Kinetic Mechanism of Phosphorylase b

RATES OF INITIAL VELOCITIES AND OF ISOTOPE EXCHANGE AT EQUILIBRIUM*

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SUMMARY

In order to assign a kinetic mechanism to phosphorylase b, initial rate studies were conducted in conjunction with isotope exchange studies at equilibrium. Initial velocity rates were measured with varied concentrations of both substrates in each direction, in the presence of saturating levels of AMP. Data were analyzed with double reciprocal plots and second-order replots of intercepts and slopes. The resulting kinetically derived dissociation constants agreed reasonably well with those determined by independent means. These results indicate that the kinetic mechanism of phosphorylase b is rapid equilibrium random $bi bi$ in nature.

The rate equation for this mechanism has been modified to take into account the fact that one of the substrates, glycogen, gives rise to a product which is chemically and kinetically indistinguishable under the conditions used to determine initial rates. This phenomenon, equivalent to having one of the two products present at all times, results in the number two being introduced into the rate equation, so the kinetically derived dissociation constant for glycogen is half the true value while the observed $K_m$ values for phosphate and glucose 1-phosphate are twice the theoretical values. This rate equation should apply wherever an enzyme synthesizes or degrades a homopolymer by 1 unit at a time.

To confirm the mechanism suggested by initial velocity experiments, isotope exchange studies at equilibrium were performed for the phosphorylase b system, again in the presence of saturating levels of AMP. The $^{14}C$-glucose-1-P $\approx$ glycogen equilibrium reaction rate increased as the concentrations of either glucose-1-P and orthophosphate or glycogen were increased, and reached a plateau as the concentration of varied substrates became saturating. The same results were obtained for $^{32}P_i \approx $ glucose-1-P exchange. Thus, there was no evidence of an inhibition of the exchange of one pair of substrates when the concentration of the other substrate pair was raised. Similar exchange rates were observed in either direction, indicating that rapid equilibrium conditions apply. A reasonable agreement existed between the maximum velocities calculated from the initial rate data and those determined from the isotope exchange rates, assuming a rapid equilibrium random $bi bi$ mechanism.

Both the initial velocity and the equilibrium isotope exchange studies support a reaction mechanism in which substrates bind in a noncompulsory order to the enzyme and in which the interconversions of ternary complexes are the rate-limiting steps.

The allosteric and structural properties of phosphorylase b (α-1,4-glucan: orthophosphate glucosyl transferase, E.C. 2.4.1.1) have been the subject of much extensive investigation. Analyses of its kinetic behavior have shown this enzyme to exhibit allosteric properties interpretable in terms of the concerted transition model proposed by Monod, Wyman, and Changeux (1), although the data indicate that certain modifications have to be made (2-5). In addition, physical studies have verified kinetic predictions of homotropic cooperativity for AMP binding, and heterotropic interactions among substrates, activators, and inhibitors (5, 6). Despite this progress, the actual kinetic mechanism of the catalytic reaction has not yet been presented.

Work on other glycogen phosphorylases has suggested that the kinetic mechanism is rapid equilibrium random $bi bi$, in Cleland's terminology (7). For example, Maddaiah and Madsen (8) proposed this mechanism for liver phosphorylase on the basis of a kinetic analysis by the methods of Dalziel (9). This suggestion was verified by comparison of kinetically and physically derived dissociation constants for glucose 1-phosphate (6). Lowry, Schuiz, and Passonneau (10, 11), fitted their kinetic data for phosphorylase a into this mechanism, without however, providing a proof.

On the other hand, the possibility that glycogen phosphorylase a may have an ordered $bi bi$ mechanism has been suggested by Michaelides and Helreiche (12). It would seem necessary, therefore, to verify the actual mechanism for phosphorylase b since this has profound significance for the interpretation of much kinetic data used for testing theoretical allosteric models. Implicit in the treatment of allosteric kinetics for this enzyme has been the assumption that the mechanism is rapid equilibrium random $bi bi$.

