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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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the mechanism of the reaction catalyzed by proline racemase.

Experimental Procedures

Materials. The enzyme and the substrates used were as described earlier by Fisher et al. (1986a–c). Experiments under oversaturated conditions were performed at a total proline concentration of 0.4 M, unless otherwise noted.

Methods. The methods used to follow the progress of the reaction, to measure the rate of loss of ^3H from tritiated substrate, and to estimate the deuterium content of partially deuterated substrate have been described by Fisher et al. (1986a–c) and by Belasco et al. (1986b). For the solvent isotope effect experiments, all solutions except enzyme were made up in either H_2O or D_2O (99.7%). The reactions were run at 37 °C at a substrate concentration of 404 mM, in 200 mM Tricine-HCl buffer, pH 8.0, containing dithiothreitol (1 mM), (ethylenedinitrilo)tetraacetic acid (EDTA) (8 mM), and proline racemase (19 nM).

Results and Discussion

Deuterium Washout Experiments. For competitive deuterium washout experiments [such as those illustrated in Figures 3 and 4 of the preceding paper (Belasco et al., 1986a)], the size of the maximum perturbation in the optical rotation (when the deuterated substrate is S′) is given by eq 12 of Belasco et al. (1986a):

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

where

$$\sigma' - \sigma = \frac{(s + s') - (p + p'')}{s_0' + p_0''} \quad (2)$$

$$R' = \frac{2}{1 + p_P/p_\infty}\left[\frac{x'\phi_s}{\phi_4'} + \frac{\phi_s}{\phi_{1,2,3}'}\frac{p_P}{p_\infty}\right] \quad (3)$$

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

and

$$x' = k_4'/(k_4' + k_5') \quad (4)$$

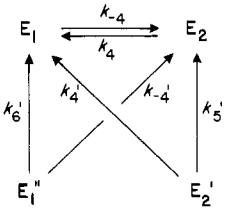
The quantity $\sigma' - \sigma$ is, from eq 2, the difference in the total concentrations of S and P (that is, the perturbation), divided by the total proline concentration, and is obtained from the measured optical rotation and the known initial substrate concentrations. The equation for R' (eq 3) is taken from the full treatment in Albery and Knowles (1986a). It may be noted that in the saturated region (where $p_\infty < p_P$) eq 3 simplifies to eq 24 of Fisher et al. (1986c), and in the oversaturated region (where $p_\infty > p_P$) eq 3 reduces to eq 6 of the preceding paper (Belasco et al., 1986a). Because there are two protonic sites in the enzyme, in this paper we must specify the site under consideration by a single prime or a double prime, as before (see footnote 2 of the preceding paper). Thus, R' and x' (eq 3 and 4) relate to the loss of isotope from the singly primed site in E_2' (by conversion of E_2' to E_1 via k_4' or by direct exchange of E_2' to E_2 via k_5'), and R'' and x'' (eq 5 and 6) relate to the loss of isotope from the doubly primed site in E_1''. These processes are illustrated in Scheme I.

$$R'' = \frac{2}{1 + s_P/s_\infty}\left[\frac{x''\phi_P}{\phi_6'} + \frac{\phi_P}{\phi_{1,2,3}''}\frac{s_P}{s_\infty}\right] \quad (5)$$

$$x'' = k_{-4}''/(k_{-4}'' + k_6'') \quad (6)$$

Values of $(\sigma' - \sigma)_{max}$ for several experiments under oversaturating conditions are reported in Table I.

Scheme I: Interconversion of Free Enzyme Forms[a]



[a] E_1 and E_2 are the two unlabeled forms of the enzyme, and E_1'' and E_2' are the two isotopically labeled forms of the enzyme.

Table I: Size of the Maximum Perturbation in the Optical Rotation under Oversaturated Conditions[a]

expt	s_0	s_0'	p_0	p_0''	$(\sigma' - \sigma)_{max}$
1	0	100	100	0	0.46_7
2	0	200	200	0	0.45_7
3	160	40	200	0	0.45_7
4	100	0	0	100	0.41_0
5	200	0	0	200	0.38_7
6	200	0	160	40	0.38_7

[a] All concentrations are in mM.

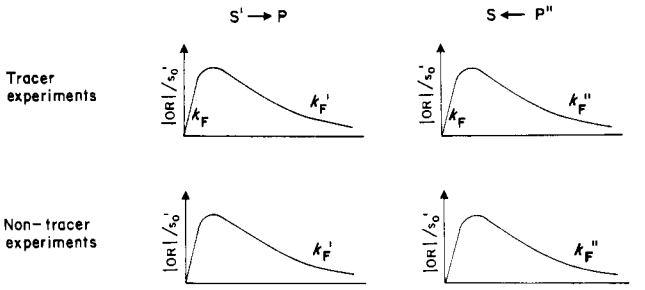


FIGURE 1: Schematic time courses for tracer and nontracer competitive deuterium washout experiments. The tracer experiments obey double-exponential expressions and yield the rate constants shown. The nontracer experiments follow an exponential decay toward the end of the perturbation, yielding the rate constants shown. The initial phase of the nontracer experiment has a more complex time course.

