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Regulation of glycogen phosphorylase by its amino-terminal region

Harris, William Richard, Ph.D.

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Regulation of glycogen phosphorylase by its amino-terminal region

by

William Richard Harris

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DEDICATION

To Molly, who keeps me on my toes.

ABBREVIATIONS USED

AMP: adenosine 5'-monophosphate

ATP: adenosine 5'-triphosphate

CaM: calmodulin

cAMP: adenosine 3':5'-monophosphate

DEAE: diethylaminoethyl

DTT: dithiothreitol

EGTA: [ethylenebis(oxyethylnitrilo)]tetraacetic acid

G-6-P: glucose-6-phosphate

HPLC: high performance liquid chromatography

IMP: inosine 5'-monophosphate

MOPSO: 3-[N-morpholino]-2-hydroxypropanesulfonic acid.

PAGE: polyacrylamide gel electrophoresis

PMSF: phenylmethylsulfonyl fluoride

SDS: sodium dodecyl sulfate

TEAE: triethylaminoethyl

UDP: uridine 5'-diphosphate

UDP-glc: uridine diphosphoglucose

GENERAL INTRODUCTION

Glycogen phosphorylase is an important enzyme in glycogen metabolism. In muscle cells, the function of phosphorylase is to degrade glycogen to provide energy for muscle contraction. The specific reaction catalyzed by phosphorylase is the reversible phosphorolysis of the α(1-4)glycosidic bonds in glycogen to form glucose-1-phosphate:

$$\text{Glycogen}_{(n)} + P_i \rightleftharpoons \text{Glycogen}_{(n-1)} + \text{Glucose-1-phosphate}$$

The equilibrium constant for the reaction as written is 0.28, but *in vivo*, the concentrations of phosphate and glucose-1-phosphate are such that the equilibrium lies far in the direction of glycogen degradation (1,2,3). Rabbit skeletal muscle is a rich source of phosphorylase, and it is this enzyme which is the subject of this dissertation.

Glycogen metabolism in skeletal muscle is controlled by a variety of extracellular stimuli, which act through a complex network of control mechanisms involving phosphorylation and dephosphorylation of several enzymes. Figure 1 shows a simplified scheme of this network. Glycogenolysis is stimulated by the activation of both cAMP-dependent protein kinase, which inactivates glycogen

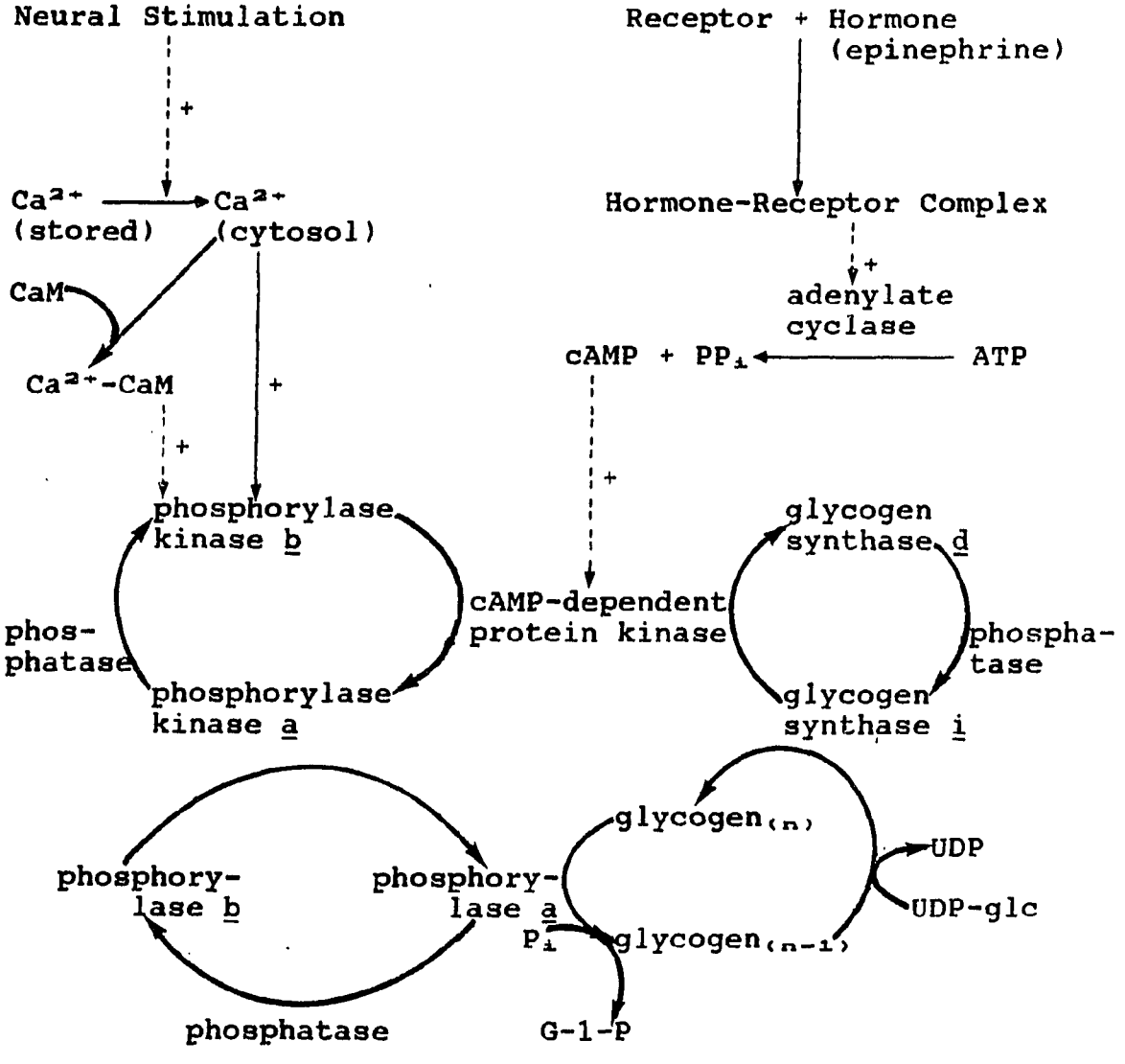


Fig. 1. Control of glycogen metabolism in muscle

synthase, and phosphorylase kinase, which activates phosphorylase. Because phosphorylase kinase can be activated either by increased Ca^{2+} or by phosphorylation, glycogenolysis can be stimulated by either neural or hormonal stimulation of the cell.

The protein kinases, cAMP-dependent protein kinase and phosphorylase kinase, catalyze the transfer of the terminal phosphate of ATP to specific serine residues in their target proteins. Glycogen synthase can be phosphorylated on several serine residues by several different protein kinases. The phosphorylated enzyme's activity depends on glucose-6-phosphate (G-6-P) to varying degrees depending on the extent of phosphorylation (4). In contrast to glycogen synthase, phosphorylase kinase is activated by cAMP-dependent protein kinase catalyzed phosphorylation. Several serine residues are phosphorylated in the α and β subunits of phosphorylase kinase, and the enzyme also undergoes autophosphorylation (5). Because of the complex nature of glycogen synthase and phosphorylase kinase phosphorylation and the scarcity of information known about their structures, it has been difficult to determine the effect of a single phosphorylation event on the structures and functions of these enzymes. The situation is similar for other proteins which are subject to control by phosphorylation and dephosphorylation.

The activation of phosphorylase by phosphorylase kinase catalyzed phosphorylation is a more simple system in which to study the effect of phosphorylation on the structure and function of an enzyme. Phosphorylase is a dimeric protein of identical subunits (6). Phosphorylase kinase catalyzes the phosphorylation of only serine-14 in each of the subunits of phosphorylase (7,8,9). Table 1 lists the significant functional differences between phosphorylase b (nonphosphorylated) and phosphorylase a (phosphorylated). Phosphorylation relieves phosphorylase from allosteric control by AMP and strengthens the subunit interactions.

Table 1. Properties of phosphorylases b and a

Phosphorylase b	Phosphorylase a
Requires AMP for activity	Active without AMP
ATP and G-6-P inhibit by competing with AMP	Not inhibited by ATP or G-6-P
Binds substrates and effectors cooperatively	Not cooperative
Dimer	Dimer $\rightleftharpoons$ tetramer

The binding site for the activator AMP is close to the amino-terminus of phosphorylase, some 30A from the catalytic site. In addition to AMP, phosphorylases b and a bind a number of other effectors. Glycogen binds at the glycogen

storage site, which is distinct from the catalytic site (10,11). Glycogen binding at the storage site increases the activity of phosphorylase (12) and causes dissociation of phosphorylase a from inactive tetramers to active dimers (13). Phosphorylase is bound tightly through this site to glycogen in intact muscle. Glucose inhibits by binding at the active site (14). Caffeine and other purine analogs inhibit by binding at a site close to the entrance to the active site cavity (15). G-6-P inhibits only phosphorylase b by binding to a site which partially overlaps the AMP binding site (16). ATP inhibits only phosphorylase b by binding in the AMP site (17).

The effects of phosphorylation on the structure of phosphorylase are less clear. Phosphorylase exists in at least two different conformations: the inactive "T" and active "R" states, following the model of Monod et al. (18). Phosphorylases a and b have been successfully crystallized for X-ray diffraction studies only in the T-state. Comparisons of the T-state X-ray crystal structures of phosphorylase b (19) (crystallized in the presence of the weak activator IMP) and phosphorylase a (14) (crystallized in the presence of the inhibitor glucose) have shown that there is little difference in the structures except in the amino-terminal 18 residues (20,21,22). These residues are not defined in the electron density map of phosphorylase b,

presumably because they are mobile. In the phosphorylase a structure, residues 5-18 are well resolved, and there are several close contacts between these residues and other residues in both subunits of the dimer. These interactions certainly add to the increased stability and stronger subunit interactions of phosphorylase a as compared to phosphorylase b. The "binding site" for the serine-14 phosphate is surrounded by several positively charged residues, which would repulse the positively charged unphosphorylated amino-terminus.

The widely accepted interpretation of these data is that the amino-terminal 18 residues act as an allosteric activator of phosphorylase. When unphosphorylated, these residues will not bind to the rest of the protein; however, when serine-14 is phosphorylated, that segment interacts with a specific binding site, causing conformational changes which are transmitted to the active site to increase the binding of substrates and increase the enzymatic activity.

Several studies of phosphorylase in solution support this hypothesis. The peptide bond between residues 16 and 17 is more susceptible in phosphorylase b than in phosphorylase a to proteolysis by both trypsin (23) and subtilisin (9,24), indicating that this part of the protein is more accessible to the proteases in phosphorylase b. Limited trypsin proteolysis of phosphorylase produces a

derivative, called phosphorylase b', which is missing the amino-terminal 16 residues (25). Phosphorylase b' requires AMP for activity, as does phosphorylase b. A phosphorylated synthetic peptide corresponding to residues 1-18 of phosphorylase a is able to confer phosphorylase a-like properties to phosphorylase b' (26) and, to a lesser extent, to phosphorylase b (27). The corresponding unphosphorylated peptide is ineffective. These results indicate that the phosphopeptide binds specifically to phosphorylase, even in the presence of the intact, unphosphorylated amino-terminus, and induces some of the same conformational changes as does phosphorylation of intact phosphorylase. These studies are consistent with the hypothesis from the X-ray diffraction studies which show that only when phosphorylated does the amino-terminus of phosphorylase bind to the rest of the protein.

Other studies, involving the specificities of phosphorylase kinase and cAMP-dependent protein kinase, raise questions about the hypothesis of a mobile amino-terminus in phosphorylase. Phosphorylase kinase is probably the only protein kinase which activates phosphorylase *in vivo*. Phosphorylase can not be phosphorylated by cAMP-dependent protein kinase (28); however, synthetic peptides with sequences derived from the amino-terminus of phosphorylase can be phosphorylated by cAMP-dependent

protein kinase with kinetic parameters similar to those obtained when phosphorylase kinase is used as the kinase (29,30). One possible explanation for this discrepancy is that other portions of the phosphorylase structure may block access of cAMP-dependent protein kinase, but not phosphorylase kinase, to serine-14 of phosphorylase. Another possibility is that the amino-terminal segment of phosphorylase is in a conformation which is not recognized by cAMP-dependent protein kinase. Because the small peptide analogs exhibit no secondary structure as measured by circular dichroism (31), and the amino-terminal segment of phosphorylase has no strong tendency to form secondary structure, as predicted by the method Chou and Fasman (32), it is unlikely that it would form any secondary structure unless it were interacting with the rest of the protein.

The assertion that the amino-terminus of phosphorylase possesses secondary structure that affects binding to protein kinases is supported by the finding that intact phosphorylase b is a much better substrate for phosphorylase kinase than are synthetic peptides with sequences corresponding to the amino-terminus of phosphorylase. A peptide with the sequence of phosphorylase residues 5-18 were phosphorylated by activated phosphorylase kinase (phosphorylated by cAMP-dependent protein kinase) with a K_M 75 times higher and V_M 2 times lower than was phosphorylase

b (33). A peptide with the phosphorylase sequence 9-24 and the 90-residue amino-terminal cyanogen bromide fragment from phosphorylase were found to be better substrates for phosphorylase kinase than previously tested peptides, but still the K_M's were at least 10 times higher and the V_M's at least 2 times lower than for phosphorylase b(31). Again, a plausible explanation is that the active site of phosphorylase kinase binds more strongly to polypeptide segments with a certain conformation, and the amino-terminal region of phosphorylase must interact with the rest of the protein to induce this conformation.

Another explanation for small peptides being worse substrates for phosphorylase kinase than phosphorylase b is that there could be a secondary binding site on phosphorylase for the kinase, and the small peptides lack this site. The secondary site might bind more tightly to the kinase than the phosphorylation site binds to the kinase active site, and this secondary binding would serve to align the phosphorylation site in the active site. This reasoning has been offered to explain the finding that G-6-P, an inhibitor of phosphorylase, inhibits phosphorylase kinase by binding to phosphorylase (34). Presuming that the amino-terminal region is mobile, one explanation for G-6-P inhibition of phosphorylase kinase is that G-6-P binding induces a conformation in phosphorylase which weakens the

binding to the kinase at a secondary site. Another explanation is that, in unliganded phosphorylase, the amino-terminal region is in a conformation favorable for binding to the kinase, but G-6-P binding changes that conformation.

In summary, there are two sets of experimental data relating to the conformation of the amino-terminal region of phosphorylase b. One set, which includes the results of X-ray crystallography and limited proteolysis, implies that the amino-terminal region is mobile. The other set of data indicates that there might be some ordered structure in this region that affects the actions of cAMP-dependent protein kinase and phosphorylase kinase.

The questions thus arise: what is the conformation of the amino-terminal region in phosphorylase b and how do effectors of phosphorylase change this conformation? These are important questions from two standpoints. First, as was mentioned before, the conformational differences among the various active and inactive forms of phosphorylase are poorly understood. The amino-terminal region plays an important role in the transitions among these conformations. Second, the phosphorylation of phosphorylase is affected by its conformation. These issues are dealt with in this dissertation in two different ways.

In the first part, the amino-terminal segment of phosphorylase was removed by limited proteolysis with the bacterial protease subtilisin. The resulting phosphorylase analog was purified and characterized to insure that the only difference between it and phosphorylase b is the removal of the amino-terminal region. The physical and enzymatic properties of the truncated analog were compared to those of phosphorylase b. If the properties of the two forms of phosphorylase are different, then this implies that amino-terminal region must interact with the rest of the protein, and this interaction must affect the properties of the enzyme.

