Mechanism and Inhibition of Prolidase*

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The pH dependence of $K_i$ for inhibition of prolidase by acetylproline, proline, and trans-1,2-cyclopentanedicarboxylic acid follows a different pattern in each case, although deprotonation of an enzymic functional group with a $pK_a$ value of 6.6 perturbs ligand binding in every instance. Results are most easily explained with prolidase active as a metalloenzyme dimer exhibiting selective cooperative interactions.

Prolidase is a ubiquitous dipeptidase which exhibits an unusual specificity for proline-containing substrates and for which a reverse protonation mechanism for substrate activation has been advanced in an accompanying article (20). Here we examine in detail some substrate analogue inhibitors for prolidase. In enzymeology, patterns of inhibitor binding are especially informative in a thermodynamic sense, for unlike $K_m$ values which commonly have a kinetic component, $K_i$ values necessarily represent equilibrium phenomena (1). We shall present evidence confirming the presence of a potent, cationic Lewis acid entity within the active site of prolidase, by investigating the pH-dependent inhibition characteristics of a collection of substrate mimics for this enzyme (2).

**MATERIALS AND METHODS**

Enzyme and Inhibitors—Porcine kidney prolidase was obtained as an ammonium sulfate suspension from Sigma and was activated and characterized as described in an accompanying article (20). Inhibitors employed were obtained from commercial sources; racemic trans-1,2-cyclopentanedicarboxylic acid was resolved into its enantiomers of known absolute configuration (3, 4) and was also converted to the cis-isomer by way of the anhydride (5).

Inhibition Kinetics—In measurements of $K_i$ for the various inhibitors, glycylproline was employed as assay substrate in buffered medium, following the protocol in an accompanying article (20). Kinetic runs were initiated by the addition of enzyme to a temperature-equilibrated (25.0 °C) solution of substrate plus inhibitor; no indication of “slow binding” of the high affinity inhibitors was noted. A nonlinear least squares direct fit of $v/[E]$ versus $[I]$ to appropriate hyperbolic expressions as given subsequently yielded inhibition constants. The pH dependence of the resulting apparent $K_i$ values necessarily represent equilibrium phenomena (1). We shall present evidence confirming the presence of a potent, cationic Lewis acid entity within the active site of prolidase, by investigating the pH-dependent inhibition characteristics of a collection of substrate mimics for this enzyme (2).

**RESULTS**

$N$-Acetylproline and Prolidase—The prototypical natural substrate for prolidase is glycylproline. The N-terminal amino group of that dipeptide has been implicated in substrate binding by the kinetic consequences of deliberate introduction of $pK_a$-perturbing nitrogen substituents and by the observation that $N$-acetylproline is not a substrate for the enzyme. In an attempt to quantify the significance of the amino group, $N$-acetylproline was examined as an inhibitor for prolidase. Employing glycylproline as substrate at a pH of 6.9, with $N$-acetylproline at several concentrations in the range 0.1–1 mM, a systematic retardation of $k_{cat}/k_m$ was noted, but no effect was seen on $k_m$, indicating strictly competitive inhibition. Directly fitting the rate data to the appropriate modification of the Michaelis equation yielded an apparent $K_i$ of 0.58 (± 0.05) mM. Because of the complicated pH dependence for catalytic hydrolysis of various substrates, an investigation of the pH profile for $K_i$ was undertaken. Fig. 1 contains the pH dependence of the competitive $K_i$ in normal and also in logarithm (inset, $pK_i$ versus pH). Quite evidently, affinity of $N$-acetylproline for the enzyme is tightest in acidic solution and diminishes with increasing pH (p$K_i$ decreases with a slope of −1.0, indicating first order hydronium ion dependence). A weighted fit of the data to the equation $K_i$ = $K_i$ (1 + $K_i$/[$H^+$])/(1 + $K_i$/[$H^+$]) yields a value of $K_i$ of 0.09 (± 0.02) mM for the limiting inhibition constant at low pH, with inflections in the profile at a $pK_a$ of 6.45 (± 0.11) and a $pK_a$ of 8.95 (± 0.04). The $pK_a$ value corresponds to a deprotonation of an enzymic functional group which evidently must be in the conjugate acid form in order for $N$-acetylproline to adhere firmly to the enzyme. The same functionality is manifested in the kinetics of substrate hydrolysis, as indicated in an accompanying article (20) and as will be elaborated subsequently. The higher $pK_a$ secured by curve-fitting might correspond to an $E$. $I$ complex, but it is of uncertain reliability since inhibitor absorption restricts spectrophotometric kinetic analysis in the pH region > 8; consequently, no interpretation should be applied to this ambiguous acid dissociation constant.

