The Liver Glucose-6-phosphatase of Intact Microsomes Is Inhibited and Displays Sigmoid Kinetics in the Presence of α-Ketoglutarate-Magnesium and Oxaloacetate-Magnesium Chelates*

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We have recently shown that the Ca*·EGTA and Mg*·EDTA complexes, but not free Ca*+ or Mg*+, inhibit the liver glucose-6-phosphatase (Mithieux, G., Vega, F. V., Beylot, M., and Riou, J. P. (1990) J. Biol. Chem. 265, 7257–7259). In this work, we report that, when complexed with Mg*+, two endogenous dicarboxylic keto acids (α-ketoglutarate (α-KG) and oxaloacetate (OAA)) inhibit the glucose-6-phosphatase activity at low concentrations of substrate. This phenomenon is specific for complexes of Mg*+ with α-KG and OAA since 1) the complexes of Mg*+ with other di- or tricarboxylic acids having high structural analogy with α-KG and OAA (oxalate, malate, succinate, citrate, aspartate, and glutamate) do not inhibit the glucose-6-phosphatase activity and 2) the Ca*·α-KG and Ca*·OAA chelates do not inhibit the glucose-6-phosphatase activity. In the presence of Mg*·α-KG or Mg*·OAA chelates, the enzyme displays sigmoid kinetic; the Hanes plots deviate from linearity, indicating the positive cooperative dependence of the velocity upon the substrate concentration. Hill coefficients (equal to 1 in the absence of the chelates) of 1.23 and 1.33 have been determined in the presence of Mg*·α-KG and Mg*·OAA complexes, respectively. The disruption of microsomal integrity by detergents abolishes the effect of Mg*·α-KG and Mg*·OAA, suggesting that the magnesium chelates inhibit the translocase component of the glucose-6-phosphatase system.

Because it contains glucose-6-phosphatase (Glc-6-Pase),† the liver is able to produce glucose, a feature that it shares only with the cortex of the kidney. Although the enzyme has been reported to be present in other tissues (1), only the Glc-6-Pase of liver (and of kidney under certain conditions) plays an important role in blood glucose homeostasis. The Glc-6-Pase catalyzes the last biochemical reaction preceding the release of glucose by the liver. This key position confers upon it a unique potential role in the regulation of hepatic glucose production. Until recently, no short-term regulatory process has been described for the control of the activity of Glc-6-Pase.

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‡ The abbreviations used are: Glc-6-Pase, glucose-6-phosphatase; EDTA, ethylenediaminetetraacetic acid; Glc-6-P, glucose 6-phosphate; OAA, oxaloacetate; α-KG, α-ketoglutarate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* The suppression of hepatic glucose production following the refeeding of fasted rats with glucose (2) or during insulin infusion with maintenance of euglycemia in man (3) or in rat (4) strongly argues for a possible acute control of this enzyme. It has been recently suggested that Ca*+, a second hormonal messenger that is known to mediate certain catecholamine effects in the liver (5), directly inhibits the activity of Glc-6-Pase (6). We have recently shown that this inhibitory effect is, in fact, dependent on the presence of the Ca*·EGTA chelate and not of free Ca*+ (7). On the contrary, free Ca*+ (at millimolar concentrations) is able to activate the enzyme activity in the absence of EGTA, as did free Mg*+ (7). In the same report, we showed that other cation-chelator complexes, such as Ca·EDTA or Mg·EDTA, are also able to inhibit the Glc-6-Pase. On the basis of these data, we have hypothesized that exogenous chelates could mimic the action of a putative intracellular chelator. We have thus tested the effect of metabolic bioriented lipids (1) to establish a possible positive control of Glc-6-Pase in vivo. It has been suggested that exogenous chelates could mimic the action of a putative acetate, magnesium, and oxaloacetate plus Mg*+, inhibit the activity of Glc-6-Pase at low substrate concentrations.

