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A RE-EVALUATION OF THE MOLECULAR WEIGHT OF YEAST (SACCHAROMYCES CEREVISIAE) FRUCTOSE-1,6-BISPHOSPHATASE

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SUMMARY: In contrast with previous results that indicate that Saccharomyces cerevisiae fructose-1,6-bisphosphatase is a dimer of 56,000 molecular weight subunits, we find that the subunit M_r of the enzyme purified from baker's yeast is 40,000. The same subunit M_r was observed in immunoprecipitates of crude supernatants of baker's yeast and S. cerevisiae cultures, as well as in acid-extracts of cells detected by immunoblotting, suggesting that the native subunit indeed has a M_r of 40,000 and it has not been produced from a larger polypeptide. Complete immunoprecipitation of fructose-1,6-bisphosphatase activity with saturating concentrations of specific antibody suggests that there is only one fructose-1,6-bisphosphatase isozyme in S. cerevisiae. The M_r of the purified enzyme determined by size exclusion HPLC suggests that it has a tetrameric structure characteristic of fructose-1,6-bisphosphatases from a broad phylogenetic spectrum.

The gluconeogenic fructose-1,6-bisphosphatases from a variety of organisms appear to be highly conserved with respect to structure and function. Enzymes from a wide phylogenetic spectrum have subunit molecular weights in the range 35,000-40,000, are tetrameric, and have similar kinetic and regulatory properties (for review, see 1 and 2). Fructose-1,6-bisphosphatase (FbPase) from yeast Saccharomyces cerevisiae has been reported to have kinetic properties which are similar to other gluconeogenic FbPases (3), but an unusual characteristic of the yeast enzyme is that it can be phosphorylated in vitro by cAMP-dependent protein kinase (4,5). Phosphorylation also occurs in vivo, apparently linked to a cAMP-dependent system (6-8). Marked increases in enzyme phosphorylation were found upon the addition of glucose to gluconeogenic yeast cultures (5,6), conditions which result in rapid disappearance of FbPase and several other enzymes involved in gluconeogenesis (9). For this

Abbreviation: FbPase, fructose-1,6-bisphosphatase.

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reason phosphorylation of yeast FbPase has been viewed as a signalling mechanism for its own degradation during this process, termed catabolite inactivation (9). The only other FbPase known to be phosphorylated both in vivo and in vitro is the enzyme from rat liver (10,11) and we have recently established from sequence analysis that rat liver FbPase extends 24-26 residues beyond the COOH-terminal amino acid of other mammalian FbPases and that two phosphorylation sites are located in this extension (12,13). To determine the amino acid sequence adjacent to the phosphorylation site(s) of the yeast enzyme, we have initiated a study of FbPase isolated form S. cerevisiae. During the course of these studies we confirmed that the yeast enzyme is phosphorylated in vivo and that yeast FbPase can be phosphorylated in vitro by cAMP-dependent protein kinase. However, in contrast to previous estimates of a subunit molecular weight of 56,000 (3,5) we have evidence to conclude that FbPase from S. cerevisiae has a subunit molecular weight of about 40,000 and a tetrameric structure similar to other gluconeogenic FbPases. This evidence is presented in this communication.

MATERIALS AND METHODS: FbPase from baker's yeast was purified by a procedure which consisted of the following steps: Extraction, heating in the presence of fructose-1,6-bisphosphate, protamine sulfate treatment, ammonium sulfate precipitation, and phosphocellulose chromatography using substrate elution. Details of the purification will be published elsewhere [1]. The resulting electrophoretically pure enzyme (Fig. 1A, lane 2) had a specific activity of 70 - 80 U/mg protein and exhibited kinetic and regulatory properties similar to those reported earlier (3). Enzyme activity was assayed as described (14). Antiserum specific for purified yeast FbPase was raised in rabbits, and used to prepare immunoprecipitates of enzyme from crude yeast supernatants for analysis by gel electrophoresis as described for rat liver FbPase (12). Gel electrophoresis in the presence of sodium dodecyl sulfate was performed in slabs of 12% polyacrylamide using a minor modification (15) of the procedure of Laemmli (16). Gels were stained with Coomassie Blue, dried and autoradiographed as described (11) except that Kodak X-Omat AR film was used. Electroblotting of electrophoresed proteins onto nitrocellulose sheets and detection of FbPase by enzyme immunoassay was performed as described (15). The immunoassay was modified to include a first incubation of 6 hours with rabbit antiserum to yeast FbPase (500-fold dilution) and, after washing, a second incubation overnight with a 1000-fold dilution of sheep anti-rabbit IGG-horseradish peroxidase conjugate (Sera Products Company, Mundelein, IL).