Proline is a regular product of dipeptide scission by prolidase, and not surprisingly it too inhibits the enzyme competitively (6–8). However, its pH profile is the inverse of that for acetylproline, as may be seen in Fig. 2. Adherence to prolidase is much tighter in neutral or alkaline solution with proline as inhibitor. The data are best fitted to the equation $K_i$ = $K_i$ (1 + $K_i$/[$H^+$])/(1 + $K_i$/[$H^+$]) · ([1 + $K_i$/[$H^+$]])$^N$ which is most easily explained. The proline nitrogen ($pK_a$ (7)) releases a proton in alkaline solution. The $K_i$ value corresponds to the zwitterionic form of the imino acid (pH < 9), whereas an...
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An extrapolated \((K_i)_{\text{linus}}\) value of 0.04 \((\pm 0.24)\) mM is found for the anion of proline \((\text{pH of} >11)\). The upward inflection on the alkaline limb of the \(pK_a\) versus \(pH\) profile, characterized by a fitted \(pK_a\) value of 9.74 \((\pm 0.98)\), probably correlates with deprotonation of proline ammonium ion within the \(E.1\) complex. An eventual leveling off of the profile appears to occur at a yet higher \(pH\), and for the latter inflection the calculated \(pK_a\) of 10.6 corresponds to that recorded for \(N\)-deprotonation of free proline zwitterion. In other words, the small sigmoidal wave seen in Fig. 2 at high \(pH\) is entirely attributable to the inhibitor; the zwitterion binds approximately 8-fold less firmly than does the anionic form of the imino acid, and this produces a compensatory 0.9 \(pH\) unit acidification of the inhibitor \(R_2NH_2^+\) group upon complexation with the enzyme (as required by thermodynamics). The more dramatic perturbation of \(K_i\) at low \(pH\) corresponds to deprotonation of an enzymic functional group, which obligatorily resides in the conjugate base form for most effective binding of proline, but as the conjugate acid for smallest attachment of acetylproline. Therefore, the fitted \(pK_a\) value of 6.6 \((\text{average})\) on the acidic-limb is quite close to that of an imidazolium ion, consideration had to be given to the presence of an essential histidine residue at the active site. However, exposure of the activated enzyme to diethyl pyrocarbonate, under conditions that are standard for carbethoxylation of the histidine side chain, failed to give the anticipated permanent inactivation. (An instantaneous, but temporary, partial diminishment of activity was noted with high concentrations of pyrocarbonate; this is believed to be a noncovalent competitive inhibition which is relaxed as the reagent spontaneously solvolyzes.) This negative result permits no conclusion; the identity of the enzymic residue providing the \(pK_a\) of 6.6 will be addressed subsequently.

A limited investigation of structural variation as regards prolidase inhibition was undertaken. The \(D\)-enantiomer of proline failed to perturb the kinetics of prolidase for any \(pH\) at concentrations up to 35 mM. Accordingly, it binds at least 120-fold less tightly than does \(L\)-proline, in agreement with the enzymic recognition of only \(L\)-substrates. The \(L\)-cystine-derived analogue \(R.4\)-thiazolidinocarboxylic acid ("thiazoproline") exhibited a \(K_i\) of 0.16 \((\pm 0.02)\) mM at a \(pH\) of 7.58, a value somewhat smaller than that of \(L\)-proline at the same \(pH\). On the other hand, \(\text{trans}-4\)-hydroxyl-L-proline was found to have a \(K_i\) of 9.0 \((\pm 0.5)\) mM at a \(pH\) of 7.50, a value 27-fold higher than for \(L\)-proline at the same \(pH\). These latter observations are in accord with the catalytic specificities exhibited by the corresponding glyeryl dipeptides in an accompanying article (20).

