Expression and site-directed mutagenesis of hepatic glucokinase

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Soluble rat liver glucokinase was expressed at high levels at 22 °C in the BL21(DE3)pLysS strain of *Escherichia coli*. Aspartate-211 of yeast hexokinase has been implicated as a catalytic residue from crystallographic data. The corresponding residue in rat liver glucokinase, aspartate-205, was mutated to alanine and the expressed mutant had 1/500th of the activity of the wild type, with no change in the K_m values for glucose or ATP. The results support a role for this residue as a base catalyst in the glucokinase reaction and, most probably, a similar role in the reactions of all members of the hexokinase family.

INTRODUCTION

Hepatic glucokinase is a member of the hexokinase family and has a high K_m and specificity for glucose, whereas hexokinases I–III have low K_m values and phosphorylate other sugars [1]. In contrast with hexokinases I–III, which are widely distributed in mammalian tissues, glucokinase is expressed only in liver and pancreatic β -cells [2]. Hepatic glucokinase gene expression is regulated by dietary and hormonal factors such as insulin, which enhances transcription by an as yet unknown mechanism [3,4a]. Although the affinity and specificity for hexose substrates differ, the values of k_{cat} for glucokinase and the other hexokinases are all rather similar, suggesting a common catalytic mechanism [5]. Mammalian low- K_m hexokinases have molecular masses of ~ 100 kDa and are feedback-inhibited by glucose 6-phosphate, whereas glucokinase and yeast hexokinase have molecular masses of about 50 kDa and are not subject to product inhibition [1,2,5,6].

The complete amino acid sequences of type I hexokinase [7] and glucokinase [8] have been deduced from cloned cDNA. Glucokinase has 53% and 33% amino acid sequence identity with the C-terminal domains of rat brain enzyme and of yeast hexokinase respectively. The crystal structure of yeast hexokinase was determined prior to the elucidation of the primary structure and therefore no X-ray co-ordinates exist in the Brookhaven data bank [9]. A preliminary refinement of the crystal structure has appeared which identifies active-site residues involved in the binding of glucose and ATP [10]. All of the residues of the yeast hexokinase that hydrogen-bond to the hydroxyl groups of glucose appear to be conserved and are in similar locations in rat liver glucokinase when the sequences are aligned [8]. A high degree of conservation is also seen in the putative ATP-binding domain, which is punctuated by a conserved lysine residue 11–14 residues C-terminal to the core sequence [8]. Recently, rat liver glucokinase has been expressed in *Escherichia coli* by using an expression system based on T7 RNA polymerase, but the yield of protein was low [11]. The purified expressed protein had properties identical to those of the wild-type enzyme. In this paper we report conditions for overexpression of rat liver glucokinase in *E. coli* to levels 10-fold greater than previously reported, and the use of site-directed mutagenesis to investigate the role of the conserved residue, Asp-205, in catalysis by glucokinase.

EXPERIMENTAL

Materials

Restriction enzymes and bacteriophage T4 DNA ligase were obtained from New England BioLabs. Q-Sepharose Fast Flow and Sephadex G-100 Superfine were from Pharmacia LKB Biotechnology Inc. Glucose-6-phosphate dehydrogenase was from Boehringer.

Oligonucleotide-directed mutagenesis

Oligodeoxynucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems model 381 A synthesizer and purified on oligonucleotide purification cartridges (OPC) according to the Applied Biosystems manual. Standard DNA manipulations were carried out as described [12]. Mutagenesis was carried out using the Eckstein method [13,14] with the Oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham; RPN 1523). Single-stranded glucokinase cDNA GK.Z1 [8] was used as the template and was annealed to an oligonucleotide (GGCCACTGTGGCATTCCACCAT) which coded for alanine at position 205. The sequence of the mutant, pGK.Z1-D205A, was determined by the Sanger dideoxy method [15] and the double-stranded clone was digested with *AocI* and *AflII*. The resulting 786 bp fragment containing the mutation site was cloned into the wild-type glucokinase expression vector pEGK [11] similarly digested with *AocI* and *AflII*, creating pEGK-D205A.

