

Research at ICP

CARBOHYDRATE METABOLISM

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For many years, the interest of our group has been focused on the regulation of carbohydrate metabolism, two important contributions being the discovery of fructose 2,6-bisphosphate (in 1980, in collaboration with L Hue, HORM unit, and HG Hers, former head of this group; 1) and that of the regulatory protein of glucokinase (in 1989; 2). We have also kept an interest in inborn errors of metabolism and our lab has contributed the identification of several “new” enzymatic deficiencies, including that of phosphomannomutase (in collaboration with Prof. J. Jaeken, Leuven; 3). As a result of this, part of our work has been devoted to the biochemical characterization of some of the enzymes involved in these deficiencies. More recently, the study of the mechanism of formation of the intriguing phosphate ester, fructose 3-phosphate, has led us to identify fructosamine 3-kinase(4). This has brought us into very different fields, those of protein repair and diabetic complications.

Molecular studies on glucokinase and its regulatory protein

M. Veiga-da-Cunha, M. Moukil, E. Van Schaftingen

Glucokinase is the enzyme that phosphorylates glucose in the liver and in the β -cells of pancreatic islets. Contrary to other hexokinases, it displays a low affinity for glucose and is not inhibited by physiological concentrations of glucose 6-phosphate. In 1988 our group discovered in rat liver a regulatory protein (GKRP) that inhibits glucokinase competitively with respect to glucose. The effect of GKRP is reinforced by fructose-6-P

(F6P) and antagonized by fructose-1-P (F1P), which act therefore as glucokinase inhibitor and activator, respectively (Fig.1). These effectors act by binding to GKRP and by modulating its affinity for glucokinase, presumably by promoting distinct conformational changes of GKRP (represented by squares and circles in Fig. 1). The effect of fructose-1-P accounts for the fact that fructose stimulates glucose phosphorylation in rat liver. Fructose can therefore serve as a nutritional signal telling the liver that simple sugars are taken up by the intestine.

Recent efforts on this subject have been devoted to the identification of GKRP residues involved in the binding of fructose 6-phosphate and fructose 1-phosphate (5). GKRP is homologous to bacterial proteins of unknown

function, as well as, more distantly, to glucosamine-6-P synthase (GLMS). This enzyme, which uses fructose 6-phosphate and glutamine to produce glucosamine 6-phosphate and glutamate, contains a “glutaminase domain” as well as an “isomerase” domain to which GKR is homologous. This homology enabled us to identify GKR residues that could potentially interact with fructose-6-P. Mutations of several of these residues led to proteins that had a reduced effect on glucokinase and that had much less affinity not only for fructose 6-phosphate but also for fructose 1-phosphate. These results indicate that GKR has one single binding site for fructose-6-P and fructose-1-P. This binding site is predicted to be at the interface of two “SIS subdomains” similar to those found in GLMS.

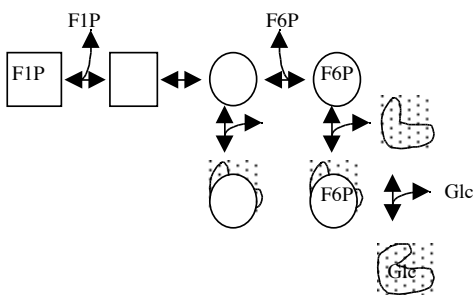


Fig.1.

Glucose-6-phosphatase

I. Gerin, M. Veiga-da-Cunha, E. Van Schaftingen

One of the important functions of the liver and of the kidneys is to produce glucose. This ability critically depends on the presence of glucose-6-phosphatase, an enzyme of the endoplasmic reticulum that hydrolyses glucose-6-P to glucose and inorganic phosphate. According to the substrate-transport model, glucose-6-phosphatase is a rather non-specific hydrolase, whose catalytic site is oriented towards the lumen of the endoplasmic reticulum. Glucose-6-P is transferred from the cytosol to the lumen of the endoplasmic reticulum by a specific transporter (Fig. 2), which confers to glucose-6-phosphatase its specificity. This model accounts for several observations, including the fact that chlorogenic acid and some of its derivatives

(like S3483, used in the studies described below) inhibit the hydrolysis of glucose-6-P in intact but not in detergent-treated microsomes. These compounds are thought to be inhibitors of the glucose-6-P translocase. We reported in 1997 the cloning of a cDNA encoding a putative translocase (6) and showed that it is mutated in glycogen storage disease type Ib (7), in which there appears to be a defect in the microsomal transport of glucose-6-P.

