Expression of Bovine Heart Fructose 6-Phosphate,2-kinase:Fructose 2,6-Bisphosphatase and Determination of the Role of the Carboxyl Terminus by Mutagenesis†

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ABSTRACT: Bovine heart fructose 6-P,2-kinase:fructose 2,6-bisphosphatase was expressed in *Escherichia coli*. In order to determine the role of the carboxyl-terminal peptide, 49 and 78 amino acids from the C-terminus were deleted using oligonucleotide-directed mutagenesis. The expressed wild-type and mutant enzymes were purified to homogeneity, and the steady-state kinetics of the mutant enzymes were compared to those of the wild-type enzyme. Deletion of 49 residues (Del 49) resulted in a 35% decrease in K_m^{Fru6P}, a 36% increase in V_{max}, and a 2-fold increase in k_{cat}/K_m of the kinase. There was no change in the kinetic properties of the phosphatase activity. Deletion of 78 residues (Del 78) resulted in a 4.5-fold decrease in K_m^{Fru6P}, a 2.5-fold increase in V_{max}, a 12-fold increase in k_{cat}/K_m of the kinase, and a 3-fold increase in k_{cat}/K_m of the phosphatase. Phosphorylation of the wild-type and Del 49 enzymes resulted in decreased K_m^{Fru6P} and activation of the kinase without affecting the phosphatase activity. Thermal inactivation rates of the wild-type and Del 49 enzymes were similar, but the rate of Del 78 was more rapid. The phosphorylated wild-type and Del 49 enzymes were more sensitive to thermal inactivation than the dephospho forms. Urea inactivation of the kinase and phosphatase of wild-type and Del 49 were similar, but Del 78 was more sensitive to urea. All phosphorylated enzymes were more susceptible to urea inactivation. These results suggest that the C-terminal peptide of the enzyme, especially the region Phe^{453}–Asn^{482}, containing protein kinase A and C phosphorylation sites, is important in maintaining less active (T) states of the kinase and the phosphatase domains. Phosphorylation of the peptide converts the kinase to a more active (R) state without affecting the phosphatase, but deletion of the peptide results in activation of the phosphatase to R state.

The synthesis and the degradation of fructose 2,6-bisphosphate (Fru 2,6-P_2),[1] a potent activator of phosphofructokinase, are catalyzed by a bifunctional enzyme, Fru 6-P,2-kinase:Fru 2,6-Pase. The concentration of Fru 2,6-P_2 is determined by the relative activities of the kinase and the phosphatase. Different isozymic forms of the bifunctional enzymes exhibit different kinase/phosphatase ratios. Thus far, three major types of the isozymes are known to occur in mammalian tissues, namely, heart, liver, and testis. These isozymes are homodimers, consisting of subunit molecular weights ranging from 54 000 to 60 000. The amino acid sequences of the liver (Darville *et al.*, 1987; Lively *et al.*, 1988), skeletal muscle (Crepin *et al.*, 1989), heart (Sakata & Uyeda, 1990), and testis (Sakata *et al.*, 1991) have been determined. A comparison of the amino acid sequences reveals that the kinase domains (N-terminal half) and the phosphatase domains (C-terminal half) are well conserved (Scheme 1). However, the extreme amino and carboxyl termini are completely different, and these differences are responsible at least in part for varying kinase/phosphatase ratios among the isozymes. Moreover, these terminal peptides contain phosphorylation sites for protein kinases subject to regulation. The liver and muscle Fru 6-P,2-kinase: Fru 2,6-Pases are the same gene products except that the muscle enzyme lacks 23 amino acids at the N-terminus and the subsequent 9 amino acids are different (Crepin *et al.*, 1989). These differences result in 3-fold higher Fru 2,6-Pase activity without affecting Fru 6-P,2-kinase in the muscle enzyme (Kitamura *et al.*, 1989). Furthermore, the liver enzyme is phosphorylated on the N-terminus by protein kinase A (P_A), which results in inhibition of the kinase and activation of the phosphatase (Furuya *et al.*, 1982; El-Maghrabi *et al.*, 1982; van Schaftingen & Hers, 1981).

The rat testis bifunctional enzyme is similar to the liver enzyme; its C-terminus is identical except for a few conservative amino acid substitutions (Sakata *et al.*, 1991). The amino-terminal peptide is approximately the same length as that of the liver enzyme (Darville *et al.*, 1987; Lively *et al.*, 1988) but contains no phosphorylation site. However, a phosphorylation site for protein kinase A can be introduced in the testis enzyme by mutagenesis at Ser^{27}, which is located a similar distance from the N-terminus as in the liver enzyme, and the phosphorylated testis enzyme exhibits similar changes in the activities of the bifunctional enzyme (Abe & Uyeda, 1994).

