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What Limits the Rate of an Enzyme-Catalyzed Reaction?

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In recent years there has been some controversy about how enzymes manage to catalyze chemical reactions as fast as they do. Lost sight of in these arguments, however, has been the fact that the actual chemical reaction is seldom what limits the rate of an enzymatic reaction.

It is the purpose of this Account to show that conformation changes in the protein and release of products (or more likely, the conformation change that permits product release) are the common rate-limiting steps. We present several well-documented cases and the kinetic evidence that allows us to deduce the relative rates of the various steps in the mechanism. Finally we show that the real upper limit on the rate of an enzyme-catalyzed reaction of a thermodynamic one.

Basic Theory. When the concentration of one substrate is varied and the levels of others held constant, most enzyme-catalyzed reactions obey the rate law

$$v = \frac{V[A]}{K + [A]}$$

where [A] is substrate concentration, V the maximum obtainable velocity at infinite substrate concentration, and K the Michaelis constant, which is the level of substrate at which the velocity is half of V and is thus the apparent dissociation constant for the enzyme–substrate complex under steady-state conditions. When [A] is infinite, $v = V$, but as [A] approaches zero, $v = (V/K)[A]$, so that V/K is the apparent first-order rate constant at low substrate concentration. V and V/K are the two fundamental constants that determine the kinetics, and they vary independently with the concentrations of other substrates, products, inhibitors, or activators, or with pH, temperature, ionic strength, etc. The variation of K with all these factors is simply determined by the ratio of V and V/K.

Analysis of effects on V and V/K is the basic tool of the kineticist, and by varying the concentrations of substrates, products, inhibitors, and the like one can deduce the order of addition of substrates and release of products, and thus the sequence of events that occurs. This essentially qualitative information determines the "kinetic mechanism", and since these techniques have been summarized in detail elsewhere[1] we will not go into them here. For our present purposes we need to analyze the data quantitatively, and again we will look at V and V/K separately.

Rate-Limiting Steps in Ordered Sequential Mechanisms. When one substrate must add before the second, and the second product cannot leave until the first has dissociated, the mechanism is ordered. Several (but not all) dehydrogenases, as well as a number of other enzymes, show this type of mechanism. A number of methods exist for telling where the rate-limiting step occurs. First, the position of the crossover points in the initial velocity patterns can be used to tell whether the rate-limiting step for V is the release of the second product or is in the portion of the reaction sequence after addition of the second substrate and up to and including release of first product (this includes the catalytic step[2]). The sum of the vertical coordinates of the crossover points of the two initial velocity patterns for forward and reverse reactions, divided by the sum of reciprocal maximum velocities in both directions, is a dimensionless number varying from 0 for a mechanism where second product release is solely rate limiting to 1 for a rapid equilibrium ordered mechanism where catalysis and/or first product release is solely rate limiting (Figure 1). Where the two V's are not equal, this number determines the rate-determining step in the slow direction only. For glycerokinase, where glycerol adds before MgATP (the magnesium complex of adenosine triphosphate), this number is 5%, corresponding to release of glycerol five times faster than catalysis or release of MgATP in the reverse (slower) direction.[3] This method has the advantage of

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(1) W. W. Cleland, *Enzymes, 3rd Ed.*, **2**, 1 (1970).

(2) In this Account we use the words "catalysis", "catalytic step", and "catalytic reaction" to refer to the actual chemical reaction between substrates, or between substrate and a group on the enzyme. Adsorption and desorption of reactants, and conformation changes, while certainly part of the overall process catalyzed by the enzyme, are thus excluded by this narrow definition.

(3) C. A. Janson and W. W. Cleland, *J. Biol. Chem.*, **249**, 2562 (1974).

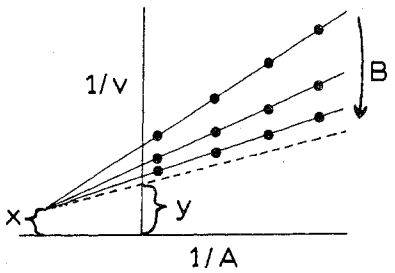


Figure 1. Initial velocity pattern for an ordered sequential mechanism with A and B as substrates. v = initial velocity; $y = 1/V$; x = vertical coordinate of crossover point. The dotted line corresponds to infinite [B], while the solid lines correspond to experimental data. y is obtained from the linear plot of vertical intercepts vs. 1/[B]. The crossover point may be below the horizontal axis (thus giving x a negative value) for one, but not both, initial velocity patterns.

not being affected by isomerization of the binary complexes in the mechanism.

