Energetics of Proline Racemase: Double Fractionation Experiment, a Test for Concertedness and for Transition-State Dominance†

Joel G. Belasco,‡ W. John Albery,§ and Jeremy R. Knowles*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received September 20, 1984; Revised Manuscript Received December 19, 1985

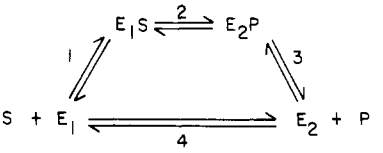
ABSTRACT: To test whether a reaction involving the making and/or breaking of two bonds at two sites is concerted (and proceeds through a single transition state) or is stepwise (and involves a reaction intermediate in which only one bond has been made or broken), we have measured the isotopic fractionation at one site as a function of isotopic substitution at the other site. In the case of proline racemase, the discrimination against solvent deuterium in the product when the reaction is run in mixed H_2O–D_2O is measured for the reaction both of [2-^{1}H]proline and of [2-^{2}H]proline. The isotopic fractionation at the solvent site may in principle be smaller, the same, or larger, when the ^{2}H-labeled substrate is used rather than the ^{1}H substrate, and—depending upon the nature of the catalyzing groups—this information indicates whether the reaction is stepwise, or concerted, or whether an isotopically *insensitive* transition state is partially rate determining. Experimentally, we have found that the discrimination against solvent deuterium in the product L-proline is the *same*, whether D-[2-^{1}H]proline or D-[2-^{2}H]proline is the substrate. This result requires that the substrate and product "on–off" steps are faster than the racemization step and that the racemization reaction proceeds either in a concerted manner or in a stepwise fashion involving enzyme catalytic groups (e.g., thiols) having ground-state fractionation factors around 0.5.

The interconversion of L- and D-proline catalyzed by proline racemase is known from the work of Abeles (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975) to proceed by a "two-base" mechanism. One catalytic group acts as a general base and abstracts the C-2 hydrogen from substrate proline while the conjugate acid of the second catalytic group delivers a solvent-derived proton to C-2 from the opposite face of the proline skeleton. In the preceding paper (Fisher et al., 1986c) we showed that the transition state(s) for the substrate interconversion involves (involve) proton motion at both protonic sites. The fractionation factors for the two sites were shown to be $\phi_{1,2,3}' = 0.37_5$ (for the proton from L-proline) and $\phi_{1,2,3}'' = 0.44$ (for the proton from D-proline). The nature of the transition state(s) cannot, however, be deduced merely from the sizes of these fractionation factors, and the experiments described in this paper were designed to establish whether the two protons are transferred synchronously in a single transition state or whether the reaction follows a stepwise path.

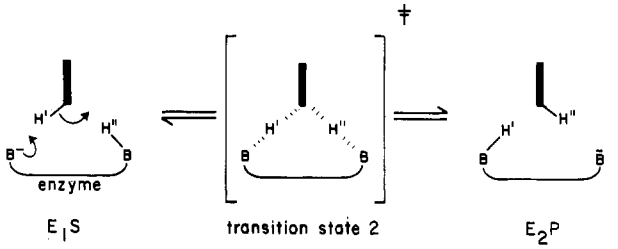
There are two possibilities for the interconversion of E_1S and E_2P (via transition state 2, Scheme I): either the reaction is a concerted one in which the two protons are in flight simultaneously and the single transition state (Scheme II) is that of an S_E2 reaction, or the reaction proceeds in two steps via a carbanion intermediate (Scheme III). We may in principle distinguish between these two mechanisms by employing a new type of isotopic experiment, the "double fractionation" experiment. We shall show how this method can be used not only as a test for concertedness but also to discover whether transition state 2 (Scheme I) is cleanly rate limiting in the reaction through steps 1–3. A portion of this work has been published (Belasco et al., 1983).

Although we discuss the theory of the double fractionation experiment for the case of two protonic sites, as found in

Scheme I: Pathway for the Interconversion of L- and D-Proline (S and P, respectively) Catalyzed by Proline Racemase (E)

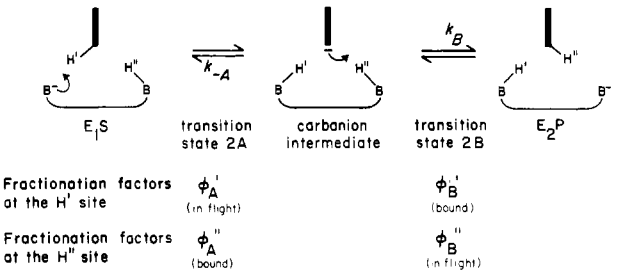


Scheme II: Interconversion of E_1S and E_2P by a Concerted Mechanism[a]



[a] B symbols are enzyme bases, and proline is represented edge-on as a heavy bar.

Scheme III: Interconversion of E_1S and E_2P by a Stepwise Mechanism[a]



[a] B symbols are enzyme bases, and proline is represented edge-on as a heavy bar.

† This work was supported by the National Science Foundation and Merck Sharp & Dohme.

‡ National Science Foundation Predoctoral Fellow. Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

§ Present address: Department of Chemistry, Imperial College of Science & Technology, London SW7 2AY, England.