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[1] Abbreviations: WT, wild type; *E. coli*, *Escherichia coli*; Fru 6-P,2-kinase:Fru 2,6-Pase, fructose 6-P,2-kinase:fructose 2,6-bisphosphatase; Fru 2,6-P_2, fructose 2,6-P_2.

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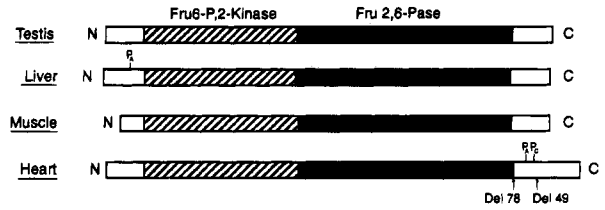
Scheme 1



The heart enzyme is unique among Fru 6-P,2-kinase:Fru 2,6-Pases in that it contains a long carboxyl terminus, 61 amino acids longer than the liver or the testis enzymes (Sakata & Uyeda, 1990). This difference may contribute partly to the lower Fru 2,6-Pase activity compared to the others. More importantly in regulation, the C-terminal region contains phosphorylation sites for protein kinases A (P_A, Scheme 1) and C (P_C, Scheme 1) at Ser^{466} and Thr^{475}, respectively (Kitamura *et al.*, 1988), and as a result of phosphorylation, the kinase is activated (Kitamura & Uyeda, 1989). This response to phosphorylation is the opposite of the response seen with liver enzyme. One of our goals is to understand the molecular mechanisms for these opposite effects on the enzyme activities induced by the phosphorylation on the opposite termini. In this paper, we report overexpression of bovine heart Fru 6-P,2-kinase:Fru 2,6-Pase enzymes in *E. coli*. To determine the significance of the C-terminus of the heart enzyme, we constructed deletion mutants from the bifunctional enzyme lacking 49 (Del 49) and 78 (Del 78) amino acid residues from the C-terminus and characterized these enzymes. The effect of phosphorylation by protein kinase A was also investigated.

EXPERIMENTAL PROCEDURES

Materials. The pKK223-3 vector DNA, restriction enzymes, and bacteriophage T4 DNA ligase were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Muta-Gene M13 *in vitro* mutagenesis kit was purchased from Bio-Rad Laboratories (Hercules, CA). The pT7-7 RNA polymerase/promoter plasmid was a gift of Dr. Stan Tabor (Harvard Medical School). The catalytic subunit of bovine heart cAMP-dependent protein kinase was purchased from Promega (Madison, WI). All other chemicals were reagent grade and obtained from commercial sources.

Construction of Expression Vector. Unless otherwise noted, molecular biology methods were from Sambrook *et al.* (1989). To construct an expression vector carrying the desired bovine heart Fru 6-P,2-kinase:Fru 2,6-Pase gene (BH2K), the overall method of Watanabe *et al.* (1989) was used in which the cDNA was first cloned into an RNA polymerase expression vector and then subcloned along with the regulatory signals into a high copy number vector.

The cDNA was prepared as described before (Sakata & Uyeda, 1990). An *Nde*I site was introduced into cDNA at the initiation codon using two complementary oligonucleotides: 5′-TATGTCCGGGAATCCTGCCTCTTCC-3′ and 5′-TGAGGAAGAGGCAGGATTCCCGGACA-3′. This DNA was ligated into *Nde*I−*Eco*RI doubly-digested pT7-7 DNA, and *E. coli* BL21 was transformed with the ligation mixture, selecting ampicillin-resistant colonies. Following confirmation that the clone carried the required cDNA, this plasmid (BH2K/pT7-7) was digested with *Nde*I. The recessed 3′ terminus created by the digestion was filled using DNA polymerase I, thus creating a blunt-end fragment. This DNA was then digested with *Hin*dIII (which removed most of the pT7-7 vector including a regulatory signal), producing a fragment carrying BH2K DNA with one blunt end and one *Hin*dIII end.

The vector pKK233-3 (Brosius & Holy, 1984) was digested with *Eco*RI, with mung bean nuclease to create blunt ends, and finally with *Hin*dIII. The BH2K and vector fragments were ligated and transformed into *E. coli* as described above.

To increase the copy number, this derivative was doubly-digested with *Sph*I and *Sca*I (Watanabe *et al.*, 1989), producing a 3.1 kb fragment containing the Tac promoter, the ribosomal binding site, and the full-length BH2K DNA. This DNA was ligated with *Sph*I−*Sma*I doubly-digested pUC18 vector DNA (Norrander *et al.*, 1983), and the mixture was transformed into *E. coli* BL21 (DE3). Following confirmation that the derivative contains the desired plasmid, i.e., BH2K DNA at the Tac promoter and ribosomal binding site from pKK223-3 cloned into pUC18, the *E. coli* strain carrying this plasmid (named BH2K/pKK/pUC18) was grown at 22 °C for 16 h in 2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM potassium phosphate (pH 7.5), 30 μM isopropyl β-D-thiogalactoside, and 50 μg/mL ampicillin.

