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Energetics of Proline Racemase: Fractionation Factors for the Essential Catalytic Groups in the Enzyme–Substrate Complexes†

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ABSTRACT: The fractionation factors of protons bound to the essential catalytic groups in proline racemase have been determined by comparison of the time courses of two competitive deuterium washout experiments. The rate of achievement of the maximum perturbation in the optical rotation has been measured in the oversaturated region (that is, at high substrate concentrations) under two conditions: in the first, we start with an equimolar mixture of deuterated substrate S′ and of unlabeled product P; in the second, we again start with equal concentrations of substrate and product, but the concentration of the deuterated material S′ is less than 20% that of S. The different concentrations of deuterated substrate produce different levels of deuteration of the enzyme's catalytic groups, the kinetic consequence of which allow the fractionation factors of these enzymic groups to be determined. The observed values for the fractionation factors of the enzyme's groups of 0.55 ± 0.1 are only consistent with these groups' being thiols. This conclusion is supported by results of measurements of the solvent isotope effect determined in the unsaturated regime. These findings confirm the earlier suggestion of Abeles and his group that two cysteine residues mediate the catalysis of proline racemization by this enzyme.

As is discussed in the preceding paper (Belasco et al., 1986a), to make further progress in elucidating the mechanism of proline racemase, we need to define the nature of the essential catalytic groups of the enzyme. Rudnick and Abeles (1975) have presented evidence on the basis of protein modification studies to suggest that both of these groups are thiols. In this paper we report measurements of the fractionation factors of protons bound to the essential catalytic groups in the enzyme–substrate complexes and show that these factors lie in the range 0.45–0.65. Of the possible catalytic functionalities in proteins, only thiols have such low fractionation factors (Schowen, 1977; Szawelski et al., 1982), and the experiments reported here provide evidence for the *actual involvement* of thiol groups (as distinct from their mere existence at the active site) in the proton abstraction and delivery steps mediated by proline racemase.

In general, it is impossible to measure the fractionation factor of a reaction intermediate that is present in insignificant proportion at the steady state, since fractionation in such an intermediate has no effect either on the overall rate of the reaction or on the distribution of products. However, the fractionation factors for intermediates that are present in kinetically significant proportions may be found by perturbing the relative stabilities of such intermediates by deuterium substitution. While in principle we could effect the required perturbation by measuring the reaction rate in D_2O, this would be undesirable because we cannot be certain, a priori, that the change in solvent will have no other effects on the rate of the enzyme-catalyzed reaction, for example, by subtly affecting the protein conformation or the pK_a values of catalytic groups. It is preferable, therefore, to introduce deuterium onto the enzyme's catalytic groups by using a labeled *substrate*, and we may then look for the effects of such deuteration on the rate of the reaction in unlabeled water. In the case of proline racemase, we may, by working at very high substrate concentrations, observe the net reaction in the oversaturated region which involves reaction from an equilibrated pool of liganded enzyme (E_1S and E_2P: see Figure 1) over a transition state describing the interconversion of the two free forms of the enzyme, E_2 and E_1. To observe fractionation on the enzyme, we must deuterate the enzyme's catalytic groups in the $E_1S \rightleftharpoons E_2P$ pool. To cancel the concomitant effects of deuteration on the rate-limiting transition state for enzyme interconversion so that attention can be focused only on fractionation in the enzyme–substrate complexes, we compare the time courses of two "competitive deuterium washout" experiments.

Competitive deuterium washout experiments are followed by monitoring the optical rotation changes that occur when enzyme is added to an equimolar mixture of one unlabeled substrate and its deuterated enantiomer (Fisher et al., 1986a). In one of the experiments—the "nontracer" experiment—we start with an equimolar mixture of unlabeled product P and

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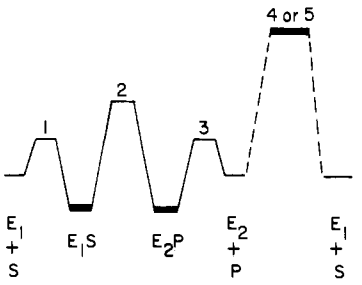
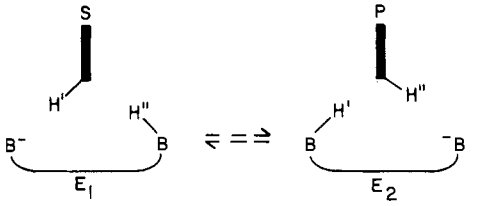


FIGURE 1: Energetics of proline racemase in the oversaturated region. The heavy lines show how the overall rate of the reaction in the oversaturated region is governed by the conversion of E_1S and/or E_2P to transition state 4 or 5 for the interconversion of the free forms of the enzyme, E_2 and E_1.

