Benzyloxycarbonylprolylprolinal, a transition-state analogue for prolyl oligopeptidase, forms a tetrahedral adduct with catalytic serine, not a reactive cysteine

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N-Benzyloxycarbonyl-L-prolyl-L-[1-¹³C]prolinal was synthesized starting with reduction of L-[1-¹³C]Pro to L-[1-¹³C]prolinol, followed by coupling with *N*-benzyloxycarbonyl-L-Pro to *N*-benzyloxycarbonyl-L-Pro-L-[1-¹³C]prolinol (Z-Pro-[1-¹³C]prolinol), and finally oxidation of the alcohol to the aldehyde with dimethyl sulphoxide. While the ¹³C NMR chemical shift of the aldehyde carbon is 202 p.p.m., that of the aldehyde hydrate is between 91.6 and 91.8 p.p.m., that of the dithiothreitol adduct is between 74.8 and 75.0 p.p.m., and that in the presence of the serine protease prolyl oligopeptidase is at 92.3 p.p.m.. The linewidth of the latter is 114 Hz, roughly consistent with the

molecular mass of 80 kDa reported for the enzyme. Inverse detection experiments gave a ¹H resonance at 5.29 p.p.m. with a linewidth of 80 Hz, also consistent with the expected chemical shift and linewidth for a hemiacetal bound to such a large enzyme, while the free hydrate gave resonances at 5.18 and 5.25 p.p.m., with very much narrower linewidths. It is concluded that Z-Pro-prolinal, a putative transition-state analogue for prolyl oligopeptidase, forms a tetrahedral complex with the enzyme at its catalytic serine, rather than at a neighbouring cysteine that was found to be highly reactive according to chemical modification studies.

INTRODUCTION

Prolyl oligopeptidase (PO; EC 3.4.21.26), previously called prolyl endopeptidase or post-proline cleaving enzyme, is a large intracellular peptidase (molecular mass 80 kDa) that preferentially hydrolyses proline-containing peptides at the carboxy end of proline residues [1,2]. It is presumably involved in the maturation and degradation of peptide hormones and neuropeptides [1]. PO is claimed to play a role in amnesia [3], and this makes it an important therapeutic target. It was previously shown that the enzyme is not related to the trypsin and subtilisin families; rather, it belongs to a new family of serine peptidases

[4,5]. Its kinetic behaviour is also different from that of the small (25–30 kDa) extracellular serine peptidases. These latter enzymes exhibit a simple pH–rate profile, controlled by a single ionizing group of p*K*_a 7, and are only slightly, if at all, affected by the ionic strength of the medium [6]. Kinetic investigations have shown that the rate-limiting step for PO catalysis is a conformational change [6,7], rather than the chemical step characteristic of the reaction of the chymotrypsin class. Unusually for serine oligopeptidases, PO hydrolyses dipeptide substrates better than tri-, tetra-, or pentapeptides [8]. PO could be inactivated with thiol reagents [1,9,10], which led to the suggestion that the enzyme is a cysteine protease [9,10]. However, while bulky

Scheme 1 Reaction of substrates and peptide aldehydes with serine and thiol proteases

The vertical undulating line indicates the possible formation of two diastereomers by addition of the enzyme Ser-OH or Cys-SH to either face of the aldehyde carbon.

Abbreviations used: PO, prolyl oligopeptidase (EC 3.4.21.26); Z-Pro-prolinal, *N*-benzyloxycarbonyl-L-prolyl-L-prolinal; DSS, 4,4-dimethyl-4-silapentanesulphonic acid sodium salt.

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reagents specific to cysteine residues inactivated the enzyme almost completely, the smaller iodoacetamide afforded only partial inhibition [6]. These observations suggest that there is a cysteine residue near the catalytic centre, but that it may not be essential for catalysis.

Understanding the mechanism of inhibition of PO by potent inhibitors may help to reveal details of its physiological role, and may be of potential therapeutic use in treating amnesia. A potent aldehyde inhibitor of PO, *N*-benzyloxycarbonyl (Z) Pro-Pro-H ($K_i = 14$ nM), has already been described [11]. However, the mechanism of inhibition has not been elucidated. Both serine and cysteine peptidases are known to be inhibited by peptide aldehydes [12,13]. It is believed, and in some cases demonstrated, that inhibition is due to formation of a tetrahedral adduct between the aldehyde and the Ser-OH or Cys-SH at the active centre of these enzymes (see Scheme 1). Structurally, the tetrahedral intermediates so formed resemble the tetrahedral intermediates and transition states through which peptide hydrolyses proceed. We have synthesized Z-Pro-Pro-H labelled with ^{13}C at its aldehydic carbon atom and used this inhibitor to address these questions: specifically, whether there is a tetrahedral complex formed between the aldehyde and PO, and whether the inhibitor reacts with the active-site serine hydroxy group or with the more nucleophilic cysteine thiol group of PO.

