Isotope Exchange at Equilibrium as a Test for Homotropic Cooperativity of Allosteric Enzymes*

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ABSTRACT: The appearance of sigmoid velocity *vs.* substrate concentration curves with regulatory enzymes can be interpreted in terms of models involving cooperativity among substrate binding sites. There are, however, certain circumstances which can lead to apparent sigmoid kinetic plots in the absence of homotropic cooperativity. One such possibility arises with enzymes exhibiting rapid equilibrium random mechanisms. If an inhibitor were to exert its effects by changing the rates of association and dissociation of substrates, the rapid equilibrium condition might no longer apply in its presence. As a result, the hyperbolic velocity *vs.* substrate concentration plot obtained in the absence of inhibitor could approach a sigmoid curve in its presence, with no requirement for site–site interaction. A simple test for such a possibility involves measurement of the kinetics of isotope exchange at chemical equilibrium in the presence and absence of modifier. Rabbit muscle glycogen phosphorylases *a* and *b* are suitable enzymes on which to test this approach because their allosteric properties are well established and we have shown previously that they exhibit rapid equilibrium random mechanisms in the absence of inhibitors. The effect of glucose on the kinetics of isotope exchange for phosphorylase *a*, and the effect of adenosine triphosphate on the same properties of phosphorylase *b*, were therefore examined, because they cause the initial velocity *vs.* substrate concentration curves to become sigmoid. For both enzymes, these inhibitors also cause the glycogen–glucose 1-phosphate and orthophosphate–glucose 1-phosphate exchange rates to show a sigmoid dependency upon substrate concentration. Moreover, these exchange rates remain equal to each other at all substrate concentrations tested. The data therefore indicate that with both enzymes the rapid equilibrium condition still prevails in the presence of inhibitors. This method may have application elsewhere in confirming cooperativity between binding sites in allosteric enzymes for which, as in the case of phosphorylase, direct binding measurements are not feasible.

The concept of allosterism as developed by Monod *et al.* (1963, 1965) (*i.e.*, the control of an enzyme's catalytic efficiency by the binding of small molecular weight effectors, not necessarily structurally related to the substrate) has been a favorite explanation for sigmoid rate *vs.* ligand concentration curves. The usual postulate involves the cooperative interaction of several catalytically active substrate binding sites, in addition to various combinations of positive and/or negative effector binding sites. Implicit in such models is the proposal that the enzyme consists of two or more subunits or protomers capable of interaction with each other. However, several investigators have been able to provide reasonable alternative explanations for apparent sigmoidal rate data without resorting to the requirement for more than one catalytic binding site, or for subunit interactions, to support their models. Examples will be cited for enzymes exhibiting rapid equilibrium random mechanisms since the isotope-exchange method may be used to advantage with such systems.

Frieden (1964) has considered the kinetic behavior of enzyme systems using the single-substrate–single-modifier case. In this simple situation, his treatment shows that the dependence of velocity upon the substrate concentration is hyperbolic only if it is assumed that the attachment of the substrate and modifier is in rapid equilibrium compared with the step(s) involving product formation. If this condition does not apply, the steady-state treatment yields rate equations with squared substrate terms and hence the corresponding reciprocal plots become nonlinear. Ferdinand (1966) extended the treatment to show that when alternative pathways exist for two-substrate enzymes, the apparent substrate inhibition or substrate activation which may occur (Dalziel, 1957) can cause apparent sigmoid curves. Furthermore, the rapid equilibrium condition for a random bi–bi mechanism might be lost at low substrate concentration (*e.g.*, less than 10^{-4} M), resulting in an apparent sigmoidicity (Frieden, 1964).

