Effect of Adding Phosphorylation Sites for cAMP-Dependent Protein Kinase to Rat Testis 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase†

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Received December 22, 1993; Revised Manuscript Received March 15, 1994®

ABSTRACT: In contrast to liver and heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases, the testis isozyme lacks a phosphorylation site for cAMP-dependent protein kinase. In order to determine the effect of phosphorylation site location for the protein kinase on rat testis bifunctional enzyme, consensus amino acid sequences (RRXS) were added at different distances from the N-terminus by site-directed mutagenesis. The expressed wild-type enzyme (WT) and mutant enzymes containing a phosphorylation site at Ser^{7} (mutant enzyme RT2KS7, where RT2K = rat testis 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), Ser^{15} (RT2KS15), or Ser^{30} (RT2KS30) were purified to apparent homogeneity. All the mutant enzymes served as substrates for the protein kinase, and the phosphate incorporation was over 90%. The K_m values of protein kinase A for RT2KS7, RT2KS15, and RT2KS30 were 250 μM, 110 μM, and 50 μM, respectively, and the relative rates were 1, 8, and 23. Various kinetic parameters of dephospho and phospho forms of these enzymes were determined. The kinetic constants of the dephospho form of RT2KS30 were similar to those of WT, but those of RT2KS15 and RT2KS7 showed an 8-fold increase in K_m^{Fru6P}, an approximately 30% decrease in the Fru-6-P,2-kinase activity, and a 3-fold increase in fructose-2,6-bisphosphatase activity. Phosphorylation of RT2KS30 resulted in a shift in the Fru-6-P saturation curve from Michaelis–Menten kinetics to sigmoidal, with increased K_m^{Fru6P} and activation of fructose-2,6-bisphosphatase. The kinetic constants of RT2KS15 and RT2KS7 were not altered by phosphorylation. All the mutant enzymes were more sensitive to heat inactivation than was WT. Furthermore, the phospho-RT2KS30 was more thermally labile than its dephospho form. A Stern–Volmer plot of iodide quenching of RT2KS30 tryptophan fluorescence was nonlinear, but those of the other mutant enzymes were linear. These results suggest that all tryptophans in the RT2KS15 and RT2KPS7 mutant enzymes are more exposed and accessible to iodide than RT2KS30.

The synthesis and the degradation of Fru -2,6-P_2, a potent activator of phosphofructokinase, are catalyzed by a bifunctional enzyme, Fru-6-P,2-kinase/Fru-2,6-Pase.[1] The relative activity of Fru-6-P,2-kinase and Fru-2,6-Pase determines the level of Fru-2,6-P_2 in a given tissue. Different tissues contain tissue-specific isozymes of the bifunctional enzyme, and these isozymes have differing relative activities of kinase and phosphatase [reviewed in Uyeda (1991)]. Several isozymes of various mammalian tissues have been characterized, and the amino acid sequences of the liver (Darville *et al.*, 1987; Lively *et al.*, 1988; Algaier & Uyeda, 1988), skeletal muscle (Crepin *et al.*, 1989), heart (Sakata & Uyeda, 1990), and testis (Sakata *et al.*, 1991) enzymes have been determined. All these isozymes are dimers of identical subunits with M_r ranging from 54 000 to 60 000. The amino acid sequences of both catalytic domains are well-conserved among these isozymes, but the amino and carboxyl termini are completely different. These differences in the terminal peptides appear to determine the different relative activities of the kinase and the phosphatase. For example, the skeletal muscle and the liver enzymes are the same gene products, except the former lacks 23 amino acids from the amino terminus, and the subsequent nine amino acids are different. This results in a 3-fold increase in the muscle phosphatase activity with the same kinase activity (Kitamura *et al.*, 1989). Deletion of either 24 or 30 amino acids from the N-terminus of the rat testis enzyme results in over 70% loss of Fru-6-P,2-kinase and a 2-fold increase in Fru-2,6-Pase activity compared to the WT enzyme (Tominaga *et al.*, 1993). Similar changes have been reported when 22 amino acids were deleted from the N-terminus of the rat liver enzyme (Kurland *et al.*, 1993). The activity ratio of the kinase and phosphatase can be regulated also by phosphorylation/dephosphorylation of either the N- or C-terminus. Phosphorylation of the N-terminus of the liver enzyme results in inhibition of the kinase and activation of the phosphatase (Furuya *et al.*, 1982; El-Maghrabi *et al.*, 1982; van Schaftingen *et al.*, 1981). In contrast, phosphorylation of the C-terminus of the heart bifunctional enzyme results in activation of the kinase without affecting the phosphatase activity (Kitamura & Uyeda, 1987; Kitamura *et al.*, 1988). These observations are consistent with a model in which these bifunctional enzymes consist of conserved catalytic domains to which have been appended isozyme-specific regulatory terminal peptides. One of the objectives of this investigation is to test this model by introducing a phosphorylation site for cAMP-dependent protein kinase in the N-terminus of the rat testis enzyme, which lacks such a site, and determine whether this construct has allosteric characteristics similar to those of the liver enzyme. Another objective is to determine the effect of adding ionic charges by introducing the consensus phosphorylation site peptide, ArgArgAlaSer, to a different location in the N-terminus and then analyze the effect of phosphorylation of these sites on the enzyme activities.

