Effect of Glucose 6-Phosphate on the Nucleotide Site of Glycogen Phosphorylase b

A GENERAL APPROACH FOR NEGATIVE HETEROTROPIC INTERACTIONS*

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SUMMARY

Kinetic studies indicated that glucose-6-P inhibition of glycogen phosphorylase b was partially competitive with respect to AMP, an activator. This observation supports the view that the interaction between the activator and the inhibitor in phosphorylase b is allosteric in nature, a negative heterotropic interaction. Such interaction is believed to arise from alterations at the activator binding site as a result of an inhibitor-induced conformational change of the enzyme. In order to characterize the nature of alterations at the AMP site, the effect of glucose-6-P on interactions between the enzyme and several AMP analogues were examined kinetically. Since a glutaraldehyde-modified phosphorylase b did not exhibit homotropic cooperativity, yet retained all the other kinetic properties of native phosphorylase b, the interactions between glucose-6-P and AMP analogues in this derived enzyme were also investigated to facilitate the kinetic analysis. Among the AMP analogues used in this study IMP, UMP, GMP, CMP, dAMP, and adenosine 5'-phosphoramide date were activators. Their activation of the enzyme could be competitively inhibited by glucose-6-P. In the presence of 20 mM glucose-6-P, the enzyme affinity toward these activators decreased 10- to 18-fold. The other nucleotide analogues used in this study were competitive inhibitors of phosphorylase b with respect to AMP. Their interactions with the allosteric inhibitor were analyzed by multiple inhibition kinetics. Results indicated that the mode of interaction between glucose-6-P and the inhibitory analogues depended upon the structure of the analogues. The interaction between phosphorylase b and adenosine, adenosine-3'-P, adenosine-2'-P, cyclic 2',3'-AMP or cyclic 3',5'-AMP was essentially independent of glucose-6-P, whereas the interaction of dCMP or dGMP with the enzyme was greatly impeded by the allosteric inhibitor. The results suggested that alterations at the AMP site of the enzyme during the allosteric transition involved primarily the subsite which interacted with the phosphate moiety of this nucleotide.

The inhibition of glycogen phosphorylase b (α-1,4-g lucan orthophosphate glucosyltransferase, EC 2.4.1.1) by glucose-6-P has kinetic properties of a competitive inhibition with respect to AMP, an activator (1, 2). The lack of structural similarity between the activator and the inhibitor, however, suggests that the apparent "competitive effect" is allosteric in nature, a negative heterotropic interaction (3, 4). Such a suggestion has received strong support from a more detailed kinetic study in the present communication.

Evidence has accumulated in recent years to support the concept of allosteric interactions. It is believed that a negative heterotropic interaction is due to alterations at the substrate or the activator binding site of an enzyme as a result of inhibitor-induced conformational changes. To our knowledge, no attempts have been made to delineate the nature of alterations at the binding site for any negative heterotropic interactions.

Recently, several reports on nucleotide specificity of phosphorylase b have appeared (5-7). These studies have shown that a great number of nucleotides, nucleosides, and related compounds can interact with the enzyme at the AMP binding site. Since structural analogues are useful in the characterization of the activator site, an examination of the effect of glucose-6-P on interactions between AMP analogues and phosphorylase b may provide useful information concerning the nature of the allosteric alterations at the nucleotide binding site. Thus, kinetic studies of the interactions between glucose-6-P and several of the nucleotide analogues in phosphorylase b have been undertaken in the present study.

Although interactions between an enzyme and its effectors may be studied kinetically, the existence of homotropic cooperativity in phosphorylase b has made the kinetic analysis highly complex. Recently, we have prepared and purified a glutaraldehyde-modified phosphorylase b (8). Kinetic studies have indicated that this enzyme derivative possesses all the kinetic characterization of the native enzyme with the exception of lacking homotropic cooperativity (9). This derived enzyme, therefore, was used in the present study for kinetic analysis of the interaction between glucose-6-P and the nucleotides or their analogues. The applicability of the conclusions of the kinetic analysis to native phosphorylase b was also examined.

The interaction between the nucleotide activator and glucose
Glucose-6-P Inhibition of Phosphorylase b

FIG. 1. Double reciprocal plot for glutaraldehyde-modified phosphorylase b with respect to AMP in the presence of the following levels of glucose-6-P: 0 (1), 1 (O), 4 (■), and 6 mM (△). The substrates used were 1% glycogen and 30 mM glucose-1-P.