In addition to the size of the perturbation maximum, we can analyze the time course of the optical rotation changes. Whereas in the preceding paper (Belasco et al., 1986a) we compared the time course of a tracer experiment with that of the analogous nontracer experiment, in the present paper we compare forward and reverse directions for the tracer experiments. For *tracer* experiments, we have shown [eq 10 of Belasco et al. (1986a)] that the variation of the optical rotation with time is given by a double-exponential expression. The fast rise in the optical rotation described by k_F arises from the rapid equilibration of unlabeled S and P, and the fall in the rotation, described by k_F' (or k_F''), arises from the slower washout of the isotope from S′ (or from P″). As discussed in the preceding paper, *nontracer* experiments have a more complex time course, though we can obtain k_F' and k_F'' from the tail end of the perturbations. These relationships are illustrated in Figure 1. The experimental data from Figures 3 and 4 of the preceding paper (Belasco et al., 1986a) are so fitted in Figures 2 and 3, from which we obtain the rate constants for the two processes, k_F and k_F' for experiments starting with S′ and k_F and k_F'' for experiments starting with P″. These rate constants are reported in Table II and provide, by using eq 7 and 8, values of R′ and R″.

$$k_F/k_F' = R' \quad (7)$$

$$k_F/k_F'' = R'' \quad (8)$$

It is gratifying that the values of k_F, which relate to the

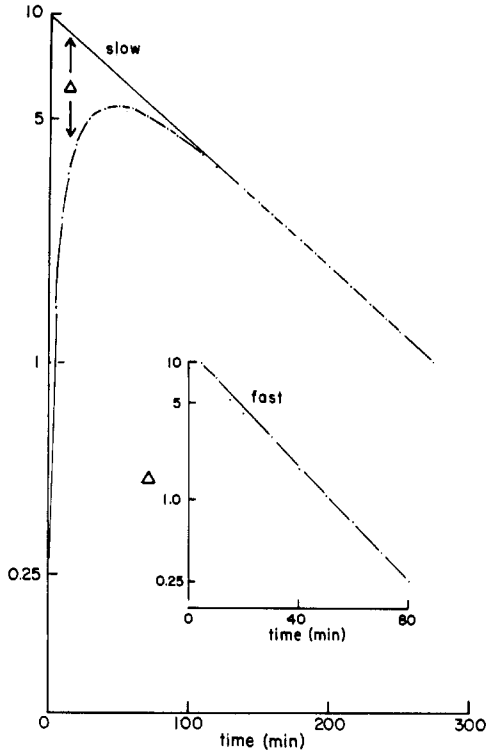


FIGURE 2: Double-exponential plots for the proline racemase catalyzed reaction of L-[2-^{2}H]proline plus D-[2-^{1}H]proline. The starting conditions were as described for experiment 3 (Table I). The absolute value of optical rotation is plotted (log scale) vs. time, and the "slow" reaction rate (k_F': for the second exponential) is derived from the late time points. The "fast" reaction rate (k_F: for the first exponential) is derived from plotting the values of Δ (log scale) vs. time (see inset). The derived rate constants are listed in Table II.

Table II: Rate Constants from Tracer Deuterium Washout Experiments

expt[a]	perturbing substrate	k_F (min^{-1})	k_F' (min^{-1})	k_F'' (min^{-1})	R'[b]	R''[b]
3	S′	49×10^{-3}	8.6×10^{-3}		5.7	
6	P″	46×10^{-3}		10.6×10^{-3}		4.3

[a] For conditions, see Table I. The values for k_F' are as cited in Table I of the preceding paper (Belasco et al., 1986a). [b] Calculated from k_F/k_F' or k_F/k_F'' (eq 7 and 8).

racemization of the undeuterated species P and S, are essentially the same whether the system is perturbed with S′ (experiment 3) or with P″ (experiment 6). In contrast, the slower rate constants k_F' and k_F'' are not identical, being in the ratio of 0.8. This ratio is the ratio of rates of the S′-to-P and the P″-to-S reaction, which can be measured more precisely in a *double*-competitive deuterium washout experiment, in which we start with equal concentrations of all labeled and unlabeled substrates: $s_0 = p_0 = s_0' = p_0''$. From this experiment [using eq 32 of Fisher et al. (1986c)] we obtain ξ, where

$$\xi = k_F'/k_F'' = R''/R'$$

The value of ξ has been measured at total proline concentrations spanning the saturated and oversaturated regions (50, 100, and 200 mM). We find that ξ is *independent* of the total concentration of substrate and remains at 0.86 as we go from the saturated region into the oversaturated region. This constancy will be discussed later. Since results from competitive experiments in which S′ and P″ compete directly in the same reaction mixture will always be more precise than a comparison of two separate experiments, we shall use 0.86 as the preferred value of ξ.

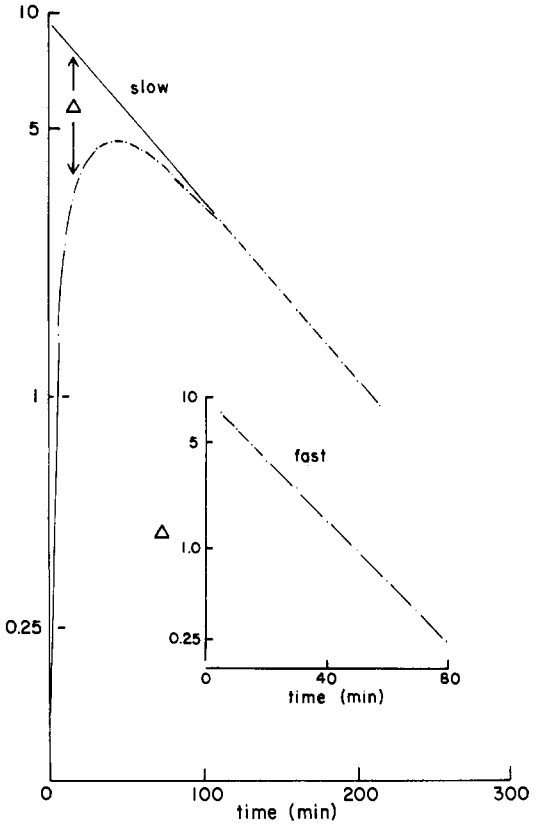


FIGURE 3: Double-exponential plots for the proline racemase catalyzed reaction of D-[2-^{2}H]proline plus L-[2-^{1}H]proline. The starting conditions were as described for experiment 6 (Table I). The absolute value of optical rotation is plotted (log scale) vs. time, and the "slow" reaction rate (k_F'': for the second exponential) is derived from the late time points. The "fast" reaction rate (k_F: for the first exponential) is derived from plotting the values of Δ (log scale) vs. time (see inset). The derived rate constants are listed in Table II.