Cyclopentanedicarboxylate—Previous evidence has suggested that prolidase contains a metal ion at the active site. \(R.2\)-Benzylocetic acid is an avidly bound inhibitor of the well characterized metalloenzyme carboxypeptidase \(A\), due to its successful mimicry of the specificity features associated with good peptide substrates of that enzyme \((i.e.\ a\ C\text{-terminal Phe residue})\), plus the presence of a second carboxylate for ligation to the active site metal ion (9). Reasoning strictly by analogy, we imagined that an appropriate isomer of cyclopentanedicarboxylic acid could behave similarly toward prolidase. For example, \(\text{trans}-L.1,2\)-cyclopentanedicarboxylic acid, \(1\), possesses the specificity-conferring 5-membered ring with properly configured carboxylate substituent of a substrate, such as 2. Furthermore, the additional carboxylate spatially occupies the location of the scissile linkage of 2 and would consequently be positioned to ligate to an active site metal ion, were such present to function as a carbamoyl-activating Lewis acid in prolidase.

When tested, \(1\) was found to constitute a potent inhibitor for prolidase, with \(K_i\) values in the submicromolar range, as will be elaborated (contemporaneously reported (10)). The \(D\)-enantiomer of \(1\) binds much less tightly; its \(K_i\) value is at least 60-fold higher. (The weaker inhibition expressed by \(D\) \(1\) may either be intrinsic or may be due to residual \(L\)-1, resulting from incomplete resolution, possibilities that are not easily distinguished.) Clearly, \(1\) conforms to the steric requirements of the enzyme active site, and the high affinity of such a small molecule for prolidase strongly suggests surrogacy for 2.

Several peculiarities characterize the binding of \(1\) to prolidase, however. As shown in Fig. 3, the inhibition appears to be only partial. A careful examination of the hyperbolic inhibition curves reveals approximately 20% residual enzyme activity after saturation of the enzyme with 1 (in the micromolar range). The solid curve in Fig. 3 is a least squares fit to a partial inhibition equation, whereas the dashed line corresponds to a best fit of the same data for putative complete inhibition \((\text{equations given in legend})\). The latter curve is clearly an inferior approximation. This pattern was consistently observed for data collected at all \(pH\) values. Furthermore, a systematic variation of both substrate and inhibitor concentration (Fig. 3, inset) shows a perturbation of \(k_{\text{cat}}\) but not of \(K_m\) for the hydrolisis of glycerylproline, indicating that the inhibition by \(1\) is noncompetitive, also in contrast to
and extremely low values of $K_2$.

Such behavior ordinarily would not indicate substrate surrogacy by L-proline, but with a significant difference. These anomalous properties extend to the pH dependence of $K_2$. In Fig. 4 may be found a pH profile for the inhibition of prolidase by 1, the pattern generally corresponds to that seen previously for acetylproline, but with a significant difference. The pattern of inhibition of prolidase by 1 is noncompetitive and only partial (with apparent residual enzymic activity even at relatively high concentrations of inhibitor), and that two enzyme deprotonations apparently regulate the binding of one inhibitor molecule in the submicromolar range, are unparalleled for the prolidase inhibitors (and substrates) previously described. To help illuminate this behavior, some analogues of 1 were investigated.

**Other Carboxylates**—In an effort to clarify the significance of the inhibition of prolidase by 1, a series of homologous mono- and dicarboxylic acids were compared as inhibitors at a nominal pH of 6, using glycylproline as the assay substrate (with $[S] \ll K_m$). The results are summarized in Table I. The $K_i$ values for the dicarboxylic acids were determined from a fit of the data to the partial inhibition equation used for 1, and each was corrected as before for the respective pH-dependent fraction of dianionic species. The $K_i$ for cyclopentanecarboxylic acid was obtained from a fit of the data to the more conventional hyperbolic complete inhibition equation. The diminished affinity exhibited by the enzyme toward cyclopentanecarboxylic acid in comparison with 1 (i.e. $K_i \sim 600$-fold larger) confirms the importance of the second carboxylate on 1. Succinic acid may also be regarded as a "fragment" of 1, since it contains two carboxylate groups on adjacent carbon atoms as does 1, but it lacks a carbocyclic moiety. The weak binding of succinic acid in comparison with 1 (i.e. $K_i \sim 2000$-fold larger) is proof of a specific requirement for a ring component in order to achieve tight binding. As previously indicated, the enantiomer of 1 shows at least 10-fold lower affinity for prolidase, proving that stereochemistry is important. Since 1 is known to have the same configuration as L-proline (which binds better than D-proline), it follows that the orientation of the carboxylate which is isostructural as L-proline matters; i.e. one of the carboxylate groups of 1 fits a portion of the binding site which recognizes the carboxylate group of L-proline. Apparently neither of the carboxylic acids having an incorrect orientation within the D-enantiomer of 1 can do so. On the other hand, the magnitudes of the apparent $K_i$ values for the meso-configuration inhibitors cis-1,2-cyclopentanedicarboxylic acid and cis-1,2-cyclohexanedicarboxylic acid, as recorded in Table I, are approximately equal to that for 1. This shows that orientation matters only at one carboxylate. The results indicate that the second carboxylate on a carbocycle contributes importantly to the extreme inhibition observed for 1, but that its steric configuration is not of major significance.

