Analogues of Intermediates in the Action of Pig Kidney Prolidase†

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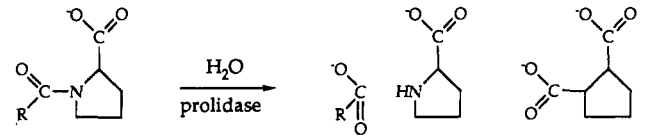
ABSTRACT: Dicarboxylic acids, resembling the collected substrates for the reverse peptide bond forming reaction, were bound several orders of magnitude more tightly than substrates, products, or previously known competitive inhibitors of reactions catalyzed by pig kidney prolidase (EC 3.4.13.9), a dipeptidase that cleaves peptide bonds to the nitrogen atom of proline. Other inhibitors containing a phosphoryl or phosphonyl group in addition to a carboxyl substituent were bound even more tightly, in a manner consistent with their possible resemblance to tetrahedral intermediates in substrate hydrolysis. These included several analogues of phosphoenol pyruvate, of which the most potent was (*Z*)-3-bromophosphoenolpyruvate (K_i = 4.6 × 10^{-9} M). K_i values were found to vary with changing pH in a manner consistent with displacement of a hydroxide ion from the active site.

Prolidase (EC 3.4.13.9) is a manganese-dependent hydrolase that cleaves dipeptides involving the nitrogen atom of proline (Bergmann & Fruton, 1937) in the trans configuration (King et al., 1989). This enzyme is present in microorganisms and many mammalian tissues, where it is believed to catalyze terminal degradation of exogenous and endogenous proteins, permitting recycling or renal excretion of proline and hydroxyproline [for a review, see Walter et al. (1980)]. In humans, a deficiency of prolidase results in a complex clinical syndrome involving mental retardation [for a review, see Kaloustian et al. (1982)].

This paper describes an effort to develop strong competitive inhibitors of prolidase. In designing inhibitors of this enzyme, our intent had been to combine structural features of the two substrates for reversal of dipeptide hydrolysis within a single molecule. Sparing an enzyme the entropic difficulty of gathering the binding determinants of two substrate molecules from dilute solution, a multisubstrate analogue inhibitor should exhibit a binding affinity considerably higher than the affinities of the two substrates combined (Byers & Wolfenden, 1972). Accordingly, it seemed possible that compounds related to 1,2-cyclopentanedicarboxylic acid (Scheme I) might serve as effective inhibitors of prolidase. In a preliminary communication (Radzicka & Wolfenden, 1990), we noted the effectiveness of this inhibitor and also the unexpectedly powerful inhibition of this enzyme by phosphoenolpyruvate, an intermediate in glycolysis. We have since uncovered several inhibitors that are even more potent than phosphoenolpyruvate and have investigated the variation of K_i with pH with results suggesting that binding is accompanied by displacement of a hydroxide ion from the active site. Inhibitors containing phosphoryl and phosphonyl substituents are found to be unusually potent, perhaps because these inhibitors resemble sp^3-hybridized oxyanion intermediates formed during substrate hydrolysis.

Scheme I



substrate products analog inhibitor

Experimental Procedures

Materials. Compounds included in the present study, referred to by number here and in subsequent text, are shown by name and structure in Table I. Compounds 3, 8–10, 12–15, 18, 20–21, 27, 29–30, 33, 35, 37–38, 41–43, 46–47, 50, 53, 55, 57, and 64 as the monopotassium salt and *cis*-1,2-cyclohexanedicarboxylic anhydride and 4-morpholineethanesulfonic acid (MES) were obtained from Aldrich Chemical Co. Compounds 2, 7, 26, 31–32, 36, 45, and 54, Gly-Pro, DEAE-Sephacel, and pig kidney prolidase were obtained from Sigma Chemical Co. Compounds 17, 22, 24–25, 28, 49, and 60–62 were purchased from Fluka Chemical Corp.. Compound 1 was purchased from EM Science Co. and compound 4 from the California Corp. for Biochemical Research; compounds 5 and 6 were purchased from Calbiochem-Behring Co. and compound 11 and manganous chloride from Allied Chemical Co.; compound 16 was obtained from Pfaltz & Bauer, Inc., compounds 23 and 44 were from Alfa Products, Inc., compound 34 was from Serva Fine Biochemicals, Inc., compound 39 from Monsanto Co., compound 48 from Fairfield Chemical Co., Inc., and Met-Pro from Bachem Bioscience Inc. Compound 19 was a gift from E. R. Squibb & Sons, Inc. Compound 52 was obtained by hydrolysis of *cis*-1,2-cyclohexanedicarboxylic anhydride, and compound 59 was prepared by the method of Fleury and Courtois (1941). Compounds 51, 56, 61, 63, 65, 66, and 67 were kindly provided by Drs. M. H. O'Leary and J. A. Peliska of the University of Wisconsin; and compounds 39 and 58 were a generous gift from Dr. D. Grobelny of the University of Kentucky.

