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Proline-Dependent Structural and Biological Properties of Peptides and Proteins

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ABSTRACT: Proline residues confer unique structural constraints on peptide chains and markedly influence the susceptibility of proximal peptide bonds to protease activity. This review presents a critical analysis of peptidases involved in the cleavage of proline-containing peptide bonds, with particular attention to the role of proline peptidases in the regulation of the lifetime of biologically active peptides. Peptidases discussed include aminopeptidase P, prolidase, dipeptidyl peptidase IV, prolyl endopeptidase, and prolyl iminopeptidase. Attention is also given to HIV-1 protease, because this key enzyme processes an Xaa–Pro peptide bond. Analysis of the above enzymes reveals that they may function as key pacemakers in the control of the activity of many peptide hormones and that they are involved in a variety of immunological processes, including T-cell-mediated immune response. The novel occurrence of *cis-trans* isomerization about Xaa–Pro bonds and the biological function of peptidyl-prolyl *cis-trans* isomerases (immunophilins) are reviewed.

KEY WORDS: *proline-containing peptides: prolyl-peptidases*, peptidyl-prolyl *cis-trans* isomerase, aminopeptidase P, dipeptidyl peptidase IV, prolyl endopeptidase, prolidase, HIV-1 protease.

I. INTRODUCTION

In the evolutionary sampling of protein and peptide structure, 20 building blocks were chosen as the optimal components of the biosynthetic chain. Among these building blocks, the α-amino acids, several subclasses exist. Unique, however, in the hierarchy is the imino acid proline, which contains the α-nitrogen atom in a cyclic ring. This "chemical modification" of the nitrogen atom affects both its basicity and the overall polarity of the proline residue. Furthermore, the cyclic nature of this amino acid places major constrains on the ϕ, ψ, and ω dihedral angles of the polypeptide backbone. Thus, inclusion of a proline residue in the sequence of a peptide or protein is expected to result in unique conformational preferences that may, or may not, be biologically significant. It is the goal of this article to review the effect of prolyl residues on the proteolytic stability of both peptides and proteins and to discuss unique enzymes that have evolved to process both X-Pro and Pro-X bonds. Consideration of this question has led us to the hypothesis that, in addition to its other biophysical characteristics, proline may serve as an important regulatory signal in determining the lifetime and degradation rates of biologically active peptides.

Numerous articles have examined the role of proline residues in cyclic peptides, reversed turns, and proteins such as collagen and elastin, and we do not discuss these topics except in passing. Despite previous attention, the structure of the proline residue is at the heart of its biological properties. This issue will therefore be briefly presented.

II. EFFECTS OF THE PROLINE RESIDUE ON THE PEPTIDE CHAIN

A. Proline Conformation

The proline residue in a peptide bond (Figure 1) manifests two important conformational char-

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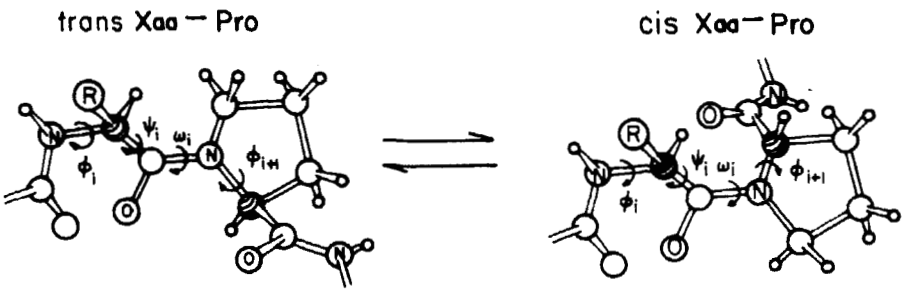


FIGURE 1.

acteristics: (1) the ϕ angle is constrained to values of −68 or −75 (ring puckered up or down) and (2) the peptide bond exhibits both *cis* and *trans* conformers. A consequence of the ring and the tertiary nitrogen of the prolyl residue is that this amino acid has been found to be a strong structure breaker.[1–3] In particular, proline is almost never found at the carboxyl terminus of an α-helix and is rarely in the center of this structure. In contrast, it is observed at or near the amine terminus of helical segments of proteins. These observations led to the conclusion that prolyl residues perturb the structure of the preceding residues due both to steric interactions and to prevention of hydrogen bond formation with carbonyls in the preceding turn of the helix.[4] A detailed theoretical analysis of this problem concluded that under certain conditions, however, proline can appear in the center of a helix in such a way that only the stereochemistry of the preceding residue is distorted. Such an α-helix is bent at the proline residue.[5–8] Moreover, a Fourier transform infrared study of leucine-containing peptides in chloroform concluded that an internal proline residue actually served as a nucleation site for the formation of helical segments.[9] Although the conclusions of this latter study are somewhat equivocal, as no information was provided on the stereochemistry of individual residues, a recent survey by Deber suggested that within the class of proteins containing transmembrane helices, prolyl residues are found only in transmembrane segments of chains that are functional.[10] This suggests that the membrane environment, that is, solvent polarity, may dramatically influence the conformational preferences of proline and that the sequences around buried prolyl residues may have biochemical characteristics.

Virtually all peptide bonds found in proteins are in the *trans* configuration. A notable exception is the X-Pro bond.[11–13] Early studies showed that poly-L-proline assumed two distinct secondary structures, both in solution and in the solid state.[14–16] These conformations, termed polyproline I and polyproline II, are typified by characteristic spectral features, readily interconvert,[14–16] and differ primarily in the ω dihedral angle. *Cis-trans* isomerism in X-Pro peptides has also been demonstrated in numerous di- and tripeptides,[17] and knowledge is available on the thermodynamic parameters that control the isomerization. In most studies, the solvent was found to exhibit a significant influence on the conformational preferences with acidic aqueous media strongly favoring the *trans* conformation.[18–20] In a series of oligoprolines, circular dichroism, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and electron transfer studies all concluded that the *trans* conformers predominated.[21,22] Interestingly, the barrier to isomerization around the X-Pro bond is in the order of 20 kcal/mol and this transformation is now thought to be one of the slow steps affecting protein folding and degradation.

B. Methods Used to Study the Conformation of Prolyl Residues

In principle, any spectroscopic method that can give information concerning the amide chromophore should provide insights into the conformations assumed by prolyl residues. Early studies on oligo- and polyprolines used polarimetry, optical rotary dispersion, and circular dichroism. This latter method gives characteristic

patterns for polyproline I and II.[16] Furthermore, this technique can be readily applied to study the formation of the triple helices that typify collagen fibers.[23,24] Nevertheless, very few proteins or peptides contain stretches of oligoproline and one must turn to NMR to examine individual prolyl residues in peptides and proteins. Both ^{1}H and ^{13}C studies have been conducted and this latter method is especially sensitive to *cis-trans* isomerism of the X-Pro bond. Unfortunately, neither of these techniques is applicable to large (>20 kDa) proteins, because of the problems encountered with spectral overlap. Nevertheless, elegant studies have been reported for linear and cyclic peptides and for small proteins. Modern two-dimensional approaches have provided detailed information on intranuclear distances and have even allowed discernment of the distribution between two distinct conformation species.[25] Analysis of this latter study reveals the dangers involved in the use of intranuclear distances determined by nuclear Overhauser enhancement measurements, without considering the possibility that more than one conformation is present in solution. With the recent development of three-dimensional (and even four-dimensional) NMR spectroscopy, it is expected that even more detailed information on proline-containing peptides will be forthcoming and that it may be possible to use these approaches to look at proline residues in proteins.

Polymerization techniques were ingeniously applied for the preparation of models, permitting the evaluation of the helix-coil stability constants of amino acids in water. In the "host-guest" method, water-soluble random copolymers of N^5-(4-hydroxybutyl)-glutamine and an amino acid are synthesized, fractionated, and characterized. Thermally induced helix-coil transition curves are obtained in water and the Zimm-Bragg parameter σ and s are deduced for the investigated amino acid. These model compounds have provided important insights into the influence of proline on polypeptide conformation[26] (see Section III).

In studying the influence that the proline residue has on the structural properties of biologically active peptides and on their biological properties, the proline residue (or the peptide bond associated with it) was replaced by surrogates. Thus, replacement of the proline residue in [Val^4]morphiceptin (Tyr-Pro-Phe-Val) by *cis*-2-aminocyclopentane carboxylic acid (*cis*-2-Ac^{5}c) results in a morphiceptin derivative in which only the *trans* peptide form has been observed between the first and second residues, because the surrogate contains a normal amide bond. The *cis-trans* isomerization about the Tyr-Pro bond was not found to be important for biological activity.[27]

Other proline analogs — such as thiazolidine, pyrrolidine, and pipecolic acid — were applied in synthesis of substrates and inhibitors of various peptidases acting on proline-containing peptides.[28–33] Peptides conformationally constrained by backbone modification are of special interest for understanding the relationship between three-dimensional structure and biological activity. The quantity of *cis* isomer of the *N*-alkyl amide bond in aqueous solution was correlated with biological activity in the case of angiotensin and thyroliberin,[34] indicating that it is the *cis* isomer that is bound to the receptor. For determining which of the rotamers, *cis* or *trans*, participate in biological recognition, an interesting surrogate of the X-Pro peptide bond, namely, the tetrazole ring system, was proposed.[35] Replacement of the X-Pro bond by this surrogate locks the dipeptide sequence analog into a geometry corresponding to the *cis* isomer.[36] The surrogate was incorporated into a model tripeptide,[36] somatostatin,[37] and bradykinin.[38] Numerous attempts have been made to replace proline in β-turns with constrained surrogates. Discussion of this literature is beyond the scope of this review.

III. SYNTHESIS OF PROLINE-CONTAINING PEPTIDES

The early studies on peptides containing proline required the laborious synthesis of model compounds. Polyprolines were prepared primarily from proline *N*-carboxy anhydride and were a heterogeneous population of different chain sizes with a fairly small dispersity. Models of collagen and elastin were prepared by condensation of active esters formed from repeating tri- , tetra- , penta- , or hexapeptides.[24,39,40] These models were of lower molecular weight than the polyprolines and were much more disperse. Almost all of these compounds were prepared by solution-phase pro-

cedures, with the notable exception of several collagen models that were prepared by the Merrifield approach.[41] Today, given the great strides in synthetic peptide chemistry, proline-containing peptides are readily synthesized either in solution or on solid supports. Although the iminopeptide bond is, in principle, more difficult to form than that from the other α-amino acids, in practice it does not present a significant complication. In the synthesis of water-soluble copolymers of N^5-(4-hydroxybutyl)-L-glutamine and a guest amino acid for structural studies, the *N*-carboxy anhydride method used with most "guest" amino acids was not applicable to proline.[26] Therefore, active ester polymerization of peptide blocks $[Glu(OBzl)]_3$ and Glu(OBzl)-Pro-Glu(OBzl) were used for polycondensation with the aid of the BOP (benzotriazolyl-*N*-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate) reagent. Aminolysis with 4-amino-1-butanol resulted in copolymers of proline and N^5-(hydroxybutyl)-L-glutamine of low proline content, which were suitable for study of the influence of proline on conformation of the nonionizable polypeptide chain.

IV. PROLYL-CONTAINING PEPTIDES IN A MEMBRANE ENVIRONMENT

Statistical and theoretical surveys of peptide and protein stereochemistry concluded that proline residues are often found in regions where the peptide chain changes direction. Thus, proline has an unusually high probability of being found in a β-turn and has also been shown to assume a γ-turn, both in solution and the solid state.[42] Because turns often appear on the surface of a protein, these findings have led to hypotheses that conformational change or proteolysis at exposed prolines is an important regulatory event. Careful surveys of membrane proteins revealed, however, that prolyl residues are often buried in transmembrane helices of "functional membrane proteins" but do not appear in transmembrane regions of polypeptide chains, which are simply used to anchor a protein in a membrane.[17,43,44] Given the fact that proline will disturb a regular α-helix and that the X-Pro peptide bond is a candidate for isomerization, a number of individuals have speculated that the region around buried prolines in transmembrane proteins may have biological significance. Recently, Deber and his co-workers[10] initiated an experimental investigation on the influence of membrane lipid on model peptides containing Leu-Pro-Phe sequences. Although these studies are preliminary, results are consistent with stabilization of the Leu-Pro-Phe unit into a folded structure by the lipid environment. Obviously, many additional studies are necessary before these results can be considered to be general and of biological import. In light of this concept, however, it is interesting that evidence was recently presented that a prolyl residue underwent a structural change during photoactivation of bacteriorhodopsin.[45] This membrane protein has three buried prolyl residues and, if one of these can be directly implicated in this transformation, important weight will have been provided to the hypothesis of Deber's group.

Substitutions of the membrane-embedded prolines 50, 91, and 186 in bacteriorhodopsin[46] indicate, in contrast to the proposals of Deber[17] and Dunker,[47] that neither *cis-trans* isomerization of the embedded prolines nor the presence of kinks at these positions are required for proton pumping. On the other hand, there is strong support for the hypothesis from recent findings in which analogues of paradaxin (33-amino acid polypeptide possessing ion channel activity) have been synthesized with L-proline at position 13 substituted by L-alanine[48] or D-proline. Although the membrane-permeating activity of paradaxin was preserved in both, the ion channel conductivity was impaired and the channel, so formed, could not be voltage activated. Moreover, substitution of Pro(7) by D–Pro prevented formation of the ion channel entirely, resulting in a detergent-like behavior of the polypeptide.[49]

V. PEPTIDASES THAT CLEAVE PROLINE-CONTAINING PEPTIDES

The presence of proline in a polypeptide chain restricts its susceptibility to most peptidases. This is primarily true for the X–Pro peptide bond and is somewhat less general for the Pro–X bond. It is especially interesting that the configuration of

the X–Pro bond can influence the cleavage of peptide bonds that are not adjacent to proline. Thus, in the sequence -Lys-Phe-Pro-, the Lys-Phe bond is only susceptible to tryptic digestion when the Phe-Pro bond is in the *trans* configuration.[50] A similar configurational influence of the X–Pro bond has also been noted for cleavage of peptides by chymotrypsin.[51] In substrates of the type X–Pro-Pro-Phe-pNA, cleavage of the Phe-pNA bond was dependent on the configuration of the X–Pro linkage. Given the high selectivity of many proteases and their inability to cleave peptide bonds involving proline, it is not surprising that specific enzymes participate in the cleavage of such bonds.[52–55,101] Although the number of proline-specific peptidases is not great (Table 1), we believe that their specificities result in unique biological consequences. In the following sections we discuss the enzymes in Table 1 from the perspective of their occurrence in the biosphere and their putative biological effects.

In discussing proline-specific enzymes, we will refer to the manner in which various substrates bind to complementary sites on the peptidases. Such analyses will use the accepted binding site nomenclature,[56] wherein different residues of the substrate are designated as P_2, P_1, P_1', P_2', etc., and the complementary subsites on the enzymes are S_2, S_1, S_1', S_2', etc. Although not truly a proline peptidase, the enzyme *cis-trans* prolyl isomerase (Table 1) recently has been shown to have important biological properties that are manifested only at bonds involving proline. Therefore, it was decided to include this enzyme in our analysis. Little definitive new work has been reported on carboxypeptidase P and carboxypeptidase II, so these enzymes are not included in this review.

A. Aminopeptidase P

1. Occurrence and Properties

Among the ubiquitous proline peptidases is aminopeptidase P (EC 3.4.11.9), which is specific for N-terminal X–Pro peptide bonds in both short and long peptides. The enzyme was first isolated from the soluble fraction of *Escherichia coli* B,[57,58] and has been found in other bacteria,[59] yeast,[60] and a variety of mammals,[61–71] including humans.[60,64,72–76] Recently, the enzyme from *E. coli* was cloned and expressed.[77,78] This allowed a significant increase in production of the peptidase and facilitated its purification. Studies on the enzyme from both bacterial and mammalian sources indicate that the active peptidase is multimeric with a molecular weight of 50 to 60 kDa for the inactive monomer from bacteria,[78,79] 71 kDa for the soluble rat brain[80] and the soluble

TABLE 1
Proline-Specific Peptidases

Enzyme	Substrate[a] P_2	P_1	P_1'	P_2'
Prolidase (3.14.13.9)	H–	Xaa– –	Pro–OH	
Aminopeptidase P (3.4.11.9)	H–	Xaa– –	Pro———	Yaa—
HIV-Proteinase (3.4.21.-)	—	Xaa– –	Pro———	Yaa—
Prolyl *cis-trans* isomerase[c]	—	Xaa– –	Pro———	Yaa—
Prolinase (3.4.13.8)	H–	Pro– –	Yaa–OH	
Proline imino peptidase (3.4.11.5)	H–	Pro– –	Yaa—	
Dipeptidyl aminopeptidase IV and III (3.4.14.5 and 3.4.14.2)	H–Xaa—Pro/	Ala– –	Yaa—	
Prolyl endopeptidase (3.4.21.26)	—Xaa———	Pro– –	Yaa—	
Carboxypeptidase P and II (3.4.12.–)	—Xaa———	Pro– –	Yaa–OH	

[a] *The substrate is cleaved at the – – bond.*
[b] Enzyme classification number is given in parenthesis.
[c] This enzyme is a rotamase, not a hydrolase.

human leukocyte enzyme,[60] and approximately 91 kDa for the membrane-solubilized enzyme from porcine kidneys.[66] The bacterial enzyme appears to form active species with a minimum of two chains.[79] The mammalian membrane-bound enzyme is glycosylated and has a glycosyl-phosphatidyl inositol anchor to the microsomal membrane.[66] The soluble aminopeptidase P forms active dimers or trimers, depending on salt concentrations.[80] An early study showed that the active tetramer from *E. coli* B dissociated on dilution and that the K_D for dimer to monomer was approximately 1 μM.[58,79] The best characterized example of aminopeptidase P is the cloned bacterial protein that has a deduced molecular weight of 49,650, is composed of 440 amino acid residues, and has four cysteine residues per subunit (see Table 2 for a summary of the properties of aminopeptidase P from various sources).