If a dead-end inhibitor combines with the binary complex of enzyme and first substrate, it gives competitive inhibition vs. the second substrate, and the inhibition constant will be the true dissociation constant if the first substrate was saturating. On the other hand, the inhibition constant calculated from the effect on V for the reverse direction (both substrates saturating) varies from the true dissociation constant, when release of second product limits the reverse reaction, to infinity, when catalysis or first product release is rate limiting. With glycerokinase, CrATP was competitive vs. MgATP (K_i = 0.5 μM) with glycerol saturating, but uncompetitive vs. MgADP (K_i = 1.4 μM) when glycerol-3-P was saturating.[3] The ratio of 2.8 here suggests that release of glycerol is 1.8 times faster than catalysis or release of MgADP. This method is not completely valid if the first substrate dissociates from a different form of the binary complex than that which combines with the second substrate (and presumably also the inhibitors which mimic it), but because of its simplicity and accuracy is still useful to give some idea of where the slow steps lie.

By altering substrates, one may change the rate-limiting step, and thus help tell where it is with the normal substrate. With glyceraldehyde-3-P dehydrogenase and the normal substrate, GAP, there is no

$$DPN^+ + \text{glyceraldehyde-3-P (GAP)} + P_i \rightleftharpoons$$
$$\text{1,3-diphosphoglycerate} + DPNH + H^+$$

difference in V when arsenate is substituted for phosphate, and DPNH release appears rate limiting on the basis of various inhibition studies. When 2-deoxy-GAP was used as substrate, V was one-half that for GAP in the arsenate system, but only 7% of the GAP value with P_i as substrate.[4] Glyceraldehyde gives a V that is 7% of the GAP value with arsenate and 5% with phosphate. Along with the sixfold drop in V in going from arsenate to phosphate with 2-deoxy-GAP, the inhibition constant of an inhibitor combining with E-DPNH rose sixfold, and substrate inhibition caused by combination of 2-deoxy-GAP with E-DPNH became undetectable. Thus with 2-deoxy-GAP the rate-limiting step with arsenate is apparently one-half DPNH release, and one-half a step involved in the reaction of arsenate with the acyl enzyme formed by dehydrogenation. This latter step has become eleven times slower with P_i replacing arsenate. With glyceraldehyde (and other nonphosphorylated aldehydes) the rate-limiting step is presumably dehydrogenation, or more likely the thiohemiacetal formation that precedes it.

The Use of Deuterium Isotope Effects to Find Rate-Limiting Steps in Dehydrogenases. When an enzymatic reaction involves H transfers, substrates containing deuterium in the transferred position often show different rates of reaction than the H-containing molecules. These isotope effects are usually expressed as v_H/v_D, and for most reactions the value will be greater than or equal to one, although inverse isotope effects are known. We can analyze the effects of deuterium substitution on both V and V/K,[5] and the resulting information, particularly for V/K, is very useful in determining the extent to which catalysis is rate limiting, since only the catalytic step where C–H bonds are broken will show an appreciable isotope effect. Before we look at specific enzymes, however, we must consider in greater detail the way in which individual rate constants determine V/K and V.

V/K is the product of four factors. The first is the bimolecular rate constant for combination of enzyme and substrate to form a complex. In many cases this value is limited by diffusion, and if the following factors are rigorously taken into account, limitation by diffusion is probably the rule. The second factor is the proportion of substrate in the correct form to react. This can involve the proper state of ionization or chelation by metal, as well as the proper conformation of the molecule in solution, and is really a correction on the level of active substrate present in solution. This factor is probably largely responsible for the apparent values of bimolecular rate constants being smaller than those calculated from maximum rates of diffusion, since many molecules have sufficient conformational freedom in solution so that only a small proportion will be in the correct form to react. The third factor is the proportion of enzyme in the correct form to react. This is the factor that allows identification of ionizable catalytic groups on the enzyme when pH is varied. Also many enzymes have several possible conformation states, and if only one can combine with substrate, V/K is proportional to the fraction of this conformation present. If other substrates involved in the reaction are not saturating, the proportion of enzyme in the correct form to react with the variable substrate may be correspondingly reduced.

The fourth factor is what we will call "yield", and represents the proportion of the first collision complex between enzyme and substrate which goes on to produce products, as opposed to dissociating to E and A. This is the factor which will concern us in discussing isotope effects on V/K. It involves all steps following substrate addition up to and includ-

(4) B. A. Orsi and W. W. Cleland, *Biochemistry*, 11, 102 (1972).

(5) The deuterium label should be 95% or higher, or correction for the H-containing molecules must be made. When the label is tritium, or any other isotope present only as a small percentage of the total, one sees only the V/K effect (since the only way of determining the isotope effect is to measure the depletion of isotope in the product formed), and it is not possible to determine effects on V. Some compounds are available commercially with nearly carrier-free tritium label, but as far as this author is aware they have not been used to determine isotope effects on V.

ing the first irreversible step, which normally is release of the first product, and includes the catalytic reaction itself, as well as all conformation changes that precede and follow catalysis. As an example of what we mean by yield, consider the mechanism

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E' + P$$

$$E' \xrightarrow{k_7} E$$

where k_7 includes release of any further products. The step associated with k_5 is irreversible if P is not present (a valid assumption when measuring initial velocities), but the "net rate constant" from EA to E'P will be

$$k_3' = k_3\left(\frac{k_5}{k_4 + k_5}\right)$$

since this step is reversible and the partitioning of E'P forward and backward must be taken into account. V/K is now

$$V/K = \frac{k_1 k_3'[E]_t}{k_2 + k_3'}$$

and the expression $k_3'/(k_2 + k_3')$ is the yield.[6] Note that V will not be a function of k_2, since [A] will be infinite, but it will be a function of k_7 (V is the reciprocal of the sum of reciprocals of the net rate constants, which in this case are k_3', k_5, and k_7.).