Site-Directed Mutagenesis. For site-directed mutagenesis, BH2K DNA was recloned into M13mp18 as follows. The DNA BH2K/pT7-7 (pT7-7 carrying BH2K cDNA, described above) was purified and digested with enzyme *Xba*I, producing a 1.8 kb fragment containing the full-length BH2K cDNA, which was then ligated into the *Xba*I site of M13mp18. JM109 was transformed with the ligation mixture, selecting Amp^r. Following confirmation by restriction mapping that the derivative carried BH2K DNA, the single-stranded derivative was transfected into CJ236 (dut^-ung^-), yielding the M13 clone carrying BH2K DNA. The oligonucleotide-directed *in vitro* mutagenesis was performed as described by Kunkel (1985) using the Muta-gene M13 *in vitro* mutagenesis kit (BioRad, Hercules, CA). Synthetic oligonucleotide primers were prepared: 5′-CCAA-GAAATTAGAGTGTTGGGTGACGGCCCCTC-3′ (Del 49), in which codon TAC (tyrosine 482) was mutated to TAG, and 5′-ACTAACAACTGACCCAAGAGCTAAAC-CCCTGTA-3′ (Del 78), in which codon TTT (phenylalanine 453) was mutated to TGA. Mutant derivatives were identified by DNA sequencing. Plasmid DNAs carrying the mutated genes were digested with *Xba*I and subcloned into the *Xba*I site of vector pT7-7 using the standard cloning techniques described above. To increase copy number, the mutated DNA was recloned into pUC18 (as described above for the WT gene). In this case, each plasmid was doubly-digested with *Eco*RV, which recognizes a site in the middle of the BH2K DNA, and *Sma*I, which recognizes a site in the vector. The 0.7 kb fragments thus produced, which contained the mutated regions Del 49 and Del 78, were purified for ligation with a 5.1 kb *Eco*RV−*Sma*I vector fragment purified from BH2K/pKK/pUC18 (the pUC18 derivative carrying WT BH2K DNA, whose construction was described above). The desired clones would thus substitute Del 49 and Del 78 DNA for the WT carboxyl terminus of the encoded enzyme. Following transformation of the ligation mixture into JM109, selecting for Amp^r, the structure of purified clones was confirmed by DNA sequencing. *E.*

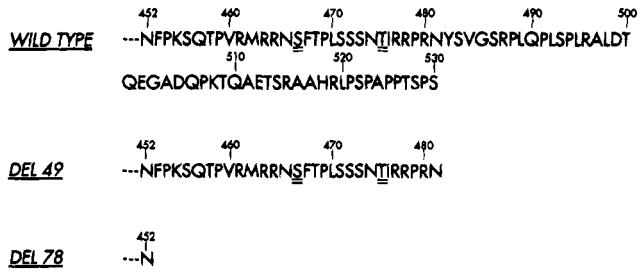


FIGURE 1: Primary structure of the carboxy-terminal amino acid sequence. Ser^{466} and Thr^{475} are phosphorylation sites for protein kinases A and C, respectively.

coli BL21 (DE3) was transformed with the DNA of these constructs which contained DNA encoding bovine heart Fru 6-P,2-kinase:Fru 2,6-Pase that lacked either the C-terminal Phe^{482}–Ser^{530} (Del 49/pKKpUC18) or the C-terminal Tyr^{453}–Ser^{530} (Del 78/pKK/pUC18) (Figure 1). The cells were grown as described above.

Assay Method for Fru 6-P,2-kinase. The assay is based on the determination of Fru 2,6-P_2 and is the method described previously (Furuya & Uyeda, 1981) with slight modification. The reaction mixture contained 100 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 5 mM ATP, 1 mM Fru 6-P, and 10 mM $MgCl_2$ in 0.1 mL. The reaction was initiated with addition of the enzyme. The mixture was incubated at 30 °C; at various time intervals, aliquots (10 μL) were transferred into 90 μL of 0.1 N NaOH, and the diluted solution was heated for 1 min at 90 °C to stop the reaction. Suitable aliquots of the heated reaction mixture were then assayed for Fru 2,6-P_2 as described (Uyeda *et al.*, 1981). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 2,6-P_2/min under these conditions. Double-reciprocal plots were used for the determination of the apparent K_m and V_{max} values.

Assay Method for Fru 2,6-Pase. This coupled fluorometric assay measures continuously the formation of Fru 6-P coupled to NADPH formation using phosphoglucose isomerase and Glu-6-P dehydrogenase as described previously (Tominaga *et al.*, 1993). The reaction mixture contained in a final volume of 0.6 mL 100 mM Tris/HCl (pH 7.5), 0.2 mM EDTA, 100 μM NADP, 17 μM Fru 2,6-P_2, 0.4 unit of Glu-6-P dehydrogenase, and 1 unit of phosphoglucose isomerase. The reaction was initiated with addition of Fru 6-P,2 kinase:Fru 2,6-Pase, and it was followed at room temperature fluorometrically at excitation and emission wavelengths at 354 and 452 nm, respectively.