All forms of aminopeptidase P examined to date appear to be metalloproteins and are especially sensitive to metals such as Mn^{2+}, Co^{2+}, and Zn^{2+}.[66,84] The latter ion is inhibitory and was found to be associated with the cloned bacterial peptidase. Not surprisingly, a number of metal chelators, such as EDTA and 1,10 phenanthroline, inhibit activity. A fuller understanding of the properties of the protein awaits its crystallization and the docking of various substrates and inhibitors into the active site. With the availability of milligram quantities of the cloned peptidase, such results should soon be forthcoming.

TABLE 2
Properties of Aminopeptidase P from Various Sources

Property	*E. coli*[58]	*E. coli* HB101[a,78]	Hog Kidney[66] (membranal)	Bovine lung[61] (membranal)	Rat brain[80] (soluble)	Guinea pig serum[82] (soluble)	Human platelets[83] (soluble)	Human leucocytes[60] (soluble)
Molecular weight, kDa								
at high conc.	200,[b] 230[c]	200[d]	280[e]	360	143,[f] 218[g]	217	223	140[e]
at very low conc.	100–120[h]	—	—	—	—	—	—	—
by SDS-PAGE	60[79,85]	50[i]	91[i]	95	71[k]	89/81.5	71	70[k]
Carbohydrate, %	—	—	25[l]	—	—	—[m]	—	—
Isoelectric point, pH	—	5.3	—	—	—	—	4.9[n]	—
Optimal pH	8.6[o]	8.5[p]	—[q]	6.8	7.6–8.0[r]	—	—	7–8.5[s]
Activating cations	Mn, Co, Cd, Ni	Mn^{t}	Mn, Co	—	Mn	Zn, Hg, Cu, Pb, Ni	Cu, Zn, Hg	Mn, Co
Inhibitory cations	—	—	Ni, Cu, Zn		Co, Zn			Ni, Zn, Cd[u]
Inhibitors	EDTA	EDTA, CH_3–Hg–Cl	EDTA, DTT, PHMB, PHMBS, Phenant	EDTA, 2ME, 2-MGP, CMPS, Pro-Pro-Ala	Iodoacetamide, Phenant	EDTA, PMSF, Phenant, captopril	EDTA,[v] Phenant	EDTA, DTT, Phenant

[a] Aminopeptidase P was cloned and the gene was expressed in *E. coli* JM83.
[b] Calculated from sedimentation and diffusion studies, 0.407 *M* ammonium sulfate, pH 6.5, 0.1 *M* Na-acetate.
[c] From sedimentation equilibrium, under same conditions as in previous footnote.
[d] By low angle laser light scattering.
[e] By gel filtration.
[f] Gel filtration through Sephacryl S-300, with 0.14 *M* NaCl.
[g] Gel filtration through Sephacryl S-300, without NaCl.
[h] Aminopeptidase P dissociates at low conc. (1.0 μg ml) with simultaneous increase in specific activity.
[i] The molecular weight calculated for the subunit from amino acid sequence derived from the nucleotide sequence is 49,650.
[j] Under reducing conditions, consists of up to 25% N-linked sugars.
[k] Under reducing conditions.
[l] N-Linked sugars.
[m] Biantennary and high mannose fraction.
[n] Vanhoof et al., unpublished results.
[o] Poly-L-proline substrate.
[p] Gly-Pro-2-NNap substrate.
[q] Not reported, assays were performed at pH 8.0.
[r] Arg-Pro-Pro substrate.
[s] Lys(Dnp)-Pro-Pro-NH-CH_2-CH_2-CO-ABz fluorogenic substrate.
[t] The purified enzyme contained about 0.2 gram atom Zn per subunit.
[u] Cd caused 100% inhibition at 10^{-5} *M* concentration.
[v] In absence of Mn^{2+}.

Note: Abbreviations: DTT, dithiothreitol; Phenant, 1-10-phenanthroline; PHMB, 4-hydroxymercuribenzoate; PHMBS, 4-hydroxymercuribenzenesulfonate; 2ME, 2-mercaptoethanol; 2-MGP, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; CMPS, *p*-chloromercuriphenylsulfonic acid.

2. Structural Specificity

Most of the detailed analyses on structural specificity have been carried out on purified bacterial aminopeptidase P.[57,58,77–79,85,86] Although less complete, studies on aminopeptidase P from mammalian sources lead to similar conclusions. The bacterial enzyme hydrolyzes a variety of peptides from dipeptides to proteins. There is an absolute requirement for an X−−Pro bond, where X must have a free α-amino group (including the imine of Pro) and the penultimate residue must be Pro. Even hydroxyproline is not acceptable in the penultimate position and the bond must be in the *trans* configuration.[88] In general, dipeptides are hydrolyzed more slowly than higher peptides. Interestingly, however, Gly-Pro-Hyp — which is used as a standard substrate for the study of mammalian aminopeptidase P — is a poor substrate for the bacterial enzyme.

A novel proof of the high specificity of aminopeptidase P, and the inability of general aminopeptidases to cleave X–Pro bonds, was demonstrated using immobilized bacterial aminopeptidase P and immobilized *Clostridial* aminopeptidase.[86,89] The latter enzyme is a general aminopeptidase that cannot cleave X–Pro bonds.[90] When it was placed in a solution of bradykinin (RPPGFSPFR), absolutely no hydrolysis occurred. Removal of the immobilized *Clostridial* enzyme by filtration and replacement with immobilized aminopeptidase P resulted in the release of one equivalent of Arg and of Pro. Further hydrolysis was only effected when the immobilized aminopeptidase P was replaced with the general aminopeptidase. Similar results were found using tuftsin (TKPR), Substance P (RPKPQQFFGLM-NH_2), and Poly(Pro-Gly-Pro) as substrate. These results give unequivocal proof for the unique specificity of aminopeptidase P and also provide a "coupled" enzyme system that could be used for sequential degradation of proline-containing peptides.

The requirement for a prolyl residue in the P_1' position of the substrate is supported by the fact that several hydrolysis products are strong competitive inhibitors (Table 3). In comparison to Pro-Phe-Lys, which is an excellent inhibitor, Ala-Phe-Lys exhibits no inhibition at 200-fold greater concentration. In analyzing contributions to binding, the role of an aromatic residue in the P_2' position is also quite evident; Pro-Phe is a 13-fold better inhibitor than Pro-Ala (Table 3), and Gly−−Pro-2NNap, Gly−−Pro-pNA, and Gly−−Pro-MCA are excellent substrates.[92] Finally, under comparable conditions, Gly-Pro, Ala-Pro, Val-Pro, and Pro-Pro were hydrolyzed approximately 200 times more slowly than Pro-Pro-

TABLE 3
Kinetic Constants for Substrates and Inhibitors of Aminopeptidase P from *E. coli*[58]

P_1	P_1'	P_2'	K_m ($M \times 10^{-4}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1} \times 10^6$)	K_i ($M \times 10^{-4}$)
Substrates[a]						
Pro ——	–Pro——	Pro—Pro—Pro_n	0.9	105	12	—
Pro ——	–Pro——	Ala	11	1210	10	—
Phe(pNO_2)–	–Pro——	Eda—ABz	30	135	0.45	—
Phe(pNO_2)–	–Pro——	Pro——Eda——ABz	2.2	20	0.91	—
Inhibitors						
	Pro—	Phe	—	—	—	0.75
	Pro—	Ala	—	—	—	10
	Pro—	Phe—Lys				
	Ala—	Phe—Lys	—	—	—	1.2
						>200

Note: Abbreviations: Phe(pNO_2), *p*-nitrophenylalanine; Eda, ethylenediamine; ABz, 2-aminobenzoyl.

[a] Substrates are cleaved at the −− bond.

Ala.[57,58] Thus, occupation of subsite S_2' is important for good binding. Once this subsite is occupied, however, further elongation of the chain does not significantly contribute to the binding energy; Pro-Phe and $(Pro)_n$ have similar K_m and K_i values. If one can assume K_m reflects binding, we can conclude that the binding domain on the enzyme does not go further than S_2'.

A unique aspect of the structural specificity of prolyl peptidases is their sensitivity to the configuration of the X−Pro peptide bond. Aminopeptidase P exhibits an absolute specificity for the *trans* form of the N-terminal bond.[88,91] Indeed, early studies, using polyproline as the substrate, revealed strikingly different hydrolysis kinetics when the starting substrate was polyproline I or polyproline II.[88] Because the *cis* form of the X−−Pro bond must isomerize before hydrolysis can occur, one would expect biphasic kinetics for the hydrolysis of both Phe-Pro and Gly-Pro-Ala, by high concentrations of bacterial aminopeptidase P, is biphasic.[88] The fast phase, which depends on enzyme concentration, corresponds to the rapid hydrolysis of the *trans* form, whereas the slow phase, which has a rate constant independent of enzyme concentration, corresponds to the rate-determining *cis-trans* isomerization.[88] The slow-phase rate constant was identical for Phe-Pro in the cationic and the zwitterionic forms, as well as when the solid form of this peptide was dissolved directly without preequilibration. In contrast, the amplitude of the slow phase, which corresponds to the starting amount of *cis* isomer, was 26, 76, and 100%, respectively. These results are in full agreement with NMR studies and show that enzymatic approaches are useful in measuring the *cis* content of peptide bonds.[17,51] In a novel application of this concept, aminopeptidase P was used in combination with endopeptidases for the determination of the percentage of nonterminal *cis* X−−Pro bonds in proteins.[17,51] As the interest in *cis-trans* isomerization in proteins is growing because of its importance in protein turnover, conformational regulation, and immunological events, the application of proline peptidases to such studies will most likely proliferate in the near future.

3. Assays of Aminopeptidase P

The isolation of an enzyme activity requires the availability of specific substrates. Early work on aminopeptidase P used polyproline as the substrate.[58] Although the polypeptide is susceptible to the bacterial enzyme, polyproline was shown to be resistant to purified mammalian aminopeptidase P.[60,80] It is also cleaved by proline iminopeptidase and is, therefore, far from an ideal substrate. More recent analyses have used oligopeptides and derivatized oligopeptides as substrates. Although some groups have used dipeptide substrates to follow aminopeptidase P activity, we caution against this approach because it can result in the purification or copurification of prolidase (see Section V.B). As a completely specific substrate for aminopeptidase P, we prefer peptides with the sequence X−−Pro-Pro. Such peptides are resistant to dipeptidyl aminopeptidases (see Section V.D) and to most carboxypeptidases. To get high sensitivity and to be able to measure activity directly in complex biological fluids (e.g., serum, cell extracts), it is desirable to have an assay that is not obscured by background components such as proteins, small peptides, and amino acids. Thus, although there are many assays in the literature based on cleavage of proline-containing peptides,[57,58,61–63,72,92–94] we have developed a sensitive method using intramolecularly quenched fluorogenic substrates such as Phe(p-NO_2)-Pro-Pro-Eda-ABz and Lys(Dnp)-Pro-Pro-Eda-Abz (where Eda = ethylene diamine, ABz = 2-aminobenzoyl, and Dnp = 2,4-dinitrophenyl).[64,73] In both substrates the fluorescence of the ABz group is quenched by the nitrophenyl moieties. This intramolecular quenching is eliminated when aminopeptidase P cleaves the X−Pro bond and the fluorescence generated is a sensitive monitor for the enzyme activity. These fluorogenic substrates have been extremely useful in measuring the distribution of aminopeptidase P in mammalian tissues and a variety of human fluids.[76] Recently, a radioassay was developed using Arg−−Pro-Pro-[^{3}H]benzylamide as the substrate.[95]

Aminopeptidase P has also been assayed using certain chromogenic substrates together with an auxiliary enzyme. For example, Gly-Pro-Pro-(chromophore)[62] or Gly-Pro-(chromophore)[92] have been used together with either dipeptidyl peptidase IV or proline iminopeptidase, respectively. In such applications, care must be taken to eliminate cleavage by peptidases with overlapping specificity. Nevertheless, this approach was applied successfully during the isolation of cloned aminopeptidase P.[77] Such coupled enzyme assays are a powerful tool for screening cells for specific peptidases.

4. Tissue Distribution of Aminopeptidase P

As mentioned briefly before, aminopeptidase P is widely distributed in higher life forms. Studies in mammals have revealed that both the soluble and membrane-bound enzyme are present in almost all tissues examined. In the analysis of enzyme activities in complex biological systems, it is essential that proper substrates are used. For example, in studies on aminopeptidase P from erythrocytes and human lung, the activity was followed primarily using X−Pro dipeptides.[68,72] Careful analysis of these studies has led us and others[63] to conclude that the preparations isolated and the activities reported may have been primarily those of prolidase. Despite such misgivings, aminopeptidase P has been found in calf,[73] rats,[64,70,96] guinea pigs,[71] rabbits, cats, cows, pigs, and humans.[60,64,72,73,76,97–99] Moreover, it is present in all organs examined, albeit with distributions that vary in different species.[69]

Especially rich sources of the enzyme include blood and blood components. Careful analysis of the activity in components of human blood shows that the enzyme is present in erythrocytes, granulocytes, lymphocytes, monocytes, and platelets.[72,97–99] In many of these studies, fluorogenic substrates such as Phe(NO_2)-Pro-Pro-Eda-ABz or Lys(Dnp)-Pro-Pro-Eda-ABz, which had high specificity for aminopeptidase P and that ruled out cleavage by prolidase or other endopeptidases, were used. Recently, these approaches allowed analysis of as little as 10 μl of untreated human serum.[64]

Several investigations clearly showed that the vast majority of the activity in blood components was intracellular and soluble in nature; lysis of human erythrocytes leads to a 1000-fold increase in aminopeptidase P activity,[97] the cell-associated activity was 100 to 200 times greater than that circulating in the serum,[99] and the specific activity of aminopeptidase P in platelets is quite high.[98] A particularly intriguing result was found on analysis of normal and mitogen-stimulated lymphocytes. Whereas the specific activity of a general aminopeptidase, leucine aminopeptidase, did not change with cell proliferation, the specific activities of both aminopeptidase P and dipeptidyl peptidase IV increased by 100 to 200% with stimulated lymphocytes.[99] The biological significance of this result will be discussed elsewhere in this review (see Section V.D.1.f).

In addition to the soluble aminopeptidase P previously described, a membrane-bound enzyme has been detected in a variety of organs, including swine kidney, rat intestine, and bovine lung.[61,63,67] Indeed, during a 2 to 3-sec transit through perfused lung, Arg-Pro-Pro-benzylamide is extensively degraded.[70] The microsomal enzyme from swine kidneys is particularly interesting in that it has been purified to homogeneity and its structural properties were extensively characterized.[66] The membrane-bound aminopeptidase P shows characteristics consistent with that of a kininase in that the preparation from bovine lung efficiently released Arg from bradykinin. Although minor differences have been reported between the properties of membrane-bound aminopeptidase P and its soluble counterpart, they are, for the most part, identical in their substrate specificities.

5. Potential Biological Relevance of Aminopeptidase P

Unlike several of the other proline-peptidases, only one report of a disease linked to hu-

man aminopeptidase P deficiency has been reported.[100] However, the unusual specificity of aminopeptidase P suggests that, along with other proline-peptidases, this enzyme may have a unique biological role. Careful perusal of the literature reveals that a number of biologically active polypeptides — including hormones, growth factors, kinins, neurotransmitters, coagulating proteins, toxins, and enzymes — contain an N-terminal X–Pro sequence (Table 4).[101] This list is growing and is by no means complete. Some of the molecules in Table 4 lose activity on cleavage of the X–Pro bond; others remain active after such peptidolysis. It is possible that removal of the X–Pro linkage results in a major change in protein stability and, thereby, the lifetime of the remaining polypeptide. Based on this thinking, aminopeptidase P could have, at a minimum, two major cellular functions: it may be the primary regulator of the activity of certain peptides and proteins and/or it may function as an initiating switch that triggers the process of protein turnover. Furthermore, many biosynthetic precursors must be enzymatically trimmed to the mature protein. Given the specificity of aminopeptidase P, it is quite likely that this enzyme is involved in the processing of all proteins that have an N-terminal proline. Recently, advantage was taken of this activity for processing proteins that start with Met-X-Pro; aminopeptidase M and aminopeptidase P were used consecutively to generate human granulocyte macrophage-stimulating factor.[102]

The high aminopeptidase P activity associated with various blood components, including erythrocytes and platelets, along with the high affinity of the enzyme for bradykinin, have been used to argue that the peptidase is a cell-associated circulating kininase that regulates the action of the nonapeptide.[83,97,98] Indeed, under properly controlled conditions, it appears that aminopeptidase P cleavage is an important step in bradykinin metabolism. Thus, in perfused lungs, radiolabled bradykinin (RPPGFSPFR) is degraded from the N-terminus by aminopeptidase P, followed by dipeptidyl peptidase IV to give Pro-Pro. However, when aminopeptidase P activity is inhibited, formation of this dipeptide ceases.[103,104]

Recently, we found that aminopeptidase P activity was high in all blood cells we examined.[98,99] An analysis of platelets and serum showed that, in contrast to exopeptidases (such as angiotensin I converting enzyme, carboxypeptidase N, and dipeptidyl peptidase IV), which are present mainly in the serum and are thought to participate in the metabolism of bradykinin, aminopeptidase P activity is primarily inside the platelets. These findings support the thesis that aminopeptidase P is involved in regulating cardiovascular and pulmonary functions. In this respect, it is pertinent that aminopeptidase P also acts *in vitro* on the antiarrhythmic peptide (Gly-Pro-Hyp-Gly-Ala-Gly) and substance P.[71]

Studies in transformed lymphocytes imply that aminopeptidase P may have an immunological function. In this context, it is significant that several cytokines (Table 4) are potential substrates for the enzyme. Further discussion of this possibility will be reserved for our analysis of dipeptidyl peptidase IV (see Section V.D.1). Finally, degradation of collagen by collagenase generates peptides that are substrates for aminopeptidase P. It is quite reasonable that this latter peptidase is involved in the normal resorption of collagen and aids in recirculating the amino acids of this protein.[61]

B. Prolidase

1. Occurrence and Properties

Closely related to aminopeptidase P is the dipeptidase prolidase (peptidase D, iminopeptidase, EC 3.4.13.9), which acts on the *N*-acyl linkage of aminoacylprolines. This enzyme also cleaves X–Pro bonds but its substrates are primarily dipeptides. This difference was obscured in the past, when enzyme preparations acting on peptide chains longer than dipeptides were also termed "prolidase."[105,106] Although many dipeptidases exist in cells, most of these cannot cleave X–Pro dipeptides.