of deuterated substrate S′. In the other experiment—the "tracer" experiment—we start with equal concentrations of P and of S + S′, where the concentration of S′ is less than 20% of the concentration of S. In both cases, the optical rotation |OR| begins[1] at zero, but because the deuterated S′ is converted to P more slowly than the unlabeled P is converted to S, |OR| rises. As the reaction proceeds, the deuterium label washes out into the solvent, and |OR| reaches a maximum and then falls back to zero as all the deuterium is lost to the medium and chemical equilibrium is reestablished. If the fractionation factors for the enzyme–substrate and enzyme–product complexes differ significantly from the factor for free substrate (ϕ_S), the proportion of enzyme in different forms (both free and bound) will be different in the tracer and the nontracer experiments. This will lead to the time course for the two experiments being different. In other language, if there is a significant equilibrium isotope effect for the transfer of a hydrogen from carbon 2 of substrate to the catalytic base of the enzyme, then the time course of the deuterium washout experiment will depend upon the proportion of the substrate (and therefore of the enzyme) that is deuterated. To obtain the maximum perturbation in the proportions of reaction intermediates by the use of deuterated substrate, we work in the oversaturated region, where the overall rate of reaction is determined by the reaction of the enzyme–substrate complexes E_1S and/or E_2P over the transition state(s) for the interconversion of the two forms of the free enzyme, E_2 and E_1 (see Figure 1). Under these conditions, all the enzyme species are part of a single equilibrated pool in which the free enzyme forms are less stable than those containing bound substrates (see Figure 1). That the time courses of the tracer and nontracer experiments are different in practice is illustrated in Figures 2 and 3, where the results for two such experiments are presented. In order to compare the two experiments directly, the optical rotation axis has been scaled to take into account the different starting levels of the perturbing deuterated substrate, S′. It is immediately evident from Figures 2 and 3 that while the *size* of the maximum value of the optical rotation is directly proportional to the starting concentration of S′, the *time* taken for the system to reach that maximum is different. The reasons for this behavior are outlined in what follows, and the derivation of the fractionation factors for the catalytic groups of the enzyme is presented under Results and Discussion.

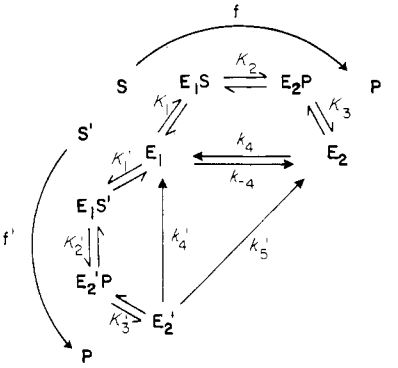
[1] In the S′ → P direction, where S is L-proline and P is D-proline, the optical rotation passes through a minimum, while in the P″ → S experiment the rotation passes through a maximum. In order to use the same language for both experiments, therefore, the results in this paper are reported and discussed in terms of |OR|, where OR is the optical rotation.

Scheme I: Protonic Sites for the Proline Racemase System[a]



[a] S is L-proline, P is D-proline, and E_1 and E_2 are the two forms of the enzyme (see footnote 2).

Scheme II: Competitive Deuterium Washout Experiment Starting with S′ and P[a]



[a] The fluxes of material through the two pathways are shown by f and f'. k_n are rate constants, and K_n are equilibrium constants.

THEORY

In a competitive deuterium washout experiment, the isotopic label in the deuterated substrate S′ is lost to the solvent after its transfer to the enzyme, E_2' (see Schemes I and II). The isotope in E_2' can in principle be washed out either in a "crossover" process that generates E_1 or in a direct exchange reaction that produces E_2. This distinction is made in the following paper (Belasco et al., 1986b), and for present purposes we define a crossover parameter, x, which, for the singly primed site[2] in E_2', is

$$x = k_4'/(k_4' + k_5') \tag{1}$$

When $x = 1$ the isotope is washed out with crossover, and E_2' only goes to E_1. When $x = 0$, the isotope is lost by direct exchange to give E_2. [Strictly, loss of isotope from the singly primed site in E_2' is described by x' and from the doubly primed site in E_1'' is described by x''. For the purposes of this paper, however, we may omit the primes: the distinction between the two x parameters is not required until the following paper (Belasco et al., 1986b).]

Using the notation developed earlier (Fisher et al., 1986b), we can write for the flux f_4 through transition state 4:

$$f_4 = k_4e_2 - k_{-4}e_1$$

$$f_4 = K_{1,2,3}k_4e_1(s/p) - k_{-4}e_1$$

$$= k_{-4}e_1(s/p - 1) \tag{2}$$

since for a racemase, $K_{1,2,3,4} = 1.0$.

[2] It should be remembered that the two protonic sites are specified by a single or a double prime (see Scheme I). The fractionation factor of the 2-proton of free L-proline is ϕ_S and that of the substrate proton when L-proline is bound to the enzyme is ϕ_{ES}'. Analogously, the 2-proton of D-proline has the fractionation factor ϕ_P (for the free substrate) and ϕ_{EP}'' (for the enzyme-bound substrate). The enzyme's catalytic site in E_2 that bears the proton is the singly primed site, and fractionation at this site in the free enzyme is given by ϕ_E'. Fractionation at the protonated site in E_1 is expressed by ϕ_E''. Correspondingly, fractionation at the enzyme catalytic site in the E_2P complex is expressed by ϕ_{EP}' and in the E_1S complex by ϕ_{ES}''.

