Formation and Stability of the Enolates of N-Protonated Proline Methyl Ester and Proline Zwitterion in Aqueous Solution: A Nonenzymatic Model for the First Step in the Racemization of Proline Catalyzed by Proline Racemase†

Glenn Williams, E. Peter Maziarz III, Tina L. Amyes, Troy D. Wood, and John P. Richard*

Department of Chemistry, University at Buffalo, State University of New York, Buffalo, New York 14260-3000

Received April 14, 2003

ABSTRACT: Rate constants for the hydrolysis of L-proline methyl ester to form proline and methanol in D_2O buffered at neutral pD and 25 °C and the deuterium enrichment of the proline product determined by electrospray ionization mass spectrometry are reported. The data give $k_{DO} = 5.3 \pm 0.5$ M^{-1} s^{-1} as the second-order rate constant for carbon deprotonation of N-protonated proline methyl ester by deuterioxide ion in D_2O at 25 °C and $I = 1.0$ (KCl). The data provide good estimates of carbon acidities of $pK_a = 21$ for N-protonated proline methyl ester and $pK_a = 29$ for proline zwitterion in water and of the second-order rate constant $k_{HO} = 4.5 \times 10^{-5}$ M^{-1} s^{-1} for carbon deprotonation of proline zwitterion by hydroxide ion at 25 °C. There is no detectable acceleration of the deprotonation of N-protonated proline methyl ester by the Brønsted base 3-quinuclidinone in water, and it is not clear that such Brønsted catalysis would make a significant contribution to the rate acceleration for deprotonation of bound proline at proline racemase. A comparison of the first-order rate constants $k_{HO}[HO^-] = 4.5 \times 10^{-11}$ s^{-1} for deprotonation of free proline zwitterion in water at pH 8 and $k_{cat} = 2600$ s^{-1} for deprotonation of proline bound to the active site of proline racemase at pH 8 shows that the enzymatic rate acceleration for proline racemase is ca. 10^{13}-fold. This corresponds to a 19 kcal/mol stabilization of the transition state for deprotonation of the enzyme-bound carbon acid substrate by interaction with the protein catalyst. It is suggested that (1) much of the rate acceleration of the enzymatic over the nonenzymatic reaction in water may result from transfer of the substrate proline zwitterion from the polar solvent water to a nonpolar enzyme active site and (2) the use of thiol anions rather than oxygen anions as Brønsted bases at this putative nonpolar enzyme active site may be favored, because of the smaller energetic price for desolvation of thiol anions than for desolvation of the more strongly solvated oxygen anions.

Several amino acid racemases catalyze the interconversion of D- and L-amino acids by abstraction of the α-amino proton of the enzyme-bound substrate to form an achiral enol(ate) intermediate which is then reprotonated nonstereospecifically to form a mixture of the D- and L-amino acids (*1*). The corresponding nonenzymatic racemization of amino acids is extremely slow and is usually monitored under harsh conditions at temperatures of 100 °C and higher (*2*−*6*). However, we have shown that it is possible to monitor the incorporation of deuterium from solvent D_2O into the α-amino carbon of the achiral amino acid glycine and its derivatives at room temperature (*7*−*10*). We have now extended this work to the chiral amino acid derivative L-proline methyl ester, because deprotonation of the α-amino carbon of proline to form the proline carbanion is the first step in the reaction catalyzed by proline racemase (*11*−*13*). It is therefore of great interest to quantify the kinetic and thermodynamic barriers to the corresponding nonenzymatic reaction in aqueous solution and to determine the magnitude of the enzymatic rate acceleration.

We have chosen to compare the rate constants for deprotonation of N-protonated glycine methyl ester (*9*, *10*) and N-protonated L-proline methyl ester, because these reactions can be easily monitored at room temperature and physiological pH. The second-order rate constant for deprotonation of N-protonated L-proline methyl ester by deuterioxide ion in D_2O at 25 °C determined here is $k_{DO} = 5.3$ M^{-1} s^{-1}, which is very similar to $k_{DO} = 6.0$ M^{-1} s^{-1} for deprotonation of N-protonated glycine methyl ester reported in our earlier work (*9*, *10*). This shows that the carbon acidity of these two N-protonated amino acid esters is similar, and it provides strong evidence that the rate and equilibrium constants for deprotonation of proline zwitterion in water are similar to those for deprotonation of glycine zwitterion reported in our earlier work (*9*).

There is a fairly detailed knowledge of the essential catalytic residues for several amino acid racemases that catalyze the direct deprotonation of an enzyme-bound amino acid (*12*, *14*−*19*). However, the mechanism for stabilization of the enzyme-bound transition state relative to substrate is not well understood. A substantial lowering of the energy of this transition state relative to that of the substrate proline zwitterion would result from transfer of this substrate zwitterion from the polar solvent water to a nonpolar enzyme active site (*9*), and we propose that this catalytic strategy is adopted by proline racemase.

† This work was supported by Grant GM 39754 from the National Institutes of Health. The manuscript was prepared during the term of J.P.R. as a SFI Walton Fellow at University College, Dublin, Ireland.

* To whom correspondence should be addressed. Tel: (716) 645-6800 ext 2194. Fax: (716) 645-6963. E-mail: jrichard@chem.buffalo.edu.

10.1021/bi0345992 CCC: $25.00
Published on Web 06/20/2003

MATERIALS AND METHODS

L-Proline methyl ester hydrochloride, 3-quinuclidinone hydrochloride, deuterium chloride (37 wt %, 99.5% D), and potassium deuterioxide (40 wt %, 98+% D) were purchased from Aldrich. Deuterium oxide (99.9% D) was purchased from Cambridge Isotope Laboratories. 3-Quinuclidinone hydrochloride was recrystallized from ethanol, and the acidic protons were exchanged for deuterium as described previously (*20*). All other organic and inorganic chemicals were of reagent grade and were used without further purification.

3-Quinuclidinone buffers were prepared by dissolving the acidic form and KCl in D_2O followed by addition of the appropriate amount of a solution of KOD in D_2O to give the desired acid/base ratio at $I = 1.0$ (KCl).