Phosphorylation of the Bifunctional Enzymes by cAMP-Dependent Protein Kinase. The reaction mixture contained in a final volume of 0.1 mL 50 mM Tris/phosphate (pH 7.5), 0.2 mM [γ-^{32}P]ATP (1000 cpm/pmol), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol, and the heart bifunctional enzyme (0.1 mg). The reaction was initiated by addition of the catalytic subunit of cAMP-dependent protein kinase (5 units), and the mixture was incubated at 30 °C for 30 min. An aliquot of the reaction mixture was removed, and [^{32}P]phosphate incorporation was determined according to the procedure of Roskoski (1983). Under these conditions, at least 0.9 mol of P_i incorporated per mole of subunit.

Heat Denaturation. The stability of Fru 6-P,2-kinase:Fru 2,6-Pase at 50 °C was determined by incubating the enzyme at 0.1 mg/mL (1 μM) in 50 mM Tris/phosphate (pH 7.5), 0.5 mM EDTA, and 2 mM dithiothreitol in a constant-temperature bath. Aliquots of the enzyme were removed at given time intervals, chilled in ice immediately, and assayed for residual activity.

Urea Inactivation. Fru 6-P,2-kinase:Fru 2,6-Pase (0.1 mg/mL) in 50 mM Tris/phosphate (pH 7.5), 0.5 mM EDTA, 2 mM dithiothreitol, and varying concentrations of urea was incubated at 0 °C for 1 h. Aliquots of the enzyme were removed and assayed for the kinase and the phosphatase activities.

Other Methods. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on a Phast Gel gradient (4–15%) using Phast System (Pharmacia–LKB, Piscataway, NJ).

RESULTS AND DISCUSSION

Overexpression of Bovine Heart Fru 6-P,2-kinase:Fru 2,6-Pase. A variety of expression vectors and host cells were examined in order to maximize the expression of the heart bifunctional enzyme. Those vectors included pT7-7, pQE9, pMal-C2, pKK223-3, and pGEX-CS, and the bacterial cells included BL21 (DE3), BL21 (LysS), M15, JM83, JM109, and HB101. Cell growth conditions were also varied, but these systems resulted in expression of the enzyme in less than 0.1% of total soluble proteins in the cell extracts. Part of the poor expression might be related to the extreme susceptibility of the heart isozyme to protease digestion in these cells even in the "protease-less" cells. However, the use of the modified vector, pKK223-3/pUC18 (Watanabe *et al.*, 1989), as described under Experimental Procedures, greatly improved the expression of the enzymes, yielding 1–3% of the soluble protein in the cell extracts, which was comparable to the expression of rat testis enzyme in BL21 cells (Tominaga *et al.*, 1993).

Purification of Bovine Heart Bifunctional Enzymes. (A) Extract. E. coli cells containing the cloned genes were grown as described under Experimental Procedures, and 48 g of the cells was suspended in 96 mL of homogenizing solution containing 50 mM Tris/phosphate (pH 8.0), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 2 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride, and 0.1 μg/mL leupeptin. Lysozyme (96 mg) was added to the suspension, and the suspension was incubated at 4 °C for 30 min. Following incubation, the mixture was sonicated 3 times for 30 s each. All operations were performed at 4 °C. The homogenate was centrifuged at 46000*g* for 20 min.

(B) Protamine Sulfate. To the supernatant solution (114 mL) was added 14 mL of 2% protamine sulfate with mixing, and the mixture was allowed to stand for 5 min at 4 °C. The precipitate was removed by centrifugation at 46000*g* for 10 min.

(C) Poly(ethylene glycol) Precipitation. Sufficient poly(ethylene glycol) (M_r = 8000) was added to the supernatant solution to bring the concentration to 3.5%, and after 20 min, the enzyme solution was centrifuged at 46000*g* for 20 min.

(D) Green Sepharose Chromatography. The supernatant solution was applied to a Green Sepharose column (Green-5, Sigma Chemical Co.) (2.2 cm × 5 cm), which had been equilibrated with 50 mM Tris/phosphate (pH 7.5), 0.5 mM

Table 1: Steady-State Kinetic Parameters of Bovine Heart Fru 6-P,2-kinase:Fru 2,6-Pase and the Mutant Enzymes[a]