Table III: Deuterium Content of Proline at the Perturbation Maximum and Values of the Crossover Parameter, x

expt[a]	σ_{max}'	R'	R''	x'[b]	x''[e]
1	0.698	6.0[c]		0.99	
2	0.688	5.6[c]		0.92	
3		5.7[d]		0.95	
4	0.656		4.7[f]		0.81
5	0.635		4.1[f]		0.74
6			4.3[d]		0.81

[a] For conditions, see Table I. [b] Calculated from eq 11. [c] Calculated from eq 9. [d] Value from Table II. [e] Calculated from the equation (for the doubly primed site) analogous to eq 11. [f] Calculated from the equation (for the doubly primed site) analogous to eq 9.

There is more kinetic information contained in the washout experiments shown in Figures 3 and 4 of the preceding paper (Belasco et al., 1986a), since we may measure the *deuterium content* of the proline at the time of the maximum in the perturbation of the optical rotation. Equation 11 of the preceding paper (Belasco et al., 1986a) states that this quantity, σ_{max}', is

$$\sigma_{max}' = (s'/s_0')_{max} = R'^{1/(1-R')} \qquad (9)$$

Results for σ_{max}' and the derived values of R' (where S′ is the perturbant) and R'' (where P″ is the perturbant) are given in Table III. There is good agreement between the value of R' of 5.7 obtained from the ratio of rate constants (eq 7) in the tracer experiment (experiment 3) and the value of 5.6 obtained under the same conditions of oversaturation from the value of σ_{max}' (eq 9) (experiment 2). Similarly good agreement is found for the values of R″: we obtain 4.3 from the rate

Table IV: Values of ϕ_4' and ϕ_4'' from Deuterium Washout Experiments

expt[a]	ϕ_4'/ϕ_S[b]	expt[a]	ϕ_4''/ϕ_P[b]
1	0.34	4	0.47
2	0.37	5	0.54
3	0.36	6	0.51
mean value	0.36 ± 0.01		0.51 ± 0.02
preferred values[c]	0.36		0.42

ϕ_4'[d]	$\phi_{1,2,3}'$[e]	ϕ_4''[d]	$\phi_{1,2,3}''$[e]
0.42	0.37_5	0.49	0.44

[a] For conditions, see Table I. [b] Calculated from eq 3 and 5. [c] The x' data are considered more accurate than the x'' data,[1] and ϕ_4'' is based upon $\phi_4'/\phi_4'' = 0.86$ (which derives from a direct competitive experiment). [d] Using $\phi_S = 1.17$ (Belasco et al., 1986a). [e] Fisher et al. (1986c).

constant ratio of the tracer experiment (experiment 6) and 4.1 from the measurement of σ_{max}' (experiment 5).

Crossover Parameters x' and x''. As discussed earlier (Belasco et al., 1986a), measurement of the size of the perturbation maximum in the oversaturated region and of the deuterium content of the substrate at that maximum allows us to determine the crossover parameters x' and x'' and discover how isotope is lost from E_2' or E_1''. Had the system been completely oversaturated, then eq 1 could have been used to obtain x. Since, however, at the highest accessible substrate levels there is still some contribution from saturated terms, we must use the full equation for $(\sigma' - \sigma)_{max}$ [eq 7.16 of Albery and Knowles (1986a)]:

$$(\sigma' - \sigma)_{max} = \frac{2R'^{R'/(1-R')}}{1 + p_P/p_\infty}\left[\frac{p_P}{p_\infty}\left(\frac{\phi_S}{\phi_{1,2,3}} - 1\right) + x'\left(\frac{\phi_S}{\phi_4} - 1\right)\right] \quad (10)$$

Under cleanly oversaturated conditions ($p_\infty \gg p_P$), this equation reduces [using eq 6 of Belasco et al. (1986a)] to the simpler eq 1 of this paper. Now, substitution of eq 3 in eq 10 gives

$$x' = \frac{R'(1 + p_P/p_\infty)}{2}[1 - R'^{1/(R'-1)}(\sigma' - \sigma)_{max}] - \frac{p_P}{p_\infty} \quad (11)$$

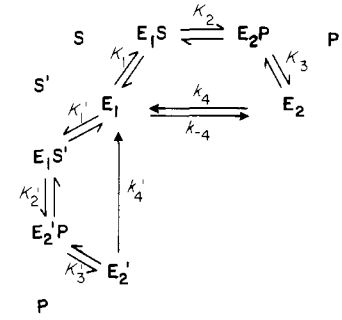
Using the values of R' and R'' already derived and the values of $(\sigma' - \sigma)_{max}$ from Table I, we obtain x' and x'' as listed in Table III.

The values of x' and x'' in Table III are all close to unity, which indicates that the isotope in E_2' is lost by crossover to E_1 and the isotope in E_1'' is lost by crossover to E_2. While the values of x'' are somewhat further from unity than those for x', in view of the overall symmetry of the proline racemase system and in the light of the invariance of ξ (the ratio of the transition state factors for loss of isotope from E_1'' and E_2'), we attribute these differences to experimental uncertainty. We conclude that isotope is washed out from E_1'' and E_2' to form E_2 and E_1, respectively. In terms of Scheme I, k_5' and k_6' are insignificant.

Transition-State Fractionation in Enzyme Interconversion. From the above discussion, we know that only the k_4 terms in Scheme I are important for the interconversion of the unliganded enzyme forms, and the fractionation factors obtained for enzyme interconversion are therefore ϕ_4' and ϕ_4''. These results are listed in Table IV.[1] The double-competitive

[1] In general, it appears that results using S′ (i.e., labeled L-proline) are more reliable than those using P″ (i.e., labeled D-proline). This may be because the enantiomeric purity of S and S′ is usually higher, by virtue of the opportunity to remove traces of P and P″ by using D-amino acid oxidase.