Solution pH or pD was determined at 25 °C using an Orion Model 720A pH meter equipped with a Radiometer GK2321C combination electrode that was standardized at pH 7.00 and 10.00. Values of pD were obtained by adding 0.40 to the observed reading of the pH meter (*21*). The concentration of deuterioxide ion at any pD was calculated from eq 1, where $K_w = 10^{-14.87}$ is the ion product of D_2O at 25 °C (*22*) and $\gamma_{OL} = 0.79$ is the apparent activity coefficient of lyoxide ion under our experimental conditions (*23*). An apparent pK_a of 9.0 in D_2O at 25 °C and $I = 1.0$ (KCl) was determined for the protonated amino group of L-proline methyl ester by potentiometric titration of a 13 mM solution of the substrate with KOD.

$$[DO^-] = \frac{10^{pD-pK_w}}{\gamma_{OL}} \quad (1)$$

^{1}H NMR Spectroscopy. ^{1}H NMR spectra at 500 MHz were recorded at 25 °C on a Varian Unity Inova 500 NMR spectrometer. The relaxation times for the α-amino proton and the protons of the methyl group of proline methyl ester and for the protons of a methylene group of 3-quinuclidinone, which were used as an internal standard, were determined under the experimental conditions of the hydrolysis reactions and were found to be in the range $T_1 = 4-6$ s. In all cases the relaxation delay between pulses was at least 10-fold longer than the longest T_1 for the protons of interest. Chemical shifts are reported relative to HOD at 4.67 ppm. The baselines of the NMR spectra were subjected to a second-order correction before determination of integrated peak areas.

Electrospray Mass Spectroscopy. All experiments were performed on a Bruker (Billerica, MA) BioApex 30es Fourier transform mass spectrometer. The instrument design, along with a description of the 3.0 T magnet and pumping system, has been reported elsewhere (*24*). This instrument is equipped with an Analytica of Branford (Branford, CT) electrospray ionization (ESI) source that contains an rf only Iris hexapole ion guide that can be used to externally accumulate ions (1 s). A Cole-Palmer (Vernon Hills, IL) Series 74900 infusion pump was used to inject analyte samples continuously into the ESI source at a rate of 60 μL h^{-1}. Nitrogen countercurrent drying gas (250 °C) at a flow rate of 10−15 L min^{-1} was used to assist in desolvation of droplets produced by ESI. A potential of between −3.2 and −3.8 kV relative to the grounded needle was applied to the metal-capped glass capillary, and the resulting ions were injected electrostatically into the ion trap cell at a trapping potential of 1.5 V. A broadband excitation was applied, and ions were detected in direct mode from time domain data sets of 128K using 100 scans per experiment. The data sets were apodized with a Gaussian function, subjected to a Fourier transform, and displayed in magnitude mode. The area of each isotope peak was determined by integration using the XMASS 3.0 software package. The ratios of the areas of the parent MH^+ (A_P) and $MH^+ + 1$ (A_{P+1}) peaks determined in different mass spectral analyses of the same sample agreed to better than ±5%.

Hydrolysis Reactions of L-Proline Methyl Ester. The hydrolysis reactions of L-proline methyl ester in D_2O at 25 °C and $I = 1.0$ (KCl) were monitored by ^{1}H NMR spectroscopy at 500 MHz. Reactions were initiated by the addition of solid L-proline methyl ester hydrochloride to a solution of 3-quinuclidinone buffer in D_2O at pD 7.4 or 8.4 ($I = 1.0$, KCl) to give a final substrate concentration of 20 mM. The solution pD was immediately adjusted to the initial pD of the buffer by the addition of a small aliquot of KOD. The hydrolysis of proline methyl ester produces D_3O^+. Therefore, the pD of the reaction mixture was monitored closely during the hydrolysis reaction, and a constant pD (±0.04 unit) was maintained by the periodic addition of a small aliquot of 1.5 M KOD. At timed intervals an aliquot was removed from the reaction mixture and was transferred to an NMR tube for immediate analysis by ^{1}H NMR.

The progress, R, of the hydrolysis reaction of proline methyl ester was calculated using eq 2, where A_{Me} is the integrated area of the singlet at 3.80 ppm due to the protons of the methyl group of proline methyl ester and A_{std} is the area of the multiplet at 2.73 ppm due to the protons of a methylene group of 3-quinuclidinone buffer, which served as an internal standard. Observed first-order rate constants for hydrolysis, k_{hyd} (s^{-1}), were determined as the slopes of semilogarithmic plots of reaction progress against time, which were linear for 3 half-times, according to eq 3. Rate constants determined in different experiments were reproducible to ±10%.

$$R = \frac{A_{Me}}{A_{std}} \quad (2)$$

$$\ln R = -k_{hyd}t \quad (3)$$

After the hydrolysis reaction was complete, the solution pH/pD was adjusted to ca. 12 by the addition of KOH, and the free base form of 3-quinuclidinone was immediately removed by extraction into chloroform. The solution pH/pD was then adjusted to ca. 7 by the addition of HCl, and the sample was freed of KCl by passage over an analytical C_{18} reverse-phase HPLC column, eluting with 100% water. The resulting salt-free solution of the proline hydrolysis product was prepared for electrospray ionization mass spectroscopic analysis by the addition of water, methanol, and acetic acid to give a final solution of ca. 50 μM proline in 49/49/2 methanol/water/acetic acid.

RESULTS

Figure 1 shows representative partial ^{1}H NMR spectra at 500 MHz of L-proline methyl ester obtained during the first 85% of its hydrolysis to form proline and methanol in the presence of 0.60 M 3-quinuclidinone buffer (pD 7.4) in D_2O

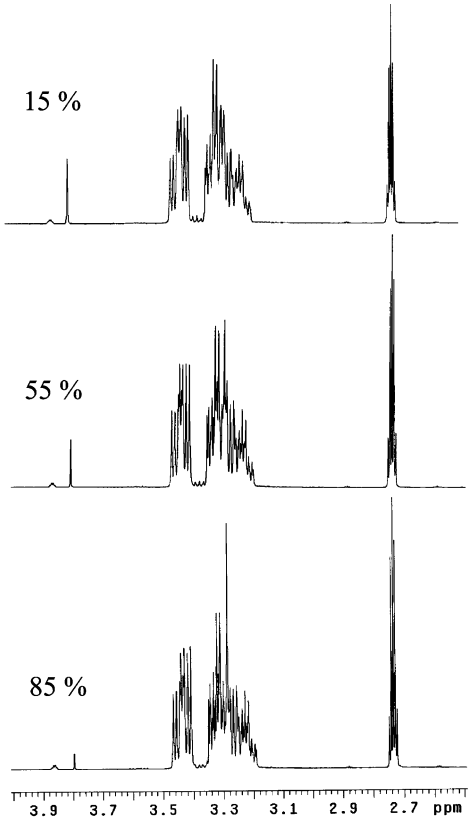
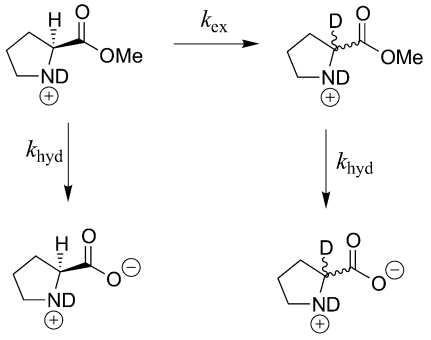


FIGURE 1: Representative partial ^{1}H NMR spectra at 500 MHz obtained during the hydrolysis of L-proline methyl ester (20 mM) to form proline and methanol in the presence of 0.60 M 3-quinuclidinone buffer (pD 7.4) in D_2O at 25 °C and $I = 1.0$ (KCl). The singlet at 3.80 ppm is due to the protons of the methyl group of proline methyl ester. The multiplet at 2.73 ppm ($J = 3.0$ Hz) is due to the protons of a methylene group of the 3-quinuclidinone buffer, which served as an internal standard to determine the relative concentration of the remaining proline. The labels above each spectrum indicate the amount of ester hydrolysis that has occurred.

