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The interaction of skeletal muscle phosphofructokinase with a variety of acidic proteins including calmodulin and troponin C and with nucleic acids (RNA and DNA) is manifest in a reversible, time-dependent loss of catalytic activity. The inactivation is affected by a number of factors. Substrates (fructose-6-phosphate and ATP), positive effectors (fructose-2,6-bisphosphate, ADP, AMP, and inorganic phosphate), and a negative effector (citrate) of phosphofructokinase all diminish inactivation while a decrease in pH or an increase in temperature favor the inactivation process. Proteins which bind calmodulin or troponin C -- such as troponin I, melittin, and protamine -- reduce the inactivation, possibly by competing with the enzyme. The inactivation can be reversed by the addition of fructose-2,6-bisphosphate, ATP, or ADP. Studies with specific proteolytic fragments of troponin C indicate that the residues near calcium-binding site III are involved in

the inactivation of phosphofructokinase. A general kinetic model for the inactivation has been developed mathematically and applied to the data. A novel electrophoresis procedure demonstrates directly the specific interaction of phosphofructokinase with calmodulin or troponin C.

The specific calcium-dependent association of phosphofructokinase with calmodulin or troponin C is manifest in large increases in the rate of and extent of *in vitro* phosphorylation of the enzyme catalyzed by cAMP-dependent protein kinase. Two distinct phosphorylation sites in phosphofructokinase have been identified. The first, corresponding to the known C-terminal site -- which has the sequence His-Ile-Ser-Arg-Lys-Arg-Ser(P)-Gly-Glu -- is demonstrated both in the control and in enzyme samples which have been phosphorylated in the presence of calmodulin or troponin C. The second is a novel site found in the middle of the phosphofructokinase molecule with the sequence Lys-Leu-Arg-Gly-Arg-Ser(P)-Phe-Met. Phosphorylation at this site is induced by calmodulin or troponin C in the presence of calcium. Preliminary studies indicate that phosphorylation has little effect on the activity of phosphofructokinase as determined by standard assays on samples taken from the phosphorylation mixtures.

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DEDICATION

To My Parents

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Table of Contents

I General Introduction

1. Phosphofructokinase: a Key Enzyme in the Glycolysis Process..... 1
2. Calcium and Calcium-Binding Proteins..... 6
3. Protein Kinase and Protein Phosphorylation..... 7
4. Objectives and Significance..... 11

II Regulation of PFK by a Reversible Inactivation Mechanism 13

Abstract..... 13
Introduction..... 15
Materials and Methods..... 19
Results..... 26
Discussion..... 62
Supplementary Material..... 68

III Effects of Calmodulin and TnC on the Phosphorylation of Phosphofructokinase 70

Abstract..... 70
Introduction..... 72
Materials and Methods..... 76
Results..... 83
Discussion..... 114

IV Reversible Inactivation of Phosphofructokinase in the Presence of Nucleic Acids 120

Abstract..... 120
Introduction..... 122
Materials and Methods..... 123
Results..... 129
Discussion..... 158

V Conclusion 163

Bibliography 167

Appendices 180

1. Purification of Phosphofructokinase..... 180
2. Preparation of TnC Fragments..... 190
3. Protein-Bound Phosphate Determination..... 202

List of Figures

II-1 Inactivation of phosphofructokinase occurring in the presence of calmodulin or troponin C..... 27

II-2 Structure of Troponin C Fragments..... 35

II-3 Determination of the dissociation constant of phosphofructokinase tetramer to dimer..... 42

II-4 Effect of troponin I on the phosphofructokinase inactivation induced by calmodulin or troponin C..... 49

II-5 Effect of melittin and myosin light chain kinase on phosphofructokinase inactivation induced by calmodulin..... 51

II-6 Effects of protamine on the inactivation of phosphofructokinase induced by calmodulin and troponin C..... 54

II-7 Reactivation of phosphofructokinase..... 56

II-8 Electrophoretic Study of the Interaction between phosphofructokinase and calmodulin or troponin C..... 60

III-1 Time course of the phosphorylation of phosphofructokinase catalyzed by cAMP-dependent protein kinase..... 84

III-2 Effects of varying pre-incubation time (A) and troponin C concentration (B) on phosphofructokinase phosphorylation..... 87

III-3 Radioactivity monitoring of the reverse-phase HPLC separation of the CNBr fragments of ^{32}P-labeled phosphofructokinase..... 92

III-4 Reverse phase HPLC peptide mapping of phosphofructokinase fragments..... 94

III-5 Isolation of the C-terminal phospho-peptide..... 97

III-6 C18 reverse-phase HPLC profile of the C-terminal peptide eluted from the sequencer filter disc after passage through 5 cycles of the Edman degradation..... 100

III-7 Determination of the individual rates of phosphorylation of Ser^{376} and Ser^{774}..... 103

III-8 Inactivation and phosphorylation of phosphofructokinase..... 106

III-9 Electrophoretic study of the interaction of phosphorylated phosphofructokinase with calmodulin and troponin C..... 112

IV-1 UV spectrum of the partially purified liver RNA fraction..... 132

IV-2 RNA gel electrophoreses of the partially purified liver RNA fraction..... 134

IV-3 Inactivation of phosphofructokinase by the rabbit liver RNA fraction..... 137

IV-4 Glycerol gradient centrifugation of phosphofructokinase and the rabbit liver RNA fraction after pre-incubation..... 144

IV-5 Separation profile of a mixture of phosphofructokinase and liver RNA fraction mixture on a Sephacryl S-300 gel filtration column..... 146

IV-6 Fluorescence intensity change of dansylcalmodulin in the presence of phosphofructokinase and DNA.... 149

IV-7 Effect of phosphofructokinase on the digestion of DNA with DNase I..... 151

IV-8 Aggregation of phosphofructokinase resulting from the digestion of DNA..... 154

IV-9 Effect of phosphofructokinase on the digestion of chicken chromatin core particle DNA by micrococcal nuclease..... 156

IV-10 Primary structure and Helix prediction of the C-terminal part (700-779) of rabbit muscle phosphofructokinase..... 161

A-1 DEAE-51-52-53 series column separation profile of PFK purification..... 185

A-2 9-19% $NaDodSO_4$ polyacrylamide gel electrophoresis of samples from PFK purification..... 187

A-3 Gel Electrophoresis of proteolytic fragments of troponin C..... 193

A-4 Reverse-phase HPLC profile of the proteolytic fragments of troponin C..... 196

A-5 Standard curve of phosphate determination..... 204

List of Tables

I-1 Classification of Common Serine and Threonine Protein Kinases..... 9

II-1 Effect of Glycerol on the Reversible Inactivation of Phosphofructokinase..... 31

II-2 Troponin C-Induced Inactivation Observed with Phosphofructokinase from Different Sources..... 32

II-3 Inactivation of Phosphofructokinase Induced by Troponin C Fragments..... 34

II-4 Inactivation of Phosphofructokinase in the Presence of Various Acidic Proteins..... 36

II-5 Kinetic Parameters for the Inactivation of Phosphofructokinase..... 39

II-6 Effects of Temperature and pH on the Interaction of Phosphofructokinase..... 45

II-7 Effects of Low Molecular Weight Ligands on the Inactivation of Phosphofructokinase..... 47

III-1 Calcium-Dependent Effects of Calmodulin and Troponin C on the Phosphorylation of Rabbit Muscle Phosphofructokinase by cAMP-Dependent Protein Kinase..... 89

III-2 Effects of Small Ligands of PFK on the Phosphorylation Obtained in the Presence of Troponin C..... 90

III-3 Phosphorylation of PFK by cGMP-Dependent Protein Kinase and by Protein kinase-C..... 109

III-4 Sequence Comparisons of Potential Phosphorylation Sites in Rabbit Muscle and Mouse Liver Phosphofructokinase..... 117

IV-1 Purification of Phosphofructokinase Inactivator... 131

IV-2 Properties of the Purified PFK Inactivator from Rabbit Liver..... 133

IV-3 Inactivation of Phosphofructokinase by Nucleic Acids..... 139

IV-4 Factors Affecting the Inactivation of Phosphofructokinase by Liver RNA Fraction..... 141

IV-5 Reactivation of Phosphofructokinase..... 142

A-1 Summary of the Purification of PFK from Rabbit Skeletal Muscle..... 189

A-2 Amino Acid Composition of the Proteolytic Fragments of Troponin C..... 201

Regulation of Phosphofructokinase by Reversible Inactivation

Chapter I

General Introduction

1. Phosphofructokinase: a Key Enzyme in the Glycolysis Process

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of ATP. Among the regulated steps in this process, the one catalyzed by phosphofructokinase (PFK) is the most important control point in the glycolytic pathway of mammals. The enzyme catalyzes the transfer of the γ-phosphoryl group of ATP to the number one carbon of D-fructose-6-phosphate to produce D-fructose-1,6-bisphosphate, which represents essentially a thermodynamically irreversible process.

Phosphofructokinase has been purified and characterized from a variety of sources. Purification procedures have been described originally for phosphofructokinase from E. coli (Griffin *et al.*, 1967), from yeast (Sols and Salas, 1966), from liver (Brock, 1969), from rabbit muscle (Ling *et al.*, 1965; Parmeggiani and Krebs, 1965), and from erythrocytes (Sols and Salas, 1969). The enzyme prepared from different sources exhibits varying kinetic properties. However, in general, all the enzymes from mammalian sources

are characterized by a sigmoid dependence of the reaction rate on the concentration of the substrate, fructose-6-phosphate, and by the dual role of ATP, which acts as both substrate and inhibitor. Positive effectors -- fructose-2,6-bisphosphate (Uyeda *et al.*, 1981; Hers and van Schaftingen, 1982), fructose-1,6-bisphosphate, ADP, AMP (Tornheim and Lowenstein, 1976), phosphate (Tejwani and Mouse, 1981), OH^-, and 6-phosphogluconate (Sommercorn and Freedland, 1982) -- activate phosphofructokinase by shifting the sigmoidal curve to the left (increasing the enzyme's affinity for fructose-6-phosphate) and by diminishing the inhibitory effect of ATP. In contrast, negative effectors -- such as citrate, H^+, phosphoenolpyruvate, creatine phosphate, long chain fatty acids, and glycerol-3-phosphate -- shift the curve to the right (decreasing the enzyme's affinity for fructose-6-phosphate) and enhance the inhibition by ATP (Claus *et al.*, 1982).

Among the allosteric effectors of phosphofructokinase, fructose-2,6-bisphosphate is the most potent activator. It is effective in the micromolar concentration range. Its tissue distribution was determined in rat by Kawajima and Uyeda (1982). The liver, containing 20 nmoles/g, has the highest concentration followed in decreasing order by brain, heart muscle, kidney, testis, and skeletal muscle -- which has only 2.6 nmoles/g. Fructose-2,6-bisphosphate is formed by the phosphorylation of fructose-6-phosphate, in a

reaction catalyzed by phosphofructokinase 2 (PFK2), a different enzyme from PFK1. It is hydrolyzed to fructose-6-phosphate by a specific phosphatase, fructose bisphosphatase 2 (FBPase2). The liver enzymes have been well characterized. A striking feature is that both PFK2 and FBPase2 are present in a single polypeptide chain, which is called a tandem enzyme (Pilkis *et al.*, 1984). Fructose-6-phosphate accelerates the synthesis of fructose-2,6-bisphosphate and inhibits its hydrolysis. Furthermore, this bifunctional enzyme can be reciprocally regulated by phosphorylation of a single serine residue. The phosphorylation decreases the activity of PFK2 and increases the activity of FBPase2. The phosphorylation is controlled by hormones such as glucagon and epinephrine (van Schaftingen *et al.*, 1982; Richards *et al.*, 1981; Hue *et al.*, 1981; Kawajima *et al.*, 1982). Unlike rat liver enzyme, bovine heart PFK-2 contains little fructose-2,6-bisphosphatase activity. It was not inactivated by cAMP-dependent protein kinase. In fact, the heart enzyme is a good substrate of protein kinase C and a relatively poor substrate of cAMP-dependent protein kinase (Rider *et al.*, 1985; Rider & Hue, 1986). Like the liver enzyme, rat muscle PFK-2 (El-Maghrabi *et al.*, 1986; Rider and Hue, 1987) and pigeon muscle PFK-2 (van Schaftingen and Hers, 1986) contain significant FBPase-2 activity and therefore represents a bifunctional enzyme. However, its bisphosphatase activity is less sensitive to

Fru-6-P inhibition than the liver PFK-2. The fact that muscle PFK-2 is not inactivated by cAMP-dependent protein kinase suggests that it is more akin to the heart enzyme. PFK-2 and FBPase-2 are separable in yeast. In contrast to the liver enzyme, the kinase is activated by cAMP-dependent protein kinase phosphorylation while the bisphosphatase is inactivated (Kretschmer *et al.*, 1987).

Animal tissues contain multiple phosphofructokinase isozymes with different immunologic, chromatographic, and electrophoretic properties (Kahn *et al*, 1979; Vora, 1981; Foe and Kemp, 1984; Vora *et al.*, 1985; Dunaway and Kasten, 1985a,b; Dunaway *et al.*, 1986; Dunaway *et at.*, 1988; Khoja, 1988; Khoja and Abuekgassim, 1989). Three fundamental isozymes are well known. They are phosphofructokinase A (or muscle type, M_4), B (or liver type, L_4), and C, which is found in brain, thymus and several other tissues along with types A and B. A fourth type of isozyme (phosphofructo-kinase D) has been identified in rat intestinal mucosa (Khoja and Kellett 1983; Khoja 1986).

Phosphofructokinase has a complex oligomeric structure. It also displays a great variability of structure among prokaryotic and eukaryotic organisms. The enzymes from prokaryotes are generally tetrameric and built up of subunits of identical size. PFK-I of E. coli is a tetramer with a molecular weight of 140,000 (Blangy, 1968). However, the active form of E. coli PFK-II, minor isozyme, is

dimeric (Kotlarz and Buc, 1981). The mammalian enzyme undergoes self association and dissociation (Brand and Söling, 1974; Reinhard and Lardy, 1980; Pilkis *et al.*, 1982; Hesterberg and Lee, 1981, 1982; Luther *et al.*, 1985, 1986;). The tetramer, with a molecular weight of 320,000 to 380,000, is the smallest active form of the enzyme. The yeast enzyme contains two types of subunits, α and ß, assembled in an octameric structure of $\alpha_4\beta_4$ (Tijane *et al.*, 1979; Chaffotte *et al.*, 1984). The three dimensional structure of phosphofructokinase from B. stearothermophilus and from E. coli has been determined in two forms: an R-state (active, from crystals grown in the presence of fructose-6-phosphate) and a T-state (inactive, from crystals grown in the presence of the inhibitor 2-phosphoglycerol or simply in the absence of activator) (Evans and Hudson, 1979; Evans *et al.*, 1981; Shirakihara and Evans, 1988; Rypniewski and Evans, 1988). The primary sequence of rabbit skeletal muscle phosphofructokinase has been determined by amino acid sequencing. The sequence of this muscle enzyme shows both an internal homology and homology to the bacterial enzymes, which suggests that it has evolved by the gene duplication of an ancestral gene now represented by the bacterial gene (Poorman *et al.*, 1984). This sequence was confirmed later by the DNA sequence (Lee *et al.*, 1987). The primary structure of another mammalian muscle enzyme, human muscle phosphofructokinase, has also been determined by cloning of

cDNA. It shows 96% homology to the rabbit muscle enzyme (Nakajima *et al.*, 1987; Sharma *et al.*, 1989). In addition, mouse liver phosphofructokinase has been determined from its mRNA sequence and comparison with the rabbit muscle enzyme indicates a homology of 68% (Gehnrich *et al.*, 1988).

2. Calcium and Calcium-Binding Proteins

Calcium concentrations in the cytoplasm are maintained at levels about 10,000-fold less than that of the extracellular fluid. Accurate control of the cytosolic calcium concentration represents a fundamental property of all living cells. As a second messenger, the calcium ion is an important regulator of a wide range of cellular functions. These include stimulus-response events such as muscle contraction and the release of hormones and neurotransmitters, as well as the regulation of enzyme activities, transport phenomena, and membrane permeabilities.

Many of these actions of calcium are thought to be mediated by the calcium-dependent regulatory protein known as calmodulin (Klee and Vanaman, 1982). Calmodulin, a ubiquitous, high-affinity calcium-binding protein of molecular weight of 16.7 kD, has been shown to play a pivotal role in diverse calcium-dependent biological process, including activation of several species of protein

kinases and phosphatases (cf. review, Cheung, 1980). It is a ubiquitous regulatory protein serving as an intracellular calcium receptor in almost all eukaryotic cells. Calmodulin has a very highly conserved sequence, containing a total of 148 amino acid residues (Watterson *et al.*, 1980). It consists of two similar globular lobes joined by an eight-turn α-helix. Each lobe have calcium-binding sites, the so-called EF-hand units (Babu *et al.*, 1985). The binding of calcium to the low affinity sites activate calmodulin and enable it to stimulate a variety of enzymes.

Troponin C is another member of the family of calcium-binding protein. It is the calcium-sensing subunit of the troponin complex, which together with tropomyosin constitutes the thin filament-linked regulatory system of skeletal muscle (cf. review, Cooke, 1986). Like calmodulin, the troponin C molecule contains two homologous domains. The N-terminal and C-terminal globular domains are also connected by a long eight- or nine-turn α-helix. Each domain contains two binding sites for calcium. The ones in the C-terminal domain have high affinity for calcium (K_d = 0.1 μM), whereas those in the N-terminal domain have low affinity (K_d = 10 μM) (Herzberg and James, 1985).

3. Protein Kinase and Protein Phosphorylation

Among the many types of reversible posttranslational

modification -- acetylation, methylation, adenylylation, uridylylation, phosphorylation etc. -- phosphorylation may be the most common. Phosphorylation-dephosphorylation, as an almost universal mechanism, regulates the function of proteins -- not only of those that display enzymatic activity but also of proteins involved in other biological processes. The reactions of protein phosphorylation and dephosphorylation can be expressed by the following two equations:

$$\text{Protein} + n\text{NTP} \longrightarrow \text{Protein-P}_n + n\text{NDP}$$

$$\text{Protein-P}_n + n\text{H}_2\text{O} \longrightarrow \text{Protein} + n\text{P}_i$$

The most commonly used and physiologically significant phosphate donor in nearly all instances is ATP. The phosphate receptor in the protein can be serine, threonine, or tyrosine (cf. review, Krebs, 1986).

The known protein kinases constitute a very diverse group of enzymes, the number of which is still growing (Hanks *et al.*, 1988). These enzymes are commonly regulated through their interactions with "second messengers" generated within cells in response to hormones and other extracellular agents. According to the phosphate acceptor amino acid, protein kinases can be classified into: 1. Serine-threonine, 2. tyrosine, and 3. histidine-lysine protein kinase. The first type contains the largest number of kinases and is well characterized. Table I lists some the most common serine and threonine protein kinases.

Table I

Classification of Common Serine and Threonine Protein Kinases

Group	Regulator	Specific Enzyme
1	Cyclic nucleotides	Type I cyclic AMP-dependent protein kinase
		Type II cyclic AMP-dependent protein Kinase
		Cyclic GMP-dependent protein kinase
2	Ca^{2+}-Calmodulin	Phosphorylase kinase
		Skeletal muscle myosin light chain kinase
		Smooth muscle myosin light chain kinase
		Multifunctional Ca^{2+}-calmodulin dependent protein kinase
3	Diacylglycerol-Ca^{2+}	Protein kinase-C

cAMP-dependent protein kinases are the best known of these enzymes. According to their elution order from anion exchange resins, they are classified as type I and type II. Both consist of two kinds of subunits: a regulatory (R) subunit, which can bind cAMP, and a catalytic (C) subunit. The latter is identical in the two enzyme types, which differ only in their R subunits. In the absence of cAMP, the enzyme is in its holoenzyme form -- R_2C_2 -- which is enzymatically inactive. Binding of cAMP to each of the R subunits leads to the dissociation of the R_2C_2 complex into the R_2 dimer and two C subunits, which are active (Krebs and Beavo, 1979). cAMP-dependent protein kinase plays a major regulatory role in eucaryotic cells. Many enzymes involved in basic metabolism are controlled through the phosphorylation catalyzed by this protein kinase. For example, in carbohydrate metabolism, phosphorylase kinase (Malencik and Fischer, 1982), glycogen synthase (Cohen, 1986; Roach, 1986), pyruvate kinase (Engstrom *et al*, 1986), and liver phosphofructokinase 2 (van Schaftingen *et al*., 1982) can be phosphorylated by cAMP-dependent protein kinase in response to hormones such as glucagon, epinephrine, etc.. The phosphorylation activates the first enzyme but inactivates other three.

Phosphofructokinase 1 is also known to be phosphorylated by cAMP-dependent protein kinase. The major known phosphorylation site of rabbit muscle phosphofructo-

kinase proved to be a serine residue located at the 6th position from the C-terminus of the enzyme (Kemp *et al.*, 1981). Foe & 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 are small. So far, the physiological significance of the phosphorylation remains unknown.

4. Objectives and Significance

The primary intent of this thesis to examine the mechanism of regulation of PFK. The area of investigation includes 1. studies on the regulation of PFK through the reversible inactivation which results from its interaction with other macromolecules and 2. control of PFK through covalent phosphorylation catalyzed by protein kinases.

One of the fundamental regulatory mechanisms of PFK is reversible inactivation. The first chapter of this thesis describes the inactivation of PFK which results from its interactions with various acidic proteins and peptides including calmodulin, troponin C, troponin, α-actinin, two smooth muscle myosin light chains, S-100, parvalbumin, poly(Glu-Tyr), and specific proteolytic fragments of troponin C. For the calcium-binding proteins, varying conditions involving both the presence and absence of calcium are investigated. The variables studied include the

presence of substrates (ATP and fructose-6-phosphate) and of low-molecular-weight allosteric effectors of the enzyme -- such as fructose-2,6-bisphosphate, ADP, AMP, citrate, and inorganic phosphate. The effects of varied pH and temperature and of added calmodulin- and troponin C- binding proteins are also examined. A kinetic model for the inactivation is developed mathematically and applied to the data. Finally, the specific calcium-dependent interaction of the enzyme with calmodulin or troponin C is demonstrated by the co-migration of the proteins in urea polyacrylamide gel electrophoresis.

The specific calcium-dependent effects of calmodulin and troponin C on the phosphorylation of PFK are reported in chapter II. The factors affecting the reversible inactivation are also shown to influence the rate and extent of the phosphorylation reaction. Two distinct phosphorylation sites are identified by sequencing. Preliminary studies are undertaken to determine the effect of the phosphorylation on the activity of the enzyme and on its interactions with the calcium-binding proteins.

In chapter III, an unusual interaction of PFK with nucleic acids -- including both RNA and DNA is described. The association leads to the inactivation of the enzyme and is influenced by a number of variables. The interaction between the nucleic acids and PFK is shown directly in gel filtration and gradient ultracentrifugation experiments.

CHAPTER II

Regulation of Phosphofructokinase by a Reversible Inactivation Mechanism

Abstract

The interaction of rabbit skeletal muscle phosphofructokinase with a variety of acidic proteins -- including calmodulin, skeletal muscle troponin C, troponin, the two smooth muscle myosin light chains, α-actinin, and S-100 -- is manifest in a reversible, time-dependent loss of catalytic activity. The effects of the isolated tryptic and thrombic fragments of troponin C suggest that the enzyme inactivation process involves amino acid side chains located near calcium binding site III of the intact troponon C molecule. Enzyme substrates (fructose-6-phosphate and ATP), positive effectors (fructose-2,6-bisphosphate, ADP, AMP, and inorganic phosphate), and a negative effector (citrate) all exert protective effects. Proteins which bind calmodulin or troponin C -- such as troponin I, melittin, myosin light chain kinase, and protamine -- also influence the inactivation, possibly by competing with the enzyme. The activity losses can be reversed by the addition of fructose 2,6-bisphosphate, ATP, or ADP but not of melttin, troponin I, or protamine. Decreases in pH or increases in

temperature favors the inactivation.

We have developed a kinetic model, based on the interconversion of two dimer isoforms of phosphofructokinase, which is consistent with the inactivation time courses. In addition, the calcium-dependent interaction of phosphofructokinase with calmodulin or troponin C is shown by co-migration of the proteins in urea gel electrophoresis.

Introduction

Rabbit muscle phosphofructokinase (PFK, 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. Phospho-fructokinase also undergoes posttranslational phosphorylation (Brand and Söling 1975, Hofer and Furst 1976). However, the physiological significance of this modification remains unclear since there is little effect on enzymatic activity (Söling and Brand 1981, Clark and Patten 1984, Foe and Kemp 1982, Sakakibara and Uyeda 1983). 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 (Hesterberg & Lee, 1981, 1982; Luther *et al.*, 1983, 1985, 1986; Shnyrov *et al.*, 1988). At pH 7.0, the association is best described by the following 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 also 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. The binding is reversed either by high ionic strength, or by effectors of phosphofructokinase, such as ATP and fructose-6-phosphate. 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 influence of F-actin binding on the catalytic properties of phosphofructokinase was originally described by Liou and Anderson (1980). Kuo *et al.* (1986) and Luther and Lee (1986) have since reported that the phosphorylated form of phosphofructokinase has a

higher affinity for F-actin than the dephosphorylated enzyme. More recently, Roberts and Somero (1987, 1989) have demonstrated the binding of phosphofructokinase to F-actin by electron microscopy. Phosphofructokinase also associates with the thick filament of muscle. This phenomenon was first described by Starr and Offer (1982), who co-purified phosphofructokinase with myosin. Further studies by Freidina *et al.* (1987) showed that phosphofructokinase, in a dimeric form, binds to the hinge region of myosin. Since the latter contains predominantly acidic amino acid residues (Lu & Wong, 1985), electrostatic interaction may have a significant role in phosphofructokinase binding by myosin.

Phosphofructokinase is known to undergo inactivation in the presence of several different proteins. For example, it binds to the acidic amino terminus of the purified erythrocyte membrane band 3 protein, with concomitant dissociation to the inactive dimer (Jenkins *et al.*, 1985). A Zn^{2+}-dependent inactivating protein purified from rat liver causes a reversible inactivation of rat liver phosphofructokinase through preferential association with the dimer (Brand and Söling, 1986). Still another protein inhibitor of the enzyme has been detected in the mucosa of rat small intestine (Kellett and Robertson, 1984). Interestingly, calmodulin binds to phosphofructokinase with moderate to high affinity (Mayr and Heilmeyer 1983). The association induces a shift in the equilibrium from the

highly active enzyme tetramer towards an inactive dimer (Mayr 1984). Two calmodulin binding fragments of phosphofructokinase have since been identified and sequenced (Buschmeier *et al.*, 1987).

This chapter demonstrates that the reversible inactivation of rabbit muscle phosphofructokinase occurs in the presence of a number of acidic proteins. These include calmodulin (CaM), troponin C (TnC), troponin, S-100, α-actinin, the two smooth muscle myosin light chains, parvalbumin, and specific proteolytic fragments of troponin C. Major areas of investigation include 1. kinetic studies of the inactivation; 2. examination of the influence of phosphofructokinase substrates and effectors; 3. comparison of the inactivation tendencies of purified phosphofructokinase from rabbit skeletal muscle, rabbit liver, and yeast; 4. determination of the effects of calmodulin- or troponin C-binding ligands; 5. demonstration of the calcium-dependent interaction of rabbit muscle phosphofructokinase with calmodulin or troponin C by gel electrophoresis; 6. mathematical development of a general model for the reversible inactivation.

Materials and Methods

Materials

Fructose-6-phosphate, fructose-2,6-bisphosphate, ATP, ADP, AMP, dithiothreitol, ß-mercaptoethanol, NADH, aldolase, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, protamine sulfate from salmon, trypsin, soybean trypsin inhibitor, melittin, and phenyl-agarose, all in highest available grade, were purchased from Sigma Chemical Company. DEAE-51, DEAE-52, and DEAE-53 were obtained from Whatman. All other reagents were analytical grade. Distilled water that had been further purified with a Milli-Q reagent water system was used throughout.

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 was 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. Phosphofructokinase was stored in a pH

8.0 buffer containing 50 mM Tris-phosphate, 5 mM pyrophosphate, 0.2 mM EDTA, 1 mM ß-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 contains 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 units aldolase, 0.4 units α-glycerol-phosphate 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 and assayed for 3-4 minutes.

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); turkey gizzard smooth muscle myosin light chain kinase, according to Malencik and Anderson (1986); bovine brain S-100, according to Kincaid and Couloin (1985); dogfish parvalbumin, according to Heizmann *et al.* (1974). Thrombin was a gift from Prof. Earl Davie.

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 trypsin per 100 mg troponin C, and then fractionated on a phenyl-agarose column as described by Brzeska *et al.* (1983). The digestion of troponin C by thrombin and subsequent fractionation on Sephadex G-75 column were performed according to Leavis *et al.* (1978a). The purities of all the fragments were checked by $NaDodSO_4$ gel electrophoresis employing a tricine buffer system (Schagger and von Jagow, 1987) in conjuction with a 16-27% gradient of polyacrylamide.

Each of the two purified tryptic fractions showed two very sharp, closely spaced bands upon the electrophoresis (data not shown). Amino acid analyses performed according

to Malencik *et al.* (1990) verified that the phenyl-agarose binding fraction corresponds essentially to the N-terminal half of troponin C (TR1) and that the non-binding fraction represents the C-terminal half (TR2). Each of these 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. 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). That is, TH1 and TR2 retain troponin I binding ability, while TR1 and TH2 do not.

The procedures for the preparation and identification of the proteolytic fragments of troponin C are detailed under Appendix 2.

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 which 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 ß-mercaptoethanol, 1.0 mM dithiothreitol, and 50% glycerol, and allowed to sit at the incubation temperature for one hour. 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 -- such as allosteric ligands, troponin I, melittin, and protamine -- are added to the inactivating system prior to mixing with phosphofructokinase. The incubations are usually performed

for 20 minutes. 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 and 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 X 10 cm) using 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 and 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 to 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 ß-mercaptoethanol for one hour 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 X 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.

Results

Inactivation of Phosphofructokinase in the Presence of Calmodulin or Troponin C

Fig. II-1 shows the time courses of phosphofructokinase inactivation occurring at different concentrations of calmodulin or troponin C and in the presence of either 0.1 mM Ca^{2+} or 0.1 mM EGTA. The activity of phosphofructokinase is represented as the ratio of measured residual activity (A) to that of the initial activity (A_0). A_0 was obtained by extrapolation of the control inactivation to zero time. Note that it represents the activity of an enzyme population which is expected to be in a pre-existing state of rapid equilibrium between tetramer and dimer.