Other workers have attempted to use limited trypsin digestion toward this end, building on previous work (35,36), which showed that trypsin cleaved from phosphorylase a a peptide that included the phosphorylation site in the amino-terminal region. Graves et al. (25) isolated the truncated protein, named phosphorylase b', from trypsin digestion of phosphorylase a and studied its properties. They found that phosphorylase b' displayed AMP-dependent activity, but none of the homotropic and heterotropic allosteric interactions characteristic of phosphorylase b. In contrast, Bot et al. (37) found that their preparation of phosphorylase b' did display allosteric interactions. Huminski et al. (38), however, found that at least two forms

of phosphorylase b' with different properties could be produced by using different trypsin concentrations. Their preparations of phosphorylase b' showed multiple bands in SDS polyacrylamide gel electrophoresis, so it is possible that the other groups were also working with phosphorylase b' preparations with multiple cleavage sites. Raibaud and Goldberg (24) found that subtilisin cleavage of the peptide bond between residues 264 and 265 causes inactivation of phosphorylase, even though the two fragments stay together. Trypsin probably also cleaves peptide bonds other than the 16-17 bond, producing phosphorylase derivatives with altered properties. The different preparations of phosphorylase b' used previously might have had different extents of internal cleavage. The work in the first part of this dissertation was initiated in order to clarify this issue by studying the properties of a pure, well characterized phosphorylase analog which is missing only a small portion of the amino-terminal region.

In the second part of this dissertation, phosphorylase kinase is used as a "probe" for conformational changes in one subunit of phosphorylase caused by phosphorylation of the other subunit. The approach is to compare phosphorylase b (unphosphorylated) with phosphorylase ab (one subunit phosphorylated) as substrates for the kinase. If they are different, then phosphorylation of one subunit causes

conformational changes in the other subunit which make it either a better or worse substrate for the kinase. If phosphorylase binds only through its amino-terminal region to phosphorylase kinase, then any conformational changes are probably manifested in the amino-terminal region.

Fisher et al. (39) first obtained direct evidence for the formation of an intermediate "hybrid" during the interconversions between phosphorylases b and a catalyzed by phosphorylase kinase and phosphorylase phosphatase. They followed both phosphate incorporation in phosphorylase and phosphorylase activity at high substrate concentration and observed 100% activity at 50% phosphate incorporation. If G-6-P was present in the phosphorylase activity assays, the activity curve followed the phosphate incorporation curve. These results suggested that there is an intermediate which has the same activity as phosphorylase a at high substrate concentration, but which, unlike phosphorylase a, is sensitive to G-6-P inhibition. In the same study, Fisher's group separated by anion-exchange chromatography three forms of phosphorylase from a phosphorylase kinase reaction mixture stopped before the conversion was complete. When the peak thought to be phosphorylase ab was collected and rechromatographed, only phosphorylases b and a were recovered, indicating that phosphorylase ab is unstable and dissociates and recombines to form the more stable

phosphorylases b and a. This work proved that phosphorylase ab is formed during the interconversions between phosphorylases b and a. This argues against a mechanism in which the two subunits of phosphorylase bind simultaneously to the multimeric phosphorylase kinase or phosphorylase phosphatase and are not released until both subunits are phosphorylated.

Other workers have studied phosphorylase ab formation in vivo and the properties of phosphorylase ab. Heilmeyer et al. (40) found evidence for phosphorylase hybrid formation in glycogen particles isolated from muscle, suggesting that such a hybrid may be an important form of phosphorylase in vivo. Bot et al. (41) found that the AMP-stimulated activity of their preparation of phosphorylase ab was not affected by caffeine, unlike phosphorylase b, which is completely inhibited by caffeine. This property was used to develop an assay for phosphorylase ab, and extensive formation of phosphorylase ab was found during electrical stimulation of skeletal muscle (42) and hormonal stimulation of heart (43). Feldman et al. (44) formed phosphorylase ab by interacting phosphorylase a monomers covalently bound to Sepharose with soluble phosphorylase b monomers. This matrix-bound phosphorylase ab had 42% of the AMP-independent activity of phosphorylase a, and AMP or G-1-P approximately doubled this activity. The work in the second part of this

dissertation studies another property of phosphorylase ab by comparing it with phosphorylase b as a substrate for phosphorylase kinase.

Explanation of Dissertation Format

This dissertation is organized into two sections. The first section deals with limited proteolysis of phosphorylase b, and the properties of the phosphorylase analog produced thereby. The experiments of Figures 1 and 3 in Section I are repetitions of preliminary experiments done by Joyce Miller. The rest of the experiments and the writing of the manuscript were done by the author. This work has been published in Archives of Biochemistry and Biophysics (45). The second section of this dissertation deals with the comparison of phosphorylase b and phosphorylase ab as substrates for phosphorylase kinase. Section II will be submitted for publication to The Journal of Biological Chemistry.

SECTION I:

PURIFICATION AND CHARACTERIZATION OF A GLYCOGEN PHOSPHORYLASE ANALOG MISSING THE AMINO-TERMINAL REGION

William R. Harris, Joyce F. Miller and Donald J. Graves,
Department of Biochemistry and Biophysics
Iowa State University
Ames, Iowa

SUMMARY

A glycogen phosphorylase analog missing only the amino-terminal 16 to 18 residues, which include the phosphorylation site, was produced by subtilisin Carlsberg cleavage of phosphorylase b in the presence of caffeine. The analog, named phosphorylase b_S^I, was purified, and its enzymatic properties were compared with those of phosphorylase b. The K_M's for glucose-1-phosphate are similar, but phosphorylase b_S^I has a V_M 43% higher than that of phosphorylase b. Also, phosphorylase b_S^I is less sensitive to inhibition by glucose-6-phosphate and stimulation by sodium fluoride than is phosphorylase b. The subunit interactions in the two enzyme forms were also compared. The monomer-monomer interactions in phosphorylase b_S^I are weaker than in phosphorylase b, as evidenced by a faster rate of resolution of the coenzyme, pyridoxal phosphate, from phosphorylase b_S^I. The dimer-dimer interactions are also weaker in phosphorylase b_S^I than in phosphorylase b, because phosphorylase b_S^I does not form tetramers or crystals as readily as does phosphorylase b. Because removal of the amino-terminal segment changes the properties of the enzyme, this segment must be interacting with other parts of the protein. This statement conflicts with previous interpretation of X-ray crystallographic data that suggest that the amino-terminal region of phosphorylase b is freely mobile.

INTRODUCTION

Glycogen phosphorylase catalyzes the degradation of glycogen under physiological conditions, and the regulation of its enzymatic activity has been intensively studied. In rabbit muscle phosphorylase, the amino-terminal segment plays an important role in this regulation. Phosphorylation of serine-14 by phosphorylase kinase converts phosphorylase b, which requires AMP for activity, into phosphorylase a, which is active without AMP. In X-ray crystallographic studies of phosphorylase b (crystallized with the weak activator IMP), the amino-terminal 19 residues are not resolved (1). In phosphorylase a (crystallized with the inhibitor glucose), all but the first 4 residues are resolved, and several of the amino acids in this region interact with other parts of the protein (2). The conclusions from these observations are that, in phosphorylase b, the amino-terminal segment is freely mobile in the solvent and that phosphorylation of serine-14 allows this segment to form specific interactions with other parts of the protein. This interaction causes conformational changes which are transmitted to the active site to change the enzymatic activity (3). For a recent review of structure-function relationships in phosphorylase, see Madsen (4).

To further investigate the structure and role of the amino-terminal segment of phosphorylase b, it would be useful to have a derivative that is missing this piece. The object of the work presented in this paper was to produce such a pure, well-characterized derivative. Other workers have attempted to use limited trypsin digestion toward this end, building on previous work (5,6), which showed that trypsin cleaved from phosphorylase a a peptide that included the phosphorylation site in the amino-terminal region. But different groups showed different effects of trypsin digestion on phosphorylase (7,8,9), and the derivatives they produced probably were mixtures resulting from multiple cleavage sites.

Raibaud and Goldberg (10) used subtilisin to produce two complementary fragments of phosphorylase that later proved useful in solving its amino acid sequence (11). A minor side reaction in this proteolysis was cleavage close to the amino terminus. We (12) and others (13) have found that subtilisin will activate phosphorylase b in the presence of the phosphorylase inhibitor caffeine. We also showed that the amino-terminal portion is cleaved under these conditions (12). In this paper, we report the purification and characterization of the phosphorylase derivative produced under these conditions. We have named

this derivative phosphorylase b_S to distinguish it from the tryptic derivative, phosphorylase b'.

EXPERIMENTAL PROCEDURES

Phosphorylase b was prepared according to the method of Fisher and Krebs (14), except that DTT was used throughout instead of cysteine. Enzyme crystallized three or four times and treated with Norit A to remove AMP was used for all experiments. Phosphorylase was stored at -20°C in 40 mM glycerol-2-phosphate, 5 mM DTT, pH 6.8, with 50% glycerol. Before use, the enzyme was dialyzed against the same buffer without glycerol.

Rabbit skeletal muscle was purchased from Pel-Freeze. Subtilisin Carlsberg, subtilisin BPN', trypsin (treated with L-1-Tosylamide-2-phenylethylchloromethyl ketone), and shellfish glycogen were purchased from Sigma Chemical Company. Glycogen was purified by the method of Anderson and Graves (15). Centricon ultrafiltration devices were purchased from Amicon. All chemicals were of reagent grade.

Phosphorylase concentrations were determined spectrophotometrically by using an $E^{1\%}_{280}$ of 13.2. Phosphorylase activity was assayed with 16 mM glucose-1-phosphate, 1% glycogen and 1 mM AMP as in Krebs et al. (16). Kinetic parameters were obtained by fitting kinetic data to initial rate equations using a computer program written in the OMNITAB language (17).

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (18). The gels were stained either with Gelcode silver stain from Pierce Chemical Company or with 0.05% Coomassie Blue-R250 in 25% 2-propanol, 10% acetic acid. In the latter case, the gels were destained with 10% 2-propanol, 10% acetic acid. Densitometry of gels was performed on a Zeinah scanning densitometer from LKB Instruments.

Ultracentrifugation was performed on a Spinco Model E analytical ultracentrifuge at 22±1°C with a rotor speed of 52,000 rpm. Sedimentation coefficients, determined with the aid of a Nikon model 6C microcomparator, were corrected for the viscosity and density of the buffer to water at 20°C.

Samples were hydrolyzed for amino acid analysis by incubating them for 24 hr at 110°C in constant-boiling HCl under vacuum. Amino acid analysis was performed on a Durrum D-400 Amino Acid Analyzer.

Protein sequencing was performed on an Applied Biosystems Model 470A protein sequencer with an on-line Model 120A phenylthiohydantoin amino acid analyzer.

HPLC was performed on a Beckman Model 332M liquid chromatograph with an Altex Model 100-40 variable-wavelength detector. The column used was a Bio-Rad TSK-DEAE-5PW (7.5mm inner diameter X 7.5cm long). Elution was accomplished at a

flow rate of 1.0 ml/min. in 20 mM Tris, pH 7.8, with a linear gradient from 0-0.3 M NaCl in 20 min.

RESULTS

Fig. 1 shows the effects on the activity of phosphorylase b of subtilisin Carlsberg attack at pH 8.5 and 0°C, in the presence of different effectors. These results are similar to those of Dombrádi et al., obtained with subtilisin BPN'(13), except that they observed a maximum of 20% activation. With no effectors or with AMP present, the activity is destroyed. With caffeine present, with or without AMP, subtilisin attack increases the activity by about 50%. Caffeine inhibition of phosphorylase was not a factor in the assays, because the cleavage reaction mixtures were diluted for assay to the point that the caffeine concentration in the assay mixtures was 0.002 mM, well below the K_i (0.08 mM) of phosphorylase b for caffeine.

In order to isolate the products of subtilisin attack on phosphorylase b, the reaction mixtures from the experiment in Fig. 1 were subjected to anion-exchange HPLC (Fig. 2). In each condition, except cleavage with no effectors present, the products eluted in a single peak with a longer retention time than that of phosphorylase b. With no effectors present, the major product of the cleavage eluted in an irregular peak with a retention time close to that of the peaks from the cleavage with effectors present.

Fig. 1 Time course of activity during subtilisin cleavage of phosphorylase b with different effectors present. Phosphorylase b (15 mg/ml in 50 mM Tris, 5 mM DTT, 1 mM EDTA, pH 8.5) was cleaved with subtilisin Carlsberg with either 5 mM caffeine (●), 5 mM caffeine + 1 mM AMP (○), 1 mM AMP (□), or no effectors (■) present. Where caffeine was present, subtilisin concentration was 0.064 mg/ml. Where caffeine was absent, subtilisin concentration was 0.14 mg/ml. At time points, an aliquot of the reaction mixture was added to 1/10th volume of 10 mM PMSF in n-propanol, then assayed as in Experimental Procedures. The activities measured before the addition of subtilisin were taken as 100%

