Energetics of Proline Racemase: Tracer Perturbation Experiments Using [^{14}C]Proline That Measure the Interconversion Rate of the Two Forms of Free Enzyme†

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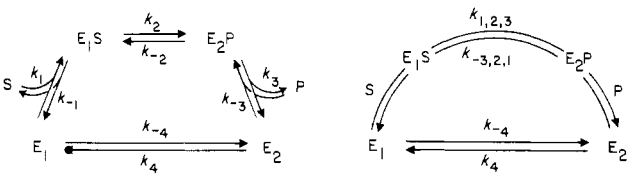
ABSTRACT: Proline racemase exists in two states, one of which binds and isomerizes L-proline and the other of which binds and isomerizes D-proline. In the enzyme-catalyzed racemization of proline at high substrate concentrations, the interconversion of the two forms of the free enzyme becomes rate limiting. The tracer perturbation method of Britton (1966, 1973) vividly demonstrates the kinetic importance of this enzyme interconversion under oversaturating conditions and allows an estimate of the rate constant for this reaction of 10^5 s^{-1}. It is further shown that the enzyme is bound state saturated and that the peak-switch concentration, c_P, is 125 mM. At substrate concentrations higher than 125 mM the enzyme becomes oversaturated, and the reaction rate is limited by the transition state for the interconversion of the two forms of the free enzyme. It seems likely that the two free enzyme forms differ only in the protonation states of the acidic and basic groups at the active site.

The early studies of Abeles and his group on proline racemase (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975) have delineated many of the fundamental characteristics of the catalysis mediated by this enzyme. The catalyzed reaction involves two enzyme bases: one (in its basic form) abstracts the substrate's 2-proton and the other (in its acidic form) donates a solvent-derived proton to the substrate's 2-carbon atom from the opposite side. Further, Rudnick and Abeles (1975) reported that there are two forms of the enzyme, one of which only binds L-proline and the other of which only binds the D enantiomer. This conclusion was based upon the following observations. At saturating substrate levels, the initial rate of the enzyme-catalyzed release of ^{3}H from L-[2-^{3}H]-proline into the solvent is independent of the initial L-proline concentration, whereas the initial rate of enzyme-catalyzed ^{3}H release from DL-[2-^{3}H]proline *decreases* with increasing DL-proline concentration. These results are nicely accommodated by a pathway involving the ordered release of the proline product and then of the substrate-derived proton. At high product proline concentrations, the substrate-derived proton (which is transiently bound to the enzyme after product dissociation) can be captured by the binding of a new product molecule before the isomerization of the enzyme. In terms of Scheme I, E_2 may be captured by P more rapidly than it isomerizes to E_1.

The existence of a detectable [and, as shown in the preceding paper (Fisher et al., 1986), kinetically important] isomerization of free proline racemase prompted us to apply the elegant method of Britton (1966, 1973) both to determine the rate of isomerization of the free enzyme forms and further to define the nature of the three kinetic regimes displayed by this enzyme. The tracer perturbation method of Britton (1966, 1973) involves the measurement of the time-dependent distribution of radiolabeled substrate and product initially at equilibrium, when the system is perturbed by the addition of a relatively large amount of an *un*labeled substrate. After the perturbation, the distribution of radiolabeled substrate and product is followed as the reaction proceeds and equilibrium is reestablished.

Scheme I: Pathway of the Proline Racemase Reaction[a]



[a] The two forms of the free enzyme, E_1 and E_2, catalyze the interconversion of the two substrates S and P.

Experimental Procedures

Materials

In addition to the materials identified in Fisher et al. (1986), D-amino acid oxidase (hog kidney) and catalase (bovine liver) were from Sigma, L-[U-^{14}C]proline (270 mCi mmol^{-1}) was obtained from Amersham/Searle, Kodak *X*-Omat XR5 film was from Eastman Kodak, Bio-Rex 40 (H^+ form, 100–200 mesh) was from Bio-Rad Laboratories, Dowex-1 (Cl^- form, 8% cross-linked, 200–400 mesh) was from Sigma, and aluminum-backed silica gel thin-layer chromatography (TLC) plates were from E. M. Merck.