The time course of inactivation is typically not first order but is instead biphasic, with an initial rapid phase followed by a slow phase. The slow phase reaches an approximate equilibrium after a certain period of time which is dependent on the concentration of calmodulin or troponin C. Both the equilibrium phosphofructokinase activity and the rate of inactivation are related to the concentration of the inactivating protein. Calmodulin and troponin C at molar concentrations equivalent to that of phosphofructokinase (in terms of tetramer) cause significant inactivation, both in the presence and absence of Ca^{2+}. In

Fig. II-1: Inactivation of phosphofructokinase occurring in the presence of calmodulin or troponin C. Phosphofructokinase (20 μg/mL) was incubated with different concentration 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 ß-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: ▽ ▼ , 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).

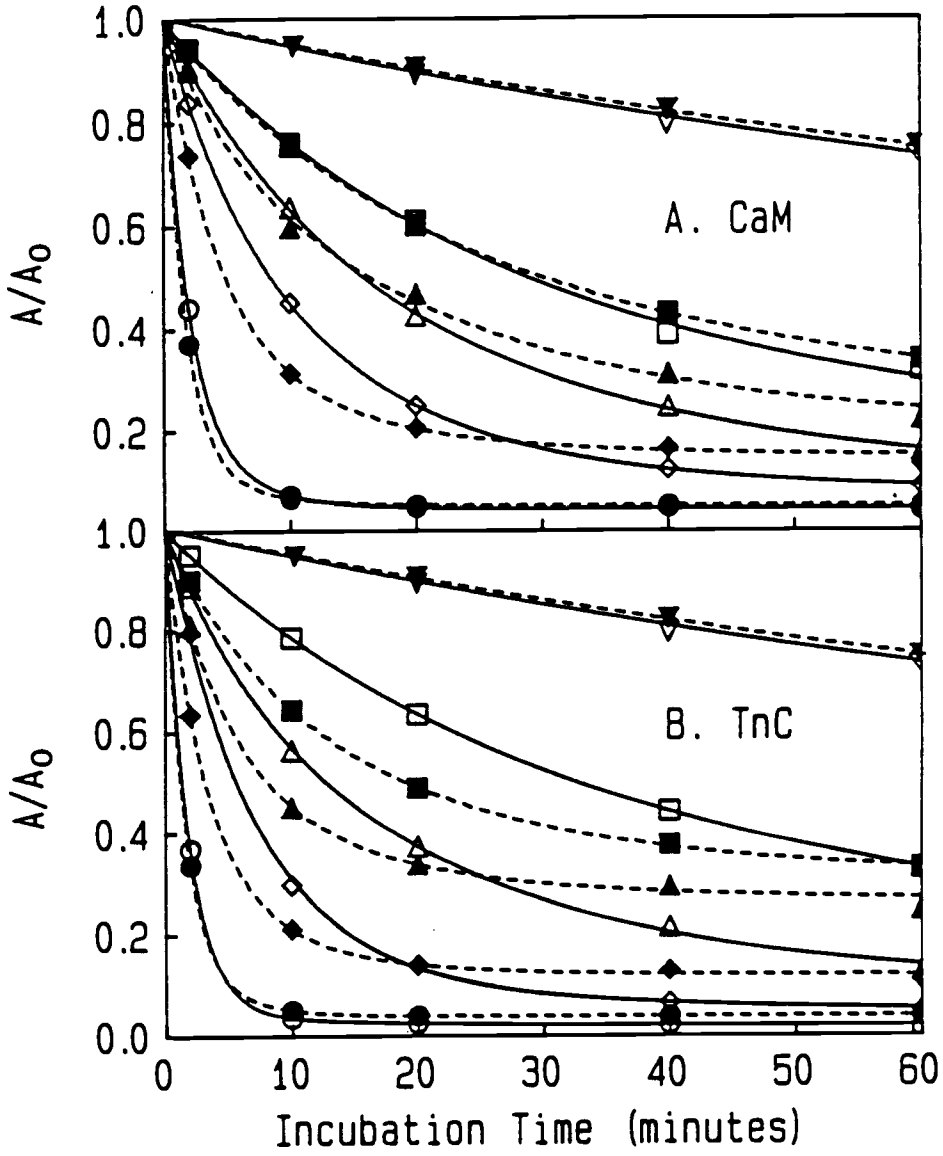

1.0
0.8
0.6
0.4
0.2
A/A0
A. CaM
1.0
0.8
0.6
0.4
0.2
0.0
A/A0
B. TnC
0
10
20
30
40
50
60
Incubation Time (minutes)

Figure II-1

the presence of calcium, the initial rate is not as fast as that in the absence of calcium. However, the former leads to a more extensive inactivation than the latter. At low levels of either calmodulin or troponin C, the initial inactivation rate is almost directly proportional to the protein concentration. 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 hours of incubation. Calmodulin and troponin C apparently inactivate phosphofructokinase by: (1) accelerating an intrinsic inactivation process and (2) shifting the equilibrium towards more extensive inactivation.

The results of our inactivation studies differ from Mayr's (1984) 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. With our inactivation conditions, in which the solutions contain 25% glycerol and no ATP, phosphofructokinase can be efficiently inactivated by a variety of acidic proteins. The presence of glycerol in the medium significantly decreases both the background

rate of inactivation in the control and slows 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 (Table II-1).

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-2, 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.

Effects 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). The thrombic fragment known as TH1 contains troponin C residues 1-120 while TH2 contains residues 121-159. As discussed in Materials and Methods, each of the tryptic fragments comprises a set of two closely related polypeptides. TR1 apparently contains sequences 8-84 and 8-88 while TR2 corresponds to sequences

Table II-1

Effect of Glycerol on the Reversible Inactivation of Phosphofructokinase.

Glycerol	Inactivation				Reactivation	
	5 min		60 min			
	Ca^{2+}	EGTA	Ca^{2+}	EGTA	Ca^{2+}	EGTA
0%	0.028	0.03	0.008	0.015	0.018	0.07
10%	0.10	0.105	0.026	0.064	0.14	0.28
25%	0.79	0.63	0.55	0.12	0.69	0.77

Data in the table represent the relative remaining or recovered activity of the enzyme, with the initial activity defined as 1. Phosphofructokinase (0.02 mg/mL) was incubated for either 5 min or 60 min with 0.1 μM troponin C under standard inactivation conditions, except for varying glycerol concentrations. Reactivation was started by addition of 1 mM ATP and 0.1 mM fructose 2,6-bisphosphate after one hour of incubation. Recovered activity was determined 2 hrs later.

Table II-2

Troponin C-Induced Inactivation Observed with Phosphofructokinase from Different Sources

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

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 minutes of incubation. The activity in the control is defined as 1.

85-159 and 89-159. Table II-3 summarizes the inactivation of phosphofructokinase obtained both with the fragments and with intact troponin C. The data represent the fragment concentrations (in μM) required for 50% inactivation of enzyme samples which have been incubated for 20 minutes under the conditions described in Materials and Methods. 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).

The varying degrees 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 II-4. Note that significant calcium dependence was detected with troponin, the 20,000 dalton light chain of smooth muscle myosin, and possibly α-actinin. Low calcium dependence was found for S-100, a small Ca^{2+}-binding protein of unknown function (Kligman and Hilt, 1988). Parvalbumin

Table II-3

Inactivation of Phosphofructokinase Induced by Troponin C Fragments

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

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 of ATP was included. Phosphofructokinase activity was measured after a 20 minute incubation period. The structures of the fragments are given in Figure II-2.

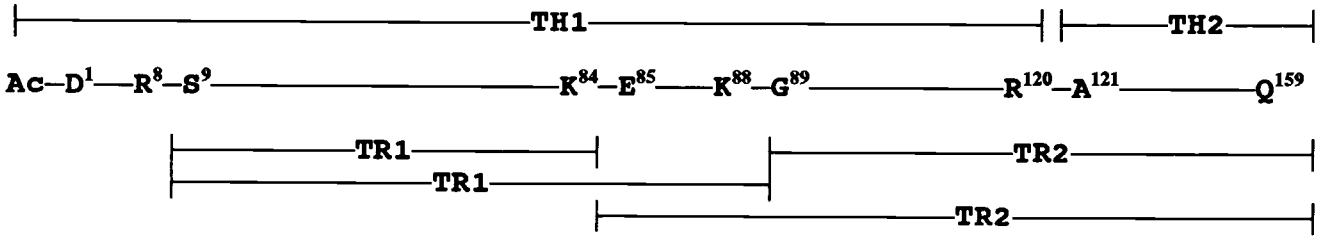


Figure II-2. Structure of Troponin C Fragments

Table II-4

Inactivation of Phosphofructokinase in the Presence of Various Acidic Proteins

Acidic Proteins(1.0 μM)	0.1 mM Ca^{2+}	0.1 mM EGTA
Control	1.000	1.000
Troponin C (18kD)	0.106	0.100
Calmodulin (16.8kD)	0.115	0.110
Troponin (75kD)	0.267	0.435
MLC (17kD)	0.303	0.322
MLC (20kD)	0.345	0.523
S-100 (16kD)	0.333	0.362
α-Actinin (96kD)	0.308	0.415
Poly(Glu-Tyr) (39kD)	0.330	0.330
Parvalbumin (12kD)	0.790	0.775
SBTI (22.5kD)	0.880	0.880

Data in the table represent the relative activity of phosphofructokinase remaining after 20 minutes 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, soy bean trypsin inhibitor.

is another Ca^{2+}-binding protein with an isoelectric point similar to that of calmodulin or troponin C (Heizmann *et al.*, 1974). However, it is considerably less effective than any of the preceding proteins, with a parvalbumin concentration as high as 6.7 μM needed to produce 50% inactivation within 20 min of incubation. Additionally, the maximum inactivation is only 70% and the results are independent of Ca^{2+}. Soybean trypsin inhibitor, a protein containing 17% acidic amino acid residues (Kim *et al.*, 1985), also shows a degree of phosphofructokinase inactivating ability. A relatively high concentration (8.9 μM) is required to attain 50% inactivation within 20 min, with a maximum inactivation of only 75%. As an extreme case, we tested a synthetic polypeptide (with an average molecular weight of 39,000) containing 51% of glutamyl residues - poly(NaGlu-Tyr). As expected, the presence of this extremely acidic copolymer results in very rapid inactivation. The concentration required for 50% inactivation within 20 min is only 51 nM, a level lower than that determined for troponin C or calmodulin. However, the maximum total inactivation is just 70%.

Kinetic Modeling of the Inactivation

Considering the known rapid dissociation and association of phosphofructokinase and the possible

existence of enzyme conformers (Luther *et al.*, 1983, 1985; Mayr, 1984), we consider the following kinetic model:

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

Scheme 1

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 interconversion of the two dimers. As detailed in the supplement to this article, we have derived the following equation relating the fraction of remaining activity (A/A_0) to the period of incubation (t).

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

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

In equation (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 equation (1) to the data, as shown in Table II-5. Note that only the first 60 minutes of the inactivation time course is considered here. In fact, the inactivation does not reach a

Table II-5. Kinetic Parameters for the Inactivation of Phosphofructokinase

Conditions	$P_1(min^{-1})$	$P_2(min^{-1})$	r^2	$k_2(min^{-1})$	$k_{-2}x10^3(min^{-1})$	K^*
Control-Ca^{2+}	0.0029	0.0045	0.995	0.0026	0.33	7.96
Control-EGTA	0.0027	0.0042	0.996	0.0024	0.30	7.87
.025μM CaM-Ca^{2+}	0.015	0.014	0.999	0.014	0.99	14
.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
.025μM CaM-EGTA	0.015	0.019	1.00	0.014	1.4	10
.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
.025μM TnC-Ca^{2+}	0.013	0.012	1.00	0.012	0.82	14
.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
.025μM TnC-EGTA	0.022	0.045	0.999	0.024	3.2	7.5
.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

Nonlinear regression analysis of the inactivation data in Figure II-1 according to equation (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, and P_2, and the estimated value of K = 4.5 nM.

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) and discussed later. Because the aggregation process is much slower than the calmodulin- or troponin C-induced transformation between D and D^*, we simply ignore it in our kinetic model. Aggregation is believed responsible for the deviation of the activities observed after one hour.

In order to calculate the rate constants, the dissociation constant K was first estimated by application of the method described by Jenkins *et al.* (1985). Phosphofructokinase was diluted at room temperature from 0.2 mg/mL to six different concentrations ranging from 0.0025 to 0.08 mg/mL with the enzyme buffer containing 50 mM Mops-KOH, 25% glycerol, 5.0 mM ß-mercaptoethanol, 0.5 mM dithiothreitol, pH 7.0. Since the dissociation of the tetramer T to the dimer D is a fast process (Luther *et al.*, 1985), the catalytic activity measurements (performed at an enzyme concentration of 0.2 µg/mL under the optimal conditions described in Materials and Methods) were carried out as soon as possible after the initial dilution (usually within 30 seconds). According to the equation $C_0/A = I + 0.5\sqrt{IK/A}$ (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 $r^2 = 0.9905$ (Figure II-3). From this linear plot, a dissociation constant $K = 4.5 \times 10^{-9} \pm 0.5$ M was estimated. This value represents an approximate upper limit for K since a certain amount of additional dissociation may occur during the early stages of the assay. 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 $(NH_4)_2SO_4$, 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 II-5 shows the fit of the model for phosphofructokinase inactivation to the kinetic data. Nonlinear regression analysis of the inactivation time course yielded the parameters P_1 and P_2 along with the correlation coefficient, r^2. By assuming that K = 4.5 nM, k_2, k_{-2}, and K^* were calculated from their relationships to P_1 and P_2. The absolute value of K mainly affects k_{-2} and K^* but has little effect on k_2. It is clear that calmodulin and troponin C not only change the rate constants k_2 and k_{-2}, but also 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

Figure II-3: Determination of the dissociation constant of phosphofructokinase tetramer to dimer. Phosphofructokinase was diluted at room temperature from 0.2 mg/mL to six different concentrations ranging from 0.0025 to 0.08 mg/mL with the enzyme buffer containing 50 mM Mops-KOH, 25% glycerol, 5.0 mM ß-mercaptoethanol, 0.5 mM dithiothreitol, pH 7.0. The catalytic activity measurements (performed at an enzyme concentration of 0.2 μg/mL under the optimal conditions described in Materials and Methods) were carried out 30 seconds after the initial dilution. C_0 represents the total phosphofructokinase concentration; A, the catalytic activity determined after dilution.

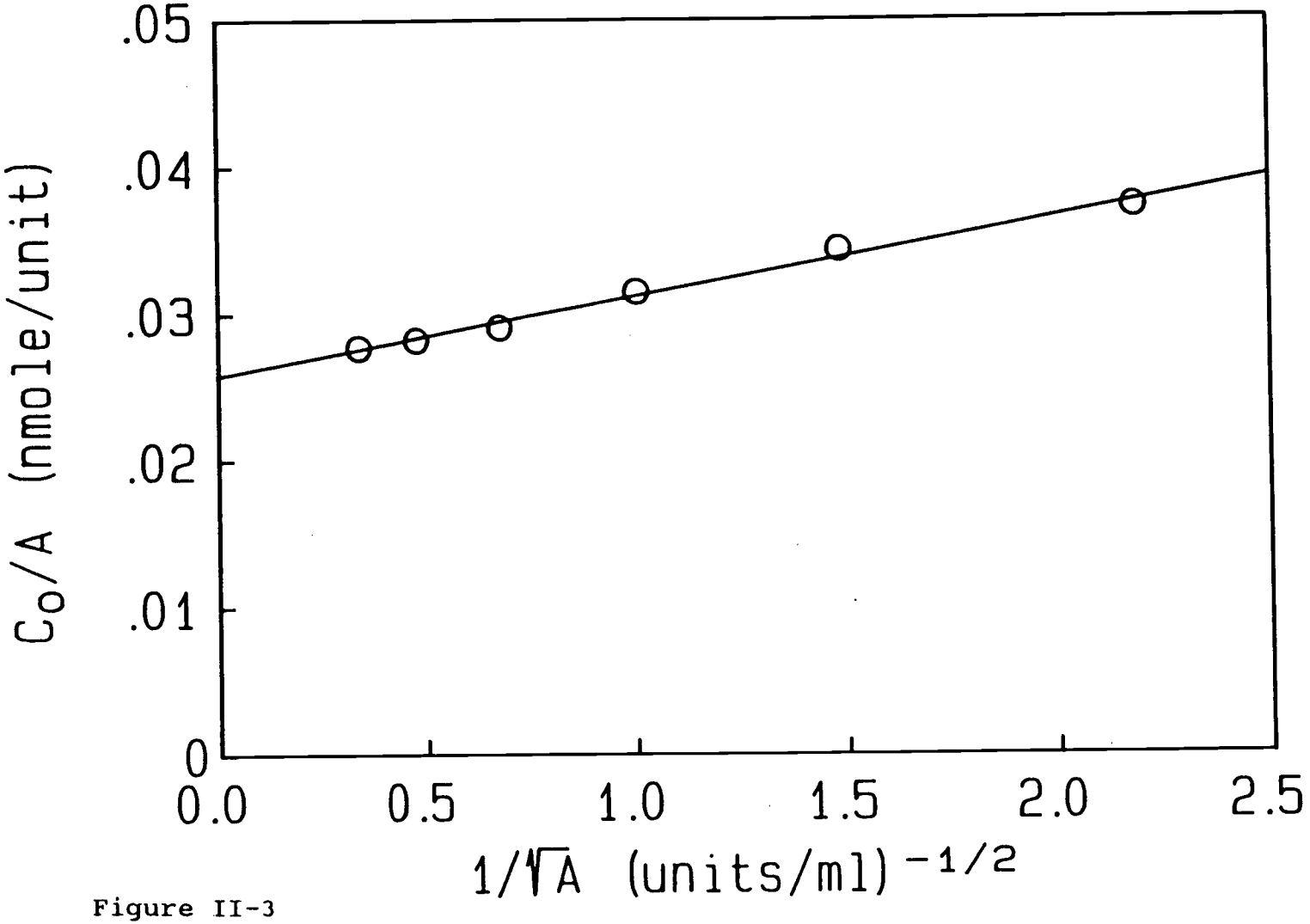

.05
.04
.03
.02
.01
0
C_o/A (nmole/unit)
0.0
0.5
1.0
1.5
2.0
2.5
$1/\sqrt{A}$ (units/ml)$^{-1/2}$

Figure II-3

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.

Factors Affecting the Inactivation of Phosphofructokinase

Temperature and pH

Table II-6 shows the effects of temperature and pH variation on the inactivation of phosphofructokinase. As shown by the parameters P_1 and P_2 of equation (1) and the remaining activity (A_{eq}) at theoretical equilibrium, increasing the temperature from 0 °C to 36 °C speeds up the inactivation rate (with a greater than ten-fold increase in P_1), and shifts the equilibrium in the direction of inactivation.[1] This increase in both the rate and extent of inactivation with temperature is opposite to the cold lability reported by Bock and Frieden (1976b), suggesting that the rate of inactivation of phosphofructokinase depends not only on the equilibrium between tetramer and dimer, but more importantly on the interaction of phosphofructokinase with the proteins. Changes in pH from 8.0 to 6.5 lead to

---

[1]Individual values of k_2, k_{-2}, and K^* could not be calculated without determination of the value K.

Table II-6

Effects of Temperature and pH on the Interaction of Phosphofructokinase

	CaM or TnC	$P_1(min^{-1})$	$P_2(min^{-1})$	r^2	A_{eq}
Temperature		(pH = 7.0)			
0 °C	0.1 μM CaM	0.013	0.062	0.993	0.65
16 °C	0.1 μM CaM	0.033	0.052	0.978	0.31
24 °C	0.1 μM CaM	0.090	0.10	0.993	0.21
36 °C	0.1 μM CaM	0.16	0.12	0.998	0.11
0 °C	0.1 μM TnC	0.012	0.037	0.999	0.51
15 °C	0.1 μM TnC	0.036	0.053	0.994	0.28
24 °C	0.1 μM TnC	0.11	0.11	1.00	0.18
36 °C	0.1 μM TnC	0.17	0.10	0.996	0.076
pH		(Temperature = 24 °C)			
pH 6.5	0.05μM CaM	0.042	0.034	0.999	0.13
pH 7.0	0.05μM CaM	0.030	0.028	0.998	0.16
pH 7.5	0.05μM CaM	0.019	0.019	0.996	0.17
pH 8.0	0.05μM CaM	0.016	0.020	0.993	0.21
pH 6.5	0.05μM TnC	0.062	0.054	1.00	0.14
pH 7.0	0.05μM TnC	0.054	0.054	0.998	0.17
pH 7.5	0.05μM TnC	0.052	0.056	0.995	0.18
pH 8.0	0.05μM TnC	0.033	0.037	0.999	0.20

The results in the table were obtained from nonlinear regression analyses of the time courses of phosphofructokinase inactivation determined in the absence of calcium at different temperatures (pH 7.0) or at varying pH values, with a fixed temperature of 24°C.

slight increases in both the initial rate and extent of inactivation, similar to those found in buffer alone (Bock and Frieden, 1976a).

Low-Molecular-Weight Effectors of Phosphofructokinase

All the low-molecular-weight effectors of phosphofructokinase exert protective effects when the enzyme is incubated with calmodulin or troponin C. The data in table II-7 show the concentrations of ligand required for 50% of maximum protection of phosphofructokinase activity, with the usual 20 minute 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.

Table II-7

Effects of Low Molecular Weight Ligands on the Inactivation of Phosphofructokinase

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)	.04	.045
ATP (μM)	8.0	20.5
ADP (mM)	0.2	0.93
AMP (mM)[1]	0.1	0.3
Phosphate(mM)	2.7	9.5
Citrate (mM)	1.6	3.3

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 minutes.

[1] The maximum protection with AMP is only 35% in the presence of calcium and 30% in the absence of calcium.

Calmodulin- and Troponin C-binding Proteins

Troponin I is a strongly basic troponin C- and calmodulin-binding protein. Each troponin C molecule can bind up to two molecules of troponin I in the presence of calcium (Grand *et al.*, 1982). When present at several fold molar excess and either with or without calcium, troponin I totally protects phosphofructokinase from the inactivation which would otherwise occur in solutions containing calmodulin or troponin C (Figure II-4). Control experiments showed that troponin I has no effect on the activity of the enzyme alone. The enzyme inactivation induced by the fragments of troponin C also can be prevented by the addition of excess troponin I (not shown).

Melittin forms a highly stable, calcium-dependent complex with calmodulin (Barnette *et al.*, 1983; Maulet and Cox, 1983). It also proved to be good a protector of phosphofructokinase activity. As shown in Figure II-5A, melittin exerts a greater effect in the presence of calcium, where only stoichiometric amounts of melittin relative to calmodulin are required for effective protection. In the absence of calcium, in contrast, a concentration of melittin which is in several fold excess over the concentration of calmodulin is required for significant effects. These results are consistent with the known calcium dependence of melittin binding by calmodulin (Maulet and Cox, 1983).

Fig. II-4: Effect of troponin I on the phosphofructokinase inactivation induced by calmodulin or troponin C. Different concentrations of troponin I as indicated were introduced into the standard phosphofructokinase inactivation system, with either 0.5 μM calmodulin (panel A) or 0.25 μM troponin C (panel B). After 20 minutes of incubation, phosphofructokinase activities were measured under optimal assay condition at 24 °C. Symbols: □, results determined in the presence of 0.1 mM Ca^{2+}; ■, results determined in the presence of 0.1 mM EGTA. Other conditions are given under Figure II-1.

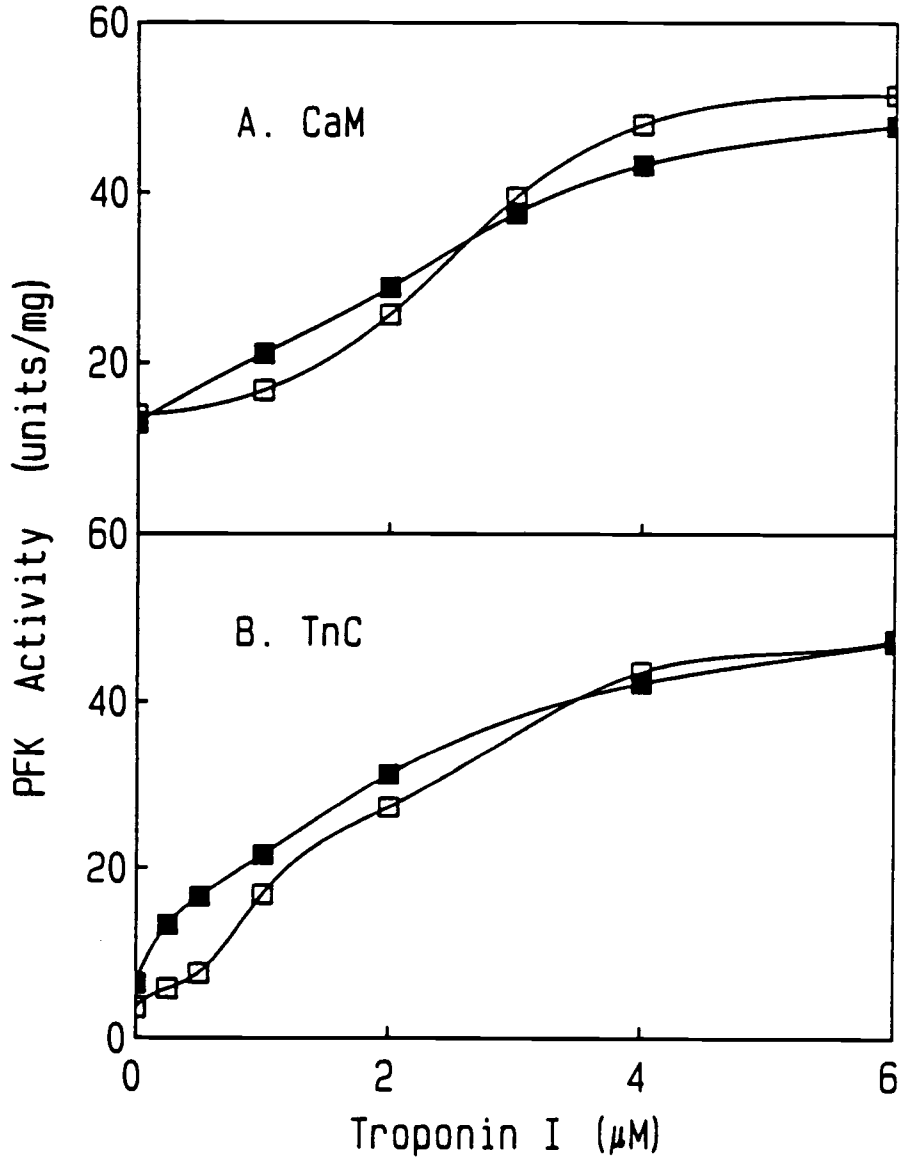
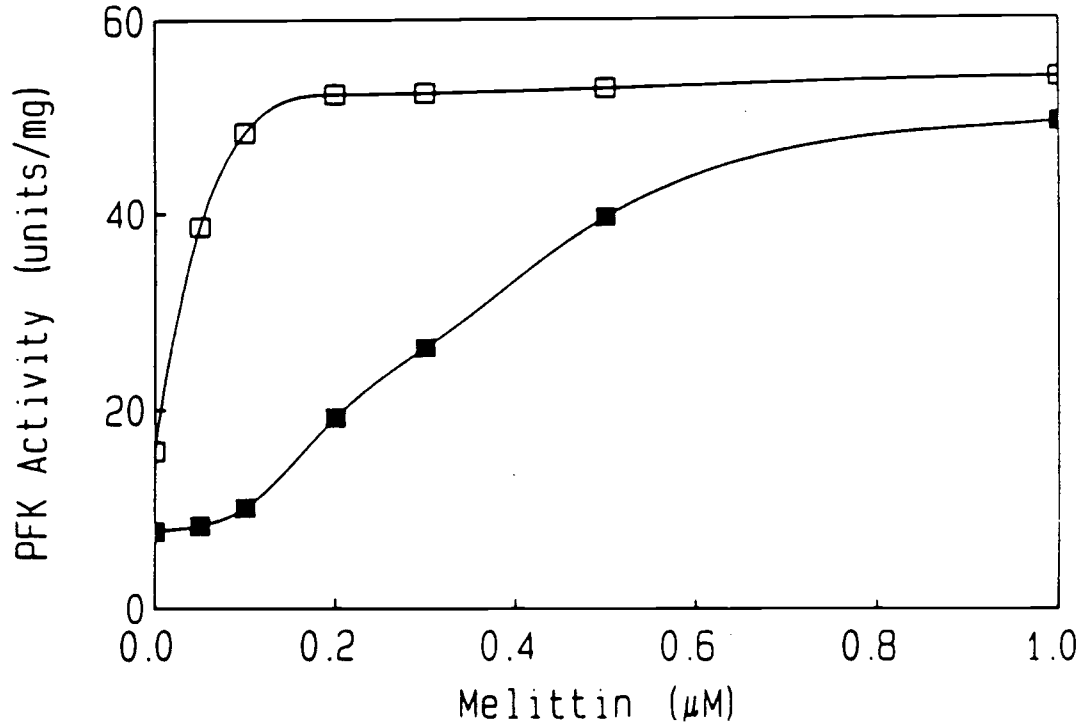

60
40
20
A. CaM
60
40
20
0
B. TnC
PFK Activity (units/mg)
0
2
4
6
Troponin I (μM)

Figure II-4

Figure II-5: Effect of melittin (A) and myosin light chain kinase (MLCK) (B) on phosphofructokinase inactivation induced by calmodulin. Different concentrations of melittin or MLCK were included in standard phosphofructokinase inactivation system containing 0.1 μM calmodulin. After 20 minutes of incubation, phosphofructokinase activities were measured under optimal assay condition at 24 °C. Symbols: □, results obtained in the presence of calcium; ■, results obtained in the absence of calcium. Other conditions are given under Figure II-1.


