaction. A ratio of enzymic activity at pH 6.8 to activity at pH 8.2 of approximately 1.0 was obtained by this method (Hayakawa *et al.*, 1973b).

Ultracentrifugal Analyses. All sedimentation experiments were performed in a Beckman Model E ultracentrifuge equipped with an RTIC temperature control unit, Schlieren and Rayleigh interference optics, and an ultraviolet system with a monochromator and photoelectric scanner. Photographic plates were measured with a Nikon profile projector, Model 6C. The molecular weight of the treated phosphorylase kinase was determined by the sedimentation equilibrium method according to the meniscus depletion technique of Yphantis (1964). For the equilibrium runs, a 12-mm six-channel Kel-F centerpiece was employed. Molecular weights were calculated from the average of those obtained from each of three black interference fringes. The partial specific volume ($\bar{v}$ = 0.730 ml/g) that was calculated for nonactivated phosphorylase kinase (Hayakawa *et al.*, 1973b) was assumed for trypsin-activated phosphorylase kinase. No correction for the density of the buffer was made in the calculation of the molecular weight.

Other Methods. Sucrose gradient centrifugation was done by an adaptation of the procedure of Martin and Ames (1961). Linear gradients were prepared from 5–20% sucrose in different buffers and centrifugation was carried out at 5° for 16.5 hr at 30,000 rpm in a swinging bucket rotor, No. 40, using a Beckman L265B ultracentrifuge. Phosphorylase and phosphorylase kinase assays and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were carried out as described previously (Hayakawa *et al.*, 1973a,b).

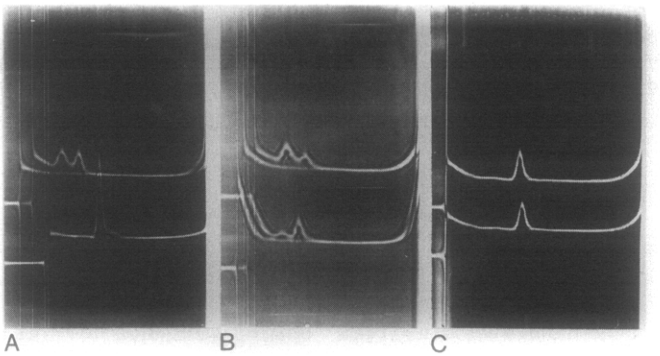

FIGURE 1: Effect of nucleotides on the ultracentrifugal pattern of trypsin-activated phosphorylase kinase. (A) Trypsin-activated phosphorylase kinase, 2.8 mg/ml, in 20 mM glycerol phosphate–9 mM mercaptoethanol–0.7 mM EDTA, pH 6.9; upper curve, with 20 mM ATP; lower curve, without ATP. The two peaks of the upper pattern have *s* values of 13.1 and 20.0. The control in the lower pattern had an *s* value of 22.4. The photograph was taken with a phase plate angle of 65° after the samples in double-sector cells had been at a speed of 52,000 rpm for 17 min with the temperature controlled at 5°. (B) Trypsin-activated phosphorylase kinase, 2.7 mg/ml, in 18 mM glycerol phosphate–0.9 mM mercaptoethanol–0.2 mM EDTA, pH 6.9; upper curve, with 20 mM ATP; lower curve, with 20 mM ATP–0.10 M Mg^{2+}. The upper pattern shows the partially dissociated enzyme with the 13.2S and 20.7S peaks in an approximate ratio of 3:2. The lower pattern shows the partially dissociated enzyme with the 14.2S and 20.9S peaks in an approximate ratio of 2:3. The photograph was taken with a phase plate angle of 60° after the samples in double-sector cells had been at a speed of 48,000 rpm for 20 min with the temperature controlled at 9°. (C) Trypsin-activated phosphorylase kinase, 2.0 mg/ml, in 20 mM glycerol phosphate–45 mM mercaptoethanol–0.8 mM EDTA, pH 6.9; upper curve, with 20 mM ADP; lower curve, with 20 mM AMP. The upper pattern shows the nondissociated enzyme with an *s* value of 20.3. The lower pattern shows the nondissociated enzyme with an *s* value of 20.7. The photograph was taken with a phase plate angle of 70° after the samples in single-sector cells had been at a speed of 56,000 rpm for 24 min with the temperature controlled at 5°. Sedimentation is from left to right in all figures. *s* values in A–C have been corrected to 20° but have not been corrected for solvent.

