Fructose-1,6-bisphosphatase. Primary structure of the rabbit liver enzyme. 'Intermediate' variability of an oligomeric protein

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Abstract The primary structure of rabbit liver fructose-1,6-bisphosphatase was determined by peptide analysis of digests with different proteases. The results establish the primary structure, complete data bank entries, and show that this enzyme variant is indeed homologous with other liver fructose-1,6-bisphosphatases. Residue differences with the enzymes from other mammals are 9–15%, with those from plants and yeasts about 50%, and with those from characterized prokaryotes up to 70%, showing an enzyme variability intermediate between those of 'variable' and 'constant' oligomeric dehydrogenases. Structural relationships, conformations and catalytic mechanisms are consistent within the family of fructose-1,6-bisphosphatases, and the rabbit protein is a typical rather than an aberrant form of the enzyme.

Key words: Fructose-1,6-bisphosphatase; Primary structure; Species variability; Gluconeogenesis; Evolutionary change

1. Introduction

Fructose-1,6-bisphosphatase (EC 3.1.3.11) catalyzes the hydrolysis of fructose bisphosphate to fructose 6-phosphate, a key regulatory step of gluconeogenesis in mammals, and also serves a gluconeogenic function in yeasts and prokaryotes. In plants, a gluconeogenic fructose-1,6-bisphosphatase in the cytosol takes part in sucrose synthesis from triose phosphates while a chloroplastic isozyme is necessary for regeneration of ribulose bisphosphate in the pentose phosphate pathway leading to photosynthetic carbon dioxide assimilation. The enzyme also functions in the Calvin cycle in photosynthetic and chemoautotrophic bacteria.

The active form of the mammalian enzyme is a homotetramer with a subunit mass of about 36 kDa [1]. A divalent metal ion, such as Mg^{2+}, Mn^{2+}, or Zn^{2+}, is required for activity, and two metal binding sites per subunit have been suggested; AMP is an allosteric inhibitor, and fructose-2,6-bisphosphate a competitive and probably also an allosteric inhibitor [2–5]. The primary structures have been reported for the enzyme from a wide variety of sources, including mammals [6–9], plants [10–12], yeasts [13,14] and bacteria [15–17]. The tertiary structure of the pig apoprotein [18] and its complexes with inhibitors, product, substrate, and substrate analogs [19–21] have been determined.

Although the structural studies were initiated with the rabbit protein (cf. [22]), that structure has never been completed. Lack of a complete sequence has frustrated attempts to study the crystal structure of this form and existing data bank entries indicate a 229-residue protein [23]. Not only has the sequence been unknown for large internal regions, but some regions appeared to have many more differences than were expected based on homology between other mammalian sequences. These differences become particularly puzzling when rabbit protein electron density maps are examined. Sequence analysis of rabbit liver fructose-1,6-bisphosphatase was undertaken to resolve these ambiguities. It also establishes the protein at large to be of an intermediately variable pattern in relation to other oligomeric proteins.

2. Materials and methods

2.1. Protein

The enzyme was purified as described [24], dissolved in 6 M guanidine/HCl, 0.4 M Tris, pH 8.15, 2 mM EDTA, reduced with dithiothreitol, and carboxymethylated with neutralized ^{14}C-labeled iodoacetate [25]. Subsequent desalting was performed by gel filtration on Ultropac TSK G2000PW (7.5×600 mm, Pharmacia, Sweden).

2.2. Peptide generation and purification

The lyophilized, carboxymethylated protein was dissolved in 9 M urea, diluted with 0.1 M ammonium bicarbonate (pH 8.1) to 0.9 M urea, and cleaved in separate batches with *Achromobacter* Lys-specific protease (Wako Chemicals, Neuss, FRG), *Pseudomonas* Asp-specific protease and *Staphylococcus aureus* strain V8 Glu-specific protease (Boehringer-Mannheim) at protease:substrate ratios of 1:50 to 1:20. Peptides obtained were separated by reverse-phase HPLC (Waters 440 system) on a C-18 column, UltroPac TSK ODS-120T, 5 μm (4.6×250 mm) (Pharmacia, Sweden), using linear gradients of acetonitrile in 0.1% trifluoroacetic acid.