FIG. 2. Dixon plot for the inhibition of phosphorylase b by glucose-6-P (G-6-P) at 1% glycogen, 30 mM glucose-1-P, and 5 (1), 0.2 (O), and 0.08 mM (△) AMP.

FIG. 3. Dixon plot for the inhibition of phosphorylase b by glucose-6-P (G-6-P) at 1% glycogen, 30 mM glucose-1-P, and 5 (1), 0.2 (O), and 0.08 mM (△) AMP.

Materials and Methods

Glycogen phosphorylase b was prepared by the procedure of Fischer and Krebs (13) and was recrystallized four times. The recrystallized enzyme was treated with Norit A to free it from nucleotides. Glutaraldehyde-modified phosphorylase b was prepared and purified by a previously described procedure (8). Glucose-1-P, shellfish glycogen, glucose-6-P, nucleotides and their analogues with the exception of GMP and dGMP were purchased from Sigma Chemical Company, GMP and dGMP were from Calbiochem. All the nucleotides and their analogues were the highest grade obtainable. Their purity was established by a paper chromatography procedure which could detect as low as 0.2% contamination of AMP in the various analogues (14). Glycogen was purified by treatment with Norit A (15) to remove contaminating nucleotides.

Initial velocity of phosphorylase b was measured at 30°C in the direction of glycogen synthesis with a procedure similar to that of Illingworth and Cori (16).

Results

In the presence of glucose-6-P, glycogen phosphorylase b exhibits a decreased affinity toward the activator AMP, without changing the V_max (1, 2). Thus, this inhibition may be considered as competitive with respect to the nucleotide. Fig. 1 shows that glucose-6-P is also a competitive inhibitor for the glutaraldehyde-modified phosphorylase b. The inhibition constant calculated from the data in Fig. 1 is 0.9 mM. All the double reciprocal plots with respect to AMP are linear irrespective of the presence of the inhibitor. The result supports the view that this enzyme derivative does not show homotropic cooperativity (8, 9). Madsen and Schechosky (2) have shown that glucose-6-P enhances the homotropic cooperativity between AMP molecules in native phosphorylase b.

In order to differentiate between a fully and a partially competitive inhibition, it is necessary to examine the inhibition
kinetics over a wide range of inhibitor concentration. One approach is to determine the dependence of initial velocity upon inhibitor concentration at fixed levels of the competing activator. The results are then plotted in the form of a Dixon plot (17) as in Fig. 2. The kinetic equation for a partially competitive inhibition may be written as

\[
\frac{1}{v} = \frac{1}{V} + \frac{K_a}{V[A]} \times \left( \frac{1 + [I]}{K_i} \right) + \frac{K'_a}{K'_i K_i} \tag{1}
\]

where [A] and [I] represent concentrations of the competing activator and inhibitor, \(K_a, K_i, \) and \(K'_a\) represent the dissociation constants for \(E_A, E_I, \) and \(EIA\) (dissociating to \(E I \) and \(A\)), respectively, \(K'_a\) being much greater than \(K_a,\) and maximum velocity \(V\) is independent of the binding of inhibitor. If the ternary complex \(EAI\) does not form in the enzyme reaction (e.g. \(K'_a = \infty\)), the term \(I/K_i \times K_a/K'_a\) will be eliminated and Equation 1 is simplified to the kinetic expression for a fully competitive inhibition. Thus, for a fully competitive inhibition, the Dixon plot should consist of a family of straight lines. For a partially competitive inhibition, the lines are approximately linear at low inhibitor concentrations where \(I/K_i \times K_a/K'_a\) is much smaller than unity. Upon increase in inhibitor concentration, the lines curve downwardly and asymptotically. If Equation 1 is rearranged into Equation 2, it can be seen readily that the finite values these lines approach are expressed by \((1/V) + (K'_a/V[A])\), a function of activator concentration.