Liver Alcohol Dehydrogenase. This enzyme catalyzes the direct transfer of hydride from ethanol to DPN according to the equation:

$$DPN^+ + \text{ethanol} \rightleftharpoons DPNH + \text{acetaldehyde} + H^+$$

The kinetic mechanism is predominately ordered, with DPN adding before ethanol and DPNH being released after acetaldehyde.[7] When fixed levels of DPNH with D in the transferable position and normal DPNH were used as substrates and the concentration of acetaldehyde was varied, it was found that the maximum velocity was the same for both substrates, but there was an isotope effect of 2.5 on V/K.[8] Lack of an isotope effect on V is consistent with the rate-limiting step for the overall reaction being release of DPN from the enzyme, while the effect of 2.5 on V/K shows that hydride transfer is at least partly rate limiting for the portion of the reaction sequence that determines V/K (addition of acetaldehyde, catalysis, and release of ethanol).

A primary isotope effect should be larger than 2.5, but there are two reasons why low values such as this are observed. (1) If, for any intermediate from the first complex formed with acetaldehyde up to and including the intermediate that undergoes hydride transfer, the net rate constant for forward reaction is much larger than the rate constant for reverse reaction, this intermediate becomes committed to continue the reaction regardless of isotope effects on later steps. This situation is possible only in the thermodynamically favored reaction direction with the higher V (as here), and cannot be of major importance in the direction with the slower V.[9] (2) If a slow step occurs after hydride transfer and before (or is identical with) release of ethanol so that the hydride-transfer step is in a preequilibrium, no isotope effects would be seen.[12]

In the present case it is not certain which of these effects is causing the low isotope effect. In the reverse (and slower) direction the "burst" of DPNH formation on the enzyme seen in stopped-flow studies shows an isotope effect of 5.2,[13] so it seems probable that with acetaldehyde the first effect described above is probably responsible. Unfortunately the isotope effect on V/K with ethanol-*1-d* has not been measured for comparison, although at 8 mM ethanol (about 14 × K) an effect of 1.34 is seen.[8] Since V is determined solely by DPNH release, either there is an isotope effect of 1.34 on this step, or there is an isotope effect on V/K of about 6, or lesser effects on both.

These questions should be partly resolved shortly by the very important discovery of Northrop that comparison of the V/K isotope effects caused by deuterium and tritium permits calculation of the true isotope effects on the bond-breaking steps according to the equation

$$\frac{\dfrac{(V/K)_H}{(V/K)_D} - 1}{\dfrac{(V/K)_H}{(V/K)_T} - 1} = \frac{a - 1}{a^{1.44} - 1}$$

where a is k_H/k_D for the bond-breaking step, and $a^{1.44}$ is k_H/k_T.[14] Comparison of the true and observed isotope effects should then provide a more solid picture of the relative rates of steps preceding and following the catalytic one.

Malic Enzyme. This enzyme catalyzes the oxidative decarboxylation of malate by TPN (triphosphopyridine nucleotide, sometimes symbolized NADP):

$$\text{malate} + TPN^+ \rightleftharpoons CO_2 + \text{pyruvate} + TPNH$$

The kinetic mechanism is ordered, with TPN adding first, and CO_2, pyruvate, and TPNH released in that order.[15] We have recently studied the isotope effects

(6) For more complex mechanisms the procedure is similar. One starts with the first irreversible step and moves back to the left, calculating the net rate constant for each step until one reaches the first collision complex. A more detailed discussion of net rate constants and partition analysis has been submitted for publication in *Biochemistry*.

(7) C. C. Wrattan and W. W. Cleland, *Biochemistry*, **2**, 935 (1963).

(8) K. Bush, V. J. Shiner, Jr., and H. R. Mahler, *Biochemistry*, **12**, 4802 (1973).

(9) A similar situation is probably responsible for the absence of an observed ^{13}C isotope effect on the oxidative decarboxylation of isocitrate catalyzed by isocitrate dehydrogenase.[10] Since the isotope-exchange studies to be discussed below[11] indicate that catalysis is much faster than release of TPN, TPNH, or isocitrate, one would expect the net rate constant for forward reaction of E-TPN-isocitrate to be considerably larger than the rate constant for release of isocitrate from this complex, so that no isotope effect on V/K for isocitrate would be observed. In the related malic enzyme reaction where the release of malate is not so slow we have recently found a ^{13}C isotope effect.

(10) M. H. O'Leary, *Biochim. Biophys. Acta*, **235**, 14 (1971).

(11) M. L. Uhr, V. W. Thompson, and W. W. Cleland, *J. Biol. Chem.*, **249**, 2920 (1974).