Enzyme Activation and Assay. Porcine kidney prolidase was activated in 0.05 M Tris-HCl buffer, pH 8.0, containing $MnCl_2$ (27 mM) and glutathione (1 mM), at 37 °C for 45 min (Smith et al., 1944; Davies & Smith, 1957). The activity of prolidase was assayed by monitoring the disappearance of the peptide chromophore at 222 nm, where Δe_M for Gly-Pro was -904. Assays were routinely performed in 0.01 M K^+-MES buffer, pH 6.0, at 25 °C with 0.7–1.3 units of enzyme in a volume of 2 mL, with Gly-Pro as the substrate (K_m = 1.2 × 10^{-3} M). MES buffers were used in all experiments, because the activity of the enzyme was strongly inhibited in buffers such as acetate and phosphate but was not much affected by K^+-MES even at 10-fold higher concentrations than those used

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Table I: Inhibitors of Pig Kidney Prolidase

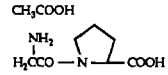
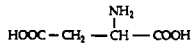
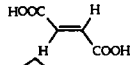
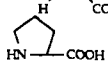
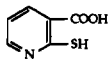
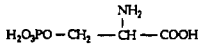
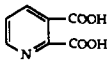
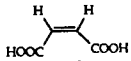
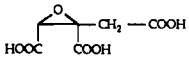
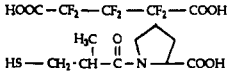
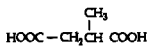
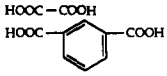
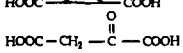
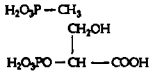
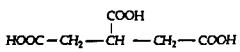
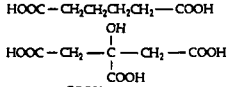
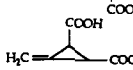
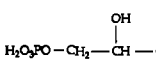
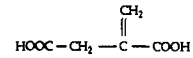
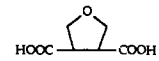
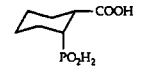
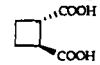
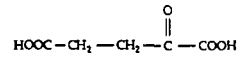
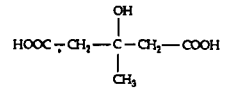
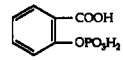
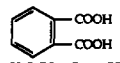
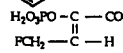
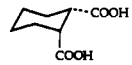
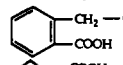
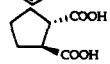
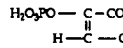
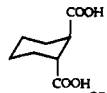
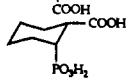
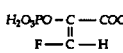
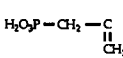
#	Name	Structure	K_i [M]
1.	Acetic acid	CH_3COOH	1.1×10^{-2}
2.	Gly-Pro		1.2×10^{-3}
3.	Ammonium sulphate	$(NH_4)_2SO_4$	9.6×10^{-3}
4.	1-Aminocyclopentane--1-carboxylic acid		$>5\times10^{-3}$
5.	DL-Aspartic acid		1.3×10^{-3}
6.	Fumaric acid		$>1\times10^{-3}$
7.	L-Proline		7.0×10^{-4}
8.	3-Mercaptopropionic acid	$HS-CH_2-CH_2-COOH$	6.3×10^{-4}
9.	2-Mercaptonicotinic acid		5.6×10^{-4}
10.	DL-Serine phosphate		5.1×10^{-4}
11.	Succinic acid	$HOOC-CH_2-CH_2-COOH$	4.9×10^{-4}
12.	Acetylenedicarboxylic acid	$HOOC-C\equiv C-COOH$	4.5×10^{-4}