60
40
20
0
PFK Activity (units/mg)
0.0
0.2
0.4
0.6
0.8
1.0
Melittin (μM)

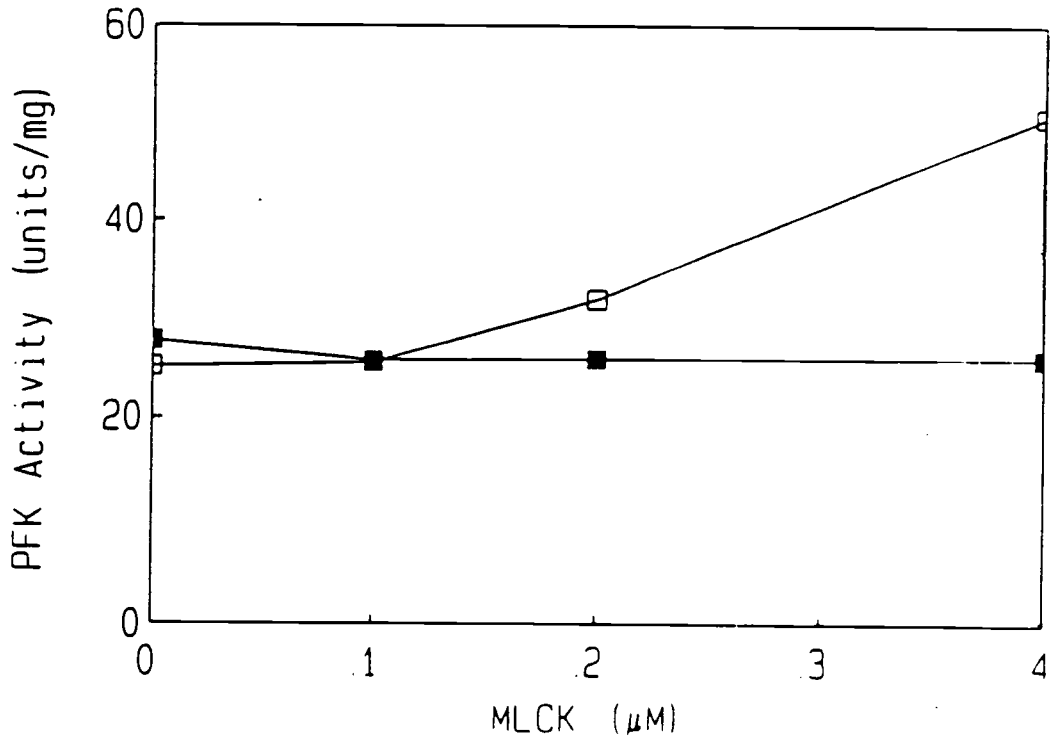

60
40
20
0
PFK Activity (units/mg)
0
1
2
3
4
MLCK (μM)

Figure II-5

Myosin light chain kinase (MLCK) forms tight complex with calmodulin in the presence of calcium (Malencik *et al.*, 1982). As expected, in the presence of calcium, MLCK, at a molar concentration twice that of calmodulin, significantly reduces the inactivation of phosphofructokinase induced by calmodulin (Figure II-5B). In the absence of calcium, no effect was observed. MLCK has little effect on the inactivation induced by troponin C, indicating a relatively poor binding to the latter.

Protamine, a strongly basic low molecular weight polypeptide (MW = 4.8 kD) (Grazi & Magri, 1982), totally protects phosphofructokinase from inactivation when present at molar concentrations equivalent to those of calmodulin or troponin C. Protamine, at concentrations of 0.1 μM, also protects the enzyme from the gradual inactivation which occurs in the absence of the other proteins (Figure II-6). This may be due to the interaction of protamine itself with the enzyme.

Reactivation of Phosphofructokinase

Phosphofructokinase activity can be restored by the addition of certain effectors (Fig. II- 7). 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

Figure II-6: Effects of protamine on the inactivation of phosphofructokinase induced by calmodulin and troponin C. The incubation was performed with different concentrations of protamine under conditions described in Materials and Methods except the absence of calcium. Enzymatic activities were assayed 20 minutes of incubation. Symbols : □, control; Δ , 0.1 μM calmodulin; ◇ , 0.1 μM troponin C.

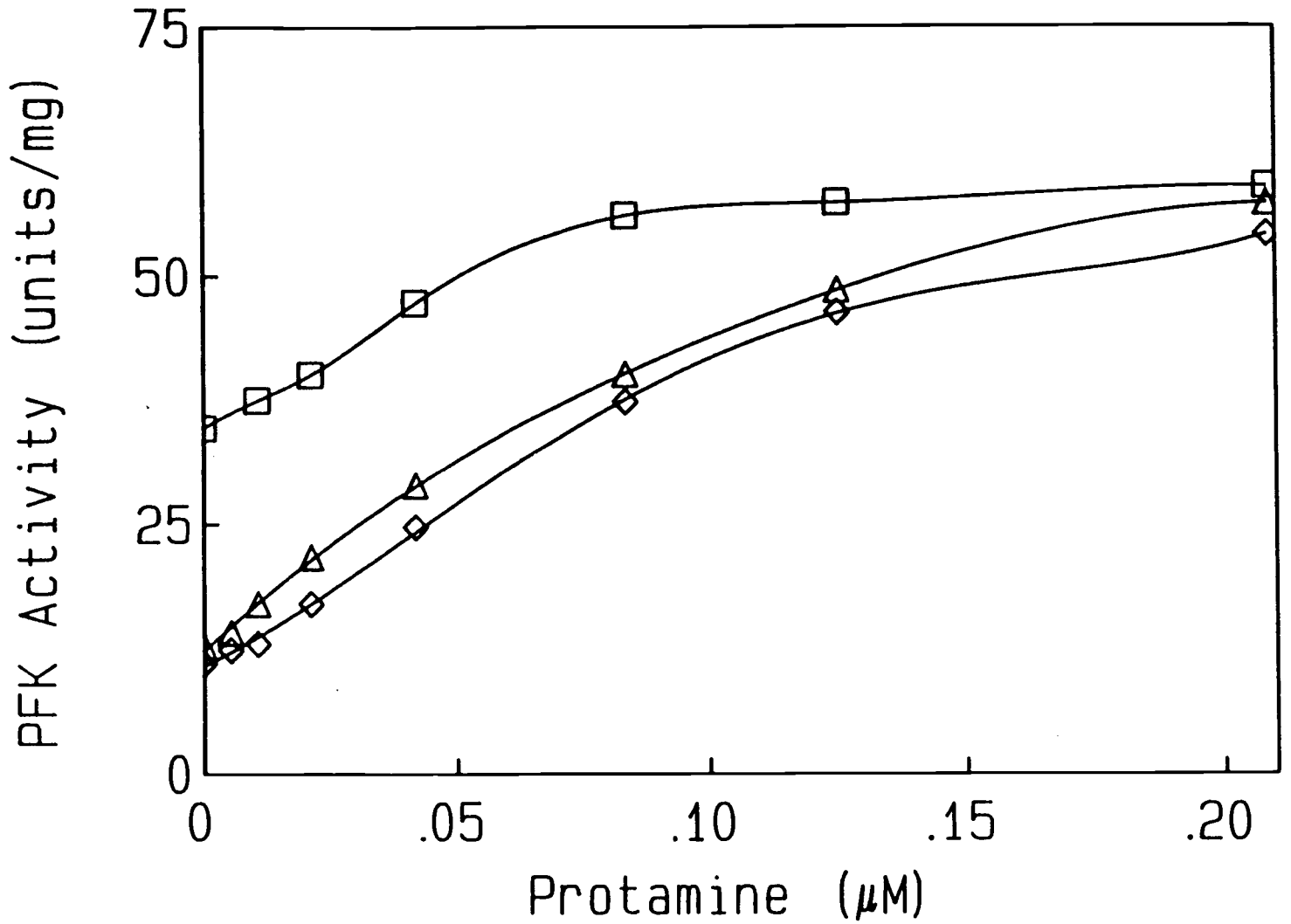


Figure II-6

Figure II-7: Reactivation of phosphofructokinase. Phosphofructokinase was pre-incubated 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 minute incubation periods, minimal volumes of the following substances were added to give the indicated final concentrations: □, 1.0 mM ATP; Δ, 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 II-1.

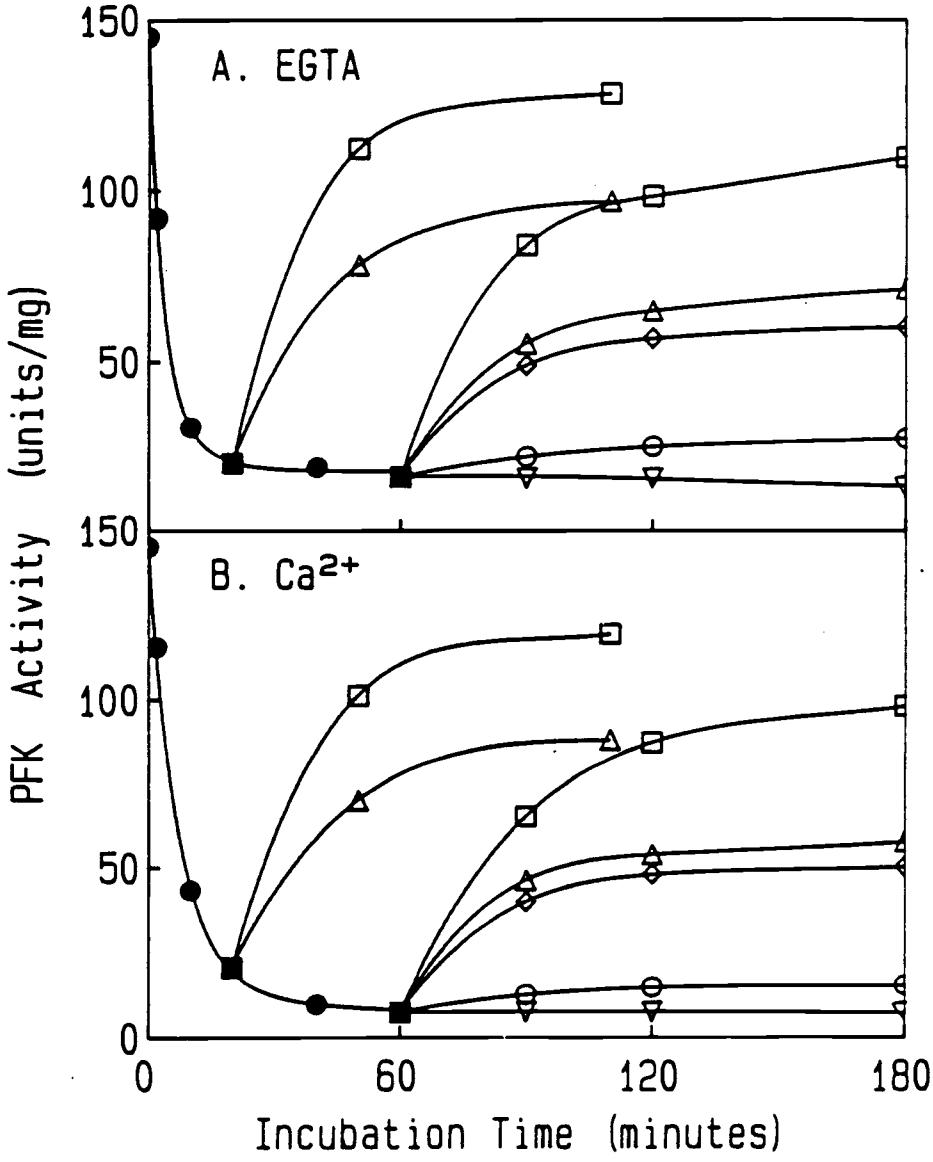

A. EGTA
B. Ca2+
PFK Activity (units/mg)
Incubation Time (minutes)
150
100
50
0
60
120
180

Figure II-7

fructose-6-phosphate and inorganic phosphate). Calmodulin- and troponin C-binding proteins -- troponin I, melittin, MLCK, and protamine -- also fail to elicit enzyme recovery. 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 minutes incubation than after 20 minutes, especially when calcium is present. After prolonged incubation (4 hours or more) in the presence of calcium, the inactivation becomes almost irreversible. The addition of glycerol (25%) to the inactivation system favors subsequent reactivation. In the absence of glycerol, and even in the presence of 10% glycerol, the inactivation reaction goes much faster while the reactivation process is significantly slowed down -- with much lower maximum reactivation achieved (Table II-1). For simplicity, Figure II-7 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 II-1).

Interaction of Phosphofructokinase with Calmodulin and Troponin C as Shown by Polyacrylamide Gel Electrophoreses

The interactions between phosphofructokinase and calmodulin or troponin C were demonstrated directly by urea gel electrophoresis, employing a 6% stacking gel in combination with a 10% resolving gel as described in Materials and Methods. The photograph (Figure II-8A) shows that the enzyme alone does not enter the stacking gel -- possibly because of aggregation. In contrast, samples which have been incubated with calmodulin or troponin C in the presence of calcium 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. Linear 9-19% gradient $NaDodSO_4$ gel electrophoresis performed on the bands which had been excised from the stacking gel, without prior fixing or staining, clearly shows the presence of troponin C or calmodulin along with phosphofructokinase (Figure II-8B). In the absence of calcium, the inactivated enzyme does not enter the gel -- indicating dissociation of the protein complex by the addition of 6 M urea and/or application of the electric field.

Figure II-8: Electrophoretic Study of the Interaction between phosphofructokinase and calmodulin or troponin C. 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 (lane 6, 7, 8, 9) or 0.3 mg/mL (lane 2, 3, 4, 5) of calmodulin or troponin C, as described in Materials and Methods. 15 μL aliquots of the mixtures were then loaded onto the gel. Lane 1, phosphofructokinase control; 2 and 6, troponin C control; 3 and 7, phosphofructokinase + troponin C; 4 and 8, calmodulin control; 5 and 9, phosphofructokinase + calmodulin. Lower photograph (B) shows the results of 9-19% $NaDodSO_4$ polyacrylamide gel electrophoresis performed on phosphofructokinase bands which had been individually excised from an unstained and unfixed urea gel. Lanes 1 and 5, protein markers; 2, 3, and 4, calmodulin-inactivated phosphofructokinase bands a, b, and c, respectively, from urea gel electrophoresis; 6, 7, and 8, troponin C-inactivated phosphofructokinase bands a, b, and c, respectively.

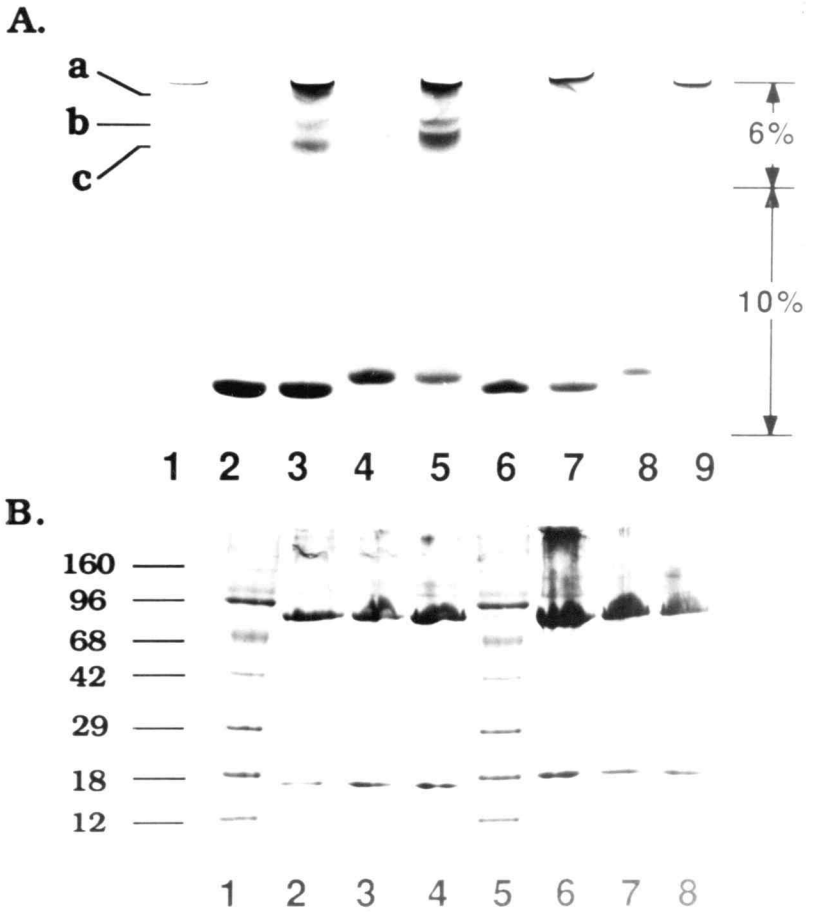


Figure II-8

Discussion

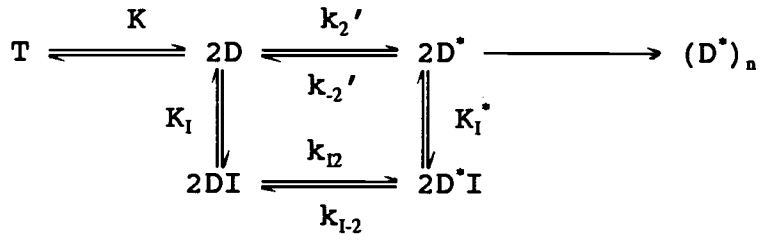
Of all the proteins which we have investigated, calmodulin and troponin C stand out for their marked abilities to enhance both the rate and extent of inactivation of rabbit skeletal muscle phosphofructokinase. Among enzymes which bind calmodulin, phosphofructokinase is unusual in that nearly equal effects are obtained with calmodulin and troponin C. Most calmodulin-dependent enzymes do not associate with the latter. Even in the case of phosphorylase kinase, which interacts extrinsically with either protein, the association with troponin C is about two order of magnitude less effective than that found with calmodulin (Cohen *et al.*, 1979; D. A. Malencik and S. R. Anderson, unpublished results). The effects 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 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 on the N-terminal side of 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 present data suggest a general mechanism for the inactivation of phosphofructokinase by a number of acidic proteins and peptides, including calmodulin and troponin C. It may apply to other examples of the inactivation of the enzyme by acidic proteins, such as that reported for the Zn^{2+}-dependent inactivating protein from rat liver (Brand and Söling, 1986) and the band 3 protein of the erythrocyte membrane (Jenkins *et al.*, 1985). The contrasting sensitivities of muscle phosphofructokinase and either liver or yeast phosphofructokinase to the inactivation may be physiologically relevant. Although skeletal muscle has a high concentration of the enzyme (0.2 mg/mL), the catalytic activity observed in resting muscle is several hundred-fold lower than that predicted from the assays performed on the purified enzyme (Passonneau & Lowry, 1962). The activity of phosphofructokinase in skeletal muscle may be subject to a highly effective inactivation mechanism involving one or more acidic proteins: troponin C, troponin, calmodulin, α-actinin, myosin, etc. In contrast, liver and yeast contain much less phosphofructokinase than muscle (Kemp, 1975; Stellwagen and Wilgus, 1975), and in addition, lack the high concentration of acidic proteins characteristic of muscle.

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:



Scheme 2

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 calmodulin 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 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 1) can be represented by the constants and concentrations in the more general model (Scheme 2) in following ways:

$$k_2 = k_2' + k_{I2} \cdot K_I \cdot [I] \qquad k_{-2} = k_{-2}' + k_{I\text{-}2} \cdot K_I^* \cdot [I]$$

$$K^* = K_0 \cdot (1 + K_I^* \cdot [I]) \ / \ (1 + K_I \cdot [I]) \qquad K_0 = [D^*]/[D]$$

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 pseudo-constants in Scheme 1 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 II-4). 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 -- as also shown in the nonlinear regression analysis.

Small ligands of phosphofructokinase have distinctly different effects on the simple dimer-tetramer equilibrium and the changes which are induced by the addition of calmodulin or troponin C. 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 (cf. Fig. II-7), 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, of variations in temperature and pH, or of calmodulin- and troponin C-binding proteins.

Excess concentrations of various proteins which bind calmodulin or troponin protect phosphofructokinase from inactivation (Figure II-4, 5, 6). These proteins may

compete with the enzyme for common binding sites or possibly alter the conformations of the inactivating proteins. However, despite their high affinities for calmodulin or troponin C, none of these proteins -- even when present at high concentration -- can reverse the inactivation. This finding is consistent with evidence that calmodulin has an essential role in the reactivation of the enzyme by preventing the polymerization of dimer D* (Mayr, 1984 a,b).

Increases in both the extent and rate of the calmodulin- or troponin C-induced inactivation with temperature (Table II-6) also contrast with the behavior of the simple dimer-tetramer system (Luther *et al.*, 1986). This suggests that the temperature dependence of the inactivation process is determined largely by the binding of calmodulin or troponin C and/or the subsequent interconversion of the dimer isoforms. This result agrees with that reported by Jenkins *et al.* (1985) for the inactivation of phosphofructokinase by the erythrocyte membrane band 3 protein. The effects of pH variation, on the other hand, follow previous reports (Bock and Frieden, 1976a) on the stabilization of the tetramer with increasing pH.

Chapter III describes the effects of the calcium-dependent interactions of phosphofructokinase with calmodulin and troponin C on the phosphorylation of the enzyme catalyzed by cAMP-dependent protein kinase.

Supplementary material

Derivation of the Kinetic Equation

Kinetic Model:

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

Assume T and D in rapid equilibrium.

So $K = D^2/T$

$$T_0 = T + 1/2\ (\ D + D^*\ ) = D^2/K + 1/2\ (\ D + D^*\ )$$

$$D^* = 2T_0 - 2D^2/K - D = 2D_0^2K^{-1} - 2D^2K^{-1} - D \qquad (1)$$

According to the model,

$$dD/dt = -k_2D + k_{-2}D^* \qquad (2)$$

$$dD^*/dt = -k_{-2}D^* + k_2D \qquad (3)$$

Substitute (1) into (2)

$$dD/dt = 2k_{-2}D_0^2K^{-1} - (k_2 + k_{-2})D - 2k_{-2}D^2K^{-1} \qquad (4)$$

$$\int_{D_0}^{D} \frac{dD}{2k_{-2}D_0^2K^{-1} - (k_2 + k_{-2})D - 2k_{-2}D^2K^{-1}} = \int_0^t dt \qquad (5)$$

According to equation

$$\int \frac{dx}{a + bx - cx^2} = \frac{1}{\sqrt{b^2 + 4ac}} \log \frac{\sqrt{b^2 + 4ac} - b + 2cx}{\sqrt{b^2 + 4ac} + b - 2cx} + C \qquad (6)$$

equation (5) becomes:

$$\log \frac{\{P + (k_2 + k_{-2}) + 4k_{-2}K^{-1}D\}\{P - (k_2 + k_{-2}) - 4k_{-2}K^{-1}D_0\}}{\{P - (k_2 + k_{-2}) - 4k_{-2}K^{-1}D\}\{P + (k_2 + k_{-2}) + 4k_{-2}K^{-1}D_0\}} = Pt \qquad (7)$$

where $P = \sqrt{(k_2 + k_{-2})^2 + 16k_{-2}^2K^{-1}T_0} = \sqrt{(k_2 + k_{-2})^2 + 16k_{-2}^2K^{-2}D_0^2}$

$$\log \frac{D_0\{4k_{-2}K^{-1}D_0 - P - (k_2+k_{-2})\} + D\{P - (k_2+k_{-2}) - 4k_{-2}K^{-1}D_0\}}{D_0\{4k_{-2}K^{-1}D_0 + P - (k_2+k_{-2})\} - D\{P + (k_2+k_{-2}) + 4k_{-2}K^{-1}D_0\}} = Pt \qquad (8)$$

$$\frac{D_0\{4k_{-2}K^{-1}D_0 - P - (k_2+k_{-2})\} + D\{P - (k_2+k_{-2}) - 4k_{-2}K^{-1}D_0\}}{D_0\{4k_{-2}K^{-1}D_0 + P - (k_2+k_{-2})\} - D\{P + (k_2+k_{-2}) + 4k_{-2}K^{-1}D_0\}} = e^{Pt} \qquad (9)$$

Rearrange equation (9) into:

$$D = D_0 \frac{\{4k_{-2}K^{-1}D_0 - (k_2+k_{-2}) + P\} + \{(k_2+k_{-2}) - 4k_{-2}K^{-1}D_0 + P\}e^{-Pt}}{\{4k_{-2}K^{-1}D_0 + (k_2+k_{-2}) + P\} - \{(k_2+k_{-2}) + 4k_{-2}K^{-1}D_0 - P\}e^{-Pt}} \qquad (10)$$

Let $P_1 = k_2 + k_{-2}$, $P_2 = 4k_{-2}K^{-1}D_0$, so $P = \sqrt{P_1^2 + P_2^2}$

$$T = \frac{D^2}{K} = \frac{D_0^2}{K} \left\{\frac{(P_2 - P_1 + P) + (P_1 - P_2 + P)e^{-Pt}}{(P_1 + P_2 + P) - (P_1 + P_2 - P)e^{-Pt}}\right\}^2 \qquad (11)$$

$$T_0 = D_0^2/K$$

Therefore

$$\frac{T}{T_0} = \left\{\frac{(P_2 - P_1 + P) + (P_1 - P_2 + P)e^{-Pt}}{(P_1 + P_2 + P) - (P_1 + P_2 - P)e^{-Pt}}\right\}^2 \qquad (12)$$

With $P_1 = k_2 + k_{-2}$, $P_2 = 4k_{-2}K^{-1}D_0 = 4k_{-2}\sqrt{T_0/K}$, $P = \sqrt{P_1^2 + P_2^2}$

CHAPER III

Effects of Calmodulin and Troponin C on the Phosphorylation of Skeletal Muscle Phosphofructokinase

Abstract

Calmodulin and troponin C exert significant calcium-dependent effects on the *in vitro* phosphorylation of rabbit skeletal muscle phosphofructokinase catalyzed by the isolated catalytic subunit of cAMP-dependent protein kinase. The influence of these calcium-dependent regulatory proteins is manifest in large increases in the rate of phosphorylation and in maximum stoichiometries which are in excess of one mol phosphate/mol enzyme protomer. The phosphorylation is dependent on the time period of pre-incubation of phosphofructokinase with calmodulin or troponin C and on the molar ratios of the proteins. It is also affected by ATP, fructose-6-phosphate, fructose-2,6-bisphosphate, ADP, AMP, citrate, and inorganic phosphate.

We have identified two distinct phosphorylation sites in phosphofructokinase. Radiolabeling of the known C-terminal site, which has the sequence His-Ile-Ser-Arg-Lys-Arg-Ser(P)-Gly-Glu, (Kemp, R. G., Foe, L. G., Latshaw, S. P., Poorman, R. A., and Heinrikson, R. I. (1981) *J. Biol. Chem. 256*, 7282) occurs both in the control and in enzyme

samples which have been phosphorylated in the presence of calmodulin or troponin C. However, in either of the two latter cases, the maximum extent of reaction (0.84 mols phosphate/site) is significantly greater than that obtained in the former (≤ 0.4 mols phosphate/site). In addition, a novel site in the middle of the phosphofructokinase molecule -- with the sequence Lys-Leu-Arg-Gly-Arg-Ser(P)-Phe-Met -- becomes phosphorylated in samples which have been incubated with calmodulin or troponin C. Interestingly, troponin C stimulates both a higher rate and greater extent of phosphate incorporation (0.59 mol phosphate/site) into this position than does calmodulin (0.13 mol/mol). Preliminary studies indicate that phosphorylation has little effect on the activity of phosphofructokinase as determined by standard assays on samples taken from the phosphorylation mixtures. Substoichiometric levels of phosphorylation of phosphofructokinase were obtained with protein kinase-C (≤ 0.1 mol/mol protomer) and with cGMP-dependent protein kinase (≤ 0.4 mol/mol protomer).

Introduction

Phosphofructokinase (PFK, ATP : D-fructose-6-phosphate-1-phosphotransferase) can be phosphorylated both *in vivo* and *in vitro*. A number of laboratories have reported that the extent of phosphorylation of phosphofructokinase is dependent on both physiological state and the methods of tissue extraction used in the purification of the enzyme. Varying phosphate levels from 0.08 to 1.17 mole phosphate per mole protomer have been reported for the isolated enzyme from resting muscle (Hussey *et al.*, 1977; Riquelme *et al.*, 1978; Uyeda *et al.*, 1978; Hofer and Sorensen-Ziganke, 1979; Söling and Brand, 1981; Kuo et al., 1986). Electrical stimulation of muscle increases the phosphate content of phosphofructokinase up to 1.0-2.0 mole phosphate per mole protomer (Hofer and Sorensen-Ziganke, 1979). However, a small stoichiometry of 0.54-0.96 mole phosphate per mole 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, with differing degrees of phosphorylation once again reported. Pilkis *et al.* (1982) observed the incorporation of 0.9 to 1.0 mol phosphate/mol 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 phosphate/mol protomer for the latter case. Similar low stoichiometries have been presented for purified phosphofructokinase from rabbit brain (Foe and Kemp, 1984), rabbit skeletal muscle (Sale and Denton, 1985), and rat liver (Domenech *et al.*, 1988). The major known phosphorylation site of rabbit muscle phosphofructokinase proved to be a serine residue located at the 6th 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 & 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 are small. The changes observed do not seem to be physiologically relevant, particularly since hormones that raise cAMP levels -- *e.g.* epinephrine -- enhance glycolysis in muscle (Clarke & Patten, 1981). 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 one mol phosphate/mol 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 and 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. In all, after more than twelve years of investigation, the maximum possible extent of phosphofructokinase phosphorylation is uncertain and the physiological significance of the phosphorylation reaction remains unknown.

In following up our observations on the interactions of rabbit muscle phosphofructokinase with calmodulin and troponin C (Chapter II), we have examined the effects of these proteins on the phosphorylation of the enzyme as catalyzed *in vitro* by the cAMP-dependent protein kinase. This chapter describes: 1. the calcium-dependent effects of

calmodulin and troponin C on the phosphorylation; 2. the influence of enzyme substrates and allosteric effectors; 3. the identification of a second phosphorylation site; 4. preliminary studies on the effects of phosphorylation on enzymatic activity and on the interactions of the enzyme with calmodulin and troponin C.

Materials and Methods

Materials

γ-^{32}P-ATP was purchased from New England Nuclear (3000 Ci/mmole) and diluted to 100 Ci/mole 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 microfibre 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.

Preparation of Protein Kinases and Phosphofructokinase

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 condition by employing chicken histone H1 as a substrate. Purification of cGMP-dependent protein kinase followed the procedure of Glass and Krebs (1979). Rabbit skeletal muscle

phosphofructokinase was purified as described by Kuo *et al.* (1986). The pooled enzyme fraction used in this work had an endogenous phosphate content around 0.15 mol/mol protomer as determined by the method of Hasegawa *et al.* (1982).

Phosphorylation of Phosphofructokinase

Phosphofructokinase (0.125 mg/mL) was pre-incubated 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 pre-incubation at 30 °C, phosphorylation was started by bringing the system to 0.25 mM γ-^{32}P-ATP (100 μCi/μmole), 0.1 mg/mL phosphofructokinase, 4 μM calmodulin or troponin C, 5 mM $MgCl_2$, 0.8 mM Ca^{2+}, and 5 μg/mL of the 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. 55 μL of this mixture was then spotted on a 2.1 cm diameter glass filter disc and subsequently precipitated and washed with a solution containing 10% (w/v) trichloracetic acid and 2% (w/w) sodium pyrophosphate. Following final washing with ethanol and ether, the radioactivity on the filter was determined in 3

mL scintillation fluid using 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-X100 and 0.3% (w/v) Permablend III (United Technologies Parkard, 91% PPO (2,5-diphenyloxazole), 9% bis-MSB (1,4-Bis(2-methylstryryl) benzene) in tolulene (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.