0006-2960/86/0425-2552$01.50/0

proline racemase, it must be emphasized that the approach is a rather general one. The method can be used for any two isotopic substitutions on separate sites providing that one isotopic substitution can be specified (e.g., H or D) while the fractionation on the other site (e.g., H/D, $^{12}C/^{13}C$, $^{14}N/^{15}N$, or $^{16}O/^{18}O$) is measured. Indeed, this method has been independently devised and extensively applied by Cleland and his group (Hermes et al., 1982, 1984a,b) to a number of enzyme reactions that involve the breaking of a bond to hydrogen and the making of a bond to hydrogen or a heavier element.

Theory

Double Fractionation Experiment. Consider a reaction that involves hydrogens on two protonic sites, designated by a single or a double prime (H′ and H″), where there is no exchange of isotope anywhere along the reaction coordinate. The experiment is designed so that the singly primed site, H′, is occupied throughout by H or by D: this is called the *specified* site. We then measure the fractionation factor ϕ'' at the doubly primed site, H″: this is called the *fractionating* site. These designations are illustrated for a concerted and for a stepwise reaction in Schemes II and III, respectively.

Consider first a *two-step* reaction as shown in Scheme III, with transition states 2A and 2B of comparable free energy so that each transition state is kinetically significant. The mixed fractionation factor for the fractionating site $\phi_{A,B}''$ when H occupies the specified site is given by (Albery & Knowles, 1976)

$$(\phi_{A,B}'')_{H'} = \frac{1 + \kappa}{\kappa(\phi_A'')^{-1} + (\phi_B'')^{-1}} \quad (1)$$

where κ is the partition ratio (k_B/k_{-A}) for the intermediate in the absence of isotopes. Equation 1 assumes that the specified site is always hydrogen, this fact being indicated by the subscript H′. But let us now suppose that the specified site is occupied by deuterium, which would be achieved (in Scheme III, for instance) by using deuterated substrate. In this case, the fractionation factor for the fractionating site would become

$$(\phi_{A,B}'')_{D'} = \frac{1 + \kappa\phi_B'/\phi_A'}{(\kappa\phi_B'/\phi_A')(\phi_A'')^{-1} + (\phi_B'')^{-1}} \quad (2)$$

When deuterium occupies the specified site, the partition ratio may be altered, thereby changing the proportions in which ϕ_A'' and ϕ_B'' contribute to the mixed fractionation factor $\phi_{A,B}''$. The value of this mixed fractionation factor may therefore be affected by a change in the relative kinetic importance of the two transition states A and B. Isotopic substitution at the specified site may therefore provide a lever that causes $(\phi_{A,B}'')_{D'}$ to differ from $(\phi_{A,B}'')_{H'}$. These relationships are illustrated in Figure 1.

From eq 1 and 2 we obtain the shift parameter ζ, which measures the change in the mixed fractionation factor $\phi_{A,B}''$ when the isotope at the specified site changes from H to D:

$$\zeta = \frac{(\phi_{A,B}'')_{H'}}{(\phi_{A,B}'')_{D'}} - 1 = \frac{(\phi_A' - \phi_B')(\phi_A'' - \phi_B'')}{(\kappa\phi_B' + \phi_A')(\phi_B'' + \kappa^{-1}\phi_A'')} \quad (3)$$

The evaluation of ζ in the double fractionation experiment therefore requires the measurement of $(\phi_{A,B}'')_{H'}$ and $(\phi_{A,B}'')_{D'}$. If there is a difference between the two fractionation factors, ζ will be nonzero, and we may conclude that the mechanism involves at least two kinetically significant transition states.

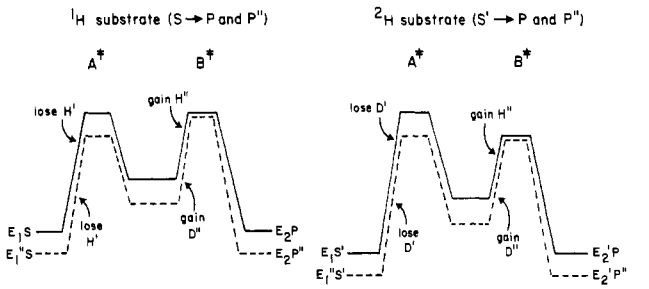


FIGURE 1: Free energy profiles for a two-step proton transfer reaction via a carbanionic intermediate (e.g., Scheme III, running left to right). In this direction, H′ is the specified site and H″ is the fractionating site. For illustration, the partitioning of the carbanionic intermediate, κ, is taken as unity for the all-hydrogen case. For the ^{1}H substrate (left-hand profile), ^{1}H occupies the H′ site, and for the ^{2}H-labeled substrate (right-hand profile), ^{2}H occupies the H′ site. Fractionation occurs since either ^{1}H or ^{2}H can occupy the H″ site. With the ^{2}H-labeled substrate (right-hand profile), transition state A becomes more cleanly rate limiting, the kinetic significance of transition state B is diminished, and there is less fractionation.