An assessment of the kinetic mechanism for an allosteric
enzyme presents theoretical and practical difficulties not encountered in a more conventional system. For example, reciprocal plots are curved at low substrate concentrations, so it is necessary to work at substrate concentrations above the nominal $K_m$ so that the velocity versus substrate relationship is rectangular hyperbolic. One might, therefore, object that only a partial picture is being presented. In addition, product inhibition studies are not possible with glycogen phosphorylase because one of the substrates (glycogen) is changed to an essentially indistinguishable product, while the equilibrium between the other two substrates (P$_i$ and glucose-1-P) is close to unity. Fortunately, the development and verification of enzymic equilibrium reaction rate theory by Boyer (13) and Boyer and Silverstein (14) provides a useful means of confirming possible kinetic mechanisms suggested by initial rate studies. We have, therefore, investigated the rates of isotope exchange at equilibrium to complement the data obtained from conventional initial rate studies.

**EXPERIMENTAL PROCEDURE**

Phosphorylase b was prepared from rabbit muscle by the method of Fischer and Krebs (15) and recrystallized a minimum of three times. Before use, crystals were centrifuged out of suspension, dissolved in 0.04 M β-glycerophosphate-0.001 M EDTA-0.005 M mercaptoethanol, pH 6.8, and purified on a column of Sephadex G-25. Chemicals and auxiliary enzymes, purchased from Sigma, were the highest grade available. The rabbit liver glycogen was routinely passed through a Dowex 1-chloride column. It was assayed against a glucose standard by the method of Dicke, as described by Ashwell (16), and its concentration is expressed as the molar equivalent of its glucose residues.

**Initial Velocity Assays**—Phosphorylase activities are expressed as micromoles of P$_i$ esterified (or released from glucose-1-P) per min per mg of protein, in the presence of saturating levels of AMP, at 30°C. Phosphorylase was incubated with AMP and glycogen prior to activity measurements in either reaction direction. Activities in the direction of glycogen synthesis were assayed by following the release of inorganic phosphate using the Fiske-SubbaRow test (17). In a typical experiment, the reaction mix contained 5 mM sodium glycophosphate, pH 6.8; 0.55 mM mercaptoethanol, 0.1 mM EDTA; 1 mM AMP; and 5 to 10 μg of protein. Initial reaction rates were calculated from the pseudo first order reaction constants by multiplying the latter by the concentration of P$_i$ calculated to be present when equilibrium is attained. Glycogen and glucose-1-P concentrations are given for each experiment. Appropriate precautions were taken to ensure that the average increase of chain length did not exceed 2 glucose units (8).

The phosphorylation of glycogen was carried out to a large excess of phosphoglucomutase and glucose-6-P dehydrogenase so that the rate-limiting step in the reduction of NADP was the phosphorylase activity. Assay conditions were essentially those described by Maddaiah and Madsen for liver phosphorylase (8), with the glycogen and P$_i$ concentrations given for each experiment. The buffer used was the same as for the glycogen synthesis, with 10 mM imidazole added, and the pH was carefully controlled at 6.8.

**Isotope Exchange Measurements**—P$_i$-labeled (Atomic Energy of Canada Limited, Ottawa, Ontario) and 14C-glucose-1-P (uniformly labeled) (Calbiochem or Amersham Searle) were used without added carrier, and the respective radioactive products were counted in a Beckman CPM-100 liquid scintillation counter, using Bray's solution as a counting medium. 32P$_i$ and 32P-glucose-1-P were separated by means of the isobutyl alcohol-benzene extraction procedure for phosphomolybdate (18). 32P-glycogen and 32P-glucose were separated, and the 14C-glycogen was isolated by a modification of the method of Gold and Segal (19).

