The reaction mechanism of rat hepatic fructose-2,6-bisphosphatase involves the formation of a phosphohistidine intermediate. In order to determine the sequence around the active site histidine, the enzyme was incubated with [2-\textsuperscript{32}P]fructose 2,6-bisphosphate, denatured, and treated with trypsin or endoproteinase Lys-C. The resultant labeled \textsuperscript{32}P-phosphopeptides were purified by gel filtration, anion exchange chromatography, and reverse phase high pressure liquid chromatography. The sequence of the tryptic peptide was determined to be HGESELNL, while the partial sequence of the endoproteinase Lys-C peptide was IFDGTRYMVNVRQDHVIQRATAYLMHNHTPRSLYRHLHGESEL. The active site sequence was compared with the active site sequence of other enzymes that catalyze phospho group transfer via a phosphohistidine intermediate. Active site sequences of phosphoglycerate mutase and bisphosphoglycerate synthase were highly homologous with the active site of fructose-2,6-bisphosphatase implying a structural similarity and a common evolutionary origin.

Both the synthesis and the degradation of Fru-2,6-P\textsubscript{2} are catalyzed by a single enzyme protein (1-4). 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.105/3.1.3.46) is an important enzyme in the regulation of hepatic carbohydrate metabolism since its activity determines the steady state concentration of Fru-2,6-P\textsubscript{2}, an activator of 6-phosphofructo-1-kinase and an inhibitor of fructose-2,6-bisphosphatase (1-4). Regulation of this bifunctional enzyme in intact cells is a complex function of both covalent modification via phosphorylation/dephosphorylation and the influence of substrates and low molecular weight effectors (5, 6).

The weight of experimental evidence supports a two-site model for catalysis, where the opposing reactions of the enzyme occur at two discrete active sites (5-7). In the case of the bisphosphatase reaction, an active site phosphoenzyme intermediate (E-P) has been demonstrated upon incubation of the enzyme with [2-\textsuperscript{32}P]Fru-2,6-P\textsubscript{2} (8, 9). The radiolabeled residue was N\textsuperscript{-}phosphohistidine (9). The kinetic competence of the E-P has also been established by comparing its turnover rate with the overall reaction rate and examining the effects of regulatory ligands, such as P\textsubscript{i}, and \alpha-glycerol-P, and cAMP-dependent phosphorylation on its formation and breakdown (10, 11). We now report the primary sequence around the active site histidine for the bisphosphatase and an unexpected homology of this sequence with the active site of phosphoglycerate mutase.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Isolation of a Phosphohistidine-containing Peptide from Rat Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase—We have reported previously that \textsuperscript{32}P phosphohistidine can be isolated from alkali hydrolysates of the bifunctional enzyme after incubation with [2-\textsuperscript{32}P]Fru-2,6-P\textsubscript{2} (8). A number of proteases were tested for their ability to generate a labeled phosphohistidine-containing peptide. An endoproteinase Lys-C (Achromobacter protease I) digest of [2-\textsuperscript{32}P]Fru-2,6-P\textsubscript{2}-labeled enzyme yielded a peptide with a molecular weight of approximately 7500 as determined by size exclusion on a TSK column in the presence of 6 M guanidinium HCl in 10 mM P\textsubscript{i}, at pH 6.8 (data not shown). Attempts to isolate this peptide using HPLC at acid pH led to the loss of labeled peptide and very low yields as a result of the acid lability of the N-P bond. A preliminary experiment with trypsin as the proteolytic agent is shown in Fig. 1. Enzyme was labeled at the bisphosphatase active site by incubation with [2-\textsuperscript{32}P]Fru-2,6-P\textsubscript{2} or at the cAMP-dependent protein kinase site by incubation with [\gamma-\textsuperscript{32}P]ATP and cAMP-dependent protein kinase. After denaturation, the enzyme was digested with trypsin and then subjected to paper electrophoresis at pH 2.0 and 2°C. The cAMP-dependent phosphorylation site peptide essentially did not move from the origin, while the active site phosphopeptide migrated toward the anode. The sequence of the tryptic fragment containing the cAMP-dependent phosphorylation site is RGSSIPQFTNSPR (14). This composition is consistent with little or no migration at pH 2.0. The migration of the active site peptide indicates that it is an acidic peptide which should bind to an anion exchange column at neutral pH. [2-\textsuperscript{32}P]Fru-2,6-P\textsubscript{2} was also subjected to paper
electrophoresis, and it migrated toward the anode beyond the active site peptide. These results further show that active site labeling does not merely involve tight binding of Fru-2,6-P₂ to the enzyme or to a peptide, since no [2-³²P]Fru-2,6-P₂ was seen in the active site lane. These results suggested that a tryptic phosphopeptide might serve as a source for sequence analysis of the bisphosphatase active site provided problems of acid lability could be avoided.