L-Proline. Specially pure L-proline was prepared as follows. Commercial L-proline (4 g) and NH_4HCO_3 (100 mg) were dissolved in water (20 mL), and the pH was adjusted to 8.3 with 2N NH_4OH. To this solution was added FAD (0.5 mg), catalase (1 mg), and D-amino acid oxidase (6.4 units); the flask was flushed with O_2 and the mixture incubated in the dark for 24 h at 37 °C under slight O_2 pressure and gentle agitation. More enzyme (2 units) was then added and the incubation continued for a further 48 h. The mixture was then freeze-dried, and methanol (50 mL) and charcoal (~1 g) were added to the residue. After warming to 60 °C, the mixture was filtered, and the filtrate was evaporated to dryness. Two recrystallizations from EtOH gave L-proline (1.6 g).

N-(Trifluoroacetyl)-L-prolyl chloride was prepared according to the method of Dabrowiak and Cooke (1971). The

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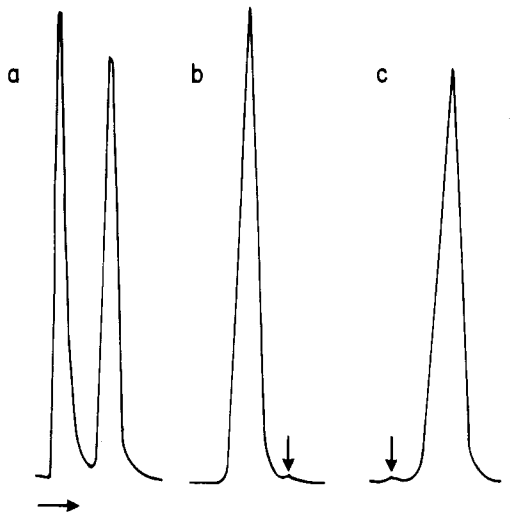

FIGURE 1: Gas chromatographic traces of the diastereoisomeric dipeptides resulting from the reaction of samples of the methyl ester of proline with *N*-(trifluoroacetyl)-L-prolyl chloride: (a) DL-proline, (b) D-proline, and (c) L-proline. For details, see Experimental Procedures.

product was dissolved in dry $CHCl_3$ and transferred under N_2 in portions to a number of vials, which were stored at −20 °C. To check the optical purity of the *N*-(trifluoroacetyl)-L-prolyl chloride and for any epimerization in the coupling reaction with proline methyl ester, a sample of the chloride was coupled with L-proline methyl ester made from the purified L-proline sample used in the synthesis of the *N*-(trifluoroacetyl)-L-prolyl chloride. The coupling reaction was done according to the procedure of Iwase and Murai (1974). The resulting protected dipeptide was analyzed by gas–liquid chromatography (GLC),[1] which showed <0.3% contamination from *N*-(trifluoroacetyl)-L-prolyl-D-proline methyl ester. The diastereoisomeric dipeptide derivatives were separated on a glass column (8 ft × 0.25 in.) containing 3% OV22 (65% phenyl methyl silicone) on 80/100 mesh Supelcoport run at 230 °C (injector at 220 °C, detector at 235 °C) with N_2 as the carrier gas (see Figure 1). Enantiomeric compositions of proline obtained by integration of the GLC peaks for *N*-(trifluoroacetyl)-L-prolyl-L-proline methyl ester and its L–D diastereoisomer were in excellent agreement with those expected from the optical rotation of the proline samples used.

Methods

Analytical and kinetic methods were as described by Fisher et al. (1986). All incubations and measurements of optical rotation were done at 37 °C. Scintillation counting was carried out in a Beckman LS-233 scintillation counter. Gas chromatographic analyses were done on a Varian Aerograph Series 1400 instrument.

