Allosteric Properties of Phosphorylase b

II. COMPARISON WITH A KINETIC MODEL*

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SUMMARY

Kinetic data for the inhibition of phosphorylase b by adenosine di- and triphosphate and glucose 6-phosphate have been compared with predictions from the theoretical model for allosteric transitions proposed by Monod, Wyman, and Changeux (6).

The substrate, inorganic orthophosphate, shows homotropic cooperativity at low concentrations, and this cooperativity is increased as the concentration of the activator, AMP, is decreased. The homotropic cooperativity of the substrates, Pi or glucose-1-P, is expressed more strongly and at higher concentrations as the above inhibitors are added, and these effects are proportional to the concentration of the inhibitor.

The activator shows homotropic cooperativity at low concentrations. Decreasing the substrate concentration does not appear to affect the strength of this cooperativity although the apparent $K_c$ values are increased. Adding an inhibitor appears to increase the homotropic cooperativity of AMP and causes it to be expressed at a higher concentration.

The inhibitor, ATP, shows homotropic cooperativity which, measured indirectly by kinetic methods, appears to increase as $P_i$ or AMP is decreased. AMP and substrate act in concert to antagonize the inhibition by ATP.

Uridine diphosphate glucose, which is a competitive inhibitor for $P_i$ or glucose-1-P, activates the enzyme in the presence of ATP at low substrate concentrations.

With some exceptions in the realm of the homotropic cooperativity of activator or inhibitor, these results are in qualitative agreement with the theoretical model cited above. Quantitative agreement has been demonstrated for several situations.

* This investigation was supported in part by United States Public Health Research Grant AM-07294 from the National Institute of Arthritis and Metabolic Diseases, and in part by Grant MT-1414 from the Medical Research Council of Canada. For Paper I of this series, see Reference 1. Part of this work, together with relevant data on binding studies, was presented by O. Avramović and N. B. Madsen at the Fifty-first meeting of the Federation of American Societies for Experimental Biology, Chicago, April 1967.

The glycogen phosphorylases of muscle ($\alpha$-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) have been the subject of intensive investigation in recent years for two main reasons. First, the control of the rate of breakdown of tissue glycogen is exerted at the molecular level by various physiological factors acting on these enzymes. Recent evidence suggests that the enzyme in resting muscle is in the form of phosphorylase b (2), and that under certain conditions phosphorylase b is capable of catalyzing glycogen phosphorylase in vivo (3). Thus the possibility that the enzyme may be controlled by the concentrations of various natural metabolites, i.e. substrates, activators, and inhibitors, must be considered. Other factors, such as metal ions (4), ionic strength (5), and pH, may also have a bearing.

Second, investigations on the nature of its inhibition by such compounds as adenosine triphosphate (1) indicate that phosphorylase b belongs quite properly to the class of enzymes which we refer to as allosteric, in the nomenclature of Monod, Changeux, and Jacob (7). Indeed, Monod et al. (6, 7) have used phosphorylase b as an example and have pointed out that the discovery of its activation by AMP by Cori, Colowick, and Cori in 1938 (8) established this enzyme as the first to be recognized as possessing allosteric properties.

During their investigations of the control of phosphorylase activity in the heart, Parmeggiani and Morgan found that phosphorylase b is inhibited by ATP, glucose-6-P, and ADP (9, 10). Of a large variety of other natural metabolites tested, only inosine triphosphate, guanosine triphosphate, reduced diphasphopyridine nucleotide, and adenosine tetraphosphate exhibited significant inhibition. In general, these compounds showed little effect on phosphorylase a. The possible significance of these findings was discussed with respect to the control of glycolysis in vivo.

We have published a preliminary report on the kinetics of the inhibition of phosphorylase b by ATP in which it was shown that, in the presence of ATP, a plot of activity with respect to substrate (glucose-1-P) concentration describes a sigmoidal curve instead of the normal rectangular hyperbola (1). In our subsequent studies on this and related phenomena, we have found it advantageous to compare our data with the predictions of the theoretical model proposed by Monod, Wyman, and Changeux (6). Other models for allosteric transitions have, of course, been
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FIG. 1. Hill plot of the effect of 10 mM ATP on the activity of phosphorylase b at various glucose-1-P (G-1-P) concentrations. Activity was measured at 30° at pH 6.7 in the presence of 28 mM glycogen and 1 mM AMP.