EXPERIMENTAL

Preparation and assay of PO

PO was isolated from pig muscle and the activity was assayed fluorimetrically with Z-Gly-Pro-2-naphthylamide as substrate by using a Jasco FP 777 spectrofluorimeter, as described earlier [2]. The excitation and emission wavelengths were respectively 340 and 410 nm.

Purification and preparation of samples for NMR experiments

After purification, the solution of PO was made 3% (w/v) in sucrose, then freeze-dried and stored. To remove the sucrose, before the NMR experiments, the freeze-dried enzyme containing 20–30 mg of PO was dissolved at 4 °C in 1–2 ml of 20 mM potassium phosphate, pH 7.0, containing 1 mM EDTA, 0.5 mM dithiothreitol and 0.01% (w/v) NaN_3 , and chromatographed on a Sephadex G-25 (Sigma, St. Louis, MO, U.S.A.) column equilibrated with the same buffer. The eluate was concentrated to 0.3–0.7 ml in an Amicon Centricon 30 concentrator at 4 °C.

NMR experiments

These were carried out at 100 and 125 MHz for ^{13}C on a VARIAN VXR 400-89 or VARIAN Inova 500 spectrometer operated at the temperatures indicated in the Figure legends. The ^{13}C NMR experiments were run under broad-band decoupling conditions. Samples of enzyme were run in a Wilmad 513-PP 10 mm OD tube with an insert to reduce sample volume (100 MHz) or in 535-PP 5 mm tubes (125 MHz). The concentration of PO was approx. 0.2 mM in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1 or 4:1, v/v), and transients were accumulated for as long as 3 days on each sample at 5 or 10 °C. DSS (4,4-dimethyl-4-silapentane-sulphonic acid sodium salt) was used as internal chemical shift reference for the ^{13}C and ^1H chemical shifts.

Synthesis of *N*-benzyloxycarbonyl-L-prolyl-L-[1- ^{13}C]prolinol (Z-Pro-[1- ^{13}C]prolinol) from L-[1- ^{13}C]proline

The synthesis of this compound was modified compared with that reported by Wilk and Orlowski [11] so that L-[1- ^{13}C]proline

could be used as the starting material. The structure of the labelled product was confirmed by independent synthesis of unlabelled Z-Pro-prolinol according to [11].

Reduction of L-[1- ^{13}C]proline to L-[1- ^{13}C]prolinol

A solution of trimethylsilyl chloride (3.7 g, 34.3 mmol) was added under Ar to a solution of LiBH_4 (0.37 g, 17.2 mmol) in tetrahydrofuran (8.5 ml) over 2 min [14]. A precipitate of LiCl formed. L-[1- ^{13}C]Proline (1 g, 8.6 mmol; from Isotec, Miamisburg, OH, U.S.A.) was added portion-wise over 5 min. After stirring for 24 h at room temperature, 130 ml of methanol was added cautiously and volatile compounds were removed *in vacuo*. The residue was treated with 20 ml of 20% (w/v) KOH solution and extracted with dichloromethane (3 × 22 ml). The combined organic layers were dried (MgSO_4) and the solvent was removed to yield L-[1- ^{13}C]prolinol in 60–70% yield.

Z-Pro-L-[1- ^{13}C]prolinol

In a 50 ml Erlenmeyer flask was mixed Z-Pro-*N*-succinimide ester (0.69 g, 2 mmol; from Bachem Bioscience, King of Prussia, PA, U.S.A.) and 11 ml of tetrahydrofuran [11]. Next, 2 mmol of L-[1- ^{13}C]prolinol was added and the mixture was stirred at room temperature for 24 h. The tetrahydrofuran was removed by flash evaporation and the residue was dissolved in 20 ml of ethyl acetate. The solution was washed with 10 ml of 1 M Na_2CO_3 and dried (MgSO_4). The ethyl acetate was evaporated, the residue redissolved in a minimum amount of ethyl acetate, and light petroleum (b.p. 32.8–54.9 °C) added to precipitate the product. The precipitate (crude yield 83%) was filtered and recrystallized from ethyl acetate to yield 55–60% of product, m.p. 109–111 °C ([11] quotes a melting point of 94–95 °C). The product was analysed for purity by HPLC on an SSI series chromatograph using an IB-SIL 5 (4.6 mm × 150 mm) C-18 BD5 reversed-phase column equilibrated with a 2:3 (v/v) mixture of acetonitrile and water. Typically, 20 μl of a 1 mM solution of the peptide was injected and the effluent was monitored at 224 nm; the sensitivity of the monitor was set at 0.05 A units full-scale. The flow-rate was 1.0 ml/min. A single peak was detected with a retention time of 4.12 min.

