

# Properties of Normal and Mutant Recombinant Human Ketohehexokinases and Implications for the Pathogenesis of Essential Fructosuria

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Alternative splicing of the ketohehexokinase (fructokinase) gene generates a "central" predominantly hepatic isoform (ketohehexokinase-C) and a more widely distributed ketohehexokinase-A. Only the abundant hepatic isoform is known to possess activity, and no function is defined for the lower levels of ketohehexokinase-A in peripheral tissues. Hepatic ketohehexokinase deficiency causes the benign disorder essential fructosuria. The molecular basis of this has been defined in one family (compound heterozygosity for mutations Gly40Arg and Ala43Thr). Here we show that both ketohehexokinase isoforms are indeed active. Ketohehexokinase-A has much poorer substrate affinity than ketohehexokinase-C for fructose but is considerably more thermostable. The Gly40Arg mutation seems null, rendering both ketohehexokinase-A and ketohehexokinase-C inactive and largely insoluble. The Ala43Thr mutant retains activity, but this mutation decreases the thermal stability of both ketohehexokinase-A and ketohehexokinase-C. At physiologic temperature, this results in significant loss of ketohehexokinase-C activity but not of ketohehexokinase-A. Affected individuals who carry both mutations therefore probably have a selective deficiency of hepatic ketohehexokinase, with peripheral ketohehexokinase-A being preserved. These findings raise the possibility that ketohehexokinase-A serves an unknown physiologic function that remains intact in essential fructosuria. Further mutation analysis in this rare disorder could illuminate the question of whether ketohehexokinase-A activity is, unlike that of ketohehexokinase-C, physiologically indispensable. *Diabetes* 52:2426–2432, 2003

**K**etohehexokinase (KHK; fructokinase, E.C. 2.7.1.3) catalyzes the phosphorylation of the ketose sugar fructose to fructose-1-phosphate. A number of other furanose sugars can also act as KHK substrates (1). KHK activity is highest in the liver, followed by renal cortex and small intestine (2). Its primary role in these sites seems to be clearance of dietary

fructose through a specialized pathway involving aldolase B and triokinase.

In other tissues, the role of KHK is not well defined but might be significant. In the lung, for example, fructokinase activity is demonstrable (3), and in insulin-deficient states, a modest level of fructose metabolism through fructose-1-phosphate is preserved, even when peripheral glucose utilization is significantly depressed (4). Metabolic labeling experiments also indicate a significant contribution of KHK to fructose metabolism in the parotid gland, as well as in the pancreatic islet (5).

One particular process in which KHK could also be involved is the modulation of glucokinase activity through its allosteric regulatory protein GCKR. The KHK product fructose-1-phosphate relieves the binding of glucokinase to GCKR, increasing glucokinase availability and promoting its translocation from sequestered nuclear to cytoplasmic localization (6–9). GCKR regulates glucokinase primarily in the liver but may play a similar role in the pancreatic islet and hypothalamus (10,11). In both these sites, GCKR has been proposed to modulate glucose sensing. However, individuals with essential fructosuria (see below), resulting from KHK deficiency, are not known to show impaired glucose tolerance, as might be predicted if reduced fructose-1-phosphate levels resulted in greater glucokinase inhibition. This may argue that KHK-derived fructose-1-phosphate does not, in fact, play an important role in regulating glucokinase activity. Unfortunately, though, molecular and genetic analysis of KHK has been minimal, precluding a definitive statement on this question.

The human *KHK* gene on chromosome 2p23 has nine exons spanning 14 kb (12). Two exons (referred to as 3a and 3c, each 135 bp long) arose by an intragenic duplication and are mutually exclusively spliced into mRNA. The resulting two predicted protein isoforms (KHK-A and KHK-C) differ at 32 positions between amino acid residues 72 and 115. The biochemically well-characterized KHK-C, usually referred to as fructokinase, is encoded by the mRNA that includes exon 3c. It is abundant in liver, renal cortex, and small intestine and also is present in the pancreatic islet (12,13).

Deficiency of hepatic KHK underlies the benign autosomal recessive disorder essential fructosuria (MIM 229800). Affected individuals display an abnormally large and persistent rise in blood fructose concentration after ingestion of fructose, sucrose, or sorbitol and excrete part of the load in the urine (14). In the only family in which muta-

