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Effect of Glycerol on Glucokinase Activity: Loss of Cooperative Behavior with Respect to Glucose

DENISE POLLARD-KNIGHT,[1] BERNARD A. CONNOLLY,[2] ATHEL CORNISH-BOWDEN, AND IAN P. TRAYER[3]

Department of Biochemistry, The University of Birmingham, P.O. Box 363, Birmingham B15 2TT, United Kingdom

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Glucose phosphorylation catalyzed by rat liver glucokinase measured at saturating concentrations of $MgATP^{2-}$ shows a cooperative response with respect to glucose in the concentration range 0.25–5 mM with a Hill coefficient of 1.6. In this range of glucose concentrations, the degree of cooperativity was dependent on the presence of glycerol in the assay mixture, and it decreased progressively and disappeared completely as the glycerol concentration reached about 20% (v/v) glycerol. If attention was confined to concentrations above 5 mM, no cooperativity could be detected either in the absence or in the presence of glycerol. The limiting velocity of the glucokinase reaction (measured at saturating concentrations of glucose and $MgATP^{2-}$), and the half-saturation concentration for glucose and $MgATP^{2-}$ were all decreased by about 50–60% as the glycerol concentration was raised from zero to 30% (v/v). The presence of glycerol had no effect on the qualitative inhibition patterns of $MgADP^{2-}$, glucose 6-phosphate, or *N*-acetylglucosamine, and only slight effects on the quantitative half-saturation values and inhibition constants. All of these effects caused by glycerol were fully reversible by decreasing the concentration of glycerol by dilution. Simulation studies based on the "mnemonical" model of glucokinase action proposed earlier [A. C. Storer and A. Cornish-Bowden (1977) *Biochem. J.* **165**, 61–69] show that the effects of glycerol on glucokinase-catalyzed glucose phosphorylation can simply be explained assuming the glycerol favors the existence of the conformation of the enzyme with a higher affinity for glucose and thus supports the model.

Glucokinase, the principal glucose-phosphorylating enzyme in liver (1–3), shows positive cooperativity with respect to glucose (4–6) and is inhibited only weakly and nonallosterically by glucose 6-phosphate. This fits well with its proposed role in regulating the concentration of glucose in the blood (7) where its cooperative response to glucose, with a half-saturation rate in the physiological range (5 mM), renders the enzyme more sensitive to changes in glucose uptake and less sensitive to changes in MgATP concentration.

The enzyme is monomeric under all assay conditions (8–10) with only a single binding site for glucose (11). Hence, the cooperativity cannot be explained in terms of quasiequilibrium models postulating subunit interactions (12, 13), by reversible association of the enzyme (14), or by models involving two or more interacting sites per polypeptide chain. Storer and Cornish-Bowden (15) proposed a "mnemonical" model to account for the kinetics of glu-

[1] Present address: Department of Biochemistry, University of California, Berkeley, Calif. 94720.

[2] Present address: European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, West Germany.

[3] To whom correspondence should be addressed.

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cokinase. This type of mechanism is a development of the idea of enzyme memory first put forward by Rabin (16) and previously applied to wheat germ hexokinase type L1 (17, 18). The essential postulate is that the free enzyme exists in two forms that are interconvertible but are not necessarily at equilibrium with one another under steady-state conditions; these bind glucose with different affinities to produce the same enzyme-glucose complex. The cooperativity is a consequence of the changing proportions of the different enzyme forms as the glucose concentration changes. The model, which has since received support from isotope exchange experiments (19), is given in Fig. 3. As it is reviewed more fully elsewhere (20) it need not be described in detail here.

The present report stems from our observation that when glucokinase is assayed at high glycerol concentrations, originally introduced to preserve enzyme stability during storage (21–23), the cooperative behavior with respect to glucose is completely lost. As we shall show, this behavior can easily be explained in terms of the mnemonical model, and it can thus be taken as additional evidence for existence of multiple forms of the free enzyme.

