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THE BINDING SITES FOR SUBSTRATE AND EFFECTORS ON GLYCOGEN PHOSPHORYLASE *b*

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SUMMARY

1. The influence of glucose, glucose 6-phosphate and ATP on the saturation curves for glucose 1-phosphate and AMP for two derivatives of rabbit muscle glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) has been examined. The derivatives were: (a) a dinitrophenylated enzyme reported to be desensitised of the cooperativity of glucose 1-phosphate sites (Soman, G. and Philip, G. (1973), Biochim. Biophys. Acta 321, 149–155) and (b) a derivative reportedly desensitised of the cooperativity of AMP sites and obtained by treatment of the enzyme with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry 7, 3590–3607).

2. With both the derivatives glucose 6-phosphate behaves as a true competitive inhibitor of glucose 1-phosphate while glucose and ATP induce cooperativity of the glucose 1-phosphate sites.

3. The DTNB-treated enzyme shows strong cooperativity between AMP sites in the presence of either glucose or glucose 6-phosphate and between glucose 1-phosphate sites in the presence of glucose or ATP.

4. The use of kinetic studies with partially desensitised allosteric enzymes is shown to be a good tool in order to understand the nature of the binding and the identity of the binding sites. The results are shown to be consistent with different modes of binding of glucose 1-phosphate and glucose 6-phosphate and of AMP and ATP on the same sites rather than binding at distinctly different sites.

5. Dinitrophenylation under a variety of conditions was not successful in desensitising the cooperativity of glucose 1-phosphate sites as seen in the presence of glucose.

6. The inadequacy of the model of Monod, Wyman and Changeux to account for the observations in this paper is pointed out and a modified model based on the different modes of binding of the ligands is suggested.

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, dinitrophenyl-.

INTRODUCTION

The activity of rabbit muscle glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) has been found to be influenced by a number of metabolites and other ligands, important among them being glucose, glucose-1-*P*, glucose-6-*P*, AMP and ATP [1]. Although interconversion between phosphorylase *a* and *b* plays a definite role in the control of glycogen breakdown [2] it is generally believed that the efficient control device involves the influence of various ligands and their intracellular concentrations [1, 3]. Considerable amounts of information are available about the influence of these ligands on the activity of the enzyme but very little is known about the nature of binding and control by these ligands.

We find that kinetic studies with partially desensitised enzymes provide useful information about the nature of binding of substrate and effectors. For this, two different derivatives of phosphorylase *b* have been employed. An attempt has been made in this paper to understand the allosteric control of phosphorylase *b* on a molecular level.

MATERIALS AND METHODS

Crystalline phosphorylase *b* was prepared from rabbit skeletal muscle according to Fischer and Krebs [4] but using mercaptoethanol wherever cysteine was necessary. AMP, glucose-1-*P* (disodium salt), shell fish glycogen and cysteine–HCl were products from E. Merck and DTNB was from the Sigma Chemical Co. Glucose-6-*P* and bovine serum albumin were from Koch-Light Laboratories and ATP was from the V. P. Chest Institute, New Delhi. FDNB was from the British Drug House. All other chemicals used were of analytical grade.

The concentrations of phosphorylase *b* were determined using an absorbance index of 13.2 at 280 nm for a 1 % solution [5] and those of the derivatives by the use of Folin reagent [6] calibrated against bovine albumin and crystalline phosphorylase. The molar concentrations of the enzyme were calculated using a molecular weight of 185 000 [5].

For dinitrophenylation, the crystalline enzyme was first dialyzed against 0.025 M Tris–HCl buffer, pH 7.6 [7]. Phosphorylase *b* thus prepared (5 mg/ml) was dinitrophenylated with a 25-fold molar excess of FDNB as described earlier [8] in the presence of required concentrations of the appropriate ligands at 30 °C and when the inactivation was 50–60 %, an excess of 0.03 M cysteine–0.04 M glycerophosphate buffer, pH 6.8, was added. The desired extent of inactivation was found from a previously determined time curve of inactivation. The mixture was passed through a column of Sephadex G-15 to remove the small molecules. The removal of AMP was accomplished by treatment with Norit A. The enzyme solutions were incubated in 0.03 M cysteine–0.04 M glycerophosphate buffer, pH 6.8, for at least 1 h prior to the assay. The time course of inactivation with FDNB was more or less the same with different batches of enzyme preparations. However, the K_m values obtained for the DNP derivatives have been found to change with the age of the enzyme within a given batch but the relative changes in the K_m values in the presence and absence of effectors were very much the same. Therefore, the results reported in this paper with the DNP–

enzyme with the exception of those in Table II were obtained with the same batch of enzyme from experiments performed in two days.

