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Studies of the Structure of Fructose-6-phosphate 2-Kinase:Fructose-2,6-bisphosphatase†

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ABSTRACT: Some physicochemical properties of a homogeneous preparation of a bifunctional enzyme, fructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase, were determined. The molecular weight of the enzyme is 101 000 as determined by high-speed sedimentation equilibrium. The molecular weight of dissociated enzyme is 55 000 in 6 M guanidinium chloride by sedimentation equilibrium and in sodium dodecyl sulfate by polyacrylamide gel electrophoresis. A value of 4.7 was observed for the isoelectric point. Tryptic peptide maps and high-performance liquid chromatography of the trypsin-digested enzyme revealed approximately 60 peptides. Amino acid analysis of the enzyme shows that it contains 27 lysine and 36 arginine residues per 55 000 daltons. No free N-terminal amino acid residue was detectable, suggesting that it is blocked. Hydrolysis of the enzyme by carboxypeptidases A and B releases tyrosine followed by histidine and arginine, indicating that the amino acid sequence at the carboxyl terminus is probably -Arg-His-Tyr. Tryptic digestion of [^{32}P]phosphofructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase yields a ^{32}P-labeled peptide detected by tryptic peptide mapping and high-performance liquid chromatography. Thermolysin digestion of CNBr-cleaved ^{32}P-enzyme also yields a single ^{32}P-peptide. These results indicate that fructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase is a dimer of 55 000 daltons and the subunits are very similar, if not identical.

The synthesis of fructose 2,6-bisphosphate (fructose-2,6-P_2) from fructose 6-phosphate (fructose-6-P) and ATP is catalyzed by fructose-6-P-2-kinase (Fru-6-P-2-kinase) (eq 1) (Furuya

$$\text{fructose-6-P} + \text{ATP} \rightleftharpoons \text{fructose-2,6-P}_2 + \text{ADP} + \text{H}^+ \quad (1)$$

& Uyeda, 1981; El-Maghrabi et al., 1981; Van Schaftingen & Hers, 1981a). The degradation of fructose-2,6-P_2 is catalyzed by fructose-2,6-bisphosphatase (Fru-2,6-bisphosphatase) (eq 2) (Richards et al., 1982; Van Schaftingen et al., 1982;

$$\text{fructose-2,6-P}_2 + \text{H}_2\text{O} \rightarrow \text{fructose-6-P} + \text{P} \quad (2)$$

Furuya et al., 1982; El-Maghrabi et al., 1982a), and both enzyme activities reside in the same protein (Van Schaftingen et al., 1982; El-Maghrabi et al., 1982a; Sakakibara et al., 1984a).

The enzyme has been purified to apparent homogeneity (El-Maghrabi et al., 1981; Sakakibara et al., 1984a). By molecular sieve filtration on Sephadex G200, we have earlier determined the molecular weight of the partially purified enzyme as 100 000 using nine marker proteins (Furuya et al., 1982b). El-Maghrabi et al. (1981) reported a molecular weight value of 85 000-90 000 by gel filtration on Sephadex G100. The molecular weight of the enzyme subunit has been estimated as 55 000 by polyacrylamide gel electrophoresis under denaturing conditions (Furuya et al., 1982b). However, we calculate a value of 51 500 from the data presented by El-Maghrabi et al. (1984a).

Fructose-6-P-2-kinase:Fru-2,6-bisphosphatase is regulated in part by phosphorylation and dephosphorylation. The en-

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zyme can be phosphorylated by cAMP-dependent protein kinase (Furuya et al., 1982b; El-Maghrabi et al., 1982c; Van Schaftingen & Hers, 1981b) which results in an increased $K_{0.5}$ for fructose-6-P of the kinase (Furuya et al., 1982b; Van Schaftingen & Hers, 1981b; Sakakibara et al., 1984a) and a decreased $K_{0.5}$ for fructose-2,6-P_2 of the phosphatase (Furuya et al., 1982a; Sakakibara et al., 1984a). The amino acid sequence around the phosphorylation site has been reported (Murray et al., 1984), but the location of the site in the enzyme is not known. We have shown previously (Sakakibara et al., 1984b) that proteolysis of the enzyme by trypsin proceeds in two stages in which the first and second cleavages yield products of M_r 53 000 and 50 000, respectively. Both of these cleavage products retain the phosphorylation site, indicating that the site is at least 30 amino acid residues away from either the N- or the C-terminus of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase (Sakakibara et al., 1984b). El-Maghrabi et al. (1982b) have reported the amino acid composition of the enzyme based on a single hydrolysis time of one sample of the enzyme. According to their data, the enzyme contains unusually high concentrations of serine and glycine, 73 and 89 residues/55 000 daltons, respectively.

Since much of the work thus far reported is preliminary, and the work may have been performed with preparations of uncertain purity, we have undertaken more extensive study of the structure of this bifunctional enzyme. The present report deals with the physicochemical properties of the enzyme, including the molecular weight, the amino acid composition, the N- and C-terminal amino acids, the amino acid sequence at the C-terminus, and the identity of the subunits.