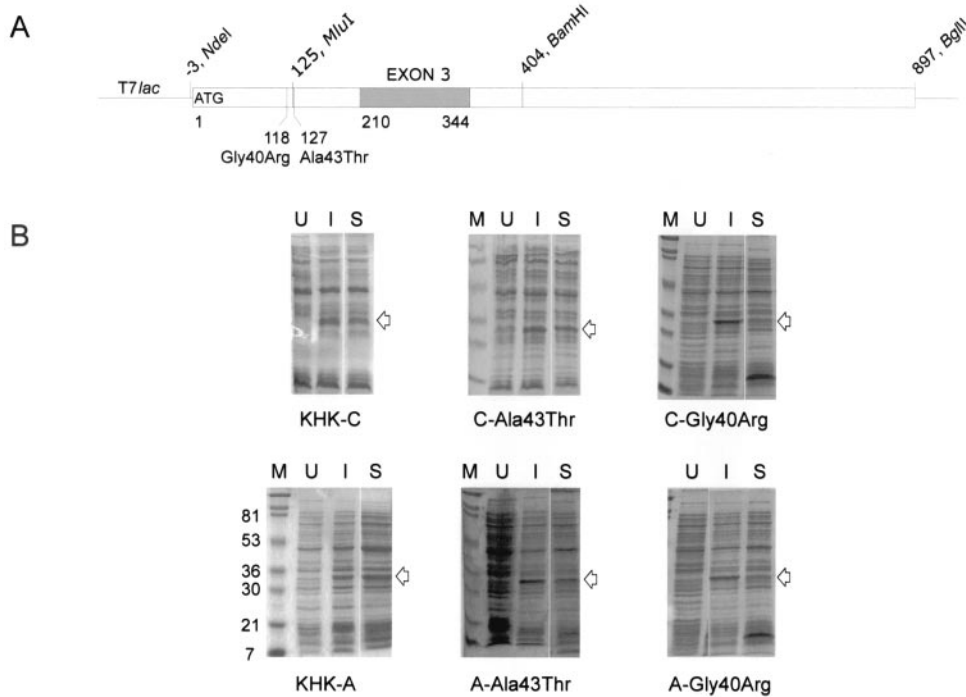
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KHK, ketohehexokinase.

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**FIG. 1.** Expression of human KHK in *E. coli*. **A:** Diagram of pET-KHK expression constructs. Initially, KHK-A cDNA was cloned between the *NdeI* and *BamHI* sites of a modified pET11a expression vector (18). Other derivatives were made by exchanging the *MluI*-*BamHI* cassette containing the alternative exon 3 sequences (shaded) and by site-directed mutagenesis at nt 118 and 127. The numbering is relative to 1 = A of the ATG initiator codon and for restriction sites indicates the most 5' residue in the recognition sequence. **B:** Induction and solubility of recombinant proteins. SDS-PAGE gels show total protein from bacteria before (*U*) and after (*I*) induction, and soluble proteins (*S*) extracted with lysis buffer as described in RESEARCH DESIGN AND METHODS. *M*, size markers (kDa). Arrows indicate KHK protein bands. Decreased recovery of recombinant proteins carrying Gly40Arg is apparent, accompanied by the appearance of a presumed degradation product after lysis.

tion analysis has been performed, affected individuals are compound heterozygotes for two mutations, predicting the amino acid substitutions Gly40Arg and Ala43Thr (15).

The alternative predicted isoform, KHK-A, has never been characterized biochemically, and indeed, there is no existing evidence that this mRNA splice variant encodes a functional protein. RNA studies, however, indicate tissue specificity of the splicing pattern. Those high-expressing tissues listed above contain KHK-C mRNA, with little or no KHK-A. In other tissues, KHK-A mRNA is present at low level, but KHK-C cannot be detected (12). There is also a shift from KHK-A to KHK-C in the liver and kidney during fetal development, which correlates with earlier observations that fructokinase activity is very low in fetal liver (16). Thus, in all situations investigated so far, presence of significant levels of fructokinase activity correlates with predominant or exclusive expression of the KHK-C mRNA.

In the present report, we have expressed and purified recombinant human KHK-C and KHK-A in *Escherichia coli*. We find that the KHK-A protein is indeed catalytically active. Furthermore, the biochemical effects of the two essential fructosuria mutations leave open the interesting possibility that KHK-A may have as-yet-undefined independent functions that remain preserved in individuals with essential fructosuria.

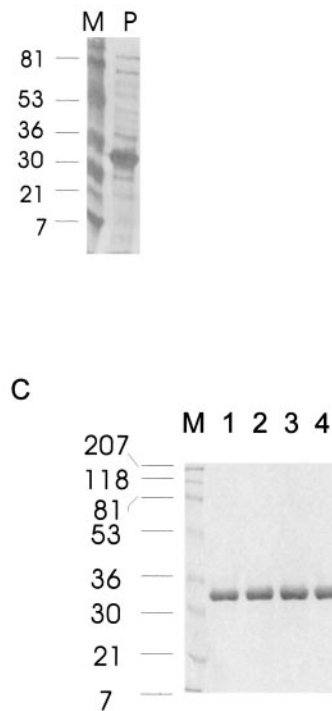
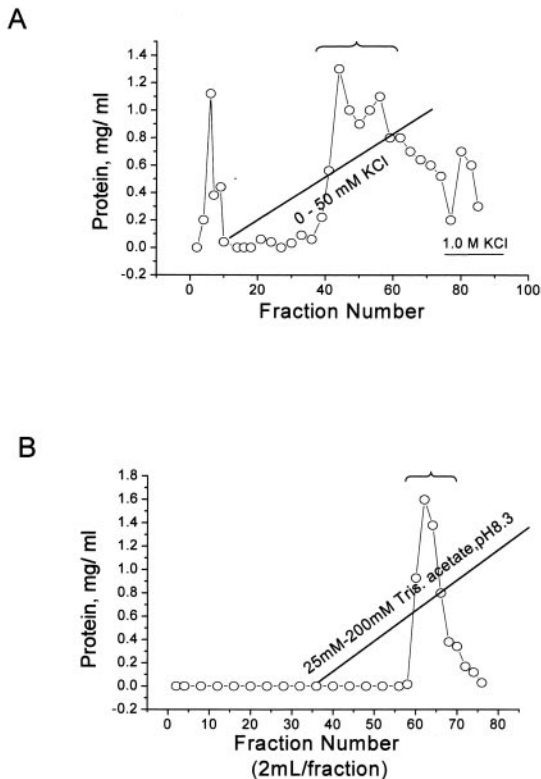
## RESEARCH DESIGN AND METHODS