Results in Fig. 2, therefore, indicate that the kinetics of glucose-6-P inhibition is of the partially competitive type. Similarly, the inhibition of native phosphorylase \(b\) by glucose-6-P also appears to be partially competitive with respect to AMP. Fig. 3 depicts the Dixon plot of glucose-6-P inhibition with respect to AMP for the native enzyme. The plot consists of lines curving downward at high concentration of the inhibitor. Although these features are in agreement with the kinetics of a partially competitive inhibition, the kinetic equations (Equations 1 and 2) are not directly applicable to these results. At low inhibitor concentration, the lines in Fig. 3 show upward curvature instead of straight lines as predicted by the equations. An upward curvature, however, is in agreement with the existence of homotropic cooperativity between glucose-6-P molecules in phosphorylase \(b\).

In addition to Dixon plot, the replot of slope versus inhibitor concentration from a primary plot similar to Fig. 1 may be used to differentiate the type of competitive inhibition. It can be seen from Equation 1 that such replot for a fully or a partially competitive inhibitor is linear or hyperbolic, respectively. This approach is not suitable for the kinetics of native phosphorylase \(b\) since its primary plot consists of curved lines (2). For the modified enzyme, the replot of Fig. 1 is essentially linear, presumably because of the low range of inhibitor concentration used. Results in Fig. 2 indicate that apparent deviation from linearity is to be expected at glucose-6-P concentrations higher than 10 \(\text{mM}\).

Recently, it has been shown that phosphorylase \(b\) may be activated by a number of other nucleotides in addition to AMP (5–7). The effect of glucose-6-P on the enzyme activation by some of these nucleotides was, therefore, examined. The activators were so selected that AMP analogues with modifications on each of the three moieties, adenine base, ribose, and phosphate, were all represented, and that both purine and pyrimidine nucleotides were included. In all these cases, glucose-6-P was found to inhibit phosphorylase \(b\) competitively.

### Table I

| Nucleotide | \(K_a\) for phosphorylase \(b\) | \(K_a\) for modified enzyme \(b\) |
|------------|------------------|------------------|
| Without glucose-6-P | 20 mM glucose-6-P | Without glucose-6-P | 20 mM glucose-6-P |
| AMP | 0.045 | 0.71 | 0.063 | 1.03 |
| IMP | 1.670 | 27.20 | 1.300 | 24.30 |
| CMP | 2.310 | 39.20 | 3.100 | 44.40 |
| 2′-dAMP | 0.900 | 11.70 | 0.920 | 13.50 |
| GMP | 1.660 | 30.80 | 1.430 | 25.00 |
| Adenosine 5′-phosphamidate | 3.570 | 64.70 | 3.130 | 58.60 |

\(^a\) Experimental conditions for AMP activation were as in the legend of Fig. 1; those for other nucleotide activation were as in the legend for Fig. 4.

\(^b\) Nucleotide concentrations at 50\% \(V_{\text{max}}\) were used as \(K_a\) values.
Glucose-6-P Inhibition of Phosphorylase b

Fig. 5. Kinetic plot for the multiple inhibition of glutaraldehyde-modified phosphorylase b: a, using glucose-6-P (G-6-P) as variable inhibitor at 0 ( ), 5 ( ), and 10 mM of adenosine ( ); and b, using adenosine as variable inhibitor at 0 ( ), 2 ( ), and 6 mM glucose-6-P ( ). Other reactants were 0.05 mM AMP, 1% glycogen and 30 mM glucose-1-P.

with respect to the activators. For the glutaraldehyde-modified enzyme, the Lineweaver-Burk plots for the activators were linear irrespective of the presence of the inhibitor. Thus, the values for $K_a$ and apparent $K_a$ for these activators could be readily calculated for the enzyme derivative. Lineweaver-Burk plots for the native enzyme, however, show pronounced curvature in the presence of glucose-6-P. As an example, the kinetic plots for both glutaraldehyde-modified and native phosphorylase b using CMP as an activator is presented in Fig. 4. Similar plots have been obtained for all the activators tested. In Table I, the values of $K_a$ and apparent $K_a$ at 20 mM glucose-6-P are listed for these activators. The ratio of $K_a$ to respective apparent $K_a$ are all in the range of 10-18.

In addition to the activating nucleotides, a great number of AMP analogues can inhibit phosphorylase b competitively with respect to AMP (6, 7). The effect of glucose-6-P on the interactions between phosphorylase b and some selected inhibitory AMP analogues has also been examined. Yonetani and Theorell (10) have devised graphic methods to analyze the kinetics of interaction between two competitive inhibitors. The graphic method developed by these authors consists of plotting $1/v$ against $[I_2]$ at several fixed levels of $[I_1]$, thus giving rise to a family of straight lines. These linear lines intersect at an abscissa value which equals $K'_{I_1}$.