Materials and Methods

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Corp. The catalytic subunit of cAMP-dependent protein kinase, poly(ethylene glycol) (M_r 300), trypsin (bovine pancreas, 7765 BAEE units/mg), phenylthiohydantoin (PTH) amino acids, carboxypeptidase A (type I-DFP, 50 units/mg), and carboxypeptidase B (type I-DFP, 100 units/mg) were obtained from Sigma Chemical Co. The reagents for the Edman degradation method were the products of Pierce Chemical Co. All other chemicals were of reagent grade and obtained from commercial sources.

Fru-6-P-2-kinase:Fru-2,6-bisphosphatase was purified to homogeneity as described previously (Sakakibara et al., 1984a). Assay methods for Fru-6-P-2-kinase and Fru-2,6-bisphosphatase were as before (Furuya & Uyeda, 1981; Sakakibara et al., 1984a).

Preparation of Phosphorylated Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. The reaction mixture contained in a final volume of 0.9 mL, 50 mM tris(hydroxymethyl)aminomethane–phosphate (Tris–phosphate), pH 7.5, 0.1 mM [γ-^{32}P]ATP (1×10^6 cpm/nmol), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 0.83 mg of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. The reaction was initiated by the addition of 2000 units of catalytic subunit of cAMP-dependent protein kinase. The reaction mixture was incubated at 30 °C for 90 min. The phosphorylated enzyme was separated from the protein kinase and other reagents by gel filtration on a Sephadex G100 column (2.5 × 40 cm). The incorporation of [^{32}P]phosphate was 0.7 ± 0.04 mol/mol of enzyme subunit.

Ultracentrifugation. Fru-6-P-2-kinase:Fru-2,6-bisphosphatase (0.46 mg/mL) was dialyzed overnight against 1000 volumes of a solution containing 50 mM Tris–phosphate, pH 8, 2 mM dithiothreitol, 0.2 mM EDTA, and 1% poly(ethylene glycol). A high-speed sedimentation equilibrium run was performed according to the procedure of Yphantis (1964). The run was performed in a 3-mm liquid column using a 12-mm double sector cell with sapphire windows. The centrifugation was at 13 000 rpm at an average temperature of 4.6 °C using a Beckman Model E analytical ultracentrifuge. The enzyme was also dissociated by dialysis against two changes of 6 M guanidinium chloride containing 0.1 M 2-mercaptoethanol. The centrifugation was at 26 000 rpm at 22.0 °C. The equilibrium distribution was photographed with a Rayleigh interference optical system after 24, 50, and 62 h after reaching the speed. The fringe displacement was measured by a Nikon two-coordinate comparator with digital micrometers. Molecular weight values were obtained from plots of ln of fringe number against radius squared.

Tryptic Digestion and Peptide Mapping. Fru-6-P-2-kinase:Fru-2,6-bisphosphatase (0.3 mg) was dialyzed against 10% acetic acid overnight and then lyophilized. The dried enzyme was dissolved in 15 mM NH_4HCO_3, pH 8.0, and heated in a boiling water bath for 10 min. The denatured enzyme was then digested with 6 μg of trypsin for 15 h at 37 °C, and the digestion was repeated with the same amount of trypsin. An aliquot of the digest containing 30 μg of peptides was applied on a thin-layer plate coated with cellulose (MN 300 Brinkmann Instruments). The plate was then subjected to electrophoresis and chromatography as described before (Karadsheh et al., 1977). The peptide spots were developed with fluorescamine spray and detected under a UV lamp.

Determination of the Carboxyl-Terminal Amino Acid. Five reaction mixtures were prepared, each containing 10 μg (0.2 nmol) of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase, 0.2 M *N*-ethylmorpholineacetate (pH 8), and 1 mM $ZnCl_2$. The reaction was initiated with the addition of 0.2 μg of carboxypeptidase A in 1 μL to all the reaction mixtures except for a zero time control sample, and the mixtures were incubated at 37 °C. At 1 min, the reaction was terminated in one sample, and likewise another sample at 5 min. After 5 min, 0.2 μg of each carboxypeptidase B (1 μL) was added to the remaining two samples, and these reaction mixtures were incubated an additional 5 and 30 min at which time the reaction was terminated. The amino acids in these samples were subjected to fluorescence labeling prior to the analysis by treating with *o*-phthalaldehyde in the presence of β-mercaptoethanol as described (Kangawa et al., 1983).

Separation of Peptides by High-Performance Liquid Chromatography (HPLC). The peptides obtained after trypsin digestion of [^{32}P]phospho-Fru-6-P-2-kinase:Fru-2,6-bisphosphatase or the C-terminus peptides were separated by HPLC. The reverse-phase chromatography was performed with a Nova Pak C_{18} column (3.9 mm × 15 cm, Waters Associates) or an Ultrasphere ODS column (Beckman Instruments) using a Waters liquid chromatograph system equipped with an automatic solvent programmer and a Perkin-Elmer UV detector. The elution of the peptides was performed with a linear gradient of acetonitrile from 1% to 37% in the presence of 0.1% trifluoroacetic acid, pH 2.48–2.0. The eluted peptides were detected at 210 or 214 nm.