In order to investigate possible changes in phosphofructokinase activity resulting from phosphorylation, the reaction was initiated without pre-incubation. After the indicated periods of incubation of the enzyme with protein kinase and calmodulin or troponin C, aliquots were removed for protein-bound radioactive phosphate determination and for activity assay under optimal conditions as described in the preceding chapter (Chapter

II). For reactivation studies, a minimal volume of fructose-2,6-bisphosphate was added to the mixture to give a final concentration 0.2 mM. This was followed by activity assays performed at indicated intervals.

To detect the effects of the phosphorylation of phosphofructokinase on its interaction with calmodulin and troponin C, the urea gel system used in Chapter II was adopted here. Phosphofructokinase (1.25 mg/mL) was first incubated with 15.25 μM of calmodulin or TnC in a buffer containing 50 mM Mops-KOH, pH 7.0, 1.0 mM $Ca(oAC)_2$, and 1.0 mM dithiothreitol for one hour. Phosphorylation was started by the addition of 0.25 mM ATP, 5.0 mM $MgCl_2$, and 10 μg/mL catalytic subunit of cAMP-dependent-protein kinase, which brings the concentration of PFK to 1.0 mg/mL. In control experiments, samples to which no protein kinase had been added were incubated in the presence of ATP-Mg^{2+} and *vice versa*. After one hour of phosphorylation at 30 °C, the reactions were terminated by the addition of a half volume of 8.0 M urea. Equal volumes of sample containing identical amounts of calmodulin or TnC were loaded to the urea gel. After electrophoresis, the gel was stained with Coomassie blue G-250. The amounts of calmodulin and TnC on the gel were quantitated by gel scanning with a Helena Laboratories Quick Scan Jr. gel scanner equipped with a peak area integrator. A 525 nm filter was used in the scanning.

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 two 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 hours 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 set-up used in this study consists of two Beckman 110B pumps, Beckman 421A controller, Beckman manual injector, and an Isco V4 detector. Reverse-phase HPLC was performed on a Vydac C18 column. A small precolumn (20 mm x 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 phospho-peptide) was sequenced directly. The second fraction was further digested with Staphylococcus aureus Strain V8 protease, employing a ratio of 1 mg protease per 30 mg peptide in 50 mM sodium phosphate (pH 8.0) at 30 °C for 4 hours (Drapeau 1978). The products of the V8 protease digestion were separated by the same HPLC system used to obtain the CNBr phospho-peptide. 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 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 phospho-peptide) 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.

Results

Phosphorylation of Phosphofructokinase by cAMP-Dependent Protein Kinase

As shown in Fig. III-1, the rate of the phosphorylation catalyzed by cAMP-dependent protein kinase increases many fold after pre-incubation of phosphofructokinase with either calmodulin or troponin C. Within a short period of time, which corresponds to the rapid phase of phosphorylation, an average of more than one mole 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 hrs of reaction (inset to Fig. III-1) verifies that phosphofructokinase is the only protein which has been phosphorylated. The rate of phosphorylation is also dependent on the length of the pre-incubation period and on the ratio of calmodulin or troponin C to the enzyme (Fig. III-2). With 0.1 mg/mL phosphofructokinase plus 4 μM troponin C, the maximum initial rate of phosphorylation is obtained after 10 minutes of pre-incubation. Shorter pre-incubation times give a relatively lower initial phosphorylation rate but with higher final phosphate incorporation. Longer periods of pre-incubation lead to a higher initial phosphorylation rate combined with a lower

Figure III-1: Time course of the phosphorylation of phosphofructokinase catalyzed by cAMP-dependent protein kinase. Phosphofructokinase (0.125 mg/mL) had been pre-incubated for one hour with 5 μM of either calmodulin or troponin C 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 of the 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 hours of phosphorylation. From left to right, 1, control; 2, with calmodulin; 3, with troponin C.

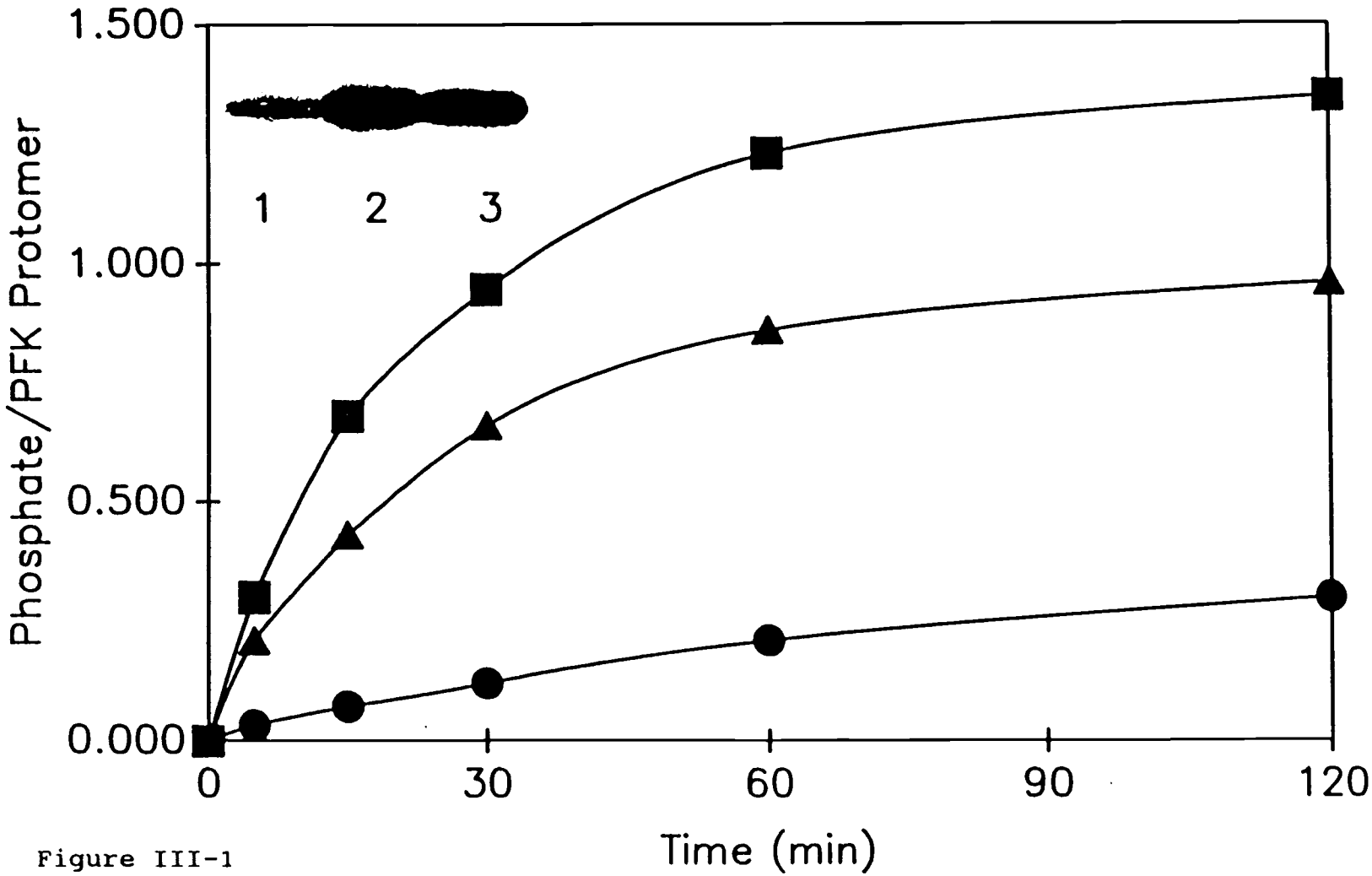

1.500
1.000
0.500
0.000
Phosphate/PFK Protomer
1
2
3
0
30
60
90
120
Time (min)

Figure III-1

final phosphate incorporation (Fig. III-2A). With a fixed 20 minute pre-incubation time, the phosphorylation rate initially increases almost linearly with increasing troponin C concentrations, approaching a plateau at troponin C/enzyme ratios above 5 (Fig. III-2B). Note that calcium is absolutely required for the effects of calmodulin or troponin C (Table III-1). In the absence of calcium, pre-incubation with calmodulin or troponin C has little effect on the phosphorylation of phosphofructokinase -- even though inactivation has occurred (Chapter II).

The addition of effectors of phosphofructokinase to the pre-incubation medium inhibits the phosphorylation reaction obtained in the presence of calmodulin or troponin C (Table III-2). Fructose-2,6-bisphosphate and fructose-1,6-phosphate produce the most marked effects. Note that the presence of a 0.1 mM concentration of either ligand almost totally abolishes the effect of calmodulin or troponin C on the phosphorylation of phosphofructokinase. The specific effects of ATP, a substrate of protein kinase, are difficult to ascertain. Other effectors -- AMP, phosphate, ADP, and citrate -- all decrease the phosphorylation rate, with the latter two having the least effect. These results are consistent with the results of the inactivation studies (Chapter II). 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

Figure III-2: Effects of varying pre-incubation time (A) and troponin C concentration (B) on phosphofructokinase phosphorylation. Phosphofructokinase (0.125 mg/ml) was pre-incubated with 5.0 μM troponin C for varying periods of time (A) or with differing concentrations of troponin C for 20 minutes (B). Conditions for pre-incubation and phosphorylation are given under Figure III-1 and in Materials and Methods. Part A shows the quantity of phosphate incorporated after 10 min (□), 30 min (Δ), and 60 min (◇) of phosphorylation. In B, phosphate incorporation was measured 2 hours after initiation of the reaction.

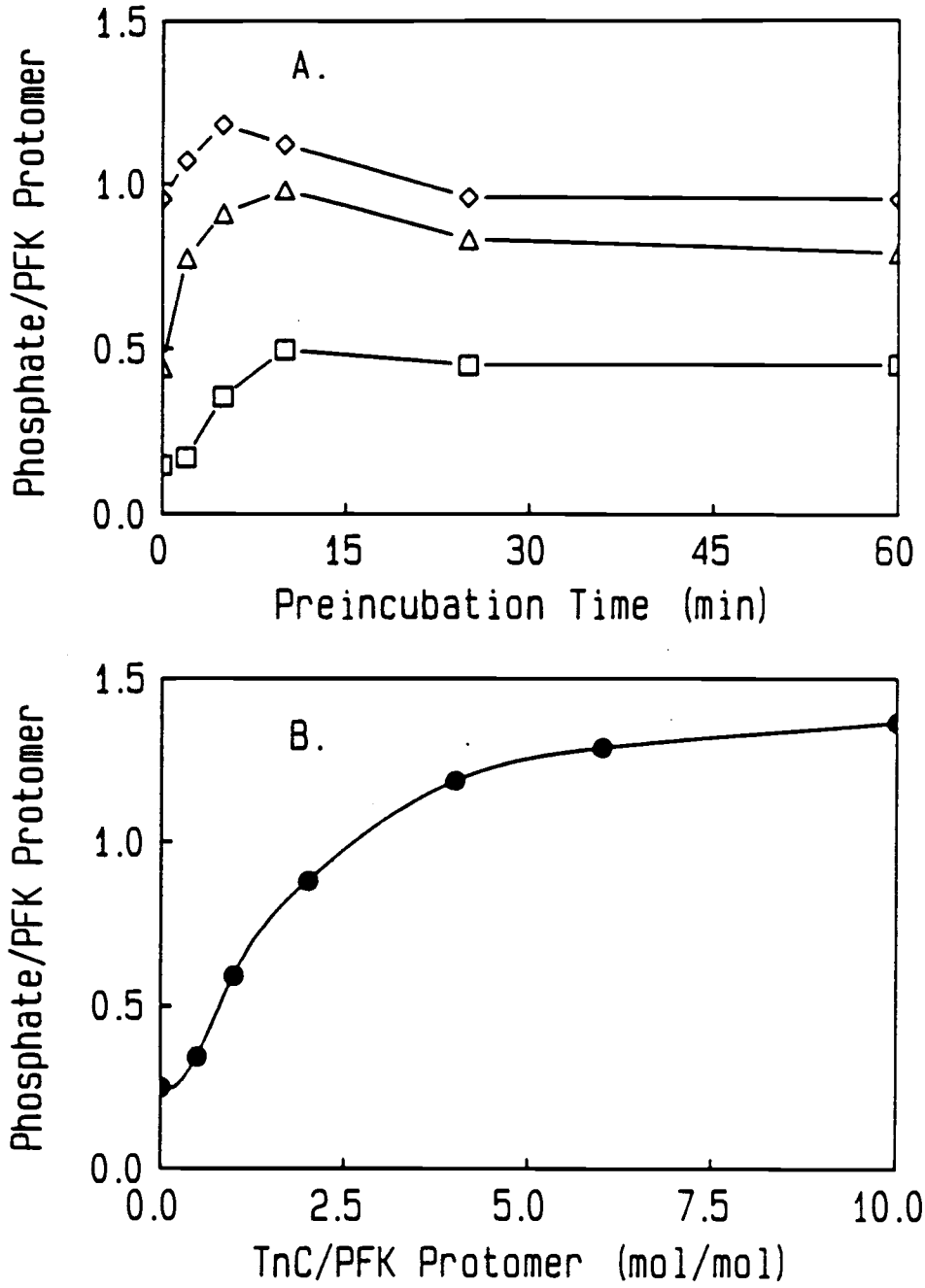

A.
Phosphate/PFK Protomer
1.5
1.0
0.5
0.0
0
15
30
45
60
Preincubation Time (min)
B.
Phosphate/PFK Protomer
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
TnC/PFK Protomer (mol/mol)

Figure III-2

Table III-1

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

Conditions	mol phosphate/mol enzyme protomer	
	after 1 hour	after 2 hours
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

PFK (0.125 mg/mL) was pre-incubated for 30 min with either 5 μM calmodulin or 5 μM troponin C as described in 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 subunits of cAMP-dependent protein kinase. Phosphate incorporation was measured after 1 hr of reaction and again after 2 hrs.

Table III-2. Effects of Small Ligands of PFK on the Phosphorylation Obtained in the Presence of Troponin C.

Additions	mol phosphate/ mol PFK monomer	
	1 hour	2 hours
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)[1]	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)[2]	0.58	0.98

PFK (0.125 mg/ml) was pre-incubated 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 hr and 2 hrs after the initiation of phosphorylation as described in Materials and Methods.

[1] Since radioactive ATP was present in the pre-incubation system, the phosphorylation reaction was started by the addition of protein kinase (5 μg/mL).

[2] With citrate, 2.0 mM Ca^{2+} was present in the pre-incubation system.

rate by 80%, 15%, and 30%, respectively; that 1.0 mM ADP and 1.0 mM citrate decrease the rate by 30% and 40%, respectively; and that 10 mM phosphate has no effect. These results agree with earlier findings of Kemp *et al.* (1981). Clearly, fructose-2,6-bisphosphate, AMP, and phosphate influence the phosphorylation in the presence of troponin C by effecting the enzyme-protein interaction. ADP and citrate may effect 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 III-3 and Figure III-4A show the separation of the CNBr fragments which were prepared from enzyme samples which had been subjected to two hours of phosphorylation. In the case of the control (Fig III-3A), 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 (Fig. III-3B and 3C). 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.

Figure III-3: 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 which had been pre-incubated with calmodulin or troponin C before phosphorylation while panel A illustrates the results determined with the control, which contained no calmodulin or troponin C. The radioactivity peak at the injection mark (0% acetonitrile) corresponds to residual ATP while the phospho-peptide fractions elute at 35% and 53% acetonitrile. The dashed line represents the gradient profile. Other experimental details are supplied in the text.

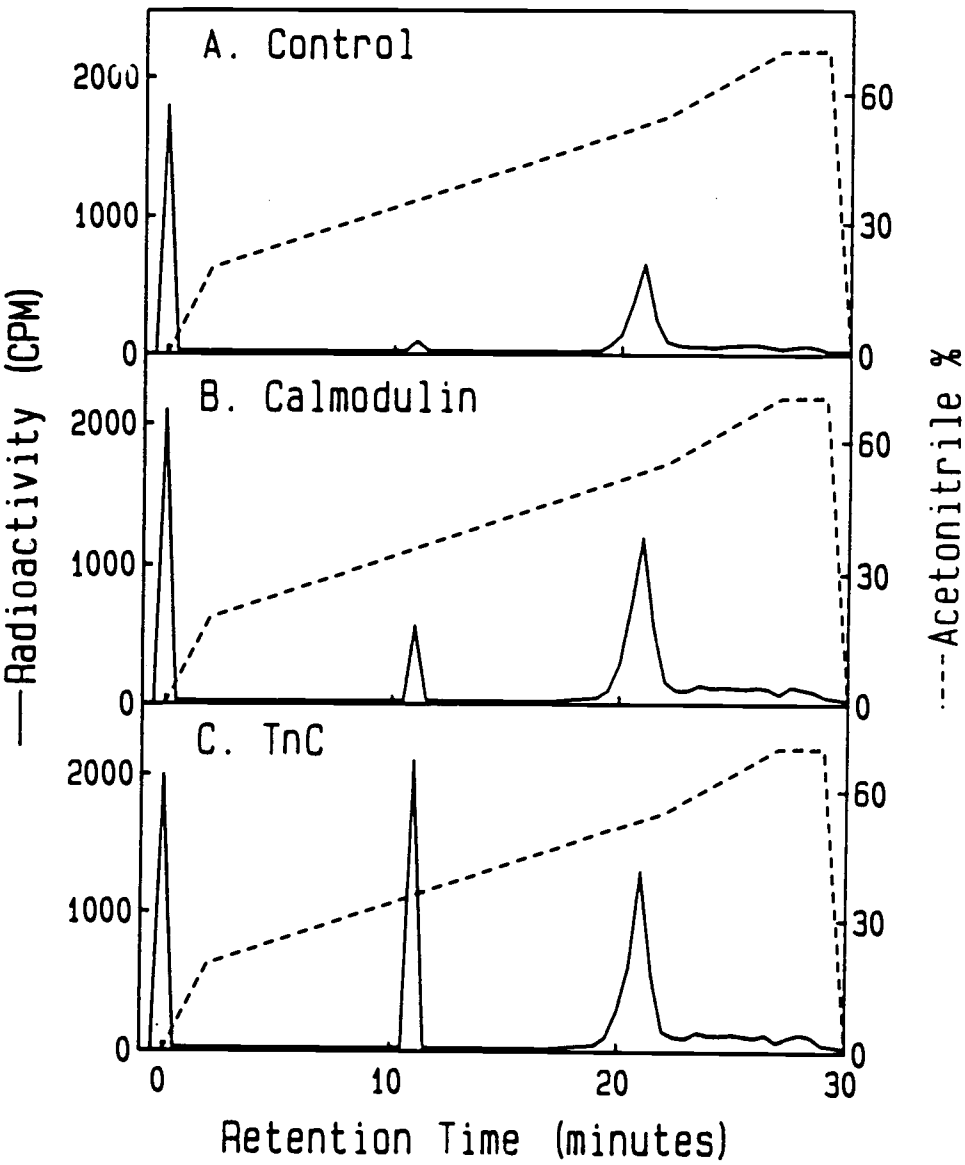

A. Control
B. Calmodulin
C. TnC
2000
1000
0
60
30
0
10
20
30
—Radioactivity (CPM)
---Acetonitrile %
Retention Time (minutes)

Figure III-3

Figure III-4: Reverse phase HPLC peptide mapping of phosphofructokinase fragments. Phosphofructokinase was subjected to two hours of phosphorylation catalyzed by cAMP-dependent protein kinase in the presence of troponin C plus calcium. (A) CNBr digestion of the enzyme prepared as described in Materials and Methods. (B) V8 protease digestion of the radioactive fraction eluted at 53% acetonitrile shown in (A). The absorbance was monitored at 220 nm. The same running phase gradient used in Figure III-3 was applied here.

* indicates the radioactive peak.

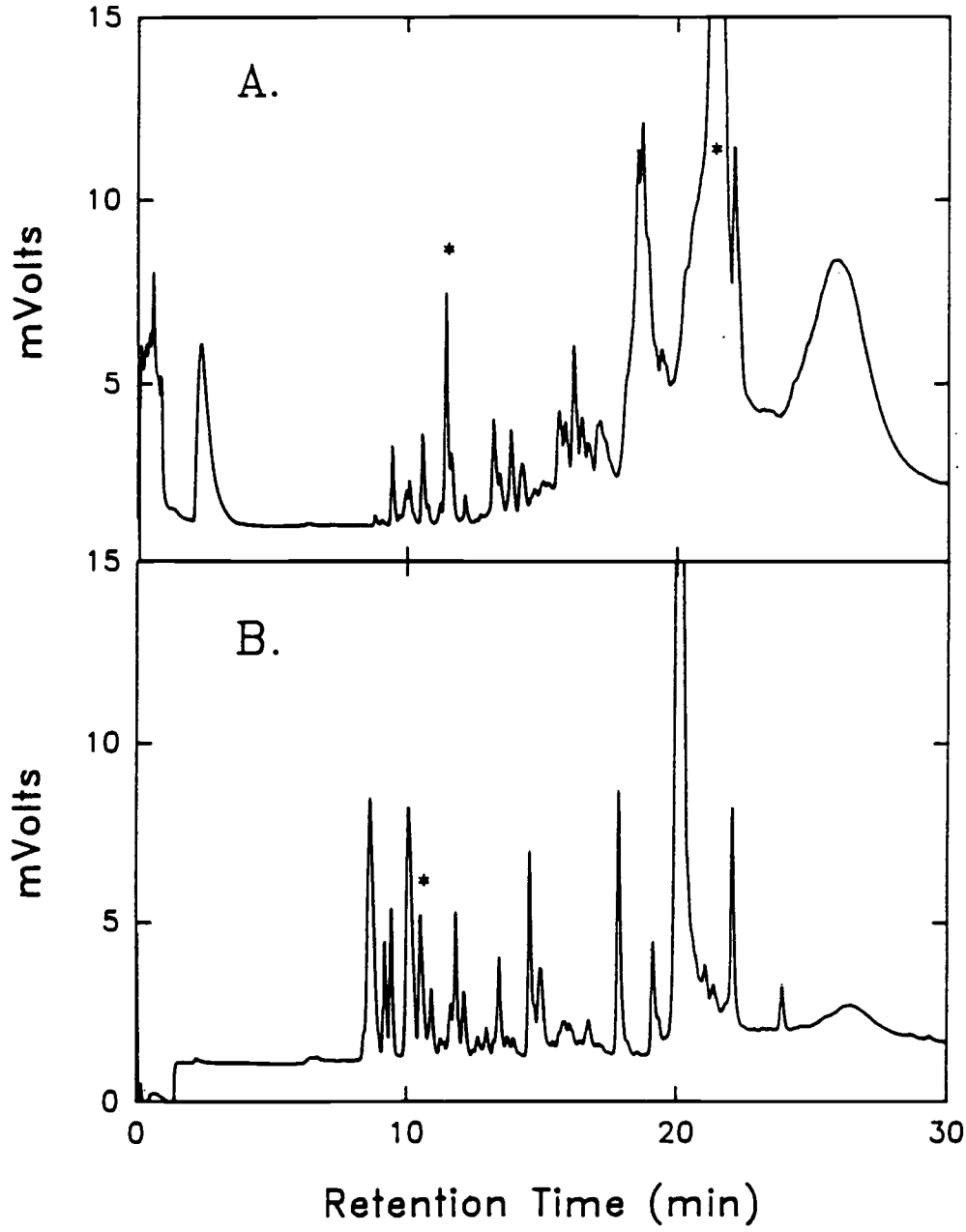


Figure III-4

Isolation and Sequencing of the Phosphorylated Fragments of Phosphofructokinase

The phospho-peptide 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 phospho-peptide. The second peak (eluting at 53% acetonitrile) is a heterogenous 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 by reverse-phase HPLC. Only one radioactive peak was detected, as shown in Fig III-4B and Fig. III-5A. This fraction was further purified on a TSK SP cation exchange HPLC column, with the phospho-peptide eluting at 0.43 M NaCl (Fig. III-5B). This fraction then was reloaded on the C18 column in order to remove salt and minor contaminating peptides. About 0.5 nmoles of each of this purified peptide (designated as the V8 phospho-peptide) and the CNBr phospho-peptide 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 background of PTH-amino acids. The initial pentapeptide sequences of the two fragments proved to be:

CNBr phospho-peptide: Lys-Leu-Arg-Gly-Arg

V8 phospho-peptide: His-Ile-Ser-Arg-Lys

Figure III-5: Isolation of the C-terminal phospho-peptide. The second radioactive peptide fraction obtained by CNBr cleavage (see Figure III-3) was further digested by treatment with the V8 protease. The resulting phospho-peptide was purified by application of the digest to a C18 reverse-phase HPLC column (A) followed by final fractionation on a TSK-SP cation exchange column (B). Other details are provided in the text.

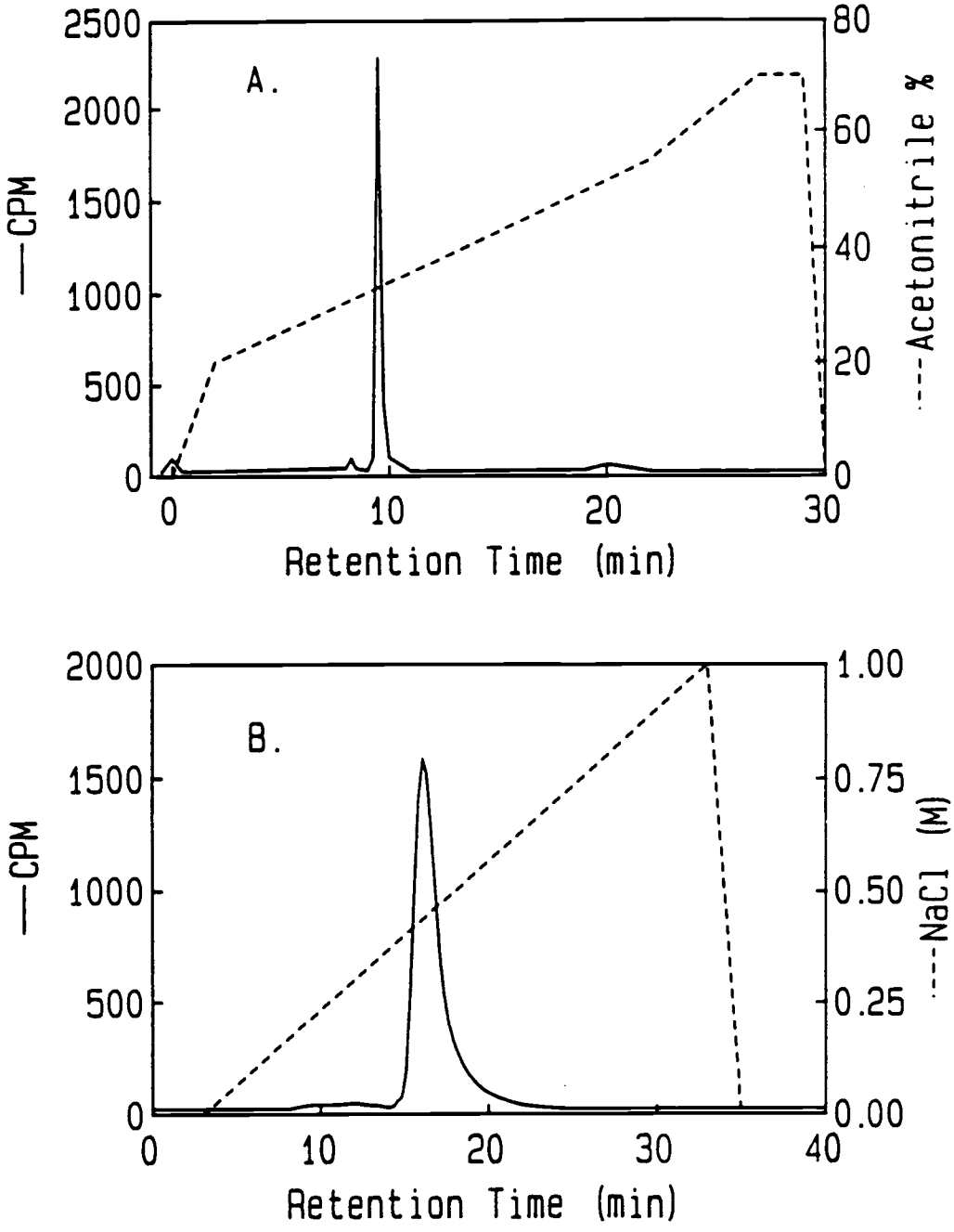

A.
2500
2000
1500
1000
500
0
CPM
80
60
40
20
0
Acetonitrile %
0
10
20
30
Retention Time (min)
B.
2000
1500
1000
500
0
CPM
1.00
0.75
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
Retention Time (min)

Figure III-5

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 phospho-peptide must correspond to the sequence Lys-Leu-Arg-Gly-Arg-Ser-Phe-Met (representing amino acid residues 371-378) and the V8 phospho-peptide, to the sequence His-Ile-Ser-Arg-Lys-Arg-Ser-Gly-Glu (corresponding to positions 768-776). In the CNBr phospho-peptide, Ser^{376} is the only possible phosphorylation site. However, in the V8 phospho-peptide, there are two possible phosphorylation sites: Ser^{770} and Ser^{774}. To identify the exact site, the filter disc containing the V8 phospho-peptide was removed from the sequencer after 5 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 recovered. After concentration in the speed vacuum concentrator, the extract was fractionated by application to a reverse-phase HPLC C18 column and eluted as shown in Figure III-6. Note that the majority of the radioactivity appears at 28% acetonitrile, with the remainder coming out later. No radioactivity is found at the injection mark (0% acetonitrile), which is the elution position for inorganic phosphate -- which would be released if the phosphoserine had been hydrolyzed during the Edman degradation (Wang *et al.*, 1988). It is clear that all the phosphate remains on the peptide after 5 cycles of the Edman

Figure III-6: C18 reverse-phase HPLC profile of the C-terminal peptide eluted from the sequencer filter disc after passage through 5 cycles of the Edman degradation. Other details appear in text.