	WT		Del 49		
	dephospho	phospho	dephospho	phospho	Del 78
		Fru 6-P,2-kinase			
K_m^{Fru6P} (μM)	23 ± 2.2	9.6 ± 1.1	15 ± 1.2	9.5 ± 1.4	4.9 ± 0.4
K_m^{ATP} (μM)	83 ± 3	74 ± 8	86 ± 7	84 ± 10	52 ± 2
V_{max} (mU/mg)	42 ± 4.4	74 ± 4.1	57 ± 3.1	77 ± 0.4	106 ± 4.7
k_{cat}/K_m^{F6P} (M^{-1} s^{-1}) ($\times 10^3$)	3.1	13	6.3	14	36
k_{cat}/K_m^{ATP} (M^{-1} s^{-1}) ($\times 10^3$)	0.85	1.7	1.1	1.5	3.4
		Fru 2,6-Pase			
$K_m^{Fru26P2}$ (μM)	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.00
V_{max} (mU/mg)	17 ± 1.3	17 ± 0.4	16 ± 1.6	17 ± 2.0	49 ± 0.4
k_{cat}/K_m^{F26P2} (M^{-1} s^{-1}) ($\times 10^5$)	3.1	4.0	3.3	3.5	10

[a] Fru 6-P,2-kinase was assayed as described under Experimental Procedures except that for the determination of K_m^{Fru6P}, 2 mM ATP was used, and 5 mM Fru 6-P was used for K_m^{ATP} determination. Fru 2,6-Pase activity was determined as described under Experimental Procedures. These values are the average ± SE of at least three determinations. mU, milliunits.

EDTA, 0.5 mM EGTA, and 1% poly(ethylene glycol) (M_r = 300) (buffer A). The column was washed with 50 mM Tris/phosphate (pH 7.5), 0.2 M KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% poly(ethylene glycol) (M_r = 300), 5% glycerol, 2 mM dithiothreitol, 2 mM benzamidine, and 0.1 μg/mL leupeptin (buffer A) until the A_{280} was less than 0.1. The enzyme was eluted with buffer A containing 1.5 M KCl and concentrated using an Amicon concentrator equipped with a YM-30 membrane. The enzyme was desalted by passing through a Sephadex G50 column (2.2 cm × 18.5 cm) which had been equilibrated with buffer A.

(E) Phosphocellulose Chromatography. The desalted enzyme was adsorbed onto a phosphocellulose column (P11) (1.6 cm × 9 cm) which had been equilibrated with buffer A, and the column was washed with buffer A containing 0.07 M potassium phosphate (pH 7.5). The enzyme was eluted from the column with a linear gradient of potassium phosphate (pH 7.5), from 0.07 to 0.15 M in a total volume of 100 mL. The fractions containing a major portion of the enzyme were pooled and concentrated with an Amicon concentrator equipped with a YM-30 membrane. This purification procedure usually yielded WT Fru 6-P,2-kinase: Fru 2,6-Pase with a specific Fru 2,6-Pase activity of 12 milliunits/mg in 10% yield.

Purification of Mutant Enzymes. The cell lysis, protamine sulfate treatment, poly(ethylene glycol) precipitation, and Green Sepharose chromatography were performed as with the WT enzyme. After the Green Sepharose chromatography step, the enzyme was desalted on a Sephadex G50 column which had been equilibrated with 15 mM Tris/phosphate (pH 7.5), 0.5 mM EDTA, and 10 mM dithiothreitol (buffer B).

(A) Yellow-3 Chromatography. The desalted enzyme was adsorbed onto an active yellow-3 column (column volume = 20 mL) (Sigma Chemical Co., St. Louis, MO) which had been equilibrated with buffer B, and the column was washed with 50 mL of buffer B. The mutant enzyme, Del 49, was eluted from the column with a linear gradient of potassium phosphate from 0 to 0.3 M, concentrated, and desalted on a Sephadex G50 column which had been equilibrated with buffer A. The Del 78 was eluted from the column with linear gradient of ATP−Mg^{2+} from 0 to 10 mM.

(B) DEAE Chromatography. The desalted Del 49 or the eluted Del 78 from the yellow-3 column was applied to a DEAE (Toyopearl, TosoHaas-Montgomeryville, PA) column (1.6 × 7.5 cm) which had been equilibrated with buffer A. The column was washed with the same solution until the A_{280} is less than 0.1, and the enzyme was eluted with a linear gradient of potassium phosphate (pH 7.5), from 0 to 0.15 M. The fractions containing a major portion of the enzyme were pooled and concentrated with the Amicon concentrator. The concentrated enzyme was stored at −70 °C.

The WT and the mutant enzymes were purified to apparent homogeneity. Upon storage or repeated freezing and thawing, small amounts of lower molecular weight proteins were often formed. The estimated molecular weights of WT, Del 49, and Del 78 were 60 000, 56 000, and 53 000, respectively, compared to the theoretical molecular weights of 60 700, 55 500, and 52 100.

All enzymes were desalted before use by column centrifugation (Penefsky, 1977) on a Sephadex G50 column (1 mL) which had been equilibrated with 50 mM Tris/phosphate (pH 7.5), 0.05 mM EDTA, and 2 mM dithiothreitol.