(12) The equilibrium constant of the hydride-transfer step will probably show a small isotope effect, with deuterium substitution favoring ethanol, but this has not been measured for this enzyme. (We have recently determined effects of 1.2–1.3 in favor of malate for the malic enzyme and malate dehydrogenase reactions.) These primary equilibrium isotope effects are caused by the different bending frequencies of C-H bonds in =CHOH and $=CH_2$ groups (S. R. Hartshorn and V. J. Shiner, Jr., *J. Am. Chem. Soc.*, **94**, 9002 (1972)). In addition, one might expect secondary isotope effects from deuterium in nontransferable positions as the result of alteration of the catalytic equilibrium in favor of the tetrahedral over the planar carbon. Thus acetaldehyde-*1-d* might show an inverse secondary-isotope effect.

(13) J. D. Shore and H. Gutfreund, *Biochemistry*, **9**, 4655 (1970).

(14) D. B. Northrop, *Biochemistry*, in press.

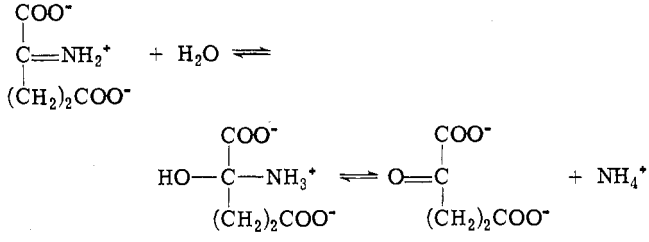
(15) R. Y. Hsu, H. A. Lardy, and W. W. Cleland, *J. Biol. Chem.*, **242**, 5315 (1967).

as a function of pH with malate and malate-2-*d* as variable substrates.[16] The effect on V/K is small (1.5–1.8) and nearly independent of pH. The effect on V, on the other hand, was absent at pH 7–8, where independent kinetic evidence indicates that TPNH release is solely rate limiting,[17] but increased at both low and at high pH, and reached a value of about 3 at high pH. Thus it appears that hydride transfer is partially rate limiting for the sequence of dehydrogenation, decarboxylation, and CO_2 release which limits V/K, and apparently also V at low and high pH. The lower isotope effect on V/K than on V at high pH proves that the forward net rate constant for the first E–TPN–malate complex is larger than the rate constant for malate release from this complex, since other factors which would lower the isotope effect on V/K would also affect V equally.

Glutamic Dehydrogenase. This still more complex dehydrogenase catalyzes the reaction

$$TPN^+ + \text{glutamate} \rightleftharpoons NH_4^+ + \alpha\text{-ketoglutarate} + TPNH + H^+$$

The primary dehydrogenation in this case should produce enzyme-bound iminoglutarate, which must be hydrolyzed to NH_4^+ and α-ketoglutarate prior to release of these reactants. Fisher has recently observed in stopped-flow studies a "burst" of enzyme-bound TPNH with a blue-shifted spectrum, and then the production of a red-shifted spectrum (E–TPNH or E–TPNH–glutamate) prior to actual release of TPNH.[18] The burst showed an isotope effect of 1.5–1.8 when L-glutamate-2-*d* was used. Cross attributed the blue-shifted spectrum formed in the burst to E–TPNH–α-ketoglutarate,[19] which has a similar spectrum, although there is no way to rule out E–TPNH–iminoglutarate, or the carbinolamine which should be intermediate between iminoglutarate and α-ketoglutarate and NH_4^+:



We looked for an isotope effect in the steady state with glutamate-2-*d*, but there was no appreciable effect on either V or V/K.[20] No effect on V was anticipated, since the rate-limiting step at high glutamate has been thought to be largely release of TPNH from the E–TPNH–glutamate complex formed after NH_4^+ and α-ketoglutarate dissociated and glutamate combined in its place (the kinetic mechanism is random sequential). The lack of any isotope effect on V/K, however, is somewhat surprising, and suggests that since this is the thermodynamically unfavorable, and thus slower, direction, the dehydrogenation must be at equilibrium, and some subsequent step prior to or identical with the first release of product must be rate limiting for V/K. The first product released is probably NH_4^+, and its release is very unlikely to be rate limiting, so this leaves iminoglutarate hydrolysis as the probable rate-limiting step for V/K. Subsequent release of α-ketoglutarate from E–TPNH–α-ketoglutarate (2 sec^{-1}),[21] and of TPNH from E–TPNH–glutamate (0.8 sec^{-1}),[21] would limit V.

At pH 9 glutamic dehydrogenase accepts monocarboxylic acids as substrates, and with 2-aminovalerate-2-*d* we recently found isotope effects on V and V/K of 6 and 3.2. These high values suggest that hydride transfer, rather than iminovalerate hydrolysis or product release, is largely rate limiting with this monocarboxylic substrate.

These conclusions have now been confirmed by finding a ^{15}N isotope effect of 1.046 with glutamate as substrate, but no isotope effect with norvaline.

