MUTATION IN BRIEF

Biochemical Characterization of Two GALK1 Mutations in Patients with Galactokinase Deficiency

Federica Sangiuolo[1], Mauro Magnani [2], Dwight Stambolian [3], and Giuseppe Novelli[1]*

[1]Department of Biopathology, Human Genetics Unit, Tor Vergata University of Rome, Italy; [2]Institute of Biochemistry "G.Foprnanini", University of Urbino, 61029 Urbino, Italy; [3]Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

*Correspondence to: Giuseppe Novelli, Giuseppe Novelli, Department of Biopathology, Tor Vergata University, via Montpellier 1, 00133 Rome, Italy; Tel.: +39-06-72596080; Fax: +39-06-20427313;
E-mail: novelli@med.uniroma2.it

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Galactokinase (GALK1) deficiency is an autosomal recessive disorder, which causes cataract formation in children not maintained on a lactose-free diet. Galactokinase deficiency results from mutation in the GALK1 gene mapped on 17q24. Since GK1 cDNA was cloned about 20 mutations (prevalently deletions and missense) have been reported to date. Most of these reported mutations are confined to single families, and only one of them, P28T, has been referred as the founder Romani mutation. In this paper we report two novel missense mutations in GALK1 gene, identified in two unrelated patients with galactokinase deficiency. One mutation, g.575G>A, substitutes a valine for a methionine at amino acid 32 (p.V32M), while the other mutation, g.2839G>A, results in the arginine to glutamine substitution p.R239Q (GenBank sequence L76927). Biochemical studies demonstrate that these mutations led to a drastic modification in GALK activity when individual mutant cDNAs were expressed in an *E. coli* system. These findings indicate the pathogeneticity of these mutations causing GALK deficiency. © 2004 Wiley-Liss, Inc.

KEY WORDS: GALK1; galactokinase deficiency; functional analysis

INTRODUCTION

Galactokinase (E.C.2.7.1.6) catalyse the conversion of galactose to galactose-1-phosphate, the first step of the Leloir metabolic pathway through which galactose enters glycolysis. Galactokinase deficiency (MIM# 230200) is an inborn error in the first step of galactose metabolism. Its major clinical manifestation is the development of cataracts during the first months of life. It has also been suggested that, also depending on milk consumption later in life, carriers of the deficiency are predisposed to presenile cataracts developing at age 20-50 years (Beutler et al., 1973; Stambolian et al., 1986; Novelli et al., 2000). The main symptom of this disease is early onset cataracts although mental retardation is also seen in some patients. Galactose accumulation in the lens of the eye is converted in galactitol by aldose reductase. High levels of galactitol in lens fibre cells cause the uptake of water by osmosis, swelling of the cells, cells lysis and ultimately cataracts (Segal & Berry, 1995). The condition can be treated by removal of galactose and lactose from the diet. (Ai et al., 2000). Newborn screening data suggest that

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the gene frequency is very low worldwide (the incidence ranges between 1:150,000 and 1:1,000,000) with an east-to-west gradient appears to be present across Europe, but is higher among the Roma in Europe (Kalaydjieva et al., 1999). Since the cloning of the galactokinase gene (GALK1; GenBank accession number L76927) in 1995 (Stambolian et al.), about 20 disease-causing mutations have been identified (Kolosha et al., 2000; Hunter et al., 2001). A variety of different mutations have been observed including insertions, deletions, and single base changes. Here we present the finding of two missense mutations in GALK1gene: g.575G>A substitution in exon 1 (p.V32M) characterized in a homozygous galactokinase deficient individual with cataracts (Kolosha et al., 2000) and g.2839G>A transition in exon 6 (p.R239Q), identified in heterozygosity in an individual whose clinical characteristic was previously reported (Magnani et al., 1982). Both amino acid substitutions were cloned and expressed in *E. coli* to determine their effect on enzyme function and thermal stability to the normal allele. Utilizing a coupled enzyme based on a spectrophotometric based assay system, the kinetic constants of p.R239Q mutant was determined, while p.V32M variant was found to have no enzyme activity. p.R239Q has a decreased enzyme activity and thermal stability compared to normal allele. A disruption of the three dimensional conformation may explain the observed instability and loss of activity in this mutant enzyme.

MATERIALS AND METHODS

DNA analysis

The entire coding regions of GALK1 gene was amplified from genomic DNA and analyzed by sequence analysis. Mutation nomenclature is based on the numbering agreement to the following GenBank codes: cDNA accession number L76927; protein ID AAB51607. Both mutations were excluded in a control panel of 150 healthy individuals of the same ethnicity (Caucasian).

