Fructose 1,6-bisphosphatase was purified from the livers of C57BL/KsJ mice to apparent homogeneity. The enzyme was shown to be a tetramer of a subunit Mr = ~35,000. The purified enzyme showed maximal activity at neutral pH; high affinity for its substrate, fructose 1,6-bisphosphate; requirement for a divalent cation (Mg²⁺ or Mn²⁺); inhibition by AMP; susceptibility to limited proteolysis by subtilisin; and activation by K⁺ or NH₄⁺. Thallium and lithium, two potential NMR probes to study the enzyme - monovalent metal interactions, affected fructose 1,6-bisphosphatase activity. Thallium was found to be an activator. Vₘₐₓ with Ti⁺ equals that obtained with K⁺, but the affinity of the enzyme for Ti⁺ (Kₘ = 16mM) was about 3 times greater than for K⁺. Lithium was strongly inhibitory. The apparent Kₘ values for Li⁺ were 0.8 and 0.3 mM in the presence of saturating concentrations of Mg²⁺ and Mn²⁺, respectively. Mouse liver fructose 1,6-bisphosphatase, either in its native state or in 1.6 M urea, was not a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase. This is in contrast with the results obtained with rat liver fructose 1,6-bisphosphatase. The rat liver enzyme was phosphorylated by the above kinase and the same maximal phosphate incorporation (about 1 mol of phosphate/mole of enzyme protomer) was obtained in either the presence or absence of urea.

Fructose 1,6-bisphosphatase was found to be increased in the livers of the genetically diabetic mouse (C57BL/KsJ-db strain). The enzyme from these animals was also purified to homogeneity. No difference in either molecular or enzymatic properties was found between the two purified enzymes.

Purification and Properties of Liver Fructose 1,6-Bisphosphatase from C57BL/KsJ Normal and Diabetic Mice*

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Frank Marcus and M. Marlene Hasey
From the Department of Biochemistry, University of Health Sciences, The Chicago Medical School, Chicago, Illinois 60612

Fructose 1,6-bisphosphatase (EC 3.1.3.11, d-fructose-1,6-bisphosphate 1-phosphohydrolase), the enzyme that catalyzes the hydrolysis of fructose 1,6-bisphosphate, is a regulatory enzyme that plays a key role in the control of gluconeogenesis (1, 2). The mammalian liver and kidney enzymes have been purified from several sources and the most extensively studied Fru-P₂ase has been the one isolated from rabbit liver (for reviews, see Refs. 2 and 3). Studies of the enzyme isolated from the liver of the rat, an animal more commonly used in metabolic studies of gluconeogenesis, have also been reported (4-6). Although some information on the enzyme isolated from livers of outbred mice has been published (7, 8), the present study selected the C57BL/KsJ mouse as the source of liver Fru-P₂ase because of its relevance to diabetes research. A mutation which occurred spontaneously in this mouse strain produced the genetically diabetic mouse (C57BL/KsJ-db strain), a laboratory animal which exhibits symptoms similar to maturity-onset diabetes in humans (for a review article, see Ref. 9). Enhanced gluconeogenesis appears to be a major factor in the hyperglycemia of diabetic mice, and changes in metabolite levels are consistent with facilitation of the Fru-P₂ase reaction or restraint of the phosphofructokinase reaction in the livers of the diabetic mice, or both (10). Thus, it appeared important to purify and study both Fru-P₂ase and phosphofructokinase from the livers of normal and diabetic C57BL/KsJ mice. The present report deals with fructose 1,6-bisphosphatase.