Despite these and other findings, the substrate-transport model is not unanimously accepted. Recent work has been therefore devoted to the demonstration of the occurrence of glucose-6-P transport into liver microsomes. In one study, we have taken advantage of the fact that glucose-6-phosphatase catalyses the synthesis of glucose-6-P when microsomes are incubated in the presence of glucose and a phosphate donor such as carbamoyl-phosphate. Under these conditions, we found that S3483 caused the intravesicular accumulation of glucose-6-P and inhibited the appearance of extravesicular glucose-6-P (8).

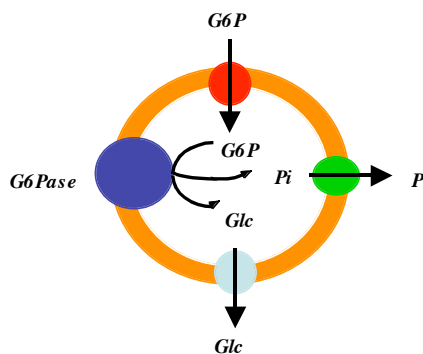


Fig.2.

This result indicates that the catalytic site of glucose-6-phosphatase is oriented towards the lumen of microsomes. In another study, we showed that S3483 inhibits the conversion of glucose-6-P to 6-phosphogluconate that occurs inside microsomes in the presence of electron acceptors such as NADP or metyrapone. This reaction is catalysed by hexose-6-phosphate dehydrogenase, a form of glucose-6-P dehydrogenase that is present in the endoplasmic reticulum. Inhibition of the oxidation of glucose-6-P by S3483 indicates that the glucose-6-P transporter furnishes substrate not only to glucose-6-phosphatase, but also to hexose-6-phosphate dehydrogenase. These findings are further proof for the substrate-transport model.

Fructosamine metabolism

Gh. Delpierre, F. Collard, J. Fortpied, E. Wiame, E. Van Schaftingen

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. The link between the elevated concentration of glucose and the development of these complications is not yet clear. One of the theories on this link emphasises the role of fructosamines.

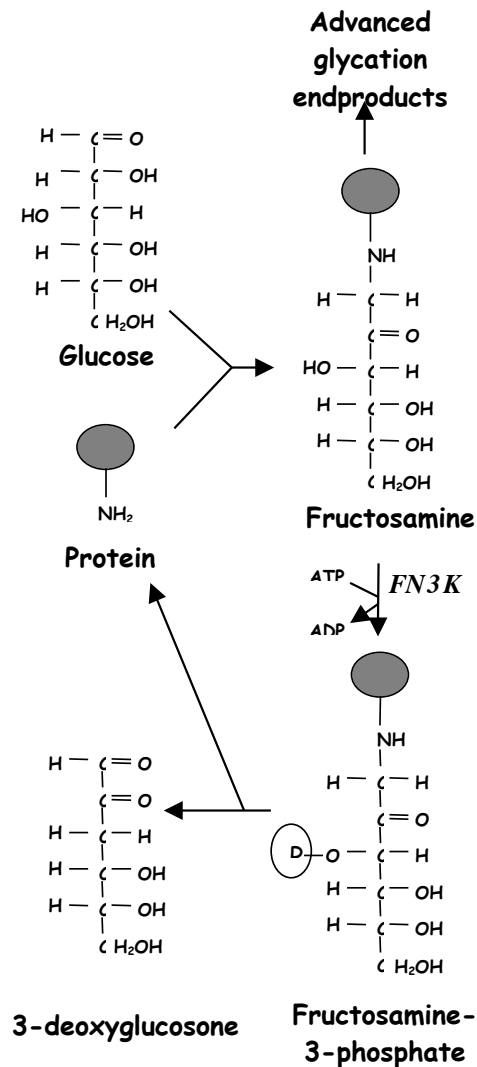


Fig.3.

We have now found that incubation of human erythrocytes with 200 mM glucose not only caused the progressive formation of glycated haemoglobin, but also increased the level of an anionic form of haemoglobin containing alkali-labile phosphate, to about 5 % of total haemoglobin.

Deoxymorpholinofructose (DMF), a competitive inhibitor of fructosamine-3-kinase, increased twofold the rate of accumulation of glycated haemoglobin, but markedly decreased the amount of haemoglobin containing alkali-labile phosphate. We could therefore conclude that the latter corresponds to haemoglobin with a fructosamine-3-P group (FN3P-Hb).