Scheme 1



at 25 °C and $I = 1.0$ (KCl). The hydrolysis reaction was monitored by following the decrease in the integrated area of the singlet at 3.80 ppm due to the methyl group of proline methyl ester, relative to the integrated area of the multiplet at 2.73 ppm ($J = 3.0$ Hz) due to the protons of a methylene group of the 3-quinuclidinone buffer, which served as an internal standard (eq 2). The observed first-order rate constants for hydrolysis of proline methyl ester (k_{hyd}, Scheme 1) are reported in Table 1.

Figure 2A shows the partial electrospray ionization mass spectrum of proline obtained from the hydrolysis of L-proline methyl ester in the presence of 0.10 M triethylamine in H_2O. A parent peak with a normalized area $A_P = 100$ at $m/z =$ 116.1 (MH^+) and a second peak with area $A_{P+1} = 6.16 \pm 0.73$[1] at $m/z = 117.1$ ($MH^+ + 1$) due to proline that contains one atom of ^{13}C are observed. Figure 2B shows the partial

Table 1: Kinetic and Product Data for the Hydrolysis and Deuterium Exchange Reactions of L-Proline Methyl Ester at Neutral pD in D_2O[a]

	[buffer]/M	k_{hyd}/ 10^{-6} s^{-1} [b]	f_D[c]	k_{ex}/ 10^{-7} s^{-1} [d]	k_{DO}/ M^{-1} s^{-1} [e]
pD = 7.4,	0.30	5.5	0.043	2.5	5.9
$f_{BD} = 0.98$	0.40	5.9	0.037	2.3	5.5
	0.50	6.2	0.030	1.9	4.5
	0.60	6.9	0.033	2.4	5.7
pD = 8.4,	0.30	43	0.039	17	4.9
$f_{BD} = 0.80$	0.40	44	0.042	19	5.5

[a] At 25 °C and $I = 1.0$ (KCl). [b] The observed first-order rate constant for hydrolysis of proline methyl ester determined by monitoring the disappearance of the methyl group of the ester by ^{1}H NMR. [c] Fractional deuterium enrichment of the proline product obtained from the hydrolysis of proline methyl ester in D_2O determined by electrospray ionization mass spectrometry according to eq 4. The standard error in these enrichments is ±30%, which was calculated by propagation of the standard errors in $(A_{P+1})_{HOH}$ (±0.73, absolute error) and $(A_{P+1})_{DOD}$ (±10%, fractional error); see text. [d] The first-order rate constant for the deuterium exchange reaction of L-proline methyl ester calculated from the values of k_{hyd} and f_D according to eq 5. [e] The second-order rate constant for the deuterioxide ion-catalyzed deuterium exchange reaction of N-protonated proline methyl ester calculated from the values of k_{ex} and the fraction of proline methyl ester present in the reactive N-protonated form, f_{BD}, according to eq 6. The average of these values is $k_{DO} = 5.3 \pm 0.5$ M^{-1} s^{-1}.

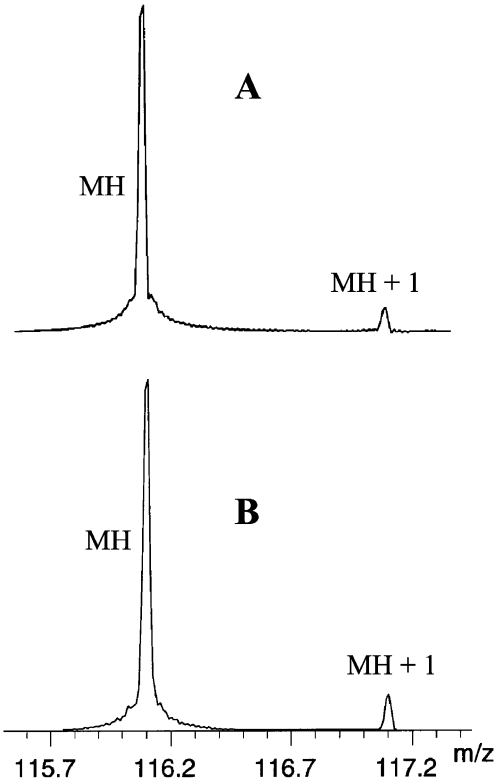


FIGURE 2: Representative partial electrospray ionization mass spectra of proline obtained from the hydrolysis of L-proline methyl ester. These spectra show a parent peak at $m/z = 116.1$ due to MH^+ and a peak at $m/z = 117.1$ due to $MH^+ + 1$. (A) Proline obtained from the hydrolysis of L-proline methyl ester (20 mM) in the presence of 0.10 M triethylamine in H_2O at 25 °C. (B) Proline obtained from the hydrolysis of L-proline methyl ester (20 mM) in the presence of 0.30 M 3-quinuclidinone buffer (pD 7.4) in D_2O at 25 °C and $I = 1.0$ (KCl).

electrospray ionization mass spectrum of proline obtained from the hydrolysis of L-proline methyl ester in the presence of 0.30 M 3-quinuclidinone buffer (pD 7.4) in D_2O at 25 °C and $I = 1.0$ (KCl), again with a normalized area $A_P = 100$ for the peak at $m/z = 116.1$ (MH^+). In this case, the area of the peak at $m/z = 117.1$ ($MH^+ + 1$) is $A_{P+1} = 10.69 \pm 1.1$.[2] The larger relative area of the peak corresponding to $MH^+ + 1$ observed for proline prepared by hydrolysis of L-proline methyl ester in buffered D_2O than for proline prepared by hydrolysis in H_2O shows that there is significant incorporation of deuterium from solvent into proline methyl ester during its hydrolysis in buffered D_2O.