Racemase Reaction. L-Proline (32.6 mM) and L-[U-^{14}C]proline (10 μCi) were incubated in 200 mM Tris-HCl buffer (2.2 mL), pH 8.0, containing 2-mercaptoethanol (20 mM) and EDTA (8 mM). Proline racemase (2.38 units in 50 μL of the above buffer) was then added, and the optical rotation of a portion (1 mL) of the mixture was monitored at 365 nm. When the optical rotation had fallen to zero, a sample (100 μL) was quenched in 0.1 N NaOH (0.5 mL) and retained for radiochemical analysis. To start the perturbation reaction, a solution of L-proline (517 mM) in Tris-HCl buffer (1 mL), pH 8.0, containing 2-mercaptoethanol (20 mM) and EDTA (8 mM), preincubated at 37 °C, was added to 2.0 mL of the above solution of equilibrated DL-[^{14}C]proline. (The initial concentrations of proline were the following: D-proline, 10.8 mM; L-proline, 183 mM.) The optical rotation of a portion (1 mL) of the mixture was monitored at 365 nm. At appropriate intervals, samples (100 μL) of the reaction mixture were quenched in 0.1 N NaOH (500 μL) and stored frozen at −20 °C until analyzed.

Proline Isolation. To free the samples from buffer components, the mixture was applied to a column (1.0 cm × 0.6 cm) of Dowex-1 (OH^- form). The column was washed with water (20 mL), and the proline was eluted in 2 N acetic acid (6 mL). The proline sample was applied to a column (1.0 cm × 0.6 cm) of Bio-Rex 40 (H^+ form). The column was washed with water (20 mL), and the proline was eluted in 2 N NH_4OH (6 mL). The proline was isolated by evaporating the appropriate fractions to dryness. The recovery of proline (based on radioactivity) was reproducibly 93% (~5.1×10^5 cpm, ~20 μmol).

Resolution. To half of each sample of proline (10 mmol, ~2.5×10^5 cpm) was added methanolic HCl [1.5 mL, made by adding redistilled thionyl chloride (1 mL) to anhydrous methanol (9 mL) at −77 °C], and the solutions were quickly transferred to screw cap vials (capacity 3.5 mL) fitted with Teflon–silicone septa. After incubation on a sand bath at 66–70 °C for 2.5 h, the vials were cooled, and the solvent was removed by inserting a hypodermic needle through the septum of each vial and then placing the vials in a vacuum desiccator. When all the solvent had evaporated, a solution (100 μL) of *N*-(trifluoroacetyl)-L-prolyl chloride (170 mM) in anhydrous $CHCl_3$, followed by anhydrous triethylamine (5 μL), was then added to each vial. The solution in each vial was mixed by vortexing and left at room temperature for 15 min. These solutions of *N*-(trifluoroacetyl)-L-prolyl-L(and D)-proline methyl esters were used directly for analysis by TLC. For GLC analysis, the samples were shaken with water (100 μL) for 30 s, and the organic phase was then removed and dried over anhydrous Na_2SO_4. Since any chiral derivatizing reagent will react at different rates with the two enantiomers with which it is being coupled, it is essential that an excess of derivatizing reagent be used and that the extent of coupling be checked. Explicit checks on synthetic mixtures of L-proline and D-proline of known composition were performed by using a 1.9-fold molar excess of *N*-(trifluoroacetyl)-L-prolyl chloride; the proper proportions of the product diastereoisomers (assessed by GLC analysis) were formed in every case.