Results

Preliminary Attempts to Dissociate Nonactivated Phosphorylase Kinase. A variety of conditions were tested in an attempt to find a mild method for dissociating nonactivated phosphorylase kinase into its subunits. At a concentration of 2 mg/ml under conditions of high ionic strength, *e.g.*, 2.5 M NaCl, or in the presence of $NaClO_4$ (0.25, 0.5, and 1.0 M) at pH 7, the enzyme aggregated as evidenced either by visible precipitation or by analytical ultracentrifugation. Inclusion of 10% sucrose in the above solutions reduced aggregation but no evidence was obtained for enzyme dissociation. Also, 1 M KI did not change the sedimentation properties of nonactivated phosphorylase kinase, although this salt was known to be very effective in dissociating a high molecular weight fraction of the pyruvate dehydrogenase complex (Hayakawa *et al.*, 1969). With cationic substances such as spermine (10 mM) or Mg^{2+} (1 mM) or Ca^{2+} (2 mM), aggregation of the enzyme again took place. Different buffers at low ionic strength such as glycerol phosphate, HCO_3^-, Hepes,[1] Tris, and glycine from pH 6.0 to 10, the chelating agents, EDTA or EGTA, carbohydrates, 5% hydrolyzed amylose (average degree of polymerization of 40 glucosyl units) or 1% maltotetraose, and phosphorylase *b* (3.4 mg/ml) also were tried but were ineffective.

Effect of ATP on the Dissociation of Phosphorylase Kinase. The effect of free ATP as a possible dissociating agent was studied since this nucleotide had been found to cause the dissociation of glyceraldehyde-3-phosphate dehydrogenase (Constantinides and Deal, 1969; Stancel and Deal, 1969), glycogen phosphorylase (DeVincenzi and Hedrick, 1970), and phosphofructokinase (Parmeggiani *et al.*, 1966; Paetkau and Lardy, 1967). With nonactivated or protein kinase-activated phosphorylase kinase, however, ATP had no effect; but with the trypsin-activated enzyme ATP did bring about dissociation as seen by the sedimentation pattern (Figure 1). The lower pattern of Figure 1A shows that trypsin-activated phosphorylase kinase sediments as a single component with a sedimentation coefficient of around 22 S. After incubation with ATP, components with sedimentation coefficients of 20 and 13 S are seen (Figure 1A, upper pattern). Figure 1B shows that the substrate of phosphorylase kinase, the MgATP complex, is less effective than ATP in promoting dissociation. Figure 1C shows that not all adenine nucleotides will cause a change in sedimentation inasmuch as AMP and ADP had no effect. It seems probable that the modification of the structure of trypsin-activated kinase that results in dissociation is related to the binding of the highly negatively charged polyphosphate moiety of ATP.

Further characterization of trypsin-modified phosphorylase kinase was carried out by analytical ultracentrifugation in

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate.