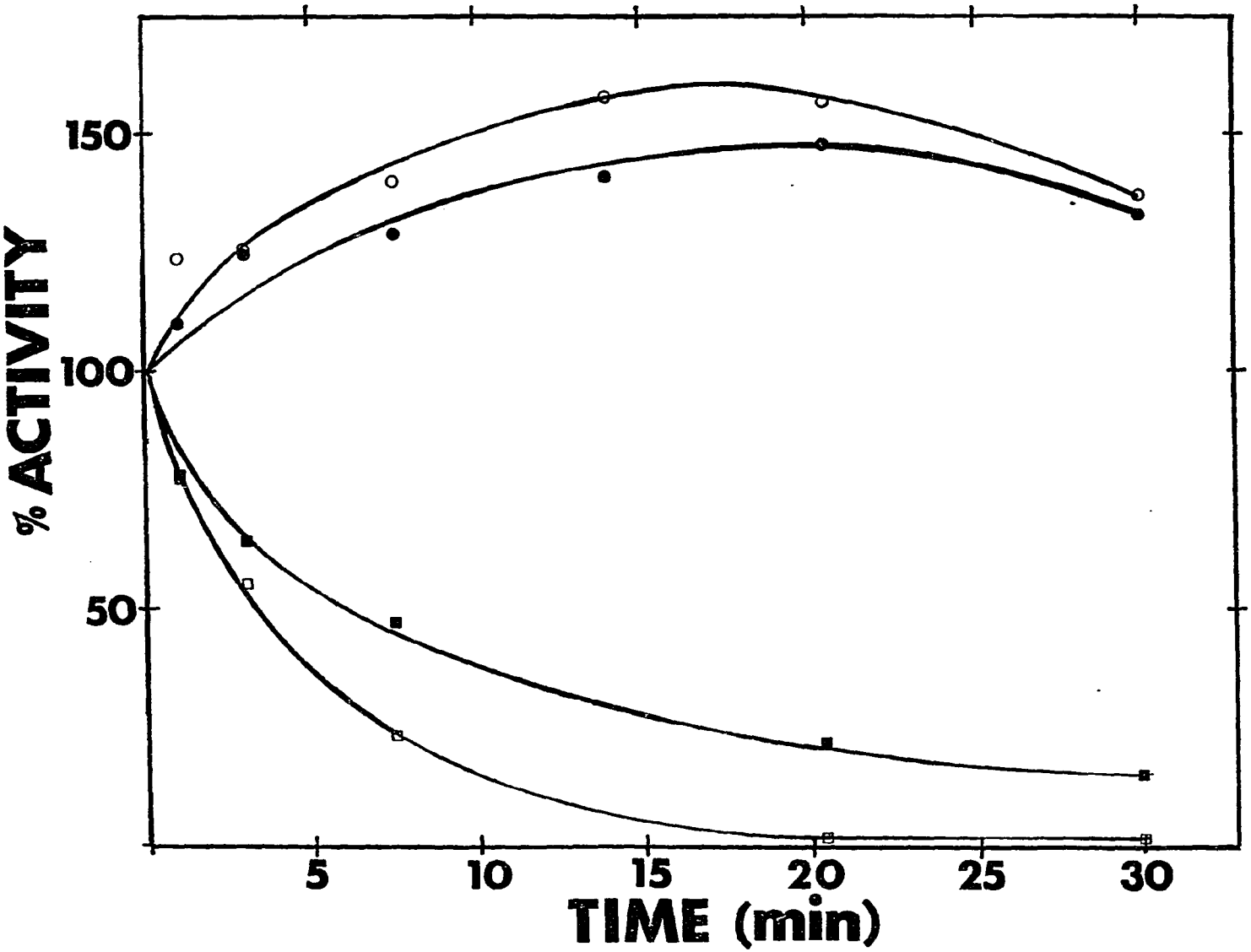
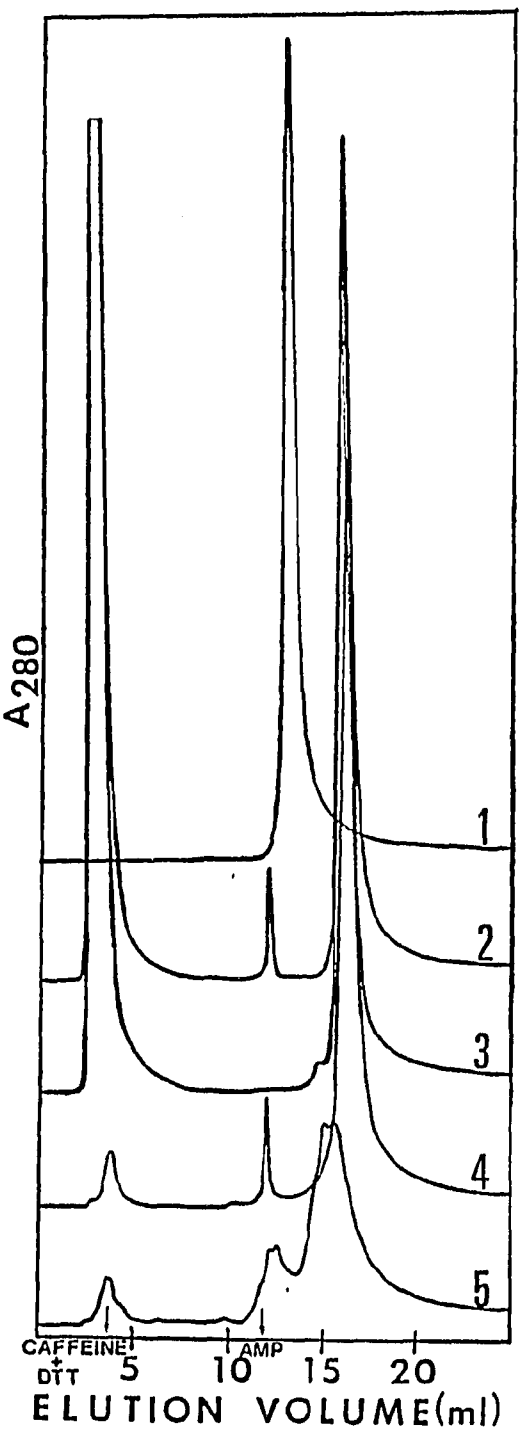

150
100
50
% ACTIVITY
5
10
15
20
25
30
TIME (min)

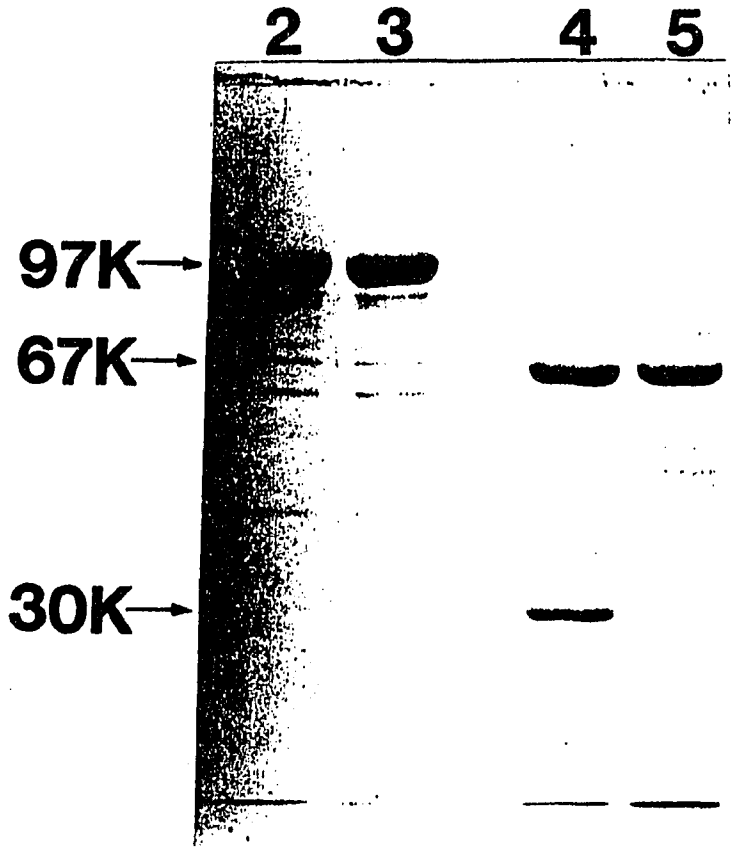
Fig. 2 Anion-exchange HPLC profiles of reaction mixtures from Fig. 1. At 31 min reaction time, an aliquot of each reaction mixture in Fig. 1 was added to 1/10th volume of 10 mM PMSF in n-propanol, and filtered; then, 0.15 ml (2 mg) was subjected to HPLC as in Experimental Procedures. 1 - phosphorylase b. 2 - reaction mixture with caffeine + AMP. 3 - reaction mixture with caffeine only. 4 - reaction mixture with AMP only. 5 - reaction mixture with no effectors


A280
1
2
3
4
5
CAFFEINE + DTT
AMP
5
10
15
20
ELUTION VOLUME(ml)

The product peaks from the HPLC in Fig. 2 were collected and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3). When caffeine was present, with or without AMP, the major product of the cleavage was a protein with the same R_f as phosphorylase b. The HPLC peak from the cleavage with only AMP present consisted mainly of two fragments, with molecular weights of 67kDa and 30kDa. Evidently, these fragments are held together noncovalently, since they elute in a single peak in anion-exchange HPLC. This gel pattern is similar to that obtained by Raibaud and Goldberg from the subtilisin BPN' cleavage of phosphorylase a (10). They also could not separate the fragments by chromatography (gel filtration) unless denaturants were included in the elution buffers. The main HPLC peak from the cleavage with no effectors present contained only a 67kDa fragment. Evidently, AMP protects the 30kDa fragment from further proteolytic degradation. This result is similar to that of Dombrádi et al. (13).

The new results from these experiments are that subtilisin cleavage of phosphorylase b in the presence of caffeine removes only a small part of the protein and increases the activity by about 50%. Subtilisin Carlsberg cleavage in the presence of the phosphorylase inhibitor glucose produced results similar to cleavage in the presence

Fig. 3 SDS-PAGE profiles of HPLC peaks from Fig. 2 0.01 mg of the protein peaks collected from the HPLC runs in Fig. 2 were electrophoresed on a 10% polyacrylamide SDS gel; the gel was stained with Coomassie Blue and destained as in Experimental Procedures. Letters above lanes correspond to the letters on the chromatograms in Fig. 2. Molecular weights of the major bands are labelled as calculated from a plot of log M_r vs. R_f for standards


2
3
4
5
97K
67K
30K

of caffeine, as did cleavage in the presence of caffeine when subtilisin BPN' or trypsin was used as the protease.

The product of subtilisin cleavage of phosphorylase b in the presence of caffeine was named phosphorylase b_S. Phosphorylase b_S was routinely prepared by the following procedure: Phosphorylase b was diluted to 15 mg/ml in 50 mM Tris, 5 mM DTT, 1 mM EDTA, pH 8.5; then, AMP and caffeine were added to concentrations of 1 mM and 5 mM, respectively. Subtilisin Carlsberg (0.022 mg) was added to 1.5 ml of this solution, and this was incubated at 0°C for 8 min, after which 0.15 ml of 10 mM PMSF in n-propanol was added to stop the proteolysis. This incubation period was not long enough to convert all the phosphorylase b. The longer the incubation period, the more impurities were generated, seen as lower molecular weight bands in SDS-PAGE (see Fig. 3). The incubation period chosen was an arbitrary compromise between yield and purity. The solution was then filtered and immediately subjected to anion-exchange HPLC as in Fig. 2. Several aliquots of phosphorylase b were cleaved and chromatographed in succession, and the phosphorylase b_S peaks were collected, pooled, and concentrated with Centricon-30 concentrators. The sample was diluted to about 20 mg/ml, filtered, and rechromatographed as before. The collected and pooled phosphorylase b_S peaks were dialyzed against 40 mM glycerol-2-phosphate, 5 mM DTT, pH 6.8, over-

night at 4°C with one buffer change. The protein was concentrated in a Centricon-30 to at least 80 mg/ml and stored on ice, where it was stable for at least 2 weeks, losing only about 10% of the specific activity during that time.

To determine the purity of phosphorylase b_S^I, prepared as above, SDS polyacrylamide gels were overloaded with phosphorylase b and phosphorylase b_S^I. Densitometry of the gel shows that the phosphorylase b_S^I is about 93% pure.

When the cleavage with caffeine present is stopped before the activity reaches 150% of control, the reaction mixture produces an anion-exchange HPLC profile such as the one in Fig. 4. Not only are the peaks corresponding to phosphorylases b (peak 1) and b_S^I (peak 3) present, but also a peak that elutes between these is seen (peak 2). Fig. 5 shows time courses of the amounts of the three anion exchange HPLC peaks during the cleavage. Phosphorylase b is a dimer of identical subunits, and these results suggest that peak 2 is a hybrid of phosphorylases b and b_S^I, with one subunit cleaved and the other intact. The complete cleavage of the dimer probably goes through such an intermediate, because the simultaneous cleavage of both subunits is unlikely.

Fig. 4 HPLC profile of partially-cleaved phosphorylase b. Phosphorylase b was cleaved as in Fig. 1, with 5 mM caffeine present. The reaction was stopped at 4 min by adding 1/10th volume of 10 mM PMSF in n-propanol. The reaction mixture was filtered and subjected to HPLC as in Experimental Procedures, except that the duration of the NaCl gradient was 30 min

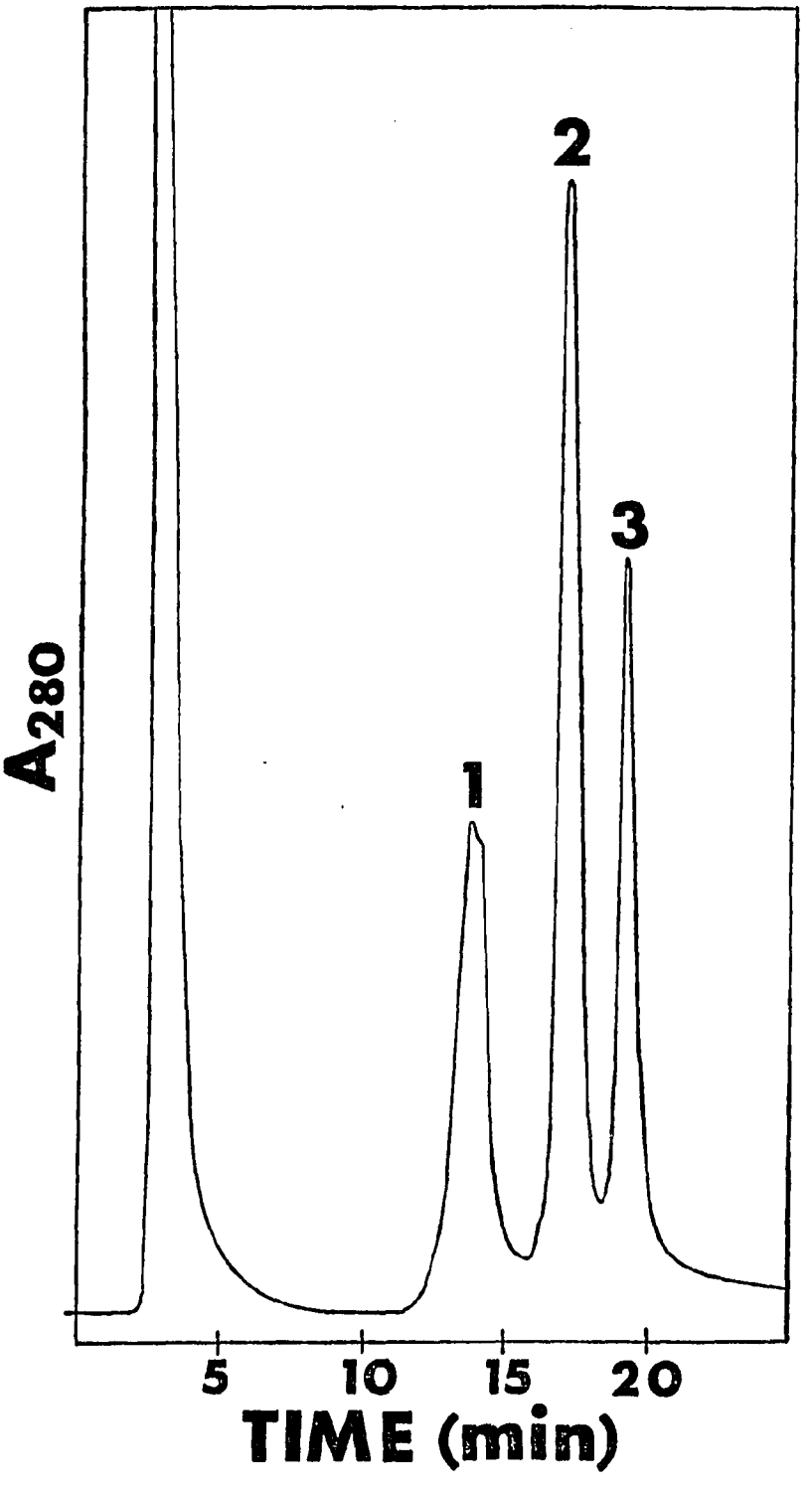
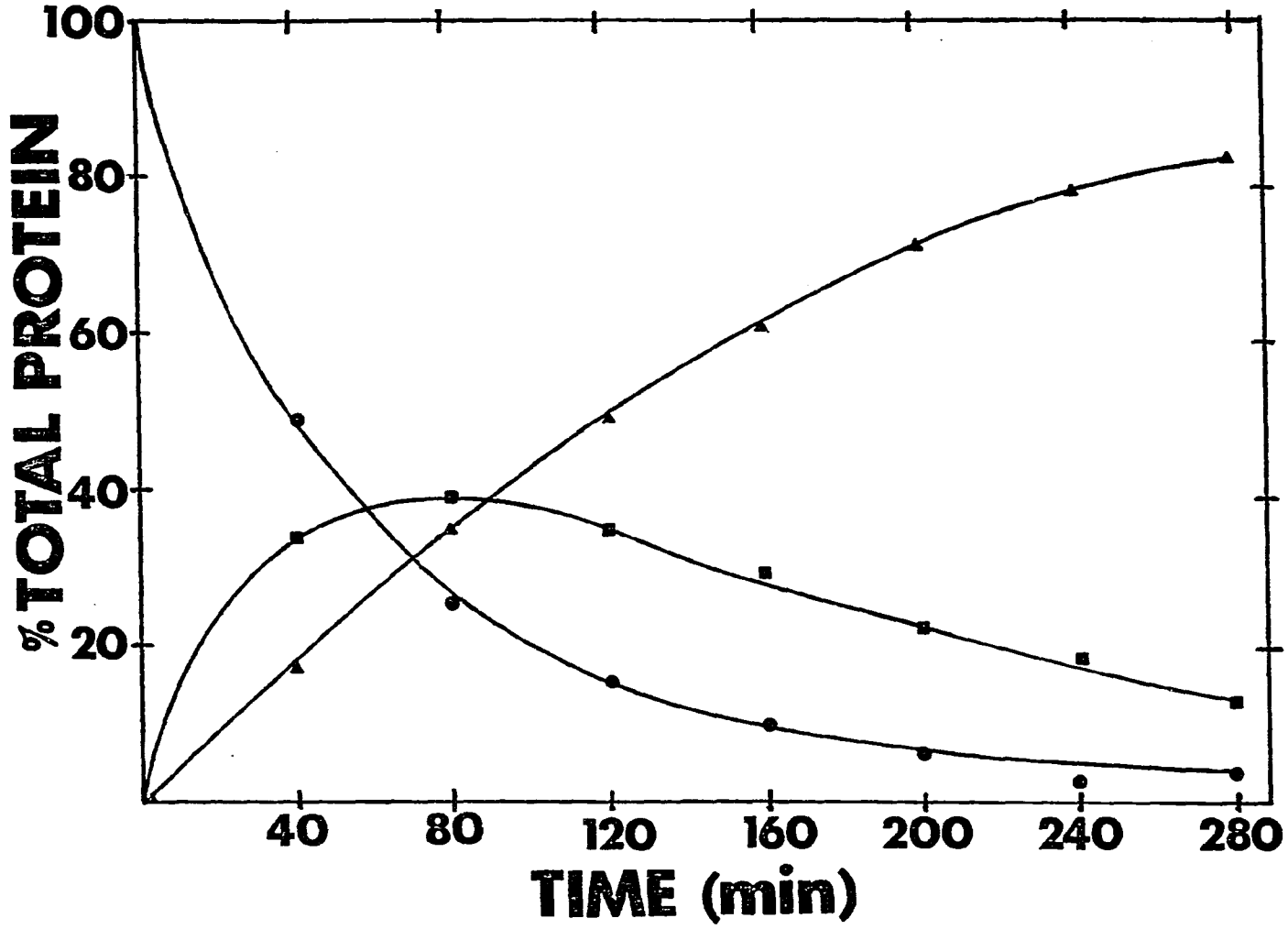

