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CONTROL OF GLUCOSE PHOSPHORYLATION IN *EUGLENA GRACILIS*

I. PARTIAL CHARACTERIZATION OF A GLUCOKINASE

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SUMMARY

In *Euglena gracilis* strain Z, grown either under autotrophic or heterotrophic conditions, glucose and fructose phosphorylation occurs by means of two distinct enzymatic activities, which may be completely separated by centrifugation at 105 000 × *g*.

Fructose is phosphorylated by a soluble fructokinase (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4) whereas a particulate glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2), with high K_m (8 mM) for glucose, is the only enzyme able to phosphorylate glucose. Kinetic analysis indicates that glucokinase is activated allosterically by orthophosphate. Activation by orthophosphate occurs, *in vitro*, in at least two ways: (1) by relieving inhibition by Mg–ATP complex; (2) by direct activation of the enzyme.

A hexokinase activity capable of phosphorylating both glucose and fructose has already been detected in *Euglena gracilis* v. bacillaris[1]. This activity, which seems to be correlated with the utilization of glucose in the culture medium, has been partially characterized in crude extracts[2].

This paper deals with a further characterization of this activity. Cells of *E. gracilis* strain Z were grown in the dark on culture medium[3] supplemented with 60 mM glucose as the sole organic substrate. Homogenates were obtained in 50 mM Tris–maleate, pH 7.5, 58 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, by sonication (22 kcyles/sec, 6 μm peak-to-peak) for 1 min at 4°. Two other procedures for breaking the cells were tested (French pressure cell, freeze–thawing) but neither of them gave a significant increase in the final yield, nor appreciable differences in the pattern of the enzymes studied. In crude extracts (800 × *g* supernatant) of Euglena cells it was then shown that glucose and fructose are phosphorylated by two distinct enzymatic activities, which can be completely separated by centrifugation of the homogenates at 105 000 × *g* for 2 h. Table I shows that 97% of the fructose phosphorylating activity is found in the supernatant, whereas 92% of the glucose phosphorylating activity sediments under these conditions. Both activities were recovered quantita-

TABLE I

SEPARATION OF GLUCOKINASE AND FRUCTOKINASE ACTIVITY FROM *Euglena gracilis* STRAIN Z HOMOGENATE

Experimental conditions: Heterotrophic Euglena cells (81 g wet weight corresponding to $2 \cdot 10^8$ cells) were disrupted by sonication in 50 mM Tris buffer; 58 mM K^+; 10 mM $MgCl_2$; 1 mM EDTA, pH 7.5. Assay system: (1) Glucokinase: 50 mM Tris, pH 8.2; 6 mM $MgCl_2$; 5 mM ATP; 0.5 mM NADP; 20 mM orthophosphate; 100 mM glucose; excess glucose-6-phosphate dehydrogenase. (2) Fructokinase: 50 mM Tris, pH 8.2; 6 mM $MgCl_2$; 5 mM ATP; 0.5 mM NADP; 5 mM fructose; excess glucose-6-phosphate dehydrogenase and phosphoglucose isomerase.

Fractions	*Protein (mg)*	*Glucokinase activity (nmoles/min)*			*Fructokinase activity (nmoles/min)*		
		Total	*per mg protein*	*Recovery (%)*	*Total*	*per mg protein*	*Recovery (%)*
(A) 800 × *g* sediment	—	trace	—	—	0	0	0
(B) 800 × *g* supernatant	1 395	49 300	35.26	100	51 500	36.9	100
(C) 800–105 000 × *g* supernatant	870	3 150	3.6	6.4	50 000	57.0	97.0
(D) 105 000 × *g* sediment	521	45 300	86.6	92.0	1 290	3.6	2.5
(E) 105 000 × *g* supernatant of (D) treated with 1% Triton	179	33 220	186.0	67.0	—	—	—

tively after this fractionation procedure. This allows one to exclude the presence, in the crude homogenate, of an aspecific hexokinase, equally active on both glucose and fructose, which would disappear after the separation procedure.