The fractional deuterium enrichment f_D (Table 1) of the proline product obtained from hydrolysis of L-proline methyl ester in D_2O was calculated using eq 4, where $(A_{P+1})_{DOD}$ and $(A_{P+1})_{HOH}$ are the areas of the peaks corresponding to $MH^+ + 1$ in the electrospray ionization mass spectrum of proline obtained from hydrolysis in D_2O and H_2O, respectively, and $A_P = 100$ for the corresponding parent ion (MH^+) in each case.

$$f_D = \frac{(A_{P+1})_{DOD} - (A_{P+1})_{HOH}}{100 + [(A_{P+1})_{DOD} - (A_{P+1})_{HOH}]} \quad (4)$$

Table 1 gives values of the first-order rate constant k_{ex} (s^{-1}) for exchange for deuterium of the α-amino proton of L-proline methyl ester in D_2O at 25 °C and $I = 1.0$ (KCl) that were calculated from the experimental values of k_{hyd} (s^{-1}) for the hydrolysis of proline methyl ester and the fractional deuterium enrichment of the proline hydrolysis product, f_D, using eq 5 derived for Scheme 1 (*9*).

$$k_{ex} = k_{hyd}\left(\frac{f_D}{1 - f_D}\right) \quad (5)$$

The data in Table 1 provide no evidence for an increase in k_{ex} (s^{-1}) with increasing concentrations of 3-quinuclidinone buffer due to buffer catalysis of the exchange reaction so that the observed deuterium exchange arises solely from the solvent-catalyzed reaction. The rate constants for exchange for deuterium of the first α-amino proton of glycine methyl ester in D_2O at neutral pD are directly proportional to the fraction of this substrate in the N-protonated form, because protonation of the amino acid ester at nitrogen increases the reactivity of the α-amino proton toward abstraction by lyoxide ion by ca. 3000-fold (*9*). Therefore, values of the second-order rate constant k_{DO} (M^{-1} s^{-1}; Table 1) for the deuterioxide ion-catalyzed deuterium exchange reaction of L-proline methyl ester were calculated according to eq 6, where f_{BD} is the fraction of proline methyl ester present in the reactive N-protonated form calculated using $pK_{BD} = 9.0$ for the amino group of proline methyl ester in D_2O at 25 °C and $I = 1.0$ (KCl) (see Materials and Methods). The

$$k_{DO} = \frac{k_{ex}}{f_{BD}[DO^-]} \quad (6)$$

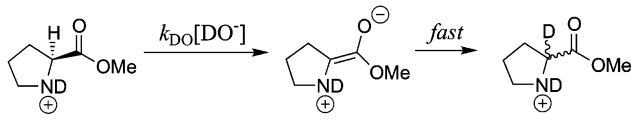
observation that this procedure gives essentially the same value of k_{DO} (M^{-1} s^{-1}) for deuterium exchange at pD 7.4 and 8.4 (Table 1) confirms that the deuterium exchange reaction occurs primarily by proton transfer from the positively charged N-protonated L-proline methyl ester to deuterioxide ion. An average value of $k_{DO} = 5.3 \pm 0.5$ M^{-1} s^{-1} can be calculated from the values of k_{DO} in Table 1.

DISCUSSION

Reactive Species and Reaction Mechanisms. Our earlier work on the deuterium exchange reactions and carbon acidity of amino acids focused on the achiral amino acid glycine and its derivatives ($^+R_3N-CH_2-CO_2R'$) (*7*−*10*). Here, separate ^1H NMR signals are observed for the α-amino protons of $^+R_3N-CH_2-CO_2R'$ and $^+R_3N-CHD-CO_2R'$, and the latter deuterium exchange product can be detected at levels as low as 1% of the total amino acid (*7*−*10*). In our first experiments on L-proline methyl ester, we observed a ca. 10% *decrease* in the integrated area of the singlet ^1H NMR signal due to the α-amino proton relative to that for the protons of the methyl group during the first 2 half-times for hydrolysis of the methyl ester in D_2O at neutral pD. However, there are large errors in the deuterium enrichment of proline methyl ester calculated from such small decreases in relative peak areas. We therefore followed deuterium incorporation into proline methyl ester that accompanies its hydrolysis in D_2O by analyzing the deuterium enrichment of the proline hydrolysis product by mass spectrometry (Scheme 1). Electrospray ionization mass spectrometry was used to obtain the deuterium enrichment by the direct analysis of the amino acid, without the requirement for its conversion to a volatile derivative. The rate constant for the deuterium exchange reaction of proline methyl ester, k_{ex} (s^{-1}), can then be calculated from the rate constant for the hydrolysis reaction, k_{hyd} (s^{-1}), and the deuterium enrichment of the proline hydrolysis product, f_D, according to eq 5 derived for the mechanism shown in Scheme 1.

The exchange for deuterium of the α-protons of ethyl acetate (*23*) and N-protonated glycine methyl ester (*9*, *10*) catalyzed by deuterioxide ion proceeds by direct deprotonation of these carbon acids by DO^- to give the corresponding free ester enolates. We therefore conclude that the very similar deuterium exchange reaction of N-protonated L-proline methyl ester catalyzed by deuterioxide ion also occurs by direct deprotonation of the ester by DO^- to form the free ester enolate, which then undergoes proton transfer from D_2O to give racemic proline methyl ester containing deuterium at the α-position (Scheme 2).

Scheme 2

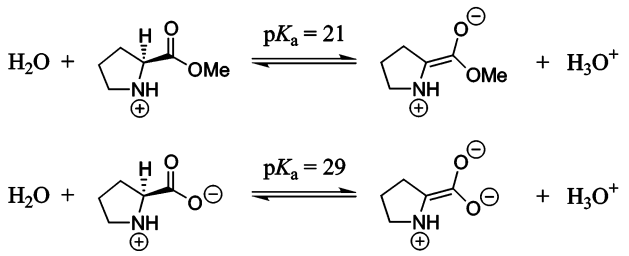


About 10% of the first α-amino proton of N-protonated glycine methyl ester is exchanged for deuterium during hydrolysis of this ester in buffered D_2O (*9*, *10*), but there is a smaller 3−4% exchange of the α-amino proton during hydrolysis of N-protonated proline methyl ester in buffered D_2O (f_D; Table 1). This reflects a larger value of k_{hyd} (s^{-1}) for ester hydrolysis *relative* to k_{ex} (s^{-1}) for deuterium

[1] Standard error calculated from the results of five replicate mass spectral analyses.

[2] The standard error of ±10% in A_{P+1} is a generous estimate made from the range of values obtained in replicate mass spectral analyses.

Scheme 3



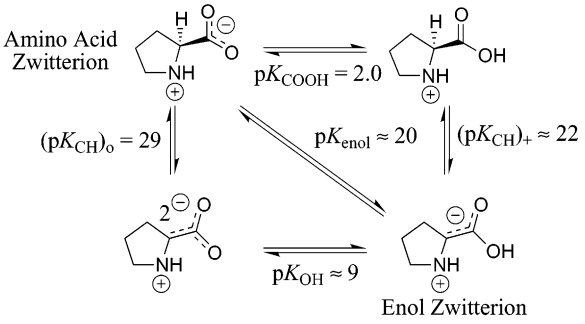
exchange for N-protonated proline methyl ester than for N-protonated glycine methyl ester.

