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Glycogen Phosphorylase from Normal and Leukemic Human Leucocytes: Kinetic Parameters of the Active Form *

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Glycogen phosphorylase of human leucocytes exists in two forms interconvertible by phosphorylation-dephosphorylation. The active form of the enzyme from normal and leukemic human leucocytes has been obtained by preincubation of the 14,000 *g* crude extracts at 30° C in the presence of ATP and magnesium ion. The enzyme associated itself to a glycogen particulate fraction obtained by centrifugation at 90,000 *g* from the crude extracts. Kinetic characteristics of the glycogen fraction enzyme were similar when obtained from normal or leukemic leucocytes. Apparent K_m values of the active enzyme for glucose-1-phosphate and glycogen were $2\text{-}3 \times 10^{-3}$ M and 0.50-0.65 mg/ml respectively. Glucose, glucose-6-phosphate, uridine diphosphoglucose, 2-deoxyglucose, fluoride, Mg^{+2} and Ca^{+2} have been shown to be inhibitors of the enzyme.

Glycogen phosphorylase from normal and leukemic leucocytes exists in two interconvertible forms (6, 10) designated *a* (phosphoenzyme) and *b* (dephosphoenzyme). The inactive form is converted to the active form by a specific kinase in a reaction requiring Mg^{++} and ATP. Active leucocyte phosphorylase is inactivated by a phosphorylase phosphatase (6, 10). Skeletal muscle phosphorylase *b* requires AMP for activity, becoming fully active in the presence of the nucleotide, is inhibited by sulfate and is activated by cysteine. Inactive liver phosphorylase is not significantly affected by AMP, is activated by sulfate and is not affected by cysteine (1, 3, 11, 13). Several studies

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dealing with glycogen phosphorylase of leucocytes suggest that this enzyme is more closely related to liver than to muscle phosphorylase since the inactive form of the leucocyte enzyme is incompletely stimulated by AMP, unaffected by cysteine and activated by sodium sulfate (6, 10, 14).

The regulation of leucocyte phosphorylase activity is mediated through interconversions between active and inactive forms, and is exerted at the phosphorylase kinase and the phosphorylase phosphatase steps. Glucose and glucose-6-phosphate enhance the dephosphorylation reaction, while AMP counteracts these effects (6, 10).

Materials and Methods

Blood samples. Normal human lymphocytes and polymorphonuclear leucocytes were isolated by the method of BOYUM (2) modified as it has been described (6). Final contamination with other white blood cells was usually between 5 and 10 %. Contamination with platelets and erythrocytes was negligible. Leukemic leucocytes were isolated by differential sedimentation, hypotonic shocks and differential centrifugation as described (4).

Preparation of leucocyte extracts. The isolated cells were suspended in 50 mM Tris-maleate pH 7.4 to a final concentration of $3\text{-}6 \times 10^8$ cells/ml and homogenized by means of a teflon pestle homogenizer. After freezing and thawing once, the homogenate was centrifuged at $14{,}000 \times g$ for 15 minutes at 40° C and the clear supernatant was used for the assays.

Preparation of a partially purified phosphorylase a. Glycogen phosphorylase exists largely in the active form as isolated from normal leucocytes, while the enzyme found in extracts of leukemic leucocytes seems to be in an inactive form which can be reactivated by ATP-Mg^{++} addition (6).

Enzyme extracts obtained from normal and leukemic leucocytes were preincubated for 10-20 minutes at 30° C with final concentrations of 5 mM ATP and 10 mM $MgCl_2$ in order to obtain active phosphorylase. Figure 1 shows that addition of 5 mM ATP-10 $MgCl_2$ gave full activation of the enzyme (phosphorylase *a*) in a few minutes of incubation. The extracts were centrifuged at $90{,}000 \times g$ for 90 minutes after preincubation. Metabolites and part of soluble protein remained at the supernatant while the protein-glycogen complex settled. This fraction consisted mainly of glycogen particles to which were associated about 70 % of phosphorylase, phosphorylase kinase and phosphorylase phosphatase among other enzymes. The pellet was suspended in 50 mM Tris-maleate, pH: 7.4, 5 mM EDTA, 50 mM NaF, and gently homogenized.