Scheme II: Reaction Scheme for the Reaction of Unlabeled Substrate (S) or Labeled Substrate (S′) with the Enzyme[a]



[a] E_1 and E_2 are the two unlabeled forms of the free enzyme. E_2' is the labeled form of E_2. k_n are the rate constants, and K_n are equilibrium constants.

deuterium washout experiment measures ξ, the ratio of the fractionation factors for the rate limiting transition state, which in the oversaturated region is ϕ_4'/ϕ_4''. As mentioned above, ξ is 0.86, and the preferred values of ϕ_4' and ϕ_4'' which obey this ratio are quoted in Table IV. For comparison, the values of $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ are also listed. It is evident that the values of ϕ_4 (which describe the transition-state fractionation in the process that interconverts free E_1 and free E_2) are rather similar to the values of $\phi_{1,2,3}$ (which describe the transition-state fractionation in the process that interconverts E_1 and E_2 and S and P via E_1S and E_2P). The similarity of these factors suggests some analogy between the two processes, which is discussed in the following paper (Albery & Knowles, 1986b)

Tritium Washout Experiments. Further confirmation of the above conclusions is provided by studying the loss of tritium from S′ as a function of the conversion of S to P (starting with $p_0 = 0$) in the oversaturated region. Scheme II shows the relevant species in such an experiment. Since the tritium is present at tracer levels and since the system is oversaturated, the enzyme is predominantly in the form of E_1S and E_2P. Furthermore, since we know (Fisher et al., 1986b) that $K_2 = 1$, the concentrations of E_1S and E_2P are each $e_\Sigma/2$, where e_Σ is the total enzyme concentration. Consideration of the fluxes then allows us to write

$$f = -\frac{1}{2}\frac{d(s-p)}{dt} = \frac{e_\Sigma}{2}\left(\frac{k_4K_3}{p} - \frac{k_{-4}K_1^{-1}}{s}\right) = \frac{e_\Sigma}{2}\frac{k_4k_3(s-p)}{sp} \quad (12)$$

and

$$f' = -\frac{ds'}{dt} = \frac{e_\Sigma}{2}k_4K_3\frac{\Phi_4'}{\Phi_S}\frac{s'}{sp} \quad (13)$$

From eq 12 and 13 we find

$$d \ln (s') = (\Phi_4'/2\Phi_S)\, d \ln (s - p)$$

Integration with the boundary conditions that at $t = 0$, $s' = s_0'$ and $p = 0$, gives

$$\ln (s'/s_0') = (\Phi_4'/2\Phi_S) \ln (1 - p/p_\infty) \quad (14)$$

While in practice the system is not completely oversaturated, the necessary corrections are considered in the Appendix and shown to be negligible.

Figure 4 shows plots of the tritium washout results according to eq 14 for the reaction of S′ (L-[2-^{3}H]proline) at 101 and 198 mM. The observed linearity of these plots confirms the validity of eq 14, and the gradients of the lines give values of Φ_4'/Φ_S of 0.194 and 0.200, respectively. Application of the

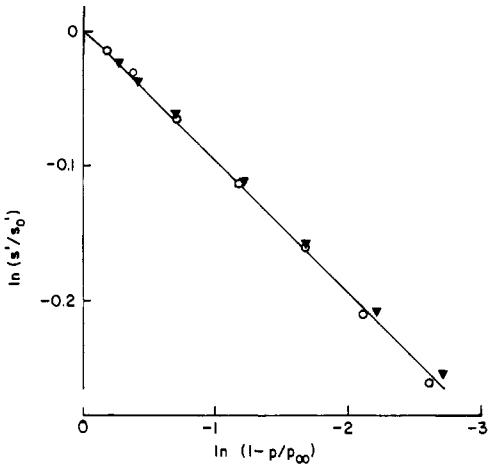


FIGURE 4: Tritium release from L-[2-^{3}H]proline as a function of the extent of reaction. The logarithm of the tritium content of the substrate [expressed by s'/s_0'] is plotted vs. the logarithm of the extent of reaction [expressed by $(1 - p/p_\infty)$] (see eq 14). Concentrations of L-[2-^{3}H]proline: (○) 198 mM; (▼) 101 mM. The line for the experiments at 101 mM is not drawn, for clarity. All experiments were performed in 200 mM Tris-HCl buffer, pH 8.0.

Swain–Schaad relation (Swain et al., 1958) [*pace* any small deviation arising from the fact that Φ_4' may be a mixed factor (Northrop, 1975; Albery & Knowles, 1977)] yields a value of ϕ_4'/ϕ_S of 0.33, in good agreement with the value of this parameter obtained from the deuterium washout experiments in Table IV.

Catalysis of the Enzyme Interconversion. As has been mentioned earlier (Fisher et al., 1986b), the use of certain buffers such as ammonium, hydrazinium, and hydrogen sulfide appears to accelerate the interconversion of the two forms of the free enzyme, thus removing the effects of oversaturation (which depend upon the enzyme interconversion being at least partly rate limiting). When the first-order rate constant k_1 for the isomerization is plotted as before vs. the total substrate concentration (Fisher et al., 1986a), we find that the catalyzing buffers have no effect on k_I in the saturated region but do cause an increase in this rate constant under oversaturating conditions.

