Protein-Induced Inactivation and Phosphorylation of Rabbit Muscle Phosphofructokinase†

Zhizhuang Zhao, Dean A. Malencik, and Sonia R. Anderson*
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331
Received April 24, 1990; Revised Manuscript Received August 10, 1990

ABSTRACT: Several previously untested proteins promote the reversible inactivation of rabbit skeletal muscle phosphofructokinase. Grouped in decreasing order of effectiveness, they include the following: skeletal muscle troponin C > troponin, the two smooth muscle myosin light chains, α-actinin, and S-100 ≫ parvalbumin and soybean trypsin inhibitor. The efficiency of troponin C in this process may even exceed that previously reported for calmodulin. Sequences near calcium binding site III are apparently involved in the troponin C–phosphofructokinase interaction. Troponin C and calmodulin exert calcium-dependent effects on the physical and chemical properties of muscle phosphofructokinase. When calcium is present, comigration with either protein allows the enzyme to enter the stacking gel during urea–polyacrylamide gel electrophoresis. Both enhance the phosphorylation of phosphofructokinase catalyzed by the cAMP-dependent protein kinase, with phosphate incorporations approaching 2 mol of P/mol of protomer. Reaction occurs at Ser^{774} and at Ser^{376}—a novel site whose phosphorylation is highly sensitive to troponin C and less so to calmodulin. Maximum phosphorylation has slight effect on the catalytic activity of the enzyme under standard assay conditions. The troponin C induced or calmodulin-induced phosphorylation of phosphofructokinase requires calcium and is strongly inhibited by either fructose 2,6-bisphosphate or fructose 1,6-bisphosphate. Inactivation occurs in the presence or absence of calcium, with generally higher concentrations of effectors required for protection in the latter case. Liver and yeast phosphofructokinases shows little activity loss in the presence of either calmodulin or troponin C. We have developed and tested a general mathematical model for the protein-induced inactivation of phosphofructokinase which may find application to other systems.

Rabbit muscle phosphofructokinase (PFK;[1] ATP:D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) is a key glycolytic enzyme characterized by allosteric kinetics, a complex oligomeric structure, and multiple modes of regulation. Phosphofructokinase is regulated in a pH-dependent manner by a number of ligands including substrates, reaction products, and various cellular metabolites (Uyeda, 1979; Goldhammer & Paradies, 1979; Kemp & Foe, 1983). Of these, fructose 2,6-bisphosphate, fructose 1,6-bisphosphate, and AMP are efficient positive effectors while ATP and citrate are potent inhibitors. Additionally, phosphofructokinase has the ability to undergo a rapid association and dissociation which is influenced by protein concentration, ionic strength, pH, temperature, and a number of ligands (Hesterbert & Lee, 1981, 1982; Luther et al., 1983, 1985, 1986; Shnyrov et al., 1988). At pH 7.0, the association is best described by the equilibria:

$$4M \rightleftharpoons 2M_2 \rightleftharpoons M_4 \rightleftharpoons {}^1/_4M_{16}$$

in which M_4 represents the tetramer of molecular weight 340 000 (Luther et al., 1983). Moreover, the association state is related to catalytic activity, with the tetramer being the smallest active form of phosphofructokinase.

Phosphofructokinase interacts with a variety of both soluble and structural proteins. Gerlach and Hofer (1986) found that selected glycolytic enzymes including phosphoglucose isomerase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase, lactate dehydrogenase, phosphorylase, creatine kinase, and adenylate kinase bind to immobilized rabbit muscle phosphofructokinase. Experiments with reconstituted thin filaments of muscle and muscle press juice extracts have demonstrated in vitro adsorption of several glycolytic enzymes, with phosphofructokinase being the most strongly adsorbed (Clarke & Masters, 1976). F-Actin is believed to be the major site for phosphofructokinase association in this system (Arnold & Pette, 1968; Arnold et al., 1971). The stimulatory effect of F-actin on the catalytic activity of phosphofructokinase was first demonstrated in vitro by Liou and Anderson (1980). More recently, Freidina et al. (1987) showed that phosphofructokinase, in a dimeric form, binds to the hinge region of myosin, the major protein component of the thick filament.

Phosphofructokinase is known to undergo inactivation in the presence of several different proteins. These include the erythrocyte membrane band 3 protein (Jenkins et al., 1985) and the Zn^{2+}-dependent inactivating protein purified from rat liver (Brand & Söling, 1986). Both proteins associate preferentially with the enzyme dimer. Still another protein inhibitor of the enzyme has been detected in the mucosa of rat small intestine (Kellett & Robertson, 1984). Interestingly, calmodulin undergoes a moderate- to high-affinity interaction with phosphofructokinase which is considered to be both specific and physiologically relevant (Mayr & Heilmeyer 1983). The association induces a shift in the equilibrium from the highly active enzyme tetramer toward an inactive dimer (Mayr, 1984a,b).

Phosphofructokinase also undergoes posttranslational phosphorylation (Brand & Söling, 1975; Hofer & Furst, 1976).

† This work was supported by research grants from the National Institutes of Health (DK13912).
* Address correspondence to this author.

[1] Abbreviations: PFK, phosphofructokinase; TnC, troponin C; TnI, troponin I; CaM, calmodulin; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/91/0430-2204$02.50/0

The endogenous phosphate content of purified phosphofructokinase depends on both the original physiological state and the methods of tissue extraction that have been used. Varying phosphate levels from 0.08 to 1.17 mol of phosphate/mol of protomer have been reported for the isolated enzyme from resting muscle (Hussey et al., 1977; Riquelme et al., 1978; Uyeda et al., 1978; Hofer & Sorensen-Ziganke, 1979; Söling & Brand, 1981; Kuo et al., 1986). Electrical stimulation of muscle increases the phosphate content of phosphofructokinase up to 1.0–2.0 mol of phosphate/mol of protomer (Hofer & Sorensen-Ziganke, 1979). However, a smaller stoichiometry of 0.54–0.96 mol of phosphate/mol of protomer was reported later by the same group (Hofer, 1985).

Phosphofructokinase also can be phosphorylated in vitro through a reaction catalyzed by the cAMP-dependent protein kinase. Pilkis et al. (1982) observed the incorporation of 0.9–1.0 mol of phosphate/mol of rat liver phosphofructokinase protomer. A similar stoichiometry was described by Mendicino et al. (1978) for the purified rat kidney enzyme and by Riquelme et al. (1978) for the rabbit skeletal muscle enzyme. However, Foe and Kemp (1982) subsequently determined a maximum incorporation of only 0.4–0.5 mol of phosphate/mol of protomer for the latter case. Similar low stoichiometries have been presented for purified phosphofructokinase from rabbit brain (Foe & Kemp, 1984), rabbit skeletal muscle (Sale & Denton, 1985), and rat liver (Domenech et al., 1988; Mieskes et al., 1987). The major known phosphorylation site of rabbit muscle phosphofructokinase proved to be a serine residue located at the sixth position from the C-terminus of the enzyme (Kemp et al., 1981). A single phosphorylation site, apparently corresponding to that of the rabbit muscle enzyme, also has been identified for each of the three isozymes of phosphofructokinase from rabbit brain (Valaitis et al., 1989).

The physiological significance of the phosphorylation of phosphofructokinase has been a major point of investigation ever since the discovery of covalently bound phosphate in the purified enzyme. Foe and Kemp (1982) and Kitajima et al. (1983) reported that the phosphoenzyme is more sensitive to ATP inhibition than is the dephosphorylated enzyme. However, the differences in catalytic activity are small [see also Söling and Brand (1981), Clark and Patten (1984), and Sakakibara and Uyeda (1983)]. Hussey et al. (1977) and Uyeda et al. (1978) reported that purified phosphofructokinase fractions with varying phosphate content differ in their sedimentation behavior. However, the phosphate content in either case was far below the ideal minimum of 1 mol of phosphate/mol of protomer. Foe and Kemp (1982) later found no difference in either the sedimentation properties, determined under a variety of conditions, or the stabilities of phosphorylated and dephosphorylated enzyme preparations. Yet Kitajima et al. (1983) subsequently demonstrated that phosphorylation affects the pH-dependent cold inactivation of the enzyme. More recently, studies from our group (Kuo et al., 1986) and others (Luther & Lee, 1986) showed that the phosphorylation of rabbit skeletal muscle phosphofructokinase results in a small enhancement of the binding of the enzyme to F-actin, a positive effector. Clearly, after more than 12 years of investigation, both the maximum possible extent and the physiological significance of the phosphorylation of phosphofructokinase remain unknown.

The results to be presented in this article deal with interconnections between the association of rabbit muscle phosphofructokinase with other proteins and its status as a substrate for protein kinases. First, we explore the reversible association of the purified enzyme with a number of proteins which have not been examined previously in this context. These proteins include skeletal muscle troponin C and its proteolytic fragments. Second, we develop and test a general mathematical model for the reversible inactivation of phosphofructokinase which could be useful in studies of other systems. Third, we demonstrate that troponin C and calmodulin exert calcium-dependent effects on both the rate and extent of phosphorylation of phosphofructokinase catalyzed in vitro by the cAMP-dependent protein kinase.

Materials and Methods

Materials. Fructose 6-phosphate, fructose 2,6-bisphosphate, ATP, ADP, AMP, dithiothreitol, β-mercaptoethanol, NADH, aldolase, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, trypsin, soybean trypsin inhibitor, and phenyl-agarose, all in highest available grade, were purchased from Sigma Chemical Co. DEAE-51, DEAE-52, and DEAE-53 were obtained from Whatman. [γ-^{32}P]ATP was purchased from New England Nuclear (3000 Ci/mmol) and diluted to 100 Ci/mol with cold ATP. *Staphylococcus aureus* strain V8 protease and CNBr were from Sigma. Acetonitrile (HPLC grade) was purchased from Baker. Trifluoroacetic acid (HPLC grade) was from Pierce. Glass microfiber filters (GF/B) for the radioactivity measurement were supplied by Whatman. Highly purified water was obtained from a Milli-Q system (Millipore). All other reagents were of the highest grade available.

Phosphofructokinase Preparation and Activity Assay. Rabbit muscle phosphofructokinase was purified from fresh rabbit skeletal muscle according to the method of Kemp (1975), with modifications described previously (Kuo et al., 1986) in which DEAE-51, DEAE-52, and DEAE-53 series ion-exchange chromatography is used as the final purification step. The purified phosphofructokinase showed a single band on $NaDodSO_4$ gel electrophoresis and had a specific activity around 200 units/mg when assayed at room temperature under optimal conditions. The pooled enzyme fraction used in this work had an endogenous phosphate content around 0.15 mol/mol of protomer as determined by the method of Hasegawa et al. (1982). Phosphofructokinase was stored in a pH 8.0 buffer containing 50 mM Tris–phosphate, 5 mM pyrophosphate, 0.2 mM EDTA, 1 mM β-mercaptoethanol, and 50% glycerol at −80 °C.

