Energetics and Mechanism of Proline Racemase†

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ABSTRACT: The results from the previous six papers are collated so as to allow the construction of the complete free energy profile for the reaction catalyzed by proline racemase. This profile includes the step that involves the isomerization of the two forms of free enzyme, which can become rate limiting at very high substrate levels (in "oversaturation"). The mechanism of the reaction has been defined, the results being best accommodated by a route that involves a transition state or unstable intermediate in which the proline carbanion is flanked by the two catalytic thiols of the enzyme.

In the preceding six papers (Belasco et al., 1986a–c; Fisher et al., 1986a–c) we have reported the results of a series of experiments designed to define the energetics and mechanism of proline racemase. As the pioneering work of Abeles and his group has shown (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975), the enzyme mediates the catalyzed reaction by the transfer of protons (rather than of hydrogen atoms or hydride ions) from and to the bound substrate. Binding and chemical modification studies suggested that proline racemase has a single substrate binding site for every two identical subunits and that each subunit provides one thiol at the active site. There are two forms of the enzyme, E_1 and E_2: E_1 binds L-proline (S), and E_2 binds D-proline (P), and we may represent these two forms of the enzyme as a pair of appropriately positioned thiol groups, the catalytically active forms differing primarily (and possibly solely) by which thiol is protonated and which is deprotonated. In this paper, we collate the experimental findings from the previous six papers, consider the evidence for the pathway outlined, and discuss the kinetic and mechanistic consequences for catalysis by proline racemase.

Results and Discussion

Reaction Scheme. The results from the previous papers in this series (Belasco et al., 1986a–c; Fisher et al., 1986a–c) allow the formulation of the mechanistic sequence for proline racemase illustrated in Figure 1. Each of the two forms of the enzyme E_1 and E_2 can bind either a proline enantiomer or water and then be converted into the other form either by the concomitant inversion of the chiral center of the substrate proline or by the mediation of a water molecule. In the preceding paper (Belasco et al., 1986c) we have shown that the mechanism of the water-mediated enzyme interconversion involves "crossover", so that when a hydrogen isotope is lost from (say) labeled E_2', E_1 is formed.[1] Conversely, isotope is lost from E_1'' to yield E_2. This lower route of interconversion of E_1 and E_2 (Figure 1) is clearly analogous to the upper route, in which the conversion of one proline enantiomer into the other is axiomatically accompanied by the interconversion of enzyme forms. The inclusion in Figure 1 of the intermediate species in which either the substrate carbanion or an hydroxide ion is bound to the diprotonated form of the enzyme will be discussed more fully later in this paper.

Table I: Kinetic Constants for the Proline Racemase Reaction[a]

$k_{-2}/k_2 = K_2 = 1.0$
$k_{-1}/k_1 = k_3/k_{-3} = 1.5$ mM
$k_1 = k_{-3} = 5 \times 10^8$ M^{-1} s^{-1} [b]
$k_{-1} = k_3 = 7.3 \times 10^5$ s^{-1}
$k_{2A} = k_{-2B} = 5.2 \times 10^3$ s^{-1}
$k_9 = k_{-10} = 2.2 \times 10^5$ s^{-1}
$k_{-2A} = k_{2B} = k_{-9} = k_{10} = 6 \times 10^{12}$ s^{-1}
$k_{2A}/k_{-2A} = k_{-2B}/k_{2B} = 8 \times 10^{-10}$
$k_9/k_{-9} = k_{-10}/k_{10} = 3.4 \times 10^{-8}$

[a] See Figure 1. The primary data are from Table III of Fisher et al. (1986a); see text. [b] This value is based upon the known range of enzyme–substrate association rates (Hammes & Schimmel, 1970) and upon the value for k_{cat}/K_m for triosephosphate isomerase of 4×10^8 M^{-1} s^{-1} (Knowles & Albery, 1977) which is known from viscosity variation experiments (R. Raines, unpublished work) to involve a diffusive transition state.

Collection of the Kinetic Results. In Table I we collect together the values for the rate constants given in Figure 1. To derive these microscopic constants from the measured rate constants, we start with the important result that transition states 1 and 3 (i.e., those that involve substrate and product binding steps) are never rate limiting: the interconversion of E_1 and E_2 via the upper pathway is dominated by transition states 2A and 2B (Belasco et al., 1986a). Further, since we have found (Fisher et al., 1986a) that $k_{cat^+} = k_{cat^-}$, and since transition states 2A and 2B are dominant, the intermediate species E_1S and E_2P must have the same free energy. That is, K_2 (the equilibrium constant between E_1S and E_2P) equals 1. Since the free energies of the enantiomers S and P are equal, this in turn suggests that $E_1{\cdot}H_2O$ and $E_2{\cdot}H_2O$ also have the same free energy (that is, $K_{1,2,3} = K_{9,10} = 1$, and $k_{1,2,3} = k_{-3,2,1}$). We may now evaluate $k_{1,2,3}$ and $k_{-3,2,1}$ using eq 13 of Fisher et al. (1986a)[2] from k_U, the second-order rate constant in the unsaturated region, which is equivalent to k_{cat}/K_m:

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[1] The two protonic sites are specified by a single or a double prime (see Figure 2). The fractionation factor for the 2-proton of free L-proline is ϕ_S and that for the proton where L-proline is bound to the enzme is ϕ_{ES}'. Analogously, the 2-proton of D-proline has the factor ϕ_P (for the free material) and ϕ_{EP}'' (for the bound material). The enzyme thiol in E_2 that bears the proton is the singly primed site, and the thiol in E_1 that bears the proton is the doubly primed site.