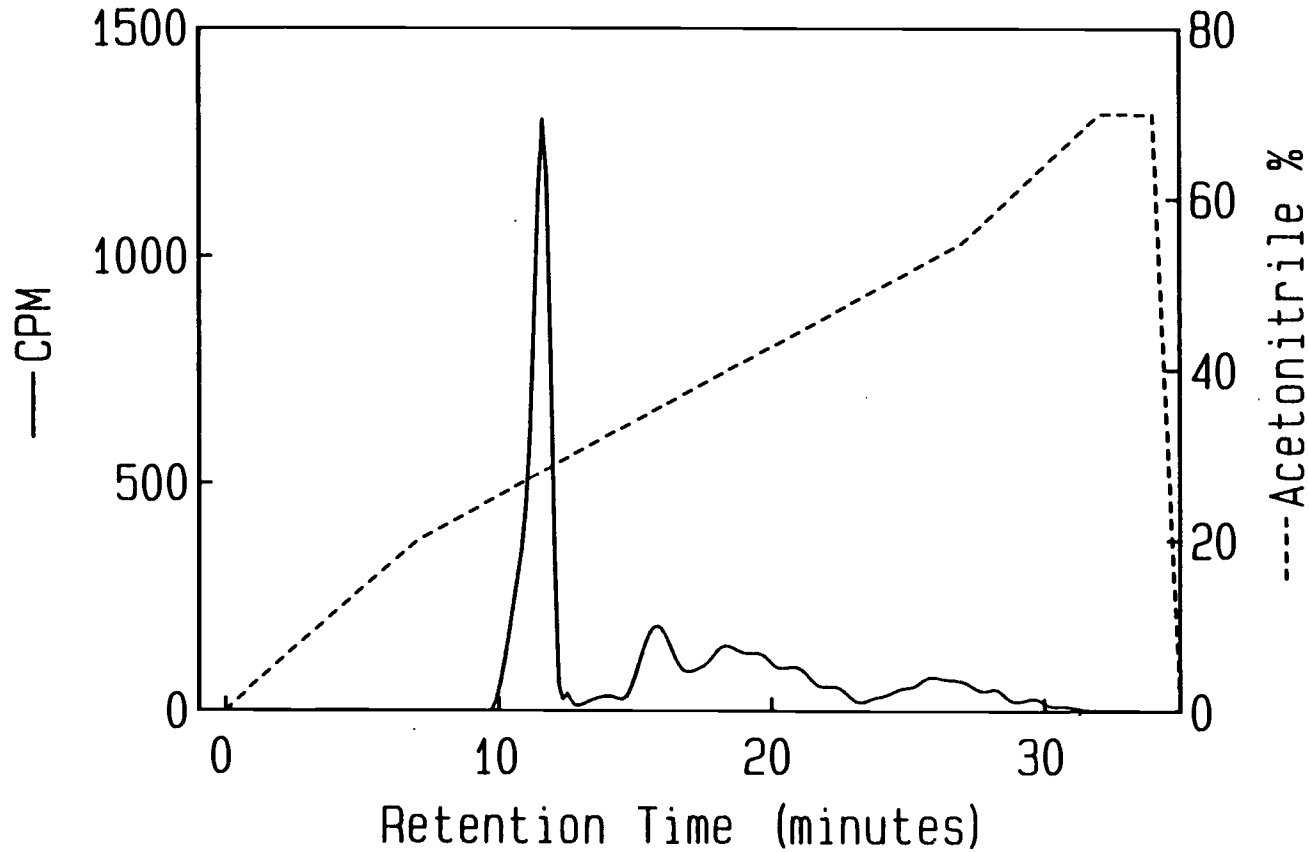

1500
1000
500
0
—CPM
80
60
40
20
0
---Acetonitrile %
0
10
20
30
Retention Time (minutes)

Figure III-6

degradation, demonstrating that the phosphorylation site on the V8 phospho-peptide is Ser^{774} -- the same 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 Fig. III-3 were applied to samples which had been removed from the phosphorylation mixture at various time intervals ranging from 5 min to 2 hrs. The radio-activities 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 is 80-85%. At least 95% of the applied radioactivity was eluted from the reverse-phase HPLC column. The phosphate content is calculated according to the radioactivities of each individual fraction and the total phosphate incorporation of the intact enzyme determined with scintillation counting on glass filter paper, based on the assumption that CNBr cleavage is complete and that the two phospho-peptides are equally recovered. The results in Fig. III-7 show that calmodulin

Figure III-7: 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 in Materials and Methods for Fig. III-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 (Δ , ▲), control (○ , ●).

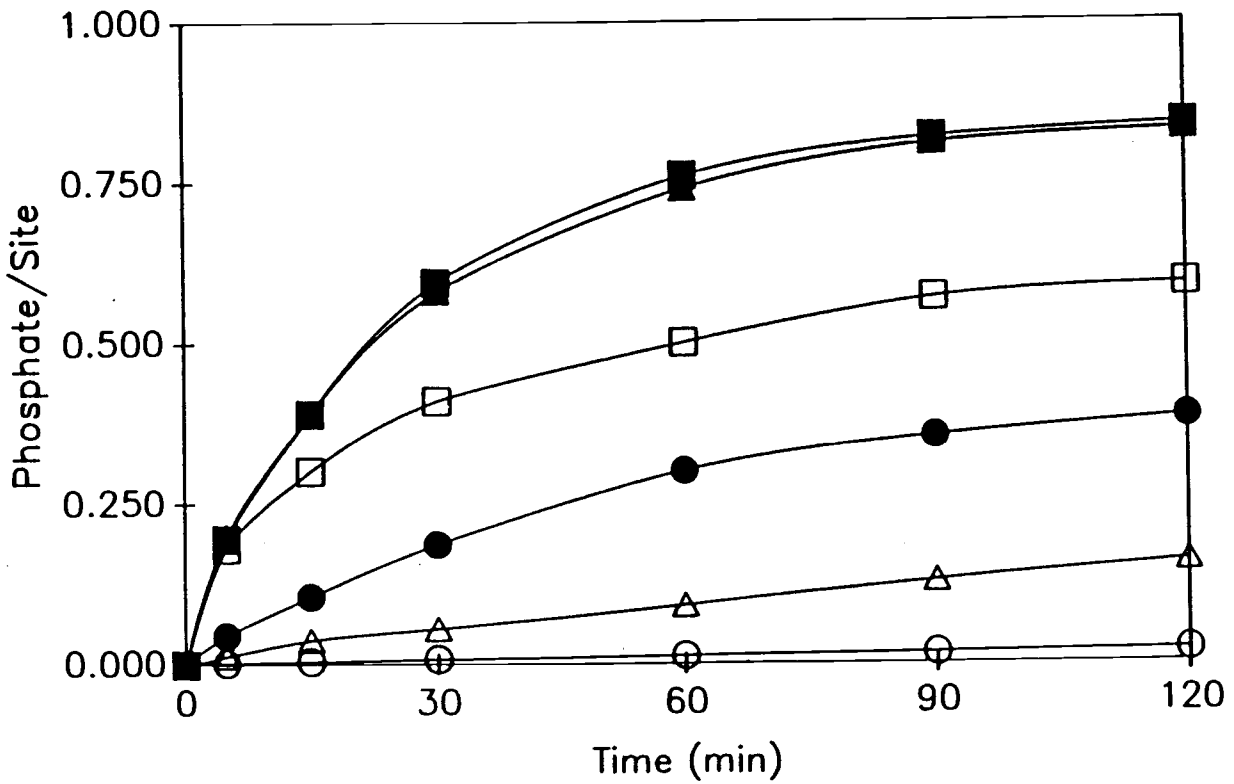

1.000
0.750
0.500
0.250
0.000
Phosphate/Site
0
30
60
90
120
Time (min)

Figure III-7

and troponin C enhance the rate and extent of phosphorylation of the C-terminal site about equally, with the incorporation of about 0.84 mol phosphate/site after 2 hours of reaction. In the absence of calmodulin or troponin C, the C-terminal site incorporates only 0.4 equivalents of ^{32}P -- a fractional stoichiometry which agrees with prevailing reports (Foe and Kemp, 1982; Sale and Denton, 1985; Domenech *et al.*, 1988). In contrast, the phosphorylation of Ser^{376} is extremely sensitive to varying conditions. The corresponding phosphate incorporation measured after two hours of incubation ranges from 0.02 mol/mol in the control experiment and up to 0.16 mol/mol in the presence of calmodulin and up to 0.59 mol/mol for enzyme samples containing added calmodulin or troponin C, respectively.

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 which had been subjected to phosphorylation for varying periods of time (Fig. III-8). In this case, calmodulin or troponin C was added immediately before initiation of phosphorylation. Note that, as anticipated

Figure III-8: 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 $MgCl_2$ (Δ), 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 hour of incubation.

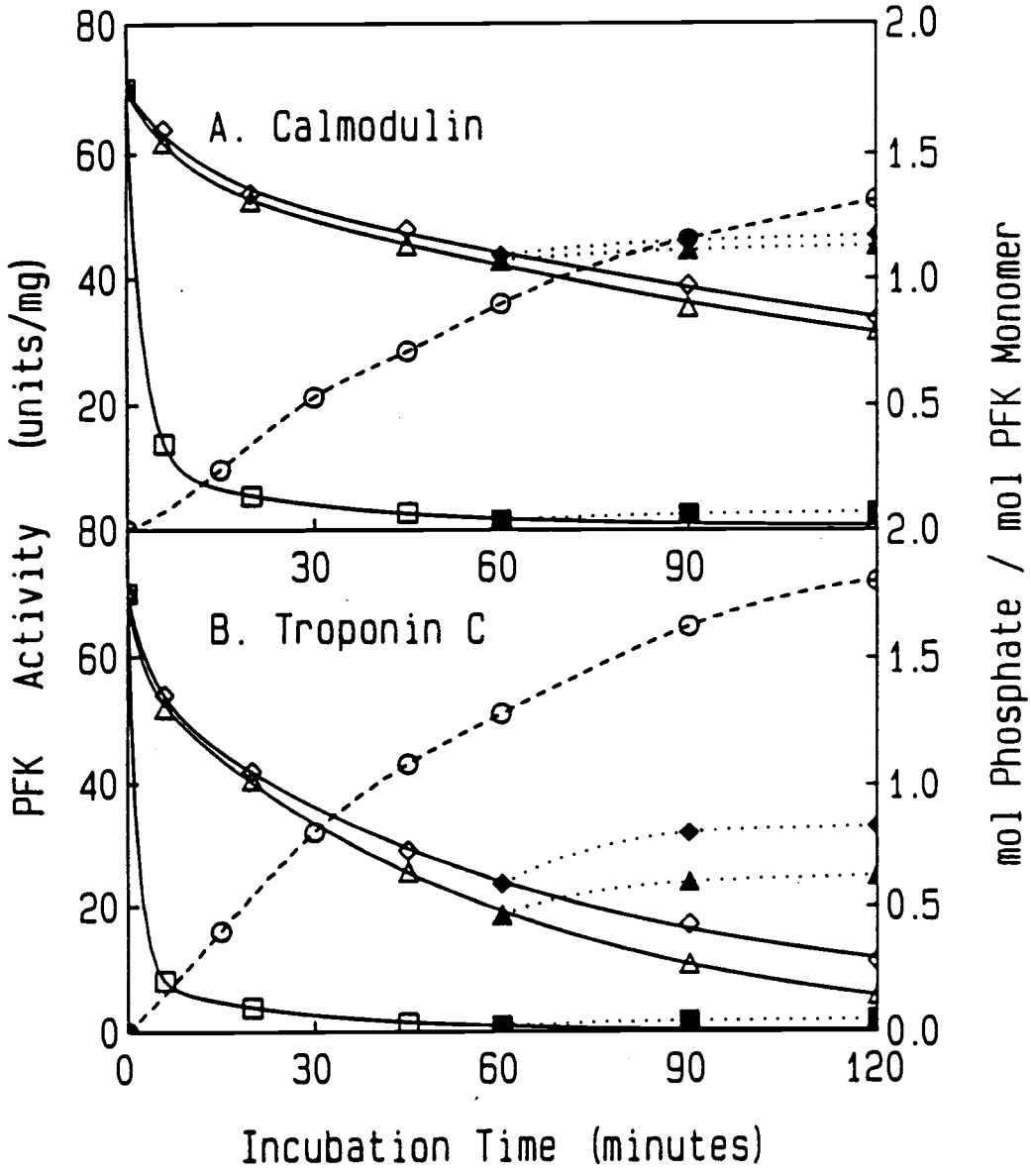

A. Calmodulin
B. Troponin C
PFK Activity (units/mg)
mol Phosphate / mol PFK Monomer
Incubation Time (minutes)
0
20
40
60
80
0.0
0.5
1.0
1.5
2.0
30
60
90
120

Figure III-8

from the results in the previous studies (Chapter II), 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.

Phosphorylation of Phosphofructokinase by cGMP-dependent Protein Kinase and by Protein Kinase-C

Table III-3 shows the extent of phosphorylation obtained in reactions catalyzed by cGMP-dependent protein kinase and by protein kinase-C. Note that 0.31 mol phosphate/mol enzyme protomer are incorporated after 2 hours 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 phosphate/mol enzyme protomer detected after a two hr incubation in either presence and absence of troponin C.

Table III-3

Phosphorylation of PFK by cGMP-Dependent Protein Kinase and by Protein kinase-C

Protein kinase	mol phosphate/mol 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

Phosphorylation was performed according to the description in Methods and Materials. The data in the table represent the mols of phosphate incorporated per mol enzyme protomer after 2 hours of reaction. All samples contained 1 mM $CaCl_2$.

Effects of Phosphorylation on Calmodulin and Troponin C Binding

In preliminary attempts to investigate the effect of phosphorylation on the interactions of phosphofructokinase with calmodulin and troponin C, we applied the urea gel electrophoresis system used in the preceding chapter (Chapter II) (Figure III-9). As shown for the untreated enzyme, phosphorylated phosphofructokinase (containing 1.2-1.5 mol phosphate/mol protomer) readily enter the stacking gel provided that either calmodulin or troponin C plus calcium are also present. Addition of ATP-$MgCl_2$ alone to the inactivation system did not show significant effects. The amounts of dissociated calmodulin detected in the resolving gel were indistinguishable in the two cases. A marginally greater dissociation (15%) of troponin C was found with the phosphorylated enzyme than with the control. The difference between calmodulin and troponin C may be due to the different phosphorylation of phosphofructokinase in the middle site induced by the two calcium-binding proteins.

In addition, we performed fluorescence titrations with dansylcalmodulin (cf. Malencik and Anderson, 1982; Malencik *et al.*, 1982). The fluorescence enhancement attained with the phosphorylated enzyme differs only slightly from that found with the control. Since dilution to the nanomolar concentration range (which is at our sensitivity limit)

proved necessary to obtain appreciable dissociation, we were unable to determine equilibrium constants or to distinguish between the two forms of the enzyme (data also not shown).

Figure III-9: Electrophoretic study of the interaction of phosphorylated phosphofructokinase with calmodulin and troponin C. Photograph (A) shows the separation obtained with a 12.5% urea gel layered with 6% stacking gel. The phosphorylation reaction was described in Materials and Methods. (B) shows the resolved calmodulin or troponin C band intensity obtained by scanning the original gel horizontally. Data above each peak represent the relative peak area. 0, PFK control; 1, calmodulin control; 2, PFK + calmodulin; 3, PFK + calmodulin + ATP-Mg^{2+}; 4, PFK + calmodulin + phosphorylation; 5, TnC control; 6, PFK + TnC; 7, PFK + TnC + ATP-Mg^{2+}; 8, PFK + TnC + phosphorylation. The polyacrylamide gel containing 6 M urea was stained with Coomassie blue G-250 reagent.

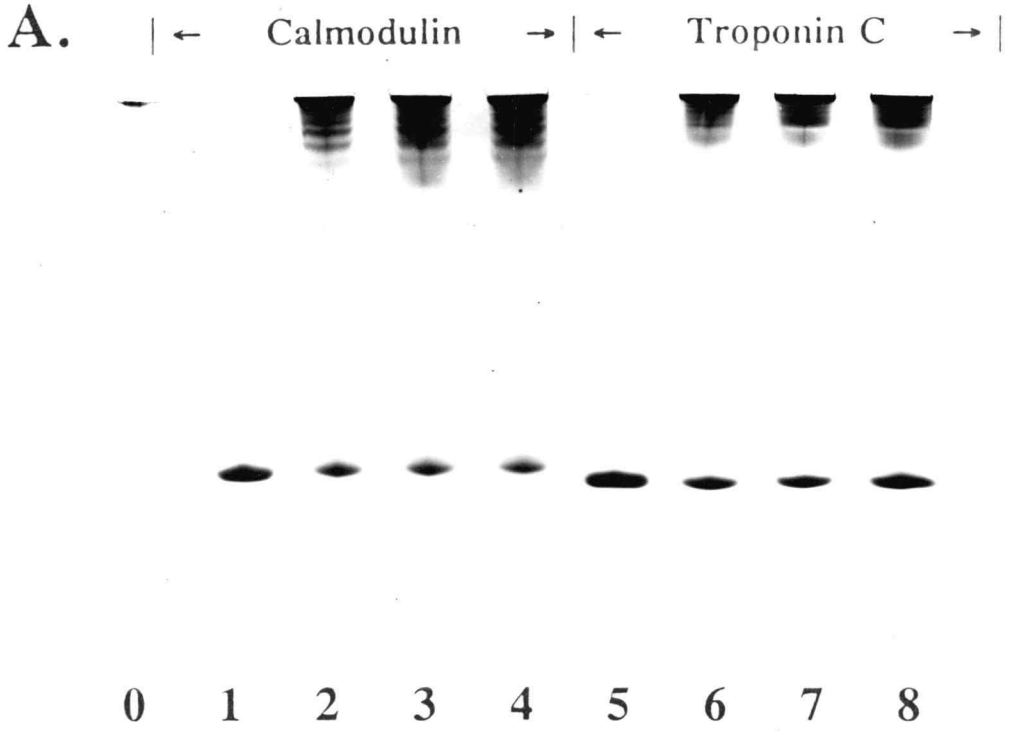

A.
Calmodulin
Troponin C
0 1 2 3 4 5 6 7 8
B.
Calmodulin
TnC
1.0
0.54
0.51
0.49
1.0
0.71
0.79
0.94
Relative Intensity
10
5
0
1 2 3 4 5 6 7 8
Calmodulin and TnC Bands

Figure III-9

Discussion

When calcium is present, the addition of either calmodulin or troponin C significantly increases both the rate and extent of *in vitro* phosphorylation of rabbit muscle phosphofructokinase catalyzed by the cAMP-dependent protein kinase. The dependence of the phosphorylation on the pre-incubation of phosphofructokinase with troponin C or calmodulin and the influence of enzyme effectors demonstrate that these proteins modulate the phosphorylation through a calcium-dependent interaction with phosphofructokinase rather than with the protein kinase. Calmodulin and troponin C increase the rate and extent of phosphorylation occurring at the C-terminal site (Ser^{774}) and, in addition, induce phosphorylation at a newly discovered site in the middle of the enzyme molecule (Ser^{376}). This result suggests a possible incorporation 2 mols phosphate/mol enzyme protomer. The maximum radiolabeling attained in our work represents 1.85 mol phosphate/mol enzyme protomer (cf. Fig. III-8B). Since the endogenous phosphate content of our purified phosphofructokinase is approximately 0.15 mols phosphate/mol protomer, the total amount of phosphate in the reacted enzyme corresponds closely to that expected.

Although calmodulin and troponin C have similar effects on both the rate and extent of phosphorylation occurring at the C-terminal site (Ser^{774}) of phosphofructokinase, they

have significantly different effects on the phosphorylation of Ser^{376}. Our measurements (Fig. III-7) show that troponin C is much more effective than calmodulin in promoting the phosphorylation of the latter site, which occurs in the hinge region connecting the N- and C-terminal domain of the enzyme model proposed by Poorman *et al.* (1984). Two specific calmodulin-binding sites in phosphofructokinase were identified by Buschmeier *et al* (1987), who prepared calmodulin-binding fragments M13 (comprising Gln^{379}-Met^{405}) and M22 (corresponding to Arg^{713}-Val^{779}). Interestingly, the interaction sites represented by these fragments are in close proximity to the phosphorylation sites identified by us. This observation 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 effect each other (Malencik and Anderson, 1982). Since previous work suggests that calmodulin and troponin C interact similarly with rabbit skeletal muscle phosphofructokinase (Chapter II), we believe that the binding sites for the two proteins are either identical or closely overlapping. Note, that the effect of calmodulin on the phosphorylation of phosphofructokinase is different from that found with chicken gizzard myosin light chain kinase (Conti and Adelstein, 1981) or with rabbit skeletal muscle phosphorylase kinase (Cox and Edstrom, 1981). In the later cases, the binding of calmodulin

prevents phosphorylation. All three examples suggest that one aspect of the regulation of protein kinase activity involves changes in the status of the target protein rather than of the kinase itself.

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 III-4). This includes a coincidence of serine residues and of two neighboring arginine residues, reflecting a common theme observed in phosphorylation sequences recognized by cAMP-dependent protein kinase (cf. review by Krebs & Beavo, 1979). The highly conserved character of this sequence suggests physiological relevance. 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 liver and rabbit skeletal muscle phosphofructokinase (Kemp *et al.*, 1981; Pilkis *et al.*, 1982; Valaitis *et al.*, 1989).

The existence of two phosphorylation sites in 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

Table III-4

Sequence Comparisons of Potential Phosphorylation Sites in Rabbit Muscle[1] and Mouse Liver[2] 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

S represent the affected serine in the phosphorylation site.

: indicates amino acid homology.

References:

[1] Lee *et al.* (1987)

[2] Gehnrich *et al.* (1988)

catalyzes the phosphorylation of rabbit skeletal muscle phosphofructokinase, both at Ser^{774} and at one or more unidentified additional sites. Enzyme activation, reflecting a decrease in the K_m for fructose-6-phosphate, occurred concomitantly. However, our studies show that 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 phosphate/mol protomer. This result is consistent with that reported by Mieskes *et al.* (1987). In addition, the presence of calmodulin or troponin C made no difference in the phosphorylation obtained with protein kinase-C. We note that a much less homogenous preparation of protein kinase-C was employed by Hofer *et al.* (1985). Our results 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 calcium-dependent phosphorylation of phosphofructokinase is consistent with the abundance of troponin C in skeletal muscle and with the finding that electrical stimulation of muscle increase the average phosphate content of the enzyme (Hofer and Sorensen-Ziganke, 1979). However, many questions about the role of phosphorylation in the regulation of phosphofructokinase remain unanswered.

Unfortunately, major irreversible losses of activity occur during the time periods required to approach maximum phosphorylation. However, preliminary measurements (Fig. III-8) suggest that phosphorylation *per se* results in only modest changes in the catalytic activity determined under standard optimal assay conditions. In addition, urea gel electrophoresis experiments and fluorescence titration of dansylcalmodulin (not shown) indicate that the fully phosphorylated phosphofructokinase retains high affinity for calmodulin and troponin C. We believe that additional factors -- possibly involving other proteins known to bind the enzyme, such as F-actin (cf. Arnold and Pette, 1968; Clarke and Masters, 1976; Liou and Anderson, 1980) or myosin (Freidina *et al.*, 1987) -- will need to be explored in order to fully understand the physiological significance of the phosphorylation induced by calmodulin and troponin C.

Chapter IV

Reversible Inactivation of Phosphofructokinase in the Presence of Nucleic Acids

Abstract

An inactivator of rabbit muscle phosphofructokinase has been partially purified from rabbit liver and identified as a mixture of RNA species. Studies show that a variety of nucleic acids -- including samples of pure tRNA, rRNA, and DNA -- can promote the inactivation of phosphofructokinase. This process is affected by a number of ligands of the enzyme. The substrates (ATP and fructose 6-phosphate) and allosteric effectors -- such as fructose 2,6-bisphosphate, ADP, AMP, citrate, phosphate, ammonium, and pyrophosphate -- all exert stabilizing effects. A nucleic acid-binding protein -- protamine -- also protects the enzyme from inactivation. The activity losses can be reversed by the addition of ATP, fructose 2,6-bisphosphate, phosphate, ADP, and AMP. Release of DNA from the enzyme complex by DNase I digestion leads to the aggregation of the enzyme and permanent loss of enzymatic activity.

According to the results of glycerol gradient centrifugation and gel filtration studies, the mechanism of the inactivation involves the dissociation of

phosphofructokinase which results from the preferential binding of nucleic acids to its inactive dimer. Both kinetic and fluorescence measurements suggest that the enzyme-nucleic acid interaction is rapid and that the binding affinity is relatively low. The presence of the enzyme also exerts certain effects on the nucleic acids. It retards the digestion of salmon DNA by DNase I and protects a certain fraction of chromatin core particle DNA from digestion by micrococcal nuclease. The sequence of phosphofructokinase contains a potential helical region near its C-terminal end which closely resembles a structural motif seen in DNA binding proteins -- the leucine zipper.

Introduction

Phosphofructokinase undergoes reversible inactivation in the presence of a number of acidic proteins which include the purified erythrocyte membrane band 3 protein (Jenkins *et al.*, 1985), the Zn^{2+}-dependent inactivating protein purified from rat liver (Brand and Söling, 1986), calmodulin (Mayr 1984a,b; also cf. Chapter II), and troponin C (cf. Chapter II). The general mechanism for the protein-induced inactivation involves the dissociation of the active tetramer of phosphofructokinase to the inactive dimer, as suggested by several authors (Jenkins *et al.*, 1985; Brand and Söling, 1986; Mayr 1984a,b) and elucidated in Chapter II of this thesis. In attempts to purify a Zn^{2+}-dependent inactivating protein from rabbit liver, we obtained an RNA-like phosphofructokinase inactivator. Later, we found that the inactivation is relatively nonspecific and that RNA and DNA have similar effects on the enzyme. This Chapter describes both the partial purification and characterization of the phosphofructokinase inactivator from rabbit liver and the results of studies on the general interaction between rabbit skeletal muscle phosphofructokinase and nucleic acids.

Materials and Methods

Materials

Rabbit liver tRNA, yeast tRNA, salmon testes DNA, bovine pancreas ribonuclease A, and micrococcal nuclease were purchased from Sigma Chemical Company. Bovine pancreas deoxyribonuclease I was from Millipore Corporation. E. coli 23S rRNA and single strand DNA probe 520 were supplied by Drs. Henry Schaup and Mirza Al-Mehdi. Chicken chromatin core particle DNA was supplied by Dr. Feng Dong. Oligo-DNA (12 BP) was a gift from Dr. Yeong Wang. Rabbit skeletal muscle phosphofructokinase was purified and assayed according to Kuo *et al.* (1986).

Purification and Identification of PFK-inactivating RNA from Rabbit Liver

100 g frozen rabbit liver was homogenized in a blender with three volumes of buffer containing 25 mM Tris-HCl (pH 7.5) , 2 mM ß-mercaptoethanol, and 1 mM EDTA (extracting buffer). The resulting crude extract was centrifuged at 24,000g. The turbid supernatant obtained was boiled for 5 minutes and clarified by centrifugation at 4 °C. HCl was added to this yellowish solution to give a concentration of 0.1 M. The precipitate was collected by centrifugation and

dissolved in 50 mL of extracting buffer. After adjustment of the pH to 7.5 with Trizma base, the solution was loaded on to a DEAE 52 column which have been equilibrated with the extracting buffer. The column was washed with five volume of 0.2 M NaCl in the same buffer and then eluted with 0.5 M NaCl. The inactivator-containing fractions were concentrated over Amicon filter YM2, dialyzed against water overnight, and finally lyophilized.

Nucleic acid ribose was determined by the orcinol reaction (Cooper 1978). Phosphate content was determined according to the procedure described by Hasegawa et al. (1982) for protein bound phosphate, except that the trichloroacetic acid precipitation step was eliminated. Deoxyribose were determined by the diphenylamine reaction according to Burton (1956).

Inactivation of Phosphofructokinase by RNA and DNA

As described for the two step inactivation studies performed with calmodulin and troponin C (Chapter II), samples of phosphofructokinase (0.04 mg/mL in a buffer containing 50 mM Mops-KOH (pH 7.0), 5.0 mM ß-mercapto-ethanol, 1.0 mM dithiothreitol, and 50% glycerol) were mixed with equal volumes of RNA or DNA solution dissolved in water alone. Control experiments involved dilution of the enzyme with water. At the designated times, 10-25 µL samples of

the mixtures were removed for activity measurements under optimal conditions (Chapter II). Factors affecting the inactivation -- such as allosteric ligands and protamine -- were added to the inactivating system prior to mixing with phosphofructokinase. The incubations were usually performed for 10 minutes at room temperature. The reactivation of phosphofructokinase was carried out through the addition of a minimal volume of the indicated activating reagents.

Glycerol Gradient Centrifugation and Gel Filtration

Phosphofructokinase (0.2 mg/mL) was incubated with 4.0 A_{260} units/mL of the liver RNA fraction in a buffer containing 25 mM Mops-KOH (pH 7.0) and 1.0 mM dithiothreitol. After half hour pre-incubation periods, the mixtures were subject to glycerol gradient centrifugation and column gel filtration. A 0.2 ml sample of the mixture was applied to a 12 mL glycerol gradient (10 - 25%) solution made up in the preceding buffer. Ultracentrifugation was performed for 24 hours at 35,000rpm in a Beckman Model L8-B ultracentrifuge equipped with SW40TI rotor. The gradients were fractionated from the bottom into 0.5 mL samples. Gel filtration was accomplished by application of a 2 mL aliquots of the enzyme-RNA mixture to a 90 cm X 1.6 cm Sephacryl S-300 column equilibrated with 25 mM Tris-HCl (pH 7.0), 1.0 mM EDTA, and 1.0 mM dithiothreitol. The column

elution rate was 30 mL/hour. The absorbance of each fraction was monitored at 260 nm. The enzymatic activities of each fraction of the RNA-treated enzyme were assayed after a one hour pre-incubation with 1.0 mM ATP. The enzyme activities of the control enzyme samples were assayed directly without pre-incubation with ATP.

Fluorescence Experiment

Dansylcalmodulin was prepared according to Malencik and Anderson (1982). The incubation of phosphofructokinase (0.5 μM in protomer), dansylcalmodulin (0.5 μM), and salmon DNA (0.01 mg/mL, 0.2 A_{260} units/mL) was carried out in a buffer containing 25 mM Mops-KOH (pH 7.0), 1.0 mM dithiothreitol, 1.0 mM $CaCl_2$, and 10% glycerol. Two different orders of mixing were used: 1) addition of DNA to enzyme which had been pre-incubated for 10 min with dansylcalmodulin; 2) addition of dansylcalmodulin to enzyme which had been pre-incubated for 10 min with DNA. The time courses of the fluorescence intensity changes of dansylcalmodulin were recorded with a Hitachi Perkin-Elmer MPF2A fluorometer using fixed excitation and emission wavelengths of 340 and 460 nm, respectively.