Expression system

The proteins were expressed using the T7 expression system described by Studier & Moffatt [16]. Expression was controlled by the T7 RNA polymerase promoter and translation initiation signals for the major capsid protein of bacteriophage T7. Expression plasmids were transformed into *E. coli* BL21(DE3) or BL21(DE3)pLysS, where the gene for T7 RNA polymerase is under the control of the inducible lac UV5 promoter. Cultures were spread on ampicillin (50 µg/ml)-only plates and ampicillin (50 µg/ml) plus chloramphenicol (30 µg/ml) plates [17] respectively. Single colonies were picked from each plate and grown overnight.

Abbreviations used: IPTG, isopropyl-1-thio- β -D-galactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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Table 1. Purification of rat liver glucokinase expressed in *E. coli*

Step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Extract	2752	3046	0.9	—	—
(NH ₄) ₂ SO ₄ (40–65%)	1534	432	3.6	4.0	55
Sephadex G-100	1252	254	4.9	5.5	45
Q-Sepharose	950	10.2	93	103	35

Protein induction

A 5 ml portion of overnight culture was added to 1 litre of M9 medium containing ampicillin (100 µg/ml) for the BL21(DE3) strain, or 1 litre of M9 medium containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml) for the BL21(DE3)pLysS strain. Cultures were grown at 37 °C until the absorbance at 500 nm reached 0.7. Each strain was then divided into two flasks and grown at 37 °C or 22 °C for 30 min. The inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to final concentration of 0.4 mM. The culture was grown at 37 °C for 4 h, and the culture at 22 °C was grown for 48 h.

Purification of expressed rat liver glucokinase

Glucokinase was purified by a modification of the method of Andreone *et al.* [8]. Resuspended (NH₄)₂SO₄ precipitates were gel-filtered on a 1.5 cm × 20 cm Sephadex G-100 column in a buffer containing 50 mM-potassium phosphate, 100 mM-KCl, 0.1 mM-EDTA, 0.1 mM-dithiothreitol (DTT), pH 7.5, and then chromatographed on a 0.9 cm × 10 cm Q-Sepharose column equilibrated with the same buffer. Glucokinase was eluted by a linear KCl gradient (100–600 mM) at a concentration of 350 mM. The *N*-acetylglucosamine-Sepharose column was unnecessary, since the enzyme represented more than 3 % of the soluble *E. coli* extract protein. A purification scheme is shown in Table 1. Glucokinase was homogeneous by the criteria of SDS/PAGE. Mutant and wild-type enzymes behaved similarly during the purification scheme.

Estimation of total induced protein

Cells (100 µl) were taken from each strain at two different temperatures and harvested by centrifugation just before induction and at the indicated times after induction. The cell pellets were dissolved in SDS/PAGE loading buffer (1 % SDS, 10 % sucrose, 10 mM-Tris, pH 8, 1 mM-EDTA, 40 mM-DTT, 0.001 % pyronin Y) and boiled for 5 min. The samples were subjected to SDS/PAGE and the gels were stained with Coomassie Blue. The amount of glucokinase was estimated from the stained gels using purified glucokinase [11] as a standard.

Estimation of soluble enzyme activity and total soluble protein

Cells (10 ml) were taken just before induction and at each time point thereafter. Cells were centrifuged, washed in buffer B [50 mM-potassium phosphate, pH 7.5, 100 mM-KCl, 1 mM-DTT, 1 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) and 2.5 µg of leupeptin/ml], pelleted again, and dissolved in 0.5 ml of buffer B containing lysozyme (1 mg/ml). Cells were then subjected to freezing/thawing three times and treated with 5 mg of DNAase I plus 5 ml of 5 mM-MgSO₄ at 4 °C for 1 h. Cell debris was centrifuged down (12000 *g* for 5 min) and the supernatants were assayed spectrophotometrically for soluble glucokinase activity [18]. Total soluble protein was estimated by

measuring the absorption at 280 nm of the supernatant and/or Lowry protein determination [19].