For f', the flux describing the washout of isotope, we can write

$$f' = -ds'/dt = (k_4' + k_5')e_2' = K_{1,2,3}'(k_4' + k_5')e_1(s'/p) = K_{1,2,3}'k_4'e_1[s'/(px)] \quad (3)$$

Summing the fluxes at E_2 (or at E_1), we obtain

$$f_4 = f + (1 - x)f' \quad (4)$$

where f is the flux through E_1S and E_2P. Elimination of f_4 and e_1 from eq 2–4 gives

$$\frac{f}{f'} = \frac{R}{2}\frac{(s - p)}{s'} - (1 - x) \quad (5)$$

where

$$R = 2x\phi_S/\phi_4 \quad (6)$$

[Strictly, we should use R' when S' is the perturbing substrate and R'' when P'' is the perturbing substrate. As with x, however, we omit the primes in this paper, since this distinction is not required until the following paper (Belasco et al., 1986b).]

Now we consider the competitive deuterium washout experiment where there is only a tracer amount of S′, and the starting conditions are

$$s_0' + s_0 = p_0$$

Equation 5 is similar to eq 11 of Fisher et al. (1986a), and we can follow the same arguments that were used to derive eq 23 of that paper to obtain

$$d\sigma/d\sigma' = R(\sigma/\sigma') + 1 - 2x$$

where

$$\sigma = (p - s)/s_0'$$

$$\sigma' = s'/s_0'$$

Integration then gives

$$\sigma' - \sigma = \frac{R - 2x}{R - 1}[\sigma' - (\sigma')^R] \quad (7)$$

where

$$\sigma' - \sigma = (s' + s - p)/s_0' \quad (8)$$

For a tracer experiment where the concentration of S′ is much less than that of S or P, the concentrations of E_1 and P in eq 3 are effectively clamped, and we can integrate eq 3 to obtain

$$\sigma' = \exp(-k_F't) \quad (9)$$

where

$$k_F' = K_{1,2,3}'k_4'e_1/(px)$$

Substitution of eq 9 into eq 7 shows that the time course of the perturbation in the optical rotation (which is proportional to $\sigma' - \sigma$) for such a tracer experiment is given by the difference between the two exponential terms:

$$\sigma' - \sigma = \frac{R - 2x}{R - 1}[\exp(-k_F't) - \exp(-Rk_F't)] \quad (10)$$

We emphasize that this double-exponential form is only found for the tracer experiment. The nontracer experiment has a more complicated time course. Differentiation of eq 7 gives the following relationship for the isotopic content at the perturbation maximum (σ_{max}') and the size of the maximum $(\sigma' - \sigma)_{max}$ as

$$\sigma_{max}' = R^{1/(1-R)} \quad (11)$$

and

$$(\sigma' - \sigma)_{max} = (R - 2x)R^{R/(1-R)} \quad (12)$$

These two relations hold for both tracer and nontracer experiments. It can be noted that eq 11 is the same as eq 25 of Fisher et al. (1986a), but here, R has the appropriate definition (eq 6) for the oversaturated region. By measuring both σ_{max}' and $(\sigma' - \sigma)_{max}$, we can obtain both R and x and thus discover (from x) how the isotopic label is lost from E_2'. This is done in the following paper (Belasco et al., 1986b).

Size of Perturbation Maximum. At the start of any competitive deuterium washout experiment, $s' = p - s$, both for the tracer experiment ($s_0' < s_0$) and for the nontracer experiment ($s_0 = 0$, $s_0' = p_0$). The size of the perturbation in the optical rotation is determined only by the relative rates of P to S and of S′ to P (f/f'), which, from eq 5 and 6, depends on the amount of the deuterated substrate S′, on the kinetic isotope effect given by ϕ_4/ϕ_S, and on the extent of crossover, x. From Scheme II one can see that the isotope starts in S′ with a fractionation factor ϕ_S and, for the case where $x = 1$, is lost in transition state 4 with a fractionation factor ϕ_4. Furthermore, from eq 12, the *size* of the perturbation is not affected by any fractionation in E_1S' or $E_2'P$. This is because, in a competitive experiment, the relative rates of the S′-to-P conversion (f') and of the P-to-S conversion (f) are not affected by changes in the stabilities of the species in the equilibrated enzyme pool. The magnitude of the perturbation is determined *only* by how S and S′ partition out of the enzyme pool, and similarly to those systems that obey the Curtin–Hammett principle, this ratio is insensitive to any ground-state effects that might influence the equilibria among different forms of the enzyme. The size of the perturbation maximum is therefore simply proportional to the concentration of the labeled substrate.

Rate of Achievement of the Perturbation Maximum. Whereas the size of the perturbation maximum is independent of the relative stability of the different forms of the enzyme, the rate of achievement of the maximum is not. Consider the very early stages of a perturbation experiment in which proline racemase is added to an equimolar mixture of S and P at oversaturating concentrations where some (the tracer experiment) or all (the nontracer experiment) of S is deuterated. The development of the perturbation will be given by $f_0 - f_0'$, where f_0 describes the initial flux of P and S, and f_0' describes the initial flux of S′ to P. Since at the start of the perturbation $s - p = -s'$, we find from eq 5 that for both the tracer experiment and the nontracer experiment

$$f_0 - f_0' = f_0'(-R/2 - 2 + x) \quad (13)$$

We now compare the development of the perturbation at time zero in the tracer (T) and nontracer (NT) experiments and normalize the fluxes for each experiment by the initial labeled substrate concentration, s_0', to obtain (using eq 13) the ratio

$$r = \frac{[(f_0 - f_0')/s_0']_T}{[(f_0 - f_0')/s_0']_{NT}} = \frac{(f_0'/s_0')_T}{(f_0'/s_0')_{NT}} \quad (14)$$

Because f_0/f_0' is the same for both the tracer and nontracer experiments, the ratio of the rates of the development of the perturbation for the two experiments (r) depends simply on the ratio of the normalized rates for the washout of the isotope from the labeled substrate.