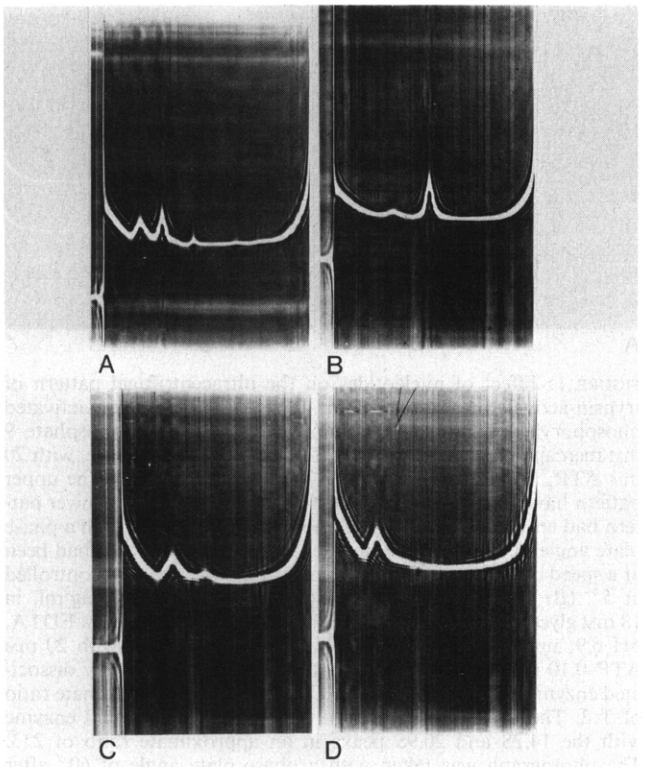

FIGURE 2: Influence of temperature and time of incubation on the ATP-induced structural change. (A) Trypsin-activated phosphorylase kinase, 2.5 mg/ml, in 20 mM glycerol phosphate–45 mM mercaptoethanol–0.8 mM EDTA, pH 6.9, with 20 mM ATP. Incubation for 2.5 hr at 0° before centrifugation at 6°. The pattern shows the partial dissociation of the enzyme in a picture taken 24 min after reaching a speed of 56,000 rpm at a phase plate angle of 55°. (B) Same conditions as in A but incubation at 20° and centrifugation at 20°. Picture taken at a phase plate angle of 60° 18 min after reaching a speed of 56,000 rpm with the temperature controlled at 20°. (C) As in A except incubation was for 8 hr before centrifugation at 6°. This pattern exhibits further dissociation of the enzyme. It was taken at a phase plate angle of 50° 24 min after reaching a speed of 56,000 rpm with the temperature controlled at 7°. (D) As in A except incubation was for 18 hr. Picture was taken at a phase plate angle of 50° 22 min after reaching a speed of 56,000 rpm with the temperature controlled at 5°. Complete dissociation to the 13S peak appears to have taken place. Sedimentation is from left to right. All samples were in a 2° Kel-F single sector centerpiece.

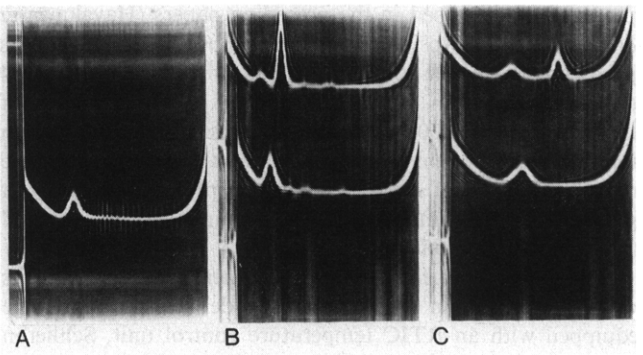

FIGURE 3: Irreversibility of enzyme dissociation after tryptic activation. (A) Trypsin-activated phosphorylase kinase, 2.0 mg/ml, in 20 mM glycerol phosphate–45 mM mercaptoethanol–0.8 mM EDTA, with 20 mM ATP at pH 6.0. Incubation was for 3 hr at 0° before centrifugation. Picture was taken 24 min after reaching 56,000 rpm at a phase plate angle of 55°. Temperature was controlled at 6.5°. (B) Lower pattern, enzyme from A rewarmed to 20° for 1.5 hr; upper pattern, enzyme as in A except incubated at 20° for 5.5 hr. Pictures taken 8 min after reaching speed of 56,000 rpm at a phase plate angle of 50°. Temperature was controlled at 22°. (C) Lower pattern, enzyme from A rewarmed for 20 hr at 20°; upper pattern, enzyme control at 20° for 24 hr. Pictures taken 24 min after reaching speed of 56,000 rpm at a phase plate angle of 50°. Temperature was controlled at 20°. Sedimentation is from left to right. All samples were in a 2° Kel-F single sector centerpiece.