Phosphofructokinase activity was assayed spectrophotometrically by coupling fructose 1,6-bisphosphate formation to the oxidation of NADH through the use of auxiliary enzymes—aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase (Racker, 1947). The assay medium contained 50 mM glycylglycine (pH 8.2), 1 mM EDTA, 2.5 mM dithiothreitol, 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1 mM ATP, 1 mM fructose 6-phosphate, 0.16 mM NADH, 0.4 unit of aldolase, 0.4 unit of α-glycerophosphate dehydrogenase, and 2 units of triosephosphate isomerase in a total volume of 1.0 mL. The reaction was started by the addition of phosphofructokinase (usually 0.2 μg) to the assay solution.

Rabbit liver phosphofructokinase was purified according to Kemp (1975) and assayed under the same conditions as used for muscle phosphofructokinase. Yeast phosphofructokinase was purified and assayed according to Welch and Scopes (1981).

Preparation of Other Proteins. Bovine brain calmodulin was prepared according to the procedure of Schreiber et al. (1981); rabbit skeletal muscle troponin I, according to Kerrick et al., (1980); troponin C, according to Potter (1982); turkey

gizzard α-actinin, according to Craig et al. (1982); turkey gizzard smooth muscle myosin light chains, according to Malencik and Anderson (1988); bovine brain S-100, according to Kincaid and Couloin (1985); and dogfish parvalbumin, according to Heizmann et al. (1974). Thrombin was a gift from Prof. Earl Davie.

The isolated catalytic subunit of the cAMP-dependent protein kinase was prepared from bovine brain according to Peters et al. (1977). Rat brain protein kinase C was purified according to the method described for the purification of bovine brain protein kinase C (Walton et al., 1987). The nearly homogeneous protein kinase C has a specific activity of 402 units/mg as assayed under optimal conditions employing chicken histone H1 as a substrate. Purification of cGMP-dependent protein kinase followed the procedure of Glass and Krebs (1979).

Preparation of Troponin C Fragments. Troponin C was digested in the presence of calcium with tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin, employing a ratio of 1 mg of trypsin/100 mg of troponin C, and then fractionated on a phenylagarose column as described by Brzeska et al. (1983). Each of the two purified tryptic fractions showed two very sharp, closely spaced bands upon electrophoresis [NaDodSO$_4$ gel performed as described by Schagger and von Jagow (1987)]. Amino acid analyses performed according to Malencik et al. (1990) verified that the phenylagarose binding fraction corresponds essentially to the N-terminal half of troponin C (TR1) and that the nonbinding fraction represents the C-terminal half (TR2). Each of these fractions is apparently a mixture of two closely related polypeptides, with the fraction known as TR1 probably corresponding to troponin C sequence position 9–84 and 9–88 and TR2 corresponding to positions 85–159 and 89–159. This interpretation agrees with previous reports (Leavis et al., 1978a; Brzeska et al., 1983; Vogel et al., 1983). We obtained two very homogeneous thrombic fragments of troponin C through the method of Leavis et al. (1978a). Each exhibits a single sharp band on 16–27% NaDodSO$_4$–polyacrylamide gel electrophoresis. Amino acid analyses confirmed that the large fragment (TH1) corresponds to the N-terminal moiety (residues 1–120) of troponin C and the small fragment (TH2), to the C-terminal moiety (residues 121–159)—as originally reported by Leavis et al. (1978b). The troponin I binding properties of the fragments, as shown by 10% urea gel electrophoresis in the presence of Ca^{2+}, agree with those described by Leavis et al. (1978a).

Inactivation and Reactivation of Phosphofructokinase. A two-step assay was adopted in this study. In the first step, relatively concentrated phosphofructokinase (0.02 mg/mL) is incubated with the specified concentration of the inactivating protein. Then, in the second step, the activity remaining is measured under optimal assay conditions by diluting the enzyme to 0.2–0.5 μg/mL, a concentration that yields measurable reaction rates. The temperature for the inactivation is 24 °C unless indicated otherwise. In a typical inactivation experiment, the stock solution of phosphofructokinase (10 mg/mL) is diluted to 0.04 mg/mL with a solution containing 50 mM Mops–KOH (pH 7.0), 5.0 mM β-mercaptoethanol, 1.0 mM dithiothreitol, and 50% glycerol and allowed to stand at the incubation temperature for 1 h. The inactivation is started by mixing equal volumes of phosphofructokinase and the inactivating protein, which is initially dissolved in water containing either 0.1 mM Ca^{2+} or 0.1 mM EGTA. Control experiments involve dilution of the enzyme with water containing Ca^{2+} or EGTA alone. At the designated times, 10–25-μL samples of the mixture are removed for activity measurements. Factors affecting the inactivation are added to the inactivating system prior to mixing with phosphofructokinase. The incubations are usually performed for 20 min. The reactivation of phosphofructokinase is carried out through the addition of a minimal volume of the activating agent.

Protein Determination. The protein concentrations were determined from ultraviolet absorbance, employing the following extinction coefficients: $E^{1\%}_{280nm}$ = 10.7 for phosphofructokinase (Hesterberg & Lee, 1981); $E^{1\%}_{280nm}$ = 2.0 for calmodulin (Watterson et al., 1980); $E^{1\%}_{280nm}$ = 2.0 for troponin C; and $E^{1\%}_{280nm}$ = 4.0 for troponin I (Malencik et al., 1975). The concentrations of the troponin C fragments were initially approximated by the Bradford method (Bradford, 1976), employing troponin C as a standard, and later confirmed by amino acid analysis. For the calculation of molar concentrations of the proteins, the molecular weights used were PFK = 336 000 (tetramer), TnC = 18 000, CaM = 16 700, and TnI = 20 700.

Gel Electrophoresis in the Detection of Phosphofructokinase–Protein Interaction. NaDodSO$_4$–polyacrylamide electrophoresis gels were run on a linear 9–19% gradient minigel system (8 cm × 10 cm) with the proper proportions of 30% acrylamide and 0.8% bis(acrylamide). The gel buffer was essentially that of the Laemmli (1970) system except that the separating gel was made 0.75 M in Tris-HCl, pH 8.8, and the running buffer (pH 8.3) was 50 mM Tris, 60 mM boric acid, and 1 mM EDTA containing 0.1% NaDodSO$_4$ (Malencik & Anderson, 1987). A 10% urea gel with a 6% stacking gel was made up in a solution containing 80 mM glycine, 20 mM Tris, 1 mM $CaCl_2$ (pH 8.3), and 6 M urea. The running buffer was of the same composition except for the absence of urea. To detect the interaction of phosphofructokinase with calmodulin or troponin C, phosphofructokinase (0.6 mg/mL) was incubated with either protein (0.12–0.3 mg/mL) in a buffer containing 25 mM Mops–KOH, pH 7.0, 10% glycerol, 1.0 mM $CaCl_2$, and 5.0 mM β-mercaptoethanol for 1 h prior to electrophoresis. Solid urea (to 6 M) was added to each sample immediately before its application to the gel. At the end of each run, the gels were either stained with Coomassie blue or cut out without staining. The unstained gel sections (about 0.4 cm × 0.6 cm) containing phosphofructokinase were directly treated with a small volume (20 μL) of NaDodSO$_4$ gel sample buffer and loaded onto the 9–19% gradient NaDodSO$_4$ gel. After electrophoresis, this final gel was stained with Coomassie blue.

Phosphorylation of Phosphofructokinase. Phosphofructokinase (0.125 mg/mL) was preincubated with 5 μM calmodulin or troponin C in a system containing 50 mM Mops–KOH, pH 7.0, 1.0 mM Ca^{2+} or 1.0 mM EGTA, 1.0 mM dithiothreitol, and 10% glycerol. Buffer without calmodulin or troponin C was used in control experiments. After the specified periods of preincubation at 30 °C, phosphorylation was started by bringing the system to 0.25 mM [γ-^{32}P]ATP (100 μCi/μmol), 0.1 mg/mL phosphofructokinase, 4 μM calmodulin or troponin C, 5 mM $MgCl_2$, 0.8 mM Ca^{2+}, and 5 μg/mL catalytic subunit of cAMP-dependent protein kinase (all in the preceding buffer). At different time intervals, 55-μL samples were removed and mixed with 5.5 μL of solution containing 10 mg/mL bovine serum albumin and 0.15% sodium deoxycholate. A 55-μL aliquot of this mixture was then spotted on a 2.1-cm diameter glass filter disk and subsequently precipitated and washed with a solution containing 10% (w/v) trichloroacetic acid and 2% (w/w) sodium pyrophosphate. Following final washing with ethanol and ether, the radioac-

tivity on the filter was determined in 3 mL of scintillation fluid on the Beckman LS6800 scintillation counter. The counting efficiency with scintillation fluid was determined to be 100%. The scintillation fluid contains 23.8% (v/v) Triton X-100 and 0.3% (w/v) Permablend III [United Technologies Parkard, 91% PPO (2,5-diphenyloxazole), and 9% bis-MSB [1,4-bis-(2-methylstyryl)benzene]] in toluene (Baker analyzed reagent).

The phosphorylation of phosphofructokinase by cGMP-dependent protein kinase followed the same procedure used with cAMP-dependent protein kinase except that 10 μg/mL protein kinase plus 10 μM cGMP were employed. The phosphorylation by protein kinase C was performed in a solution containing 25 mM Mops–KOH, pH 7.5, 5 mm $MgCl_2$, 1 mM $Ca(OAc)_2$, 1 mM dithiothreitol, 50 μg/mL phosphatidylserine, 5 μg/mL diolein, 0.25 mM ATP, 0.25 mg/mL phosphofructokinase, 0 or 10 μM troponin C, and 2 units/mL protein kinase C.

Identification of Phosphorylation Sites. To facilitate radioactive monitoring of the separated fragments of phosphofructokinase, most of the free ATP was removed from the reaction mixtures before CNBr digestion was undertaken. The phosphorylation reaction was first terminated by the addition of an equal volume of ice-cold solution containing 70% formic acid and 10 mM EDTA. Then a small volume of a stock solution of unlabeled phosphofructokinase (10 mg/mL) was added to give a final concentration of 2.0 mg/mL. The protein was precipitated by the addition of 10% trichloroacetic acid and washed twice with 2 volumes of solution containing 10% trichloroacetic acid and 2% sodium pyrophosphate. This was followed by washing with ethanol and ether. The pellet then was dissolved in 70% formic acid. The trichloroacetic acid precipitable radioactivity of each sample was checked by scintillation counting on glass filter papers as described earlier. The chemical digestion of phosphofructokinase was performed by treating the enzyme with a 100-fold molar excess of CNBr (relative to total methionine) in 70% formic acid for 24 h in the dark (Gross, 1967). The reaction was stopped by a 10-fold dilution of the reaction mixture with water. In preparation for HPLC, the samples were then dried in a Savant speed vacuum concentrator and redissolved in a solution containing 3.0 M guanidine hydrochloride and 0.1% trifluoroacetic acid.

The HPLC setup used in this study consists of two Beckman 110B pumps, a Beckman 421A controller, a Beckman manual injector, and an Isco V4 detector. Reverse-phase HPLC was performed on a Vydac C18 column. A small precolumn (20 mm × 2 mm) packed with the same materials was used as a guard column. The column was run with a programmed gradient of A (water + 0.1% trifluoroacetic acid) and B (acetonitrile + 0.06% trifluoroacetic acid). The flow rate is controlled at 0.75 mL/min.