Prolidase was first discovered in 1937 by Bergman and Fruton[107] in pig intestinal mucosal extracts, and later found in various mammalian tissues[108] and in microorganisms.[109] The enzyme was purified from several sources,[110–112] including human erythrocytes,[113–116] human fibroblasts,[117] and human liver.[118] A number of re-

TABLE 4
Polypeptides Containing Proline at the Amino End[a]

Polypeptide	N-terminal sequence
With N-terminal Xaa-Pro sequence	
α-Bag cell peptide, marine mollusk	Ala-Pro-Arg-Leu-Arg-
Basic trypsin inhibitor, bovine	Arg-Pro-Asp-Phe-Cys-
Bradykinin, bovine	Arg-Pro-Pro-Gly-Phe-
β-casomorphin, bovine	Tyr-Pro-Phe-Pro-Gly-
Cathepsin D, porcine	Gly-Pro-Ile-Pro-Glu-
Cathepsin L, bovine	Leu-Pro-Asp-Ser-Val-
Cathepsin B, human	Leu-Pro-Ala-Ser-Phe-
Cathepsin H, bovine	Gly-Pro-Tyr-Pro-Gly-
Conalbumin (ovotransferrin)	Ala-Pro-Pro-Lys-Ser-
Corticotropin-like intermediate (α-chain)	Agr-Pro-Val-Lys-
Dendroaspis angusticeps, snake venom	Glx-Pro-Arg-Arg-Lys-
Eledoisin, octopus	pGlu-Pro-Ser-Lys-Asp-
Erythropoietin	Ala-Pro-Pro-Arg-Leu-
Fibrin α-chain	Gly-Pro-Arg-Val-Val-
Fibrinogen derived vasoactive peptide Bβ 30-43	Arg-Pro-Ala-Pro-Pro-
Fibrinogen derived vasoactive peptide Bβ 43-47	Ala-Arg-Pro-Ala-Lys[b]
Flavocytochrom b_2, yeast	Glu-Pro-Lys-Leu-Asp-
Gastric gastrin releasing peptide, porcine	Ala-Pro-Val-Ser-Val-
Growth hormone, mouse	Phe-Pro-Ala-Met-Pro-
Hageman factor (factor XII), human	Ile-Pro-Pro-Trp-Glu-
Hemopexin, human	Thr-Pro-Leu-Pro-Pro-
Hydra peptide	pGlu-Pro-Pro-Gly-Gly-
Interleukin 2	Ala-Pro-Thr-Ser-Ser-
Kentsin	Thr-Pro-Arg-Lys-OH
Lutropin α-chain, human	Ala-Pro-Asp-Val-Gln-
Morphiceptin	Tyr-Pro-Phe-Pro-NH_2
Neuropeptide tyrosine (NPY)	Tyr-Pro-Ser-Lys-Pro-
Neurotensin fragment 9-13	Arg-Pro-Tyr-Ile-Leu-
Pancreatic polypeptide, human	Ala-Pro-Leu-Glu-Pro-
Papain	Ile-Pro-Glu-Tyr-Val-
Plasminogen, human	Glu-Pro-Leu-Asp-Asp-
Prepro-arginine vasopressin-neurophysin II, bovine	Met-Pro-Asp-Ala-Thr-
Prolactin, mouse	Leu-Pro-Ile-Cys-Ser-
Promelittin	Ala-Pro-Glu-Pro-Glu-
Streptavidin	Asp-Pro-Ser-Lys-Asp-
Substance P	Arg-Pro-Lys-Pro-Gln-
Thyrotropin α chain, bovine	Phe-Pro-Asp-Gly-Glu-
Triosephosphate isomerase, human	Ala-Pro-Ser-Arg-Lys-
Trypsinogen, spiny dogfish	Ala-Pro-Asp-Asp-Asp-
Xylanase, yeast	Ala-Pro-Ala-Asp-Lys-
With penultimate Xaa-Pro sequence	
Alkaline phosphatase, human	Ile-Ile-Pro-Val-Glu-
Cystatin, human	Ser-Ser-Pro-Gly-Lys-
Homeostatic thymus hormone -HTH_β-chain, calf	Pro-Glu-Pro-Ala-Lys-
Kassinin, frog	Asp-Val-Pro-Lys-Ser-
Proricin A chain, castor bean	Ile-Phe-Pro-Lys-Gln-
Ranatensin	pGlu-Val-Pro-Gln-Trp-
Renin	Ile-Ser-Pro-Val-Val-
Sauvagine	pGlu-Gly-Pro-Pro-Ile
Stefin, human	Met-Ile-Pro-Gly-Gly-

[a] From Yaron, A., *Biopolymers*, 26, S215–S222, 1987, with permission. For literature references see Table 1 in the original article.

[b] The N-terminal alanine was shown to be unimportant for biological activity.

views on prolidase and prolidase deficiency have been published[52,55,109,119]

The amino acid sequence deduced from the cDNA of human erythrocyte prolidase was confirmed by direct sequencing of several regions of the protein.[118] The mature enzyme consists of two identical subunits, each composed of 492 residues (M_r = 54,305) in good agreement with the value of 56,000 estimated by SDS-PAGE for the subunit.[108,120] The polypeptide chain is processed at the amino terminus by removal of Met and blocking the N-terminal alanine, presumably by an acetyl group. No processing occurs at the C-end. A value of pI = 4.4 was reported for prolidases from various sources. The enzyme is optimally active at pH 7.6–7.8 and is activated by Mn^{2+} ions. The function of the Mn^{2+} ion is not clear; some assume it is involved in the catalytic mechanism, others that its primary function is to stabilize the enzyme.[121] However, the Mn^{2+}-activated form of the enzyme that was prepared did not need activation to hydrolyze substrates.[113] Inhibition by 4-chloromercuribenzoic acid suggests that a sulfhydryl group is involved in the substrate-enzyme interaction.[122,123]

2. Substrate Specificity

Few investigations on substrate specificity using pure prolidase preparations have been reported recently. Prolidases from various sources hydrolyze dipeptides in which the C-terminal amino acid proline, hydroxyproline, or sarcosine, is linked through its tertiary nitrogen to the carbonyl of an amino acid residue bearing a free α-amine. Only the *trans* isomer of X−Pro dipeptides is cleaved by the enzyme.[124] Studies comparing the hydrolysis of Gly-Pro with the desamino analog (acetyl proline) concluded that prolidase has an absolute requirement for an α-amino group. A detailed kinetic analysis on porcine kidney prolidase, however, suggests that this conclusion must be reevaluated.[121]

Comparison of data from hog kidney and human erythrocyte prolidase suggests that, whereas the branched or aromatic nature of the side chain of residue P_1 has only a minor influence on substrate susceptibility, the L-configuration is essential. Pro-Pro is cleaved 11 times less efficiently than Gly-Pro by porcine kidney prolidase (Table 5), suggesting either an effect of the ring on binding or an influence of the basicity of the terminal nitrogen. Lack of Pro-Pro cleavage was reported previously for prolidase from porcine intestine[111] and calf brain.[125] The proline residue in site P_1', which also must be in the L-configuration, can be replaced by Hyp or sarcosine with a 50-fold loss in hydrolytic efficiency. Replacement of Pro with thiazolidinecarboxylic acid (Gly-Thi) leads to a substrate that is 2.7 times more efficiently hydrolyzed than Gly-Pro (Thi = $\overline{NH\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH}$-COOH).

Although acylation of the α-amine of Gly-Pro results in a molecule that is resistant to prolidase, alkylation of this amine is tolerated by the enzyme. Furthermore, when the amino group in Gly-Pro is entirely replaced by CH_3-S- or Ph-CH_2-S-, as well as by the haloacetylprolines, good substrates result[121] (Table 5). The relative facility of the enzyme to hydrolyze these compounds indicates that the α-amino group is not an absolute specificity requirement for prolidase.

Kinetic study[121] of these substrates led to the conclusion that a functional group with a pK_a value of 6.6 is essential to the hydrolytic mechanism. The authors proposed that the group is a water molecule rendered acidic by coordination to the metalloenzyme complex. The water molecule is displaced from the active site metal ion by the substrate, thereby forming the enzyme-substrate complex. Similar conclusions were reached on the basis of a kinetic analysis of the pH dependence of the inhibition of prolidase.

Studies on inhibitors have resulted in further insights into the active site of prolidase. Examination of porcine kidney prolidase showed that Cbz-pipecolic acid is nearly as good an inhibitor as Cbz-proline.[29,30] Thus, the active site S_1' can accept a 6-numbered piperidine ring in place of a 5-membered pyrolidine ring. This was taken as an indication that subsite S_1' is a hydrophobic cleft and was used to explain the observation that Leu-Ala and Ala-Leu (10 m*M*) were hydrolyzed, albeit poorly, by porcine kidney prolidase. Hydrolysis of Leu-Ala and Lys-Ala by pig intestinal prolidase was reported previously by Sjostrom et al.,[111] and it is possible that prolidase may not be absolutely specific for iminodipeptides. However, many of these conclusions are based on

TABLE 5
Kinetics of Cleavage by Prolidase

Substrate		$(V_{max}/K_m)_{rel}$[a]	$(k_{cat}/K_m)_{lim}$[b]
Dipeptides			
	Gly-Pro	1.0	1.0
	Gly-Hyp	—	0.016
	Gly-Sar	—	0.021
	Ala-Pro	1.54	1.44
	Val-Pro	—	0.98
	Phe-Pro	0.69	—
	Met-Pro	0.69	—
	Pro-Pro	—	0.091
	D-Ala-Pro	—	<0.002
	Gly-Thi[c]	—	2.7
Proline derivatives			
	Gly-Pro		1.0
	CH_3-CH_2-CO-Pro		<0.0001
	CH_3O-CH_2-Gly-Pro		0.36
	CH_3O-CH_2-CO-Pro		0.121
	CH_3-S-CH_2-CO-Pro		2.07
	Ph-CH_2-S-CH_2-CO-Pro		1.7
	$ClCH_2$-CO-Pro		0.303
	Cl_2CH-CO-Pro		0.238
	Cl_3C-CO-Pro		0.280

[a] Calculated from data in Ohhashi et al., *Clin. Chim. Acta*, 187, 1–10, 1990. The value for Gly-Pro was taken as 1.0. Reactions were performed with highly purified prolidase from human erythrocytes at pH 7.8, 0.6 m*M* $MnCl_2$. A 10-min preincubation was performed with 1.4 m*M* $MnCl_2$. Temperature in both steps was 37°C.

[b] From Mock et al., *J. Biol. Chem.*, 265, 19600–19605, 1990. The catalytic efficiency $(k_{cat}/K_m)_{lim}$ is defined in the original article, where rates were relative to glycylproline. Reactions were performed with porcine prolidase.

[c] Thi stands for thiazolidine carboxylic acid, NH-CH_2-S-CH_2-CH-COOH.

results with commercial porcine kidney prolidase. Analysis of this enzyme has revealed some minor heterogeneity on SDS-PAGE. It would, therefore, be prudent to carry out similar studies on homogeneous prolidase prior to making definitive statements on substrate specificity of the enzyme.

A strong inhibitor of the porcine kidney enzyme is *trans*-cyclopentane-1,2-dicarboxylic acid (I), ($K_i = 5.1 \times 10^{-7}$ *M*),[126] in which the N-terminal residue of an X–Pro dipeptide is replaced by a carboxylic acid group. Interestingly, both the L- and D-enantiomers of I are inhibitors with K_i values of 0.09 and >5.5 μ*M*, respectively.[121,127] Both carboxyls of I are necessary for the inhibition, as is the ring because cyclopentane monocarbocylic acid bound to prolidase 600 times less effectively than the dicarboxylic compound, and succinic acid binds very poorly. Although both carboxyls are important for the inhibition, only one of them has to adopt the configuration that corresponds to the proline moiety of prolidase substrates. Most importantly, this strong inhibition by I was partial and noncompetitive. The authors postulate that the kinetic anomalies and the pH dependence support the involvement of a solvent water molecule that forms a complex with the enzyme (H_2O:M^{2+}Enz). This bound water has a pK_a value of 6.6 and is responsible for the pH activity profiles exhibited by prolidase. Furthermore, a metalloenzyme dimer exhibiting selective cooperativity between enzyme monomers was assumed to explain the kinetics of the inhibition of prolidase.[127] Obviously, this hypothesis warrants extensive biochemical scrutiny. The strongest inhibitor reported to date is phosphoenol pyruvate ($K_i = 8.5 \times 10^{-9}$ *M*).[126] This metabolic intermediate shares structural features with cyclopentane-1,2-dicarboxylic acid and may behave as a partial bisubstrate analog. The potency of phosphoenol pyruvate and the ubiquitous nature of this metabolite raised questions concerning its role in the *in vivo* regulation of prolidase.

As was true for aminopeptidase P, prolidase only appears to hydrolyze dipeptides with a *trans* X–Pro linkage.[124,128] Substrates with high proportion of the *trans* form and a fast rate of the *cis-trans* isomerization are hydrolyzed faster by prolidase. This conclusion is based on kinetic studies, first by Lin and Brandts[108,124] and confirmed, and further substantiated, by King et al.[128] by NMR studies. Direct ^1H and ^{13}C NMR analysis of the hydrolysis of Ala-Pro by prolidase showed that the rates of *cis-trans* isomerization of iminopeptides can be measured under steady state conditions.[129–131] The time course of the hydrolysis of Ala-Pro by prolidase showed a faster removal of the *trans* isomer as the concentration ratio of enzyme to substrate was increased. The data could be best analyzed based on a scheme that assumed absolute specificity of prolidase for

the *trans* isomer of Ala-Pro. These conclusions strengthen previous work by pH-jump kinetics.[124]

Taking into account the *trans* configuration of the peptide bond in the susceptible X–Pro dipeptides and the Mn^{2+} ion requirement for prolidase activity, King et al.[29] proposed a model for the enzyme-substrate complex in which the active site Mn^{2+}-cation is simultaneously ligated to the prolyl carboxyl group and the amido oxygen of the preceding residue of the *trans* X–Pro dipeptides. The model used bond lengths and bond angles within standard ranges and required no abnormal steric interactions. This model explained the absolute *trans* specificity of prolidase, because the *cis* isomer of the X–Pro dipeptide cannot be bound to the Mn^{2+}-cation through both the prolyl carboxylate and the amido oxygen. Smaller cations than Mn^{2+} are not acceptable as they would require distortion of bond angles. Following hydrolysis of the X–Pro bond, the fragments remain attached to the metal ion only through a single link, thereby facilitating release of the products (Figure 2).

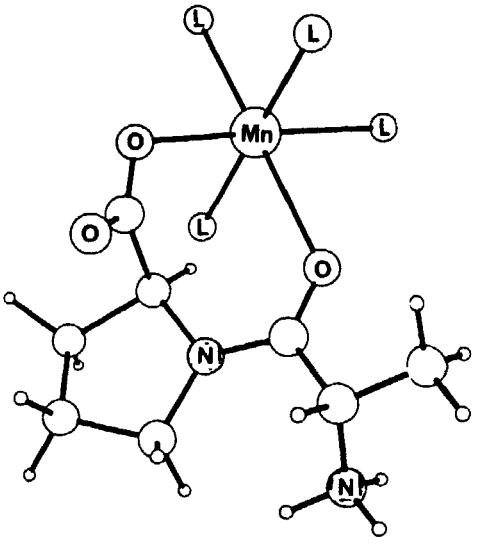


FIGURE 2. Hypothetical complex between Mn^{2+} and the *trans* isomer of Ala-Pro at the active site of prolidase. (Reproduced from King et al., *Eur. J. Biochem.*, 180, 377–384, 1989. With permission.)

3. Biological Properties of Prolidase

In analogy to aminopeptidase P, prolidase occurs in many species and in human tissues and cells. The lack of an X–Pro dipeptidase in bacteria results in poor growth on proline-containing dipeptides.[132] It is quite reasonable that a primary biological function of the enzyme in mammals involves the metabolism of collagen degradation products and the recycling of proline from X–Pro dipeptides. Prolidase deficiency results in abnormalities of the skin and other collageneous tissues, as well as in mental retardation. Massive amounts of X–Pro dipeptides are excreted in the urine of certain individuals and prolidase activity in their tissues is low. Accordingly, the disease can be diagnosed by detecting the large amounts of Gly-Pro present in the urine and by assaying prolidase activity in erythrocytes, leukocytes, and cultured skin fibroblasts.[108]

Recently, isolation and characterization of the human prolidase gene[133] permitted the analysis of DNAs from patients with prolidase deficiency. In one patient, a partial gene deletion of several hundred bases, including exon 14 (there are 15 exons present in the gene) was successfully identified. In two patients, a single nucleotide replacement of G to A at position 826 in exon 12 was found by nucleotide sequence analysis.[133] This results in replacement of aspartic acid by asparagine at amino acid residue 276. That this single substitution was responsible for the enzyme deficiency was confirmed by incorporation of normal or mutant prolidase cDNA into an expression plasmid and transfecting and expressing it in NIH 3T3 cells. Only the normal prolidase cDNA led to expression of active prolidase, although the polypeptide was expressed in both cases. Because active prolidase can be recovered in prolidase-deficient fibroblasts transfected with the expression plasmid clone containing a normal human prolidase, cDNA gene replacement therapy for individuals with this disorder can be given consideration.[133]

Prolidase is localized inside erythrocytes. It is reasonable that it functions to cleave dipeptides that penetrate the erythrocyte membrane and produce amino acids, which are exported from the red blood cell. Gly-Pro, for example, enters the intact human erythrocyte via a saturable membrane-transport system.[130] Because the erythrocyte cannot utilize amino acids, it was suggested that the principal role of the amino acid transport system present in this cell is to efflux the amino acids resulting from the hydrolysis of absorbed

peptides.[130] Prolidase is an essential component of this efflux process. Finally, the gene of human erythrocyte prolidase has been mapped to the short arm of chromosome 19 located around the centromere.[118,134] This locus is thought to be in close proximity to the locus for myotonic dystrophy,[135] and a linkage between prolidase polymorphism and myotonic dystrophy has been demonstrated.[136] Although prolidase, unlike aminopeptidase P, is not involved in hormone regulation and protein processing, its role in metabolism suggests that it serves a unique function in all cell types.