an attempt to further define the functional role of the different subunits of the enzyme in the catalytic process. In Figure 2 the effects of temperature, time, and pH are illustrated. The structural change induced by ATP is greater at lower temperatures (Figure 2A) than at 20° (Figure 2B) and in this respect is similar to the results reported for glyceraldehyde-3-phosphate dehydrogenase (Constantinides and Deal, 1969). ATP does not change the sedimentation characteristics of trypsin-activated kinase rapidly. Figures 2A, C, and D show ultracentrifugal patterns obtained after different times of incubation and show that the major light component (13 S) is completely formed only after 18 hr of incubation. The half-time for the conversion is estimated to be approximately 4 hr at 0° at this pH. The effect of ATP also depends upon pH. For example, in glycerol phosphate–EDTA buffers, the rate of change is enhanced by decreasing the pH from 7.8 to 6.9 to 6.0 to 5.6. After 4 hr of incubation at pH 5.6 or 6.0, essentially all of trypsin-treated phosphorylase kinase sediments as the 13S component in the presence of ATP. In an experiment at pH 6.0 it was determined that the dissociated enzyme still possessed 76% of its original catalytic activity, *i.e.* a particular sample of the kinase with an original activity of 50,000 units/mg at pH 6.8 or pH 8.2 now showed 37,000 units/mg.

Molecular Weight of Trypsin-Activated Phosphorylase Kinase. The structural change induced in trypsin-activated phosphorylase kinase by ATP is presumably a dissociation of the enzyme into its substructure. To define the extent of this alteration, the molecular weight of the trypsin-activated enzyme was determined by the meniscus depletion method after incubation with ATP. In this experiment the trypsin-activated kinase was stored at pH 6.9 for 18 hr at 0° with 20 mM ATP. A molecular weight of 3.46×10^5 was obtained and no obvious polydispersity was detected across the cell. No molecular weight values have been determined for the trypsin-activated kinase in the absence of ATP, but it is probably close to that of nonactivated phosphorylase kinase (1.33×10^6) (Hayakawa *et al.*, 1973b) since the sedimentation coefficient of the trypsin-activated enzyme before dissociation is similar to that of the nonactivated kinase. At a concentration of 2.7 mg/ml, under conditions essentially identical with those in the experiment shown in the lower curve of Figure 1A, nonactivated phosphorylase kinase had a sedimentation coefficient of 23.5, whereas the value for trypsin-activated enzyme was 22.4 S.

Irreversibility of the Dissociation of Trypsin-Activated Phosphorylase Kinase. The reversibility of the dissociation of trypsin-activated phosphorylase kinase induced by ATP was examined. After the enzyme was dissociated with ATP at pH 6.9, it was dialyzed against additional glycerol phosphate–EDTA buffer or passed through a column of G-50 Sephadex to remove ATP. In each instance the resulting enzyme was slightly turbid, and in sedimentation velocity experiments it was found that aggregates were present with sedimentation rates of greater than 22 S. Since temperature was known to have a large effect on enzyme dissociation, another approach was to rewarm the dissociated enzyme in the presence of ATP from 0 to 20°. Figure 3 shows, however, that the

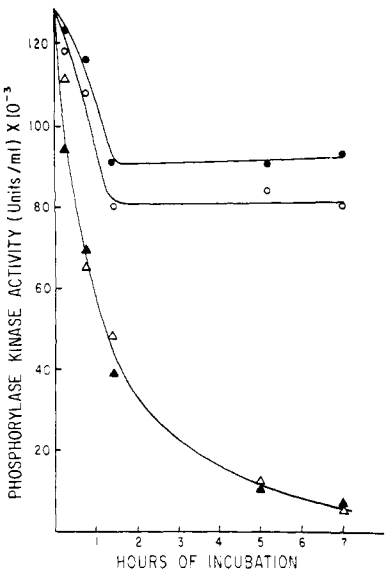

FIGURE 4: Inactivation of trypsin-activated phosphorylase kinase at low enzyme concentrations. Enzyme (22 μg/ml) was incubated in 20 mM glycerol phosphate–45 mM mercaptoethanol–0.8 mM EDTA at pH 6.9. Aliquots were removed at the various times indicated and tested for phosphorylase kinase activity at pH 6.8. Closed symbols indicate experiments at 20° and open symbols experiments at 0°. Triangles indicate the presence of 20 mM ATP.