Let us suppose that the enzyme interconversion steps can be catalyzed by both the acid HA and its conjugate base A, so that the first-order rate constant k_I (Fisher et al., 1986a) becomes

$$\frac{1}{k_I} = \frac{1}{k_{sat}} + \frac{s_0}{8(k_O + k_{HA}[HA] + k_A[A])} \qquad (15)$$

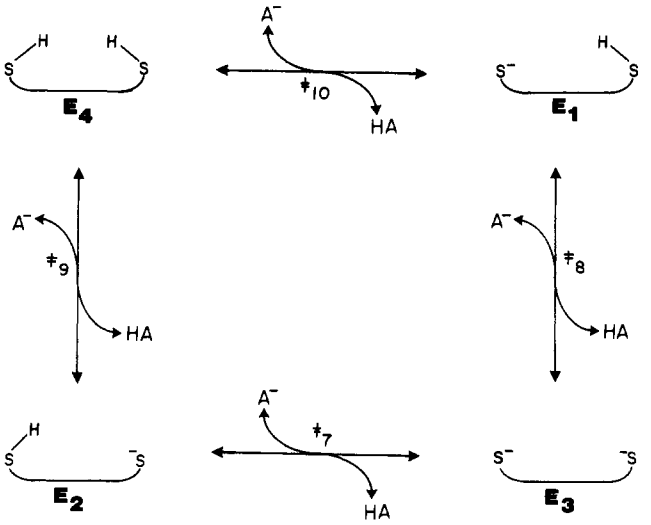
where k_{sat} is the rate constant in the saturated region (for proline racemase, $k_{sat} = 2k_{cat}$), k_O is the zero-order rate constant in the oversaturated region, k_{HA} is a first-order rate constant describing acid catalysis by HA in the oversaturated region, and k_A is a first-order rate constant describing base catalysis by A in the oversaturated region.

In eq 15 one can see how the acid and base catalysis terms (k_{HA} and k_A) describe parallel routes to the uncatalyzed enzyme interconversion reaction described by k_O. When the catalytic terms are large, there will be no oversaturation behavior.

We may now write for the flux, f_A, caused by catalysis by either HA or A:

$$f_A = k_{HA}[HA] + k_A[A] = \frac{s_0/8}{k_I^{-1} - k_{sat}^{-1}} - \left[\frac{s_0/8}{k_I^{-1} - k_{sat}^{-1}}\right]_0 \qquad (16)$$

Scheme III: Interconversion of Free Enzyme Forms[a]



[a] E_1 and E_2 are the two active monoprotonated forms of the enzyme, E_3 is the doubly deprotonated enzyme, and E_4 is the doubly protonated enzyme. HA is a general acid, and A^- is its conjugate general base.

where the subscript zero denotes the values of the rate constants determined in the *absence* of catalyzing buffer. The first-order rate constants k_{HA} and k_A describe the reaction of the bound states of the enzyme (E_1S and E_2P) to the transition state(s) for enzyme interconversion.

In Scheme III we show the different routes for the catalyzed interconversion of the free enzyme forms, where $(k_{9,10})_{HA}$ and $(k_{7,8})_A$ describe the acid- and base-catalyzed paths, respectively. Since the observed rate constants k_{HA} and k_A relate to the catalysis of the slowest step in oversaturation (from E_1S and E_2P to the transition states for the interconversion of E_2 and E_1), we may write

$$k_{HA} = \frac{(k_{9,10})_{HA}}{K_3^{-1} + K_{2,3}^{-1}} \qquad (17)$$

and

$$k_A = \frac{(k_{7,8})_A}{K_3^{-1} + K_{2,3}^{-1}} \qquad (18)$$

In earlier papers, the interconversion of E_1 and E_2 was described by a single step (step 4), and this must now be modified to accommodate the more detailed processes of Scheme III. Thus, from eq 23 Fisher et al. (1986a), the dip-switch concentration c_D was given by

$$c_D = \frac{2(1 + K_4)}{K_3^{-1} + K_{2,3}^{-1}}$$

and this becomes, to include the additional free enzyme species E_3 and E_4 of Scheme III:

$$c_D = \frac{2(1 + K_4 + K_7 + K_9)}{K_3^{-1} + K_{2,3}^{-1}} \qquad (19)$$

where $K_4 = K_{7,8} = K_{9,10}$. Each term in the numerator of this equation refers to a different form of the free enzyme: E_2, E_1, E_3, and E_4, respectively.

From the symmetry of the proline racemase system and from the rough pH–activity profile (Cardinale & Abeles, 1968) we assume that the two catalytic groups on the enzyme have the same dissociation constant K_E and that K_4 is near unity. With these simplifications

$$1 + K_4 + K_7 + K_9 \approx (1 + K_E/[H^+])(1 + [H^+]/K_E) \qquad (20)$$

Table V: Rate Constants for the Catalysis of Enzyme Interconversion by Hydrazine Buffers[a]

buffer concn (mM)	$10^{-3}k_I$ (s^{-1})	$10^{-6}(k_{9,10})_{HA}$[b] (M^{-1} s^{-1})	$10^{-6}(k_{7,8})_A$[c] (M^{-1} s^{-1})
4	0.88	7.8	4.5
15	1.12	7.3	4.3
50	1.70	7.1	4.1
200	2.90	7.3	4.3
mean		7.4	4.3

[a] k_{sat} was 4.48×10^3 s^{-1} in the absence of hydrazine and 4.24×10^3 s^{-1} in the presence of 200 mM hydrazine. A value of 4.4×10^3 s^{-1} was used. k_I was 0.78×10^3 s^{-1} in the absence of hydrazine. [b] Calculated from eq 16 and 23. Values for [HA] at pH 8.0 were calculated on the basis of a pK_a of 7.75 for hydrazinium ion. [c] Calculated from eq 16 and 22.

Table VI: Rate Constants for the Catalysis of Enzyme Interconversion by Various Buffers[a]

buffer[b]	pK_a	$10^{-3}k_I$ (s^{-1})	$10^{-6}(k_{9,10})_{HA}$ (M^{-1} s^{-1})	$10^{-6}(k_{7,8})_A$ (M^{-1} s^{-1})
ammonia	9.25	3.04	1.8	30
hydrazine[c]	7.75	2.90	7.4	4.3
hydrogen sulfide	7.04	2.84	9.0	0.90
hydroxylamine	5.73	2.50	113	0.60

[a] See footnotes to Table V. [b] At 200 mM. [c] Values from Table V.