The lack of an aspecific hexokinase constitutes a remarkable finding, since the phosphorylation of glucose is catalyzed, both in animals[4,5] and in plants[6] by hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) with low specificity and low K_m for glucose. In eukaryotes the only exception is the liver of vertebrates[4], in which the glucose is phosphorylated by hexokinase and glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) as well. In *E. gracilis* the glucose appears to be phosphorylated only by a specific glucokinase whereas the hexokinase is absent. Since glucokinase of Euglena sediments at 105 000 × *g* it is likely to be particulate and thus similar to the G_1 and G_2 fractions[7] of the liver glucokinase. The glucokinase of Euglena shows a high specificity and a high K_m for glucose, as analogues particulate and soluble activities[4-7] found in liver.

The soluble fructokinase activity (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4) from Euglena can be compared with that described in peas, in which an aspecific, particle-bound hexokinase has also been found[8]. Fructokinase can be

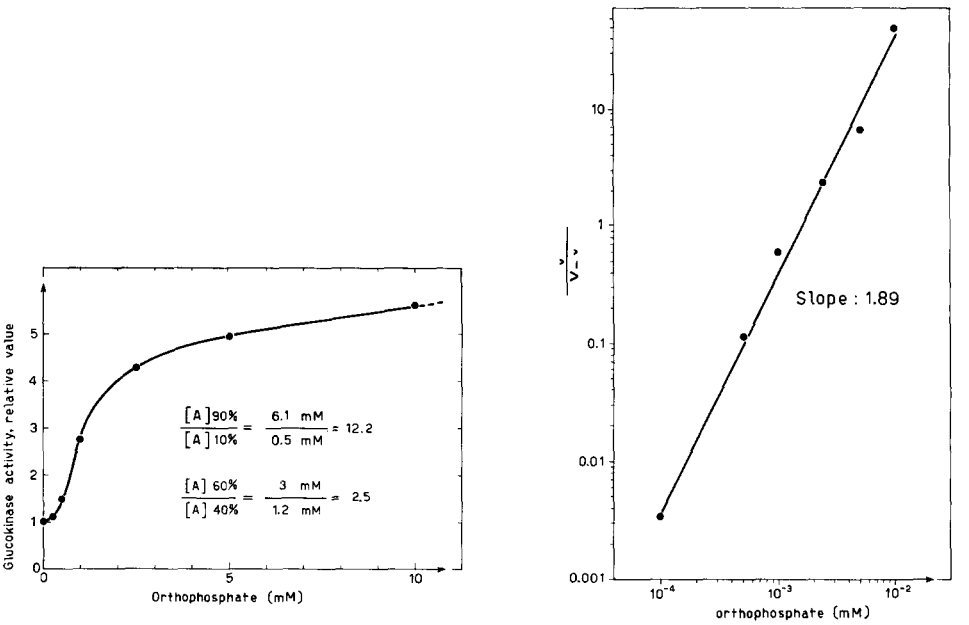


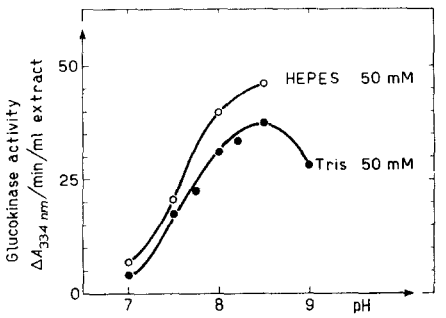
Fig. 1. Dependence of reaction velocity on the concentration of orthophosphate with Euglena particulate glucokinase. The orthophosphate concentrations ([A]) required to obtain respectively the 90% and 10% or 60% and 40% of maximal reaction velocity, at saturating orthophosphate, and their ratios are indicated. The fraction of activity in absence of orthophosphate was neglected. For assay conditions see Table I; orthophosphate concentrations were varied as indicated in the figure.

