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Inhibition of Prolidase by Phosphoenolpyruvate is Biphasic. Avoidance of Endogenous-Metabolite Inactivation by Cooperativity within an Enzyme Dimer

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Abstract. A previously reported potent competitive inhibition of the dimeric enzyme prolidase by phosphoenolpyruvate is in fact biphasic. At pH 7.1 the first (partially) inhibiting moiety binds with a K_i of 0.30 μM, but a second binds more weakly, K_i' of 0.24 mM. An explanatory cooperative interaction between enzyme monomers is postulated, so as to avoid inactivation of prolidase by prevailing physiological concentrations of PEP.

Prolidase (EC 3.4.13.9) is a widely occurring mammalian dipeptidase, that splits aminoacylprolines into the constituent amino acids. Apparently this metal ion-containing enzyme is required because many other catabolic proteases are incapable of cleaving the RCO—proline tertiary carboxamide linkage, so that C-terminal proline-containing dipeptides build to toxic concentrations in individuals identified with a genetically caused deficiency of prolidase.[1,2] Previous studies of prolidase from this laboratory have included an examination of catalytic specificity and inhibition.[3,4] Other investigators have reported that the metalloenzyme as obtained from swine kidney is inhibited potently by phosphoenolpyruvate (PEP).[5,6] Of especial interest in the latter regard was an observation that the apparent competitive K_i value, 8.5×10^{-9} M at a pH of 6.0, registered nearly four orders of magnitude below the prevailing physiological concentration of this phosphate derivative within the cytoplasm ($\sim 5 \times 10^{-5}$ M). It was suggested that in order to escape nearly complete inactivation by this comparatively abundant endogenous inhibitor, prolidase might need to be sequestered *in vivo* within a cell surface (membrane), in spite of contrary evidence indicating that it is a cytoplasmic enzyme.[7,8] The purpose of this communication is to show that an alternative mode exists for avoidance of such interference with the enzyme's function. We additionally suggest some further enzymic biomedical implications emerging from the stratagem apparently fashioned by prolidase for overcoming inhibition by an inescapable metabolic intermediate.

Prolidase was isolated from fresh pig kidney by a modification of the procedure of Manao *et al.*,[9] and was characterized by SDS polyacrylamide gel electrophoresis. The kinetics of enzymic hydrolysis of glycylproline were examined employing the method of initial rates by continuous spectrophotometric monitoring at 230 nm, a wavelength that exhibits diminishing absorption by the scissile amide linkage subsequent to an introduction of prolidase.[3] Presented in Figure 1 is a re-determination at a pH of 7.1 of the relative value of k_{cat}/K_m (the second order rate constant corresponding to the enzymic reaction under conditions of low substrate concentration) for dipeptide cleavage in the presence of various concentrations of phosphoenolpyruvate. Rate retardation in this kinetic parameter corresponds to competitive inhibition, or possibly to a noncompetitive mode.[10] It is evident that the phosphate derivative does perturb catalysis at submicromolar concentrations, but it appears that inhibition in that range is only partial, and a much higher concentration of PEP is required in order to completely quench enzymic activity (necessitating a log-scale presentation).

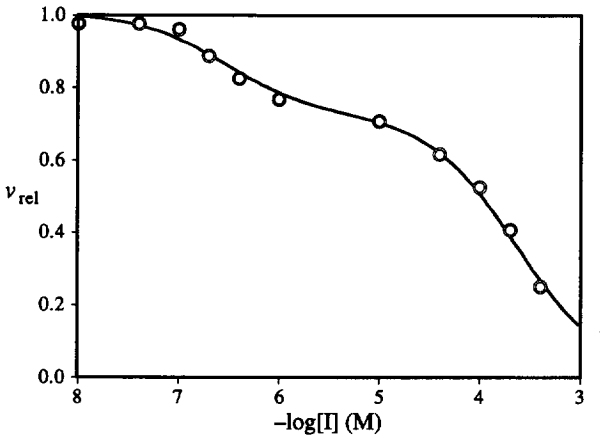

Figure 1. Relative catalytic velocity (k_{cat}/K_m) for the cleavage of glycylproline by pig-kidney prolidase in the presence of variable amounts of phosphoenolpyruvate (I), under assay conditions [GlyPro] $\leq 0.1 \times K_m$, pH = 7.1 (PIPES buffer), $v_{rel} = 1.0$ for [I] = 0.

The experimental reaction rate profile, given fractionally relative to the uninhibited enzymic reaction, has been fitted to a biphasic inhibition equation: $v_{rel} = 1/(1 + [I]/K_i + [I]^2/K_iK_i') + (k'/k)/(1 + K_i/[I] + [I]/K_i')$, which is an expression corresponding to the kinetic mechanism depicted in Scheme 1. This interpretation is premised on several demonstrations that prolidase exists in solution as an *enzyme dimer* (of identical 55 kDa sub-units).[9,11-15] The simplest explanation for the biphasic inhibition curve is that cooperative protein-protein interactions occur between the monomers, such that binding of a second phosphoenolpyruvate to the dimeric enzyme is significantly less favorable than the first. However, substrate glycylproline ($K_m \sim 1$ mM) appears to be accepted normally by the second active site, since no deviations from standard monophasic