**Construction of expression plasmids containing human KHK.** A modified pET11a vector (17) was used to express wild-type (wt) KHK-C and KHK-A and their corresponding mutant derivatives (Gly40Arg and Ala43Thr). The natural initiation and termination codons were used, so the primary structures of the recombinant proteins are predicted to be identical to those of the native proteins. The construction of the initial plasmid, pET-KHK-A (Fig. 1A), was described previously (18). All derivatives of it were isogenic (with the more common Val allele, rather than Ile, at the polymorphic amino acid residue 49). First, pET-KHK-C was made by exchanging an RT-PCR-synthesized *MluI*-*BamHI* cassette (nt 125–404) spanning the alternative third exon. Mutations were then introduced into both pET-KHK-A and pET-KHK-C by PCR using oligonucleotides containing the required nucleotide changes (details available

on request). All constructs were verified by DNA sequencing of purified plasmids.

**Expression and purification of recombinant KHK.** Growth conditions of transformed *E. coli* JM109(DE3)pLysS were optimized for maximal KHK expression using 10- to 50-ml flask cultures. Then 25-L fermenter cultures were grown in LB medium containing 4% glucose and 100 µg/ml ampicillin at 37°C for KHK-C, 30°C for KHK-A, and 25°C for all mutants. Expression was induced with 0.4 mmol/l isopropylthiogalactoside (IPTG) at an apparent  $A_{600}$  of 0.5. Cells were harvested 2–3 h later and stored at –80°C. For purification, cells were resuspended in 10 ml/g lysis buffer (50 mmol/l Tris-HCl [pH 8.0], 5 mmol/l EDTA, 0.25 mmol/l phenylmethylsulfonyl fluoride [PMSF], 1 mmol/l dithiothreitol [DTT], and 0.1% [vol/vol] Triton X-100), kept on ice for 1 h, and then passed through a French press at a pressure of 69 MPa. Debris was removed by centrifugation for 1 h at 18,000 rpm in an SS34 rotor (Sorvall) at 4°C. Streptomycin sulfate was added slowly to 11 mg/ml, and the lysate was stirred for 10 min at 4°C. The supernatant was recovered by centrifugation for 30 min at 10,000 rpm in the SS34 at 4°C. Ammonium sulfate was added to 44% saturation with stirring at room temperature for 30 min, and the suspension was centrifuged for 30 min as before. The ammonium sulfate saturation was raised to 55% and the precipitate was isolated, again by centrifugation at 10,000 rpm for 30 min at 4°C. Additional steps were performed at 4°C. The 44–55% ammonium sulfate pellet was dissolved in a minimum volume (~20 ml for a 25-l culture) of 20 mmol/l BisTris-HCl, pH 6.5 (binding buffer), dialyzed against binding buffer for 16 h, and centrifuged at 10,000 rpm for 15 min. The supernatant was passed through 0.4-µm filters and then chromatographed on DEAE-cellulose (DE-52, Whatman, 15-ml bed) equilibrated in binding buffer. Proteins were eluted using a linear gradient of 0–50 mmol/l KCl in binding buffer (Fig. 2A). Fractions containing the 33-kDa protein with KHK activity were pooled and dialyzed against 25 mmol/l Tris-acetate (pH 8.3). The dialysate was added to 5 ml of Blue Sepharose (Pharmacia) equilibrated in the same buffer and kept on ice for 1–2 h with occasional shaking. The supernatant, containing KHK, was recovered by centrifugation at 4,000 rpm at 4°C for 15 min, filtered through 0.2-µm filters, and again chromatographed on DEAE-cellulose (5-ml column). KHK was eluted with a linear gradient of 25–200 mmol/l Tris-acetate, pH 8.3 (Fig. 2B). Fractions showing a single 33-kDa band on SDS-PAGE were pooled. No modifications to this purification scheme were needed for KHK-A or KHK-C or their Ala43Thr mutant derivatives.

**Protein and enzyme assays.** SDS-PAGE was by standard methods (19). Protein concentration was determined using a Coomassie microassay (Bio-Rad Laboratories, Hercules, CA). Enzyme activity in column fractions was assayed using a pyruvate oxidase-coupled method (20), with 10 mmol/l D-fructose as substrate. For kinetic studies, activity was measured at 25°C by the use of coupling enzymes pyruvate kinase and lactate dehydrogenase. The typical assay contained 50 mmol/l Na.PIPES (pH 7.0), 6 mmol/l MgCl<sub>2</sub>, 1 mmol/l phosphoenolpyruvate, 100 mmol/l KCl, 5 mmol/l ATP, 5 mmol/l



**FIG. 2. Purification of recombinant KHK-C.** Vertical axes indicate protein concentration. **A:** First DEAE-cellulose column chromatography. After sample loading, the 15-ml column was washed with 75 ml of binding buffer. Proteins were then eluted with a 100-ml linear gradient of 0–50 mmol/l KCl. Fraction volumes were 10 ml (1–10) or 1.25 ml (11–90). Those indicated by the brace were pooled, and SDS-PAGE of this material (*P*) is shown. **B:** Second DEAE-cellulose column chromatography. After loading, the 5-ml column was washed with 30 ml of 25 mmol/l Tris-acetate (pH 8.3) before elution with a 50-ml linear gradient of 25–200 mmol/l Tris-acetate (pH 8.3). The brace indicates the 2-ml fractions that were pooled. **C:** SDS-PAGE of final preparations of the four purified recombinant KHK proteins. *M*, prestained size markers (kDa; BioRad). Five micrograms of protein was loaded in each lane: 1, KHK-C-Ala43Thr; 2, KHK-C; 3, KHK-A-Ala43Thr; 4, KHK-A.