The isolation of an active site tryptic fragment of the enzyme is shown in Fig. 2. Under optimal conditions, when 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase was incubated with [2-³²P]Fru-2,6-P₂, the enzyme could be phosphorylated to a maximum level of 1.8 mol of phosphate/mol of enzyme (10, 11). After incubation with trypsin, the entire digest was applied to a Sephadex G-25 superfine column (1.5 × 100 cm) equilibrated with 20 mM TEA-HCO₃, pH 8.2. As shown in the top panel of Fig. 2, the gel filtration profile of the tryptic digest revealed a peak of radioactivity coincidental with absorbance at 280 nm (fractions 29–32). This peak of radioactivity eluted at an elution/void volume of about 1.50, suggesting that the peptide had a molecular weight of 800–1200. The second peak of radioactivity is ³⁵S, eluting at the inclusion volume of the column. The peptide-associated radioactivity was applied directly to a DEAE-Sephadex A-25 column (0.9 × 10 cm) equilibrated with 20 mM TEA-HCO₃, pH 8.2. As expected, the ³²P-phosphopeptide bound to DEAE-Sephadex and was eluted with a linear gradient of TEA-HCO₃ (20–600 mM) at 300 mM TEA-HCO₃ (Fig. 2B). Only one peak of radioactivity was eluted from the DEAE-Sephadex column, and the pooled fractions were dried down by rotary evaporation. The residue was dissolved in 10 mM ethylenediamine, pH 6.8. The final purification step employed high pressure liquid chromatography on a Hamilton PRP-1 reverse phase column (4.1 × 150 mm) in 10 mM ethylenediamine, at pH 6.8, with two gradients of 0–20% CH₃CN, followed by 20–100% CH₃CN (Fig. 2C). A single peak of ³²P, radioactivity eluted at 13% CH₃CN. The radioactive peak was the first peptide to elute from the column, probably reflecting its highly charged nature. Since the buffer used to elute the ³²P-phosphopeptide contained an amine, it was necessary to completely remove it from the peptide prior to sequencing. Rotary evaporation did not completely remove ethylenediamine, but it was removed by passing the peptide over a P-2 (0.9 × 10 cm) column equilibrated with 1% acetic acid and run at 0 °C. The overall recovery of ³²P radioactivity was 24%. Similarly, the endoprotease Lys-C peptide containing the active site histidine was also purified by the same procedure (data not shown).

**Amino Acid Composition of Fructose-2,6-bisphosphatase Active Site Peptides**—The peptides were concentrated by rotary evaporation, and an aliquot was subjected to hydrolysis in 6 N HCl for 24 h at 100 °C. The amino acid composition of the peptides is shown in Table I. The composition of the tryptic peptide indicated a 9-residue peptide with 1 residue of histidine per mol of peptide. Since phosphohistidine is found in the labeled enzyme after incubation with [2-³²P]Fru-2,6-P₂, the histidine found in this peptide must be the phosphoacceptor. Table I also shows that the endoprotease Lys-C peptide contains 3 histidines per mol of peptide.

**Sequence Determination of Fructose-2,6-bisphosphatase Active Sites**—When these two peptides were subjected to automated sequence analysis using a gas phase sequencer, the sequences shown in Table II were obtained. The tryptic peptide yielded the following sequence: HGESELNLH. The active site histidine is, therefore, the 1st residue, and, since this is a tryptic peptide, the next amino-terminal residue must be either an arginine or a lysine. As expected, the peptide contains 2 glutamic acids, as well as the phosphohistidine, and is quite acidic. A partial sequence of the endoprotease Lys-C peptide revealed the tryptic peptide at the carboxyl end and further extended the sequence in the amino-terminal direction. The 1st arginyl residue of the tryptic peptide was residue 39 of the endoproteinase Lys-C peptide, and the last identifiable residue was a leucine, the 6th residue of the tryptic peptide. The sequences obtained were consistent with the amino acid compositions of both peptides and positive identification of all residues was obtained.

