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Subunit interactions in pig-kidney fructose-1,6-bisphosphatase: Binding of substrate induces a second class of site with lowered affinity and catalytic activity

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ABSTRACT

Background: Fructose-1,6-bisphosphatase, a major enzyme of gluconeogenesis, is inhibited by AMP, Fru-2,6-P_2 and by high concentrations of its substrate Fru-1,6-P_2. The mechanism that produces substrate inhibition continues to be obscure.
Methods: Four types of experiments were used to shed light on this: (1) kinetic measurements over a very wide range of substrate concentrations, subjected to detailed statistical analysis; (2) fluorescence studies of mutants in which phenylalanine residues were replaced by tryptophan; (3) effect of Fru-2,6-P_2 and Fru-1,6-P_2 on the exchange of subunits between wild-type and Glu-tagged oligomers; and (4) kinetic studies of hybrid forms of the enzyme containing subunits mutated at the active site residue tyrosine-244.
Results: The kinetic experiments with the wild-type enzyme indicate that the binding of Fru-1,6-P_2 induces the appearance of catalytic sites with lower affinity for substrate and lower catalytic activity. Binding of substrate to the high-affinity sites, but not to the low-affinity sites, enhances the fluorescence emission of the Phe219Trp mutant; the inhibitor, Fru-2,6-P_2, competes with the substrate for the high-affinity sites. Binding of substrate to the low-affinity sites acts as a "stapler" that prevents dissociation of the tetramer and hence exchange of subunits, and results in substrate inhibition.
Conclusions: Binding of the first substrate molecule, in one dimer of the enzyme, produces a conformational change at the other dimer, reducing the substrate affinity and catalytic activity of its subunits.
General significance: Mimics of the substrate inhibition of fructose-1,6-bisphosphatase may provide a future option for combatting both postprandial and fasting hyperglycemia.

1. Introduction

The two irreversible reactions that interconvert Fru-6-*P* and Fru-1,6-P_2 constitute a major crossroads of metabolism. Phosphorylation of Fru-6-*P* is catalyzed by PFK, and hydrolysis of Fru-1,6-P_2 is catalyzed by FBPase [1]. Both enzymes are regulated by Fru-1,6-P_2 (product activation for PFK, substrate inhibition for FBPase) and by a series of effectors, of which we shall be concerned with AMP and Fru-2,6-P_2 [1–7] in this paper (Fig. 1). The opposite effects of AMP and Fru-2,6-P_2 on the two enzymes are readily rationalized, as both act as signals of ATP concentration, stimulating glycolysis when this is low, and simultaneously inhibiting gluconeogenesis. However, there is another symmetry in Fig. 1 that is more surprising: both enzymes are subject to inhibition by excess of substrate, PFK by ATP, and FBPase by Fru-1,6-P_2. For PFK this is to be expected, because although ATP is a substrate it is also a major product of glycolysis, so the substrate inhibition can be regarded as a form of feedback inhibition. The superficially similar substrate inhibition of FBPase by Fru-1,6-P_2 is more puzzling, as there is no obvious physiological function.

The inhibition of FBPase by excess substrate has been known for many years [8], and two different hypotheses have been proposed, to explain it, on the one hand that it is strictly a kinetic process [9]; and on the other that it is due to allosteric substrate inhibition [10], though crystallographic studies of the enzyme–Fru-2,6-P_2 complex do not support the existence of an allosteric site [11]. The mechanism of inhibition of FBPase by Fru-2,6-P_2 involves binding of the two fructose bisphosphate molecules at the same site, albeit with different binding determinants [12]. The substrate inhibition of FBPase occurs at rather

Abbreviations: FBPase, fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11); PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); Fru-1,6-*P*2, fructose 1,6-bisphosphate; Fru-2,6-*P*2, fructose 2,6-bisphosphate; Glu-tag, C-terminal extension of nine glutamate residues

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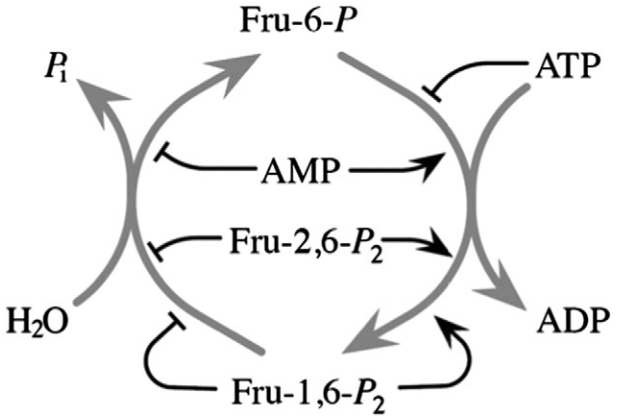

Fig. 1. Regulation of FBPase and phosphofructokinase. The two enzymes are regulated in opposite directions by AMP and Fru-2,6-P_2, and both are also inhibited by excess substrate, FBPase by Fru-1,6-P_2 and phosphofructokinase by ATP. Although the latter is well understood in terms of metabolic regulation, the role of the former remains unclear.

non-physiological concentrations of Fru-1,6-P_2, but its conservation in widely different organisms [2,13–19] suggests the existence of a function. Moreover, although inhibition by excess substrate is found in numerous enzymes with multiple substrates it is rare in one-substrate enzymes and hydrolases [20].

The general structure of pig-kidney FBPase is illustrated schematically in Fig. 2. It is a tetramer with D_2 symmetry with identical 37 kDa subunits designated C1, C2, C3 and C4, organized as a dimer of dimers C1C2 and C3C4 [21,22]. Each subunit has two folding domains, an AMP domain in residues 1–200 and a catalytic domain in residues 201–337. There are two quaternary structures, the R and the T states, with the R to T transition triggered by the binding of AMP to an allosteric site [21–24].