EXPERIMENTAL PROCEDURES

Materials: The following materials were purchased from the sources indicated: NADP, glucose-6-phosphate dehydrogenase, and phosphoglucomutase from Sigma; tris(hydroxymethyl)aminomethane (Tris) and urea from Bio-Rad Laboratories, Richmond, Va.; triethanolamine from J. T. Baker Co., Phillipsburg, N.J.; methyl sulfonyl fluoride, subtilisin Carlsberg, phosphorylase-a, and fluorescein-diacetate from Boehringer Mannheim; ammonium sulfate (ammonium grade), glutathione, ATP, ammonium chloride, urea, and constant boiling HCL sequential grade from Pierce; AMP, ATP, malonic acid, histone type II A, fructose 1,6-bisphosphate (Müller, St. Louis), glycerol, methyl sulfonyl fluoride, subtilisin Carlsberg, phosphorylase-a, and fluorescamine from Sigma; triethanolamine-HCl, dithiothreitol, and thallium salts from Trison Chemical, Nacyaquine NY II; catalase (Cel-Imp) from Bio-Rad, (C7677) to from Arosphan. All other reagents were purchased from common commercial sources and were of the highest purity available. Spectra/Por 3 membranes and spectroscopic closures from Spectrum Medical Industries, Los Angeles, California.

Rat liver Fru-P₂ase was purified as described by Tejwani et al. (5) and the pig kidney enzyme as described by Colomb and Marcus (39). The catalytic subunit of cyclic AMP-dependent protein kinase was purified from bovine liver as described by Sedlak et al. (40). Male C57BL/Js and C57BL/KsJ misty diabetic mice were obtained at 7-8 weeks of age from the Jackson Laboratory, Bar Harbor, Maine. The animals were housed in plastic cages within a Puffer-Hubbard Environment chamber thermostated at 21° and maintained on a 12 hours light (8 am to 8 pm), 12 hours dark (8 pm to 8 am) cycle. They were fed Purina rat chow and water ad libitum.

Methods: Enzyme Assays - Fru-P₂ase activity was determined spectrophotometrically by following the rate of formation of NADPH at 340 nm in the presence of excess P₆-glucosaminerase and glucose-6P dehydrogenase. Unless otherwise stated, the reaction mixture contained a 30 mM triethanolamine- dithiothreitol buffer, 0.15 mM fructose 1,6-bisphosphate, 3.3 mM NADP, 150 mM KCl, 0.1 mM EDTA, 0.3 mM AMP, phosphoglucomutase (2.2U/ml), glucose-6-P dehydrogenase (0.6 U/mI), and Fru-P₂ase. Assays were carried out at 30°. A unit of Fru-P₂ase activity is defined as that amount of enzyme which catalyzes the formation of 1 umol of fructose-6-P/min under the conditions described above. Specific activity is expressed in terms of umol of protein. The protein concentration was determined by absorbance at 280 nm assuming a ε280 value of 7.0 for the purified mouse liver enzyme. ε280 values ranging from 6.30 to 7.63 (5, 41) have been determined for other purified liver Fru-P₂ases.

Purification of Mouse Liver Fructose 1,6-Bisphosphatase

1. Extraction - Six mice of strain C57BL/KsJ were killed by cervical dislocation, the livers were quickly removed, weighed, and homogenized in a Potter-Elvehjem tissue grinder with 5 volumes of ice-cold 1 mM EDTA (pH 8). Three livers were homogenized at a time. The homogenate was centrifuged at 100,000 g for 30 min at 17,000 rpm and the supernatant was collected after filtration through cotton wool (extract).

2. Heat Step - The extract was transferred to a polypropylene centrifuge tube, and heated with constant stirring in a water bath at 80°. When the

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1 The abbreviation used is: Fru-P₂ase, fructose 1,6-bisphosphatase.
temperature of the solution reached 60°C (within approximately 2 min), the tube was placed in a water bath at 60°C for 5 min. The solution was then cooled in an ice bath, centrifuged at 4°C for 30 min at 17,000 g, and the supernatant was collected (by gel filtration).

3. Phosphoglycerolosphate Chromatography. To the heated fraction, 20 μM fructose 1,6-bisphosphatase was added to achieve a final concentration of 2 μM, and the mixture was equilibrated with 20 mM potassium phosphate (pH 7.4) at 23°C for 30 min. After 0.5 ml of each fraction was collected, the enzyme was purified by gel filtration on a Sephadex G-50 column equilibrated with 20 mM potassium phosphate (pH 7.4) at 23°C. The fraction was collected by gel filtration on a Sephadex G-50 column equilibrated with 20 mM potassium phosphate (pH 7.4) at 23°C. The fraction was collected and analyzed for enzyme activity.