Separation of Phenylthiohydantoin (PTH)-Amino Acids. PTH-amino acids were separated by HPLC chromatography with the same C_{18} (Nova Pak) column as described above. The gradient for the elution was as described in Waters "Application Brief" M3500 and consisted of solvent A [25 mM sodium acetate (pH 5.15)/CH_3CN (80:20)] and solvent B [solvent A/isopropyl alcohol/H_2O (50:25:25)]. The column was developed first with solvent A, and after 1 min into the chromatography, the elution was continued with solvent B.

Table I: Amino Acid Composition of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase[a]

amino acid	residues/55 000 daltons from this work	El-Maghrabi et al. (1982b)[b]
aspartic acid	45.6 ± 1.1	38.5
threonine[c]	23.8 ± 0.9	21.1
serine[c]	26.1 ± 2.6	66.9
glutamic acid	75.0 ± 1.6	82.5
proline	20.0 ± 0.1	13.8
glycine	28.8 ± 2.9	81.6
alanine	31.1 ± 2.4	38.5
valine	31.3 ± 1.3	23.8
methionine	4.8 ± 0.1	5.5
isoleucine	28.8 ± 2.0	14.7
leucine	44.8 ± 2.1	22.0
tyrosine[c]	25.2 ± 0.7	13.8
phenylalanine	14.0 ± 0.5	9.2
tryptophan	ND[d]	9.2
lysine	27.0 ± 1.1	33.9
histidine	14.0 ± 1.5	14.7
arginine	35.9 ± 0.5	24.8
half-cystine	3.7 ± 0.1	8.3

[a] The procedure of the amino acid analysis is described under Materials and Methods. The values and the standard deviations are calculated from the data at 24, 48, and 72 h of hydrolysis; the determinations were performed in triplicate. [b] Corrected values of El-Maghrabi et al. (1982b) obtained by multiplying by 55 000/60 000. [c] Extrapolated to zero hour of hydrolysis. [d] ND, not determined.

The PTH-amino acids were detected at 254 nm.

Amino Acid Analysis. Fru-6-P-2-kinase:Fru-2,6-bisphosphatase was dialyzed extensively against several changes of 5 mM NH_4HCO_3, lyophilized, and dried in vacuo at 50 °C. Hydrolysis of the protein in triplicate was carried out at 110 °C in evacuated tubes containing constant-boiling 6 N HCl for periods of 24, 48, and 72 h. Cysteine and cystine were determined after performic acid oxidation of the protein according to the procedure of Moore (1963). Amino acid analyses were performed by Glen Wilson at The University of Iowa, Iowa City, IA.

Isoelectric Focusing. The isoelectric focusing was performed by using precast gels (pH 4–6.5) provided by LKB Instruments Co. and following the directions given by the manufacturer. The marker proteins used were human carbonic anhydrase B (pI = 6.55), bovine carbonic anhydrase (pI = 5.85), β-lactoglobulin A (pI = 5.20), trypsin inhibitor (pI = 4.55), glucose oxidase (pI = 4.15), methyl red dye (pI = 3.75), amyloglucosidase (pI = 3.50), and pepsinogen (pI = 2.80).

Other Methods. Protein concentration was routinely determined with the Bradford method (Bradford, 1976) using crystalline bovine serum albumin as a standard. For more precise measurement, the protein concentration was determined with an analytical ultracentrifuge equipped with interference optics (Babul & Stellwagen, 1969). The values obtained by these methods agree within 10% variation. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970).

Results and Discussion

Molecular Weight of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. Fru-6-P-2-kinase:Fru-2,6-bisphosphatase was subjected to high-speed sedimentation equilibrium according to the method of Yphantis (1964). Figure 1A shows the plot of ln J (J, fringe numbers) vs. r^2 (r, radial distance), and the inset shows molecular weight plotted against J. As shown in the figure, most of the points fall on a straight line, indicating the enzyme is homogeneous. The molecular weight of the enzyme calculated from the plot is 101 000 based on a partial