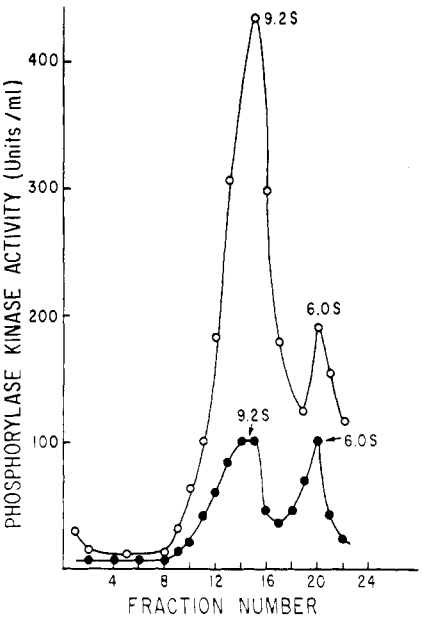

FIGURE 5: Sucrose gradient centrifugation of trypsin-activated phosphorylase kinase. Trypsin-activated kinase (2.4 mg/ml) was incubated in 20 mM glycerol phosphate–10 mM mercaptoethanol–0.8 mM EDTA, pH 6.9, with and without 20 mM ATP for 8 hr at 0° prior to centrifugation. The sucrose gradient (5–20%) was made in the above buffer without ATP. Enzyme (300 μg) was applied to the gradient and centrifugation was carried out at 5° for 16.5 hr at 30,000 rpm. Samples were removed after centrifugation by the use of a polystatic pump, collected in a fraction collector, and tested for phosphorylase kinase activity at pH 8.2: open circles, enzyme without ATP; closed circles, enzyme with ATP.

enzyme dissociated at 0° (A) did not reassociate at 20° and sedimented as a single component (13 S) even after being rewarmed to 20° for 1.5 hr (B, lower pattern), 5.5 hr (B, upper pattern), or 20 hr (C, lower pattern). Interestingly the enzyme control kept at 20° in the presence of ATP also dissociated to a 13S component but at a much slower rate (C, upper pattern). The fact that reassociation does not occur upon rewarming could be due to the 22S species being a quasistable form, while the 13S component of trypsin-activated kinase is a more favored species thermodynamically.

Phosphorylase Kinase Stability. In order to determine the conditions for carrying out density gradient centrifugation experiments (see below), the effects of ATP, protein concentration, temperature, buffers, and time of incubation on enzyme activity were tested. Figure 4 shows the effect of incubation of a dilute solution of trypsin-activated phosphorylase kinase. Inactivation occurred to nearly equal extents at 0 and 20° but was greatly pronounced in the presence of ATP. Inactivation that occurred in the absence of ATP leveled off after about 1 hr suggesting that a new equilibrium was reached. In an experiment with 2.5 times as much enzyme as in Figure 4 (not illustrated), inactivation was definitely more pronounced at 0° than at 20° in the presence of ATP. With a concentration of 2.2 mg/ml (1,000× higher than in Figure 4), no inactivation was observed after 4 hr at 0 or 20° or at 20° with ATP. At 0° in the presence of ATP, a 30% loss of enzymic activity occurred. The type of buffer also influenced the activity of trypsin-activated phosphorylase kinase. In glycerol phosphate, enzyme activity appears roughly proportional to protein concentration, whereas in Hepes buffer there was a marked deviation from linearity indicating that perhaps enzyme dissociation might occur at low protein concentrations to give a less active or inactive enzyme form.

As trypsin-activated phosphorylase kinase was found to be particularly sensitive to storage at low protein concentrations at 0° with ATP, the effect of these conditions on the activity of nonactivated and protein kinase-activated phosphorylase kinase was also tested. However, only enzyme which was trypsin activated (Table I) was found to be cold labile and sensitive to ATP.