Expression and purification of recombinant GALK alleles

Wild-type full length GK1 cDNA and both mutant were cloned into pET 30b expression vector (Novagen). Recombinant constructs were transformed into BL21 cells and grown at 37°C. Proteins were purified by Ni^{+2} chromatography and the soluble fraction was used for kinetic analysis.

GALK activity

The galactokinase activity was determined by following the formation of ADP through a series of coupled reactions, linked to the oxidation of NADH, that cause the decrease in absorbance at 340 nm. Galactokinase catalyzes the first step in the metabolism of galactose by phosphorylating the galactose at the first carbon (Galactose + ATP? Galactose - 1 - P + ADP). ADP formed in the galactokinase reaction from ATP and galactose was measured in a coupled enzyme assay by following the rate of oxidation of NADH as shown in reaction schemes (2), (3), and (4). The coupling enzymes were pyruvate kinase and lactate dehydrogenase which utilize ADP to convert phosphoenolpyruvate into ATP and pyruvate. The conversion of pyruvate and NADH to lactate and NAD is measured by an absorbance change at 340 nm (Ballard, 1966).

galactokinase
galactose + ATP ----------> galactose - 1 phosphate + ADP (2)

pyruvate kinase
ADP + phosphoenolpyruvate ----------> pyruvate + ATP (3)

lactate dehydrogenase
pyruvate + NADH ----------> lactate + NAD (4)

Readings were made spectrophotometrically for 300 seconds total at 12 seconds intervals. The kinetic constants were determined using a hyperbolic regression computer program (Heinrich 1964; Platt et al., 2000). Six reactions were prepared with various concentrations of galactose and the optimal amount of galactokinase determined previously. Velocity was determined from the slope of the change in absorbance versus time and

converted to µm substrate per second per µg galactokinase. The same procedure was used to determine the affinity of ATP, with galactose concentration constant at 3.3 mM. Specific activity was determined in terms of units/mg enzyme. One unit is the amount of enzyme in a mg required to convert 1 µmol substrate per minute.

RESULTS AND DISCUSSION

While rGALK (wild-type protein) and p.R239Q variant were expressed at high levels in the soluble fraction, p.V32M variant had a 10 fold lower level of expression with no activity. All proteins had a similar molecular weight of 48,723 daltons (data not shown).

Effect of pH: While rGALK shows the greatest activity with a pH optimum between 7.75 and 8.0 (Fig. 1A), p.R239Q variant shows a pH optimum for enzyme activity at pH 7.5 (Fig 1B). The K_M values for p.R239Q are also low for both galactose and ATP at pH 7.0 and increase as the pH increase, although the increase of ATP K_M does not reach the level observed for galactose.

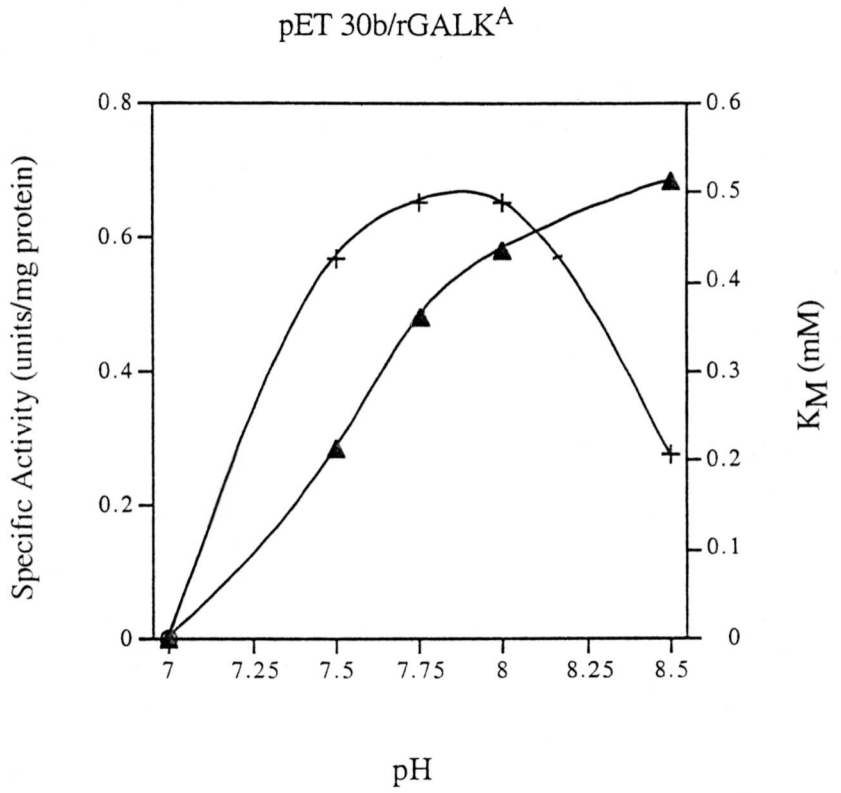

Figure1A. Effect of pH on enzyme specific activity (+) and on galactose K_M (?) of rGALK (A). Optimum pH is between 7.75 and 8.0. Means of three experiments are shown.

