Effect of glucose-6-P on the catalytic and structural properties of glycogen phosphorylase *a*

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Kinetic studies of muscle phosphorylase *a* in cationic buffer (pH 6.8) demonstrate that glucose-6-P competitively inhibits the binding of the substrate, glucose-1-P, to the enzyme. The inhibitory effect of glucose-6-P is largely overcome by glycerol-2-P. AMP counteracts inhibition of the enzyme by glucose-6-P, while glucose and glucose-6-P can interact to produce a synergistic inhibition of phosphorylase *a* activity. Preincubation of phosphorylase *a* with glucose-6-P at 20°C results in ~3-fold increase in activity, while ultracentrifugation experiments carried out under the same conditions showed that phosphorylase *a* can be converted to dimers by glucose-6-P.

Phosphorylase a *Inhibition* *Activation* *Dimerization* *Structure–function*

1. INTRODUCTION

It has been reported that glucose-6-P does not significantly influence the enzymatic activity of skeletal muscle glycogen phosphorylase *a* (EC 2.4.1.1) [1]. However, it is known that glucose-6-P activates phosphorylase phosphatase in vitro, and this activation is attributed to glucose-6-P induced conformational changes of the substrate phosphorylase *a* [2,3]. Furthermore, this ligand causes marked changes in the fluorescence properties of acetamidosalicylate-phosphorylase *a* or in the electron-spin resonance spectrum of a spin-labelled phoshorylase *a*, and formally competes with glycerol-2-P [4].

Experiments presented here demonstrate that in the absence of glycerol-2-P, a common buffer salt used in kinetic studies of phosphorylase, glucose-6-P considerably affects phosphorylase *a* activity, while, for the first time, ultracentrifugation provides a direct evidence for the dissociation of phosphorylase *a* tetramer to dimers by glucose-6-P.

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2. MATERIALS AND METHODS

Phosphorylase *b* was isolated from rabbit skeletal muscles as in [5]. The 4-times crystallized enzyme was treated as in [6] to remove bound AMP. Phosphorylase *a* was prepared from phosphorylase *b* by phosphorylation with rabbit muscle phosphorylase kinase [7]. The enzyme was recrystallized at least 3 times in 50 mM glycerol-2-P, 50 mM 2-mercaptoethanol, 1 mM EDTA buffer (pH 6.8). The crystals were dissolved in a buffer containing 5 mM glycerol-2-P (pH 6.8), 5 mM 2-mercaptoethanol, and 1 mM EDTA, treated twice with acid-washed Norit (1 mg/mg protein), and purified on a column of Sephadex G-25 equilibrated with the same buffer. Possible contamination of enzyme by phospho–dephospho-phosphorylase hybrid was checked as in [8]. Enzyme assayed under standard conditions (16 mM glucose-1-P) could not be further activated with 75 mM glucose-1-P. Protein concentration was determined from absorbance measurements at 280 nm as in [9].

Initial reaction rates were determined in the direction of glycogen synthesis. Reaction mixtures

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were 0.5 ml and contained 3–5 μg/ml phosphorylase *a*, 1% glycogen, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 5 mM triethanolamine/HCl buffer (pH 6.8). Enzyme and glycogen were preincubated for 15 min at 30°C before initiating the reaction with glucose-1-P, care being taken to convert $\leqslant 20\%$ of glucose-1-P during the assay. P_i released in the reaction was measured as in [10].

Sedimentation velocity experiments were carried out using an MSE Centriscan 75 preparative and analytical ultracentrifuge, with a 10-mm single-sector cell at 60000 rev./min and 20 ± 0.5°C. Sedimentation coefficients obtained from direct measurements of the scanner traces were corrected for viscosity and density of the buffer to water at 20°C. The percentage of components with different sedimentation coefficients was estimated as in [11].

Oyster glycogen (BDH Chemicals) was freed of AMP as in [12]. AMP, glucose-1-P (dipotassium salt), and glucose-6-P (monosodium salt) were products of Sigma Chemical Co. (St Louis MO). Caffeine and glucose were purchased from Serva, and glycerol-2-P was obtained from E. Merck.

