Cloning, functional expression and partial characterization of the glucose kinase from *Renibacterium salmoninarum*

Margarita I. Concha *, Gloria León

_Instituto de Bioquímica, Facultad de Ciencias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile_

Received 6 January 2000; received in revised form 3 March 2000; accepted 6 March 2000

**Abstract**

The complete *gck* gene from the fish pathogen *Renibacterium salmoninarum*, encoding a glucose kinase, was analyzed and expressed. The partial characterization of the recombinant enzyme confirmed that it belongs to a group of glucose kinases involved in carbon catabolite repression. Multiple sequence alignments were used to deduce a new consensus sequence for this family of bacterial proteins, characterized by several conserved Cys residues. This sequence was more specific and allowed the detection of the first eukaryotic protein of this family. The recombinant enzyme was inhibited by *N*-ethylmaleimide and the substrates protected the enzyme from this inhibition, suggesting the presence of Cys residues in or close to the active site. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords**: Glucose kinase; ROK family; Consensus sequence; *Renibacterium salmoninarum*

1. Introduction

Bacterial kidney disease (BKD) is one of the most prevalent diseases among cultured salmonid fish. Its etiological agent, *Renibacterium salmoninarum*, is a slow-growing and poorly characterized Gram-positive bacterium, which is internalized both in vivo and in vitro into the host cells [1,2].

In previous work, we isolated a recombinant clone, containing an *R. salmoninarum* DNA fragment apparently able to confer an invasive phenotype to *Escherichia coli* HB101 [3]. This fragment contained an open reading frame (ORF) that showed considerable sequence similarity (43% identity) with a glucose kinase (GlcK) from *Streptomyces coelicolor*, which is involved in the regulation of carbon catabolite repression [4]. This enzyme also belongs to a family of bacterial proteins (ROK family), constituted by repressors, ORFs of unknown function, and sugar kinases [5]. In more recent years, three other glucose kinases (GlcKs) that share these features have been described [6–8]. These findings suggest that both the catalytic activity and the regulatory role of these enzymes might be conserved among Gram-positive bacteria. Considering the lack of information concerning the sugar metabolism of *R. salmoninarum*, we analyzed and partially characterized the cloned gene and the corresponding protein, demonstrating that it corresponds to a glucose kinase that is expressed in the pathogen. To our knowledge, this is the first GlcK from an intracellular bacterium that has been characterized to date.

2. Materials and methods

2.1. Bacterial strains, and growth conditions

*R. salmoninarum* ATCC 33209 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was cultured in KDM-2 agar plates at 16°C as previously described [3]. All *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth. Ampicillin (100 μg ml⁻¹) was added when needed for selection. For expression experiments, *E. coli* strain BL21, transformed with plasmid pGSTGlcK, was grown as usual except for an additional incubation with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside), for a further 3 h at 37°C. The GlcK⁻ *E. coli* strain ZSC103 (gpt⁻2gk⁻7strA) was obtained from the *E. coli* Genetic Stock Center. All the plasmids constructed and used are described in Table 1.

---

* Corresponding author. Tel.: +56 (63) 221108; E-mail: mconcha@uach.cl

0378-1097/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

PII: S0378-1097(00)00124-5
2.2. DNA isolation and manipulation

Chromosomal DNA from *R. salmoninarum* was isolated as previously described [3], digested with *Eco*RI, ligated into pUC19 and used to transform Max efficiency DH5α® competent cells (Gibco-BRL). DNA fragments were isolated and subcloned according to standard procedures [9].

2.3. DNA sequencing and computer analyses

The nucleotide sequence of the insert contained in the pMIC5 plasmid was determined on both strands by the chain termination method [10] and deposited in the EMBL databank under accession number X89964. Computer-assisted sequence analysis was carried out using the PC Gene software package (Intelligenetics, Inc.).

2.4. RNA isolation and RT-PCR

Total RNA from *E. coli* and *R. salmoninarum* was isolated [9] and RT-PCR was performed as previously described [11]. Briefly, total RNA treated with amplification grade DNase I was incubated with Superscript II reverse transcriptase (Gibco BRL). The cDNA was then amplified by PCR (35 cycles at 94°C, 60°C and 72°C, for 1 min each) using the following pair of primers: 5′-CAGTA-GATGGCGATGCAGAT and 5′-CCGCCCACAAATCA-CGAAACAT. The amplification product (169 bp) was separated by agarose gel electrophoresis.

