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Biochemical and Biophysical Research Communications 347 (2006) 373–379
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Synthesis, kinetic evaluation, and utilization of a biotinylated dipeptide proline diphenyl phosphonate for the disclosure of dipeptidyl peptidase IV-like serine proteases

Brendan F. Gilmore *, Louise Carson, Laura L. McShane, Derek Quinn, Wilson A. Coulter [1], Brian Walker

School of Pharmacy, Queens University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, UK

Received 19 June 2006
Available online 27 June 2006

Abstract

In this study, we report on the synthesis, kinetic characterisation, and application of a novel biotinylated and active site-directed inactivator of dipeptidyl peptidase IV (DPP-IV). Thus, the dipeptide-derived proline diphenyl phosphonate NH_2-Glu(biotinyl-PEG)-Pro-$^{P}(OPh)_2$ has been prepared by a combination of classical solution- and solid-phase methodologies and has been shown to be an irreversible inhibitor of porcine DPP-IV, exhibiting an over all second-order rate constant (k_i/K_i) for inhibition of 1.57×10^3 M^{-1} min^{-1}. This value compares favourably with previously reported rates of inactivation of DPP-IV by dipeptides containing a P_1 proline diphenyl phosphonate grouping [B. Boduszek, J. Oleksyszyn, C.M. Kam, J. Selzler, R.E. Smith, J.C. Powers, Dipeptide phophonates as inhibitors of dipeptidyl peptidase IV, J. Med. Chem. 37 (1994) 3969–3976; B.F. Gilmore, J.F. Lynas, C.J. Scott, C. McGoohan, L. Martin, B. Walker, Dipeptide proline diphenyl phosphonates are potent, irreversible inhibitors of seprase (FAPα), Biochem, Biophys. Res. Commun. 346 (2006) 436–446.], thus demonstrating that the incorporation of the side-chain modified (*N*-biotinyl-3-(2-(2-(3-aminopropyloxy)-ethoxy)-ethoxy)-propyl) glutamic acid residue at the P_2 position is compatible with inhibitor efficacy. The utilisation of this probe for the detection of both purified dipeptidyl peptidase IV and the disclosure of a dipeptidyl peptidase IV-like activity from a clinical isolate of *Porphyromonas gingivalis*, using established electrophoretic and Western blotting techniques previously developed by our group, is also demonstrated.

Keywords: Diphenyl phosphonate; Dipeptidyl peptidase IV; *Porphyromonas gingivalis*, Affinity-based probe

Numerous biologically important peptides and proteins contain highly conserved proline residues. In many cases, the presence of proline residues in peptide chains renders them resistant to proteolytic degradation, since most mammalian peptidases are unable to hydrolyse Xaa-Pro peptide bonds. As a result, post-translational modification of such peptides and proteins by limited proteolysis, or proteolytic ''trimming'', requires a class of highly specific, post-proline cleaving peptidases. Indeed, an increasing number of such peptidases have been described recently in the literature, including serine integral membrane peptidases (SIMPs) dipeptidyl peptidase IV [1,2], dipeptidyl peptidase II/VII (also known as quiescent cell proline aminodipeptidase) [3,4], seprase (surface expressed protease) [5–7], and DPP8 [8].

Dipeptidyl peptidase IV (DPP-IV) is a serine protease whose catalytic residues are arranged in a ''non-classical orientation'' (Ser-Asp-His). It is a member of the type II transmembrane glycoprotein, prolyl oligopeptidase family and is expressed at the cell surface of epithelial and endothelial cells as well as activated lymphocytes, as a 220-kDa

* Corresponding author. Fax: +44 28 90 247 794.
E-mail address: b.gilmore@qub.ac.uk (B.F. Gilmore).
[1] Present address: School of Medicine and Dentistry, Queens University Belfast, Grosvenor Road, Belfast BT12 6BP, UK.

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doi:10.1016/j.bbrc.2006.06.113

homodimer of two identical, non-covalently linked 110-kDa subunits [9].

