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FOR THE RECORD

Crystallization and preliminary X-ray analysis of fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase

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Abstract: Diffraction-quality crystals of the bifunctional enzyme fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase from rat testis have been obtained. The crystals were grown in the presence of ATPγS, fructose 6-phosphate, the detergent *n*-octylglucoside, and the precipitant polyethylene glycol 4000. The crystals have the symmetry of the trigonal space group $P3_{1/2}21$ with $a = b = 83.0$ Å and $c = 130.6$ Å. Flash-frozen crystals diffract to beyond 2.2 Å, and native data have been collected.

Keywords: bifunctional enzyme; enzyme mechanism; kinase; phosphatase glycolysis regulation

Fructose 2,6-bisphosphate is the most potent activator of phosphofructokinase, which is a key regulatory enzyme of glycolysis. Synthesis and degradation of this unique sugar bisphosphate are catalyzed by fructose 6-phosphate, 2-kinase (Fru 6-P + ATP ⇌ Fru 2,6-P_2 + ADP) and fructose 2,6-bisphosphatase (Fru 2,6-P_2 → Fru 6-P + P_i). Both these activities reside on a single protein, fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase. Different tissues contain tissue-specific isozymes of the bifunctional enzyme, and these isozymes have differing relative activities of kinase and phosphatase (Uyeda, 1991). Several of these isozymes from various mammalian tissues have been characterized. They are all homodimers, consisting of subunits with M_r ranging from 54,000 to 60,000 Da. The amino acid sequences of the enzymes from liver (Darville et al., 1987; Algaier & Uyeda, 1988; Lively et al., 1988), skeletal muscle (Crepin et al., 1992), heart (Sakata & Uyeda, 1990), testis (Sakata et al., 1991), and brain (Watanabe et al., 1994) have been determined. The amino acid sequences of both catalytic domains are highly conserved among these isozymes, but the amino and carboxyl termini are completely different. These differences in the terminal peptides appear to regulate the relative activities of the kinase and the phosphatase, which in turn determine the Fru 2,6-P_2 concentrations in each tissue. The activity ratio of the kinase can also be modulated by phosphorylation/dephosphorylation of either the N- or the C-terminus. Phosphorylation of the N-terminus of the liver enzyme results in inhibition of the kinase and activation of the phosphatase (van Schaftingen et al., 1981; el-Maghrabi et al., 1982; Furuya et al., 1982). In contrast, phosphorylation of the C-terminus of the heart enzyme results in activation of the kinase without any effect on the phosphatase activity (Kitamura & Uyeda, 1987; Kitamura et al., 1988). Other isozymes including the rat testis bifunctional enzyme lack such phosphorylation sites. It is known that the kinase domain resides in the amino-terminal half and the phosphatase domain in the carboxyl-terminal half of the enzyme. The kinetics of the reaction catalyzed by Fru 6-P,2-kinase follow ternary complex formation (Kitajima et al., 1984).

Enzyme mechanisms can be understood through knowledge of catalytic residues and substrate binding sites (Knowles, 1991). We are attempting to solve the crystal structure of Fru 6-P,2-kinase:Fru 2,6-Pase in order to more clearly determine the mechanism of this bifunctional enzyme and to understand the interplay of the two opposing activities. Lee et al. (1994) reported the crystallization of a truncated form of the rat liver enzyme consisting of amino acid residues 227–470. This truncated en-

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Abbreviations: Fru 6-P,2-kinase:Fru 2,6-Pase, fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase; Fru 6-P, fructose 6-phosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; Tris, tris(hydroxymethyl)aminomethane; ATPγS, adenosine-5′-(γ-thio)triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; PEG, polyethylene glycol; *n*-ocylglucoside, 1-*o*-*n*-octyl-β-D-glucopyranoside; MES, morpholinoethanesulfonic acid.

zyme contains only the Fru 2,6-Pase domain and lacks the Fru 6-P,2-kinase domain. Here we report the crystallization of the full-length Fru 6-P,2-kinase:Fru 2,6-Pase from the rat testis in the presence of ATPγS and Fru 6-P. The crystals diffract to high resolution and are suitable for the determination of the molecular structure of the enzyme.

