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COMPLEMENTARITY IN THE REGULATION OF PHOSPHOGLUCOMUTASE, PHOSPHOFRUCTOKINASE AND HEXOKINASE; THE ROLE OF GLUCOSE 1,6-BISPHOSPHATE

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Summary

ATP and citrate, the well known inhibitors of phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), were found to inhibit the activities of the multiple forms of phosphoglucomutase (α-D-glucose 1,6-bisphosphate: α-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) from rat muscle and adipose tissue. This inhibition could be reversed by an increase in the glucose 1,6-bisphosphate (Glc-1,6-P_2) concentration. Other known activators (deinhibitors) of phosphofructokinase, viz. cyclic AMP, AMP, ADP or P_i, had no direct deinhibitory action on the ATP or citrate inhibited multiple phosphoglucomutases. Cyclic AMP and AMP, could however lead indirectly to deinhibition of the phosphoglucomutases, by activating phosphofructokinase which catalyzes the ATP-dependent phosphorylation of glucose 1-phosphate to form Glc-1,6-P_2, the latter then released the multiple phosphoglucomutases from ATP or citrate inhibition. The Glc-1,6-P_2 was also found to exert a selective inhibitory effect on hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) type II, the predominant form in skeletal muscle. This selective inhibition by Glc-1,6-P_2 was demonstrated on the multiple hexokinases which were resolved by cellogel electrophoresis or isolated by chromatography on DEAE-cellulose. Based on the in vitro studies it is suggested that during periods of highly active epinephrine-induced glycogenolysis in muscle, the Glc-1,6-P_2, produced by the cyclic AMP-stimulated reaction of phosphofructokinase with glucose 1-phosphate, will release the phosphoglucomutases from ATP or citrate inhibition, and will depress the activity of muscle type II hexokinase.

Introduction

Previous studies done in this laboratory [1] have revealed that ATP is a potent inhibitor of the multiple forms of phosphoglucomutase (α-D-glucose

1,6-bisphosphate: α-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) from rat adipose tissue. We suggested that this control of phosphoglucomutase activities by ATP may complement phosphofructokinase regulation. In the present investigation we looked for other common effectors of these two enzymes. These experiments revealed the regulatory role of glucose 1,6-bisphosphate (Glc-1,6-P_2) on the phosphoglucomutase activities and shed further light on the complementarity between the phosphoglucomutases and phosphofructokinase.

Since Glc-1,6-P_2 was also reported to be a positive effector of the two key enzymes in glycolysis, namely, phosphofructokinase [2,3] and pyruvate kinase [4], we found it of interest to study whether it has any effect on the activities of hexokinase, the third key enzyme in glycolysis. The results reported here reveal that Glc-1,6-P_2 inhibits selectively type II hexokinase. The complementarity in the regulation of phosphoglucomutase, phosphofructokinase and hexokinase will be discussed in view of the results obtained.

Materials and Methods

Materials

Cellogel strips for Beckman Microzone Electrophoresis were obtained from Chemetron (via Gustavo Modena, 24-Milano, Italy). Glucose 1-phosphate, glucose 1,6-bisphosphate ATP, $NADP^+$, adenosine cyclic 3′:5′-monophosphate, phenazine methosulfate, DEAE-cellulose (fine), muscle phosphofructokinase (type III) and yeast glucose 6-phosphate dehydrogenase (type V), were purchased from Sigma Chemical Company. AMP was obtained from Serva. Nitroblue tetrazolium was from Fluka. Imidazole was the low fluorescence grade from Sigma.

Extraction, electrophoresis and assay of the multiple forms of phosphoglucomutase

Extracts of epididymal fat pads from male albino rats (120—150 g) were prepared as described previously [1]. Leg muscles extracts from the same rats and from rabbits (2.5 kg) were similarly prepared by homogenizing the tissues in cold double distilled water (2 ml water per g of muscle) for 15 s in an ice bath using a Willems-Polytron-Homogenizer (Model PT 10 ST "OD", Kinematica) at a setting of 5. The homogenate was centrifuged at 4° C for 15 min at 27 000 × *g*. Aliquots of 0.25 μl (1—3 μg protein) of the clear supernatant were placed on cellogel strips using the Beckman microzone electrophoresis cell (Model R-101). Electrophoresis was conducted at 250 V for 60—80 min in $7 \cdot 10^{-4}$ M citric acid and $43 \cdot 10^{-4}$ M Na_2HPO_4 (pH 7.0). After electrophoresis the cellogels were cut into several parts, each part was then stained for phosphoglucomutase activity under the conditions given in the figure legends. Controls were run simultaneously in which the substrate, coenzyme or the auxiliary enzyme was omitted.

Another part of the clear supernatant solution was diluted with three volumes of cold double distilled water and 10-μl aliquots (10—30 μg protein) were used for spectrophotometric measurements of total enzymatic activity. The assay was a modification of the spectrophotometric method used by Joshi

et al. [5]. The assay medium was similar to the staining medium (see figure legends) except for omission of the phenazine methosulfate and nitro-blue tetrazolium. The final volume was 1.0 ml. Activity was determined at room temperature by measuring the rate of increase in optical density at 340 nm. One unit of enzyme activity catalyzed the reduction of 1 μmol of $NADP^+$ per min. It should be noted that the activity of the auxiliary enzyme (glucose 6-phosphate dehydrogenase) which was added in excess, was not affected by the different conditions investigated in the present study.