Sucrose Gradient Centrifugation Studies. With information on the effect of temperature, protein concentration, buffers,

TABLE I: Effect of Temperature and ATP on the Stability of Nonactivated, Protein Kinase-Activated, and Trypsin-Activated Phosphorylase Kinase.[a]

Enzyme Form	Activity after Incubation at 20°	Activity after Incubation at 0°	Activity after Incubation at 20° with 20 mM ATP	Activity after Incubation at 0° with 20 mM ATP
Nonactivated phosphorylase kinase	49,000	47,000	57,000	52,000
Protein kinase-activated phosphorylase kinase	80,000	92,000	97,000	94,000
Trypsin-activated phosphorylase kinase	70,000	53,000	37,000	23,000

[a] Enzyme was incubated for 3 hr in 20 mM glycerol phosphate–20 mM mercaptoethanol–0.8 mM EDTA, in 0.2 M NaCl at pH 6.9. Protein concentrations of the enzyme forms were, respectively, 44, 26, and 44 μg/ml. After incubation aliquots were removed, diluted, and tested for activity at 30° at pH 8.2. Activities are reported in phosphorylase kinase units/milligram.

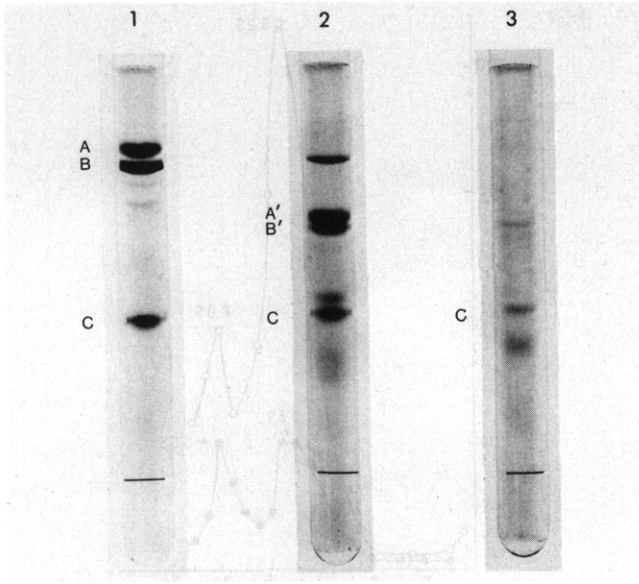

FIGURE 6: Gel electrophoresis of phosphorylase kinase. Electrophoresis was at pH 9.3 in 0.1% sodium dodecyl sulfate. Gel 1, phosphorylase kinase activated by extensive treatment (Hayakawa *et al.*, 1973a) with protein kinase and [γ^{32}P]ATP; gel 2, trypsin-activated kinase; gel 3, fractions (14–16) from sucrose density gradient centrifugation (see Figure 8). Proteins were stained with Coomassie Brilliant Blue and destained by diffusion in methanol–acetate solution reported by Weber and Osborn (1969).