MATERIALS AND METHODS

Materials. ATP (disodium salt), glucose-6-phosphate dehydrogenase (type VII from Bakers' yeast), pyruvate kinase (type III from rabbit muscle), and lactate dehydrogenase (type XI from rabbit muscle) were obtained from Sigma (Poole, Dorset, U. K.), as were all other fine biochemicals (NADH, phosphoenolpyruvate, $NADP^+$, etc.). All other reagents and solvents were Analar or best quality reagent grade as supplied by BDH (Poole, Dorset, U. K.) or Fisons (Loughborough, Leics., U. K.).

Glucokinase preparations. Glucokinase was purified to a specific activity of 140 μmol min^{-1} mg^{-1} (2.5 kat kg^{-1}) from the livers of well-fed rats essentially by the method of Holroyde *et al.* (8). After purification, glucokinase was stored in 20 mM triethanolamine-HCl, pH 7.0, 0.3 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 50 mM glucose, 30% glycerol, at −20°C. For kinetic studies glucokinase was equilibrated from the storage buffer to 25 mM triethanolamine-HCl, pH 7.0, 100 mM KCl, 5 mM glucose, 0.5 mM dithiothreitol, 30% (v/v) glycerol by gel filtration on a column (25 × 2 cm) of Sephadex G-25. The most active fractions were pooled and kept on ice. The inclusion of 5 mM glucose in this buffer prolonged the stability of the enzyme. The small amounts of glucose introduced into the assay cuvette with the enzymes were always allowed for.

Standard assay solutions. Concentrated stock solutions, prepared fresh each day, were used to add glucose, glucose 6-phosphate, ATP, and ADP to the assay cuvettes. The concentrations of glucose and glucose 6-phosphate in these solutions were determined by the ferricyanide assay for reducing sugars (24). Any glucose contamination of the glucose 6-phosphate solution was determined using a glucose assay kit (Blood sugar GOD-Perid method; Boehringer-Mannheim). Contamination levels of 0.2% were routinely found. The concentration of nucleotides was determined spectrophotometrically using an A_{260} of 15,000 liters mol^{-1} cm^{-1} (25). The final Mg^{2+} concentration in the assay cuvette depended on the combination of ATP, ADP, and glucose 6-phosphate present. In all cases the Mg^{2+} levels used and the ensuing concentrations of Mg-nucleotide complexes were determined by reference to Storer and Cornish-Bowden (5, 15, 26).

Enzyme assays. Most initial rates were determined using a coupled assay based on that of DiPietro and Weinhouse (2), using glucose-6-phosphate dehydrogenase as the coupling enzyme, under the detailed conditions given previously (27). The reaction was followed by measuring the change in A_{340} in a Gilford recording spectrophotometer, Model 2400-S.

When studying the product inhibition due to glucose 6-phosphate, another coupled assay was used in which the formation of ADP was coupled to the oxidation of NADH (0.25 mM) by addition of pyruvate kinase (40 units), lactate dehydrogenase (40 units), and excess phosphoenolpyruvate (1 mM). The reaction was carried out at 30°C in a total volume of 0.75 ml 50 mM glycylglycine-KOH buffer, pH 8.0, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 0.5 mM dithiothreitol, and other additions as indicated. The mixture was preincubated for 5 min to remove traces of ADP present in the ATP, and the absorbance change was measured as previously. The values of V_{app} used for Hill plots (log $(V_{app}/v - 1)$ against log [glucose]) were obtained from the limiting slopes at high glucose concentrations of plots of [glucose]/v against [glucose].

Analysis of results. The reaction was always initiated by the addition of glucokinase, and the rates observed were linear over at least the first 3 min. Initial rates were obtained from the recorder traces using a ruler and pencil. All the graphs and kinetic constants given under Results were obtained using least-squares regression analysis.

The inhibition constants, K_{ic} and K_{iu}, are defined

as recommended by the International Union of Biochemistry (28) in relation to the following equation:

$$v = \frac{V[S]}{K_m\left(1 + \frac{[I]}{K_{ic}}\right) + [S]\left(1 + \frac{[I]}{K_{iu}}\right)}$$

in which S is the substrate whose concentration is varied and I is the inhibitor being studied, and V and K_m are the parameters of the Michaelis–Menten equation. The K_{ic} values were determined by the plot of $1/v$ against [I] (29) and K_{iu} values by the plot of $[S]/v$ against [I] (30). These values were obtained from assays carried out at glucose concentrations from 10 to 100 mM, where deviations from the Michaelis–Menten equation were not visible, even in the absence of glycerol.