Reaction with DTNB was carried out as described by Kastenschmidt et al. [9]. Crystalline enzyme was first passed through a column of Sephadex G-15 equilibrated with 50 mM sodium glycerophosphate–2 mM EDTA buffer, pH 6.8, to remove mercaptoethanol. The enzyme (4 mg/ml) was then incubated with a 10-fold molar excess of DTNB at 28 °C till about three groups were modified (about two hours) as measured spectrophotometrically. The excess reagent was removed by immediately passing the mixture through a Sephadex G-15 column equilibrated with the glycerophosphate–EDTA buffer.

The initial reaction velocities were measured in the direction of glycogen synthesis and the liberated inorganic phosphate was estimated colorimetrically by the method of Fiske and SubbaRow [10]. Substrates containing 2% glycogen and varying concentrations of AMP or glucose-1-*P* as indicated were mixed with an equal volume of suitably diluted enzyme solution and the initial rates measured. Dinitrophenylated enzyme was assayed in 0.015 M cysteine–0.02 M glycerophosphate buffer, pH 6.8, and the DTNB-treated enzyme in 0.025 M glycerophosphate–1 mM EDTA buffer, pH 6.8.

RESULTS

We have shown that specific modification of rabbit muscle glycogen phosphorylase *b* with FDNB in the presence of AMP and orthophosphate resulted in an enzyme derivative devoid of cooperativity between the glucose-1-*P* sites [8]. The results were analysed with the use of Hill plots and it was shown that the value of the Hill coefficient for glucose-1-*P* in the presence of 10 mM glucose-6-*P* was 1 for this derivative whereas the value was 1.8 for the native enzyme [8]. We find that when glucose is used as the inhibitor the saturation curve for glucose-1-*P* for this derivative becomes sigmoidal and the reciprocal plots non-linear. This observation has necessitated a detailed study of the influence of the various inhibitors on the kinetics with respect to glucose-1-*P* and AMP. Since Hill plots have been found to be very effective in determining the strength of cooperativity of the binding sites of phosphorylase *b* [8, 11] we have employed them for analysis of the data reported in this paper.

Phosphorylase *b* was dinitrophenylated in the presence of 1 mM AMP and 10 mM orthophosphate until the inactivation was 50–60%. Fig. 1 shows the influence of glucose, glucose-6-*P* and ATP on the kinetics of this derivative for glucose-1-*P* in the form of a Hill plot. The reciprocal plots (inset to Fig. 1) show clearly that while glucose-6-*P* behaves as a competitive inhibitor, glucose acts as an allosteric inhibitor indicating that these two ligands bind on the enzyme at different sites or differently. The n value with 10 mM glucose-6-*P* is 1.0 whereas the values with 10 mM glucose and 10 mM ATP are 1.36 and 1.35, respectively. ATP has been shown to increase the cooperativity of glucose-1-*P* sites in native enzyme ($n = 1.6$ with 10 mM ATP) [11]. Therefore, as far as the effect of ATP is concerned the DNP derivative differs from the native enzyme only in the strength of cooperativity of the glucose-1-*P* sites.

The influence of glucose, glucose-6-*P* and ATP on the kinetics with respect to the activator for the DNP–enzyme is shown in Fig. 2. The native enzyme has been found to exhibit homotropic cooperativity for the AMP sites in the concentrations of AMP employed here [11]. Thus the results of Fig. 2 indicate that the modified enzyme