Nuclease Digestion of DNA

DNase I activity was assayed spectrophotometrically at 260 nm according to the method of Hitchcock *et al.* (1977). Different concentrations of phosphofructokinase (0 - 0.2 mg/mL) were pre-incubated with 0.5 A_{260} units/mL of salmon DNA for 10 minutes in a buffer which contains 25 mM Tris-HCL (pH 7.0), 1.0 mM $MgCl_2$, and 0.1 mM $CaCl_2$. The reaction was started by the addition of DNase I to a final concentration of 0.2 μg/mL. For the study of the DNase I-induced aggregation of the enzyme, the reaction was performed at 36 °C. Room temperature (24-25 °C) was used for all the other incubations. The aggregation process was monitored spectrophotometrically at 550 nm (Pandian *et al.*, 1983).

To determine whether phosphofructokinase protects DNA from nuclease digestion, the enzyme (0 or 1.0 mg/mL) was pre-incubated for 10 minutes with chicken chromatin core particle DNA (0.1 mg/mL, 2 A_{260} units/mL) dissolved in a buffer containing 50 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. Then micrococcal nuclease was added to 0.25 units/mL. After specified periods of time, samples were removed and the digestion were stopped by adding 10 mM EDTA. 2 μL aliquots of each sample were loaded on 4% DNA gel.

Gel Electrophoresis

10% and 15% RNA gel were made up by using proper proportions of 30% acrylamide and 1.5% bis-acrylamide in a buffer containing 50 mM Tris-40 mM boric acid (pH 8.3), 1.0 mM EDTA, and 6.6 M urea. Running buffer is the same composition except the absence of urea. The gels were stained with Stainsall solution (Donis-Keller, 1979).

4% DNA gel was prepared from the same acrylamide/bis-acrylamide (30:1.5) and buffer as used for the RNA gels except the absence of urea in the gel buffer. Running buffer has the same composition. After electrophoresis, the gel was stained with ethidium bromide and viewed under UV light (Ausio *et al.*, 1987).

Results

Purification and Identification of PFK-inactivating RNA from Liver

In the procedure originally developed by Brand and Soling *et al.* (1986) for the purification of the Zn^{2+}-dependent inactivator of phosphofructokinase from rat liver, the liver was homogenized with 2 volumes of buffer containing 20 mM triethanolamine-HCl (pH 7.5) plus several protease inhibitors. This is followed by: (1) precipitation of the inactivator at pH to 5.0; (2) passage through a Sephadex G-50 column to remove low molecular substances; (3) fractionation on DEAE-52; (4) ammonium sulfate precipitation; (5) separation on phenyl-Sepharose column; (6) final purification with an FPLC anion exchange column. In attempted purification of a rabbit liver inactivator according to the above procedure, we could not detect inactivating activity until after the DEAE-52 fractionation. No further purification was obtained with subsequent steps. Moreover, the activity is heat-stable and protease resistant. With this information in mind, we simplified the procedure by introducing the heat treatment and acid precipitation steps as described in Methods and Materials. With the modified methods, 1650 A_{260} units (around 36 mg) of the inactivator was usually obtained from

100 grams of rabbit liver. The inactivating activity is detectable after heat treatment and is amplified with further purification possibly because of the removal of contamination proteins. Table IV-1 summarizes the purification. The final fraction is almost free from proteins as determined by using either the Bradford method (Bradford, 1976) or the silver staining method (Krystal *et al.*, 1985). It also displays the UV absorption characteristics of a nucleic acid (Figure IV-1). Table IV-2 shows that its inactivating ability is resistant to protease and DNase digestion but is sensitive to RNase A digestion. It contains ribose but little deoxyribose. Its ribose and phosphate contents, relative to its absorbance at 260 nm, are close to those expected for RNA. RNA electrophoresis gels (Figure IV-2) indicates that it is a heterogenous mixture of RNAs with a size below 60 bases. RNA sequencing from the 3'-end accomplished by Dr. Henry Schaup and his group gave the following partial sequence for the major component of the liver RNA fraction:

5'GAGACCCCACCUU(U/G)GGUUUGACUGUAAU-----GUAAGUAAUUAGCA3'

in which the "/" means either or and "-" represent a blank zone on the gel where a band was expected. A few bases on both 5'- and 3'-end were not identified. Nucleic acid sequence analysis employing Bionette genebank database computer software indicates that this is a ribonuclease resistant fragment.

Table IV-1

Purification of Phosphofructokinase Inactivator

Steps	Volume (ml)	Absorbance A_{260}[1]	Activity Units[2]	Activity units/A_{260}
Crude	290	35	0	0
Heat Treatment	230	23	26,450	5
HCl Precipitation	150	22.2	40,000	12
DE-52 + Dialysis	20	82.5	54,450	33

[1] Absorbance was measured with 1.0 cm path.

[2] One unit is defined as the activity which leads to the loss of 1 unit of phosphofructokinase activity within one minutes in the incubation condition as described in Materials and Methods.

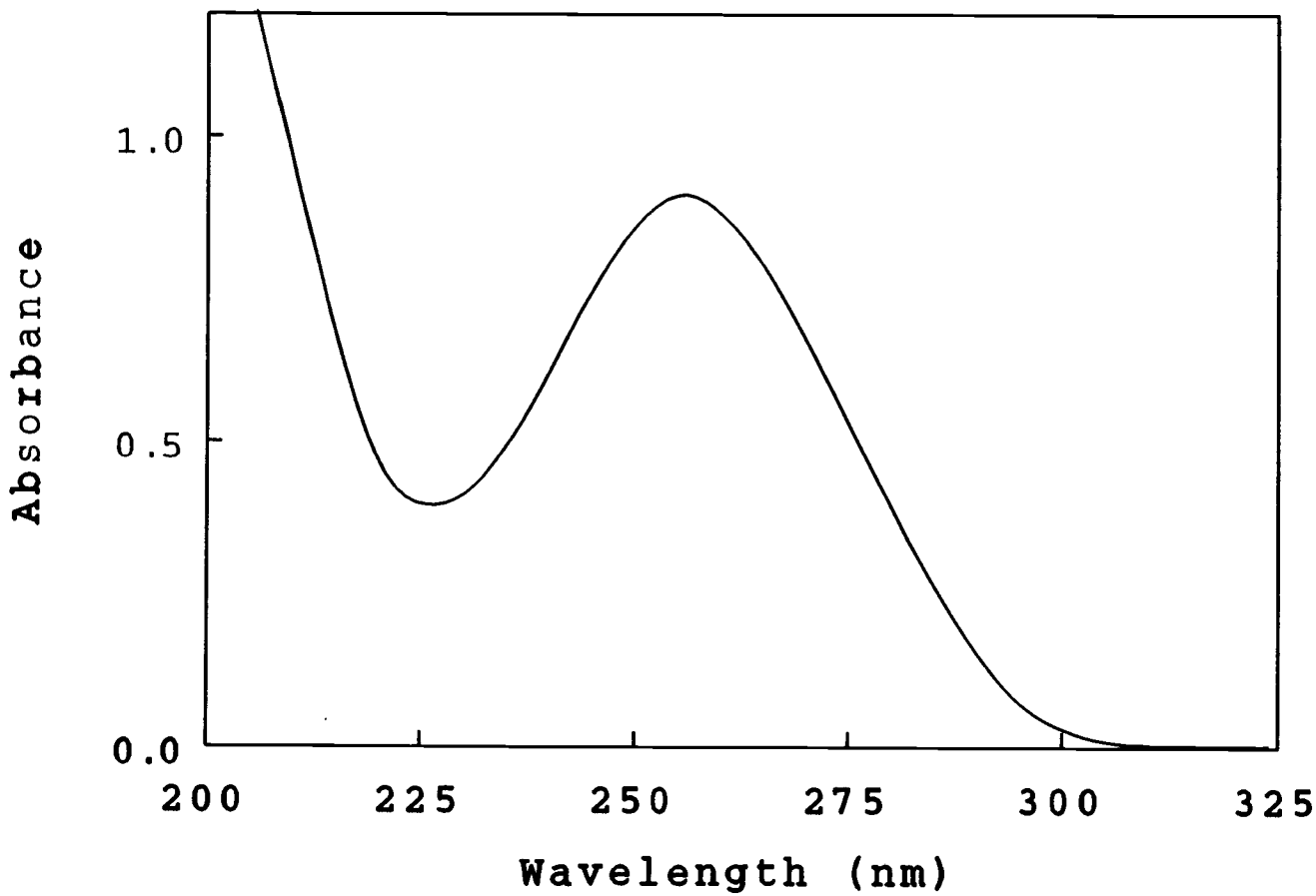


Figure IV-1: UV spectrum of the partially purified liver RNA fraction

Table IV-2

Properties of the Purified PFK Inactivator from Rabbit Liver

Activity	Reversible inactivation of phosphofructokinase
UV absorbance	maximum at 256 nm, A_{280}/A_{260} = 0.44; A_{230}/A_{260} = 0.47
phosphate	0.14 ± 0.02 μmole/A_{260} unit
ribose	0.12 ± 0.01 μmol/A_{260} unit
deoxyribose	≤ 5 nmol/A_{260} unit
Trypsin treatment	no effect
DNase I treatment	no effect
RNase A treatment	loss of inactivating activity
Size	10-60 bases

Figure IV-2: RNA gel electrophoreses of the partially purified rabbit liver RNA fraction. The 10% (A) and 15% (B) RNA gels were made up by using the proper proportions of 30% acrylamide and 1.5% bis-acrylamide in a buffer containing 50 mM Tris-40 mM boric acid (pH 8.3), 1.0 mM EDTA, and 6.6 M urea. Running buffer is of the same composition except for the absence of urea. The gels were stained with Stainsall solution. Lane 1, partially purified liver RNA fraction; lane 2, yeast tRNA.

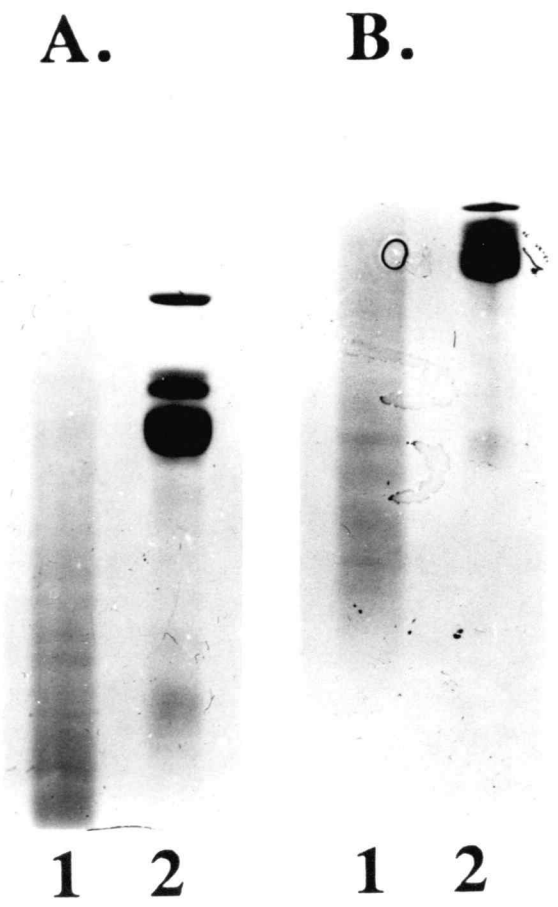


Figure IV-2

Inactivation of Phosphofructokinase by Nucleic Acid

Figure IV-3 shows the inactivation of phosphofructokinase induced by the partially purified liver RNA fraction. Note that the extent of inactivation increases almost linearly with increasing concentrations of RNA up to A_{260} = 0.02. Higher concentrations have little additional effect, with 35% of the original enzyme activity persisting at A_{260} = 0.08. The inactivation rate observed with the partially purified RNA is much faster than that induced by calmodulin or troponin C (Chapter II). The reaction reaches an apparent equilibrium within 2 minutes under the conditions used in this study (inset to Figure IV-3). Several other nucleic acid samples -- such as salmon testis DNA, chromatin core particle DNA, rabbit liver tRNA, and E. coli 23S rRNA -- have generally similar inactivating properties (Table IV-3). The values of both A_{max} (maximum extent of inactivation) and $C_{½}$ (concentration for half maximum inactivation) indicate that 23S rRNA has a distinctly higher inactivating ability than tRNA and DNA. The relative inabilities of the short double stranded oligo-DNA and the single stranded DNA probe to inactivate phosphofructokinase suggests a requirement for minimum nucleic acid size.

Figure IV-3: Inactivation of phosphofructokinase by the rabbit liver RNA fraction. Phosphofructokinase (0.02 mg/mL) was incubated with different concentrations of RNA under conditions described in Materials and Methods. The residual enzymatic activity was measured after 10 minutes of incubation. The inset shows the time course of inactivation obtained with 0.04 A_{260} units/mL RNA.

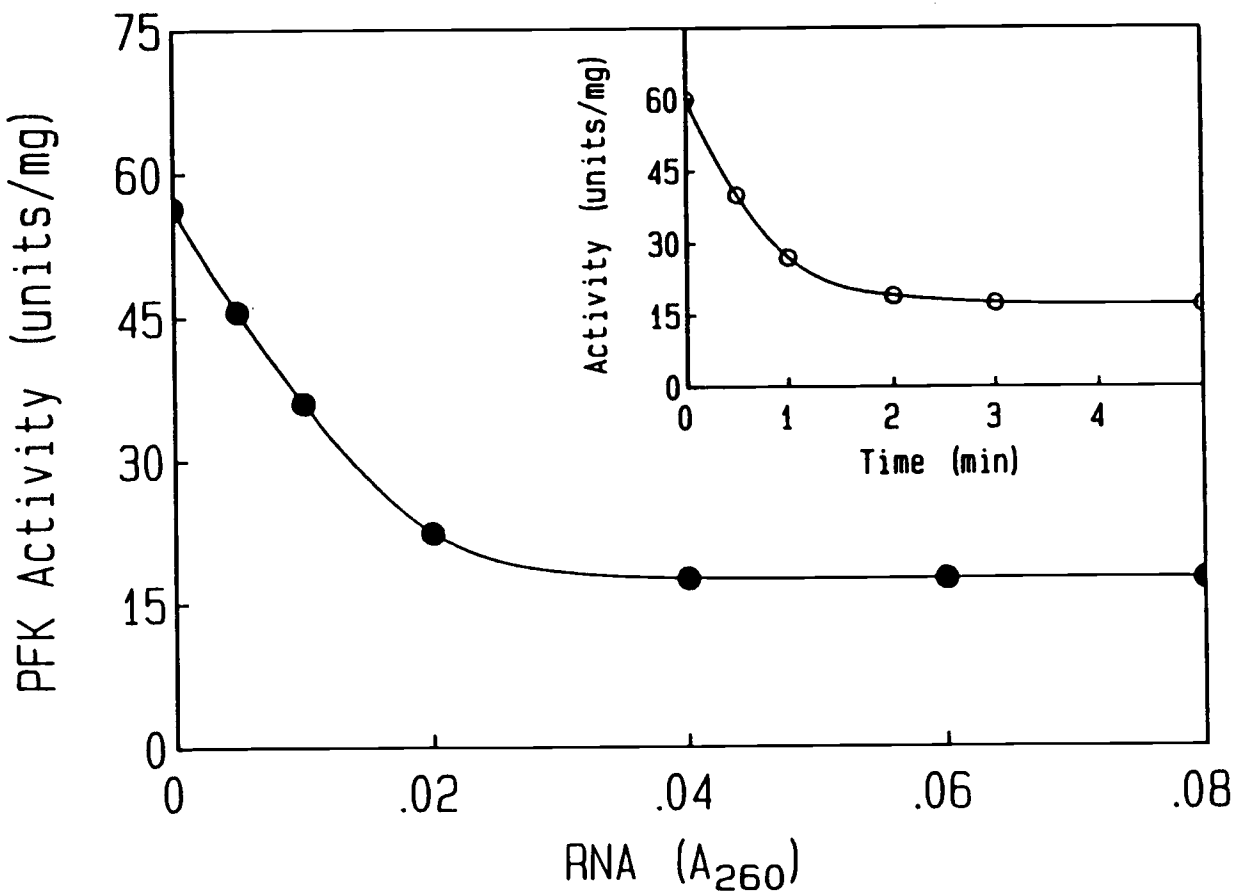

PFK Activity (units/mg)
75
60
45
30
15
0
0
.02
.04
.06
.08
RNA (A_{260})
Activity (units/mg)
60
45
30
15
0
0
1
2
3
4
Time (min)

Figure IV-3

Table IV-3

Inactivation of Phosphofructokinase by Nucleic Acids

Nucleic Acids	$C_{½}$ (A_{260} unit/mL)	A_{max} (%)
Liver RNA fraction	0.012	65
Rabbit liver tRNA	0.011	62
E. coli 23S rRNA	0.010	84
Salmon testes DNA	0.010	67
Chromatin core particle DNA	0.012	64
Oligo DNA (12 BP)	0.25	15
Single strand DNA (probe 520, 11 Bases)	0.70	10

Inactivation was performed for 10 minutes as described in Materials and Methods. A_{max} stands for the percentage of inactivation of phosphofructokinase induced by nucleic acid within 10 minutes of incubation. $C_{½}$ represents the concentration of nucleic acid (in A_{260} unit) required to achieve half maximal inactivation of the enzyme.

Factors Affecting the Inactivation of Phosphofructokinase

The inactivation of phosphofructokinase by nucleic acids is influenced by a number of factors. Table IV-4 summarizes the effects obtained with the substrates and allosteric effectors of the enzyme and a nucleic acid binding protein -- protamine. Low-molecular-weight ligands, including both positive and negative allosteric effectors of the enzyme, confer total protection when present at high enough concentrations. Fructose-2,6-bisphosphate is the most effective of these effector, with a concentration for half maximum protection as low as 0.1 μM. Protamine affects the inactivation possibly by binding to nucleic acid.

The effects of temperature on the inactivation are similar to those observed for calmodulin and troponin C in previous studies (Chapter II). Variation of pH from 7.0 to 9.0 does not effect the inactivation significantly (data not shown for simplicity).

Among these effectors, ATP, ADP, AMP, fructose 2,6-bisphosphate, and phosphate stimulate the recovery of enzymatic activity, with ATP having the largest effect (Table IV-5). Other effectors listed in table IV-4 can not reverse the inactivation. Interestingly, the RNase A digestion products of the partially purified liver RNA also promote enzyme recovery.

Table IV-4

Factors Affecting the Inactivation of Phosphofructokinase by Liver RNA Fraction

Effector	$C_{½}$
Fructose-2,6-bisphosphate (μM)	0.11
Fructose-6-phosphate (μM)	25
ATP (mM)	0.15
ADP (mM)	0.26
AMP (mM)	0.34
Phosphate (mM)	4.0
Pyrophosphate (mM)	3.1
Citrate (mM)	5.5
NH_4^+ (mM)	10
Protamine sulfate (μM)	0.11

Phosphofructokinase was incubated with 0.1 A_{260} units/mL of RNA for 10 minutes in the presence of the individual factors as described under Methods and Materials. $C_{½}$ represents the concentration of each effector required to obtained half maximum protection of the enzyme activity.

Table IV-5

Reactivation of Phosphofructokinase

Ligand	% Recovery
ATP (1.0 mM)	70%
RNAse A digest of liver RNA	60%
Fructose-2,6-bisphosphate (0.1 mM)	30%
Phosphate (10.0 mM)	31%
ADP (1.0 mM)	10%
AMP (1.0 mM)	11%
0 (no addition)	0

Phosphofructokinase (0.02 mg/mL) was pre-incubated with the liver RNA fraction (0.1 A_{260} units/mL) for 10 minutes. This was followed by the addition of the indicated concentration of ligand. After 45 minutes of further incubation, the enzyme activity was determined under optimal conditions. The data in the table represent the percent recovery of enzymatic activity.

Glycerol Gradient Centrifugation and Gel Filtration

Figure IV-4 shows the separation obtained upon glycerol density gradient centrifugation of a phosphofructokinase control and of an enzyme sample which had been pre-incubated with the liver RNA fraction. In the case of the control, the peak of enzymatic activity was found at 17.5% glycerol while the RNA-treated sample proved almost totally inactive. However, after pre-incubation of each fraction with 1.0 mM ATP, the ATP-recovered activity peak of the latter is become evident at 15% glycerol. This difference in sedimentation rate clearly indicates the dissociation of the treated enzyme tetramer to a smaller species.

The gel filtration study shown by Figure IV-5 gives similar results. ATP-recovered enzymatic activity of the sample treated with RNA has a higher retention volume than the untreated active enzyme. In the former case, the peak eluted at the same position as would rabbit skeletal muscle glycogen phosphorylase b -- which has a molecular weight of 197,000. In addition, there is a coincident A_{260} peak which obviously represents the absorbance of RNA. The enzyme, present at a concentration below 0.1 mg/mL, could not contribute more than 0.1 to the absorbance at 260 nm. Meanwhile, the area under the major A_{260} peak decreases. According to the retention volumes, the approximate molecular weights of the enzyme-RNA complex and of the liver

Figure IV-4: Glycerol gradient centrifugation of phosphofructokinase and the rabbit liver RNA fraction after pre-incubation. Symbols: □ activity of the control assayed directly after fractionation; ■ activity of the RNA-treated enzyme following incubation with ATP. The dashed line represents the glycerol gradient.

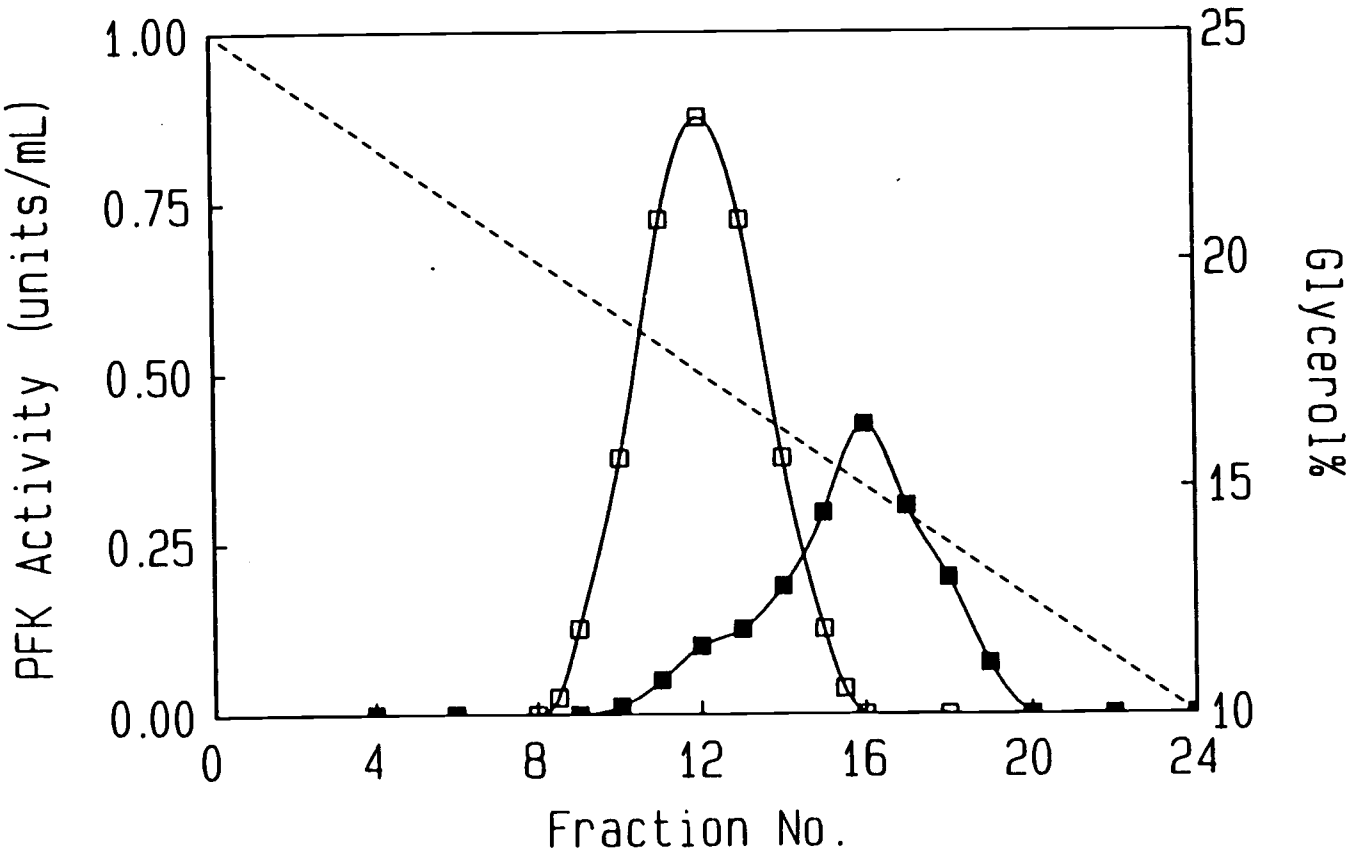

1.00
0.75
0.50
0.25
0.00
PFK Activity (units/mL)
25
20
15
10
Glycerol%
0
4
8
12
16
20
24
Fraction No.

Figure IV-4

Fig. IV-5: Separation profile of a mixture of phosphofructokinase and liver RNA fraction mixture on a Sephacryl S-300 gel filtration column. Symbols: □ enzyme activity assayed without incubation with RNA; ■ ATP-recovered activity after incubation with RNA. The dashed line represents the absorbance of the RNA control at 260 nm; dotted line, A_{260} after incubation with phosphofructokinase. The fraction size was 2.5 mL. The elution profile for glycogen phosphorylase b (MW = 194,000) and cytochrome C (MW = 12,000) are included in the figure as standard molecular weight markers.

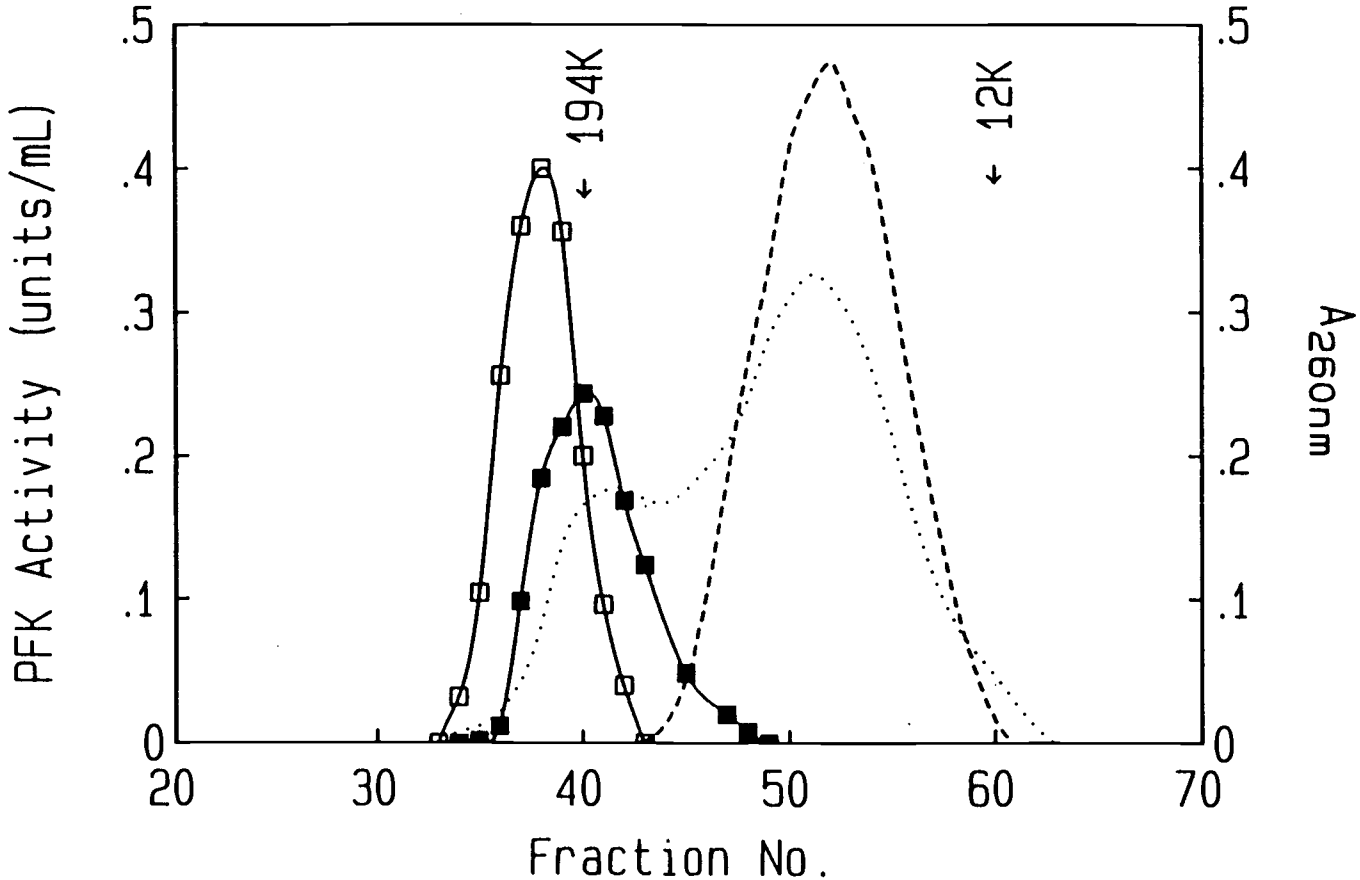

PFK Activity (units/mL)
A260nm
Fraction No.
194K
12K
.5
.4
.3
.2
.1
0
20
30
40
50
60
70

Figure IV-5

RNA fraction are 200,000 and 39,000, respectively. These results suggest the dissociation of the enzyme from the tetramer to the dimer and binding of the inactive form of the enzyme by RNA.

Competition of DNA with Calmodulin for Phosphofructokinase Binding

As shown in Figure IV-6, the binding of dansylcalmodulin by phosphofructokinase increases the fluorescence intensity of the former protein by 2-fold. The equilibrium is achieved within 15 minutes. Addition of salmon DNA to the protein-enzyme complex causes a rapid 8% decrease of the intensity. However, pre-incubation of the enzyme with DNA significantly decreases the rate of the fluorescence change occurring upon the addition of dansylcalmodulin. Yet DNA has no effect on dansylcalmodulin alone. Even after 3.5 hrs of incubation, the fluorescence is 15% less than that observed when DNA is added last.