Equilibrium mixes were set up for each concentration of substrates desired. The P$_i$ to glucose-1-P ratios were consistent with the anticipated equilibrium constant at pH 6.8 ($K = 0.21$) (Trevelyan, Mann, and Harrison (20)). AMP was present in all experiments at saturating levels (1 mM). Equilibrium mixes were incubated at 30°C for at least 24 hours prior to the addition of isotopes, in order to ensure the attainment of equilibrium.

Reactions were started by the addition of either 10 or 20 μl of radioactive substrate (32P$_i$ or 14C-glucose-1-P), to 0.5-ml aliquots of the stock equilibrium mix. In this way, very small volume changes occurred and the amount of labeled substrate added was insignificant when compared with the total substrate concentration, thus avoiding net reaction. Reactions were stopped, after appropriate time intervals, with 1 ml of 45% KOH for the 14C-glucose-1-P := glycogen exchange and 2 ml of acid molybdate for the 32P$_i$ := glucose 1 P exchange. The radioactive products, either 32P-glycogen or 32P-glucose-1-P, were then isolated and counted as described above.

Equilibrium reaction rates were calculated from the exchanging substrate concentrations, time of reaction, and the amount of radioactivity incorporated into product, using reactions in which not more than 80% of the radioactive label appeared in the product (13, 14). Equilibrium reaction rates are expressed as micromoles of product produced per min per mg of protein.

**RESULTS AND DISCUSSION**

**Initial Velocity Studies**—Phosphorylase carries out a rather unique reaction in which, in a single catalytic event, one glucose moiety is added onto, or removed from, one of the end groups of an enormous branched polymer of glucose. Of the glucose units of rabbit liver glycogen, 6.8% are at these end groups and the outer branches have an average length of 9 residues (21). Thus, the product of the phosphorylase reaction is essentially equivalent to the substrate and, in fact, as shown by Hestrin (22), glycogen does not affect the chemical equilibrium (ratio of P$_i$ to glucose 1 P) until it is either degraded to a limit dextrin or the chains are elongated considerably. In either case, the reaction rate slows down (22–24). In our experiments, care has been taken to ensure that an average of 2 or less glucose units per end group are added to or subtracted from the glycogen.

Therefore, under the conditions of the initial velocity studies reported below, one of the products of the reaction is present at all times, at a concentration equivalent to one of the substrates. Furthermore, as shown below and as demonstrated earlier by Maddaiah and Madsen (8), the dissociation constant is the same when measured from either direction. In Cleland's nomenclature (7), $P = A$ and $K_{1P} = K_{1P}$. Making these substitutions in Cleland's general rate equation for a rapid equilibrium random bi bi mechanism, and substituting the terminology used in Fig. 1, one obtains Equation 1, shown in the legend for Fig. 1. The most important practical effect of the number 2 in the numerator of the third expression on the right is that the kinetically derived values for $K_1$ and $K_2$ are actually one-half of the true dissociation constant for glycogen and the free enzyme, if one were to use the usual rate equation. As will be seen below, the corrected values for $K_1$ and $K_2$ do agree reasonably well with the dissociation constant for glycogen which was determined by physical methods.
Fig. 1. Proposed rapid equilibrium random bi bi kinetic mechanism for muscle phosphorylase b. E, G, and P are, respectively, enzyme-AMP complex, glycogen, and Pi. G' is glycogen with one less terminal glucose residue while P' is glucose-1-P. The eight dissociation constants and two rate constants are indicated. The overall rate equation relating the initial velocity ($V_0$), total enzyme concentration (E) and substrate concentrations for the forward direction is, as explained in the text

$$\frac{E}{V_0} = \frac{1}{k_1} + \frac{K_4}{k_4} + \frac{2K_5}{k_5} + \frac{K_3K_6}{k_3k_6}$$

(1)

which can be written in the general form of Dalziel (9)

$$\frac{E}{V_0} = \phi + \frac{\phi_1}{(G)} + \frac{\phi_2}{(P)} + \frac{\phi_3}{(G)(P)}$$

(2)

Analogous equations may be written for the reverse reaction, with the appropriate constants for Equation 1 and adding primes in Equation 2.