2.3. Structural analysis

Total compositions were determined with a 4151 Alpha plus analyzer (Pharmacia, Sweden) after hydrolyses with 6 M HCl/0.5% phenol in evacuated tubes for 24 h at 110°C. Sequence degradations were performed on an ABI 470A sequencer and a MilliGen Prosequencer 6600, both with on-line analyzers. For detection of the blocked N-terminal amino acid residue, the protein was deblocked by incubation with trifluoroacetic acid/methanol (1:1) at room temperature for 24 h [26]. The sample was subsequently lyophilized and applied directly to sequencer degradation.

3. Results

3.1. Primary structure of the rabbit protein

Amino acid sequence determination by peptide analysis (Fig. 1) revealed a 337-residue structure (Fig. 2). The determination is based mainly on analysis of a Lys-specific digest of the ^{14}C-carboxymethylated enzyme. Two other aliquots provided overlapping fragments, obtained by treatment with Asp-N-specific and Glu-C-specific proteases, respectively. The N-terminus was found to be blocked, as expected [22], and was deblocked by limited hydrolysis in organic solvents which

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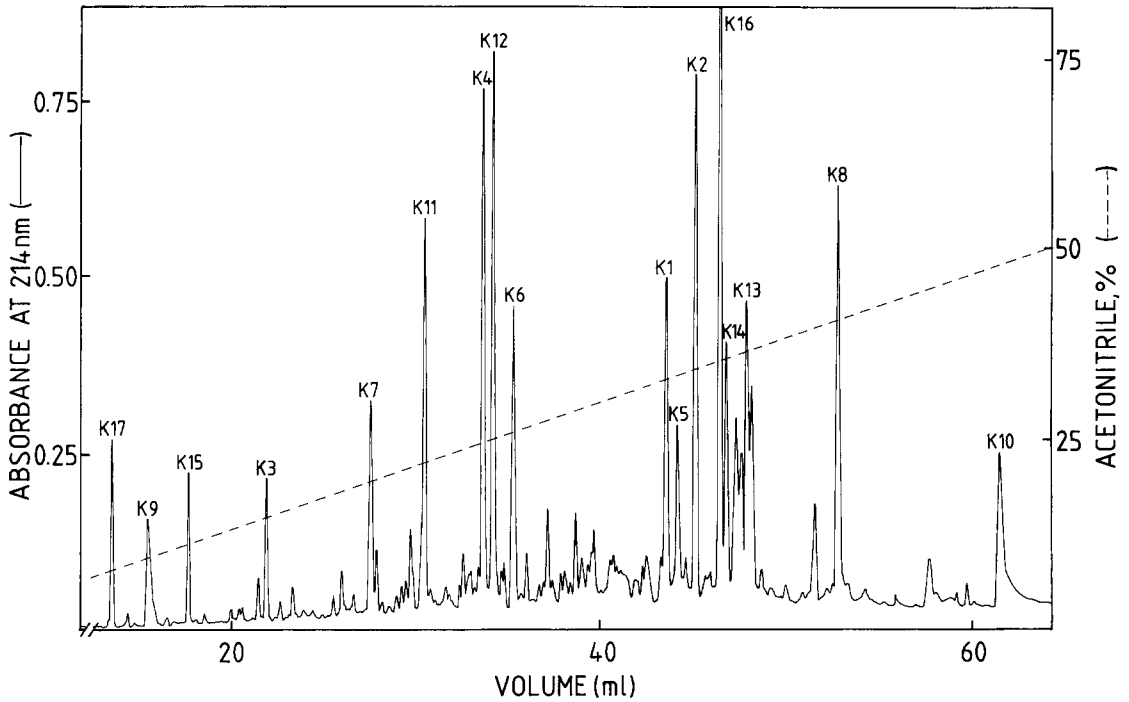

Fig. 1. Reverse-phase HPLC separation of the Lys-C protease generated peptides from carboxymethylated fructose-1,6-bisphosphatase. Peptides in the eluate are indicated with the same nomenclature as that used in Fig. 2.