Similar rate constants are observed for deuterioxide ion-catalyzed exchange of a single α-proton of N-protonated glycine methyl ester [k_{DO} = 3.0 M^{-1} s^{-1} (*9*)][3] and N-protonated L-proline methyl ester (k_{DO} = 5.3 M^{-1} s^{-1}; this work). A value of k_{HO} = 3.6 M^{-1} s^{-1} for deprotonation of N-protonated proline methyl ester by hydroxide ion in H_2O can be calculated from k_{DO} = 5.3 M^{-1} s^{-1}, with the assumption that the secondary solvent deuterium isotope effect is the same as that, k_{DO}/k_{HO} = 1.46, for the deprotonation of acetone (*25*). The large kinetic acidity of cationic N-protonated proline methyl ester (k_{DO} = 5.3 M^{-1} s^{-1}) compared with that of the neutral α-carbonyl carbon acid ethyl acetate [k_{DO} = 0.0017 M^{-1} s^{-1} (*23*)] results from stabilization of the transition state for proton transfer by intramolecular interactions between the developing negative charge at the α-amino carbon and the cationic nitrogen (*9*). A similar, large kinetic carbon acidity has been noted for cationic ketones (*26*, *27*).

Carbon Acidity of N-Protonated Proline Methyl Ester and of Proline Zwitterion. We estimate that the carbon acid pK_a for N-protonated proline methyl ester (Scheme 3) is the same as pK_a = 21 ± 1 for N-protonated glycine methyl ester reported in our earlier work (*9*, *10*). This is because the very similar statistically corrected second-order rate constants for deprotonation by hydroxide ion of the α-amino carbons of N-protonated glycine methyl ester [k_{HO} = 2.1 M^{-1} s^{-1} (*9*)] and N-protonated proline methyl ester (k_{HO} = 3.6 M^{-1} s^{-1}; this work) should fall at the same position on the good linear correlation between log k_{HO} for deprotonation of the α-carbonyl carbon of cationic ketones and esters and the pK_a of the carbon acid (*7*−*9*). Although we have not examined the deuterium exchange reaction of the parent amino acid L-proline zwitterion, the very similar reactivity of N-protonated glycine methyl ester and N-protonated proline methyl ester toward deprotonation by lyoxide ion shows that the effect of the bridging trimethylene group at proline zwitterion on carbon acidity is small. We conclude that the values of k_{HO} and pK_a for carbon deprotonation of proline zwitterion should be similar to k_{HO} = 4.5 × 10^{-5} M^{-1} s^{-1} and pK_a = 28.9 ± 0.5 for glycine zwitterion that were reported in our earlier work (*9*). Therefore, we assign pK_a = 29 ± 1 as the carbon acidity of proline zwitterion in aqueous solution (Scheme 3).

The Burden Borne by Proline Racemase. The similarity in the kinetic parameters for enzymes that approach perfection in catalysis (*28*, *29*) can easily mask very large differences in the "burden borne" by these proteins in stabilizing the transition state for the corresponding reaction in solution (*30*). For example, the large difference between the catalytic performance of simple [non pyridoxal phosphate dependent] amino acid racemases and that of other enzymes that catalyze the deprotonation of α-carbonyl carbon (*31*) is not widely appreciated. The deprotonation of dihydroxyacetone phosphate is relatively "easy" and occurs with a half-time of ca. 30 min in the presence of 0.8 M 3-quinuclidinone buffer at pH 7.5 (*32*), while the estimated half-time for deprotonation of proline zwitterion at the same pH is ca. 1500 years.[4] The deprotonation of bound proline is partly rate-determining in the racemization reaction catalyzed by proline racemase at saturating concentrations of proline (*11*, *13*, *33*). A rate acceleration for this enzyme of k_{cat}/k_{non} = 6 × 10^{13} at pH 8 can be calculated from k_{cat} = 2600 s^{-1} for the deprotonation of bound proline (*34*), and $k_{non} = k_{HO}[HO^-]$ ≈ (4.5 × 10^{-5} M^{-1} s^{-1})(10^{-6} M) = 4.5 × 10^{-11} s^{-1} for solvent-catalyzed deprotonation of proline zwitterion (see above). This corresponds to a 19 kcal/mol stabilization of the transition state for the enzyme-catalyzed deprotonation of bound proline compared with its deprotonation in water at pH 8. By comparison, the rate acceleration for deprotonation of bound dihydroxyacetone phosphate by triosephosphate isomerase has been calculated as 10^6-fold (*32*), which corresponds to a transition state stabilization of 8 kcal/mol.

Scheme 4



The large kinetic barrier to deprotonation of proline zwitterion in water is due primarily to the large thermodynamic barrier to the conversion of proline zwitterion to its enol or enolate (Scheme 4). The thermodynamic barriers to the processes shown in Scheme 4 were estimated as follows:

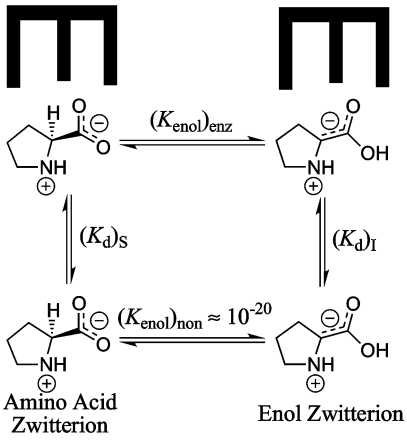
(1) The value of $(pK_{CH})_+ \approx 22$ for carbon deprotonation of cationic proline to form the enol zwitterion was estimated from the value of pK_a = 21 for carbon deprotonation of N-protonated proline methyl ester (this work), and the one unit higher pK_a for carbon deprotonation of acetic acid [pK_a = 26.6 (*35*)] than for deprotonation of ethyl acetate [pK_a = 25.6 (*23*)].

(2) The value of $(pK_{CH})_o$ = 29 for carbon deprotonation of proline zwitterion to form the anionic enolate is assumed

[3] The second-order rate constant for deprotonation of N-protonated glycine methyl ester, which has two exchangeable α-protons, by deuterioxide ion is k_{DO} = 6.0 M^{-1} s^{-1} (*9*). However, a value of k_{DO} = 3.0 M^{-1} s^{-1} is used in this comparison because proline methyl ester has only one exchangeable α-proton.