In Figure 2 we illustrate the free energy profile for both the hydrogen and deuterium systems under oversaturating conditions where the rate-limiting transition states for the net turnover of substrate are those (labeled 4) involving the interconversion of unliganded enzyme forms on the right- and left-hand sides. It is clear from Figure 2 that the conversion

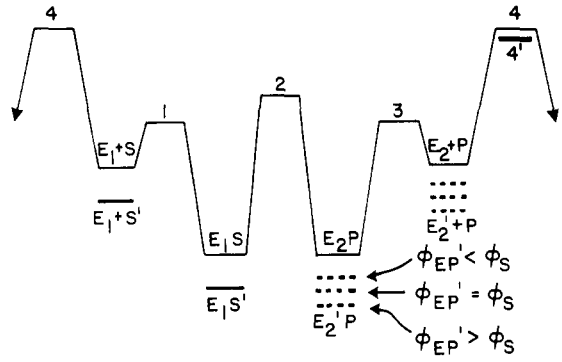


FIGURE 2: Energetics of proline racemase in the oversaturated region with deuterated and undeuterated substrate. The thin line represents the reaction of unlabeled substrate, from an equilibrated pool of liganded enzyme (E_1S and E_2P) over the transition state (4) for the interconversion of E_1 and E_2. The heavy lines represent the free energies of deuterated species, which react from an equilibrated pool of liganded enzyme (E_1S' and $E_2'P$: three possible cases for the fractionation in $E_2'P$ are shown) over the transition state (4') for the conversion of E_2' to E_1. All free energy differences are exaggerated for clarity.

of E_1 to E_2 (over the left-hand transition state 4) is accompanied by the net conversion of P to S, and the conversion of E_2' to E_1 (over the right-hand transition state 4') is accompanied by the net conversion of S' to P. All the enzyme species between the two flanking transition states are in equilibrium with one another. In particular, the mixing of the two pools of liganded enzyme species, $E_1S + E_2P$ (the protonated pool) and $E_1S' + E_2'P$ (the deuterated pool), will take place through E_1 according to

$$E_1S + S' \rightleftharpoons E_1S' + S \tag{15}$$

Since the values of f_0' are proportional to the amounts of enzyme in the deuterated pool (i.e., $E_1S' + E_2'P$), eq 14 becomes

$$r = \frac{[(e_1s' + e_2'p)/s'_0]_T}{[(e_1s' + e_2'p)/s_0']_{NT}} \tag{16}$$

That is, the ratio of the rates of achievement of the perturbation maxima is equal to the relative size of the deuterated pool in the tracer and nontracer experiments, scaled by the initial concentrations of labeled substrate in each instance.

Now, at the start of the *nontracer* experiment, the concentration of S is zero, and (from eq 15) all the enzyme is in the deuterated pool ($E_1S' + E_2'P$), and so

$$(e_1s' + e_2'p)_{NT} = e_\Sigma \tag{17}$$

where e_Σ is the total enzyme concentration. In contrast, in the *tracer* experiment, nearly all the enzyme is in the protonated pool ($E_1S + E_2P$). The small fraction of enzyme in the deuterated pool ($E_1S' + E_2'P$) is determined by the fraction of S that is deuterated, modified by any fractionation deriving from the fact that the deuterium is not only on the substrate in E_1S' but also on the enzyme in $E_2'P$. We can therefore write

$$(e_1s' + e_2'p)_T \approx e_\Sigma(s_0'/s_0)_T(\phi_{ES,EP}'/\phi_S) \tag{18}$$

where $\phi_{ES,EP}'$ is the mixed fractionation factor (Albery & Knowles, 1976) describing fractionation at the singly primed site[2] on the liganded enzyme species E_1S' and $E_2'P$, and

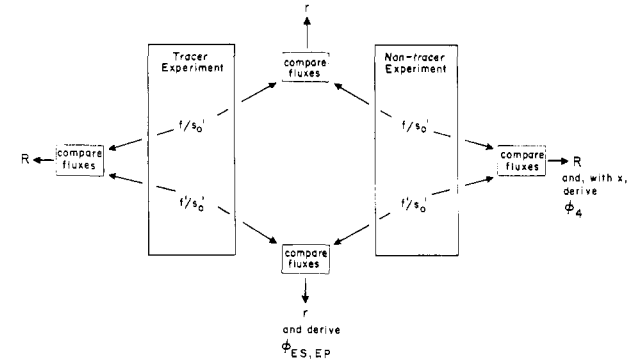
$$\phi_{ES,EP}' = (\phi_{ES}' + \phi_{EP}')/2 \tag{19}$$