MATERIALS AND METHODS

Enzyme Purification and Assay—Microsomes were purified from the liver of rats fasted for 48 h as previously described (7). Briefly, the liver was rapidly perfused with cold 0.15 m NaCl, removed, and homogenized in 0.25 m sucrose, pH 7.4 (9 ml/g of fresh weight), using a Teflon glass homogenizer. The homogenate was centrifuged at 20,000 × g for 10 min, and the resulting supernatant, at 100,000 × g for 1 h. The microsomal pellet was washed once in the homogenization buffer. The microsomes were suspended in the same medium (0.9 ml/g of fresh weight). The activity of Glc-6-Pase was performed at pH 7.3 at 37 °C. The reaction mixture (600 μl) was comprised of 20 mm Tris·HCl, pH 7.3, 20 mm CaCl2 or MgCl2, various concentrations of organic acids, pH 7.3, Glc-6-P, and microsomes (about 100 μg of protein). After 10 min, protein was precipitated by the addition of 2 ml of ascorbic acid/trichloroacetic acid (2%/10%, w/v) and 0.3 ml of 0.36 (mean, 0.34) μmol of Pi/min/mg of protein at 37 °C and pH 7.3, respectively. The Glc-6-Pase activity was increased by 250–280% upon disruption of microsomes by deoxycholate (1 mm Glc-6-P).

Experimental Approach The major problem that arose when starting this study was the weakness of the association of organic acids with metal cations. In the case of the exogenous chelates used in our previous study (Ca·EGTA, Co·EDTA, and Mg·EDTA), the association constants are high and the proportion of free species is negligible with regard to complexed species (7). In the present study,
the association constants are low. For instance, $K_1 = 91 \text{ M}^{-1}$ at physiological pH for the Mg-OAA complex (9). So, the proportion of free species must be considered at all concentrations of reactants. This could impede a clear interpretation of the results, since we previously reported that free Ca$^{2+}$ and Mg$^{2+}$ activated Glc-6-Pase at millimolar concentrations (7). To obviate this difficulty, we have chosen to test the effect of organic acids in the absence and in the presence of Ca$^{2+}$ or Mg$^{2+}$. This strategy offers several advantages. 1) This concentration of cation has no effect on the activity of the Glc-6-Pase (see Fig. 1). 2) It allows the formation of a significant proportion of complex even if the concentration of the chelator is low. 3) Even if the free cation concentration is decreased to 15 mM (following a chelating process), the resulting activation of Glc-6-Pase would be very moderate (Fig. 1), which will not hinder an inhibiting effect from being revealed.

RESULTS

We tested the action of a number of di- and tricarboxylic organic acids (oxalate, malate, oxaloacetate, succinate, citrate, and $\alpha$-ketogluartate) and dicarboxylic amino acids (aspartate and glutamate) on the activity of Glc-6-Pase, at the concentrations of 0.01, 0.1, 1.0, and 10 mM, either in the absence or in the presence of CaCl$_2$ or MgCl$_2$ (data not shown). Two of them (a-KG and OAA) were found to inhibit the Glc-6-Pase activity in the presence of Mg$^{2+}$, whereas all others had no effect on the enzyme, irrespective of the conditions (absence or presence of Ca$^{2+}$ or Mg$^{2+}$). Consequently, we carried out a dose dependent study of the effect of OAA and a-KG, in the absence and in the presence of Ca$^{2+}$ and Mg$^{2+}$.