† This work was supported by grants from The Department of Veterans Affairs and NIDDK (DK16194).

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® Abstract published in *Advance ACS Abstracts*, May 1, 1994.

[1] Abbreviations: Fru-6-P,2-kinase/Fru-2,6-Pase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; WT, wild type; RT2K, rat testis 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

0006-2960/94/0433-5766$04.50/0

Various proteins are known to be phosphorylated by cAMP-dependent protein kinase. Information concerning how the protein kinase recognizes a specific phosphorylation site peptide comes from substrate specificity studies using a variety of synthetic peptides [reviewed in Pearson and Kemp (1991), Zetterqvist *et al.* (1990), and Kennely and Krebs (1991)]. More recently, the 3-D structures of a protein kinase–inhibitor complex has been elucidated by X-ray crystallography (Knighton *et al.*, 1991). However, there has been little information regarding protein–substrate specificity; for example, how does moving the location of the phospho site affect the ability of a protein to act as a substrate for the kinase protein. Thus, it is of interest to compare those constructs of the bifunctional enzyme described here as potential substrates for the protein kinase.

MATERIALS AND METHODS

Muscle phosphofructokinase was prepared as described (Uyeda *et al.*, 1978). The cDNA encoding rat testis Fru-6-P,2-kinase/Fru-2,6-Pase was prepared as described (Sakata & Uyeda, 1990). 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). Catalytic subunit of bovine heart cAMP-dependent protein kinase was purchased from Promega (Madison, WI). All other chemicals were reagent grade and were obtained from commercial sources.

Preparation of Mutants by Site-Directed Mutagenesis. Plasmid RT2K/pT7-7, containing the rat testis Fru-6-P,2-kinase/Fru-2,6-Pase (RT2K) gene cloned in a pT7-7 vector (Sakata & Uyeda, 1990), was digested with *Xba*I and *Pst*I, and the isolated 1.7-kilobase fragment was ligated into the *Xba*I–*Pst*I site of M13mp19 (M13/RT2K). The ligation mixture was used to transform *Escherichia coli* JM109-competent cells. The phage harboring M13/RT2K was harvested and transfected into *E. coli* CJ236 (dut^- ung^-). The purified recombinant M13/RT2K phage was used to prepare uracil-containing single-stranded template. Synthetic oligonucleotide primers 5′-GGAGGCCCGCCCGGCGCGCCAGCCAACG (corresponding to nucleotides 99–126 of RT2K except 111 and 114 were altered), 5′-AATCCCCTGAGGAGGATCTCGATGCCATAC (corresponding to nucleotides 62–92 of RT2K except 71–82 were altered), and 5′-GCGTCCCCACGGAGAGCGAGCCAGAATCCC (corresponding to nucleotides 38–67 of RT2K except 47–58 were altered) were prepared. The oligodeoxynucleotide-directed *in vitro* mutagenesis was performed as described by Kunkel (1985) using the Muta-Gene M13 *in vitro* mutagenesis kit. The synthesized double-stranded DNA was used to transform *E. coli* MV1190-competent cells. Mutant derivatives were identified by DNA sequencing (RT2KS30/M13, RT2KS15/M13, and RT2KS7/M13), and the DNAs were digested with *Xba*I and *Bam*HI. The DNA fragments (0.5 kb) containing the mutated RT2K genes were subcloned into the *Xba*I–*Bam*HI sites of RT2K/pT7-7 and expressed in *E. coli* BL21 (DE3) as before (Tominaga *et al.*, 1993). The WT and mutant Fru-6-P,2-kinase/Fru-2,6-Pase enzymes were purified as described previously (Tominaga *et al.*, 1993) and stored at −70 °C. The enzyme was desalted before use by column centrifugation (Penefsky, 1977) on a Sephadex G-50 column (1 mL) which had been equilibrated with 50 mM Tris/phosphate, pH 7.5, 0.5 mM EDTA, and 2 mM dithiothreitol.