D-fructose, 0.2 mmol/l NADH, 40 units/ml pyruvate kinase, and 40 units/ml lactate dehydrogenase. Absorbance was measured at 340 nm. Initial reaction rates were determined using varying concentrations of the sugar substrate at a saturating [Mg.ATP] (5 mmol/l) or with variable [Mg.ATP] and fixed [D-fructose] (5 mmol/l for KHK-C and C-Ala43Thr; 10 mmol/l for KHK-A and A-Ala43Thr). Individual  $K_m$  and  $V_{max}$  estimates were made using regression fits to the Michaelis-Menten equation; the values in Tables 1 and 2 represent mean  $\pm$  SE from the number of independent experimental series indicated. Although KHK is dimeric (20),  $k_{cat}$  was calculated as activity per KHK monomer, because the structure of the distantly related *E. coli* ribokinase (21) suggests two active sites per KHK dimer. Stability of recombinant proteins (in the presence or absence of added glycerol) was tested on fresh *E. coli* extracts. Crude lysates from 50-ml cultures (see above) were centrifuged for 30 min at 14,000 rpm at 4°C in a microcentrifuge rotor. Supernatants were adjusted to 0.4 mg/ml protein, with glycerol at 0 or 30% vol/vol. These samples were then incubated at 37°C; 10- to 20- $\mu$ l aliquots were removed at 0- to 60-min time points and stored on ice until assayed. Thermostability was further assessed on extracts containing 30% glycerol by incubating at various temperatures (25–65°C) and times (0–30 min).

## RESULTS

**Expression of wt and mutant forms of human ketohehexokinase in *E. coli*.** For investigating the biochemical effects of the *KHK* mutations previously found in three subjects with essential fructosuria, these mutations (Gly40Arg) and (Ala43Thr) were engineered into both the C and A isoforms of KHK, expressed in *E. coli* (17). Both wt isoforms were also expressed, because no comparison of their properties has previously been made (in fact, the predicted KHK-A isoform has not previously been shown to possess enzymatic activity).

Figure 1 shows SDS-PAGE of protein extracts from uninduced (U) and induced (I) cultures carrying each expression construct. In all cases, a similar level of expression of the 33-kDa recombinant protein was seen ( $\sim$ 10% of total protein in *E. coli* lysates). Furthermore, both the KHK-C and KHK-A wt isoforms showed similar fructokinase activities, as judged by a rapid pyruvate oxidase-

coupled assay (using 10 mmol/l D-fructose). This provided the first confirmation that KHK-A, the novel isoform predicted by cDNA cloning experiments, is indeed catalytically active.

The wt and Ala43Thr mutant proteins all were easily recoverable in the soluble fraction of lysates generated using the nondenaturing detergent Triton X-100 (Fig. 1B). With both of the Gly40Arg mutants, however, there was greatly reduced recovery of soluble full-length recombinant protein, even when induction was carried out at 25°C. Instead, a prominent band at  $\sim$ 15 kDa appeared, presumably a degradation product generated during the lysis period. Furthermore, whereas both of the wt and both of the Ala43Thr mutants were catalytically active, no KHK activity could be demonstrated in extracts containing either Gly40Arg mutant. We therefore conclude that the Gly40Arg mutation renders both KHK-C and KHK-A non-functional, possibly by a major effect on tertiary structure.

**TABLE 1**

Kinetic properties determined for wt and mutant recombinant human KHKs

Enzyme	$k_{cat}/s^{-1}$	$K_m$ (mmol/l) (D-fructose)	$K_m$ (mmol/l) (Mg.ATP)
KHK-C	$7.6 \pm 0.7$ (6)*	$0.80 \pm 0.18$ (6)	$0.15 \pm 0.01$ (6)
C-Ala43Thr	$1.7 \pm 0.1$ (6)	$0.84 \pm 0.10$ (3)	$0.32 \pm 0.06$ (3)
KHK-A	$6.9 \pm 0.6$ (6)	$7.0 \pm 0.8$ (6)	$0.36 \pm 0.02$ (7)
A-Ala43Thr	$5.9 \pm 0.2$ (3)	$8.2 \pm 1.4$ (3)	$0.41 \pm 0.04$ (3)

Data are means  $\pm$  SE from the number of separate experimental series indicated in parentheses. \*Mean obtained from three independent purifications of KHK-C. The maximum  $k_{cat}$  value exhibited by any one batch was  $8.4 \pm 0.2 s^{-1}$  (two estimates). Higher values are likely to reflect the true value more closely, because KHK-C lost activity quickly on storage. Batch variability in specific activity of the more stable KHK-A was less apparent.



