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EVIDENCE FOR DIFFERENT FORMS OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE

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Purified liver fructose 1,6-bisphosphatase exhibits different forms upon isoelectric focusing. The enzyme focused at pH 5.75, 5.60, and 5.44. Treatment of the enzyme preparation with the catalytic subunit of cAMP-dependent protein kinase and ATP altered the isoelectric focusing profile such that the bands at 5.75 and 5.60 were diminished, the band at 5.44 increased, and two new bands appeared at 5.30, and 5.18. Fructose 1,6-bisphosphatase may be present in rat liver in different forms, one of which is phosphorylated as the enzyme is isolated.

Rat hepatic fructose 1,6-bisphosphatase is a key regulatory enzyme in the gluconeogenic pathway. The mechanism by which fructose 1,6-bisphosphatase activity is modulated under conditions of enhanced or decreased gluconeogenic flux has been shown to involve changes in the level of fructose 2,6-bisphosphate (1). Another mechanism which has been suggested is regulation by cAMP-dependent phosphorylation (2,3). However, we have been unable to detect significant functional activity changes which correlate with cAMP-dependent phosphorylation (see ref 1 for review). One explanation could be that there are different forms of the enzyme which undergo different degrees of phosphorylation with subsequent differences in regulation of their activities. It was the purpose of this study to determine whether purified preparations of rat liver fructose 1,6-bisphosphatase contain more than one form of the enzyme and if so to characterize their phosphorylation by the cAMP-dependent protein kinase.

METHODS

Purification and Assay of Rat Liver Fructose 1,6-Bisphosphatase. Rat liver fructose 1,6-bisphosphatase was purified by a modification of the method of Riou et al. (2). A 6-12 percent polyethylene glycol fractionation step was included in the preparation prior to DEAE-Sephadex A-50 chromatography. For all concentration steps a collodion bag apparatus was utilized. The enzyme preparations contained the following protease inhibitors: 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM diisopropyl fluorophosphate (DFP), 1 mM pepstatin, and 0.5 μg/ml leupeptin. The enzyme exhibited a single

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band after sodium dodecyl sulfate gel electrophoresis with a subunit molecular weight of 41000 (2). Fructose 1,6-bisphosphatase was assayed spectrophotometrically as previously described (2).

Isoelectric Focusing. Isoelectric focusing was done utilizing a Hoeffer model SE500 vertical slab electrophoresis unit. Denaturing conditions were utilized. The gels were 4 percent acrylamide/bis acrylamide, 9M urea, with an ampholine range of pH 5-7. Gels were run at 600V (constant voltage) for 10-12 hours.

The isoelectric focusing gels were stained with $AgNO_3$ according to the method of Merril (4). The gels were then scanned utilizing the Kontees Fiber Optics densitometer, dried with the Hoeffer SE 540 slab gel dryer, and autoradiographed utilizing Kodak X-Omat AR film.

Chromatofocusing. Chromatofocusing of fructose 1,6-bisphosphatase was conducted utilizing the Pharmacia Polybuffer and gel, PBE-94. The pH range was 4.0-7.0. The gel exchanger was equilibrated with "start" buffer, 0.025 M imidazole-HCl, pH 7.4. The eluent buffer, Polybuffer, was diluted 1:8 and adjusted to pH 4.0 with HCl. The sample was equilibrated with the "start" buffer and applied to the column. The column was then eluted with Polybuffer.

RESULTS

Chromatofocusing and isoelectric focusing of rat liver fructose 1,6-bisphosphatase were conducted in order to resolve possible isoenzymic as well as phosphorylated and dephosphorylated forms of the enzyme. When the enzyme which had been purified to apparent homogeneity was chromatofocused under non-denaturing conditions within a pH range of 4.0-7.0 (as described in Methods), three protein peaks were discernable (Fig. 1). Sodium dodecyl sulfate electrophor-