Computer simulation was used to investigate the characteristics of the mnemonical model. The rate equation was derived automatically by means of a program described previously (31) after it had been modified to carry out numerical simulation with specified values of the rate constants. Computer programs were run on an International Computers Ltd 1906A and a Digital Equipment Ltd DEC 20 computer with an on-line Hewlett-Packard graph plotter.

RESULTS

Dependence of the Rate on the Glucose Concentration at Varying Glycerol Concentrations

The glucokinase-catalyzed rate of glucose phosphorylation, v, measured at 5 mM $MgATP^{2-}$ with the glucose concentration varied between 0.25 and 5 mM and at varying glycerol concentrations, is shown in Fig. 1. Below 5 mM glucose, cooperativity was most marked in the absence of glycerol but gradually disappeared as the glycerol concentration was increased to 20% (v/v). At concentrations of glucose below 5 mM the slope of the Hill plot was dependent on the glycerol concentration. In 20 and 30% (v/v) glycerol the slope of the Hill plot was unity, i.e., there was no detectable cooperativity. As the glycerol concentration was decreased, the Hill coefficient increased to a maximum value of 1.6 in the absence of glycerol.

Figure 2 summarizes the effect of the glycerol concentration on the limiting velocity, Hill coefficient, and $K_{0.5}$ for glucose.

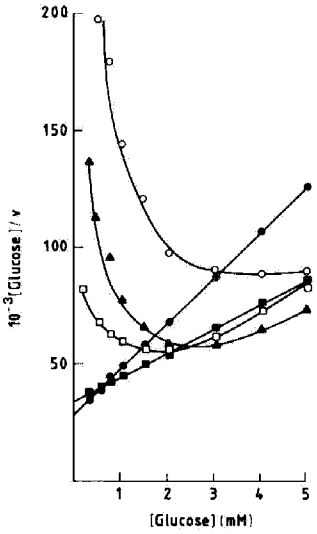

FIG. 1. Dependence of the rate of the glucokinase-catalyzed reaction on the glucose concentration and different glycerol concentrations. The $MgATP^{2-}$ concentration was fixed at 5 mM and the glucose concentrations were varied as indicated. The different glycerol concentrations (%, v/v) were 30 (●), 20 (■), 10 (□), 5 (▲), and 0% (○).

This figure shows that all three of these parameters decrease with increasing glycerol concentrations.

All of these effects caused by glycerol were fully reversible. If the higher concentrations of glycerol were decreased by dilution, the enzyme behaved as expected from previous results (26) with full cooperative effects. This observation shows that glycerol (or any impurities, e.g., aldehydes) does not cause a chemical modification of the enzyme.

$MgATP^{2-}$ Kinetics

Michaelis–Menten kinetics were obtained when the velocity of the glucokinase reaction was measured at $MgATP^{2-}$ concentrations between 0.22 and 2.1 mM, at several fixed glucose concentrations both in the absence of glycerol, as reported earlier (15), and in the presence of 30% (v/v) glycerol. The K_m for $MgATP^{2-}$ was found to be 0.28 mM in 30% (v/v) glycerol, half the value of 0.55 mM found in the absence of glycerol.

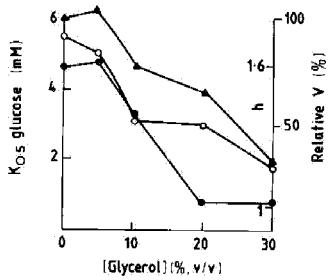

FIG. 2. A summary of the effects of varying glycerol concentration on different kinetic parameters. ○, $K_{0.5}$ for glucose; ●, Hill coefficient; ▲, limiting velocity, V, taken as 100% in the absence of glycerol and in the presence of 100 mM glucose and 5 mM $MgATP^{2-}$.

Inhibition Studies

The various kinetic constants obtained for the substrates and products of the glucokinase reaction at glycerol concentrations from 0 and 30% (v/v) are summarized in Table I. The presence of glycerol had no effect on the qualitative product inhibition patterns (15) and only minimal effects on the quantitive half-saturation values and inhibition constants.