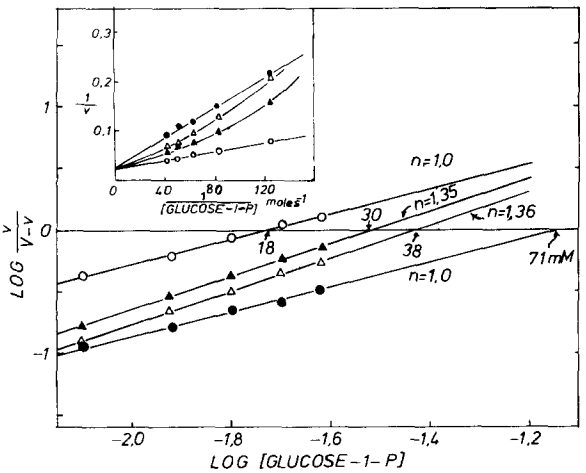

Fig. 1. Hill plots with glucose-1-*P* varied in the absence and presence of inhibitors for DNP-phosphorylase *b*. Phosphorylase *b* was dinitrophenylated in the presence of 10 mM orthophosphate and 1 mM AMP with FDNB until inactivation was 60%. The reaction was stopped by the addition of cysteine–glycerophosphate buffer (pH 6.8) and the DNP-enzyme assayed at 30 °C. ○, control (without inhibitor): ●, with 10 mM glucose-6-*P*; △, with 10 mM glucose; ▲, with 10 mM ATP. The concentrations of glycogen and AMP in the assay mixtures were 1% and 1 mM, respectively, and that of the enzyme was $3.24 \cdot 10^{-7}$ M. *Inset:* Reciprocal plots for the same data (same symbols). Velocities are expressed as μM of orthophosphate liberated/min per mg of the enzyme.

no longer shows cooperativity between the AMP sites. The same is true in the presence of the inhibitors. The reciprocal plots (not shown) in the presence of glucose and glucose-6-*P* were linear but showed different K_m and V values typical of mixed inhibition indicating that these ligands bind at different sites on the enzyme. The kinetics in the presence of ATP was competitive.

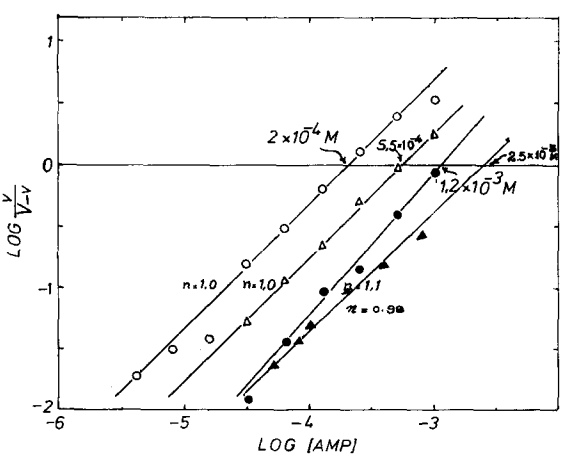

Fig. 2. Hill plots with AMP varied in the absence and presence of inhibitors for DNP–phosphorylase *b*. The DNP–phosphorylase *b* was obtained and assayed as in Fig. 1: ○, control (without inhibitor); ●, with 10 mM glucose-6-*P*; △, with 10 mM glucose; ▲, with 10 mM ATP; concentration of glucose-1-*P* was 16 mM. Other details were as in Fig. 1.

In view of the striking difference in behavior of glucose and glucose-6-*P* on the kinetics of glucose-1-*P* in the DNP–enzyme it was of interest to examine another derivative of phosphorylase *b*. Kastenschmidt et al. have reported that modification of 2–3 SH-groups of phosphorylase *b* by treatment with DTNB would result in an enzyme derivative showing a linear reciprocal plot for AMP under conditions when the native enzyme gives non-linear plots [9]. We have subjected this derivative to studies similar to those illustrated above for the DNP derivative.

Phosphorylase *b* was treated with DTNB till about 3 SH-groups were modified. The influence of various ligands on the kinetics with respect to AMP for the DTNB-treated enzyme is presented in Fig. 3. As reported by Kastenschmidt et al. [9] the