When the CNBr fragments were separated by gradient reverse-phase HPLC, two radioactive fractions were found. Each was collected and dried in the speed vacuum system. The first fraction (referred to as the CNBr phosphopeptide) was sequenced directly. The second fraction was further digested with *S. aureus* strain V8 protease, employing a ratio of 1 mg of protease/30 mg of peptide in 50 mM sodium phosphate (pH 8.0) at 30 °C for 4 h (Drapeau, 1978). The products of the V8 protease digestion were separated by the same HPLC system used to obtain the CNBr phosphopeptide. The single radioactive peak was collected and further purified on a TSK-SP HPLC cation-exchange column, with a gradient of NaCl (0–1.0 M) in 25 mM sodium acetate (pH 4.5) employed for elution. The radioactive fraction (eluting at 0.43 M NaCl) was collected, dried, redissolved in a small volume of water containing 0.1% trifluoroacetic acid, and reloaded on the reverse-phase HPLC C18 column. The only radioactive fraction eluted from this column (referred to as the V8 phosphopeptide) was collected and sequenced. Amino acid sequencing of the phosphofructokinase fragments was accomplished by an Applied Biosystem 475A gas-phase protein sequencer maintained at the Oregon State University Center for Gene Research and Biotechnology.

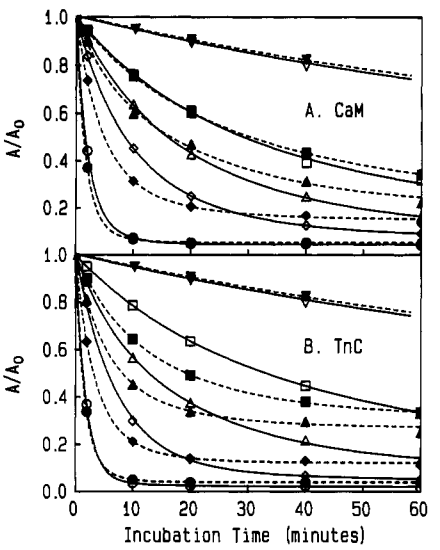


FIGURE 1: Inactivation of phosphofructokinase occurring in the presence of troponin C or calmodulin. Phosphofructokinase (20 μg/mL) was incubated with different concentrations of CaM (A) or troponin C (B) in solutions containing either 0.1 mM Ca^{2+} (open symbols with solid lines) or 0.1 mM EGTA (closed symbols with dashed line). The buffer contained 25 mM Mops–KOH, pH 7.0, 25% glycerol, 5 mM β-mercaptoethanol, and 0.5 mM dithiothreitol. Incubation temperature is 24 °C. At the indicated time intervals, aliquots were removed for activity assay at 30 °C. The calmodulin and troponin C concentrations were as follows: (▽, ▼) zero; (□, ■) 0.025 μM; (△, ▲) 0.05 μM; (◇, ◆) 0.1 μM; (○, ●) 0.4 μM. The activity was expressed by A/A_0, with A representing the measured activity and A_0 representing the activity at zero time of inactivation. (A_0 = 118 units/mg.)

RESULTS

Inactivation of Phosphofructokinase in the Presence of Troponin C and Calmodulin. Troponin C is evolutionarily related to calmodulin but does not generally interact with calmodulin-dependent enzymes [cf. Vanaman (1980)]. Experiments with troponin C may shed light on its potential role in the regulation of phosphofructokinase and on the uniqueness of the reported association of the enzyme with calmodulin (Mayr & Heilmeyer, 1983). Figure 1, illustrating the measured residual activity (A/A_0) of phosphofructokinase determined after varying periods of preincubation with either calmodulin (panel A) or troponin C (panel B), shows that similar rates of enzyme inactivation occur with the two proteins.

The time course of inactivation is typically not first order but is instead biphasic, with an initial rapid phase followed by a slow phase (Figure 1). Both the equilibrium phosphofructokinase activity and the rate of inactivation are related to the concentration of the inactivating protein. Troponin C and calmodulin both cause significant inactivation when present at molar concentrations equivalent to that of phosphofructokinase (in terms of tetramer). The initial rate is slower in the presence of calcium than in its absence. However, the former condition leads to a more extensive inactivation than the latter. At low levels of either troponin C or calmodulin, the initial inactivation rate is almost directly proportional to

Table I: Effects of Small Ligands on the Inactivation of Phosphofructokinase Induced by Troponin C[a]

ligands (units of $C_{1/2}$)	with 0.1 mM Ca^{2+}	with 0.1 mM EGTA
fructose-6-P (μM)	70	148
fructose-2,6-P_2 (μM)	0.04	0.045
ATP (μM)	8.0	20.5
ADP (mM)	0.2	0.93
AMP (mM)[b]	0.1	0.3
phosphate (mM)	2.7	9.5
citrate (mM)	1.6	3.3

[a] The data in the table represent the concentration of each ligand required for 50% of maximum protection when 0.02 mg/mL of phosphofructokinase is incubated in the presence of 0.25 μM troponin C for 20 min. [b] The maximum protection with AMP is only 35% in the presence of calcium and 30% in the absence of calcium.

the protein concentration. This observation is discussed in our development of a kinetic model for the inactivation. Phosphofructokinase activity in the control experiments also decreases with time, but at a much slower rate. During the first hour of incubation, the activity in the control declines almost linearly, allowing extrapolation to A_0. The inactivation of the control sample eventually slows down, finally attaining a relative equilibrium with 38% activity remaining after 6 h of incubation. Troponin C and calmodulin apparently inactivate phosphofructokinase by (1) accelerating an intrinsic inactivation process and (2) shifting the equilibrium toward more extensive inactivation.

The results of our inactivation studies with calmodulin differ from Mayr's (1984a,b) in the degree of calcium dependence found. This may be due to the different conditions used. In Mayr's experiments, in which the solutions contained 0.1 M KCl and 50 μM ATP, the inactivation obtained in the absence of calcium was relatively low. The presence of glycerol in the medium significantly decreases both the background rate of inactivation in the control and the rate of the induced inactivation, thus facilitating the determination of complete time courses. It also increases the percent recovery of phosphofructokinase activity in subsequent reactivation experiments.

All the low-molecular-weight effectors of phosphofructokinase exert protective effects when the enzyme is incubated with troponin C. The data in Table I show the concentrations of ligand required for 50% of maximum protection of phosphofructokinase activity, with the usual 20-min incubation period. In brief, the inactivation is prevented by both positive and negative allosteric effectors of the enzyme. Among these factors, fructose 2,6-bisphosphate, ATP, and fructose 6-phosphate are very effective, while ADP, citrate, and phosphate are less so. AMP—the potent allosteric activator of phosphofructokinase—shows relatively little effect. The maximum protection obtained with AMP is only 35% but is more than 90% with all the other factors. In the absence of calcium, the effector concentrations required for protection are generally higher than those required in the presence of calcium, except in the case of fructose 2,6-bisphosphate.

Finally, we have examined the inactivation of purified phosphofructokinase obtained from three different sources: rabbit skeletal muscle, rabbit liver, and yeast. As summarized in Table II, liver phosphofructokinase shows little sensitivity to 1.0 μM troponin C while yeast phosphofructokinase is totally resistant to inactivation. Similar results were obtained in the presence of calmodulin.

Effect of Proteolytic Fragments of Troponin C and of Other Acidic Proteins. Studies with the purified tryptic and thrombic fragments of troponin C may help to identify interaction sites for phosphofructokinase. These particular fragments have been well characterized, both structurally and functionally (Leavis et al., 1978a,b). The thrombic fragment known as TH1 contains troponin C residues 1–120 while TH2 contains residues 121–159. As discussed under Materials and Methods, tryptic fragment TR1 apparently contains sequences 8–84 and 8–88 while TR2 corresponds to sequences 85–159 and 89–159. Table III summarizes the inactivation of phosphofructokinase obtained both with the fragments and with intact troponin C. The data represent the fragment concentrations (in micromolar) required for 50% inactivation of enzyme samples within the usual 20-min incubation period. Note that all the fragments promote enzyme inactivation, but with varying degrees of effectiveness. The differences among them are most pronounced when the incubation buffer contains 5 μM ATP. TH2 and TR1 both show low inactivating ability and high sensitivity to ATP protection. TR2 is almost as effective as intact troponin C under all conditions examined. TH1 is distinguished by effectiveness in solutions containing EGTA, either with or without ATP. Interestingly, the phosphofructokinase inactivating abilities of the fragments correlate with their known affinities for troponin I (Leavis et al., 1978a).

Table II: Troponin C Induced Inactivation Observed with Phosphofructokinase from Different Sources[a]

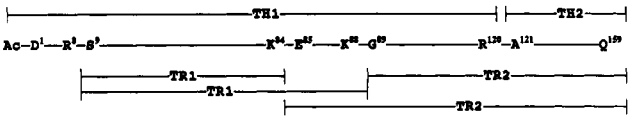
PFK source	1.0 μM TnC–Ca^{2+}	1.0 μM TnC–EGTA
rabbit muscle	0.11	0.10
rabbit liver	0.93	0.94
yeast	1.0	1.0

[a] PFK (20 μg/mL) was incubated with 1.0 μM troponin C in the presence of either 0.1 mM Ca^{2+} or 0.1 mM EGTA under standard inactivation conditions (see Materials and Methods). Data in the table represent the relative activities of phosphofructokinase remaining after 20 min of incubation. The activity in the control is defined as 1.