Extraction and electrophoresis of the multiple forms of hexokinase

Tissues from male albino rats (100—130 g) were homogenized in an equal volume (fat pads, in 0.1 volume, w/v) of cold 0.1 M Tris · Cl buffer (pH 7.4) containing 5 mM EDTA and 5 mM 2-mercaptoethanol, for 10—15 s in an ice bath using a Willems-Polytron-Homogenizer (Model PT 10ST "OD", Kinematica) at a setting of 5. The homogenates were centrifuged at 4°C for 35 min at 27 000 × *g*. Aliquots of 1.25—1.75 μl (0.020—0.040 mg protein) of the clear supernatant were placed on cellogel strips using the Beckman microzone electrophoresis cell (Model R-101). Electrophoresis was conducted at 250 V for 50 min in 0.02 M sodium barbital buffer (pH 8.4) containing 2.7 mM EDTA and 5 mM 2-mercaptoethanol. Cellogels were stained for hexokinase activity at 37°C in the dark for 30—45 min in a reaction mixture similar to that described by Katzen [6] with some modifications; it consisted of 0.1 M Tris · Cl buffer (pH 7.4), 0.75 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM ATP, 2 mM KCN, 0.6 international units · ml^{-1} glucose 6-phosphate dehydrogenase (Sigma), 0.1 M glucose, 0.1 mM phenazine methosulfate and 0.4 mM nitro-blue tetrazolium. The developed cellogels were washed and preserved in 40% methanol.

Spectrophotometric assay of hexokinase activities

The assay for hexokinase activities was a modification of the spectrophotometric method used by Sharma et al. [7]. The assay mixture was similar to the staining mixture (see above) except for omission of the phenazine methosulfate, nitro-blue tetrazolium and KCN. The final volume was 1.0 ml. Reactions were conducted at 30°C and continuous recording of the increase in optical density at 340 nm resulting from $NADP^+$ reduction were made in a Gilford model 6400 spectrophotometer. Controls were recorded for each experiment in which the enzyme, the substrates or the auxiliary enzyme were omitted. It should be noted that the activity of the auxiliary enzyme (glucose 6-phosphate dehydrogenase), which was added in excess, was not affected by the Glc-1,6-P_2 investigated in the present study.

One unit of hexokinase activity is defined as the amount of enzyme which transforms 1 μmol of substrate per min at 30°C under the described assay conditions. Specific activities are expressed as units per mg protein.

DEAE-cellulose column chromatography of hexokinases

DEAE-cellulose chromatography of the multiple hexokinases from extracts of epididymal fat pads or leg muscles from young (100—130 g) male rats, was conducted according to the procedure of Katzen et al. [8], which is a slight modification of the procedures previously described [9,10].

Protein determination

Protein was measured by the method of Lowry et al. [11] with crystaline bovine serum albumin as a standard.

Determination of glucose 1,6-bisphosphate

Glucose 1,6-bisphosphate was measured by the fluorometric method of Passonneau et al. [12].

Number of experiments

Each of the experiments illustrated in Figs 1—8 is representative of 10—20 experiments all of which gave identical results.

Results

We found earlier [1] that the fastest migrating anodal form of phosphoglucomutase on cellogel electrophoresis (form 1) from the rat adipose tissue, in contrast to the other two forms, did not require added Mg^{2+} for maximal activity. As shown in Fig. 1 this form was not found in the rat skeletal muscle. The rat muscle contained only two forms, both of which were activated by added Mg^{2+}. Their electrophoretic migration corresponded to forms 2 and 3 of the rat adipose tissue. Occasionally an additional band, which stained very weakly, was detected in the rat adipose tissue and muscle; its migration was between form 1 and 2 and very close to the latter. This might be a dephosphorylated form of one of the major forms [5]. Joshi et al. [5] have similarly separated two forms of phosphoglucomutase from rat, rabbit and human muscle by column chromatography. The cellogel electrophoretic patterns of the

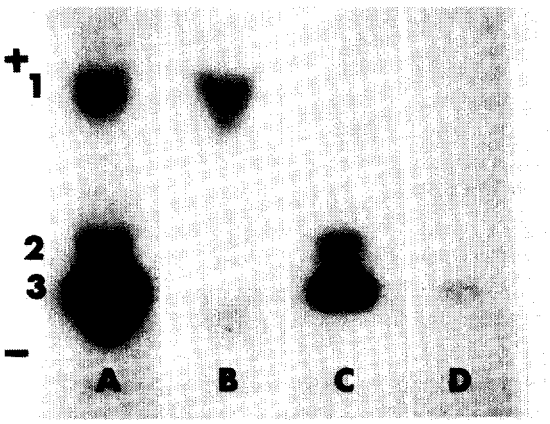

Fig. 1. Comparison between the electrophoretically distinct forms of phosphoglucomutase from rat muscle and rat adipose tissue and their response to Mg^{2+}. A, adipose tissue activities with Mg^{2+}, and B, without Mg^{2+}; C, skeletal muscle activities with Mg^{2+}, and D, without Mg^{2+}. Extraction and electrophoresis as described in Materials and Methods. After electrophoresis the cellogels were cut into four parts; Parts A and C were developed for phosphoglucomutase activity at 37° C for 20 min in a reaction mixture consisting of 40 mM imidazole-HCl (pH 7.2), 3 mM $MgCl_2$, 1.5 mM EDTA, 2 mM glucose 1-phosphate, 1 μM Glc-1,6-P_2, 3 units · ml^{-1} glucose 6-phosphate dehydrogenase, 0.5 mM $NADP^+$, 0.1 mM phenazine methosulfate and 0.4 mM nitro-blue tetrazolium. Parts B and D were developed in an identical medium, except for absence of $MgCl_2$.

phosphoglucomutases from rat muscle or adipose tissue were not changed when extraction of the enzymes was performed in the presence of 2-mercaptoethanol.