[2] We replace K_4 and k_4 of eq 13 of Fisher et al. (1986a) by $K_{9,10}$ and $k_{9,10}$, respectively, since the latter refer to the preferred pathway of enzyme interconversion through $E_4{\cdot}OH^-$ (Belasco et al., 1986c).

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$$k_{1,2,3} = k_{-3,2,1} = 2k_U \quad (1)$$

Now, since

$$k_{1,2,3} = k_1 k_{cat}/k_{-1} \quad (2)$$

we have

$$k_1/k_{-1} = k_{-3}/k_3 = 2k_U/k_{cat} \quad (3)$$

Since transition states 1 and 3 are kinetically insignificant, we cannot measure k_1 and k_{-3}. We may assume, however, that these "on" steps are diffusion controlled, and this then allows the estimation of k_{-1} and k_3 given in Table I. Finally, in the upper pathway of Figure 1, it is obvious that the intermediate $E_4{\cdot}C^-$ is of high free energy. The enzyme-bound carbanion will abstract a proton from one of the two neighboring enzyme acid groups in an essentially activationless proton transfer. That is, $E_4{\cdot}C^-$ will collapse to E_1S or to E_2P, where $k_{-2A} = k_{2B} \approx kT/h$. This means that transition states 2A and 2B will have the same free energy, and

$$k_{2A} = k_{-2B} = 2k_{cat} \quad (4)$$

We could also arrive at the same conclusion by arguing from the overall symmetry of the proline racemase system.

Looking now at the lower route of Figure 1, we have [eq 14 of Fisher et al. (1986a)][2]

$$k_{9,10} = 2k_O k_{-3}/k_3$$

As argued in the preceding paper (Belasco et al., 1986c), $E_4{\cdot}OH^-$ is also of high free energy, so that $k_{-9} = k_{10} \approx kT/h$, and

$$k_9 = k_{-10} = 4k_O k_{-3}/k_3 \quad (5)$$

Values for the relevant rate constants are listed in Table I.

We next collect together the values for the fractionation factors of the two protonic sites (see Figure 2) in intermediates and transition states of the system. These are listed in Table II. For a racemase, the fractionation factors for *substrate* and *product* are obviously identical ($\phi_S = \phi_P$), and as we showed earlier (Belasco et al., 1986b), mere binding of the substrate or the product to the enzyme does not alter the fractionation factor, and so

$$\phi_{ES}' = \phi_S = \phi_{EP}'' = \phi_P$$

In evaluating the fractionation at the *enzyme* site in the enzyme–substrate complexes, ϕ_{ES}'' and ϕ_{EP}' (Belasco et al., 1986b), we assumed that $K_2 = 1$ and that transition state 2 was cleanly rate limiting in the conversion of $E_1 + S$ to $E_2 + P$ (through transition states 1–3). Both of these assumptions have now been justified (see above). Moreover, since transition state 2 dominates in steps 1–3, we may write $\phi_{2A,2B}' = \phi_{1,2,3}'$ and $\phi_{2A,2B}'' = \phi_{1,2,3}''$.

We now examine the evidence that supports the pathway for proline racemase catalysis shown in Figure 1.

Nature of the Catalytic Groups. The suggestion from the chemical modification work of Rudnick and Abeles (1975) that the catalytic groups of proline racemase are thiols is confirmed in this work (Belasco et al., 1986b) by the direct measurement of the fractionation factors ϕ_{EP}' and ϕ_{ES}'' for the proton that is on the enzyme's catalytic base when substrate is bound. The observed values of about 0.55 are well below the value near unity expected for a proton on an oxygen or a nitrogen base such as carboxylate, imidazole, or an amine but are close to those of thiols, which have fractionation factors near 0.5. The conclusion that the enzyme's catalytic groups are thiols is supported by the observed value for the fractionation factor of these groups in the *free* enzyme, $\bar{\phi}_E$, which was found to be approximately 0.44 (Belasco et al., 1986b).