Removal of fructose 1,6-bisphosphatase then permitted the absorption of the protein solutions was performed according to Davis (43). Acrylamide gel electrophoresis in 7.5% gels according to Davis (43). Acrylamide gel electrophoresis was performed in 7.5% gels according to Davis (43).

Determination of Phosphoglycerol phosphate and Serine Phosphate. The serine phosphate content of the purified enzyme was determined according to Davis (43). The serine phosphate content was determined according to Davis (43). The serine phosphate content was determined according to Davis (43). The serine phosphate content was determined according to Davis (43).

Enzyme Purification and Properties— The data of Table 1 summarize the purification of mouse (C57BL/KsJ) liver Fructose-Pase. The purification procedure heavily relied on the well known interaction of Fructose-Pase with a cation exchanger (i.e., P- or CM-cellulose) and the specific alteration of the enzyme-exchanger interaction by the substrate fructose 1,6-bisphosphatase (11). In this study, the "diagonal" procedure with phosphenol at pH 7.5 was used. The first chromatographic step, 2 μM fructose 1,6-bisphosphatase was added to the protein solution to prevent the binding of the enzyme to P-cellulose at pH 7.5. Fructose-Pase was then eluted with 0.5 mM fructose 1,6-bisphosphatase. The purification procedure yielded a homogeneous enzyme preparation. A single protein band was observed upon polyacrylamide gel electrophoresis of the purified enzyme either in the absence or presence of sodium dodecyl sulfate. From the latter experiments, a subunit Mr = 35,000 was calculated (12). This value is in agreement with the subunit molecular weight of other Fructose-Pases (2). The molecular weight of the purified enzyme determined by gel filtration on Sephadex G-200 was about 140,000.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Recovery (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>45</td>
<td>167</td>
</tr>
<tr>
<td>Heated</td>
<td>38</td>
<td>133</td>
</tr>
<tr>
<td>P-Cellulose I</td>
<td>9.5</td>
<td>127</td>
</tr>
<tr>
<td>P-Cellulose II</td>
<td>6</td>
<td>72</td>
</tr>
</tbody>
</table>

aThe initial amount of mouse liver was 9.5 g. Assays were performed at pH 7.3 and 30°C as described under "Experimental Procedures." Protein concentrations were determined by absorbance at 280 nm, except for the extract and the heated fraction, the protein contents of which were estimated by the biuret method (46).

(b) Amino Acid Composition of Fructose-Pase - S-peptide

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Milk</th>
<th>Liver</th>
<th>Rabbite</th>
<th>Pig kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>3.2</td>
<td>2.7</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>His</td>
<td>1.2</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td>2.7</td>
<td>1.9</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Met</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Asp</td>
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<td>3.2</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Thr</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Ser</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Glu</td>
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<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Pro</td>
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<td>1.2</td>
<td>1.2</td>
<td>1.0</td>
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<tr>
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<td>0.6</td>
<td>0.6</td>
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<td>Ala</td>
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<tr>
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<td>2.3</td>
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<tr>
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<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Leu</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Tyr</td>
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<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Thr</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>38.0</td>
<td>38.0</td>
<td>38.0</td>
<td>38.0</td>
</tr>
</tbody>
</table>

The values for the amino acid composition of the mouse liver Fructose-Pase were calculated from data obtained after 22 hours of hydrolysis, and based on the assumption that the peptide contained two phosphenylalanine residues. The values in parenthesis are rounded off to the nearest integral value. Tryptophan was not determined.

The initial amount of mouse liver was 9.9 g. Assays were performed at pH 7.3 and 30°C as described under "Experimental Procedures." Protein concentrations were determined by absorbance at 280 nm, except for the extract and the heated fraction, the protein contents of which were estimated by the biuret method (46).