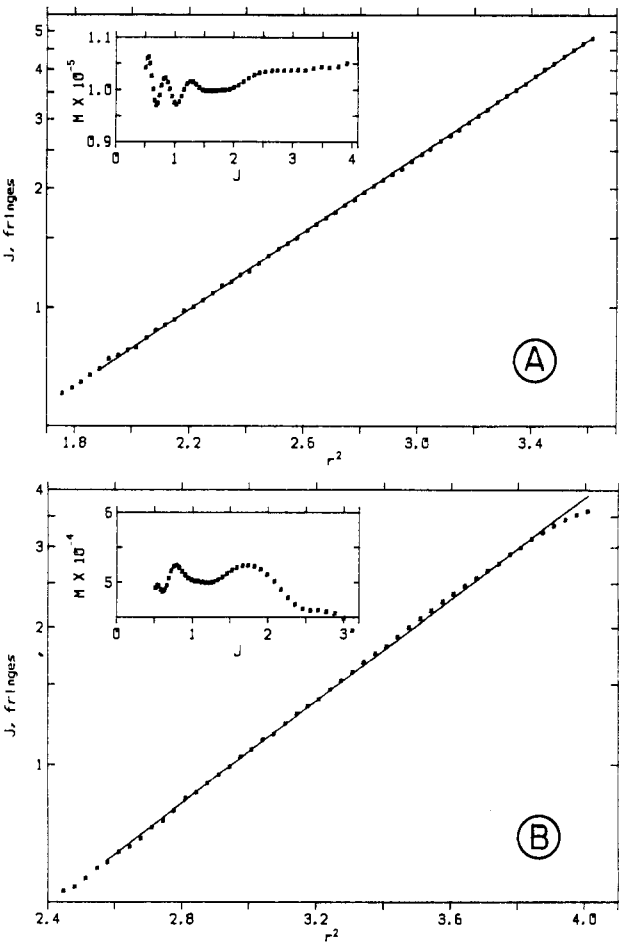

FIGURE 1: High-speed sedimentation equilibrium data of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. The experimental conditions were as described under Materials and Methods. The figures show plots of ln J (J, fringe number) vs. r^2 (r, radial distance), and the insets are plots of molecular weight vs. J. (A) Native enzyme, (B) dissociated enzyme in guanidinium chloride.

specific volume of 0.723 mL/g calculated from the amino acid composition (Table I) (Cohen & Edsall, 1943). This value for the molecular weight is in excellent agreement with that (100 00) we have reported previously (Furuya et al., 1982b) which was obtained by molecular sieve filtration on Sephadex G200 using several marker proteins. A closer examination of the plot of the molecular weight vs. J (inset) suggests a small amount of self-association beyond a concentration of two fringes (0.5 mg/mL).

Figure 1B shows the plot of ln J vs. r^2 of dissociated enzyme in guanidinium chloride. From the slope, the molecular weight is estimated as 50 000. Closer examination of the plot of the molecular weight vs. J (inset) shows that the solution is nonideal, with a molecular weight extrapolated to zero concentration of 55 000.

Acrylamide slab gel electrophoresis of the dissociated Fru-6-P-2-kinase:Fru-2,6-bisphosphatase in sodium dodecyl sulfate reveals a single band. The molecular weight of the subunit was estimated as 55 000 from the gel electrophoresis pattern using several marker proteins (data not shown). The value is similar to those reported previously (Furuya et al., 1982). The observations suggest that the enzyme consists of two subunits with the same molecular weight. The apparent discrepancy between the observed subunit molecular weight of 55 000 and the expected 50 000 (half of the native enzyme) could be due to preferential binding of guanidinium chloride. The same difference observed for the subunit in sodium do-