Finally, there is the possibility that modifiers in certain systems may induce a change in a given kinetic mechanism which results in the appearance of sigmoid curves that are not the result of homotropic cooperativity. For example, an enzyme which exhibits a rapid equilibrium random mechanism in the absence of modifier may lose the rapid equilibrium condition in the presence of modifier, due to effects on certain rate constants. In fact, Sanwal *et al.* (1965, 1966) have shown a modifier-dependent change in kinetic mechanism for the NAD-dependent isocitrate dehydrogenase of *Neurospora crassa*. In this system the mechanism was found to be ordered in the presence of the activator AMP, but in the absence of AMP it became random, with the addition steps partially rate limiting. As a result the reciprocal plots for NAD^+ in the absence of AMP became nonlinear. The authors found no evidence for the binding of more than one NAD^+ molecule to the enzyme surface.

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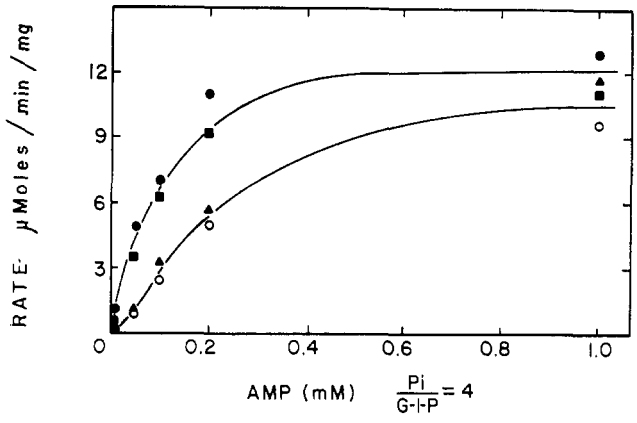

FIGURE 1: The effect of ATP on equilibrium isotope-exchange rates with muscle phosphorylase *b*. The 0.5-ml equilibrium reaction mixes contained 11.4 mM glycogen (end groups), 1 mM glucose-1-P, 4 mM P_i, with AMP as shown, in 5 mM sodium glycerophosphate, 1.5 mM EDTA, and 2 mM mercaptoethanol (pH 6.8). The upper curve represents results obtained in the absence of ATP, with 7 μg/ml of phosphorylase *b*. (●) [^{32}P]P_i ⇌ glucose-1-P and (■) [^{14}C]glucose-1-P ⇌ glycogen. The lower curve represents results obtained in the presence of 1 mM ATP, with 21 μg/ml of phosphorylase *b*. (▲) [^{32}P]P_i ⇌ glucose-1-P and (○) [^{14}C]glucose-1-P ⇌ glycogen.

All of these causes for non-Michaelian kinetics may be ruled out if it can be proven that the rate-limiting step remains the interconversion of the ternary complexes. This proof can be unambiguously provided by the isotope-exchange method so that homotropic cooperativity remains the obvious cause of the sigmoid curve.

Madsen, and also Helmreich and Cori, showed in 1964 that the activator AMP and the substrate glucose 1-phosphate appeared to exhibit homotropic cooperativity between their binding sites on rabbit muscle phosphorylase *b*. This enzyme was subsequently cited by Monod and his group (Ullman *et al.*, 1964; Morod *et al.*, 1965) as a model enzyme for their allosteric theories. Recently, it has been shown that the kinetic mechanism of both phosphorylases *b* and *a* from rabbit muscle is rapid equilibrium random bi-bi (Engers *et al.*, 1969, 1970a,b). However, the possibility remained that in the presence of allosteric effectors such as ATP the rapid equilibrium conditions break down, resulting in apparent sigmoidal curves. Since it was implicitly assumed in Monod's model that rapid equilibrium conditions prevail throughout, it was decided to examine the kinetics of isotope exchange at equilibrium for these enzyme forms in the presence of their allosteric effectors. These experiments indicate, in the case of phosphorylase, that the sigmoid data are in fact due to cooperativity between substrate binding sites rather than the result of a breakdown in the rapid equilibrium conditions of the mechanism.

Methods

Phosphorylase *b* was prepared from rabbit muscle by the method of Fischer and Krebs (1962), and recrystallized a minimum of three times. Crystalline rabbit muscle phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase according to the method of Krebs and Fischer (1962).