3. RESULTS

Fig.1 shows the inhibitory effects of increasing concentrations of glucose-6-P on phosphorylase *a* activity at various concentrations of glucose-1-P. An apparent competitive inhibition is indicated, since extrapolation of these plots to the ordinate, yields the same value for V (66.6 μmol.min^{-1}.mg^{-1}). The double-reciprocal plots also show concave upward curvature, suggesting a homotropic cooperativity between substrate binding sites [13]. Thus, the result of adding increasing concentrations of glucose-6-P is to increase the apparent K_m values for the substrate and to increase also the value of Hill coefficient (fig.1 inset, table 1).

It was found that glucose-6-P inhibition of phosphorylase *a* except for triethanolamine–HCl, can be equally well demonstrated in Tris–HCl and also imidazole–HCl buffers (pH 6.8). On the contrary, this inhibition is largely reversed by glycerol-2-P and maleate. In the presence of 2 mM glucose-6-P, increasing concentrations of glycerol-2-P in the assay decrease both the apparent K_m value for glucose-1-P and the Hill coefficient (table 1). However, glycerol-2-P alone in the concentrations tested has no effect on the enzyme activity. Practically, the enzyme is not inhibited by 2 mM glucose-6-P in the presence of 20 mM glycerol-2-P in agreement with [1].

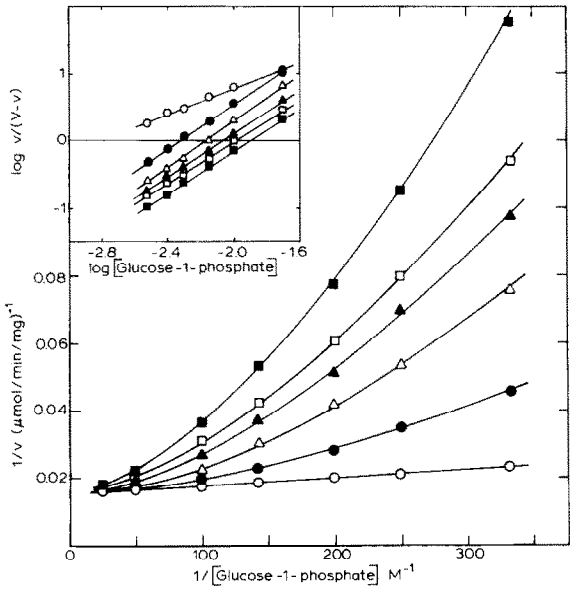

Fig.1. Kinetics of glucose-6-P inhibition of phosphorylase *a* with respect to glucose-1-P. Double-reciprocal plots for glucose-1-P binding in the absence (○) or presence of 1 (●), 2 (△), 3 (▲), 4 (□) and 5 mM (■) glucose-6-P. The enzyme was assayed at 30°C in 5 mM triethanolamine–HCl buffer (pH 6.8), containing 0.5 mM dithiothreitol and 0.5 mM EDTA, with 1% glycogen. [Glucose-1-P] was varied from 3–40 mM. Inset: Hill plots for glucose-1-P. The V value with respect to glucose-1-P was 66.6 μmol.min^{-1}.mg^{-1}. Log $v/(V-v)$ was plotted against log(glucose-1-P) which yields Hill n (Hill coefficient) as the slope and the apparent K_m values [14].