Fig. 2. Velocity of muscle phosphorylase b activity as a function of glycogen concentration at six levels of Pi concentrations. (1/v in this and all other reciprocal plots is in units of milligram of protein × min per µmole of product, either glucose-1-P or Pi.) Concentrations of Pi, reading from the upper line downward, and corresponding apparent Michaelis constants for glycogen, are as follows: a, 0.97 mM Pi, 1.55 mM glycogen; b, 1.5 mM Pi, 1.25 mM glycogen; c, 2.0 mM Pi, 1.1 mM glycogen; d, 3.0 mM Pi, 0.86 mM glycogen; e, 5.2 mM Pi, 0.54 mM glycogen; f, 11.2 mM Pi, 0.38 mM glycogen.

The other effect of the modified rate equation for glycogen phosphorylase is that the Michaelis constants for phosphate and glucose-1-P ($K_4$ and $K_5$, respectively), determined with saturating levels of glycogen, are twice the theoretical values. This result is that expected from having equal concentrations of a product and substrate, with equal dissociation constants, present at all times when the activity of the enzyme is being measured. The phenomenon is probably of only academic interest in the case of phosphorylase. However, for those enzymes which carry out analogous reactions on both a homopolymer or a heteropolymer, the Michaelis constants for given monomer substrates

Fig. 3. Velocity of muscle phosphorylase b activity as a function of Pi concentrations at five levels of glycogen. Concentrations of glycogen and corresponding apparent Michaelis constants for Pi are as follows: circles, 1.12 mM glycogen, 4.45 mM Pi; X, 2.25 mM glycogen, 3.33 mM Pi; △, 3.75 mM glycogen, 3.00 mM Pi; ●, 5.6 mM glycogen, 2.80 mM Pi; □, 28 mM glycogen, 2.50 mM Pi.

may well vary depending on the type of polymer which is being synthesized or degraded. Naturally, the discussion about the dissociation constants for the polymer itself would also apply. An example of an enzyme for which these considerations should be borne in mind when evaluating the kinetics is polynucleotide phosphorylase (EC 2.7.7.8).

Initial rate data for the phosphorolysis of glycogen at varying concentrations of both glycogen and Pi are shown in the primary plots of Figs. 2 and 3. In Fig. 2, the intersection point in the upper left-hand quadrant yields the dissociation constant for glycogen and enzyme-AMP, and corresponds to the appropriate constant in Fig. 1. Similarly, in Fig. 3, the point of intersection yields the dissociation constant for Pi and enzyme-AMP. The upward curvature of these lines at the lower substrate concentrations is typical for phosphorylase b and is most likely due to an expression of homotropic cooperativity between the Pi binding sites (3). However, it could also be due to a breakdown of the rapid equilibrium condition for a random bi bi mechanism, resulting in the appearance of squared substrate concentration terms in the rate equation (25). Experiments to distinguish between these two possibilities are reported below, but, for the time being, the first explanation will be assumed. Where the lines on reciprocal plots are curved, the extrapolations have been drawn from the data for the higher range of substrate concentrations.

The initial rate data for the reaction in the direction of glycogen synthesis at varying concentrations of glycogen and glucose-1-P are given in Figs. 4 and 5. The dissociation constants for glycogen derived from Figs. 2 and 4 are very similar, i.e. $K_1 = K_7$, as would be expected for this enzyme.

The intercepts and slopes from the double reciprocal plots were replotted according to the method of Dalziel (9). Internal consistencies of the data were indicated by common intercepts on the intercept plots and parallel lines on the slope plots. The replots were essentially linear.

Table I summarizes the various kinetic coefficients derived from the replots, which are generalized entities applicable to any
kinetic mechanism. The specific kinetic constants, applied to the kinetic mechanism in Fig. 1, are also given in Table I.