Experiments with cultured yeast were carried out with the Fleishmann strain of Saccharomyces cerevisiae (a gift of Dr. P. Valenzuela, Chiron Corporation, Emeryville, CA) or where indicated with S. cerevisiae strain

[1] J. Rittenhouse and F. Marcus, in preparation.

S288C (Yeast Genetic Stock Center, Berkeley, CA). Cells were grown overnight to high density at 30° with shaking in Wickersham's rich medium (17). The cells were diluted into defined medium (17) containing 2% ethanol as carbon source to an A_{660} of approximately 0.15, and shaken for 4 to 5 hours. At this time the cells were in active growth and contained derepressed levels of FbPase. Extracts were prepared by vigorously Vortexing cell pellets 3 times for 40 sec with 2 volumes of glass beads (Type V, Sigma) and 1 volume of 2 mM EDTA containing 5% of a 6 mg/ml solution of phenylmethylsulfonyl fluoride in 95% ethanol. The beads were washed with approximately 3 volumes of homogenization solution, and the washings centrifuged 40 min at 100,000g. For use in *in vivo* [^{32}P]phosphate incorporation experiments, derepressed cells were washed twice by centrifugation, resuspended in the same volume of phosphate-free defined medium containing 2% ethanol, and shaken 15 min at 30°. Cells (22 ml) were added to a 50 ml flask containing 4 mCi [^{32}P]phosphate and immediately split into two 10 ml cultures. After 15 minutes glucose was added to the experimental culture to a final concentration of 2%. Cells were pelleted 15 min later, homogenized, and centrifuged as described for unlabeled cells except that the EDTA solution was replaced by 50 mM KF containing 5 mM EDTA and 5 mM EGTA. Pure yeast FbPase (10 µg) was added as carrier to an aliquot of each supernatant containing 0.4 mg protein (approximately 0.5 ml). FbPase was immunoprecipitated at 4° for 1 h after addition of 20 µl of anti-FbPase antiserum and 0.25 ml of 300 mM Na phosphate, pH 7.5, containing 0.3 mM EDTA and 0.3% Triton-X100. Precipitates were washed and electrophoresed as described (12).

In vitro phosphorylation of pure yeast FbPase was performed for 60 min at 30° in 35 mM K phosphate, pH 6.8, containing 5 mM $MgSO_4$, 0.3 mM ATP, and 5.5 µM fructose 2,6-bisphosphate, which contained additionally in 50 µl: 18 µg yeast FbPase, 1 µCi [γ-^{32}P]ATP (Amersham) and 180 units of cAMP-dependent protein kinase catalytic subunit, prepared as described (18). The reaction was terminated by addition of 1/2 volume of concentrated electrophoresis sample buffer and heating 3 min at 100°.

The molecular weight of native yeast FbPase was measured by size exclusion HPLC on a Bio-Sil TSK-250 column (Bio-Rad) connected to a Spectra-Physics SP8700 Solvent Delivery System. The mobile phase buffer was 0.1 M Na_2SO_4 - 0.02 M NaH_2PO_4, pH 6.8. Samples were diluted in mobile phase buffer and injected in 10 or 20 µl volumes. The flow rate was maintained at 0.5 ml/min and the column effluent was monitored by absorbance at 280 nm. Molecular weight standards were bovine immunoglobulin G and ovalbumin (Bio-Rad), rabbit muscle creatine kinase (Type I, from Sigma), and pig kidney (19) and spinach chloroplast FbPase. Spinach chloroplast FbPase was prepared by a modification of earlier published methods (20,21). Rat liver FbPase was prepared as described (14).

RESULTS AND DISCUSSION: The subunit M_r of purified yeast FbPase determined by gel electrophoresis in the presence of sodium dodecyl sulfate was found to be about 40,000 (Fig. 1A, lane 2). Because our molecular weight estimate is lower than that reported previously (3), we considered that our purified enzyme may have been proteolytically modified during the isolation procedure, resulting in a reduction of the subunit size from 56,000 to 40,000. In order to test the subunit size of the enzyme present at the beginning of the purification procedure, rabbit antibody raised against purified yeast FbPase was