EXPERIMENTAL PROCEDURE

Phosphorylase b was prepared from rabbit muscle by the method of Fischer and Krebs (13) and was recrystallized four or five times. Before use, crystals were centrifuged out of suspension, dissolved in an appropriate buffer, and passed through a column of Sephadex G-25 gel in order to remove AMP, magnesium, and other impurities. Chemicals and auxiliary enzymes were purchased from Sigma or Calbiochem and were the highest grade obtainable. The rabbit liver glycogen was routinely passed through a Dowex 1-chloride column.

Phosphorylase activity was measured in the direction of glycogen synthesis with glucose-1-P as the substrate, or as the phosphorolysis of glycogen with Pi as the substrate, by the methods previously described (14). In either case, the glycogen concentration was held constant at 28 mM (glucose residues).

RESULTS

Inhibition by ATP—As noted by a number of authors (7, 11, 15), the Hill plot (16) is a useful tool for analyzing some allosteric effects, and the values of the slopes obtained are considered to be a measure of the number of interacting sites as well as the strength of their interaction. When the interaction is very strong, the value of the slope of the Hill plot approaches the number of binding sites for the ligand. The effect of 10 mM ATP on the activity of phosphorylase b at various concentrations of glucose-1-P is shown in Fig. 1, where the data are arranged in the form of a Hill plot. In the absence of inhibitor the $K_m$ is 3.7 mM and the value of $n$ is 1.0, a typical result for a substrate which obeys Michaelis-Menten kinetics and does not show cooperative effects. The presence of ATP increases the apparent $K_m$ to 11 mM and the value of $n$ to 1.75. As has been suggested before, the data in Fig. 1 may be interpreted as indicating that the presence of ATP allows the homotropic cooperativity of the glucose 1-P binding sites to be expressed at substrate concentrations where it does not normally occur. In Fig. 2 the data from Fig. 1 are replotted as $Y$ with respect to $x$ according to the equation of Monod et al. (6). $G$-1-P, glucose-1-P.

Fig. 2. Plot of data from Fig. 1 as $Y$ with respect to $x$ with theoretical lines calculated from the equation of Monod et al. (6).

Fig. 3. Hill plot of the effect of 10 mM ATP on the activity of phosphorylase b at various concentrations of $P_i$. The concentration of AMP was 1 mM.
method of Monod et al. (6). It may be seen that the data fit the theoretical lines calculated from the formula given by the latter authors when it is assumed that there are two binding sites for glucose-1-P and the value of \( L' \), the "apparent allosteric constant," is taken as 10.

Figs. 3 and 4 illustrate the inhibition of phosphorylase \( b \) by ATP in experiments in which the rate of phosphorylosis of glycogen was determined at various concentrations of inorganic phosphate. The data have been treated in the same manner as for Figs. 1 and 2. Differences noted from the experiments with glucose-1-P include a slightly lower value of \( n \) for the same concentration of ATP and a higher value for the apparent allosteric constant (28 instead of 10). In addition, it may be seen that the velocities fall below the predicted values at high concentrations of phosphate for both control and inhibited experiments. This may indicate a mild degree of substrate inhibition, which is also suggested by the fact that, when the control data are plotted by the method of Lineweaver and Burk (17), an upward curvature is seen near the ordinate.

The experiment depicted in the Hill plot of Fig. 5 shows that one can observe homotropic cooperativity of the substrate Pi in the absence of an inhibitor when the rate is measured at very low substrate concentrations. As predicted by the model of Monod et al. (6), each increase in the concentration of inhibitor (ATP) results in an increase in the cooperativity of the substrate, as shown by the increasing value for \( n \). In Fig. 6, the same data have been plotted by the method used by Taketa and Pogell (18). The results show that another prediction of the model of Monod et al. is fulfilled, namely, that the inhibitor will show a strong homotropic cooperativity. The apparent increase in this cooperativity as the substrate concentration is decreased will be discussed below. Finally, the fit of the data against the theoretical lines calculated from the formula of Monod et al. (6) is shown in Fig. 7. The fit is again poor at higher phosphate concentrations, possibly because of substrate inhibition or a general effect of nonspecific phosphate binding to the protein. It may be seen that the value of the apparent allosteric constant increases with increasing levels of ATP.