Rate-Limiting Steps in Random Sequential Mechanisms. Many reactions in which there is a direct transfer between two substrates adsorbed on the enzyme show random sequential kinetics; that is, both substrates form binary as well as ternary complexes with the enzyme, although the presence of one may influence both the dissociation constant and the rate constants for combination of the other. Rate equations for these mechanisms are generally derived on the assumption that substrate combination is at equilibrium, and the rate-limiting step is catalysis, or at least precedes or is actually the first product-release step. This "rapid equilibrium assumption" is generally made to simplify what are otherwise very complex and unwieldy rate equations, but how does one tell whether the rapid equilibrium assumption holds and where the rate-limiting steps lie?

Several methods have been applied. First, if the assumption does not hold for *both* substrates, the initial velocity patterns (see Figure 1) may not clearly intersect as they should, but look nearly parallel.[22] Further, all quantitative information derived from the apparent slopes of the plots will be in error, and not agree with values derived from study of the reverse reaction, or from binding studies. This is the case for phosphofructokinase[23] which catalyzes the phosphorylation of fructose-6-P by MgATP to give fructose-1,6-P_2, and probably also for other kinases such as mammalian hexokinase[24] and arginine kinase,[25] which have nearly parallel initial velocity patterns.

If the rapid equilibrium assumption holds for one substrate, but not for the other, the initial velocity pattern may look normal, and the calculated dissociation constant for the substrate that dissociates

(16) M. I. Schimerlik and W. W. Cleland, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **34**, 495 (1975).

(17) At pH 7 the K_i for pyruvate as an uncompetitive product inhibitor with both malate and TPN saturating is equal to the dissociation constant of pyruvate as a substrate when TPNH is saturating and CO_2 and pyruvate the variable substrates. This can only be true if E–TPNH is the only enzyme form present at appreciable concentration at saturating malate and TPN.

(18) H. F. Fisher, J. R. Bard, and R. A. Prough, *Biochem. Biophys. Res. Commun.*, **41**, 601 (1970).

(19) D. G. Cross, *J. Biol. Chem.*, **247**, 784 (1972).

(20) C. L. Myers and W. W. Cleland, in preparation.

(21) A. di Franco, *Eur. J. Biochem.*, **45**, 407 (1974).

(22) For the non-rapid-equilibrium case discussed here, the reciprocal plots are actually nonlinear, concave up, but in the region where the data would normally be obtained the curvature would hardly be noticeable.[23]

(23) J. Bar-Tana and W. W. Cleland, *J. Biol. Chem.*, **249**, 1263 1271 (1974).

(24) H. J. Fromm and V. Zewe, *J. Biol. Chem.*, **237**, 1661 (1962); T. L. Hanson and H. J. Fromm, *ibid.*, **240**, 4133 (1965).

(25) M. L. Uhr, F. Marcus, and J. F. Morrison, *J. Biol. Chem.*, **241**, 5428 (1966); E. Smith and J. F. Morrison, *ibid.*, **244**, 4244 (1969).

slowly will be correct. For the other substrate, however, the calculated dissociation constant may be drastically different from the true one, as with MgATP and yeast hexokinase where the values differ by a factor of 50 (see below).

The second method of detecting rate-limiting steps in random mechanisms involves measuring rates of isotopic exchange at chemical equilibrium between a labeled substrate and product. If the rate-limiting step is truly catalysis or some step closely associated with it, all possible exchanges will go at equal rates.[26] This is the case for creatine kinase,[27] but it appears unique in this respect, and kinases with sugar substrates (hexokinase,[28] galactokinase,[29] fructokinase[30]) have all shown faster MgATP–MgADP than sugar–sugar-P exchanges by ratios of 1.5–3. For these enzymes release of sugar must be at least partly rate limiting for exchange,[31] and for the reverse reaction. The hexokinase mechanism is discussed in greater detail below.

A changing ratio between the exchanges at high and low reactant levels has recently been used as a criterion for determining when catalysis was rate limiting in random mechanisms. With TPN–isocitrate dehydrogenase:

$$TPN^+ + \text{isocitrate} \rightleftharpoons CO_2 + \alpha\text{-ketoglutarate} + TPNH$$

CO_2–isocitrate and TPN^+–TPNH exchanges were measured with TPN^+ and TPNH, or α-ketoglutarate and isocitrate being varied in constant ratio.[11] When TPN^+ and TPNH were varied, the CO_2–isocitrate exchange was always faster than the TPN^+–TPNH exchange, but had a lower apparent K, so that the ratio between the exchange rates was much greater at low than at high TPN^+ and TPNH. When α-ketoglutarate and isocitrate were varied, the CO_2–isocitrate exchange was fastest at high α-ketoglutarate and isocitrate levels, but because the apparent K for the TPN^+–TPNH exchange was 40-fold lower than that for the other exchange, TPN^+–TPNH exchange became the faster one at low α-ketoglutarate and isocitrate. These patterns show that catalysis is much faster than release of either set of reactants, as well as that the mechanism is random. The reason for the different apparent K's seen in the two exchanges is that the enzyme must be half-saturated by the varied reactants in order to support exchange between them, while for the other exchange a much lower degree of saturation will suffice to keep catalysis faster than reactant release. This phenomenon is not seen with fructokinase, where the apparent K's for the two exchanges are identical.[30] Presumably here catalysis (or at least steps which occur while both substrates and products are present) is mainly rate limiting, with release of fructose being slow enough to cause fructose–fructose-1-P exchange to be threefold slower than MgATP–MgADP exchange.