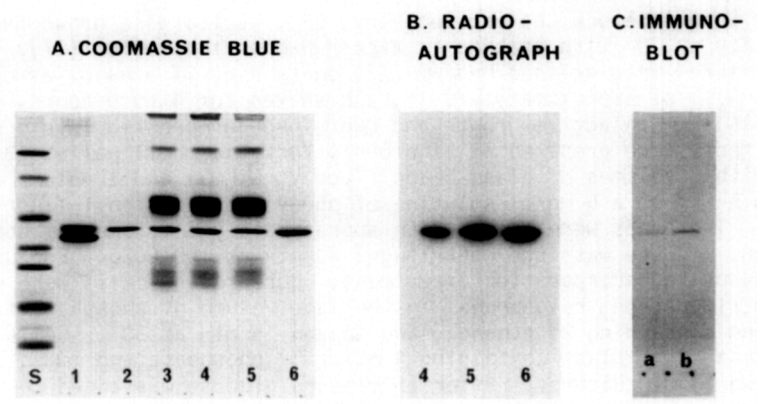

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of yeast fructose-1,6-bisphosphatase. Panel A shows the Coomassie Blue-stained gel of pure and immunoprecipitated FbPases: lane 1, upper band, rat liver FbPase (M_r, 40,000) and, lower band, pig kidney FbPase (M_r 36,500); lane 2, purified baker's yeast FbPase; lane 3, log-phase gluconeogenic S. cerevisiae cell supernatant immunoprecipitated with antiserum to baker's yeast FbPase; lane 4, log-phase gluconeogenic S. cerevisiae cell supernatant labeled for 30 min with [^{32}P]phosphate and immunoprecipitated with antiserum to baker's yeast FbPase; lane 5, same as lane 4 but 2% glucose added 15 min after [^{32}P]phosphate addition; lane 6, in vitro [^{32}P]-labeled baker's yeast FbPase; lane S, molecular weight markers: phosphorylase b, 97,000; bovine serum albumin, 68,000; ovalbumin, 43,000; lactate dehydrogenase, 35,000; carbonic anhydrase, 29,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400. Panel B shows the radioautograph from the gel illustrated in panel A with corresponding lane numbers 4-6. Panel C shows immunoassay of yeast FbPase in electroblotted material: lane a, pure baker's yeast FbPase; lane b, acid-extract of gluconeogenic (Fleishmann strain) S. cerevisiae cells. The extract was prepared by vigorous vortexing of cell pellets with 2 volumes of glass beads and 1 volume of 10% trichloroacetic plus 4% sulfosalicylic acid. After centrifugation, the pellet was washed twice with cold acetone and resuspended in electrophoretic sample buffer and heated 3 min at 100°.

incubated with crude baker's yeast supernatants. Analysis of the resulting immunoprecipitates by gel electrophoresis in the presence of sodium dodecyl sulfate showed only one enzyme band from the yeast supernatant (Fig. 1A, lane 3), and this band migrated the same as the pure enzyme (Fig. 1A, lane 2). The same result was obtained with the strain S288C of S. cerevisiae. Thus, at the start of the purification procedure the yeast enzyme appears to have a subunit M_r of 40,000. However, it could be argued that the enzyme was degraded during extract preparation and immunoprecipitation, or during the gel electrophoretic procedure. In order to minimize opportunities for proteolytic attack of the enzyme, yeast cells were homogenized in the presence of a mixture of trichloroacetic and sulfosalicylic acids. The precipitated material was electrophoresed and electroblotted to a nitrocellulose sheet. Only one predominant enzyme band was detected by enzyme immunoassay (Fig. 1C, lane b) which had the

same migration as the purified FbPase (Fig. 1C, lane a). Thus, all presented evidence indicates that the in vivo form of FbPase in yeast has a subunit molecular weight of 40,000.

The possiblility that yeast may have a second isozyme of FbPase with a subunit molecular weight of 56,000 which was not recognized by our antibody was considered. Immunotitration of crude supernatants of baker's yeast and derepressed yeast cultures of the S288C and Fleishmann strains resulted in complete removal of the supernatant FbPase activity in each case (not shown). Thus, the presence of a second FbPase isozyme seems unlikely.