**Activation by AMP**—Just as an allosteric inhibitor should show antagonism toward both the substrate and an activator, so an
activator should cooperate with the substrate. Such effects were demonstrated by Helmreich and Cori (19) when they showed that AMP and Pi have reciprocal effects on each other's apparent $K_m$ value, that is, an increase in the concentration of AMP results in a decrease in the $K_m$ for Pi, and vice versa. The same has been shown to be true when the varied substrate is glucose-1-P (1). In addition, the model proposed by Monod et al. (6) predicts that this cooperation between activator and substrate ought to manifest itself by a change in the strength of the homotropic cooperativity of the substrate as the concentration of the activator is changed. Data bearing out this prediction are shown in the form of a Hill plot in Fig. 8, where it may be seen that the value of $n$ increases when the concentration of AMP is decreased. As required by the results of Helmreich and Cori (19), the value of the apparent $K_m$ for Pi also increases as AMP is decreased. TPN is present in the reaction mixture and could possibly act as an allosteric inhibitor. It may be seen that increasing the concentration of TPN from 1.0 to 3.5 mM causes a slight inhibition but does not change the value of $n$, suggesting that it is merely a weak competitive inhibitor for Pi, as are a large number of organic phosphates (14).

It has been well established that phosphorylase b has an absolute requirement for AMP, and in our experiments the activity in the presence of optimal concentrations of the substrates but without added AMP was less than 0.4% of $V_{max}$. Even this small activity could be due to contamination of substrates or enzymes with AMP. The activation by AMP was formerly thought to follow normal Michaelis-Menten kinetics for an activator (8), but, more recently, deviation from orthodox behavior has been found by several investigators. For example, Helmreich and Cori (19) found a departure from linearity in Lineweaver-Burk plots at low concentrations of AMP, as have other investigators (20, 21). Fig. 9 shows that, when the activation is depicted on a Hill plot, there is a change from the slope of 1 at high concentrations of AMP, and an $n$ value of 1.5 is obtained. In the presence of ATP the whole line has a slope of 1.4, and the inhibition is reflected in a shift of the line to the right and a 6-fold increase in the apparent $K_m$ for AMP. Fig. 10 is a separate experiment which confirms the homotropic cooperativity of the AMP-binding sites in the absence of ATP. The inhibition by ATP was investigated at two concentrations of the substrate, glucose-1-P, and it may be seen that decreasing the substrate concentration increased the apparent $K_m$.

In Fig. 11, the data from Figs. 9 and 10 are plotted as $F$ with respect to $y$ according to the method of Monod et al. Theoretical lines calculated from the formula suggested by these authors are also shown, the constants having been chosen to give reasonable fits to one of the control curves and one of the inhibited curves. In the absence of inhibitor, the theoretical line fits the experimental data at high concentrations of AMP but does not
allow for sufficient expression of the sigmoid character of the data at low AMP concentrations. Furthermore, the formula of Monod et al. does not yield zero activity in the absence of activator. For example, the calculated value of $Y$ at zero AMP was 0.08, whereas the experimental value was 0.004. A similar result may be seen in Fig. 7 of Monod et al. (6). On the other hand, the line calculated for the case in which ATP was added shows a much greater correspondence to the experimental data.

The model predicts that the homotropic cooperativity of the activator should increase in proportion to the concentration of the inhibitor. Fig. 12 presents data which are in agreement with this prediction except that none of the slopes obtained in the presence of ATP is as steep as that obtained in the absence of ATP at low concentrations of AMP. This apparent anomaly may be related to another seen repeatedly with AMP, the breaks in the lines in the Hill plots.