This last equation is in a form essentially the same as the kinetic equation used by Yonetani and Theorell (10) for the multiple inhibition kinetics for two fully competitive inhibitors. The graphic method developed by these authors consists of plotting $1/v$ against $[I_2]$ at several fixed levels of $[I_1]$, thus giving rise to a family of straight lines. These linear lines intersect at an abscissa value which equals $K'_{I_1}$. The mode and extent of the interaction between the two inhibitors has been expressed by a constant, $\alpha$, interaction constant, which relates to the dissociation constants of the various enzyme inhibitor complexes, $\alpha = K_{I_1}/K_{I_1} = K_{I_2}/K_{I_2}$. Thus, this constant can be obtained by simultaneous determination of either $K_{I_1}$ and $K_{I_1}$ or $K_{I_2}$ and $K_{I_2}$.

Although the affinity of glycogen phosphorylase b toward the various nucleotides decreases by more than 10-fold in the presence of 20 mM glucose-6-P, the multiple inhibition kinetics indicate that an interaction between the enzyme and glucose-6-P does not always have an adverse effect upon the interaction between the enzyme and an inhibitory AMP analogue. Many of the inhibitory analogues interact with phosphorylase b essentially
independently of glucose-6-P. As an example, Fig. 5 depicts the kinetic plot of the interaction between glucose-6-P and adenosine in the glutaraldehyde-modified phosphorylase b. A family of straight lines is obtained when \( 1/v \) is plotted against the concentration of adenosine at several constant levels of glucose-6-P (Fig. 5b). From the intersection of these lines, the value of \( K_i \) is calculated to be 5.7 mM. This value is essentially the same as the dissociation constant for adenosine-enzyme complex \( (K_{D}) \) determined from a Dixon plot. Similar results have been obtained by using glucose-6-P as the variable inhibitor at several levels of adenosine. The ratio of \( K_{D} \) to \( K_i \) has been calculated to be 0.94 from the data in Fig. 5a and a Dixon plot. The interactions between a number of other inhibitory AMP analogues and glucose-6-P have been investigated using either the sugar derivative or the analogue as variable inhibitor. As shown in Table II, several other AMP analogues also interact with the glutaraldehyde-modified phosphorylase b independently of glucose-6-P. The interaction constants, \( \alpha \), for the analogues listed in the table are all in the range of 0.9 to 1.1. In contrast to these analogues, dCMP and dGMP exhibit pronounced antagonistic interaction with glucose-6-P in the glutaraldehyde-modified phosphorylase b. Fig. 6 shows that the multiple inhibition kinetics for these analogues consist of lines which are essentially parallel, thus, indicating that the \( \alpha \) values are very large. These results suggest that the inhibitory analogues may be divided into two categories: those interacting with the glutaraldehyde-modified phosphorylase b independently of glucose-6-P and those showing pronounced antagonistic interaction with the sugar derivative.

In Fig. 7, the kinetic plots for the interaction between glucose-6-P and adenosine in native phosphorylase b are depicted either using adenosine (Fig. 7a) or glucose-6-P as the variable inhibitor.

### Table II

| Analogue          | Interaction constant \( (\alpha) \) |
|-------------------|------------------------------------|
| Adenosine         | 0.94                               |
| Adenosine         | 0.99                               |
| 2'-AMP            | 1.03                               |
| 3'-AMP            | 0.95                               |
| Cyclic 2',3'-AMP  | 1.05                               |
| Cyclic 3',5'-AMP  | 0.93                               |

*a* Using glucose-6-P as variable inhibitor; other conditions as in legend of Fig. 5.

