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INHIBITION OF RABBIT MUSCLE GLYCOGEN PHOSPHORYLASE BY α-D-GLUCOPYRANOSE 1,2-CYCLIC PHOSPHATE

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Summary

α-D-glucopyranose 1,2-cyclic phosphate is a reversible inhibitor of rabbit muscle phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) and the *a* and *b* forms are inhibited to approximately equal extents. Inhibition of phosphorylase *a* is competitive with respect to α-D-glucopyranose 1-phosphate and orthophosphate, and non-competitive with respect to glycogen (in the direction of synthesis). The data indicates that the cyclic phosphate binds to the enzyme · AMP · glycogen complex with a dissociation constant of 0.5 mM, to the enzyme · AMP complex with a constant of approx. 0.2 mM, and to the enzyme · glycogen complex with a constant of 0.3 mM. The similar affinities for the enzyme · glycogen complex in the presence and absence of AMP suggests that, unlike some other inhibitors that are competitive with respect to glucose-1-*P*, the cyclic phosphate binds to the active conformation of the enzyme. Binding does not occur with any enzyme form containing glucose-1-*P* or inorganic phosphate. Glucose 1,2-cyclic phosphate binds to the enzyme with an affinity five times that of glucose-1-*P* and eight times that of inorganic phosphate (in the case of the enzyme · AMP · glycogen complex). It is likely that the cyclic phosphate is locked in a conformation that mimics the conformation of glucose-1-*P* in the enzyme · substrate complex.

Introduction

α-D-Glucopyranose 1,2-cyclic phosphate has some potential as an active-site probe for the study of enzymes that utilize glucose-1-*P* as a substrate. One pos-

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Abbreviations: glucose-1-*P*, α-D-glucopyranose 1-phosphate; glucose 1,2-cyclic phosphate, α-D-glucopyranose 1,2-cylic phosphate.

sibility is that of forming a stable covalent 2-phosphoglucosyl enzyme with enzymes that normally form transient glucosyl enzyme intermediates. Another is that the cyclic phosphate is a rigid analog of one of the rotational isomers of glucose-1-*P* and might be useful for elucidating the conformation of enzyme-bound glucose-1-*P*. A recent publication by Kokesh et al. [1] has explored the interaction of the cyclic phosphate with polysaccharide phosphorylase from potato. In this case, the cyclic phosphate is neither an irreversible inhibitor nor a substrate, but is a relatively good reversible inhibitor competitive with respect to glucose-1-*P*. No firm conclusion was reached, but the possibility that the cyclic phosphate mimics the conformation of enzyme-bound glucose-1-P was discussed.

In the present work we have found, similarly, that the cyclic phosphate is not an irreversible inhibitor of glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) from rabbit muscle, but is a good reversible inhibitor. In experiments with phosphorylase *a* the cyclic phosphate proved to be competitive with respect to both glucose-1-*P* and inorganic phosphate and non-competitive with respect to glycogen. Since phosphorylase *a* has a rapid equilibrium random bi-bi mechanism [2—4] the present evidence indicates that the cyclic phosphate binds to the enzyme · AMP complex, the enzyme · glycogen complex, and the enzyme · AMP · glycogen complex, but not to enzyme forms containing either glucose-1-*P* or P_i. Therefore, the cyclic phosphate occupies the phosphate binding subsite of the enzyme active site [5], and probably also occupies the subsite that normally binds the glucose residue that is transferred in the catalyzed reaction. Compared to the situation with potato phosphorylase [1], binding of cyclic phosphate to the enzyme · AMP · glycogen complex is relatively weak: the ratio of affinities between cyclic phosphate and glucose-1-*P* is approx. 5, in contrast to 26 for potato phosphorylase [1].

Materials and Methods

Phosphorylase *b* was prepared from frozen rabbit muscle (Pel-Freez, type I) by the method of Fischer and Krebs [6]. Phosphorylase *a* was prepared by the method of Krebs and Fischer [7] using a crude preparation of phosphorylase kinase, and was recrystallized three times. Glucose-1-*P* (dipotassium salt), AMP, and shellfish glycogen were products of Sigma Chemical Corp. Glucose 1,2-cyclic phosphate was prepared by cyclization of glucose-1-*P* with dicyclohexylcarbodiimide in aqueous pyridine solution [8,9]. This material was crystallized as the barium salt and gave a single spot of R_F 0.63 on paper chromatography in isopropanol/NH_3/H_2O (7 : 1 : 2, v/v) [10]. This value for the R_F excludes the possibility of the presence of unreacted glucose-1-*P* or an *N*-phosphorylurea derivative, which could have been a side-product of the reaction [8].