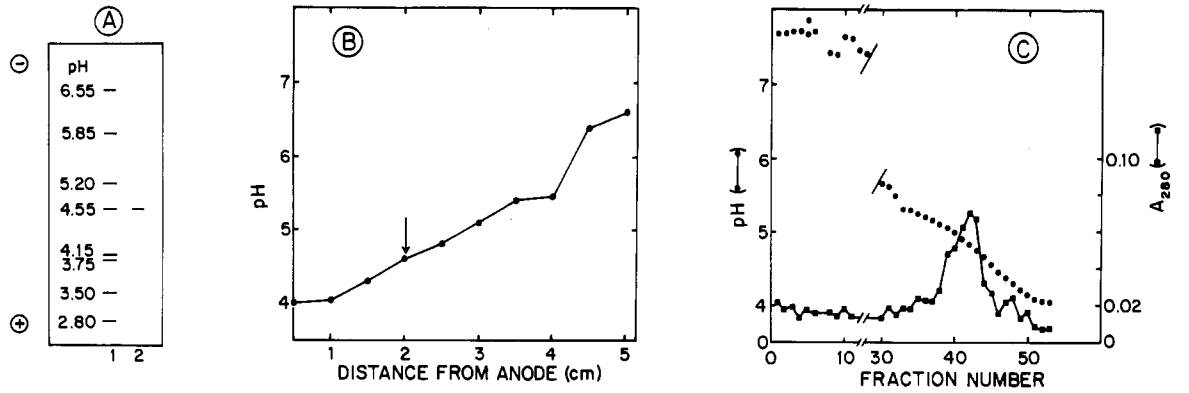

FIGURE 2: Isoelectric focusing of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. (A) Fru-6-P-2-kinase:Fru-2,6-bisphosphatase (16 μg) was used (lane 2). The marker proteins (lane 1) with isoelectric points (in parentheses) are (from the top) human carbonic anhydrase B (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.20), trypsin inhibitor (4.55), glucose oxidase (4.15), methyl red dye (3.75), amyloglucosidase (3.50), and pepsinogen (2.80). The other conditions are as described under Materials and Methods. (B) Isoelectric focusing in 8 M urea was carried out according to the procedure of O'Farrell (1975). The pH gradient was from 3 to 10, and 20 μg of the enzyme was used. An identical gel was used to determine the pH. The arrow points to the position of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. (C) Chromatofocusing of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. The enzyme (72 μg in 0.5 mL of 25 mM imidazole hydrochloride, pH 7.4, 1 mM dithiothreitol, and 3 mM P_i) was applied on a PBE94 exchanger (Pharmacia) column (0.54 cm × 13 cm) which had been equilibrated with 25 mM imidazole hydrochloride, pH 7.4. The enzyme was eluted from the column with 48 mL of polybuffer PB74 (Pharmacia) (diluted 1/8 containing 3 mM H_3PO_4) and 1 mM dithiothreitol, pH 4.0, at a flow rate of 7 mL/h. The pH of the eluate was determined immediately, and the protein concentration was determined from the absorbance at 280 nm.

decyl sulfate can be attributed to uncertainty in the shape of the sodium dodecyl sulfate–protein complex.

Careful examination of our gel pattern shows that the subunit band of our enzyme preparation lies close to halfway between two marker proteins (Sakakibara, 1984a). However, a more recent report by El-Maghrabi et al. (1984a) shows that their subunit band falls below the halfway point between the same two marker proteins, and the molecular weight calculated from their data is 51 500. We have shown previously that a mild tryptic digestion of the enzyme reveals a stepwise degradation with cleavage products of 53 000, 50 000, and 48 000 daltons (Sakakibara, 1984b). Thus, their enzyme preparation appears to be proteolytically digested.

Isoelectric Point. The isoelectric point of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase is 4.7 as determined by isoelectric focusing on a gel using several marker enzymes (Figure 2A). The same value was obtained under denaturing conditions in 8 M urea (Figure 2B). By chromatofocusing, the value is 4.8 (Figure 2C). This is in contrast to the results of Garrison & Wagner (1982), who reported the isoelectric point as 6.6 in a urea-containing gel. El-Maghrabi et al. (1982a) reported a value of 6.6 with no description of the experimental procedure. Garrison and Wagner prepared the enzyme according to the previously published procedure (El-Maghrabi et al., 1982a) for this determination. In view of the large difference in the amino acid composition as discussed below and a lower molecular weight of the subunit which may be due to proteolytic digestion, it is not surprising that the isoelectric point is greatly different from the value that we determined.

Tryptic Peptide Map. To establish whether the subunits are identical, tryptic peptide mapping was performed. The result, as depicted in Figure 3, shows that little protein remains at the origin, indicating that tryptic digestion was essentially complete. The peptide maps obtained from two digestion periods for three different preparations of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase yielded approximately 60 ± 5 spots. The HPLC separation of the tryptic peptides from ^{32}P-phosphorylated Fru-6-P-2-kinase:Fru-2,6-bisphosphatase revealed a comparable number of peptides (50–60) as discussed later. Since the enyzme contains 27 lysine and 36 arginine residues per 55 000 daltons, the observed number of peptides

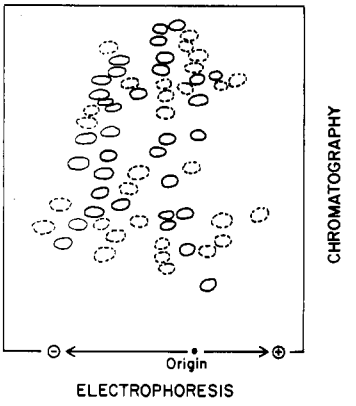

FIGURE 3: Tryptic peptide map of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. The conditions for tryptic digestion and for thin-layer electrophoresis and chromatography are described under Materials and Methods. Solid lines are intense fluorescence spots, and dotted lines are weak fluorescence spots.

in the tryptic digest suggests that both subunits are either identical or very similar.

Amino Acid Composition. The amino acid composition of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase is presented in Table I. The results of El-Maghrabi et al. (1982b) are also included in the table for comparison. The recovery of the total amino acids is 95% on the basis of the dry weight of the protein, and the variation in the composition among three preparations of the enzyme was less than 10%. The amino acid composition reported by El-Maghrabi et al. (1982b) is considerably different from our result, more than could be accounted for by experimental error. For example, they reported 9 cysteine, 73 serine, and 89 glycine residues per 55 000 daltons, which are 2.5–3 times higher, and 16 isoleucine, 24 leucine, and 15 tyrosines, which are half of the values we found. Their values for lysine and arginine residues are reversed compared to our results, and most of the other amino acids also differ by 10–30%. A calculation of their data shows that the total weight of the protein based on their reported amino acid composition is 60 600 daltons rather than 55 000 daltons as they stated. However, this error does not account for the large discrepancy between our results and theirs. The fact that their result is based on a single determination after 20-h hydrolysis

of one sample of the enzyme may account for only part of the differences. In any case, it is clear that their preparation of the enzyme is different from ours.