Steady-State Kinetic Properties of C-Terminal Deletion Mutants. The steady-state kinetic parameters of the WT and the mutant dephosphoenzymes and the phosphorylated enzymes are summarized in Table 1. Fru 6-P,2-kinase activities (V_{max}) of Del 49 and Del 78, respectively, were 1.3× and 2.5× greater than that of WT. The K_m^{ATP} values of these enzymes were similar, but the K_m^{Fru6P} values of Del 49 and Del 78 were decreased by 35% and 79%, respectively, of that of the WT. Similarly to the kinase activity, the Fru 2,6-Pase activity of Del 78 was 3-fold higher than WT and Del 49 enzymes, but the $K_m^{Fru26P2}$ value was unchanged. The specificity constants (k_{cat}/K_m) of Del 49 and Del 78 for the kinase increased by 2-fold and 12-fold, respectively. Thus, the deletion of 49 amino acid residues of the C-terminus of bovine heart Fru 6-P,2-kinase:Fru 2,6-Pase caused small changes in the kinetic properties, i.e., a 2-fold increase in the k_{cat}/K_m of the kinase and no change in the phosphatase activities. However, the deletion of 78 residues caused drastic changes in the activities as demonstrated by a 12-fold increase in the k_{cat}/K_m of the kinase and a 3-fold increase in the k_{cat}/K_m of the phosphatase. This large increase in the k_{cat}/K_m of the kinase was a result of a 2-fold increase in the V_{max} of the kinase and a nearly 5-fold decrease in the K_m for Fru 6-P. The increased catalytic efficiency of the deletion mutants was probably the result of a combination of factors, including the increased rate of binding, k_1, of Fru 6-P to the kinase binding site, the decreased rate of dissociation, k_{-1}, of Fru 6-P, or a combination of both, and the acceleration of one or more of the catalytic steps. Thus, removal of the entire peptide produced an enzyme with higher catalytic

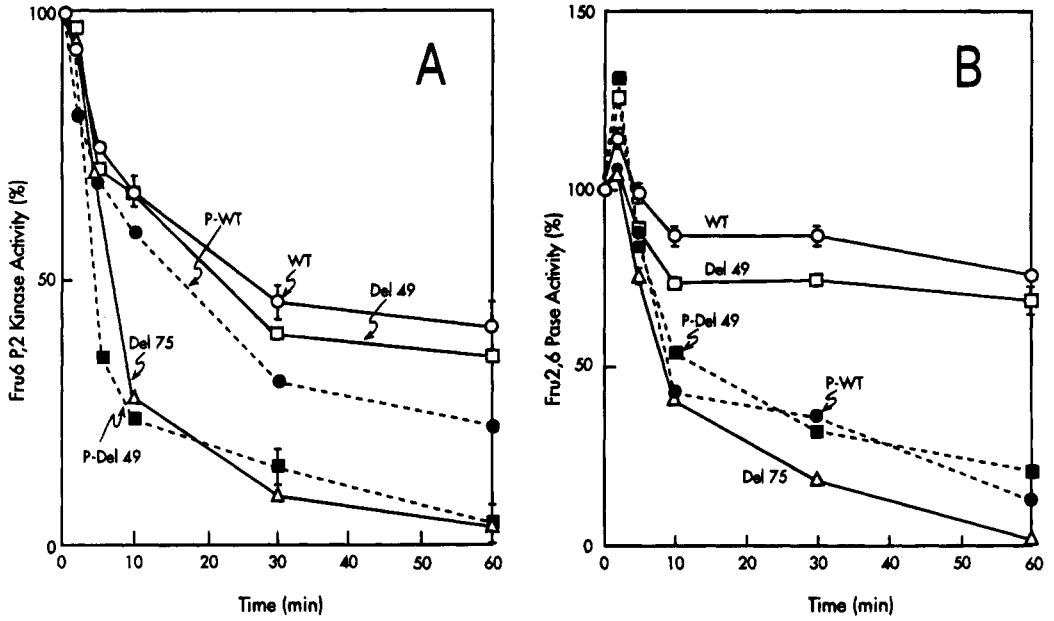


FIGURE 2: Thermal inactivation of dephospho and phospho forms of bovine heart Fru 6-P,2-kinase:Fru 2,6-Pase and the mutant enzymes at 50 °C. (A) Fru 6-P,2-kinase activity; (B) Fru 2,6-Pase activities. The experimental procedure is described under Experimental Procedures. WT (○); Del 49 (□); Del 78 (△); P-WT (phosphorylated WT) (●); and P-Del 49 (phosphorylated Del 49) (■).

efficiency than the WT. This is highly unusual that both the K_m and V_{max} improved by the mutation. In most cases, mutation of enzymes results in an increased K_m and/or a decreased V_{max}. However, this bifunctional enzyme is an allosteric enzyme, and the peptide containing 29 amino acid residues between Asn^{452} and Gln^{481} of the C-terminus of the heart bifunctional enzyme is important in regulating the kinase activity. It is highly significant that this peptide contains two unique phosphorylation sites at Ser^{466} and Thr^{475} for protein kinases A and C, respectively (Kitamura *et al.*, 1989).