Table II: Fractionation Factors for the Proline Racemase Reaction[a]

$\phi_S = \phi_P = \phi_{ES}' = \phi_{EP}'' = 1.17$[b]	
$\phi_{EP}' \approx \phi_{ES}'' \approx 0.55$[c]	
$\bar{\phi}_E \approx 0.44$[c,d]	
$\phi_{2A,2B}' = 0.38$[b]	$\phi_{2A,2B}'' = 0.44$[b]
$\phi_{9,10}' = 0.42$[e]	$\phi_{9,10}'' = 0.49$[e]
$\phi_{2B}' = \phi_{2A}'' = \phi_{10}'' = \phi_9' \approx 0.55$	
$\phi_{2A}' = 0.29$[f]	$\phi_{2B}'' = 0.37$[f]
$\phi_{10}' = 0.34$[f]	$\phi_9'' = 0.44$[f]

[a] These fractionation factors (see Figure 2) relate to the intermediates and transition states for the reactions shown in Figure 1 (see also footnote 1). All fractionation factors are measured with respect to HDO as the standard. [b] Fisher et al. (1986c). [c] Belasco et al. (1986b). [d] This value derives from a solvent isotope effect experiment in D_2O and is considered less precise than the value of 0.55 deriving from the difference in initial rates in the perturbation experiments under tracer and nontracer conditions (Belasco et al., 1986b). [e] Belasco et al. (1986c). [f] Obtained from the mixed fractionation factors, and the value of 0.55 for the proton at rest on the enzyme.

It is gratifying that, using the methods developed in earlier papers (Belasco et al., 1986b,c), we can now probe the fractionation at sites in enzyme-bound *intermediates*, as well as at sites in the transition states of the catalyzed reaction. In the present case, we have thereby proved the *direct catalytic involvement* of the two thiols at the active site of the racemase.

Mechanisms of Enzyme Interconversion. When the two forms of the enzyme interconvert by the upper route of Figure 1 with the concomitant interconversion of S and P, we have shown (Belasco et al., 1986a) that transition states 1 and 3 are not rate limiting: the substrate and product on–off steps are relatively rapid. The double fractionation experiment allowed the further conclusion that the two proton transfers involved in the conversion of E_1S to E_2P are either concerted or, if stepwise, must involve enzymic thiols (Belasco et al., 1986a). Inspection of Table II shows that the kinetic isotope effects in the substrate-mediated enzyme interconversion (Figure 1, upper route) and in the solvent-mediated enzyme interconversion (Figure 1, lower route) are very similar, so that $\phi_{2A,2B}' \approx \phi_{9,10}'$ and $\phi_{2A,2B}'' \approx \phi_{9,10}''$. Even more striking are the results from the double-competitive deuterium washout experiment (Belasco et al., 1986c), which show that the *ratio* of the fractionation factors for the two protonic sites, ϕ'/ϕ'', is unchanged at 0.86 on going from the saturated region (where transition states 2A and 2B of the upper route are rate limiting) into the oversaturated region (where transition states 9 and 10 of the lower route are rate limiting). These similarities, as well as the fact that E_1 goes to E_2 (or E_2 to E_1) whether the interconversion is mediated by substrate or by water, strongly suggest that the upper and lower routes for enzyme interconversion involve very similar mechanisms, the details of which we may now evaluate.

In Figure 3 we show the map [after Albery (1967) and More O'Ferrall (1970)] for the three possible routes of enzyme interconversion, two stepwise and one concerted. The reaction is mediated by HX, which may be the substrate (proline), a water molecule, or a small catalyzing acid having a pK_a of 7 ± 2 such as ammonia, hydrazine, or hydrogen sulfide. In discriminating among the three routes in Figure 3, we may first eliminate the stepwise reaction via the bottom right-hand corner, through $E_3{\cdot}H_2X^+$. While this route is possible for the water-mediated interconversion of E_1 and E_2 (via $E_3{\cdot}H_3O^+$), it is impossible for the substrate-mediated reaction since that would involve a pentacovalent carbocation, and we require a common mechanism for both water- and substrate-mediated routes. We are therefore left with the acid-catalyzed route via $E_4{\cdot}X^-$ and the concerted route across the middle of the map. From what follows, it appears that the route via $E_4{\cdot}X^-$ is the