![Fig. 11. Plot of data from Figs. 9 and 10 as $Y$ with respect to $\gamma$ with lines calculated from the equation of Monod et al. (6), as written in Fig. 2, with $L' = L(1 + \theta)^2/(1 + Y)^2$. ○, control from Fig. 10, 24 mm glucose-1-P ($\alpha = 7; K = 4.5 \times 10^{-4} M; L = 587$); ●, data from Fig. 10, 24 mm glucose-1-P with 7.6 mm ATP ($\alpha = 7; K = 4.5 \times 10^{-4} M; L = 3300$); X, data from Fig. 9, 24 mm glucose-1-P with 9 mm ATP ($\alpha = 7; K = 3.5 \times 10^{-4} M; L = 3300$).](http://www.jbc.org/)

![Fig. 12. Hill plot with AMP varied, showing the effect of increasing concentrations of ATP. Substrate was 2.3 mm P1. The apparent $K_m$ values, reading from the top, are 0.13, 0.71, 1.9, 2.5, and 5.0 mm AMP.](http://www.jbc.org/)

The model proposed by Monod et al. predicts that the homotropic cooperativity of the activator should increase as the concentration of the substrate decreases, and that this will be reflected in increasing slopes of Hill plots when log $v/(V_{max} - v)$ is plotted against the logarithm of the activator concentration. The data of Fig. 8, when plotted in this manner, yield slopes with a value of 1.6 which do not vary with the substrate concentration. Sealock and Graves (20) reported an average value for $n$ of 1.5 ± 0.13 for seven determinations over a range of glucose-1-P concentrations from 9.6 to 64 mm. Furthermore, the excellent data in Fig. 5 of Lowry, Schuls, and Passonneau (21) also yield a value for $n$ of approximately 1.6, which is not changed significantly by decreasing the concentration of P1 or glycogen or both.

**Inhibition by Glucose-6-P and ADP**—A Hill plot illustrating the effect of these two compounds on the activity of phosphorylase b when the variable substrate is glucose-1-P is shown in Fig. 13. The addition of 2 mm glucose-6-P changes the apparent $K_m$ from 2.7 mm to 9.2 mm, and the value of $n$, from 1 to 1.3. The result of adding increasing concentrations of ADP is to increase the apparent $K_m$ values and to further increase the value of $n$. Fig. 14 shows a similar experiment carried out with P1 as the variable substrate. It is apparent that, as the concentration of ADP is increased, there is a continual increase in the homotropic cooperativity of the substrate, a result similar to that obtained with ATP.

![Fig. 13. Hill plot with glucose-1-P (G-1-P) varied, showing the effect of 2 mm glucose-6-P (G-6-P) or increasing concentrations of ADP. AMP at 1 mm was present.](http://www.jbc.org/)

**Activation by Competitive Inhibitor (UDP-glucose)**—Monod et al. (6) have pointed out that a true competitive inhibitor which can bind at the same site as the substrate itself should
FIG. 14. Hill plot with Pi varied, showing the effect of increasing concentrations of ADP, with 1 mM AMP present. The apparent $K_m$ values, reading from the top, are 1.8, 5.1, 13, 23, and 36 mM Pi.

FIG. 15. Hill plot with AMP varied, showing the effect of ATP and 2 mM glucose-6-P (G-6-P). The substrate was 24 mM glucose-1-P.

behave, like the substrate, as an antagonist of the allosteric inhibitor. This effect was first described for aspartate transcarbamylase (22). UDP-glucose has been shown to be an inhibitor which competes with either Pi or glucose-1-P for phosphorylases from several sources (14, 23). Fig. 16 shows that low concentrations of UDP-glucose increase the activity of the enzyme when ATP is present but not when it is absent. The substrate used here was Pi, but a similar result was obtained when the substrate was glucose-1-P.

**DISCUSSION**

The use of the model for allosteric transitions proposed by Monod et al. (6) permits the generation of curves which fit the experimental data obtained for phosphorylase b to a fairly satisfactory degree, as shown in Fig. 2. The apparent substrate inhibition exhibited by Pi complicates the fit to the model when that substrate is the variable ligand. It is of interest that M. C. Michaelides and E. Helmreich found that 50 mM Pi caused inhibition of the binding of AMP to phosphorylase a, whereas 10 mM Pi had little effect. The fit of the data to the model was least satisfactory for the case in which the activator, AMP, was the variable ligand in the absence of the inhibitor, because the model did not permit the expression of zero activity in the absence of the activator. The kinetic results in this paper, as well as those obtained by other investigators (19), suggest that, in the absence of AMP, glucose-1-P and Pi are bound so loosely, if at all, that they are unable to effect an allosteric transition.