13.	2,3-Pyridinedicarboxylic acid		4.4×10^{-4}
14.	Maleic acid		4.1×10^{-4}
15.	DL-*trans*--1,2-Cyclopropanedicarboxylic acid		4.0×10^{-4}
16.	Malonic acid	$HOOC-CH_2-COOH$	4.0×10^{-4}
17.	(±)-*cis*-Epoxytricarballylic acid		3.5×10^{-4}
18.	Hexafluoroglutaric acid	$HOOC-CF_2-CF_2-CF_2-COOH$	3.1×10^{-4}
19.	Captopril		2.7×10^{-4}
20.	Methylsuccinic acid		2.2×10^{-4}
21.	Oxalic acid	$HOOC-COOH$	2.1×10^{-4}
22.	Isophthalic acid		2.0×10^{-4}
23.	Ethylenediphosphonic acid	$H_2O_3P-CH_2-CH_2-PO_3H_2$	1.7×10^{-4}
24.	2,3-Epoxysuccinic acid		1.6×10^{-4}
25.	Oxalacetic acid		1.6×10^{-4}
26.	Methylphosphonic acid	$H_2O_3P-CH_3$	1.6×10^{-4}
27.	D(+)-2-Phosphoglyceric acid		1.3×10^{-4}
28.	Tricarballylic acid		1.3×10^{-4}
29.	Phosphonoformic acid	$H_2O_3P-COOH$	1.2×10^{-4}
30.	Diglycolic acid	$HOOC-CH_2-O-CH_2-COOH$	1.2×10^{-4}
31.	Adipic acid	$HOOC-CH_2CH_2CH_2CH_2-COOH$	8.3×10^{-5}
32.	Citric acid		7.4×10^{-5}
33.	3-Methylenecyclopropane-*trans*-1,2-dicarboxylic acid		7.2×10^{-5}
34.	DL-3-Phosphoglyceric acid		7.1×10^{-5}
35.	Cyclopentanecarboxylic acid		6.2×10^{-5}
36.	Glutaric acid	$HOOC-CH_2-CH_2-CH_2-COOH$	6.1×10^{-5}
37.	Itaconic acid		4.7×10^{-5}
38.	3,4-Furandicarboxylic acid		3.8×10^{-5}
39.	2-Phosphinocyclohexanecarboxylic acid		2.4×10^{-5}
40.	N-(Phosphonomethyl)-glycine	$H_2O_3P-CH_2-NH-CH_2-COOH$	2.3×10^{-5}
41.	Methylenediphosphonic acid	$H_2O_3P-CH_2-PO_3H_2$	1.8×10^{-5}
42.	Thiodiglycolic acid	$HOOC-CH_2-S-CH_2-COOH$	1.5×10^{-5}
43.	(±)*trans*-1,2-Cyclobutanedicarboxylic acid		1.5×10^{-5}
44.	Propylenediphosphonic acid	$H_2O_3P-CH_2-CH_2-CH_2-PO_3H_2$	1.3×10^{-5}
45.	2-Ketoglutaric acid		1.2×10^{-5}
46.	3-Hydroxy-3-methylglutaric acid		1.1×10^{-5}
47.	*cis*-1,2-Cyclobutanedicarboxylic acid		8.5×10^{-6}
48.	4-Phosphonobutyric acid	$H_2O_3P-CH_2-CH_2-CH_2-COOH$	2.4×10^{-6}
49.	2-Carboxyphenyl phosphate		1.7×10^{-6}
50.	Phthalic acid		1.2×10^{-6}
51.	(*Z*)-3-Fluoromethylphosphoenolpyruvic acid		9.7×10^{-7}
52.	*cis*-1,2-Cyclohexanedicarboxylic acid		7.9×10^{-7}
53.	Phosphonoacetic acid	$H_2O_3P-CH_2-COOH$	5.8×10^{-7}
54.	Homophthalic acid		5.3×10^{-7}
55.	*trans*-DL-1,2-Cyclopentanedicarboxylic acid		5.1×10^{-7}
56.	(*E*)-3-Cyanophosphoenolpyruvic acid		2.0×10^{-7}
57.	*trans*-DL-1,2-Cyclohexanedicarboxylic acid		1.3×10^{-7}
58.	2-Phosphonocyclohexanecarboxylic acid		9.25×10^{-8}
59.	Phosphoglycolic acid	$H_2O_3PO-CH_2-COOH$	8.8×10^{-8}
60.	3-Phosphonopropionic acid	$H_2O_3P-CH_2-CH_2-COOH$	7.2×10^{-8}
61.	(*Z*)-3-Fluorophosphoenolpyruvic acid		4.5×10^{-8}
62.	2-(Phosphonomethyl)-acrylic acid		1.3×10^{-8}
63.	(*Z*)-3-Chlorophosphoenolpyruvic acid		9.6×10^{-9}
64.	Phosphoenolpyruvic acid		8.5×10^{-9}
65.	(*Z*)-3-Methylphosphoenolpyruvic acid		6.2×10^{-9}
66.	Phospholactic acid		5.0×10^{-9}
67.	(*Z*)-3-Bromophosphoenolpyruvic acid		4.6×10^{-9}

