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Chemical Modification Locates Guanidinyl and Carboxylate Groups within the Active Site of Prolidase

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Summary: Reagents phenylglyoxal or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate inactivate the enzyme prolidase, with protection conferred by the competitive inhibitor *N*-acetylproline. The presence of arginine and carboxylate (aspartic/glutamic acid) residues at the active site of this metallodipeptidase may be inferred.

The enzyme prolidase (EC 3.4.13.9) exists in most mammalian tissues; its function is to cleave aminoacylproline dipeptides into the constituent amino acids. This "house-keeping" role is apparently necessitated by the inability of many other proteases to hydrolyse the acyl–proline linkage. Deficiency in the enzyme has severe consequences for individuals so afflicted, arising from the accumulation of C-terminal proline dipeptides to toxic levels. Although long known (1) and recently sequenced (2), prolidase has been poorly characterized mechanistically. The human enzyme consists of a homodimer of 492 amino acid residue subunits; the dimeric nature appears to be a common feature for prolidases from a number of sources (3). Although the sequence for prolidase has been suggested to be unique as regards known proteases (2), its inspection reveals the presence of a feature common to the zinc-containing collagenases.† Recent specificity and inhibition studies from this laboratory tend to confirm the metalloenzyme character of prolidase, and indicate a reverse-protonation mechanism for normal substrate binding and activation (5, 6). Because of several other intriguing features of prolidase, including Mn(II) activation and some puzzling kinetic complications affecting the recognition of synthetic substrates and inhibitors, a fuller investigation of this enzyme is in order.

Materials and Methods

Swine kidney prolidase was obtained from Sigma Chemical Co. (No. P 6675). Buffers employed were *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) or tris-

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†A comparison of the sequences of human erythrocyte prolidase and of human skin collagenase suggests an evolutionary relationship. In particular, residues 212-237 of collagenase [LHRV–AAHELGHSLGLHSTDIGALMYP] show a ~50% homology with residues 358-383 of prolidase [LGAVFMPHGLGHFLGI–DVHDVGG––YP]. This region incorporates the 5-residue "dihistidine loop" (underlined) that is thought to serve as the binding locus for an active site metal ion [Zn(II)?]. Several other apparently matching sequence regions within these enzymes may be found as well (4).

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[hydroxymethyl]aminomethane (TRIS), at a concentration of 50 mM. The enzyme was "activated" for 60 min with 0.021 M Mn(II) (pH 7.5, 37 °C) prior to use. For most chemical modification studies the excess Mn(II) was then sequestered by addition of nitrilotriacetate (0.028 M) in order to avoid interference with subsequent kinetic assay, following precedent (5). However, in the case of the carbodiimide reaction supererogatory carboxylate addition was avoided. Enzyme solutions so obtained showed no loss of activity over extended time periods in the absence of chemical modification reagents. During the course of the inactivation reactions (~4.5 mg/ml of enzyme, 0-50 mM phenylglyoxal, 0-20 mM carbodiimide), remaining enzyme activity was measured periodically by withdrawing aliquots and spectrophotometrically (220 nm) assaying for hydrolysis of prolidase substrate glycylproline at a pH of 7.5 and 25 °C, using the method of initial rates. For enzyme protection by competitive inhibitors such as *N*-acetylproline (K_i value of 1 mM at pH 7.5, diminishing at lower pH) (6) or *N*-carbobenzyloxyproline (K_i value of 0.035 mM at pH 7.4) (7), conditions were selected such that the dilution accompanying the transfer from inactivation mixture to assay solution (200-fold) was sufficient to dissociate the inhibitor from the enzyme, permitting prompt assay. Tolerances reported are standard errors from nonlinear least squares analysis.

Results

As part of the characterization of prolidase, and as a prelude to possible development of new types of mechanism-based inactivators of this category of enzyme, a detection of active site residues by chemical modification was undertaken. A number of reagents have been developed over the years which are selective in aqueous solution for one or a few of the side chain functionalities of peptides. Should enzymic active site residues become so modified, their presence may be revealed by the catalytic consequences. Application of this technique has been successful with prolidase in the following instances.

Arginine. The guanidine moiety capping the side chain of the amino acid arginine characteristically adds to 1,2-dicarbonyl compounds (8). The results of incubation of a buffered solution of porcine kidney prolidase with 50 mM phenylglyoxal are shown in Figure 1. A time-dependent incapacitation of the enzyme is noted when it is assayed with the prototypical substrate glycylproline.