We further assume that the dissociation constants for the stably bonded proton of transition states 9 and 10 (to give transition states 8 and 7, respectively; see Scheme III) are also given by K_E, so that

$$k_A[A]/(k_{HA}[HA]) = K_E/[H^+] \quad (21)$$

From eq 16–19 we obtain

$$(k_{7,8})_A = \frac{2f_A(1 + K_E/[H^+])}{c_D[A]} \quad (22)$$

and

$$(k_{9,10})_{HA} = \frac{2f_A(1 + [H^+]/K_E)}{c_D[HA]} \quad (23)$$

On the reasonable basis that E_1 and E_2 are the catalytically active forms of the free enzyme, and since all our experiments have been conducted at pH 8.0 [which is at or close to the pH optimum of the enzyme (Cardinale & Abeles,1968)], pK_E is about 8, and $[H^+] \approx K_E$. We may now use eq 16, 22, and 23 and c_D = 2.9 mM (Fisher et al., 1986b) to calculate values for base catalysis $(k_{7,8})_A$ and acid catalysis $(k_{9,10})_{HA}$ of enzyme interconversion by hydrazine buffers. These results are given in Table V.

It is gratifying that the values of both rate constants (for acid and base catalysis) are independent of the buffer concentration. Further, the absolute values of these constants ($\sim 10^7$ M^{-1} s^{-1}) are entirely reasonable for proton-transfer reactions between acids of similar pK_a (Albery, 1980). It is most probable that the proton transfers at issue occur between hydrazine (pK_a 7.7) and an active site thiol of pK_E near 8.0.

The other three buffer catalysts that we have studied have been investigated at a single buffer concentration (200 mM), and the catalytic constants (see Table VI) are presented as a Brønsted plot in Figure 5. While the data are limited, they lie very satisfactorily on the theoretical lines of slope 0.5 predicted for proton transfers between acids of similar pK_a. These results show conclusively that the enzyme interconversion steps that are rate limiting in the proline racemase reaction at oversaturating levels of substrate involve proton transfers and that the interconversion is subject to general acid–base catalysis.[2]

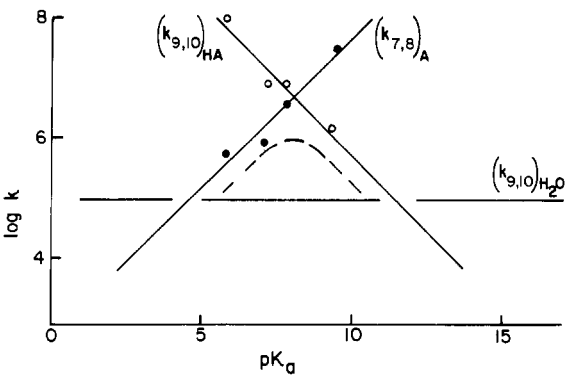


FIGURE 5: Brønsted plot for the catalyzed interconversion of the two forms of free enzyme, E_1 and E_2. The logarithms of the rate constants for general acid catalysis, $(k_{9,10})_{HA}$ (open circles), and for general base catalysis, $(k_{7,8})_A$ (filled circles), from Table VI are plotted vs. the pK_a values of the catalyzing buffer components. The observed *un*catalyzed rate of 10^5 s^{-1} is plotted as a horizontal line. The dashed curve represents the first-order rate constant for general catalysis (by both HA and its conjugate base A) by 200 mM buffer at pH 8.0.

In an earlier paper (Fisher et al., 1986b), we reported the rate constant for the interconversion of the two forms of free enzyme under conditions where the process is not catalyzed to be about 10^5 s^{-1}. This uncatalyzed rate constant is plotted as the horizontal line in Figure 5. We also plot in Figure 5 (dashed curve) the first-order rate constant for general catalysis:

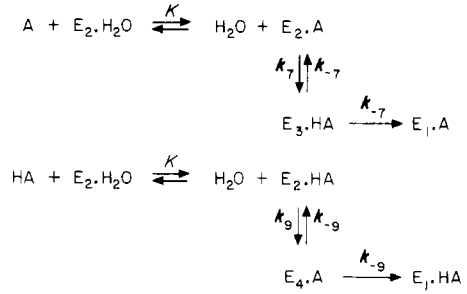
$$k_{gc} = (k_{7,8})_A[A] + (k_{9,10})_{HA}[HA] \quad (24)$$

when catalysis may occur both by a general acid HA and by its conjugate base A in a buffer concentration of 200 mM at pH 8.0. It is interesting that *each* term on the right-hand side of eq 24 gives rise to the same bell-shaped curve. This arises as follows. To the left of the maximum, most of the buffer is present as A ($pK_a < 8$) while to the right of the maximum most of the buffer is present as HA ($pK_a > 8$). Consider a case where $k_{gc} = (k_{7,8})_A[A]$. To the left of the maximum [A] is relatively constant, log $(k_{7,8})$ increases with pK_a with a gradient of 0.5, and log (k_{gc}) rises. To the right of the maximum, while log $(k_{7,8})$ continues to increase with a gradient of 0.5, A is now the minor species, and log [A] falls with pK_a with a gradient of −1.0. The net effect is a decrease in log (k_{gc}) with pK_a with a gradient of 0.5. A similar analysis can be applied to the $k_{9,10}$ term of eq 24. Since each term on the right-hand side of eq 24 gives the same bell-shaped curve, we cannot distinguish between general acid and general base catalysis merely by inspection of Figure 5.