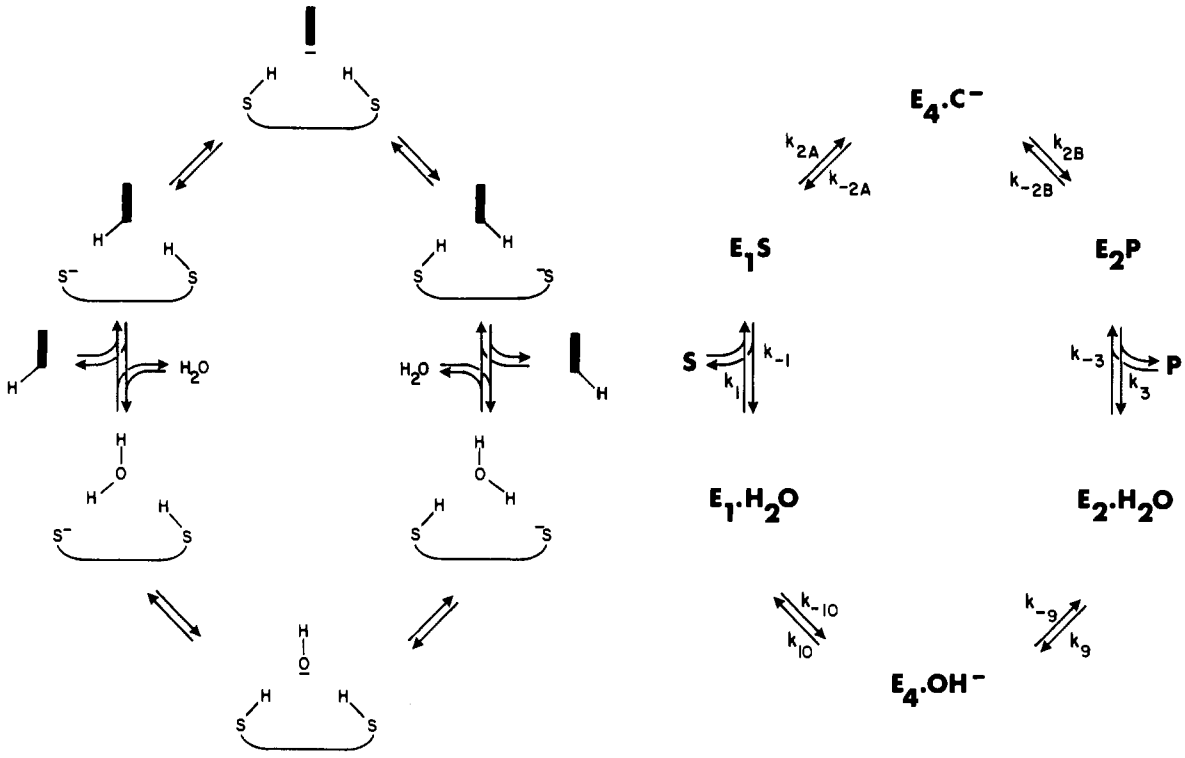


FIGURE 1: Mechanistic pathway for the proline racemase catalyzed reaction. The two catalytic thiols of the enzyme are shown, and the substrate is illustrated edge on with a heavy bar. The rate constants and symbols for intermediates are shown in the adjoining scheme.

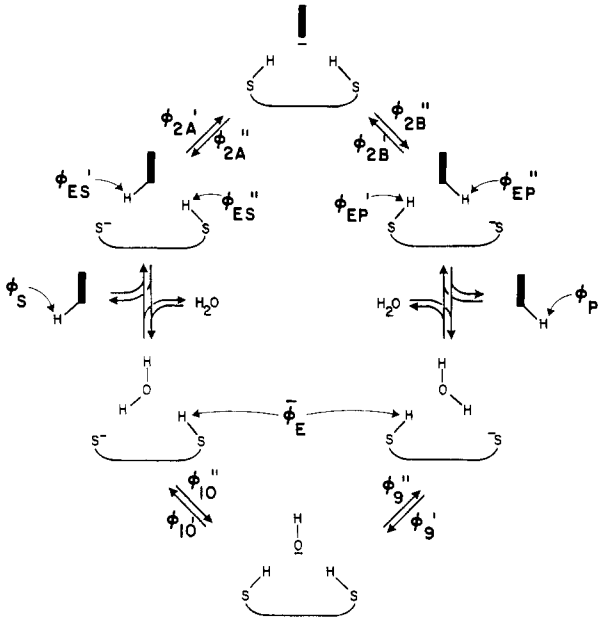


FIGURE 2: Fractionation factors for the single- and double-primed sites of proline racemase.

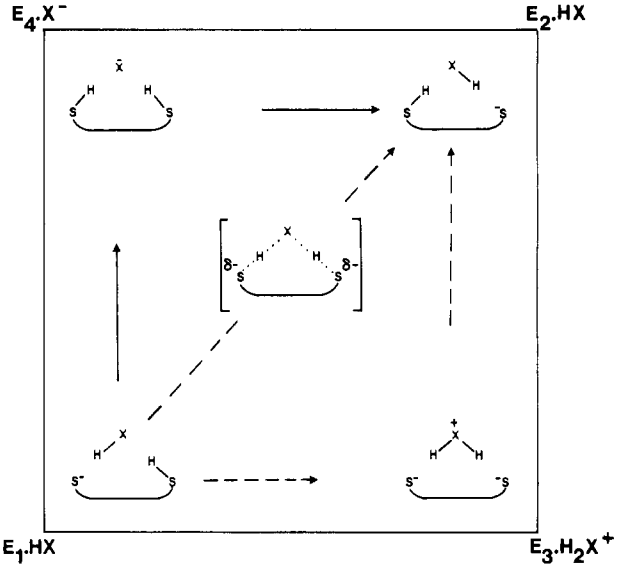


FIGURE 3: Map showing the three routes for the interconversion of enzyme forms E_1 and E_2 mediated by HX: the *acid*-catalyzed route via the intermediate $E_4 \cdot X^-$, the *base*-catalyzed route via the intermediate $E_3 \cdot H_2X^+$, and the concerted route across the diagonal.