C. HIV-1 Protease

In type 1 human immunodeficiency virus (HIV-1), the capsid and nonstructural proteins — including a protease — are synthesized as a polyprotein precursor that is the product of the *gag* and *pol* gene (Figure 3). This polyprotein contains the HIV-1 protease sequence, is processed proteolytically by the virus-encoded protease,[138,139] and release of HIV-1 protease from its precursor is believed to occur autocatalytically.[140] Although the mechanism of release is unknown, protease maturation involves cleavage of the Phe-Pro bonds present at the N- and C-termini of the polypeptide precursor.[137,138,141–144] Recently, evidence supporting the suggestion that a *gag-pol* product dimer can initiate a sequence of proteolytic events was presented. The protease was produced as a fusion protein in which the enzyme (mol wt of 10,793 Da) was preceded by two copies of a modified IgG binding domain derived from protein A. The IgG binding domain was linked to the protease by an Asp-Pro peptide bond that could not be cleaved by the viral protease. A dimer of the 25,400-Da fusion protein was catalytically active, specifically cleaving a substrate peptide at the correct Tyr-Pro bond.[145] The viral proteinase catalyzes all of the processing steps required for the formation of all viral proteins, except for the initial cleavage of the glycoproteins of the viral envelope. These molecules are also translated as polyproteins, but their maturation is catalyzed by cellular proteinases.

From the perspective of this review, the uniqueness of the HIV-1 proteinase is that it has a high specificity for X–Pro sequences in the middle of a polypeptide. Thus, it is representative of a rare class of proteases: an endopeptidase that cleaves the X–Pro tertiary amide. Obviously, the major reason for the tremendous interest in the HIV-1 protease is that it represents a potential target in the treatment of AIDS. Indeed, intensive efforts have been expended to develop specific inhibitors of the enzyme. Such efforts are, for the most part, beyond the scope of this review. Studies on the HIV-1 protease, however, have revealed some interesting and unique insights into the capability of aspartic proteases to cleave X––Pro containing peptides. The structural requirements of this event will be explored in detail. A review on the HIV proteases appeared recently.[146]

Viral proteinases have been purified from virions and biochemically characterized for a number of avian[147–149] and mammalian[150–152] retroviruses. They have also been expressed in bacteria,[140,142,143,153–165] and the HIV-1 protease has been prepared by total chemical synthesis.[141,162,166,167] Efficient purification methods for

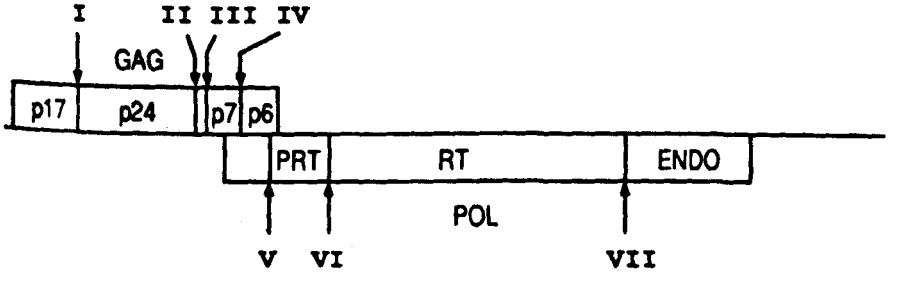


FIGURE 3. Diagram of the *gag* and *pol* reading frames. Numbered arrows indicate proteolytic processing sites within the polyprotein products. PRT stands for HIV-1 proteinase; RT stands for reverse transcriptase. (Reproduced from Darke et al., *Biochem. Biophys. Res. Commun.*, 156, 297–303, 1988. With permission.)

HIV-1 and HIV-2 were developed, such as affinity purification using pepstatin-agarose.[168] The HIV-1 protease is composed of 99-amino acid residues and has been crystallized and examined by X-ray techniques.[169–174] The enzyme contains the sequence Asp-Thr(Ser)-Gly, corresponding to the catalytic center of cellular aspartic proteinases, and it has significant homology to other aspartyl proteases such as pepsin, chymosin, cathepsin D, cathepsin E, and renin.[159,170,175,176] Indeed, computer modeling of the retroviral protease structure has led to the proposal that if the HIV-1 protease existed as a dimer of identical subunits, it would resemble more closely the bilobal structure of the other aspartic proteases.[177] Molecular weight determination by hydrodynamic techniques, by analytical gel filtration, and by chemical cross-linking supported the dimeric structure of the HIV-1 protease,[158] and this structure has been verified by crystallographic analysis of recombinant and chemically synthesized HIV-1 protease (Figure 4).[169,170]

As expected for an aspartic protease, mutation of the Asp to Thr[159] or Ala[144] resulted in a loss of catalytic activity. The pH optimum of the HIV-1 protease is on the acidic side, 5.5, and the enzyme is inhibited by aspartic proteinase inhibitors such as pepstatin (K_i = 1.1 μM) and acetyl pepstatin (35 nM, at pH 5.0).[179] The reader is referred to a number of recent investigations for additional details on the structure of the protease.[146,169–173,180–183]

A second retrovirus, human immunodeficiency virus type 2 (HIV-2), is also associated with immune dysfunction in humans.[184,185] The two viruses share an overall 50% nucleotide sequence identity. The amino acid sequence of HIV-2 is identical to that of HIV-1 enzyme at 48 positions with 2 additional conservative amino acid changes. A 99-amino acid protein having the deduced sequence of the HIV-2 protease was synthesized[186] and, like the HIV-1 protease, this protein catalyzed specific processing of the *gag* precursor and acted on the same cleavage sites. Despite the apparent similarities, the second cleavage site in the *gag*-coded precursors of the HIV-1 and -2 protease are widely divergent [IPFAA––AQQKG vs SATIM––MQRGN]. This suggests that higher order structural features influence interactions between these enzymes and their substrates.

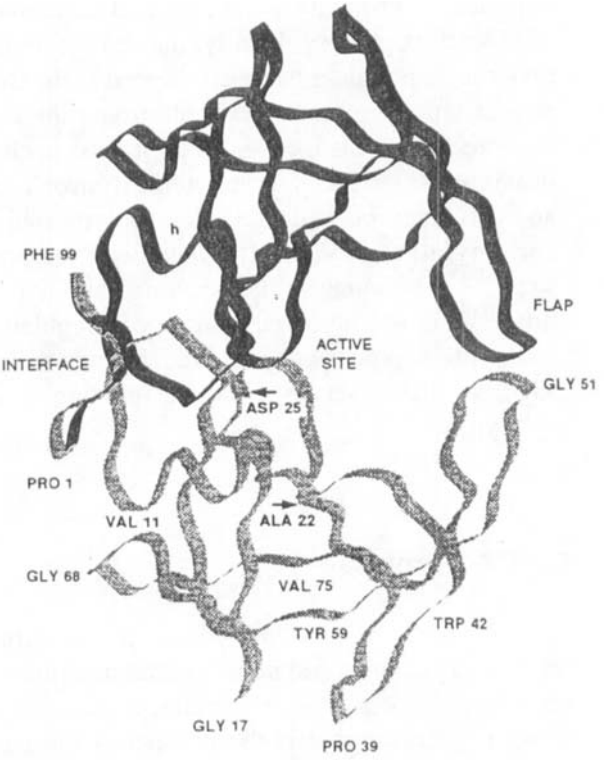


FIGURE 4. Ribbon tracing of the X-ray crystal structure of HIV-1 PR dimer based on data from Wlodawer et al. (*Science*, 245, 616–621, 1989. With permission.) Names of regions and amino acid sequence numbers important for the discussion of dynamical structure in the protein are indicated. (Reproduced from Harte et al., *Proc. Natl. Acad. Sci. U.S.A.*, 87, 8864–8868, 1990. With permission.)

1. Specificity of HIV-1 Protease

The natural substrates for HIV-1 protease are the seven cleavage sites that are involved in the maturation of the viral proteins coded for by the *GAG* and *POL* genes (Figure 3, Table 6). Of these seven sites, three possess a consensus sequence,[187] also required by other retorviral proteases; (Ser/Thr)-Xaa-Xaa-(Tyr/Phe)-Pro, in which cleavage occurs before proline. The other cleavages occur at Leu-Ala, Met-Met, Phe-Leu, and Leu-Phe linkages. It is unusual that a single peptidase cleaves both X–Pro bonds and peptide bonds involving a primary amino acid. The specificity of the substrate cleavage sites was demonstrated using the appropriate synthetic peptides,[141,142,157,158,161,167,179,183,188–191] and the possibility that contaminating activities were present

TABLE 6
Cleavage Sites in the *gag/pol* Gene Product of HIV-1

Cleavage site	HIV-1 site	Sequence	K_m (m*M*)	V_{max} (mmol/min/mg)
I	*gag* 124-138	...HSSQVSQNY--PIVQNI...	N.D.	>275
II	*gag* 357-370	...GHKARVL--AEAMSQV...	2.3	100
III	*gag* 370-383	...VTNTATIM--MQRGNF...	0.16	682
IV	*gag* 440-453	...SYKGRPGNF--LQSRP...	13.9	382
V	*pol* 59-72	...DRQGTVSFNF--PQIT...	0.70	954
VI	*pol* 162-174	...GCTLNF--PISPIET...	N.D.	>120
VII	*pol* 721-734	...AGIRKIL--FLDGIDK...	6.1	145

From Darke et al., *Biochem. Biophys. Res. Commun.*, 156, 297–303. 1988, with permission. For cleavage sites see Figure 3.

in the protease is excluded because identical results were obtained with purified viral protease and the chemically synthesized enzyme.

It is important to note that the cleavage specificity of the proteinase is determined both by the two residues forming the cleavable bond and by the sequence of the flanking peptide chain. Thus, a decapeptide containing a Tyr-Pro site, but flanked by sequences derived from the avian sarcoma leucosis virus (ASLV), was completely stable to the HIV proteinase. In contrast, the same Tyr-Pro bond present in the decapeptide containing the cleavage site I domain (Figure 3, Table 6)[142] of the HIV precursor is cleaved by the enzyme and the enzyme cleaved Phe–Pro linkages in decapeptides corresponding to junction domains (V) and (VI) in the HIV *gag-pol* precursor.[137,142] An additional example of the high selectivity of the enzyme was the hydrolysis of a synthetic octadecapeptide containing the N-terminal cleavage site of the protease (residues −10 to +8). Synthetic HIV-1 protease cleaved this peptide only at the natural cleavage site (Phe-Pro), generating two equimolar fragments.[141]

As illustrated in Table 6, the primary amino acid sequences spanning the cleavage sites in the HIV-1 proteinase precursor are heterogeneous. Therefore, it is difficult to establish the subsite requirements that determine the cleavage specificity of the enzyme. An interesting approach to this problem used mutational analysis of the Tyr/Pro site (I), which generates the amino terminus of the viral capsid protein, and the Phe/Pro site (VI), which results in the carboxyl end of the proteinase. Single amino- acid substitutions were made by mutational techniques and the "mutated" substrates were expressed along with the HIV-1 proteinase in the form of a truncated *gag/pol* precursor. Cleavage of such constructs at the Tyr/Pro site was severely inhibited by substitutions within the P_4, P_2, P_1, and P_2' positions. In contrast, the Phe/Pro site in these constructs exhibited far greater tolerance to amino acid substitution. It appears that, at least in the case of sites I and VI, the primary amino acid sequence around a scissile bond is more critical for cleavage of the Tyr/Pro site than for the Phe/Pro site.[192]

In an attempt to determine the minimal size of a substrate for the viral protease, synthetic oligopeptides homologous to cleavage sites (Figure 3) were prepared and subjected to the protease.[161,188] A 20-peptide, P_{10}-P_{10}' containing cleavage site I, was hydrolyzed only at the Tyr-Pro bond(P_1-P_1') by synthetic protease.[141] Interestingly, a 15-peptide (P_9-P_6'), comprising junction I, was cleaved only slightly faster than the 7-peptide (P_4-P_3').[137] In other studies, model peptides consisting of 18 amino acids were cleaved with efficiencies similar to those for hexapeptides.[189] Peptides shorter than the hexapeptide were not cleaved. The determinants required for binding and specific cleavage are therefore contained in the heptapeptide sequence Ser-Gln-Asn-Tyr-Pro-Ile-Val (from the MA/CA domain spanning cleavage site I), which corresponds to the sequence from residue 128 to 135 in the HIV *gag*

protein.[193] Kinetic constants (K_m = 2.5 m*M* and V_{max} = 275 nmol/min/mg) were determined for this heptapeptide by HPLC analysis.[157]

Using *N*-acetyl carboxamide derivatives, the smallest peptide cleavable by the protease was the hexapeptide Ac-S-Q-N-Y-P-V-NH_2, although elongation of the C-terminus with Val (P_3') increased the efficiency markedly. Further elongation at either end did not result in a significant increase in V_{max}/K_m.[137,167,190] The recombinant HIV-1 protease cleaved the nonapeptide Ac-R-A-S-Q-N-T-P-V-V-NH_2 with a V_{max} value of 160 nmol/min/μg protease, which is about 250 times faster than the specific activity reported for a similar substrate hydrolyzed by synthetic protease.[167] Evidently, not all the molecules in the synthetic product were fully active. Replacement of Tyr by Phe in P_1 site did not affect K_m but decreased the rate about 5 times. Similar conclusions concerning the minimum size required for cleavage were reached using peptides that mimicked the MA/CA junction (cleavage site I, Table 6).[137,157,191,194] In these studies, both the HIV-1 protease and avian sarcoma-leucosis virus (ASLV) protease required 6 to 7 residues for specific enzymatic cleavage.

In a series of synthetic peptides composed of the cleavage site between the α and pp32 domains of the ASLV of reverse-transcriptase–integration polyprotein, changes in the amino acid residues that flank the cleavage site of the peptide bond alter the capacity of the peptides to serve as substrates.[183] The protease cleaves between Tyr and Pro in H-T-F-Q-A-Y--P-L-R-E-A-OH. Substitution at P_1 by Phe resulted in a good substrate, by Ile a poor substrate, and by Ala a resistant peptide. Replacement of Pro (P_1') by Gly or Asp reduced the cleavage efficiency by 80 to 90%, but did not abolish it completely. It is evident from these studies that both amino acids adjacent to the cleavage site as well as those up to three residues removed can influence binding to HIV-1-like proteases. It remains to be determined whether conformational factors play a role in the interaction of the peptide substrate with the extended binding domain on the protease.

The HIV-1 protease belongs to the family of aspartic acid proteases, which include renin, cathepsin D, and pepsin. Therefore, it was of interest to find out whether other aspartic acid protease could cleave the X–Pro bond. In an investigation of avian myeloblastosis virus, it was found[194] that the decapeptide T-F-Q-A-Y--R-L-R-E-A is cleaved at the Y--P site by the ASLV protease as well as by pepsin. At high salt concentrations, some cleavage by the ASLV protease was also observed when the Tyr residue was replaced by Ala or by Ile. Such cleavage was not observed at low salt concentration. The iodinated analogue of this decapeptide is cleaved at the Tyr(5)(I)-Pro(6) bond by pepsin, renin, and cathepsin D. Cathepsin D and renin also cleaved the iodinated decapeptide at the bond between Gln(4) and Ala(5). With increasing salt concentration, the cleavage at Gln-Ala disappeared. The authors concluded that, unlike HIV-1 PR and pepsin, the specificities of renin and cathepsin D for the target on the Tyr(I)-Pro decapeptide is altered by high ionic strength.

The study on the HIV-1 protease has thus resulted in two unexpected findings. First, that an X–Pro endopeptidase can efficiently cleave certain peptide bonds containing primary α-amino acids. Second, that several members of the aspartyl protease family share this characteristic. Full comprehension of this phenomenon and the reason for the narrower specificity observed with aminopeptidase P awaits detailed X-ray analyses of enzyme-substrate or enzyme-inhibitor complexes.

2. Biological Function of HIV-1 Protease: Development of Inhibitors

The HIV-1 protease is an essential component in the life cycle of this virus, being necessary for replication[138,144,195] and cleavage at unique sites of the HIV-1 polyprotein.[138,159] When this retrovirus is made deficient in the viral protease, it remains immature and infection incompetent.[159,195–199] Thus, the HIV-1 protease is an excellent target for rational drug design. Indeed, it is the substrate specificity of the protease and the fact that normal cellular proteinases cannot cleave the *gag-pol* gene product that has resulted in extensive efforts by pharmaceutical companies on the development of treatments for AIDS. These

efforts were aided by the large body of work directed toward the development of inhibitors for aspartic proteases such as pepsin and renin.

Most substrates for the HIV-1 protease have K_m values 100-fold higher than those for good substrates of pepsin and other aspartic proteases. Modification of these substrates to produce acceptable inhibitors must incorporate structural features that increase the binding affinity by a factor of 10^5 or 10^6 (K_i about 10^{-9} *M*). In the search for HIV-1 peptide inhibitors, the X–Pro bond would be a reasonable starting point to carry out medicinal chemistry. Indeed, a great deal of work has addressed the modification of this bond by both peptide chemistry and peptidomimetic approaches. The result of these inhibitor development programs has been the preparation of some outstanding candidates for drugs that will specifically inhibit the enzyme at micromolar and even nanomolar, concentrations. It is, however, becoming quite clear that the best of these drug candidates do not require a proline-like side chain in the P_1' position of the inhibitor. Thus, these studies do not yield a great deal of information that is directly relevant to our review, and the reader is referred to the extensive and rapidly growing literature for further details on inhibitors.

One interesting result that has been generated by the inhibitor search is that the HIV-1 protease forms a binding site with nearly perfect C_2 symmetry and that symmetric inhibitors can cocrystallize with the enzyme to retain this symmetry feature.[171] Indeed, the hypothesis has been put forth that the HIV-1 protease attempts to force asymmetric inhibitors to bind in a symmetric fashion. If this hypothesis is correct, it may explain the unique specificity of the enzyme. That is, X–Pro bonds might normally perturb the symmetry of the polypeptide chain, thereby rendering most X–Pro bonds in polypeptides resistant to the protease. Only those X–Pro bonds that, because of their surrounding sequence, can interact symmetrically with the enzyme can act as substrates. The correctness of this concept needs to be addressed by X-ray investigations on HIV-1 protease cocrystallized with inhibitors that closely mimic the natural cleavage sites (Figure 5).