Analysis of Diastereoisomers. The mixtures of diastereoisomeric dipeptide derivatives were separated by TLC of small samples (5 or 10 μL containing ~2.5×10^4 cpm) on aluminum-backed plates (5 × 20 cm) of silica gel. The plates were eluted with ethyl acetate, dried, marked with ^{14}C-labeled ink, and subjected to radioautography for 24 h. The developed films were used to locate the radioactive dipeptide derivatives (R_f of L–L derivative, 0.37; R_f of L–D derivative, 0.30), which were recovered by cutting out pieces of the TLC plate of uniform size containing them. These pieces were subjected directly to scintillation counting by using a toluene-based cocktail [toluene, 2460 mL; ethanol, 1120 mL; naphthalene, 210 g; 2,5-diphenyloxazole, 105 g; 2,2′-*p*-phenylenebis(phenyloxazole), 0.42 g]. An uncharacterized byproduct of the coupling reaction of R_f 0.56 did not interfere with the analysis of the radioactivity distribution.

Theory

Using the composite rate constants of Scheme I, we can

[1] Abbreviations: GLC, gas–liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

Table I: Tracer Perturbation Experiment for Proline Racemase in 200 mM Tris-HCl buffer, pH 8.0 at 37 °C[a]

optical rotation[b] (deg)	concn of L-proline[c] (s) (mM)	^{14}C in L-proline[d] (cpm)	^{14}C in D-proline[d] (cpm)	fraction of radioactivity in L-proline (s^*/c^*)	c_P (mM)
0.0	10.9	5605	5875	0.488	
−5.048	181	8670	8275	0.512	181[e]
−4.909	179	9130	8080	0.531	138
−4.648	174	10330	8050	0.562	126
−4.146	166	9532	6267	0.603	127
−3.543	156	10890	6030	0.644	115
−3.130	149	10900	5770	0.654	115
−2.598	140	9115	5990	0.651	118
−2.072	132	9850	5430	0.646	105
−1.155	116	9760	6800	0.589	125
0.0	97	7730	8520	0.482	
					121 ± 4

[a] The buffer contained 2-mercaptoethanol (20 mM) and EDTA (8 mM). Concentrations of proline before perturbation: L-[^{14}C]proline = D-[^{14}C]proline = 16.3 mM. Concentrations of proline immediately after perturbation: L-proline = 183 mM; D-proline = 10.9 mM. [b] Measured at 365 nm, 10-cm path length. [c] Calculated from the optical rotation measurements. [d] Determined after TLC separation of the diastereoisomeric *N*-trifluoroacetyl-L-prolyl[^{14}C]proline methyl ester derivatives. [e] This first value was omitted from the calculation of the mean value of c_P.

write down the following expression for the flux f of the isotopically unlabeled species:

$$f = k_{1,2,3}e_1 \cdot s - k_{-3,2,1}e_2 \cdot p \quad (1)$$

$$f = k_4 e_2 - k_{-4} e_1 \quad (2)$$

Similarly, the flux f^* of the labeled species is given by

$$f^* = k_{1,2,3}e_1 \cdot s^* - k_{-3,2,1}e_2 \cdot p^* \quad (3)$$

where s^* and p^* are the concentrations of labeled S and P, respectively. Elimination of e_1 and e_2 from eq 1–3 gives

$$\frac{f^*}{f} = \frac{2(s^*p - sp^*) + c_P(s^* - p^*)}{c_P(s - p)} \quad (4)$$

where the peak-switch concentration, c_P (Albery & Knowles, 1986; Fisher et al., 1986), is given by

$$c_P = 2k_4/k_{-3,2,1} = 2k_{-4}/k_{1,2,3}$$

and describes the substrate concentration at which the rate-limiting transition state shifts from a catalytic step (steps 1–3) to the enzyme interconversion step (transition state 4). Writing as before the total concentration of unlabeled subtrates ($s + p$) as c, and the total concentration of labeled substrates ($s^* + p^*$) as c^*, eq 4 becomes

$$\frac{d(s^* - p^*)}{d(s - p)} = \frac{(c + c_P)(s^* - p^*)}{c_P(s - p)} - \frac{c^*}{c_P} \quad (5)$$

Since c and c^* are constant throughout the reaction, we can integrate ($s^* - p^*$) with respect to ($s - p$) to obtain

$$\frac{s^* - p^*}{s - p} = \frac{c^*}{c}\left[1 - \left(\frac{s - p}{s_0 - p_0}\right)^{c/c_P}\right] \quad (6)$$

using the boundary condition that at the start of the reaction when $s = s_0$ and $p = p_0$, the labeled species are in their equilibrium proportions, which for a racemase means that $s^* = p^*$.