Isotope Partition Method. Rose has recently introduced a novel kinetic technique for determining certain rate constants in enzyme mechanisms.[32] He incubated glucose-^{14}C and yeast hexokinase in high enough concentration so that all of the enzyme was in the binary E–glucose complex, and then added this sample to a solution containing a great excess of unlabeled glucose and various levels of MgATP. After several seconds (time for at least the first turnover) the enzyme is denatured and the radioactivity in glucose-6-P (after a small correction for the turnover of the diluted glucose) is a measure of the amount of E–glucose which reacted with MgATP to give glucose-6-P, as opposed to dissociating to E and glucose. By varying MgATP, one can determine by comparison of the yield at infinite MgATP and the enzyme level whether glucose is released from the ternary E–glucose–MgATP complex at an appreciable rate relative to V (it was not), and from comparison of the apparent K for the isotope partition with the K for the chemical reaction, one can calculate the ratio of the rate constant for glucose dissociation from E–glucose to the V for reaction to form glucose-6-P (0.29, in this case).

This method has tremendous potential for showing whether substrates dissociate more slowly than V, especially in the thermodynamically favorable direction. It is equally applicable to ordered mechanisms (to the first substrate only), or to random mechanisms (to either substrate). With fructokinase, we have recently found that fructose dissociates from E–fructose 80 times faster than V, but from E–fructose–MgATP at a rate that is of the same order of magnitude as V.[30]

Mechanism of Yeast Hexokinase. For some time enzymologists have been puzzled by the fact that the dissociation constant of glucose from binding studies agreed reasonably well with that from kinetic studies (30–100 μM), while MgATP appeared not to bind, despite a value of 100 μM from kinetic studies.[33] Some workers even postulated that the mechanism was ordered (glucose adding first) to explain these results, although the fact that the enzyme has ATPase activity clearly shows that MgATP can combine. The K for the ATPase activity is 5 mM, and this is presumably the true dissociation constant of MgATP, while the K for phosphorylation is 50 times less.

In this laboratory we have been using the inert chromium complexes of nucleotides as dead-end inhibitors, since the metal and nucleotide remain firmly bound to each other instead of being in a rapid equilibrium, as with Mg nucleotides.[34] CrATP is an excellent inhibitor of yeast hexokinase (K_i = 1 μM for the active β,γ-bidentate isomers), and when initial velocities are measured shows equal affinity for free enzyme and E–glucose.[35] When CrATP is present,

(26) For creatine kinase, for example, one can measure creatine–creatine-P, MgATP–MgADP, and MgATP–creatine-P exchanges, with the first two between similar molecules being the ones usually used. Since ^{14}C or ^{32}P labels would be used, there would be no appreciable isotope effects on the rates caused by the isotope itself.

(27) J. F. Morrison and W. W. Cleland, *J. Biol. Chem.*, **241**, 673 (1966).

(28) H. J. Fromm, E. Silverstein, and P. D. Boyer, *J. Biol. Chem.*, **239**, 3645 (1964).

(29) J. Gulbinsky and W. W. Cleland, *Biochemistry*, **7**, 566 (1968).

(30) F. M. Raushel and W. W. Cleland, in preparation.

(31) In theory, sugar-P release could also limit the exchange rate, but since K_{eq} is high and there is much more sugar-P than sugar in such experiments, sugar release will be the only important step.

(32) I. A. Rose, E. L. O'Connell, S. Litwin, and J. Bar-Tana, *J. Biol. Chem.*, **249**, 5163 (1974).

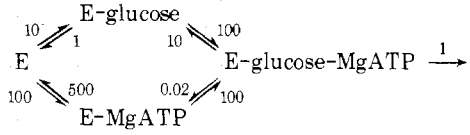
(33) For a review of previous kinetic work on hexokinase, see D. I. Purich, H. J. Fromm, and F. B. Rudolph, *Adv. Enzymol.*, **39**, 249 (1973).

(34) M. L. DePamphilis and W. W. Cleland, *Biochemistry*, **12**, 3714 (1973); M. I. Schimerlik and W. W. Cleland, *J. Biol. Chem.*, **248**, 8418 (1973); C. A. Janson and W. W. Cleland, *ibid.*, **249**, 2562, 2567, 2572 (1974).

(35) K. D. Danenberg and W. W. Cleland, *Biochemistry*, **14**, 28 (1975).