Inhibition of the multiple phosphoglucomutases by ATP and by citrate, and its reversal by glucose 1,6-bisphosphate (Glc-1,6-P_2)

As shown in Fig. 2, ATP strongly inhibited the activities of both forms of phosphoglucomutase from rat muscle; this is similar to the effect we found in rat adipose tissue [1]. ATP inhibition could be reversed by an increase in the Glc-1,6-P_2 concentration (Fig. 2C). This deinhibition was studied under different degrees of ATP inhibition and with varying concentrations of Glc-1,6-P_2. Spectrophotometric measurements revealed that the total activity of the muscle phosphoglucomutase decline to 15 ± 2% of the control values in the presence of 3.5 mM ATP and was reversed to 73 ± 4% of the control values by increasing the concentration of Glc-1,6-P_2 to 50 μM (values are means ±S.E. for 10 experiments, $p < 0.001$). At lower degrees of ATP inhibition, less Glc-1,6-P_2 was required for complete reversal of the inhibition.

Glc-1,6-P_2 has also been reported [2,3] to be one of the strongest activators and deinhibitors of phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11). The similarity in the response of phosphoglucomutase and phosphofructokinase to ATP and to Glc-1,6-P_2, prompted us to investigate whether there are additional common effectors of these two enzymes. As shown in Figs 2D and 2E, neither cyclic AMP nor AMP could reverse the ATP inhibited phosphoglucomutases, nor did ADP or P_i (not shown). (The deinhibition was studied under various levels of ATP, between 1—3.5 mM, and with varying concentrations of the adenine nucleotides or P_i). However, on the other hand, citrate was found to be another common inhibitor of phosphofructokinase and the phosphoglucomutases. It inhibited both forms of phosphoglucomutase from rat muscle (Fig. 3B) as well as all the three forms from rat adipose tissue (Figs 4B and 4C). The inhibitory effect of citrate was more pronounced in Tris-HCl buffer than in imidazole buffer. The total activity of phosphoglucomutase measured in mouse muscle was also reported to be inhibited by citrate [13]. These investigators suggested that the inhibitory action of citrate was due to the removal of the Mg^{2+} necessary for the activity

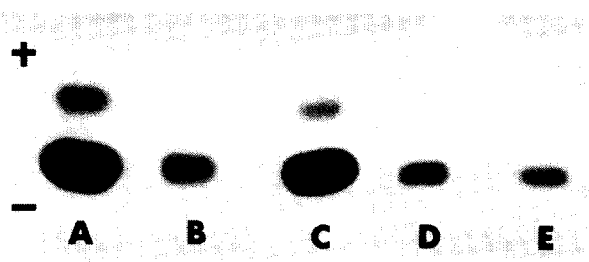

Fig. 2. Inhibition of the muscle phosphoglucomutases by ATP and deinhibition by Glc-1,6-P_2. Conditions as for Fig. 1C. After electrophoresis the cellogels were cut into 5 parts, each developed for phosphoglucomutase activity under the following conditions: A, control, without ATP; B, with ATP (3.5 mM); C, with ATP (3.5 mM) and Glc-1,6-P_2 (50 μM); D, with ATP (3.5 mM) and cyclic AMP (0.2 mM); E, with ATP (3.5 mM) and AMP (0.5 mM).

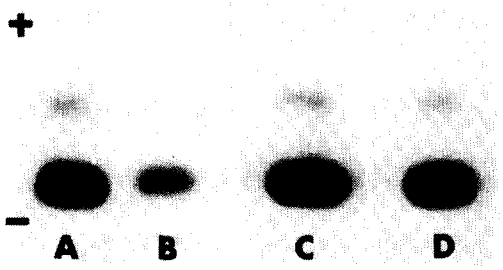

Fig. 3. Inhibition of the muscle phosphoglucomutases by citrate and deinhibition by Glc-1,6-P_2 or by Mg^{2+}. A, control, without citrate; B, with citrate (2.5 mM); C, with citrate (2.5 mM) and Glc-1,6-P_2 (50 μM); D, with citrate (2.5 mM) and Mg^{2+} (2.5 mM). Extraction and electrophoresis as described in Materials and Methods. After electrophoresis the cellogels were cut into 4 parts; one part (A) was developed for phosphoglucomutase activity at 37°C for 20 min in a reaction mixture consisting of 50 mM Tris · HCl (pH 7.2), 0.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM glucose 1-phosphate, 1 μM Glc-1,6-P_2, 3 units · ml^{-1} glucose 6-phosphate dehydrogenase, 0.5 mM $NADP^+$, 0.1 mM phenazine methosulfate and 0.4 mM nitro-blue tetrazolium. The other three parts were developed in an identical medium, except for the presence of 2.5 mM sodium citrate (B); 2.5 mM sodium citrate and 50 μM Glc-1,6-P_2 (C); 2.5 mM sodium citrate and 2.5 mM $MgCl_2$ (D).