Before use, phosphorylase *b* crystals were centrifuged out of suspension, dissolved in 0.04 M sodium β-glycerophosphate–0.001 M EDTA–0.005 M mercaptoethanol (pH 6.8), and purified on a column of Sephadex G-25. Phosphorylase *a* was diluted to the desired concentration in a similar buffer, except that the sodium β-glycerophosphate concentration was 0.02 M.

Chemicals, where possible, were purchased from Sigma, and were the highest grade available. The rabbit liver glycogen was routinely passed through a Dowex 1-chloride column and assayed against a glucose standard by the method of Dische, as described by Ashwell (1957). Its concentration is expressed as the molar equivalent of its glucose end groups; *i.e.*, 2% glycogen is equal to 10.1 mM end groups.

[^{32}P]P_i and [^{14}C]glucose-1-P were used without added carrier, and the respective radioactive products were isolated and counted as described previously (Engers *et al.*, 1969).

Equilibrium mixes of 0.4 ml containing all components except glycogen were incubated at 30° for at least 1 hr, then the desired concentration of glycogen in 0.1 ml was added and the complete equilibrium mix incubated for a further 5 min prior to the addition of isotope. This order of addition of substrates prevented extensive disproportionation of the glucose end groups on the glycogen molecules (Engers *et al.*, 1969). For the experiments reported in this paper, where the glycogen concentration is high (>2%) and the effective concentration of glycogen calculated in terms of end groups, experiments were set up so as to ensure that first-order conditions prevail. Reaction times were adjusted such that less than 15% of the radioactive label was incorporated into the isolated product. Under these conditions, the possibility of disproportionation effects are reduced to a minimum.

Equilibrium reaction rates were calculated from the exchanging substrate concentrations, time of reaction, and the amount of radioactivity incorporated into product (Boyer, 1959). Equilibrium reaction rates are expressed as micromoles of product produced per minute per milligram of protein.

Results

Figure 1 illustrates the results obtained for an isotope-exchange experiment using phosphorylase *b* in which glucose-1-P, P_i, and glycogen were held constant at 1, 4, and 11.4 mM (end groups), respectively. The activator AMP was varied from 0.01 to 1 mM in the presence and absence of the inhibitor ATP. The control curve in the absence of ATP shows that both exchange rates, *i.e.*

$$[^{32}P]P_i \rightleftharpoons \text{glucose-1-P} \quad \text{and} \quad [^{14}C]\text{glucose-1-P} \rightleftharpoons \text{glycogen}$$

are identical, and exhibit a normal hyperbolic saturation curve, with one-half maximal rate of exchange occurring at a value of approximately 0.13 mM AMP, as determined from a plot of 1/rate *vs.* 1/AMP. This value is in good agreement with the K_m values obtained in initial velocity experiments, using either glucose-1-P or P_i as substrate (Madsen and Shechosky, 1967, Figures 10 and 12). In addition, this value of 0.13 mM is in the same range as the values determined by Avramović and Madsen (1968) for the dissociation constant of AMP binding to enzyme, as measured by equi-

