Expression, purification and preliminary crystallographic studies of human ketohexokinase

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Ketohexokinase (HKH: EC 2.7.1.3) catalyses the (reversible) phosphorylation of fructose to fructose-1-phosphate. HKH is the first enzyme in a specialized catabolic pathway metabolizing dietary fructose to the glycolytic intermediate glyceraldehyde-3-phosphate. Mutations inactivating HKH underlie the metabolic disorder essential fructosuria. The primary structure of HKH shows no significant homology to other mammalian hexokinases. It is most similar to prokaryotic ribokinases, but catalyses a distinct phosphorylation reaction. Recombinant human HKH has been crystallized in the orthorhombic form (space group P2_12_12) or P2_12_1_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. HKH 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; EC 4.1.2.13) and triokinase (EC 2.7.1.28) complete the conversion to glyceraldehyde-3-phosphate, an intermediate in the glycolytic and gluconeogenic pathways. HKH 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 HKH (Bonthron et al., 1994). Although the liver is the major site of HKH 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 HKH 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 HKH 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 HKH isoforms revealed that the 3c-containing isoform is present in adult tissues expressing HKH at high levels, while the 3a-containing isoform is present in adult tissues expressing HKH 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.

HKH has no significant primary structure similarity to other mammalian hexokinases. It is a member of the family of prokaryotic ribokinases and fumarase sugar kinases, having ~20–25% sequence identity with them. HKH contains a match (residues 252–263) to the conserved sequence DTGAGDxFx(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 (Szychala et al., 1996; Bork et al., 1993). Unlike all these enzymes, however, HKH 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 (SphI to EagI), which eliminated an unwanted *MluI* site. The cDNA insert (3a exon, Val49) was generated from the plasmid pHKHK3a (Bonthon et al., 1994) by PCR using oligonucleotides dGCC-TCGACATGGAAAGGAGCGAATC-TCTGTC and dCTCGAGAGATCG-CACACGATGGCATCAAGCGAGTCGG, which incorporate novel *NdeI* (CATATG, including the initiator codon) and *BglII* sites. The *NdeI* + *BglII* digested product was then cloned into the *NdeI* and *BamHI* 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 *BamHI*-Sse83871 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 Rauschel & 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 OD₆₀₀ between 0.4 and 0.5. Protein expression was induced by the addition of IPTG to a final concentration of 0.4 mM. After a further 3–3.5 h, the bacteria were pelleted and resuspended in 50 mM Tris–HCl pH 8, 5 mM EDTA, 0.25 mM PMSF, 1 mM 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 mM bis-Tris pH 6.5 containing 0.25 mM PMSF and 1 mM 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 mM for α-type protein).

Enzyme activity was assayed by measuring the change in absorbance at 340 nm in a 1 ml solution containing 50 mM PIPES pH 7, 100 mM KCl, 5 mM fructose, 6 mM MgCl₂, 5 mM ATP, 100 μg lactate dehydrogenase, 100 μg pyruvate kinase, 1 mM phosphoenolpyruvate and 0.2 mM 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 Centriprep-10 concentrators. The protein concentration, determined by UV absorption at 280 nm, was 10 mg ml⁻¹ in 10 mM 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 mM HEPES pH 7.5. Bipramidal 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.

2.4. Diffraction experiments

The intensity data were recorded at room temperature using a 300 mm MAR Research image-plate scanner and Cu Ka 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 θ(θ) > 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 Rint = 0.089 and ⟨(λ/θ)/θ⟩ = 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 1](image1.png) Single crystals of human ketohexokinase.

![Figure 2](image2.png) Typical diffraction pattern and its enlargement (oscillation range 1°).
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 \(P2_12_2_1\). The 00l reflections are missing from the recorded data set. The unit-cell parameters are \(a = 93.4, b = 121.5, c = 108.4\ \text{Å}\). An analysis of the Matthews volume (Matthews, 1968) indicates that most likely four monomers are present in the asymmetric unit, corresponding to \(V_M = 2.35 \text{ Å}^3\ \text{Da}^{-1}\).

The research of MJ was supported in part by an International Research Scholar’s award from the Howard Hughes Medical Institute. Work in the DTB laboratory was supported by grant G9403693MB from the Medical Research Council.

References