We therefore find that

$$r \approx \phi_{ES,EP}'/\phi_S \tag{20}$$

In eq 18, the factor s_0'/s_0 arises from the equilibrium in eq 15, and the factor $\phi_{ES,EP}'/\phi_S$ describes the consequences of deuterium substitution. In Figure 2 we have assumed that the binding of the enzyme to S' does not affect the fractionation, so $\phi_{ES}' = \phi_S$ (this assumption is valid: see Results and Discussion). If, also, $\phi_{EP}' = \phi_S$ (that is, if there is no change in fractionation when the deuterium is transferred from substrate in E_1S' to enzyme in $E_2'P$), then $\phi_{ES,EP}' = \phi_S$, and r is unity. Under these conditions, the fraction of enzyme in the deuterated pool ($E_1S' + E_2'P$) simply matches the amount of deuterium in the substrate, and the early time courses of the tracer and nontracer experiments are identical. If, however, $\phi_{EP}' < \phi_S$, $E_2'P$ will be less stable than E_1S' (see Figure 2), and in a tracer experiment the small fraction of enzyme in the deuterated pool ($E_1S' + E_2'P$) will be *less* than that expected from the statistical ratio of s_0'/s_0, because the binding of S' to the enzyme will be energetically less favorable than the binding of S. This lower fraction means that the flux f_0' in the tracer experiment will be lower, and r will be less than 1. In effect, S will occupy a disproportionately large fraction of the enzyme and impede the turnover of S', thereby slowing the progress of the perturbation in the tracer experiment. Conversely, if $\phi_{EP}' > \phi_S$, the fraction of enzyme in the deuterated pool will be larger than the statistical ratio, r will be greater than 1, and the perturbation maximum will be reached faster in the tracer experiment.

Scheme III: Flux Comparisons and Derived Parameters from Tracer and Nontracer Competitive Deuterium Washout Experiments



The comparisons described above are illustrated in Scheme III. The size of the perturbation in either the tracer or the nontracer experiment depends upon the ratio of the fluxes of labeled and unlabeled material and provides R, from which we can obtain ϕ_4, the transition-state fractionation for the interconversion of the free forms of the enzyme. In contrast, comparison of the fluxes of either labeled or unlabeled substrate in the tracer and nontracer experiments provides r, from which we obtain $\phi_{ES,EP}$, the ground-state fractionation factor for the enzyme's catalytic groups. There are, however, two further problems we must consider. First, in the tracer experiment it is not possible in practice to achieve the condition that s_0' is negligible with respect to s_0. The initial concentration of the deuterated substrate S' has to be a significant fraction (say 0.1 to 0.2) of that of S in order to produce a perturbation that is large enough to measure accurately. Second, in deriving eq 20 we have assumed that the system is completely oversaturated, whereas in practice there are terms that describe behavior in the saturated region that, even at a total proline concentration of 400 mM, are still significant. Both of these problems can be solved by using the equations from the general treatment (Albery & Knowles, 1986), which are presented in the Appendix.

Experimental Procedures

Materials. All materials used in this work were as described earlier (Fisher et al., 1986a–c).

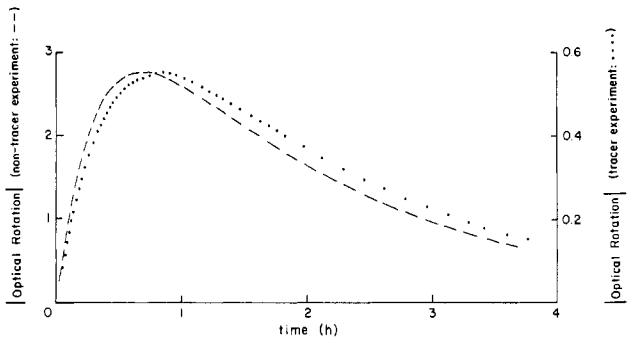


FIGURE 3: Competitive deuterium washout experiments with L-[2-^{2}H]proline in the oversaturated region. At the start of the nontracer reaction (---), the cell (at 37 °C) contained L-[2-^{2}H]proline (200 mM) and unlabeled D-proline (200 mM) in 200 mM Tris-HCl buffer, pH 8.0, containing EDTA (8 mM), 2-mercaptoethanol (20 mM), and proline racemase (~26 units). The optical rotation at 365 nm was followed. In the tracer (···) experiment, the cell contained L-[2-^{2}H]proline (40 mM), unlabeled L-proline (160 mM), and unlabeled D-proline (200 mM).

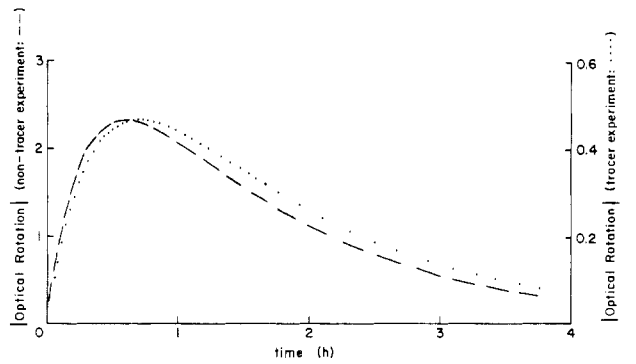


FIGURE 4: Competitive deuterium washout experiments with D-[2-^{2}H]proline in the oversaturated region. The conditions were as for the experiments in Figure 3, except that D-[2-^{2}H]proline was the labeled enantiomer, and L-proline was unlabeled. Tracer experiment (···); nontracer experiment (---).