Table II summarizes certain relationships between the kinetic coefficients and kinetic constants. The equilibrium constant calculated from initial rate data using the Haldane relationship (7, 9) does not agree with that obtained by direct measurement (20), as it did for other phosphorylase systems. Thus, the major difference in the kinetic constants of phosphorylase b which leads to the incorrect Haldane relationship concerns the relative binding affinities of the anionic substrates; glucose-1-P not showing the markedly greater binding affinity and lower Michaelis constant compared with P$_i$ that is seen in the other phosphorylases. However, it is admittedly hard to see how the insertion of these extra isomerization steps into a supposedly symmetrical kinetic mechanism could lead to an alteration in the calculated equilibrium constant.

Table III illustrates the kinetically determined dissociation

with its allosteric properties. For example, it was suggested by Avramovic and Madsen (6), on the basis of chemical inactivation studies, that the binding of P$_i$ or glucose-1-P to phosphorylase b already saturated with AMP is correlated with a conformational change. This suggestion received support from the studies of Kastenschmidt, Kastenschmidt, and Helmreich (5), who proposed two $R$ states which differ in their affinity for AMP and glucose-1-P. Black and Wang (4) also suggested that, a two-stage allosteric transition is involved in the activation of the enzyme. Thus, extra enzyme isomerization steps might have to be added to the scheme in Fig. 1. Significantly, the major difference in the kinetic constants of phosphorylase b which leads to the incorrect Haldane relationship concerns the relative binding affinities of the anionic substrates; glucose-1-P not showing the markedly greater binding affinity and lower Michaelis constant compared with P$_i$ that is seen in the other phosphorylases. However, it is admittedly hard to see how the insertion of these extra isomerization steps into a supposedly symmetrical kinetic mechanism could lead to an alteration in the calculated equilibrium constant.

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Dissociation constants for substrates and phosphorylase b
Units for enzyme saturated with AMP are millimolar except as noted.

<table>
<thead>
<tr>
<th>Source of dissociation constant</th>
<th>Glucose-1-P</th>
<th>P_i</th>
<th>Glycogen</th>
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<tbody>
<tr>
<td>Initial velocity kinetics</td>
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<tr>
<td>From Table I</td>
<td>7.4</td>
<td>15</td>
<td>4.6</td>
</tr>
<tr>
<td>From two other similar</td>
<td>(7.8, 8.0)</td>
<td>(10, 10)</td>
<td>(3.9, 4.4)</td>
</tr>
<tr>
<td>experiments</td>
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<tr>
<td>Physical studies</td>
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<td></td>
<td>10^4</td>
<td>5</td>
<td>10^9</td>
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<tr>
<td></td>
<td>20^4</td>
<td>11</td>
<td></td>
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<tr>
<td>Chemical modification studies*</td>
<td></td>
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<td>From this paper.</td>
<td>6.3</td>
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<td>11</td>
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</tbody>
</table>

* This paper.
+ Bae and Bae (27).
- Kastenschmidt, Kastenschmidt, and Helmreich (29), for the apoenzyme in the absence of AMP. The holoenzyme gave similar results.
- Bresler and Firsov (28).
- Avramovic and Madsen (6).

Constants for phosphorylase b as compared with those obtained by independent physical methods. The $K_{diss}$ of 7.4 mM for glucose-1-P observed kinetically agrees well with the value of 6 mM determined by Avramovic and Madsen (6). The kinetic $K_{diss}$ for P_i, 10 to 15 mM, also agrees with the 11 mM value obtained from the isocyanate inactivation studies (6). It should be noted that the values published by other investigators for the P_i dissociation constant also fall in this range (27, 28).