Assay Method for Fru-6-P,2-kinase. The assay is based on the determination of Fru-2,6-P_2 and is the same as 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, and 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.

Assay Method for Fru-2,6-Pase. This coupled assay measures continuously the formation of Fru-6-P coupled to NADPH formation using phosphoglucose isomerase and Glu-6-P dehydrogenase as described previously (Kitajima *et al.*, 1984) with slight modification. 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 desalted Glu-6-P dehydrogenase, and 1 unit of desalted phosphoglucose isomerase. To remove phosphate in the enzyme solution, Fru-6-P,2-kinase/Fru-2,6-Pase was desalted by column centrifugation on a Sephadex G-50 column (1 mL) which had been equilibrated with 15 mM Tris/sulfate, pH 7.5, 0.5 mM EDTA, and 2 mM dithiothreitol. The reaction was initiated with the addition of the desalted 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 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, and 2 mM dithiothreitol. The reaction was initiated by addition of the catalytic subunit of cAMP-dependent protein kinase, and the mixture was incubated at 30 °C for 30 min. At the indicated times, suitable aliquots of the reaction mixture were removed, and [^{32}P]phosphate incorporation was determined according to the procedure of Roskoski (1983).

Heat Denaturation. The stability of Fru-6-P,2-kinase/Fru-2,6-Pase at 42 °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 a given time, chilled in ice, and assayed immediately for residual activity.

Fluorescence Quenching. The reaction mixture contained, in a final volume of 0.1 mL, 50 mM Tris/phosphate, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol, varying concentrations of potassium iodide, and the enzyme at 0.1 mg/mL. Additionally, ammonium sulfate was added with the iodide solution to maintain constant ionic strength. The emission spectra of the mixtures were then measured. To prevent I^{3-} formation, 0.1 mM sodium thiosulfate was included in the stock potassium iodide solution (4 M), and this mixture was prepared fresh each time. The reaction mixture was incubated at 20 °C for 5 min.

Other Methods. Protein concentration was determined with the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. For the determination of stoichiometry of phosphate incorporation into enzyme, a more precise protein concentration was determined by amino acid analysis. Poly-

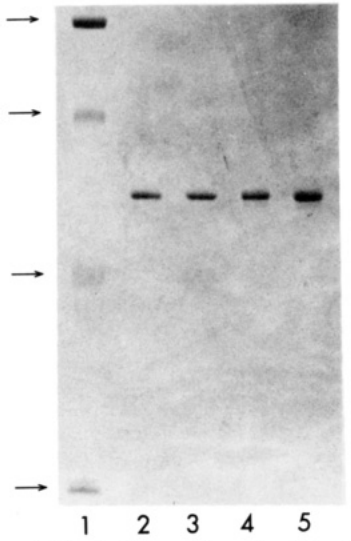

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of RT2K (lane 2), RT2KS30 (lane 3), RT2KS15 (lane 4), and RT2KS7 (lane 5). Lane 1, marker enzymes (from top): phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and carbonic anhydrase (M_r 30 000).

Wild Type (WT)	ASPRELTQNPLKKIWMPVSNGRPALHASQR---
RT2K S30	ASPRELTQNPLKKIWMPVSNGRPARRASQR---
RT2K S15	ASPRELTQNPLRRISMPVSNGRPALHASQR---
RT2K S7	ASPRRASQNPLKKIWMPVSNGRPALHASQR---

FIGURE 2: Primary structure of amino-terminal amino acid sequence. Double-underlined residues: consensus phosphorylation site sequences.

acrylamide gel electrophoresis was performed in 10% acrylamide containing 0.1% sodium dodecyl sulfate according to the procedure of Laemmli (1970).