The results of the kinetics of glucose inhibition of phosphorylase *a* obtained under the same conditions are summarized in table 1. Since it appears that glucose-6-P acts in a similar way as glucose which also increases the homotropic cooperativity between substrate molecules [14], we examined the joint action of these two inhibitors on the enzyme activity. A marked synergistic effect of the two in inhibiting phosphorylase *a* occurs as indicated in fig.2a. In fig.2b, the intercept on the x-axis gives $-\alpha K_i$ (the inhibition constant for glucose in the presence of glucose-6-P) [15]. Thus, from the plot,

Table 1

Kinetic parameters for phosphorylase *a*

Additions	K_m (mM)	*n*
None	1.6 ± 0.2	1.0
Glucose-6-P (1 mM)	4.6	1.6
Glucose-6-P (2 mM)	6.9	1.7
Glucose-6-P (3 mM)	8.5	1.7
Glucose-6-P (4 mM)	10.3	1.55
Glucose-6-P (5 mM)	12.3	1.6
Glucose-6-P (2 mM) + glycerol-2-P (2.5 mM)	6.0	1.5
Glucose-6-P (2 mM) + glycerol-2-P (5 mM)	4.8	1.4
Glucose-6-P (2 mM) + glycerol-2-P (10 mM)	3.5	1.25
Glucose (10 mM)	4.2	1.3
Glucose (20 mM)	7.0	1.35
Glucose (30 mM)	9.6	1.45

The apparent K_m and *n* (Hill coefficient) values with respect to glucose-1-P were calculated from the data of fig.1 as described in the legend. Kinetic experiments of glucose inhibition or glycerol-2-P effect on glucose-6-P inhibition of phosphorylase *a* were performed under the same conditions as in fig.1, and the data obtained were plotted as Hill plots [14] to calculate the kinetic parameters. In the absence of inhibitor the mean K_m is quoted together with the standard deviation of the mean (10 determinations)

αK_i = 1.2 mM, and if we take K_i = 6 mM (an approximate value obtained from the data of table 1, based on the assumption that $K_{m,app.} = K_m(1 + I/K_i)$), then α, an interaction factor, is 0.2. However, we cannot calculate the inhibition constant for glucose-6-P in the presence of glucose, because the replot of the slopes *vs* the corresponding glucose-6-P concentrations yields a curve (fig.2b, inset). It is worth noting that the synergism between glucose and glucose-6-P exists even in the presence of glycerol-2-P, but in this case higher concentrations of glucose-6-P are required.

In table 2, the effects of glucose-6-P, glucose and caffeine on phosphorylase *a* activity are presented. Caffeine, which is known to competitively inhibit the binding of glucose-1-P to the enzyme and also to act synergistically with glucose [15], can also interact with glucose-6-P to produce an apparent synergistic inhibition of phosphorylase *a* activity. Furthermore, in the presence of the