The phosphorylation of WT and Del 49 resulted in a higher Fru 6-P,2-kinase activity, confirming our previous results obtained with the heart enzyme isolated from bovine heart (Kitamura *et al.*, 1989), but no significant change was observed in the phosphatase activity compared to the corresponding dephospho forms. The K_m^{Fru6P} values of the phosphorylated WT and Del 49 were decreased by 58% and 37%, respectively, compared to the dephospho forms. Moreover, the phosphorylated WT and Del 49 forms had higher specificity constants than the corresponding dephospho forms of the enzymes. The phosphatase activity of Del 78 showed higher k_{cat}/K_m value than those of WT and Del 49 mutant enzymes. The observation that the deletion of this phosphorylation site peptide activated Fru 2,6-Pase suggests that the C-terminus of the heart bifunctional enzyme somehow holds the enzyme in a less active (both kinase and phosphatase) conformation, and the phosphorylation only converts it to a more active conformation with respect to the kinase without affecting the phosphatase. When the entire C-terminus is removed, then the phosphatase domain is also converted to a more active conformation.

In contrast to the heart bifunctional enzyme, deletion of the N-terminal peptides (regulatory domains of those enzymes) from rat testis (Tominaga *et al.*, 1993) and rat liver (Kurland *et al.*, 1993) causes a significant decrease in the kinase activity and an increase in the phosphatase activity.

Thermal Inactivation. Fru 6-P,2-kinase activities of both WT and Del 49 were inactivated at 50 °C in approximately 30 min (Figure 2). However, the kinase activity of Del 78 was considerably more labile than that of WT or Del 49, losing over 85% in 30 min.

Fru 2,6-Pase activities of WT and Del 49 were more resistant to the heat treatment than the kinase activity, losing only 20% in 60 min. However, Del 78 lost 85% of the phosphatase activity in 30 min.

A comparison of the phosphorylated WT with the dephospho enzyme revealed that the kinase activity of the former enzyme was slightly more sensitive to the thermal inactivation (Figure 2A). However, the phosphorylation of Del 49 increased the thermal lability nearly 2-fold (Figure 2B). The phosphatase activities of phosphorylated WT and Del 49 were also more sensitive to the inactivation, and both enzymes were inactivated with approximately the same rates.

Effect of Urea. Fru 6-P,2-kinase activity of WT and Del 49 did not change significantly (up to 3 M urea), and 50% inactivation occurred at 4.4 M urea (Table 2). In contrast, rat testis bifunctional enzyme is considerably more sensitive to urea inactivation than the heart enzyme, as 50% inactivation occurs at 2.7 M urea (Tominaga *et al.*, 1993). The kinase activity of Del 78 decreased significantly above 2 M urea, and 50% inactivation occurred at 3.3 M, demonstrating decreased stability of Del 78 enzyme in urea. The phosphorylated WT and Del 49 were more sensitive to urea denaturation than the dephospho forms, and 50% inactivation occurred at 3.7 and 3.9 M, respectively. The phosphatase activity of phosphorylated WT enzyme increased 35% at 2 M urea, while phospho Del 49 increased less than 10%.

As was the case with the effect on the enzyme activities, the 49 amino acid residues from the C-terminus appear not to have any significant stabilizing effect against heat or urea inactivation. However, the peptide containing the phosphorylation sites (Asn^{452}–Glu^{481}) was essential for stability, suggesting that the peptide containing several basic residues interacts with some groups (probably acidic residues) on the enzyme surface to stabilize the enzyme. Previous studies (Tominaga *et al.*, 1993, 1994) have shown that rapid loss of both kinase and phosphatase activities of the rat testis enzyme

Table 2: Urea Concentration (M) at Which 50% Inactivation Occurred[a]

	WT		Del 49		
	dephospho	phospho	dephospho	phospho	Del 78
Fru 6-P,2-kinase	4.4	3.7	4.4	3.9	3.3
Fru 2,6-Pase	4.4	4.1	4.3	4.0	3.5

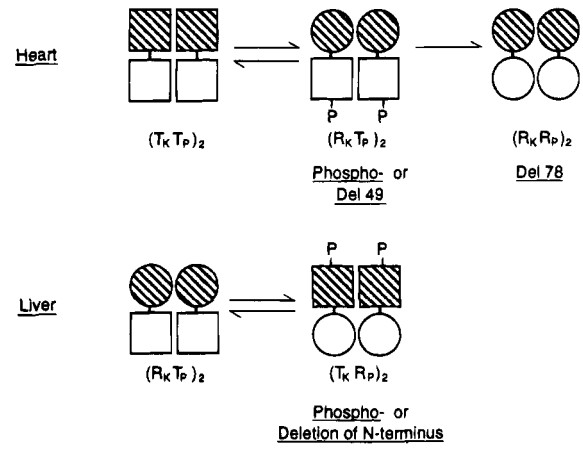
[a] Inactivation in varying concentrations of urea was performed as described under Experimental Procedures.