The activity of Glc-6-Pase was not altered in the presence of a-KG at concentrations ranging from 1 to 15 mM, both in the absence and in the presence of 20 mM Ca$^{2+}$. On the contrary, in the presence of 20 mM a-KG, Ca$^{2+}$ induced a significant ($p < 0.01$) inhibitory effect on Glc-6-Pase at all the concentrations tested (Fig. 2A). The Glc-6-Pase activity was not altered in the presence of OAA alone at concentrations equal to or lower than 7.5 mM, but it was slightly inhibited in the presence of 10 and 15 mM OAA. In the presence of OAA and Ca$^{2+}$, the Glc-6-Pase activity was slightly but not significantly ($p > 0.1$) inhibited, except at the highest OAA concentration, for which the inhibition effect was significant ($p < 0.01$). In the presence of OAA and Mg$^{2+}$, the Glc-6-Pase was significantly ($p < 0.01$) inhibited at all OAA concentrations, as compared with the activity measured in the presence of OAA alone (Fig. 2B).

Since the decrease of the concentrations of free species did not result in a decrease of the enzyme activity (see Fig. 1 and the curves of Glc-6-Pase activity versus Mg$^{2+}$ and OAA alone of Fig. 2), the occurrence of the inhibitory effect observed in the presence of both OAA and Mg$^{2+}$ and a-KG and Mg$^{2+}$ could only be attributed to the formation of Mg-OAA and Mg-a-KG complexes. The amount of Mg-OAA complex formed under our experimental conditions was calculated from the stability constant of the 1:1 OAA dianion-Mg$^{2+}$ complex, $K_s = 91 \text{ M}^{-1}$ (9), assuming that OAA was totally under dianionic form. The percentage of inhibition of Glc 6 Pase activity ((activity in the presence of OAA alone) - (activity in the presence of OAA and Mg$^{2+}$)) plotted versus the concentration of Mg-OAA complex present in the medium exhibited the shape of a saturation curve (Fig. 3). The maximal inhibition observed plateaued at about 30% of the control activity, and the concentration of Mg-OAA complex that induced 50% of the maximal inhibition was 1 mM. Since the stability constant of Mg-a-KG complex was not available, the concentration of Mg-a-KG complex was calculated on the basis of the Mg-OAA complex stability constant. This assumption relies on the fact that the stability constants of organic dicarboxylic acids with Ca$^{2+}$ and Mg$^{2+}$ are in the same order of magnitude (10, 11) and on the structural analogy of both keto diacids, which differ only by one carbon chain length. The plot of the degree of inhibition of Glc-6-Pase versus the Mg-a-KG complex concentration also exhibited

**FIG. 1.** Effect of CaCl$_2$ and MgCl$_2$ on Glc-6-Pase activity. Glc-6-Pase was assayed in the presence of 1 mM Glc-6-P and CaCl$_2$ (0.1-40 mM) (•) or MgCl$_2$ (0.1-40 mM) (○). The results are expressed as the percentage of control activity measured in Tris-HCl buffer, pH 7.3, in the absence of salt (mean ± S.D., n = 3).

**FIG. 2.** Effect of a-KG and OAA on the Glc-6-Pase activity. Panel A, Glc-6-Pase was assayed in the presence of a-KG (1-15 mM) alone (●) in the presence of 20 mM MgCl$_2$ (○), and in the presence of 20 mM CaCl$_2$ (□). The Glc-6-P concentration was 1 mM. Results are expressed as the percentage of the control activity (mean ± S.D., n = 5 (●) and n = 3 (○)). Panel B, the same experiments are represented using the same symbols as in A, but a-KG was replaced by OAA (n = 5 (●) and n = 3 (□)). The stars mean that the differences were significant with regard to the control value measured in the presence of a-KG or OAA alone at the same concentration (*, $p < 0.01$; **, $p < 0.001$).