**DISCUSSION**

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase is one of only a handful of enzymes whose reaction mechanism has been shown to involve covalent catalysis in which a phosphohistidine has been implicated. The first such enzyme was succinyl-CoA synthetase from E. coli (20). Three proteins in the sugar transport system of bacteria, Enzyme I, HPr, and enzyme-III₂₅₆, have been shown to catalyze the sequential transfer of the phosphoryl group of P-enolpyruvate to a phosphotransferase sugar (21), all via a phosphohistidine intermediate (22). Interestingly, while the site of P-enolpyruvate-dependent phosphorylation in the primary structure of HPr is a histidine, these proteins have also recently been shown to be subject to phosphorylation by a protein kinase on a seryl residue (22). This phosphorylation of the HPr on serine can modulate the rate of sugar transport and determines the hierarchical utilization of phosphotransferase sugars and may play a role in catabolite repression (22). Thus, HPr and the hepatic bifunctional enzyme are both regulated by a protein kinase-mediated phosphorylation on a serine residue and both involve a phosphohistidine in phosphoryl group transfer. In addition to the liver bifunctional enzyme, mammalian enzymes that involve phospho group transfer via a histidyl residue include diphosphoglycerate mutase (23), bisphosphoglycerate synthase (23), glucose-6-phosphatase (24), and nucleoside diphosphokinase (25).

The amino acid sequence around the active site histidine is known for some of these proteins and are compared in Table III. His-15 is the site of P-enolpyruvate-dependent phosphorylation in HPr proteins from three different gram-positive bacteria, as well as E. coli (22). Ser-46 is the target amino acid of the ATP-dependent kinase (22). There is no obvious homology between the active site histidine of the HPr proteins and the rat liver bifunctional enzyme, nor with the active site sequence of succinyl-CoA synthetase. There is, however, a striking homology between the fructose-2,6-bisphosphatase active site sequence and the active site sequences of two phosphoglycerate mutases and two phosphoglycerate synthases (Table III). All five enzymes contain 6 residues (boxed region) that are essentially identical or require a single nucleotide base change in their respective codons, except for the threonine in rabbit phosphoglycerate mutase which requires two base changes. The active site histidine in the yeast mutase is located near the amino terminus (His-8), while in the case of the hepatic bifunctional enzyme, the active site histidine is in the middle of the subunit (14). There is a 37.5% identity between 75 amino acids at the amino terminus of the yeast mutase and a 75-amino acid region in the rat liver enzyme which contains the bisphosphatase active site (14).³ The finding of active site homologies between these enzymes is striking but perhaps not surprising in view of the multifunctional

³Preliminary experiments revealed that a commercial preparation of rabbit muscle phosphoglycerate mutase from Dr. S. Pilki (Proteins X-ray Mannheim) did not catalyze hydrolysis of Fru-2,6-P₂ (A. Tauler and S. Pilki, unpublished results).
nature of both enzymes. The synthase and mutase catalyze three reactions: the conversion of 1,3-diphosphoglycerate to 2,3-diphosphoglycerate; the interconversion of 3- and 2-phosphoglycerates; and the dephosphorylation of 2,3-diphosphoglycerate. Although there is some suggestive evidence for involvement of 2 phosphohistidine residues in the phosphoglycerate mutase and bisphosphoglycerate synthase reactions, the current consensus is that all three reactions are catalyzed at a single active site (26-28). Thus, the phosphoglycerate mutase, bisphosphoglycerate synthase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase all catalyze transfer of phosphate from a bisphosphatase to water and have essentially homologous active sites containing a histidinyl residue. The strong active site homology suggests an evolutionary link between these enzymes.

The active site homology between these enzymes also reflects the importance of the respective bisphosphates whose steady state concentration they control. In red blood cells, the bisphosphoglycerate synthase catalyzes the synthesis and also the hydrolysis of 2,3-bisphosphoglycerate, an important regulator of the oxyhemoglobin dissociation curve. Fru-2,6-P₂ plays a pivotal role in determining glycolytic flux in liver (1-6). In extrahepatic tissues, where a switching mechanism between glycolysis and gluconeogenesis is unnecessary, the role of Fru-2,6-P₂ in regulating glycolysis is less certain (15). For example, in heart, it has been claimed that there is little or no bisphosphatase activity associated with 6-phosphofructo-2-kinase (15). It remains to be determined whether the heart 6-phosphofructo-2-kinase contains the hepatic bisphosphatase active site sequence and/or whether it is changed or modified in some manner as to preclude efficient hydrolysis of Fru-2,6-P₂. Work is also currently in progress to determine whether the hepatic bisphosphatase active site sequence is present in Fru-2,6-P₂ metabolizing enzymes in other extrahepatic tissues.