Dipeptidyl peptidase IV possesses a well-characterised, relatively restricted exopeptidase activity, cleaving Xaa-Pro↓ (and to a lesser extent Xaa-Ala↓) dipeptides (arrow indicates scissile bond) from the N-terminal of peptides and proteins [1,2], including many bioactive and regulatory peptides. Whilst proline and alanine are almost exclusively present at the P_1 position of substrates, peptides containing hydroxyproline, dehydroxyproline, serine, glycine, and valine at this position are also hydrolysed by DPP-IV, but at greatly reduced rates [10]. In the P_2 position any amino acid residue is accepted, provided it has a protonated amino group. Peptides that contain proline or hydroxyproline in the P'_1 position are resistant to cleavage by DPP-IV [11].

In addition to being expressed by eukaryotic cells, DPP-IV-like activity has also been detected in prokaryotes such as bacterium including *Chryseobacterium meningosepticum* [12,13] and *Porphyromonas gingivalis* [14–16]. Indeed, there is a high degree of homology between eukaryotic and prokaryotic DPP-IV [17]. The significance of proteases as bacterial virulence factors may be illustrated by those microorganisms associated with periodontal disease. Periodontopathogens have long been known to express a battery of protease activities resulting predominantly in inflammation and destruction of periodontal tissue [18,19]. *Porphyromonas gingivalis* (*P. gingivalis*), a gram-negative anaerobe, is recognized as a major pathogen in adult periodontitis, and is consistently associated with clinical manifestations of the disease (reviewed in Refs. [20,21]). *P. gingivalis* is known to secrete a number of proteases, including dipeptidyl peptidase IV, and whilst the varied roles of eukaryotic DPP-IV have been exhaustively reported [22–25], very little is known about the exact role(s) of bacterial DPP-IV in the development of periodontitis. However, since rat DPP-IV, apart from acting as an exopeptidase, also displays endopeptidase activity against denatured fibrillar collagens [26], and given the high degree of homology between DPP-IV species across all genera, it is tempting to speculate that *P. gingivalis* DPP-IV might play a direct role in periodontal tissue destruction; a hallmark of adult periodontitis.

One approach to delineating the roles of DPP-IV in normal and pathological processes (including periodontitis) would be through the use of active site-directed inhibitors that exhibit selectivity of action against this peptidase and closely related homologues. A number of templates have been utilized for the generation of inhibitors against DPP-IV including peptides containing a P_1 proline diphenyl phosphonate grouping [27] or amino acid-derived nitriles [28,29], and more recently, we have reported on the inactivation of the closely related DPP-IV homologue seprase, by peptides containing a P_1 proline diphenyl phosphonate grouping [30]. Protease inhibitors based upon peptides containing α-aminoalkyl diphenyl phosphonate esters in the P1 position were originally introduced by Powers' group [31,32], they exhibit exquisite selectivity for the serine proteases, bringing about phosphonylation of the active site serine residue and their concomitant irreversible inactivation. Our group introduced the concept and methodologies for profiling serine protease species by producing biotinylated derivatives of α-aminoalkyl diphenyl phosphonates for the detection of elastase-like proteases and trypsin-like proteases produced by human breast tumour cells [33a,b] and trematode parasites [34], respectively. Powers and coworkers have also developed fluorescent and biotinylated derivatives directed against other proteases [35,36].

We now wish to report on the synthesis, kinetic characterisation, and application of the biotinylated diphenyl phosphonate derivative NH_2-Glu(biotinyl-PEG)- $Pro^P(OPh)_2$ as a novel active site-directed inactivator of dipeptidyl peptidase IV and demonstrate how it can be employed for the disclosure of active dipeptidyl peptidase-IV-like serine proteases produced by *P. gingivalis*.