Initial velocity in the direction of glycogen synthesis was determined by a procedure described by Hu and Gold [11]. Reaction mixtures contained 25 mM potassium maleate (pH 6.8), 0.1 mM EDTA, 3.5 mM dithiothreitol, and 1 mM AMP (except where indicated) in a total volume of 50 μl; they also contained glucose-1-*P*, shellfish glycogen, enzyme, and glucose 1,2-cyclic phosphate as indicated. Glycogen concentrations are expressed as molar concentra-

tion of glucose residues. Incubation was carried out for 5—10 min and inorganic phosphate was determined. Initial velocity in the direction of glycogen degradation was carried out under similar conditions, substituting [^{32}P]orthophosphate for glucose-1-*P*. Isotopic glucose-1-*P* synthesis was estimated by the method described by Gold et al. [2]. All rate determinations were carried out at 30°C, pH 6.8. Points shown in the figures are usually averages of duplicates, while the lines are drawn from least-squares treatment of the data before averaging duplicates.

Results and Discussion

Preliminary experiments on the steady-state inhibition of phosphorylase *a* and *b* by glucose 1,2-cyclic phosphate were carried out in the direction of glycogen degradation using high substrate concentrations (20 mM P_i and 10 mM glycogen) and low substrate concentrations (0.1 mM P_i and 0.05 mM glycogen). The two forms of the enzyme showed similar degrees of inhibition, so the phenomenon was investigated in detail using phosphorylase *a*, which tends to give more reproducible results in our hands.

Kinetics of inhibition by glucose 1,2-cyclic phosphate with respect to glucose-1-*P*, P_i, and glycogen are shown as double reciprocal plots in Figs. 1, 2 and and 3, respectively. It is clear that the cyclic phosphate inhibits competitively with respect to glucose-1-*P* and P_i, and non-competitively with respect to glycogen. Experiments were carried out to examine the inhibition by glucose 1,2-cyclic phosphate with respect to glucose-1-*P* in the absence of AMP (unpublished data); inhibition was competitive under conditions similar to those described in the legend to Fig. 1.

Several experiments were carried out with phosphorylase *b* to demonstrate that glucose 1,2-cyclic phosphate is not an irreversible inhibitor of this enzyme.

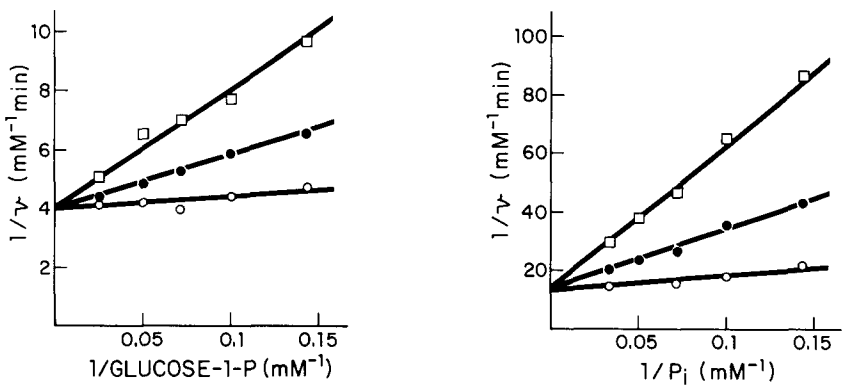

Fig. 1. Phosphorylase *a* activity as a function of concentrations of glucose-1-*P* and glucose 1,2-cyclic phosphate. Conditions are described in the experimental section. Concentration of glycogen is 10 mM (glucose residues) and AMP is 1.0 mM. Cyclic phosphate concentrations are: ○, none; ●, 1.89 mM; □, 3.78 mM.