Aside from the difference in the amino acid composition, significant differences also exist in the various kinetic parameters and the partial reactions catalyzed by Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. For example, Pilkis et al. (1984) reported that the enzyme catalyzes the Fru-6-P:Fru-2,6-P_2 exchange reaction and also the ATP–ADP exchange reaction at 50% of the rate of the overall kinase reaction. Their enzyme preparation did not catalyze the reversal of the forward kinase reaction, and a phosphoryl-enzyme was formed when the enzyme was incubated with ATP or Fru-2,6-P_2 (Pilkis et al., 1983). Contrary to those results, we (Kitajima et al., 1984) have demonstrated that our preparation of the enzyme catalyzes the ATP–ADP exchange at only 3% of the overall reaction, and it does not catalyze the Fru-6-P:Fru-2,6-P_2 exchange reaction at any significant rate even in the presence of phosphate. We also showed that the enzyme does catalyze the reversal of the Fru-6-P-2-kinase reaction at half the rate of the forward reaction (Kitajima et al., 1984). Furthermore, there is no evidence of phosphoryl-enzyme formation during the course of the kinase or the phosphatase reaction. However, we showed that cruder enzyme preparations do catalyze high ATP–ADP exchange activity. These results strongly suggest that the preparation of the enzyme used by El-Maghrabi et al. (1982b) was contaminated by other protein(s). More recent publications from this group support this conclusion. They (El-Maghrabi et al., 1984a) have now modified their purification procedure to include a Blue Sepharose chromatography step, similar to the procedure described by Sakakibara et al. (1984a). Using this preparation, they have reported that the ATP–ADP exchange is 15% of the overall reaction rate instead of 50% and the reaction is indeed reversible (El-Maghrabi et al., 1984b). Thus, the large discrepancy in the amino acid composition reported by El-Maghrabi et al. (1982b) and by us is probably due to the presence of other proteins in their preparation.

N-Terminus of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. No free N-terminal amino acids were detected by the manual Edman degradation method (Blomback et al., 1966; Gelman & Begg, 1967) with the phenylthiohydantoin-amino acids analyzed by HPLC on a C_{18} reverse-phase column. Under the same conditions, we were able to obtain a quantitative yield of three N-terminal amino acid residues in sequence, namely, alanine, serine, and serine from pig heart citrate synthase, in accord with the known sequence (Bloxham et al., 1981). Murray et al. (1984) also reported lack of a free N-terminus.

We have shown previously that when Fru-6-P-2-kinase:Fru-2,6-bisphosphatase was subjected to mild proteolysis by trypsin in the presence of Fru-2,6-P_2, a major cleavage product ("T_1", M_r 53 000) and a minor product ("T_2", M_r 50 000) were formed (Sakakibara et al., 1984b). A possibility existed that trypsin might have cleaved sequentially from the N-terminus of the enzyme, thus exposing a new N-terminal residue. The N-terminal analysis of a mixture of the cleavage products, consisting of 75% T_1 and 25% T_2, did not reveal any phenylthiohydantoin-amino acid. Thus, we conclude that the cleavage by trypsin occurred at the C-terminus of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. Furthermore, we conclude that Fru-6-P-2-kinase:Fru-2,6-bisphosphatase possesses blocked N-terminal residue(s). The same conclusion was reached by Murray et al. (1984). The nature of the blocking group remains to be determined, but the complete absence of a free N-terminal residue is consistent with the idea that both subunits are identical or very similar. The blocking group may be acetyl since many glycolytic enzymes as well as other cytoplasmic enzymes are known to contain N-acetylated amino acid [see review by Wold (1981)].

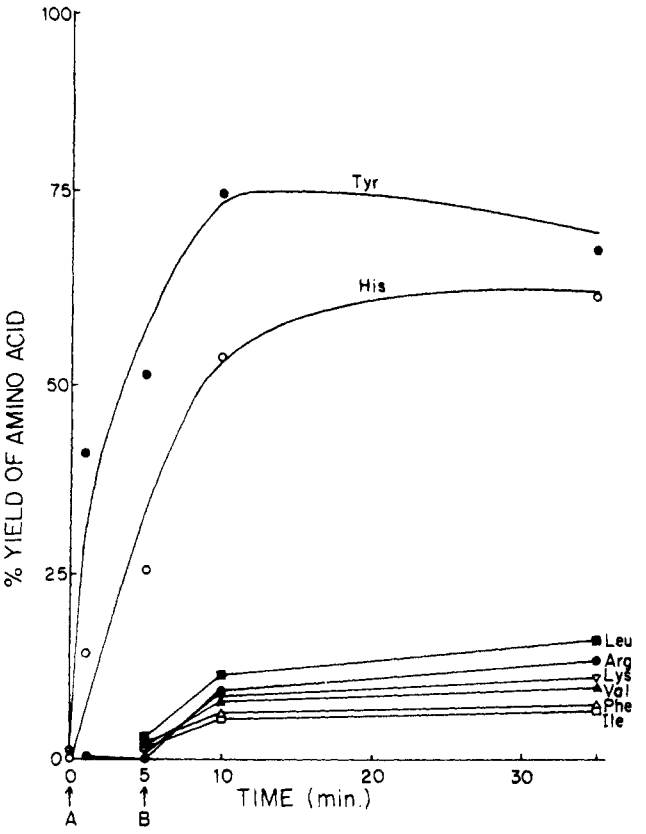

FIGURE 4: Rate of release of amino acids from Fru-6-P-2-kinase:Fru-2,6-bisphosphatase by carboxypeptidases A and B. The experimental conditions are described under Materials and Methods. "A" and "B" indicate the addition of carboxypeptidases A and B, respectively.