[4] Calculated from the estimated observed rate constant of $k_{HO}[HO^-]$ = (4.5 × 10^{-5} M^{-1} s^{-1})(3.2 × 10^7 M) for deprotonation of proline zwitterion at pH 7.5. In contrast with dihydroxyacetone phosphate, no Brønsted general base catalysis is expected for deprotonation of proline zwitterion, because the lower carbon acidity of proline zwitterion (pK_a = 29) than of N-protonated proline methyl ester (pK_a = 21) should result in a decrease, not an increase, in the significance of buffer catalysis of deprotonation of the carbon acid in solution (*9*, *52*).

Scheme 5



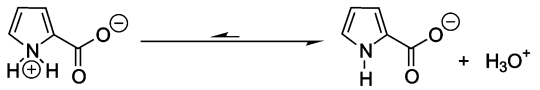
to be the same as $pK_a = 28.9$ for carbon deprotonation of glycine zwitterion (*9*).

(3) The value of $pK_{enol} \approx 20$ for keto−enol tautomerization of proline zwitterion to form the enol zwitterion was calculated from $(pK_{CH})_+ \approx 22$ and $pK_{COOH} = 2$ for ionization of the carboxylic acid group of cationic proline (*36*).

(4) The value of $pK_{OH} \approx 9$ for oxygen ionization of the zwitterionic enol was calculated from $(pK_{CH})_o = 29$ and $pK_{enol} \approx 20$.

Strategies for Catalysis of Amino Acid Deprotonation. There is a large disparity between our knowledge of the structure and the essential catalytic groups of amino acid racemases (*16*−*19*, *37*, *38*) and our relatively poor understanding of the mechanism by which these enzymes stabilize the very high energy transition states for the corresponding nonenzymatic reactions in solution. The principal barrier which must be lowered to achieve the ca. 19 kcal/mol stabilization of this transition state effected by enzymes such as proline racemase (see above) is the 27 kcal/mol thermodynamic barrier to conversion of the amino acid zwitterion to the zwitterionic enol ($K_{enol} \approx 10^{-20}$; Scheme 4). This large barrier to keto−enol tautomerization of proline zwitterion in water can be reduced at an enzyme active site provided the enzyme binds the enol zwitterion intermediate *more tightly* than it binds the substrate amino acid zwitterion, so that $(K_d)_S \gg (K_d)_I$ (Scheme 5). There are several mechanisms by which such selectivity for binding of the intermediate of the reaction catalyzed by proline racemase may be achieved:

(1) The observation that proline racemase binds pyrrole-2-carboxylate (**1**) with a ca. 300-fold larger affinity than it binds proline zwitterion (*12*) provides evidence that the enzyme forms tighter contacts with substituents at the planar C-2 carbon of pyrrole than with these substituents at the tetrahedral α-carbon of proline. Proline racemase should bind selectively to proline zwitterion, because the zwitterion is the dominant species in solution at neutral pH and protonation at nitrogen activates the α-carbon toward proton transfer. If proline racemase is also selective for binding of the N-protonated form of **1**, then the affinity of the enzyme for this zwitterion will be much higher than the *observed* affinity of the enzyme for **1**, because the N-protonated zwitterionic form of **1** is present in negligible amounts at neutral pH (*39*, *40*).[5] Stabilization of a zwitterionic enol intermediate relative to the substrate amino acid zwitterion might be favored at an enzyme active site that has a high degree of structural complementarity to the charged groups of the bound intermediate at which C-2 is trigonal planar, if, for example, the change from planar to tetrahedral geometry results in an increase in the separation between interacting groups at the ligand and enzyme.



1

(2) We assume that the enolic intermediate of the reaction catalyzed by proline racemase is largely monoprotonated at oxygen and is the zwitterionic enol rather than the negatively charged enolate, because protonation of the oxygen dianion at the negatively charged enolate ($pK_{OH} \approx 9$; Scheme 4) is favored at neutral pH (*41*, *42*). The enzyme-catalyzed keto−enol tautomerization of proline zwitterion converts the weakly acidic α-amino proton at proline ($pK_a = 29$; this work) to a more strongly acidic hydroxyl proton ($pK_{OH} \approx 9$) at the 1,1-enediolate (Scheme 5), and the latter may form a hydrogen bond to the enzyme catalyst that is not present at the reactant complex. The structure and strength of hydrogen bonds at enzymes are controversial (*43*−*46*); however, their stability at an active site of low dielectric constant may be 5 kcal/mol or larger (*45*).

(3) There is a strengthening of the stabilizing intramolecular electrostatic interactions between the cationic and anionic centers upon keto−enol tautomerization of proline zwitterion to give the enol zwitterion, because this tautomerization reduces the separation of the opposing interacting charges by one carbon atom (*47*). The binding of these ligands at an enzyme active site of low dielectric constant should result in a greater intramolecular electrostatic stabilization of both the amino acid zwitterion and the enol zwitterion relative to that in water (*47*), but also a *larger difference* in these electrostatic stabilizations, so that $(K_{enol})_{enz} > (K_{enol})_{non}$ (Scheme 5).

We are unable to present a detailed analysis of the effect on K_{enol} of a decrease in the "effective" dielectric constant at an enzyme active site relative to $D = 79$ for solvent water (Scheme 5). However, there is good evidence that the *difference* in the stabilizing electrostatic interactions between closely spaced opposite charges in the gas phase and in water is very large and that this difference is attenuated by changing the separation between the unlike charges. For example, the addition of an α-NMe_3^+ group to acetate anion results in only a 4 kcal/mol decrease in the basicity of the carboxylate group in water (*9*), but it results in a much larger 109 kcal/mol decrease in gas-phase basicity, from 348.5 kcal/mol for $CH_3CO_2^-$ to 239.3 kcal/mol for $^+Me_3NCH_2CO_2^-$ (*48*, *49*).

[5] To the best of our knowledge the pK_a of the cationic nitrogen of pyrrole-2-carboxylate zwitterion has not been determined. A value of $pK_a = -3.8$ (*39*) has been reported for pyrrole, which undergoes protonation at C-2 rather than at nitrogen (*40*), and the pK_a of the N-protonated aromatic heterocycle must be even more negative than this. The stabilizing intramolecular electrostatic interaction between the protonated nitrogen and the carboxylate anion at pyrrole-2-carboxylate zwitterion would increase the basicity of the nitrogen of pyrrole-2-carboxylate relative to that of pyrrole, but the effect should be no larger than the ca. 3 unit acidifying effect of the cationic α-NH_3^+ substituent on the pK_a for oxygen ionization of acetic acid (*9*). This suggests $pK_a < 0$ for the cationic nitrogen of pyrrole-2-carboxylate zwitterion, so that this zwitterion is an exceedingly minor species at neutral pH.