It is clear from Figure 5 that general catalysis can only be observed by buffers at 200 mM when k_{gc} (shown by the dashed line in Figure 5) is comparable to or greater than the uncatalyzed water rate (shown by the horizontal line). Buffer catalysis can therefore *only* be observed for buffers with pK_a values between 5 and 11. This explains why we have observed little or no catalytic effect by the following substances at 200 mM: triethylamine (pK_a = 10.7), methylamine (10.6), acetic acid (4.76), methoxyamine (4.72), or methanol ($\sim$16). Yet

[2] We have considered whether an extra catalytic group on the enzyme could mediate the required proton transfers, but this seems unlikely because catalysis by buffers such as hydrazine at 200 mM is observed. The effective concentration of such an extra catalytic group at the active site would be 10 or 100 times this value, and it would be unlikely that an added catalyst such as hydrazine could compete effectively with such a group.

Scheme IV: Interconversion of Free Enzyme Forms[a]



[a] The catalytic reactions involving either the general acid HA or the general base A are broken down into an associative preequilibrium (K) followed by rate-limiting proton transfers.

we might have expected catalysis by tris(hydroxymethyl)-aminomethane (Tris) (pK_a = 8.3), Bicine (8.35), Tricine (8.15), triethanolamine (7.57), bicarbonate (6.35), phosphate (6.49), ethanolamine (9.50), and dithiothreitol (9.0). These buffers are, however, without catalytic effect, and we attribute their failure to steric and other factors that preclude the appropriate fit into the active site of the enzyme that allows the rearrangement of protons and the isomerization of one form of the enzyme ($B^-\cdots HB$) to the other ($BH\cdots B^-$).

Marcus Treatment of the Catalysis of Enzyme Interconversion. Proton-transfer reactions between hydrogen-bonded bases have been shown to obey the Marcus theory (Marcus, 1968; Albery, 1980), and we now apply this treatment to the results shown in Figure 5, first to examine the relationship between the water-catalyzed rate constant of 10^5 s^{-1} and the rate constants for buffer catalysis and second to see if we can distinguish between general acid and general base catalysis.[3] To apply the Marcus treatment we must divide the reaction into an associative step followed by a catalytic step, as shown in Scheme IV. In this scheme we assume that $\mathbf{k}_{-7} = \mathbf{k}_8$ and $\mathbf{k}_{-9} = \mathbf{k}_{10}$ (where $\mathbf{k}$ values are the first-order rate constants). We can then relate the second-order rate constants $k_{7,8}$ and $k_{9,10}$ (Scheme III) to the first-order rate constants $\mathbf{k}_7$ and $\mathbf{k}_9$ (Scheme IV), as

$$k_{7,8} = K\mathbf{k}_7/2 \tag{25}$$

and

$$k_{9,10} = K\mathbf{k}_9/2 \tag{26}$$

For the uncatalyzed water-mediated reaction, K = 1, and we obtain

$$\mathbf{k}_{7,H_2O} = \mathbf{k}_{9,H_2O} = 2 \times 10^5\ s^{-1} \tag{27}$$

The Marcus expression can be written as

$$\log \mathbf{k} = \log \mathbf{k}_\bullet + \tfrac{1}{2} \log \mathbf{K}_\bullet + (\log \mathbf{K}_\bullet)^2/[16 \log [\mathbf{k}_\bullet h/(kT)]] \tag{28}$$

where $\mathbf{k}_\bullet$ is the value of $\mathbf{k}_7$ or $\mathbf{k}_9$ for an acid of pK_a = 8 and $\mathbf{K}_\bullet = \mathbf{K}_7$ or $\mathbf{K}_9$ in Scheme IV. From the data in Figure 5 and eq 25 and 26

$$\mathbf{k}_\bullet = 6 \times 10^8\ s^{-1} \tag{29}$$

In eq 28 the values of $\log \mathbf{K}_7$ and $\log \mathbf{K}_9$ for the water-catalyzed reactions are given by the differences between the pK_{He} for the enzyme thiol of about 8, and $pK_{H_3O^+} = -1.7$ and $pK_{H_2O} = 15.7$:

$$\log \mathbf{K}_7 = pK_{H_3O^+} - pK_{He} = -9.7 \tag{30}$$

and

$$\log \mathbf{K}_9 = pK_{He} - pK_{H_2O} = 7.7 \tag{31}$$

Substitution of eq 29 and either eq 30 or 31 into eq 28 gives the following results for the Marcus treatment:

$$\mathbf{k}_{7,H_2O} = 3 \times 10^2\ s^{-1} \tag{32}$$

and

$$\mathbf{k}_{9,H_2O} = 1 \times 10^4\ s^{-1} \tag{33}$$

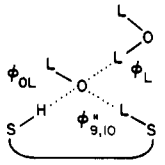
Comparison of these values with the observed value of 2×10^5 s^{-1} shows better agreement for $\mathbf{k}_{9,H_2O}$ (when water acts as an acid) than for $\mathbf{k}_{7,H_2O}$ (when water acts as a base). The reason for the difference between the values in eq 27 and 33 may lie in the assumption of a simple statistical value for the association constant K. Thus, K for the catalytic buffer species may be rather less than the statistical value, owing to the constraints of the active site. As discussed, it appears that catalytic species as large or larger than bicarbonate ion are excluded from the active site altogether.

Water-Mediated Reaction. The observed rate of the uncatalyzed water-mediated reaction (eq 27) is equivalent to a free energy of activation of 45 kJ/mol, and this can be compared with the free energy difference between $E_4{\cdot}OH^-$ and $E_2{\cdot}H_2O$ given by $\mathbf{K}_9$ (eq 31) of 46 kJ/mol. This similarity shows that the transition state for the water-mediated reaction is close in free energy to $E_4{\cdot}OH^-$ and, therefore, that the collapse of $E_4{\cdot}OH^-$ (to either $E_1{\cdot}H_2O$ or $E_2{\cdot}H_2O$) has little or no free energy of activation. The value of $\mathbf{k}_{-9}$ is about 10^{12}–10^{13} s^{-1} and is indeed close to kT/h. One might then argue that the species $E_4{\cdot}OH^-$ is of such high free energy and its lifetime is so short that it is not really a reaction intermediate. It is, however, unprofitable to worry whether there is a small dip in the free energy surface or not. What is important is that the results indicate that the interconversion of E_2 and E_1 proceeds through a species the structure of which is very close to E_4 (Scheme III).