Experimental

Materials

Dipeptidyl peptidase IV (from porcine kidney) was supplied by the Sigma Life Sciences (Poole, England), H-Gly-Pro-(7-amido-4-methylcoumarin)AMC was supplied by Bachem (UK) Ltd. (Meyerside, UK). Fmoc-Glu(biotinyl-PEG)-OH and piperidine-4-carboxylic acid polyamine resin were purchased from Novabiochem, Nottingham, UK. Dimethylformamide (DMF) was supplied by BDH Chemicals Ltd. (Poole, England). Sodium peroxidosulphate, pyrrolidine, and isopropenyl chloroformate were supplied by Fluka Chemika Ltd. (Dorset, England). Triphenyl phosphite was supplied by the Aldrich Chemical Co. (Gillingham, Dorset, England). Isobutyl chloroformate was supplied by the Sigma Chemical Co. (Poole, England). Magnesium sulphate was supplied by Lancaster Synthesis UK (Morecambe, Lancashire, England). Silver nitrate was supplied by Prolabo (Rue Pelée, Paris, France). Diisopropylethylamine (DIPEA) was supplied by PerSeptive Biosystems Ltd. (Warrington, England). All chemicals and solvents were of the highest purity and were subjected to no further purifications prior to use. Horseradish peroxidase streptavidin was obtained from Vector Laboratories Ltd., Burlingame, CA, USA. SuperSignal West Pico Chemiluminescent Substrate was supplied by Pierce Biotechnology Inc., Rockford, IL, USA. Anaerobe Basal Broth and Anaerobic Gas Generating Kit for maintenance of bacterial strains were obtained from Oxoid Ltd., Hampshire, UK.

Synthesis of probe

The probe was synthesized primarily by classical solution-phase methodologies; The dipeptide proline diphenyl phosphonate was synthesized by an adaptation of the method described by Boduszek et al. [27]. Essentially, Fmoc-Glu(biotinyl-PEG)-OH (0.39 mmol, 312 mg) was dissolved in DMF (5 ml), to which were added HATU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uranium hexafluorophosphate) (0.39 mmol, 149 mg) and diisopropylethylamine (1.56 mmol, 314 μl). The mixture is stirred at ambient temperature for 20 min before addition of one molar equivalent of diphenyl pyrrolidine-2-phosphonate (proline diphenylphosphonate) (119 mg, 0.39 mmol). The reaction mixture was stirred gently at ambient temperature, overnight. Excess solvent was subsequently removed under reduced pressure. Following standard work-up the crude product was retrieved as a viscous oil, and the identity of the N-terminal protected Fmoc-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ confirmed by electrospray mass spectroscopy $\{(M+H)^+ = 1083.3\}$.

The crude product was then dissolved in dichloromethane (15–20 ml) and a fourfold excess of the polymer-bound base, piperidine-4-carboxylic

acid polyamine resin (1.56 mmol), is added to cleave the Fmoc-protecting groups from the product [37]. The reaction was allowed to proceed for 24 h, at ambient temperature, with gentle agitation. After this period, the reaction mixture was filtered through a sintered glass frit, rinsed with dichloromethane (5 ml) and methanol (5 ml). The filtrate was collected and excess solvent removed by rotary evaporation, under reduced pressure. Since the majority of the dibenzofulvene produced by the Fmoc deprotection of the product is not scavenged and remains in solution, the product from the previous step is taken up into approximately 50 ml dichloromethane, washed with an 10% aqueous solution of HCl (3× 25 ml). The aqueous layer is basified with potassium hydroxide, liberating the product as the free base, which is taken up into the organic layer during subsequent washing of the aqueous layer with dichloromethane (3× 25 ml) and ethyl acetate (2× 25 ml). The organic fractions are pooled, dried over anhydrous $MgSO_4$, filtered and excess solvent removed under reduced pressure. The product is recovered as a faintly coloured viscous oil in moderate yield (63%), homogeneous by TLC (chloroform/methanol 20:80) ($R_f = 0.53$), the structure of which is detailed in Fig. 1. Identity of the product was confirmed by electrospray mass spectroscopy $\{(M+H)^+ = 861.3\}$.

Kinetic techniques

Inactivation studies: (a) continuous assay. A 10 mM stock solution of H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ was prepared in DMF and stored at −20 °C until required for kinetic evaluation. Ten millimolar of stock solutions of each inhibitor were diluted in DPP-IV assay buffer (25 mM Tris, 140 mM NaCl, and 10 mM KCl, pH 7.9) to yield final working concentrations (in each well) of 10, 25, 50, and 100 μM. These were then evaluated for inhibitory activity against DPP-IV in the microtitre plate assay. A working solution of DPP-IV was prepared in DPP-IV assay buffer and 5 μl (containing ~20 ng DPP-IV) of this working stock enzyme solution was added to each well to initiate the reaction. A stock solution of commercial DPP-IV substrate, H-Gly-Pro-NHMec (10 mM) in DMF, was prepared and stored, protected from light, at −20 °C. From this stock solution, a 200 μM working solution was prepared such that when added to each well (25 μl) a final concentration of 50 μM was achieved. The final volume of reaction mixture in each well was 100 μl.