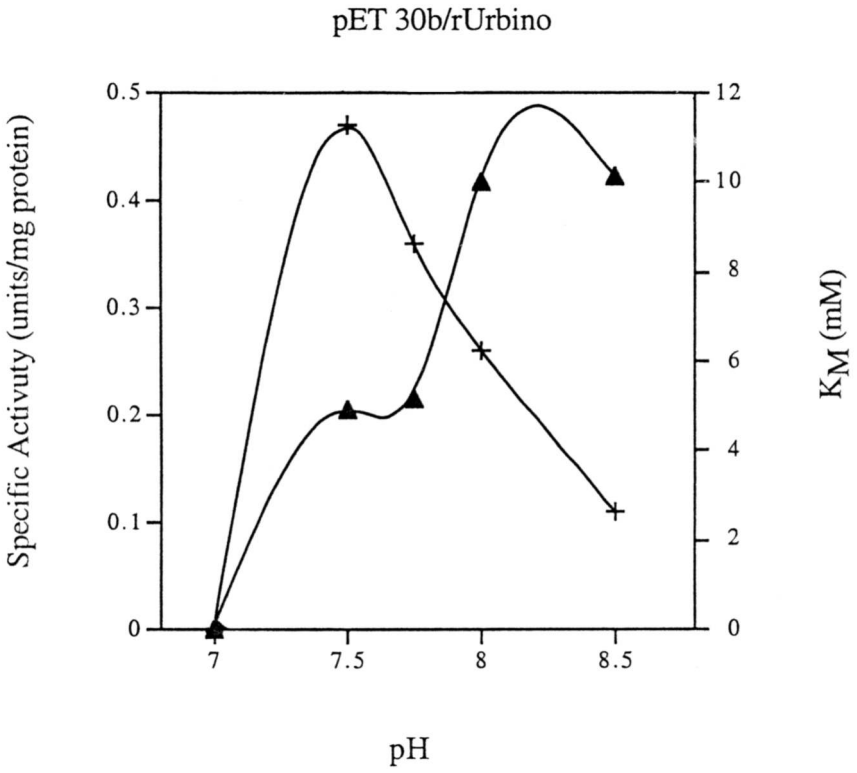

Figure1B. Effect of pH on enzyme specific activity (+) and on galactose K_M (?) of p.R239Q mutant. Optimum pH is 7.5 for p.R239Q. Means of three experiments are shown.

Moreover p.R239Q have a higher galactose K_M than rGALK due to the effect of the arginine to glutamine substitution at codon 239, respectively 4912 µM and 436 µM.

Specific activity: The specific activity for rGALK enzyme at pH 8.0 is 0.652 units/mg protein, while the highest activity for p.R239Q is 0.47 units/mg protein at pH 7.5. Measurements for three experiments were averaged and are shown in Fig 1A (deviations are smaller than symbols).

Recombinant protein saturation level: when both substrates are held at 7.5 X K_M in buffer pH 8.0, protein activity increases linearly with respect to increasing protein concentration up to 100 µg/ml. Beyond 100 µg/ml of protein, the velocity begins to plateau as seen in Fig 2. Therefore to test the stability of the protein 68 µg/ml protein was used.