TABLE 2  
Comparison of kinetic parameters of recombinant human KHK-A and KHK-C using various substrates

	KHK-A		KHK-C	
	$k_{\text{cat}}/\text{s}^{-1}$	$K_m$ (mmol/l)	$k_{\text{cat}}/\text{s}^{-1}$	$K_m$ (mmol/l)
D-xylulose	$11.1 \pm 0.17$ (3)	$1.0 \pm 0.05$ (3)	$7.6 \pm 0.17$ (3)	$0.44 \pm 0.07$ (3)
D-ribulose	$7.6 \pm 0.3$ (2)	$32.7 \pm 5.3$ (2)	$4.8 \pm 0.4$ (2)	$1.8 \pm 0.2$ (2)
D-ribose	$1.55 \pm 0.07$ (2)	$201 \pm 8.5$ (2)	$8.2 \pm 0.6$ (2)	$434 \pm 62$ (2)
L-sorbose	$5.2 \pm 0.1$ (2)	$19.3 \pm 3.5$ (2)	$6.5 \pm 0.4$ (2)	$5.8 \pm 0.8$ (2)

Data are means  $\pm$  SE from the number of separate experimental series indicated in parentheses. All were performed using the same purified batch of KHK-A or KHK-C. At D-ribose concentrations  $>500$  mmol/l, considerable enzyme inhibition was apparent; these values were omitted when estimating the kinetic parameters, so the estimates given are derived from a limited substrate concentration range below the apparent  $K_m$ .

**Purification of wt and mutant recombinant human KHKs.** The wt and Ala43Thr mutant forms of KHK each were purified to  $>99\%$  homogeneity as judged by SDS-PAGE (Fig. 2A–C). Final yields ranged from 0.5 to 1.5% of initially solubilized protein. The purified proteins migrated in the region of 33 kDa, consistent with the 32.7 kDa predicted from the sequences.

**Kinetic properties of mutant forms of KHK.** Some kinetic properties of the purified wt and mutant KHKs were examined. With D-fructose as substrate, recombinant KHK-C had a mean specific activity of 14 units/mg ( $k_{\text{cat}} = 7.6 \text{ s}^{-1}$ ). The corresponding enzyme purified from human liver had a value of 9.4 units/mg at  $37^\circ\text{C}$  (2), which would correspond to  $\sim 4.1$  units/mg at  $25^\circ\text{C}$ , judging from the rat enzyme's thermal  $Q_{10}$  of  $\sim 2$  (16). This is low, compared with 18 (16) or 12 units/mg (20) for the rat enzyme and 17 units/mg for the bovine enzyme (1). The higher activity of the recombinant human enzyme may therefore indicate some loss of activity in the human liver enzyme obtained by Bais et al. (2). Consistent with this, we do observe variability in specific activity of recombinant KHK-C, the highest value for any one freshly purified preparation being 15.5 units/mg ( $k_{\text{cat}} = 8.4 \text{ s}^{-1}$ ). Stability of KHK-C is discussed further below.

The substrate concentration dependence of activity was determined for fructose and ATP (Fig. 3 shows data for ATP; Table 1 summarizes kinetic parameters for D-fructose and ATP). The  $k_{\text{cat}}$  values of the two wt isoforms are comparable, but KHK-A has a much higher  $K_m$  for fructose (7 mmol/l) than does KHK-C (0.8 mmol/l). There is also a less marked difference between the two isoforms'  $K_m$  for ATP (KHK-C, 0.15 mmol/l; KHK-A, 0.36 mmol/l).

As mentioned above, whereas the Gly40Arg mutation abolished activity of both KHK-A and KHK-C, Ala43Thr did not initially seem to have a major effect on recombinant enzyme activity in "spot" assays. This was surprising because  $^{31}\text{P}$ -NMR studies of one of the essential fructosuria patients had shown total unresponsiveness of intrahepatic ATP,  $P_i$ , and fructose-1-phosphate on fructose infusion, suggesting a virtually complete absence of liver fructokinase (22). The properties of the purified KHK-Ala43Thr mutants offer a possible explanation, however (Table 1). The Ala43Thr mutation has little effect on the  $K_m$  for D-fructose of either KHK-C or KHK-A. There is a slight increase in  $K_m$  for ATP, although at 0.3 mmol/l, the  $K_m$  of mutant KHK-C is still well below the estimated fasting ATP concentration in liver of  $\sim 2$  mmol/l (22). The major effect of Ala43Thr seemed to be a substantial reduction in the  $k_{\text{cat}}$  of mutant KHK-C. However, in contrast, the  $k_{\text{cat}}$  of mutant KHK-A was virtually unaffected.

During these experiments, we observed that the purified KHK proteins suffered different degrees of activity loss during storage. Further studies suggested that the apparent differences in  $k_{\text{cat}}$  between mutant KHK-C and KHK-A originate in differential effects of Ala43Thr on the stabilities of the recombinant proteins.