(8) Except for the differences indicated in the table, activity was measured as described in Methods.
cation procedure has been repeated more than 15 times and is very reproducible. Animals varying in age from 8 to 28 weeks have been used. No significant differences in either the initial amount of enzyme (average, 17.2 units/g of liver) or in the specific activity of the purified enzyme (about 30 units/mg) have been observed. The method described above was also applicable to mouse liver extracts made in 1 mM EDTA plus 10 mM KF, pH 8. The final specific activity, the recovery of pure enzyme, and the properties of the purified enzyme were identical with that prepared from EDTA extracts without fluoride.

Activity studies at saturating concentrations of the substrate, fructose 1,6-bisphosphate ($K_s = 7 \mu M$), and of $3.2 \mu M$ Mg$^{2+}$ showed that the purified enzyme had maximal activity in the neutral pH range (Fig. 1) and may thus be considered as a "neutral" Fru-P$_2$ase (for a review, see Ref. 2). However, as with other Fru-P$_2$ases, limited proteolysis can lead to the formation of an enzyme form with a more alkaline pH optimum. This is also exemplified in Fig. 1, which shows the pH activity curve of subtilisin-treated mouse liver Fru-P$_2$ase. Subtilisin cleaved the native $M_r = 35,000$ proteomer into a fragment of $M_r = 29,000$ (the "S-subunit") plus a peptide of $M_r = 6,000$ (the "S-peptide"). The S-peptide was purified by gel filtration on a Sephadex G-75 column in 9% HCOOH (13), and the amino acid composition was determined. It was found to be very similar to those reported for two other S-peptides, namely the rabbit liver and the pig kidney Fru-P$_2$ase S-peptides (Table II). The S-peptide fragments are derived from the NH$_2$ terminus of Fru-P$_2$ase, and the amino acid sequence of both the rabbit liver (14) and the pig kidney (15) S-peptide has been determined. A detailed analysis of the sites of cleavage of rabbit liver Fru-P$_2$ase by subtilisin has led to the suggestion that the region of the molecule including residues 57 to 67 may exist as an exposed peptide susceptible to proteolytic attack (16). The cleavage of the mouse liver enzyme by subtilisin with the formation of a peptide of 63 amino acids, as well as the results obtained with pig kidney Fru-P$_2$ase (15), clearly supports the above suggestion.

Mouse liver Fru-P$_2$ase requires a divalent metal activator such as Mg$^{2+}$ or Mn$^{2+}$. Sigmoid kinetics was observed for the activation by magnesium (Fig. 2) in agreement with kinetic data of other Fru-P$_2$ases (17, 18). From a Hill plot of the data of Fig. 2 (Fig. 2, inset) values of $n = 1.85$ and $K_s = 0.29 \mu M$ were obtained. Much lower free Mn$^{2+}$ concentrations (20 to 40 $\mu M$) were required for maximal activity. The value of $K_s$ for Mn$^{2+}$ was below 10 $\mu M$ and significant inhibition was observed at high Mn$^{2+}$ concentrations. Activity at 0.5 $\mu M$ Mn$^{2+}$ was 30% of that measured at 0.033 $\mu M$. All of the above experiments were performed in the presence of 0.1 mM EDTA. The activation of Fru-P$_2$ases by either EDTA or histidine is due to the chelation of inhibitory heavy metals (19) and the inhibitory metal has been identified as Zn$^{2+}$ (20, 21). Mouse liver Fru-P$_2$ase was inhibited by Zn$^{2+}$. In the presence of 0.3 $\mu M$ Zn$^{2+}$, the specific activity of the enzyme (measured at pH 7, 150 mM KCl, 1.7 $\mu M$ MgSO$_4$) was only about 1 unit/mg, compared to 30 units/mg obtained under similar conditions but in the presence of 0.1 mM EDTA.