Methods. The methods used in this work were as described by Fisher et al. (1986a–c). For the solvent isotope experiments, all solutions except enzyme were made up in either H_2O or D_2O (99.7%). The reactions were run at unlabeled L-proline concentrations of 3.03 mM (D_2O) or 3.14 mM (H_2O) in 397 mM Tricine-HCl buffer, pH 8.0, containing dithiothreitol (1 mM), (ethylenedinitrilo)tetraacetic acid (EDTA) (8 mM), and KCl (400 mM). Values for k_1 (Fisher et al., 1986a) were calculated.

Results and Discussion

Figures 3–5 show the variation of the optical rotation with time, for pairs of experiments where one experiment starts with equal initial concentrations of deuterated and protonated proline enantiomers ($s_0' = p_0$) and the other is a tracer experiment ($s_0' + s_0 = p_0$ and $s_0' < s_0$). Either L-proline is deuterated ($s_0' = p_0$: Figure 3) or D-proline is deuterated ($s_0 = p_0''$: Figure 4). In Figures 3 and 4 the total concentration of proline is 400 mM, and the enzyme is very largely *oversaturated* (Fisher et al., 1986b). Figure 5 shows a control experiment under *saturating* conditions, where the oversaturation phenomenon has been eliminated by using a lower total proline concentration in 200 mM ammonium bicarbonate buffer (Fisher et al., 1986c). In each of Figures 3–5, the optical rotation has been scaled by the concentration of the perturbing substrate (s_0' or p_0'') so as to allow a clearer visual comparison to be made between the tracer and the nontracer experiments.

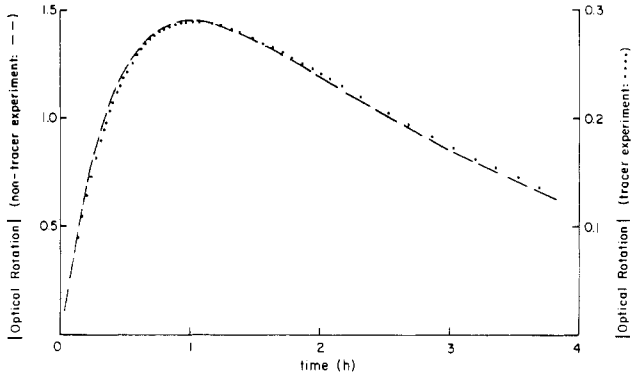


FIGURE 5: Competitive deuterium washout experiments with L-[2-^{2}H]proline in the saturated region. At the start of the nontracer (---) reaction, the cell (at 37 °C) contained L-[2-^{2}H]proline (100 mM) and unlabeled D-proline (100 mM) in 200 mM ammonium bicarbonate buffer, pH 8.0, containing EDTA (8 mM), 2-mercaptoethanol (20 mM), and proline racemase (~5 units). The optical rotation at 365 nm was followed. In the tracer (···) experiment, the cell contained L-[2-^{2}H]proline (20 mM), unlabeled L-proline (80 mM), and unlabeled D-proline (100 mM).

Consider first the results for the reaction run under *saturating* conditions. Inspection of Figure 5 shows that, within experimental error, the progress curves are identical, so $r = 1$. From eq A2 of the Appendix, we see that $\phi_{ES}'/\phi_S = 1$. That is, the fractionation factor of the bound substrate (when the proton still resides on carbon) is the same as that of the free substrate. While this result is unsurprising, since it is unlikely that the fractionation factor of the substrate's C-2 proton would be changed by the substrate being bound to the enzyme, it provides a satisfactory check on the whole method.

Turning next to the results in the *oversaturated* region in Figures 3 and 4, it is clear that in both cases the tracer experiment is significantly slower than the corresponding nontracer reaction. That is, r (eq 14) is less than 1, and the mixed factor $\phi_{ES,EP}'$ is smaller than ϕ_S (eq 20). From the arguments presented in the introduction, this shows that the enzyme–substrate intermediates are *destabilized* by deuterium substitution. Since we have shown above that ϕ_{ES}' is equal to ϕ_S, we must conclude that it is the ϕ_{EP}' component of the mixed factor $\phi_{ES,EP}'$ that is less than ϕ_S. Analogously, for the doubly primed site, ϕ_{ES}'' is less than ϕ_P. In passing, it is gratifying that, in every comparison (Figures 3–5), the *size* of the maximum in the perturbation of the optical rotation (scaled by the initial concentration of the deuterated substrate) is the same for the tracer and nontracer experiments. As discussed and as predicted by eq 12, the size of the perturbation depends only on the relative rates of the two conversions, of S′-to-P and of P-to-S.

In this paper, we wish to derive the fractionation factors ϕ_{EP}' and ϕ_{ES}'' from the differences in the initial velocities of the tracer and nontracer experiments observed in Figures 3 and 4. We note first that toward the end of both the tracer and the nontracer experiments, when the deuterium isotope has largely washed out into the solvent, good first-order kinetics with the *same* first-order rate constant, k_F', are observed for both experiments. The values of k_F' derived from the tail ends of the tracer and nontracer reactions of S′ (Figure 3) are given in Table I. From the ends of the reactions starting with P″ as the deuterated substrate (Figure 4), we obtain the first-order rate constant k_F'', which is analogous to k_F'. The values of k_F'' are also given in Table I. [A more complete treatment shows that, because of the residual effect of the deuterium substitution, the nontracer rate constant should actually be about 2% larger than the tracer rate constant.] The agreement