D. Dipeptidyl Peptidases

The enzymes reviewed in previous sections cleaved peptide bonds involving the proline nitrogen. There are additional classes of enzymes that have relatively high selectivity for peptide bonds involving the proline carbonyl group (Pro––Y). Among these are dipeptidyl peptidases such as DPP-II and DPP-IV, which cleave the Pro––Y bond only when Pro is the penultimate residue at the N-terminus of a polypeptide chain. Thus, such enzymes release X–Pro dipeptides, which are potential substrates for prolidase and aminopeptidase P. DPP-IV is of significant current interest owing to its apparent involvement in immunology.

1. Dipeptidyl Peptidase IV

Dipeptidyl peptidase IV (EC 3.4.14.5, DPP-IV) is a serine type peptidase[200] that cleaves dipeptides from the amino end of peptide chains if the penultimate residue is proline or, although less efficiently, alanine[52,55] or hydroxyproline.[32] The enzyme acts at weakly basic conditions, whereas dipeptidyl peptidase II is active at acidic pH. DPP-IV was first identified in rat liver[201] and later found in many sources. It was purified from a number of animal tissues[52,55,200,202–206] and, more recently, from human placenta,[207] human kidney,[208] human urine,[209] human kidney cortex,[210] human lymphocytes,[211,212] and human seminal plasma.[213] Highest activity is found in the kidney and the intestinal brush-border membrane.[214–217] In the liver, high concentrations are present in bile canalicular membrane domains[202,204,217–220] and the lysosomal membranes.[221] It is found on the cell surface of many epithelial cells, endothelial cells, and T lymphocytes.[222–227] DPP-IV was reported also in extracts of the venom gland of queen bees,[228] bacteria,[109,229–231] and the vacuolar membrane of yeast,[232] and is apparently ubiquitous in living cells.

The cell-associated enzyme is an intrinsic membrane sialoglycoprotein[207,214] that is anchored to the plasma membrane through a hydrophobic domain, most of the molecular mass being ex-

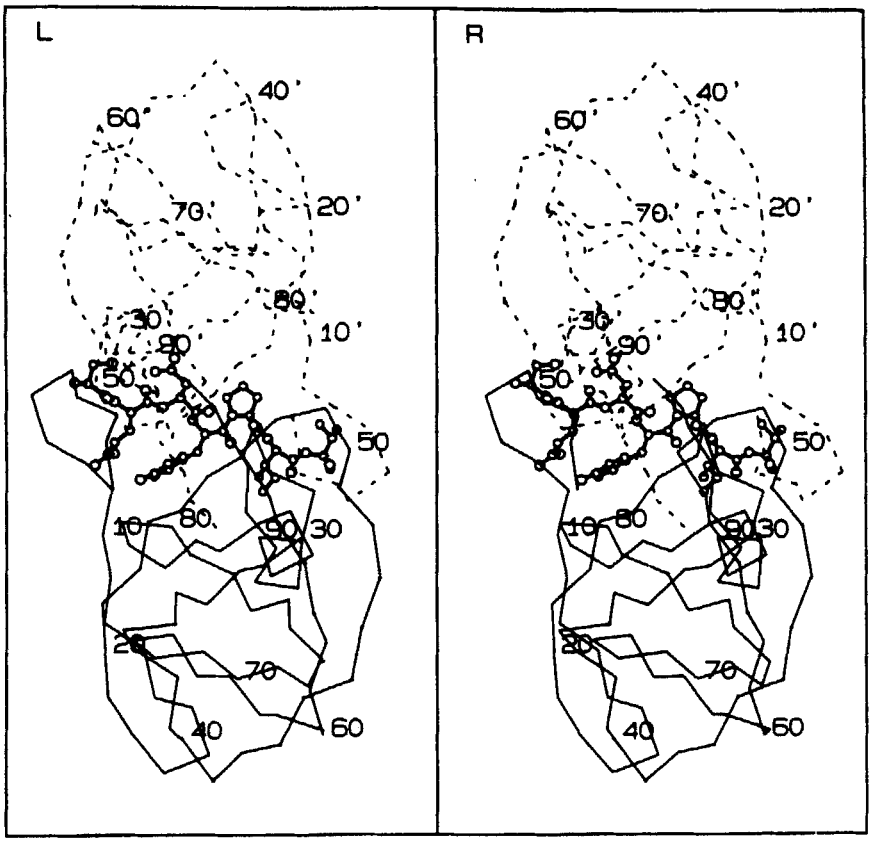


FIGURE 5. A stereoview of the active dimer of the model HIV-1 PR with the substrate, Ser-Gln-Asn-Tyr-Pro-Ile-Val. Substrate residues P_4 to P_1 before the scissile bone and P_1' to P_3' after the scissile bond are shown. The substrate is indicated by dark lines in a ball-and-stick representation. (Reproduced from Weber et al., *Science*, 243, 928–931, 1989. Copyright 1989 by the AAAS. Used by permission.)

posed to the outside of the cell.[206,215,233,234] The native enzyme consists of two identical subunits of 110 to 130 kDa.[200,202,207,208,214,215,217,235–237] This molecular weight may require correction because of the abnormal behavior of glycoproteins in SDS-PAGE. Membrane-bound DPP-IV and papain-solubilized DPP-IV extracted from hog arteries or purified from rat liver plasma membranes, although immunologically indistinguishable,[234] exhibit different electrophoretic mobilities and N-terminal domains.[206,215] Apparently papain treatment removes a hydrophobic amino-terminal peptide resulting in the conversion of mDPP-IV to the soluble sDPP-IV.[206]

The primary structure of rat liver DPP-IV was deduced from its cDNA.[206] Complete sequence analysis demonstrated an open reading frame of 2301 nucleotides that encodes the total primary structure of the enzyme. The molecular mass calculated for the 767-residue polypeptide (88,107 Da) is in good agreement with the M_r determined by SDS-PAGE for the mature protein (110 to 130 kDa) if glycosylation of the polypeptide chain of the mature liver enzyme is taken into account,[206] and with the SDS-PAGE value of 87 kDa obtained with DPP-IV immunoprecipitated from cell-free translation products.[200,202,206] Evidence for glycosylation was obtained by demonstrating formation of a 103-kDa component in products translated in the presence of dog pancreas microsomes.

The polypeptide corresponding to mDPP-IV starts with a putative signal peptide that has a hydrophobic core domain preceded by lysine.

Apparently, the putative signal sequence in mDPP-IV is not cleaved off during biosynthesis, but remains as the potential membrane-spanning domain. Removal of this segment by papain hydrolysis results in a chain lacking highly hydrophobic regions and in a soluble form of the enzyme (sDPP-IV) that starts with an Ala residue at position 35 from the amino terminus. Thus, DPP-IV may be anchored in the membrane by a single segment located at the amino terminus.[206] This segment also acts as a translocation signal. In contrast to many secretory proteins, the signal sequence in DPP-IV is not removed. Comparison of the chain lengths of various signal peptides in proteins revealed that in proteins with uncleaved signal peptides, the chain length is at least 22 amino residues, whereas in the cleavable chains, the chain length averaged 13 (7 to 16) residues. The length of the hydrophobic core region may, therefore, be one of the factors affecting cleavage by the signal peptidases. The cDNAs specific for the human T-cell-activating antigen CD26[238] and for the human intestinal DPP-IV[239] were also isolated and sequenced. In both cases, the identity of the CD26 antigen with DPP-IV was demonstrated. The deduced amino acid sequences were highly homologous with the rat liver DPP-IV and with the mouse thymocyte-activating antigen, respectively. Independent studies showed that membrane glycoprotein gp 110 from bile canaliculus of the rat is identical with DPP-IV.[240,241] Studies on this glycoprotein have provided information on the topology of DPP-IV in the membrane.[216,233,242]

Highly purified, soluble DPP-IV was obtained from terminally differentiated rat epidermal cells.[243] Similar in behavior to membranal detergent-solubilized DPP-IV, it was shown to bind to concanavalin A, displayed a pH optimum of 7.5, was inhibited by diisopropylphosphofluoridate, metal ions (such as Zn, Co, Ni, Cd, and Hg), and by Diprotin A. It was not inhibited by *N*-ethylmaleimide, iodoacetamide, EDTA, *o*-phenanthroline, *N*-tosyl-L-Phe chloromethyl ketone, *N*-α-tosyl-L-lysine chloromethylketone, amastatine, bestatine phosphoramidone, leupeptin, antipain, soybean trypsin inhibitor, or antitrypsin. A bacterial DPP-IV activity was isolated to high purity from a *Bacteroides gingivalis* using Gly-Pro-pNA as the substrate.[109] The molecular weight by SDS-PAGE was 80 kDa and by gel filtration was 75 kDa. It showed pH optimum and metal ion inhibition similar to DPP-IV from mammalian cells.

a. Substrate Specificity

DPP-IV cleaves off dipeptides from peptide chains consisting of three or more amino acid residues, and from dipeptides linked to chromophoric amines, forming chromogenic amide-substrates such as 4-nitroanilides, 2-naphthylamides,[201,244,245] 4-phenylazoanilides,[246] methyl coumarine amides,[247] or 4-methoxy-2-naphthylamides.[248,249] Substrates of DPP-IV require the presence of a proline in the penultimate position at the α-amino end. Although position P_1 can also be occupied with alanine or hydroxyproline,[32] proline peptides are by far the best substrates (Table 7). A free N-terminus in the protonated form is necessary for enzymatic hydrolysis.[251] The bond between the nitrogen of proline (P_1 residue) and the N-terminal P_2 residues must be in the *trans* configuration.[252] Increasing the distance between the α-amine and the carbonyl group in P_2, as in β-alanine and γ-aminobutyric acid, reduces the catalytic efficiency by several orders of magnitude. No cleavage occurs with ε-aminohexanoic acid (see Table 7).

DPP-IV has an absolute requirement for the L configuration of the amino acid residue, both in the penultimate and the N-terminal position, when Pro occupies position P_1. X-Pro-pNA compounds were not hydrolyzed when X was D-Ala, D-Phe, or D-Tyr. With alanine in P_1, D residues and achiral amino acids (such as α-aminoisobutyric acid) at the N-terminus are hydrolyzed slowly. It should be noted that the mechanism of catalysis is different for Pro and Ala substrates.[32] An extensive investigation of the structural requirements for pig kidney DPP-IV hydrolysis of X-Pro-pNA yielded kinetic constants [k_{cat}, K_m, and k_{cat}/K_m] at pH 7.6 for various amino acids (see Table 7). In a series of 16 compounds, the K_m values ranged from 0.92×10^{-5} to 13×10^{-5} *M* and k_{cat}/K_m values ranged from 4.3×10^5 to $5.6 \times 10^6\ s^{-1}M^{-1}$. In general, amino acids with aliphatic side chains at P_2 are favored by DPP-IV, although, except for Gly and Asn at this

TABLE 7
Kinetic Constants for the Hydrolysis of *p*-Nitroanilides by Dipeptidyl Peptidase IV[a]

X-Y sequence in X-Y-pNA	K_m ($\times 10^{-5}$ *M*)	k_{cat}/K_m (μM^{-1} s^{-1})
Pro-Pro	0.92	5.56
Abu-Pro	1.53	4.78
Leu-Pro	1.75	3.74
Val-Pro	1.28	3.51
Ala-Pro	1.66, 2.6[b]	3.29, 3.62[b]
Ile-Pro	1.23	2.32
Glu-Pro	2.10	1.88
Phe-Pro	4.27	1.67
Tyr-Pro	4.03	1.56
Ser-Pro	3.99	1.53
Gln-Pro	4.90	1.42
Arg-Pro	3.0[b]	3.42[b]
Lys-Pro	5.16	1.06
Gly-Pro	10.2, 13.0[b], 9.7[c]	0.77, 0.86,[b] 0.54[c]
Asp-Pro	5.85	0.51
Asn-Pro	11.8	0.43
Sar-Pro	13.0	0.75
DMG-Pro	145	0.000613
TMG-Pro	7040	0.0000208
DMT-Pro	1135	0.0000287
β-Ala-Pro	154	0.004.38
Abu-Pro	352	0.000278
Ahx-Pro	—[d]	—[d]
Gly-Dhp	14.3	2.06
Gly-Pip	8.13	0.0505
Ala-Hyp	579	0.0124
Phe-Ala	85	0.0183
Lys-Ala	231	0.00814
Ala-Ala	830, 100[b]	0.00537, 0.0154[b]
Ser-Ala	1700	0.00255

Note: Abbreviations. pNA, *p*-nitroanilide; Sar, sarcosine; DMG, *N,N*-dimethylglycine; TMG, *N,N,N*-trimethylglycine; DMT, *S,S*-dimethylthioglycine; Abu, γ-aminobutyric acid; Ahx, ε-aminohexyl; Dhp, dehydroproline; Pip, pipecolic acid.

[a] Enzyme from pig kidney was purified according to Wolf, B., et al., *Acta Biol. Med. Germ.*, 37, 409–420, 1978. A single band was seen in PAGE; the preparation was free of alanine aminopeptidase activity and the assumed molecular weight was 115 kDa per subunit. The reactions were performed at pH 7.6, 30°C, and ionic strength of 0.125. (From Heins, J., et al., *Biochim. Biophys. Acta*, 954, 161–169, 1988.)

[b] Enzyme was purified from soluble fractions of rat epidermal cells. (From Kikuchi, M., et al., *Arch. Biochem. Biophys.*, 266, 369–376, 1988.) For calculation of k_{cat} values, a M_r of 190,000 was assumed. Rates were measured in 100 m*M* Tris-HCl buffer, pH 7.5 at 37°C.

[c] Enzyme from lamb kidney was purified according to Koida and Walter, *J. Biol. Chem.*, 254, 7593–7599, 1976. Rates were measured by Yoshimoto, T., et al., (*J. Biol. Chem.*, 253, 3708–3716, 1978.) at pH 7.8, 37°C. For calculation of k_{cat}, a M_r of 115,000 was assumed.

[d] Not hydrolyzed.

position, all substrates are within a factor of 5 in terms of K_m and catalytic efficiency. Although monoalkylation of glycine had little effect on kinetic constants, the di- and trimethylation of the α amine of Gly-Pro-pNA resulted in substrates that were cleaved very slowly. Interestingly, even *S,S*-dimethylglycolylproline-pNA was hydrolyzed by DPP-IV. As shown recently,[253] pig kidney DPP-IV hydrolyzed peptides of the type Ala-X-pNA, in which X stands for the prolyl residue or its surrogates with altered ring structure. In the compounds tested, X was a residue of proline, (*S*)-azetidine-carboxylic acid, *S*-oxazolidine-4-carboxylic acid,(R)-thiazolidine-4-carboxylic acid, and (*S*)-pipecolic acid. Except for pipecolic acid ($k_{cat}/K_m = 0.71 \times 10^6\ M^{-1s-1}$), all the derivatives were hydrolyzed with values of k_{cat}/K_m about $5 \times 10^6\ M^{-1s-1}$.

Increasing the side-chain length of residues in position X resulted in an improved hydrolytic coefficient in the order Gly, Ser, Glu, Met.[254] However, because factors in addition to the physical size of the residues may be involved in the interactions, this interpretation must be accepted with caution.

Studies on inhibitors (Table 8) have provided some additional information concerning binding to DPP-IV. In one study, the K_i values for Gly-Pro, Ala-Ala, Pro-Ala, and Pro-D-Ala were 1.83, 3.6, 0.27, and 0.96 m*M*, respectively.[236] If one assumes that these dipeptides occupy the S_2 and S_1 subsites on the enzyme, it is difficult to correlate these K_i values with the K_m values for substrates in Table 7. Thus, an Ala residue in the P_1 site of a substrate is unfavorable, whereas an Ala residue at the carboxyl terminus of the above inhibitors is acceptable. It is possible that inhibitors and substrates occupy the active site of DPP-IV in decidedly different modalities or that the observed differences in K_m and K_i values reflect the different mechanism that are known to exist for X-Pro--Y and X-Ala--Y substrates.

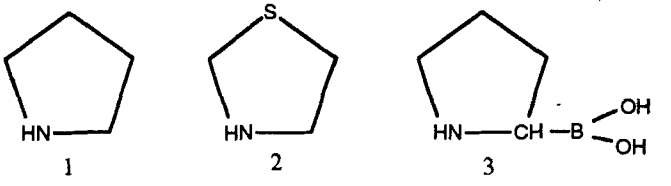
TABLE 8
Dipeptide Inhibitors of DPP-IV Hydrolysis of Gly-Pro-2-NNap[a]

Inhibitor	K_i (m*M*)
Val-Pro	0.05[b]
Leu-Pro	0.10[b]
Pro-Pro	0.25[c]
Pro-Ala	0.8[b]
	0.27[d]
Met-Pro	0.45[b]
Phe-Pro	0.60[b]
Pro-Pro-Pro-Pro	0.60[c]
Pro-D-Ala	0.96[d]
Ala-Pro	2.43[b]
Gly-Pro-Hyp	7.6[c]
Gly-Pro	1.83[d]
	13.6[c]
Val-Ala	0.05[b]
Leu-Ala	0.20–0.50[b]
Met-Ala	0.50[b]
Lys-Ala	2.40[b]
Ala-Ala	3.6[d]
Phe-Ala	1.25[b]

[a] Data were compiled from three sources (as specified in following footnotes), and the rates were determined at slightly varying conditions.
[b] From Bella et al. (*Arch. Biochem. Biophys.*, 218, 156–162, 1982.), DPP-IV was from rat intestinal brush border and rates were measured at pH 8.4, 37°C. No inhibition was observed by Bella et al. with Gly-Pro, Gly-Ala, Z-Gly-Pro, Ala-Ala, Z-Ala-Ala, Ser-Ala, Ser-Pro, Asp-Ala, Z-Pro-Ala, Pro-Pro, Val-βAla, or Z-Phe-Pro.
[c] From Harada et al. (*Biochim. Biophys. Acta*, 705, 288–290, 1982.) DPP-IV was from pig kidney and rates were measured at pH 7.5, 37°C. Harada et al. found no inhibition with Pro-Gly or Pro-Gly-Gly.
[d] From Yoshimoto et al. (*J. Biol. Chem.*, 253, 3708–3716, 1978, with permission), DPP-IV was from lamb kidney and rates were measured at pH 7.8, 38°C.