The inhibitory potency of each inhibitor was determined at four individual concentrations using a standard microtitre plate assay where the rate of substrate hydrolysis at 37 °C, in each instance, was monitored every 60 s over a period of 90 min by measuring the rate of increase of fluorescence at (excitation 395 ± 25 nm, emission 460 ± 40 nm) in a CYTOFLUOR® Multi-well plate reader Series 4000 spectrofluorimeter.

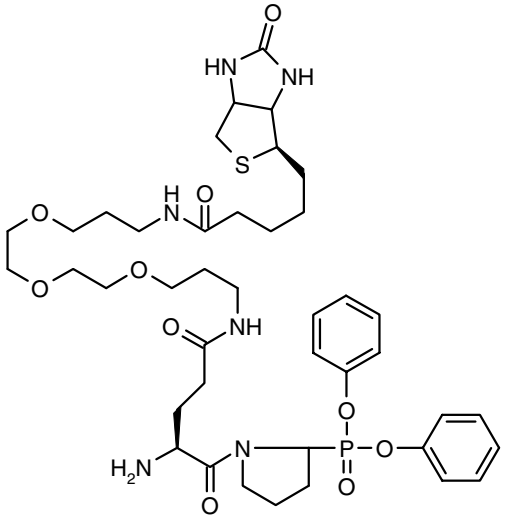

Fig. 1. H_2N-Glu(biotinyl-PEG)- $Pro^P(OPh)_2$.

Strains

All *P. gingivalis* strains (ATCC 33277, W83, and BH 18/10) were supplied by Dr. Wilson Coulter from the culture collection of Dr. Charles Shelburne, School of Dentistry, University of Michigan. All strains were grown in basal anaerobe broth (Oxoid) under anaerobic conditions in a anaerobic culture jar employing a gas generating kit such that final carbon dioxide concentrations are in the range 7–10% v/v and the volume of hydrogen produced (~1800 ml) ensures complete anaerobiosis.

Evaluation of DPP-IV-like activity from P. gingivalis crude sonicates

After 48 h, the broth cultures (150 ml) were removed from the anaerobic jar and centrifuged (4 °C, 10,000*g* for 10 min) to pellet *P. gingivalis* cells. The supernatant was removed and discarded. The cell pellet was subjected to several cycles of freeze–thaw at −20 °C and 4 °C, respectively. Immediately, 1 ml of DPP-IV assay buffer was added to the cell pellet, gently mixed, and sonicated on ice (three times, 2 min, using 100 W, vibracell sonicator (Jencons, Bedfordshire, UK)) before centrifugation at 10,000*g* for 10 min. This procedure was repeated up to six times. Following retrieval of the supernatant from each strain, the protein content of each was determined using a standard BCA assay (Pierce Biotechnology, Rockford, IL), according to manufacturer's protocol. Following total protein determination all crude sonicates were adjusted with DPP-IV assay buffer to a final total protein concentration of 500 μg/ml. A sample of each sonicate was immediately added, in triplicate, to the wells of a black microtitre plate containing 40 μl DPP-IV assay buffer and 50 μl H-Gly-Pro-NHMec in assay buffer (final substrate concentration, 50 μM). The rate of substrate hydrolysis at 37 °C, in each instance, was monitored every 60 s over a period of 30 min exactly as previously described.

Characterisation of labelled peptidases

Labelling of peptidases. To a solution (100 μl) of purified DPP-IV (~500–600 ng) in DPP-IV assay buffer, pH 7.9, was added a solution (100 μl) of H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ (200 μM) in assay buffer containing 1% DMF to give a final concentration of inactivator of 100 μM. The solution was then incubated at 37 °C for 1 h, with gentle agitation. The general procedure is detailed in [33a].

In the case of crude sonicates of *P. gingivalis*, strain W83 was selected for further analysis, since of all strains evaluated for DPP-IV-Like activity with the fluorogenic substrate H-Gly-Pro-NHMec (as described above), W83 consistently exhibited the highest degree of dipeptidylpeptidase-IV-like activity (data not shown). To a sample (99 μl) of the crude sonicate of *P. gingivalis* W83 (total protein ~50 μg), prepared as detailed above, was added a solution (1 μl) of H_2N-Glu(biotinyl-PEG)- $Pro^P(OPh)_2$ (5 mM) in DMF, yielding a final inhibitor concentration of 50 μM.