Table II: Influence of pH on the Activity of Prolidase on Gly-Pro and Its Inhibition by 3-Phosphonopropionic Acid at 25 °C[a]

pH	5.5	6.0	6.5	7.0	7.5
V_{max} (μmol min^{-1} mg^{-1})	382	1230	1390	1150	915
K_m (Gly-Pro) (M)	1.8×10^{-3}	1.7×10^{-3}	1.2×10^{-3}	6.7×10^{-4}	5.2×10^{-4}
V_{max}/K_m	4.7×10^{-2}	1.6×10^{3}	2.6×10^{3}	3.9×10^{3}	4.0×10^{3}
K_i (60)[b] (M)	5.7×10^{-8}	5.8×10^{-8}	3.3×10^{-7}	3.2×10^{-6}	$>1.8 \times 10^{-4}$

[a] Values were measured in K$^+$-MES buffers (0.01 M), I = 0.1, containing $MnCl_2$ (3×10^{-3} M) and glutathine (1.25×10^{-5} M). [b] Values measured for 3-phosphonopropionic acid (60), uncorrected for the concentration of the active form of the inhibitor.

in the assay. Substrate and inhibitor solutions were normally prepared in the assay buffer, except in the case of a few compounds whose solubility in water was limited, which were taken up in methanol. In these cases 3 μL of solvent methanol was introduced per milliliter of assay solution, but these levels of methanol were found to produce no significant inhibition. K_i values (Table II) were obtained from double-reciprocal plots of the reaction rate as a function of substrate concentration ranging from 2.5×10^{-4} to 2×10^{-3} M.

Purification of Prolidase. Commercial pig kidney prolidase (10 mg, 205 units/mg of protein, suspended in saturated ammonium sulfate) was dissolved in 4 mL of 0.05 M Tris-HCl buffer, pH 8.0, dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, at 4 °C, and applied to a 18 × 250 mm DEAE-Sephacel column. The enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in 0.05 M Tris-HCl buffer (2 × 250 mL), pH 8.0, at 4 °C. A flow rate of 6 mL/h was maintained, and elution was monitored at 280 nm. Each fraction was divided into two parts. The first set of fractions was assayed for prolidase I activity according to Myara (1987) (see Results); after addition of manganese and glutathione, each fraction was incubated as described above and assayed with Gly-Pro as a substrate. The second set of fractions was assayed for prolidase II activity according to Myara (1987) with Met-Pro as a substrate. In this assay manganese and glutathione were added to the enzyme directly before mixing with the substrate, to prevent prolidase II from losing activity. In other respects, the assay was similar to the assay for prolidase I activity.

Determination of K_m, V_{max}, and K_i as a Function of pH. Values for K_m and V_{max} (Table II) were obtained from double-reciprocal plots of the reaction rate as a function of substrate concentration at pH values from 5.5 to 7.5, with other conditions as described above. These experiments were performed in 0.01 M K$^+$-MES buffers of ionic strength 0.1 (maintained by addition of KCl) containing 0.3 mM $MnCl_2$ and 0.012 mM glutathione. The concentration of Gly-Pro varied from 2.5×10^{-4} to 2×10^{-3} M. K_i values of 3-phosphonopropionic acid (compound 60, pK_a 2.26; 4.63; 7.75) (Heubel et al., 1979) were determined under the same conditions, as a function of changing pH.