**FIG. 3.** Effect of Mg-a-KG and Mg-OAA chelates on the Glc-6-Pase activity. The percentage of inhibition induced by the presence of both a-KG (or OAA) and 20 mM MgCl$_2$ i.e. the activity in the presence of a-KG (or OAA) alone minus the activity in the presence of a-KG (or OAA) plus 20 mM Mg$^{2+}$ is plotted versus the concentration of Mg-a-KG (or Mg-OAA) complex present in the incubation medium. This concentration was calculated from the association constant of the Mg-OAA complex (see text). Panel A, percentage of inhibition as a function of Mg-a-KG complex (mean ± S.D., n = 5). Panel B, percentage of inhibition as a function of Mg-OAA complex (mean ± S.D., n = 5).
the shape of a saturation curve. The maximal inhibition also
plateaued at 30%, and the concentration of Mg-α-KG inducing
50% of the maximal effect was about 1 mM.
In order to characterize the inhibition, we studied the
kinetics of the enzyme in the absence and in the presence
of the chelates at a concentration that yielded the plateau
of inhibition at 1 mM Glc-6-P. A representative experiment
is reported in Fig. 4. In the absence of chelate, the kinetics
were hyperbolic. The Hanes plot (i.e. substrate concentration
divided by the velocity versus the substrate concentration)
was linear. In the presence of magnesium chelates, the kinetics
were sigmoidal (Fig. 4, A and C). They revealed that the Glc-
6-Pase was inhibited at low concentrations of Glc-6-P (lower
than or equal to 4 mM) and not at high Glc-6-P concentrations
(8 mM and higher). The $K_{0.5}$ values, determined from the plots
of the velocity versus the Glc-6-P concentration, were signif-
icantly increased in the presence of Mg-α-KG and Mg-OAA
complexes (Table I). The Hanes plots, emphasizing the sig-
moidicity, significantly deviated from the linearity (Fig. 4, B
and D). This indicated the cooperative dependence of the
velocity upon the Glc-6-P concentration. The Hill representation
(Fig. 5) allowed us to obtain some indication of the degree of cooperativity of the Glc-6-Pase upon its
substrate in the presence of the inhibitory magnesium cho-

![Fig. 4. Kinetics of Glc-6-Pase in the presence of Mg-α-KG
and Mg-OAA complexes. Panel A, the Glc-6-Pase was assayed
in the absence (•) and in the presence (○) of 7.85 mM Mg-α-KG
chelate. Panel B, Hanes plot (velocity/substrate versus substrate)
of the data of panel A. Panel C, kinetics of the Glc-6-Pase in the absence (•)
and in the presence (○) of 7.85 mM Mg-OAA. Panel D, Hanes plot of the
panel C data. The $K_{0.5}$ values determined from panels A and C were
2.2, 2.6, and 3.6 mM in the absence and in the presence of Mg-α-KG
and Mg-OAA complexes, respectively.](http://www.jbc.org/)

| $K_{0.5}$ (mM) | Control $n = 6$ | Mg-α-KG $n = 6$ | Mg-OAA $n = 5$
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<td>2.23 ± 0.12</td>
<td>2.95 ± 0.76$^*$</td>
<td>4.4 ± 1</td>
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<tr>
<td>1.00 ± 0.04</td>
<td>1.23 ± 0.08$^a$</td>
<td>1.33 ± 0.06$^a$</td>
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$^*$ Significantly different from the control, $p < 0.05$.
$^a$ Significantly different from the control, $p < 0.01$.

**Table I**

**Effect of Mg-α-KG and Mg-OAA chelates on the kinetic constants of Glc-6-Pase**

The $K_{0.5}$ values were determined from the plots of the velocity versus the Glc-6-P concentration in the absence (control) and in the presence of Mg-α-KG and Mg-OAA chelates at a concentration of 7.85 mM. The Hill coefficients ($h$) were determined from the Hill plots of the same experiments. The results are expressed as mean ± S.D.

**FIG. 5. Determination of Hill coefficients of Glc-6-Pase in
the presence of Mg-α-KG and Mg-OAA chelates.** The Glc-6-
Pase was assayed as described in the legend to Fig. 4 in the absence
(○) and in the presence of Mg-α-KG chelate (□) and Mg-OAA chelate (○). The maximal velocity ($V_{max}$) was obtained from Lineweaver-
Burk plots of the control experiment. The Hill coefficients deter-
mined from this plot were 0.99 (control), 1.23 (Mg-α-KG), and 1.42
(Mg-OAA).