Table III: Inactivation of Phosphofructokinase Induced by Troponin C Fragments[a]

fragments	Ca^{2+}	EGTA	Ca^{2+} + ATP	EGTA + ATP
TnC	0.078	0.072	0.13	0.098
TR1	0.60	0.31	5.42	4.74
TR2	0.14	0.14	0.27	0.41
TH1	0.36	0.091	1.22	0.23
TH2	0.95	1.42	6.98	10.0

[a] The data in the table represent the concentrations of troponin C or its fragments in μM required for 50% of inactivation. Varying concentrations of troponin C or its fragments were incubated with 0.02 mg/mL of phosphofructokinase in the presence of either 0.1 mM Ca^{2+} or 0.1 mM EGTA. "+ATP" means 5 μM ATP was included. Phosphofructokinase activity was measured after a 20-min incubation period. Troponin C fragments:



The extent of inactivation determined after incubation of phosphofructokinase with a variety of individual proteins, each maintained at a fixed concentration of 1 μM when present, is summarized in Table IV. Note that moderate inactivation, with varying degrees of calcium dependence, occurs in the presence of α-actinin, troponin, the 17- and 20-kDa myosin light chains, and S-100. Parvalbumin, a Ca^{2+}-binding protein with an isoelectric point similar to that of calmodulin or troponin C (Heizmann et al., 1974), is considerably less effective than any of the preceding proteins. A parvalbumin concentration as high as 6.7 μM is needed to produce 50% inactivation within 20 min of incubation. Additionally, the maximum inactivation is only 70%. Soybean trypsin inhibitor, a protein containing 17% acidic amino acid residues (Kim et al., 1985),

Table IV: Inactivation of Phosphofructokinase in the Presence of Various Acidic Proteins[a]

acidic proteins (1.0 μM)	0.1 mM Ca^{2+}	0.1 mM EGTA
control	1.000	1.000
troponin C (18 kDa)	0.106	0.100
calmodulin (16.8 kDa)	0.115	0.110
troponin (75 kDa)	0.267	0.435
MLC (17 kDa)	0.303	0.322
MLC (20 kDa)	0.345	0.523
S-100 (16 kDa)	0.333	0.362
α-actinin (96 kDa)	0.308	0.415
poly(Glu-Tyr) (39 kDa)	0.330	0.330
parvalbumin (12 kDa)	0.790	0.775
SBTI (22.5 kDa)	0.880	0.880

[a] Data in the table represent the relative activity of phosphofructokinase remaining after 20 min of incubation with a 1.0 μM solution of the indicated protein under standard conditions (see Materials and Methods). Abbreviations: MLC, smooth muscle myosin light chain; SBTI, soybean trypsin inhibitor.

also shows a low degree of phosphofructokinase inactivating ability. As an extreme case, we tested a synthetic polypeptide (with an average molecular weight of 39000) containing 51% of glutamyl residues—poly(NaGlu-Tyr). The concentration of this acidic copolymer required for 50% inactivation within 20 min is only 51 nM. However, the maximum total inactivation is just 70%.

Kinetic Modeling of the Inactivation. A mathematical model for the reversible inactivation of phosphofructokinase could be valuable, both for the interpretation of our data and for its potential applications to other systems. In view of the known rapid dissociation and association of phosphofructokinase and the possible existence of enzyme conformers (Luther et al., 1983, 1985; Mayr, 1984a,b), we consider the kinetic model shown in Scheme I.

Scheme I

$$T \underset{}{\overset{K}{\rightleftharpoons}} 2D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 2D^*$$

K is the dissociation constant for the equilibrium between tetramer T and dimer D. The transformation of the two conformers of the dimer, D and D*, is the rate-limiting step—with k_2 and k_{-2} representing the forward and reverse reaction rate constants, respectively. $K^* = k_2/k_{-2}$ stands for the equilibrium constant for the interconversin of the two dimers. As detailed in the supplementary material, we have derived the following equation relating the fraction of remaining activity (A/A_0) to the period of incubation (t).

$$A/A_0 = \left[\frac{(P_2 - P_1 + P) + (P_1 - P_2 + P)e^{-Pt}}{(P_1 + P_2 + P) - (P_1 + P_2 - P)e^{-Pt}}\right]^2 \quad (1)$$

$$P_1 = k_2 + k_{-2} \qquad P_2 = 4k_{-2}\sqrt{T_0/K} \qquad P = \sqrt{P_1^2 + P_2^2}$$

In eq 1, $A/A_0 = T/T_0$, where T and T_0 represent the molar concentrations of phosphofructokinase tetramer at time t and 0, respectively. Unweighted nonlinear regression analysis (using Statgraphics computer software) of the inactivation time course gives a very good fit of eq 1 to the data shown in Figure 1. Table V shows the values of the parameters P_1 and P_2 along with the correlation coefficient, r^2. Note that only the first 60 min of the inactivation time course is considered here. In fact, the inactivation does not reach a definite equilibrium but continues at a slow rate. We attribute this to the further transformation of D* to aggregated forms, as noted by other authors (Mayr, 1984a). Because the aggregation process is much slower than the calmodulin or troponin C induced transformation between D and D*, we ignore it in our kinetic model. Aggregation is believed responsible for the deviation of the activities observed after 1 h.

In order to approximate the rate constants, the dissociation constant K was first estimated by application of the method described by Jenkins et al. (1985) to the results of activity measurements performed at enzyme concentrations ranging from 0.0025 to 0.08 mg/mL. According to the equation $C_0/A = I + 0.5(IK/A)^{1/2}$ (in which C_0 represents the total phosphofructokinase concentration; A, the catalytic activity determined after dilution; K, the dissociation constant; and I, a constant), linear regression analysis yields a plot of C_0/A versus $1/\sqrt{A}$ with a correlation coefficient $r^2 = 0.9905$ (not shown). From this linear plot, a dissociation constant $K = (4.5 \pm 0.5) \times 10^{-9}$ M was calculated. We note that the sedimentation equilibrium experiments of Luther et al. (1986), which were performed in a buffer containing 25 mM Tris-carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA, pH 7.0, provided dissociation constants of 7.7×10^{-9} M and 3.6×10^{-11} M corresponding to the two models $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_8$ and $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$, respectively.

Table V shows the values of k_2, k_{-2}, and K^* which were calculated from their relationships to P_1 and P_2 by assuming $K = 4.5$ nM. The absolute value of K mainly affects k_2 and K^* but has little effect on k_2. It is clear that troponin C and

Table V: Kinetic Parameters for the Inactivation of Phosphofructokinase[a]

conditions	P_1 (min^{-1})	P_2 (min^{-1})	r^2	k_2 (min^{-1})	$k_{-2} \times 10^3$ (min^{-1})	K^*
control-Ca^{2+}	0.0029	0.0045	0.995	0.0026	0.33	7.96
control-EDTA	0.0027	0.0042	0.996	0.0024	0.30	7.87
0.025 μM CaM-Ca^{2+}	0.015	0.014	0.999	0.014	0.99	14
0.05 μM CaM-Ca^{2+}	0.025	0.016	0.999	0.028	1.2	20
0.1 μM CaM-Ca^{2+}	0.045	0.027	1.00	0.043	1.9	22
0.4 μM CaM-Ca^{2+}	0.22	0.099	1.00	0.22	7.1	31
0.025 μM CaM-EGTA	0.015	0.019	1.00	0.014	1.4	10
0.05 μM CaM-EGTA	0.027	0.031	0.994	0.025	2.2	11
0.1 μM CaM-EGTA	0.082	0.075	0.999	0.077	5.4	14
0.4 μM CaM-EGTA	0.28	0.13	1.00	0.27	9.5	28
0.025 μM TnC-Ca^{2+}	0.013	0.012	1.00	0.012	0.82	14
0.05 μM TnC-Ca^{2+}	0.031	0.022	0.999	0.029	1.5	19
0.1 μM TnC-Ca^{2+}	0.067	0.029	0.999	0.065	2.1	31
0.4 μM TnC-Ca^{2+}	0.27	0.078	1.00	0.26	5.6	47
0.025 μM TnC-EGTA	0.022	0.045	0.999	0.024	3.2	7.5
0.05 μM TnC-EGTA	0.057	0.081	0.998	0.051	5.8	8.8
0.1 μM TnC-EGTA	0.13	0.10	0.999	0.12	7.1	17
0.4 μM TnC-EGTA	0.31	0.13	1.00	0.30	9.1	33

[a] Nonlinear regression analysis of the inactivation data in Figure 1 according to eq 1 yields parameters P_1 and P_2 with correlation factor r^2. k_2, k_{-2}, and K^* were calculated from the value of P_1, P_2, and the estimated value of $K = 4.5$ nM.

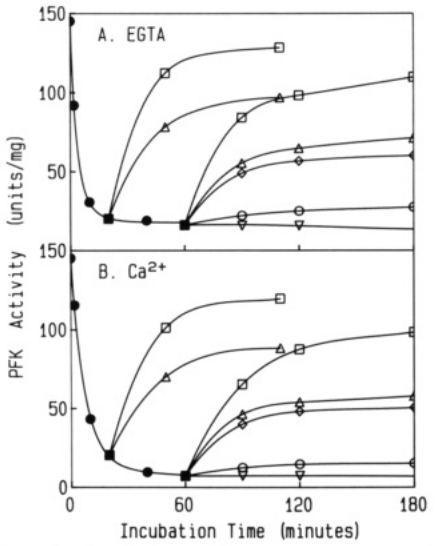


FIGURE 2: Reactivation of phosphofructokinase. Phosphofructokinase was preincubated under standard conditions with 0.1 μM troponin C in the presence of either 0.1 mM EGTA (panel A) or 0.1 mM Ca^{2+} (panel B). After 20- and 60-min incubation periods, minimal values of the following substances were added to give the indicated final concentrations: (□) 1.0 mM ATP; (△) 0.1 mM fructose-2,6-P_2; (◇) 1.0 mM ADP; (○) 1.0 mM AMP; (▽) 1.0 mM citrate. The recovered enzyme activity was measured under optimal assay conditions at 30 °C at the indicated times. Other conditions are explained under Figure 1.

calmodulin not only change the rate constants k_2 and k_{-2} but also change the equilibrium constant K^* between the two conformers of the dimer. The nearly proportional increase in the apparent value of k_2 suggests a linear relationship between the initial rate of inactivation and the concentration of the inactivating protein. Calcium ion shows its effects in moderately lower values of k_2 and higher values of K^*. The kinetic model also can be applied to the inactivation of phosphofructokinase induced by the other proteins. For poly(Glu-Tyr), for example, relative higher values of k_2 and lower values of K^* are obtained.

Reactivation of Phosphofructokinase after Incubation with Troponin C. Phosphofructokinase activity can be restored by the addition of certain effectors (Figure 2). Of these, ATP, ADP, and fructose 2,6-bisphosphate are especially efficient in reactivation while fructose 6-phosphate, AMP, phosphate, and citrate give no reactivation (data not shown for fructose 6-phosphate and inorganic phosphate). Note that the presence or absence of Mg^{2+} has no effect on the enzyme reactivation induced by ATP or ADP. The final extent of reactivation decreases both with the duration of inactivation and with the concentration of the inactivating protein (data not shown). A significantly lower recovery is obtained after 60-min incubation than after 20 min, especially when calcium is present. After prolonged incubation (4 h or more) in the presence of calcium, the inactivation becomes almost irreversible. For simplicity, Figure 2 shows only the results obtained with troponin C. Higher recoveries are obtained with calmodulin, following the generally greater effectiveness of troponin C in the inactivation process (cf. Figure 1).