Z-Pro-L-[1- ^{13}C]prolinol

To a stirred solution of oxalyl chloride (0.765 ml, 8.8 mmol) in dry dichloromethane (2.235 ml) at –63 °C (solid CO_2 /chloroform) was added a solution of dry DMSO (0.142 ml, 2 mmol) in dichloromethane (3 ml) over 10 min [15]. Immediately following the addition, a solution of Z-Pro-L-[1- ^{13}C]prolinol (313 mg, 0.95 mmol) in dichloromethane (4 ml) was added over 10 min, resulting in a cloudy solution that was stirred for 20 min. Triethylamine (0.558 ml, 4 mmol) was then added over 5 min, generating first a clear solution and then a precipitate after stirring for 20 min at –63 °C. After the cooling bath was removed, 20% KHSO_4 (4 ml) and hexanes (11.5 ml) were added, and the resulting mixture was stirred vigorously while warming, generating two phases. The layers were separated and the aqueous phase was extracted with diethyl ether (11.5 ml). The combined organic layers were washed with saturated NaHCO_3 (2 × 4 ml), water (3 × 4 ml) and saturated NaCl (2 × 4 ml), then dried (MgSO_4), filtered and concentrated at or below room temperature, yielding nearly 100% recovery. To avoid air-oxidation, the aldehyde is stored below 0 °C in chloroform under N_2 .

RESULTS AND DISCUSSION

No X-ray structure has yet been published for any of the members of the PO family. In order to probe the active centre of this new class of serine peptidases, we have studied the interaction of a specific peptide aldehyde inhibitor, Z-prolylprolinal, with PO. We expected to obtain answers to the following questions: (i) is formation of a tetrahedral complex between the inhibitor and the enzyme in evidence from the ^{13}C spectrum? and (ii) does the chemical shift reflect interaction with an oxygen nucleophile, i.e. the active centre Ser-OH, or with the highly reactive Cys-SH located near the active site of the enzyme, as indicated by inactivation experiments?

^{13}C NMR of Z-Pro-L-[1- ^{13}C]prolinal

The spectrum was recorded in both $[\text{2H}]\text{chloroform}$ (Figure 1, top) and in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (4:1, v/v). In $[\text{2H}]\text{chloroform}$ the aldehyde carbonyl carbon chemical shift is 198 p.p.m., typical of aliphatic aldehydes. There are smaller peaks to the high-frequency side of the principal aldehyde peak, that could be attributed to *cis-trans* isomers (with respect to the Xaa-Pro peptide bond) of the compound. There are two amide bonds to Pro, and peptide bonds to proline are well known to have high barriers to rotation on the NMR time scale [16]. This *cis-trans* isomerization phenomenon is even more pronounced in the spectrum of Z-Pro-L-[1- ^{13}C]prolinal in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (4:1, v/v) (Figure 1, bottom). The chemical shift of the aldehyde is 202–203 p.p.m. (referenced to the water-soluble silane standard DSS), not very different from that in chloroform. With time, a second prominent resonance became evident at 91.6–91.8 p.p.m., that we attribute to the formation of the aldehyde hydrate, also present as a mixture of *cis-trans* isomers with respect to the Xaa-Pro peptide bond. The chemical shift of the hydrate is very

useful, as it serves as an excellent model for the chemical shift of the complex expected to be formed between the serine of PO and the aldehyde carbon of the inhibitor.

Model for a cysteine adduct of Z-Pro-[1- ^{13}C]prolinal

Since the purification and storage of the enzyme used 0.5 mM dithiothreitol to protect the cysteine from oxidation, the spectrum of the adduct formed between dithiothreitol and Z-Pro-[1- ^{13}C]prolinal was also recorded. Two resonances with unequal areas and with chemical shifts at 74.8 and 75.0 p.p.m. were observed (results not shown). These could be the result either of *cis-trans* isomers along peptide bonds preceding the Pro residues, or of the two diastereomers generated at the aldehyde carbon in the hemithioacetal adducts formed with dithiothreitol. In either case, it can be concluded with confidence that, at the aldehydic carbon, a cysteine adduct of the Z-Pro-[1- ^{13}C]prolinal (i.e. the hemithioacetal carbon) will have a chemical shift of approx. 16–17 p.p.m. lower frequency than a serine adduct (i.e. the hemiacetal carbon). Inverse detection experiments (see below) indicated that the proton at the hemithioacetal carbon has a chemical shift of 6.40 p.p.m.