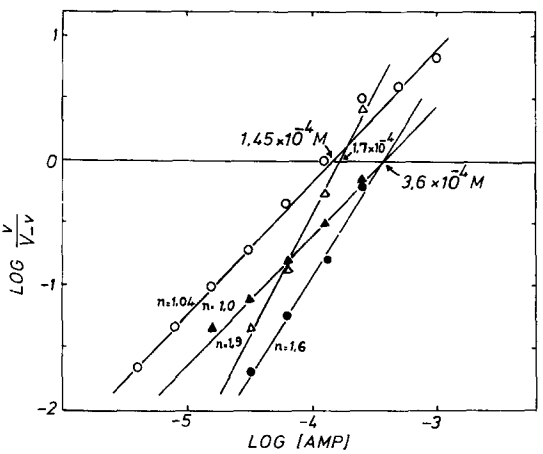

Fig. 3. Hill plots with AMP varied in the absence and presence of inhibitors for the DTNB-treated phosphorylase *b*. Phosphorylase *b* was treated with DTNB until three groups were modified. The reaction mixture was passed through a Sephadex G-15 column. The enzyme derivative was assayed in the glycerophosphate–EDTA buffer (pH 6.8) at 30 °C: ○, control (without inhibitor); ●, with 2 mM glucose-6-*P*: △, with 5 mM glucose: ▲, with 2 mM ATP. Concentration of the enzyme was $1.35 \cdot 10^{-7}$ M and those of glucose-1-*P* and glycogen were 16 mM and 1%, respectively.

derivative shows lack of cooperativity for AMP sites ($n=1$) in the absence of inhibitors. However, in the presence of glucose and glucose-6-*P* the *n* value increases indicating return of cooperativity of the AMP sites. It may be noted that in the presence of ATP the kinetics for AMP are as expected for a desensitised enzyme and the reciprocal plots (not shown) show competitive kinetics for AMP and ATP.

Fig. 4 shows Hill plots with glucose-1-*P* as the varying substrate in the presence of the inhibitors for the DTNB-treated enzyme. The influence of glucose-6-*P* is quite similar to that observed with the DNP derivative, that is, it acts as a competitive inhibitor of glucose-1-*P*. But clear differences in the effect of glucose and glucose-6-*P* are seen here also. With ATP as the inhibitor the strength of cooperativity of the glucose-1-*P* sites is nearly the same as that of the native enzyme. The *n* values with the modified and native enzymes are summarised in Table I to facilitated comparison.

The results presented thus far show that desensitisation of the DNP derivative and the DTNB-treated enzyme is only partial and that the same sites could behave as if desensitised or fully sensitised under the influence of different ligands.

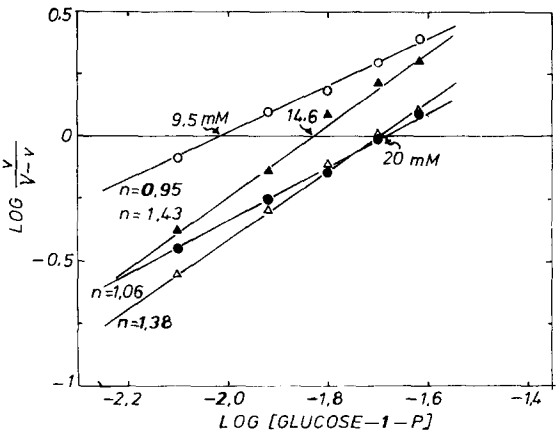

Fig. 4. Hill plots with glucose-1-*P* varied in the presence and absence of inhibitors for the DTNB-treated phosphorylase *b*. The DTNB-treated enzyme was obtained and assayed as in Fig. 3. ○, control (without inhibitor); ●, with 10 mM glucose-6-*P*; △, with 10 mM glucose; ▲, with 10 mM ATP. Concentration of AMP was 1 mM. Other details were as in Fig. 3.

The distinctly different behavior of glucose and glucose-6-*P* has interested us to obtain a derivative which shows no cooperativity for glucose-1-*P* sites in the presence of glucose. Since dinitrophenylation was successful in obtaining partially desensitised enzyme we have attempted the same method in the presence of various ligands alone and in combination. The results were analysed using Hill plots and are summarised in Table II along with the K_m values for glucose-1-*P* in the absence and presence of glucose. As can be seen in Table II some derivatives show decrease in the strength of cooperativity. However, complete desensitisation could not be achieved

TABLE I

HILL COEFFICIENTS FOR NATIVE, DINITROPHENYLATED-, AND DTNB-TREATED PHOSPHORYLASE *b* IN THE ABSENCE AND PRESENCE OF INHIBITORS

The experimental details were as in Figs 1 and 3. Each experiment was repeated 3–4 times with different batches of enzyme preparations and in some cases with different concentrations of inhibitors. The *n* values shown are the average values calculated from 3 experiments.