2
3
1
A280
5
10
15
20
TIME (min)

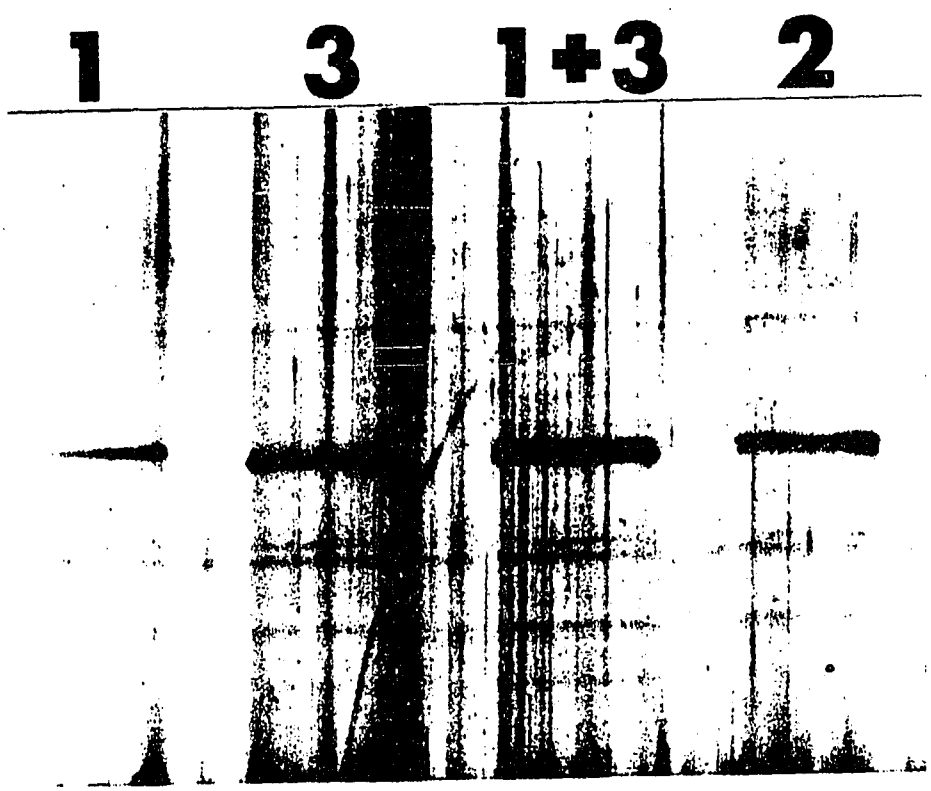
Fig. 5 Time course of subtilisin cleavage of phosphorylase b. Phosphorylase b (4.9 mg/ml in Buffer B with 10 mM caffeine) was cleaved with subtilisin (0.007 mg/ml). At each time point, an aliquot of the reaction mixture was stopped by adding it to 1/10th volume of 10 mM PMSF in n-propanol. The samples were filtered and subjected to HPLC as in Fig. 4. The areas of the peaks were estimated with the aid of a polar planimeter. (●) peak 1, Fig. 4; (■) peak 2; (▲) peak 3


100
80
60
40
20
% TOTAL PROTEIN
40
80
120
160
200
240
280
TIME (min)

To confirm that peak 2 is phosphorylase with a small portion cleaved from one subunit and that peak 3 has the cleavage in both subunits, the three HPLC peaks were analyzed with SDS-polyacrylamide gel electrophoresis (Fig. 6). When phosphorylase b (lane 1) and phosphorylase b_S^i (lane 2) were mixed (lane 3), two bands were resolved. Two bands are also present in peak 2 (lane 4), proving that peak 2 is a phosphorylase b-b_S^i hybrid. When larger amounts of protein are applied to the gel and stained with Coomassie Blue, the cleaved and uncleaved subunits are unresolved.

An attempt was made to isolate the phosphorylase b-b_S^i hybrid and determine its activity. This was complicated, however, by the discovery that, if a small section of the middle of the hybrid peak is collected from a preparative HPLC run and then rechromatographed, significant amounts of the phosphorylase b and phosphorylase b_S^i peaks appear. This is not due to contamination from the phosphorylase b and b_S^i peaks, because the amounts of these peaks increase with increasing incubation time of the hybrid peak before rechromatography. Similarly, if pure phosphorylase b and phosphorylase b_S^i are mixed and allowed to stand for as little as 5 min, then subjected to HPLC, the hybrid peak appears in the chromatogram. This suggests that the subunits of the different enzyme forms recombine with each

Fig. 6 SDS-PAGE of peaks from HPLC of partially cleaved phosphorylase b. The numbers above the lanes correspond to the numbers of the peaks in Fig. 4. 30 ng of proteins (70 ng for peak 2) were electrophoresed on an 8% polyacrylamide SDS gel and silver stained as in Experimental Procedures. Peak 2 was collected during the preparation of phosphorylase b_S^I then rechromatographed as before. From this run, only a small portion of the middle of peak 2 was collected for electrophoresis, to preclude contamination from peaks 1 or 2


1
3
1+3
2

other, probably through dissociation-association. This hybridization does not occur during the chromatography. If phosphorylases b and b_S^I were injected into the column separately, then eluted with the NaCl gradient, no hybrid peak was seen in the chromatogram. An estimate of the hybrid activity was made by collecting a narrow portion of the middle of the hybrid peak from a preparative HPLC run and assaying it immediately. This gave an activity for the hybrid midway between that of phosphorylases b and b_S^I.

The peptide(s) missing in phosphorylase b_S^I could be from either the amino or carboxyl end of phosphorylase, or from both ends. To determine if part of the amino-terminus is missing, phosphorylase b_S^I was subjected to automated Edman sequence analysis. Whereas phosphorylase b cannot be sequenced by this method because of its acetylated N-terminus, ten consecutive cycles of Edman degradation of phosphorylase b_S^I yielded mixtures of two amino acids corresponding to a mixture of phosphorylase sequences 17-26 and 19-28. The first cycle of Edman degradation yielded alanine (residue 19) and glycine (residue 17) in an approximately 60:40 ratio, respectively. This is the best estimate of the ratio of the two sequences, because incomplete yields from the Edman degradation complicate interpretation of the results from subsequent cycles.

To determine whether phosphorylase $b_S^{'}$ is missing part of the carboxyl-terminus of phosphorylase b, amino acid analysis was performed on the peptide products of the cleavage reaction that produces phosphorylase $b_S^{'}$. The cleavage reaction mixture was subjected to ultrafiltration with a Centricon-10, which has an exclusion limit of 10 kDa. The concentrated protein was washed with buffer and refiltered to minimize noncovalent binding of cleaved peptides to the protein. The results of amino acid analysis of the filtrate are contained in Table 1. The experimental ratios of the amino acids were calculated from

$$\text{Ratio} = \frac{\text{moles amino acid X 15}}{\text{total moles all amino acids (except Gly and Leu)}}$$

The reason that glycine and leucine are excluded from the formula is that these are residues 17 and 18, and these residues are present in only 60% of the cleaved peptides. Because leucine is also present in the peptide [1-16], only 15 residues are used in the calculation; thus the factor of 15 in the numerator of the formula. The composition of the peptides released corresponds closely to the composition expected from a 60:40 mixture of phosphorylase sequences [1-18] and [1-16], respectively. This shows that no peptides

Table 1. Amino acid analysis of peptides released by subtilisin cleavage of phosphorylase b. 0.2 ml of phosphorylase b (96 mg/ml in 20 mM Tris, 5 mM DTT, 1 mM EDTA, 5 mM caffeine, pH 8.5) was incubated for 4 min at 0°C with subtilisin Carlsberg (0.1 mg/ml). The reaction was stopped by the addition of 1.8 ml of 5 mM Tris, 1.2 mM DTT, 0.25 mM EDTA, 1 mM PMSF,pH 8.5. The sample was filtered with a Centricon-10 (exclusion limit=10kDa) to about 0.1 ml; then, another 2 ml of the stopping buffer was added, and the sample filtered to dryness. The filtrate was lyophilized and subjected to amino acid analysis as in Experimental Procedures. A blank sample was also prepared, in which inactivated subtilisin was added to the reaction mixture after the stopping buffer

Amino Acid	Ratio from Amino Acid Analysis[a, b]	Ratio Expected[c]
Arg	3.32	3
Asp+Asn	1.07	1
Gly	0.51	0.6
Glu+Gln	2.90	3
Ile	0.98	1

Leu	1.74	1.6
Lys	1.92	2
Pro	0.96	1
Ser	2.80	3
Val	1.06	1

[a]Ratios were calculated from the formula given in the text.

[b]Average of two experiments.

[c]Ratio expected from a 60:40 mixture of phosphorylase sequences [1-18] and [1-16], respectively (see text). The sequence of the amino-terminal region of phosphorylase is:

```
                              5                   10
N-acetyl-Ser-Arg-Pro-Leu-Ser-Asp-Gln-Glu-Lys-Arg-

                        15                  20
Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu-Ala-Gly-Val-Glu-
```

are cleaved by subtilisin from the carboxyl-terminus of phosphorylase b. The conclusion from this experiment and the sequencing experiment is that the phosphorylase b_S^1 preparation is a mixture of phosphorylase derivatives, about 60% of which is the derivative missing only the amino-terminal 18 residues, with the rest being the derivative missing only the amino-terminal 16 residues.

To compare the monomer-monomer interactions in phosphorylases b and b_S^1, the rate of resolution of the coenzyme, pyridoxal phosphate, from each enzyme form was measured. Shaltiel et al. (19) showed that incubation of phosphorylase b in high concentrations of imidizolium salts and cysteine caused the pyridoxal phosphate to be exposed to the solvent, where it reacted with cysteine and was removed from the enzyme, inactivating it. The imidizolium salts alone also caused monomerization of phosphorylase b without resolution of the coenzyme. Hedrick et al. showed that the rate of resolution is related to the rate of monomerization (20). Separate solutions of phosphorylase b and phosphorylase b_S^1 (each 2 mg/ml in 40 mM glycerol-2-phosphate, 5 mM DTT, pH 6.8) were diluted 1:1 with 0.4 M imidizolium-citrate, 0.1 M cysteine, pH 6.5 and the solutions were incubated on ice. At time points (1.5, 10, 20, 30, 40, 50, and 60 min), aliquots were removed and assayed as in Experimental Procedures. The half-time for

resolution was 23 min for phosphorylase b and 4.3 min for phosphorylase b_S^I. Incubation of the enzymes in the same buffer with 2-mercapto-ethanol substituted for cysteine caused no loss of activity of either enzyme. The conclusion is that the monomer-monomer interactions are weaker in phosphorylase b_S^I than in phosphorylase b.

To compare the dimer-dimer interactions in phosphorylases b and b_S^I, ultracentrifugation studies were done. With no effectors present, both forms exist as a dimer (8.7S). When saturating amounts of AMP and glucose-1-phosphate are present, phosphorylase b exists as the tetramer (12.4S), whereas phosphorylase b_S^I gives a lower sedimentation coefficient (10.8S), indicating that it is in equilibrium between the dimer and tetramer. Another indication of altered subunit interactions is the observation that phosphorylase b_S^I does not crystallize under conditions in which phosphorylase b readily crystallizes (1 mM AMP + 10 mM Mg^{2+} at 0°C).

Initial-rate kinetic studies were done to compare the enzymatic properties of phosphorylases b and b_S^I. The K_M's for glucose-1-phosphate at saturating AMP and glycogen are similar, 5.7 mM for phosphorylase b_S^I and 6.0 mM for phosphorylase b. The V_M of phosphorylase b_S^I, 102 I.U./mg, is 43% higher than that of phosphorylase b, 71 I.U./mg. The reciprocal plots for AMP (assayed with 16 mM glucose-1-

phosphate and 1% glycogen) with both enzyme forms show upward curvature, characteristic of cooperative binding. The Hill coefficient for AMP with both forms of the enzyme is 1.4.