C-Terminus. When Fru-6-P-2-kinase:Fru-2,6-bisphosphatase is digested with carboxypeptidase A, tyrosine is released rapidly, followed by histidine (Figure 4). The yields of tyrosine and histidine were 75% and 63%, respectively. Addition of carboxypeptidase B to the reaction mixture containing carboxypeptidase A results in the release of arginine and several other amino acids including leucine, isoleucine, valine, phenylalanine, and lysine. However, the yield of those amino acids is comparable to that of arginine and less than 15%. Digestion of the enzyme with trypsin for 3 h does not release any detectable amount of tyrosine, indicating that arginine is not adjacent to tyrosine. These results indicate that the C-terminal amino acid is tyrosine and histidine is the second amino acid. The third amino acid is probably arginine because other amino acids were not formed until the arginine residue was released by carboxypeptidase B. However, since the yield of arginine is low, one cannot be certain. Thus, the result that a single C-terminal amino acid, tyrosine, is detected further supports the idea that the subunits of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase are identical.

[^{32}P]Phosphopeptide. Fru-6-P-2-kinase:Fru-2,6-bisphosphatase was phosphorylated with [^{32}P]ATP by cAMP-dependent protein kinase. The phosphate incorporation was 0.7 ± 0.04 mol/mol of subunit. The ^{32}P-labeled enzyme was separated from the protein kinase and other reagents by gel filtration on Sephadex G100 and digested with trypsin at an enzyme/trypsin ratio of 50/1 for 15 h at 30 °C. The radioautograph of the tryptic peptide map of the phosphoenzyme

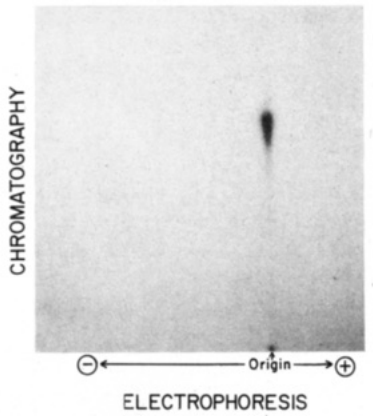

FIGURE 5: Radioautograph of tryptic peptide map of ^{32}P-labeled Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. [^{32}P]Phospho-Fru-6-P-2-kinase:Fru-2,6-bisphosphatase (700 μg, 9.43 × 10^6 cpm) in 70 μL of 5 mM NH_4HCO_3, pH 8.0, was digested with 14 μg of trypsin at 30 °C for 15 h. The reaction was terminated by heating at 100 °C for 5 min. An aliquot (1 μL) of the digested enzyme was spotted on a thin-layer plate, and the peptide mapping was performed as described under Materials and Methods. A radioautograph of the plate was prepared by exposing the plate to Kodak X-Omat AR film for 2 h at 25 °C.

reveals one major and possibly a minor spot (Figure 5). Figure 6A shows a typical elution pattern of the [^{32}P]-phosphopeptides by HPLC on a C_{18} reverse-phase column as described under Materials and Methods. The results show that a single ^{32}P-labeled peptide was eluted at 68 min, in agreement with the results from the tryptic peptide map. These results may also indicate that the subunits are identical or very similar. This conclusion is further supported by the observation that when the ^{32}P-labeled enzyme was cleaved by CNBr, a single ^{32}P-labeled peptide was eluted upon chromatography on a Bio-Gel P6 column Furthermore, thermolysin digestion of the isolated CNBr-cleaved ^{32}P-peptide yielded a single ^{32}P-labeled peptide by HPLC reverse-phase chromatography on a C_{18} column (Figure 6B).

The total number of peptides eluted by this method is approximately 50–60 including several minor peaks. These results are in good agreement with those from tryptic peptide maps and provide additional evidence in support of the view that the subunits of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase are identical.