Phosphorylase assay. Glycogen phosphorylase activity was measured according to the method of GILBOE *et al.* (7). The assay mixture contained: 1 % glycogen, 50 mM [$U^{14}C$] glycose-1-phosphate (0.002 μCi/uM), 50 mM Tris-maleate, pH: 7.4 and enzyme (0.03 ml) in a total volume of 0.09 ml.

Kinetic assays. Reaction velocities were determined as a function of glucose-1-phosphate and glycogen concentrations. Time course of the reaction was separately determined for each set of concentrations. Special care was taken to assure that less than 10 % of the substrate was depleted during the reactions. Concentrations of glucose-1-phosphate ranged from 2×10^{-2} M to 5×10^{-4} M (10^5 cpm/μM). Glycogen concentrations ranged from 0.1 to 5 mg/ml. In addition, reaction mixtures contained 35 mM imidazol, pH: 7.4, 4 mM mercaptoethanol, 0.4 mM EDTA. Enzyme was diluted in 50 mM Tris-maleate, pH: 7.4. Reaction started by adding 0.03 ml of enzyme fraction to 0.06 ml

of assay mixture. At appropiate times, aliquots were removed and treated according to the method of GILBOE *et al.* (7). Duplicates were usually run for each point. Data were plotted as reciprocal of initial velocity versus reciprocal of the variable substrate. Apparent Michaelis constants were calculated by the intercept with the abcisa axis. Inhibition constants were estimated by plotting the curves slope versus the respective concentration of inhibitor.

Chemicals. [U^{14}C] glucose-1-phosphate was obtained from The Radiochemical Centre (Amersham). UDP-Glucose, rabbit liver glycogen, glucose-1-phosphate, glucose-6-phosphate, 2-deoxyglucose and ATP were purchased from Sigma. All other reagents were of analytical grade.

Results

Michaelis constants for the active form of leucocyte phosphorylase. Table I shows that the apparent K_m values obtained for glucose-1-phosphate and glycogen were of the same order of magnitude for glycogen phosphorylase *a* of polymorphonuclear leucocytes, lymphocytes or leukemic leucocytes. Lineweaver-Burk plots were linear in all cases.

Table I. *Apparent Michaelis constants (±S.D.) of leucocytes glycogen phosphorylase for the substrate glucose-1-phosphate and glycogen.* Number of determinations is given in parentheses.

Type cell	K_m for G1P (mM)	K_m for glycogen (mg/ml)
PMN *	2.7±0.6 (3)	0.49±0.20 (2)
Lymphocytes	1.9±0.7 (6)	0.59±0.17 (4)
CML	2.6±0.5 (7)	0.63±0.11 (6)
AML	3.0±0.8 (5)	0.49±0.20 (4)
CLL	2.1±0.9 (6)	0.62±0.18 (4)
ALL	2.6±0.6 (5)	0.58±0.31 (3)

* PMN: polimorphonuclears; CML: chronic myeloid leukemia; AML: Acute myeloid leukemia; CLL: chronic lymphoid leukemia; ALL: Acute lymphoid leukemia.

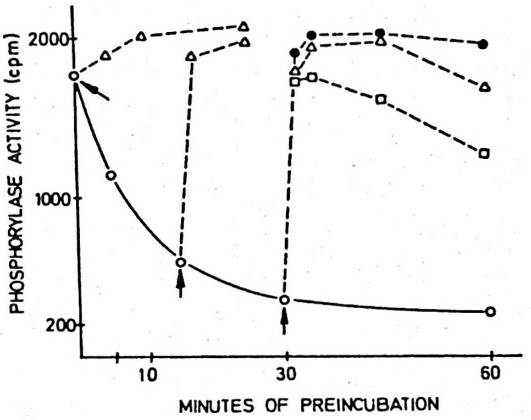

Fig. 1. *Activation of lymphocyte glycogen phosphorylase by ATP-Mg^{+2} in crude extracts.* Lymphocyte crude extract was preincubated in the presence of 5 mM dithiotreitol for 1 hour. At the times indicated, ATP and Mg^{+2} were added to aliquots. Final concentrations of ATP-Mg^{+2} were 5-10 mM (△), 10-20 mM (●), 2.5-5 mM (□).