We have confirmed that the enzyme is phosphorylated both in vivo and in vitro (Fig. 1B, lanes 4-6), the latter being catalysed by the catalytic subunit of bovine heart cAMP-dependent protein kinase. In vitro incorporation by pure enzyme ranged from 0.8 to 0.9 moles of phosphate per mole of enzyme subunit, similar to previous findings (4). Incorporation of [^{32}P]phosphate by yeast cells into FbPase was measured under conditions of glucose catabolite inactivation (9), in which the FbPase specific enzyme activity was observed to drop by 80 to 90% at 60 min after glucose addition. Yeast cells actively growing gluconeogenically were incubated for 30 min with [^{32}P]phosphate and immunoprecipitates of crude supernatants were electrophoresed and autoradiographed. FbPase became labeled in the absence of added glucose (Fig. 1B, lane 4) and the addition of glucose to the culture for the last 15 minutes of the labelling period produced approximately a 2-fold increase in radiolabel incorporated into the enzyme (Fig. 1B, lane 5). The large amount of label incorporated in the absence of glucose coupled with the small stimulation by glucose addition contrasts with earlier findings (6,7) and may be due to differences in yeast strain or labelling conditions.

Recently, Pohlig, et al. (5) have reported finding that in vitro-phosphorylated S. cerevisiae FbPase appears at molecular weight 40,000 upon gel electrophoresis in the presence of sodium dodecyl sulfate. These authors have postulated that an enzyme-bound protease which becomes activated in the presence of sodium dodecyl sulfate degrades the enzyme from 56,000 to 40,000

with high efficiency. However, our immunoblot experiments with acid-extracted yeast cells in which cellular protease activity would be inactivated do not support this interpretation. In addition, it should be noted that FbPases purified from two other yeasts, Candida utilis (22), and Kluyveromyces fragilis (23)[2], are reported to have a subunit molecular weight of 35,000 and native molecular weights of 130,000 and 150,000, respectively. The K. fragilis enzyme was further shown to be phosphorylated in vitro by a yeast cAMP-dependent protein kinase (23).

The molecular weight of the pure FbPase from baker's yeast measured by size exclusion HPLC (Figure 2) was estimated to be 135,000. However, the

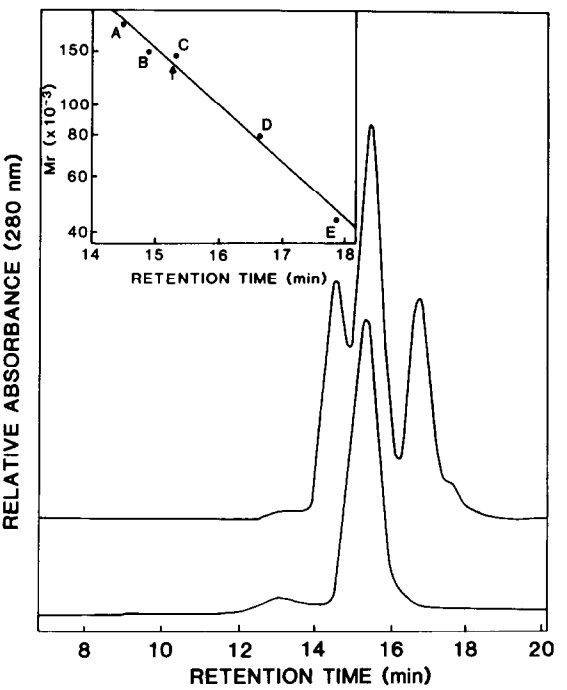

Fig. 2. Molecular weight determination of purified yeast fructose-1,6-bisphosphatase by size exclusion HPLC. A mixture of 2.5 μg spinach chloroplast FbPase, 5 μg pig kidney FbPase, and 2.5 μg rabbit muscle creatine kinase was chromatographed as described in Methods. The elution profile for this mixture is shown by the upper tracing with the chloroplast enzyme eluting first followed by pig kidney FbPase and creatine kinase. The lower tracing shows the chromatographic behavior of 10 μg of pure yeast FbPase. Inset: The retention times of various reference proteins were plotted as a function of their respective molecular weights (M_r). The retention time of pure yeast FbPase is shown by the arrow and corresponds to a molecular weight of 135,000. Molecular weight markers were: (A), spinach chloroplast FbPase, 180,000; (B), bovine immunoglobulin G, 150,000; (C), pig kidney FbPase, 146,000; (D), rabbit muscle creatine kinase, 81,000; (E), ovalbumin, 43,000.

[2] Y. Toyoda and J. Sy, personal communication.

retention time was the same as that seen for pig kidney FbPase, which has been shown by sequence analysis to have a molecular weight of 146,000 (19). A mixture of the yeast and pig kidney enzymes eluted as a single peak without measurable band broadening. These data suggest that a reasonable composition for the native yeast FbPase is a tetramer of four identical subunits. Thus, it appears that S. cerevisiae and other yeasts have FbPases that are similar in size and subunit composition to the general class of gluconeogenic FbPases.

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