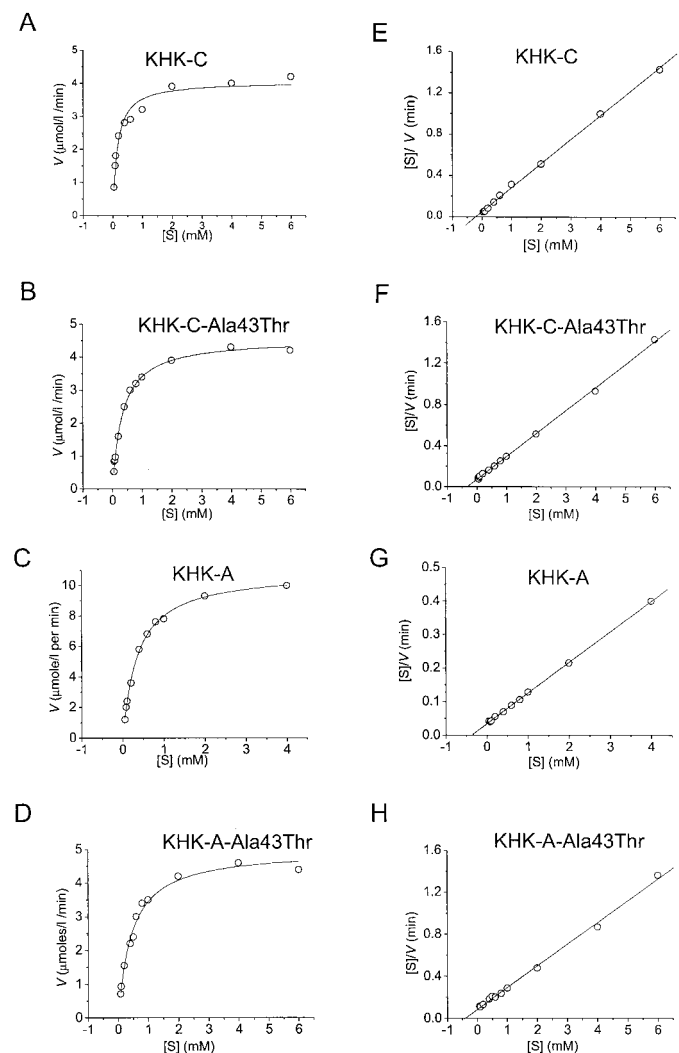
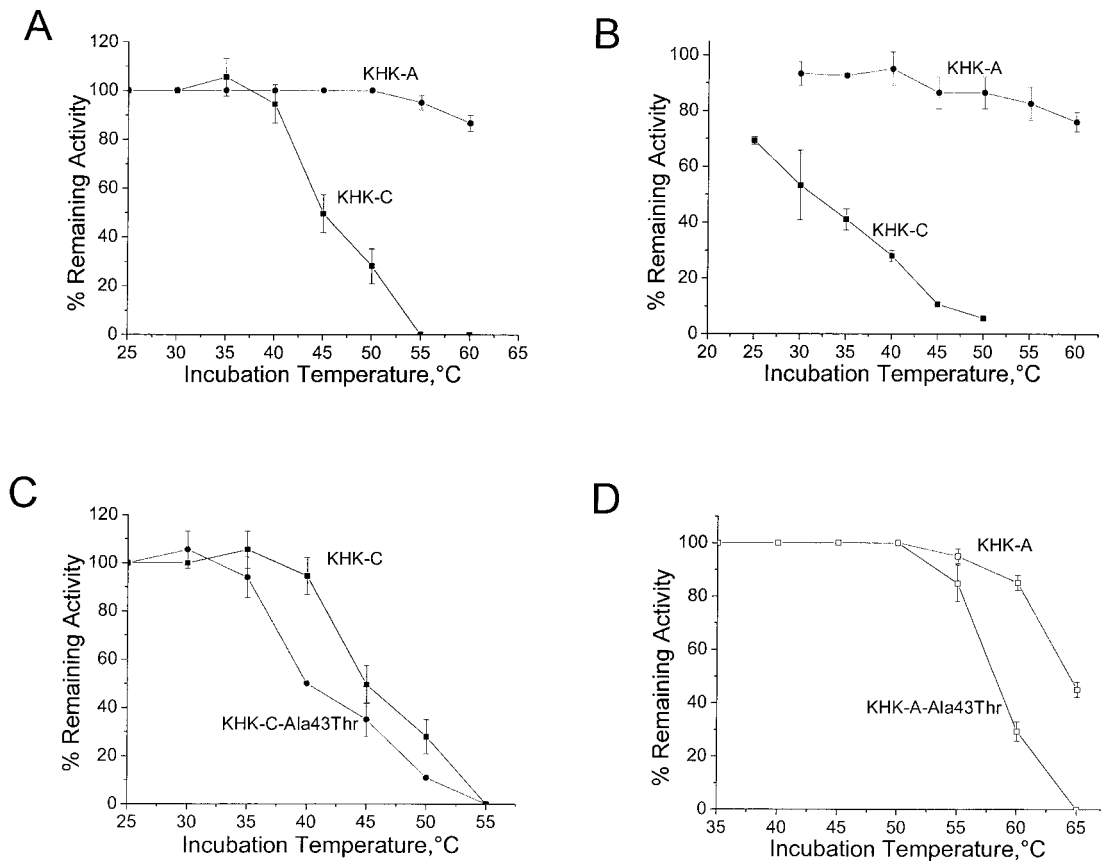


FIG. 3. Kinetic analysis of recombinant KHKs. Each graph shows one experimental series used to obtain a single estimate of  $K_m$  for ATP. (The number of such independent estimates used and their means and SE are shown in Tables 1 and 2.) A–D: Nonlinear regression fit to Michaelis-Menten equation. E–H: Hanes-Woolf plots fitted by linear regression. The kinetic parameters ( $K_m$  and  $V_{\text{max}}$ ) from both fits were in close agreement.