Moving these charges one atom closer together at the enol zwitterion could easily increase the magnitude of their interaction by 2-fold (*47*), and it would result in a net *increase* in the stabilizing intramolecular electrostatic interaction of ca. 109 kcal/mol in the gas phase but only 4 kcal/mol in water. This represents a *differential stabilization* of the zwitterionic enol of ca. 100 kcal/mol in the gas phase relative to water. The effective dielectric constant at the enzyme active site will be considerably larger than $D = 1$ for the gas phase, and this will result in a reduction in this "medium" effect on the free energy change for keto−enol tautomerization to below the maximum value of ca. 100 kcal/mol. However, a value of just 10% of this maximum could account for more than half of the rate acceleration for the deprotonation of bound proline zwitterion by proline racemase.

A large medium effect on the deprotonation of hydroxybenzylthiamin diphosphate at the benzylic carbon upon transfer of the zwitterionic carbanion product from water ($D = 79$) to the active site of pyruvate decarboxylase with an estimated dielectric constant of $D \approx 13-15$ has been proposed to account for the more than 9 unit lower pK_a of the enzyme-bound than of the free carbon acid (*50*). This corresponds to a 12 kcal/mol stabilization of the zwitterionic carbanion relative to the cationic carbon acid. Calculations based upon the X-ray structural data for amino acid racemases (*19*, *38*), with the goal of determining whether the effective dielectric constants of their active sites are significantly different from the value of $D = 79$ for water, would be of considerable interest.

Catalysis of Proton Transfer: Role of Amino Acid Side Chains. It is often assumed that amino acid side chains which function as Brønsted acids and bases in the catalytic cycle for enzyme-catalyzed reactions make a large contribution to the enzymatic rate acceleration (*51*). However, there is no detectable advantage to deprotonation of N-protonated proline methyl ester by 0.60 M of the small Brønsted base 3-quinuclidinone (p$K_{BH} = 7.5$) over its deprotonation by solvent species at neutral pH (Table 1).[6] No such Brønsted general base catalysis is expected for deprotonation of proline zwitterion, because the lower carbon acidity of proline zwitterion (p$K_a = 29$) than of N-protonated proline methyl ester (p$K_a = 21$) should result in a decrease, not an increase, in the significance of buffer catalysis of deprotonation of the carbon acid in solution (*9*, *52*). This suggests that the major role of the cysteine thiol anion bases at the active site of proline racemase (*12*) and related amino acid racemases (*16*, *17*, *37*) is simply to accept the proton that is lost from the acidic carbon of substrate and that they do not play a critical role in lowering the kinetic barrier to deprotonation of the enzyme-bound substrate.

The evolutionary choice of the thiol anion side chains of cysteine residues as catalytic bases at proline racemase (*12*) and related amino acid racemases (*16*, *17*, *37*) is curious, because thiol anions are notoriously poor catalysts of deprotonation of α-carbonyl carbon. For example, the second-order rate constant for deprotonation of a model ketone by $HOCH_2CH_2S^-$ (p$K_{BH} = 9.6$) is ca. 30-fold smaller than that for deprotonation by phenoxide ion (p$K_{BH} = 9.9$), despite the very similar equilibrium basicities of these bases (*53*). If such Brønsted base catalysis makes an important direct contribution to the enzymatic rate acceleration for amino acid racemases, then there should be an advantage to the evolution of an enzyme which utilizes carboxylate anions rather than thiol anions as the catalytic bases.

The evolution of active sites with different catalytic bases for enzymes that catalyze the deprotonation of amino acid zwitterions (thiol anions) and those that catalyze the deprotonation of neutral α-carbonyl carbon acids (carboxylate anions) may be a result of the different imperatives for catalysis of these two reactions. The substantial Brønsted general base catalysis observed for deprotonation of neutral α-carbonyl carbon acids in solution (*32*, *54*) favors the use of carboxylate anion side chains as catalytic bases by enzymes such as triosephosphate isomerase (*55*−*58*) and ketosteroid isomerase (*59*, *60*). By comparison, there is no advantage to the use of carboxylate anions as bases for deprotonation of amino acid zwitterions, which may account for the fact that such side chains are not employed in the active sites of amino acid racemases.

We propose that the active sites of amino acid racemases have a low effective dielectric constant which favors conversion of the substrate amino acid zwitterion to a zwitterionic enol intermediate (Scheme 5). In such an environment, thiol anions will be the preferred catalytic base, because the transfer of anions from the polar solvent water to a nonpolar enzyme active site requires the partial "desolvation" of negative charge, and this will be easier for anionic sulfur than for anionic oxygen, which is more strongly solvated (*61*). In other words, the smaller energetic price for generating anionic sulfur compared to anionic oxygen at a nonpolar enzyme active site with a low effective dielectric constant may favor the utilization of thiol anions as the active site bases by amino acid racemases.

[6] The deprotonation of N-protonated glycine methyl ester is subject to weak Brønsted general base catalysis by oxyanion and tertiary amine bases (*9*, *10*). For example, a 50% increase in the observed rate constant for the deuterium exchange reaction of N-protonated glycine methyl ester is expected in the presence of 1 M phosphate buffer (pK_{BD} = 7.0) at pD 7.40 (*9*). The failure to observe general base catalysis of deuterium exchange into N-protonated proline methyl ester must be qualified by setting a limit on the rate acceleration from this catalysis that might have escaped detection in our experiments, which in this case is the uncertainty (±30%) in the values of k_{ex} (s^{-1}) calculated from the fractional deuterium enrichment f_D of the proline hydrolysis product (Table 1). The data for both glycine methyl ester and proline methyl ester show that little stabilization of the transition state for amino acid racemization should result from the simple recruitment by an enzyme catalyst of a catalytic Brønsted base to abstract the α-amino proton.

REFERENCES

1. Adams, E. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 69−138.
2. Baum, R., and Smith, G. G. (1986) *J. Am. Chem. Soc. 108*, 7325−7327.
3. Steinberg, S. M., Masters, P. M., and Bada, J. L. (1984) *Bioorg. Chem. 12*, 349−355.
4. Stroud, E. D., Fife, D. J., and Smith, G. G. (1983) *J. Org. Chem. 48*, 5368−5369.
5. Smith, G. G., Evans, R. C., and Baum, R. (1986) *J. Am. Chem. Soc. 108*, 7327−7332.
6. Smith, G. G., and Reddy, G. V. (1989) *J. Org. Chem. 54*, 4529−4535.
7. Rios, A., Richard, J. P., and Amyes, T. L. (2002) *J. Am. Chem. Soc. 124*, 8251−8259.
8. Rios, A., Crugeiras, J., Amyes, T. L., and Richard, J. P. (2001) *J. Am. Chem. Soc. 123*, 7949−7950.

