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Expression, purification and preliminary crystallographic studies of human ketohexokinase

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Ketohexokinase (KHK; E.C. 2.7.1.3) catalyses the (reversible) phosphorylation of fructose to fructose-1-phosphate. KHK is the first enzyme in a specialized catabolic pathway metabolizing dietary fructose to the glycolytic intermediate glyceraldehyde-3-phosphate. Mutations inactivating KHK underlie the metabolic disorder essential fructosuria. The primary structure of KHK shows no significant homology to other mammalian hexokinases. It is most similar to prokaryotic ribokinases, but catalyses a distinct phosphorylation reaction. Recombinant human KHK has been crystallized in the orthorhombic form (space group $P2_12_12$ or $P2_12_12_1$). Single crystals of this polymorph suitable for X-ray diffraction have been obtained by vapour diffusion using 2-propanol and MPD as precipitants (pH 7.5). The crystals have unit-cell parameters a = 93.4, b = 121.5, c = 108.4 Å. Diffraction data were collected to 4.3 Å resolution. The asymmetric unit contains four protein molecules.

1. Introduction

In mammals, dietary fructose is primarily metabolized through a pathway distinct from that responsible for glucose metabolism. KHK is the first enzyme in this pathway, catalysing the phosphorylation of fructose to fructose-1-phosphate. Subsequently, the enzymes aldolase B (fructose-1-phosphate aldolase; E.C. 4.1.2.13) and triokinase (E.C. 2.7.1.28) complete the conversion to glyceraldehyde-3-phosphate, an intermediate in the glycolytic and gluconeogenic pathways. KHK is most abundant in the liver, where it comprises up to 0.6% of total protein (Donaldson *et al.*, 1993). It has been purified from bovine (Raushel & Cleland, 1977), rat (Donaldson *et al.*, 1993) and human livers (Bais *et al.*, 1985). It is believed to be active as a dimer and requires K^+ and ATP for activity.

Essential fructosuria (Lasker, 1941), a benign inborn error of metabolism, is characterized by a large and persistent rise in blood fructose level after the ingestion of fructose, sucrose or sorbitol and the excretion of 10–20% of the ingested load in the urine. The hepatic fructokinase deficiency which underlies this condition is caused by mutations which inactivate KHK (Bonthron *et al.*, 1994). Although the liver is the major site of KHK activity, somewhat lower levels are also found in the kidney, small intestine and pancreas (Donaldson *et al.*, 1993), while much lower levels are found in the heart, brain and muscle (Bais *et al.*, 1985).

We have characterized the genomic structure of the human and rat *KHK* genes (Hayward & Bonthron, 1998) and shown that the mRNA includes eight exons encoding a protein of 298 amino acids. There are two alternative third exons (3a and 3c) which are mutually exclusively spliced, generating two distinct isoforms of the KHK protein. Exons 3a and 3c are identical in size and similar in sequence, reflecting an ancient intragenic duplication predating the divergence of the rodent and primate lines. Our analysis of the expression pattern of the two KHK isoforms revealed that the 3c-containing isoform was present in adult tissues expressing KHK at high levels, while the 3a-containing isoform was present in adult tissues expressing KHK at low levels and in all tissues during early fetal development. In addition to the isoform heterogeneity, one common polymorphism has been identified (Bonthron *et al.*, 1994), resulting in a conservative substitution (Val/Ile) at amino-acid residue 49.

KHK has no significant primary structure similarity to other mammalian hexokinases. It is a member of the family of prokaryotic ribokinases and furanose sugar kinases, having ~20–25% sequence identity with them. KHK contains a match (residues 252–263) to the conserved sequence DT*x*GAGD*x*F*x*(G/A)-(G/A) which is found in ribokinases and human adenosine kinase and is postulated to be involved in the binding of the sugar moiety of adenosine (Spychala *et al.*, 1996; Bork *et al.*, 1993). Unlike all these enzymes, however, KHK catalyses the transfer of a phosphate group to the 1-position of the sugar ring, whereas the others catalyse the transfer of a phosphate group to either the 6-position (plant

and prokaryotic fructokinases; mammalian hexokinases) or the 5-position (ribokinases and adenosine kinase).