	Native enzyme	DNP–enzyme	DNTB-treated enzyme
n Values for glucose-1-*P* sites			
Without inhibitor	0.96	0.99	0.95
With glucose-6-*P* (10 mM)	1.8	1.0	1.03
With glucose (10 mM)	1.7	1.33	1.36
With ATP (10 mM)	1.68	1.38	1.39
n Values for AMP sites			
Without inhibitor	1.35	1.04	1.03
With glucose-6-*P* (2 mM)	1.6	1.0	1.85
With glucose (5 mM)	1.55	1.06	1.65
With ATP (10 mM)	1.65	0.97	1.0

TABLE II

INFLUENCE OF DINITROPHENYLATION ON THE HILL COEFFICIENT AND K_m VALUES OF PHOSPHORYLASE *b*

Phosphorylase *b* was dinitrophenylated with FDNB as detailed in Materials and Methods until the inactivation was 50–60%. The reaction was stopped by the addition of excess cysteine–glycerophosphate buffer (pH 6.8). The DNP–enzyme was preincubated in the same buffer for one hour prior to rate measurements. The initial rates were measured with glucose-1-*P* as the varying substrate. The concentrations of AMP and glycogen in the assay mixtures were 1 mM and 1%, respectively.

Dinitrophenylated in the presence of:	*n* values for glucose-1-*P*		K_m values for glucose-1-*P* (mM)	
	No ligand	With glucose (12.5 mM)	No ligand	With glucose (12.5 mM)
None	1.0	1.3	9.1	22.9
AMP (1 mM)	1.0	1.3	24.0	33.9
Orthophosphate (10 mM)	1.0	1.3	14.1	31.6
AMP (1 mM) and orthophosphate (10 mM)	0.93	1.5	11.0	33.1
Glucose (10 mM)	0.90	1.3	14.1	26.3
Glucose (10 mM) and AMP (1 mM)	0.95	1.3	25.7	44.0
Glucose-1-*P* (40 mM)	1.0	1.3	7.7	28.2
Glucose-1-*P* (40 mM) and AMP (1 mM)	0.92	1.4	7.4	19.5
Unmodified (native) enzyme	0.93	1.7	7.4	17.0

by this method. It is possible that glucose interacts with different sites on the enzyme; the glycogen site is a possible one. Kinetic studies with phosphorylase *a* in the presence of glucose have been shown to yield results consistent with the different sites of interaction for glucose [12].

It has been shown that UDP glucose which is a competitive inhibitor of glucose-1-*P* has increased the activity of phosphorylase *b* in the presence of ATP [11]. Since with the DNP–enzyme reported here glucose-6-*P* functions as a competitive inhibitor of glucose-1-*P* we have studied the influence of various concentrations of glucose-6-*P* (1–10 mM) on the activity of this derivative in the presence of ATP and glucose separately but no activation was observed. Similar experiments performed with the DTNB-treated enzyme also did not show any increase of activity.

DISCUSSION

Since the DTNB-treated enzyme and the dinitrophenylated enzyme do not exhibit cooperativity under some conditions but show strong cooperativity of sites under the influence of certain ligands, both these derivatives cannot be considered as fully desensitised. This paper thus stresses the need for detailed kinetic studies before ascertaining the loss of allosteric properties in modified enzymes.

The contrasting behavior of glucose and glucose-6-*P* in the dinitrophenylated enzyme indicates differences in their binding sites. Since these ligands show differences

only in the strength of cooperativity of sites in the native enzyme the separation of sites could not be understood from studies with the native enzyme. The results with the derivatives are, however, different; glucose-6-*P* showing competitive kinetics and glucose exhibiting sigmoidal kinetics for glucose-1-*P*. While glucose-6-*P* is a competitive inhibitor of glucose-1-*P* in the DTNB-treated enzyme ATP increases the homotropic cooperativity of glucose-1-*P* sites (see Fig. 4), suggesting differences also in the binding sites of these ligands. Kurganov et al. [14] comparing the synergism and antagonism exhibited by various combinations of these ligands during the tryptic digestion of phosphorylase *b* have arrived at a similar conclusion about the spacial separation of binding sites. The results presented in this paper thus show that detailed kinetic studies of the influence of various ligands on partially desensitised enzyme derivatives provide a useful tool in delineating the separation of binding sites of substrate and effectors.