Fig. 2. Phosphorylase *a* activity as a function of concentrations of orthophosphate and glucose 1,2-cyclic phosphate. Conditions are described in the experimental section. Concentration of glycogen is 4.0 mM (glucose residues) and AMP is 1.0 mM. Cyclic phosphate concentrations are: ○, none; ●, 1.89 mM; □, 3.78 mM.

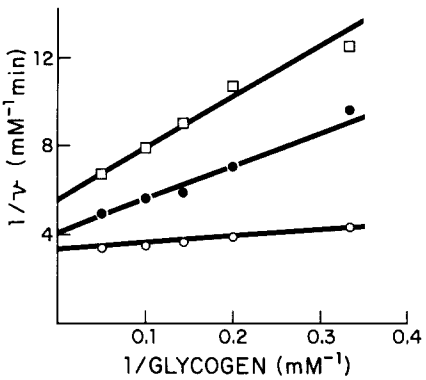

Fig. 3. Phosphorylase *a* activity as a function of concentrations of glycogen and glucose 1,2-cyclic phosphate. Conditions are described in the experimental section. The reaction was carried out in the direction of glycogen synthesis at a glucose-1-*P* concentration of 10 mM and 1.0 mM AMP. Cyclic phosphate concentrations are: ○, none; ●, 2.0 mM; □, 4.0 mM. Concentrations of glycogen are expressed in terms of total glucose residues.

The enzyme was incubated under conditions similar to those used for the steady-state kinetics with 20 mM glucose 1,2-cyclic phosphate for 30 min. Incubations were carried out in the absence of substrates, in both the presence and absence of 0.5 mM AMP, and in the presence of 0.05 mM glycogen with and without AMP. Enzyme activity was determined by diluting the reaction mixture and assaying the rates of both glycogen synthesis and degradation. Glycogen synthesis was determined with 20 mM glucose-1-*P* and 10 mM glycogen; glycogen degradation was determined with 20 mM P_i and 10 mM glycogen and again with 0.1 mM P_i and 0.05 mM glycogen. In all cases, there was no observable inhibition of activity when compared with enzyme that had been treated similarly, but without the cyclic phosphate.

Because the cyclic phosphate was used in the form of its barium salt, it was necessary to demonstrate that Ba^{2+} does not inhibit the enzyme. Phosphorylase *b* was tested in the presence of 10 mM glucose-1-*P*, 10 mM glycogen, 1 mM AMP, and $BaCl_2$ at concentrations as high as 10 mM. There was no significant inhibition of phosphorylase *b* at any concentration of $BaCl_2$.

Kinetic constants for the inhibitor can be calculated from the data in Figs. 1—3 by an analysis that has been described in detail [5]. The kinetic data in the presence of AMP indicate that the inhibitor binds only to the enzyme · AMP complex and the enzyme · AMP · glycogen complex; enzyme forms containing glucose-1-*P* or P_i cannot bind the cyclic phosphate. Rate equation 1, based on the known kinetic mechanism of phosphorylase *a* [2—4], can be derived by using the system of nomenclature introduced by Cleland [12].

$$v = \frac{V_1AB - V_1AP/K_{eq}}{K_{ia}K_b(1 + I/K_I) + K_bA(1 + I/K_{AI}) + K_aB + AB + (K'_aP + AP)V_1/V_2K_{eq}} \quad (1)$$

A, *B*, and *P* represent the concentrations of glycogen, glucose-1-*P*, and P_i, respectively. The constants K_a and K'_a are Michaelis constants for glycogen in the directions of synthesis and degradation, respectively. K_I is the equilibrium constant for dissociation of the inhibitor from the enzyme · AMP · inhibitor

complex, and K_{AI} is that for dissociation of inhibitor from the enzyme · AMP · glycogen · inhibitor complex. Because the kinetic mechanism is rapid equilibrium random bi-bi the Michaelis and inhibition constants for substrates represent macroscopic dissociation constants of appropriate enzyme forms [2—4]; the constants have been determined under conditions similar to those used here [2]. Other relationships that pertain to the rate equation are expressed in Eqns. 2—4.