Simulation Studies

Although the experimental data do not contain sufficient information to allow the rate constants of the mechanism shown in Fig. 3 to be evaluated, computer simulation has shown that the effects of glycerol can be explained in terms of this mechanism and has shed light on the quantitative nature of the effects. Formally, the sign of the Hill coefficient is fully determined by the sign of $(k_3 - k_2)$, the difference between the *on* rate constants for binding of glucose to E and E′, respectively, as found previously for the original form of the mnemonical mechanism with a different index of cooperativity (17). In principle, therefore, the loss

TABLE I

A SUMMARY OF THE KINETIC PARAMETERS OF THE GLUCOKINASE REACTION OBTAINED IN THE ABSENCE AND PRESENCE OF GLYCEROL

		Kinetic parameter	
		Glycerol concentration (%, v/v)	
Substrate or inhibitor[a]	Parameter[b]	0	30
Glucose	$K_{0.5}$	5.5 mM	1.5-2.1 mM
	h	1.6	1.05
$MgATP^{2-}$	K_m	0.5 mM	0.28-0.35 mM
	V	100%	40%
$MgADP^{-}$/Glucose	K_{ic}	10-15 mM	8-10 mM
	K_{iu}	2.1 mM	2 mM
$MgADP^{-}$/$MgATP^{2-}$	K_{ic}	1.4 mM	1.0 mM
	K_{iu}	4.0 mM	1.8 mM
Glucose 6-phosphate/glucose	K_{ic}	60 mM	200 mM
	K_{iu}	60 mM	45 mM
Glucose 6-phosphate/$MgATP^{2-}$	K_{ic}	11.5 mM	6 mM
	K_{iu}	∞	∞
N-Acetylglucosamine/glucose	K_{ic}	0.3 mM	0.3 mM
	K_{iu}	∞	∞

[a] Where two compounds are shown, the first is the inhibitor studied and the second is the substrate whose concentration is varied.

[b] In the absence of glycerol, the concentration of the glucose was varied between 10 and 100 mM in these experiments. In this range deviations from Michaelis-Menten kinetics are not observed.

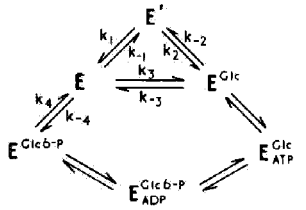

FIG. 3. Proposed mnemonical model for glucokinase action (14). Only the steps referred to in the text have been assigned rate constants.

of cooperativity in the presence of glycerol requires only that these two rate constants become equal. However, in order to get good qualitative agreement between the observations and the simulated curves it appeared necessary to postulate that this change in the *on* rate constants was a consequence of stabilization by glycerol of E, the form of free enzyme released at the end of the catalytic cycle, and that interconversion of E and E′ becomes slower in the presence of glycerol. Thus k_1, k_{-1}, and their ratio k_1/k_{-1} are decreased by glycerol.

These effects alone would lead one to expect that glycerol would have little effect on the rate at high concentrations of glucose and $MgATP^{2-}$, because under these conditions there is insufficient time for E to relax to E′, regardless of solvent. The fact that this is not observed, i.e., that the maximum velocity decreases as the concentration of glycerol increases, suggests that at least one later step in the mechanism becomes slower in the presence of glycerol. The most likely candidate, which was assumed in our simulations, was the release of glucose 6-phosphate, which has been shown to be the slowest step at saturation (19), i.e., we suppose that k_4 decreases in the presence of glycerol.

A mechanism for glucokinase cooperativity involving random binding of substrates (but not a mnemonical transition) has also been proposed (10) and was also considered in computer simulations to explain the effects of glycerol. We could not, however, find conditions that caused a decrease in the $K_{0.5}$ for glucose, the K_m for $MgATP^{2-}$, and the maximum velocity by varying rate constants within about 20- to 30-fold. These investigations were not pursued extensively as flux ratio studies and experiments designed to trap binary complexes in the steady state have shown that the glucokinase reaction proceeds via a predominantly ordered process with glucokinse binding first and glucose 6-phosphate released last (19).