The reaction between the peptidase and inhibitor was terminated in each case by the addition of an equal volume of Laemmli treatment buffer followed immediately by boiling for 10 min at 100 °C.

Electrophoresis. Electrophoresis in the presence of SDS was performed by a modification of the method of Laemmli [38]. SDS–PAGE was performed on NuPage® Novex 4–12% Bis-Tris gels using an X-Cell Surelock™ mini-gel electrophoresis unit connected to an external PowerEase® DC power supply unit (Invitrogen Ltd.). Labelled proteins were denatured by boiling for 10 min in Laemmli treatment buffer. SeeBlue® Plus2 prestained molecular weight standards were included in each gel.

Western blotting. Electro-blotting of the separated proteins was carried out using a modification of the methodology described first by Towbin et al. [39], using the Trans-Blot® SD electrophoretic transfer cell (Bio-Rad Laboratories). Separated proteins were electro-blotted onto Immobilon™-P polyvinylidene difluoride (PVDF) membranes (Millipore) in NuPage Transfer Buffer (Invitrogen Ltd.). The running time was 2 h, at 200 mA, at room temperature.

Detection of labelled peptidases. After protein transfer, all unbound sites on the Immobilon™-P PVDF membrane were blocked by a non-specific, non-reactive protein by incubation of the blot membrane in a 3% (w/v) solution of bovine serum albumin (BSA) in Tris-buffered saline

(TBS; 0.05 M Tris/HCl, 0.1 M NaCl, pH 7.4) for at least 1 h at room temperature with gentle agitation. The membrane was then gently washed with TBS containing 0.1% Tween 20 (5× 10 ml portions). After washing, the solution was discarded and replaced with streptavidin/horseradish peroxidase (streptavidin-HRP) (Vector Laboratories) prepared at a dilution of 1 in 10,000 in blocking solution containing 0.3% BSA. The PVDF membrane and streptavidin-HRP were incubated together, with gentle agitation, at room temperature for 45 min. The membrane was washed at least six times with TBS containing 0.1% Tween 20 and excess liquid removed. Horseradish peroxidase activity was detected by overlay with SuperSignal® West Pico chemiluminescent substrate (2 ml) for five minutes. After removal of the substrate, the blot is placed between acetate sheets and overlaid with photograph film in a film cassette. Films were exposed for ~30 s, developed, and fixed.

Results and discussion

Inactivation studies

Fig. 2 shows the typical time course for the formation of product (7-amino-4-methylcoumarin) from the substrate H-Gly-Pro-AMC by the action of porcine DPP-IV in the presence of increasing concentrations of H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$. Unsurprisingly, these curves are indicative of the action of an active site-directed irreversible inhibitor. Linear transformation of the data in Fig. 2 was performed using the GraFit™ data analysis software package (Erithacus Software Ltd.) to yield apparent second order rate constants, A, for each inhibitor concentration employed in the assay. Fig. 3 shows the graph of the reciprocal of apparent second order rate constant ($1/A$) vs. inhibitor concentration [I]. We observed a linear relationship between $1/A$ and [I], indicative of an irreversible inhibitor acting through a complexing mechanism, where $1/A$ is dependent on [I] (Tian and Tsou, Walker and Elmore) Fig. 4.

The determined values for second order rate constant, [A], for each inhibitor concentration are used to determine