RESULTS

Mutant Enzymes. WT and the mutant enzymes (RT2KS30, RT2KS15, and RT2KS7) were purified with a previously published procedure (Tominaga *et al.*, 1993). Judging from the gel electrophoresis patterns (Figure 1), these preparations were homogeneous. The molecular weights of these enzymes (lanes 3 and 4) were approximately 55 000, the same as that of the WT enzyme (lane 2). The mutation was confirmed by nucleic acid sequencing, and the deduced amino acid sequences are shown in Figure 2.

Mutant Enzymes as Substrates for Protein Kinase A. The altered enzymes containing phosphorylation sites at varying distances from the N-terminus were tested as substrates for cAMP-dependent protein kinase. Initial rates of phosphorylation of these substrates (Figure 3) and K_m values were determined. As previously shown (Sakata *et al.*, 1991), the testis enzyme (WT) is not phosphorylated by protein kinase A. However, all the mutant enzymes were substrates for the protein kinase, and the phosphate incorporation into all these enzymes was stoichiometric. The results also showed that the K_m values for RT2KS30, RT2KS15, and RT2KS7 were 250 μM, 110 μM, and 50 μM, respectively, and the relative rates were 1, 8, and 23, respectively. These K_m values are 2–63 times larger than those of heart (4 μM) (Kitamura *et al.*, 1988) or liver (4 μM or 22 μM) (Kitamura *et al.*, 1988; Murray *et al.*, 1984) bifunctional enzymes but comparable to synthetic peptides corresponding to the phosphorylation site

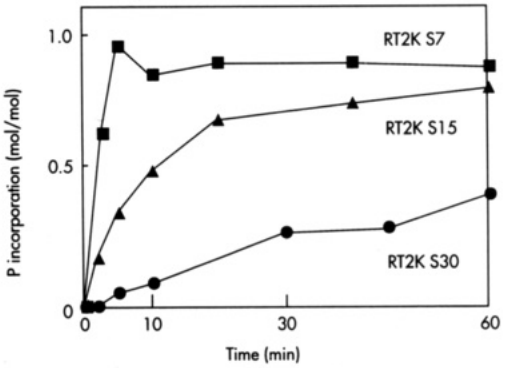

FIGURE 3: Phosphorylation of RT2KS30, RT2KS15, and RT2KS7. The reaction mixture contained, in final volume of 0.1 mL: 0.1 mg of a mutant enzyme, 50 mM Tris/phoshate, pH 7.5, 0.2 mM [γ-^{32}P]-ATP (1000 cpm/pmol), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, and 2 mM dithiothreitol. The reaction was initiated by addition of catalytic subunit of cAMP-dependent protein kinase (5 units). The reaction mixture was incubated at 30 °C. At the indicated times, aliquots (5 μL) were removed, and [^{32}P]phosphate incorporation was determined.

of the liver enzyme (Glass *et al.*, 1986). These results show that RT2KS7, whose phosphorylation site was closest to the N-terminus at Ser^7, was the best substrate for the protein kinase, and RT2KS30, whose site was the farthest at Ser^{28}, was the poorest substrate. The K_m and the phosphorylation rate of RT2KS15, whose site was on Ser^{15}, were in between these two extremes. The amino acid sequence of the phosphorylation site of RT2KS15 was different (RRIS) from those (RRAS) of the other enzymes, which may account for it being a poorer substrate than RT2KS7. These differences may also depend on how these sites are exposed on the enzyme surface and how accessible they are to the protein kinase.

Kinetic Parameters. Kinetic constants of dephospho and phospho forms of WT Fru-6-P,2-kinase/Fru-2,6-Pase and the mutant enzymes are presented in Table 1. Comparison of the dephospho forms of these enzymes indicates that the kinetic properties of the kinase and the phosphatase of RT2KS30 and the WT were similar, but RT2KS15 and RT2KS7 were significantly different. RT2KS15 and RT2KS7 showed an approximate 8-fold increase in the K_m for Fru-6-P and decrease in V_{max} of the kinase, and increase in K_m for Fru-2,6-P_2, and over a 3-fold increase in V_{max} of the phosphatase compared to the values of WT. These results suggest that the introduction of net two positive charges by replacing $E^5 \rightarrow R^5$ in RT2KS7 brought about those changes in the kinetic parameters. It was not expected that the conversion of $K^{12}KIW$ to $R^{12}RIS$ (RT2KS15), where the overall charge was the same, would cause a large change in the kinetic constants of RT2KS15. Furthermore, the substitutions of $L^{25}H^{26} \rightarrow RR$ (RT2KS30), where two positive charges were added, did not affect the activities. However, addition of negative charges by phosphorylation affected the activity as discussed below. Thus, it is clear that the electronic charge at these locations was not the only factor(s) important in the interaction of the N-terminal peptide with the surface of the enzyme molecule, and other forces, including hydrophobic interaction, may be more important.