Here, we report the initial results of experiments aimed at determining the crystal structure of ketohexokinase. Catalytically active recombinant human KHK was produced in *Escherichia coli* and purified to homogeneity. Preliminary X-ray diffraction data obtained from crystals of this material are presented.

2. Materials and methods

2.1. Plasmid construction

For KHK overexpression in *E. coli*, the cDNA was cloned into a modified pET11a T7 expression plasmid so as to produce a protein identical in sequence to the native protein (Sambrook *et al.*, 1989). pET11a was first modified by the removal of bases 654–2251 (*Sph*I to *Eag*I), which eliminated an unwanted *Mlu*I site. The cDNA insert (3a exon, Val49) was generated from the plasmid pHKHK3a (Bonthron *et al.*, 1994) by PCR using oligonucleotides dGCCTCGAGCATATGGAAGAGAAGCAGATCCTGTG and dCTCGAGAGATCTCACACGATGCCATCAAAGCCCTGC, which incorporate novel *Nde*I (CATATG, including the initiator codon) and *Bgl*II sites. The *Nde*I + *Bgl*II digested product was then cloned into the *Nde*I and *Bam*HI sites of the modified pET11a vector. Sequencing this construct revealed a PCR-generated error in codon 272, which was repaired by the replacement of a *Bam*HI–*Sse*8387I fragment (codons 135–293) with its equivalent from pHKHK3a.

2.2. Protein expression and purification

The *E. coli* strain JM109/DE3 was used to express the recombinant KHK protein. Purification of the protein was based on a protocol modified from Raushel & Cleland (1977). From a freshly streaked colony, 2 l of culture (Luria–Bertani broth, 310 K, 200 rev min^{-1} shaking) was grown to an OD_{600} between 0.4 and 0.5. Protein expression was induced by the addition of IPTG to a final concentration of 0.4 m*M*. After a further 3–3.5 h, the bacteria were pelleted and resuspended in 50 m*M* Tris–HCl pH 8, 5 m*M* EDTA, 0.25 m*M* PMSF, 1 m*M* DTT, 20 µg ml^{-1} lysozyme at 6 ml resuspension buffer per 1 g of pellet. The suspension was incubated overnight at 277 K.

The supernatant was recovered and streptomycin sulfate added slowly to a final concentration of 11 mg ml^{-1}. The supernatant was fractionated using ammonium sulfate at room temperature. The 40–50% ammonium sulfate fraction was resuspended in 4 ml of 20 m*M* bis-Tris pH 6.5 containing 0.25 m*M* PMSF and 1 m*M* DTT.

At 277 K, the preparations were fractionated through a Sephacryl 200HR column and selected fractions were then bound to a DEAE-cellulose column and eluted using a linear KCl gradient (150–400 m*M* for 3a-type protein).

Enzyme activity was assayed by measuring the change in absorbance at 340 nm in a 1 ml solution containing 50 m*M* PIPES pH 7, 100 m*M* KCl, 5 m*M* fructose, 6 m*M* $MgCl_2$, 5 m*M* ATP, 100 µg lactate dehydrogenase, 100 µg pyruvate kinase, 1 m*M* phospho(enol)pyruvate and 0.2 m*M* freshly prepared NADH.

2.3. Crystallization

The crystallization experiments were conducted at 293 K using only 2 mg of the protein (isoform 3a with the Val variant at codon 49). The vapour-diffusion method was applied in the hanging- and sitting-drop variants (McPherson, 1982). The protein fraction was desalted and then concentrated using Centricon-10 concentrators. The protein concentration, determined by UV absorption at 280 nm, was 10 mg ml^{-1} in 10 m*M* HEPES buffer pH 7.5. Initial crystallization conditions (precipitant and pH) were established by the sparse-matrix method (Jancarik & Kim, 1991) using Crystal Screen II (Hampton Research, California, USA). 5 µl protein samples were mixed on siliconized cover slips (for hanging-drop experiments) or on polypropylene bridges (for sitting-drop experiments) with equal amounts of reservoir solutions. The droplets were equilibrated against 1 ml reservoir solutions in 24-well cell-culture plates. The best crystals were obtained when the reservoir contained 20% 2-propanol, 20% MPD in 100 m*M* HEPES pH 7.5. Bipyramidal crystals (Fig. 1) appeared after about 10 d and reached maximum dimensions of 0.25 × 0.2 × 0.2 mm within four weeks. The crystals for X-ray diffraction experiments were mounted in thin-walled quartz capillaries with a small amount of mother liquor.