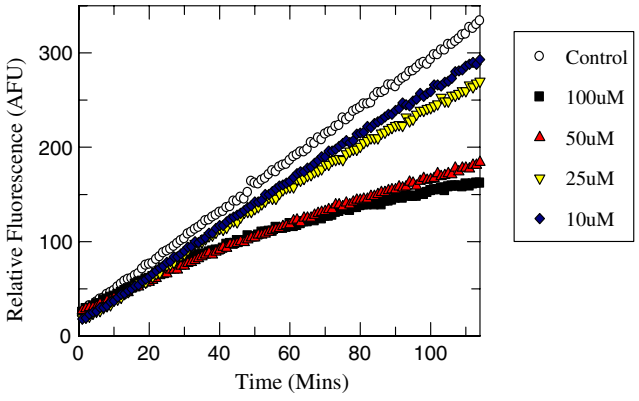

Fig. 2. Progress curves for the generation of 7-amino-4-methylcoumarin from the dipeptidyl peptidase IV catalysed hydrolysis of H-Gly-Pro-NHMec in the presence of H_2N-Glu(biotinyl-PEG)- $Pro^P(OPh)_2$. The figures represent the best fit of data to the equation for irreversible inhibition (Walker and Elmore, 1984) of DPPIV (porcine) by H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ in the presence of H-Gly-Pro-NHMec. The inactivation studies were carried out at a fixed substrate concentration (50 μM) and varying concentrations of inactivator as indicated above.

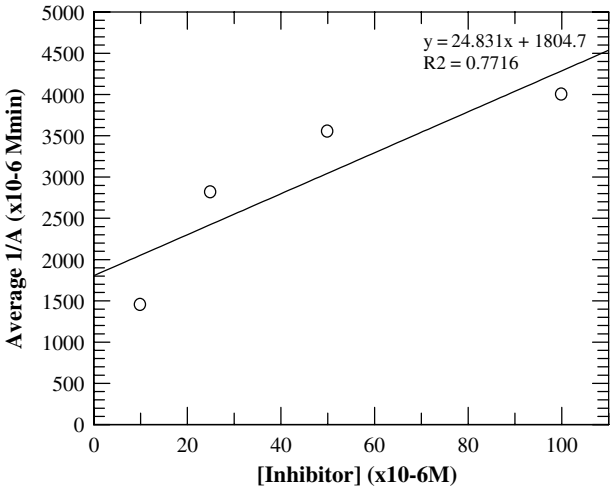

Fig. 3. Graph of $1/A$ vs. inhibitor concentration for H_2N-Glu(biotinyl-PEG)- $Pro^P(OPh)_2$ indicative of an irreversible inhibitor acting through a complexing mechanism, where the reciprocal of second order rate constant A is dependent on [I].

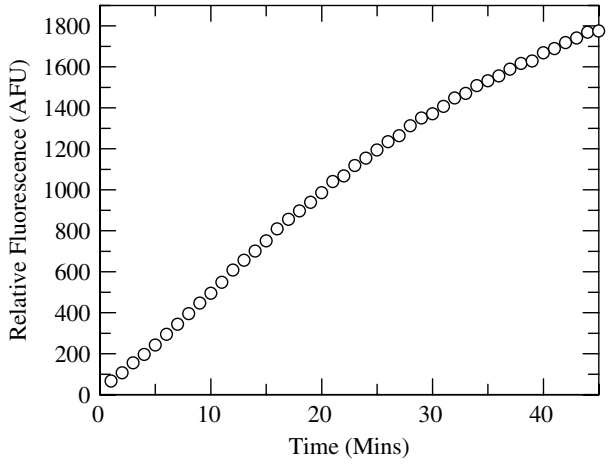

Fig. 4. Typical progress curves for the formation of product (7-amino-4-methylcoumarin) from the substrate H-Gly-Pro-AMC by the DPPIV-like action of crude sonicates of *P. gingivalis* W83 cultures (average of three measurements).

the kinetic constants, K_i and k_i for the kinetic scheme I, taken from Walker and Elmore [40]. Evaluation of the inhibitor constant K_i and the apparent first order rate constant, k_i, for the irreversible inactivation was carried out using the methods of Tian and Tsou [41]. The calculated kinetic parameters for the inactivation of porcine DPP-IV by H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ are listed in Table 1. Since the kinetic parameters are evaluated in the presence of competing substrate, the true second order rate constant k_i/K_i would be increased by a factor of $(1 + [S]/K_m)$ yielding a value of $1.57 \times 10^3\ M^{-1}\ min^{-1}$.