Initially, we attempted to crystallize wild-type rat testis Fru 6-P,2-kinase:Fru 2,6-Pase expressed in *Escherichia coli* (Sakata et al., 1991) and purified as described by Tominaga et al. (1993). Previously published crystallization conditions for various kinases were tried on the bifunctional enzyme. Several crystals of the enzyme were obtained using modified crystallization conditions that have yielded rabbit muscle pyruvate kinase crystals (Schmidt-Bäse et al., 1991). However, these crystals diffracted to only moderate resolution, and the crystallization was difficult to reproduce. A mutant form of the rat testis Fru 6-P,2-kinase:Fru 2,6-Pase in which all four Trp residues were changed to Phe is expressed in much higher levels in *E. coli* than the wild-type enzyme. The mutant enzyme shows kinetic properties nearly identical to the wild-type enzyme; the only differences are a twofold higher K_m for Fru 6-P of Fru 6-P,2-kinase and a twofold higher V_{max} for Fru 2,6-P_2 of Fru 2,6-Pase (F. Watanabe, D.M. Jameson, & K. Uyeda, Enzymatic and fluorescent studies of four single tryptophan mutants of rat testis fructose 6-phosphate, 2-kinase:fructose-2,6-bisphosphatase, in prep.). Using this mutant enzyme, we were able to grow well-diffracting crystals more reproducibly. The best crystals were obtained at 4 °C using the sitting drop vapor diffusion method. Typically, a 10-mg/mL protein solution in 50 mM Tris-PO_4, pH 7.5, 5–10% glycerol, 0.5 mM EDTA, 1% PEG 400, 10 mM dithiothreitol, 3 mM $MgCl_2$, 1 mM ATPγS (tetralithium salt), 1 mM Fru 6-P was mixed with an equal volume of the vapor diffusion reservoir containing 50 mM succinate, pH 6.0–6.3, 10 mM HEPES, pH 7.0, 12–18% PEG 4000, 1–2% *n*-octylglucoside. Some crystals were also grown without 0.5 mM EDTA and 1% PEG 400 in the protein buffer. The crystals are diamond-shaped or rhombic and grow to maximal dimensions of 0.6 × 0.25 × 0.15 mm within 2–7 days (Fig. 1). Crystals also grow without the kinase substrate Fru 6-P and when the succinic acid/HEPES buffer is replaced by 100 mM MES, pH 7.0, but not if ATPγS is omitted from the solution. The detergent appears to be essential for reproducible crystal growth. When it is not included in the crystallization solution, the protein tends to precipitate. It is possible that hydrophobic surfaces on the enzyme cause it to aggregate at high concentrations, and the detergent may interact with these hydrophobic surfaces and shield them from one another.

X-ray diffraction experiments were performed on a Xuong-Hamlin area detector using graphite monochromated CuKα radiation from a Rigaku RU-300 rotating anode generator. The generator was operated at 50 kV and 100 mA. At room temperature, the crystals diffracted beyond 2.8 Å, but decayed rapidly. Upon soaking the crystals in cryoprotectant solutions (50 mM Tris-PO_4, pH 7.5, 10 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM succinic acid, pH 6.2, 10 mM HEPES, pH 7.0, 0.5 mM EDTA, 1% PEG 400, 16% PEG 4000) containing increasing concentrations of glycerol (from 10% to 25%) for 5 min in each solution, we were able to flash-freeze the crystals in liquid nitrogen at 120 K. The frozen crystals diffract beyond 2.2 Å resolution. A native data set was collected and the space group was determined as $P3_{1/2}21$. The unit cell constants of the crystals as determined by the program XDS (Kabsch, 1988) are $a = b = 83.0$ Å, $c = 130.6$ Å, and $\alpha = \beta = 90°$, $\gamma = 120°$. For the native data set, 144,982 reflections were measured and reduced to 26,474 unique reflections with the program Scalepack (Otwinowski, 1993) with R_{merge}[6] = 0.071. Assuming one Fru 6-P,2-kinase:Fru 2,6-Pase molecule per asymmetric unit, the Matthews coefficient is 2.41 Å^3/Da, which corresponds to a solvent content of 48.9% (Matthews, 1968). Several heavy-atom derivatives of the Fru 6-P,2-kinase:Fru 2,6-Pase crystals have been prepared as part of our efforts to solve the structure by the method of multiple isomorphous replacement.

Fig. 1. Crystals of fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase. The crystals were grown at 4 °C in a 5.4-μL drop initially containing 5 mg/mL Fru 6-P,2-kinase:Fru 2,6-Pase in 25 mM Tris-PO_4, pH 7.5, 2.5% glycerol, 5 mM dithiothreitol, 1.5 mM $MgCl_2$, 0.5 mM ATPγS, 0.5 mM Fru 6-P, 0.25 mM EDTA, 0.5% PEG 400, 25 mM succinic acid, pH 6.3, 5 mM HEPES, pH 7.0, 7% PEG 4000, 0.5% *n*-octylglucoside, equilibrated against a 1-mL reservoir containing 14% PEG 4000, 50 mM succinic acid, pH 6.2, and 10 mM HEPES. Dimensions of the centered crystal are 0.6 × 0.25 × 0.15 mm.