Phosphorylases b and b_S^1 are affected differently by glucose-6-phosphate and sodium fluoride. When assayed with 5 mM glucose-1-phosphate, 1% glycogen, saturating (1 mM) AMP and 5 mM glucose-6-phosphate, phosphorylase b was inhibited 69%, but phosphorylase b_S^1 was inhibited only 23%. When assayed at low (0.01 mM) AMP and 0.1 M NaF, phosphorylase b was activated 207%, but phosphorylase b_S^1 was activated only 24%. With respect to these two properties, phosphorylase b_S^1 resembles phosphorylase a, which is not affected by glucose-6-phosphate or sodium fluoride.

DISCUSSION

The main result in this paper is that removal of only the amino-terminal region of phosphorylase b causes an increase of 43% in the V_M of the enzyme. The specific activity of phosphorylase $b_S^{\prime}$ is close to that of phosphorylase a, although phosphorylase $b_S^{\prime}$ still requires AMP for activity and phosphorylase a does not. If the amino-terminal segment of phosphorylase b were freely mobile, as has been previously assumed from the X-ray crystallographic studies, then its removal would have no effect on the properties of the enzyme. The results in this paper indicate that the amino-terminal segment of phosphorylase b interacts with the rest of the protein and that this interaction lowers the V_M and sensitizes the enzyme to glucose-6-phosphate inhibition. One possible explanation for the contradiction between these results and the X-ray crystallographic results is that the phosphorylase b used for the X-ray studies was crystallized with IMP, which promotes the inactive "T" conformation. In the active "R" conformation, the position of the amino-terminal segment may be quite different. Experiments are under way to probe this region by determining whether various effectors of phosphorylase affect the rate of proteolysis in the amino-terminal region.

There are two main sites of subtilisin cleavage in phosphorylase b. When caffeine is present, the cleavage is mainly in the amino-terminal region. When caffeine is absent, cleavage occurs between residues 264 and 265 (10), and also in the amino-terminal region. The evidence for the amino-terminal cleavage in the absence of caffeine is that the product of the cleavage with only AMP present coelutes in HPLC with phosphorylase $b_S^{\prime}$, which has only the amino-terminal cleavage. Also, when cleavage with only AMP present is stopped before complete inactivation occurs, the SDS-PAGE pattern of the product shows two bands in the 30 kDa region, which includes the amino-terminal region (data not shown). Although we did not attempt to correlate the rates of cleavage at the two sites with the rates of activation or inactivation, it is likely that the cleavage in the amino-terminal region causes activation and altered HPLC elution, and that cleavage between residues 264 and 265 causes inactivation. Both cleavages probably occur under all conditions, and different effectors alter the exposure of the different sites.

The interactions between subunits, both monomer-monomer and dimer-dimer interactions, are weaker in phosphorylase $b_S^{\prime}$ than in phosphorylase b. The amino-terminal 19 residues in phosphorylase b are not resolved by X-ray crystallography (1), so it is not known whether these

residues interact directly with the opposite subunit, as they do in phosphorylase a (2). This region must be involved in strengthening the subunit association in phosphorylase b, however, because its removal weakens the association. The amino-terminal segment of phosphorylase b may interact directly with the opposite subunit, or it may be that the removal of this segment changes the overall conformation, resulting in weaker subunit association.

The Hill coefficients for AMP activation of phosphorylases b and b_S are the same, indicating that removal of the amino-terminal segment does not affect the homotropic cooperativity. Previous reports on phosphorylase b', the product of limited trypsin proteolysis of phosphorylase a, gave conflicting results on this issue. In these works, phosphorylase b' was assumed to be phosphorylase missing only the amino-terminal 16 residues. The preparation of phosphorylase b' in our laboratory did not display cooperative AMP binding (7). Bot et al. found that their preparation of phosphorylase b' did display cooperativity (8). Huminski et al., however, found that at least two forms of phosphorylase b' with different properties could be produced by using different trypsin concentrations (9). Their preparations of phosphorylase b' showed multiple bands in SDS polyacrylamide gel electrophoresis, so it is possible that the other groups

were also working with phosphorylase b' preparations with multiple cleavage sites. Raibaud and Goldberg (10) found that subtilisin cleavage of the peptide bond between residues 264 and 265 caused inactivation of phosphorylase, even though the two fragments stayed together. Trypsin probably also cleaves peptide bonds other than the 16-17 bond, producing phosphorylase derivatives with altered properties. The different preparations of phosphorylase b' used previously might have had different extents of internal cleavage. The preparation of phosphorylase b_S^1 in the present work is 93% pure by SDS-polyacrylamide gel electrophoresis, so any differences between phosphorylase b and phosphorylase b_S^1 are due to removal of the amino-terminal portion.

When Raibaud and Goldberg treated phosphorylase a with subtilisin BPN', they observed a minor cleavage at a site close to the amino-terminus (10). This site was later shown to be between residues 16 and 17 (11). The additional cleavage site observed in our work (between residues 18 and 19) is likely due to the use of a different protease, subtilisin Carlsberg.

Phosphorylases b and b_S^1 readily form a hybrid when mixed together, so both forms of the enzyme must dissociate to monomers to some extent. Davis et al. (21) reached a similar conclusion from their observation that two of the

phosphorylase isozymes of rabbit heart hybridize when mixed together.

The fact that the bb$_s$ hybrid can be separated from both phosphorylases b and b$_s$ by ion-exchange HPLC suggests that, possibly, an ab hybrid could be separated from phosphorylases a and b by HPLC. Earlier studies (22,23) suggested that such hybrids are formed during the interconversions of phosphorylases a and b by phosphorylase kinase and phosphorylase phosphatase. Fisher et al. separated these three forms of phosphorylase by TEAE-cellulose chromatography, but did not isolate the hybrid because it evidently recombined to form phosphorylases a and b (24). Preliminary experiments in our laboratory indicate that such a separation is possible by ion-exchange HPLC, and that the phosphorylase ab hybrid can be stabilized. We are working to isolate the ab hybrid in pure form and study its properties.

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SECTION II:

KINETIC ANALYSIS OF THE SEPARATE PHOSPHORYLATION EVENTS IN THE PHOSPHORYLASE KINASE REACTION

William R. Harris and Donald J. Graves

From the Department of Biochemistry and Biophysics
Iowa State University
Ames, Iowa

SUMMARY

Glycogen phosphorylase, a dimer of identical subunits, is activated by phosphorylase kinase catalyzed phosphorylation of one serine residue in each subunit. In this paper, the effect of phosphorylation of one subunit on the phosphorylation of the other subunit was studied. The three forms of phosphorylase, phosphorylase b (nonphosphorylated), phosphorylase ab (one subunit phosphorylated) and phosphorylase a (both subunits phosphorylated) were separated by anion-exchange high performance liquid chromatography (HPLC). Purified phosphorylase ab was found to be stable under the conditions of the phosphorylase kinase assay. Phosphorylase kinase was found to have a lower K_M for phosphorylase ab (3.9 $\pm$ 0.24 μM) than for phosphorylase b (14.9 $\pm$ 2.6 μM), which implies that phosphorylation of the first subunit causes conformational changes that make the other subunit bind more tightly to the kinase. Using the HPLC separation as a simultaneous assay for the 3 forms of phosphorylase during the phosphorylase kinase reaction, the apparent first order rate constants for the first (k_1) and second (k_2) were measured. It was found that AMP reduced the ratio k_2/k_1 from 3.7 without AMP to 1.4 with AMP. This implies that AMP reduces the conformational differences between

phosphorylases b and ab. The ratio k_2/k_1 was reduced from 3.7 with the multimeric holophosphorylase kinase to 2.1 when the monomeric $\gamma\delta$ complex of phosphorylase kinase subunits was used as the enzyme. This indicates that simultaneous phosphorylation of the subunits of phosphorylase may occur with the holophosphorylase kinase.

INTRODUCTION

Enzyme catalyzed phosphorylation-dephosphorylation of proteins is an important control mechanism in many aspects of the metabolism of virtually all cells. The activities of the various protein kinases and phosphatases are controlled not only by factors that affect the kinases and phosphatases themselves, but also by factors that affect the conformations of their protein substrates.

Glycogen phosphorylase offers a relatively simple system in which to study substrate control of phosphorylation-dephosphorylation. Phosphorylase kinase catalyzes the phosphorylation of only serine-14 in each of the identical subunits of the phosphorylase dimer (1,2,3), converting phosphorylase b, which requires AMP for activity, to phosphorylase a, which is active without AMP.

Some effectors of phosphorylase change its conformation in such a way as to affect the phosphorylation of serine-14. An early study on phosphorylase kinase showed that glycogen activated and glucose-6-phosphate inhibited the kinase with phosphorylase b as substrate (4). Others later showed that these effects were substrate directed (5,6). AMP and glucose-1-phosphate had no effect on the phosphorylase kinase reaction(4).

Phosphorylation of serine-14 changes the conformation of phosphorylase, but it is not known whether the phosphorylation of one subunit of the phosphorylase dimer changes the conformation so as to affect the phosphorylation of the other subunit. One purpose of this study is to address this question by comparing phosphorylase b (unphosphorylated) and phosphorylase ab (one subunit phosphorylated) as substrates for phosphorylase kinase, using initial rate kinetics.

Fisher et al. (7) first obtained direct evidence for the formation of an intermediate "hybrid" in the interconversions between phosphorylases b and a. They purified phosphorylase ab by anion-exchange chromatography, but found that it was unstable, dissociating and recombining to form phosphorylases b and a. We have purified phosphorylase ab by anion-exchange HPLC[1], and have shown it to be stable under the conditions of the phosphorylase kinase assay.

The HPLC separation of phosphorylases b, ab and a was used as a simultaneous assay for these proteins during the time course of phosphorylase b to a conversion. This assay was used to determine the influence of effectors on the relationship between the phosphorylation of the first and second subunits of phosphorylase.

The fact that substantial amounts of the intermediate are formed during the phosphorylase kinase reaction argues against a mechanism in which the two subunits of phosphorylase fit simultaneously into two active sites of the multimeric phosphorylase kinase and are not released until both subunits are phosphorylated. Nevertheless, some extent of simultaneous phosphorylation of both subunits of phosphorylase is still a possibility. For this reason, the HPLC assay was used to compare intact phosphorylase kinase with the complex between the catalytically active γ subunit of phosphorylase kinase and calmodulin. The similar $\gamma\delta$ complex of phosphorylase kinase subunits has been shown to exist as the heterodimer (8), so simultaneous phosphorylation of both phosphorylase subunits is improbable with this enzyme form. If simultaneous phosphorylation does not occur, then the relationship between the phosphorylation of the first and second subunits of phosphorylase should be the same with the two enzyme forms.

EXPERIMENTAL PROCEDURES

Reagents

All chemicals were of reagent grade and used without further purification, except glucose. Unpurified glucose caused large baseline peaks in the HPLC runs. Glucose was purified by stirring 1 l of a 1.5 M solution about 150 g of Amberlite MB-3 mixed-bed ion-exchanger (Mallinkrodt) for several hours. The solution was then filtered through glass microfiber filters and stored frozen. [γ-^{32}P]ATP was purchased from New England Nuclear.

Enzymes

Phosphorylase b was prepared from frozen rabbit skeletal muscle (Pel-Freez) according to the method of Fisher and Krebs (9), except that DTT was used throughout instead of cysteine. Enzyme crystallized 3 or 4 times and treated with Norit A to remove AMP was used for all experiments. Phosphorylase concentrations were determined spectrophotometrically by using an $E^{1\%}_{280}$ of 13.2.

Phosphorylase ab and phosphorylase a were purified by HPLC from reaction mixtures containing partially phosphorylated phosphorylase. The 1.5 ml reaction mixtures consisted of 16.7 mg/ml phosphorylase b, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 3 mM ATP and 0.017 mg/ml phosphorylase kinase in a

buffer of 8 mM MOPSO, 25 mM Tris, 2 mM DTT, pH 8.2. The reactions were initiated by the addition of kinase, and incubated at 30°C. At 5 min, the reactions were stopped by the addition of 0.075 ml of 0.2 M EGTA. The mixtures were filtered through 0.45 μm membranes and separated on the HPLC as described later in this section, except that the NaCl gradient was from 0-0.15 M NaCl in 15 min, starting 5 min after injection. The phosphorylase ab peak, which eluted at 10 min, was collected in 3 ml fractions in tubes containing 0.03 ml of 0.1 M AMP. The phosphorylase a peak eluted at 17 min. Several reaction mixtures were chromatographed, the peaks were collected and pooled, then the pools were concentrated in Centicon 30 devices from Amicon and rechromatographed as before. The peaks were collected and pooled as before, then dialyzed against 40 mM MOPSO, 10 mM DTT, pH 6.8. The dialysis buffer for phosphorylase ab contained 0.5 mM AMP or 3 mM ATP. The dialyzed proteins were concentrated in Centricon 30s. The protein concentrations were determined spectrophotometrically as before for phosphorylase b, or, when nucleotides were present, by the method of Bradford (10), using phosphorylase b as a standard.

Phosphorylase kinase, prepared by the method of Hayakawa et al. (11) and purified as described by Cohen (12), was generously provided by Scott Kee in our

laboratory. Activated phosphorylase kinase was prepared by incubating nonactivated phosphorylase kinase at 0.5 mg/ml with 0.015 mg/ml of the catalytic subunit of cAMP-dependent protein kinase (Sigma) at pH 6.8 in the presence of 10 mM $MgCl_2$, 3 mM ATP and 1 mM $CaCl_2$ for 4 h at 30°C. The ratio of phosphorylase kinase activity at pH 6.8 to that at pH 8.2 was 0.44 for the activated enzyme, as assayed by the method of Brostrom et al. (13).

The complex between the active γ subunit of phosphorylase kinase and calmodulin was generously provided by Chiun-Jye Yuan in our laboratory. It was prepared essentially by the method of Kee and Graves (14) with some modifications. The γ subunit peak from reverse-phase HPLC was collected in silanized polystyrene tubes and stored on ice. The acetonitrile from the HPLC buffers was evaporated by a gentle stream of nitrogen. The enzymatic activity was restored by incubation on ice in a pH 8.2 mixture containing 43 mM HEPES, 25 mM Tris, 2 mM DTT, 3 mM $CaCl_2$, 20% glycerol, 0.5 mg/ml calmodulin and 0.06 mg/ml γ subunit.

Phosphorylation assays

For HPLC assays, the reaction mixtures consisted of phosphorylase at 2.0 mg/ml (unless otherwise specified), 0.1 mM $CaCl_2$, 3 mM ATP, 10 mM $MgCl_2$, 0.8-2.5 μg/ml activated phosphorylase kinase or γ-calmodulin at 1.5-2.0 μg/ml (γ

concentration), effectors at specified concentrations, and a buffer of 20 mM MOPSO, 5 mM DTT, pH 6.8. The reaction mixtures were preincubated at 30°C. The reactions were initiated by the addition of Mg-ATP, and at time points, 0.1 ml aliquots were removed and added to 0.1 ml of ice-cold, filtered 20 mM EGTA, then immediately frozen in liquid nitrogen or dry ice-ethanol. Samples were thawed just before injection of 0.18 ml into the HPLC.

HPLC

HPLC was performed on a Beckman 332M liquid chromatograph with a Beckman 165 detector and a Nelson Analytical Model 3000 data system. The column used was a TSK-DEAE-5PW (7.5 mm inner diameter X 7.5 cm length) from Bio-Rad. Elution was accomplished at a flow rate of 1.0 ml/min in a buffer of 30 mM glycerol-2-phosphate, 150 mM glucose, pH 6.8, with a gradient from 0-0.1 M NaCl in 10 min starting at injection of the sample. After 10 min the NaCl gradient went to 0.5 M in 5 min.