Table I: Results for ϕ_{EP}' and ϕ_{ES}'' from the Initial Velocity Ratios

L-[2-^{2}H]proline (S')		D-[2-^{2}H]proline (P'')	
k_F'[a] (min^{-1}) (tracer experiment)	8.6×10^{-3}	k_F''[b] (min^{-1}) (tracer experiment)	1.06×10^{-2}
k_F'[a] (min^{-1}) (nontracer experiment)	8.6×10^{-3}	k_F''[b] (min^{-1}) (nontracer experiment)	1.11×10^{-2}
r[c]	0.81	r[d]	0.85
p_∞ (mM)	200	s_∞	200
$(s_0')_T/p_\infty$	0.20	$(p_0'')_T/s_\infty$	0.20
ϕ_{EP}'[e]	0.48	ϕ_{ES}''[e]	0.61

[a] From the tail ends of the reaction time courses shown in Figure 3. [b] From the tail ends of the reaction time courses shown in Figure 4. [c] The ratio of the normalized initial velocities of the tracer and nontracer experiments shown in Figure 3. [d] The ratio of the normalized initial velocities of the tracer and nontracer experiments shown in Figure 4. [e] Calculated from eq A1 (from the Appendix).

between the pairs of k_F' values and k_F'' values in Table I shows that under oversaturated conditions the differences between the curves in Figures 3 and 4 are much too large to be explained by experimental imprecision. This conclusion is reinforced by the absence of any significant difference between the two curves under *saturated* conditions (see Figure 5).

To find ϕ_{EP}' and ϕ_{ES}'' from the curves in Figures 3 and 4, we measure the ratio of the *initial* velocities of the perturbations. The limiting initial velocities are obtained by writing for the optical rotation, OR, at various times, t, near the beginning of the perturbation:

$$(OR/t) = [dOR/dt]_{t\to 0} - at \qquad (21)$$

where a is some constant. The values of (OR/t) are plotted vs. time and extrapolated to zero time to find the initial velocity. We then derive the required ratio r (eq 14) by dividing each velocity by the appropriate value of s_0' or p_0''. The values of r are given in Table I. Now, we have shown above that there is no change in substrate fractionation on binding to the enzyme ($\phi_{ES}'/\phi_S = 1$), and we may then use eq 19 and the full eq A1 from the Appendix to obtain values of ϕ_{EP}' and ϕ_{ES}'' on the basis of the observed values of r (Table I), $\phi_S = 1.17$ (Fisher et al., 1986a), $p_P = 62.5$ mM (Fisher et al., 1986c), $p_\infty = 200$ mM, and the fact that $\phi_4' \approx \phi_2'$ and $\phi_4'' \approx \phi_2''$ [see Belasco et al. (1986b)]. Because the ratios of the initial velocities, r, are not far from 1, the values of ϕ_{EP}' and ϕ_{ES}'' are rather imprecise, but we find that

$$\phi_{EP}' \approx \phi_{ES}'' \approx 0.55 \pm 0.1$$

That is, the *enzyme-bound* protons in the enzyme–substrate and enzyme–product complexes have unusually low fractionation factors. From compilations of known fractionation factors [e.g., see Schowen (1977)], the only stable protonic sites having fractionation factors much less than 1 are thiols, which have values near 0.5. Recent experiments by Szawelski et al. (1982) have shown that the fractionation factor for the thiol of 2-mercaptoethanol is 0.55, and theoretical calculations of Gabbay and Rzepa (1982) support such a low value. On this basis, therefore, *we conclude that both E_1S and E_2P have one of the two protons on a thiol group, the other being the carbon-bound proton of the substrate.* That is, the two catalytic bases of proline racemase are thiols. This finding is entirely consistent with the suggestion, on the basis of chemical modification studies, that the two active groups of proline racemase are cysteine thiols, one contributed by each of the two protein subunits (Rudnick & Abeles, 1975). While such modification experiments can only prove the existence of particular groups at the active site as distinct from their functional importance in catalysis, our present experiments indicate that these thiols are indeed responsible for proton abstraction and delivery in the enzyme-catalyzed reaction.

The conclusion that thiol groups are directly involved in the action of proline racemase is reinforced by the *solvent* isotope effect on the time course to equilibrium of the racemization of L-proline in the unsaturated region in H_2O and D_2O. Under these circumstances the rate-determining process is the reaction of free enzyme over the transition states of steps 1–3. Experimentally, we find

$$\frac{(k_I)_{D_2O}}{(k_I)_{H_2O}} = \frac{(k_{1,2,3}')_{D_2O}}{(k_{1,2,3}')_{H_2O}} = 1.02 \pm 0.05 \qquad (22)$$

That is, there is *no* detectable solvent isotope effect under these conditions. Ignoring medium effects and assuming that fractionation on sites other than those involved in the catalytic action cancels out, we have

$$(k_{1,2,3}')_{D_2O}/(k_{1,2,3}')_{H_2O} \approx \phi_{1,2,3}''/\bar{\phi}_E \qquad (23)$$

where $\bar{\phi}_E$ is a mixed fractionation factor describing the fractionation on the two forms of the free enzyme. Since we have shown earlier (Fisher et al., 1986a) that $\phi_{1,2,3}'' = 0.44$, we have from eq 22 and 23, $\bar{\phi}_E \approx 0.44$. This confirms the fact that the catalytic groups in the free enzyme have unusually low fractionation factors and that enzyme thiol groups are responsible for proton abstraction and delivery at the active site. Indeed, it is gratifying that the independent measurement of $\phi_{ES,EP}$ and of $\bar{\phi}_E$ leads to the same conclusion about the nature of the catalytic groups of proline racemase. Further implications of the absence of a solvent isotope effect under unsaturated conditions are discussed in the following paper (Belasco et al., 1986b).