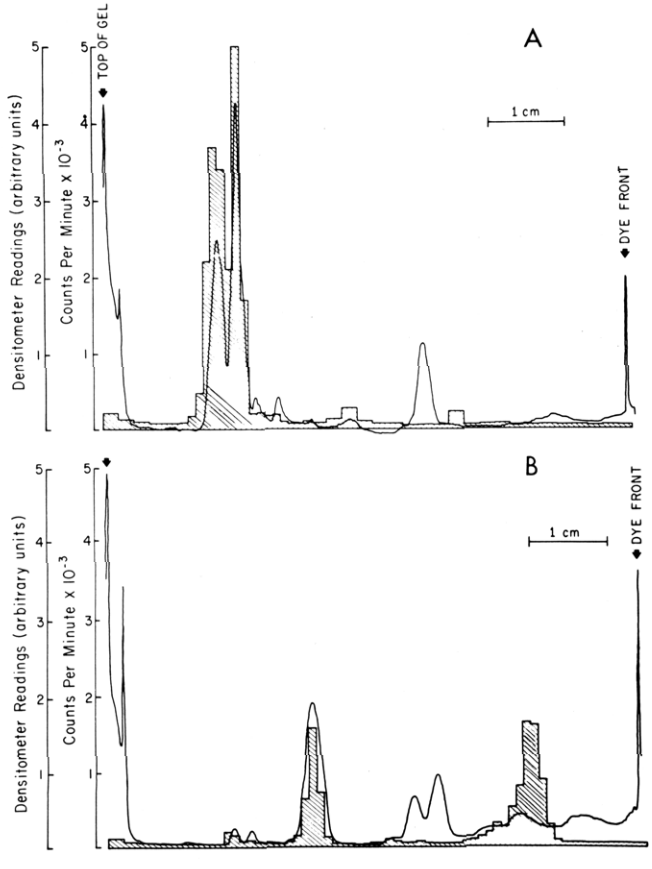

FIGURE 7: Distribution of ^{32}P radioactivity on sodium dodecyl sulfate electrophoresis of protein kinase-activated phosphorylase kinase before and after trypsin treatment: (A) before trypsin treatment; (B) after trypsin treatment. Gels were scanned after staining using a densitometer and slices were counted for radioactivity (Hayakawa *et al.*, 1973a).

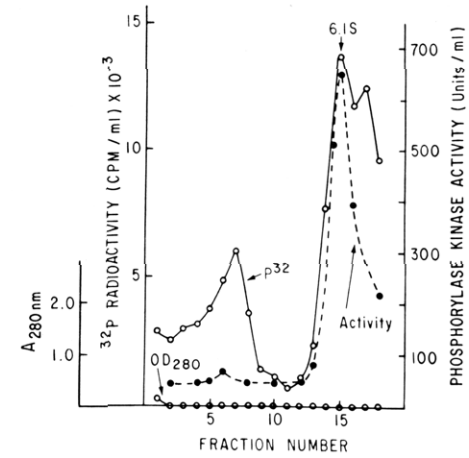

FIGURE 8: Sucrose gradient centrifugation of trypsin-activated phosphorylase kinase prepared from protein kinase-activated enzyme. Enzyme (300 μg) was applied to a sucrose gradient (5–20%) in 20 mM Hepes–45 mM mercaptoethanol buffer, pH 6.9. Centrifugation, collection of fractions, and assay was as in Figure 5. ^{32}P distribution was determined by counting an aliquot of the fracions in a scintillation counter.

and ATP on the stability of trypsin-activated phosphorylase kinase, sucrose gradient centrifugation studies were initiated in order to investigate the behavior of the enzyme at very low concentrations. In the absence of ATP, a 9.2S and 6.0S species of phosphorylase kinase could be identified on the basis of enzymic activity (Figure 5). The same peaks were present when enzyme was preincubated with ATP but lower activities were observed. Relatively more of the 6.0S species was recovered. Thus, trypsin-activated phosphorylase kinase which is dissociable to a 13S component at higher protein concentrations undergoes further dissociation at low concentrations to give new enzyme forms which appear to be catalytically active. That dilution and not sucrose caused dissociation of the 13S component was shown by carrying out analytical ultracentrifuge experiments at higher concentrations in the presence of sucrose.

Density gradient centrifugation studies were extended to include an examination of trypsin-treated phosphorylase kinase which had been activated (phosphorylated) by treatment with protein kinase and [^{32}P]ATP. This "^{32}P-labeled" phosphorylase kinase, prior to trypsin treatment (Figure 6, gel 1), showed the typical A (mol wt = 118,000), B (mol wt = 108,000), and C (mol wt = 41,000) subunit pattern of this enzyme as revealed by sodium dodecyl sulfate gel electrophoresis (Hayakawa *et al.*, 1973b). All of the incorporated ^{32}P was present in subunits A and B as anticipated (Figure 7A) (Hayakawa *et al.*, 1973a). After trypsin treatment the ^{32}P was present as two main radioactive peaks corresponding to molecular weights of ~80,000 and 25,000 (Figure 7B). The first of these radioactive peaks corresponded to the position of protein bands A′ and B′ seen in the stained gel pattern (Figure 6, gel 2).[2] The radioactive bands contained only 38% of the original ^{32}P that had been incorporated into the enzyme (Figure 6A), indicating that most of the ^{32}P was probably lost as low molecular weight rapidly migrating peptides. An experiment supporting this conclusion was the finding that

[2] Because of the incomplete separation of the new components, A′ and B′, it was not possible to ascertain the relative radioactivities of these species (Figure 6B).

trypsin treatment of [^{32}P]phosphorylase kinase gives rise to readily dialyzable [^{32}P]peptides.