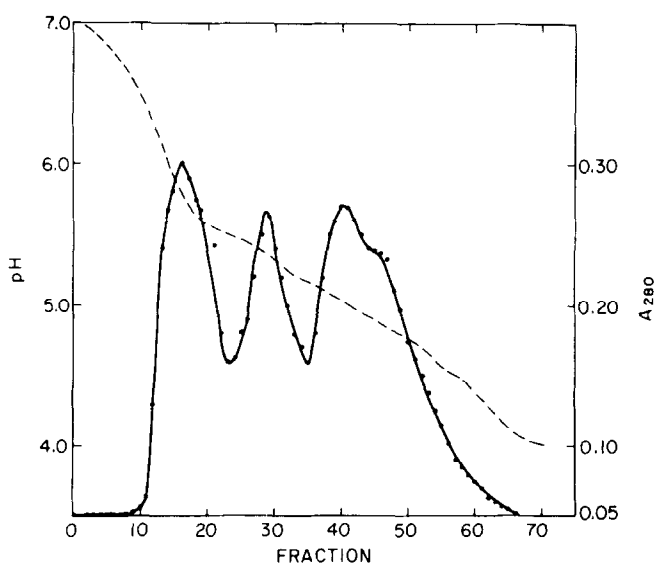

Figure 1. Rat liver fructose 1,6-bisphosphatase which had been purified to homogeneity was chromatofocused as described in Methods. Enzyme activity was detectable in the peak at pH = 5.75 and the peak at pH = 5.3, but not in the peak at pH = 5.0. When each of these peak fractions were run on SDS polyacrylamide disc gels a single band was detected in each gel, each with a relative mobility corresponding to that of the 41000 molecular subunit of rat liver fructose 1,6-bisphosphatase (data not shown).

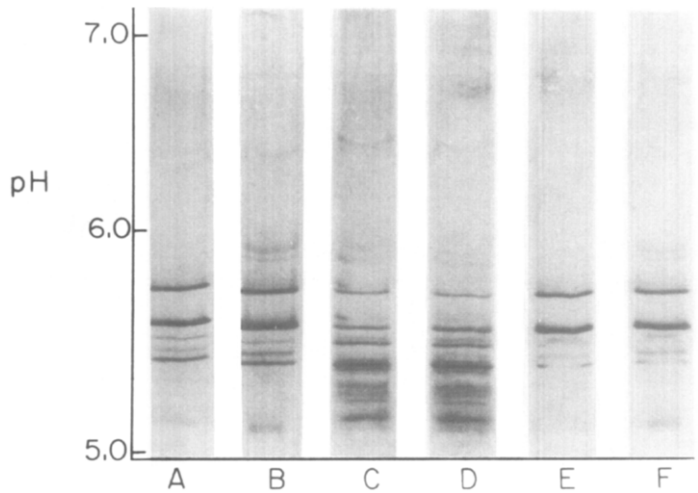

Figure 2. Isoelectric focusing of fructose 1,6-bisphosphatase purified from rats in two different nutritional states. Isoelectric focusing and $AgNO_3$ staining were conducted as described in Methods. Two separate enzyme preparations were processed in tandem under identical conditions from rats fed either a normal diet or rats fasted for 48 hours and refed a high carbohydrate diet for 72 hours. Lane (A) native enzyme, (B) native enzyme from fasted/refed rats, (C) phosphorylated enzyme, (D) phosphorylated enzyme from fasted/refed rats, (E) alkaline phosphatase-treated enzyme, and (F) alkaline phosphatase-treated enzyme from fasted/refed rats.

esis of each peak showed an apparent subunit molecular weight of 41,000 suggesting that the different forms were not a result of proteolysis during chromatofocusing. However, it was not possible to completely separate one form from the other and the form eluting at pH 5 and below had lost most of its activity. The chromatofocusing results were corroborated by isoelectrofocusing of the enzyme (Figure 2A). Isoelectric focusing revealed three major bands with pIs of 5.75, 5.60, and 5.44 (Figure 2A) and densitometric scans indicated that they were in the proportions of 2:2:1 (30 percent: 34 percent: 17 percent), respectively (Fig. 3A). When fructose 1,6-bisphosphatase was incubated with the cAMP-dependent protein kinase and (γ-^{32}P)ATP, 3 mols of ^{32}P were incorporated per mole of tetramer and the isoelectric focusing pattern of the enzyme was altered (Figure 2C). Densitometric scanning indicated that the bands at 5.75 and 5.60 were diminished to 6 and 7 percent, respectively, of the total area, whereas the band at 5.44 was increased to 26 percent of the total area (Figure 3C). Furthermore, a diffuse broad band in the region of pH 5.30 and a band at pH 5.18 appeared (25 percent and 21 per-