It may be noted that our results and those of Kurganov et al. [14] do not distinguish between distinctly different binding sites and different modes of binding by the ligands on same sites. The native enzyme with fully cooperative glucose-1-*P* sites as seen in the presence of glucose-6-*P* on modification of the 2,3-amino acid residues yields derivatives which show not only lack of cooperativity of the substrate sites but also competitive kinetics for glucose-1-*P* and glucose-6-*P*. This transition of the sites implicate different modes of binding by these ligands on same sites rather than distinctly different binding sites for them in native phosphorylase *b*. The same may be said of the sites for AMP and ATP (see Fig. 3). These imply that the groups incorporated in the enzyme by chemical modification have interfered with the otherwise possible mode of binding of glucose-6-*P* and ATP. With DNP–phosphorylase apolar binding of DNP groups prior to covalent bond formation has been suggested from spectral and circular dichroism measurements [15]. DTNB treatment has been shown to modify only cysteinyl residues [9] and dinitrophenylation modifies both cysteinyl and lysyl residues [8]. Considering the structural features of the incorporated groups it is tempting to speculate that the specific influence of these groups is in shielding the binding site in the native enzyme which promotes different modes of binding of glucose -1-*P* and glucose-6-*P* or AMP and ATP. This argument is supported by the observations of Battell et al. that modification of cysteinyl residues with iodoacetamide has no effect on the allosteric properties of the enzyme [16], showing thereby that it is the conformational change induced by the incorporated groups and not simple chemical modification which is responsible for affecting the allosteric properties of phosphorylase *b*.

The two-state transition model of Monod et al. [17] does not explain the observations that 1. glucose-6-*P* shows true competitive inhibition for glucose-1-*P* kinetics but increases the cooperativity of the AMP sites in the DTNB-treated enzyme; 2. the contrasting influence of glucose-6-*P* which being an allosteric inhibitor of the native enzyme (and hence binds to regulatory site) becomes a competitive inhibitor of glucose-1-*P* in the modified enzymes; and 3. the behavior of glucose in the kinetics with respect to AMP and glucose-1-*P* in the DTNB-treated enzyme. Many differences between the experimental observations and predictions of the model have been found by several authors [11, 18–21]. Attempts, therefore, have been made to accommodate the disparities by incorporating further assumptions and assigning additional conformational states [19–22]. While this model predicts the homotropic and heterotropic

effects to be interlinked, Wang and Tu have observed that treatment of phosphorylase *b* with glutaraldehyde would result in an enzyme derivative in which all homotropic effects are abolished but all heterotropic interactions are retained [21]. These authors find that the model proposed by Koshland et al. [23] accounts for these results more satisfactorily.

In view of the results of this paper which are consistent with the different modes of binding of some ligands on same sites of the enzyme we find that a model involving "right and wrong" binding of substrate and effectors could explain the observations with phosphorylase *b*. Right and wrong binding as mechanisms for specificity have been proposed for different enzymes (e.g., ref. 24, 25). Since there are two sites each on phosphorylase *b* dimer for glucose-1-*P* and AMP and the monomers are similar (but not necessarily identical) [26] glucose-1-*P* and AMP may be assumed to bind across the dimer (right mode of binding). Thus with glucose-1-*P* its glucose moiety or part of it binds on one monomer and its phosphate morelyor park of it onto the other. A similar binding may be assumed for AMP also. The exact nature of the monomer–monomer interaction site of phosphorylase is not known at present and with this in mind we have initiated some studies of the solvent effects on the kinetic properties of this enzyme.

Because of the structural features of the substrates and the effectors of phosphorylase *b* the possibility of right and wrong binding is inherent in the model. It may be noted that in the presence of a ligand that binds in a wrong fashion a conformational change will have to be forced on the enzyme in order for the substrate or activator to bind in the right fashion. Thus, in the presence of the inhibitor the kinetics with respect to the substrate or activator will become strikingly cooperative as observed. It may also be noted that the different modes of binding proposed in this paper would lead to different conformational states for the enzyme. It appears that evidence for this model must come from studies with hybrid forms of phosphorylase.

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