**DISCUSSION**

The data reported in this paper show that the activity of the liver microsomal Glc-6-Pase is inhibited by two endoge-
nous organic acid-magnesium chelates, i.e. Mg-OAA and Mg-
α-KG complexes. There exist several differences between
these inhibitory effects and the inhibitory effects of the ex-
ogenous Mg-EDTA chelates that we reported previously (7). 1) The inhibitory effect induced by endogenous magnesium
chelates (about 30% at a concentration of 5 mM for a Glc-6-
P concentration of 1 mM) is smaller than that induced by the
Mg-EDTA complex (about 60% under the same conditions).
This could be due to the occurrence of a concomitant activa-
tion effect induced by the presence of millimolar free cations
in the first case and not in the second (see above). 2) The
Mg-EDTA chelate presents a threshold concentration under
which it is ineffective; a significant inhibition effect can only
be observed at a concentration equal to or higher than 2.5
mM. On the contrary, the lowest complex concentration of
the endogenous magnesium chelates studied here (about 0.6
mM) had a significant inhibitory action on Glc-6-Pase. 3) The
Mg-EDTA-induced inhibition of Glc-6-Pase is not specific
either with regard to the chelator or with regard to the cation.
The exogenous chelates (Ca-EDTA and Ca-EGTA) have the
same (and an even slightly more potent) effect on the enzyme
activity as does the Mg-EDTA complex. On the contrary,
numerous other chelates cannot mimic the Mg-OAA and Mg-
alpha-KG-induced inhibition of Glc-6-Pase. With regard to the chelator, various molecules having high structural analogy with OAA and alpha-KG (oxalate, malate, succinate, citrate, glutamate, and aspartate) have no effect on Glc-6-Pase in the presence of Mg2+. With regard to the cation, alpha-KG does not inhibit and OAA inhibits slightly but nonsignificantly the enzyme activity in the presence of Ca2+.

The absence of effect of the other organic acid-cation combinations cannot be attributed to the nonoccurrence of the complexation phenomenon. Indeed, the stability constants of the complexes of Ca2+ with OAA, alpha-KG, succinate, and malate and of Mg2+ with OAA, malate, and succinate are in the same order of magnitude as the Mg-OAA complex (10, 11). It is noteworthy that citrate and oxalate, which had been shown to inhibit the Glc-6-Pase partially purified from deoxycholate-treated microsomes at acidic pH but not at physiological pH (12), do not inhibit Glc-6-Pase in intact microsomes in the presence of Mg2+ and Ca2+ at pH 7.3. However, they complex both Ca2+ and Mg2+ in a stronger manner than the other molecules studied here (11). The reason OAA alone inhibits the Glc-6-Pase at high concentrations is unclear. The possibility that it could be due to the presence of significant amounts of endogenous cation was ruled out since this inhibition effect also takes place in the presence of 10 mM EDTA (data not shown).