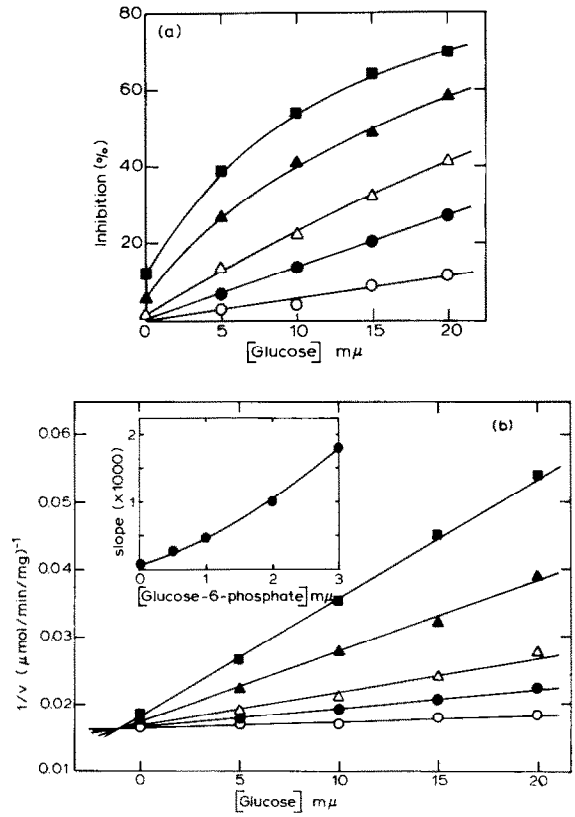

Fig.2. (a) Inhibition of phosphorylase *a* activity by glucose, glucose-6-P and a combination of both inhibitors. The enzyme was assayed with 20 mM glucose-1-P and 1% glycogen in 5 mM triethanolamine–HCl buffer (pH 6.8), containing 0.5 mM dithiothreitol and 0.5 mM EDTA, at 30°C. Glucose concentrations were as indicated in the figure. Glucose-6-P concentrations were 0 (○), 0.5 (●), 1 (△), 2 (▲) and 3 mM (■). (b) Dixon plots of $1/v$ *vs* [glucose] at different fixed [glucose-6-P] (the above mentioned). Inset: The replot of slopes *vs* [glucose-6-P].

3 ligands together, enzyme activity is nearly eliminated. However, AMP is able to completely reverse these inhibitory actions.

Fig.3 shows that preincubation of phosphorylase *a* (5 mg/ml) with 5 mM glucose-6-P at 20°C results in a marked increase of enzymatic activity. At 30 min, activation with glucose-6-P is ~3-times greater than that observed without glucose-6-P. The data in fig.3 also show that stimulation of enzymatic activity can also be demonstrated if enzyme is preincubated with 50 mM glucose in agreement with [16], while the addition of 50 μM AMP

Table 2

Synergistic inhibition of phosphorylase *a* activity by glucose-6-P, glucose, and caffeine

Additions	Activities (%)	
	− AMP	+ 50 μM AMP
None	100 (53)	100 (73)
Glucose-6-P (1 mM)	67	98
Glucose (5 mM)	86	96
Caffeine (0.5 mM)	57.5	97
Glucose-6-P (1 mM) + glucose (5 mM)	27	99
Glucose-6-P (1 mM) + caffeine (0.5 mM)	16	96
Glucose (5 mM) + caffeine (0.5 mM)	16	93
Glucose-6-P (1 mM) + glucose (5 mM) + caffeine (0.5 mM)	2.5	93

The enzyme was assayed with 5 mM glucose-1-P and 1% glycogen in the absence or presence of AMP as in section 2. Activities indicated are the percentage remaining when compared to controls taken as 100%. Values in parentheses are the control activities in $\mu mol \cdot min^{-1} \cdot mg^{-1}$

to phosphorylase *a* which had been preincubated for 44 min with glucose-6-P results in an instantaneous decrease of activity. To relate alterations of enzymatic activity to quaternary structure of phosphorylase *a*, sedimentation velocity experiments were carried out under the same conditions of stimulation of activity (20°C). The following results were obtained (table 3):

(i) Phosphorylase *a* in the presence of 5 mM glucose-6-P sediments mainly as a dimer ($s_{20,w}$ = 8.8), while a minor fast-sedimenting component is formed with an $s_{20,w}$ of value of 13.4 corresponding to the tetramer [16];

(ii) The identified dimeric form of phosphorylase *a* in the presence of glucose-6-P is able to be associated to the tetrameric form with low concentrations of AMP;

(iii) Glucose (10 mM) and glucose-6-P (2 mM) when added independently to the enzyme cause partial dissociation to dimers, while a cooperative promotion of dimer formation when the two ligands are added together can be observed.