For a *concerted* reaction[1] with a single transition state (Scheme II) the protons at both sites are simultaneously in flight. Classically, one expects that multiple sites in a single transition state will behave independently with respect to isotopic substitution (Kresge, 1964), so that the fractionation at one site (in our case, $\phi_{A,B}''$) will be the same regardless of the isotope at the other site. That is, $(\phi_{A,B}'')_{H'}$ will equal $(\phi_{A,B}'')_{D'}$, and ζ in eq 3 will be zero. More recently, however, it has become clear that when the two isotopic substitutions involve hydrogen, there may be coupling between the two protonic motions. Thus, Limbach et al. (1982) have studied several systems where such coupling is expected, and Cleland has demonstrated coupling in several enzymatic reactions where two hydrogens are in flight at the transition state (Hermes et al., 1984a). In theoretical work, Limbach et al. (1982), Huskey and Schowen (1983), and Saunders (1985) have invoked tunneling to explain the observed deviations from cumulative behavior. The above notwithstanding, we will explore the expected effects on ζ from the classical point of view, while recognizing that if two hydrogens (as distinct from one hydrogen and one heavy element) are involved, changes in the value of ζ may be harder to interpret.

Even if the observed value of ζ is zero, we may not conclude that the reaction has only one kinetically significant transition state, because it is possible for the right-hand side of eq 3 to equal (or be close to) zero for a stepwise reaction also. First, if either κ or κ^{-1} (eq 3) is much greater than 1, ζ will be negligible. This is expected, since to see an effect on $\phi_{A,B}''$ of changing the isotope at the specified site, *both* transition states 2A and 2B must be partly rate limiting. Second, if either $\phi_A'' = \phi_B''$ or $\phi_A' = \phi_B'$ (this is equivalent to saying that the fractionation factor for the proton is the same whether it is in flight or bound to the enzyme), then again ζ is zero (see eq 3). In order, then, to see a change in $\phi_{A,B}''$ on changing the isotope at the singly primed site and so prove the existence of more than one kinetically significant transition state, we need $\kappa \approx 1$, $\phi_A'' \neq \phi_B''$, and $\phi_A' \neq \phi_B'$.

[1] We do not, here, distinguish between concertedness and synchroneity. Dewar (1984) has said that in a *synchronous* reaction all bond-making and bond-breaking processes have proceeded to comparable extents at the transition state, whereas a *concerted* reaction is simply one that occurs in a single step without the incursion of a reaction intermediate. Synchroneity is clearly more restrictive, and the quantitative predictions concerning the behavior of ζ strictly relate to concerted processes.

Test for Concertedness. We now describe the application of the double fractionation experiment to proline racemase. The reaction involves the abstraction of a carbon-bound proton from the substrate and the delivery of a solvent-derived proton from the conjugate acid of an enzymic base (Schemes II and III) to the same carbon center.[2] When, for example, the proline racemase catalyzed isomerization of L-[2-^{1}H]proline (^{1}H at the specified site, H′) is run in a mixed H_2O–D_2O solvent, then if the step that involves the delivery of H″ (a proton or deuteron) to C-2 (to make D-[2-^{1}H]- or D-[2-^{2}H]-proline) is at least partly rate limiting, we shall observe a discrimination against the formation of product that is deuterated. The observed discrimination will provide the transition-state fractionation factor $(\phi_{A,B}'')_{H'}$. The experiment is then repeated with L-[2-^{2}H]proline as substrate (^{2}H on the specified site, H′) to obtain $(\phi_{A,B}'')_{D'}$. From these values we obtain (eq 3) the parameter ζ.

It should be noted that proline racemase satisfies the condition that there be no exchange between either of the protonic sites and the solvent while the substrate is bound (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975). Further, the three requirements for a change in $\phi_{A,B}''$ (namely, $\kappa \approx 1$, $\phi_A' \neq \phi_B'$, and $\phi_A'' \neq \phi_B''$) are likely to be met by proline racemase. From the overall symmetry of the racemase reaction [the overall equilibrium constant is 1.0, and since $k_{cat^+} \approx k_{cat^-}$ (Fisher et al., 1986a) we know that $K_2 \approx 1.0$ (Fisher et al., 1986b)], we expect that if the reaction proceeds in a stepwise manner, the two transition states for proton transfer (2A and 2B in Scheme III) will have similar free energies, so that κ (the partition ratio for the putative carbanion intermediate) will be close to unity. In fact, the observation of substantial fractionation at both protonic sites when the proline racemase reaction is run in each direction in single fractionation experiments (Fisher et al., 1986c) ensures that, for a stepwise mechanism, the value of κ is sufficiently close to unity so as to allow the application of the double fractionation experiment. It can be shown from eq 1 and 3, together with

$$(\phi_{A,B}')_{H''} = \frac{1 + \kappa}{\kappa(\phi_A')^{-1} + (\phi_B')^{-1}}$$

that

$$\zeta = \frac{(\phi_{A,B}'')_{H'}}{\phi_A''} + \frac{(\phi_{A,B}')_{H''}}{\phi_B'} - \frac{(\phi_{A,B}'')_{H'}(\phi_{A,B}')_{H''}}{\phi_A''\,\phi_B'} - 1 \quad (4)$$

There are no terms involving κ in this equation because they have become subsumed in the mixed transition state factors $(\phi_{A,B}'')_{H'}$ and $(\phi_{A,B}')_{H''}$ observed in single fractionation experiments. These latter two values will be substantially less than 1 [as they are for proline racemase (Fisher et al., 1986c)] only if κ is reasonably close to unity.