Returning erythrocytes incubated with 200 mM glucose and DMF to a low-glucose medium devoid of DMF caused a decrease in the amount of glycated haemoglobin, a transient increase in FN3P-Hb and a net decrease in the sum (glycated haemoglobin + FN3P-Hb). These effects were prevented by DMF, indicating that fructosamine-3-kinase is involved in the removal of fructosamine residues. The second step of this "deglycation" process is most likely a spontaneous decomposition of the fructosamine-3-P residues to a free amine, 3-deoxyglucosone and Pi. This is supported by the finding that 2-keto-3-deoxygluconate, the product of 3-deoxyglucosone oxidation, is formed in erythrocytes incubated for two days with 200 mM glucose in sufficient amount to account for the removal of fructosamine residues from proteins (9).

While overexpressing mammalian fructosamine 3-kinase in *Escherichia coli*, we noted that control bacterial extracts contained low activities of an enzyme capable of phosphorylating fructoselysine. This stimulated us to study the metabolism of fructoselysine in *E. coli*. We found that these bacteria indeed grow on fructoselysine as an energetic substrate at a rate of about 1/3 of that observed with glucose. Extracts of cells grown on fructoselysine catalysed the ATP-dependent phosphorylation of fructoselysine to a product that was converted to hexose phosphates and other phosphorylated intermediates. Database searches allowed us to identify an operon containing a putative kinase (YhfQ) belonging to the PfkB/ribokinase family, a putative deglycase (YhfN), homologous to the isomerase domain of glucosamine-6-P synthase and a putative cationic amino acid transporter (YhfM). The proteins encoded by YhfQ and YhfN were overexpressed in *E. coli*, purified and shown to catalyse the ATP-dependent phosphorylation of fructoselysine to fructoselysine-6-P (YhfQ), and the reversible conversion of fructoselysine-6-P and water to lysine and glucose-6-P (YhfN). The kinase and

the deglycase were both induced when *E. coli* was grown on fructoselysine, and reached then activities sufficient to account for the rate of fructoselysine utilization. The two-step pathway that we have identified accounts therefore for fructoselysine utilisation by *E. coli*. Free fructoselysine is most probably produced through digestion of glycosylated protein. It is apparently not absorbed by the gut, but degraded by the microbial flora in the intestine. Our results indicate that *E. coli* participates in this metabolism.

Reaction mechanism of phosphoglycerate mutase

J.F. Collet, E. Van Schaftingen in collaboration with V. Stroobant, Ludwig Institute

The conversion of 3-phosphoglycerate to 2-phosphoglycerate is catalysed by two types of phosphoglycerate mutases (PGM), which differ in their mechanism, amino acid sequence and three-dimensional structure. The first type, present in vertebrates, certain invertebrates, yeast, fungi and some bacteria, uses 2,3-bisphosphoglycerate as a cofactor. The catalysed reaction is inter-molecular and follows a ping-pong mechanism in which a histidine residue is phosphorylated. By contrast, the cofactor-independent PGM (PGMi) does not require 2,3-bisphosphoglycerate. It is found in plants, in some microorganisms including bacteria and *Trypanosoma brucei*. It is a monomeric enzyme of about 60 kDa that catalyses an intramolecular reaction and requires a divalent metal cation for activity. Several data plead for the formation of a phosphoenzyme intermediate, which has, however, not been isolated until now.

We have now reported (10) that recombinant, cofactor-independent phosphoglycerate mutase from *T. brucei*, is inactivated by incubation with EDTA and reactivated by Co^{2+} much more than by Mn^{2+} or Fe^{2+} . It displays a minor (maximally 0.01 % of the mutase activity) phosphoglycerate phosphatase activity, which is stimulated by Mn^{2+} more than by Co^{2+} and displays a K_M of 5 μM in the presence of Mn^{2+} . Upon incubation with [^{32}P]-phosphoglycerate, radioactivity was incorporated into the enzyme; this reaction was stimulated by Mn^{2+} and Fe^{2+} much more than by Co^{2+} . The phosphorylated residue was identified by tandem mass spectrometry as Ser74, a residue in homologous position as the phosphorylated serine in alkaline phosphatase. The formation of the radiolabelled phosphoenzyme and its disappearance upon

addition of cold substrate were slow events that took several minutes to be complete, contrasting with the k_{cat} of the mutase reaction (28 s^{-1}). The K_M for the formation of the phosphoenzyme (5 μM) was about 20-fold lower than the K_M for 3-phosphoglycerate in the mutase reaction, but similar to the value observed with the phosphatase activity. These results indicate that the observed phosphoenzyme is an intermediate in the minor phosphatase activity, but not in the phosphomutase reaction.

Inborn errors of metabolism

T. de Barsey, E. Van Schaftingen

In 2001, samples from about 100 patients were analysed, allowing the diagnosis of carnitine deficiency (3 cases), of aldolase B deficiency (2 cases), of various forms of glycogen storage disease (6 cases) and of phosphomannomutase deficiency (20 cases).

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