Effect of glucose and some of its derivatives on kinetic constants. The presence of glucose, glucose-6-phosphate or 2-deoxyglucose in the leucocyte extract during preincubation increases the rate of inactivation of phosphorylase (6). As it has been suggested that glucose stabilized phosphorylase in a form more susceptible to the action of phosphatase (6) it seemed of interest to evaluate the effect of glucose and some of its derivatives on phosphorylase activity. When glucose (figure 2), glucose-6-phosphate (figure 3), uridine diphosphoglucose (UDPG) or 2-deoxyglucose (results not shown) were included in the phosphorylase reaction mixture, there was found a competitive inhibitory effect for both glucose-1-phosphate and glycogen. Inhibition by glucose and its derivatives was constantly observed in all the studied cells (normal or leukemic). Uridine diphosphoglucose (apparent K_i: 0.75 mM) was the most potent inhibitor whereas K_i for glucose and glucose-6-phosphate were 4.3 mM and 7.2 mM respectively.

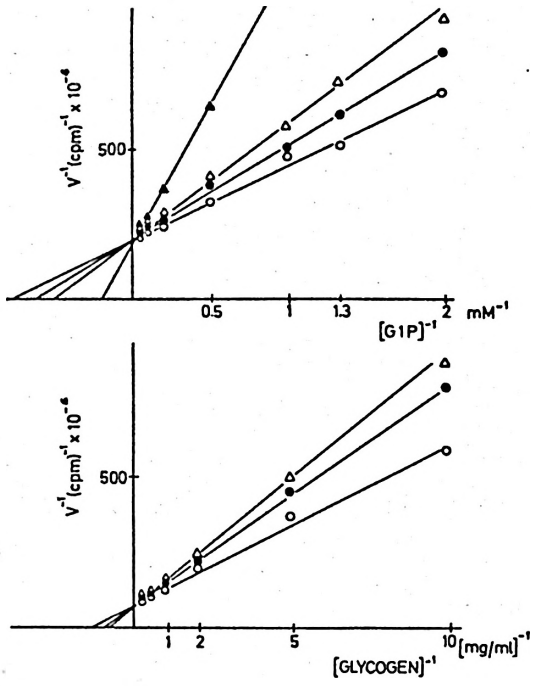

Fig. 2. *Effect of glucose on apparent K_m of phosphorylase a for glucose-1-phosphate and glycogen.*
Glucose final concentrations were: 2 mM (●), 5 mM (△), 10 mM (▲), no glucose (○).

Influence of various ions on kinetic constants. Magnesium, fluoride and calcium (the latter not shown) showed an inhibitory effect on leucocyte active phosphorylase (figure 4). Fluoride is currently used as phosphorylase phosphatase inhibitor in phosphorylase assay mixtures and buffers. Nevertheless, fluoride inhibitor effect on phosphorylase *a* assayed in the presence of 50 mM glucose-1-phosphate was negligible.

Discussion

Glycogen phosphorylase activity in crude extracts of leukemic leucocytes is lower than that of normal leucocytes. However, it is possible to activate the leukemic leucocyte enzyme in the presence of ATP-$MgCl_2$, the levels of activity reached under these conditions being closer to those of normal leucocytes (6). Glucose-1-phosphate and glycogen Michaelis constants for glycogen phosphorylase active form have been found to be similar when the enzyme was obtained from normal lymphocytes, normal polymorphonuclear leucocytes and cells of chronic myeloid, chronic lymphoid, acute myeloblastic and acute lymphoblastic leukemia. This fact stresses our opinion that glycogen phosphorylase exists largely in the active form as isolated from normal leucocytes while the enzyme is inactivated in extracts of leukemic leucocytes. This