Rabbit liver glycogen (Sigma) was fractionated in the preparative ultracentrifuge to remove light fractions which did not sediment at 96,000 $\times$ g for 60 min. This material was then used to determine the dissociation constant for glycogen and the enzyme saturated with AMP by the ultracentrifugal separation method of Madsen and Cori (24). The results, shown in Table III, are similar to those obtained by Kastenschmidt et al. (29), for corn phytoglycogen and the enzyme in the absence of AMP, and also for enzyme from which the pyridoxal phosphate had been removed. There is reasonable agreement between the results from these physical measurements and those derived from the kinetic studies.

The favorable comparison of kinetic dissociation constants with those determined by independent physical methods supports the assignment of the rapid equilibrium random bi bi mechanism (7) to phosphorylase b.

Isotope Exchange Studies—Preliminary experiments showed that the rate of isotope exchange was directly proportional to the concentration of enzyme present. In order to confirm the fact that the reaction is rapid equilibrium, one equilibrium mix was divided into several 0.5-ml aliquots and the $P_i$ = glucose-1-P rate followed in one-half of them, the glucose-1-P = glycogen rate in the remainder. It can be seen in Fig. 6 that the two rates of incorporation are very similar. This experiment gives support to the concept that the interconversion of ternary complexes is the rate-limiting step in the conversion of substrate to products.1

Fig. 6. Isotope exchange rates measured from a single equilibrium mix containing 20 μg of phosphorylase b per ml, 1.17 mM glucose-1-P, 3.94 mM P_i, 55.5 mM glycogen, 1 mM AMP, in 3 mM sodium-glycerophosphate, 1.5 mM EDTA, 2.5 mM mercaptoethanol, pH 6.8. ○, incorporation of $^{14}$C-glucose-1-P into glycogen; ▲, incorporation of $^{32}$P into glucose-1-P.

The approach to equilibrium of the $^{32}$P = glucose-1-P exchange followed first order kinetics as would be expected. However, the rate of the $^{14}$C-glucose-1-P = glycogen exchange slowed down with time, so that successive rate constants decreased as shown in Fig. 7. For the experiments reported in this paper, this deviation from first order kinetics was not serious enough to prevent using the rates calculated from the early periods. The ratio of isotopes found in the two exchanging substrates after equilibrium has been established is that expected from the chemically derived equilibrium constant for the phosphorylase reaction (20). For example, approximately 20% of the total $^{32}$P added an ordered mechanism with glycogen adding first in a rapid equilibrium step, regardless of the relative rate of ternary complex interconversion (14).
is found in the form of glucose-1-P, while approximately 80% of the total $^{14}$C is found in the form of glycogen.

The deviation of the glucose-1-P $\rightleftharpoons$ glycogen exchange may be due to the disproportionate attack of phosphorylase on the outer chains of glycogen. As shown by Illingworth, Brown, and Cori (30), the previous incubation period would have allowed the enzyme to lengthen some of the outer chains of glycogen and to shorten others. Examination of the iodine color of our incubation mixes showed a shift in the spectra similar to that reported by these authors (30) and suggested that, by the time isotope was added, the outer chain glucose units have become asymmetrically distributed. During the isotope incorporation, the loss of $^{14}$C-glucose moieties most recently incorporated might then be greater than expected from statistical considerations.

If this hypothesis is correct, then elimination of the prior incubation period should allow the glucose-1-P $\rightleftharpoons$ glycogen exchange to achieve a first order character. An experiment to test this is shown in Fig. 7. Without prior incubation, both the exchanges are similar and follow first order kinetics for 75% of the path toward isotope equilibrium. With prior incubation, the $^{14}$C exchange began to deviate from first order as early as 11% of the attainment of equilibrium, whereas the $^{32}$P$_1$ exchange rate (not shown) remained constant.