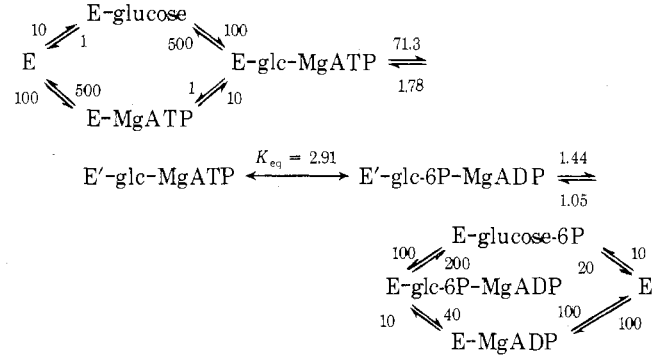
however, the reaction rate slows down over several minutes to a new rate that is far less, and this change occurs with all sugars and sugar analogs that are good substrates for the enzyme. When the enzyme is preincubated for 10 min with MgATP, CrATP, and lyxose (an inhibitor that cannot undergo reaction), and reaction then started by addition of an excess of glucose, the reaction rate is linear, and the inhibition constant for CrATP has decreased by a factor of 40. While CrATP in the absence of sugar is reversibly bound to hexokinase (it is removed by Sephadex chromatography), the tight CrATP–glucose–hexokinase complex formed during incubation for several minutes does not dissociate during Sephadex chromatography, and contains one glucose and one CrATP per active subunit of mol wt 50,000. When this complex (labeled with glucose-^{14}C) is denatured with acid, 25% of the glucose is released as free sugar and 75% as a CrADP–glucose-6-P complex in which both ADP and glucose-6-P are liganded to chromium. Further, this CrADP–glucose-6-P complex is slowly released from the enzyme at a rate of about 4%/min, so that CrATP is acting as a very slow and tightly bound substrate for hexokinase. The slow step is not catalysis, however, since the 3:1 ratio of CrADP–glucose-6-P to free glucose upon denaturation of the isolated tight E–CrATP–glucose complex is independent of incubation time. Thus the conformation changes that permit catalysis and then permit release of CrADP–glucose-6-P are rate limiting.[36]

The studies with CrATP suggest that the hexokinase mechanism involves (1) reversible binding of substrates in an independent fashion; (2) a conformation change with K_{eq} = 40; (3) catalysis with K_{eq} = 3; (4) a conformation change with a K_{eq} around unity; (5) reversible release of products. We reached very similar conclusions for MgATP as a substrate from the following observations.[35] First, the isotope partition study of Rose indicated that glucose was released from E-glucose at a rate slightly less than V, and from E-glucose–MgATP at a rate less than 5% of V.[32] A simulation study was then made to discover what combinations of rate constants for random combination of substrates would yield linear reciprocal plots for both substrates (crossing on the horizontal axis as observed) and still fit the isotope partition data and the observed kinetic constants and binding constants. These criteria are very restrictive, and led to the following choice of rate constants (unimolecular ones relative to V; bimolecular ones chosen to yield kinetic constants in mM):



This mechanism also provides an explanation for the partial substrate inhibition by MgATP that is induced by the presence of lyxose[37] if it is assumed that lyxose and MgATP each make the other bind more tightly (lyxose lowers the K for ATPase activity by a factor of 40[38]).

An explanation for the 50-fold lower unimolecular constants for release from the ternary, as opposed to binary, complexes in the above scheme can be provided as follows. When ATP-(6-glucose) was prepared as a possible transition-state analog, it was competitive vs. MgATP, but apparently uncompetitive vs. glucose, showing that it bound much more tightly to E-glucose than to free enzyme.[39] Similar observations have been made for the analog of ATP containing glucose in place of ribose.[40] Thus the first step after combination of both sugar and nucleotide seems to be a step with a K_{eq} of at least 40. Presumably this step sets the stage for catalysis, if the sugar is a substrate, and one would also expect a similar step after catalysis to permit products to leave. By assuming that: (1) the dissociation constants for sugar and nucleotide are independent of each other (as with CrATP), (2) the bimolecular rate constants for combination of enzyme and glucose or glucose-6-P are the same, and the same is true for the nucleotides, (3) the catalytic step is not rate limiting, but is fast in both directions, (4) by taking the rate constants in the above scheme and the observed kinetic constants in both forward and reverse directions as a starting point, one can solve for a unique set of rate constants for the entire mechanism as follows:



The close agreement between the observed value of 3 for K_{eq} for the catalytic step with CrATP and the value of 2.91 here (which was calculated before the CrATP value was known) is striking, even though it may be partly fortuitous. Clearly the free energy in the reaction is dissipated largely in tight binding of sugar (as opposed to sugar-P) and the conformation change that follows addition of sugar and nucleotide, rather than in the catalytic step itself.

It is instructive to analyze the rate constants derived for this model to determine which steps really are rate limiting in both directions. The net rate constants in the forward direction for the steps starting with E-glucose–MgATP are: 50, 4.17, 1.43, and for overall product release, 21. Since V is 1.00, it is

(36) The reason why these conformation changes are so slow for CrATP and so fast for MgATP is not known, but can possibly be explained by postulating that deformation of the coordination sphere of the metal is involved. Such deformation should be facile enough with Mg, but require considerable input of energy for Cr.