It has now been possible to demonstrate homotropic cooperativity of the substrate, the activator, and the inhibitor, as would be expected from a consideration of the assumptions inherent in postulating the model. The heterologous cooperativities are of the type predicted by the model. However, the heterologous effects on the homotropic cooperativities of the activator and inhibitor are not necessarily those predicted. For example, the results in Fig. 6, in which a decrease in the concentration of the substrate caused an increased homotropic cooperativity of the inhibitor, appear paradoxical and do not agree with the discussion about these situations given by Monod et al. (6). Hill plots

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1 E. Helmreich, personal communication.
generated from the model and plotted as log \( (Y_0 - Y/Y) \) with respect to log inhibitor result in constant slopes of 1.6 ± 0.03 when \( L \) is assumed to be 600, \( \alpha \) is 5 or 0.5, and \( \gamma \) is 20 or 1. It may be that this essentially indirect kinetic approach to measuring the homotropic cooperativity of the inhibitor is not appropriate and that a resolution of the problem must await direct binding studies.

It is also surprising that the homotropic cooperativity of the activator, AMP, does not appear to be increased by a decrease in the substrate concentration. In this case, activity measurements should be a better approximation of actual binding of AMP than was the case for ATP. Direct binding studies by equilibrium dialysis indicate that there is cooperation between the two AMP-binding sites on phosphorylase \( b \), and slopes of 1.4 are found on Hill plots. The addition of ATP causes a highly significant decrease in the amount of AMP which can be bound. Further attempts are under way, where technically feasible, to assess the kinetic results in this paper by direct physical methods.

The kinetic model considered in this paper assumes that there is exclusive binding of each type of ligand to only one of the two (hypothetical) conformational states of the enzyme. Nonexclusive binding has recently been considered by Rubin and Changeux (15), but it is doubtful whether this more complicated model needs to be applied to phosphorylase \( b \) in a consideration of substrate effects, because there is no activity in the absence of the activator. Other forms of phosphorylase, such as phosphorylase \( a \) of muscle and liver phosphorylase, may well exhibit nonexclusive binding of the substrate. It must be noted, however, that the greatest fidelity to the model was obtained for substrate effects, and that the homotropic interactions of the activator and inhibitor did not always behave as predicted. Certainly a more sophisticated treatment must be eventually worked out for these latter cases, and nonexclusive binding may have to be invoked.

One of the most important features of the kinetic model of Monod et al. is the assumption that there is an equilibrium between two conformational states of the protein which can be shifted by the binding of various allosteric ligands. In the case of phosphorylase \( b \), we would expect substrate and activator to cooperate in shifting this equilibrium toward an active \( (R) \) state, while the inhibitors would favor the inactive \( (T) \) state. The literature abounds with assertions information which suggests that phosphorylase can exist in various conformational states, and that the active form is protected against the action of various harmful agents by the presence of substrates or activator, or both. For example, glucose-1-P and AMP protect against the inactivation by isocyanate or \( p \)-chloromercuribenzenzoate, the protection being greater when both are present (24). Both these agents cause conformational changes which lead to the dissociation of the molecule. Ullman, Vagelos, and Monod (25) recently showed that the binding of bromothymol blue by phosphorylase \( b \) was increased in the presence of AMP. AMP has also been shown to protect phosphorylase against thermal denaturation (26) and the action of trypsin or phosphate-removing enzyme (27). Optical rotatory dispersion also indicates an effect of AMP on the conformation of phosphorylase \( b \) (28).

All of this information suggests strongly that AMP and possibly some substrates do cause conformational changes in phosphorylase, but these changes have not been specifically tied in with the existence of the hypothetical \( R \) and \( T \) states suggested by the kinetics. It appears clear, then, that future work must be directed toward this objective.

Acknowledgment — We are indebted to Miss Marianne Chan for performing three of the experiments reported in this paper.

REFERENCES

20. SEALOCK, R. W., AND GRAVES, D. J., Biochemistry, 6, 201 (1967).
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