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REFERENCES
1. Pilkis, S. J., El-Maghrabi, M. R., McGrane, M., Pilkis, J., Fox, E., and Claus, T. H. (1982) Mol. Cell. Endocr. 25, 245-266.
2. Hers, H.-G., and Van Schaftingen, E. (1982) Biochem. J. 206, 1-12.
3. Uyeda, K., Furuya, E., Richards, C. S., and Yokoyama, M. (1982) Mol. Cell. Biochem. 48, 97-125.
4. Pilkis, S. J., Chrisman, T., Burgess, B., McGrane, M., Colosia, A. D., Pilkis, J., Claus, T. H., and El-Maghrabi, M. R. (1983) Adv. Enzyme Regul. 21, 147-173.
5. Pilkis, S. J., Regen, D. M., Stewart, B. H., Chrisman, T., Pilkis, J., Kountz, P. D., McGrane, M., El-Maghrabi, M. R., and Claus, T. H. (1983) Mol. Aspects Cell. Regul. 3, 85-122.
6. Claus, T. H., El-Maghrabi, M. R., Regen, D. M., Stewart, H. B., McGrane, M., Kountz, P. D., Nylander, F., Pilkis, J., and Pilkis, S. J. (1984) Curr. Top. Cell. Regul. 23, 57-86.
7. El-Maghrabi, M. R., and Pilkis, S. J. (1984) J. Cell. Biochem. 26, 1-17.
8. Pilkis, S. J., Regen, D. M., Stewart, H. B., Pilkis, J., Pate, T. M., and El-Maghrabi, M. R. (1984) J. Biol. Chem. 259, 948-958.
9. Pilkis, S. J., Walderhaug, M., Murray, K., Beth, A., Vankataraman, S. D., Pilkis, J., and El-Maghrabi, M. R. (1985) J. Biol. Chem. 258, 6135-6141.
10. Stewart, H. B., El-Maghrabi, M. R., and Pilkis, S. J. (1985) J. Biol. Chem. 260, 12935-12941.
11. Stewart, H. B., El-Maghrabi, M. R., and Pilkis, S. J. (1986) J. Biol. Chem. 261, 4793-4797.
12. Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., Claus, T. H., and Cumming, D. A. (1981) J. Biol. Chem. 256, 3171-3174.
13. El-Maghrabi, M. R., Pate, T. M., Murray, K., and Pilkis, S. J. (1984) J. Biol. Chem. 259, 13906-13910.
14. Lively, W. O., El-Maghrabi, M. R., Pilkis, J., D’Angelo, G., and Pilkis, S. J. (1987) J. Biol. Chem., in press.
15. El-Maghrabi, M. R., Correa, L. J., Heit, P., Pate, T. M., Cobb, C. E., and Pilkis, S. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5005-5009.
16. Colosia, A. D., Lively, M. O., El-Maghrabi, M. R., and Pilkis, S. J. (1987) Biochem. Biophys. Res. Commun. 143, 1092-1098.
17. Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. (1983) Methods Enzymol. 91, 399-413.
18. Field, P. O. H., Rosebrough, N. J., Part, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
19. Roskoski, R., Jr. (1983) Methods Enzymol. 99, 3-6.
20. Wang, T., Jurášek, L., and Bridger, W. A. (1972) Biochemistry 11, 2067-2070.
21. Saier, M. H., Jr. (1985) Mechanisms and Regulation of Carbohydrate Transport in Bacteria, Academic Press, Orlando, FL.
22. Reizer, J., and Peterkofsky, A. (1987) Regulatory Mechanisms for Sugar Transport in Gram Positive Bacteria, Academic Press, Orlando, FL, in press.
23. Rose, Z. B. (1982) Methods Enzymol. 87, 42-51.
24. Feldman, P., and Bulte, S. G. (1972) Biochim. Biophys. Acta 266, 698-710.
25. Colombo, M. G., Cheruy, A., and Vignais, P. V. (1972) Biochemistry 11, 3378-3386.
26. Britton, H. G., and Clarke, J. B. (1972) Biochem. J. 130, 397-410.
27. Breathnach, R., and Knowles, J. (1977) Biochemistry 16, 3064-3066.
28. Blattler, W. A., and Knowles, J. R. (1980) Biochemistry 19, 738-743.
Active Site Sequence of Hepatic Fructose-2,6-bisphosphatase: Homology in Primary Structure with Phosphoglucomutase