Fig. 2. Hill plot respect to concentration of orthophosphate with Euglena particulate glucokinase. Log scales v = reaction velocity, V = maximal reaction velocity at saturating orthophosphate. The velocity values are corrected by subtraction of the basal velocity in absence of orthophosphate.

purified about 10-fold by fractionation of the 105 000 × *g* supernatant with 40–60% $(NH_4)_2SO_4$.

Glucokinase is released from the 105 000 × *g* sediment by treatment with 1% Triton X100 (Table I). After centrifugation at 105 000 × *g*, about 70% of the glucokinase activity formerly present in the sediment was then recovered as soluble enzyme. This procedure gave a 5-fold, and over, purification with respect to the crude extract.

Besides specificity and subcellular localization, the two kinase activities differ in their sensitivity to inorganic phosphate. Fructokinase activity is only slightly activated by inorganic phosphate *in vitro*, whereas glucokinase activity was found to increase about 5-fold, and over, in the presence of phosphate. The effect of phosphate on glucokinase appears to be reversible: a high glucokinase activity is detected in the crude preparation after extraction in phosphate buffer. This activity disappears after dialysis against Tris or imidazole buffer and is almost completely restored upon addition of phosphate to the assay mixture. In the range 0–10 mM phosphate, the activation of the enzyme follows a sigmoid curve (Fig. 1), the ratio between the phosphate concentration required to give 90% *V* and that required to give 10% *V* being 12.2; this value corresponds to $n = 1.82$ (ref. 9). A cooperative type of inter-



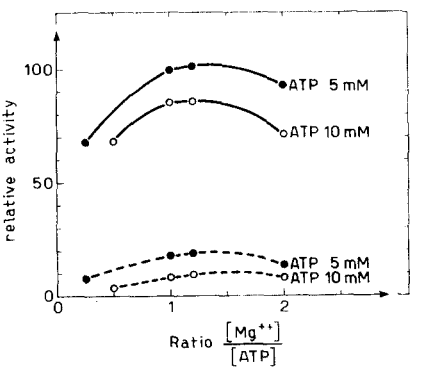


Fig. 3. Rate of reaction catalyzed by particulate glucokinase as pH function.

Fig. 4. Relationship between Mg/ATP ratio and glucokinase activity in the presence (——) or in the absence (- - - -) of orthophosphate. Assay conditions were as described for Table I; concentrations of Mg and ATP were varied as indicated in the figure.

action is further suggested by the Hill plot: log $(v/V - v) = n$ log [orthophosphate] − log k (Fig. 2), which gives a value for n ("interaction coefficient") of 1.89. These results suggest that the activation of glucokinase by phosphate is due to an allosteric transition. The kinetics of the glucokinase reaction as a function of the Mg–ATP complex have been investigated. At pH 8.5, *i.e.* the optimum for the reaction under these experimental conditions (Fig. 3), and with 100 mM glucose, the K_m for glucose being 8 mM with 5 mM Mg–ATP, the optimum Mg/ATP ratio was found to be 1.2, both in the presence and in the absence of phosphate (Fig. 4). This ratio was maintained in all further experiments. The K_m value for the Mg–ATP complex was 1 mM at different phosphate concentrations (Fig. 5). High concentrations of Mg–ATP inhibit glucokinase activity. From the data reported in Fig. 5 it

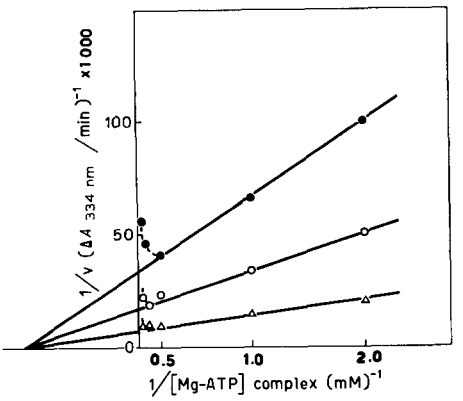


Fig. 5. Effect of orthophosphate concentration on particulate glucokinase activity at various Mg–ATP complex concentrations. Orthophosphate was varied as follows: ●—●, 0.1 mM; ○—○, 1 mM; △—△, 10 mM.

can be concluded that the activation by phosphate occurs in at least two ways: (1) by counteracting the inhibition by Mg–ATP; (2) by activating the enzyme directly, regardless of the Mg–ATP concentration.