The required fractionation factor inequalities also seem probable. Since ϕ_B'' and ϕ_A' relate to protons in flight and ϕ_A'' and ϕ_B' to protons at rest (Scheme III), we expect $\phi_B'' < \phi_A''$ and $\phi_A' < \phi_B'$. In the case of the stepwise mechanism, the substitution of deuterium at the specified site (that is, the use of deuterated substrate) will make transition state 2B (for proton transfer at the fractionating site) *less* rate determining, with a consequent reduction in the relative contribution of ϕ_B'' to the mixed fractionation factor $\phi_{A,B}''$ (Figure 1). Because $\phi_B'' < \phi_A''$, the value of the mixed fractionation factor will increase, and ζ will be *negative* (eq 3). Provided, therefore, that the above conditions hold, a value of $\zeta = 0$ would indicate a concerted mechanism (Scheme II) while a significantly negative value of ζ would prove a stepwise mechanism (Scheme III).[3]

Test for Transition-State Dominance. We assumed above that transition state 2 (Scheme I) was cleanly rate limiting and was the "dominant" transition state in steps 1–3. A frequent and important question in enzymology is whether a single transition state *is* dominant, or whether two or more transition states are partially rate limiting. Thus, for Scheme I, we may ask whether transition states 1 and 3 are of similar free energy to that of transition state 2. In certain cases, the double fractionation experiment can provide an answer to this question also. For a stepwise mechanism (Scheme III) in which the fractionation factors for protons in flight (ϕ_A' and ϕ_B'') are substantially less than the fractionation factors for enzyme-bound protons at rest (ϕ_B' and ϕ_A''), ζ will be negative regardless of whether transition states 1 and 3 are partially rate determining. This will be so because, as before, the transition state (2B) for proton transfer at the fractionating site will be made less rate determining by the substitution of deuterium at the specified site. On the other hand, for a concerted mechanism, ζ will have a *positive* value if transition states 1 and 3 are partially rate limiting. The reason for this is that the single transition state (2) involving proton transfer will become *more* rate determining (than 1 or 3) by the substitution of deuterium at the specified site.

Summary. We may summarize the information obtained from measurement of the shift parameter ζ as follows:

(1) If $\zeta < 0$, the simplest conclusion is that the reaction proceeds by a stepwise mechanism in which each isotope moves in its own kinetically significant transition state (Scheme III). The possibility remains, however, that there is coupling between isotopic motions at the transition state (as discussed under Theory).

(2) If $\zeta = 0$, then either the reaction is concerted (Scheme II) or the fractionation fractors for either site in the two transition states of a stepwise reaction are equal ($\phi_A'' \approx \phi_B''$, or $\phi_A' \approx \phi_B'$). Furthermore, if $\zeta = 0$, it is clear that the transition state(s) involving proton transfer (e.g., step 2 in Scheme I) is (are) dominant.

(3) If $\zeta > 0$, then the transition state(s) involving protonic motion is (are) not dominant and other isotopically insensitive steps (e.g., steps 1 and 3 in Scheme I) are also partially rate limiting.

In this paper we report the results of a double fractionation experiment with proline racemase, in which the discrimination against solvent deuterium is examined with both deuterated and undeuterated samples of proline to obtain values of the two mixed fractionation factors and of ζ.

Experimental Procedures

Materials

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

Methods

All methods (aside from those described below) were as described by Fisher et al. (1986a–c).

[2] The transition state(s) for the interconversion of the two forms of *free* enzyme (step 4, Scheme I) can be made kinetically insignificant by choosing saturating (not oversaturating) substrate concentrations and using a buffer that catalyzes the free enzyme interconversion (Fisher et al., 1986a,b). These conditions simultaneously ensure the isotopic equilibration of the bases of the free enzyme with the solvent protons.

[3] In the discussion so far we have made the H′ site the specified site and looked for fractionation at the H″ site, and one may ask whether additional information can be obtained if these designations are switched. For proline racemase this would be achieved by using D-[2-^{1}H]proline and D-[2-^{2}H]proline as the two (specified) reactants, instead of using the two L enantiomers. The symmetry of eq 4 shows, however, that the same value of ζ will be obtained from running the reaction in either direction.

Preparative gas chromatography was performed on a Varian Model 920 instrument fitted with a thermal conductivity detector.

The isotopic purity of samples of D-[2-^{1}H]- and D-[2-^{2}H]-proline was assessed by derivatization of the corresponding methyl esters with *N*-(trifluoroacetyl)-L-prolyl chloride, separation of the L–L and L–D dipeptide derivatives by preparative gas chromatography, and subjection of these samples to mass spectrometric analysis. The deuterium content of the D-[2-^{2}H]proline was found to be 98.8%.