In the same study,[236] no inhibition was found with Trasylol trypsin inhibitor, leupeptin, antipain, chymostatin, bestatin, pepstatin, phosphoramidon, and elastatinal. In a separate investigation,[256] the resistance of Gly-Pro-Hyp and Gly-Pro-Pro hydrolysis by DPP-IV led to the examination of prolyl peptides as inhibitors of the pig kidney enzyme. Using the substrate Gly-Pro-pNA in Tris-buffer, pH 7.5, competitive inhibition was established for the following peptides (K_i values in millimolar are given in parenthesis): Pro-Pro (0.25), Pro_4 (0.60), Gly-Pro-Hyp (7.6), and Gly-Pro (13.6). No inhibition was observed with Pro-Gly and Pro-Gly-Gly. Thus, an unacylated N-terminal proline cannot substitute for a prolyl residue in competition for site S_1, and Pro in the S_2 site is highly favorable.

Understanding of the catalytic mechanism of DP-IV and interest in the biological role of the enzyme resulted in the development of specific DPP-IV inhibitors. Four types of these specific inhibitors[31,257] include: (1) diacylhydroxylamine derivatives of Xaa-Pro dipeptides;[258–260] (2) oligopeptides with the N-terminal Xaa-Pro sequence;[253,261] (3) dipeptides of the type Xaa-pyrrolidide and Xaa-thiazolidide[31] in which an amino acid is linked to pyrrolidine (see Scheme, 1) or thiazolidine (Scheme, 2) which mimic the essential part of the proline residue; and (4) dipeptides of the type Xaa-boroPro in which boroPro is an amino boronic acid analog of proline (Scheme, 3).[257,262]



1 2 3

The diacylhydroxylamine derivatives are enzyme-activated substrate analog inhibitors containing a peptidyl part Xaa-Pro, recognized by DPP-IV, linked to a structure that, through interaction with the enzyme-active site, undergoes a change resulting in irreversible inactivation. Such "suicide" inhibitors are known for the serine- and cysteine-type of proteinases and were adapted for the design of specific inhibitors for DPP-IV in the form Xaa-Pro-NHO-CO-C_6H_4-pNO_2.[31] With Xaa = Ala and Phe, an irreversible inhibition of the hydrolysis of Gly-Pro-pNA to an extent of 50% was caused at a concentration of 30 and 20 μ*M* (IC_{50}), respectively.[31] Among oligopeptides, the well-known inhibitors of DPP-IV are Ile-Pro-Ile (diprotin A) and Val-Pro-Leu (diprotin B),[261] which are known to be hydrolyzed by DPP-IV.[253] Very potent are the amino acyl-pyrrolidides and thazolidides in which the amino acyl- is an ε-benzyloxycarbonyllysine residue, with IC_{50} = 2 and 2.7 μ*M*, respectively, or Ile-, Val-, Leu-, Phe-, and Ala- having IC_{50} values in the range of 2.8 to 87 μ*M*.[31] Specific

inhibitors for DPP-IV, having K_i values in the nanomolar range, are Ala-boroPro and Pro-boroPro dipeptides containing boroPro as the C-terminal residue ($K_i = 2 \times 10^{-9}$ *M*). These inhibitors exhibit slow-binding kinetics, like the previously investigated peptide boronic acid inhibitors of serine-type proteases. No inhibition was detected with *N*-Boc-Ala-boroPro.

DPP-IV was found to be a strong catalyst for transpeptidation reactions. During incubation of Ala_3 with the enzyme, transient formation of higher peptides, mainly of Ala_5, was observed. In analogy to known transpeptidation reactions, this pentapeptide was assumed[236] to be formed by reaction of the amino terminal dipeptide-enzyme complex with the tripeptide substrate rather than with water. Extensive kinetic studies performed showed that although the rate-limiting step for the hydrolysis of substrates with alanine in P_1 is the acylation reaction, with a proline residue in that position, the deacylation reaction becomes rate-determining.[235,251]

b. Natural Substrates

DPP-IV hydrolyzes biologically active peptides such as substance P[263–265] and β-casomorphin.[207,243,266–268] Using the highly purified soluble DPP-IV from differentiated epidermal cell[243] (see Table 9), β-casomorphin consisting of 7 amino acid residues was found to be a better substrate than the 11 residue long substance P. Elongation of the N-terminal fragment of substance P, Arg-Pro-Lys-Pro, from 4 to the full 11-peptide of substance P caused a decrease of the catalytic efficiency from 1322 to 168 $s^{-1}mM^{-1}$. Hence, in this substance P series, the efficiency

TABLE 9
Hydrolysis of Peptides by Dipeptidyl Peptidase IV[a]

Substrate	K_m (m*M*)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Gly-Pro-Ala	0.26[b], 0.28, 0.31[c]	272[b], 202, 265[c]
Ala-Pro-Gly	2.94[b], 0.17[c]	222[b], 272[c]
Tyr-Pro-Phe	0.05	1750
Gly-Pro-Leu-Gly	0.55[b]	668
Leu-Pro-Gly-Gly	0.12[c]	690[c]
Gly-Pro-Gly-Gly	0.30, 0.28[c]	262, 112[c]
Tyr-Pro-Phe-Pro	0.05	1816
Tyr-Pro-Phe-Pro-Gly	0.05	2040
Tyr-Pro-Phe-Pro-Gly-Pro-Ile (β-casomorphin, bovine)	0.05	1428
Tyr-Pro-Phe-Val-Glu-Pro-Ile (β-casomorphin, human)	0.03	4132
Arg-Pro-Lys-Pro	0.05	1322
Arg-Pro-Lys-Pro-Gln-Gln-Phe	0.15	415
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly	0.18	335
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (substance P)	0.30	168
Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	0.09	981
Ala-Ala-Ala	1.54[b], 2.50[c]	25.1[b], 6.0[c]
Gly-Ala-Ala	0.59[b]	76.5[b]
Leu-Leu-Gly	1.25[c]	1.2[c]
Leu-Leu-Leu	0.35[c]	7.7[c]

[a] Unless otherwise noted, data are for soluble dipeptidyl peptidase IV from rat epidermal cells.[243] M_r of 190,000 was assumed for calculation of k_{cat} values. Rates were measured at pH 7.5, 37°C.

[b] Dipeptidyl peptidase IV from lamb kidney was purified according to Yoshimoto, T. and Walter, R., *Biochim. Biophys. Acta*, 485, 391–401, 1977. Rates were measured at pH 7.8, 37°C.[236] M_r of 115,000 was assumed for calculation of k_{cat} values.

[c] Dipeptidyl peptidase IV from rat intestinal brush border membrane.[255] Rates were measured at pH 8.4, 37°C.[255] M_r of 230,000 was assumed for calculation of k_{cat} values.

decreases with increasing chain length. This is mainly due to the improvement of the Michaelis constant on shortening the peptide chain. In contrast, shortening of the seven-residue chain of β-casomorphin to the N-terminal pentapeptide did not affect the K_m. However, the catalytic efficiency increased from 1428 to 2040 $s^{-1}mM^{-1}$. Further decrease in chain length had practically no effect. Similar results were obtained with pig kidney DPP-IV.[266] Several of the natural peptides have K_m values below 40 μM, including substance P, prolactin, and trypsinogen activation peptide, indicating that such structures may represent natural substrates hydrolyzed by DPP-IV *in vivo*. Other biologically active substrates cleaved by DPP-IV include monomeric fibrin, renin inhibitor, kentsin, pro-melittin, and hGHRH.[207,228,264,269] More recently, the list of bioactive peptides and proteins that are cleavable by DPP-IV was expanded. DPP-IV from human placenta cleaved the following proteins and peptides: human gastrin-releasing peptide, human chorionic gonadotropin, human pancreatic polypeptide, sheep prolactin, aprotinin, corticotropin-like intermediate lobe peptide, and (Tyr-)melanostatin.[270] Not unexpectedly, the polypeptides, used in their native state, were cleaved much more slowly than the smaller oligopeptides. The possible biological relevance of these degradations is discussed in the next section.

c. Biological Role of Dipeptidyl Peptidase IV

The widespread occurrence of DPP-IV and its unique substrate specificity have attracted attention from different scientific disciplines. Evidence is available to indicate participation of the enzyme in a number of biochemical processes, such as renal transport and intestinal digestion[271] of proline-containing peptides, immunological activation of immunocompetent cells, and fibronectin-mediated adhesion. It is also possible that DPP-IV is involved in activation or deactivation of biologically relevant peptides. For example, the placental blood contains high levels of substance P and this tissue is a rich source of the peptidase. It is possible, therefore, that DPP-IV is involved in contractile processes of the placenta via the vasoactive properties of substance P.[207] It has been known for some time that DPP-IV cleaves a dipeptide from monomeric fibrin and may interfere with blood clotting.[272] Furthermore, casein, which is resistant to most proteinases and peptidases, contains alternating proline residues in the sequences of this protein that do not contain casomorphin. If such precursors reach the blood, DPP-IV might digest them to yield the morphine-like activity of casomorphin.[268] These suggestions are somewhat speculative and there is no evidence that the enzyme acts on these peptides under physiological conditions. However, studies have shown that it is the DPP-IV activity that cleaves Tyr-Ala from the amino terminus of hGHRH.[269] Although the biological relevance of this event remains to be established, it is clear that this cleavage inactivates the releasing hormone. It is possible that the rapid degradation is necessary to limit the activity of the releasing hormone to localized targets.[269]

A role of DPP-IV in the conversion of precursors of biologically active peptides into the end products by stepwise cleavage of the precursor was proposed.[228] Promelittin is the precursor of the main constituent of honey-bee venom. In the amino acid sequence of the "pro" region of this precursor, every second residue is either proline or alanine. DPP-IV activity, shown to be present in extracts from venom glands of queen bees, cleaved off in a stepwise manner the Xaa-Pro/Ala type dipeptides from the amino end of the "pro" region, Ala(22), until reaching the last even-spaced proline, Pro(41), not entering the following melittin sequence. The sequence of pre-pro-melittin is Met(1)––Ala(22)-Pro-Glu-Pro-Glu-Pro-Ala-Pro-Glu-Pro-Glu-Ala-Glu-Ala-Asp-Ala-Glu-Ala-Asp-Pro-Glu-Ala(43)-Gly-Ile–––Gly(70). It is not obvious why the enzyme does not cleave the Glu-Ala(43) dipeptide sequence. Similar processing may occur to generate mature antifreeze protein from winter flounder[273] and this type of cleavage is probably widely used to generate mature hormones from their polypeptide precursors.

d. Renal Transport of Prolyl and Hydroxyprolyl Peptides

DPP-IV is an integral protein constituting as much as 4% of the renal brush border membrane

protein. As such, it may play a role in processing of peptides present in the glomerular filtrate. Di- and tripeptides, formed by DPP-IV hydrolysis from oligopeptides that pass into the filtrate, may be reutilized by reabsorption through the renal brush border peptide transport system. The obligatory role of DPP-IV in the hydrolysis and absorption of β-casomorphin [1–5] has been demonstrated.[274,275] In these studies, advantage was taken of a novel rat strain with a genetic deficiency of DPP-IV and the fact that the recently discovered renal transport system accepts di- and tripeptides, but longer peptides are excluded.

Thus, insufficient processing of prolyl peptides in the DPP-IV-negative animals resulted in a failure of amino acids in casomorphin [1–5] to accumulate in kidney vesicles. In addition, a large increase in the amount of proline- and hydroxyproline-containing peptides excreted in the urine was detected. Positive controls behaved as expected; the peptide was cleaved and transported. Thus, the obligatory role of DPP-IV in the renal processing of proline- and hydroxyproline-containing peptides was established. It is likely that renal peptide transport results in conservation of amino acid nitrogen by reabsorbing small peptides present in the tubular fluid.

In this study, it is not clear why the N-terminal X–Pro bonds of the substrates used were not cleaved by aminopeptidase P, which is known to be present in the renal brush border with its catalytic site facing the extracellular space.[66] It is possible that aminopeptidase P does not play a role in hydrolysis of oligopeptides or that the tetrapeptide generated by action of this enzyme on casomorphin [1–5] is still not accepted by the peptide transport system or that the vesicles prepared from the DPP-IV negative animals have an impaired activity of aminopeptidase P as well. The latter could result from the conditions under which the membranes were prepared. It might be worthwhile to carry out these experiments under *in vivo* conditions.

e. Fibronectin-mediated Hepatocytes-DPP-IV Interaction

As discussed earlier, DPP-IV has been shown to be identical to a cell surface glycoprotein, gp 110.[240,241] Recent studies using radiolabeled DPP-IV reveal direct interaction of the enzyme with fibronectin.[276] This interaction does not involve the DPP-IV catalytic site and competitive inhibitors of the enzyme stimulate fibronectin binding to hepatocytes *in vitro*. Furthermore, substrates of DPP-IV affect the rate of hepatocyte[277,278] and fibroblast[279] spreading on matrices of collagen and fibronectin. The tight binding of DPP-IV to rat liver biomatrix[218] and electron microscopic localization of this enzyme and fibronectin to the hepatocyte sinusoidal membrane suggest an important role for these proteins in the interaction of hepatocytes with the extracellular matrix *in vivo*.[218,276]

f. Immunological Aspects

The role of DPP-IV in the immune system became of interest when its presence on the surface of human peripheral lymphocytes as an ectoenzyme was demonstrated.[222,247,280] The association of DPP-IV with human T-lymphocyte activation markers was the culmination of a number of independent attempts to prepare antibodies against lymphocyte surface proteins. Monoclonal antibodies against surface antigens that were expressed during mitogenic stimulation of lymphocytes often recognized a target designated CD26 (originally Ta1).[281] These antibodies crossreacted with DPP-IV and it was eventually concluded that another leukocyte activation marker (Tp 103) was also identical with DPP-IV.[284] Thus, DPP-IV is an example of a leukocyte differentiation antigen that possesses enzymatic activity. Among blood and bone marrow cells, mainly the T lymphocytes contain DPP-IV,[223,224,285,291] although presence of the enzyme was also described in monocytes[287] and platelets.[98] Study of the correlation between expression of the surface-associated DPP-IV with other surface markers revealed that the majority of DPP-IV positive cells are CD4 positive (CD4 monoclonal antibodies comprise the T-helper-cell subset), but that there is a minor fraction of CD8 cells that express the enzyme (CD8 monoclonal antibodies comprise the T-suppressor subset).

The expression of DPP-IV on T cells was found to be associated with the capacity of these

cells to produce large amounts of interleukin 2 (IL-2) and to proliferate strongly in response to mitogenic or alloantigenic stimulation.[99,288–292] Inhibition of the enzyme impaired mitogen-induced DNA synthesis and B-cell differentiation. *In vitro* induction of IL-2 and interferon by activated T cells, as well as the IL-2 driven lymphocyte proliferation, were shown to be dependent on the functional activity of DPP-IV in T lymphocytes.[290] Hence, the DPP-IV catalytic activity plays a key role in the T-cell-mediated immune response[290] and, because of its distribution, it may serve as a T-cell surface marker[223,224,288,289] for IL-2-producing cells.

Further studies showed that modulation of the CD26 antigen in response to treatment with the monoclonal anti-CD26 enhanced CD3ζ phosphorylation, $p56^{lck}$ activity, calcium mobilization, and IL-2 production.[293,294] Observation of the association of CD26 with the lower molecular weight isoforms of CD45 led to the proposed mechanism[294] by which the CD26 interaction with CD45, a known membrane-linked protein tyrosine phosphatase, could account for the enhanced ζ chain phosphorylation and $p56^{lck}$ activity.[295]

Involvement of the CD26 antigen in activation of T cells was recently demonstrated by recombinant methods.[238,239] The antigen from activated T cells was cloned and expressed in the human leukemic T-cell line, Jurkat, which originally lacks CD26. The transfectant acquired new functions, namely, calcium mobilization and production of IL-2 in response to crosslinking of the expressed CD26 and of CD3 with their respective monoclonal antibodies.[238] The predicted sequence of the antigen was 85% homologous with that of rat liver DPP-IV.[206] The use of a cDNA specific for human intestinal DPP-IV made it possible to confirm the identity of DPP-IV with a mouse thymocyte activation antigen, the mouse counterpart of the human CD26.[239] Dang et al.[296] demonstrated that the proliferation of CD4 T cells can be induced by anti-CD3 and collagen. Interaction with two surface antigens, namely, 1F7 (which is DPP-IV) and VLA-3 (which is a member of the integrin family), was required for collagen-induced activation. Inhibition of the response to collagen was achieved using anti-DPP-IV and with peptides containing sequences common to the collagen triad Gly-Pro-Y, namely, Gly-Pro-Gly-Gly,Gly-Pro-Ala, and Gly-Pro-Hyp. It was concluded that DPP-IV serves as a cell surface receptor for collagen or as an accessory molecule that facilitates collagen binding to its receptor and is therefore involved in the interaction of CD4 cells with the extracellular matrix.

Although DPP-IV plays a key role in the T-cell-mediated immune response, precise details on the mechanism and the nature of the physiological substrates are still unknown. It was speculated that the most probable function of DPP-IV lies in the cleavage and modification of biologically active peptides with an X–Pro N-terminal sequence in the signaling process of lymphocytes. Because DPP-IV is exposed on the outside of the T cell, a putative substrate for DPP-IV in T-cell activation should also be exposed on the cell surface of either the T cell itself, or of the accessory or target cell. In this respect, it is of interest that DPP-IV is able to modulate the activity of biologically active peptides such as substance P,[263,264] promelittin,[228] yeast pro-α-factor,[297] frog procerulein, and the antifreeze proprotein of the winter flounder.[273] Finally, recent studies showed that the proliferative response to mitogen stimulation of lymphocytes was accompanied by an increase of the specific activity of DPP-IV. As shown by Hendriks et al.,[99] a parallel increase in the specific activity of aminopeptidase P and of DPP-IV occurred in cultures of mitogen-activated human lymphocytes. No such increase was seen with Leu-*p*-nitroanilide-cleaving aminopeptidase,[99,298] which remained unchanged during the course of cultivation. It is possible that DPP-IV and aminopeptidase P act in concert during lymphocyte activation.