Assay of enzyme activity

Glucokinase activity was measured by the formation of glucose 6-phosphate in a glucose-6-phosphate dehydrogenase/NADP⁺-coupled assay as previously described, or by coupling ADP formation to the pyruvate kinase and lactate dehydrogenase reactions and monitoring the decrease in NADH absorbance at 340 nm [18].

Immunoblot analysis

Immunoblot analyses were performed essentially as described by Towbin *et al.* [20] with a 1:500 dilution of glucokinase antiserum as described previously [11].

RESULTS

Effect of temperature on expression of rat hepatic glucokinase in *E. coli*

The T7 RNA polymerase expression system is capable of expressing a wide variety of genes from both prokaryotic and eukaryotic sources [17]. In this system, the expression of the protein is controlled by the Φ10 promoter of the T7 RNA polymerase and the strong translation initiation signals for the major capsid protein of bacteriophage T7. Addition of IPTG to a growing culture induces T7 RNA polymerase, which in turn transcribes the target DNA in the expression plasmid. This expression system has been successfully used for overexpressing a number of low-abundance proteins of the hepatic glycolytic/gluconeogenic pathway, including glucokinase [11], fructose-1,6-bisphosphatase [21] and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and its separate kinase and bisphosphatase domains [22,23]. In all of these cases, however, the yield of enzyme found in the soluble fraction is low, about 1–5 % of the total protein produced. A number of reports have suggested that the yield of active soluble protein can be dramatically increased by lowering the induction temperature [24–26]. Presumably, lowering the temperature decreases the rate of synthesis of the protein and allows it to fold properly [27]. The rate of enzyme production can also be decreased by using a strain of *E. coli* that contains a plasmid expressing T7 lysozyme (pLysS), a natural inhibitor of T7 RNA polymerase [16,17,28].

In an attempt to improve the yield of soluble active glucokinase, we tested the effect of IPTG induction as a function of time in two different strains at 22 °C and 37 °C (Fig. 1). Both the BL21(DE3) strain and the pLysS-containing strain produced increased amounts of a protein that migrated with the same mass (50 kDa) as rat liver glucokinase. Western blotting analysis revealed that the 50 kDa peptide reacted with antiserum to the rat liver glucokinase (results not shown). Over the 4 h induction period at 37 °C, the amount of glucokinase increased steadily, reaching a value of 10 mg/litre in the BL21(DE3) strain and a value of 25 mg/litre in the BL21(DE3)pLysS strain (Figs. 1*a* and 1*c*). Induction in BL21(DE3) was seen as early as 30 min, while in the presence of pLysS no induction was seen until 60 min. Although T7 lysozyme is a natural inhibitor of T7 RNA polymerase, the presence of pLysS caused only a short lag in the appearance of the enzyme, but at later times the amount of enzyme was actually higher in the pLysS strain. This was not due to changes in the expression plasmid copy number, which was the same in the BL21(DE3) and BL21(DE3)pLysS strains (results not shown). However, as also shown in Figs. 1*a* and 1*c*, only a very small fraction of the protein was soluble at 37 °C. The percentage soluble protein at 2 h was about 6 % for the pLysS-containing strain and about 3 % for the BL21(DE3) strain.

Fig. 1. Time course of expression and synthesis of rat liver glucokinase in *E. coli*

Either BL21(DE3) or BL21(DE3)pLysS was transformed with the pET/glucokinase expression plasmid, and protein synthesis was induced by the addition of 0.4 mM-IPTG. Total protein was estimated from Coomassie-Blue-stained SDS/PAGE gels using BSA as a standard. Active enzyme was determined spectrophotometrically as described in the Experimental section. ●, Total glucokinase protein; ○, active glucokinase protein. (a) Induction in BL21(DE3) at 37 °C; (b) induction in BL21(DE3) at 22 °C; (c) induction in BL21(DE3)pLysS at 37 °C; (d) induction in BL21(DE3)pLysS at 22 °C.