**FIG. 4.** Stability of wt and mutant recombinant human KHKs as a function of temperature. Soluble *E. coli* extracts containing wt or mutant forms of KHK were incubated for 30 min in the presence of 30% glycerol at the temperatures shown on the x-axis, after which residual KHK activity was determined. *A*: Comparison between KHK-A and KHK-C in fresh lysates. *B*: Comparison between purified KHK-A and KHK-C. *C*: Effect of Ala43Thr mutation on KHK-C. *D*: Effect of Ala43Thr mutation on KHK-A. Bars (omitted if smaller than symbol size) indicate  $\pm 1$  SE of four (*A*, *C*, and *D*) or two (*B*) experiments.

**Thermolability of KHK proteins.** In fresh *E. coli* lysates incubated for 30 min in the presence of 30% glycerol, KHK-A lost little activity at temperatures up to 55°C, whereas KHK-C was inactivated completely at this temperature (Fig. 4A). This is unlikely to reflect differential susceptibility to proteolysis in the crude lysates, because an even more marked difference in stability was seen using purified KHK-A and KHK-C (Fig. 4B). The much greater thermolability of KHK-C than KHK-A was further apparent in time-course experiments (Fig. 5A and C).

The Ala43Thr mutation had the effect of reducing the thermostability of both KHK-C and KHK-A, in each case shifting the temperature curve leftward by  $\sim 5^\circ\text{C}$  relative to the corresponding wt enzyme (Fig. 4C and D). The time courses confirmed that the Ala43Thr mutant in each case lost activity faster than its wt parent at the same temperature. However, the temperature window over which this effect is apparent is different for KHK-C than for KHK-A. At 40°C, for example, a large destabilizing effect of Ala43Thr on KHK-C is apparent (Fig. 5B), whereas a temperature of 60°C is needed to show a similar effect of this mutation on KHK-A (Fig. 5D). We also examined inactivation of the wt and mutant KHKs at physiologic temperature (37°C), in the absence of glycerol (Fig. 6). As expected from the previous data, the Ala43Thr mutant of KHK-C rapidly lost activity under these conditions. Although the half-life of KHK in vivo and the nature of any intracellular factors that might affect its stability are not known, the above data on

in vitro stability of recombinant enzymes do suggest that Ala43Thr is likely to be pathogenic primarily as a result of its destabilization of the KHK-C enzyme.

**Activity toward other substrates.** Because, compared with KHK-C, KHK-A has a much higher  $K_m$  for D-fructose, we considered the possibility that it might have evolved preferential activity toward other substrates. Table 2 shows kinetic parameters determined for D-xylulose, D-ribulose, D-ribose, and L-sorbose, all of which were previously shown to be phosphorylated by bovine fructokinase (1). Only for D-ribose was the KHK-A  $K_m$  value lower than that of KHK-C, although this is not within a physiologically relevant concentration range. KHK-A does display a higher  $k_{cat}$  and lower  $K_m$  (1 mmol/l) for D-xylulose than for D-fructose. Nonetheless, the  $K_m$  of KHK-C for D-xylulose (0.4 mmol/l) is lower still, so it is difficult to conclude that KHK-A is specially adapted for phosphorylation of this sugar.

## DISCUSSION

Before our studies of the human and rodent *KHK* genes (12,15), the existence of alternative KHK isoforms was not suspected. The alternative splicing of *KHK* mRNA is tissue-specific, with liver, renal cortex, and small intestine producing mostly the C isoform and other tissues the A isoform. This splicing pattern also correlates with the expression level in that tissues that show the A splice form also express the gene only at very low levels. These RNA