of the enzyme since the inhibition could be reversed by adding Mg^{2+}. We could indeed reverse the citrate inhibition of the multiple forms of phosphoglucomutase by an increase in the Mg^{2+} concentration (Figs 3D and 4E). Similarly, the ATP inhibition could also be reversed by increasing Mg^{2+} concentration. However, since we found that ATP [1] as well as citrate (Figs 4B and 4C) also inhibited the activity of form 1 from rat adipose tissue, which does not require added Mg^{2+} for maximal activity (see Fig. 1), it therefore seems most unlikely that these two ligands inhibit this form by complexing Mg^{2+} (we reported earlier [1] that the activity of this form, in the absence of Mg^{2+}, did not change even when EDTA was added in concentrations 10—20 times higher). The simple reversal of the ATP or citrate inhibition of all the multiple forms of phosphoglucomutase by added Mg^{2+} does not in itself demonstrate that these anions

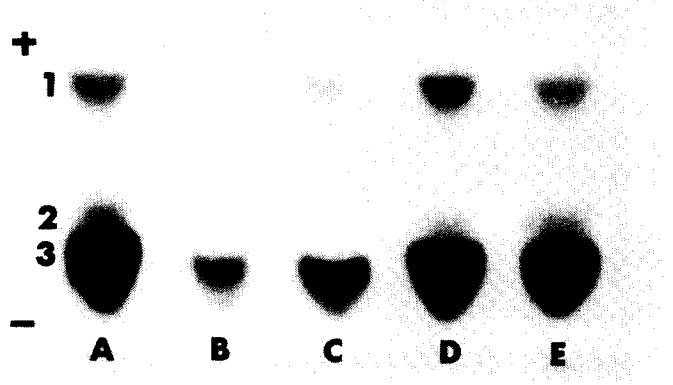

Fig. 4. Inhibition of the adipose tissue phosphoglucomutases by citrate and deinhibition by Glc-1,6-P_2 or by Mg^{2+}. Conditions as for Fig. 3. A, control, without citrate; B, with citrate (5 mM); C, with citrate (2.5 mM); D, with citrate (2.5 mM) and Glc-1,6-P_2 (50 μM); E, with citrate (2.5 mM) and Mg^{2+} (2.5 mM).

inhibit by chelating Mg^{2+}; the effect may instead be anionic inhibition competitive with Glc-1,6-P_2, which is relieved by formation of the anion · Mg complex. The ATP inhibition as well as the citrate inhibition of all the multiple forms of phosphoglucomutase, could also be reversed by an increase in the Glc-1,6-P_2 concentration (Figs 2C and 3C and 4D). Spectrophotometric measurements revealed that 2.5 mM citrate caused a 63 ± 2% and 61 ± 1% inhibition of the total phosphoglucomutase activity from rat muscle and adipose tissue respectively (the values are means ± S.E. for 10 experiments, $p < 0.001$). This inhibition was completely reversed by increasing the concentration of Glc-1,6-P_2 to 50 μM.

The release of the multiple phosphoglucomutases from ATP or citrate inhibition mediated through activation of phosphofructokinase

The experiments presented above reveal that the activities of the multiple forms of phosphoglucomutase are inhibited by ATP and by citrate and that this inhibition can be reversed by an increase in the Glc-1,6-P_2 concentration. If we assume that such regulation occurs in vivo, the question then arises what is the possible source of Glc-1,6-P_2 in cells, outside the phosphoglucomutase reaction itself [12,14,15], which will release the phosphoglucomutase activities from ATP or citrate inhibition. Two different mechanisms for the formation of Glc-1,6-P_2, outside of the phosphoglucomutase reaction itself, were proposed in the literature: one is by the glucose 1-phosphate kinase reaction [16] and the other by the glucose 1-phosphate transphosphorylase reaction [17]. More recently it has been reported [18,19] that the "glucose 1-phosphate kinase" which catalyzes the ATP-dependent phosphorylation of glucose 1-phosphate to Glc-1,6-P_2 is most probably identical with phosphofructokinase. According to these authors this reaction is the main route for Glc-1,6-P_2 synthesis in the skeletal muscle.

In the experiments shown in Fig. 5 we tested this possible source of Glc-1,6-P_2 synthesis by substituting the Glc-1,6-P_2 in the developing mixture for phosphoglucomutase activities (containing purified glucose 1-phosphate) with highly purified muscle phosphofructokinase and low concentration of

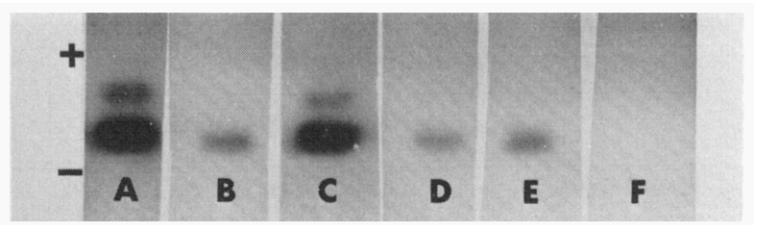

Fig. 5. Activation of the muscle multiple phosphoglucomutases, in the absence of Glc-1,6-P_2, by the addition of purified muscle phosphofructokinase and low concentrations of ATP. Extraction and electrophoresis as described in Materials and Methods. After electrophoresis the the cellogels were cut into six parts. Part A was developed for phosphoglucomutase activity in the presence of Glc-1,6-P_2, as described in Fig. 1C; Part B was developed in an identical medium except for absence of Glc-1,6-P_2. The medium contained purified glucose 1-phosphate (a lot from Sigma essentially free of Glc-1,6-P_2); Part C was developed in an identical medium as part B, in the absence of Glc-1,6-P_2, except for the addition of purified muscle phosphofructokinase (Sigma) 1.4 units · ml^{-1} and ATP (0.1 mM). Parts D, E and F, were developed in an identical medium as part C except for omission of phosphofructokinase (part D), or ATP (part E), or glucose 1-phosphate (part F).