2. Dipeptidyl Peptidase II

Like DPP-IV, DPP-II (EC 3.4.14.2) also releases dipeptides of the type Xaa-Pro and Xaa-Ala from the N-terminus of peptides.[54] However, DPP-II acts at acidic pH, has a subunit M_rs of 54 to 64 kDa, and a lysosomal localization. It is widely distributed in various tissues and is found in membrane-associated and soluble form. For the most part, this enzyme has a substrate specificity and other characteristics quite similar to those of DPP-IV. Interestingly, brain DPP-II

cleaves X-Pro--Pro sequences in contrast to DPP-IV.[299] The enzyme from porcine ovary, classified as DPP-V,[300] should be classified, on the basis of its specificity, as DPP-II.[299]

E. Prolyl Endopeptidase

Prolyl endopeptidase (EC 3.4.21.26) was discovered by Walter et al.[301] in homogenates of human uterus as an oxytocin-degrading enzyme that cleaved the Pro-Leu bond. The enzyme was shown to act on Pro–X bonds in a number of other biologically active peptides, including angiotensin II and bradykinin.[250] Several enzymes — termed TRH-deamidase, brain kinase B, endo-oligopeptidase B, and postproline endopeptidase — that cleave peptide bonds on the carboxyl side of the proline residue have the same specificity as the enzyme originally named postproline-cleaving enzyme.[250] Comparison of their properties[302] and application of immunochemical techniques[303–305] has led to the conclusion that the same enzyme is responsible for all of the previously mentioned activities. The term "prolyl endopeptidase" (PEPase) is at present generally accepted for this proline-specific endopeptidase.[302] A review on PEPase appeared in 1983 (Reference 302; see also 52–54).

1. Physical Properties

PEPase from a variety of sources, including eggs and sperm from the ascidian *Halocynthia roretzi*,[306] lamb brain and kidney,[307] and various mammals have been characterized.[250,271,302,304,308–311] In general, the molecular weights and pH optima of most PEPases are quite similar. The bacterial enzyme[229,312–314] differs from mammalian PEPases, having a higher pI and lacking cysteine in the free form. The primary structure of porcine kidney PEPase was deduced[315] from its cDNA, which encoded a polypeptide chain of 710 amino acid residues, corresponding to a molecular mass of 80,751 Da. The deduced sequence shows no homology with the known serine proteases. The active site Ser-554 is surrounded by an amino acid sequence different from other serine proteases. However, it still conforms to the consensus sequence, Gly-X-Ser-X-Gly, in active sites of serine-type of proteases and esterases.[316] The PEPase from *Flavobacterium meningosepticum* has a similar structure with a deduced sequence of 705 amino acid residues and contains a signal peptide at its amino terminus with the active site Ser localized to position 556. In the mature enzyme, it becomes Ser-536 and is part of the sequence -Gly-Arg-Ser-Asn-Gly-Gly- .[313,314] The molecular weight of preparations from different animal organs range from 62 to 77 kDa and the enzyme is composed of a single polypeptide chain. As a serine type of peptidase, the PEPase is inhibited by diisopropyl-phosphofluoridate, but surprisingly, it is resistant to phenylmethane sulfonylfluoride. The optimal pH with different substrates is in the neutral to mild alkaline range, 7.0 to 7.8; the pI is 4.5 to 4.9, and metal ions such as Zn^{2+} and Cu^{2+} inhibit certain, but not all PEPases.[308]

2. Specificity

PEPase is an endopeptidase cleaving a Pro–Y bond in a structure that consists of at least an acyl-X-Pro--Y sequence. A free α-amine in an N-terminal sequence Xaa-Pro-Y and Pro-Y prevents the hydrolysis of the Pro-Y bond. In contrast, the Pro–Y sequence will be cleaved when present in the C-terminal position, the enzyme then acting as a carboxypeptidase.

The Pro residue can be replaced by Ala, but the catalytic efficiency is much lower,[52] as was seen in a comparison of peptide substrates with C-terminal proline amides and esters. Proline can be replaced by alanine, *N*-methylalanine and sarcosine (*N*-methylglycine) in Cbz-Gly-X--Leu-Gly substrates without significantly affecting the K_m or k_{cat}/K_m values found for PEPase from *Flavobacterium meningosepticum* (Table 10). In comparison with the Pro-substrate, the Ala-substrate had a k_{cat}/K_m value that was 6.1 times lower. The effect of replacement of Pro by Ala was much larger in Cbz-Gly-Pro-2-NNap with the k_{cat}/K_m value reduced 83-fold in the Ala compound.[229] This was most likely caused by the presence of the bulky aromatic group in position P_1'. However, different reaction conditions, unspecified in the kinetic study of substrates with free carboxyl groups,[318] cannot be excluded. The common structural feature of the Cbz-Gly-

TABLE 10
Effect of Position P_1 on PEPase[a] Substrates

Xaa in Cbz-Gly-Xaa—Leu-Gly	K_m (m*M*)	k_{cat}/K_m (mM^{-1} s^{-1})
Pro	0.54	32.4
Ala (N-Me)	0.51	13.9
Sar	0.73	7.74
Ala	0.86	5.31
Gly	1.33	0.02

[a] PEPase from *Fl. meningosepticum*.[318]

X--Leu-Gly substrates (Table 10) is the complete or partial skeleton of a five-membered ring for residue X. When an amino acid side chain extends beyond the space corresponding to the ring (Hyp or Ser), poor substrates are obtained. Still larger substituents result in resistance to PEPase [pipecolyl, α-aminoisobutyl, N-methyl-Val, N-methyl-Leu, Hyp(OBzl), and Ser(OBzl)].[318] It can also be concluded that the unique nature of the Xaa-Pro bond is not an absolute requirement for PEPase. This is not surprising, because the tertiary amide is not the bond cleaved by the enzyme. The cleavage of the Pro−Y bond is, however, dependent on the configuration of the Xaa-Pro bond, which has to be in the *trans* configuration.[319]

The Pro−Y sequence is best cleaved when Y is a hydrophobic residue, the rates decreasing with basic residues and dropping further with acidic residues. The P_1' position cannot be occupied by Pro. In contrast to the residue in P_1', the residue in P_2 (X-Pro) had only a minimal effect on kinetic constants[305] when Cbz-X-Pro--MeONapNH was the substrate.

The active site of PEPase was mapped by determining the kinetic parameters, K_m and k_{cat}, for the hydrolysis of peptides in which the chain length was systematically changed by modification of an acyl-Xaa-Pro-Y dipeptide derivative, where Y is a group such as -ONp, -NNap, or an amino acid residue (Table 11), or by elongating Gbz-Gly-Pro-Leu on the carboxyl terminus (Table 12). The results of these studies, which used PEPase from several sources, clearly show[229,308,317,320] that the active site of the enzyme is composed of five binding subsites: S_3, S_2, S_1, S_1', and S_2'. Analysis of the data indicates that when S_2' is not occupied, and the enzyme behaves as a carboxypeptidase, the catalytic efficiency is greatly reduced. The enzyme is also highly stereospecific; subsites S_2, S_1, and S_1' cannot accept D-residues.[308,320] Thus, a variety of "substrates" containing D-Ala or D-Pro at these positions (P_2, P_1, P_1') are either not hydrolyzed or are hydrolyzed poorly. The information gained from mapping the active site served as a valuabe guide in the design of inhibitors for PEPase.

3. Inhibitors

Specific inhibitors can be especially useful in providing additional insights into the active site of PEPase. Furthermore, certain inhibitors can greatly aid studies directed at uncovering the physiological role of this enzyme. Inhibitors of PEPase fall into two separate categories: enzyme reagents and substrate analogs. Reagents such as inhibitors of serine proteases, thiol reagents, metal chelating agents, and metallic ions all have an effect on PEPase activity. As mentioned earlier, both the peptidase and esterase action of PEPases are stoichiometrically inhibited by diisopropylphosphofluoridate, classifying this enzyme as a serine protease. However, the enzyme was not inhibited by PMSF (Phe CH_2SO_2F), which is also a serine protease reagent. Cbz/Tos-Gly-Pro-CH_2-Cl and Cbz-Gly-Gly-Pro-CH_2-Cl were good inhibitors of peptidase and esterase activity that alkylate the histidyl residue in the active site of PEPase.[321]

Except for the bacterial enzyme, which does not contain cysteine, PEPases are inhibited by thiol reagents such as *p*-chloromercuribenzoate and *N*-ethyl maleimide, but not, or only weakly, by iodoacedic acid or its amide. Titration of three-SH groups by *p*-chloromercuribenzoate caused complete loss of activity of PEPase from bovine brain,[308] which was restored with the help of the sulfhydryl compounds dithiothreitol, 2-mercaptoethanol, or cysteine. The size of the introduced substituent was taken as the factor responsible for the inhibitory power of *p*-chloromercuribenzoate vs. lack of it in the case of iodoacetic acid.

TABLE 11
Effect of Residues in Positions P_2 and P_3 on PEPase Hydrolysis

Cbz-peptide-[a]	-ONp Lamb kidney[320] K_m (m*M*)	-ONp Lamb kidney[320] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	-2NNap *Fl. meningosepticum*[317] K_m (m*M*)	-2NNap *Fl. meningosepticum*[317] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	-2NNap Bovine brain[308] K_m (m*M*)	-2NNap Bovine brain[308] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Cbz-Gly-Pro-	0.07	488	0.14	1212	0.68	585
Cbz-Ala-Pro-	0.07	884	0.08	834	0.0862	915
Cbz-D-Ala-Pro-	—	—[b]	0.2	0.73	—[c]	—[c]
Cbz-Gly-Gly-Pro-	0.27	98.2	0.133[d]	645[d]	—	
Cbz-Ala-Gly-Pro-	0.38	78.9	0.29	664	—	—
Cbz-D-Ala-Gly-Pro-	1.02	37.5	0.14	271	—	—

[a] The invariable proline is in position P_1. The preceding amino acid residues are in positions P_2 and P_3, respectively.
[b] Negligible rate of hydrolysis.
[c] Not hydrolyzed.
[d] The substrate was Cbz-Gly-Gly-Pro-ONp.

TABLE 12
Hydrolysis of Peptides by PEPase

Substrate[a]	Lamb kidney[320] K_m (m*M*)	Lamb kidney[320] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	*Fl. meningosepticum*[317] K_m (m*M*)	*Fl. meningosepticum*[317] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	Bovine brain[308] K_m (m*M*)	Bovine brain[308] k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Cbz-Gly-Pro--Leu	0.17	150	0.22	104	0.038	474
Cbz-Gly-Pro--Leu-Gly	0.06	852	0.32	1600	0.042	1360
Cbz-Gly-Pro--Leu-Gly-Gly	0.13	214	1.4	500	—	—
Cbz-Gly-Pro--D-Ala	0.25	6.1	[b]	[c]	[c]	[c]
Cbz-Gly-Pro--Leu-D-Ala	0.37	124	1.5	1070	0.19	114

[a] Substrates are cleaved at the - - bond.
[b] Negligible rate of hydrolysis.
[c] Not hydrolyzed.

However, it is not established whether the SH group is near the active site or whether SH modification affects the tertiary structure of PEPase.

Substrate analogs containing a carboxy terminal prolinal group (proline aldehyde) have been found to be especially potent inhibitors of PEPase.[28,322,323] Such compounds inhibit serine and cysteine proteases. The K_i value of Cbz-Pro-prolinal (3 to 14 n*M*) for PEPase is more than three orders of magnitude lower than that of the corresponding acid and alcohol, suggesting that this derivative functions as a transition state inhibitor. It is believed that Cbz-Pro-prolinal occupies enzyme subsites S_2, S_1, and S_1'. In analogy to substrates, P_1 need not be Pro and Cbz-X-prolinal where X = Val, Phe, Ile, or Ala are all inhibitors albeit less potent than when X = Pro.[324,325] Supporting the stereospecificity requirement of S_1 and S_2 is the finding that Cbz-L-Phe-D-prolinal and Cbz-D-Phe-L-prolinal are three to four orders of magnitude less potent inhibitors for ascidian enzyme.

Interestingly, introducing a sulfur atom into the proline ring of prolinal resulted in the highly potent inhibitors Cbz-Pro-thioprolinal (K_i = 3.5 n*M*) and Cbz-Pro-thiazolidine (K_i = 39 n*M*). The latter compound is still a very good inhibitor, despite the lack of the aldehyde moiety, thus demonstrating the influence of introducing the sulfur atom into the pyrrolidine ring. This is evident by comparison with Cbz-Pro-pyrrolidine (K_i = 2400 N*M*), which lacks the sulfur atom.[28]

When a sulfur atom was introduced into each of the two rings present in Cbz-Pro-prolinal, an extremely potent inhibitor (Cbz-thiopro-thioprolinal) with a K_i value of 0.01 n*M* was obtained. This compound is 300 times more efficient than that of the parent compound.[28]

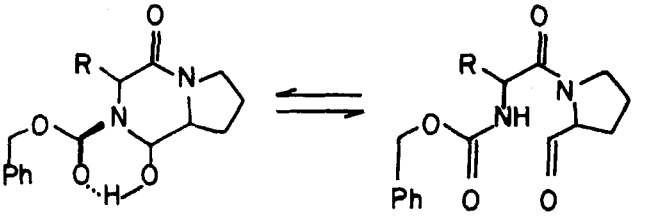
^{1}H-NMR analysis of prolinal peptides suggest that a cyclic structure may be present in solution (Figure 6). This may be stabilized by the six-membered ring formed by the reaction of the aldehyde group with the α-nitrogen atom of the preceding residue as well as by the possible hydrogen bond formation between the carbonyl oxygen of the benzyloxycarbonyl group and the hydroxyl.[325] The presence of an equilibrium between a cyclic and open form of the prolinal inhibitors is supported by inhibition studies. For determination of IC_{50} values it was necessary to preincubate the enzyme with the inhibitor, and prolonged preincubation of prolinal inhibitors with the ascidian and the *F. meningosepticum* enzyme was essential for strong inhibition to occur. A slow conversion of the cyclic (inactive) to the open structure (active) would explain this time-dependent process of inhibition. Furthermore, Cbz-Pro-prolinal is a 200 times better inhibitor than Cbz-Val-prolinal.[150] Although the time course of the inhibition was not determined in this study, the strong inhibition can be accounted for by absence of the cyclized form, which cannot be formed with the prolyl nitrogen. At present, the question of whether the inhibition of PEPase by prolinal inhibitors is noncompetitive[322,326] or competitive[325] remains undecided.

Cbz-Gly-Pro-diazomethyl ketone (Cbz-Gly-Pro-CHN_2) inactivated bovine brain PEPase completely and irreversibly at low concentrations (0.3 μ*M*), without affecting other proteolytic enzymes such as DPP-IV, pyroglutamate aminopeptidase, and trypsin. Substrates of PEPase, such as luteinizing hormone-releasing hormone and Cbz-Gly-Pro-Ala, protected the enzyme against inactivation by the inhibitor. This specific active site-directed inhibitor, when administered intraperitoneally to rats, completely inactivated PEPase in all tissues studied, including the brain. Because peptidyl diazomethyl ketones are chemically unreactive and known to enter cells by pinocytosis, they are particularly valuable tools for *in vivo* studies of the PEPase function in the metabolism of its neuropeptide substrates.[327] Most importantly, the lipophilic nature of this inhibitor permits it to cross the blood brain barrier and also inactivate the brain enzyme.

4. Distribution and Biological Function

PEPase has been purified from plants, microorganisms, invertebrates, and is widely distributed in animal tissues.[302,328] An extensive comparison of PEPase in tissues of various species showed activity in the testis, liver, skeletal muscle, brain, lung, and kidney, with the level of distribution varying in different mammals. Activity was distributed throughout all brain regions, with hippocampus and striatum showing highest enzyme levels,[302] and was also distributed in astrocytes, oligoendrocytes, and neurons.[329]

Bovine brain PEPase cleaved specifically the Pro(4)-Gln(5) bond in substance P. The N-terminal tetrapeptide Arg-Pro-Lys-Pro and the



cyclized form ⇌ open form

FIGURE 6. Structure of Z-X-prolinal. R represents the side chain of amino acid X; the dotted line is a possible hydrogen bond. (Reproduced from Nishikata et al., *Chem. Pharm. Bull.*, 34, 2931–2936, 1986. With permission.)

C-terminal heptapeptide were the only products. The K_i value of 10^{-6} *M* for competitive inhibition of the PEPase-catalyzed hydrolysis of Cbz-Gly-Pro-pNA by substance P indicates the possible role of the enzyme in the regulation of a synaptic action of substance P.[330] Assuming a k_{cat} value of 10 to 50 s^{-1}, known for various PEPase substrates, a catalytic efficiency of 10^7 to 10^8 M^{-1} s^{-1} was calculated for the hydrolysis of substance P. This is comparable to the values found for the neurotransmitter-degrading-enzyme acetylcholinesterase.