Scheme 1. Mechanism accounting for biphasic inhibition of prolidase (E) by phosphoenolpyruvate (I).

$$\begin{array}{lll} E_2 & \xrightarrow[k]{\text{GlyPro}} & \text{Gly + Pro} \\ K_i \;\|\!\!\|\; I & & \\ E_2I & \xrightarrow[k']{\text{GlyPro}} & \text{Gly + Pro} \\ K_i' \;\|\!\!\|\; I & & \\ E_2I_2 & \text{inactive} & \end{array}$$

Michaelis-Menten behavior were noted in a careful survey of the catalytic kinetics.[3] The fitted least-squares parameters for the equation for the curve in Figure 1 are $K_i = 0.30$ ($\pm$ 0.06) μM (the constant for the first-encountered partial inhibition)[16] and $K_i' = 0.24$ ($\pm$ 0.02) mM (for binding of the second inhibitor molecule, which is requisite for complete extinction of enzymic activity). The residual activity of the enzyme dimer containing only one bound phosphoenolpyruvate, expressed as a fraction of that for the uninhibited enzyme, comes out to be $k'/k = 0.72$ ($\pm$ 0.02), which is actually somewhat greater than the 50% of activity that might have been anticipated for blockage of one active site within a protein dimer. The pertinent point is that this latter catalytic capacity should be largely unimpeded by ambient phosphoenolpyruvate *in vivo*, since the value of the second inhibition constant K_i' apparently exceeds the prevailing cytoplasmic concentration of that species. Consequently, no additional strictures are required in order for prolidase to function with enough efficiency to prevent circulating aminoacylproline dipeptides from building to toxic concentrations in normal individuals.[17]

We suggest that prolidase has adopted a dimeric protein structure explicitly for this purpose. A number of sequentially homologous metalloenzymes which function similarly to prolidase in an aminopeptidase capacity, but which probably are not bothered by such inhibition, instead exist in the form of monomers.[18,19] Induction of cooperative interactions between appropriately coupled active sites of the prolidase-dimer complex, mediated through protein conformational changes, appears to present an expedient evolutionary solution to the problem of eluding an endogenous inhibitor such as phosphoenolpyruvate, when the prevalent concentration of that metabolic intermediate may not be reduced because it is essential for physiological roles within an organism.[20] Comparable enzymic oligomerization is unexceptional within cellular metabolic cycles, wherein modulation of catalytic activity through cooperative interactions is required for regulation of steady-state concentrations of chemical intermediates. Although prolidase appears to be merely a catabolic enzyme, its dipeptide-degrading activity in healthy individuals is indispensable.[1,2] Broadly viewed, metabolite cross-inhibition as here examined might actually be a frequent impediment within integrated biochemical systems, and the apparent means of avoidance adopted by prolidase may be more widespread than is presently recognized. Moreover, certain inherited disease states might be specifically associated with a breakdown in that mechanism of active-site collaboration. In such cases a viable treatment strategy might lie in restoration of the defective co-operativity. For example, an analogue of the offending metabolite might be sought, which would bind enzymically in preference to it, but which would also induce the desired protein-protein interactions so as to allow the second active site to sustain residual catalytic activity. Recognition of such a potential avenue of inquiry (by appropriate kinetic examination of an isolated mutant enzyme) could constitute a first step to devising new remediations for otherwise intractable metabolic diseases.

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10. The first phase of the inhibition curve (K_i) necessarily appears noncompetitive,[4] because of the residual activity expressed in the second phase. It was experimentally unfeasible to check for noncompetitive behavior in the millimolar range of PEP concentration (K_i') because its spectral absorption interfered with the kinetic assay.
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16. The disparity in the magnitude of this inhibition constant, relative to the slightly lower value previously reported, may be largely a pH effect; K_i values have been shown to increase with pH for anionic (active site metal ion-coordinating) inhibitors of prolidase.[4,6] The pH of 7.1 that we have employed was chosen because it is closer to the physiological value than that previously employed (pH of 6.0).[6] Prolidase obtained from a commercial source yielded variant inhibition results (positive rather than negative cooperativity); however, SDS–PAGE analysis of such material suggested that it was nonhomogeneous, with apparent contamination by proteolytically degraded enzyme. Our intact prolidase, consisting of a dimeric single polypeptide chain as secured from a modified isolation procedure (incorporating $C_6H_5CH_2SO_2F$ to block serine proteases), has experienced minimal processing (the post-isolation nonphysiological aqueous-Mn^{2+} incubation previously advocated[9] has also been omitted as unnecessary). Consequently, it is believed to reflect the activity of the native species.
17. Partial inhibition of prolidase by other tightly bound inhibitors has been observed, but only the ordinary monophasic pattern is noted with weak inhibitors.[4] The phenomenon may also be substrate-dependent, since nothing requires that E_2I have the same specificity in Scheme 1 as E_2. Picolinylprolines as substrate analogues are also cleaved readily by prolidase, but inhibition of that catalytic activity does not appear to show comparable biphasic behavior (Y. Liu, unpublished work).
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20. Of course there is no proof that the first of the PEP inhibitor molecules participates through binding to an active site; that interpretation is only a plausible inference. It is also kinetically permissible that the second active site in the prolidase dimer is normally masked, and only becomes active upon the first PEP binding.

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