| Inhibitor* | $(K_i)_{apparent}$ | pH |
|-----------|------------------|----|
| trans-1,2-Cyclopentanedicarboxylic acid | 0.09 (±0.03) | 5.93 |
| trans-1,2-Cyclohexanedicarboxylic acid | ≥5.5 (±0.7) | 5.93 |
| cis-1,2-Cyclopentanedicarboxylic acid | 0.08 (±0.004) | 6.01 |
| cis-1,2-Cyclohexanedicarboxylic acid | 0.14 (±0.02) | 6.08 |
| Succinic acid | 176 (±48) | 6.06 |
| Cyclopentanecarboxylic acid | 51 (±1) | 5.95 |

*For carboxylates, $K_i$ adjusted to correct for second acid dissociation of each species as given individually (assuming that the dianion is exclusively active).

$pK_{an} = 5.85$ (11).
$pK_{an} = 6.77$ (12).
$pK_{an} = 6.61$ (13).
While each of the categories of prolidase inhibitor examined here in detail (proline, acetylproline, and cyclopentanedicarboxylate) has its own unique pH dependence, all are strongly influenced by the protonation state of an enzymic residue with a pKᵣ value of ~6.6. This clearly is the same proton donor which participates in the binding or activation of dipeptide substrates, as described in an accompanying article (20). At minimum, the present evidence points to this moiety being cationic in its conjugate acid form. In weakly acidic solution, zwitterionic proline as an inhibitor is rejected by the enzyme, an apparent consequence of its presenting a positively charged R₂NH⁺ group to this cationic enzyme residue at the active site. On the other hand, diaminoc 1 is bound most firmly in acidic solution and several orders of magnitude more tightly than acetylproline. The most obvious candidate for an enzymic cation with a pKᵣ of 6.6 would be a histidine residue, but no such species could be detected. We rather suggest that the inhibition patterns observed are most readily accommodated by means of the mechanistic explanation advanced to rationalize the substrate specificities exhibited by prolidase. We hypothesize the existence within the active site of an essential metal ion, which in the absence of substrate or inhibitor coordinates to a solvent water molecule and acidifies it to a pH of ~6.6 (e.g. H₂O:M⁺⁺Enz). The metal ion is positioned to interact with the carbonyl oxygen of the scissile amide linkage of a substrate, so as to activate it in Lewis acid fashion for nucleophilic addition. Proline does not displace this water molecule; consequently, it experiences an electrostatic repulsion that is relaxed only above a pH of 6.6, when hydroxide becomes the metal ligand. Tightest binding of acetylproline or of 1, involving coordination to the metal ion, may only be observed at a pH below 6.6, since only H₂O (and not HO⁻) may be displaced. The relative basicities of a carboxylate (in 1) and a carboxamide (in acetylproline) explains the 1000-fold tighter ligation of the cyclopentanedicarboxylates. This proposal may be reconciled with the apparent absence of stereospecificity as regards inhibition of prolidase by the cis and trans forms of 1. Were the enzymic metal ion positioned to interact chelatively with a bound substrate in the plane of the scissile carboxamide linkage, it would necessarily reside in the plane of the 5-membered ring also. Consequently, a carboxylate projecting either above or below that plane should be capable of reaching the metal ion, as depicted in Scheme 1.