Results

Earlier assays of prolidase were based on the analysis of reaction mixtures for proline at timed intervals by a variety of discontinuous methods (Chinard, 1952; Mayer & Nordwig, 1973; King et al., 1986). For convenience in searching for prolidase inhibitors, we developed a continuous spectrophotometric assay based on the disappearance of the Gly-Pro peptide chromophore at 222 nm in K$^+$-MES buffer at pH 6.0 (Radzicka & Wolfenden, 1990). The enzyme's activity on this substrate over the pH range between 5.5 and 7.5 is shown in Table II.

Human erythrocytes have been reported to contain two forms of prolidase that differ in specificity with respect to the N-terminal amino acid and can be separated chromatographically (Myara, 1987). It was therefore of interest to determine whether two forms of the enzyme were also present in the pig kidney prolidase purchased from Sigma Chemical Co. When this preparation was desalted and subjected to chromatography on DEAE-Sephacel by Myara's procedure, with a linear salt gradient in Tris-HCl buffer, only one peak was eluted from the column, at 0.14 M NaCl. The relative activities of all fractions on two different substrates were as follows: Gly-Pro after preincubation of the enzyme with Mn^{2+} and Met-Pro without such preincubation were found to be invariant and consistent with "prolidase I" character as defined by Myara. Prolidase II, if that had been present, should have been eluted in the neighborhood of 0.26 M NaCl and should have been active against Met-Pro without preincubation with Mn^{2+}. Thus, the enzyme used in the present studies was considered equivalent to prolidase I as defined by Myara.

Double-reciprocal plots of the initial reaction velocities against substrate concentration, determined in the presence and absence of inhibitors, were linear, intersecting at the ordinate as expected for competitive inhibition. Table I shows the results obtained with various carboxylic, phosphoric, and phosphonic acid inhibitors in MES buffer at pH 6.0, arranged in order of increasing effectiveness.

K_m and V_{max} values for the substrate Gly-Pro (pK_a values = 2.8 and 8.65) (Perrin, 1965), in K$^+$-MES buffers at several pH values, are shown in Table II along with K_i values for 3-phosphonopropionic acid (60). The enzyme showed maximal activity at pH values above 7.5, and the effects of varying pH were consistent with the conversion of the enzyme to an inactive form below a pK_a value in the neighborhood of 6.3. Inhibition became more effective with decreasing pH, suggesting that the inhibitor might be bound in a protonated form, or with the release of a hydroxide ion from the enzyme as discussed below.

Discussion

trans-1,2-Cyclopentanedicarboxylic acid (55), considered a potential bisubstrate analogue inhibitor (Scheme I), was found to exhibit a K_i value several orders of magnitude lower than that of the product L-proline (compound 7, Table I) or that reported for carbobenzoxy-Pro (King et al., 1989). On the basis of this initial observation, we examined a variety of carboxylic, phosphoric, and phosphonic acids, with the results shown in order of increasing affinity (or decreasing K_i value) in Table I.

Within the group of compounds containing only carboxylic acid functions, tightest binding was observed in dicarboxylic acids (compare compound 1 with 16, compound 35 with 55, and compound 28 with 36). Within the group of acyclic dicarboxylic acids, optimal effectiveness was observed when the carboxyl carbon atoms were separated by four bond lengths (for example, compare compound 11 with compounds 16 and 21 and compound 31 with 36). When three bond lengths separated these carbon atoms, activity was little affected by the introduction of a 2,3 double bond in the cis arrangement but was reduced by a trans arrangement (compare compound 6 and compounds 11 and 14). Within the group of alicyclic dicarboxylic acids, inhibitory effectiveness was found to approach an optimum as the size of the ring increased to five

members, remaining approximately the same for a six-membered ring (compare compound 15 with 43 and compound 43 with compounds 55 and 57). In their comparison of carbobenzoxy amino acids as prolidase inhibitors, King et al. (1989) observed a similar trend, although inhibition was markedly weaker than in the present series.