Comparison of the kinetic properties of the phospho and dephospho forms of the mutant enzymes revealed that only the phosphorylated RT2KS30 showed changes in the kinetic constants as a result of phosphorylation (Table 1). All the phosphorylated enzymes employed in the kinetic studies contained at least 0.9 mol of P/mol of subunit. As shown in Figure 4A, the phosphorylation of RT2KS30 resulted in a shift in the Fru-6-P saturation curve from Michaelis–Menten

Table 1: Kinetic Constants of Rat Testis Fru-6-P,2-kinase/Fru-2,6-Pase and the Mutant Enzymes[a]

	RT2K	RT2KS30		RT2KS15		RT2KS7	
	dephospho	dephospho	phospho	dephospho	phospho	dephospho	phospho
Fru-6-P,2-kinase							
K_m^{Fru6P} (μM)	29	30	49	250	250	250	250
K_m^{ATP} (μM)	80	90	90	83	83	84	84
V_{max} (mU/mg)	43	41	41	28	28	31	31
Fru-2,6-Pase							
$K_m^{F2,6P_2}$ (μM)	0.037	0.038	0.038	0.039	0.039	0.038	0.038
V_{max} (mU/mg)	19	22	29	60	59	58	60

[a] Fru-6-P,2-kinase was assayed as described under Materials and Methods, except that for the determination of K_m^{Fru6P}, 2 mM ATP was used, and for K_m^{ATP} determination, 5 mM Fru-6-P was used. Fru-2,6-Pase activity was determined as described under Materials and Methods. mU = milliunits.

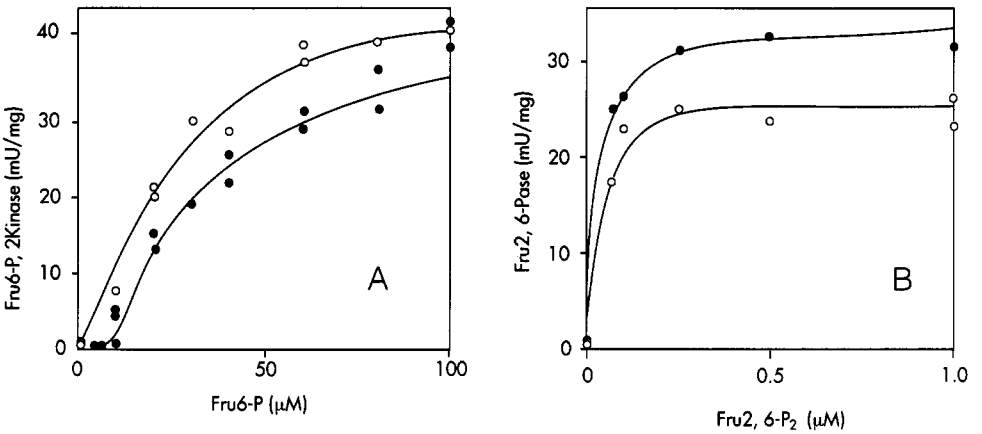

FIGURE 4: Plot of initial reaction velocity (V_0) of dephospho (○) and phospho (●) RT2KS30. (A) Fru-6-P,2-kinase with respect to Fru-6-P concentration; and (B) Fru-2,6-Pase with respect to Fru-2,6-P_2. The assay conditions were as described under Materials and Methods except the ATP concentration was 2 mM, and varying concentrations of Fru-6-P were used.

to sigmoidal, indicating positive cooperativity for the substrate. Phosphorylation led to an increase in the $K_{0.5}^{Fru6P}$ from 30 μM to approximately 50 μM, but the K_m^{ATP} was unchanged. In addition, there was an increase in V_{max} of Fru-2,6-Pase from 22 ± 2 to 29 ± 2 milliunits/mg without affecting $K_m^{Fru26P2}$ (Figure 4B). These kinetic changes were qualitatively similar to those of the phosphorylated liver enzyme (Furuya *et al.*, 1982; El-Maghrabi *et al.*, 1982; van Schaftingen *et al.*, 1981), but they were smaller in magnitude. The phosphorylation of RT2KS15 or RT2KS7 did not affect their kinetic properties (Table 1). These results demonstrated that the testis enzyme could be converted to the liver-type enzyme and that the phosphate must be introduced at or near the 28th amino acid residue from the N-terminus to be effective as an allosteric regulator. Other locations were ineffective. The phosphorylation site of the rat liver enzyme is on Ser^{32} (Lively *et al.*, 1988) which is nearly identical to the altered site in RT2KS30 enzyme.