preferred one. First, the striking similarity in the fractionation factors for the proline- and the water-mediated interconversions is better accommodated by the stepwise route via the top left-hand corner of Figure 3 than by the concerted route across the diagonal. This is because the relative free energies of the $E_3 \cdot H_2X^+$ and $E_4 \cdot X^-$ corners of Figure 3 are very different when HX is proline and when HX is water. For water, $E_3 \cdot H_3O^+$ and $E_4 \cdot OH^-$ will have roughly the same free energy, and we could accept a lowest energy route between E_1 and E_2 across the diagonal. For proline, however, the fact that H_2X^+ would be pentacovalent means that the $E_3 \cdot H_2X^+$ corner will be of *very* much higher free energy than the $E_4 \cdot X^-$ corner, and the lowest energy route from E_1 to E_2 would inevitably pass close to the $E_4 \cdot X^-$ corner (Bruice, 1976). Since it would be very unlikely that two transition states located at very different points on the map (one close to the center and one close to the $E_4 \cdot X^-$ corner) would have such similar fractionation factors, it is clearly preferable to propose that the transition states for *both* the proline- and the water-mediated reactions are close to the E_4 corner.

Second, when a Brønsted plot for the catalyzed enzyme interconversion is constructed (Belasco et al., 1986c), we find a slope of 0.5, which is the expected slope for reactions between bases of roughly equal strength ($pK_a \sim 6$–9), and is consistent with the route through $E_4 \cdot X^-$ (Figure 3). A concerted pathway would have a much lower slope (Grunwald & Meiboom, 1963;

Albery, 1967; Hegarty & Jencks, 1975). On this basis, then, we must prefer the stepwise route where the substrate (or water) acts as an acid. This preference is reinforced by the fact that the absolute values of the rate constants for the catalyzed and the uncatalyzed enzyme interconversion can be explained by reaction through $E_4 \cdot X^-$ (Belasco et al., 1986c).

Third, in addition to the above arguments, the absolute values of the fractionation factors also agree with enzyme interconversion via $E_4 \cdot X^-$. For both the substrate-mediated and the water-mediated enzyme interconversion, the transition states in the $E_4 \cdot X^-$ route must lie close in structure and free energy to the unstable intermediates, $E_4 \cdot C^-$ (for the substrate-mediated reaction) and $E_4 \cdot OH^-$ (for the water-mediated conversion). At each transition state, one proton is bound to one of the two enzyme thiols with the fractionation factor that we have observed in the enzyme–substrate complexes of 0.55 (Table II). Knowing the observed mixed transition-state fractionation factors ($\phi_{2A,2B}'$ and $\phi_{2A,2B}''$), we can calculate the factor for the other (in-flight) proton (ϕ_{2A}' and ϕ_{2B}''). These values, listed in Table II, vary between 0.29 and 0.37 and are reasonable for a transition state in which the in-flight proton is quite close to the thiol. In contrast, we should expect that a single concerted transition state would have individual fractionation factors of 0.5 or less at each site, giving an overall product for the two sites of less than 0.25. This does not agree with the observed factors, which are between 0.38 and 0.44 (Table II). Precisely analogous arguments apply to the water-mediated reaction, through transition states 9 and 10. Finally, we have shown that the solvent isotope effect results are also accommodated by the route through $E_4 \cdot X^-$ (Belasco et al., 1986c).

In summary, the following facts support the view that the substrate interconversion reaction proceeds via an intermediate in which the proline carbanion is bound to the diprotonated form of the enzyme E_4 and that, analogously, the water-mediated interconversion of enzyme forms goes through the unstable $E_4 \cdot OH^-$ intermediate: (i) The value of the Brønsted slope for the catalyzed interconversion of E_1 and E_2 (Belasco et al., 1986c); (ii) the absolute values of the rate constants for enzyme interconversion by water and by other acid catalytsts; (iii) the values of the kinetic isotope effects for the transferring protons (including those probed by the solvent isotope effect); (iv) the close similarity between the fractionation factors for the substrate-mediated and the water-mediated interconversion of enzyme forms; (v) the fact that when an isotopic label is lost from E_2', only E_1 (and no E_2) is produced, and analogously, only E_2 is formed from E_1'' (Belasco et al., 1986c); (vi) the results of the double fractionation experiment (Belasco et al., 1986b).

Now, it can be argued that both $E_4 \cdot C^-$ and $E_4 \cdot OH^-$ are species of such high free energy and their lifetimes so short that it is unhelpful to refer to them as reaction intermediates. It would indeed be difficult to distinguish between a single transition state toward the top left-hand corner of Figure 3 and a very short-lived intermediate actually *at* the corner, and it is probably pointless to worry about whether there is a small dip in the free energy profile or not.[3] What is important, however, is that the discussion above has shown that the lowest energy path does go close to the $E_4 \cdot X^-$ corner and does not cut diagonally across Figure 3 in a synchronous reaction. It is evident that even if there is no discernible $E_4 \cdot X^-$ intermediate, the highest point on this part of the free energy profile contains a bound species that is close to being anionic (i.e., a substrate carbanion, or OH^-), with each of the two relevant protons lying close to the flanking sulfur centers.