The mechanism of inhibition by Fru-1,6-P_2 needs to be understood, because the search for therapeutic inhibitors of FBPase is an active

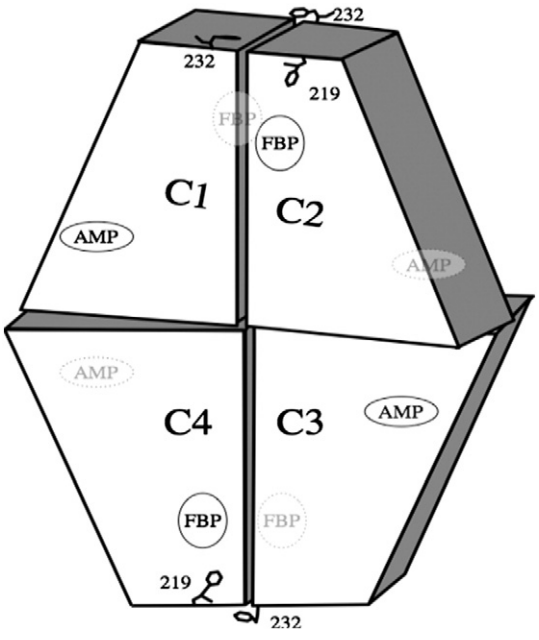

Fig. 2. Schematic drawing of FBPase. The active site, i.e. the site of binding Fru-1,6-P_2, is labeled FBP, and the AMP binding site is labeled AMP. Dotted ovals with gray labeling indicate the ligand binding sites on the faces of the tetramer that are hidden from view. The tetramer is shown in the T-state conformation. The location of residues 219 and 232 involved in the Phe219Trp and Phe232Trp mutants used in the fluorescence experiments are marked, together with diagrams indicating the Phe side chains schematically. The location of residue 244 involved in the Tyr244Phe mutant is not explicitly labeled as it is in the active site and thus in the same position as the FBP labels.

area of research [25–29], on account of the importance of gluconeogenesis in type 2 diabetes [30,31] and other serious illnesses. We have shown that nuclear accumulation of FBPase *in vivo* is impaired in diabetic rat liver [32] and this may be an important regulatory mechanism that could account for the increased gluconeogenic glucose production in the livers of diabetic rodents. Analogs of Fru-2,6-P_2 or Fru-1,6-P_2 are likely to be more suitable as drugs than analogs of AMP, because they can be expected to be more specific.

In this paper we shall present kinetic and fluorescence data, as well as studies of hybrid tetramers with mixed subunits, to clarify the cause of the inhibition.

2. Materials and methods

2.1. Biochemicals and chemicals

Fru-1,6-P_2, Fru-2,6-P_2, NAD^+, AMP, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were purchased from Sigma or Boehringer Mannheim and other chemicals came from Merck. All of them were of the highest available purity.

2.2. Site-directed mutagenesis

The Phe219Trp, Phe232Trp and Tyr244Phe FBPases were obtained using the Altered Sites II in vitro Mutagenesis Systems kit (Promega, Madison,WI), and the corresponding mutagenic oligonucleotides as previously described [22]. The following mutagenic oligonucleotide (with altered bases shown in bold) was used for mutation of Tyr in position 244: 5′-CCCTACGGGGCCAGG**TTC**GTGGGCTCCATGGTG-3′ (Tyr244Phe). Glu-tagged FBPase was obtained by adding nine Glu codons to the pig kidney cDNA by PCR. The oligonucleotide required as primer for the site-specific mutagenesis for Glu-tagged FBPase was 5′-TGGATCCTCA**TTCTTCTTCTTCTTCTTCTTCTTC**GCCTGCAGCCTTGGCT GCGTGCTTCTG-3′. The mutations were confirmed by sequencing. Protein expression and purification of Phe219Trp and Phe232Trp FBPases were performed as previously described [22]. For expression, the fragments encoding the wild-type, Glu-tagged FBPase and Tyr244Phe FBPase were excised from the corresponding plasmid and cloned into the vector pET22 (Novagen, San Diego, CA) as described [33]. The purification of Glu-tagged FBPase was performed by sequential chromatography on Cibacron Blue Sepharose CL-6B and DEAE-Sephacel. The purification of Tyr244Phe FBPase was made by sequential chromatography on Cibacron Blue Sepharose CL-6B and CM-Sepharose. Protein purity and concentration throughout the purification were monitored by SDS-PAGE and by Bio-Rad protein assay. The wild-type FBPase and the Phe219Trp, Phe232Trp and Tyr244Phe mutants, showed identical electrophoretic mobilities (37 kDa) whereas the FBPase mutant tagged with a 9-residue polyglutamyl extension at the C-terminal (Glu-tagged FBPase) shows an apparent mass of 38 kDa. All enzymes were >96% homogeneous using SDS-PAGE as a criterion (data not shown).

2.3. Activity assay

The spectrophotometric coupled enzyme assay was used to measure FBPase activity, as described previously [33]. Digital absorbance values were collected on a Hewlett Packard 8453 spectrophotometer and the data were fitted to a straight line on a coupled computer using the UV–visible Chem Station Program. Because FBPase exhibits substrate inhibition, the K_m and k_{cat} values were obtained by fitting the experimental data to a modified form of the Michaelis–Menten equation that incorporates a term for substrate inhibition [33,34]. The K_a value and Hill coefficient for Mg^{2+} were determined by fitting the saturation curves to the Hill equation. The inhibition curves elicited by AMP or Fru-2,6-P_2 were fitted to the equation of Taketa and Pogell [8].