In addition to its action on substance P, PEPase acts on a number of other biologically active peptides that have a basic amino acid residue preceding a proline. These include oxytocin, vasopressin, gonadoliberin, α-melanocyte-stimulating hormone, thyroliberin, bradykinin, neurotensin, oxidized insulin B chain, angiotensin II, thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, α-melanocyte stimulating hormone, and dynorphin. Collagen, gastrin, and adrenocorticotropin-releasing hormone are not hydrolyzed by this endopeptidase. Eight peptides of M_r = 3000 isolated from muscle extracts were digested by PEPase. Data suggest that these might be endogenous substrates for this enzyme.[331,332] Thus, the wide distribution of PEPase and the high rate of degradation of biologically active peptides indicate a function of this enzyme for *in vivo* regulation of the action of these peptides. The enzyme appears to participate in processing angiotensin I, as judged by using Cbz-Pro-prolinal inhibition.[333] This is an additional example of a proline peptidase as a member of the blood pressure regulation family. Interestingly, intraperitoneal or oral administration of Cbz-Pro-prolinal reduced PEPase activity in rat and mouse brain and protected these animals from amnesia induced by scopolamine or electric shock.[334] This suggests that certain PEPase substrates are involved in memory consolidation and the learning process.[28,335]

PEPase activity in rat brain varies with age and is maximal in 2-week-old rats.[336] In addition, an endogenous inhibitor was purified from rat brain, and its concentration also varies with the age of the animal.[337] The presence of this highly specific protein inhibitor (M_r = 7000; K_i = 2 μ*M*) and its correlation to PEPase activity may indicate a role in the development of brain neurons. Although this thesis is highly speculative, PEPase inhibitors impair ascidian fertilization and endogenous PEPase inhibitors have been purified from the sperm of ascidian[310] and pancreas of pigs.[338] Thus, the enzyme may have a general role in developmental biology. PEPase activity is depressed in delayed hypersensitive guinea pig skin lesions induced by bovine γ-globulin as an antigen. The depression is caused by an inhibitor generated by inflammation.[339]

In summary, it is becoming increasingly clear that PEPase has important and specific biological functions. Nevertheless, despite the remarkable *in vitro* results, we caution that additional *in vivo* analysis is required before the role of this enzyme in brain and other physiological functions can be unequivocally defined. Further studies on the precise localization of the enzyme should help to clarify some of these aspects.[53]

F. Proline Iminopeptidase and Prolinase

Included among the enzymes that cleave Pro−Y bonds, in which the proline has the secondary amine unsubstituted, are proline iminopeptidase (PIP, EC 3.4.11.5) and prolinase (EC 3.4.13.8). The former enzyme was first detected in *E. coli* using polyproline as the substrate.[340,341] It is an aminopeptidase that cleaves N-terminal proline from low- and high-molecular-weight peptides, including polyproline, salmine, Pro-Gly, Pro-Gly-Gly, and Pro-Leu-Gly-Lys (MIF). It cannot cleave X−Pro peptide bonds except when X is Pro. Similar activities have been purified from *Bacillus coagulans*, *Neisseria gonorrhoeae*, apricot seeds, and other sources.[59,342–345] To date, no homogeneous PIP from a mammalian source has been unequivocally demonstrated. Although an electrophoretically pure PIP from bovine kidney was reported,[346] this activity was not verified using leucine aminopeptidase substrates and the presence of a general aminopeptidase of broad substrate specificity in this preparation cannot be excluded. Moreover, a mammalian PIP was actually shown to be identical with leucine aminopeptidase.[347,348] Given some of the uncertainties that still exist on PIP and the lack of in-depth studies on the biological role of this en-

zyme, the reader is referred to the literature for additional details on the properties of PIP-like enzymes.[55,347]

Similar to PIP, prolinase cleaves Pro–Y bonds involving N-terminal proline. Its action, however, is restricted to dipeptides and it is also known as iminodipeptidase. However, all prolinase preparations also cleave dipeptides that do not contain an N-terminal proline and it is likely that prolinase is a broad specificity dipeptidase.[349,350] Again, because of the lack of specificity and the absence of studies evaluating the biological importance of prolinase, we will not discuss this activity in detail.

G. Peptidyl-Prolyl *Cis-Trans* Isomerase

As discussed earlier (see Section I), in contrast to peptide bonds from the 19 α-amino acids, those involving the nitrogen of a prolyl residue can exist as a mixture of *cis* and *trans* rotamers. Although the *trans* form is preferred in most cases, the proportion of the *cis* rotamer present in an equilibrium mixture can be very high.

Isomerization between the two energetically similar rotamers[11] is characterized by slow kinetics and a high energy barrier ($\Delta G \neq$ 17 to 20 kcal/mol).[351–353] This slow interconversion may not satisfy the demands of many important biological processes and it was postulated that the *cis-trans* isomerization about the X-Pro peptide bond may require catalysis by a rotamase.[354] Such rotamases were indeed discovered and because they act on the acylproline peptide bond they will be reviewed briefly. It should be noted that the impetus for many studies in this area is the identity of rotamases with immunophilins.[355,356] This is a rapidly growing field and we will restrict our survey to studies that provide insights into the mechanism of action of *cis-trans* isomerases. Details on relevance to immunosuppressants will not be reviewed.

Fisher et al.[354] isolated an enzyme that catalyzed the *cis-trans* isomerization of prolyl bonds in oligopeptides. The enzyme promoted a 180° rotation around the X-Pro peptide bond without cleavage or formation of a covalent bond[353,357] and was termed peptidyl-prolyl *cis-trans* isomerase (PPIase), rotamase, or conformase. Detection of PPIase activity was aided by the isomeric specificity of α-chymotrypsin, which cleaves the Phe--NHNp bond in peptides of the general structure X-Pro-Phe--NHNp only if the X-Pro link is in the *trans* conformation. In the presence of a sufficient concentration of chymotrypsin, the *trans* isomer in the substrate is hydrolyzed rapidly. This burst is followed by a slower rate-determining conversion of the *cis* conformer, which can be used to deduce the kinetic constant k_c/K_m for PPIase.[51,358] Using the oligopeptide Glt-Ala-Ala-Pro-Phe--NHNp as the substrate, PPIase was detected in a variety of organisms and is now known to be ubiquitous and highly conserved in all species. The enzyme originally purified from hog kidney has a M_r of 17 kDa, and was strongly inactivated by *p*-chloromercuribenzoate, SDS, Hg^{2+}, and Cu^{2+}, but not by metal chelators, diisopropylphosphofluoridate and TPCK.[51]

1. Cyclophilin and the FK506-Binding Protein are PPIases

Advances in the area of organ transplants and bone marrow therapy have been greatly aided by the use of immunosuppressants such as cyclosporin A and the recently discovered FK506 and rapamycin. These life saving drugs appear to interact with receptor proteins that are located in the cytoplasm. These receptors, known as cyclophilin and FK506 binding protein, are members of a class of molecules termed immunophilins. Pertinent to this review, both cyclophilin and FK506-binding protein have PPIase activity. The medical relevance of the immunophilins has, therefore, been a driving force for new insights into *cis-trans* isomerases.

Cyclophilin-like activities, originally isolated from bovine thymus,[359] were detected in yeast,[360] neurospora,[361] and human tissues and cell lines.[359,362,363] and appear to be abundantly expressed in many eukaryotes.[364] The protein was cloned from *Saccharomyces cerevisiae* and human T cells,[363] and has a deduced molecular weight of approximately 17 kDa. Cyclophilins studied from a variety of organisms have a high sequence homology. The FK506-binding protein activities cloned from mammalian or yeast cells

had a deduced molecular weight of 10 to 14 kDa.[365,366] Comparison of nucleotide sequences indicated that the cyclophilins and FK506-binding proteins evolved independently. In addition, these immunophilins are antigenically distinct. Nevertheless, both molecules exhibit PPIase activity and have a region of sequence similarity that has been associated with a domain responsible for the similar biological activities of the two proteins.[367] The cyclophilin from a Jurkat T cell lymphoma had a very high rotamase activity ($k_{cat}/K_m = 1.4 \times 10^7\ M^{-1}\ s^{-1}$ at 10°C).

2. Structure of FK506-Binding Protein/ PPIase

The availability of large amounts of recombinant cyclophilin and FK506-binding protein have permitted structural analysis of these proteins, in both the bound and free state. Complementary studies have been performed on cyclosporin A and FK506. Analysis of FK506-binding protein/PPIase in solution by NMR[368,369] revealed a five-stranded, concave β-sheet whose cavity was covered by an α-helix segment. The interior of the cavity contained the hydrophobic aromatic rings of three phenylalanines, two tyrosines, and one tryptophan. This cavity proved to be the drug-binding pocket. Binding of FK506 to FK506-binding protein was analyzed by X-ray crystallography.[370] The cyclohexyl, pipecolic acid, and pyranose moieties of FK506 were buried in the hydrophobic pocket and the rest of the molecule was exposed to the environment. Interestingly, the bonds to the 2-carboxyl and *N*-amide groups on the six-membered pipecolic acid ring occur as the *trans* rotamer in bound drug, whereas they are *cis* in the unbound FK506. The pyranose ring is rotated to the outside of the unbound macrocycle, whereas it is driven inside on binding to FK506-binding protein. The highly hydrophobic binding pocket may be a characteristic of the immunophilins. Interestingly, an *E. coli* rotamase, which was not specifically inhibited by cyclosporin A, had a much lower Phe content (6%) than most cyclophilins (9%) and lacked a Trp residue conserved in eukaryotic cyclophilins.[357]

The mechanism for the catalysis of *cis-trans* isomerization has been a major goal of studies on this enzyme. Early studies suggested that the activity of porcine PPIase was dependent on a single thiol group. An inverse secondary kinetic effect in X-Pro substrates, in which the hydrogen linked to the α-carbon in residue X is substituted by deuterium, indicated transient formation of a covalent intermediate, possibly a hemiortho-thioamide.[371] However, the deduced amino acid sequence of a *Candida albicans* homolog of human cyclophilin gene, which was expressed in *E. coli* and shown to possess PPIase activity sensitive to inhibition by cyclosporin A *in vitro*, contained only two cysteine residues that did not align with any of the four cysteines conserved among mammalian cyclophilins.[372] Moreover, in human cyclophilin expressed in *E. coli*, individual mutation of the four cysteines to alanine resulted in mutant proteins that possessed full affinity for cyclosporin A and equivalent catalytic efficiency as a rotamase. It was concluded that cysteines play no essential role in catalysis or in cyclosporin-A binding.[357] This rules out the previously proposed mechanism involving the formation of tetrahedral hemithioorthoamide.[371] Whereas mechanisms that embody other tetrahedral intermediates may be operative, an alternative mechanism was considered, that involves distortion of bound substrate with a twisted (90°) peptidyl-prolyl amide bond (Figure 7).[357] This mechanism is lent credence by crystal structures of FK506 and cyclosporin A that reveal substructures that mimic the twisted amide bond of the peptide substrate.[373] Specifically, the ketone carbonyl next to the pipecolic acid group in FK506 is structurally similar to the twisted X-Pro bond, and it was suggested that both FK506 and rapamycin have ground-state geometries similar to the transition state assumed during the isomerization (Figure 8).

Although the above studies on cyclophilin and FK506-binding protein provide mechanistic insights into PPIase, it is important to note that the catalysis by cyclophilin of prolyl *cis-trans* isomerization, as measured with Suc-Ala-Ala-*cis*-Pro-Phe-pNA, was relatively inefficient.[358] A 5-m*M* concentration would have to be attained to achieve a 100-fold acceleration of the unca-

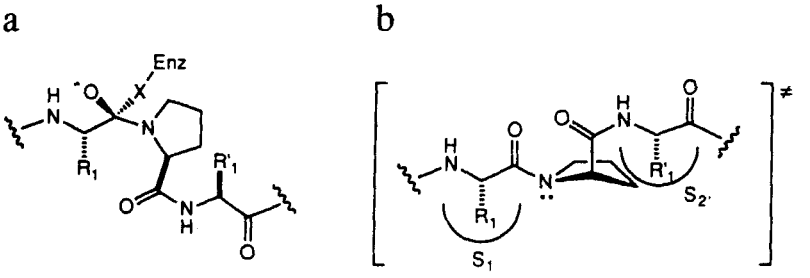


FIGURE 7. Two alternative mechanisms for rotamase-catalyzed peptidyl-prolyl *cis-trans* isomerization. (a) Covalent catalysis in which a nucleophile, X, in the enzyme active site attacks the carbonyl carbon to form a tetrahedral intermediate (X ≠ S). (b) Noncovalent catalysis in which the enzyme utilizes its binding energy to selectively stabilize the transition state with a 90° rotation of the amide bond out of planarity (induced fit mechanism). (Reproduced from Liu et al., *Proc. Natl. Acad. Sci. U.S.A.*,[87] 2304–2308, 1990. With permission.)

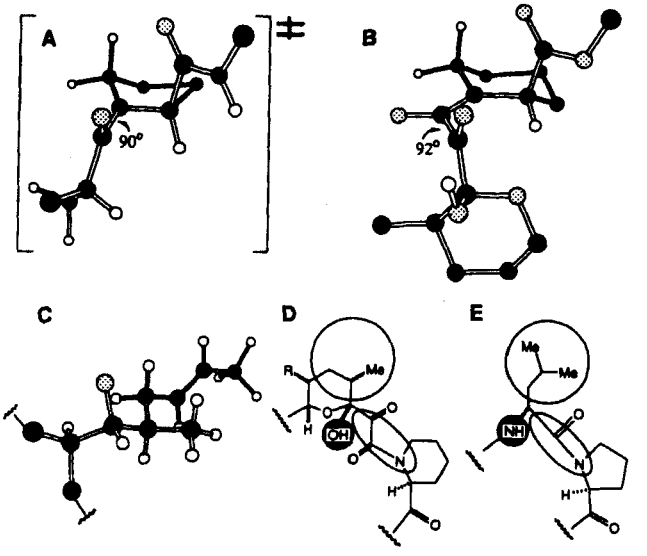


FIGURE 8. (A) Model for the transition state structure of a twisted peptidyl-prolyl amide bond that is stabilized by the rotamase enzymes cyclophilin and FKBP. (B) Substructure of FK506 and (C) CsA (both from X-ray) that is proposed to mimic the twisted amide bond of a peptide substrate. (D) Substructure of FK506 (R = OMe) and rapamycin (R = H) proposed to mimic a twisted leucyl-prolyl amide bond of a peptide substrate. (E) Leucyl-prolyl fragment indicating structural similarities to immunosuppressant substructures and suggesting that FK506 and rapamycin may be transition state analogs of a leucyl-(twisted amide)-prolyl peptide substrate for FKBP. (Reproduced from Schreiber, *Science*, 251, 283–287, 1991. Copyright 1991 by the AAAS. Used by permission.)

talyzed reaction, having a halftime of less than 5 s. Even less efficient was FK506-binding protein, raising questions about physiological relevance. Therefore, a search for better substrates was performed with a series of compounds of the general structure Suc-Ala-X-Pro-Phe-pNA, where X was Gly, Ala, Val, Leu, Phe, His, Lys, or Glu. Little dependence of k_c/K_m for cyclophilin-

catalyzed isomerization on X was found. The isomerization catalyzed by the FK506-binding protein, on the other hand, displayed a dependence of k_c/K_m on X varying over three orders of magnitude. With X being Leu (k_c/K_m 640,000 $M^{-1}s^{-1}$), it became possible to measure the K_i value of 1.7 ± 0.6 n*M* for the tight binding FK506 to FK506-binding protein. The inhibition constants of PPIase activity of cyclophilin by cyclosporin A are K_i = 5.6 n*M* and 2.6 n*M*.[358,371]

Careful analysis of the structure and substrate specificity of cyclophilin and FK506-binding protein supports the conclusion that these molecules are members of a family of peptidyl *cis-trans* isomerases that possess distinct substrate specificities. It is likely that detailed characterization of the individual members of this family will result in the discovery of a number of new, and perhaps unexpected, biological roles for these molecules.

3. Biological Role of Peptidyl-Prolyl Cis-Trans Isomerase

Several functions have been proposed for the immunophilines-peptidyl-prolyl *cis-trans* isomerases. The rotation around the X-Pro bond has been demonstrated as one of the major steps in the *in vitro* refolding of denatured proteins[351,374-378] and has been implicated in the interconversion of conformers in native proteins.[379] Furthermore, as noted throughout this review, *cis* X−Pro bonds are not hydrolyzed by several peptidases and also restrict hydrolysis of adjacent peptide bonds. Thus, the turnover of protein chains may depend on the conversion of a *cis* rotamer into its *trans* state.[17,319,381] PPIases may also help refold proteins that have changed their conformations during transport through membranes.[357,380] Thus, the rotamases may have a role in protein transport in a wide variety of organisms.

It is, of course, tempting to implicate PPIase in the immune response. However, the direct correlation of rotamase activity and T-lymphocyte activation is not supported by the divergent activities of FK506, rapamycin, and several synthetic analogs. Whereas FK506 inhibits the production of IL-2 by the T cell, rapamycin-binding does not inhibit IL-2 production, but inhibits some events in a later stage of the T-cell activation process. Because binding of FK506 and of rapamycin to FK506-binding protein inhibits the rotamase activity of the protein, and studies have shown that both inhibitors bind through their cyclohexyl-pipecolic acid-pyranose sequence, the different functions must reside in the nonbinding portion of the immunodepressants, which constitute effector elements. This conclusion is strongly supported by the synthesis of FK506BD, an inhibitor designed to have a binding activity but to lack the putative effector elements. In fact, this latter molecule has no effect on T-cell activation but binds to FK506-binding protein and inhibits rotamase activity.[382]

VI. CONCLUDING REMARKS

It should be clear that the presence of proline in a polypeptide chain imposes restrictions on its susceptibility to most peptidases. These restrictions are related both to the presence of an Xaa-Pro bond and the isomeric state of this bond. The unique effect of proline in a polypeptide chain suggests that this residue functions in a regulatory capacity to protect the peptide and perhaps to initiate the processing of biologically active peptides.[53,101,383] The involvement of this residue in regulatory events is supported by the recent discoveries of the *cis-trans* prolyl isomerases. It is anticipated that future studies on the structure of these enzymes, on their action on proline-containing peptide chains, and on their biological significance will yield important insights into the conformational foundations of biological regulation.

Further studies of the role of the proline residue in metabolic stability of biologically active peptides and in regulation of their activity by enzymatic hydrolysis may result in the discovery of new regulatory mechanisms and additional biologically active peptides. An understanding of recognition by enzymes of the sequences surrounding proline is needed to predict cleavage sites in proteins and peptides and for the eventual detection of new active peptides by highly informed reading of the protein sequence. Studies on the dependence of the HIV-1 proteinase cleav-

age sites on its sequence should prove useful toward this goal and might prove invaluable in attempts to develop clinical approaches for AIDS therapy.

Disclosure of identity between DPP-IV and the surface antigen CD26 points out the broad biological potential of proline peptidases. At present there is only scant information about proline peptidases acting on the carboxyl end of polypeptides. It is likely, therefore, that the list of enzymes acting on proline-associated peptide bonds will expand in the near future. A thorough definition of the specificity and localization of these peptidases will contribute further to the understanding of the biological role of the enzymes and to the importance of the prolyl residue as well.

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