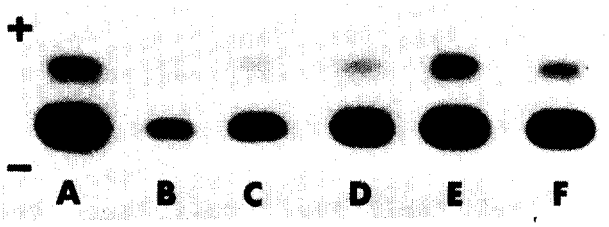

Fig. 6. Inhibition of the muscle multiple phosphoglucomutases by high ATP and deinhibition by cyclic AMP or AMP in a medium containing phosphofructokinase. A, the muscle phosphoglucomutases which were developed in a reaction mixture as for Fig. 5C, containing phosphofructokinase and low concentration of ATP (0.1 mM) and no added Glc-1,6-P_2; B, as A except that the concentration of ATP was increased to 1 mM; C—E, as B, with the high ATP (1 mM), but the medium also contained cyclic AMP at 5 μM (C), 10 μM (D) and 25 μM (E); F, as B, with the high ATP (1 mM), but the medium also contained 0.3 mM AMP.

ATP. In this system an activation of both forms of the muscle phosphoglucomutase was found (Fig. 5C). Their staining intensity was similar to that obtained in the presence of Glc-1,6-P_2 (Fig. 5A). Identical results were obtained with the two forms of phosphoglucomutase from rat or the rabbit skeletal muscle. It is evident that this activation of the phosphoglucomutases resulted from the Glc-1,6-P_2 which was synthesized by the ATP-dependent phosphorylation of glucose 1-phosphate catalyzed by the phosphofructokinase, from the controls which were simultaneously developed (Figs 5D, E, F) and in which phosphofructokinase, or ATP or glucose 1-phosphate were omitted respectively; no activation of the phosphoglucomutases occurred in these controls. Fluorometric measurements of Glc-1,6-P_2 revealed directly that Glc-1,6-P_2 was indeed produced by the action of the added phosphofructokinase on glucose 1-phosphate; the actual levels of Glc-1,6-P_2 formed under the experimental conditions employed in Fig. 5C were found to be 7.0 ± 0.5 μM (mean ± S.E. for 10 experiments), which are more than adequate to bring about a full activation of the phosphoglucomutases.

Increasing the concentration of ATP in the reaction mixture containing the phosphofructokinase, caused an inhibition of the activity of both forms of phosphoglucomutase (Fig. 6B). This inhibition could be released by adding cyclic AMP (Figs 6C—E) or AMP (Fig. 6F). The reaction was extremely sensitive to cyclic AMP; deinhibition by cyclic AMP could already be detected at concentrations of 2—5 μM which are within the concentration range found in skeletal muscle after epinephrine treatment [20]. ADP or P_i had no significant deinhibitory action. Since, as shown before (Fig. 2), the cyclic AMP or AMP exerted no direct deinhibitory action on the ATP-inhibited activities of phosphoglucomutase, it is evident that the deinhibition of the phosphoglucomutases in the present system (Fig. 6) by added cyclic AMP or AMP, was mediated through activation (deinhibition) of the phosphofructokinase by these effectors; the activated phosphofructokinase catalyzed the synthesis of Glc-1,6-P_2 from glucose 1-phosphate, and Glc-1,6-P_2 released both forms of phosphoglucomutase from ATP inhibition.

As shown in Fig. 7, citrate also caused an inhibition of the activities of both muscle phosphoglucomutases developed in the medium in which the Glc-

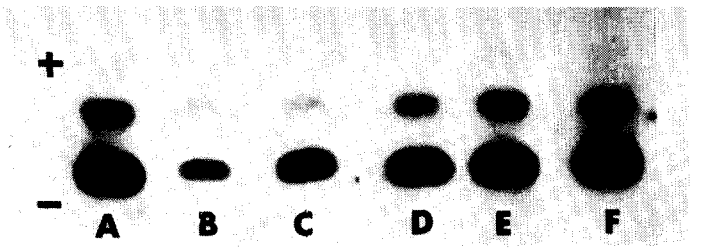

Fig. 7. Inhibition of the muscle multiple phosphoglucomutases by citrate and deinhibition by cyclic AMP or AMP in a medium containing phosphofructokinase and low concentration of ATP. A, the muscle phosphoglucomutases which were developed in a reaction mixture as for Fig. 5C, containing phosphofructokinase and low concentration of ATP (0.1 mM) and no added Glc-1,6-P_2; B, as A except that citrate (1.5 mM) was added; C—E, with citrate (1.5 mM) and cyclic AMP at 5 μM (C), 10 μM (D) and 25 μM (E); F, with citrate (1.5 mM) and AMP (0.3 mM).

1,6-P_2 was substituted with purified muscle phosphofructokinase and low concentration of ATP. The citrate inhibition like the ATP inhibition, could be reversed by cyclic AMP or AMP, by a similar mechanism mediated through activation (deinhibition) of phosphofructokinase, since we found that these effectors did not exert any direct deinhibitory effect on the citrate-inhibited phosphoglucomutases.