in urea or guanidine is closely associated with dissociation of a dimer to monomers. Deletion of the N-terminus from rat testis enzyme also results in an enzyme that is more susceptible to protein concentration dependent dissociation (Tominaga *et al.*, 1993). Although we have not demonstrated the dissociation of the heart enzyme, it is reasonable to assume that the dissociation of heart enzyme also caused loss of the enzyme activities, and the C-terminal was probably important in stabilizing the dimeric structure. Phosphorylation of this peptide also resulted in increased sensitivity to urea and thermal inactivation, suggesting that it is affecting the subunit interaction. These results strongly suggest that the regulatory peptide is important for stability, perhaps by strengthening the subunit−subunit interaction.

General Conclusions. Since the three-dimensional structure of the bifunctional enzyme is not yet available, a precise explanation for the role of the C-terminus of the heart enzyme for the stability and allosteric regulation of the enzyme activities cannot be offered. However, the following unique properties of the C-terminal peptide may provide a possible explanation for the observed results. It is important to note that the entire C-terminus of the heart enzyme (78 amino acid residues) is highly basic, containing 9 Arg, 2 Lys, 2 Asp, and 2 Glu, especially the regulatory peptide (29 residues) which contains 5 Arg and 1 Lys but no acidic residue (Figure 1). In contrast, all other isozymes (testis, liver, and muscle) of the bifunctional enzyme have short C-terminal peptides (18 amino acids long), which are acidic, containing 1 Asp and 2 Glu and no basic amino acids. It is possible that the basic C-terminal peptide of the heart enzyme interacts with some acidic residues on the enzyme surface which results in an enzyme conformation with a high K_m for Fru 6-P and a low V_{max} or T state with respect to the Fru 6-P,2-kinase domain $(T_KT_P)_2$ (Scheme 2). The phosphorylation of the peptide weakens some of those ionic interactions by neutralizing one or more positive charges which converts the kinase to the more active low K_m form of the enzyme or R state $(R_KT_P)_2$ (Scheme 2). The weakened interaction is suggested by the fact that the phosphorylation resulted in a more unstable form of the enzyme. Furthermore, the effect of the phosphorylation was seen only with the kinase domain, and the phosphatase domain was unaffected. Thus, the phosphorylated regulatory peptide apparently still has strong interaction with the enzyme surface to maintain the phosphatase domain in the T state. However, the deletion of the entire C-terminus (as in Del 78), including the regulatory peptide, resulted in an activation of both phosphatase and kinase, suggesting that both domains of this enzyme were converted to R states $(R_KR_P)_2$. This resulted in a large increase in the catalytic efficiencies of the kinase and the phosphatase.

These conformational changes in the heart enzyme contrast with those observed for the testis and the liver isozymes.

Scheme 2: Comparison of Possible Conformational States of the Heart and Liver Bifunctional Enzymes upon Phosphorylation by Protein Kinase and by Deletion of N- or C-Terminal Deletion[a]



[a] Open and slashed symbols are the Fru 2,6-Pase domain and the Fru 6-P,2-kinase domain, respectively. R (○) and T (□) states of Fru 6-P,2-kinase are indicated as R_K and T_K, while those of Fru 2,6-Pase are indicated as R_P and T_P.

As discussed in the introduction, phosphorylation of the liver enzyme or the truncated testis enzyme results in inhibition of kinase and activation of phosphatase $(T_KT_P)_2$ (Scheme 2). Deletion of the N-terminus of the testis and liver enzyme also causes similar reciprocal changes in the enzyme activities. Thus, there exists important differences in the roles that these regulatory peptides (N-terminus of the liver enzyme and the C-terminus of the heart enzyme) play. It appears that the effect of any modification of the liver N-terminal peptide leads to one large conformational change which affects both catalytic domains in a concerted manner (Scheme 2). On the other hand, modifications of the C-terminal heart enzyme produce changes in one domain at a time in a sequential manner, first altering the kinase domain followed by the phosphatase domain, resulting in activation of both domains, rather than reciprocal changes. Thus, these results further emphasize important differences in allosteric communication between two catalytic domains mediated by the N- or C-terminal regulatory peptides of liver or testis versus heart Fru 6-P,2-kinase:Fru 2,6-Pases. The large differences in the subunit−subunit interaction between these enzymes, as demonstrated by the difference in the urea inactivation, may contribute in part to the differences in the enzyme conformational changes.

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