It is particularly satisfying to observe from the data presented here, that the presence of the side-chain modification of the glutamic acid residue at the P_2 position (*N*-biotinyl-3-(2-(2-(3-aminopropyloxy)-ethoxy)-ethoxy)-propyl) is compatible with inhibitor efficacy, giving rise to

Table 1
Calculated kinetic parameters for the inactivation of porcine DPPIV by the active site-directed probe H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$

Kinetic parameter	Calculated value
A	$0.691 \times 10^3\ M^{-1}\ min^{-1}$
A_{true}	$1.2 \times 10^3\ M^{-1}\ min^{-1}$
k_i	$40.3 \times 10^{-3}\ min^{-1}$
K_i	$43.16 \times 10^{-6}\ M$
k_i/K_i	$0.934 \times 10^3\ M^{-1}\ min^{-1}$

an inhibitor with moderate potency comparable to similar reported inhibitors of DPP-IV [27,30]. In keeping with earlier observations, the Fmoc-protected reagent exhibited no inhibitory activity towards DPP-IV (data not shown).

Evaluation of DPP-IV-like activity in crude sonicates from P. gingivalis W83

As discussed in Experimental, of all strains of *P. gingivalis* evaluated for DPP-IV-like activity in crude culture sonicates, strain W83 consistently exhibited the highest levels of DPP-IV-like activity, and was thus selected for labelling studies. Numerous strains of *P. gingivalis* are classified as either avirulent/non-invasive (such as ATCC33277) or virulent/invasive (i.e. W83) [42]. Therefore, it seems reasonable that DPP-IV activity in crude sonicates might prove one possible indicator of virulence/invasiveness and therefore reagents capable of selective labelling of such activity may well have utility as diagnostic markers. Typical progress curves for the formation of product (7-amino-4-methylcoumarin) from the substrate H-Gly-Pro-AMC by the DPP-IV-like action of crude sonicates are shown in Fig. 5.

Labelling studies

Fig. 5a and b shows the results of the labelling studies of both purified DPP-IV from porcine kidney and from the probing of crude sonicates of *P. gingivalis* W83 cultures with the affinity probe, H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$.

Fig. 5a and b shows the same blot, at film exposure times of 1 and 5 min, respectively. These figures show that incubation of purified DPP-IV with the biotinylated probe results in the labelling of one main species of molecular mass ~110 kDa in each instance. Lanes 1 and 2 represent the no probe control, lanes 3 and 4 DPP-IV (1 μg) preincubated with probe (100 μM), lanes 5 and 6 DPP-IV (100 ng) preincubated with probe (100 μM). The determined molecular mass is exactly in keeping with the reported value for DPP-IV. Unfortunately, it was not possible to detect DPP-IV at quantities of 10 ng or below using this probe (data not shown). The limits of detection of this probe are however comparable with the observed results of earlier experiments utilizing diphenylphosphonate-based probes for the disclosure of other serine protease, such as trypsin [43]. In Fig. 5a and b, lanes 7 and 8, DPP-IV (1 μg) is pretreated