Interaction of Phosphofructokinase with Troponin C and Calmodulin As Shown by Polyacrylamide Gel Electrophoresis. An unusual property of rabbit muscle phosphofructokinase—viz., the total inability to enter either nondenaturing or urea-containing polyacrylamide gels—was used to demonstrate its interactions with troponin C and calmodulin. Urea gel electrophoresis, employing a 6% stacking gel in combination with a 10% resolving gel, was performed as described under

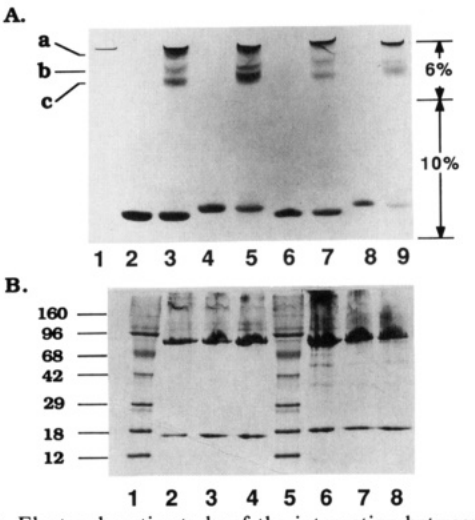


FIGURE 3: Electrophoretic study of the interaction between phosphofructokinase and troponin C or calmodulin. Upper photograph (A) shows the separation obtained with a 10% urea gel layered with 6% stacking gel. Phosphofructokinase (0.6 mg/mL) or a control solution without phosphofructokinase was incubated with 0.12 mg/mL (lanes 6–9) or 0.3 mg/mL (lanes 2–5) troponin C or calmodulin in the presence of calcium, as described under Materials and Methods. Aliquots (15 μL) of the mixtures were then loaded onto the gel. Lane 1, phosphofructokinase control; lanes 2 and 6, troponin C control; lanes 3 and 7, phosphofructokinase + troponin C; lanes 4 and 8, calmodulin control; lanes 5 and 9, phosphofructokinase + calmodulin. Lower photograph (B) shows the results of 9–19% $NaDodSO_4$–polyacrylamide gel electrophoresis performed on phosphofructokinase bands that had been individually excised from an unstained and unfixed urea gel. Lanes 1 and 5, protein markers; lanes 2, 3, and 4, calmodulin-inactivated phosphofructokinase bands a, b, and c, respectively, from urea gel electrophoresis; lanes 6, 7, and 8, troponin C inactivated phosphofructokinase bands a, b, and c, respectively.

Materials and Methods. The photograph in Figure 3A shows the inability of the enzyme alone to enter the stacking gel, possibly reflecting aggregation. In contrast, samples that have been incubated with troponin C or calmodulin in the presence of calcium readily enter the stacking gel with a concomitant decrease or even complete disappearance of the calmodulin or troponin C bands. In these cases, the enzyme usually appears as three components in the stacking gel. In order to show the original colocalization of troponin C or calmodulin with phosphofructokinase, linear 9–19% gradient $NaDodSO_4$ gel electrophoresis was performed on the bands which had been excised from the stacking gel, without prior fixing or staining (Figure 3B). When urea–polyacrylamide gel electrophoresis is performed in the absence of calcium, the inactivated enzyme fails to enter the stacking gel and all the troponin C or calmodulin is found in the lower gel (not shown).

Phosphorylation of Phosphofructokinase by cAMP-Dependent Protein Kinase. As shown in Figure 4, the rate of phosphorylation catalyzed by cAMP-dependent protein kinase increases manyfold after preincubation of phosphofructokinase with either calmodulin or troponin C in the presence of calcium. Within a short period of time, which corresponds to the rapid phase of phosphorylation, an average of more than 1 mol of phosphate is incorporated into each mole of enzyme protomer. Autoradiography of the $NaDodSO_4$–polyacrylamide electrophoresis gel containing samples removed from the phosphorylation mixture after 2 h of reaction (inset to Figure 4) verifies that phosphofructokinase is the only protein which has been phosphorylated. The rate of phosphorylation is also dependent on the length of the preincubation period and on the ratio of calmodulin or troponin C to the enzyme. With 0.1 mg/mL phosphofructokinase plus 4 μM troponin C, the maximum initial rate of phosphorylation is obtained after

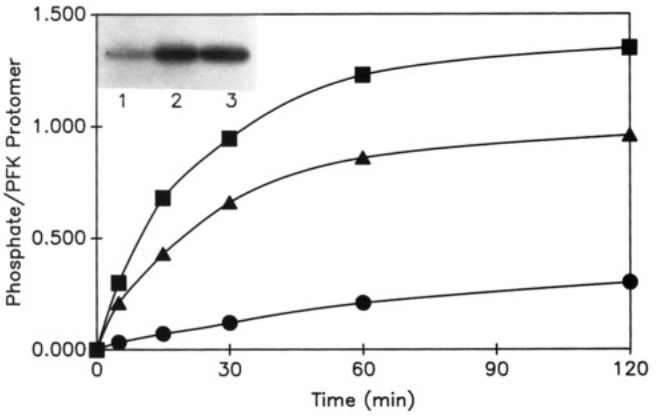


FIGURE 4: Time course of the phosphorylation of phosphofructokinase catalyzed by cAMP-dependent protein kinase. Phosphofructokinase (0.125 mg/mL) had been preincubated for 1 h with 5 μM of either troponin C or calmodulin in a solution containing 50 mM Mops–KOH, pH 7.0, 1.0 mM $CaCl_2$, 1.0 mM dithiothreitol, and 10% glycerol (30 °C). Phosphorylation was initiated at zero time by the addition of 0.25 mM ATP, 5.0 mM $MgCl_2$, and 5 μg/mL catalytic subunit of protein kinase. Troponin C (■); calmodulin (▲); control (●). The inset shows the autoradiograph of the 9–19% $NaDodSO_4$–polyacrylamide electrophoresis gel containing 1.5-μg samples of phosphofructokinase taken after 2 h of phosphorylation. From left to right: 1, control; 2, with calmodulin; 3, with troponin C.

Table VI: Calcium-Dependent Effects of Calmodulin and Troponin C on the Phosphorylation of Rabbit Muscle Phosphofructokinase by cAMP-Dependent Protein Kinase

conditions	mol of phosphate/mol of enzyme protomer after 1 h	after 2 h
PFK + 1 mM Ca^{2+}	0.18	0.35
PFK + calmodulin + 1 mM Ca^{2+}	0.85	1.01
PFK + troponin C + 1 mM Ca^{2+}	1.20	1.31
PFK + 1 mM EGTA	0.15	0.33
PFK + calmodulin + 1 mM EGTA	0.17	0.38
PFK + troponin C + 1 mM EGTA	0.17	0.40

[a] PFK (0.125 mg/mL) was preincubated for 30 min with either 5 μM calmodulin or 5 μM troponin C as described under Materials and Methods. Phosphorylation was initiated by the addition of 0.25 mM ATP, 5.0 mM $MgCl_2$, and 5 μg/mL of the catalytic subunit of cAMP-dependent protein kinase. Phosphate incorporation was measured after 1 h of reaction and again after 2 h.

10 min of preincubation. Shorter preincubation times give a relatively lower initial phosphorylation rate while longer periods of preincubation lead to a higher initial phosphorylation rate and a ~20% lower final phosphate incorporation. With a fixed 20-min preincubation time, the phosphorylation rate initially increases almost linearly with increasing troponin C concentrations, approaching a plateau at troponin C/enzyme ratios above 5. In the absence of calcium, troponin C and calmodulin have little effect on phosphate incorporation (Table VI). Effectors of phosphofructokinase inhibit the induced phosphorylation reaction (Table VII). The presence of a 0.1 mM concentration of either fructose 2,6-bisphosphate or fructose 1,6-bisphosphate almost totally abolishes the influence of either troponin C or calmodulin. The specific effects of ATP, a substrate of protein kinase, are difficult to resolve. Other effectors—AMP, phosphate, ADP, and citrate—decrease the phosphorylation rate, with the latter two having the least effect. These results are consistent with the inactivation studies (Table I). Control experiments without troponin C showed that 0.1 mM fructose 2,6-bisphosphate, 0.1 mM fructose 1,6-bisphosphate, and 1.0 mM AMP increase the phosphorylation rate by 80%, 15%, and 30%, respectively; that 1.0 mM ADP

Table VII: Effects of Small Ligands of PFK on the Phosphorylation Obtained in the Presence of TnC[a]

additions	mol of phosphate/mol of PFK monomer 1 h	2 h
control (no addition)	0.20	0.35
TnC (5 μM)	0.84	1.26
TnC + Fru-2,6-bisphosphate (0.1 mM)	0.21	0.41
TnC + Fru-1,6-bisphosphate (0.1 mM)	0.24	0.39
TnC + ATP (0.31 mM)[b]	0.65	1.30
TnC + ADP (1.0 mM)	0.53	0.90
TnC + AMP (1.0 mM)	0.52	0.69
TnC + phosphate (10.0 mM)	0.28	0.63
TnC + citrate (1.0 mM)[c]	0.58	0.98

[a] PFK (0.125 mg/mL) was preincubated with 5 μM troponin C in the presence of 1.0 mM Ca^{2+} and the indicated ligands for 30 min at 30 °C. Phosphate incorporation was determined both at 1 and 2 h after the initiation of phosphorylation as described under Materials and Methods. [b] Since radioactive ATP was present in the preincubation system, the phosphorylation reaction was started by the addition of protein kinase (5 μg/mL). [c] With citrate, 2.0 mM Ca^{2+} was present in the preincubation system.

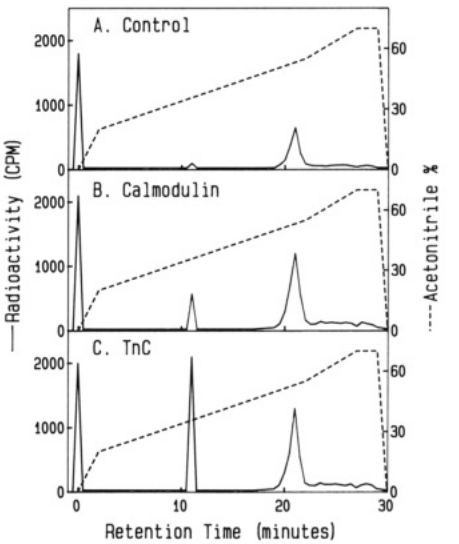


FIGURE 5: Radioactivity monitoring of the reverse-phase HPLC separation of the CNBr fragments of ^{32}P-labeled phosphofructokinase. Panels B and C illustrate the distribution obtained with enzyme samples that had been preincubated with troponin C or calmodulin before phosphorylation while panel A illustrates the results determined with the control, which contained no troponin C or calmodulin. The radioactivity peak at the injection mark (0% acetonitrile) corresponds to residual ATP while the phosphopeptide fractions elute at 35% and 53% acetonitrile. The dashed line represents the gradient profile. Other experimental details are supplied in the text.

and 1.0 mM citrate decrease the rate by 30% and 40%, respectively; and that 10 mM phosphate has no effect—in agreement with earlier findings by Kemp et al. (1981). Clearly, fructose 2,6-bisphosphate, AMP, and phosphate influence the phosphorylation in the presence of troponin C by affecting the enzyme–protein interaction. ADP and citrate may affect both the enzyme–protein interaction and the enzyme itself.

Peptide Mapping of the Phosphorylated Phosphofructokinase. Peptide mapping of the CNBr digests of ^{32}P-labeled phosphofructokinase was performed by reverse-phase HPLC. Figure 5 shows the separation of the CNBr fragments which were prepared from enzyme samples which had been subjected to 2 h of phosphorylation. In the case of the control (Figure 5A), one radioactive peptide peak, eluting at 53% acetonitrile, predominates. The calmodulin and troponin C treated

phosphofructokinase samples yield this peak plus an additional major component, eluting at 35% acetonitrile (Figure 5B,C). Note the enhancement of both peaks which is demonstrated in the digests of the protein-treated enzyme and, in particular, the distinctive effects of troponin C on the component eluting at 35% acetonitrile.