Discrimination against Solvent Deuterium. A solution of D-[2-^{1}H]- or D-[2-^{2}H]proline (120 mM) in D_2O (1.50 mL) was warmed to 37 °C. To this was added a prewarmed solution in H_2O (0.50 mL) of 800 mM ammonium bicarbonate, pH 8.0, containing 2-mercaptoethanol (80 mM), (ethylenedinitrilo)tetraacetic acid (EDTA) (4 mM), and proline racemase (1.33 units/mL for D-[2-^{1}H]proline; 4.0 units/mL for D-[2-^{2}H]proline). The reaction was allowed to proceed at 37 °C and was monitored by following the fall in optical rotation at 365 nm. When the optical rotation had fallen to 70% of its initial value, the reaction mixture was quenched by adding it to Dowex-50 (H^+ form, 2 g). The suspension was then poured into a small column and washed with water (35 mL). The proline was eluted from the column with 2 M ammonium hydroxide (8 mL). The eluate was divided into two parts, and each was evaporated to dryness. One part was dissolved in 200 mM triethylammonium bicarbonate, pH 8.3 (2.0 mL), to which was added FAD (2 μg), catalase (1200 units), and D-amino acid oxidase (1.2 units). After the reaction was allowed to proceed for 8 h at room temperature under oxygen, the solution was evaporated to dryness. The residue was dissolved in methanol, and the solution was then treated with charcoal and filtered through Celite. The filtrate was evaporated to dryness. The residue was refluxed in methanol (5 mL) and thionyl chloride (200 μL) for 30 min. After evaporation to dryness, the residue was treated with *N*-(trifluoroacetyl)-L-prolyl chloride in chloroform (0.17 M, 0.5 mL) for 10 min. Triethylamine (50 μL) was then added, and after 5 min the solution was washed with water and the organic layer filtered through anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, the residue was redissolved in ether, and the solution was filtered.

N-(Trifluoroacetyl)-L-prolyl-L-proline methyl ester was isolated from the ether solution by preparative gas chromatography at 185 °C on a stainless steel column (12 ft × 0.25 in.) packed with 3% OV-25 (75% phenyl methyl silicone) on 100/210 mesh Supelcoport. The injector was maintained at 220 °C and the thermal conductivity detector at 265 °C. The carrier gas (helium) flow rate was 38 mL/min.

Mass spectrometric analysis of the deuterium content of the isolated *N*-(trifluoroacetyl)-L-prolyl-L-proline methyl ester was by direct insertion at an ionizing voltage of 70 eV and a source temperature of 100–210 °C. The isotopic content was determined from the relative intensities of the molecular ion peaks at m/z 322 and 323 compared to those of an unlabeled sample.

Enantiomeric Purity of D-[2-^{1}*H*]*Proline and of* D-[2-^{2}*H*]-*Proline.* For the purposes of the double fractionation experiment described in this paper, it was essential that very small amounts of contaminating L-proline in the D-proline substrate be assessed precisely.

The derivatization of proline with *N*-(trifluoroacetyl)-L-prolyl chloride proceeds with >99% stereochemical integrity, and the diastereomeric dipeptides *N*-(trifluoroacetyl)-L-prolyl-L-proline methyl ester and *N*-(trifluoroacetyl)-L-prolyl-D-proline methyl ester can be readily separated by gas chromatography. Although tiny, the somewhat variable level of stereochemical infidelity in the derivatization reactions (evaluated on the basis of gas chromatographic analysis of the diastereomeric composition of the derivatization product of L-proline that had previously been treated with D-amino acid oxidase and recrystallized) makes the direct gas chromatographic analysis of trace enantiomeric contaminants in proline unreliable.

To bypass this difficulty, an analytical procedure was devised that involved comparison of the isotopic content of a sample of deuterated L-proline before and after the addition of a large excess of protonated D-proline accompanied by its L-[2-^{1}H]-proline contaminant.

D-[2-^{1}H]Proline (40 equiv) was combined with 1 equiv of L-[2-^{2}H]proline. After treatment with D-amino acid oxidase to remove the D-proline, the prolines were converted to their methyl esters and then coupled with *N*-(trifluoroacetyl)-D-prolyl chloride. The adduct with L-proline [*N*-(trifluoroacetyl)-D-prolyl-L-proline methyl ester] was purified by preparative gas chromatography and analyzed for its isotopic content by mass spectrometry. Because of the large excess of D-[2-^{1}H]proline used in the experiment, even a 0.1% L-[2-^{1}H]proline contaminant would constitute a substantial portion (4%) of the L-proline incorporated in the DL dipeptide. Thus, with the exception of a small contribution from the L-[2-^{2}H]proline sample (98.8% deuterated), nearly all of the undeuterated dipeptide arose from the contaminating L-[2-^{1}H]proline in the original D-[2-^{1}H]proline. On this basis, the enantiomeric purity of the D-[2-^{1}H]proline used in the solvent discrimination experiments was evaluated. A mirror experiment (41 equiv of D-[2-^{2}H]proline plus 1 equiv of L-[2-^{1}H]-proline) allowed the determination of the enantiomeric purity of the D-[2-^{2}H]proline.

D-[2-^{1}H]Proline (180 μmol) was mixed with L-[2-^{2}H]proline (4.50 μmol) and dissolved in 200 mM triethylammonium bicarbonate, pH 8.3 (2.0 mL). To this solution were added FAD (2 μg), catalase (2000 units), and D-amino acid oxidase (2.4 units). After the reaction was allowed to proceed for 20 h at room temperature under oxygen, the solution was evaporated to dryness. The residue was dissolved in methanol, and the solution then treated with charcoal and filtered through Celite. The filtrate was evaporated to dryness. The residue was refluxed in methanol (5 mL) and thionyl chloride (200 μL) for 30 min. After evaporation, the residue was treated with *N*-(trifluoroacetyl)-D-prolyl chloride in chloroform (0.17 M, 0.35 mL) for 10 min. Diethylamine (25 μL) was added, and after 5 min the mixture was washed with water and the organic layer filtered through anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, the residue redissolved in ether, and the solution filtered. Analytical gas chromatography on OV-25 (220 °C) showed that essentially all of the D-proline had been oxidized. *N*-(Trifluoroacetyl)-D-prolyl-L-proline methyl ester was isolated by preparative gas chromatography on OV-25 and analyzed for its isotopic content by mass spectrometry. When this method was used, the original D-[2-^{1}H]proline sample was found to contain 0.18% L-[2-^{1}H]proline.