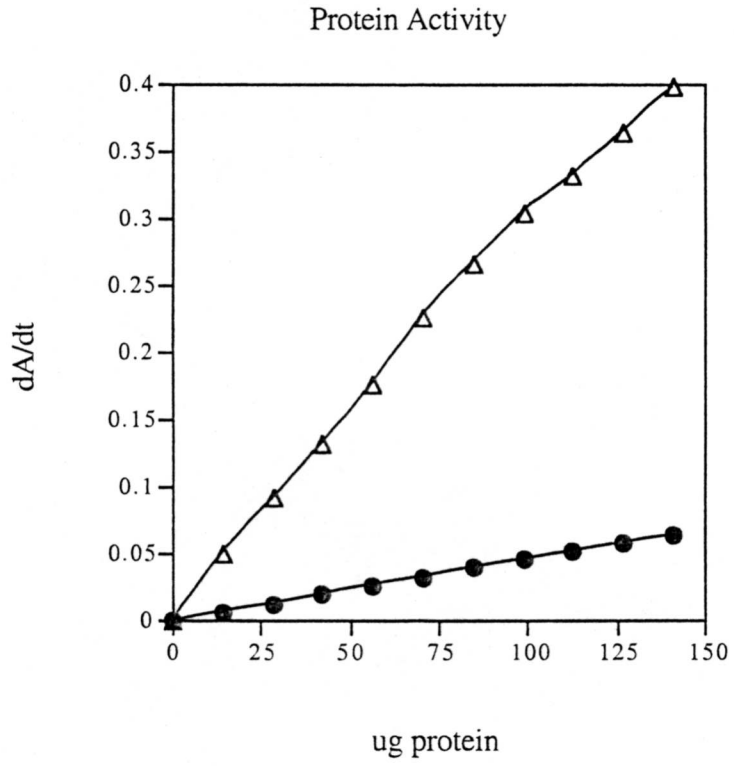

Figure 2. Enzyme activity of rGALK (?) and p.R239Q mutant (?) at pH 8.0. Galactose and ATP concentrations were held constant at 7.5X K_M as protein concentrations were varied. rGALK shows a ten fold greater activity than p.R239Q.

Protein stability: protein stability was investigated to determine the possible impact of point mutation on enzyme features. Recombinant proteins were incubated at 16°, 22°, 37° and 45° for 30 minutes and then assayed at 22°. Measurements were made with enzyme concentration at 68 µg/ml with saturating concentrations of ATP 7.5X K_M at pH 8.0, and varying galactose concentrations to determine how specific activity varied with temperature. rGALK retained full activity from 16° to 22° C incubation, lost half of its activity at 37°C, 80% at 45°C and all activity at 55°C. The p.R239Q variant was most active after the 16°C incubation, but showed greater instability loosing 60% activity at 22°C and 100% at 37°C. Each assay was performed three times and the average is reported in Fig. 3 (deviations are smaller than symbols).

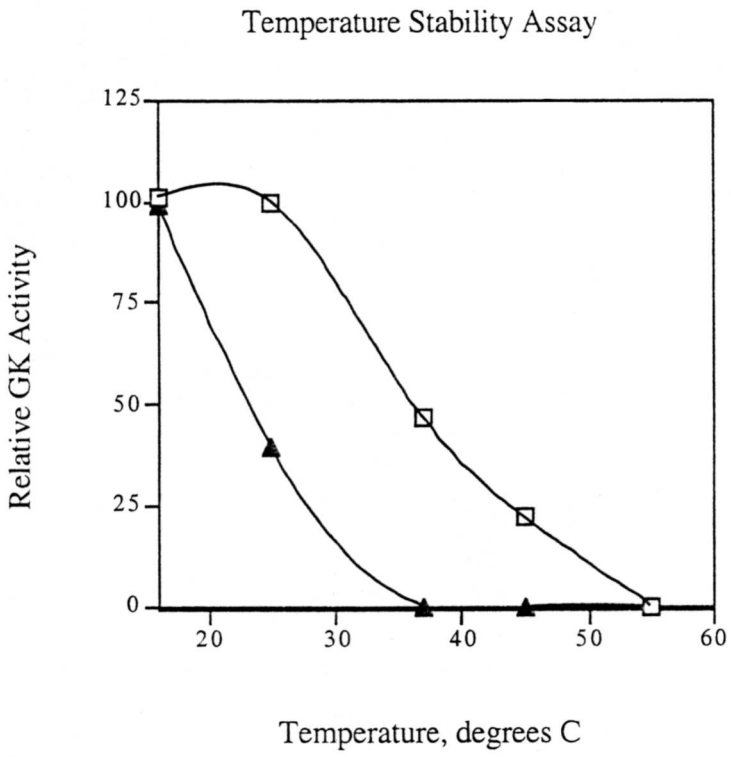

Figure 3. Temperature titration of enzyme stability was carried out at pH 8.0 with 7.5X K_M galactose and ATP. (?) represents rGALK and (?) p.R239Q mutant. Means of three experiments are shown for each case.

Human galacktokinase, GALK1, has been expressed in and purified from *E.coli.* The ability to produce good yields of active protein in this way makes it possible to study the biochemical consequences of mutations within the coding sequence of the GALK1 gene. The insolubility protein recovered in p.V32M case suggests that in some cases protein folding and/or stability of the folded state may be more important than enzymological defects (Timson & Reece, 2003). This suggests that gross structural changes have occurred in these proteins. Generally this mutation are associated with more severe clinical phenotypes, in fact, when transfected COS cells no galactokinase activity could be detected (Stambolian et al., 1995).

The kinetic characteristics of the recombinant proteins determined in this paper, using *E.coli* assay system, provide an estimate of the enzyme's activity irrespective of other associated proteins or post-translational modifications.

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