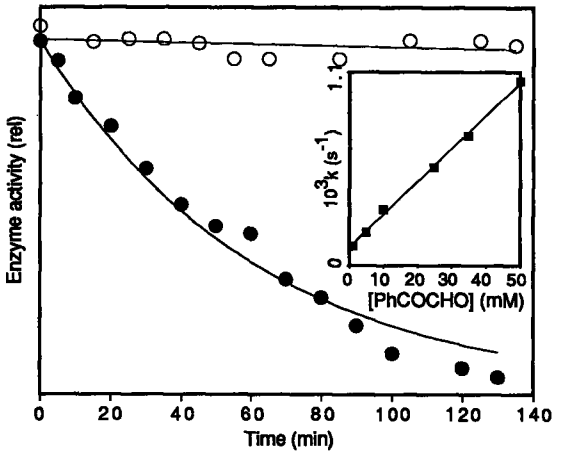

Figure 1. Relative enzyme activity (cleavage of glycylproline) as a function of time after addition of phenylglyoxal to a solution of prolidase. Filled circles, 50 mM PhCOCHO, pH 7.5 (HEPES buffer), 25 °C; open circles, same conditions, except in the presence of 0.7 mM *N*-carbobenzyloxyproline. Inset: pseudo-first order rate constant for inactivation versus concentration of reagent phenylglyoxal.

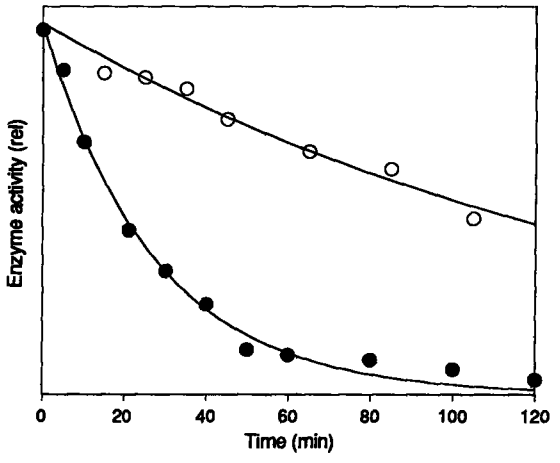

Figure 2. Relative enzyme activity as a function of time after addition of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate to a solution of prolidase. Filled circles, 20 mM reagent, pH 7.5 (TRIS buffer), 25 °C; open circles, same conditions, except in the presence of 20 mM *N*-acetylproline.

The decay in enzyme activity is exponential, extrapolating to complete inactivation. When the incubation with phenylglyoxal is carried out in the presence of competitive inhibitors *N*-acetylproline or *N*-carbobenzyloxyproline (at concentration = $20 \times K_i$), the enzyme is protected against inactivation, to an extent expected by the affinity of the inhibitor for the enzyme (6, 7). An examination of the incapacitation rate with respect to phenylglyoxal concentration is presented in the inset to Figure 1. The enzyme modification reaction displays first order (*i.e.*, 1.00 ± 0.09) dependence on phenylglyoxal concentration as expected, and from the data an inactivation rate constant of 0.019 ($\pm$ 0.007) $s^{-1}M^{-1}$ (25 °C, pH 7.5) may be obtained. Similar results were obtained with the alternative reagent 2,3-butanedione (50 mM) in the presence of sodium borate (50 mM). However, the inactivation was not so clean; a residual enzymic activity of approximately 6% was noted, and the *N*-acetylproline protection seemed to be less complete. The most plausible interpretation of these experiments is that an essential arginine resides at the active site of prolidase.

Glutamic or Aspartic Acid. Carbodiimides exhibit high selectivity for carboxylate residues in proteins. An initial *O*-acylisourea intermediate is formed by addition to the carbodiimide, which in aqueous solution either may react with an amine or may rearrange to form a more stable *N*-acylurea, in either case transforming the carboxylate. In Figure 2 are presented the results of subjecting prolidase to a 20 mM solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. As in the previous case a rapid and complete exponential decay in the catalytic capability of the enzyme is noted. Once again *N*-acetylproline retards inactivation, but the protection is less complete than in the case of phenylgloxal inactivation. Saturating amounts of *N*-acetylproline cause only a six-fold reduction in the rate of inactivation of the enzyme, whereas in the previous case protection was complete. Since this might have resulted from only partial masking of an active site carboxylate, *N*-carbobenzyloxyproline and proline itself were similarly tested as active site protectants, in the expectation that a larger or a smaller species might yield a different level of protection. However, in each case approximately the same six-fold rate

differential was noted. The enzyme modification reaction seems to exhibit somewhat greater than unitary dependence on carbodiimide concentration. However, because the reagent decays through spontaneous solvolysis (which has not been corrected for), we do not attempt to provide a rate constant. The alternative carboxylate-selective acyclic reagent 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide methiodide also incapacitated the enzyme, but *N*-acetylproline protection was even less effective in that case.