Fru-P$_2$ase activities are activated by some monovalent cations (17, 18, 22, 23), and the results shown in Table III demonstrate that the mouse liver enzyme is no exception to this general feature of Fru-P$_2$ases. Either with Mg$^{2+}$ or Mn$^{2+}$, activation was observed with K"$^+$ or NH$_4^+$, while Na"$^+$ was somewhat inhibitory. Lithium ions were found to be strongly inhibitory (Fig. 3). The apparent $K_s$ values for Li"$^+$ were 0.8 and 0.3 $\mu M$ in the presence of saturating concentrations of Mg$^{2+}$ or Mn$^{2+}$, respectively. The inhibition of Fru-P$_2$ase by lithium ions is reversible. The reversibility of the inhibition was demonstrated by assay of a sample in the presence of 2.3 mM Li"$^+$, a concentration that decreased activity to 13%. Dilution of the same enzyme sample into a fresh reaction mixture with no lithium resulted in a nearly complete (90%) restoration of the activity. It is important to emphasize the effect of Li"$^+$ on Fru-P$_2$ase since lithium-7 can be an effective NMR probe of monovalent cation sites in enzymes (24, 25). We have also studied the effect of thallium, another potential NMR probe for monovalent cation-activated enzymes (26), on Fru-P$_2$ase.

FIG. 1. Effect of pH on native and subtilisin-treated mouse liver Fru-P$_2$ase. Native Fru-P$_2$ase was purified as summarized in Table I. Enzyme activity (O) was determined at pH 7.05 as described under "Methods," except for the variable pH. Treatment with subtilisin was performed by incubating the purified Fru-P$_2$ase (0.18 mg/ml) in 0.1 M ammonium acetate (pH 6.5), 0.1 mM EDTA with subtilisin at a ratio of Fru-P$_2$ase to subtilisin of 133:1 (w/w). After 1 h of incubation, samples were assayed for enzyme activity (O).

FIG. 2. Effect of Mg$^{2+}$ on mouse liver Fru-P$_2$ase activity. Except for the variable Mg$^{2+}$, the assays were performed at pH 7.05 as described under "Methods." Inset, Hill plots of the same data; $n$, slope of the straight line.
increased to 78 with K_2SO_4, while is inhibited by AMP. The inhibition data (Fig. 29) demonstrated positive cooperativity for AMP inhibition as temperature increased (23, 27–29). At 30°C, in the presence of 150 mM KC\textsubscript{1} and 3.3 mM MgSO\textsubscript{4}, the concentration at which AMP inhibition was equal to that obtained with 3.3 mM MgSO\textsubscript{4} and 6.7 with 0.13 mM MnSO\textsubscript{4}.

FIG. 4 (center). Inhibition of mouse liver Fru-Pase by AMP. Enzyme activity was measured at pH 7.05 as described under “Methods,” except for the presence of AMP. Assays were performed at 30°C (○), as well as at 37°C (●). Hill plots of data were plotted according to Taketa and Pogell (37). n, slope of the straight line; K_M, AMP concentration at 50% inhibition.

FIG. 5 (right). Time course and extent of phosphorylation of Fru-Pases by the catalytic subunit of cyclic AMP-dependent protein kinase. The phosphorylation of Fru-P\textsubscript{ases} with 0.3 μM catalytic subunit of cyclic AMP-dependent protein kinase was carried out as described under “Methods.” The open symbols denote the experiments performed with 15 μM native rat liver (○) or mouse liver (●) Fru-P\textsubscript{ase}. The closed symbols show the experiments in the presence of 1.6 mM urea. The concentration of rat liver Fru-P\textsubscript{ase} (○) was 2 μM, while the concentration of mouse liver Fru-P\textsubscript{ase} (●) was either 8 or 33 μM.

Thallium activated mouse liver Fru-P\textsubscript{ase}. At 3.3 mM MgSO\textsubscript{4}, the extrapolated V_max with Tl_2SO\textsubscript{4} was equal to that obtained with K_2SO\textsubscript{4}, while K_a for Tl\textsuperscript{2+} was 16 mM compared with a K_a of 46 mM for K\textsuperscript{+}. Mouse liver Fru-P\textsubscript{ase}, like other Fru-P\textsubscript{ases}, is inhibited by AMP. The inhibition data (Fig. 4) demonstrated positive cooperativity (n = 2.6) and the typical decrease in enzyme inhibition as temperature increased (23, 27–29). At 30°C, in the presence of 150 mM KCl and 3.3 mM MgSO\textsubscript{4}, the K_i for AMP was 36 μM. At 37°C, the K_i for AMP increased to 78 μM, a value which is close to the physiologically related range of the AMP concentration in liver cells (1).