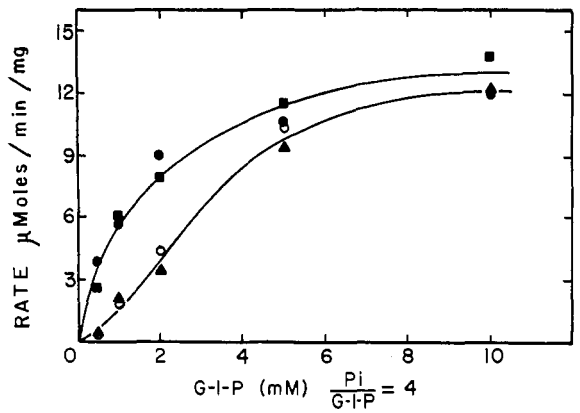

FIGURE 2: The effect of ATP on equilibrium isotope-exchange rates with muscle phosphorylase *b*. The 0.5-ml equilibrium reaction mixes contained 10.6 mM glycogen (end groups), 1 mM AMP, with glucose-1-P and P_i as shown, in 5.7 mM sodium glycerophosphate, 1.5 mM EDTA, and 2 mM mercaptoethanol (pH 6.8). The upper curve represents results obtained in the absence of ATP, with 4 μg/ml of phosphorylase *b*. (■) $[^{32}P]P_i \rightleftharpoons$ glucose-1-P and (●) [^{14}C]glucose-1-P $\rightleftharpoons$ glycogen. The lower curve represents results obtained in the presence of 9.6 mM ATP, with 12 μg/ml of phosphorylase *b*. (▲) $[^{32}P]P_i \rightleftharpoons$ glucose-1-P and (○) [^{14}C]glucose-1-P $\rightleftharpoons$ glycogen.

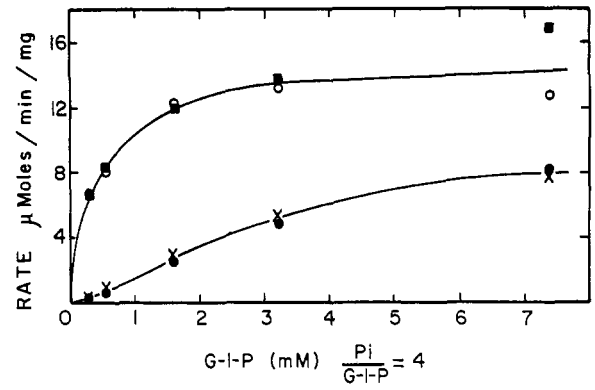

FIGURE 3: The effect of glucose on equilibrium isotope exchange with muscle phosphorylase *a* in the absence of AMP. The 0.5-ml equilibrium reaction mixes contained 10.6 mM glycogen (end groups), with glucose-1-P and P_i as shown, in 3 mM sodium glycerophosphate, 1.5 mM EDTA, and 0.8 mM mercaptoethanol (pH 6.8). The upper curve represents results obtained in the absence of glucose, with 4 μg/ml of phosphorylase *a*. (■) $[^{32}P]P_i \rightleftharpoons$ glucose-1-P; (○) [^{14}C]glucose-1-P $\rightleftharpoons$ glycogen. The lower curve represents results obtained in the presence of 50 mM glucose, with 11.1 μg/ml of phosphorylase *a*. (●) $[^{32}P]P_i \rightleftharpoons$ glucose-1-P; (×) [^{14}C]glucose-1-P $\rightleftharpoons$ glycogen.

librium dialysis and protection against inactivation by isocyanate.

The inclusion of 1 mM ATP results in a sigmoidal inhibition pattern, as is shown in the lower curve in Figure 1. The $[^{32}P]P_i$–glucose-1-P exchange rate is equivalent to the [^{14}C]-glucose-1-P–glycogen rate in the presence of the allosteric inhibitor ATP; this observation confirms the suggestion that the sigmoidicity is due to a cooperativity among binding sites (Madsen, 1964; Madsen and Shechosky, 1967), and not due to a breakdown of the random rapid equilibrium conditions.

Avramović and Madsen (1968), and also Kastenschmidt *et al.* (1968a,b), have confirmed, by the use of binding studies that there are two AMP binding sites per mole of phosphorylase *b* (Madsen and Cori, 1957), an obligate requirement for the expression of homotropic allosteric cooperativity by AMP. In fact, the binding studies demonstrated homotropic cooperativity among the AMP binding sites. Of added interest is the fact that the concentrations used in Figure 1, as well as those used in the binding studies mentioned above, approach those expected for *in vivo* conditions, and reinforce the belief that these cooperative effects may be involved as a control mechanism in living muscle.