Glc-1,6-P_2, an inhibitor of hexokinase type II

Glc-1,6-P_2 has been reported to be a positive effector of the two key enzymes in glycolysis: phosphofructokinase [2,3] and pyruvate kinase [4]. These reports as well as the experiments described above, which revealed its regulatory role on the multiple forms of phosphoglucomutase, prompted us to investigate whether Glc-1,6-P_2 also affects hexokinase, the third key enzyme in glycolysis. The effect of Glc-1,6-P_2 was investigated on the different forms of rat tissue hexokinase which were resolved by cellogel electrophoresis (Fig. 8) or partially purified by chromatography on DEAE-cellulose (Table I, Fig. 9). Fig. 8 shows the cellogel electrophoresis patterns of the multiple hexokinases from adipose tissue, brain and muscle of young (100—130 g) rats, developed in

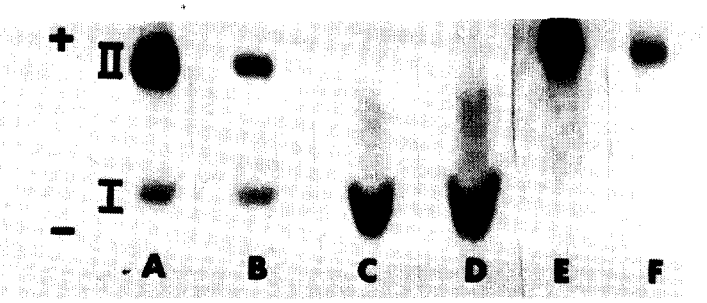

Fig. 8. The effect of Glc-1,6-P_2 on the multiple hexokinases from rat tissues resolved by cellogel electrophoresis. Extraction, electrophoresis and color development were conducted as described in Materials and Methods. The cellogels after electrophoresis were stained for hexokinase activity in the absence and presence of 100 μM Glc-1,6-P_2. A, adipose tissue; B, adipose tissue with Glc-1,6-P_2; C, brain; D, brain with Glc-1,6-P_2; E, skeletal muscle; F, skeletal muscle with Glc-1,6-P_2. Tissues were from young (100—130 g) male rats. The specificity for hexokinase activity was indicated by the lack of color development in the absence of either glucose or ATP.

TABLE I

THE EFFECT OF Glc-1,6-P_2 ON THE PARTIALLY PURIFIED HEXOKINASES FROM RAT ADIPOSE TISSUE AND SKELETAL MUSCLE

All enzyme types were isolated by chromatography on DEAE-cellulose and assayed spectrophotometrically as described in Materials and Methods, in the absence (Control) and presence of 50 μM Glc-1,6-P_2. Tissues were from young (100—130 g) male rats. Values are means ± S.E. for 7 experiments (fat pad) or 6 experiments (muscle).

Type and source	Specific activity (units/mg protein)		Inhibition by Glc-1,6-P_2	
	Control	With 50 μM Glc-1,6-P_2	%	P value
I (Fat pad)	0.07 ± 0.01	0.07 ± 0.01	0.1 ± 0.1	>0.1
II (Fat pad)	1.04 ± 0.15	0.62 ± 0.10	40.5 ± 1.7	<0.001
II (Muscle)	0.69 ± 0.07	0.38 ± 0.04	45.6 ± 2.2	<0.001

the absence or presence of Glc-1,6-P_2 in the reaction mixture. The hexokinases were designated as types I and II according to the nomenclature of Katzen and Schimke [9]. (II, being the faster migrating anodal form). Adipose tissue contained both forms but the activity of type I was proportionately less than type II (in older rats (250 g) the ratio of type I to type II was reversed, confirming the results of Katzen and Schimke [9] and Katzen [6]). Brain hexokinase was virtually all type I, whereas skeletal muscle hexokinase activity consisted mainly of type II (with heavier staining small tracers of type I in muscle and type II in brain could also be visualized). The tissue distribution of the multiple hexokinase resolved by the cellogel electrophoresis was very similar to that reported by Katzen and Schimke [9], who used the method of starch gel electrophoresis. The cellogel electrophoresis is however much simpler and less time consuming. It can be seen in Fig. 8 that Glc-1,6-P_2 exerted a selective inhibitory effect on hexokinase type II and had no effect on type I, regardless of the enzyme's tissue source. Under identical conditions experiments with liver ex-

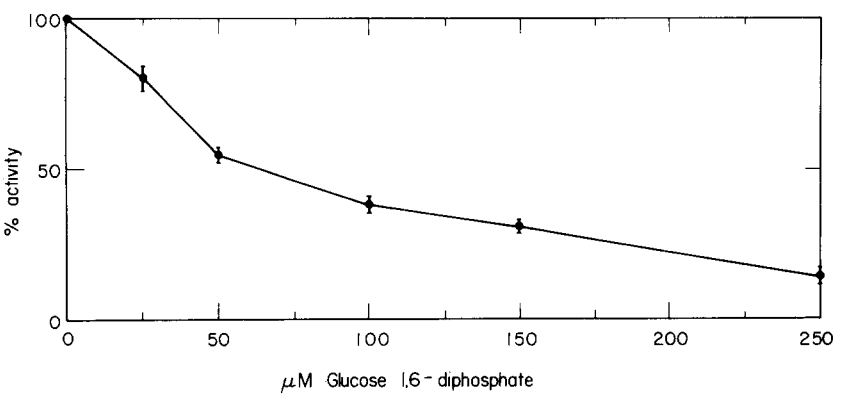

Fig. 9. The effect of varying Glc-1,6-P_2 concentration on the activity of the partially purified skeletal muscle type II hexokinase. Hexokinase type II was isolated from leg muscles of 100—130 g male rats by chromatography on DEAE-cellulose, and assayed spectrophotometrically as described in Materials and Methods, in the presence of varying concentrations of Glc-1,6-P_2. Vertical bars represent standard errors of the mean for 6 experiments.