Prolidase was found to be very strongly inhibited by both the cis (52) and trans (57) isomers of 1,2-cyclohexanedicarboxylic acid, the trans isomer being somewhat more powerful (Table I). Inspection of molecular models suggests that in the cis isomer the carboxyl carbon atoms in their equatorial and axial positions are separated by roughly 3.08 Å. In the trans isomer, with both carboxyl groups equatorial, the separation is 3.07 Å, whereas in the less abundant isomer with both carboxyl groups axial, the separation is 3.9 Å. In phthalic acid (50), a similarly effective inhibitor with both carboxyl groups in the same plane, the separation between the carboxyl carbon atoms is roughly 3.1 Å.

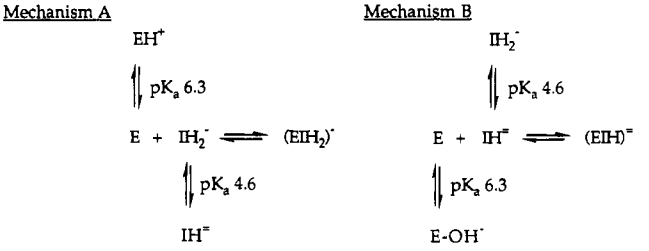
Phospho- and phosphonocarboxylic acids proved to be even more potent inhibitors than the corresponding dicarboxylic acids (compare compound 11 with 60, compound 21 with 29, and compound 37 with 62), but diphosphonic acids, although more inhibitory than dicarboxylic acids, were less effective than phospho- or phosphonocarboxylic acids (compare compound 11 with 23 and compound 23 with 60; also compare compound 36 with 44 and compound 44 with 48). The exceptional potencies conferred on carboxylic acids by the presence of a single phospho or phosphono group may be due to their higher affinities for an active-site metal ion (see below) and/or to the possibility that the single phospho or phosphono group may adopt a tetrahedral configuration at the phosphorus that resembles an sp^3-hybridized intermediate in peptide hydrolysis. In earlier work in zinc-containing peptidases (Tronrud et al., 1970; Bartlett & Marlowe, 1987; Hanson et al., 1989), very tight binding of phosphonates was ascribed to this latter property.

Among the most effective inhibitors uncovered by this study were derivatives of phosphoenolpyruvic acid, fluorinated (61), chlorinated (63), or brominated (67) at position 3. Inhibition by each of these potential derivatizing agents was found to be reversible, suggesting that active-site derivatization does not occur.

To obtain information concerning the probable state of ionization of one of the more potent inhibitors in its bound form, we investigated the influence of changing pH on inhibition by 3-phosphonopropionic acid (60). A difficulty raised by the observations in Table I is that no buffer exists that would not be expected to be capable, at least in principle, of interacting with metal ions that are presumably present at the enzyme's active site, and acetate and phosphate buffers were found to inhibit the enzyme strongly at a concentration of 0.01 M. However, we saw little evidence of inhibition by K^+-MES buffers even at concentrations (0.1 M) 10-fold higher than those used in the present experiments.

The results obtained in K^+-MES buffers, presented in Table II, show that inhibition by 3-phosphonopropionic acid [whose pK_a values are 2.3, 4.6, and 7.75 (Heubel et al., 1979)] was sharply dependent on changing pH, with its K_i value decreasing by almost two orders of magnitude as the pH was reduced from 7 to 6.[1] These findings appear to be formally consistent

Scheme II: Alternative Mechanisms of Binding Involving (A) Proton Release by the Enzyme or (B) Hydroxide Ion Release from the Enzyme That Could Lead to the pH Dependence of Binding Described in the Text



with either of two mechanisms of binding, shown in Scheme II. This scheme incorporates the additional information, from the pH dependence of V_{max}/K_m shown in Table II, that below a pK_a value of roughly 6.3 the enzyme appears to gain a proton or lose a hydroxide ion to become inactive. According to mechanism A, 3-phosphopropionic acid is bound as the doubly protonated monoanion. However, this form of the inhibitor becomes rare in solution above pH 4.6, so that this mechanism would require that the true dissociation constant of the inhibitory complex be much lower than the apparent K_i value that was determined experimentally.[2] According to mechanism B, the inhibitor is bound as a singly protonated dianion, its binding being accompanied by release of a hydroxide ion from the active site.[3] Mechanism B appears attractive because the combined evidence in Table I suggests that negatively charged carboxylate and phosphate groups are likely to serve as sites of inhibitor interaction with this metalloenzyme and because this mechanism removes the need to postulate an extremely low dissociation constant for the enzyme–inhibitor complex.