The effect of lowering the induction temperature is shown in Figs. 1(b) and 1(d). As with the case at 37 °C, upon induction both strains produced increased amounts of glucokinase. However, the increase in total glucokinase was substantially greater over the entire 48 h induction period than that obtained after 4 h of induction at 37 °C. Again there was a lag in the induction of the enzyme in the BL21(DE3)pLysS strain. In the BL21(DE3) strain the amount of enzyme produced reached a plateau (20 mg/litre) at 15 h, while in the pLysS-containing strain the plateau (40 mg/litre) was reached as early as 6 h after induction. The reason for greater levels of total protein with the pLysS strain is unknown. Figs. 1(b) and 1(d) also show that the amount of soluble protein recovered upon induction at 22 °C was increased greatly in both strains. In the pLysS-containing strain the amount of soluble active protein reached 10–12 mg/litre at 15 h, which represents about 25–30% of the total protein produced, while in the BL21(DE3) strain about 1.6 mg/litre was active soluble enzyme, which represents only about 8% of the total protein. The value for the pLysS strain is about 10-fold higher than that obtained both in the present study and previously for the BL21(DE3) strain at 37 °C after 3 h of induction. It is clear from our study that the major difference in recovery of active enzyme is seen when the pLysS strain is used at an induction temperature of 22 °C and that this system is useful for expressing and characterizing wild-type and mutant forms of hepatic glucokinase.

Role of Asp-205 in glucokinase catalysis

Analysis of the yeast hexokinase structure has suggested that Asp-211 acts as a base catalyst for the yeast enzyme [10]. However, there are at present no functional data which support this hypothesis for yeast hexokinase or for any mammalian hexokinase. The corresponding residue in the glucokinase sequence is Asp-205 [8]. In order to evaluate the role of Asp-205 in the glucokinase reaction, the residue was mutated to alanine, an amino acid which cannot act as a base catalyst in the reaction,

Fig. 2. Induction of wild-type and mutant D205A rat liver glucokinase protein

A growing culture of BL21(DE3)pLysS cells carrying either pEGK-WT (lanes 1–4) or pEGK-D205A (lanes 5–8) was induced by 0.4 mM-IPTG at an A_{600} of 0.4 and allowed to grow for 0, 8, 16 or 24 h at 22 °C. A portion of cells was dissolved in SDS and subjected to SDS/PAGE as described in the Experimental section. The position of glucokinase (GK) is shown, along with molecular mass standards in lane M.

Fig. 3. Expression of soluble rat liver glucokinase

Glucokinase was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (45–65%) from extracts prepared from BL21(DE3)pLysS cultures carrying either pEGK-WT (lanes 1–3) or pEGK-D205A (lanes 4–6) expression plasmids induced for 8, 16 or 24 h. Portions were subjected to SDS/PAGE as described in the Experimental section. The position of glucokinase (GK) is shown, along with molecular mass standards in lane M.

giving the mutant enzyme D205A. The time course of induction of wild-type enzyme and D205A in *E. coli* is shown in Fig. 2. The Coomassie-Blue-stained gel shows that the same amount of D205A as wild-type enzyme was induced. Both soluble enzyme forms were initially isolated by 45–65% $(\text{NH}_4)_2\text{SO}_4$ precipitation and the enzymes were subjected to SDS/PAGE. As shown in Fig. 3, after this single purification step glucokinase represents the major protein band, and again the same amounts of D205A and wild-type enzyme were recovered in the soluble fraction of *E. coli* extracts.