Data Analysis

The HPLC peaks were integrated by the Nelson Analytical computer program, and the fraction of the total protein area of the run was calculated for each component. A nonlinear least squares curve-fitting program written for the Apple

IIe computer (15) was used to calculate the apparent rate constants, k_1 and k_2, that gave the best fit of the data to the equations for first order consecutive reactions, given below for the conversion of phosphorylase b to phosphorylase a:

$$[\text{phosphorylase b}] = [b]_o e^{-k_1 t}$$

$$[\text{phosphorylase ab}] = [b]_o \frac{k_1}{k_2 - k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right)$$

$$[\text{phosphorylase a}] = [b]_o \left(1 - \frac{k_2}{k_2 - k_1} e^{-k_1 t} + \frac{k_1}{k_2 - k_1} e^{-k_2 t}\right)$$

The apparent rate constant k_1 calculated by the computer program was divided by 2 because phosphorylase b has 2 phosphorylation sites per dimer, while phosphorylase ab has only 1. If the reactivities of the two phosphorylation sites are the same in phosphorylases b and ab, then the measured rate constant k_1 would be 2 X k_2.

Initial rate kinetics

Substrates (phosphorylases b and ab) were prepared as follows: protein solutions in 40 mM MOPSO, 10 mM DTT, 0.5 mM AMP, pH 6.8 were drop-dialyzed (16) on Millipore VSWP membranes (0.15 ml on a 13 mm disc) against the same buffer with 3 mM ATP substituted for AMP. The protein

concentrations were determined by Bradford assay as before, and the samples diluted with the dialysis buffer to 2 times the final concentrations in the assays. Reaction mixtures containing 0.2 mM $CaCl_2$, 20 mM $MgCl_2$, 0.2-1.0 μg/ml activated phosphorylase kinase and 3 mM [γ-^{32}P]ATP (400 cpm/pmol) were preincubated for 2 min at 30°C. Reactions were initiated by the addition of an equal volume of substrate solution, containing varying concentrations of phosphorylase b or phosphorylase ab. At 3, 6 and 9 min, 30 μl of the reaction mixture was spotted on Whatman ET-31 paper squares (2 mm) and the squares were washed, dried and counted as in Reimann et al. (17), except that 1% sodium pyrophosphate was included in the first 3 wash solutions. Kinetic parameters were calculated by fitting data to initial rate equations using a computer program written in the OMNITAB language (18).

RESULTS

Isolation and stabilization of phosphorylase ab

Fig. 1 shows a chromatogram of a reaction mixture of phosphorylase partially phosphorylated by phosphorylase kinase, then subjected to HPLC as in Experimental Procedures. HPLC of standards showed that peak 1 is phosphorylase b and peak 3 is phosphorylase a. That peak 2 is phosphorylase ab was confirmed by the shape of the plot of the area of peak 2 vs. time during the reaction (see Fig. 4), characteristic of an intermediate in a two-step reaction. Also, only 1 mole of radiolabelled phosphate per $2x10^5$g (M_r of phosphorylase dimer) from ATP could be incorporated into isolated peak 2, as compared to 2 moles for phosphorylase b.

If glucose was excluded from the HPLC buffers, phosphorylase a eluted in a very broad peak. This is probably due to the dimer-tetramer equilibrium of phosphorylase a. Glucose has been shown to prevent tetramer formation (19).

Others (4,20) have separated phosphorylase ab by ion-exchange chromatography, but they found that it was unstable, dissociating and recombining to form phosphorylases b and a, as seen by rechromatography. Fig. 2 shows chromatograms of phosphorylase ab prepared as in

Fig. 1 Anion-exchange HPLC chromatogram of partially phosphorylated phosphorylase. A sample from an assay of the time course of the phosphorylase kinase reaction was subjected to HPLC as in Experimental Procedures

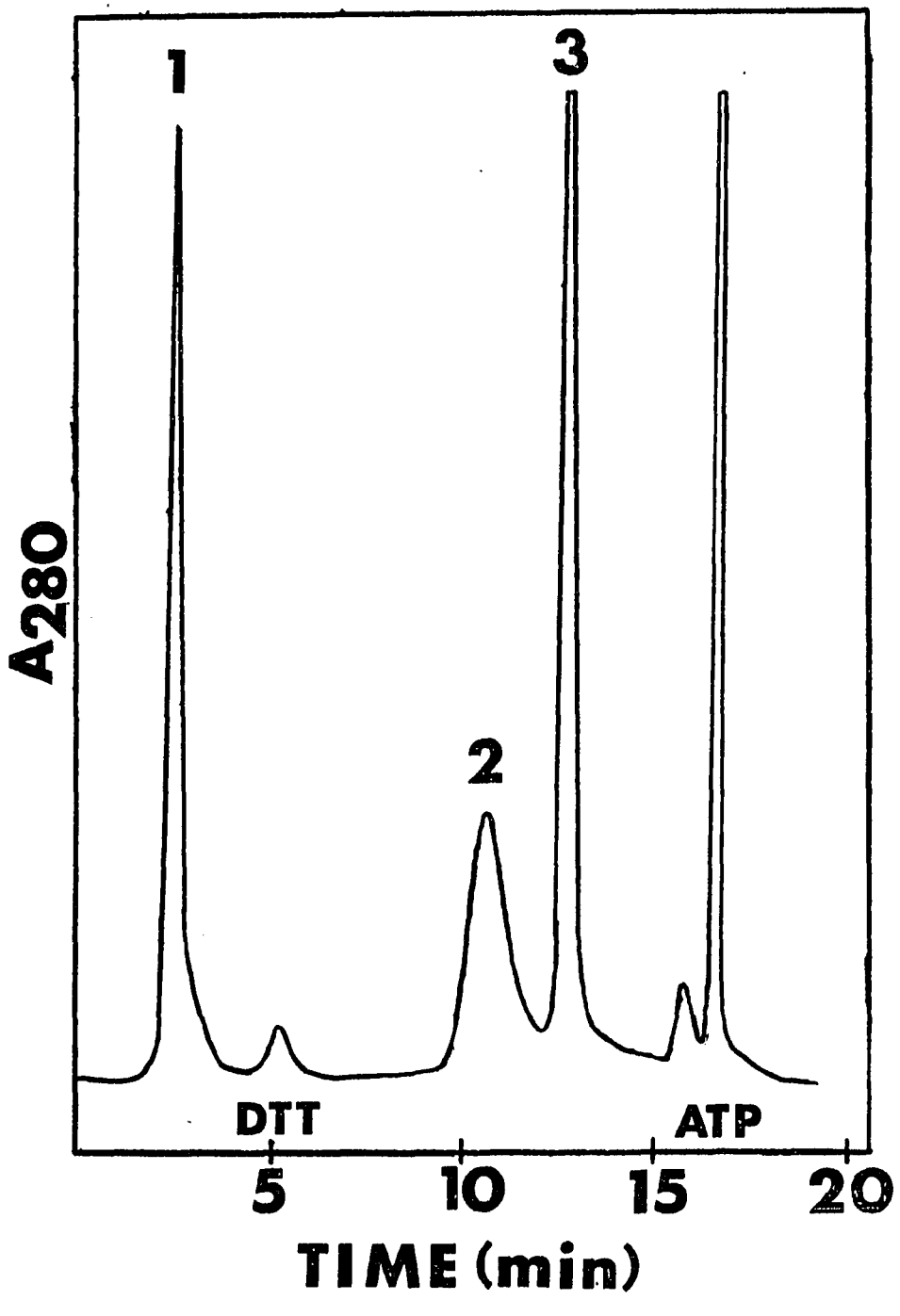

1
3
2
A280
DTT
ATP
5
10
15
20
TIME (min)

Fig. 2A Stability of phosphorylase ab. 0.085 mg of phosphorylase ab in buffer with 0.5 mM AMP was subjected to HPLC as in Experimental Procedures, except that the elution buffer was 25 mM Tris, 100 mM glucose, pH 7.8, with a gradient of 0-0.25 M NaCl from 0-20 min, starting at sample injection. The elution positions of phosphorylases b and a are indicated with arrows

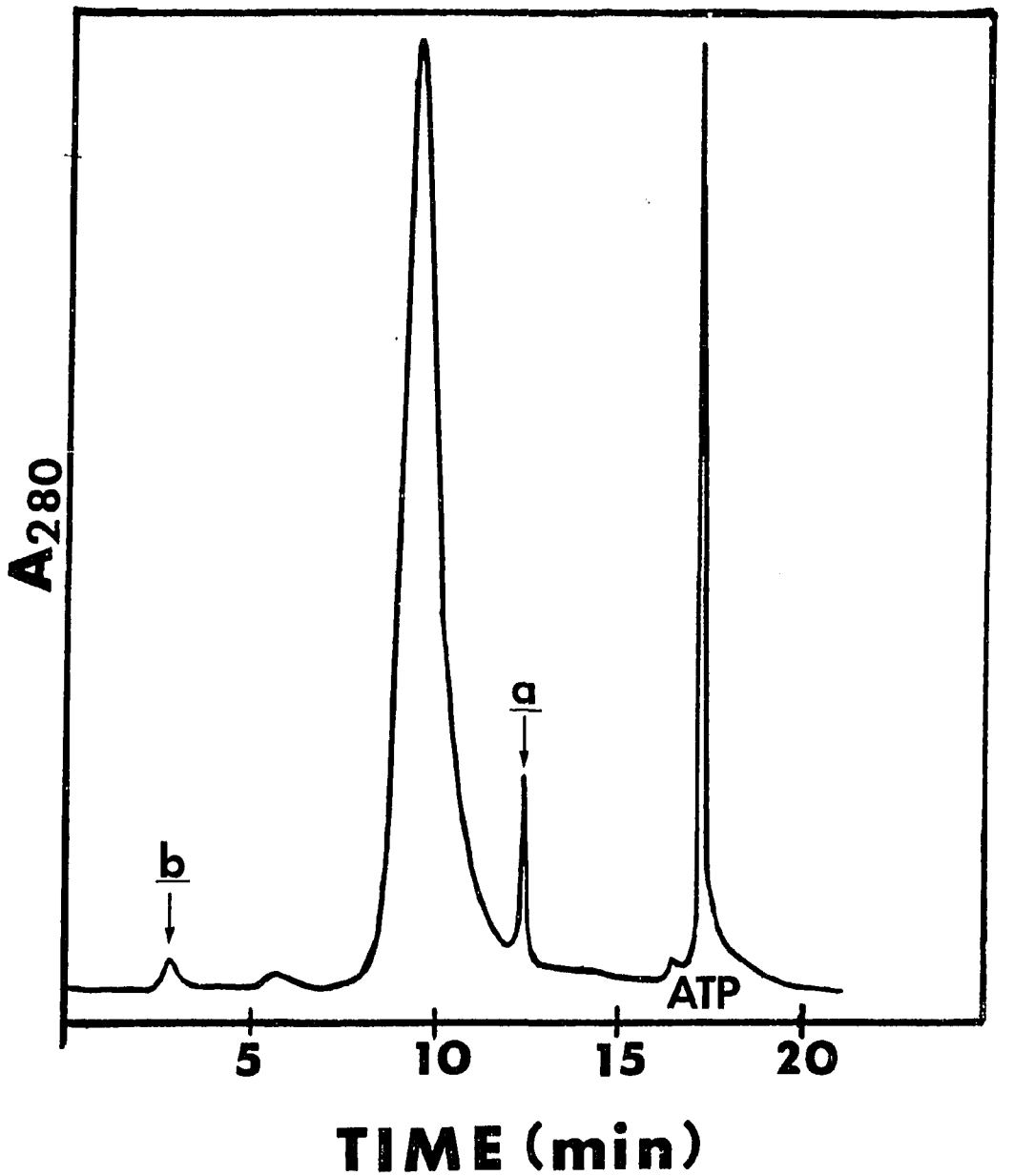
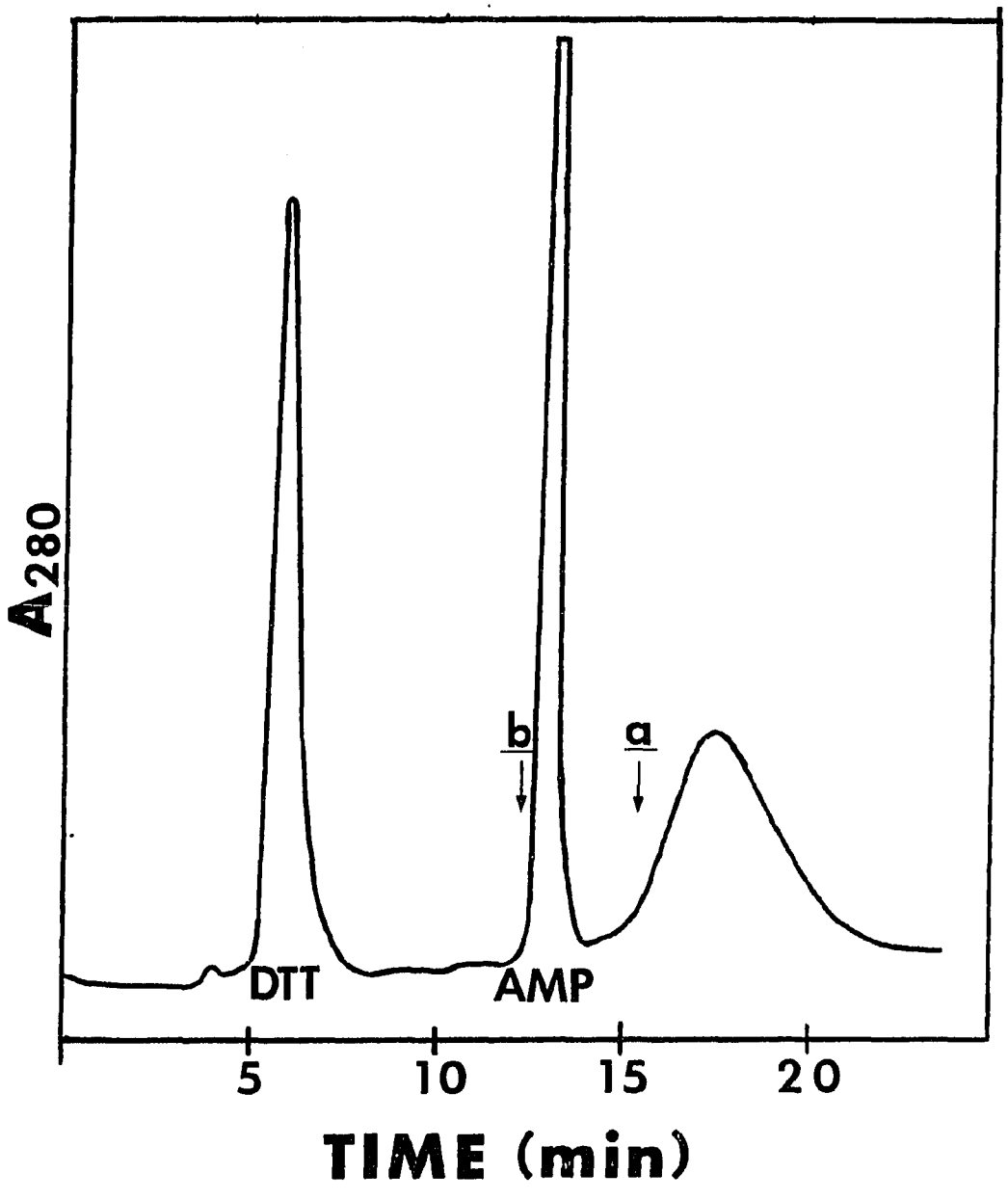

A280
b
a
ATP
5
10
15
20
TIME (min)

Fig. 2B 0.2 mg of phosphorylase ab in buffer with 3 mM ATP was subjected to HPLC as in Experimental Procedures. The elution positions of phosphorylases b and a are indicated with arrows.