Equation 6 can be rearranged to give

$$\frac{c_P}{c} = \frac{\ln[(s - p)/(s_0 - p_0)]}{\ln\left[1 + \frac{c(s^* - p^*)}{c^*(s - p)}\right]} \quad (7)$$

This equation allows one to calculate c_P for any point where the distribution of the label ($s^* - p^*$) is measured as a function of the overall conversion of S to P, which is determined by following the optical rotation.

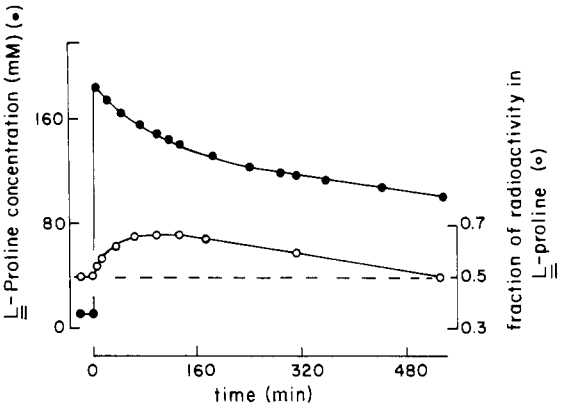

FIGURE 2: Tracer perturbation experiment for the reaction catalyzed by proline racemase at high substrate concentrations with ^{14}C-labeled substrates in 200 mM Tris-HCl buffer, pH 8.0.

RESULTS

The tracer perturbation experiment was performed as follows. An equilibrated mixture of 32.6 mM DL-[^{14}C]proline in the presence of proline racemase was perturbed by the addition of unlabeled L-proline to a concentration of 183 mM. The progress of the racemization reaction was monitored polarimetrically, and the distribution of isotopically labeled species was measured by the withdrawal of samples, from which proline was isolated and resolved and the ^{14}C content of each enantiomer was determined.

The results from the tracer perturbation reaction run in Tris-HCl buffer are summarized in Table I. When these data are plotted graphically (Figure 2) it is evident that there is a flux of radioactive material from D-proline to L-proline, *in the opposite direction* to the overall flux of proline, which is in the L to D direction as the L-proline racemizes. In contrast to this result, when the reaction is run in ammonium bicarbonate buffer, *no* perturbation of the tracer [^{14}C]proline is observed (Figure 3).

The values for c_P calculated by using eq 7 and the results from the perturbation experiment shown in Figure 2 are listed in Table I. The average value of c_P is 121 ± 4 mM. Duplication of the radioactivity measurements gave a value of 130 ± 5 mM.

DISCUSSION

In the preceding paper (Fisher et al., 1986) we have presented kinetic evidence consistent with the view that the rate of the reaction catalyzed by proline racemase is, at very high

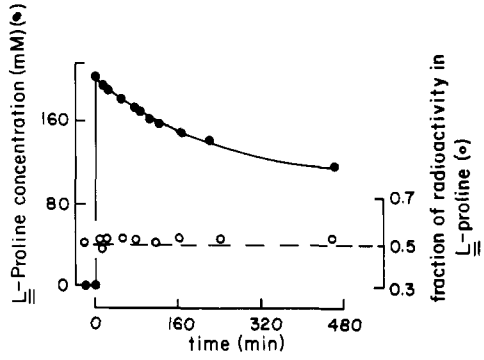

FIGURE 3: Tracer perturbation experiment for the reaction catalyzed by proline racemase at high substrate concentrations with ^{14}C-labeled substrates in 200 mM ammonium bicarbonate buffer, pH 8.0. Aside from the change in buffer, the reaction conditions and substrate concentration levels were very close to those for the experiment shown in Figure 2.