Solvent Isotope Effect. The final piece of information on the mechanism of the enzyme interconversion steps derives from the deuterium solvent isotope effect in the S-to-P reaction. In the *unsaturated* and *saturated* regions (where the enzyme interconversion steps are kinetically insignificant), no effect on k_1 is seen on going from H_2O into D_2O (Belasco et al., 1986a). In the *oversaturated* region, however, a solvent isotope effect (k_{D_2O}/k_{H_2O}) of about 0.5 is observed. These effects are entirely in accord with our expectations. In both the unsaturated and saturated regions, the solvent-derived proton in the ground state is on an active site thiol group: $\phi_{E_1''} \approx \phi_{E_2''S} \approx 0.5$ (Belasco et al., 1986b). In the transition state, this proton is in flight with $\phi_{1,2,3}'' \approx 0.44$ (Fisher et al., 1986c). The other proton is substrate-derived and is unaffected by the change from H_2O to D_2O. We do not therefore expect a significant solvent isotope effect in the unsaturated or saturated regions, and it is gratifying that none is seen (Belasco et al., 1986a). In the oversaturated region, however, the ground-state fractionation for the solvent-derived proton now relates to the equilibrated pool of $E_1''S$ and E_2P'', where in $E_1''S$ the proton is on a thiol ($\phi_{E_1S''} \approx 0.5$) but in E_2P'' the proton is on substrate with ϕ_P = 1.17 (Fisher et al., 1986c). The *mixed* fractionation factor $\phi_{ES,EP}''$ for the $E_1''S$ and E_2P'' pool (assuming K_2 = 1.0) is therefore 0.84. The transition-state fractionation factor is $\phi_{9,10}''$. Since, as discussed above, $E_4{\cdot}OH^-$ is of much higher

[3] Catalysis by H_3O^+ or by OH^- can be ruled out, since the rate constants for catalysis by these species will be at the diffusion limit of about 10^9 M^{-1} s^{-1}, and at pH 8 the predicted first-order rate constants for the interconversion would be at least 2 orders of magnitude slower than the observed rate of 10^5 s^{-1}.

Scheme V: Fractionation Factors in $E_4 \cdot OH^-$



free energy than E_1 or E_2, transition states 9 and 10 (Figure 5) have similar structures to $E_4 \cdot OH^-$. Scheme V shows the fractionation factors for those transition states. We assume that the factors ϕ_{OL} (=1.20) and ϕ_L (=0.70) are the same as those found for hydrated lyoxide ions in solution (Gold & Grist, 1972; Albery, 1975). When the isotope is washed out through transition states 9 and 10, a proton L that in the reactants was in the equilibrated pool of $E_1''S$ and E_2P'' (with a mixed fractionation factor $\phi_{ES,EP}''$ of 0.84; see above) has been replaced by a proton on free P'' with a fractionation factor ϕ_P of 1.17 (Fisher et al., 1986c). Using these values and that for $\phi_{9,10}''$ (=ϕ_4'') of 0.49 (Table IV), we find

$$\frac{k_{D_2O}}{k_{H_2O}} = \frac{\phi_P \phi_{9,10}'' \phi_{OL} \phi_L}{\phi_{ES,EP}''} = 0.57$$

This value is in good agreement with the experimental value of 0.5. The solvent isotope effects therefore confirm our conclusion that enzyme interconversion takes place through transition states close to the diprotonated form of the enzyme, E_4 (Scheme III). As will be seen in the final paper of this series (Albery & Knowles, 1986b), this pathway nicely parallels the mechanism of the interconversion of enzyme–substrate and enzyme–product complexes. The enzyme thiolate first abstracts a proton either from substrate proline (to form an intermediate in which the substrate carbanion is bound to the diprotonated enzyme E_4) or from water (to form an intermediate in which hydroxide ion is bound to E_4). The intermediate species then collapses to generate product (in the case of the enzyme–substrate complex) or the other form of the free enzyme (in the case of the enzyme–water complex).

Appendix

In this Appendix we consider the full expression for the tritium washout experiments to determine the importance of the corrections for the fact that the substrate concentrations are not cleanly oversaturating. Equation 6.2 of Albery and Knowles (1986a) gives, for a racemase where $K_{S,P} = 1$ and for a system where the crossover parameter x is unity

$$\frac{2\Phi_S}{\Phi_{1,2,3}'} \ln (s'/s_0') = \frac{\Phi_4'}{\Phi_{1,2,3}'} \frac{(1 + p_P/p_\infty)}{(1 + p_{P,\Phi}/p_\infty)} \ln (1 - p/p_\infty) + \frac{p_{P,\Phi}/p_P - 1}{1 + p_\infty/p_{P,\Phi}} \ln (1 + p/p_{P,\Phi}) \quad (A1)$$

where

$$p_{P,\Phi} = p_P(\Phi_4'/\Phi_{1,2,3}') \quad (A2)$$

Now, from Table IV, we see that $\Phi_{4'} \approx \Phi_{1,2,3}'$. Equation A2 therefore gives $p_{P,\Phi} \approx p_P$. With this approximation and the fact that under our conditions $p_\infty > p_P$, eq A1 reduces to the simpler eq 14, and no corrections are required.

Registry No. Proline racemase, 9024-09-3; ammonia, 7664-41-7; hydrazine, 302-01-2; hydrogen sulfide, 7783-06-4; hydroxylamine, 7803-49-8.

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