works even with large proteins [26], and allowed analysis of the first four N-terminal residues (Fig. 2). The structure of the N-terminal 60-residue segment is in agreement with the analyses earlier described [27]. The C-terminus of the intact protein was found to be a lysine residue, proven by identical peptide ends upon sequence degradation of the C-terminal fragments from all three digests and by total compositions of these peptides. Two further structures required special attention. One was peptide K11 (Fig. 2) from the Lys-specific digest, which produced a fairly high absorbance at 280 nm and initially was suspected to contain Trp. However, sequencer degradation did not reveal Trp, and the present structure of that peptide is also compatible with the mass, 1329 Da, obtained by FAB mass spectrometry. The absorbance of peptide K11 is therefore concluded to be derived from other material. The second special problem was the continuity between positions 150 and 151, not passed by Edman degradations (Fig. 2). This overlap was proven by MALDI-TOF mass spectrometry on a Lasermat instrument, which gave a spectrum with a peak centering at the mass 36 750 Da for the carboxymethylated protein chain. This is in agreement with the calculated mass of 36 779 Da for direct continuity at positions 150–151.

Table 1
Variability in characterized mammalian fructose-1,6-bisphosphatases at 25 functionally important positions involved in metal, substrate and inhibitor bindings in the structure of the pig enzyme [28]. Of 25 such positions, only three exhibit variations in the mammalian enzymes with one deviating residue in each case

Species	Position		
	20	30	177
Rabbit	Glu	Met	Gly
Rat	Glu	Met	Met
Pig	Gln	Met	Met
Sheep	Glu	Met	Met
Human	Glu	Leu	Met

The whole structure determined is also in agreement with the total composition by hydrolysis.

3.2. *Relationships to other fructose-1,6-bisphosphatases*

The structure now determined complements incomplete data in data banks and shows that the rabbit liver protein is identical in size and properties to other species variants. The overall residue identity is high (85–91%) toward that of other mammalian enzymes of this type, intermediate (44–57%) toward the plant and yeast enzymes and also toward characterized prokaryotic forms (30–43%).

Four residues (Glu97, Asp118, Asp121, Glu280) play important roles in metal-binding [30], while over 20 further residues (at positions 17, 20, 21, 27, 28, 29, 30, 31, 112, 113, 121, 122, 123, 124, 125, 177, 212, 215, 243, 244, 247, 248, 264, 274 and adjacent positions) are of particular importance in substrate and inhibitor binding [20]. Of these functionally important residues, only three vary among five now characterized mammalian fructose-1,6-bisphosphatases (Table 1). Considering also sub-mammalian forms characterized, the metal-binding residues (four positions, above) are still strictly conserved, as are two (Gly122 and Ser124) of the three most important residues binding the 2-phosphate group of fructose-2,6-bisphosphate, while the third exhibits only minor variation (Lys/Arg274). Similarly, three residues (Asn212, Arg243, Tyr264) of the six residues that bind the 6-phosphate group of fructose-2,6-bisphosphate are strictly conserved, the other three being conserved within mammals (Tyr215, Tyr244, Lys274) but variable in other species [20]. Most deviations are found among the AMP binding positions, where no one of the at least thirteen residues interacting with AMP [19] is completely conserved among all characterized species, and it is also those residues that involve the three mammalian variable positions (Table 1), suggesting corresponding minor differences in AMP binding.