A280
b
a
DTT
AMP
5
10
15
20
TIME (min)

Experimental procedures, dialyzed against buffer with either 0.5 mM AMP (A) or 3 mM ATP (B). HPLC of the sample with AMP had to be done with higher pH buffers, because at pH 6.8, AMP elutes with phosphorylase ab. The sample with AMP showed only phosphorylase ab, showing that it is stable under these conditions. The sample with ATP showed only very small amounts of phosphorylases b and a, indicating that ATP sufficiently stabilizes phosphorylase ab. The stabilization of phosphorylase ab by ATP is advantageous, because it permits phosphorylation assays to be performed without adding effectors other than ATP, which is required for the phosphorylase kinase reaction.

An interesting feature of Fig. 2A is that, under these conditions, phosphorylase a elutes before phosphorylase ab, in contrast to the elution order at pH 6.8, which is expected from the charges of the proteins. This shows that the elution of phosphorylase from anion-exchange columns is influenced not only by charge, but also by conformation. The idea of conformation-dependent elution helps to explain why adding a single phosphate to a protein as large as phosphorylase can change its ionic properties enough to cause a large change in the ion-exchange elution pattern. The conformational change which accompanies phosphorylation probably contributes to the change in the ionic properties.

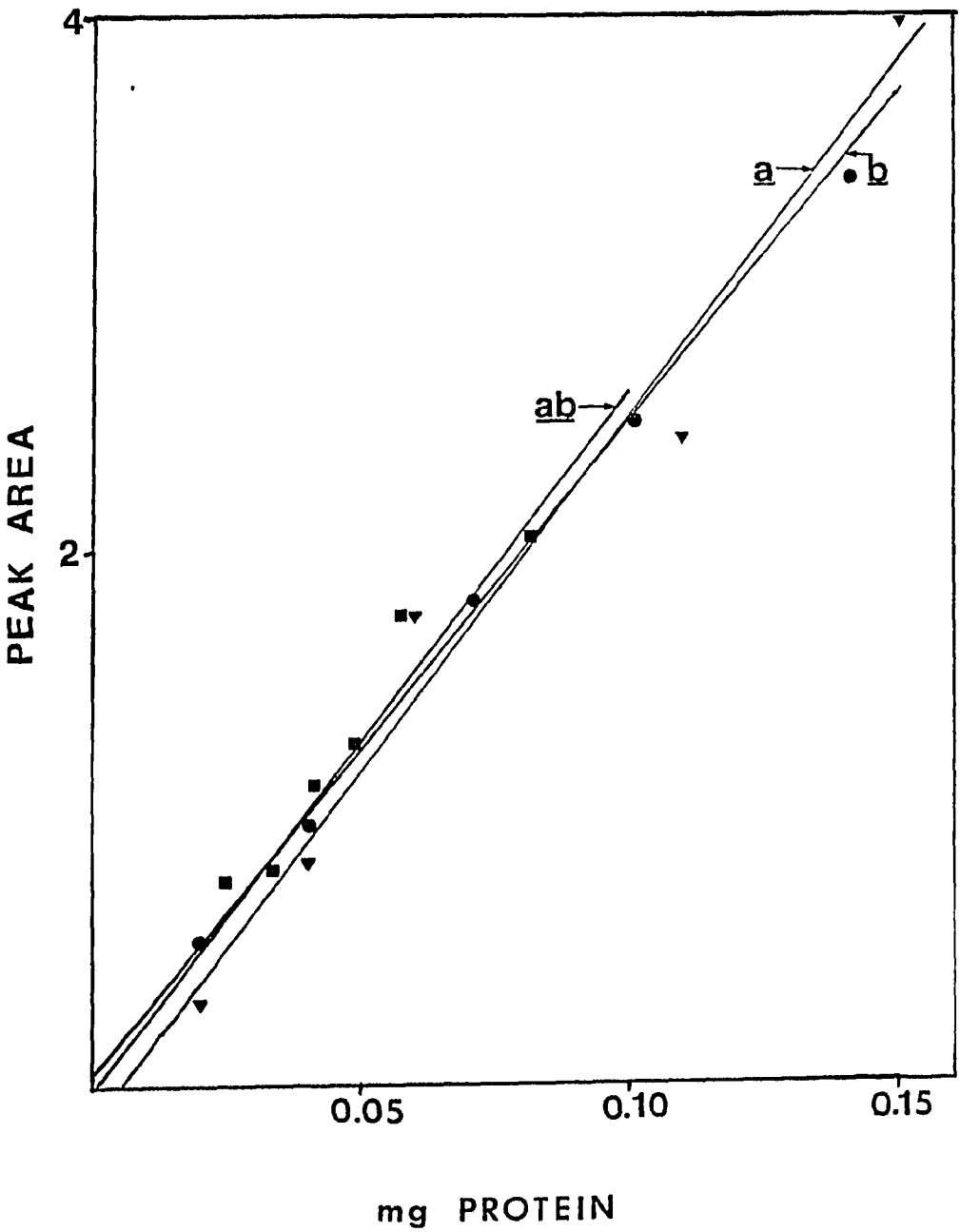
Standard curves for phosphorylases b, ab and a

To demonstrate the stability of phosphorylase ab during a phosphorylase kinase assay, standard curves for all 3 forms of phosphorylase in the presence of the components of the assay reaction mixture were determined using the HPLC assay. All 3 proteins were included in the samples for the standard curves, and the concentrations of the proteins in the samples were designed to approximately simulate a series of samples taken during a phosphorylation time course. If phosphorylase ab dissociates and recombines under these conditions, then the standard curves would be skewed. Fig. 3 shows the standard curves for the proteins in the presence of 150 mM glucose. Standard curves obtained in the absence of effectors were similar. The standard curves for the 3 forms of phosphorylase are linear, and they are close in slope to each other. This result demonstrates both the stability of phosphorylase ab in the phosphorylase kinase assay and the validity of the HPLC assay for the time course of phosphorylation.

Initial rate kinetics

The kinetic parameters of phosphorylase kinase using phosphorylase b and phosphorylase ab as substrates were determined as in Experimental Procedures. The apparent K_m's and V_M's were, respectively: with phosphorylase b, 14.9 $\pm$

Fig. 3 Standard curves for phosphorylases b (●), ab (■) and a (▼). Except for sample 1, all samples contained all 3 proteins. Amounts (in μg) of each protein the samples were: sample 1, ab-81; sample 2, b-140, ab-32, a-20; sample 3, b-100, ab-49, a-40; sample 4, b-70, ab-57, a-60; sample 5, b-40, ab-41, a-110; sample 6, b-20, ab-24, a-150


4
2
PEAK AREA
a
b
ab
0.05
0.10
0.15
mg PROTEIN

2.6 μM and 4.0 $\pm$ 0.24 μmole/min/mg, and with phosphorylase ab, 3.9 $\pm$ 0.24 μM and 1.7 $\pm$ 0.029 μmole/min/mg. The concentrations given are those of unphosphorylated phosphorylase subunits; therefore, the protein concentrations for phosphorylase ab are twice as high as those given.

These are probably not the true kinetic parameters for phosphorylase ab, because of competition between the unphosphorylated and phosphorylated subunits of phosphorylase ab for binding to the kinase. The initial-rate equation for the phosphorylation of phosphorylase ab is

$$\frac{1}{v} = \frac{K_{ba}}{V_M} \cdot \frac{1}{ab} + \frac{1}{V_M}\left(1 + \frac{K_{ba}}{K_{ab}}\right)$$

where v is the observed velocity, V_M is the maximal velocity, ab is the concentration of phosphorylase ab, K_{ba} refers to the interaction with the kinase of the nonphosphorylated subunit of phosphorylase, and K_{ab} refers to that of the phosphorylated subunit. This causes the measured K_M to be higher than the actual affinity of the unphosphorylated subunit for the kinase. The measured V_M is also lower than the actual value, because saturation with the unphosphorylated subunit cannot be obtained in the presence of an equal concentration of the phosphorylated

subunit. We have found that phosphorylase a inhibits the phosphorylation of phosphorylase b (data not shown).

The fact that the measured K_M of phosphorylase ab is 3.8 times lower, despite inhibition by its phosphorylated subunit, than that of phosphorylase b, indicates that phosphorylation of one subunit of phosphorylase b changes the conformation of the second subunit to make it bind more tightly to the kinase.

Phosphorylation time course assays

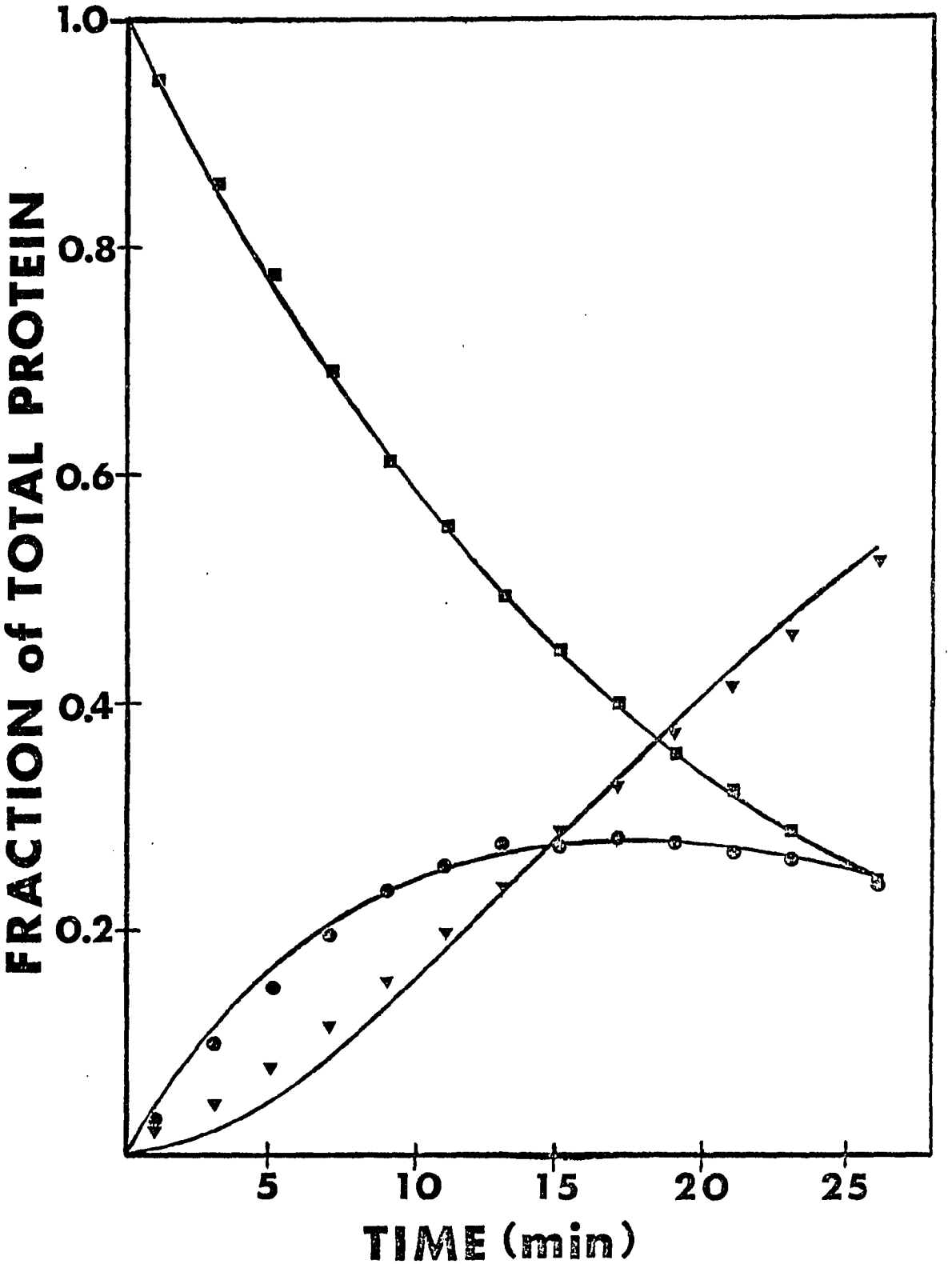
Fig. 4 is a plot of the amounts of the 3 forms of phosphorylase during phosphorylation by phosphorylase kinase. The points represent the experimental data and the curves were generated by the curve-fitting program as in Experimental Procedures. When the data were fit to the equations for first order consecutive reactions, the rate constants k_2 (for phosphorylase ab — a) and k_1 (for phosphorylase b — ab) were in the ratio k_2/k_1 = 3.6 - 4.0 in 4 replications of the experiment.

The enzyme catalyzed conversion of phosphorylase b to a is not a simple series of first order reactions. The rates of the reactions depend not only on the concentrations of phosphorylases b and ab, but also on the competition among all 3 forms of phosphorylase for binding to the kinase. But if it is presumed that the competition of both phosphorylase

a and the phosphorylated subunit of phosphorylase ab for the kinase affects phosphorylases b and ab equally, then any change in the ratio of the first order constants is due to a change in the relative K_M's and/or V_M's of the kinase with phosphorylases b and ab. Therefore the ratio, k_2/k_1, of the apparent first order constants is useful for determining the influence of phosphorylase effectors or a different form of the kinase on the relative catalytic efficiency of the kinase in the two steps of the reaction. The ratio k_2/k_1 from the experiment in Fig. 4 was 3.6, which correlates qualitatively with the initial rate data which showed that phosphorylase ab is a better substrate for phosphorylase kinase than is phosphorylase b. Therefore, the ratio of the first order rate constants is a good indicator of the relative catalytic efficiency of the kinase in the two steps of the reaction. Any change of this ratio indicates a change in this relative catalytic efficiency, and therefore a change in the conformational difference between phosphorylases b and ab.

In Fig. 4, the phosphorylase concentration was 2.0 mg/ml (20 μM), which is close to the reported K_M of activated phosphorylase kinase for phosphorylase b (6). When the phosphorylase concentration was raised ten-fold to 20 mg/ml, a similar k_2/k_1 was obtained. If the conformational difference between phosphorylases b and ab

Fig. 4 Amounts of phosphorylase b(■), phosphorylase ab(●) and phosphorylase a(▲) during phosphorylase kinase reaction


1.0
0.8
0.6
0.4
0.2
FRACTION of TOTAL PROTEIN
5
10
15
20
25
TIME (min)

results in only a lower K_M for phosphorylase ab, then raising the initial phosphorylase b concentration to saturating would be expected to alter the ratio k_2/k_1. The fact that k_2/k_1 is not altered indicates that the V_M of phosphorylase kinase is higher with phosphorylase ab than with phosphorylase b, despite the lower value of V_M for phosphorylase ab from the inital rate experiments, which is due to inhibition by the phosphorylated subunit of phosphorylase ab.