Studies of Z-Pro-[1- ^{13}C]prolinal in the presence of PO

The ^{13}C NMR spectrum was run in a 5 mm (at 500 MHz) or 10 mm (at 400 MHz) outer-diameter tube with approx. 15 mg of protein/ml of solution (0.2 mM enzyme) for 3 days at 5 or 10 °C, while the spectrum was monitored every 12 h. The enzyme was stable under these conditions according to both gel electrophoresis and activity measurements. As we are experimenting with an oligopeptidase, the absence of autolysis during the experiment is understandable. A single broad resonance was

Figure 1 ^{13}C NMR spectrum of Z-Pro-L-[1- ^{13}C]prolinal and of the aldehyde hydrate

Top spectrum in $[\text{2H}]\text{chloroform}$, chemical shift referenced to tetramethylsilane. Bottom spectrum in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (4:1, v/v) chemical shift referenced to internal DSS. Spectra were recorded on a Varian VXR 400-89 instrument at 100 MHz for ^{13}C under broad-band decoupling conditions. Spectral parameters for the top spectrum are: acquisition time, 1.6 s; delay, 1.0 s, number of scans, 1; linewidth, 10.0 μs (90°); number of points, 32768. Parameters for the bottom spectrum were the same as for the top spectrum, except the number of scans was 1000.

Figure 2 ^{13}C NMR spectrum of Z-Pro-L-[1- ^{13}C]prolinal in the presence of 0.2 mM PO in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1, v/v)

The spectrum was recorded under broad-band decoupling conditions on a 400 MHz instrument. The chemical shifts are referenced to internal DSS. Spectral parameters were: spectral frequency, 100.577 MHz; spectral width, 35 000 Hz; acquisition time, 1.3 s; pulse delay, 0.7 s; number of points, 32 768; pulse width, 10 μs (90°); number of transients, 99 000; probe temperature, 5 $^\circ\text{C}$. See the Experimental section for solution components. Line-broadening: bottom spectrum, 100 Hz; top spectrum, 5 Hz.

Scheme 2 Summary of aldehyde environments and chemical shifts

The numbers outside the parentheses indicate ^{13}C ; those inside the parentheses indicate ^1H chemical shifts at the aldehydic position.

observed (Figure 2, bottom; line-broadening is 100 Hz) at approx. 92.3 p.p.m. with a linewidth of about 114 Hz (after correction for the line-broadening used), which is much broader than the resonances near 75 p.p.m. attributed to the adduct with the dithiothreitol, that was present to maintain the reduced oxidation state of the cysteine. The linewidth of the resonances near 75 p.p.m. (corrected for the line-broadening used to achieve acceptable signal-to-noise ratios) is consistent with that expected for a freely tumbling small molecule. Figure 2 (top) shows the spectrum of the same sample with 5 Hz line-broadening, demonstrating that the 75 p.p.m. resonance apparent in the bottom spectrum is composed of two resonances, with chemical shifts identical with those observed for the dithiothreitol–Z-Pro-[1- ^{13}C]prolinal adduct. The results suggest that the resonance at 92.3 p.p.m. corresponds to the bound form of the inhibitor and its chemical shift is appropriate to an sp^3 hybridized carbon atom

with an oxygen atom added to the aldehyde, rather than to a sulphur atom. It is also important to point out that a ^{13}C spectrum of either the enzyme in buffer, or dithiothreitol in buffer, but in the absence of the inhibitor, showed no resonances in the 75–100 p.p.m. range.

The results of the ^{13}C NMR experiments were confirmed by an inverse detection experiment (heteronuclear multiple quantum correlation spectroscopy), in which the ^{13}C is detected via the proton attached to it (results not shown). In a sample of PO with excess of inhibitor, there were proton resonances observed at 5.18, 5.25 and 5.29 p.p.m. The former two had a linewidth of 12.8 and 12.5 Hz under the decoupling conditions used. The linewidth of the 5.29 p.p.m. resonance, on the other hand, was 93.2 Hz (or approx. 80 Hz, when corrected for the linewidth of a small, freely tumbling molecule in the same solution, such as the ones represented by the resonances at 5.18 and 5.25 p.p.m.).