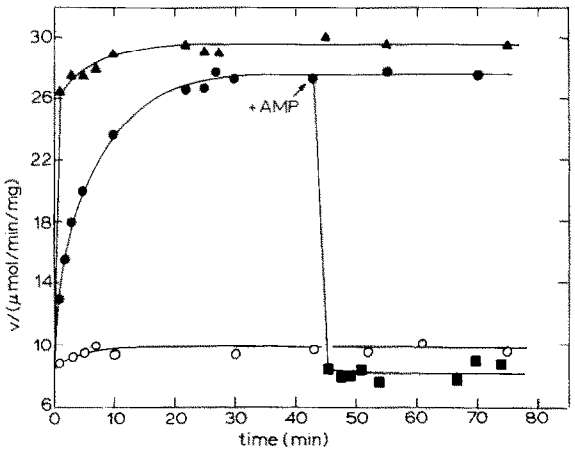

Fig.3. Effect of glucose-6-P on the activity of phosphorylase *a*. Phosphorylase *a* (5 mg/ml) was preincubated at 20°C in 15 mM triethanolamine–HCl, 0.5 mM dithiothreitol, 0.5 mM EDTA buffer (pH 6.8) in the absence (○) or presence of 5 mM glucose-6-P (●) or 50 mM glucose (▲). Aliquots were removed at various intervals for measurement of activity at 20°C. The assay mixture (0.2 ml) contained 0.3 mg/ml of phosphorylase *a*, 1% glycogen, 20 mM glucose-1-P in 15 mM triethanolamine–HCl, 0.5 mM dithiothreitol, 0.5 mM EDTA buffer (pH 6.8) (30 s assay). AMP (50 μM) was added to enzyme which had been incubated for 44 min with glucose-6-P (■).

4. DISCUSSION

These results show that in the presence of the inhibitor, glucose-6-P, the previously non-cooperative phosphorylase *a* now displays cooperative glucose-1-P binding (fig.1). The kinetics of phosphorylase *b* with respect to glucose-1-P becomes also cooperative in the presence of glucose-6-P [13], while glucose-6-P is essentially without effect on phosphorylase *b'* activity [17], indicating that the first 19 residues in phosphorylase are important in the expression of homotropic and heterotropic interactions [18]. X-ray diffraction studies on phosphorylase *a* crystals have shown that the AMP/ATP binding site is remarkably indiscriminate in binding phosphate-containing compounds. There is also evidence that the glucose moiety of glucose-6-P is firmly bound to the crystals [19]. Glucose-6-P inhibition of the enzyme is competitively reversed by increasing substrate concentration (fig.1) and this may be also due to some extent to the secondary binding of

Table 3

Effect of glucose, glucose-6-P, and AMP on the sedimentation properties of phosphorylase *a*

Additions	$s_{20,w}$	% of total
None	13.7	100
Glucose (50 mM)	8.3	100
Glucose (10 mM)	12.4	74
	8.5	26
Glucose-6-P (2 mM)	13.2	63
	8.6	37
Glucose-6-P (5 mM)	8.8	75
	13.4	25
Glucose-6-P (20 mM)	8.9	83
	13.6	17
Glucose-6-P (2 mM) + glucose (10 mM)	8.9	100
Glucose-6-P (5 mM) + AMP (25 μM)	13.7	73
	8.8	27
Glucose-6-P (5 mM) + AMP (100 μM)	13.9	100

All ultracentrifugal sedimentation velocity measurements were performed with 5 mg enzyme/ml in 15 mM triethanolamine–HCl, 0.5 mM dithiothreitol, 0.5 mM EDTA buffer (pH 6.8) at 20°C. Other components were added as indicated. Other conditions were as in section 2

glucose-1-P in the same location as AMP [20]. The inhibitory action of glucose-6-P is also reversed by glycerol-2-P, suggesting that glycerol-2-P possibly binds in the AMP/ATP binding site.

Glucose-6-P in inhibiting the enzymatic reaction acts in a similar way as does glucose. Low concentrations of these ligands alone are only slightly inhibitory in saturating concentrations of glucose-1-P. However, in combination they markedly inhibit the activity of the enzyme (fig.2). This synergistic effect indicates different binding sites for the two inhibitors [21] and the determined α-value of 0.2 for the glucose/glucose-6-P pair indicates that positive interactions exist between these two ligands in the enzyme–glucose–glucose-6-P complex [22]. It has also been reported that the inhibitors glucose and glucose-6-P are synergistic in their action on enzymatic activity and tryptic digestion of phosphorylase *b* [23,24].