Figs. 8 and 9 present the results of the isotope exchange at equilibrium experiments for the phosphorylase b system. In Fig. 8, the glycogen concentration was held constant while glucose-1-P and $P_1$ concentrations were varied at a constant ratio up to values approaching 10 times $K_m$. The $P_1$ $\rightleftharpoons$ glucose-1-P exchange rate rose and then leveled off as saturation of the enzyme with these two substrates was reached. The glucose-1-P $\rightleftharpoons$ glycogen rate paralleled the $P_1$ $\rightleftharpoons$ glucose-1-P rate. The results of Fig. 8 negate the possibility that glycogen is the initial substrate to be bound in a compulsory mechanism. If this had been so, one would have expected a depression of the glucose-1-P $\rightleftharpoons$ glycogen exchange rate at the elevated $P_1$ and glucose-1-P concentrations (14).

The unlikely possibility that glucose-1-P is the leading substrate in an ordered sequence of addition was explored as shown in Fig. 9. The results indicate that both exchange rates investigated reach a maximum rate of exchange at saturating glycogen concentrations. Since no lowering in the $P_1$ $\rightleftharpoons$ glucose-1-P exchange was observed, these results support the view that glucose-1-P does not bind first in an ordered pathway of substrate interaction with the enzyme-AMP complex.

It may be appropriate to comment on the rates calculated for the glucose-1-P $\rightleftharpoons$ glycogen exchange. The equation used is from Reference 13

$$R = \frac{-(P') (G) \ln (1 - F)}{(P') + (G)}$$

where $P'$ and $G$ are glucose-1-P and glycogen, and $F$ is the frac-
tion of isotope equilibrium attained at time, t. Because glycogen
is involved, we are unable to assign a concentration which is a re-
alistic measure of the substrate concentration available to the
enzyme. We have, for convenience, used the total molar concen-
tration of glycogen residues throughout this paper. This
probably gives unrealistically high rates of isotope exchange in
Figs. 8 and 9. It could be argued that the concentration of glu-
cose end groups should be used but this figure is probably low
because of the disproportionate attack of the enzyme on the outer
chains as discussed above. Thus, the total exchanging pool of
unit would be greater than the end groups alone.

If the concentration of glucose end groups is used in Equation
3, the calculated rates of the $^{14}C$-isotope exchange are decreased
below the values for the $^{32}$Pi exchange, but the two exchange rates
remain parallel. At very high concentrations of glycogen, the
values for G in Equation 3 would tend to cancel out and make the
choice of a value less critical. The experiment reported in Fig. 8
was repeated at 320 mM glycogen (22 mM end groups). Fig. 10
shows that, within experimental error, the two exchange rates
are equivalent, no matter which concentration of glycogen was
used to calculate the glucose-1-P $\rightleftharpoons$ glycogen exchange rate.

As discussed by Fromm, Silverstein, and Boyer (31), $R_{\text{max}}$, the
reaction rate at infinite concentrations of substrates, may be
obtained for an enzyme exhibiting rapid equilibrium random bi bi kinetics by the relationship

$$R_{\text{max}} = \frac{V_f \times V_r}{V_f + V_r}$$

where $V_f$ and $V_r$ are the maximal velocities obtained from initial
rate kinetics for the forward and reverse directions, respectively.

For phosphorylase b, under the conditions of our experiments,
the observed $V_f$ and $V_r$ values, from Table I, yield an $R_{\text{max}}$ of
218 $\mu$moles per min per mg. This compares favorably with the values for the $P_1 = \text{glucose-1-P}$ exchange rates at high substrate
concentrations.

The application of the isotope exchange studies to phosphoryl-
ase a yields results which are similar to those reported in this
paper and suggests that the two forms of the enzyme have the
same kinetic mechanism. A detailed study of the kinetics of
phosphorylase a is underway. While this work was in progress,
we have, for convenience, used the total molar concentration
of isotope equilibrium attained at time, t. Because glycogen
units would be greater than the end groups alone.

While this manuscript was being processed, a paper on the
kinetic mechanism of maltodextrin phosphorylase from Escher-
ichia coli was published by Chao, Johnson, and Graves (33).
Their approach was similar to ours, and they concluded that their
data are consistent with a rapid equilibrium random bi bi kinetic
mechanism.

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