9. Rios, A., Amyes, T. L., and Richard, J. P. (2000) *J. Am. Chem. Soc. 122*, 9373−9385.
10. Rios, A., and Richard, J. P. (1997) *J. Am. Chem. Soc. 119*, 8375−8376.
11. Albery, W. J., and Knowles, J. R. (1986) *Biochemistry 25*, 2572−2577.
12. Rudnick, G., and Abeles, R. H. (1975) *Biochemistry 14*, 4515−4522.
13. Cardinale, G. J., and Abeles, R. H. (1968) *Biochemistry 7*, 3970−3978.
14. Gallo, K. A., Tanner, M. E., and Knowles, J. R. (1993) *Biochemistry 32*, 3991−3997.
15. Tanner, M. E., Gallo, K. A., and Knowles, J. R. (1993) *Biochemistry 32*, 3998−4006.
16. Glavas, S., and Tanner, M. E. (1999) *Biochemistry 38*, 4106−4113.
17. Koo, C. W., Sutherland, A., Vederas, J. C., and Blanchard, J. S. (2000) *J. Am. Chem. Soc. 122*, 6122−6123.
18. Koo, C. W., and Blanchard, J. S. (1999) *Biochemistry 38*, 4416−4422.
19. Cirilli, M., Zheng, R., Scapin, G., and Blanchard, J. S. (1998) *Biochemistry 37*, 16452−16458.
20. Amyes, T. L., and Richard, J. P. (1992) *J. Am. Chem. Soc. 114*, 10297−10302.
21. Glasoe, P. K., and Long, F. A. (1960) *J. Phys. Chem. 64*, 188−190.
22. Covington, A. K., Robinson, R. A., and Bates, R. G. (1966) *J. Phys. Chem. 70*, 3820−3824.
23. Amyes, T. L., and Richard, J. P. (1996) *J. Am. Chem. Soc. 118*, 3129−3141.
24. Padley, H. R., Bashir, S., and Wood, T. D. (1997) *Anal. Chem. 69*, 2914−2918.
25. Pocker, Y. (1959) *Chem. Ind.* 1383−1384.
26. Tobin, J. B., and Frey, P. A. (1996) *J. Am. Chem. Soc. 118*, 12253−12260.
27. Halkides, C. J., Frey, P. A., and Tobin, J. B. (1993) *J. Am. Chem. Soc. 115*, 3332−3333.
28. Albery, W. J., and Knowles, J. R. (1976) *Biochemistry 15*, 5631−5640.
29. Knowles, J. K., and Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105−111.
30. Wolfenden, R., and Snider, M. J. (2001) *Acc. Chem. Res. 34*, 938−945.
31. Richard, J. P., and Amyes, T. L. (2001) *Curr. Opin. Chem. Biol. 5*, 626−633.
32. Richard, J. P. (1984) *J. Am. Chem. Soc. 106*, 4926−4936.
33. Fisher, L. M., Belasco, J. G., Bruice, T. W., Albery, W. J., and Knowles, J. R. (1986) *Biochemistry 25*, 2543−2551.
34. Fisher, L. M., Albery, W. J., and Knowles, J. R. (1986) *Biochemistry 25*, 2529−2537.
35. Gao, J. (1996) *J. Mol. Struct. (THEOCHEM) 370*, 203−208.
36. Jencks, W. P., and Regenstein, J. (1976) in *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data* (Fasman, G. D., Ed.) pp 305−351, CRC Press, Cleveland, OH.
37. Glavas, S., and Tanner, M. E. (2001) *Biochemistry 40*, 6199−6204.
38. Hwang, K. Y., Cho, C.-S., Kim, S. S., Sung, H.-C., Yu, Y. G., and Cho, Y. (1999) *Nat. Struct. Biol. 6*, 422−426.
39. Chiang, Y., and Whipple, E. B. (1963) *J. Am. Chem. Soc. 85*, 2763−2767.
40. Joule, J. A., and Smith, G. F. (1972) *Heterocyclic Chemistry*, Van Nostrand, London.
41. Gerlt, J. A., Kozarich, J. W., Kenyon, G. L., and Gassman, P. G. (1991) *J. Am. Chem. Soc. 113*, 9667−9669.
42. Guthrie, J. P., and Kluger, R. (1993) *J. Am. Chem. Soc. 115*, 11569−11572.
43. Warshel, A., and Papazyan, A. (1996) *Proc. Natl. Acad. Sci., U.S.A. 93*, 13665−13670.
44. Warshel, A., Papazyan, A., and Kollman, P. A. (1995) *Science 269*, 102−104.
45. Gerlt, J. A., Kreevoy, M. M., Cleland, W. W., and Frey, P. A. (1997) *Chem. Biol. 4*, 259−267.
46. Guthrie, J. P. (1996) *Chem. Biol. 3*, 163−170.
47. Hine, J. (1975) *Structural Effects on Equilibria in Organic Chemistry*, John Wiley & Sons, New York.
48. Patrick, J. S., Yang, S. S., and Cooks, R. G. (1966) *J. Am. Chem. Soc. 118*, 231−232.
49. Price, W. D., Jockusch, R. A., and Williams, E. R. (1998) *J. Am. Chem. Soc. 120*, 3474−3484.
50. Jordan, F., Li, H., and Brown, A. (1999) *Biochemistry 38*, 6369−6373.
51. Richard, J. P. (1998) *Biochemistry 37*, 4305−4309.
52. Richard, J. P., Williams, G., O'Donoghue, A. C., and Amyes, T. L. (2002) *J. Am. Chem. Soc. 124*, 2957−2968.
53. Pohl, E. R., and Hupe, D. J. (1978) *J. Am. Chem. Soc. 100*, 8130−8133.
54. Zeng, B., and Pollack, R. M. (1991) *J. Am. Chem. Soc. 113*, 3838−3842.
55. Straus, D., Raines, R., Kawashima, E., Knowles, J. R., and Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2272−2276.
56. Hartman, F. C. (1970) *J. Am. Chem. Soc. 92*, 2170−2172.
57. Coulson, A. F. W., Knowles, J. R., Priddle, J. D., and Offord, R. E. (1970) *Nature (London) 227*, 180−181.
58. Waley, S. G., Miller, J. C., Rose, I. A., and O'Connell, E. L. (1970) *Nature (London) 227*, 181.
59. Kuliopulos, A., Mildvan, A. S., Shortle, D., and Talalay, P. (1989) *Biochemistry 28*, 149−159.
60. Hawkinson, D. C., Eames, T. C. M., and Pollack, R. M. (1991) *Biochemistry 30*, 10849−10858.
61. Pearson, R. G. (1986) *J. Am. Chem. Soc. 108*, 6109−6114.

BI0345992