The enantiomeric purity of D-[2-^{2}H]proline was assessed analogously by using a mixture of D-[2-^{2}H]proline (172 μmol) and L-[2-^{1}H]proline (4.16 μmol). The D-[2-^{2}H]proline was found to contain 0.08% L-[2-^{2}H]proline.

Results and Discussion

Double Fractionation Experiment. In order to provide useful mechanistic information, the values of the single site

Table I: Results from the Double Fractionation Experiment

	substrate	
	D-[2-^{1}H]proline	D-[2-^{2}H]proline
fractional deuterium content of solvent	0.749	0.749
fractional deuterium content of L-proline product[a]	0.529 ± 0.005	0.556 ± 0.002
	0.524 ± 0.002	
mean	0.526 ± 0.002	
$\phi_{1,2,3}'$ (uncorrected)[b]	0.38	0.43
fractional enantiomeric contamination[c]	1.8×10^{-3}	0.8×10^{-3}
correction factor for enantiomeric contaminant[d]	1.02	0.99
correction factor for extent of reaction[d]	0.93	0.86
$\phi_{1,2,3}'$ (corrected)	0.36 (H″)	0.37 (D″)

[a] Each result is from a separate experiment. The errors are standard deviations from repeated (≥7) scans of the mass spectrum. [b] Calculated directly from eq 6. [c] See Experimental Procedures. [d] For the details of these corrections, see the supplementary material.

mixed fractionation factors must be as precise as possible. For the proline racemase system, we have obtained the fractionation factors by measuring the deuterium content of the product from reactions of D-[2-^{1}H]proline and of D-[2-^{2}H]-proline in mixtures of H_2O–D_2O (25:75 v/v). The reactions were stopped after the optical rotation had fallen to about 70% of its initial value,[4] and the unreacted D-proline was largely destroyed with D-amino acid oxidase. The L-proline product was then isolated and its deuterium content determined mass spectrometrically. The possibility of removing the unreacted D-proline with high stereoselectivity was the reason why the fractionation factors were determined starting with D-proline rather than L-proline as substrate.[5] The deuterium content of the solvent, m, was chosen so that approximately equal quantities of L-[2-^{1}H]proline and L-[2-^{2}H]proline would be formed. The reactions were carried out in ammonium bicarbonate buffer so as to eliminate oversaturation (Fisher et al., 1986b) and to ensure that steps 1–3 (Scheme I) would be rate limiting. From the ratio of deuterated to protonated L-proline formed (s'/s) and the fractional deuterium content of the solvent, m, the fractionation factors relative to HDO were obtained from the usual equation (Albery & Davies, 1969):

$$\phi_{1,2,3}' = \frac{s'}{s}\frac{(1-m)}{m}[1 - 0.06(\tfrac{1}{2} - m)] \qquad (5)$$

where the term in square brackets is the correction for the breakdown of the rule of the geometric mean. Throughout this series, fractionation factors are referred to the standard solvent HDO.

For data of the highest precision, however, two corrections must be applied. First, the racemase reaction is reversible, and even though the reaction was quenched early (when only 15% of the starting D-proline had formed L-proline), the initial product L-proline will itself have undergone some reaction and isotopic exchange.[4] Second, the observed deuterium content of the isolated L-proline must be corrected for any small contaminant of L-proline in the starting D-proline substrate. Because only 15% of the D-proline reacts to form approximately equal amounts of S′ and S, a contaminant of only 1% of S in the starting P could lead to errors as large as 10% in the ratio of s'/s, and hence in the derived fractionation factors.

Our technique for measuring the enantiomeric purity of the starting D-proline to a few parts in 10^4 is described under Experimental Procedures. Both of these relatively small corrections can be made on the basis of known quantities, using equations presented as supplementary material (see paragraph at end of paper regarding supplementary material). The values of the two fractionation factors, $(\phi_{1,2,3}')_{H''}$ and $(\phi_{1,2,3}')_{D''}$ (see footnote 5), are given in Table I. It is gratifying to note in passing that the value of $(\phi_{1,2,3}')_{H''}$ of 0.375 is in very good agreement with that found in the previous paper (Fisher et al., 1986c) for reaction in 100% H_2O. Evidently the fractionation factor has not been altered significantly even though the solvent has changed from 100% H_2O to 75% D_2O.

From the values of the fractionation factors in Table I, and eq 3, we find that

$$\zeta = -0.03 \pm 0.05$$

That is, ζ is zero within experimental error, and deuterium substitution on the specified site (H″ for reaction from right to left in Schemes II and III)[5] has not affected the discrimination at the fractionating site (H′). As discussed above, this means either that the reaction is concerted as shown in Scheme II (and that there is no coupling between the protonic motions) or that ζ is near zero because $\phi_A' \approx \phi_B'$ or because $\phi_A'' \approx \phi_B''$.