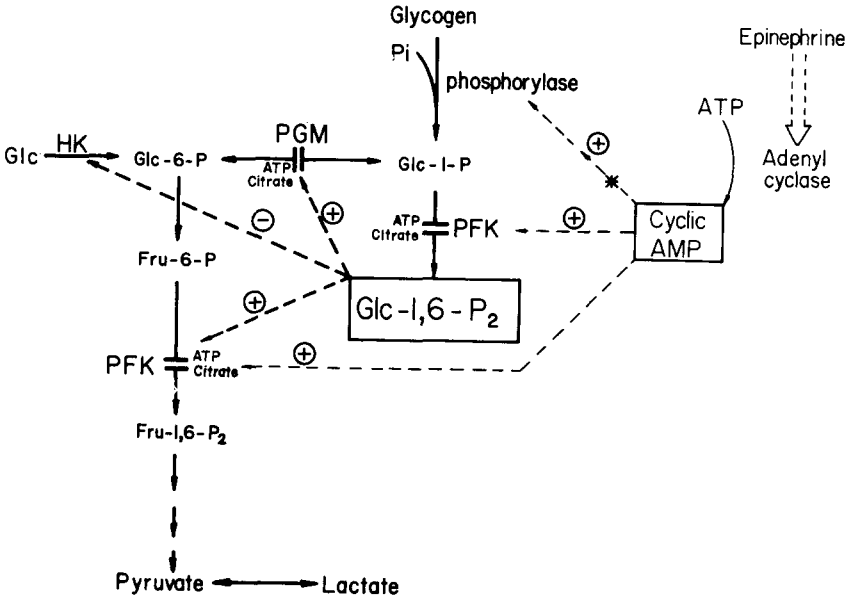

Fig. 10. Proposed scheme showing the regulatory role of Glc-1,6-P_2 on phosphoglucomutase (PGM), hexokinase (HK) and phosphofructokinase (PFK) in mammalian skeletal muscle. Inhibition by ATP and citrate is indicated by ⊖,*the well established mechanism of epinephrine action leading to the active phosphorylase a [31,32] is not shown in this scheme.

tracts (not shown) also revealed a selective inhibition of type II by Glc-1,6-P_2 and no effect on types I, III, or on the type IV (the liver specific, fastest migrating anodal form).

The effect of Glc-1,6-P_2 was also determined spectrophotometrically on the partially purified hexokinase types from rat adipose tissue and skeletal muscle obtained from DEAE-cellulose chromatography (Table I). When the partially purified hexokinase types obtained from DEAE-cellulose chromatography were examined by cellogel electrophoresis, each enzyme type was found to retain the unique mobility it possessed in crude homogenates. The specific activities of the partially purified hexokinase types obtained from the DEAE-cellulose chromatography (control values in Table I) were similar to those reported by Grossbard and Schimke [10]. It can be seen in Table I that the addition of 50 μM Glc-1,6-P_2 to the reaction mixture, caused a 45.6 ± 2.2% inhibition of the activity of the skeletal muscle type II hexokinase and a similar (40.5 ± 1.7%) inhibition of this enzyme type in fat pad. The activity of type I from the fat pad was not affected by Glc-1,6-P_2. Fig. 9 illustrates the inhibitory effect of Glc-1,6-P_2 from 0—250 μM on the partially purified skeletal muscle type II hexokinase. It can be seen that the degree of inhibition was dependent on the concentration of Glc-1,6-P_2. With 25 μM Glc-1,6-P_2 the enzyme activity was already reduced to 80% of its normal values.

Discussion

As pointed out by Mansour in his recent reviews on phosphofructokinase [21,22], the evidence is now accumulating, indicating complementarity in the

regulation of different enzymes for the benefit of cellular metabolic control. The results of the present study suggest a complementarity in the regulation of phosphofructokinase, phosphoglucomutase and hexokinase, as illustrated in Fig. 10. Phosphoglucomutase has been recently categorized as a regulatory enzyme [23] since intracellular substrate-product ratio may be significantly displaced from equilibrium [14,24]. The experiments described here reveal that the activities of the multiple forms of phosphoglucomutase are inhibited by the same negative modifiers as for phosphofructokinase, namely, ATP and citrate. It is also possible that other common inhibitors may play a role in the control of these enzymes; 3-phosphoglycerate and 2,3-diphosphoglycerate were reported to inhibit the phosphoglucomutase reaction (competitively with Glc-1,6-P_2) [14], and these two metabolites are also effective inhibitors of phosphofructokinase [3,25]. The possible physiological significance of the inhibition of phosphoglucomutase by the same negative modifiers of phosphofructokinase, is prevention of the reactivation of phosphofructokinase by Glc-1,6-P_2 (one of its strongest deinhibitors [2,3]), which might have been produced by the phosphoglucomutase reactions with 1,3-diphosphoglycerate [14,15] or with Fru-1,6-P_2 [12]. Thus both enzymes are kept under common inhibition. A similar complementary was observed in our earlier studies [26] between aldolase and phosphofructokinase with regard to ATP inhibition. It is possible that the phosphoglucomutase like phosphofructokinase [27] is in the ATP-inhibited form under conditions which might well exist in the cell.

