

Heterologous expression of an active rat regulatory protein of glucokinase

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Abstract The cDNA presumed to encode the rat liver regulatory protein of glucokinase has been expressed in *Escherichia coli* and a partially soluble protein has been obtained. This recombinant protein was partially purified and found to have the same apparent molecular mass as the regulatory protein purified from rat liver. Like the latter, it inhibited rat liver glucokinase competitively with respect to glucose and its effect was sensitive to fructose 6-phosphate and fructose 1-phosphate.

Key words: Glucokinase; Glycolysis; Fructose 1-phosphate; Fructose 6-phosphate

1. Introduction

The liver of several species contains a regulatory protein that inhibits glucokinase competitively with respect to glucose [1–2]. In mammals, the effect of this regulatory protein is reinforced by fructose 6-phosphate and antagonized by fructose 1-phosphate. Based on partial amino acid sequences of the regulatory protein, cDNAs were recently isolated from rat liver libraries [3]. Expression of the corresponding coding sequence resulted in the production of a protein that was recognized by antibodies directed against the rat liver regulatory protein, but that was completely insoluble and inactive [3]. Since then, we have discovered that a sequencing error had led us to underestimate the length of the regulatory protein by about 60 residues [4]. In this paper we report the expression and the partial purification of a recombinant protein that has properties identical with those of the protein purified from rat liver.

2. Materials and methods

Restriction and modifying enzymes were from Boehringer, GeneAmp DNA Amplification kit from Perkin Elmer Cetus and T7 sequencing kit from Pharmacia. pUCgroE was kindly provided by J. Robbins, E. Remaut and W. Fiers (University of Ghent).

2.1. Construction of the expression plasmid

The protein was expressed in the expression system of Studier and Moffatt [5]. A full length cDNA devoid of mutation (termed pBS-PR) was constructed by ligation of a *Bsp*MI/*Xho*I fragment of pBS-ZPR1 to an *Eco*RI/*Bsp*MI fragment of pBS-LPR17. The DNA corresponding to the coding region of rat liver regulatory protein was PCR-amplified from pBS-PR, using one primer (5'-GCGAATTCATATGCCAGGC-ACCAAACG-3') with a *Nde*I site containing the start codon (underlined), and the second primer (5'-ACGGATCCTCTAGAAATATCAATTCAGG-3') containing the stop codon. The amplified DNA was inserted in pBlueScript KS (+) T-vector [6], sequenced and then inserted in pET3a expression plasmid [5]. After cloning and amplification in XL1-Blue, the resulting plasmid termed pET-PR1900, was used to transform competent *E. coli* BL21(DE3) pLysS [5].

2.2. Expression, purification and assay of the recombinant protein

The cells were grown and induced as described elsewhere [3]. The cells were then disrupted and the protein was purified as described in [7]. The regulatory protein was assayed by its ability to inhibit glucokinase [8].

One unit of rat regulatory protein is defined as the amount causing half-maximal inhibition of 15 mU of glucokinase in 1 ml in the presence of 5 mM glucose and 100 μ M sorbitol 6-phosphate or 200 μ M fructose 6-phosphate [1]. Western blots were performed as in [9], using a rabbit antibody directed against rat liver regulatory protein, and revealed as in [10].

3. Results and discussion

The sequence corresponding to nucleotides 22–1905 of the rat liver cDNA [4] was inserted in an expression plasmid pET [5]. Addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside to cells harboring this plasmid resulted in the appearance of a protein that had a molecular mass of \approx 65 kDa, identical with that of authentic rat liver regulatory protein, whereas no such protein was induced in control cells. Centrifugation of the extracts at 30,000 \times g for 10 min resulted in the sedimentation of all the recombinant protein when the bacteria had been grown and induced in tryptone-phosphate medium at 23°C or 37°C or in M9 medium at 37°C. Thus, the protein produced under these conditions was presumably present only in inclusion bodies. A small amount, estimated to about 5% of the recombinant protein, was however found to be soluble when the cells were grown in M9 medium at 23°C. A similar proportion was obtained when the plasmid was expressed in cells containing pUCgroE, a plasmid encoding the chaperonin GroEL [12].

The soluble form of regulatory protein was purified from cells grown and induced in M9 medium at 23°C. The fractions eluted from the anion-exchange column at 100 mM NaCl contained the ca 65 kDa recombinant protein and inhibited glucokinase; no inhibition was found with fractions derived from control cells (not shown). From a 3 l culture, ca. 600 units of regulatory protein could be purified with a specific activity of 20 units per mg protein. A western blot performed with the partially purified protein showed that the recombinant protein reacted with antibodies directed against rat liver regulatory protein and that it had the same molecular mass as the latter (not shown).