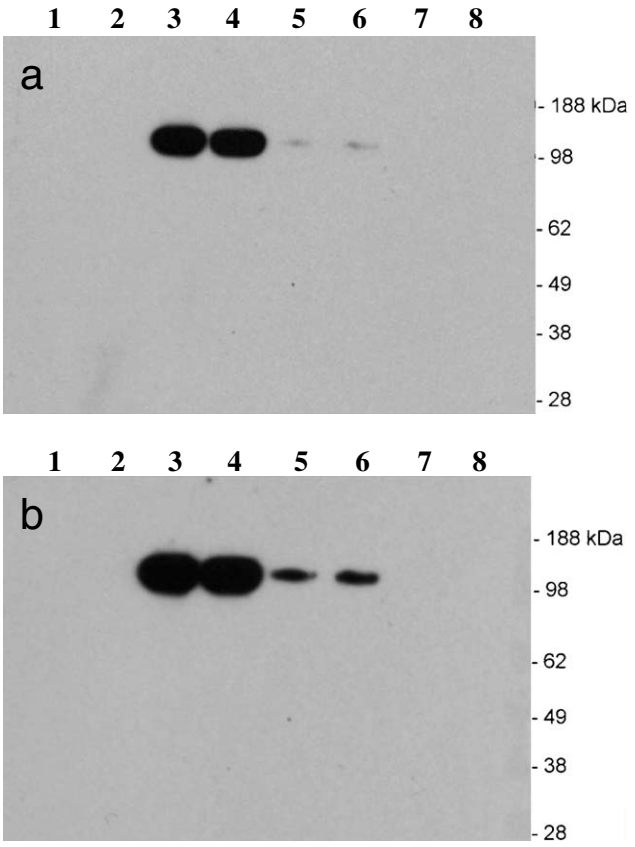

Fig. 5. (a,b) The same blot, at film exposure times of 1 and 5 min, respectively. Lanes 1 and 2 represent the no probe control (1 μg DPPIV), lanes 3 and 4 DPPIV (1 μg) preincubated with the affinity probe, H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$ (100 μM), lanes 5 and 6 DPPIV (100 ng) preincubated with affinity probe (100 μM). Lanes 7 and 8, DPP-IV (1 μg) pretreated with Pefabloc (100 μM) for 15 min at 37 °C prior to incubation with the probe (100 μM).

with Pefabloc (100 μM) for 15 min at 37 °C prior to incubation with the probe (100 μM). As can be clearly seen from the figures, labelling of DPP-IV is completely blocked by preincubation with Pefabloc, an active-site-directed inhibitor of the serine proteases, previously shown to be an inhibitor of DPP-IV [5]. This competitive blocking experiment indicates that the labelling is, as expected, active-site directed.

Fig. 6 shows the results of probing experiments on crude sonicates of *P. gingivalis* W83 cultures. As can be seen from this figure incubation of a sample of crude sonicate of *P. gingivalis* W83 48 h culture disclosed a single band in the Western blot at ~80–85 kDa (lanes 3 and 4), which was not present in the no probe control. Lanes 5 and 6 represent a 10-fold dilution of the sonicate prior to probing with the affinity probe, here we see no corresponding band, indicating that the species responsible for the single band at ~80–85 kDa was not present in sufficient quantity to detect in the diluted sample. The molecular mass observed for species and the indication, since effectively labelled by H_2N-Glu(biotinyl-PEG)-$Pro^P(OPh)_2$, that the species is a proline-specific serine protease suggest strongly that the

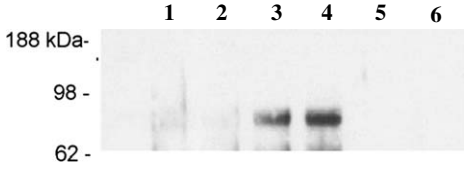

Fig. 6. Results of probing experiments on crude sonicates of *P. gingivalis* W83 cultures with the affinity probe, H_2N-Glu(biotinyl-PEG)-Pro-$^{P}(OPh)_2$. Lanes 1 and 2; no probe control, sonicate only. Lanes 3 and 4; 20 μl crude sonicate of *P. gingivalis* W83 cultures with affinity probe, H_2N-Glu(biotinyl-PEG)-Pro$^{P}(OPh)_2$ (50 μM). Lanes 5 and 6; 20 μl sonicate (1 in 10 dilution) of *P. gingivalis* W83 cultures with affinity probe, H_2N-Glu(biotinyl-PEG)-Pro$^{P}(OPh)_2$ (50 μM).

observed species may be *P. gingivalis* DPP-IV, a protein of 723 amino acids, a high degree of homology with both mouse and human DPP-IV (~30%) and with a calculated molecular mass of 81.8 kDa [15].

In this study, we have concerned ourselves primarily with demonstrating the active-site directed labelling of purified DPP-IV. This is, to the best of our knowledge, the first demonstration that DPP-IV, indeed any proline-specific serine protease, may be labelled in this manner. Given the intense interest recently in the role of proline-specific serine proteases in diseases such as diabetes and rheumatoid arthritis, we envisage that this type of activity-based profiling will have utility in a number of scenarios both clinically and in the laboratory. In a recent publication, Edosada et al. [44] demonstrated that improved selectivity for the homologous, membrane-bound peptidase seprase could be brought about by *N*-acyl-Gly-Pro templates. Taken together these data may be useful in designing specific inhibitors for individual proline-specific serine peptidases to identify them in complex biological milieu and assist in our understanding of the multifarious roles of these peptidases in disease processes.

Acknowledgments

Many thanks to Catherine Fulton for maintaining *P. gingivalis* strains. Louise Carson's work was supported by a Wellcome Trust Vacation Studentship Grant (Ref: VS/05/QUEB/A2).

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