Thermal Stability. Previous studies from this laboratory demonstrated that deletion of N-terminal peptides made the enzyme more susceptible to thermal inactivation and protein concentration-dependent dissociation (Tominaga *et al.*, 1993). The thermal stability correlated with the length of the deleted N-terminal peptide, which appears to determine the strength of the subunit–subunit interaction. To see if introduction of positive charges at different positions in the N-terminus region affects the subunit–subunit interaction, thermal stabilities of these mutants were compared with that of the WT enzyme. As shown in Figure 5, the WT enzyme was the most stable, and the substituted ArgArg residues toward the N-terminus in the peptide resulted in enzymes with increasing sensitivity toward thermal inactivation. The estimated half-lives of WT, RT2KS30, RT2KS15, and RT2KS7 were approximately 120, 60, 48, and 18 min, respectively.

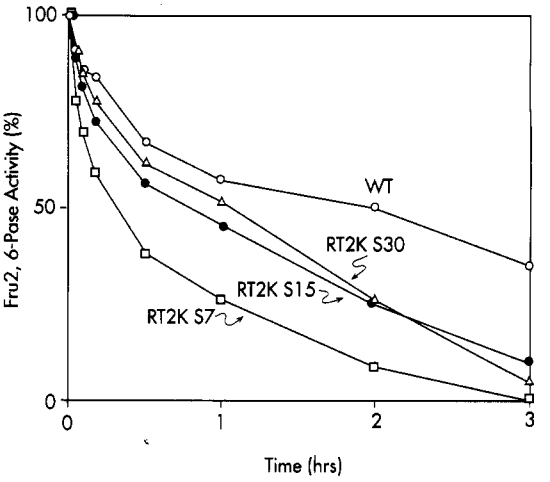

FIGURE 5: Thermal inactivation of rat testis Fru-6-P,2-kinase/Fru-2,6-Pase and the mutant enzymes. The experimental procedure employed is described under Materials and Methods.

Comparison of the phospho and dephospho forms of RT2KS30 demonstrated that the phosphorylated enzyme was nearly 2 times more sensitive to heat inactivation (Figure 6). However, there were no significant differences between the phospho and the dephospho forms of the other mutant enzymes, RT2KS15 and RT2KS7 (data not shown). These results suggested that there were differences in conformation of the phospho and the dephospho forms of RT2KS30 but not the other enzymes.

Intrinsic Fluorescence Quenching by Iodide. Iodide ions can generally quench surface-exposed tryptophan but do not effectively penetrate into the interior of a protein (Lehrer, 1971; Tandon & Horowitz, 1990). Thus, iodide quenching can be useful in estimating the extent of tryptophan exposure

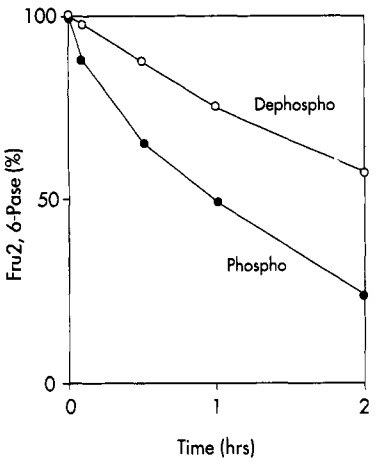

FIGURE 6: Thermal inactivation of dephospho and phospho forms of RT2KS30 at 43 °C. The experimental conditions were as in Figure 5 except 6% glycerol was added. (○), Dephospho and (●) phospho.