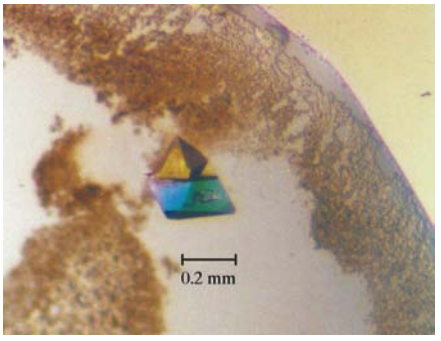

Figure 1
Single crystals of human ketohexokinase.

2.4. Diffraction experiments

The intensity data were recorded at room temperature using a 300 mm MAR Research image-plate scanner and Cu $K\alpha$ radiation generated from an SRA2 rotating-anode generator (Siemens) operated at 45 kV and 112 mA. The crystal-to-detector distance was 190 mm and the oscillation range was 1.0°. 25 734 reflections [with $I/\sigma(I) > 0.0$] were collected to 4.3 Å resolution (Fig. 2). They were merged to give a unique data set of 4888 reflections characterized by $R_{\rm int} = 0.089$ and $\langle I/\sigma(I)\rangle = 8.8$. Indexing and integration of the images was performed in *DENZO* and scaling of the intensity data in *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).

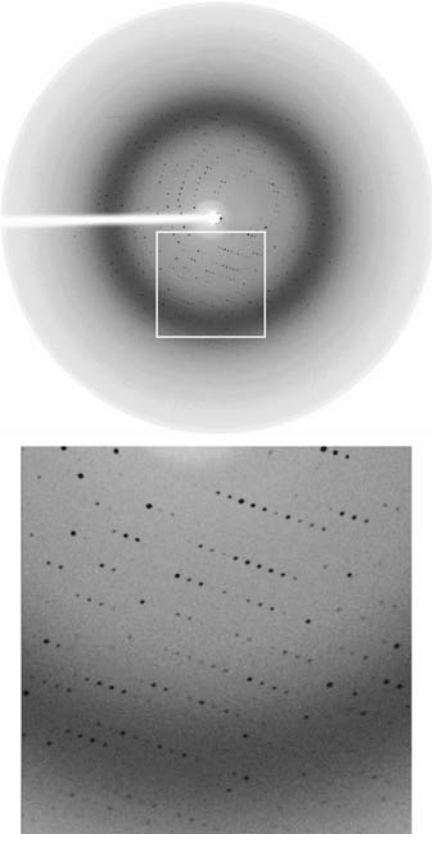

Figure 2
Typical diffraction pattern and its enlargement (oscillation range 1.0°).

3. Results and discussion

The crystals were very unstable in the X-ray beam at room temperature, allowing only incomplete data to be collected from a single specimen. Our attempts to collect diffraction data at low temperature were unsuccessful as flash-freezing (Teng, 1990) dramatically increased the mosaicity of the crystals. This damaging effect of low temperature could not be obviated by the use of cryoprotectants. The room-temperature diffraction data represent 60.8% of the theoretically possible reflections (56.4% in the last resolution shell, 4.45–4.30 Å). The crystals of the present form are orthorhombic and belong to the space group $P2_12_12$ or $P2_12_12_1$. The 00*l* reflections are missing from the recorded data set. The unit-cell parameters are a = 93.4, b = 121.5, c = 108.4 Å. An analysis of the Matthews volume (Matthews, 1968) indicates that most likely four monomers are present in the asymmetric unit, corresponding to V_M = 2.35 Å^3 Da^{-1}.

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