While the foregoing explanation seems to rationalize most convincingly the overall patterns of inhibition and substrate activation by prolidase, there are certain anomalous details in our results which require further commentary. The inhibition by 1 is noncompetitive, only partial, and exhibits a second order pH dependence, unlike the more weakly bound inhibitors (and substrates). This evidence may be rationalized with an hypothesis that prolidase is catalytically active as a dimer (14-19) exhibiting some selective cooperativity phenomena. The strong partial inhibition by cyclopentanedicarboxylate may be indicative of a firm ligation of the dianion to one active site in a dimeric enzyme, with further inhibition of a second active site only at a much higher inhibitor concentration. Such behavior can be shown to create an appearance of noncompetitive inhibition in certain circumstances. In addition, the second order pH dependence for the binding of 1 suggests that the inhibitor is most firmly bound when two enzymic functional groups with pKᵣ of 6.6 are protonated, perhaps one active site residue from each half of a dimer. This mode of cooperativity appears to be restricted to tightly bound enzymic ligands (Kᵣ values in the micromolar range), since no comparable perturbations are seen in the kinetics of dipeptide substrate hydrolysis.

In order to demonstrate the kinetic competence of this idea, we offer Scheme 2 as a mechanism for approximating our dipeptide hydrolysis data (for [S] ≪ Kᵣ). The common acid dissociation constant Kᵣ pertains to the doubly (E₂H₂) and singly (E₂H) protonated enzyme dimers (pKᵣ value of ~6.6, factors of ½ and 2 for statistical correction). The components of the dimer function independently for dipeptide substrates (as well as for the more weakly bound, competitive inhibitors). The inhibitor 1 binds only to the doubly protonated enzyme dimer with inhibition constant Kᵣ (in the micromolar range, residual catalytic activity of E₂H₁ ignored for simplicity). The influence of the substrate amino group is reflected in Kᵣ (dipeptide active only as the conjugate base). This scheme approximates the kinetic behavior at low substrate concentrations (Kᵣ/Kᵣ), provided that the previously adduced reverse-protonation mechanism pertains. The kinetic expression concerning to this mechanism may be formulated as follows: v/ [E] = 2k[S][1/(1 + Kᵣ/[H])·(1 + [H]/Kᵣ')·(1 + [I]/(Kᵣ·(1 + Kᵣ/[H])))). It may be seen that the more puzzling features of prolidase catalysis are reproduced, namely, the bell-shaped pH profile for substrate hydrolysis and the second order pH dependence for the apparent value of Kᵣ with 1.

This scheme for prolidase as a dimeric enzyme may be further extended conjecturally to explain certain differences in the hydrolyses of dipeptides and nonphysiological substrates such as chloroacetylproline and methylthioacetylproline (as described in an accompanying article (20)). The latter species (lacking of pKᵣ') are readily cleaved enzymically, but with a kᵣ/Kᵣ versus pH profile that has a much narrower bell shape than for glycylproline, and that is best fitted with coincident acidic- and alkaline-limb pH vs. kᵣ values of 6.6. This would be explained if for these substrates the half-protonated dimer (E₂I) were significantly more active than the unprotonated (E₂) or the fully protonated enzyme (E₂H₂); i.e. cooperative interactions between enzyme monomers are manifested with the latter substrates as well. As previously described, a dissimilar pH profile in kᵣ/Kᵣ for alternate substrates requires either different productive enzyme conformations or different first committed steps in the enzyme mechanism or some similar such explanation. While the root causes for the apparent differing enzymic affinities (cyclopentanedicarboxylate preferring E₂H₂, chloroacetylproline preferring E₂H, glycylproline accepted by either) are not readily discernable, selective cooperativity between enzyme mono-
mers presents in principle the most parsimonious rationalization for the reactivity patterns that are observed. Of course, we would not exclude more complex explanations for the phenomena. Finally, a recent report suggests a possible reason for this enzymic behavior. The ubiquitous biochemical intermediate phosphoenolpyruvate is reported to inhibit prolidase analogously to cyclopentanedicarboxylate and at concentrations well below its prevailing physiological concentration (10). We suspect that it too will prove to be only a partial inhibitor (as are all the examples of micromolar concentration active inhibitors that we have encountered). We broadly suggest that enzymic cooperative interactions are triggered by tight-binding ligands (which are perhaps best considered as transition state analogues) and are designed to ensure an acceptable minimum level of prolidase activity in the presence of such unavoidable endogenous inhibitors. The artifact kinetic consequences that we have observed may merely be a reflection of conformational changes within an enzyme mechanism operating under these constraints.

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