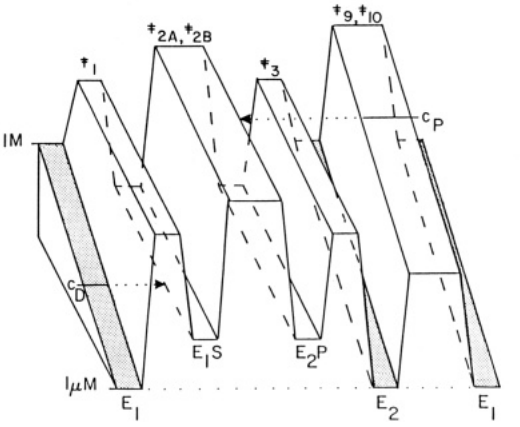


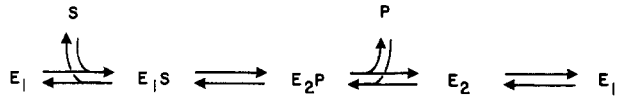
FIGURE 4: Free energy profile for proline racemase (calculated from the results in Table I) as a function of substrate concentration. At the front of the diagram ([S] = [P] = 1 μM) the system is unsaturated, and the rate-limiting free energy difference is between E_1 and ‡2. At the back of the diagram ([S] = [P] = 1 M) the system is oversaturated, and the rate-limiting free energy difference is between E_1S or E_2P and ‡9 or ‡10. Between the dip-switch and peak-switch concentrations (c_D and c_P) the system is bound state saturated with the rate-limiting free energy difference between E_1S and ‡2.

Free Energy Profile for Proline Racemase. From the rate constants assembled in Table I, we may construct the free energy profile for the proline racemase reaction. This is shown in Figure 4, in which the consequence of varying the substrate concentration is shown in the third dimension. The profile shows the "dip-switch concentration" (Fisher et al., 1986a,b) where the free energies of the free enzyme forms E_1 and E_2 match those of the liganded states E_1S and E_2P. At yet higher substrate levels, we come to the "peak-switch concentration" where the free energy of the highest transition state of steps 1–3 (the upper route of Figure 1) matches that of the highest transition state of steps 9 and 10 (the lower path of Figure 1). It is these defined concentrations that divide the behavior of the enzyme into the three regimes: unsaturated, saturated, and oversaturated. In earlier papers (Fisher et al., 1986a,b) we have developed the methods for finding these behavioral boundaries and have shown for proline racemase that, under reversible conditions, interconversion of the two free enzyme forms indeed becomes rate limiting in the oversaturated region.

In general, we know that saturated behavior for an enzyme (where the reaction rate is independent of substrate concentration) may arise when the slowest step is the conversion of an enzyme–substrate complex to a transition state also involving liganded enzyme. This is the classical explanation of the saturation phenomenon, which we may call bound state saturated. Alternatively, however, precisely the same steady-state behavior will be seen for the entirely different situation in which the rate of reaction becomes independent of substrate concentration because the rate-limiting transition state is that for the interconversion of the two forms of free enzyme. This is called free state saturated. We have emphasized (Albery & Knowles, 1986a; Fisher et al., 1986b) the importance of the tracer perturbation method, originally developed by Britton and his collaborators (Britton, 1966, 1973), in distinguishing between these two types of saturation behavior. This approach has shown that proline racemase shows bound-state saturation behavior, in which the dip switch

[3] If there is no intermediate, the fractionation factors $\phi_{2A,2B}'$, $\phi_{2A,2B}''$, $\phi_{9,10}'$, and $\phi_{9,10}''$ are no longer mixed factors but relate simply to the fractionation of the appropriate proton in the single transition state. The actual values of these factors (Table II) are, of course, quite consistent with the protons being largely bound to the enzyme thiol groups in such a transition state.

Scheme I: Minimal Scheme for an Enzyme Catalyzing the Interconversion of a Single Substrate and a Single Product



concentration is lower than the peak switch concentration (see Figure 4).