It should also be pointed out that any mechanism for glucokinase action that involves two interconvertible conformations of the free enzyme, with differing affinities for glucose, even if it is considered that both of these can accomplish a complete catalytic cycle (32), would account for the observed results by either of the models given above. A more thorough discussion of such "hysteretic" mechanisms is given by Neet and Ainslie (33). The mnemonic model of glucokinase action preferred by ourselves can be viewed as a special formulation of this hysteretic mechanism (33) since there is no information at the moment that allows us to distinguish between them.

There are several analogous aspects between the mechanism proposed for rat liver glucokinase and that of yeast hexokinase, which catalyzes the same chemical reaction, that lend support to our treatment of these data. A "two-tiered hysteretic" mechanism has been proposed for the yeast enzyme (34) and substrate binding is also thought to proceed in a preferred manner [(35) and references therein]. In addition, structural studies on yeast hexokinase (35, 36, 37) have demonstrated that large changes in conformation occur, although the best characterized is induced by glucose (37).

DISCUSSION

Meunier *et al.* (18) found that low concentrations of urea suppressed the cooperativity of wheat germ hexokinase, and this was interpreted by them as direct evidence in favor of the mnemonical enzyme concept. They considered that such treatment would most probably alter differentially either or both of the free forms of the enzyme. Glycerol is the only agent

reported so far to reversibly modify the kinetics of glucokinase, as urea, Triton X-100, and various chemical treatments (10) and specific inhibitors (6, 38) were found not to significantly affect the Hill coefficient. Thus, although the changes in glucokinase kinetics in glycerol solutions compared to those in water were puzzling at first sight, they do fit well with the proposed mnemonical model as an explanation of glucokinase cooperativity.

The nature of the interactions has only recently been investigated although glycerol (and other polyhydric compounds, such as sugars) have been used for many years by biochemists to stabilize enzyme activity and protein structure (21–23). Gekko and Timasheff (39, 40) have found that in aqueous solutions glycerol causes proteins to become preferentially hydrated and is itself excluded from the domain of the protein. The addition of glycerol therefore raises the chemical potential of the system, a situation which is thermodynamically unfavorable. This effect can be regarded as distributed over the entire protein-solvent interface and so any reduction of this interface will be thermodynamically favorable. Conversely, any increase in the surface contact between protein and solvent would be even more thermodynamically unfavorable in the presence of glycerol than in pure water, especially if this involves the exposure of additional hydrophobic residues, as would be likely during protein unfolding. Thus, glycerol would tend to favor the more folded or compact state.

If the two proposed free enzyme conformations of glucokinase exist, then some alteration in hydrophobic bonding may be involved in their interconversion. For example, E may have a more compact structure with less exposed hydrophobic groups than E′ and so glycerol would tend to favor the structure of E to a greater extent than E′ giving rise to the observed kinetics. Results from other studies give further support of these proposals. If H_2O is replaced as the solvent by ^2H_2O, there are changes in the kinetics of the glucokinase reaction similar to those resulting from the presence of glycerol (41). Although there is some controversy over the effects of ^2H_2O on protein interactions, the general opinion is that hydrophobic interactions between amino acid side chains are stronger in ^2H_2O (42, 43). The two sets of results in the two solvents may therefore be interpreted similarly and are consistent with the possible involvement of hydrophobic interactions in the mnemonical transition.

Buc *et al.* (44) have provided fluorescence evidence that two free forms of the wheat germ hexokinase exist and, furthermore, they postulate that the interconversion involves a disordering or unfolding of the polypeptide chain increasing the accessibility of the hydrophobic regions of the enzyme to solvent. Since both the wheat germ and liver enzymes are monomeric enzymes that catalyze the same reaction, and both deviate from Michaelis-Menten kinetics, albeit in opposite directions, it would seem most probable that similar types of protein-solvent interactions are involved in the mnemonical transition in each case. This would lend support to the proposal that the model best explains the results in glycerol and that the transition for glucokinase involves hydrophobic interactions.

We therefore believe that the kinetics of the glucokinase reaction in glycerol further support the mnemonical model as an explanation of glucokinase cooperativity. These results also suggest the possible involvement of hydrophobic interactions in this model, a proposal which may be shown by future studies involving direct measurements of protein conformational changes.

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