("oversaturated") substrate levels, limited by an obligatory isomerization of the free enzyme. The kinetic results also showed that, in the saturated region, the catalyzed reaction rate is determined *either* by the substrate interconversion (steps 1–3) *or* by the enzyme isomerization (step 4). The data did not allow us to define which range of transition states dominate the saturated region, that is, whether the enzyme showed "bound-state" or "free-state" saturation. In the present paper, we present further evidence that proves the kinetic importance of enzyme interconversion at oversaturating substrate concentrations and that defines which range of transition states controls the kinetics in the saturated region.

When an equilibrated mixture of radioactive substrate and product is perturbed by the addition of a large amount of one unlabeled substrate, if the enzyme is relatively slow to return from its "product" state to its "substrate" state, then one may observe a flux of radiolabeled material that is in the opposite direction to the (much larger) flux of unlabeled materal. This was first recognized by Britton (1966, 1973), who has used the phenomenon to diagnose kinetically significant enzyme isomerizations for several enzymes (Britton & Clarke, 1968, 1972; Britton et al., 1971, 1972). From Figure 2, it is clear that proline racemase shows this behavior, which confirms the functional importance of enzyme isomerization in this system. [As pointed out by Britton (1973), the only mechanism other than a relatively slow enzyme isomerization that would lead to such "countertransport" is one where active sites interact in an oligomeric protein. Proline racemase, although a homologous dimer, has only one active site that comprises elements from each subunit (Rudnick & Abeles, 1975), so this alternative is not a possible one.] When the reaction is performed in ammonium bicarbonate buffer, countertransport is not observed (Figure 3), which agrees with the absence of oversaturation behavior in the kinetics of isomerization of unlabeled proline when the reaction is run in this buffer (data not shown).

The parameter c_P is the "peak-switch concentration" and describes the substrate concentration at which the rate-limiting transition state shifts from that of a catalytic step (1, 2, or 3) to that for the enzyme interconversion (4). In terms of the rate constants in Scheme I

$$c_P = 2k_{-4}/k_{1,2,3} = 2k_4/k_{-3,2,1} \quad (8)$$

That is, c_P is twice the ratio of the net rate constants for the two paths by which E_2 returns to E_1 (free enzyme isomerization, and reaction with P to produce S).

As was pointed out in the previous paper (Fisher et al., 1986), the rate constants for the unsaturated (k_U), saturated (k_{cat^+} and k_{cat^-}), and oversaturated (k_O) regions are related by

$$\frac{c_P}{2k_O} + \frac{2}{k_U c_P} = \frac{1}{k_{cat^+}} + \frac{1}{k_{cat^-}} \quad (9)$$

Since this is a quadratic equation, there is not a unique solution for c_P. However, provided that the saturated region exists over a reasonable concentration range (that is, that the two roots to the quadratic equation are significantly different from one another), then the two roots are