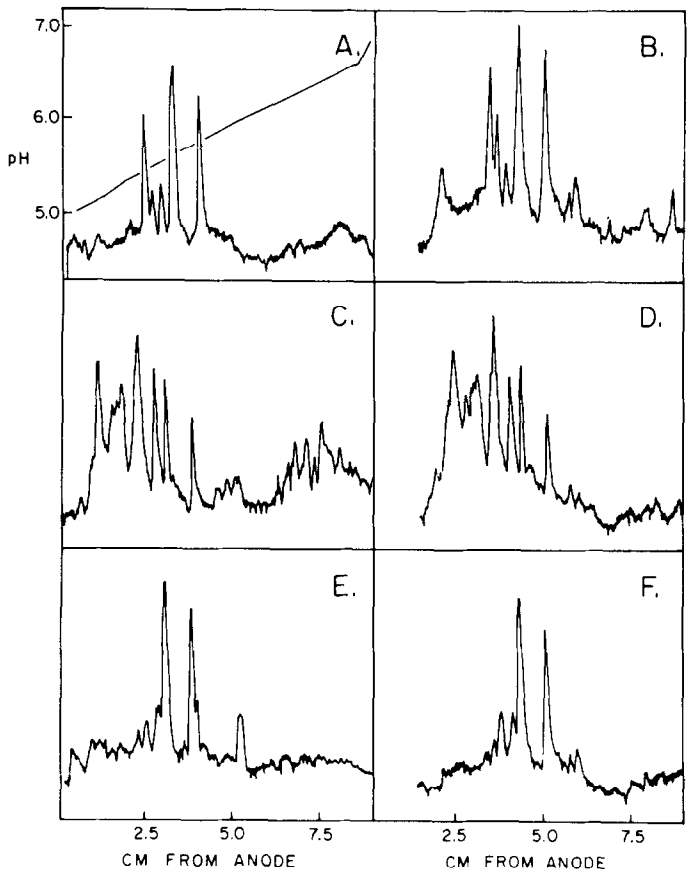

Figure 3. Densitometric scan of isoelectric focusing gel in Figure 1.

cent, respectively, of the total area). When the native enzyme was incubated with alkaline phosphatase, densitometric scans indicated that the peak at pH 5.44 was diminished to 8 percent of the total area (Figure 2E, 3E) and essentially only the forms migrating at pH 5.75 and 5.6 were seen.

In order to determine if these charge shifts upon phosphorylation were a result of proteolysis during the incubation with protein kinase, the nonphosphorylated enzyme was incubated under identical conditions in the absence of ATP. This treatment did not affect the ioselectrofocusing profile of the enzyme, i.e., it resembled that of the native enzyme (data not shown). Autoradiograms of the gels indicated that the native enzyme incubated in the presence of (γ-^{32}P)ATP but in the absence of the cAMP-dependent protein kinase, did not incorporate any phosphate. Fructose 1,6-bisphosphatase which was

Table I

pI	Phosphorylated fructose 1,6-bisphosphatase	Phosphorylated Fructose 1,6-bisphosphatase immunoprecipitated with antibody
	percent Total Area of Integrate Peaks	
5.52	11.7	16.2
5.40	26.3	23.1
5.30	24.9	23.0
5.18	34.8	36.6

Rat liver fructose 1,6-bisphosphatase was incubated with the cAMP-dependent protein kinase and (γ-^{32}P)ATP as described in Methods. The phosphorylated enzyme was then immunoprecipitated with rat liver fructose 1,6-bisphosphatase antibody as described in Methods. The densitometric scan was conducted on the autoradiogram of the isoelectric focusing gel.

incubated in the presence of (γ-^{32}P)ATP and the cAMP-dependent protein kinase incorporated phosphate into bands at pH = 5.52, 5.44, 5.30, and 5.18 (Table 1).

To insure that none of the bands we identified as fructose 1,6-bisphosphatase were contaminants of the enzyme preparations, we precipitated the phosphorylated and nonphosphorylated fructose 1,6-bisphosphatase with rabbit anti-rat liver fructose 1,6-bisphosphatase serum. The antigen-antibody complex was dissolved in sample buffer containing 9 M urea and subjected to isoelectric focusing. All of the $AgNO_3$-staining bands were precipitable with anti-fructose 1,6-bisphosphatase serum (Table 1).

A potential problem which could result in microheterogeneity, was the effect of the enzyme's endogenous metal ion content on migration of the enzyme in isoelectric focusing gels. We have shown that the purified enzyme contains four moles of tightly bound Mg^{++} and Zn^{++} per tetramer (5). Ampholines present in the isoelectric focusing gel could differentially chelate these metal ions and cause a shift in the migration of the enzyme. However, when we incubated the native form of the enzyme in the presence of an excess of EDTA (5 mM) the isoelectric focusing profile was unchanged (data not shown).