“in Vitro” Phosphorylation Experiments—The demonstration of in vitro phosphorylation of rat liver Fru-P\textsubscript{ase} by the catalytic subunit of cyclic AMP-dependent protein kinase has led to the suggestion that cyclic AMP-dependent protein kinase may catalyze the in vivo phosphorylation of Fru-P\textsubscript{ase} (6). Thus, it appeared important to test whether purified mouse liver Fru-P\textsubscript{ase} was a substrate in the phosphorylation reaction. As illustrated by the experiment shown in Fig. 5, purified mouse liver Fru-P\textsubscript{ase} was not a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase under experimental conditions identical with those described for the in vitro phosphorylation of rat liver Fru-P\textsubscript{ase} (6).

Although no phosphorylation was observed using mouse liver Fru-P\textsubscript{ase} as substrate (Fig. 5, ○), the control experiment (Fig. 5, □) indeed showed that rat liver Fru-P\textsubscript{ase} is a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase. The results of the time course of phosphorylation of the rat liver enzyme are in excellent agreement with the data of Riou et al. (cf. Fig. 1 of Ref. 6). Negative results of in vitro phosphorylation of purified mouse liver enzyme were obtained with Fru-P\textsubscript{ase} prepared from extracts with or without 10 mM KF. Competition experiments showed that purified mouse liver Fru-P\textsubscript{ase} (1.9 to 7.6 μM) did not diminish the phosphorylation of 3.8 μM rat liver Fru-P\textsubscript{ase} by the catalytic subunit of cyclic AMP-dependent protein kinase, indicating that the mouse liver Fru-P\textsubscript{ase} neither was an inhibitor of the kinase nor contained protein kinase inhibitory activity. In addition, altering the conditions of the phosphorylation reaction such as including 2 mM fructose 1,6-bisphosphate or omitting EDTA did not result in incorporation of phosphate from ATP into mouse liver Fru-P\textsubscript{ase}. It is well established that the sites of phosphorylation by cyclic AMP-dependent protein kinase are determined by specific local amino acid sequences (30, 31). In addition, it is known that in some proteins (i.e. lysozyme) phosphorylation sites only become accessible to the kinase after the protein is unfolded or denatured (32). Thus, it appeared important to establish whether a site(s) of phosphorylation in mouse liver Fru-P\textsubscript{ase} could be unmasked after unfolding the enzyme. We therefore examined phosphorylation of both rat and mouse liver Fru-P\textsubscript{ase} in 1.6 mM urea after unfolding with 8 mM urea. As shown in Fig. 5 (●), rat liver Fru-P\textsubscript{ase} in 1.6 mM urea became a better substrate for the catalytic subunit of cyclic AMP-dependent protein kinase. The rat liver enzyme in urea was phosphorylated more rapidly than in the absence of urea and the incorporation of about 1 mol of phosphate/mol of rat liver Fru-P\textsubscript{ase} protomer occurred in 30 min. The same maximal phosphate incorporation into rat liver Fru-P\textsubscript{ase} (about 1 mol of phosphate/mol of enzyme protomer) was obtained in either the presence or absence of urea. A separate experiment demonstrated that the phosphorylation in 1.6 mM urea of rat liver Fru-P\textsubscript{ase} previously phosphorylated in its native state to the extent of 0.84 mol of phosphate/mol of enzyme protomer resulted in only a 10% increase (0.08 mol of phosphorus/protomer) in the total extent of phosphorylation. This indirect evidence would appear to indicate that the same site was phosphorylated in both conditions. An increase in the rate of phosphorylation (but not in the extent) also has been shown to occur when unfolded rat liver pyruvate kinase is used as a substrate of cyclic AMP-dependent protein kinase (33). In clear contrast, mouse liver Fru-P\textsubscript{ase} incorporated less than 0.2 mol of phosphate/mol of Fru-P\textsubscript{ase} protomer in phosphorylation experiments performed in 1.6 mM urea (Fig. 5, ●). Thus, we conclude that although rat liver Fru-P\textsubscript{ase} contains a phosphorylation site for cyclic AMP-dependent protein kinase, mouse liver Fru-P\textsubscript{ase} is not a substrate for the above kinase. However, it could be argued that mouse liver was not a substrate of cyclic AMP-dependent protein kinase because the isolated enzyme is already phosphorylated. This was clearly not the case, since no phosphate (either alkali-labile or total) was found in purified mouse liver Fru-P\textsubscript{ase} when assayed for phosphate as...
described under "Experimental Procedures." Samples of commercial phosphorylase a used as a control of the method showed about 0.6 mol of alkali-labile phosphate/mol of enzyme subunit. The possibility that mouse liver Fru-Pase contained covalently bound phosphate was also examined by isolating the enzyme from the livers of mice previously injected with 1.2 to 7 mCi of inorganic $^{32}$P. No radioactivity was found in Fru-Pase isolated from these animals.