2.5. Enzyme characterization

Enzyme activity was measured spectrophotometrically by monitoring the reduction of NADP in a glucose 6-phosphate dehydrogenase coupled reaction as previously described [4]. The fusion enzyme glutathione S-transferase/glucose kinase (GSTGlcK) was isolated by affinity chromatography on glutathione-Sepharose 4B, using the bulk GST purification module (Pharmacia) according to the manufacturer’s instructions. The hexose specificity screening of the fusion protein was performed incubating five units of the affinity-purified enzyme in a buffer containing 50 mM Tris–HCl (pH 7.5), 25 mM MgCl₂, 25 mM ATP, and 25 mM of either glucose, fructose, galactose, or mannose, at 37°C. Aliquots of the reaction mixtures were removed at different times, analyzed on silica-coated thin layer chromatography plates, developed and visualized as described previously [12]. Proteins were analyzed by discontinuous SDS–12%PAGE under reducing conditions according to the method described by Laemmli [13].

3. Results and discussion

In earlier work we isolated the pPMV-189 plasmid, containing an *R. salmoninarum* ORF that resembled the *S. coelicolor* GlcK [3]. The 479 bp *HindIII/Eco*RI fragment from this ORF was used to isolate, from a *R. salmoninarum* genomic library, a new clone designated DH5α (pMIC5), containing an additional 395 bp from the upstream region of this ORF (Table 1). Within this region, a −10 hexamer was predicted (Fig. 1) and in its vicinity, although separated by a non-optimal spacing (23 bp), a near consensus −35 hexamer (TTGACC) was found. Coincidentally, the *S. coelicolor* glcK gene also lacks a consensus −35 hexamer [4]. The most probable start codon for the *R. salmoninarum* glcK gene was proposed based on the following criteria: (i) frequent use of GTG as the initiation codon in high G+C Gram-positive bacteria like *R. salmoninarum* [14]; (ii) size conservation of bacterial GlcKs (33–34 kDa); (iii) presence in all these enzymes of a conserved N-terminal sequence (Fig. 1) involved in ATP binding [7]; and (iv) absence of any other predicted promoter within the upstream region. The coding region that extends from the proposed GTG to the stop codon (TAG), encodes for a protein of 319 residues with a molecular mass of 33.3 kDa.

To confirm the identity of the isolated gene, the putative GlcK was expressed as a glutathione S-transferase fusion protein of an expected molecular mass of 59 kDa (Fig. 2a). The GlcK E. coli strain (ZSC103) was transformed with pGSTGlcK to evaluate the GlcK activity, both in induced and uninduced bacterial lysates. As expected, the negligible GlcK activity detected in the mutant, increased dramatically after its transformation with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Plasmids constructed and/or used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td><strong>Description</strong></td>
</tr>
<tr>
<td>pUC19</td>
<td>M13mp19 derivative, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>pPMV-189</td>
<td>pUC19 with a 2.3 kb <em>HindIII</em> fragment containing the <em>R. salmoninarum</em> <em>glcK</em> gene under the control of the pUC19 <em>lacZ</em> promoter</td>
</tr>
<tr>
<td>pMIC5</td>
<td>pUC19 with a 0.8 kb insert containing additional 395 bp from the upstream region of the <em>R. salmoninarum</em> <em>glcK</em> gene</td>
</tr>
<tr>
<td>pGEX-4T-2</td>
<td>Gene fusion vector containing the GST gene under the control of the inducible tac promoter, translation terminator and thrombin recognition site</td>
</tr>
<tr>
<td>pGSTGlcK</td>
<td>pGEX-4T-2 with the blunted <em>HindIII/SmaI</em> fragment containing the <em>R. salmoninarum</em> <em>glcK</em> ORF, ligated in frame to the GST gene</td>
</tr>
</tbody>
</table>
pGSTGlcK, even under uninduced conditions (Fig. 2b). Furthermore, the specific activity of the recombinant enzyme present in the lysate increased two-fold after induction of ZSC103(pGSTGlcK) cells with IPTG (Fig. 2b). After the affinity chromatography purification step, the fusion protein shown in Fig. 2a, lane 3 displayed a specific activity of 4220 ± 382 U (mg protein)^{-1}, which represents a ten-fold increase with respect to the induced lysate. On the other hand, no GlcK activity could be detected in *R. salmoninarum* lysates, although the presence of the *glcK* transcript in these lysates was clearly demonstrated by RT-PCR (Fig. 2c, lane 1). In this same regard, when freshly prepared *R. salmoninarum* lysates and a purified recombinant GlcK, inactivated after storage for 2 days at −20°C, were analyzed by Western blot, the same immune-reactive fragments of the enzyme were observed in both cases, in spite of the use of several protease inhibitors during their preparation [15]. These results strongly suggest that the presence of active proteases in *R. salmoninarum* lysates could be responsible for the lack of GlcK activity observed.