$$c_P \approx \frac{2k_U^{-1}}{1/k_{cat^+} + 1/k_{cat^-}} \quad (10)$$

and

$$c_P \approx 2k_O(1/k_{cat^+} + 1/k_{cat^-}) \quad (11)$$

Since c_P describes the partitioning between the two routes by which E_2 returns to E_1 (by free enzyme isomerization or by reaction with P to form S), if the correct root is eq 10, then since k_U always involves the transition states 1–3 (see Scheme I), the k_{cat} terms will be dominated by transition state 4: the system will be "free state saturated" [see Figure 3 of Fisher et al. (1986)]. That is, the overall rate-determining transformation will be from a free enzyme form to transition state 4 involving the isomerization of the two forms of unliganded enzyme. If, on the other hand, the correct root is eq 11, then since k_O always involves transition state 4, the k_{cat} terms will be dominated by the transition states 1–3 for S and P interconversion, and the system will be "bound state saturated". Here, the overall rate-determining transformation will be from an enzyme–substrate complex to transition states 1, 2, or 3 involving the interconversion of *bound* (liganded) enzyme–substrate complexes [see Figure 3 of Fisher et al. (1986)]. From the results reported in Table III of the preceding paper (Fisher et al., 1986), the two possible values for c_P are 2.9 (from eq 10) and 125 mM (from eq 11). Since the present work gives c_P = 125 mM (Table I), we may conclude that eq 11 gives the correct root of eq 9 and that c_D, the "dip-switch concentration" (Albery & Knowles, 1986), is 2.9 mM. This means that in the saturated region the enzyme interconversion steps k_4 and k_{-4} are fast relative to the substrate interconversion steps (1–3) and that the latter range of transition states is rate determining. Proline racemase is therefore bound state saturated. This finding now allows us to use the identity of the values of k_{cat^+} and k_{cat^-} established in the previous paper (Fisher et al., 1986). We can conclude that, for proline racemase, E_1S and E_2P have the same free energy and that $K_2 = 1$.

Finally, we may estimate the *rate* of free enzyme interconversion, k_4, as follows. On the reasonable basis that the free energy difference between E_1 and E_2 is relatively small (i.e., $K_4 \approx 1$, so $k_4 \approx k_{-4}$), eq 13 of the previous paper (Fisher et al., 1986) becomes

$$k_U \approx k_{1,2,3}/2$$

from which, with eq 8, we may write

$$k_4 \approx k_U c_P$$

The values of c_P (from the present paper) and of k_U [from Fisher et al. (1986)] give $k_4 \approx 10^5$ s^{-1}. The rate of interconversion of the two forms of free proline racemase is therefore less than a hundred times faster than k_{cat} [which is 2600 s^{-1}: Table III of Fisher et al. (1986)]. This contrasts with analogous estimates obtained by Britton and his group for several phosphomutases listed in Table II, for which the enzyme isomerization rates are more than 10^3-fold larger than k_{cat}.

Table II: Rates of Free Enzyme Isomerization Determined from Tracer Perturbation Experiments

enzyme	k_{cat} (s^{-1})	rate of free enzyme interconversion (s^{-1})	ref
phosphoglucomutase	7.5×10^2	$\sim 10^7$	Britton & Clarke (1968)
phosphoglycerate mutase[a]	1.6×10^3	$>10^6$	Britton et al. (1972)
phosphoglycerate mutase[b]	2.7×10^2	$>10^6$	Britton et al. (1971)
phosphoglycerate mutase[c]	1.9×10^3	$>4 \times 10^6$	Britton & Clarke (1972)
proline racemase	2×10^3	$\sim 10^5$	this work

[a] From yeast. [b] From wheat germ. [c] From rabbit muscle.

What the enzyme isomerization involves in chemical terms for proline racemase cannot yet be defined. Clearly the least that must happen is a reorganization of the protonation states of the basic and acidic groups responsible for the abstraction and supply of protons from and to the substrate, but we cannot be sure that a protein conformational change is not also required. It is tempting, however, to suggest that the enzyme interconversion *only* involves protonation state changes, since we have shown that the interconversion is very much faster (eliminating the oversaturation regime) in ammonium bicarbonate buffer. No tracer perturbation can be detected under these conditions (Figure 3). Catalysis of prototropy between a neighboring acid–base pair by ammonium ion and/or by bicarbonate ion is both attractive and precedented, whereas it is harder to see how, in molecular terms, such ions could catalyze a protein conformational change. The question of whether, in fact, the interconversion of the two forms of proline racemase at $\sim 10^5$ s^{-1} merely involves the redistribution of protons at the active site is discussed more fully in the sixth paper of this series (Belasco et al., 1986).

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Registry No. Proline racemase, 9024-09-3; L-proline, 147-85-3; D-proline, 344-25-2.

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