*b* Using the inhibitory analogue as variable inhibitor; other conditions as in legend of Fig. 5.
The discovery of competitive interaction between structurally dissimilar enzyme ligands has played a central role in the formulation of the concept of allosteric interactions (3). This concept may be described briefly by the statement that binding of a specific metabolite to a stereospecific site on an enzyme may result in a modification at another specific enzyme site which is topographically separate from the first site. Thus, an investigation of the nature of the modification at the binding site during an allosteric transition is of fundamental importance toward the understanding of the molecular mechanism of allosteric interaction. The present study represents an attempt to delineate the effect of glucose-6-P on the AMP binding site of glycogen phosphorylase b. Since glucose-6-P and AMP are not structural analogues, their interaction in the enzyme is probably allosteric in nature. This suggestion is substantiated by the observation that inhibition of the enzyme by glucose-6-P is partially rather than fully competitive with respect to the activator AMP.

Although many AMP analogues activate phosphorylase b upon interacting at the AMP binding site (5-7), AMP exhibits by far the highest affinity toward the enzyme. Some of the analogues which represent only a minor modification of AMP structure exhibit enzyme affinities one to two orders lower than AMP (6,7). This observation suggests that either a minor or a localized modification, or both, at the AMP binding site may invoke a drastic decrease in enzyme affinity toward the nucleotide. Therefore, it is not necessary that interactions between phosphorylase b and all the AMP analogues should be equally effected by glucose-6-P. However, in spite of their variations in structure, the nucleotide activators examined in the present study have shown approximately the same extent of decrease in their respective enzyme affinities in the presence of 20 mM glucose 6 P, thus suggesting that the alteration at the AMP binding site induced by the allosteric inhibitor is equally felt by all the activating nucleotides.

One possible interpretation for the general effect of glucose-6-P on nucleotide activation of phosphorylase b may be that the induced modification at the activator binding site involves mainly a subsite which interacts with, or is occupied by a common structural entity of these activators. Since the 5'-phosphate is one of the functional groups on nucleotide essential for enzyme activation, the presence of this group is, therefore, one of the common features of these activators. From kinetic studies of the multiple inhibition of phosphorylase b by glucose-6-P and several inhibitory AMP analogues, it appears that the allosteric inhibitor exerts its effect primarily on the interaction between the enzyme and the phosphate group of the nucleotide. The interaction between phosphorylase b and adenosine, an AMP analogue lacking phosphate group, is not affected by the allosteric inhibitor. In contrast, the inhibitory AMP analogues containing free 5'-phosphate groups all show strong antagonistic interaction with glucose-6-P in the enzyme. These observations, therefore, suggest that the inhibitor-induced modification at the nucleotide binding site involved mainly the subsite which is occupied by or interacts with the 5'-phosphate group. The nature of the change at this subsite is, however, not clear. Several different types of molecular changes can all lead to a decrease in enzyme affinity toward 5'-phosphate-containing nucleotides. Thus, a change which involves a general distortion or tightening at the subsite can make the entrance and fitting of the nucleotide sterically hindered. Another possibility is that there are changes

**Fig. 8.** Kinetic plot for the multiple inhibition of phosphorylase b with dGMP levels fixed at 0 ( ), 5 (O), and 15 mM (Δ) and varying the concentration for glucose-6-P (G-6-P) (a); or with glucose-6-P fixed at 0 ( ), 1 (O), and 2 mM (Δ) at varying concentrations of dCMP (2'-deoxy-5'CMP) (b).
involving increase in anionic groups at this subsite region so that nucleotides carrying 5'-phosphate groups will be electrostatically repelled from this site. Since the phosphate group of nucleotide activator has been shown to participate in binding of the nucleotide to phosphorylase b, (6, 7) an allosteric transition involving removal of the interacting protein groups from the binding site may also lead to a decrease in enzyme affinity toward the nucleotide. The results of the present study do not allow an unequivocal choice among the various alternatives.

The molecular mechanism of allosteric interaction appears to be highly delicate. For a complex molecule such as AMP, a localized allosteric modification of the binding site suffices to regulate its activation of the enzyme. The fact that the allosteric inhibition directs its effect toward a nucleotide group essential for enzyme activation may be physiologically significant. Thus, the inhibition can be equally effective toward all possible nucleotide activators.

Since they interact with an enzyme specifically at the active center, substrate analogues are generally useful for the characterization of the active center of the enzyme. Recently, such an approach has been extended to the delineation of the allosteric site of an enzyme using analogues of an allosteric effector. A further extension of this approach is attempted in the present study to the investigation of the alteration at an enzyme binding site during a specific allosteric transition of the enzyme. The results suggest that this may be a generally useful approach for the study of negative heterotropic interactions.

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