In summary, our results confirm that the molecular weight of Fru-6-P-2-kinase:Fru-2,6-bisphosphatase is 101 000 and it consists of two similar if not identical subunits of M_r 55 000. This conclusion with regard to the identity of the subunits is based on the following observations: (a) A single band is observed in SDS–polyacrylamide gel electrophoresis and isoelectric focusing gels; (b) the phosphorylation sites on the enzyme are also similar, as judged from the tryptic peptide map and HPLC chromatography of tryptic and thermolysin

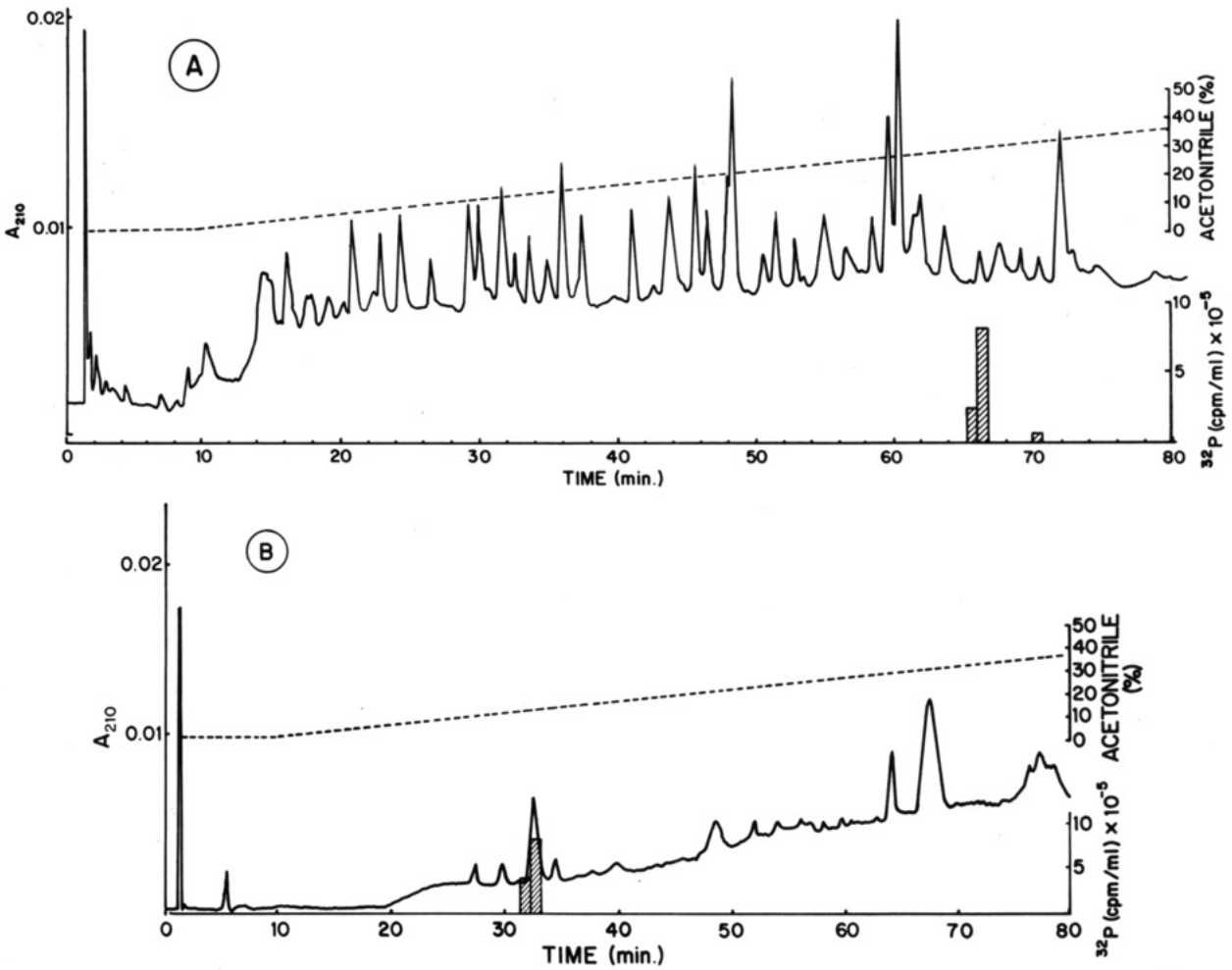

FIGURE 6: High-performance liquid chromatography of (A) a tryptic digest and (B) a thermolysin digest of CNBr-cleaved ^{32}P-labeled Fru-6-P-2-kinase:Fru-2,6-bisphosphatase. (A) The conditions for the preparation of [^{32}P]phosphoenzyme and for tryptic digestioins are the same as in Figure 4. (B) The [^{32}P]phosphoenzyme (0.8 mg, 1.6 × 10^7 cpm) was reacted with 1.8 mg of CNBr in 0.5 mL of 70% formic acid for 24 h at 4 °C. The sample was diluted with 5 mL of H_2O and lyophilized. The cleaved peptides were then passed through a Bio-Gel column (1.5 cm × 54 cm). Over 85% of ^{32}P was eluted after uncleaved protein. The CNBr-cleaved ^{32}P-peptide sample was lyophilized and digested by thermolysin as described (Heinrickson, 1977). HPLC separation of the peptides is described under Materials and Methods.

digests of the native and CNBr-cleaved peptides; and (c) a single C-terminal amino acid, tyrosine, is released by carboxypeptide A, and the amino acid sequence at the C-terminus is probably -Arg-His-Tyr.

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