The present experiments reveal also that the ATP or citrate inhibition of the multiple phosphoglucomutases, can be reversed by an increase in the Glc-1,6-P_2 concentration but not by the other activators (deinhibitors) of phosphofructokinase. The main source of Glc-1,6-P_2 in cells, outside the phosphoglucomutase reactions themselves [12,14,15] is most probably the phosphofructokinase reaction with glucose 1-phosphate. This reaction is activated (deinhibited) by cyclic AMP or AMP and the Glc-1,6-P_2 formed can then release the phosphoglucomutases from ATP or citrate inhibition.

It is possible that these regulatory mechanisms operate during periods of highly active epinephrine-induced glycogenolysis in the skeletal muscle. Bevitz et al. [28] have concluded from their measurements of the concentration of intermediary metabolites in the glycogenolytic chain, that epinephrine in skeletal muscle influences not only the glycogen → glucose 1-phosphate reaction but also stimulates the glucose 1-phosphate → glucose 6-phosphate and the fructose 6-phosphate → fructose 1,6-bisphosphate reactions. Based on the in vitro experiments presented in this paper we suggest that the sequence of metabolic events resulting from epinephrine (see Fig. 10) is as follows: epinephrine causes an increase in the level of cyclic AMP in muscle [20,29] leading to an increase in the rate of glycogen breakdown to glucose 1-phosphate through phosphorylase activation [20,29—32]. A rise in the level of glucose 1-phosphate will favor the phosphofructokinase reaction with this substrate and this reaction will be activated by the increased levels of cyclic AMP, leading to the synthesis of Glc-1,6-P_2. Glc-1,6-P_2 will then release the phosphoglucomutases from ATP or citrate inhibition, leading to the conversion of the glucose 1-phosphate to glucose 6-phosphate, which through the phosphoglucose isomerase reaction will be converted to fructose 6-phosphate. An increase in

the level of fructose 6-phosphate will now favor the phosphofructokinase reaction with this substrate, and this reaction will be further activated by Glc-1,6-P_2 [2,3,33] and by cyclic AMP [27,33,34]. It has been reported very recently [35—37] that the muscle contains a single phosphofructokinase isozyme which is distinct from the two in liver. Mansour [33] has recently provided direct evidence that phosphofructokinase from mammalian skeletal muscle, is modified to a more active form as a result of epinephrine administration. His experiments revealed that this activation is mediated through a combination of hexose phosphates and adenylate nucleotides that are known to be increased by epinephrine. The activation of phosphofructokinase which is known to act as a rate-limiting enzyme within the Embden-Meyrhoff scheme, leads to an increase in total glycolysis in the mammalian skeletal muscle. This is reflected by an increase in lactic acid production after epinephrine [28].

Thus the activities of phosphoglucomutase, phosphofructokinase (reacting with glucose 1-phosphate or fructose 6-phosphate), and glycogen phosphorylase are all synchronized to achieve an increase in total glycogenolysis and glycolysis following epinephrine administration. Experiments which are now in progress in our laboratory show that epinephrine causes a rise in the Glc-1,6-P_2 in muscle to levels close to those found effective in the present studies.

The results presented in this paper may explain the findings reported by Joshi et al. [5] who have shown that the total activity of phosphoglucomutase in rat muscle is reduced by about 50% during starvation and restored on subsequent treatment with epinephrine. Since the concentration of citrate in muscle was reported to be increased by starvation [38] it seems probably that the observed reduction in phosphoglucomutase activity during starvation was caused by an increase in the concentration of citrate which severely inhibits the phosphoglucomutase activities in the muscle (Fig. 3). Epinephrine overcomes this inhibition through the sequence of metabolic events suggested above (see Fig. 10).

The experiments described in the present paper also reveal that Glc-1,6-P_2 exerts a selective inhibitory effect on hexokinase type II, which is the predominant form in the skeletal muscle. It is possible that during periods of highly active epinephrine-induced glycogenolysis in the skeletal muscle, the Glc-1,6-P_2 produced by the cyclic AMP-stimulated reaction of phosphofructokinase with glucose 1-phosphate (see Fig. 10), will inhibit the activity of the muscle type II hexokinase. From several earlier reports [39—42] it could be deduced that epinephrine action leads to an inhibition of hexokinase reaction in the skeletal muscle. This inhibition may be mediated through a combination of Glc-1,6-P_2 and glucose 6-phosphate, a known inhibitor of the enzyme [43] which is increased by epinephrine [28]. It should be noted, however, that recent reports [44,45] have revealed that type II hexokinase exhibits a slow response to glucose 6-phosphate.

It has been recently reported [46] that P_i is also capable of inhibiting skeletal muscle type II hexokinase; it causes a severe depression of the enzyme's activity at concentrations which may be obtained during tetanic muscular contraction. However, in contrast to muscular contraction which causes a rise in the level of P_i, epinephrine was reported to decrease the concentration of P_i in the muscle [47]. Thus during periods of epinephrine-induced glyco-

genolysis in muscle, it is Glc-1,6-P_2 which most probably depresses the activity of the muscle hexokinase. Such depression will restrict glucose utilization by the skeletal muscle during epinephrine induced glycogenolysis. Consequently glucose will be conserved for tissues such as the brain which has an almost continual requirement for glucose for energy production. The present experiments reveal that the brain (type I) hexokinase is not affected by Glc-1,6-P_2.

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