(37) At K levels of substrate there is almost no E-MgATP present, but at high MgATP it becomes a predominate form. Thus the effect of lyxose, which combines appreciably only with E-MgATP, is drastically enhanced by raising MgATP. The substrate inhibition is partial because infinite MgATP can only provide E-MgATP for lyxose to combine with, and at finite lyxose, not all of it is converted to E-lyxose-MgATP. This substrate inhibition is competitive vs. glucose, since glucose and lyxose compete for the sugar binding site.

(38) G. De la Fuente, R. Lagunas, and A. Sols, *Eur. J. Biochem.*, **16**, 226 (1970).

(39) P. J. Danenberg, unpublished.

(40) D. C. Hohnadel and C. Cooper, *Eur. J. Biochem.*, **31**, 180 (1972).

clear that the conformation change that permits product release after catalysis is 70% rate limiting, and the catalytic step is 24% rate limiting, despite the fact that it is assumed to be in rapid equilibrium.[41] In the reverse direction, the net rate constants starting with E-glucose-6-P-MgADP are: 0.285, 0.536, 1.56, and for net product release, 1.0. Since V here is 0.143, the first conformation change before catalysis is 50%, catalysis is 27%, the second conformation change 9%, and product release 14% rate limiting.

Thus an increasing amount of evidence shows that the chemical steps in catalysis are usually not the major rate-limiting steps for most enzymatic reactions, but suggests that the conformation changes that set up the catalytic step, or permit the dissociation of products after the catalytic step, are rate limiting. In one case, hexokinase, the use of chromium nucleotides slows down these conformation changes to the point where they are easily followed, and hopefully will enable us to gain a greater insight into the actual workings of enzymatic catalysis.

Limits on V and V/K. We have now looked at a number of enzymes and their rate-limiting steps, but we have not asked what actually sets the upper limit on V and V/K. Is it really catalysis? No. In practice, V is limited by the thermodynamic restraints present in the Haldane relationship which relates the equilibrium constant to the kinetic constants. For a mechanism with only one substrate and product the Haldane is:

$$K_{eq} = \frac{V_1 K_p}{V_2 K_a}$$

where V_1 and V_2 are maximum velocities in forward and reverse directions, and K_a and K_p the Michaelis constants for substrate A and product P. (For more complex mechanisms, there are either Michaelis or dissociation constants for each product in the numerator, and each substrate in the denominator.[42])

To raise V_1 in this relationship, one must lower K_p, or raise V_2 and K_a.[43] The upper limit on K_a is set by the physiological level of A; if K_a exceeds this, most of the catalytic potential of the enzyme is wasted (that is, the enzyme will operate in the first-order region where V_1/K_a is the first-order rate constant, and nothing is gained by raising V_1 and K_a together). The upper limit on V_2 is set by K_a. While K_a is not the dissociation constant of A at equilibrium, it is the apparent dissociation constant in the steady state, and is thus basically the ratio of a unimolecular rate constant for release of A from the enzyme to the bimolecular rate constant for combination with enzyme. With the maximum value of K_a set by the physiological level of A, and with the bimolecular rate constant limited by diffusion, the unimolecular rate constant for release of A from the enzyme has a fixed maximum value. Since this is one of the rate constants that limit V_2, it sets an upper limit to it.

The limit on how low K_p can be is set by two factors. First, if K_p is not at least 5 to 10 times the physiological level, product inhibition will occur. Second, one may run into the same problem that limits V_2, namely that the maximum value of the unimolecular rate constant for release of P will begin to limit V_1. Thus in practice there is a definite upper limit to V_1 set by K_{eq} and the physiological levels of A and P. All of this can be summarized by saying that, if substrates are sticky enough to adsorb to the enzyme, the products will be sticky enough to come off at a rate which sets an upper limit to V. In practice, this also limits V/K, since we have seen that, for V to be as high as possible, K should be as large as possible, and thus it is not possible to lower K in an effort to raise V/K.

After this discussion it should come as no surprise to find that after three billion years of biochemical evolution, most enzymes have V's that seem to be maximized within the restraints of the Haldane relationship, and that the chemical reactions involved in catalysis have become fast enough to be only partly, if at all, rate limiting. The wide variation of V values for different enzymes reflects the values of K_{eq} and of the physiological levels in each case.

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(41) It is partially rate limiting because only three-fourths of the enzyme in the equilibrium is available for the slow step that follows.

(42) W. W. Cleland, *Biochim. Biophys. Acta*, 67, 104 (1963).

(43) We are actually referring to $V_1/[E]_t$ and $V_2/[E]_t$, the catalytic center activities. This discussion is of course teleological; however, natural selection should operate ruthlessly to increase the catalytic power of enzymes, since this lowers the energy needs of the cell for protein synthesis by requiring fewer enzyme molecules to be made. We assume that the physiological levels of reactants in the cell are essentially set by the properties of the entire metabolic system, and that a successful cell produces enough (but just enough) of each enzyme to produce or maintain the appropriate level of each reactant in the cell.