When the ^{32}P-labeled enzyme was studied by sucrose density gradient centrifugation (Figure 8) the enzymatic activity was found almost entirely in a fraction with an *s* value of 6.1 S. Although this fraction was radioactive, it can be seen that other fractions were also present which contained ^{32}P. The enzymically active fractions (14–16) were pooled, concentrated using Sephadex G-200, and analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 6, gel 3). Several protein-staining bands were present, but the pattern was much simpler than that seen in gel 2. Bands C and B′ were prominent along with some diffuse species but band A′ was missing. The level of radioactivity was low and found concentrated in components of lower molecular weight than C. From these results, it is not possible to determine whether enzymic activity seen after tryptic attack and centrifugation was due to band C or band B′ or to a combination of these two.

Discussion

Alteration of the catalytic activity and/or regulatory properties of enzymes is commonly affected by limited proteolysis. Although modification of allosteric transitions of enzymes by proteolysis is probably not a part of an "on–off" control *in vivo*, proteases can be used advantageously with isolated enzymes to study mechanisms of control.

After a limited tryptic attack, phosphorylase kinase is activated, *i.e.* the ratio of activities at pH 6.8/8.2 approaches unity and the activity at pH 8.2 increases approximately twofold (Krebs *et al.*, 1964). In the preceding paper (Hayakawa *et al.*, 1973a), it was found that subunits A and B were modified but subunit C was unchanged by trypsin. In this work, the relationship between substructure and activity was further examined.

The results obtained point out some important features about phosphorylase kinase. First, the fact that 13S, 9S, and 6S species could be derived from tryptic digestion of phosphorylase kinase and that these forms showed catalytic activity in the assay shows that the high polymeric form of phosphorylase kinase (mol wt = 1.33×10^6) is not essential for enzymatic catalysis. This result was not obtainable with nonactivated or protein kinase-activated phosphorylase kinase as these enzyme forms have not been found to dissociate by various mild methods. Further analysis of trypsin-activated kinase by gel electrophoresis in sodium dodecyl sulfate (Figure 6) showed that band A which is originally present in inactive kinase is absent. After sucrose gradient centrifugation, an active form of phosphorylase kinase was obtained which upon analysis in gel electrophoresis with sodium dodecyl sulfate showed the presence of two components, B′ and C, and some diffuse material of lower molecular weight. Band A′ is missing in this active form of the enzyme, and if it is assumed that it arises from A, the results suggest that subunit A or a derived form is not necessary for catalysis by phosphorylase kinase. From the present data, it is not possible to delineate which enzyme components are necessary for catalytic activity.

The effects of ATP, protein concentration, and cold temperatures are very similar to effects seen with phosphofructokinase (Parmeggiani *et al.*, 1966; Kono and Uyeda, 1971) and glyceraldehyde-3-phosphate dehydrogenase (Constantinides and Deal, 1969). After peptide bond cleavage of phosphorylase kinase, no doubt the interaction forces required for stabilization of the quaternary structure have been changed. The effect of cold indicates that hydrophobic interactions are indeed extremely important in this circumstance. The dissociated form (13 S) produced by ATP at enzyme concentrations of several milligrams/milliliter does not reassociate upon rewarming to the starting form (22 S). The results suggest that dissociation of trypsin-activated kinase is under kinetic control. Interestingly, glyceraldehyde-3-phosphate dehydrogenase (Teipel and Koshland, 1971) and phosphofructokinase (Alpers *et al.*, 1971), enzymes which are sensitive to ATP, show evidence for kinetic control of different conformational states.

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