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References

Algaier J, Uyeda K. 1988. Molecular cloning, sequence analysis, and expression of a human liver cDNA coding for fructose-6-P, 2-kinase:fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun 263*:839–849.

Crepin KM, DeCloedt M, Vertommen D, Foret D, Michel A, Rider MH, Rousseau GG, Hue L. 1992. Molecular forms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase expressed in rat skeletal muscle. *J Biol Chem 267*:21698–21704.

Darville MI, Crepin KM, Vandekerckhous J, van Damme J, Octave JN, Rider MH, Marchaud M, Hue L, Rousseau GG. 1987. Complete nucleotide sequence for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone. *FEBS Lett 224*:317–321.

el-Maghrabi MR, Claus TH, Pilkis J, Pilkis SJ. 1982. Regulation of

[6] $R_{merge} = \Sigma_{hkl} [\Sigma_i (|I_{hkl,i} - \langle I_{hkl} \rangle|)] / \Sigma_{hkl,i} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h, k, and l, and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection.

6-phosphofructo-2-kinase activity by cyclic AMP-dependent phosphorylation. *Proc Natl Acad Sci USA 79*:315–319.

Furuya E, Yokoyama M, Uyeda K. 1982. Regulation of fructose-6-phosphate, 2-kinase by phosphorylation and dephosphorylation: Possible mechanism for coordinated control of glycolysis and glucogenolysis. *Proc Natl Acad Sci USA 79*:325–329.

Kabsch W. 1988. Automatic indexing of rotation diffraction patterns. *J Appl Crystallogr 21*:67–71.

Kitajima S, Sakakibara R, Uyeda K. 1984. Kinetic studies of fructose 6-phosphate, 2-kinase and fructose 2,6-bisphosphatase. *J Biol Chem 259*:6896–6903.

Kitamura K, Kangawa K, Matsuo H, Uyeda K. 1988. Phosphorylation of myocardial fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. *J Biol Chem 263*:16796–16801.

Kitamura K, Uyeda K. 1987. The mechanism of activation of heart fructose 6-phosphate, 2-kinase:fructose-2,6-bisphosphate. *J Biol Chem 262*:679–681.

Knowles JR. 1991. Enzyme catalysis: Not different, just better. *Nature 350*:121–124.

Lee YH, Lin K, Okar D, Alfano NL, Sarma R, Pflugrath JW, Pilkis SJ. 1994. Preliminary X-ray analysis of a truncated form of recombinant fructose-2,6-bisphosphatase. *J Mol Biol 235*:1147–1151.

Lively MO, el-Maghrabi MR, Pilkis J, D'Angelo G, Colosia AD, Ciavola JA, Fraser BA, Pilkis SJ. 1988. Complete amino acid sequence of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 263*:839–849.

Matthews BW. 1968. Solvent content of protein crystals. *J Mol Biol 33*: 491–497.

Otwinowski Z. 1993. *Scalepack*. New Haven, Connecticut: Yale University Press.

Sakata J, Abe Y, Uyeda K. 1991. Molecular cloning of the DNA and expression and characterization of rat testes fructose-6-phosphate, 2-kinase: fructose-2,6-bisphosphatase. *J Biol Chem 266*:15764–15770.

Sakata J, Uyeda K. 1990. Bovine heart fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase: Complete amino acid sequence and localization of phosphorylation sites. *Proc Natl Acad Sci USA 87*:4951–4955.

Schmidt-Bäse K, Buchbinder JL, Reed GH, Rayment I. 1991. Crystallization and preliminary analysis of enzyme-substrate complexes of pyruvate kinase from rabbit muscle. *Proteins Struct Funct Genet 11*:153–157.

Tominaga N, Minami Y, Sakakibara R, Uyeda K. 1993. Significance of the amino terminus of rat testis fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase. *J Biol Chem 268*:15951–15957.

Uyeda K. 1991. Phosphofructokinase and fructose 6 phosphate, 2-kinase: fructose 2,6-bisphosphatase. In: Kuby SA, ed. *A study of enzymes. CRC review, vol II*. Boston: CRC Press. pp 445–456.

van Schaftingen E, Davies DR, Hers HG. 1981. Inactivation of phosphofructokinase 2 by cyclic AMP-dependent protein kinase. *Biochem Biophys Res Commun 103*:362–368.

Watanabe F, Sakai A, Furuya E, Uyeda K. 1994. Molecular cloning and tissue specific expression of fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase of rat brain. *Biochem Biophys Res Commun 198*:335–340.