Appendix

From the general treatment of Albery and Knowles (1986), the full equation for r is

$$\frac{r(1-(s_0')_T/p_\infty)}{1-r(s_0')_T/p_\infty} = \frac{\dfrac{\phi_{ES,EP}'}{\phi_S} + \dfrac{1}{2}\dfrac{\phi_4}{\phi_2'}\dfrac{\phi_{ES}'}{\phi_S}\dfrac{p_P}{p_\infty}}{1+\dfrac{\phi_4}{\phi_2'}\dfrac{p_P}{p_\infty}} + \frac{\frac{1}{2}}{1+\dfrac{p_\infty}{p_P}} \qquad (A1)$$

In deriving this equation from eq 10.8 of Albery and Knowles (1986), we have assumed that transition state 2 is rate limiting in the saturated region (Belasco et al., 1986b), that $K_2 = 1$ (Fisher et al., 1986b), and that [as will be shown in Belasco et al. (1986b)] $x = 1$. It can be seen from eq A1 that when p_∞ is much greater than the peak switch concentration p_P (i.e., when the system is completely oversaturated) and when $(s_0')_T$ is much smaller than p_∞ (i.e., when the tracer is truly at tracer levels), then eq A1 reduces to eq 20. On the other hand, in the saturated region where p_∞ is smaller than p_P, the right-hand side of eq A1 reduces to $(1 + \phi_{ES}'/\phi_S)/2$, and we can write

$$\frac{\phi_{ES}'}{\phi_S} \approx 1 + \frac{2(r-1)}{1-r(s_0')_T/p_\infty} \qquad (A2)$$

Registry No. D_2, 7782-39-0; L-proline, 147-85-3; D-proline, 344-25-2; proline racemase, 9024-09-3; L-cysteine, 52-90-4.

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Energetics of Proline Racemase: Rates, Fractionation Factors, and Buffer Catalysis in the Oversaturated Region. Nature of the Interconversion of the Two Forms of Free Enzyme†

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ABSTRACT: To probe the nature of the interconversion of the two unliganded forms of proline racemase, a number of experiments have been performed under oversaturating conditions where the rate of the enzymic reaction is mainly limited by the rate of this interconversion. Competitive deuterium washout experiments, where an equimolar mixture of D- and L-proline (in which some or all of one enantiomer is specifically deuterated at the 2-position) is allowed to reach chemical and isotopic equilibrium mediated by the enzyme, have been followed in four ways. The size and the rate of achievement of the maximum perturbation in the optical rotation have been measured, the deuterium content of the substrate at this maximum has been determined, and the final approach to equilibrium after the perturbation maximum has been followed. Further, the enzyme-catalyzed rate of tritium loss from [2-^{3}H]proline has been established. Finally, it has been shown that the enzyme interconversion reaction is catalyzed by several buffers (such as ammonium, hydrazinium, and hydrogen sulfide). These data are discussed in terms of Marcus' theory, which allows a rather detailed picture of the mechanism of free enzyme interconversion to be drawn. This process nicely parallels the mechanism of the enzyme-catalyzed interconversion of the proline enantiomers, and it is evident that substrate racemization (with the concomitant switch of the enzyme-bound protons) is mirrored by the water-mediated switch of the enzyme-bound protons that effects the interconversion of the free enzyme forms. The results favor a stepwise reaction for the interconversion of the free enzyme forms in which a proton is abstracted from a bound water molecule to give a reaction intermediate having a hydroxide ion bound to the diprotonated form of the enzyme.

In the preceding paper (Belasco et al., 1986a) we used the competitive deuterium washout experiment to provide information about the nature of the catalytic functionalities of proline racemase. This experiment starts with an equimolar mixture of D- and L-proline, some or all of one enantiomer being specifically deuterated at the 2-position. At zero time the optical rotation is zero, but since the deuterated enantiomer reacts more slowly than its unlabeled partner, an imbalance in the concentrations of the enantiomers is generated, and the absolute value of the optical rotation, |OR|, rises. Concurrently, the deuterium is lost from substrate to the medium, and as the reaction proceeds the |OR| falls again until chemical and isotopic equilibrium is reestablished. Such competitive deuterium washout experiments are a rich source of information about the fractionation factors of intermediates and transition states both in the substrate interconversion steps and in the steps involving the loss of isotope to the medium (as the free enzyme forms are interconverted). Four measurements can be made: the size of the maximum perturbation in the optical rotation, the rate of achievement of this maximum, the deuterium content of the substrate at the maximum, and the rate of the subsequent approach to equilibrium. These and other experiments performed under oversaturating conditions, including the effect of different buffer types on the rate of interconversion of the two forms of the free enzyme, the rate of tritium loss from [2-^{3}H]proline catalyzed by the racemase, and the solvent isotope effect, are reported in this paper. The results are combined to provide a rather detailed picture of

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