Isolation and Sequencing of the Phosphorylated Fragments of Phosphofructokinase. The phosphopeptide eluting at 35% acetonitrile corresponds to a sharp peak on the absorbance (220-nm) profile and proved pure enough to be directly sequenced. It will hence be referred to as the CNBr phosphopeptide. The second peak (eluting at 53% acetonitrile) is a heterogeneous fraction judged by the broad peak on the OD_{220nm} profile. It was subjected to proteolytic digestion with V8 protease, and the resulting digest was refractionated as described under Materials and Methods. About 0.5 nmol of this purified peptide (designated as the V8 phosphopeptide) and about 0.5 nmol of the CNBr phosphopeptide were subjected to five cycles of the Edman degradations in the Applied Biosystem 475A gas-phase sequencer. The homogeneity of each sample was evidenced by a negligible backgroun of PTH-amino acids. The initial pentapeptide sequences of the two fragments proved to be as follows: CNBr phosphopeptide, Lys-Leu-Arg-Gly-Arg; and V8 phosphopeptide, His-Ile-Ser-Arg-Lys.

Considering the amino acid sequence of rabbit skeletal muscle phosphofructokinase (Poorman et al., 1984; Lee et al., 1987) and the specificities of CNBr and the V8 protease, the CNBr phosphopeptide must correspond to the sequence Lys-Leu-Arg-Gly-Arg-Ser-Phe-Met (representing amino acid residues 371–378), and the V8 phosphopeptide, to the sequence His-Ile-Ser-Arg-Lys-Arg-Ser-Gly-Glu (corresponding to positions 768–776). In the former, Ser^{376} is the only possible phosphorylation site. However, there are two possible phosphorylation sites in the latter: Ser^{770} and Ser^{774}. To identify the exact site, the filter disk containing the V8 phosphopeptide was removed from the sequencer after five cycles of the Edman degradation and extracted with a mixture of water and acetonitrile (v/v = 1:1) (Wang et al., 1988). Nearly 100% of the radioactivity was thus recovered. Fractionation of the concentrated extract by reverse-phase HPLC showed that the majority of the radioactivity appears at 28% acetonitrile, with the remainder eluting later. No radioactivity was found at the injection mark (0% acetonitrile), the elution position for inorganic phosphate, which would be released if the phosphoserine had been hydrolyzed during the Edman degradation (Wang et al., 1988) (data not shown). The retention of radioactivity after five cycles of the Edman degradation demonstrates that the phosphorylation site on the V8 phosphopeptide is Ser^{774}, the position reported by Kemp et al. (1981).

Determination of the Individual Rates of Phosphorylation of the Two Sites. We also determined the individual rates of phosphorylation occurring at the two sites. The procedures described in Figure 5 were applied to samples that had been removed from the phosphorylation mixture at various time intervals ranging from 5 min to 2 h. The radioactivities of the separated fractions were counted directly in the running phase solution of the reverse-phase column. The counting efficiencies were determined to be 49.0% and 50.5% in 35% and 53% acetonitrile solution, respectively. The recovery of protein in the trichloroacetic acid precipitation was 80–85% while at least 95% of the applied radioactivity was eluted from the reverse-phase HPLC column. The specific phosphate levels were calculated from the radioactivities of the individual fractions by assuming that CNBr cleavage is complete and

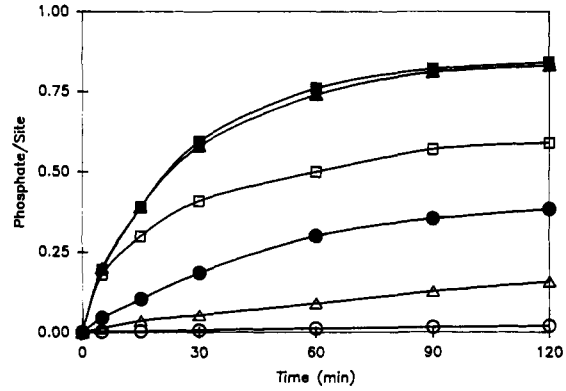


FIGURE 6: Determination of the individual rates of phosphorylation of Ser^{376} and Ser^{774}. The CNBr fragments prepared from phosphofructokinase which had been subjected to phosphorylation for differing periods of time were separated by reverse-phase HPLC following the procedure described under Materials and Methods for Figure 3. The radioactivity of each fraction was counted directly in the running phase solution. The phosphate content was calculated from the total radioactivity of each fraction, with the consideration that the recovery of protein from HPLC is 95%. The open symbols represent phosphorylation of Ser^{376}, and the closed symbols correspond to phosphorylation of Ser^{774}. Troponin C (□, ■); calmodulin (△, ▲); control (○, ●).

that the two phosphopeptides are equally recovered. The *total* phosphate incorporation of the intact enzyme was verified by scintillation counting on glass filter paper. The results in Figure 6 show that calmodulin and troponin C enhance the rate and extent of phosphorylation of the C-terminal site about equally, with the incorporation of ~0.84 mol of phosphate/mol of Ser^{774} after 2 h of reaction. In contrast, the extent of the phosphorylation of Ser^{376} ranges from 0.02 mol/mol in the control to 0.16 mol/mol when calmodulin is present, and up to 0.59 mol/mol for samples containing troponin C. In the absence of calmodulin or troponin C, the C-terminal site incorporates only 0.4 equiv of ^{32}P—a fractional stoichiometry that agrees with prevailing reports (Foe & Kemp, 1982; Sale & Denton, 1985).

Effect of Phosphorylation on the Enzymatic Activity of Phosphofructokinase. To investigate the effect of phosphorylation on enzymatic activity, we performed side by side catalytic assays and radiochemical determinations of covalently bound ^{32}P on samples that had been subjected to phosphorylation for varying periods of time (Figure 7). In this case, calmodulin or troponin C was added immediately before initiation of phosphorylation. As anticipated from the results in Table I, the inactivation rate decreases significantly in the presence of 0.25 mM ATP alone. The phosphorylation of phosphofructokinase catalyzed by the added cAMP-dependent protein kinase slightly retards the inactivation rate even further, with minimal effects on the reactivation which is induced by the addition of fructose 2,6-bisphosphate. These experiments indicate that the phosphorylation of phosphofructokinase has only modest effects on the catalytic activity determined under standard assay conditions and/or on its propensity for reversible inactivation.

Effects of Phosphorylation on Calmodulin and Troponin C Binding. We applied our urea gel electrophoresis system in a preliminary investigation of the effect of phosphorylation on the interactions of phosphofructokinase with troponin C and calmodulin. As shown for the untreated enzyme (Figure 3), phosphorylated phosphofructokinase (containing 1.2–1.5 mol of phosphate/mol of protomer) readily enters the stacking gel provided that either calmodulin or troponin C plus calcium are also present. The amounts of dissociated calmodulin de-

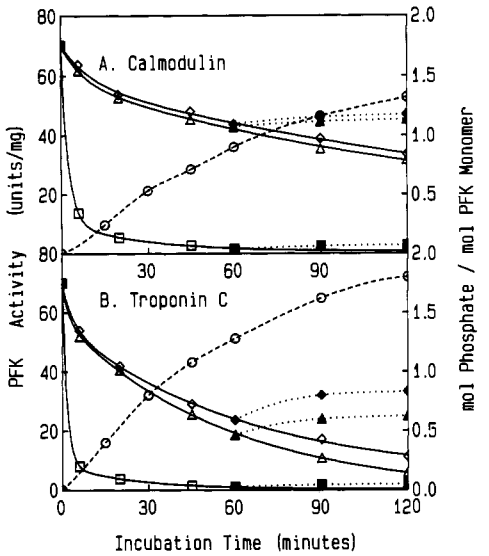


FIGURE 7: Inactivation and phosphorylation of phosphofructokinase. Phosphofructokinase (0.1 mg/mL) was incubated with 4 μM of either calmodulin (A) or troponin C (B) at 30 °C in a solution containing 50 mM Mops–KOH, pH 7.0, 10% glycerol, 1.0 mM dithiothreitol, and 0.8 mM $Ca(OAc)_2$. The time courses were determined in the absence of Mg^{2+}-ATP (□), in the presence of 0.25 mM ATP–5 mM $MgCl_2$ (△), and in the presence of 0.25 mM ATP–5 mM $MgCl_2$ plus 5 μg/mL of the catalytic subunit of cAMP-dependent protein kinase (◇, ○). The solid and dashed lines indicate the time course of inactivation and phosphorylation, respectively. The dotted lines with closed symbols illustrate the reactivation occurring upon the addition of 0.2 mM fructose-2,6-bisphosphate after 1 h of incubation.

Table VIII: Phosphorylation of PFK by cGMP-Dependent Protein Kinase and by Protein Kinase C[a]

protein kinase	mol of phosphate/mol of enzyme protomer	
	Ca^{2+} + 0 TnC	Ca^{2+} + 5 μM TnC
cGMP-dependent protein kinase	0.31	0.07
protein kinase C	0.09	0.08

[a] Phosphorylation was performed according to the description under Methods and Materials. The data in the table represent the moles of phosphate incorporated per mole of enzyme protomer after 2 h of reaction. All samples contained 1 mM $CaCl_2$.

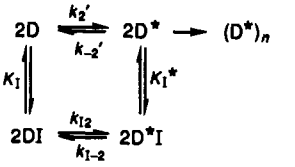
tected in the resolving gel were indistinguishable with phosphorylated and nonphosphorylated enzyme. However, a marginally greater dissociation (10–15%) of troponin C was found with the phosphorylated enzyme than with the control. These results are not illustrated in view of their similarity to those shown in Figure 3.

Phosphorylation of Phosphofructokinase by cGMP-Dependent Protein Kinase and by Protein Kinase C. Table VIII shows the extent of phosphorylation obtained in reactions catalyzed by cGMP-dependent protein kinase and by protein kinase C. Note that 0.31 mol of phosphate/mol of enzyme protomer are incorporated after 2 h of incubation with cGMP-dependent protein kinase in the absence of troponin C and that the introduction of 4.0 μM troponin C significantly decreases the phosphorylation (both samples contained 0.8 mM calcium acetate). The enzyme is poorly phosphorylated by protein kinase C, with less than 0.1 mol of phosphate/mol of enzyme protomer detected after a 2-hour incubation in either the presence or absence of troponin C.

Discussion

Our survey of a variety of previously unprobed contractile and other acidic proteins reveals that skeletal muscle troponin C has a pronounced ability to enhance both the rate and extent

Scheme II



of the reversible inactivation of purified rabbit skeletal muscle phosphofructokinase. The effectiveness of troponin C may even exceed that of calmodulin, whose calcium-dependent interaction with the enzyme was first reported by Mayr and Heilmeyer (1983). Troponin C was not necessarily expected to affect phosphofructokinase since it does not interact with most calmodulin-dependent enzymes. Even in the case of phosphorylase kinase, which associates reversibly with either protein, the binding of troponin C is about 2 orders of magnitude less effective than that of calmodulin (Cohen et al., 1979). Observations on other proteins that are ancestrally related to calmodulin and troponin C help to define the degree of specificity inherent in the phosphofructokinase interactions. Note that the two smooth muscle myosin light chains and S-100 are moderately effective in promoting the inactivation while parvalbumin is not (Table IV).