Importance of Oversaturation for Enzymes Working under Reversible Conditions. The state of an enzyme as it releases its product is necessarily different from the state that binds substrate. While this difference may not be large and may only involve the protonation state of catalytic residues or their precise position, the fact that products are chemically different from substrates demands differences in the forms of enzyme that accept them. Even for isomerases or racemases where substrates and products are very similar to one another, small but real differences in the nature of the enzyme at the beginning and the end of the reaction are inevitable. Strictly, therefore, we should always represent an enzyme-catalyzed reaction minimally as shown in Scheme I. Such a representation may seem merely pedantic, since if the rate of interconversion of the two enzyme forms E_1 and E_2 is faster than that of all other steps, the free enzyme is just a rapidly equilibrating pool of species the interconversion of which is kinetically insignificant. This has certainly been the prevailing view, and it has normally been assumed, for example, that the rates of protonation and deprotonation of acids and bases on an enzyme are indeed much faster than the actual catalytic steps of the reaction. Our work on proline racemase shows that this assumption can be false. When the interconversion of free enzyme forms is rate limiting, one would never discover this from steady-state kinetics under irreversible conditions (that is, where the product concentration is very low, either because only the first few percent of the reaction is monitored or because a coupling enzyme is used). Classical saturation behavior with increasing substrate concentration would still be observed, but V_{max} would no longer represent the reaction of E_1S to E_2 + P ("bound state saturated") but would represent reaction of E_2 to E_1 ("free state saturated"). To limit kinetic investigation, therefore, to reactions under irreversible conditions can be seriously misleading, in the sense that the V_{max} may measure a process that has nothing to do with the reaction of substrate to product. If, in contrast, reactions are studied under reversible conditions where the substrate and product are present at concentrations near their equilibrium values, we may not only evaluate the nature of V_{max} at saturation, but also encounter a new regime, *over*saturation, in which the reaction rate is limited by the reaction of E_1S and E_2P over the transition state between E_2 and E_1. It should, moreover, be emphasized that the majority of enzymes operate in vivo under reversible conditions: only those enzymes that are control points in metabolic pathways experience substrate and product levels that are very different from their equilibrium values (Albery & Knowles, 1977).

It is appropriate, therefore, to explore the broader implications of catalysis under reversible conditions and to delineate the zones where the different types of behavior (unsaturated, saturated, and oversaturated) dominate the kinetic behavior. To explore the behavior of a system as a function of substrate and product concentrations (s and p, respectively), we construct a "case diagram" (Albery & Knowles, 1986b) for proline racemase. The diagram, shown in Figure 5, depicts the different types of behavior as a function of log (s) and log (p). The diagram is divided by a 45° line, along which $p = s$, and the system is at thermodynamic equilibrium and exhibits reversible behavior. To the southeast of the line, $p < s$, and there is an irreversible flux in the S to P direction, while to the northwest of the line, $p > s$, and there is an irreversible flux in the P to S direction. On the 45° line there will be the peak switch (c_P) and dip switch (c_D) concentrations (Fisher et al., 1986a), which mark the boundaries between the unsaturated, saturated, and oversaturated regions for the reversible system.

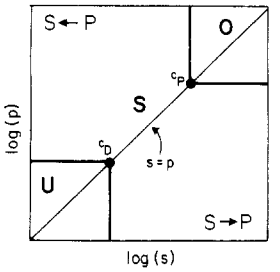


FIGURE 5: Case diagram for proline racemase. The type of behavior, unsaturated, saturated, or oversaturated, is shown by the heavy letters U, S and O, respectively. These regions are separated by the dip switch (c_D) and peak switch (c_P) concentrations, respectively.

In the southwestern corner of Figure 5, where the concentration of S and P are both low, we have the unsaturated region. In the northeastern corner, where the concentrations of both S and P are both high, we have the oversaturated region. These regions are separated from the saturated region by the lines passing through c_D and c_P, as shown in Figure 5. From this figure, it is clear how much kinetic information is lost if an enzyme-catalyzed reaction is only studied under irreversible conditions. Oversaturation is never seen, since under these conditions one is limited to experiments along either the bottom horizontal axis (S-to-P direction, with $p \rightarrow 0$) or the left-hand vertical axis of Figure 5 (P-to-S direction, with $s \rightarrow 0$). Only by an investigation along the $p = s$ diagonal does one evaluate the kinetic behavior under the reversible conditions that pertain to the functioning of most enzyme systems in vivo. Because of the symmetry of the system, the case diagram for proline racemase is particularly simple. A more extensive treatment of the consequences of oversaturation, and a discussion of case diagrams for enzymes for which the overall equilibrium constant is not unity, will be found in Albery and Knowles (1986b).

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REFERENCES

Albery, W. J. (1967) *Prog. React. Kinet. 4*, 355–398.

Albery, W. J., & Knowles, J. R. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 285–293.

Albery, W. J., & Knowles, J. R. (1986a) (submitted for publication).

Albery, W. J., & Knowles, J. R. (1986b) (submitted for publication).

Belasco, J. G., Albery, W. J., & Knowles, J. R. (1986a) *Biochemistry* (fourth paper of seven in this issue).

Belasco, J. G., Bruice, T. M., Albery, W. J., & Knowles, J. R. (1986b) *Biochemistry* (fifth paper of seven in this issue).

Belasco, J. G., Bruice, T. M., Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986c) *Biochemistry* (preceding paper in this issue).

Britton, H. G. (1966) *Arch. Biochem. Biophys. 117*, 167–183.