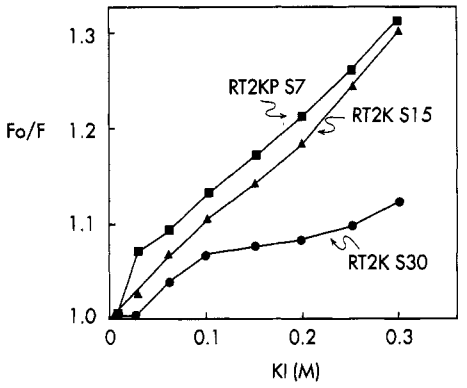

FIGURE 7: Iodide quenching of RT2KS30 (●), RT2KS15 (▲), and RT2KS7 (□). The intrinsic tryptophan fluorescence of the enzyme (1.0 μM) was quenched with increasing concentrations of potassium iodide as described under Materials and Methods.

on the protein surface. The iodide quenching of rat testis Fru-6-P,2-kinase/Fru-2,6-Pase (WT) showed a heterogeneous system of tryptophan accessibility and indicated that most of the tryptophans (total = 4), including Trp^{15} near the N-terminus, were not readily exposed on the enzyme surface.[2] In order to determine whether the tryptophans of the N-terminal peptides or any of the other tryptophans of those mutant enzymes are exposed on the enzyme surface, iodide quenching of the mutant enzyme was determined. A Stern–Volmer equation ($F_0/F = 1 + K_{SV}[X]$) was applied to the data, where F_0 and F are the fluorescence intensity in the absence and the presence, respectively, of a given concentration (X) of a quencher and K_{SV} is the Stern–Volmer quenching constant (Lehrer & Learis, 1978). As shown in Figure 7, RT2KS30 yielded a nonlinear plot that was similar to that of WT,[2] representing a heterogeneous system of tryptophan accessibility, while the plots of RT2KS15 and RT2KS7 were considerably more linear than that of WT within experimental error. The increased quenching of RT2KS15 and RT2KS7 compared to RT2KS30 suggested that Trps in the former enzymes were more exposed than the latter. Moreover, since RT2KS15 lacks Trp^{15}, the similarity of the curves with that of RT2KS7 strongly suggests that iodide quenched the interior Trps rather than the Trp^{15} of the N-terminus. There were no significant differences in iodide quenching of phospho and dephospho forms of these enzymes.

[2] N. Tominaga, D. M. Jameson, and K. Uyeda, manuscript in preparation.

DISCUSSION

Comparison of amino acid sequences of various Fru-6-P,2-kinase/Fru-2,6-Pase isozymes shows that the kinase and the phosphatase domains are very similar, while N- and C-terminal peptides are completely different. This is consistent with a model in which the isozyme-specific regulatory domains located in either terminus have been appended to the well-conserved catalytic domains. The results presented in this paper appear to support this model. By introducing a phosphorylation site for cAMP-dependent protein kinase to the N-terminus, the testis bifunctional enzyme became refractory to regulation by the covalent modification, similar to the liver isozyme. Phosphorylation of this site in the mutant enzyme (RT2KS30) resulted in inhibition of Fru-6-P,2-kinase and activation of Fru-2,6-Pase. The same reciprocal changes have been demonstrated by phosphorylation of the rat liver enzyme (Furuya *et al.*, 1982; El-Maghrabi *et al.*, 1982; van Schaftingen *et al.*, 1981), although the extent of the allosteric changes induced by phosphorylation of rat testis enzyme was smaller than those observed with the liver enzyme. This difference may be due to the sequence of the N-terminal peptide (over 25 amino acids long) of the testis enzyme being significantly different from that of the liver enzyme. It also reflects the importance of the rest of the N-terminus peptide to the changes in the enzyme conformation induced by the phosphorylation. Another possibility is that the consensus phosphorylation site sequence (ArgArgAlaSer), introduced here in rat testis enzyme, affected the degree of kinetic changes. Moreover, the present data also demonstrated the importance of the specific location of the phosphorylation site in order to induce the conformational change; the site must be approximately 28 amino acids from the N-terminus, a site nearly identical to that of the liver enzyme.

One of the obvious effects of the addition of protein kinase A phosphorylation site is the introduction of at least two positive charges in the N-terminus because of addition of two Arg residues. The observation that the kinetic constants of RT2KS30 were identical to those of WT suggested that these new positive charges at R^{25} and R^{26} have little effect on the enzyme conformation. However, substitution of $Glu^5 \rightarrow$ Arg in RT2KS7, causing a net increase of +2 charges near the N-terminus, resulted in nearly 50% decrease in the kinase activity and a 3-fold increase in the phosphatase activity. On the other hand, there was no change in the net charge in the construct, RT2KS15, yet the resulting kinetic properties were the same as those of RT2KS7. These results strongly suggested that the ionic charges were not the only factor important to the interaction of this region of the N-terminus with the enzyme surface, but that the specific location of the charges as well as the other amino acid residues in the peptide are probably more important.