The action of proteolytic fragments suggest that specific regions of the troponin C molecule are involved in the inactivation of phosphofructokinase. TR2, which apparently consists of two related fragments encompassing troponin C residues 85–159 and 89–159, and TH1 (corresponding to residues 1–120) retain the inactivating properties of troponin C while TR1 (fragments encompassing residues 9–84 and 9–88) and TH2 (corresponding to residues 121–159) do not. We note that the sequence which is common to TR2 and TH1 occurs near calcium binding site III in the intact troponin C molecule and is believed to participate in the binding of troponin I (Grand et al., 1982).

The mechanism for the inactivation of phosphofructokinase by calmodulin and troponin C may apply to other proteins, such as the Zn^{2+}-dependent inactivating protein from rat liver (Brand & Söling, 1986) and the band 3 protein of the erythrocyte membrane (Jenkins et al., 1985). In the kinetic model which we have developed mathematically and applied to the data, we assume that the inactivating proteins have catalytic functions. Taking the protein–phosphofructokinase binding equilibrium into consideration, the model can be written in a more general form, as shown in Scheme II. This model is based on the hypothesis that the phosphofructokinase dimer exists in two forms and that the inactivating proteins bind only the dimer. This scheme is similar to that which was proposed without mathematical development by Mayr (1984a). Our scheme differs from the latter in that it does not include the binding of protein by the phosphofructokinase tetramer or by the aggregated dimers. The tetramer T and dimer D are in rapid equilibrium with a dissociation constant K, as generally believed. The interactions of the dimers, D and D*, with the inactivating proteins are also rapid, with association constants K_I and K_I*, respectively. The interconversion of the two dimer isoforms, D to D*, is a slow process which is greatly accelerated in the complexes DI and D*I. The aggregation of dimer D* occurs at an even slower rate and leads to the permanent loss of phosphofructokinase activity. The existence of this last step is supported by observations that phosphofructokinase forms aggregates and undergoes irreversible activity losses after long incubation periods (several hours) at relative high phosphofructokinase concentrations (e.g., 0.1 mg/mL; data not shown). This final step shows its effects in

a failure to achieve a true inactivation equilibrium. By ignoring the slow aggregation process, the rate constants k_2 and k_{-2} and equilibrium constant K^* in the simplified kinetic model (Scheme I) can be represented by the constants and concentrations in the more general model (Scheme II) in the following ways:

$$k_2 = k_2' + k_{I2}K_I[I] \qquad k_{-2} = k_{-2}' + k_{I\text{-}2}K_I^*[I]$$
$$K^* = K_0(1 + K_I^*[I])/(1 + K_I[I]) \qquad K_0 = [D^*]/[D]$$

We assume that most of the inactivator I remains unbound, or in other words, that [I] is nearly a constant during the period of the reaction investigated. Hence, all of the pseudoconstants in Scheme I are solely dependent on the initial concentration of inactivator in the equations. For the rate constants k_2 and k_{-2}, k_2' and k_{-2}' are apparently much smaller than the remaining terms. Thus k_2 and k_{-2} are essentially directly proportional to the initial inactivator concentration [I], as demonstrated by the nonlinear regression analysis of the kinetic data (Table V). K_0 represents the equilibrium constant at [I] = 0, as in the control experiment. Since K_I^* may be greater than K_I, K^* increases with increasing concentrations of the inactivating protein.

Small ligands of phosphofructokinase have distinctly different effects on the simple dimer–tetramer equilibrium and the changes that are induced by the addition of troponin C or calmodulin. For example, several positive effectors and a negative effector, citrate, tend to block the action of troponin C—with effects on both the rate and equilibrium. Yet, negative effectors stabilize the dimer (Kono & Uyeda, 1973), which is inactive, while positive effectors stabilize the tetramer and higher polymers (Lad et al., 1974; Reinhart, 1983). The effect of citrate on the inactivation may reflect stabilization of dimer D. Nonetheless, citrate has little effect on the reactivation process. The remarkable efficiencies of fructose 2,6-bisphosphate and ATP, both as inhibitors of inactivation and as promoters of reactivation, are consistent with previous observations on calmodulin (Mayr, 1984b). Note, however, that the latter investigated only the calcium-dependent interaction of calmodulin with phosphofructokinase and did not consider the influence of negative effectors.

Binding of either troponin C or calmodulin has distinctive effects on the physical and chemical properties of the phosphofructokinase molecule which are dependent on the presence of calcium. The association enables phosphofructokinase to enter the stacking gel during urea–polyacrylamide gel electrophoresis. Analyses of the enzyme bands eluted from the stacking gel demonstrate its comigration with troponin C or calmodulin. Even more interestingly, the addition of either protein significantly affects the in vitro phosphorylation of rabbit muscle phosphofructokinase catalyzed by cAMP-dependent protein kinase. Both proteins similarly enhance the rate and extent of phosphorylation occurring at the known C-terminal site (Ser^{744}). However, they also induce phosphorylation at a newly discovered site (Ser^{376}) which occurs in the hinge region connecting the N- and C-terminal domains of the phosphofructokinase model proposed by Poorman et al. (1984). Since the most complete radiolabeling attained in our work corresponds to 1.85 mol of phosphate/mol of enzyme protomer (cf. Figure 7) and the endogenous phosphate content of our purified phosphofructokinase is ~0.15 mol/mol, the total amount of phosphate in the reacted enzyme is close to the maximum expected.

Troponin C stimulates both a higher rate and greater extent of phosphate incorporation into Ser^{376} than does calmodulin. Although the two proteins probably recognize identical or closely overlapping binding sites in phosphofructokinase, they have markedly different effects on the exposure and/or reactivity of Ser^{376}. Interestingly, two calmodulin-binding fragments containing phosphofructokinase residues 379–405 and 713–779 (Buschmeier et al., 1987) correspond to sequences in close proximity to the phosphorylation sites identified by us. This is consistent with the hypothesis that calmodulin and cAMP-dependent protein kinase may act on common polypeptide sequences and that phosphorylation and calmodulin binding may affect each other (Malencik & Anderson, 1982). Note that the effect of calmodulin on the phosphorylation of phosphofructokinase is different from that found with gizzard myosin light chain kinase (Conti & Adelstein, 1981; Malencik et al., 1982) or with rabbit skeletal muscle phosphorylase kinase (Cox & Edstrom, 1981). In the latter cases, the binding of calmodulin prevents phosphorylation.

Table IX: Sequence Comparisons[a] of Potential Phosphorylation Sites in Rabbit Muscle[b] and Mouse Liver[c] Phosphofructokinase

```
Muscle PFK (residues 357-389)   VTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAH
                                : ::::: ::::: :::::: ::: ::::::
Liver  PFK (residues 358-390)   VQKAMDEERFDEAIQLRGRSFENNWKIYKLLAH

Muscle PFK (residues 748-779)   LKILAKYEIDLDTSEHAHLEHI-SRKRSGEATV
                                :: :: : :         :::   :  :
Liver  PFK (residues 748-780)   LKMLAHYRISMADYVSGELEHVTRRTLSIDKGF
```

[a] S represents the affected serine in the phosphorylation site. A colon (:) indicates amino acid homology. [b] Lee et al., 1987. [c] Gehnrich et al., 1988.

The sequences of rabbit muscle and mouse liver phosphofructokinase have 68% overall homology (Lee et al., 1987; Gehnrich et al., 1988). Comparisons show that the liver enzyme contains an internal sequence exhibiting a high degree of homology with rabbit muscle phosphofructokinase residues Val^{357}–His^{389} (Table IX). A lower degree of homology occurs at the C-terminal ends of the two enzymes. Nonetheless, phosphorylation of the final serine residue is known to occur in both rabbit or rat liver and rabbit skeletal muscle phosphofructokinase (Kemp et al., 1981; Pilkis et al., 1982; Valaitis et al., 1989). The lower sensitivity of the liver enzyme to troponin C and calmodulin (Table II) may preclude effects of these proteins on phosphorylation. In studies of liver phosphofructokinase, Mieskes et al. (1987) demonstrated that the maximum phosphate incorporation is 0.5 mol of P/mol of protomer with either cAMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase—both of which affect the C-terminal site. For the latter enzyme, the assay mixture contained 1.5 μM calmodulin.

The existence of two phosphorylation sites in rabbit muscle phosphofructokinase raises the possibility that different protein kinases may be responsible for specific phosphorylation. Hofer et al. (1985) reported that Ca^{2+}- and phospholipid-dependent protein kinase C from rat brain catalyzes the phosphorylation of rabbit skeletal muscle phosphofructokinase, both at Ser^{744} and at one or more unidentified additional sites. However, our studies show that under all conditions examined the rabbit muscle enzyme is a very poor substrate for the nearly homogeneous protein kinase C which we have prepared from rat brain. Even after prolonged incubation, the maximum extent of reaction is only 0.1 mol of phosphate/mol of protomer. This result is consistent with that reported by Mieskes et al. (1987). We note that a much less homogeneous preparation of protein kinase C was employed by Hofer et al. (1985). Our results also indicate that phosphofructokinase can be phosphorylated by cGMP-dependent protein kinase to a limited degree. However, the effect of troponin C on the phosphorylation obtained with this protein kinase needs further study.

The physiological relevance of our results can be examined in light of existing information on the in vivo status of phosphofructokinase. First, the catalytic activity exhibited in resting muscle is several hundred-fold lower than that predicted from assays performed on the purified enzyme (Passoneau & Lowry, 1962). Phosphofructokinase could be subject to a highly effective inactivation mechanism involving one or more muscle proteins: troponin C, troponin, calmodulin, α-actinin, myosin, etc. All of these proteins[2] are capable of promoting the in vitro inactivation (cf. Tables I and IV). Our data suggest that both the activity (Tables I and IV) and phosphorylation state of the enzyme (Tables VI and VII) could be controlled by the intracellular concentrations of calcium and of specific substrates and effectors. Whether free troponin C occurs in muscle is unknown. However, considering the relative concentrations of troponin ($\sim$100 μM; Perry, 1974) and phosphofructokinase ($\sim$1 μM; de Duve, 1972) in skeletal muscle, significant amounts of troponin C could become available through unequal rates of turnover of the different subunits and/or from the dissociation tendencies of troponin [cf. Lovell and Winzor (1977) for in vitro studies].