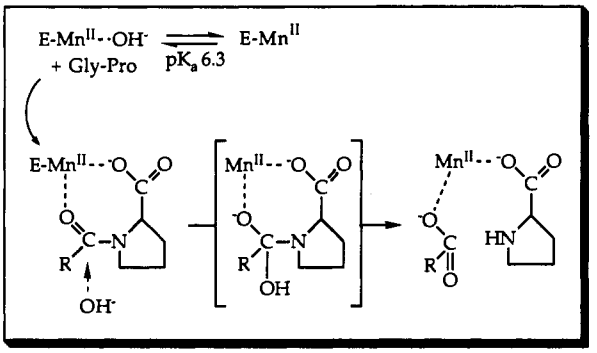
It seems reasonable to suppose that prolidase may share some mechanistic resemblance to other metallopeptidases such as thermolysin and carboxypeptidase A. King et al. (1989) have shown that prolidase is active on the trans isomer of the substrate and have speculated that the substrate may be bound through its amide oxygen and proline carboxylate groups to the octahedrally coordinated Mn^{2+} in the active site of prolidase. Oxalate, with a similar separation between its carbonyl oxygen atoms, has recently been shown to form a complex with pyruvate–phosphate dikinase involving octahedral coordination of manganese at the active site (Kofron, et al., 1988). Bound in a similar manner, the bifunctional acids of the present study might be expected to serve as strong ligands of prolidase. Furthermore, the unusual affinities of the present phosphorus-containing inhibitors raise the additional possibility that these compounds may serve as transition-state analogues as discussed above. Scheme III shows some possible binding interactions of substrates, products, and inhibitors with manganese at the active site of prolidase. All of the more potent inhibitors uncovered by the present investigation appear to be capable of adopting a configuration resembling that of

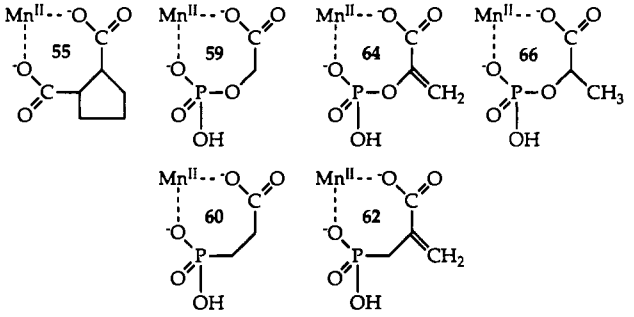
[1] In a preliminary report, of which we became aware after the present work was complete, Lacoste et al. (1989) describe the relatively weak inhibition of prolidase by compounds 26, 29, 53, and 60 at pH 8. Table II shows that at pH 8 only a small inhbitory effect would be expected for inhibitors of this type.

[2] This argument applies with greater force to the triply protonated free acid, whose concentration becomes vanishingly small at pH values much above 2.3.

[3] In view of the ability of Mn^{+2} to form strong complexes with organic acids, we considered the trivial possibility that the present inhibitors might reduce the activity of the enzyme by completely removing manganese from the active site in a reversible equilibrium process. For the more potent inhibitors, that possibility could be ruled out for stoichiometric reasons, because Mn^{+2} was present in solution at concentrations [$(1.3–3.3) \times 10^{-4}$ M] several orders of magnitude higher than those of inhibitors (10^{-7} M or lower). Accordingly, inhibition presumably occurs by complex formation at the active site.

Scheme III: One Potential Mechanism of Action of Prolidase, Showing the Resemblence of the Possible Intermediate Shown in Brackets to Enzyme Complexes Formed by Anions of Several Inhibitors That May Displace Hydroxide Ion from Manganese at the Enzymes's Active Site





an sp^3-hybridized oxyanion, shown in brackets, that may be generated during the course of substrate hydrolysis.

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