A variety of modifications of the N-terminal peptide of the testis and liver bifunctional enzymes resulted in inhibition of Fru-6-P,2-kinase by raising K_m^{Fru6P}, and activation of Fru-2,6-Pase activity. These modifications include the following: deletion of 22 amino acids from the N-terminus (Kurland *et al.*, 1993) and introduction of one or more negative charges by either phosphorylation (Furuya *et al.*, 1982; El-Maghrabi *et al.*, 1982; van Schaftingen *et al.*, 1981) or conversion of $Ser^{32} \rightarrow$ Asp (Kurland *et al.*, 1992) in the liver enzyme; deletion of 24 and 30 amino acids from the N-terminus of the testis enzyme (Tominaga *et al.*, 1993); introduction of ArgArg-AlaSer sequences near the N-terminus and phosphorylation of these sites; and treatment in dilute urea (Tominaga *et al.*, 1993). These results may be explained by assuming two

conformational states (R and T) of the enzyme based on the concerted transition model (Monod *et al.*, 1963). The native enzyme occurs in the R state with high Fru-6-P,2-kinase activity (low K_m^{Fru6P}) and low Fru-2,6-Pase activity, and modified enzymes occur in the T state with low kinase (high K_m^{Fru6P}) and high phosphatase activities. The following lines of evidence suggest that the R to T transition involves partial local unfolding and weakened subunit–subunit interaction (probably as a result of the partial unfolding): (a) as found previously (Tominaga *et al.*, 1993), tryptophan fluorescence maximum red-shifts in dilute urea; (b) the N-terminus deletion mutants (Tominaga *et al.*, 1993) and those mutants described here were more sensitive to thermal inactivation, dissociation in urea, and protein concentration-dependent dissociation; (c) the phosphorylated RT2KS30 was more susceptible to heat denaturation; and (d) the tryptophan residues of RT2KS15 and RT2KS7 were more exposed to a fluorescence quencher. However, the molecular mechanism of how the N-terminal peptide affects the subunit–subunit interaction which results in the allosteric changes will not be known until 3-D structures of these enzymes are known.

Another objective was to examine how well protein kinase A recognizes protein substrates with phosphorylation sites located at different regions of the N-terminus. Although many proteins have been shown to be phosphorylated, extensive study on the structural requirements for the protein kinase substrates has been carried out with a variety of synthetic peptides containing consensus sequences. Knighton *et al.* (1991) describe the 3-D structure of the catalytic subunit of the protein kinase when complexed to a protein kinase inhibitor peptide. On the basis of the crystal structure they conclude that this peptide contains an amphipathic helix which is followed by a turn and an extend conformation. This extended region of the peptide corresponds to a consensus phosphorylation site of all substrates. The present altered enzymes with phosphorylation sites located near the N-terminus (RT2KS7, RT2KS15) were superior to those substrates whose sites were located more in the interior of the protein, even though they lacked the required secondary structures on the N-terminal to the phosphoacceptor serine. These results suggest that accessibility of the substrate phosphorylation site for the protein kinase active site is as important as, if not more important than, the required conformation for substrate recognition. If the phosphorylation site were located in the interior, there may be higher ordered structure of the protein, thereby causing steric hindrance. On the basis of extensive studies on the substrate specificity of protein kinases, however, it is generally accepted that protein kinases recognize a local structure around the phosphorylation site of a protein substrate and that other parts of the protein are less important in determining the specificity [reviewed in Kennely and Krebs (1991)]. This idea appears not to be supported by the above observations. However, it may explain why RT2KS30 was a poor substrate compared to rat liver Fru-6-P,2-kinase/Fru-2,6-Pase (or bovine heart enzyme). As discussed in the introduction, the majority of amino acid sequences are well conserved among those isozymes, especially the liver and the testis enzymes. The major difference is the N-termini. Thus, the reason for RT2KS30 being a poor substrate compared to the liver enzyme may be because the N-terminal peptide lacks essential extended conformation.

ACKNOWLEDGMENT

We thank Cu Nguyen for his skillful technical assistance and Dr. Sarah McIntire for critical review of the manuscript.

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