Fig. 4. Kinetics of wild-type (●) and D205A (○,▲) glucokinase activities

Activities of partially purified preparations of wild-type and D205A glucokinase were determined at 2, 5, 10, 20, 50, 90 and 100 mM-glucose in the presence of 5 mM-ATP. Inset: double-reciprocal plot of velocity and glucose concentration. ●, Wild-type glucokinase activity; ○, D205A activity measured in the absence of ATP; ▲, mutant D205A glucokinase activity measured in the presence of ATP and glucose. The kinetic data obtained for D205A shown in the inset were obtained by using 50 times more extract than was used to obtain kinetic data for the wild-type enzyme.

Table 2. Comparison of the properties of wild-type and D205A glucokinases

Property	Wild-type	D205A
K_m for glucose (mM)	8–10	3–5
K_m for ATP (mM)	0.2	0.2
Specific activity (munits/mg)	100	≤ 0.2
Inhibition by glucose 6-phosphate	No	No
Molecular mass (kDa)	50	50

The mutant and wild-type enzymes were purified to homogeneity by a modification of the method of Andreone *et al.* [8], as described in the Experimental section, and the kinetic properties of the mutant were analysed. D205A had 1/500th of the activity of the wild-type enzyme when both were measured with saturating concentrations of glucose (100 mM). The effect of the mutation appeared to be primarily on k_{cat} , since the K_m for glucose of D205A was 3–5 mM, compared with 8 mM for the wild-type enzyme (Fig. 4, Table 2). The determination of the K_m for glucose of the D205A mutant was difficult, since the glucokinase activity of this mutant was very low and because the coupling enzymes used in the glucokinase assay had a small amount of contaminating low- K_m hexokinase activity. Blanks without added glucokinase were subtracted and much higher levels of D205A mutant protein were used in the assay. There is probably no significant difference between the wild-type and D205A enzymes with respect to affinity for glucose. There was also no detectable change in the K_m for ATP (Table 2). The apparent molecular masses of the wild-type and D205A enzymes were also the same, as measured by gel filtration on Sephadex G-100 Superfine. Neither form was inhibited by glucose 6-phosphate. The mutant protein had the same urea-concentration-dependence for denaturation and concomitant inactivation as the wild-type protein (results not shown). The mutant and wild-type enzymes also showed the same time-dependent decrease

in activity during limited trypsin proteolysis (results not shown). The large decrease in k_{cat} , but small change in K_m for glucose, with no change in the affinity for ATP, are consistent with the hypothesis that Asp-205 acts as a base catalyst in the rat liver glucokinase reaction.

DISCUSSION

Determination of the tertiary protein structure of yeast hexokinase isoenzymes in the presence of glucose and glucose analogues has suggested that the specific amino acid residues Ser-158, Asp-211, Glu-269 and Glu-302 participate in the binding of glucose by hydrogen bonding to the hydroxyl groups of glucose [9,10]. Although determination of the original X-ray crystal structure of yeast hexokinase was undertaken before the primary sequence of the enzyme was known, a role for an aspartate residue in promoting the nucleophilic attack of the glucose 6-hydroxyl group on the γ -phosphate of ATP was postulated [9]. Harrison, in a refinement of the yeast structure which utilizes the known sequence, postulated that this catalytically important residue is Asp-211 [10]. The results reported in the present paper provide the first functional evidence that the corresponding Asp-205 in glucokinase is an important catalytic residue which probably acts as a base catalyst. Since this residue is conserved in all the known hexokinase sequences, including those from both mammals and yeast, it seems reasonable to predict that this residue acts as a base catalyst in all hexokinases. Thus hexokinase is like 6-phosphofructo-1-kinase [29] in having an aspartate residue that promotes nucleophilic attack of a sugar hydroxyl group on the γ -phosphate of ATP.

The finding in this paper that lowered induction temperature and the use of a pLysS-containing strain enhance the yield of active soluble glucokinase is a potentially important finding for further analysis of the structure/function relationships of this enzyme as well as other hexokinases. Production of 10–12 mg of glucokinase/litre of *E. coli* culture should also permit the isolation of adequate amounts of the enzyme for its crystallization.

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