To characterize the recombinant enzyme, the specificity for D-glucose was determined. Whilst D-glucose was efficiently phosphorylated by the enzyme, fructose, mannose or galactose, even at concentrations as high as 25 mM, were not modified (Fig. 3). Similar apparent *K_m* values for D-glucose (266 μM) and ATP (1.5 mM) were determined for the recombinant enzyme in comparison with published data for the *Bacillus subtilis* and *S. coelicolor* GlcKs [12,16]. All the above data (sequence similarity, conserved size, catalytic activity, substrate specificity and similar apparent *K_m* for the substrates) are consistent with the notion that the isolated *R. salmoninarum* gene effectively corresponds to the *glcK* gene encoding a glucose kinase that belongs to a group of regulatory proteins including several other GlcKs from Gram-positive bacteria, and sugar repressors, like NagC and XylR [4-8].

To identify residues or conserved motifs that were specific for the ROK GlcKs, multiple sequence alignments were carried out (Fig. 4a). As previously mentioned, the amino terminal sequence (box 1) is conserved in all the
bacterial GlcKs, regardless of its regulatory role. On the other hand, a motif characterized by three vicinal Cys and several other fully conserved residues (Fig. 4a, box 5) is absent in *E. coli*, *Zymomonas mobilis* and *Brucella abortus* GlcKs (Swiss-Prot accession numbers: P46880, P21908 and Q59171, respectively) which lack a regulatory function. Nevertheless, the motif is present in most identified members of the ROK family, including a transcriptional regulator from the archaeon *Archaeoglobus fulgidus* (GenBank accession number AF1968). Therefore, an alternative consensus sequence for this protein family was deduced (Fig. 4b) and used to scan the Swiss-Prot and TrEMBL databases. It is important to note that, unlike the original consensus sequence [5], this new sequence was completely specific for members of the ROK family and also more sensitive since it allowed the detection of the first eukaryotic member, corresponding to the bifunctional enzyme UDP-N-acetyl-glucosamine-2-epimerase/N-acetylmannosamine kinase from rodents and human. It has been recently proposed that the sugar kinase domain of this key enzyme is located in the C-terminal half of the protein, which also shows considerable resemblance to the ROK GlcKs [17].

Finally, since the motif characterized by fully conserved Cys residues was found in several bacterial repressors, it was tempting to speculate that it might not be essential for catalysis but instead could be involved in some regulatory function. To test this idea, we determined the effect of the sulfhydryl reagent, N-ethylmaleimide (NEM) on the catalytic activity of the recombinant GlcK, both, in the absence and presence of substrates. As illustrated in Fig. 5, the GlcK was almost completely inactivated after preincubation with NEM in the absence of substrates. In addition, a partial protection against this inhibition was observed when the enzyme was preincubated either separately or simultaneously with its substrates. Maximal protection was achieved in the presence of both substrates, but when added separately, D-glucose was much more effective than ATP. These results suggest that at least one Cys is located in or close to the active site and might be

![Fig. 3. Specificity of glucose kinase for hexoses. At the incubation times indicated, 5 ml aliquots of each reaction mixture were analyzed by thin layer chromatography. Lanes 1-5, GSTGlcK incubated with D-glucose. Lane 6, 20 nmol of fructose-6-phosphate as a standard. Lanes 7, 8 and 9, GSTGlcK incubated with mannose, galactose and fructose, respectively.](https://academic.oup.com/femsle/article-abstract/186/1/97/759317)

![Fig. 4. Multiple sequence alignment of the ROK glucose kinases. a: The complete deduced amino acid sequence of all the known glucose kinases from the ROK family were aligned with the ClustalW1.8 program, but only the most conserved blocks of amino acid residues are shown. Box 5 indicates the block that is specific for members of the ROK family. b: The specificity of the alternative ROK consensus sequence deduced was evaluated scanning the Swiss-Prot and TrEMBL databases with the ScanProsite tool available on the ExPASy WWW server.](https://academic.oup.com/femsle/article-abstract/186/1/97/759317)
involved in sugar binding. Coincidentally, a similar pattern of inhibition and protection was observed for the related \( N \) acetylglucosamine kinase [18]. Moreover, the modification of these enzymes with dithiol reagents indicated the existence of two vicinal Cys in their respective active sites [18]. Regarding this point, it is interesting to note that the only conserved vicinal Cys shared by the \( R. \) salmoninarum GlcK and the rat \( N \)-acetylmannosamine kinase are those present in the new consensus sequence. Taken together, these data suggest that these Cys residues are essential for the catalytic activity of this group of sugar kinases, and might be required for sugar binding. This would explain why this motif is also present in sugar repressors of the XylR-R. salmoninarum motif is also present in sugar repressors of the XylR-\( R. \) salmoninarum motif.

Acknowledgements

This work was supported by grants 2950080 and 1951195 from FONDECYT. We thank Dr. RodolfoAmt-hauer for helpful discussions and Prof. Oriana González for improving the English of this manuscript.

References