Influence of effectors

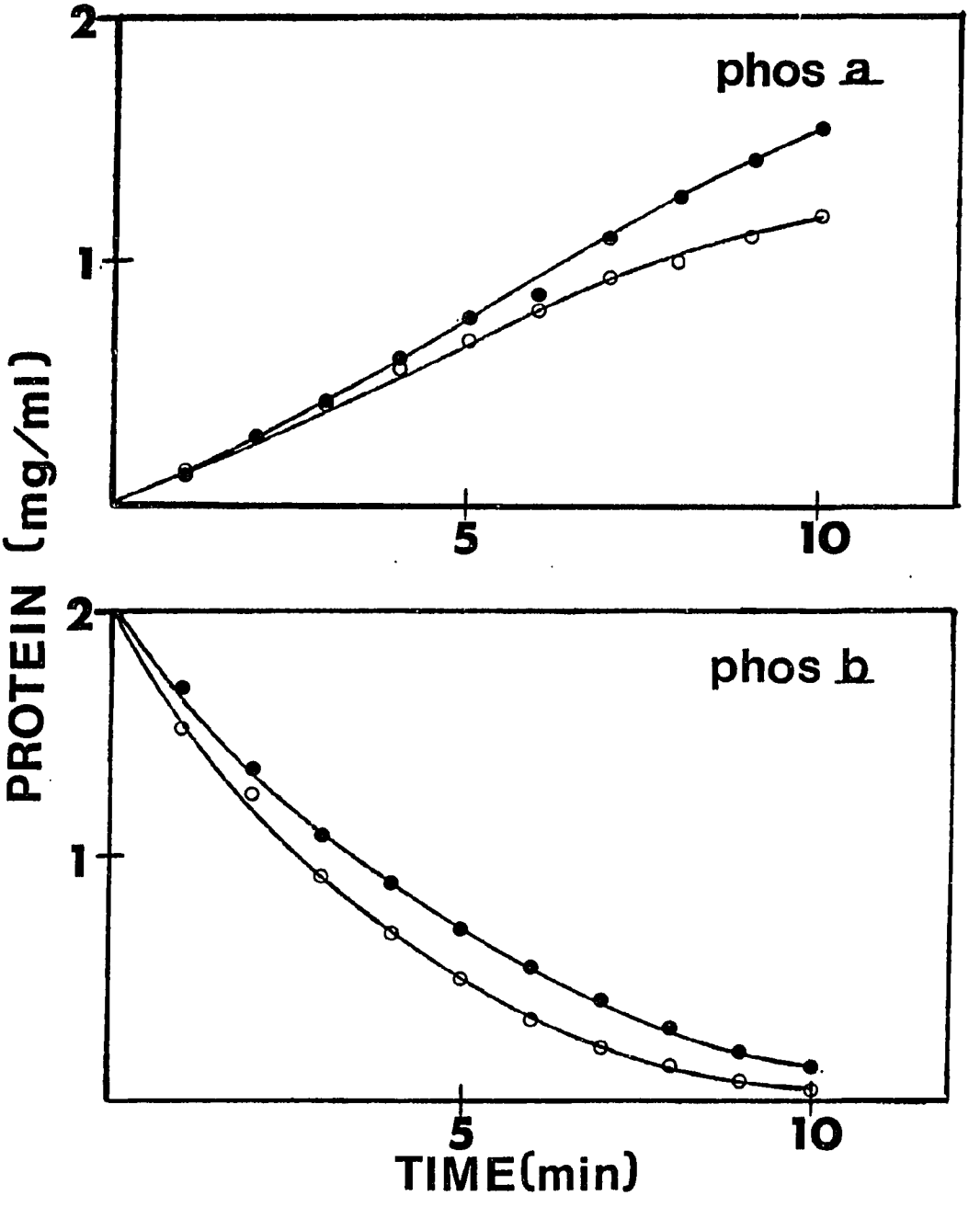
Table 1 shows the values for the apparent first order rate constants from the phosphorylation time course assay with different effectors present. Attempts were made to measure the effects of glycogen and glucose-6-phosphate, but the presence of these compounds in the samples disrupted the chromatography so that reliable results could not be obtained.

ATP inhibits phosphorylase b by binding at the AMP site and stabilizing the inactive T-conformation (21). Because ATP is required for the phosphorylase kinase reaction, it is present at a concentration of 3 mM in all of the assays, so it is the ATP-liganded form of phosphorylase which is the basis for comparison with other effectors.

Fig. 5 shows the variation in the amounts of the phosphorylases b and a during the phosphorylase kinase reaction, in the absence and presence of 1 mM AMP. At this concentration of AMP, phosphorylase b is fully active, and thus saturated with AMP, even in the presence of 3 mM ATP. With AMP present, phosphorylase b decreased more quickly and phosphorylase a increased more slowly than without AMP. Because AMP eluted with phosphorylase ab, it was impossible to determine the amount of phosphorylase ab, and therefore total protein, in the HPLC runs when AMP was present. This made the determination of the apparent rate constants less reliable, but Fig. 5 clearly shows that k_1 was greater and k_2 was smaller when AMP was present. If the phosphorylase ab concentration in each run was calculated by subtracting the concentrations of phosphorylases b and a from the average total protein concentration from the runs without AMP, then the ratio $k_2/k_1 = 1.4$ is obtained. When compared to $k_2/k_1 = 3.7$ with no effectors, this indicates that when AMP is present, the conformational difference between phosphorylases b and ab is not as great.

Glucose, which inhibits phosphorylase by binding at the active site and stabilizing the inactive T-conformation (22), had no effect on the absolute values of either k_1 or k_2 when compared to a parallel experiment with no effectors present. This shows that the presence of glucose has no

Fig. 5 Amounts of phosphorylases b and a during the phosphorylase kinase reaction in the absence (●) and presence (o) of 1 mM AMP


2
1
phos a
5
10
PROTEIN (mg/ml)
2
1
phos b
5
10
TIME(min)

effect on the conformational differences between phosphorylases b and ab. The lack of effect by glucose also shows that there is no effect on the course of phosphorylation by any changes in the quaternary structure of phosphorylase as it is converted from b to a. Phosphorylase a (23) and, to a lesser extent, phosphorylase ab (4), exist as tetramers in the absence of effectors. Glucose causes dissociation into dimers (20), so in the presence of glucose, all forms of phosphorylase would be dimers throughout the conversion of phosphorylase b to a. Because the time course of phosphorylation was identical with and without glucose, any tetramer formation which occurs in the absence of glucose has no effect on phosphorylation.

Time course of phosphorylation by γ-calmodulin

Phosphorylase kinase is a large enzyme with the subunit structure $(\alpha\beta\gamma\delta)_4$ (24). The γ subunit has been shown to possess catalytic activity (25), and the δ subunit is identical to calmodulin (26). It is possible that the two subunits of phosphorylase bind in two different active sites of the kinase and are phosphorylated before phosphorylase dissociates ("simultaneous" phosphorylation). If this were the only mechanism, then only a very small amount of phosphorylase ab would be detected during a time course of

phosphorylation, and k_2/k_1 would be very large. If simultaneous phosphorylation occurs to a lesser extent, then k_2/k_1 would be larger than if it did not occur at all. To investigate this issue, the time course of phosphorylation of phosphorylase by a complex between the γ subunit of phosphorylase kinase and calmodulin was studied. This complex has previously been shown to exist as the heterodimer (9), so simultaneous phosphorylation with this enzyme form is unlikely. As seen in Table 1, with no effectors present, $k_2/k_1 = 2.1$ with γ-calmodulin, as compared with $k_2/k_1 = 3.7$ with intact phosphorylase kinase. These results could be interpreted to mean that there is some degree of simultaneous phosphorylation by intact kinase. Another possibility is that γ-calmodulin is not as sensitive as intact phosphorylase kinase is to the conformational difference between phosphorylases b and ab. This could be because other parts of the kinase, not present in γ-calmodulin, are involved in binding to phosphorylase, or because the γ subunit has a different conformation, and therefore a different specificity, in γ-calmodulin than in the intact kinase.

DISCUSSION

In this paper, we used phosphorylase kinase as a probe for conformational differences between phosphorylases b and ab. Using initial rate kinetics, we found that the kinase has a lower K_M for phosphorylase ab than for phosphorylase b. The difference in the K_M's means that phosphorylation of one subunit of phosphorylase b causes a conformational change which makes the other subunit a better substrate for the kinase. This is analogous to cooperative binding of an allosteric effector, which causes the enzyme to be sensitive to the effector over a narrow range of effector concentration. In the case of "cooperative" phosphorylation, phosphorylase activity is increased over a narrower range of phosphorylase kinase activity than if there were no effect of phosphorylation of one subunit on the phosphorylation of the other subunit.

The conformational changes in phosphorylase which affect phosphorylase kinase binding could be either only in the amino-terminal region around the phosphorylation site or only in other parts of the protein, or both. The amino-terminal 18 residues are not resolved in the X-ray crystal structure of phosphorylase b (27), indicating that this region of the protein is mobile. If the amino-terminal region is mobile, then conformational changes in

phosphorylase that affect binding to the kinase are probably in other parts of the protein. When the amino-terminal 16 to 18 residues of phosphorylase are removed by limited proteolysis, several properties of the protein change (28), indicating that the amino-terminal region does interact with the rest of the protein. In this case, the conformation of the amino-terminal region could be an important factor in the binding of phosphorylase to the kinase. Preliminary results in our laboratory indicate that the phosphorylase analog which is missing the amino-terminal region does not inhibit the phosphorylation of phosphorylase b. This result supports the idea that only the amino-terminal region of phosphorylase interacts with phosphorylase kinase.

Because the initial rate kinetic data was difficult to fully interpret, we relied on the HPLC assay of the full time course of phosphorylation to study the influences of phosphorylase effectors on the conformational differences between phosphorylases b and ab. Because the conversion of phosphorylase b to a involves two reactions and multiple equilibria, the integrated rate equation for the process would be exceedingly complex, and its solution would require knowledge of the binding constants for the phosphorylated subunits of phosphorylases ab and a. To qualitatively compare the effects of different conditions on the relative rates of phosphorylation of the two subunits, it is useful

to use the simple model of first order consecutive reactions. The apparent rate constants obtained from this treatment are composites of several rate and equilibrium constants, and they reflect the overall catalytic efficiency of the kinase in the two steps of the reaction.

Glucose had no effect on the time course of phosphorylation. AMP caused the ratio of the apparent first order rate constants, k_2/k_1, to decrease from 3.7 without AMP to about 1.4. This indicates that AMP reduces the conformational differences between phosphorylases b and ab which are important for phosphorylation by phosphorylase kinase. We cannot say whether or not phosphorylases b and ab are the same as substrates for the kinase with AMP, only that AMP reduces the important conformational differences.

The possibility of simultaneous phosphorylation of both phosphorylase subunits by phosphorylase kinase is a factor in the time course assays. Simultaneous phosphorylation would increase the ratio k_2/k_1. The fact that γ-calmodulin, with which simultaneous phosphorylation is unlikely, gives a lower k_2/k_1 than does intact phosphorylase kinase indicates that some degree of simultaneous phosphorylation is possible. Another explanation is that γ-calmodulin has lost some of the binding specificity of the intact kinase, and is not as sensitive to the conformational differences between phosphorylases b and ab.

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GENERAL DISCUSSION

The experiments in Section II showed that phosphorylation of one subunit of phosphorylase b causes conformational changes which make the other subunit bind more tightly to phosphorylase kinase. If the amino-terminal region of phosphorylase is mobile, then this region may not be involved in those conformational changes. If, however, the amino-terminal region interacts with the rest of the protein, as the results in Section I imply, then any conformational changes in phosphorylase which affect binding to phosphorylase kinase may be manifested in this region. The significance of and relationship between the two parts of this dissertation are brought out in the following suggestions for future experiments to investigate the role of the amino-terminal region of phosphorylase in controlling its phosphorylation.

The question of whether parts of phosphorylase other than the amino-terminus bind to phosphorylase kinase could be addressed by determining whether phosphorylase b_S can inhibit the phosphorylation of phosphorylase b. If phosphorylase does have a secondary kinase binding site, then it would be present in phosphorylase b_S, which would compete for binding to phosphorylase kinase. Preliminary experiments have shown that with no effectors other than ATP

present, phosphorylase b_S^1, even when present at 10 times the concentration of phosphorylase b, does not affect the phosphorylation of phosphorylase b. This experiment should be repeated with a variety of phosphorylase effectors present, to determine if any conformations of phosphorylase b_S^1 will bind to the kinase.

Another set of experiments would be aimed at better understanding the interaction of the amino-terminal region of phosphorylase with the rest of the protein. The strength of this interaction could be measured by a direct assay for binding between phosphorylase b_S^1 and a synthetic peptide with the sequence of phosphorylase [1-18]. Janski and Graves did an analogous study with phosphorylase b', which is a trypsin proteolytic phosphorylase analog missing the amino-terminal 16 residues (27). They measured tetramer and crystal formation of phosphorylase b' induced by peptides in the presence of AMP or IMP, and found that only peptides containing a phosphate group on the serine corresponding to the phosphorylation site in phosphorylase could change the properties of phosphorylase b'. This however, was not a direct binding assay, and there is some question as to whether phosphorylase b' had internal cleavages that might have affected the quaternary structure of the protein. A direct binding assay for complementation between the well

characterized phosphorylase b_S^1 and nonphosphorylated peptides might reveal a substantial interaction.

Complementation could be measured in the presence of phosphorylase effectors. The fact that removal of the amino-terminal region increases the activity of phosphorylase implies that the amino-terminus inhibits phosphorylase and therefore stabilizes the inactive T-conformation. It might be expected that effectors which also inhibit phosphorylase would strengthen the complementation between a peptide and phosphorylase b_S^1. Especially interesting would be the effect of the inhibitor G-6-P, which also inhibits the phosphorylase kinase reaction by binding to the substrate phosphorylase (34). Also, the activity of phosphorylase b_S^1 is less sensitive than that of phosphorylase b to G-6-P inhibition. If G-6-P inhibits phosphorylation by weakening the interaction between the amino-terminus and the rest of the protein, then complementation would be weaker with G-6-P. On the other hand, if the inhibition of phosphorylation is due to causing a mobile amino-terminus to bind to the rest of the protein, then complementation would be strengthened by G-6-P.

Peptides with deletions or substitutions in the native sequence could be tested to discover which residues are important for binding to phosphorylase b_S^1. If these are the same residues which are important for phosphorylation of

these peptides by phosphorylase kinase (as reviewed by Graves (46)), this would imply that these residues are important for forming a specific conformation which the kinase recognizes or induces upon binding.

The K_M of phosphorylase kinase for phosphorylase b is at least 10 times lower than that of the best peptide substrate (31,33). The next question to investigate would be whether the interaction between the amino-terminal region and the rest of the protein induces a conformational change in the amino-terminus which makes it a better substrate for phosphorylase kinase. If this is the case, then complementation of a phosphorylase amino-terminal peptide with phosphorylase b_S^1 would increase the phosphorylation of that peptide. This experiment could be repeated in the presence of phosphorylase effectors. An important control would be to measure the effect, if any, of phosphorylase b_S^1 on the phosphorylation of a peptide whose sequence is unrelated to phosphorylase, which would be expected not to bind to phosphorylase b_S^1.

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