The above results indicate that the enzyme appears to exist in two major forms with pIs of 5.75 and 5.60. The different pI values of these forms are not accounted for by a difference in phosphate content since both forms were

observed after alkaline phosphatase treatment, nor is it likely that they arise from different amounts of bound divalent cations. One appears to be in a partially phosphorylated form and migrates with a pI of 5.44.

We purified the enzyme from rats under a different nutritional condition in an attempt to determine if there were differences in the phosphorylation state of the enzyme. Isoelectric focusing was conducted on enzyme from rats fasted for 48 hours and refed a high carbohydrate diet for 72 hours. This enzyme exhibited an isoelectric focusing profile similar to the enzyme purified from rats fed a normal diet (Figure 2B, 3B). Treatment of the enzyme from fasted/refed rats with the cAMP-dependent protein kinase or alkaline phosphatase resulted in isoelectric focusing patterns which were also similar to those of the enzyme from normal rats (Figure 2D,F and 3D, F respectively).

DISCUSSION

The demonstration of two apparently different forms of purified rat liver fructose 1,6-bisphosphatase raises the question of whether such forms exist in intact cells. Claus, et al. (6) employing immunological methods first demonstrated that glucagon enhanced ^{32}P incorporation into fructose 1,6-bisphosphatase in intact hepatocytes. Garrison and Wagner (7), using a two-dimensional gel system to identify labelled hepatocyte phosphopeptides, confirmed that glucagon enhanced ^{32}P incorporation into fructose 1,6-bisphosphatase by about 2-fold. These workers also showed that the fructose 1,6-bisphosphatase antibody precipitated two radiolabelled spots (No. 24 and 25 (see ref. 7 and 8)) suggesting that two forms of fructose 1,6-bisphosphatase may be present in rat liver cells (Dr. Jim Garrison, personal communication). Glucagon enhanced ^{32}P incorporation into only one of the two forms. These spots focus within the same pH region (5.6-5.4) described here for the purified enzyme (5.75-5.4). These findings suggest that the purification does not alter the enzyme so as to induce microheterogeneity upon isoelectric focusing and that two forms whose phosphorylation state is differentially affect by glucagon may also exist in intact cells. Rittenhouse et al. (8) have suggested that there are two cAMP-dependent phosphorylation sites at the

COOH-terminal region of the fructose 1,6-bisphosphatase subunit. The increase in the number of bands upon phosphorylation of the purified enzyme by the cAMP-dependent protein kinase (Fig. 3) may be due to the differential incorporation of either one or two moles of phosphate per enzyme subunit.

The presence of different forms of fructose 1,6-bisphosphatase, both in purified preparations of the enzyme and in intact hepatocytes, raises a number of questions as to the regulation of this activity in rat liver. The two major forms (pI 5.75 and 5.60) may represent different isoenzymic forms or they may reflect as yet unrecognized charge modifications of the protein. The two forms may also be regulated differently by protein phosphorylation. These questions will not be ultimately answered until the different forms of the protein are purified and characterized in detail. This work is in progress.

REFERENCES

1. Claus, T.H., El-Maghrabi, M.R., Regen, D.M., Stewart, H.B., McGrane, M., Kountz, P., Nyfeler, F., Pilkis, J., Pilkis, S.J. (1983) Current Topics in Cellular Regulation 23, In Press.
2. Riou, J.P., Claus, T.H., Flockhart, D.H., Corbin, J., and Pilkis, S.J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4615-4619.
3. Ekman, P. and Dahlquist-Edberg, U. (1981) Biochim. Biophys. Acta 662, 265-270.
4. Merril, C.R., Goldman, D., and Van Keuven, M.L. (1981) Methods in Enzymology
5. McGrane, M.M., El-Maghrabi, M.R., and Pilkis, S.J. (1983) J. Biol. Chem. 258, 10445-10454.
6. Claus, T.H., Schlumpf, J., El-Maghrabi, M.R., McGrane, M., and Pilkis, S.J. (1981) Biochem. Biophys. Res. Commun. 100, 716-723.
7. Garrison, J., and Wagner, J.D. (1982) J. Biol. Chem. 257, 13135-13143.
8. Rittenhouse, J., Chatterjee, T., Marcus, F., Reardon, I., and Heinrikson, R.L. (1983) J. Biol. Chem. 258, 7648-7652.