The inhibitory effect of high concentration of Mg–ATP had already been described for frog muscle[10], and also in this case the inhibition was found to be relieved by phosphate. Hexokinase activities from erythrocytes[11,12], ascite tumor cells[13], frog muscle[10] and brain[14–16] were found to be inhibited by glucose 6-phosphate and activated by phosphate by relieving this inhibition. An allosteric mechanism has been proposed[18] to explain the activation by phosphate of hexokinase from erythrocytes and ascite tumor cells which counteracts the allosteric inhibition due to glucose 6-phosphate accumulation. In Euglena the conditions for the assay of the enzyme[17] allow one to rule out the possibility of glucose 6-phosphate accumulation, and to conclude that phosphate affects the enzyme activity directly, regardless of either Mg–ATP or the glucose 6-phosphate concentrations.

Both hexokinase activities detected in heterotrophically grown cells are also found in cells grown in the light with CO_2 as the sole carbon source; they show the same subcellular distribution and the same characteristics. As far as glucokinase is concerned, the activation by phosphate is also clearly detectable in enzyme extracted from cells grown autotrophically. The hypothesis can therefore be put forward that *in vivo* as well as *in vitro* a control mechanism on glucose utilization is operating through changes in free phosphate concentrations. Such an effect has been shown *in vivo* in red blood cells[19].

REFERENCES

1 R. E. Hurlbert and S. Rittemberg, *J. Protozool.*, 9 (1962) 170.
2 M. M. Belsky and J. Schultz, *J. Protozool.*, 9 (1962) 200.
3 H. Lyman and H. W. Siegelman, *J. Protozool.*, 14 (1967) 297.
4 G. D. Walker, *Essays in Biochemistry*, Vol. 2, Academic Press, London and New York, 1966, p. 33.
5 R. K. Crane, *The Enzymes*, Vol. 6, Academic Press, London and New York, 1966, p. 47.
6 P. Saltman, *J. Biol. Chem.*, 200 (1953) 145.

7 G. BERTHILLIER, L. COLOBERT, M. RICHARD AND G. GOT, *Biochim. Biophys. Acta*, 206 (1970) 1.
8 A. MEDINA AND A. SOLS, *Biochim. Biophys. Acta*, 19 (1956) 378.
9 K. TAKETA AND B. M. POGELL, *J. Biol. Chem.*, 240 (1965) 651.
10 S. KARPATKIN, *J. Biol. Chem.*, 242 (1967) 3525.
11 I. A. ROSE AND E. L. O'CONNELL, *J. Biol. Chem.*, 239 (1964) 12.
12 S. RAPOPORT, *Essays in Biochemistry*, Vol. 4, Academic Press, London and New York, 1968, p. 69.
13 R. C. MCCOMB AND W. D. YUSHOK, *Biochim. Biophys. Acta*, 34 (1959) 515.
14 M. D. JOSHI AND V. JAGANNATHAN, *Arch. Biochem. Biophys.*, 125 (1968) 460.
15 E. A. NEWSHOLM, F. S. ROLLESTONE AND K. TAYLOR, *Biochem. J.*, 106 (1968) 193.
16 C. L. MOORE, *Arch. Biochem. Biophys.*, 128 (1968) 734.
17 R. K. CRANE AND A. SOLS, *J. Biol. Chem.*, 203 (1953) 273.
18 E. R. STADTMAN, *Adv. Enzymol.*, 28 (1966) 41.
19 I. A. ROSE, J. V. B. WARMS AND E. L. O'CONNELL, *Biochem. Biophys. Res. Commun.*, 15 (1964) 33.