The possible physiological importance of the inhibitory effect of the Mg-OAA complex on Glc-6-Pase seems rather improbable. The liver concentration of OAA is markedly lower (about 5 μM) than the concentration required to observe a significant inhibitory effect in our study and is not subjected to important metabolic variations (13). On the contrary, if the alpha-KG concentration in the liver is relatively low (under 1 mM), this concentration is expected to increase, for example, after feeding, at a time when the endogenous hepatic glucose production is inhibited, which is a phenomenon that could be related to the inhibition of the Glc-6-Pase activity. The amino acid concentration is enhanced by several times itself in the portal vein of the rat after a protein meal, whereas only slight fluctuations are observed in the arterial blood (14). The amino acid concentration in the liver may thus be transiently increased greatly over the need for the cell metabolism, as a result of the high activity of amino acid uptake of the liver (15, 16). In the hepatocyte cytosol, glutamine transaminates with a number of keto acids to yield the corresponding amino acids and alpha-ketoglutaramate, which either spontaneously cyclizes to 2-hydroxy-5-oxoproline or is hydrolyzed in an irreversible reaction by a ketoacid-ω-amidase to yield alpha-KG and ammonia (14). Glutamic acid may also give rise to the production of alpha-KG because of the action of a glumatic synthetase that incorporates ammonia in glutamic acid to produce a glutamine molecule (with consumption of ATP) that may recycle to again produce alpha-KG (14). Because of the removal of alpha-ketoglutarate (by cyclization or by irreversible hydrolysis to alpha-KG), the overall equilibrium of the cycle is displaced toward the production of alpha-KG (14). It should be noted that glutamine and glutamate have no effect on Glc-6-Pase in our in vitro assay (not shown). Thus, a rise of the amino acid concentration in the liver is likely to be accomplished by a rise of the alpha-KG concentration. Since free Mg2+ is available at millimolar concentrations in the liver cytosol, the formation of Mg-alpha-KG chelate may be considered.

It seems hazardous to quantitatively speculate on the enhancement of the alpha-KG concentration and on the proportion of Mg-alpha-KG chelate that might be assembled in a medium as complex as the hepatocyte cytosol. On the other hand, one may speculate on the degree of inhibition that could be induced by the Mg-alpha-KG chelate at physiological concentrations of Glc-6-P (about 100 nM). Indeed, from the Hill plots, which are linear, it is possible to extrapolate the value of the velocity at 0.1 mM Glc-6-P, in the absence and in the presence of magnesium chelates. We calculated that the inhibition effect, which plateaued at 30% in the presence of 1 mM Glc-6-P, would extrapolate to 75–80% in the presence of the Mg-alpha-KG chelate at a Glc-6-P concentration of 100 nM. Since the lowest concentration of Mg-alpha-KG chelate (0.6 mM) had a lowest inhibitory effect on the Glc-6-Pase at 1 mM Glc-6-P, it seems reasonable to postulate that the Mg-alpha-KG chelate might significantly inhibit in vivo the activity of the enzyme at physiological concentrations of Glc-6-P.

Data obtained in vitro using isolated hepatocytes might corroborate this hypothesis. It is known that glucose per se is a poor substrate for glycogen synthesis in isolated hepatocytes and in perfused livers of fasted rats and that higher rates of glycogen deposition are obtained in the presence of glucose and fructose or glucose and gluconeogenic substrates (17–21). In addition, glycogen synthesis is enhanced in the presence of glutamine (18–21) and other amino acids, although less efficiently (18). Since glutamate was formed from glutamine and glutamine plus glutamate from other amino acids, Katz et al. (18) hypothesized that the active compound stimulating the glycogen deposition could be glutamine or glutamate or an unknown metabolite derived from them. Low or high rates of glycogen deposition in the absence or in the presence of glutamine, respectively, could be at least in part dependent on the fact that Glc-6-Pase may function actively in the first case (the Glc-6-P formed being hydrolyzed by the enzyme and glucose being excreted from the hepatocyte) and is inhibited in the second case (the Glc-6-P formed entering into the glycogenosynthetic pathway). If this assumption is true, the inhibition of the Glc-6-Pase activity might be related to the intracellular synthesis of alpha-KG from glutamine and to the formation of the Mg-alpha-KG complex.

In conclusion, our results suggest that alpha-KG (complexed with Mg2+), a compound derived from glutamine metabolism, could, by inhibiting the Glc-6-Pase activity, play a role in the regulation of the production of glucose by the liver.

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Inhibition of Glucose-6-phosphatase by Magnesium Chelates

The liver glucose-6-phosphatase of intact microsomes is inhibited and displays sigmoid kinetics in the presence of alpha-ketoglutarate-magnesium and oxaloacetate-magnesium chelates.

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