Other Residues. Reagent acetylimidazole has been reported to be selective for the tyrosine side chain in proteins (phenolic *O*-acetylation) (9). The catalytic activity of prolidase was unaffected by exposure of the enzyme to a buffered solution of this reagent (25 mM, pH 7.5, 60 min). An identical result was obtained in attempted modification of the enzyme by iodination under conditions which reportedly *ortho* halogenate the phenolic ring (50 mM KI_3, pH 7.5, 60 min). Accordingly, no evidence for an active site tyrosine was forthcoming. The more potent acetylating agent acetic anhydride in relatively high concentration (≥0.4 M, pH 8.5) did slowly impair prolidase, but the presence of *N*-acetylproline only weakly protected the enzyme, retarding the inactivation by merely 1.5-fold. While this may be the consequence of a lysine residue in the vicinity of the active site, any such amino group does not appear to be as essential as the arginine detected with phenylglyoxal. (The inhibition was shown not to be due to acetate ion.) In our hands the cysteine-specific reagent 2,2'-dipyridyl disulfide (25 mM, pH 7.5, 100 min) was without effect on the activity of prolidase. It has previously been reported that prolidase is susceptible to iodoacetamide before activation, but not after the standard Mn(II) treatment employed prior to utilization of the enzyme (10). The latter observation has been confirmed. In view of these and the iodination observations, it does not appear that there is a free cysteine sulfhydryl at the active site. Failure to detect a catalytically important histidine residue in prolidase has also been previously reported (no inactivation with diethyl pyrocarbonate) (6), as is also suggested by the iodoacetamide negative result. Prolidase appears to be inert to diisopropyl fluorophosphate, indicating that it is not a serine protease (11).

Discussion

The presence of one or more arginine residues at the active site of prolidase is not unexpected. The existence of a binding site for the obligatory C-terminal carboxylate group of typical dipeptide substrates has been demonstrated by inhibition studies (5, 6). Were that role fulfilled by a salt linkage involving an argininyl guanidinium ion, a ready explanation would be provided for the protective effect of *N*-acetylproline in the enzyme modification reaction with phenylglyoxal. No comparable evidence for an active site lysine was forthcoming. However, the provisionally related metalloenzyme carboxypeptidase A, which is also susceptible to 1,2-dicarbonyl inactivation (12), contains several arginines at the active site. Point mutation evidence has been reported recently for the mechanistic significance of a guanidinium group (Arg 127) other than the substrate-binding arginine (Arg 145) within that enzyme (13). Similarly, a pair of mechanistically essential arginine residues has been implicated by directed mutagenesis for the metalloprotease mammalian neutral endopeptidase (14). Consequently, one may not exclude an important auxiliary role other than substrate attraction for an essential arginine of prolidase, such as electrostatic polarization of the substrate binding site. Although the inactivation reaction in our case appeared to be cleanly first

order in phenylglyoxal, this by no means excludes modification of more than one critical active site arginine.

Successful incapacitation of prolidase by a carbodiimide, with protection by proline derivatives, is taken to indicate the presence of an active site carboxylate functional group. Present evidence does not distinguish between an aspartate and a glutamate (or the protein C-terminus) for the targeted residue. Other, more fully-characterized metalloproteases commonly contain an essential active site glutamic acid residue (Glu 270 in the case of carboxypeptidase A, Glu 143 in the case of thermolysin), which may be derivatised similarly (15). The published sequence of 492 amino acid residues for human prolidase contains 37 glutamic and 27 aspartic acids (2). A case of human prolidase deficiency has been traced to the mutation of Asp 276 into Asn within the enzyme, indicating essentiality of that residue for a functional protein (16). However, there is no evidence indicating that the essential carboxylate which is susceptible to carbodiimide modification corresponds to that residue, nor that Asp 276 resides at the active site.[§] The observation that protection of the active site by competitive inhibitors is only partial (sixfold rate reduction, independent of structure of the protectant) could indicate that more than one critical carboxylate is affected, with an essential nonactive-site residue being modified more slowly. Although the normal dipeptide substrates for prolidase contain a potentially cationic amino substituent, the role for the enzymic active site carboxylate is unlikely to be ionic binding of the substrate ammonium group. Present mechanistic knowledge of the enzyme indicates that the aminoacyl terminus of a productively bound dipeptide coordinates chelatively to an active site metal ion, requiring the unprotonated amino substituent. This conclusion emerges from the observation that 2-(alkylthio)-acetylprolines are also competent substrates for prolidase, with a catalytic pH-rate profile supporting such a metal ion-promoted mechanism for the enzyme (5, 6).

In summary, the present evidence suggests a congeneric relationship for prolidase with other members of the metalloprotease family, several of which contain mechanistically consequential arginine and glutamate residues within the active site.

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[§]The 5-residue "dihistidine loop" identified in the preceding footnote contains, in the case of the collagenases and neutral endopeptidase, a glutamic acid residue shown to be essential by mutagenesis (17). However, that residue position appears to be occupied by glycine in human prolidase (2).

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