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Experimental Procedures

Tryptic Peptide

| Amino Acid | (mol%) |
|------------|--------|
| Aspartic Acid | 0.35 |
| Glutamic Acid | 1.36 |
| Serine | 1.16 |
| Threonine | 0.45 |
| Alanine | 0.48 |
| Proline | 0.76 |
| Valine | 2.19 |
| Isoleucine | 2.28 |
| Leucine | 0.15 |
| Phenylalanine | 0.30 |
| Cystine | 0.40 |

Total residues | 97

Endo Lys C Peptide

| Amino Acid | (mol%) |
|------------|--------|
| Aspartic Acid | 0.35 |
| Glutamic Acid | 1.36 |
| Serine | 1.16 |
| Threonine | 0.45 |
| Alanine | 0.48 |
| Proline | 0.76 |
| Valine | 2.19 |
| Isoleucine | 2.28 |
| Leucine | 0.15 |
| Phenylalanine | 0.30 |
| Cystine | 0.40 |

Total residues | 97

Automated Edman degradation of the tryptic and endo Lys C phosphopeptides isolated from 6-phosphofructo-2,6-bisphosphatase: The peptides were subjected to automated Edman degradation on the gas-phase sequencer (ABI 470A). The residues of the tryptic peptide and 7.5 moles of the endo Lys C peptide were applied to the glass fiber filter of the ABI 470A. Only the major sequence observed is presented. Minor residues at each cycle were less than 15 per cent the major residue. The same sequence was obtained with 2-4 different preparations. A dash indicates that a clear identification of a phosphotyrosyltyrmine-amino acid could not be made.

Amino Acid Analysis

Amino acid analysis was performed with a Waters "Ficro Tag" system equipped with DEC 280 computers for data acquisition and storage. Samples were subjected to hydrolysis on a glass ion exchange column for 30-60 h at 120°C.

Automated Edman Sequencing

Automated Edman sequencing was carried out on an Applied Biosystems 470A protein sequencer using the Edman degradation procedure of Graber et al. (17) followed by high performance liquid chromatography identification of PTH-amino acids with an Applied Biosystems PTH Analyzer (18).

Other Methods

Peptide sequencing was performed by the method of Loréy et al. (18).

Amino Acid Incorporation into Proteins

Amino acid incorporation into proteins was determined by the phosphonincellulose paper assay (19) at 4°C in order to minimize the hydrolysis of acid labile N-P bonds.

Figure 1. High Voltage Electrophoresis of the active and Phosphorylation Site J9-Tryptic Peptide. Rat liver 6-phosphofructo-2,6-bisphosphatase (55S) was incubated either with catalytic subunit of the cAMP-dependent protein kinase and 0.1 M (1.0 x 10^7 cpm/nmol) adenine (ATP) as described previously (13) or with 1μM (2.6 x 10^7 cpm/nmol) ATP. After 5 minutes the reactions were terminated by the addition of 5 percent TCA at 4°C. The precipitate was washed twice with cold TCA, once with ether, and then dissolved in 50 microliters of IM urea. The dissolved labelled enzymes were diluted to the following concentrations: 100× TDE-RM, 100× R, 2× KM, and 3× Urea. Tryptic was added and the mixture was incubated for 12 hours at 4°C. An aliquot was then added to Whatman 3MM paper and subjected to electrophoresis at pH 2.0. Radioactive peptides were detected by autoradiography. [23P] (Fr-2,6-1.4) was run as a control.

Figure 2. Purification of the 19-Active Site Tryptic Peptide of 6- phosphofructo-2,6-bisphosphatase. The enzyme was labelled with [32P] (P-Fru-2,6-1.4) complexed with trypsin and the labelled peptide was purified by gel filtration on Sephadex G-25 (10) isoelectric point chromatography on Bio-Rad chromatography on Sephadex A-25 and C) reverse phase HPLC as described under "Experimental Procedures."