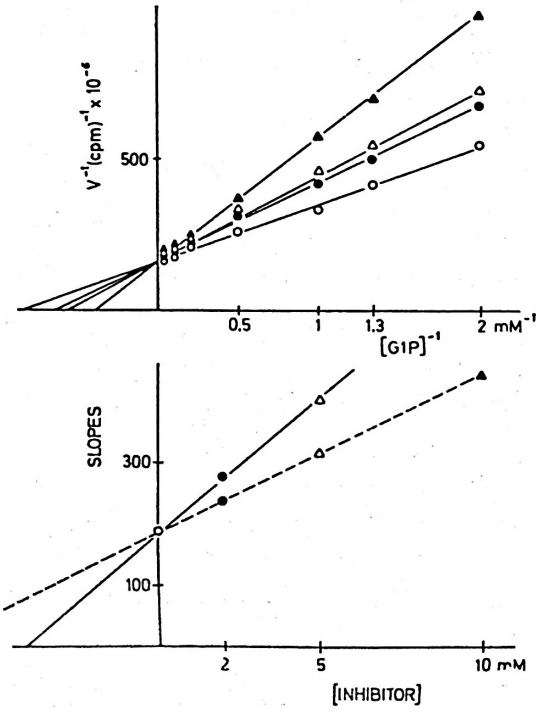

Fig. 3. *Effect of glucose-6-phosphate on apparent K_m of phosphorylase a for glucose-1-phosphate.*
Glucose-6-phosphate concentrations were: 2 mM (●), 5mM (△), 10 mM (▲), no glucose-6-phosphate (○). Inhibition constants for glucose-6-phosphate (dotted line) and glucose (continuous line) were calculated from data showed in figures 2 and 3.

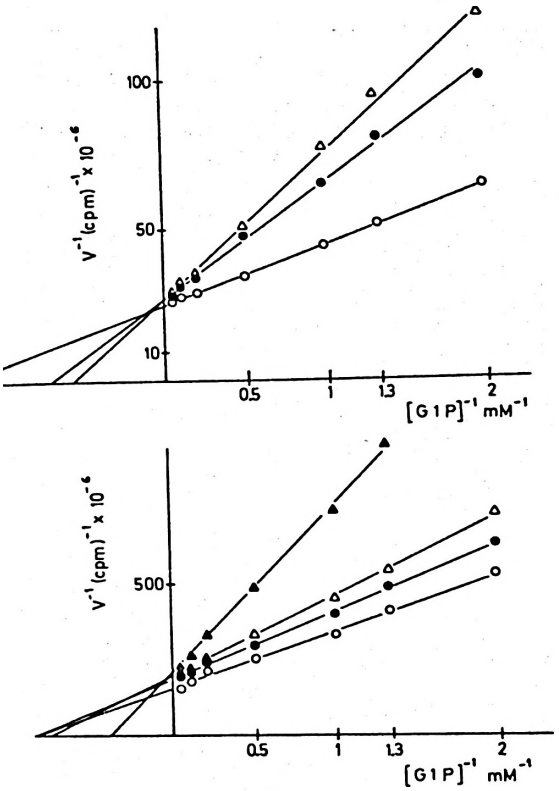

Fig. 4. *Effect of fluoride and magnesium ion on apparent K_m of phosphorylase a for glucose-1-phosphate.*
Upper: Sodium fluoride concentrations were 10 mM (●), 15 mM (△). Lower: Magnesium chloride concentrations were: 10 mM (●), 20 mM (△), 30 mM (▲). Open circles (○) show phosphorylase activity assayed without effectors.

difference could be due to the different methods employed for isolating cells or to a failing in the phosphorylase activating system at a previous step to the phosphorylase kinase reaction.

Experiments performed with leucocyte crude extracts (6, 10) as well as with the glycogen particulate fraction (results shown in this paper), show that glucose and glucose-6-phosphate are important effectors on the regulation of leucocyte glycogen phosphorylase. Both sugars inhibit competitively the phosphorylase active form with glucose-1-phosphate and glycogen. In addition, both glucose and glucose-6-phosphate stimulate the inactivation of the enzyme by dephosphorylation catalyzed by a phosphorylase phosphatase (6, 10), and glucose-6-phosphate stimulates glycogen synthase activity by acting at two levels: direct activation of the inactive form (5, 8) and promoting formation of synthase I, the active species (12).

Resumen

Se han identificado, en leucocitos humanos, dos formas de glucógeno fosforilasa interconvertibles por fosforilación-defosforilación. Se ha obtenido glucógeno fosforilasa en su forma activa por preincubación a 30° C de los extractos crudos de 14.000 *g* de leucocitos humanos, normales y leucémicos, en presencia de ATP y iones magnesio. El enzima se encuentra asociado a la fracción particulada de glucógeno obtenida por centrifugación a 90.000 *g* de los extractos crudos. Las características cinéticas del enzima asociado a la fracción particulada de glucógeno son similares tanto cuando proviene de leucocitos normales como de leucémicos. Los valores de Km aparente del enzima activo para glucosa-1-fosfato y glucógeno están en el rango de 2×10^{-3} M y 0,5 mg/ml, respectivamente. La glucosa, glucosa-6-fosfato, uridín difosfoglucosa, 2-desoxiglucosa y los iones fluoruro, magnesio y calcio se comportan como inhibidores del enzima.

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