Could the very small value of ζ derive from the identity or near identity of ϕ_A' and ϕ_B' or of ϕ_A'' and ϕ_B'' in the stepwise reaction shown in Scheme III? From symmetry arguments (proline racemase is a dimer of identical subunits, each of which contributes to a single active site), enforced by the quite similar experimental values of $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ (Fisher et al., 1986c), it is clear that $\phi_A' \approx \phi_B''$ and that $\phi_A'' \approx \phi_B'$ (see Scheme III). That is, the two in-flight protons have similar fractionation factors, as do the two enzyme-bound protons. Further, we know that $\phi_{1,2,3}' = 0.37$ (this paper; Fisher et al., 1986c), and for $\kappa = 1$, eq 1 (for the P → S direction: see footnote 5) gives

$$(\phi_A')^{-1} + (\phi_B')^{-1} = 5.4 \qquad (6)$$

For any pair of values of the two fractionation factors ϕ_A' and ϕ_B' that satisfy this equation, we can calculate the value of ζ (from eq 3). The *general* relationship between ϕ_A', ϕ_B', and ζ (based on eq 6 and $\kappa = 1$) is shown in Figure 2, which illustrates the mechanistic constraints that derive from the experimental findings of $\phi_{1,2,3}' = 0.37$ and $\zeta = -0.03$. For instance, if $\phi_A'' = \phi_B' = 1$, which would be reasonable if the two enzyme catalytic groups were oxygen or nitrogen bases, then $\phi_A' = \phi_B'' = 0.23$ (from eq 6) and $\zeta = -0.35$ (eq 3). Such a large negative value of ζ is clearly outside our experimental limits, and a simple stepwise model (as Scheme III) where B^- and BH are oxygen or nitrogen centers is ruled out. But what if, as has been suggested by Rudnick and Abeles (1975), the

[4] The extent of reaction of 15% was chosen as a compromise that provides sufficient L-proline for analysis while keeping the contribution from the back-reaction of L-proline small.

[5] It will be evident that, in terms of Schemes II and III, the reaction has been run using P (D-[2-^{1}H]proline) and P″ (D-[2-^{2}H]proline) in the right-to-left direction. H″ is therefore the specified site, and H′ is the fractionating site. As noted in footnote 3, however, the same value of ζ is obtained whichever direction the reaction is followed.

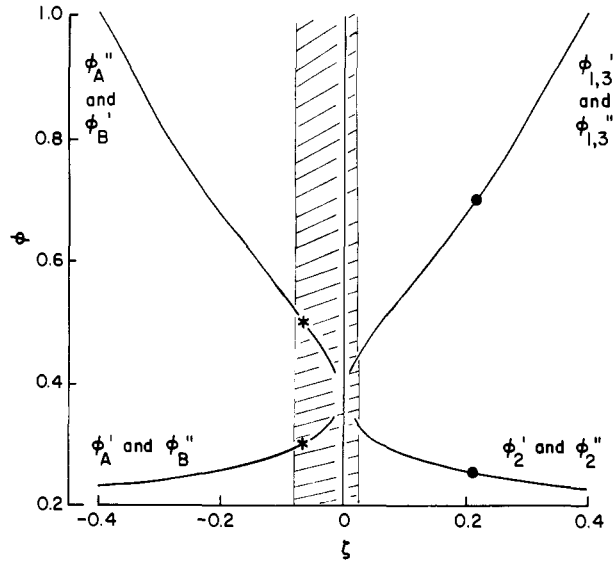


FIGURE 2: Relationship between the fractionation factors for enzyme-bound protons at rest ($\phi_A'' = \phi_B'$: Scheme III), and for in-flight protons ($\phi_A' = \phi_B''$: Scheme III), and the shift parameter ζ. The plot is constructed by using the experimental value of $\phi_{1,2,3}'$ for proline racemase of 0.37 and $\kappa = 1$. For a concerted reaction (e.g., Scheme II), $\zeta = 0$. For a stepwise reaction (e.g., Scheme III), ζ is negative, and the pairs of values of ϕ_A'' ($=\phi_B'$) and ϕ_A' ($=\phi_B''$) that are consistent with the known value of $\phi_{1,2,3}'$ are plotted on the left-hand half of this figure. For a reaction where isotopically silent transition states challenge the dominance of the isotopically sensitive transition state (e.g., 1 and 3 challenge 2, Scheme I), ζ is positive, and the appropriate pairs of fractionation factors are shown on the right-hand half of the figure. The hatched area shows the *experimental* value of ζ with its error limits. For explanations of the asterisks and the filled circles, see the text.

two enzyme functional groups are thiols? Thiols are known to have unusually low equilibrium fractionation factors around 0.50 (Szawelski et al., 1982; Schowen, 1977), and if $\phi_A'' = \phi_B' = 0.50$, then eq 6 gives $\phi_A' = \phi_B'' = 0.30$. These data produce a value for ζ of −0.06 (see the asterisks in Figure 2) which *is* consistent with our experimental findings. The stepwise mechanism involving *catalytic thiol groups* cannot therefore be eliminated.