Analysis of the phosphate content of purified pig kidney Fru-Pase also revealed no endogenous phosphate in this enzyme, and extensive attempts to phosphorylate purified pig kidney Fru-Pase also revealed no endogenous phosphate in this enzyme, and extensive attempts to phosphorylate purified pig kidney Fru-Pase in its native state by the catalytic subunit of cyclic AMP-dependent protein kinase also have been unsuccessful. However, it should be mentioned that a recent report by Mendicino et al. (34) has shown that pig kidney Fru-Pase can be phosphorylated in vitro by an undefined (as far as cyclic AMP dependence) kidney protein kinase. With regard to the phosphorylation of rat liver Fru-Pase by the catalytic subunit of cyclic AMP-dependent protein kinase, recent work by Pilks et al. (35) has established the amino acid sequence of the phosphorylation site as being Ser-Arg-Pro-[Ser(P)-Leu-Pro-Leu-Pro]. As noted by the authors, this sequence differs somewhat from the usual pattern seen in proteins phosphorylated by cyclic AMP-dependent protein kinase. In addition, it would appear that this proline-rich peptide is not a part of the NH$_2$-terminal end of Fru-Pase since the NH$_2$-terminal region is low in (or has no) proline residues (Table II) and no segment showing any resemblance to the above structure is found in the known sequences of rabbit liver (14) and pig kidney (15) Fru-Pase NH$_2$-terminal S-peptide.

Preparation and Properties of the Enzyme from the Livers of Diabetic Mice—Mouse liver Fru-Pase was also purified from the livers of the genetically diabetic (C57BL/KsJ-db-db) mice. The animals used in this study were at least 20 weeks of age. Hyperglycemia and elevated plasma glucagon levels are characteristic of the genetically diabetic mouse at this stage (36). Fru-Pase was purified as described under "Methods." The extract was made in 1 M EDTA, 10 mM KF (pH 8). No significant differences were observed during purification. The specific activity of the pure enzyme from diabetic liver was also about 30 units/mg. It showed a single protein band after polyacrylamide gel electrophoresis either in the absence or presence of sodium dodecyl sulfate. The electrophoretic mobility, as well as the subunit molecular weight, was identical with the enzyme purified from the liver of control animals. In addition, both Fru-Pases showed the same pH optimum, the same substrate affinity, and the same AMP inhibition pattern. Thus, no difference in either molecular or enzymatic properties appeared to exist between the two enzymes. The only relevant difference observed was found in the amount of enzyme present in extracts. In accordance with previous observations (10, 37, 38), Fru-Pase activity was higher in the liver of diabetic animals. On the average, the amount of enzyme (expressed as units/g of liver) was found to be increased by about 1.5-fold. Since the liver of the diabetic mouse weighs nearly twice that of a normal mouse of the same age, the total amount of Fru-Pase in the liver of a diabetic mouse was 3 times the normal amount.

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Fructose 1,6-bisphosphatase from C57BL/KsJ Mouse Liver

Purification and properties of liver fructose 1,6-bisphosphatase from C57BL/KsJ normal and diabetic mice.
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