Our results also may explain some of the inconsistencies noted in earlier phosphorylation studies of the enzyme. As summarized in the introduction, in vitro phosphorylation catalyzed by cAMP-dependent protein generally results in the incorporation of less than 0.5 mol of phosphate/mol of enzyme protomer. In the presence of calmodulin or troponin C plus calcium, we obtain complete phosphorylation at the known C-terminal site (Ser^{774}) plus phosphorylation at a novel site (Ser^{376}). The additional phosphorylation may relate to the increase in the average phosphate content of phosphofructokinase isolated from electrically stimulated skeletal muscle (Hofer & Sorensen-Ziganke, 1979). Phosphorylation at two distinct sites also occurs in rat heart phosphofructokinase. The phosphorylated enzyme isolated from epinephrine-perfused hearts is generally more active than the nonphosphorylated enzyme (Narabayashi et al., 1985; Clark & Patten, 1981). Preliminary experiments indicate that the induced phosphorylation of rabbit skeletal muscle phosphofructokinase has little effect on its catalytic activity determined under optimal assay conditions. Future experiments will explore connections between phosphorylation and the allosteric regulation of the enzyme by both protein and nonprotein effectors. A total absence of phosphorylation-dependent effects on catalytic activity could indicate some biological function other than control of enzymatic activity.

Acknowledgments

We thank Dr. Reginald McParland and the Oregon State University Center for Gene Research and Biotechnology for expert assistance in gas-phase sequencing.

Supplementary Material Available

Detailed derivation of the kinetic equations (2 pages). Ordering information is given on any current masthead page.

Registry No. Ca^{2+}, 7440-70-2; Ser, 56-45-1; soybean trypsin inhibitor, 9078-38-0; fructose 2,6-bisphosphate, 77164-51-3; fructose 1,6-bisphosphate, 488-69-7; protein kinase, 9026-43-1.

References

Arnold, H., & Pette, D. (1968) *Eur. J. Biochem. 6*, 163.
Arnold, H., Henning, R., & Pette, D. (1971) *Eur. J. Biochem. 22*, 121.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Brand, I. A., & Söling, H. D. (1975) *FEBS Lett. 57*, 163.
Brand, I. A., & Söling, H. D. (1986) *J. Biol. Chem. 261*, 5892.
Brzeska, H., Szynkicwicz, J., & Drabikowski, W. (1983) *Biochem. Biophys. Res. Commun. 115*, 87.
Buschmeier, B., Meyer, H. E., Mayr, G. W. (1987) *J. Biol. Chem. 262*, 9454.
Clark, M. G., & Patten, G. S. (1981) *J. Biol. Chem. 256*, 27.
Clark, M. G., & Patten, G. S. (1984) *Curr. Top. Cell. Regul. 22*, 127.
Clarke, F. M., & Masters, C. J. (1976) *Int. J. Biochem. 7*, 359.
Cohen, P., Picton, C., & Klee, C. B. (1979) *FEBS Lett. 104*, 25.
Conti, M. A., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 3178.
Cox, D. E., & Edstrom, R. D. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 49*, 166.
Craig, S. W., Lancashire, C. L., & Cooper, J. A. (1982) *Methods Enzymol. 85*, 317.
de Duve, C. (1972) in *Structure and Function of Oxidation Reduction Enzymes* (Akeson & Ehrenburg, Eds.) p 715, Pergamon Press, Oxford.
Domenech, C. E., Mieskes, G., & Söling, H.-D. (1988) *Eur. J. Biochem. 173*, 79.
Drapeau, G. (1978) *Can. J. Biochem. 56*, 534.
Foe, L. G., & Kemp, R. G. (1982) *J. Biol. Chem. 257*, 6368.
Foe, L. G., & Kemp, R. G. (1984) *Arch. Biochem. Biophys. 228*, 503.
Freidina, N. A., Shpagina, M. D., & Podlubnaya, Z. A. (1987) *Biochemistry (Engl. Transl.) 51*, 1718.
Gehnrich, S. C., Gekakis, N., & Sul, H. S. (1988) *J. Biol. Chem. 263*, 11755.
Gerlach, G., & Hofer, H. W. (1986) *Biochim. Biophys. Acta 881*, 398.
Glass, D. B., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 9728.
Goldhammer, A. R., & Paradies, H. H. (1979) *Curr. Top. Cell. Regul. 15*, 109.
Grand, R. J. A., Levine, B. A., & Perry, S. V. (1982) *Biochem. J. 203*, 61–68.
Gross, E. (1967) *Methods Enzymol. 11*, 238.
Hasegawa, H., Parniak, M., & Kaufman, S. (1982) *Anal. Biochem. 120*, 364.
Heizmann, C. W., Malencik, D. A., & Fischer, E. H. (1974) *Biochem. Biophys. Res. Commun. 57*, 162.
Hesterberg, L. K., & Lee, J. C. (1981) *Biochemistry 20*, 2974.
Hesterberg, L. K., & Lee, J. C. (1982) *Biochemistry 21*, 216.
Hofer, H. W. (1985) in *Regulation of Carbohydrate Metabolism*, pp 107–141, CRC Press, Cleveland.
Hofer, H. W., & Furst, M. (1976) *FEBS Lett. 62*, 118.
Hofer, H. W., & Sorensen-Ziganke, B. (1979) *FEBS Lett. 90*, 199.
Hofer, H. W., Schlatter, S., & Graefe, M. (1985) *Biochem. Biophys. Res. Commun. 129*, 892.
Hussey, C. R., Liddle, P. E., Ardron, D., & Kellet, G. L. (1977) *Eur. J. Biochem. 80*, 497.
Jenkins, J. D., Kezdy, F. J., & Steck, T. L. (1985) *J. Biol. Chem. 260*, 10426.
Kellett, G. L., & Robertson, J. P. (1984) *Biochem. J. 220*, 601.
Kemp, R. G. (1975) *Methods Enzymol. 42*, 67.
Kemp, R. G., & Foe, L. G. (1983) *Mol. Cell. Biochem. 57*, 147.
Kemp, R. G., Foe, L. G., Latshaw, S. P., Poorman, R. A., & Heinrikson, R. I. (1981) *J. Biol. Chem. 256*, 7282.

[2] The 17- and 20-kDa smooth muscle myosin light chains were examined.

Kerrick, W. G. L., Malencik, D. A., Hoar, P. E., Potter, J. D., Colby, R. L., Pocinwong, S., & Fischer, E. H. (1980) *Pflugers Arch. 386*, 207.
Kim, S.-H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K., & Kaizuma, N. (1985) *J. Biochem. 98*, 435.
Kincaid, R. L., & Couloin, C. C. (1985) *Biochem. Biophys. Res. Commun. 133*, 256.
Kitajima, S., Sakakibara, R., & Uyeda, K. (1983) *J. Biol. Chem. 258*, 13292.
Kuo, H.-J., Malencik, D. A., Liou, R.-S., & Anderson, S. R. (1986) *Biochemistry 25*, 1278.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978a) *J. Biol. Chem. 253*, 5452.
Leavis, P. C., Rosenfeld, S., & Lu, R. C. (1978b) *Biochim. Biophys. Acta 552*, 281.
Lee, C.-P., Kao, M.-C., French, B. A., Putney, S. D., & Chang, S. H. (1987) *J. Biol. Chem. 262*, 4195.
Liou, R.-S., & Anderson, S. R. (1980) *Biochemistry 19*, 2684.
Lovell, S. J., & Winzor, D. J. (1977) *Biochem. J. 167*, 131.
Luther, M. A., & Lee, J. C. (1986) *J. Biol. Chem. 261*, 1753.
Luther, M. A., Gilbert, H. F., & Lee, J. C. (1983) *Biochemistry 22*, 5494.
Luther, M. A., Hesterberg, L. K., & Lee, J. C. (1985) *Biochemistry 24*, 2463.
Luther, M. A., Cai, G., & Lee, J. C. (1986) *Biochemistry 25*, 7931.
Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3840.
Malencik, D. A., & Anderson, S. R. (1987) *Biochemistry 26*, 695.
Malencik, D. A., & Anderson, S. R. (1988) *Biochemistry 27*, 944.
Malencik, D. A., Heizmann, C. W., & Fischer, E. H. (1975) *Biochemistry 14*, 715–721.
Malencik, D. A., Anderson, S. R., Bohnert, J. L., & Shalitin, Y. (1982) *Biochemistry 21*, 4031.
Malencik, D. A., Zhao, Z., & Anderson, S. R. (1990) *Anal. Biochem. 184*, 353.
Mayr, G. W. (1984a) *Eur. J. Biochem. 143*, 513.
Mayr, G. W. (1984b) *Eur. J. Biochem. 143*, 521.
Mayr, G. W., & Heilmeyer, L. M. G., Jr. (1983) *FEBS Lett. 159*, 51.
Mendicino, J., Leibach, F., & Reddy, S. (1978) *Biochemistry 17*, 4664.
Mieskes, G., Kuduz, J., & Söling, H.-D. (1987) *Eur. J. Biochem. 167*, 383.
Narabayashi, H., Lawson, J. W. R., & Uyeda, K. (1985) *J. Biol. Chem. 260*, 9750.
Passonneau, J. V., & Lowry, O. H. (1962) *Biochem. Biophys. Res. Commun. 7*, 10.
Perry, S. V. (1974) *Biochem. Soc. Symp. 39*, 115.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691–5697.
Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., & Claus, T. H. (1982) *Arch. Biochem. Biophys. 215*, 379.
Poorman, R. A., Randolph, A., Kemp, R. G., & Heinrikson, R. L. (1984) *Nature 309*, 467.
Potter, J. D. (1982) *Methods Enzymol. 85*, 241.
Racker, E. (1947) *J. Biol. Chem. 167*, 843.
Riquelme, P. T., Fox, R. W., & Kemp, R. G. (1978) *Biochem. Biophys. Res. Commun. 81*, 864.
Sakakibara, R., & Uyeda, K. (1983) *J. Biol. Chem. 258*, 8656.
Sale, E. M., & Denton, R. M. (1985) *Biochem. J. 232*, 905.
Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368.
Schreiber, W. E., Sasagawa, T., Titani, K., Wade, R. D., Malencik, D. A., & Fischer, E. H. (1981) *Biochemistry 20*, 5239.
Shnyrov, V. L., Freydina, N. A., & Permyakov, E. A. (1988) *Biochim. Biophys. Acta 953*, 128.
Söling, H.-D., & Brand, I. A. (1981) *Curr. Top. Cell. Regul. 20*, 107.
Uyeda, K. (1979) *Adv. Enzymol. 48*, 193.
Uyeda, K., Miyatake, A., Luby, L. J., & Richards, E. G. (1978) *J. Biol. Chem. 253*, 8319.
Valaitis, A. P., Foe, L. G., Kwiatkowska, D., Latshaw, S. P., & Kemp, R. G. (1989) *Biochim. Biophys. Acta 995*, 187.
Vanaman, T. C. (1980) *Calcium Cell Funct. 1*, 41.
Vogel, H. J., Lindahl, L., & Thulin, E. (1983) *FEBS Lett. 157*, 241.
Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., & Gill, G. N. (1987) *Anal. Biochem. 161*, 425–437.
Wang, Y., Fiol, C. J., DePaoli-Roach, A. A., Bell, A. W., Hermodson, M. A., & Roach, P. J. (1988) *Anal. Biochem. 174*, 537.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962.
Welch, P., & Scopes, R. K. (1981) *Anal. Biochem. 112*, 154–157.