$$K_{ia}K_b = K_aK_{ib} \tag{2}$$

$$K_{ia}K_p = K'_aK_{ip} \tag{3}$$

$$K_{eq} = V_1K_p/V_2K_b \tag{4}$$

These equations allow us to calculate K_I and K_{AI} from the kinetic data and the known kinetic constants. Although uncertainties in the data and the need for using kinetic constants in the calculations limit the accuracy of the inhibitor dissociation constants, it is possible to obtain consistent values. A replot of slopes vs. I from Fig. 3 gives K_I = 0.2 mM, using a value of 10 mM for K_{ib} [2]. Fig. 1 yields K_{AI} = 0.5 mM using the value of K_I determined above and 1 mM as the best estimate of K_{ia} [2]. Similarly, the data in Fig. 2 give K_{AI} = 0.6 mM. Since the Michaelis constant for glucose-1-*P* is 2.7 mM, the relative affinity of the enzyme · AMP · glycogen complex for glucose 1,2-cyclic phosphate compared to glucose-1-*P* is approx. 5; this is significantly less than the value of 26 reported by Kokesh et al. [1] as the corresponding ratio for potato phosphorylase. The affinity of the cyclic phosphate for the enzyme · AMP complex appears to be considerably greater than that of glucose-1-*P*, but the high probable error in both values makes conclusions impossible.

Several inhibitors which are competitive with respect to glucose-1-*P* have been shown to bind more strongly to phosphorylase *a* in the absence of AMP than in its presence. Helmreich et al. [13] reported that an equilibrium mixture of α and β glucose has a K_{AI} of 90 mM in the presence of AMP and 7.9 mM in its absence; Ariki and Fukui [14] found that α-D-glucopyranosyl fluoride has a K_{AI} of 17 mM in the presence of AMP and 2.8 mM in its absence. Glucose-1-*P* behaves in the opposite way, binding more strongly in the presence of AMP. Both inhibitors appear to increase homotropic cooperativity in the binding of glucose-1-*P* in the absence of AMP. Crystallographic studies of Sygusch et al. [15] indicate that D-glucose binds at the same site as glucose-1-*P* in what is probably the active site of phosphorylase *a*. A reasonable explanation for these findings is that inhibitors such as glucose and glucosyl fluoride bind preferentially to an inactive conformation of the enzyme, while AMP and substrates bind more strongly to the active form [13]. Our experiments with glucose 1,2-cyclic phosphate and glucose-1-*P* as variable substrate carried out in the absence of AMP lead to the calculation of K_{AI} = 0.3 mM. This is only slightly smaller than the value of 0.5—0.6 mM obtained in the presence of AMP and indicates that this inhibitor behaves more like glucose-1-*P* than does glucose or glucosyl fluoride. It is probable that glucose 1,2-cyclic phosphate binds to the active form of the enzyme to a considerable extent.

Kokesh et al. [1] have discussed their results with potato phosphorylase in terms of alternate enthalpic and entropic explanations. The former holds that

the stronger binding of the cyclic phosphate is the result of an additional enzyme-ligand interaction, possibly a hydrogen bond. The latter suggests that the cyclic phosphate mimics the conformation of enzyme-bound glucose-1-*P* and binds more strongly because it is locked in this conformation, while glucose-1-*P* has freedom of rotation around the C—OP and CO—P bonds and must give up the corresponding entropy when binding to the enzyme. Kokesh et al. [1] did not have sufficient evidence to distinguish between these alternatives and we do not have the necessary evidence in the case of muscle phosphorylase. The additional evidence presented here appears to favor the argument that glucose-1-*P* binds to the enzyme in a conformation similar to that of the cyclic phosphate.

Glucose 1,2-cyclic phosphate occupies the phosphate-binding site of glycogen phosphorylase *a*, since it is competitive with respect to P_i. Binding at this site is sufficient to explain the competitive inhibition with respect to glucose-1-*P* because P_i and glucose-1-*P* bear a competitive relation to one another [2]. The moderately strong binding observed for the cyclic phosphate (approx. eight times the affinity of P_i for the enzyme · AMP · glycogen complex) suggests that it does not bind exclusively at the phosphate binding site, but also occupies the glucose transfer site. If true, the orientation of the phosphate group in the cyclic phosphate must approximate the conformation of glucose-1-*P* bound to the enzyme.

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