Britton, H. G. (1973) *Biochem. J. 133*, 255–261.
Bruice, T. C. (1976) *Annu. Rev. Biochem. 45*, 331–373.
Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970–3978.
Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986a) *Biochemistry* (first paper of seven in this issue).
Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986b) *Biochemistry* (second paper of seven in this issue).
Fisher, L. M., Belasco, J. G., Bruice, T. W., Albery, W. J., & Knowles, J. R. (1986c) *Biochemistry* (third paper of seven in this issue).
Grunwald, E., & Meiboom, S. (1963) *J. Am. Chem. Soc. 85*, 2047–2052.
Hammes, G. G., & Schimmel, P. R. (1970) *Enzymes* (3rd Ed.) *2*, 67–114.
Hegarty, A. F., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 7188–7189.
Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105–111.
More O'Ferrall, R. A. (1970) *J. Chem. Soc. B*, 274–277.
Rudnick, G., & Abeles, R. H. (1975) *Biochemistry 14*, 4515–4522.

Effects of Lectin Activation on Sialyltransferase Activities in Human Lymphocytes†

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ABSTRACT: The effects of phytohemagglutinin (PHA) stimulation on the activities of sialyltransferase 1 (SAT-1), and sialyltransferase 3 (SAT-3), in human lymphocytes were investigated in vitro. For SAT-1 and SAT-3, respectively, the apparent K_m values with variable CMP-NeuAc concentrations were 0.19 and 0.015 mM and with variable LacCer were 0.075 and 0.17 mM. Progressive increases in the activities of SAT-1 and SAT-3 were detected in lymphocytes stimulated with PHA, whereas no increase was observed in control lymphocytes incubated in culture medium alone. These increased activities occurred within 18–36 h of incubation and preceded optimum lymphocyte proliferation. Intact lymphocytes were needed for the lectin-stimulated increase of sialyltransferase activities because neither concanavalin A nor phytohemagglutinin added to the broken cell preparation modulated SAT-1 activity. The glycolipid products formed as a result of these enzymatic reactions in the presence of endogenous and exogenous acceptors were tentatively identified by thin-layer chromatography and autofluorography. The addition of exogenous LacCer to the SAT-1 assay resulted in the radiolabeling of a small amount of ganglioside GM1b (3.4%), but GM3 was the major labeled product (96%). When $GgOse_4Cer$ was added to the SAT-3 assay, 32% GM3 and 24.6% GM1b were detected while 44% consisted of glycolipids not labeled in assays performed without exogenous acceptors. Of the radioactivity transferred to endogenous acceptors, 81.3% was in GM3 and 14.6% in GM1b. These results demonstrate that the modulation of sialyltransferase activity occurs earlier than cellular activation.

Both glycolipids and proteins exist in sialylated and nonsialylated forms, and the degree of sialylation of glycolipids may be of biological importance (Hakomori, 1981). Gangliosides are sialylated glycolipids that have been implicated generally in the regulation of cell division and specifically in cell surface contact inhibition of proliferation (Roseman, 1970; Hakomori, 1984). We previously reported that lectin stimulation of human peripheral blood lymphocytes results in a 9-fold increase of radiolabeled precursor incorporation into gangliosides of these cells (Yates et al., 1980). Although this finding indirectly suggests that the de novo synthesis of sialylated glycolipids is probably linked to cell activation, it is possible that the apparent sialyltransferase alteration could be explained by the altered cellular transport of the radiolabeled precursor glucosamine. One strategy to assess directly whether the sialylation of cell membrane constituents might be linked to cellular activation and division is to determine if sialyltransferase activities are modulated during cell proliferation.

The major ganglioside of human lymphocytes is GM3[1] with IV^3nLc_4 occurring in lesser amounts (Macher et al., 1981). The biosynthesis of GM3 is dependent on sialyltransferase 1 (SAT-1), and IV^3nLc_4 synthesis requires sialyltransferase 3 (SAT-3) activity (Basu & Basu, 1982; Basu et al., 1982). The experiments in this paper first determined the activities of these two sialyltransferases in normal human lymphocytes. Then, the activation and division of human lymphocytes triggered

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[1] Abbreviations: SAT-1, CMP-NeuAc:LacCer α-sialyltransferase; SAT-3, CMP-NeuAc:$nLcOse_4Cer$ α-sialyltransferase; GM3, sialyl(α2–3)lactosylceramide; GM1, sialyl(α2–3)gangliotetraosylceramide or $II^3NeuAcGg_4$; GM1b, sialyl(α2–3)gangliotetraosylceramide or $IV^3NeuAcGg_4$; IV^3nLc_4, sialyl(α2–3)neolactotetraosylceramide; LacCer, lactosylceramide; $GgOse_4Cer$, gangliotetraosylceramide; $nLcOse_4Cer$, neolactotetraosylceramide; NeuAc, *N*-acetylneuraminic acid; PBMC, peripheral blood mononuclear cells; SBSS, Seligman's balance salt solution; PHA, phytohemagglutinin; Con A, concanavalin A; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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