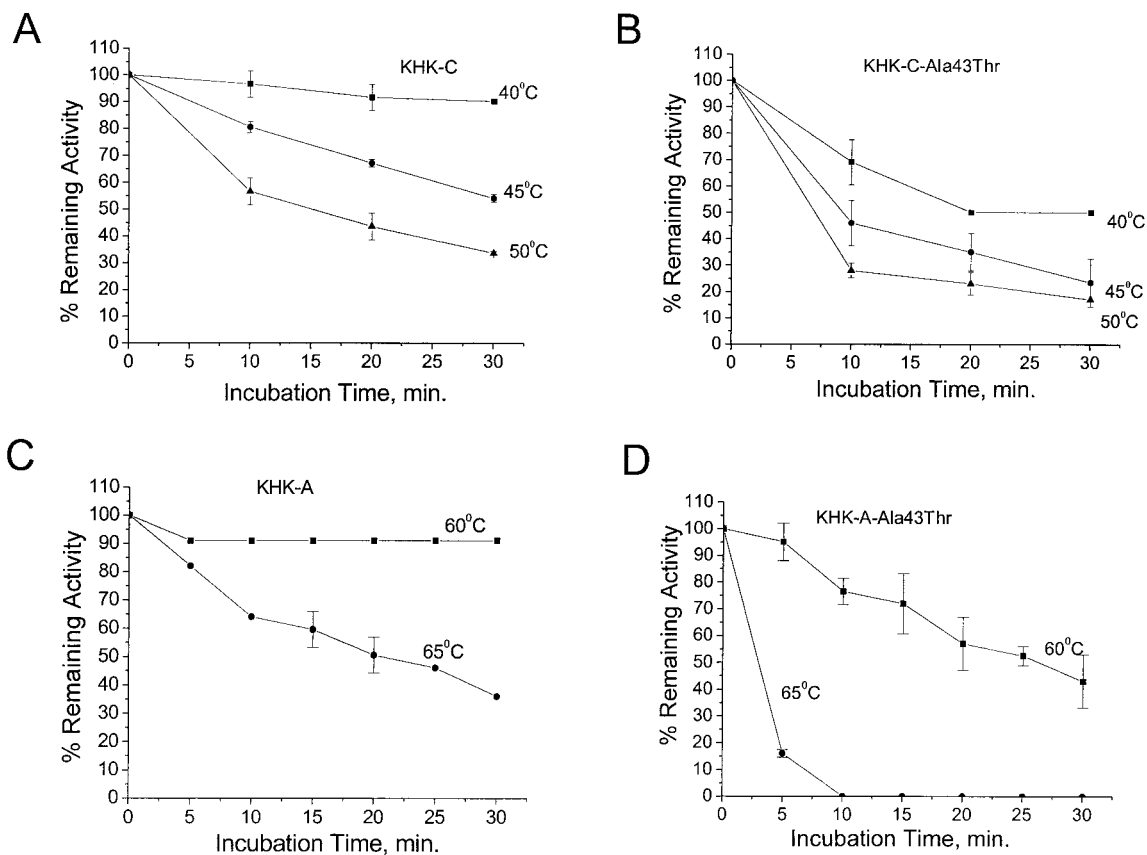


FIG. 5. Stability of wt and mutant recombinant human KHKs as a function of time. Soluble *E. coli* extracts containing wt or mutant KHK were incubated in the presence of 30% glycerol at the indicated temperatures for various periods of time (x-axis), after which residual KHK activity was determined. *A* and *B*: Effect of the Ala43Thr mutation on KHK-C stability at 40–50°C. *C* and *D*: Effect of the Ala43Thr mutation on KHK-A stability at 55–60°C. Bars (omitted if smaller than symbol size) indicate  $\pm 1$  SE of four experiments.

studies, however, gave no indication of whether the alternative splicing of KHK mRNA might have physiologic relevance. The only circumstantial evidence favoring this was its evolutionary conservation in rats, mice, and humans. Direct evidence for the functionality of KHK-A was lacking.

Previous biochemical studies have examined KHK isolated from liver, which has the highest level of activity. The properties of recombinant KHK-C purified in the present study are consistent with those of purified hepatic enzymes (1,2,16,20). Our second finding, that the KHK-A isoform is indeed biochemically active, suggests that the low levels of KHK-A expression seen in a wide range of tissues probably do fulfill some specific physiologic function. Its high  $K_m$  (7 mmol/l) for D-fructose, however, makes it somewhat unlikely that KHK-A plays a significant role in metabolism of dietary fructose. It also does not seem to display a lower  $K_m$  than KHK-C for any of the other substrates so far tested.

First recognized in 1876 (14), essential fructosuria may be the earliest-described inborn error of metabolism but has been little studied, being completely benign. Deficiency of hepatic fructokinase was shown by enzyme assay on a liver biopsy from one patient (23) and by  $^{31}\text{P}$ -NMR studies of hepatic metabolites after fructose loading in another (22). The latter patient and other affected family members are compound heterozygotes for the KHK Gly40Arg and Ala43Thr mutations (15).

Our present data show that, unexpectedly, Ala43Thr is

not a null mutation. Nonetheless, the lack of any demonstrable KHK activity in the liver of the patient with essential fructosuria suggests that the thermolability of mutant KHK-C carrying Ala43Thr is sufficient to drastically reduce the steady-state level of central KHK-C. The impact of Ala43Thr on the low levels of KHK in other tissues could be different, however. KHK in these tissues is mostly of the more thermostable A splice form. Because Ala43Thr is unlikely to have a major effect on the activity of KHK-A at physiologic temperature, the modest amounts of KHK-A in many tissues may be preserved, even though the much

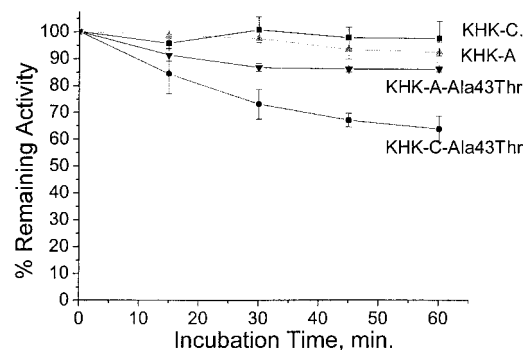


FIG. 6. Stability of wt and mutant recombinant human KHKs at 37°C in the absence of glycerol. Soluble *E. coli* extracts containing wt or mutant KHK were incubated at 37°C for various times (x-axis). Bars indicate  $\pm 1$  SE of four (KHK-C, KHK-A-Ala43Thr) or three (KHK-A, KHK-C-Ala43Thr) experiments.

greater levels of KHK-C in central viscera are drastically reduced. Whether this is of independent physiologic significance remains unproved. Mutation analysis of additional families with essential fructosuria would probably be very informative in this regard, because it might indicate clearly whether mutations that abolish KHK-A activity as well as KHK-C can be tolerated. We have so far failed, however, in our efforts to ascertain additional patients with essential fructosuria. An alternative approach to the same question is to examine mouse knockout models, because this should allow selective ablation of KHK-A or KHK-C.

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