Digestion of DNA with Nuclease in the Presence of Phosphofructokinase

The digestion of salmon DNA by DNase I is inhibited by the addition of phosphofructokinase (Figure IV-7). The inhibition curve has a sigmoid shape, with lower enzyme

Figure IV-6: Fluorescence intensity change of dansylcalmodulin in the presence of phosphofructokinase and DNA. The incubation condition are detailed in Methods and Materials. The arrow key indicates the addition of DNA to the pre-incubation system of dansylcalmodulin and phosphofructokinase. Symbols: ■ reaction started by pre-mixing the enzyme and dansylcalmodulin; ▲ reaction started by pre-incubation of the enzyme with DNA for 10 minutes before addition of dansylcalmodulin.

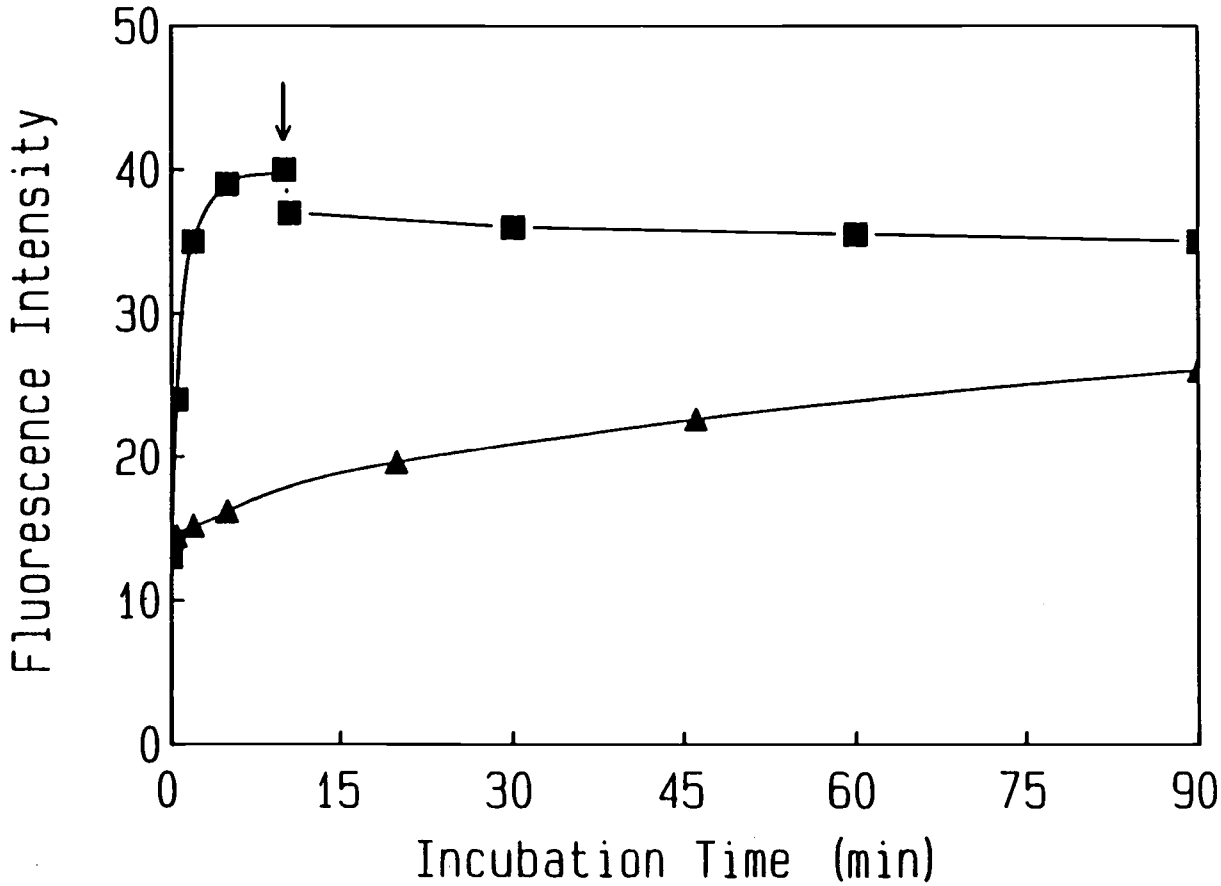

Fluorescence Intensity
50
40
30
20
10
0
0
15
30
45
60
75
90
Incubation Time (min)

Figure IV-6

Figure IV-7: Effect of phosphofructokinase on the digestion of DNA with DNase I. Reaction conditions are described in Materials and Methods. Activity was determined in the presence (■) and absence (●) of 0.1 mM fructose 2,6-bisphosphate.

Definition of the unit of DNase I activity: one munit will produce a ΔA_{260} of 0.001 per minute per mL.

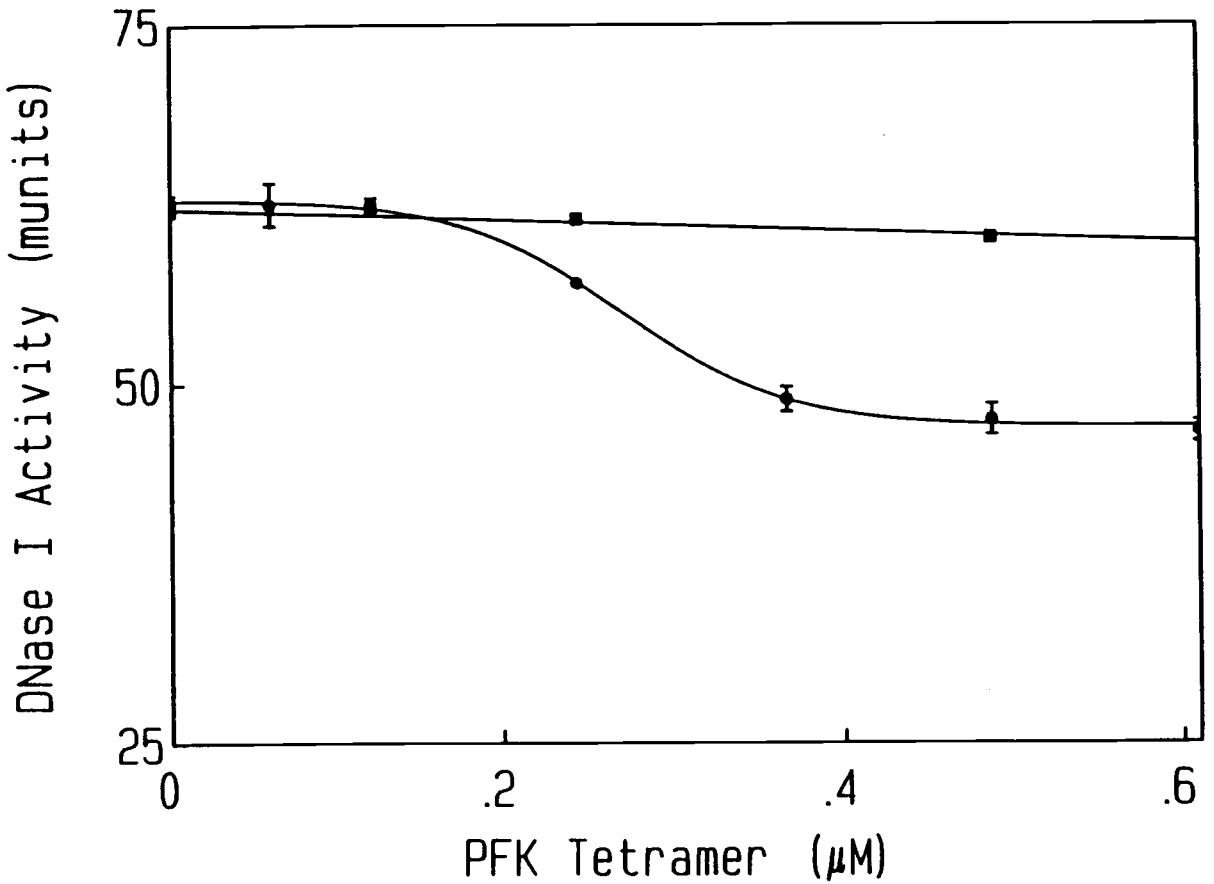


Figure IV-7

concentrations having no effect while higher concentrations give a maximum inhibition in which 75% DNase I activity remains. The saturation obtained at about 0.37 μM phosphofructokinase (in tetramer) corresponds to about 65 DNA base pair for each enzyme tetramer. Addition of fructose-2,6-bisphosphate to the reaction mixture diminishes the inhibition.

The digestion of DNA in the PFK-DNA mixture leads to the aggregation of the enzyme, evident in turbidity measurements performed at 550 nm, and permanent loss of the enzyme activity (Figure IV-8). This supports the suggestion that the inactive dimer of the enzyme tends to form inactive high polymers when the inactivator is removed (Mayr, 1984; Chapter II). Higher concentration of DNA apparently require longer times for digestion so that aggregation of the enzyme appears later.

The ability of phosphofructokinase to protect DNA from nuclease digestion was investigated with chicken chromatin core particle DNA and micrococcal nuclease. The gel photograph in Figure IV-9 clearly shows that a minor fraction of the DNA has been totally protected from digestion. There are also some DNA stays on the bottom of wells with phosphofructokinase which can not get into the gel. We do not have enough information on the structure of the protected DNA to determine whether a specific sequence is involved in protection.

Figure IV-8: Aggregation of phosphofructokinase resulting from the digestion of DNA. Phosphofructokinase (0.2 mg/mL) was pre-incubated with 0.4 A_{260} units/mL (□, ■) or 2.0 A_{260} units/mL (Δ, ▲) of DNA for 10 minutes. Then 0.2 μg/mL of DNase I was added (open symbols). Closed symbols represent the values obtained with samples to which no DNase I was added. All the reactions were performed at 36 °C.

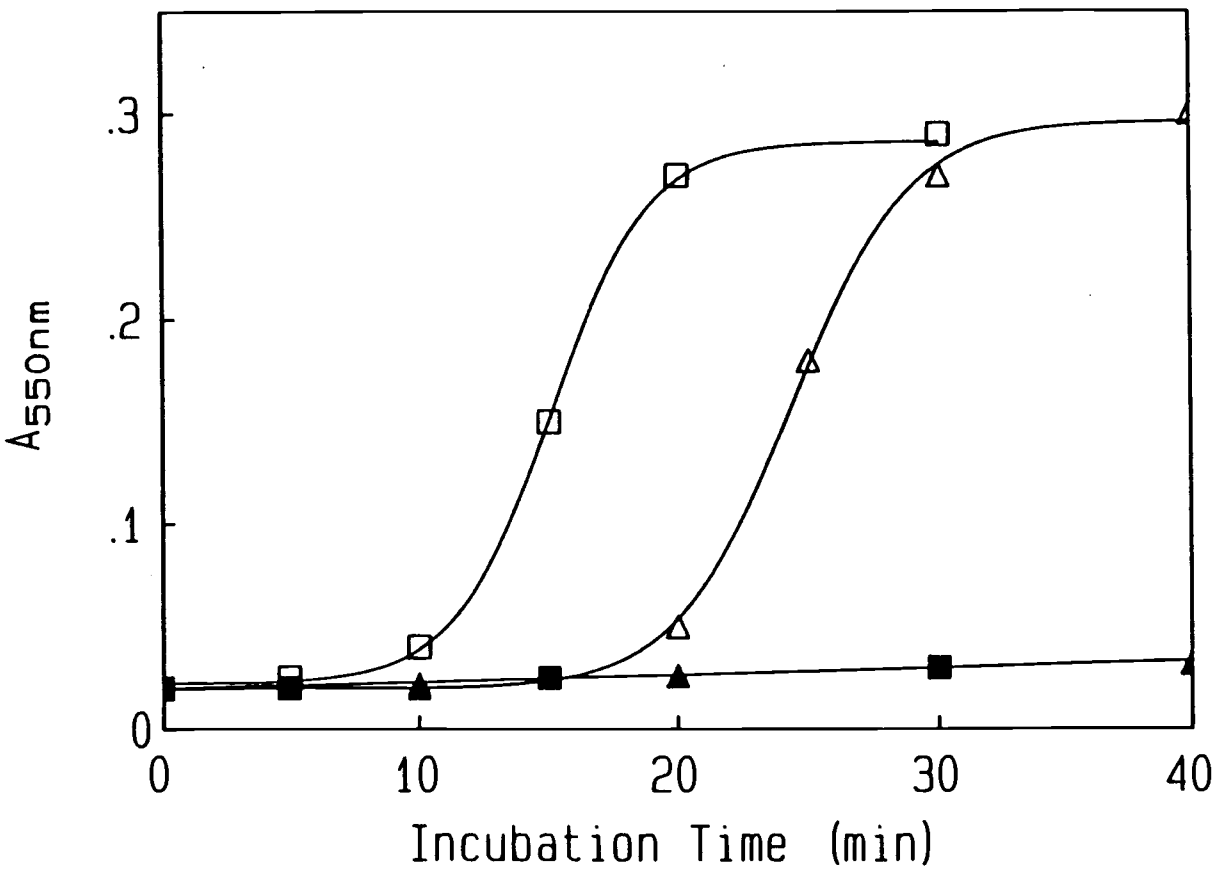


Figure IV-8

Figure IV-9: Effect of phosphofructokinase on the digestion of chicken chromatin core particle DNA by micrococcal nuclease. Panel A, control experiment done in the absence of phosphofructokinase. Panel B, the results of digestion in the presence of phosphofructokinase. Lane M, DNA markers (pBR322/Hha I); lanes 1 to 6, incubations were performed for 0, 5, 10, 25, 60, 120 minutes, respectively.

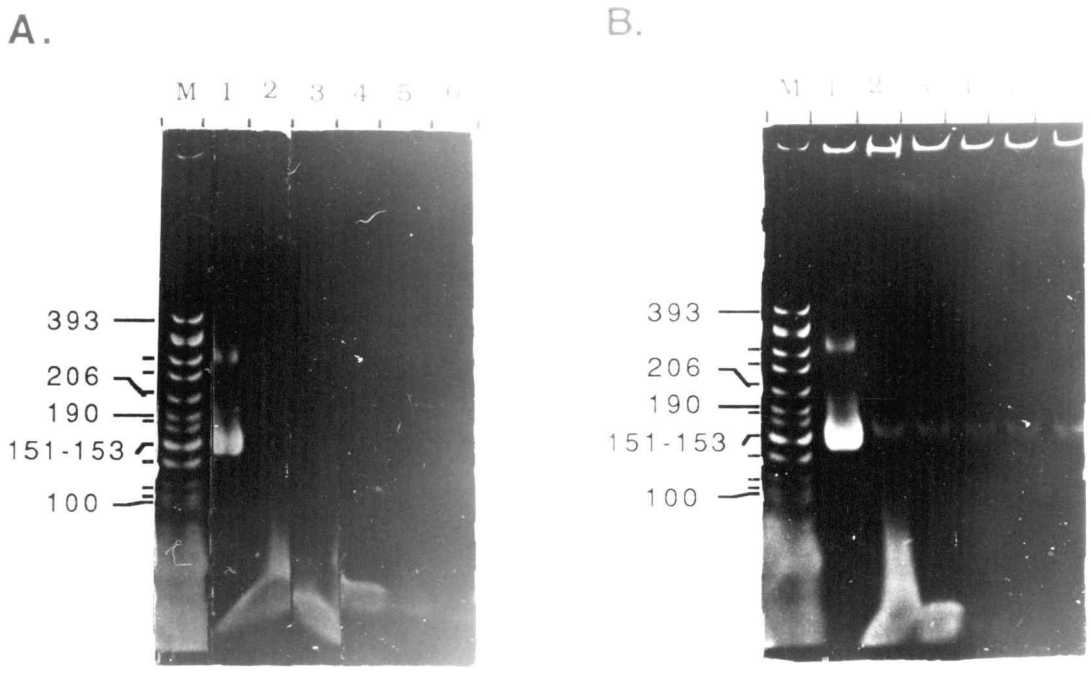


Figure IV-9

Discussion

The phosphofructokinase inactivator purified from rabbit liver exhibits chemical and physical properties which are characteristic of RNA. RNA gel electrophoresis and sequencing experiments confirmed that it is a heterogeneous mixture of RNA's ranging in size from 10-60 bases. It may consist of a mixture of small nuclear RNAs, tRNAs, rRNAs, mRNAs, and their nuclease digestion fragments. Complete resistance of the phosphofructokinase inactivating ability of this preparation to proteolytic digestion together with sensitivity to RNase A indicate that RNA is responsible for the inactivation.

The effectiveness of various types of RNA and DNA in the inactivation of phosphofructokinase suggests a relatively nonspecific association of the enzyme with nucleic acids. Electrostatic interaction may be a major factor in the inactivation, involving a region of the enzyme which is rich in positively charged amino acid residues. The interaction of such a positively charged region with the negatively charged nucleic acid may promote a conformation change of the enzyme which results in inactivation. The failure of a 12-base pair oligo DNA and an 11-nucleotide single-stranded DNA to inactivate phosphofructokinase suggest that the interaction involves fairly large regions in both the enzyme and the nucleic acids.

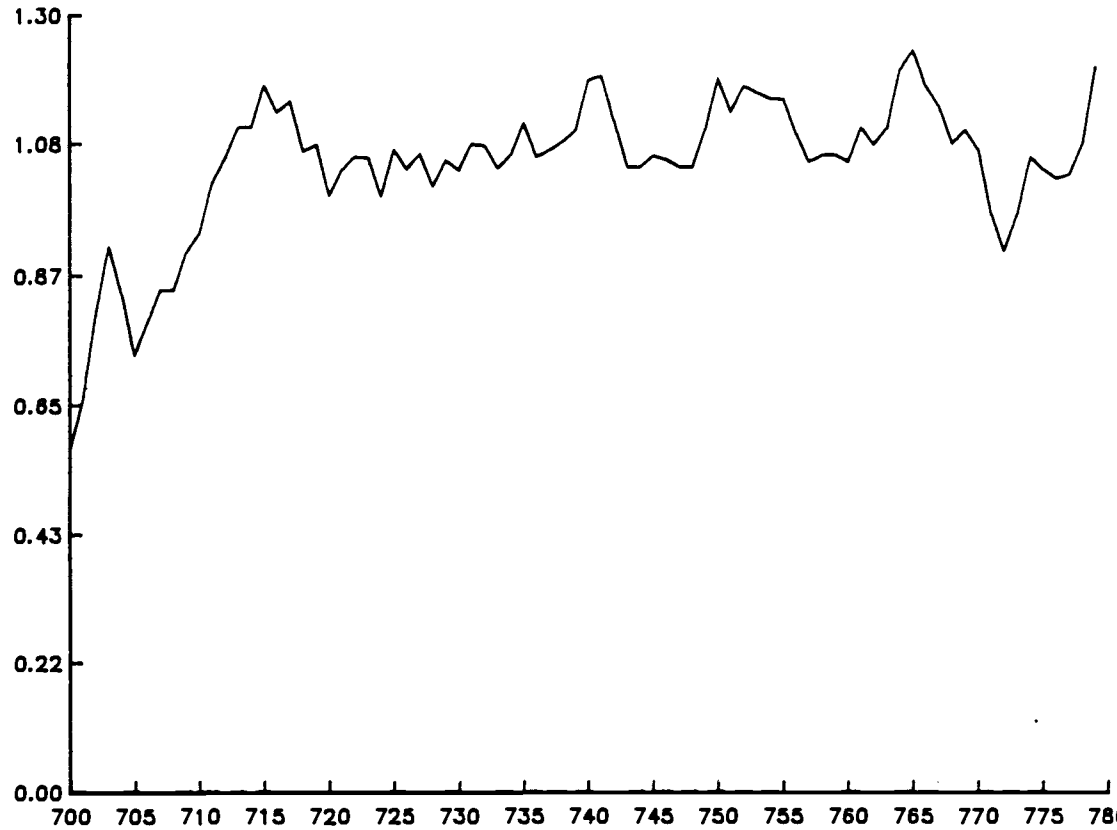
The kinetics of the inactivation are similar to those reported in our early study with the acidic proteins (Chapter II). The inactivation obtained with the nucleic acids is distinguished by a higher rate together with a lower extent of final inactivation than that reported for proteins such as calmodulin or troponin C. It is more like the inactivation induced by synthetic poly (Glu-Tyr). This may result from the highly acidic properties of the nucleic acids and their lower affinity for phosphofructokinase, as suggested in the experiments with dansylcalmodulin. Factors which affect the inactivation of the enzyme by acidic proteins show similar effects in the inactivation induced by nucleic acids. One exception is the efficient reversal of the nucleic acid-induced inactivation by phosphate.

The inactivation of phosphofructokinase induced by nucleic acids may follow the mechanism proposed for acidic proteins (Chapter II). The glycerol gradient sedimentation and gel filtration experiments clearly indicate the dissociation of the active enzyme tetramer to a smaller species -- which is probably the dimer judged by the molecular weight of the enzyme-RNA complex obtained from gel filtration. The gel filtration study also shows the association of the rabbit liver RNA fraction with the inactive enzyme. The significant slowing down of the dansylcalmodulin-phosphofructokinase association by DNA suggests the competition of DNA with calmodulin in enzyme

binding. DNA and calmodulin may bind to identical or closely overlapping regions on the enzyme. Yet the binding of DNA is kinetically favored while the binding of calmodulin is thermodynamically favored. The DNase I-induced aggregation and permanent loss of phosphofructo-kinase activity demonstrates that complexed DNA is necessary for the reactivation of the enzyme. Similar effects were previously seen in studies with calmodulin (Mayr, 1884a; Chapter II).

Although the present data suggests a relatively nonspecific interaction between phosphofructokinase and nucleic acids, a certain element of specificity may be involved. We note that the 60-residue C-terminal sequence of phosphofructokinase has a high probability of helix formation, and even more surprisingly, that it contains a leucine zipper-like structure (Figure IV-9). It has four leucine residues (sequence positions 744, 751, 758, 766) which are spaced 7 or 8 residues apart, following a pattern which is typical of the DNA binding motif known as the leucine zipper (McKninght, 1988). Since the C-terminal region of the enzyme is very sensitive to protease digestion (Kemp, 1981), it may have a high degree of exposure to the solvent and hence of accessibility for DNA binding.

Fig. IV-10: Primary structure and Helix prediction of the C-terminal part (residues 700-779) of rabbit skeletal muscle phosphofructokinase. The helix probability is calculated by using a protein secondary structure prediction computer program written by Jack Kramer and employing a window of 7 amino acid residues in the calculation.



C-terminal Sequence of PFK (700-779)

^{700}FANTPDSGCVLGMRKRALVFQPVTELQNQTDFEHRIPKEQW
WLKLRPILKILAKYEIDLDTSEHAHLEHISRKRSGEATV-COOH^{779}

Figure IV-10

Chapter V

Conclusion

The activity of phosphofructokinase, the key enzyme in the glycolytic pathway, can be regulated *in vitro* through the reversible inactivation of the enzyme which accompanies its association with a number of acidic proteins. The mechanism of the inactivation involves the dissociation of the active enzyme tetramer to inactive dimers -- through a transformation of dimer isoforms which is greatly increased, both in rate and extent, by the acidic inactivating proteins. An abundance of acidic proteins such as myosin, actin, troponin C, troponin, tropomyosin, and α-actinin is characteristic of muscle. Perhaps, these proteins induce the inactivation of the enzyme *in vivo*, accounting for the observation that a major fraction of phosphofructokinase is inactive in resting muscle (Passonneau and Lowry, 1962). The reversible inactivation of phosphofructokinase may be one of the fundamental regulatory mechanisms of muscle glycolysis. Like the enzyme itself, the inactivation process is influenced by a number of effectors. While positive and negative allosteric effectors of the enzyme have opposite functions on the enzyme itself, both protect the enzyme from the inactivation. The remarkable effectiveness of fructose-2,6-bisphosphate, both in

preventing inactivation and in promoting reactivation, underscores its cardinal role in regulation of phosphofructokinase. This is determined by the basic mechanism of the inactivation -- the two dimer transformation process. Distinctive differences in the inactivating capabilities of troponin C fragments suggest that specific sequences or amino acid side chains in the vicinity of the calcium-binding site III (or among residues 85-120) are involved in the inactivation. The conclusion may apply as well to calmodulin, which has a high degree (67%) of homology with troponin C.

The *in vitro* interaction of phosphofructokinase with various types RNA or DNA also leads to the inactivation of the enzyme. The mechanism involves the preferential binding of the enzyme dimer by the nucleic acids, as demonstrated by gel filtration and density gradient sedimentation. Competition experiments with a fluorescent derivative (dansyl) of calmodulin show that in a mixture, the interaction of phosphofructokinase with calmodulin prevails thermodynamically over association with DNA. The physiological significance of the enzyme-nucleic acid interactions is hard to ascertain.

Calcium, the important second messenger in various cellular processes, may also regulate glycolysis through its effect on the phosphorylation of phosphofructokinase. Calmodulin, the ubiquitous calcium receptor, and troponin C,

the calcium-dependent regulator controlling muscle contraction, express the second messenger through specific calcium-dependent interactions with the enzyme which affect the phosphorylation catalyzed by cAMP-dependent protein kinase. In the presence of calcium, the calcium-binding proteins not only increase the phosphorylation at the known C-terminal site (His-Ile-Ser-Arg-Lys-Arg-Ser(P)-Gly-Glu), but also induced phosphorylation at a novel site in the middle of the enzyme molecule (Lys-Leu-Arg-Gly-Arg-Ser(P)-Phe-Met). The *in vitro* phosphorylation of the enzyme catalyzed by cAMP-dependent protein kinase is consistent with reported evidence that electrical stimulation of muscle increases the *in vivo* phosphorylation of phosphofructokinase and that calcium may be involved in the process. Although our preliminary studies indicate little effect of the phosphorylation on the activity of the enzyme determined under optimal assay conditions, we believe that other factors, still unknown, may be involved in the full expression of the process.

The inactivation of phosphofructokinase by the acidic proteins may be an important regulation mechanism in the muscle glycolysis. The effects of calmodulin and troponin C on the phosphorylation of phosphofructokinase suggests that one aspect of the regulation of protein kinase involves the changes in the status of target protein rather than the kinase itself. Phosphofructokinase, the rate-limiting

enzyme in muscle glycolysis, is a major target for both neural and hormonal control. The finding that calcium affects the phosphorylation of the enzyme through its interaction with calmodulin and troponin C and, especially, the discovery of a novel phosphorylation site in the middle of the enzyme molecule gives a new direction to studies of the physiological significance of the phosphorylation of phosphofructokinase.

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Appendices

Appendix 1

Purification of Rabbit Skeletal Muscle PFK

Procedures

Rabbit skeletal muscle phosphofructokinase was purified according the procedure developed by Kuo *et al.* (1986) which combines and modifies several steps in the methods originally published by Ling *et al.* (1966) and Kemp (1975).

Step 1. Extraction. A large female New Zealand rabbit (weighing about 4 kilograms) was killed by injection of 1.0 - 2.0 mL beuthanasia-D into the external marginal vein of an ear. The animal is immediately bled by cutting the blood vessels of the neck, and the muscle of the hind legs and back are quickly removed and chilled in ice. About 700-800 grams of muscle was usually obtained. The muscle was then ground in an meat grinder with coarse plate (3/18 inch) followed by re-grinding with a fine plate (4/81 inch). All the subsequent steps were performed at 4 °C. The ground muscle was extracted by stirring in three volumes of extracting buffer containing 30 mm KF, 4 mM EDTA, and 15 mM ß-mercaptoethanol (pH 7.5) for 25 minutes. The extract was centrifuged for 10 minutes at 4,100 rpm in a Beckman model J-6B. The supernatant fraction is passed through glass wool to remove lipid particles and the pH (usually 6.6-6.7) is

adjusted to 6.8 with 2 M Trizma base.

Step 2. Precipitation with isopropanol. The supernatant extract was immersed in a dry ice-acetone bath. When the temperature of the extract drops to 0 °C, one fifth volume of ice cold isopropanol was added dropwise while maintaining efficient stirring. As the volume of isopropanol increases, the temperature of the solution was allowed to decrease to - 4 °C and was held for 20 minutes after the addition. Freezing of the solution should be avoided. The pellet was collected by centrifugation at 4,100 rpm for 20 minutes, dissolved in 0.1 M Tris-phosphate (pH 8.0), 5 mM sodium pyrophosphate, 0.2 M EDTA, 0.2 mM fructose-1,6-bisphosphate, and 1 mM ß-mercaptoethanol with a volume of about 1/15 of the crude extract. The solution was then dialyzed against the buffer for 2 - 4 hours to remove isopropanol.

Step 3. Heat Treatment. The dialyzed solution is transferred to a stainless steel beaker and placed in 90 °C water bath. The solution is rapidly stirred and its temperature was brought to 57 °C and held at 57 - 59 °C for 3 minutes. Following the heat treatment, the beaker was immediately placed in a ice-water bath with stirring until the temperature decreased to 4 °C. The suspension was then spun for 20 minutes at 14,000 rpm in Sorvall RC2-B centrifuge employing a SS-34 rotor. The pellet was resuspended with the same Tris-phosphate buffer (about 1/6

volume of the preceding supernatant) and centrifuged as before. The clear and yellowish supernatant was combined.

Step 4. Ammonium Sulfate fractionation. The combined supernatant from step 3 was brought to 38% ammonium sulfate (213 g/liter) and stirred for another 30 minutes after ammonium sulfate dissolved. The precipitate was removed by centrifugation at 14,000 rpm for 20 minutes. The enzyme in the supernatant was precipitated by further addition of ammonium sulfate to 55% (101 g/liter). The pellet was collected by centrifugation at 14,000 rpm for 20 minutes as before and dissolved in 1/30 volume (referenced to the first crude extract) buffer containing 50 mM Tris-phosphate (pH 8.0), 0.2 mM EDTA, 1.0 mM ß-mercaptoethanol, and 5 mM sodium pyrophosphate and dialyzed against the same buffer overnight.

Step 5. DEAE Ion-exchange Chromatography. DEAE-51, DEAE-52, and DEAE-53 were packed into three columns (2.5 cm X 18 cm) connected in series and equilibrated with the preceding buffer. After application of the protein sample and washing with equilibration buffer, a linear gradient of 0.1 - 0.5 M Tris-phosphate (V = 1000 mL) was applied. Appropriate fractions were collected and concentrated with Amicon YM-10 filter. The concentrated enzyme samples were dialyzed against a buffer containing 50 mM Tris-phosphate (pH 8.0), 0.2 mM EDTA, 1.0 mM ß-mercaptoethanol, 5 mM sodium pyrophosphate, and 50% glycerol. The final preparation of

the enzyme was stored at - 80 °C.

Results

Figure A-1 shows the profile of the final purification step of PFK on the DEAE 51-52-53 serial column. Typically, OD_{280nm} profile does not show a clear single peak but multiple overlapping peaks while the enzyme activity profile match up the protein profile. The different fractions of PFK show no difference on 9-19% $NaDodSO_4$ gel. In fact, the shape of the separation profile differs with differing preparations. These may be affected by the condition of the rabbit killed.