Glucose and glycogen induce an increase of enzymatic activity of phosphorylase *a* at low temperature (15–23°C) promoting the dissociation of the tetrameric form to dimers [25]. Since phosphorylase *a* activation by glucose-6-P may be correlated with the formation of a dimeric species (fig.3, table 3), this suggests that stimulation is directly related to the dimerization of phosphorylase *a*. It has been reported that tetrameric phosphorylase *a* cannot be dissociated to the dimeric form by glucose-6-P [8,26]. The contradiction between our results and those in [8,26] might be due to the presence of anionic salts (glycerol-2-P and maleate) in their ultracentrifugation experiments.

This report presents evidence that the well known stimulatory effect of glucose-6-P in the dephosphorylation of phosphorylase *a* by phosphorylase phosphatase observed at low temperature [3], could be attributed to a dissociation phenomenon.

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REFERENCES

[1] Morgan, H.E. and Parmeggiani, A. (1964) J. Biol. Chem. 239, 2440–2445.
[2] Bot, G. and Dosa, I. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. 6, 73–87.
[3] Martensen, T.M., Brotherton, J.E. and Graves, D.J. (1973) J. Biol. Chem. 248, 8329–8336.
[4] Busby, S.J.W. and Radda, G.K. (1976) Curr. Top. Cell. Reg. 10, 89–160.
[5] Fischer, E.H. and Krebs, E.G. (1962) Methods Enzymol. 5, 369–373.
[6] Ktenas, T.B., Oikonomakos, N.G., Sotiroudis, T.G., Nikolaropoulos, S. and Evangelopoulos, A.E. (1980) Biochem. Biophys. Res. Commun. 97, 415–422.
[7] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[8] Hurd, S.S., Teller, D. and Fischer, E.H. (1966) Biochem. Biophys. Res. Commun. 24, 79–84.
[9] Kastenschmidt, L.L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry 7, 3590–3608.
[10] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[11] Sotiroudis, T.G., Oikonomakos, N.G. and Evangelopoulos, A.E. (1981) Biochim. Biophys. Acta 658, 270–279.

[12] Helmreich, E. and Cori, C.F. (1964) Proc. Natl. Acad. Sci. USA 51, 131–138.
[13] Madsen, N.B. and Shechosky, S. (1967) J. Biol. Chem. 242, 3301–3307.
[14] Helmreich, E., Michaelides, M.C. and Cori, C.F. (1967) Biochemistry 6, 3695–3710.
[15] Kasvinsky, P.J., Shechosky, S. and Fletterick, R.J. (1978) J. Biol. Chem. 253, 9102–9106.
[16] Wang, J.H., Shonka, M.L. and Graves, D.J. (1965) Biochem. Biophys. Res. Commun. 18, 131–135.
[17] Graves, D.J., Mann, S.A.S., Philip, G. and Oliveira, R.J. (1968) J. Biol. Chem. 243, 6090–6098.
[18] Weber, I.T., Johnson, L.N., Wilson, K.S., Yeates, D.G.R., Wild, D.L. and Jenkins, J.A. (1978) Nature 274, 433–437.
[19] Fletterick, R.J. and Madsen, N.B. (1980) Annu. Rev. Biochem. 49, 31–61.
[20] Sygusch, J., Madsen, N.B., Kasvinsky, P.J. and Fletterick, R.J. (1977) Proc. Natl. Acad. Sci. USA 74, 4757–4761.
[21] Segel, I.H. (1975) in: Enzyme Kinetics, pp.470–473, Wiley-Interscience, New York.
[22] Yonetani, T. and Theorell, H. (1964) Arch. Biochem. Biophys. 106, 243–251.
[23] Silonova, G.V., Livanova, N.B. and Kurganov, B.I. (1969) Molekul. Biol. 3, 768–784.
[24] Kurganov, B.I., Lisovskaya, N.P. and Livanova, N.B. (1972) Biokhimiya 37, 289–298.
[25] Graves, D.J. and Wang, J.H. (1972) in: The Enzymes (Boyer, P.D. ed) 3rd edn, vol.7, pp.435–482, Academic Press, New York.
[26] Livanova, N.B., Eronina, T.B. and Silonova, G.V. (1972) FEBS Lett. 24, 82–84.