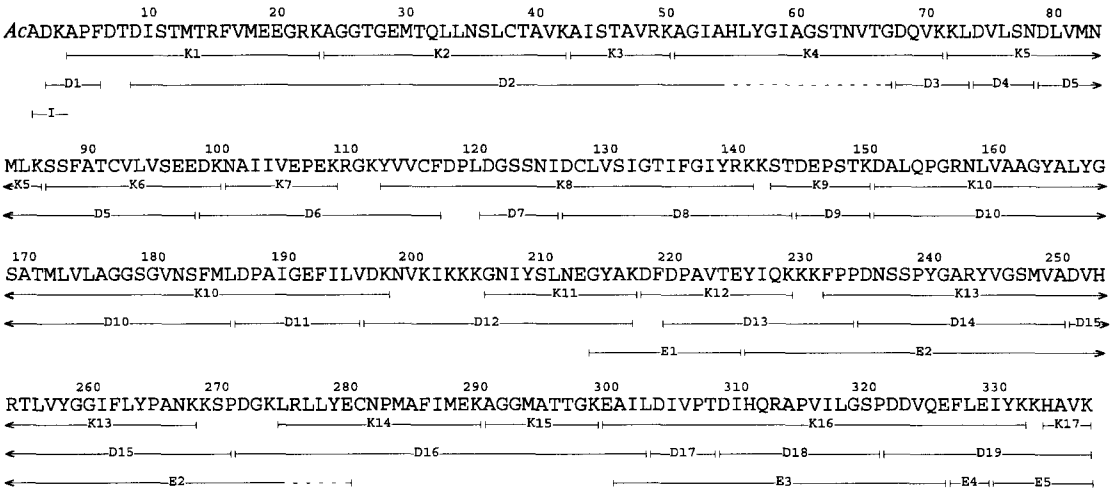

Fig. 2. Primary structure of fructose-1,6-bisphosphatase from rabbit liver. Peptides are indicated by solid lines for segments analysed by sequence degradations. Letters indicate method of cleavage, K for Lys-C protease, D for Asp-N protease, and E for Glu-C protease.

4. Discussion

The present results establish the native structure of rabbit liver fructose-1,6-bisphosphatase (Fig. 1), whose relationships were not known and have appeared contradictory although it was one of the first forms structurally studied. The present analysis establishes that the rabbit liver enzyme is indeed homologous to the enzymes from other mammalian species, eliminating the long-standing confusion.

The results establish the presence of the phosphate-interacting Asn125 [20], the two critical residues Asp121 and Glu280, forming the negatively charged, metal-binding pocket [30], and the presence of a 10-residue segment, which all appear to be missing from previous data. When compared to known vertebrate sequences, the rabbit liver protein is unique at fourteen positions. The most interesting, and possibly significant, differences from the pig kidney structure are the substitution of glycine for arginine at position 25, which is in the middle of an AMP-binding region [19], and the tripeptide Gly-Gly-Ser at residues 177–179 which replaces Met-Val-Asn. Met177 also interacts with the adenine base of the inhibitor AMP [19].

In addition to these direct functional correlations, the observed variability among fructose-bisphosphatases also allows an estimate of the extent of evolutionary change of this enzyme type. The variability pattern of the bisphosphatases is intermediate between those of other oligomeric enzymes, like the 'constant' liver alcohol dehydrogenases of class III and fundamental enzymes in glycolysis [30] on the one hand, and the 'variable' liver alcohol dehydrogenase of class I on the other (Table 2), groups which differ in evolutionary speed by a factor of approximately three [29,30]. There are no obvious segment patterns of variability among characterized bisphosphatases in contrast to those observed in the variable class I alcohol dehydrogenases [30], but there is an 8-residue segment at positions 118–125 which contains seven strictly conserved residues involved in metal and phosphate binding. However, the bisphosphatase family of enzymes resembles the medium-chain dehydrogenases (MDR) and the short-chain dehydrogenases (SDR) [31] with respect to conservation pattern for the different types of amino acid residues. In all cases, glycine is strongly over-represented among the conserved residues, 15 of 36 in MDR [31], 10 of 38 in SDR [31], and 9 of 34 in the bisphosphatases. Thus, in contrast to previous suggestions, the rabbit liver fructose-1,6-bisphosphatase shows a variability pattern that is typical of other oligomeric families.

Table 2
Overall residue differences in percent between rabbit and human forms of fructose-1,6-bisphosphatase, as well as between other variants of the enzyme, all related to corresponding differences of the 'variable' and 'constant' patterns of oligomeric medium-chain alcohol dehydrogenases [29,30]. As shown, the bisphosphatase has properties in between those of the 'variable' and 'constant' enzymes, where the constant pattern is typical also of fundamental enzymes in glycolysis [30]. ND, not determined.

Enzyme pair	Bis-phosphatase	Alcohol dehydrogenase	
		class I	class III
Human — Rabbit	9	12	ND
Human — Rat	15	18	6
Human — Yeast	54	—*	38

*Class I of the vertebrate enzyme type not present in yeast.

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