Table A-1 summarizes the purification of phosphofructokinase from rabbit skeletal muscle.

Figure A-2 shows the $NaDodSO_4$ polyacrylamide gel of the phosphofructokinase purification procedures.

The covalent bound phosphate of PFK was determined according to the procedure described in Appendix III. Low phosphate content was found for all the fractions shown in the DEAE-51-52-53 serial profile (Figure A-1). No significant difference was detected for different fractions. The average phosphate content of the purified PFK by this method is 0.15 ± 0.05 mol/mol PFK protomer. This result is somewhat different from that reported previously (Kuo *et al.*, 1986).

Figure A-1: DEAE-51-52-53 series column separation profile of PFK purification. The columns were eluted with 1000 ml linear gradient of 0.1 - 0.5 M Tris-phosphate (pH 8.0). Fraction size is 6.0 ml/tube. Solid line represents the A_{280} profile, Dashed line, PFK activity profile. Dotted line indicates the buffer gradient.

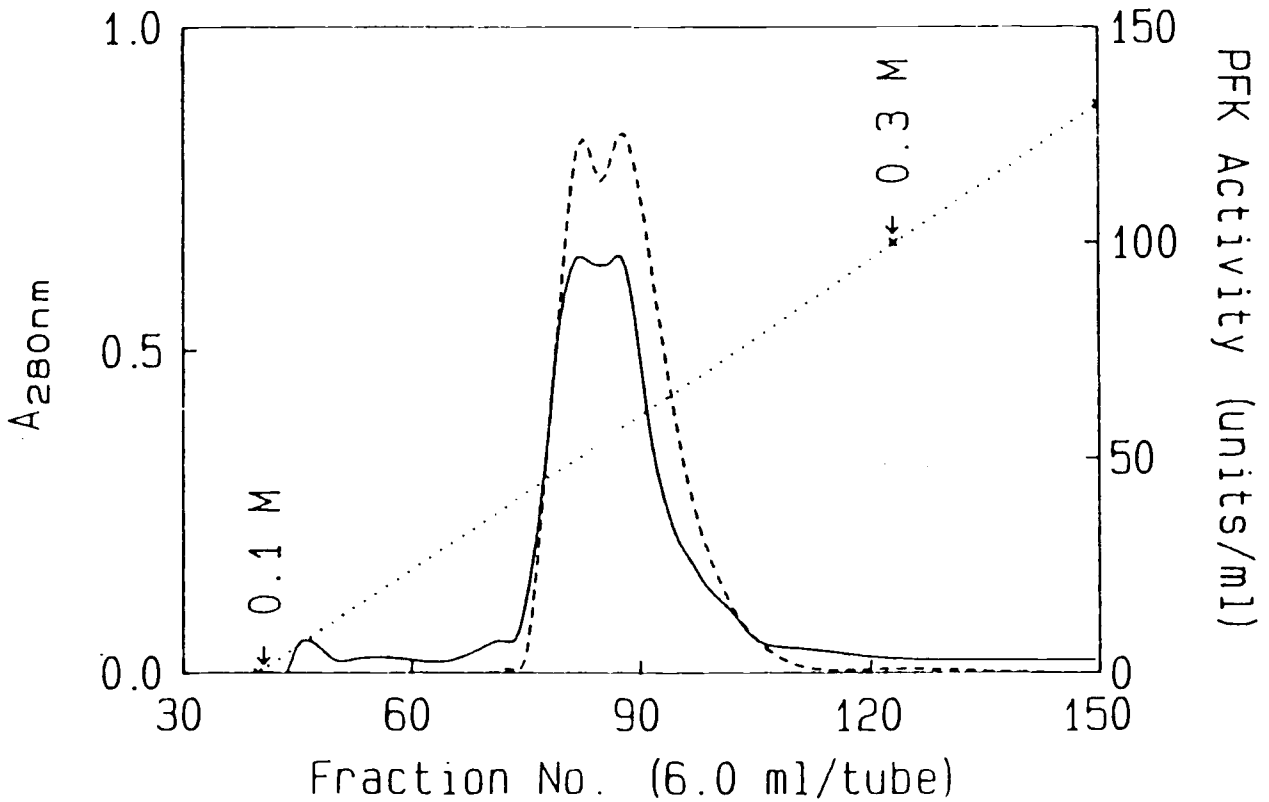
1.0
0.5
0.0
A_{280nm}
150
100
50
0
PFK Activity (units/ml)
0.1 M
0.3 M
30
60
90
120
150
Fraction No. (6.0 ml/tube)

Figure A-1

Figure A-2: 9-19% $NaDodSO_4$ polyacrylamide gel electrophoresis of samples from PFK purification. Lane 1, protein marker; 2, crude extract; 3, isopropanol precipitation pellet; 4, heat treatment supernatant; 5, 38% $(NH_4)_2SO_4$ precipitation supernatant; 6, 55% $(NH_4)_2SO_4$ precipitation pellet; 7, 8, 9, DEAE-51-52-53 series column elution fraction No. 81, 84, and 87, respectively.

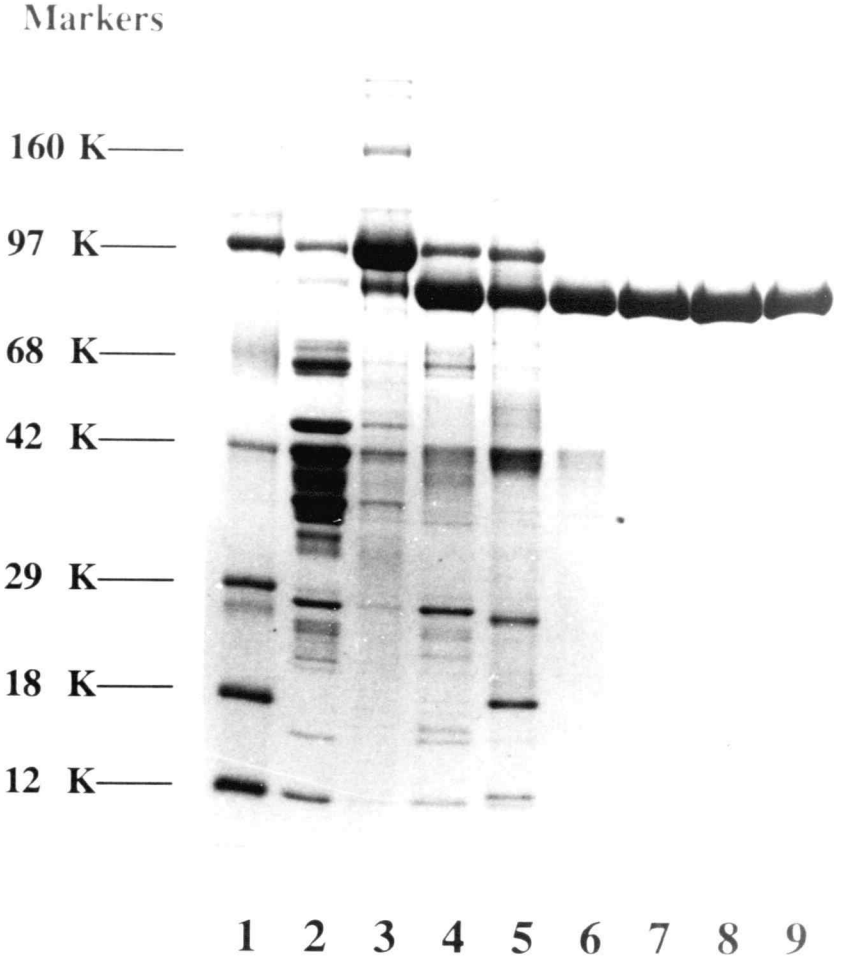

Figure A-2

Table A-1. Summary of Purification of PFK from Rabbit Skeletal Muscle

Procedure	Volume (mL)	Total units[1]	Protein (mg/mL)[2]	S.A. (units/mg)	Yield (%)	Purification (fold)
Extract	2100	66370	12.9	2.45	100	1
Isopropanol	150	43140	17.6	16.3	65	6.65
Heat Treatment	175	39822	3.76	60.5	60	24.7
Ammonium Sulfate	65	32520	2.74	182	49	74.3
DEAE-51-52-53	8.2	25220	15.0	205	38	83.7

[1] Activity was assayed under optimal condition at pH 8.0 (Kuo *et al.*, 1986).

[2] Protein concentration was determined according Bradford method (Bradford, 1976).

Appendix 2

Preparation of the Fragments of Troponin C

Proteolytic Digestion of Troponin C

Troponin C was digested in the presence of calcium with tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated soybean trypsin, employing a ratio of 1 mg trypsin per 100 mg troponin C, and fractionated on a phenyl-agarose column as described by Brzeska *et al.* (1983). TnC (1 - 2 mg/ml) was incubated with trypsin at room temperature for 30 minutes in buffer containing 20 Mm NH_4HCO_3 and 1.0 mM $Ca(OAc)_2$. The reaction was stopped by addition of soybean trypsin inhibitor with a ratio of 4 mg inhibitor/mg trypsin. The digestion mixture was separated by phenyl-agarose column equilibrated with 20 mM NH_4HCO_3 buffer containing 1.0 mm $Ca(OAc)_2$ and eluted with the buffer containing 2 mM EDTA. Both the breakthrough (TR2) and elution (TR1) of the column were collected and lyophilized.

The digestion of troponin C by thrombin and subsequent fractionation on Sephadex G-75 column were performed according to Leavis *et al.* (1978a). TnC (10 mg/mL) was incubated with 10 units/mL of thrombin in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1 mM dithiothreitol. The reaction was continued at room

temperature for 72 hours and then stopped with addition of solid urea to 6 M. The mixture was fractionated on a 1.5 X 100 cm column of Sephadex G-75 equilibrated with a buffer containing 20 mM NH_4HCO_3. Two peaks were resolved. Both the big fragment (TH1) and the small fragment (TH2) are collected and lyophilized.

Urea Gel Electrophoresis

A 10% urea 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. After running the gel was stained with coomassie blue G-250. Figure A-3A. shows the separation of the fragments of troponin C on the urea gel. Single band was shown for both tryptic fragments, with TR1 moving slower than TR2 . Single band was resolved for thrombic fragments too. In some instances, the urea gel was used for further purification of the fragments. For this purpose, small pieces of the gel containing peptides was excised without staining and eluted with a elutrap employing the same gel running buffer.

Peptide $NaDodSO_4$ Polyacrylamide Gel Electrophoresis

The purity of the peptides was checked by $NaDodSO_4$

polyacrylamide gel electrophoresis employing a tricine buffer system (Schagger and von Jagow, 1987) in conjuction with a gradient of polyacrylamide. The 16 - 27% gel (8 X 10 cm) was made up in 1.0 M Tris-HCl (pH 8.45) by using the proper proportions of 50% acrylamide and 1.33% bis(acrylamide). The anode buffers was 0.2 M Tris-HCl (pH 8.8) and the cathode buffer contained 0.1 M Tricine-0.1 M Tris (pH 8.45) and 0.1% $NaDodSO_4$. The gel was stained by Coomassie blue reagent.

As shown in Figure A-3B. Unlike the urea gel, each of the two purified tryptic fractions shows two closely spaced bands on 16-27 % $NaDodSO_4$-polyacrylamide peptide gel electrophoresis. Two homogeneous thrombic fragments of troponin C were obtained. Each exhibits a single sharp band on 16-27% $NaDodSO_4$-polyacrylamide gel electrophoresis. The high resolution of the gradient peptide gel made it possible that two bands for each of the tryptic fragments were resolved while only a broad band was shown by regular $NaDodSO_4$ gel.

Fig A-3: Gel Electrophoresis of proteolytic fragments of troponin C.

(A) 10% urea 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. Lane 1 and lane 5 represent intact troponin C; lane 2, TR1; lane 3, TR2; lane 4, mixture of tryptic digestion.

(B) 16-27% $NaDodSO_4$ polyacrylamide gel electrophoresis was made up in 1.0 M Tris-HCl (pH 8.45) by using the proper proportions of 50% acrylamide and 1.33% bis(acrylamide). The anode buffers contained 0.2 M Tris-HCl (pH 8.8) and the cathode buffer was 0.1 mM Tricine-0.1 M Tris (pH 8.45)-0.1% $NaDodSO_4$. Lane 1-7 represents TR2, TR1, peptide markers, TH2, TH1, TH1, and intact TnC, respectively.

Both gels were stained with coomassie blue G-250.

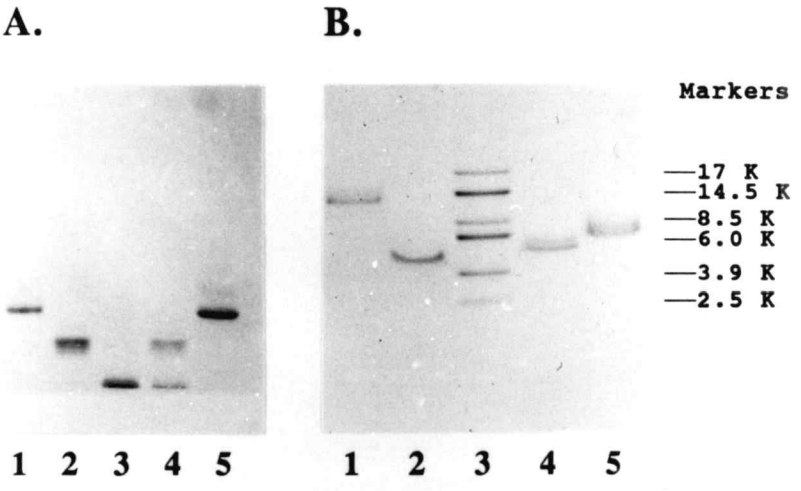

Figure A-3

Reverse-Phase HPLC

HPLC separation the peptides was performed by employing a Vydac C-18 column. The column was run with a programmed gradient of A (water + 0.1% trifluoroacetic acid) and B (acetonitrile + 0.06% trifluoroacetic acid). As shown in Figure A-4A, TR1 shows two closely spaced peaks each of which is corresponding to one band on the peptide gel. TR2 can not be separated with a peak resolved. Both thrombic fragments show a single sharp peak which is consistent with the peptide gel results (Figure A-4B).

Troponin I Binding Properties

The troponin I binding properties of the fragments were determined according to Leavis *et al.* (1978a). The mixtures of TnI with individual fragments of troponin C were separated with 10% urea gel electrophoresis in the presence of Ca^{2+}. The results agree with those reported by Leavis *et al* (1978a). That is, TH1 and TR2 retained troponin I binding ability, while TR1 and TH2 did not.

Figure A-4: Reverse-phase HPLC profile of the proteolytic fragments of troponin C. HPLC separation the peptides was performed by employing a Vydac C-18 column. The column was run with a programmed gradient of A (water + 0.1% trifluoroacetic acid) and B (acetonitrile + 0.06% trifluoroacetic acid).

(A): tryptic fragments, TR1 and TR2.

(B): thrombic fragments, TH1 and TH2.

Running phase gradient

Time (min)	B% (Acetonitrile + 0.06% TFA)
0	10
5	40
25	70
30	70
32	10

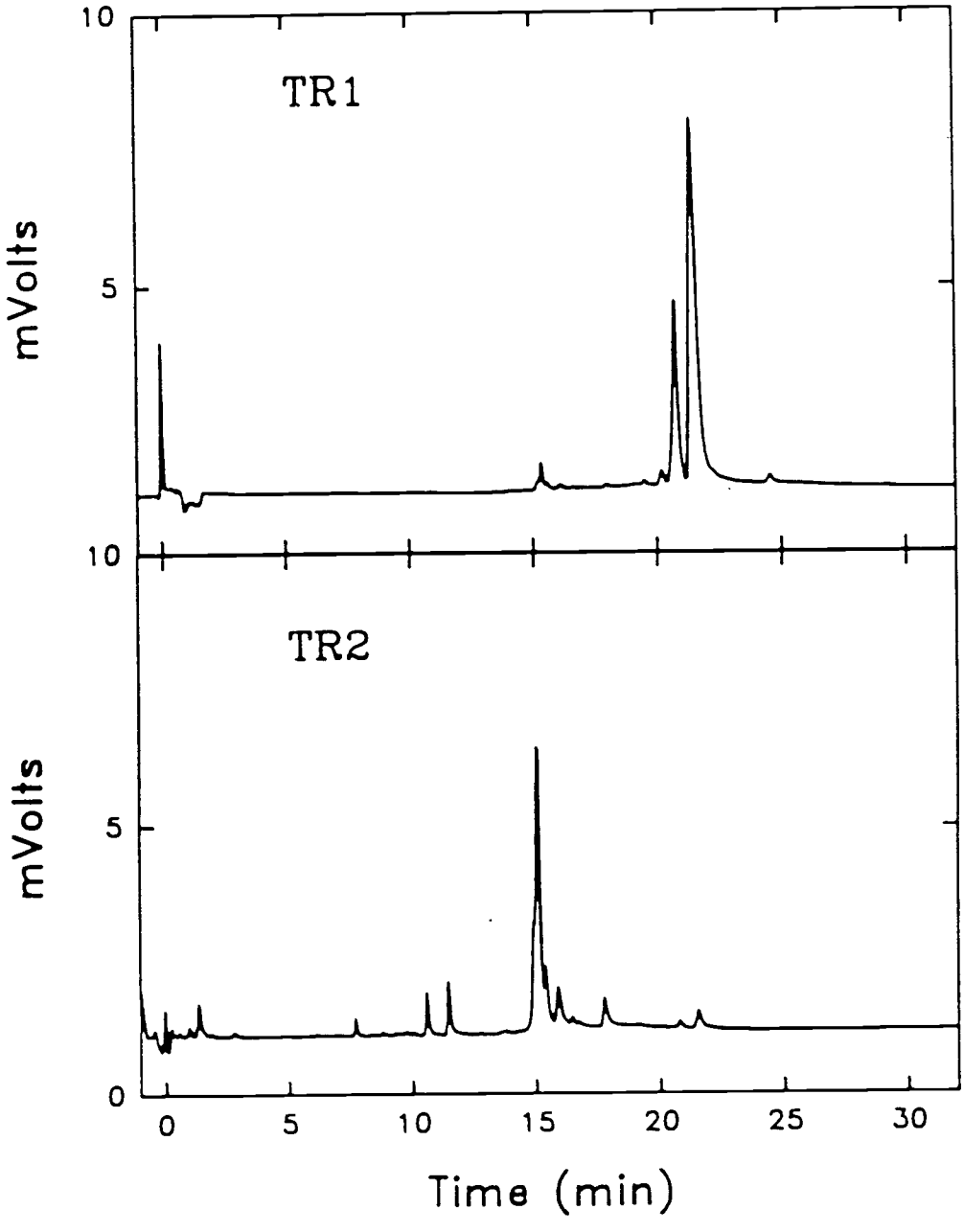

Figure A-4A

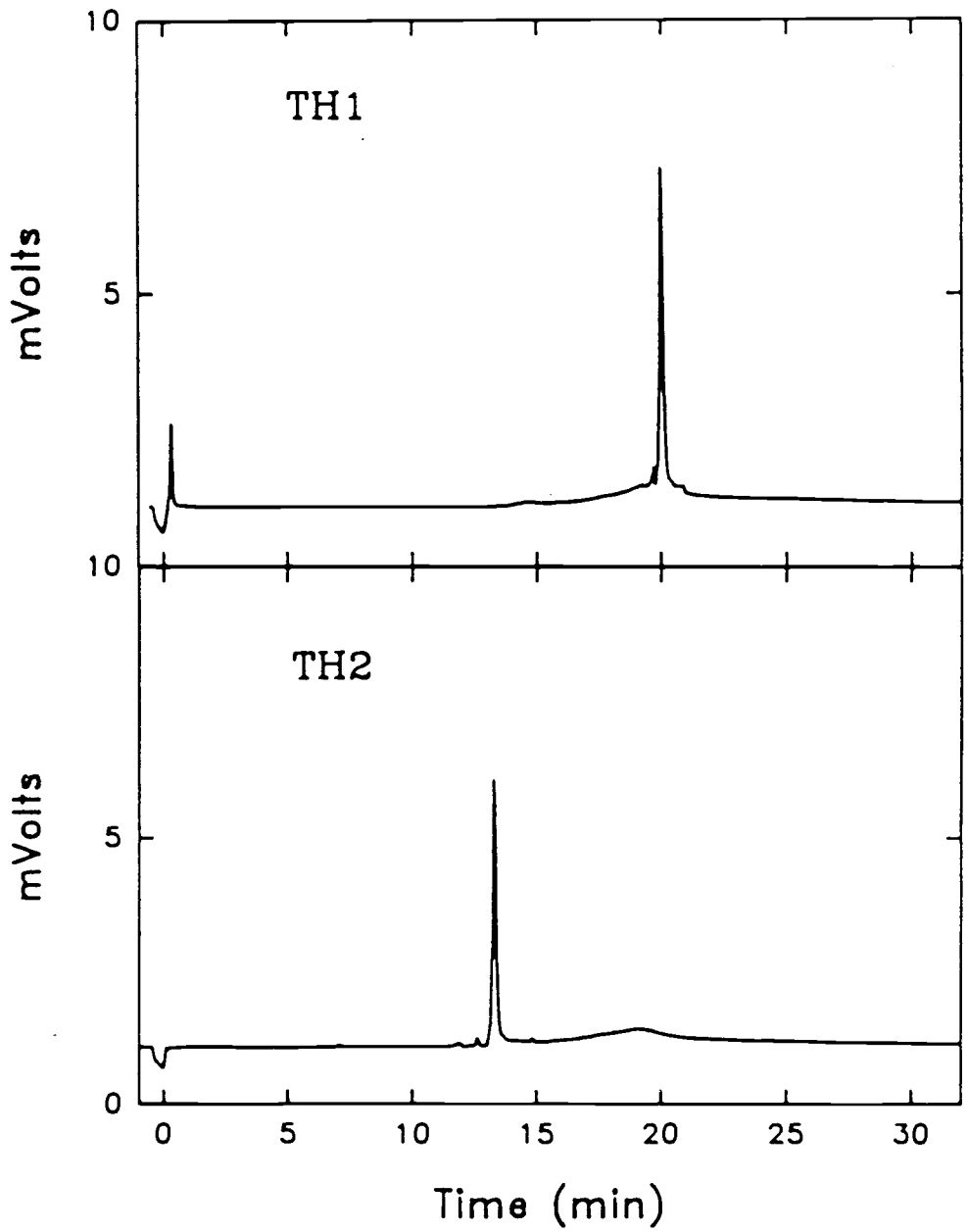

Figure A-4B

Amino Acid Sequencing of Fragment TH2

Amino acid sequencing of TH2 was accomplished by an Applied Biosystem 475A gas phase protein sequencer. The first 5 amino acid sequence of TH2 at N-terminal are:

Ala-Ser-Gly-Glu-His

which corresponds to the position 120-125 in the intact troponin C molecule. This sequence suggests that TH2 corresponds to amino acid residues 120-159 in intact troponin molecule.

Partial amino acid sequencing of TH1 has been done by Leavis *et al.* (1978b) with 3 amino acids resolved from C-terminal.

Amino Acid Composition Analysis

Amino acid analyses were performed according to the precolumn dabsylation and reverse-phase HPLC methods developed by Malencik *et al.* (1990). 4 μg HPLC purified fragments of troponin C were hydrolyzed with 6 M HCl in the vapor phase for 24 hrs at 110 °C. For derivatization, 50 μL of 50 mM $NaHCO_3$, pH 8.5, was added followed by 100 μL of 4 mM dabsyl chloride (400 nmol) in acetonitrile at 70 °C for 10 minutes. Then 50 mM sodium phosphate pH 6.8-ethanol (v:v = 1:1) was added to bring the volume to 1.0 mL. 20 μL sample was injected and analyzed on a C18 reverse-phase HPLC

column.

Table A-2 shows the results of amino acid analysis employing intact TnC as a standard.

Amino acid analysis verifies that the phenyl-agarose binding fraction corresponds essentially to the N-terminal half of troponin C (TR1) and that the non-binding fraction represents the C-terminal half (TR2). Each of these 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).

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).

Table A-2

Amino Acid Composition of the Proteolytic Fragments of Troponin C

Fragments Residues	TR1 (9-84, 9-88)	TR2 (85-159, 89-159)	TH1 (1-120)	TH2 (121-159)
Asx	7.7 (8,9)	10.0 (12,13)	15.2 (14)	7.7 (8)
Glx	12.3 (13,14)	17.0 (14,15)	26.0 (24)	7.8 (7)
Ser	3.7 (4)	2.8 (3)	5.5 (5)	2.1 (2)
Thr	3.7 (4)	1.4 (1)	5.6 (5)	1.2 (1)
Gly	7.2 (7)	5.4 (6)	8.6 (9)	3.8 (4)
Ala	5.4 (5,6)	5.5 (5,6)	11.0 (12)	1.1 (1)
Pro	1.0 (1)	0 (0)	0.5 (1)	0.4 (0)
Val	5.4 (5)	1.6 (2)	5.0 (5)	2.3 (2)
Arg	1.4 (2)	3.1 (4)	5.5 (6)	1.1 (1)
Met	5.9 (7)	2.9 (3)	7.9 (7)	3.1 (3)
Ile	5.4 (5)	4.4 (5)	7.9 (8)	2.2 (2)
Leu	5.3 (5)	3.6 (4)	7.0 (7)	2.0 (2)
Phe	4.9 (5)	4.7 (5)	6.9 (8)	1.7 (2)
Cys	0.1 (0)	0.3 (1)	0.5 (1)	0 (0)
Lys	4.7 (4,5)	3.8 (4,5)	7.4 (6)	3.3 (3)
His	0.2 (0)	1.2 (1)	0 (0)	0.7 (1)
Tyr	1.7 (1)	1.0 (1)	1.5 (2)	0 (0)

The number between brackets indicates the expected number of amino acid.

Appendix 3

Protein-Bound Phosphate Determination

The phosphate content determination was performed according the procedure of Hasegawa *et al.* (1982) with minor modification.

Step 1. Washing. Usually, 1.0-2.0 mg PFK was precipitated with 10% trichloroacetic acid. The pellet was washed three times with 1.5 ml water each and redissolved in 0.5 - 1.0 mL 15% formic acid. Heating in heat block to 50-70 °C will speed up the dissolving. No phosphate loss because of possible hydrolysis of the enzyme (which happens significantly at 100 °C) in this step was detected by using ^{32}P-labeled PFK (at both Ser^{376} and Ser^{774}). The sample was cooled and re-precipitated with 10% trichloroacetic acid followed by washing with water as mentioned before. The dissolving, precipitation, and washing were repeated one more time. The final pellet was dissolved in 1.0 mL 15% formic acid. The protein concentration was determined by measuring the absorbance at 280 nm. The extinction coefficient in 15% formic acid is determined to be the same as that in 50 mM Tris-phosphate (pH 8.0). The value is $E^{1\%}_{280nm} = 10.7$. Usually, 85% protein was recovered after the washing and precipitation procedure.

Step 2. Ashing. 100 to 500 μg of PFK was dried in

acid washed 10 x 75 mm glass tubes by using a speed vacuum concentrator. Phosphoserine -- the standard which is dissolved in water -- was treated in a similar way. After drying, 20 μL of 50% sulfuric acid and 20 μL of perchloric acid were added to each sample. The tubes are placed in a aluminum blocks heated on a hot plate. The temperature was raised to 140 °C, maintained at that temperature for 10 min, and then it was increased to 190 °C and held there until the color of the samples becomes clear. This usually takes at least 30 min. The brown color caused by carbon interferes with the color development in the following step.

Step 3. Color Development. After cooling to room temperature, the inner wall of the tubes was washed by addition of 250 μL of water followed by brief centrifugation. The color development was started by addition of 35 μL fresh ascorbic acid solution (10%, w/v) followed by 90 μL ammonium molybdate solution (1%, w/v). The tubes were covered with parafilm. After 20 min incubation at 65 °C, the samples were cooled on ice and the absorbance at 820 nm was measured. The absorbance was measured directly without dilution at 820 nm in a LKB spectrophotometer. Figure A-5 shows the standard curve obtained with phosphoserine.

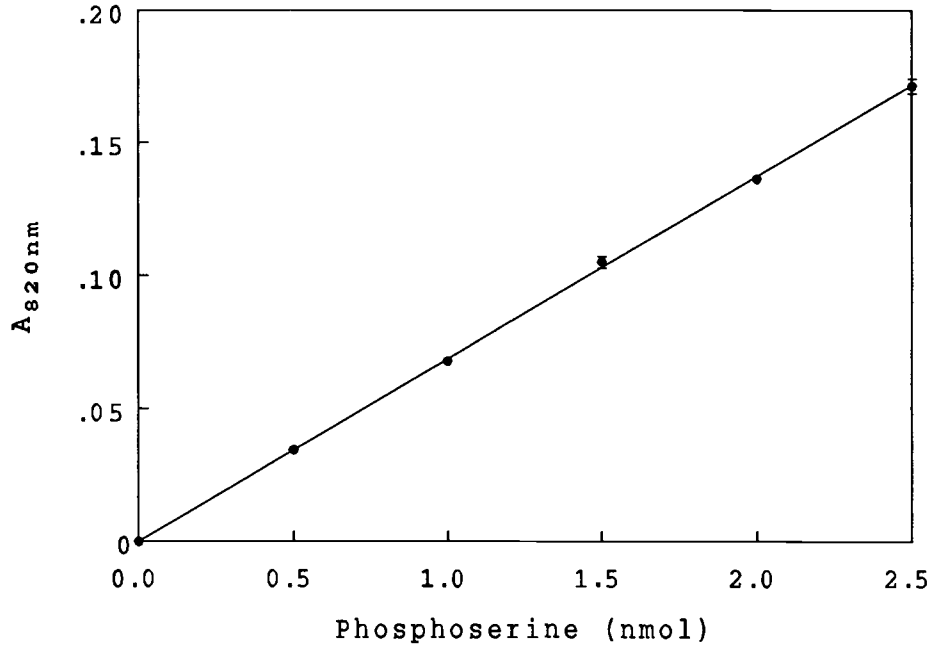

Figure A-5: Standard curve of phosphate determination. Phosphoserine was used as a standard in the protein bound phosphate determination. 0 - 2.5 nano mole of phosphoserine samples were ashed with 20 μL of 50% sulfuric acid and 20 μL of perchloric followed the procedure used for proteins. The color development was started by addition of 250 μL water followed by addition of 35 μL fresh ascorbic acid solution (10%, w/v) and 90 μL ammonium molybdate solution (1%, w/v) and incubated at 65°C for 20 min. The A_{820} was measured directly after cooling