The results shown in Figure 2 are from an experiment conducted with phosphorylase *b* in the presence and absence of 10 mM ATP. The glucose-1-P and P_i concentrations were varied at a constant ratio, with glycogen and AMP held constant. The control curve in the absence of ATP is hyperbolic, with both exchange rates identical. In the presence of ATP, the curve becomes sigmoidal in shape, analogous to those results obtained for initial velocity studies (Madsen, 1964; Madsen and Shechosky, 1967). Here again, the two exchange rates were equivalent, confirming that the mechanism is still rapid equilibrium. A Hill plot of the data in Figure 2 gives a slope of 1.1 for exchange rates obtained minus ATP and 1.75 for the data obtained in the presence of ATP. These *n* values for the Hill plots are virtually identical with those obtained for initial velocity studies, varying either glucose-1-P or P_i under similar conditions (Madsen and Shechosky, 1967). These data also support the conclusion that there exists more than one binding site per mole of enzyme for the substrates glucose-1-P and P_i.

Figure 3 illustrates the results obtained when equilibrium isotope-exchange rates were measured for phosphorylase *a* in the presence and absence of glucose, which has been shown to be an allosteric effector for phosphorylase *a* (Helmreich *et al.*, 1967). The upper curve represents the control experiment, conducted in the absence of glucose. As can be seen, the $[^{32}P]P_i$–glucose-1-P and [^{14}C]glucose-1-P–glycogen isotope-exchange rates follow a hyperbolic curve, and are the same. The lower curve shows the results obtained in the presence of 50 mM glucose. Again, in the presence of the allosteric effector, the rate curve is sigmoidal, and the isotope-exchange rates are the same. These results agree with the observed effect of glucose on the initial velocity of phosphorylase *a*, as reported by Helmreich *et al.* (1967).

Discussion

It had been shown previously for glycogen phosphorylase, by initial velocity studies in conjunction with isotope-exchange studies at equilibrium (Engers *et al.*, 1969, 1970a,b), that the kinetic mechanism of both phosphorylases *b* and *a* is random rapid equilibrium bi–bi. In this present communication, we have shown that in the presence of the allosteric effectors ATP (Madsen, 1964; Madsen and Shechosky, 1967) and glucose (Helmreich *et al.*, 1967), the kinetic mechanisms remain random rapid equilibrium, *i.e.*, the rate-limiting step in the reaction sequence is still the interconversion of ternary complexes. Hence, the sigmoidicity which results is in fact most likely due to some form of cooperativity between binding sites or possibly subunits, presumably resulting from a conformational change induced by the binding of the ligands in question.

Isotope-exchange studies at equilibrium are particularly

suited to the phosphorylase system, since they allow the circumvention of certain technical difficulties encountered when one attempts to measure initial velocities at low substrate concentrations. These technical difficulties (Helmreich, 1969) are well known to those working in the phosphorylase field, and in this laboratory initial velocity experiments at low substrate concentrations are designed in such a manner as to minimize these difficulties (Maddaiah and Madsen, 1966). However, the isotope exchange at equilibrium methods developed by Boyer (1959) allows one to work at relatively low substrate concentrations (less than 1 mM), without the inherent measurement problems, since the system is at equilibrium and the rate of incorporation of radioactive label into product can be measured quite accurately. One apparent drawback to the method is that all substrates must be present at once; therefore, any effects observed, such as results obtained in the presence of a modifier, can only be interpreted in a qualitative manner, as compared with initial velocity measurements. However, this apparent drawback may also be an advantage, since the presence of all substrates and modifiers in the system at once most likely approaches *in vivo* conditions, and hence equilibrium isotope-exchange studies may provide one with a starting point in the necessary task of applying our vast stores of enzymic knowledge obtained *in vitro* to actual enzyme function and regulation *in vivo*.

This method of investigating the interaction of an enzyme with its substrates should prove to be a useful means of confirming homotropic interactions in enzyme systems not suited for direct binding studies. The application of equilibrium isotope-exchange methods to other enzymes exhibiting sigmoidal kinetics should be a useful test for discriminating unambiguously between conditions which can result in the expression of apparent sigmoidicity, *i.e.*, cooperative allosteric effects, or a breakdown in the kinetic mechanism.

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