Consistent with the ^{13}C chemical shifts, we believe that the 5.18 and 5.25 p.p.m. resonances pertain to the *cis-trans* isomers of the hydrated aldehyde, the one at highest frequency (5.29 p.p.m.) pertaining to the hemiacetal bound to PO. This chemical shift is very different from the 6.40 p.p.m. chemical shift observed for the dithiothreitol adduct of the inhibitor. Scheme 2 summarizes the chemical shifts used in the assignments, with the proton chemical shifts obtained from the inverse detection experiments indicated in parentheses.

Because of the possible involvement of PO in serious memory loss, potent inhibitors of the enzyme are of potential therapeutic benefit. Aldehyde inhibitors of thrombin, a trypsin-like serine protease, are currently in clinical trials. In addition to the active-site serine, PO appears to have a highly nucleophilic cysteine nearby, since the enzyme is inhibited by thiol-specific reagents, such as *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetamide. The larger reagents cause virtually complete inhibition (> 90%), whereas the smaller iodoacetamide affords only about 50% inhibition. These results rule out the notion that PO is a cysteine protease, and suggest instead that the inhibition by these reagents is due to steric effects. Nevertheless, given the fact that a thiol group is intrinsically a better nucleophile than a serine, it would not be unexpected to observe reaction of the aldehyde and ketone-based inhibitors with the cysteine, rather than with the active site serine residue.

As Figures 1 and 2 demonstrate, this question can be best resolved by ^{13}C NMR. It has been known for some time that Z-Pro-Pro-H is a good inhibitor of PO [11]. Addition of the enzyme to the labelled inhibitor gave a complex characterized by a chemical shift within 0.5 p.p.m. of the one observed for the hydrate of the inhibitor, very different both from that of the free, unhydrated aldehyde form at 202 p.p.m., and that of the tetrahedral complex formed with the dithiothreitol near 75 p.p.m. We conclude that the aldehyde forms a tetrahedral adduct with the active site serine. There are several examples in the literature of aldehydes interacting with cysteine at the active centre of cysteine proteases, such as in papain [17–19] and in hepatitis A virus 3C proteinase [20], as well as with serine in serine proteases, such as in the *N*-acetyl-L-phenylalaninal-chymotrypsin [21] and leupeptin-trypsin complexes [22]. The chemical shift assignments summarized in Scheme 2 are in excellent accord with the literature values on related systems. The much greater linewidth of the resonance corresponding to the PO-inhibitor complex compared with those of the hydrate and the model hemithioacetal can be attributed to the large molecular mass (80 kDa) of PO and of the complex. Using a simple approximation for the relationship between molecular mass and linewidth [23] in Hz (molecular mass = linewidth/ $1.8 \times 10^{-3} \text{ s}^{-1}$), the 114 Hz linewidth predicts a molecular mass of 63 kDa. Considering that the constant in the denominator is a sensitive function of the effective rotational correlation time, τ , of the particular protein, the predicted molecular mass from the observed linewidth can be considered to offer further support to the assignment of the resonance to a PO-

bound inhibitor. In addition, based on the observation of a single bound resonance, one can also conclude that the active-centre serine of PO adds to one face of the aldehyde selectively (i.e. to the *re* or *si* face), forming only a single diastereomeric adduct (within the resolution afforded by the experiment). The inverse detection experiments further confirmed the assignment of the 92.3 p.p.m. ^{13}C resonance to the enzyme-bound hemiacetal.

Our demonstration that a peptide aldehyde inhibitor binds to the active-site serine of PO can serve as a basis for designing even more potent inhibitors for this enzyme. The inherently more reactive thiol group apparently will not interfere with the reaction of the essential serine residue, and the electrophilic reactive groups of other potential inhibitors are also expected to inhibit the enzyme by reacting with the active-site hydroxy group.

We thank Ms. I. Szamosi for technical assistance and are grateful to Ciba-Geigy and the Central Chemical Research Institute of the Hungarian Academy of Sciences for financial support of F.K. during his stay at Rutgers. This work was supported in part by the US-Hungarian Joint Fund (No. JF-316) to L.P. and F.J., the OTKA grant I/6 T 12752 (to L.P.) and the Rutgers University Busch Biomedical Grant and Ciba-Geigy, Ardsley, NY, U.S.A. (to F.J.).

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