Finally, the possibility that the dominance of transition state 2 is challenged by the isotopically insensitive transition states 1 and 3 (Scheme I) must be faced. As has been discussed above, such a challenge would lead to a positive value of ζ, but is it possible that this situation could give a very small positive ζ that would be consistent with our experimental value? Since transition states 1 and 3 relate to substrate binding and release, we can assume that $\phi_1' = \phi_3'' = \phi_S = 1.17$ (Fisher et al., 1986c) and that ϕ_1'' and ϕ_3' could be as low as 0.50 (if the enzyme catalytic bases are thiols). The fractionation on transition states 1 and 3 (assuming them to be of equal free energy) is given by

$$(\phi_{1,3}')^{-1} = (\phi_{1,3}'')^{-1} = \tfrac{1}{2}(\phi_1'^{-1} + \phi_3'^{-1}) = \tfrac{1}{2}(\phi_1''^{-1} + \phi_3''^{-1}) \quad (7)$$

from which we can set a lower limit on $\phi_{1,3}'$ (for the case of thiols at the active site) of 0.70. The mixed fractionation factor for steps 1–3 is (Albery & Knowles, 1986)

$$(\phi_{1,2,3}')^{-1} = \frac{(\phi_{1,3}')^{-1} + \kappa^*(\phi_2')^{-1}}{1 + \kappa^*} = 0.37^{-1} \quad (8)$$

where $\kappa^* = {}^1/_2 \exp[(\Delta G_2^{\ddagger} - \Delta G_1^{\ddagger})/(RT)]$. (The factor of $^1/_2$ in this equation arises because we are considering three transition states, two of which are of equal free energy.) Since $\phi_{1,3}'$ can be no lower than 0.70, eq 8 shows that when $\kappa^* = 1$, ϕ_2' ($\approx\phi_2''$) ≈ 0.26, from which $\zeta \approx 0.21$ (eq 3). Such a large positive value for ζ is far outside the observed limits (see the filled circles in Figure 2). Furthermore, if we ask what value of κ^* would produce (with the above values for $\phi_{1,3}'$ and ϕ_2') a ζ value within the observed range, we find that κ^* would have to be greater than 20. This would mean that transition state 2 was indeed dominant. We must conclude, therefore, that the observed value of ζ precludes any involvement of transition states 1 and 3 and that transition state 2 is cleanly rate limiting. This conclusion is consistent with the fact that k_{cat}/K_m is 9×10^5 M^{-1} s^{-1}, which is slower than the diffusion limit for substrate and product "on" rates, which in turn would determine k_{cat}/K_m if transition states 1 and 3 were rate limiting and reactions 1 and 3 were diffusion controlled.

In summary, we can conclude that (i) transition states 1 and 3 are never kinetically significant, and (ii) the reaction proceeds either in a concerted manner or in a stepwise fashion involving catalytic groups (e.g., thiols) having ground-state fractionation factors around 0.5. To define further the nature of the transition state(s) for the enzyme-catalyzed proton transfers, we must determine the ground-state fractionation factors for the enzyme's catalytic groups ϕ_E'' ($=\phi_{E_1S}''$) and ϕ_E' ($=\phi_{E_2P}'$). This experiment is described in the following paper (Belasco et al., 1986), in which we show that the catalytic groups of proline racemase are, indeed, thiols.

Supplementary Material Available

Equations that allow the small corrections to be applied for the enantiomeric contamination of substrate and for the extent of reaction (9 pages). Ordering information is given on any current masthead page.

Registry No. $^2H_2$, 7782-39-0; proline racemase, 9024-09-3.

References

Albery, W. J., & Davies, M. H. (1969) *Trans. Faraday Soc. 65*, 1059–1065.

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5588–5600.

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

Belasco, J. G., Albery, W. J., & Knowles, J. R. (1983) *J. Am. Chem. Soc. 105*, 2475–2477.

Belasco, J. G., Bruice, T. W., Albery, W. J., & Knowles, J. R. (1986) *Biochemistry* (following paper in this issue).

Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970–3978.

Dewar, M. J. S. (1984) *J. Am. Chem. Soc. 106*, 209–219.

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* (preceding paper in this issue).

Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114.

Hermes, J. D., Morrical, S. W., O'Leary, M. H., & Cleland, W. W. (1984a) *Biochemistry 23*, 5479–5488.

Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., Morrison, J. F., & Cleland, W. W. (1984b) *Biochemistry 23*, 6263–6275.

Huskey, W. P., & Schowen, R. L. (1983) *J. Am. Chem. Soc. 105*, 5704–5706.

Kresge, A. J. (1964) *Pure Appl. Chem. 8*, 243–258.
Limbach, H.-H., Henning, J., Gerritzen, D., & Rumpel, H. (1982) *Faraday Discuss. Chem. Soc. 74*, 229–243.
Rudnick, G., & Abeles, R. H. (1975) *Biochemistry 14*, 4515–4522.
Saunders, W. H. (1985) *J. Am. Chem. Soc. 107*, 164–169.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64–99, University Park Press, Baltimore, MD.
Szawelski, R. J., Wharton, C. W., & White, S. (1982) *Biochem. Soc. Trans. 10*, 232–233.

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

Joel G. Belasco,‡ Thomas W. Bruice,§ W. John Albery,‖ and Jeremy R. Knowles*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received September 20, 1984; Revised Manuscript Received December 19, 1985

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

† This work was supported by the National Science Foundation and Merck Sharp & Dohme.
‡ National Science Foundation Predoctoral Fellow. Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.
§ National Institutes of Health Postdoctoral Fellow. Present address: Department of Pathology, UCLA School of Medicine, Los Angeles, CA 90024.
‖ Present address: Department of Chemistry, Imperial College of Science and Technology, London, SW7 2AY England.

0006-2960/86/0425-2558$01.50/0