Like the native regulatory protein [1] the recombinant regulatory protein inhibited rat liver glucokinase in a competitive manner with respect to glucose (Fig. 1). Its effect was greatly reinforced by fructose 6-phosphate (Fig. 2). Using the linearisation method described in [11], we calculated a dissociation constant of 15 μ M and 5 μ M for the complexes formed by the

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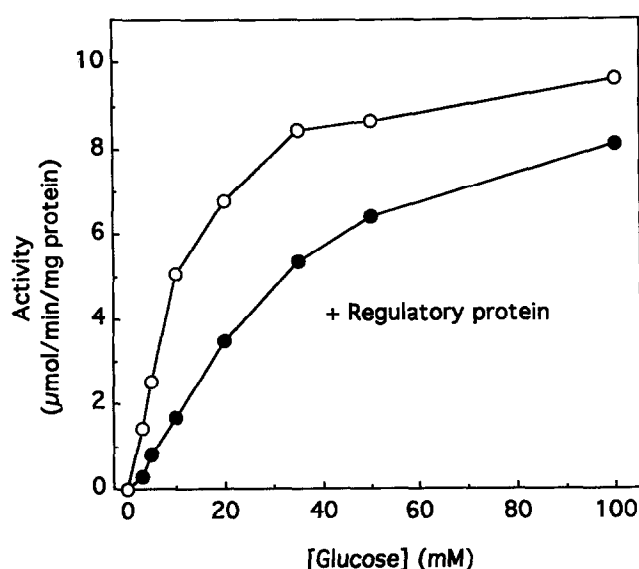


Fig. 1. Effect of the recombinant regulatory protein on the glucose saturation curve of rat liver glucokinase. The assays were carried out using the pyruvate kinase/lactate dehydrogenase coupled assay and contained 200 μ M fructose 6-phosphate and, where indicated, 2 U/ml of recombinant regulatory protein.

regulatory protein and fructose 6-phosphate or sorbitol 6-phosphate respectively, in good agreement with the results obtained with the native regulatory protein [11]. Fructose 1-phosphate antagonized the inhibition exerted by the recombinant protein and its effect was competitive with fructose 6-phosphate (Fig. 3), as previously observed for the protein purified from rat liver [1].

The recombinant protein that was produced in the present work has the same apparent molecular mass and the same kinetic effects on glucokinase as the protein purified from rat liver. These results provide therefore the final proof that the isolated cDNA encodes the fructose 6-phosphate and fructose

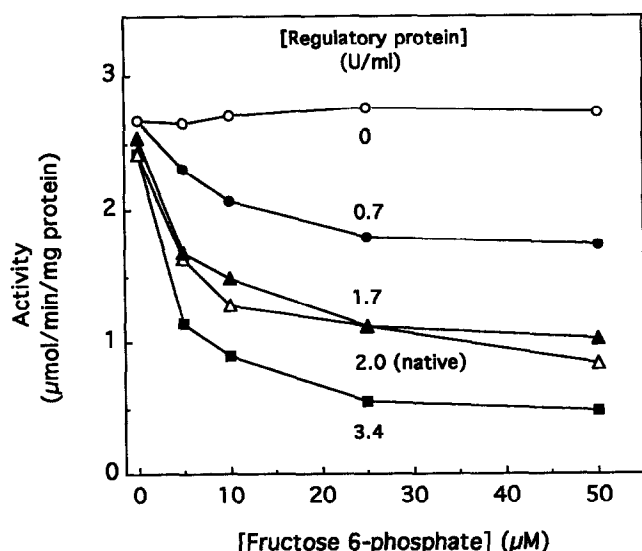


Fig. 2. Effect of fructose 6-phosphate on the activity of glucokinase measured in the presence of different concentration of recombinant and, where indicated, of native (i.e. non-recombinant) rat liver regulatory protein. Glucokinase was assayed at 5 mM glucose in the presence of the indicated concentrations of fructose 6-phosphate.

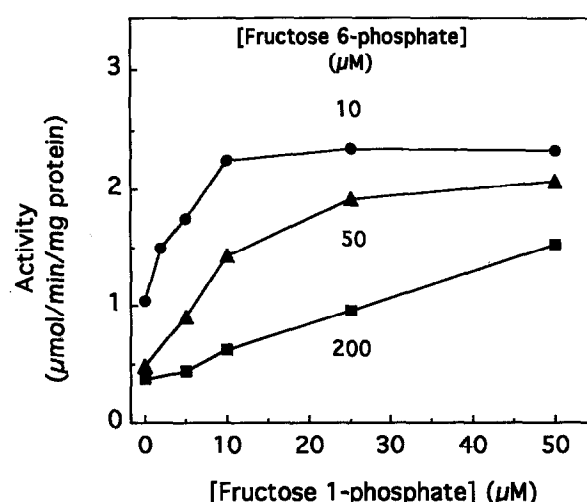


Fig. 3. Effect of fructose 1-phosphate on the activity of glucokinase measured in the presence of recombinant regulatory protein and different concentrations of fructose 6-phosphate. All assays contained 3.4 U/ml of recombinant regulatory protein.

1-phosphate sensitive protein regulator of glucokinase. The protein that was previously expressed contained only 568 amino acids of the regulatory protein plus 15 amino acids encoded by the vector. Its inactivity is best explained by the fact that it was completely insoluble. Such was also the case for a protein containing the first 512 amino acids of the regulatory protein (Detheux, unpublished results). These results suggest therefore that the carboxyterminal region of the regulatory protein is important for its solubility.

The livers of at least two amphibians and one reptile contain a protein inhibitor of glucokinase that is insensitive to fructose 6-phosphate and fructose 1-phosphate [13]. The cDNA of the *Xenopus* liver has been recently cloned and found to be homologous to the cDNA encoding rat liver regulatory protein [7]. At the protein level, the identity amounts to 57% and spreads over the whole sequence. The results of the present paper show that the sensitivity to fructose-phosphates is not conferred to the rat regulatory protein by an extraneous factor but is an intrinsic property of the polypeptide whose cDNA was cloned. These results allow therefore the conclusion that two proteins with markedly different regulatory properties have evolved from one single ancestral gene.

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