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# Peptidylglycine monooxygenase activity of monomeric species of growth hormone

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ABSTRACT

C-terminal α-amidation of peptides is an important event in the course of pro-hormone and neuropeptide processing; it is a modification that contributes to the biological activity and stability of about 25 peptides in neural and endocrine systems. This laboratory has shown that bovine growth hormone (bGH) also has a catalytic function, i.e. peptidylglycine monooxygenase activity, which is the first step in the alpha-amidation of glycine-extended peptides. We report here that the peptidylglycine monooxygenase activity of monomeric bovine pituitary GH, in the presence of ascorbate, is stimulated by combination with oligomeric forms of bGH one of which is a hetero-oligomer with metallothionein. Three species of recombinant monomeric GH (bovine, human and chicken) also catalyze this monooxygenase reaction. Tetrahydrobiopterin also functions as a reductant - with a significantly greater turnover than achieved with ascorbate. These findings clarify the role of GH in peptidylglycine monooxygenation and provide an explanation for earlier observations that peptide amidation is not totally obliterated in the absence of ascorbate, in cultured pituitary cells or *in vivo*. The evolution of bifunctional GH is also discussed, as are some of the significances of the peptidylglycine monooxygenase activity of human GH in relation to peptides such as oxytocin, glucagon-like peptide-1 and peptide PYY.

## 1. Introduction

Approximately 50% of bioactive peptides in neural and endocrine systems possess a C-terminal amide, which is important for their bioactivity and biological stability (Bradbury and Smyth, 1991). The immediate precursors of amidated peptides are glycine-extended, with the glycine residue being the signal for amidation. Peptide α-amidation occurs widely in vertebrates and invertebrates but is not known to be a post-translational modification characteristic of yeast and prokaryotes. Physiologically significant amidated peptides include oxytocin, gastrin, glucagon-like peptide-1 (GLP-1), peptide YY ($PYY_{3\text{-}36}$), and growth hormone-releasing hormone (GHRH). Peptide α-amidation is catalysed by two enzymes, acting sequentially; (a) peptidylglycine monooxygenase (EC 1.14.17.3), generating peptidyl-α-hydroxylglycine, and (b) peptidyl−α−hydroxyglycine α-amidating lyase (PAL: EC 4.3.2.5), which releases α-amidated peptide product and glyoxylate (Tajima et al., 1990; Katopodis et al., 1990). Peptidylglycine monooxygenase is known to require ascorbate and copper (Eipper et al., 1983). Significant nonenzymatic dismutation of the α-hydroxyglycine intermediate to amidated peptide has also been reported (Young and Tamburini, 1989; Takahashi et al., 2009).

It is accepted that ascorbate can act as a reductant for peptidylglycine monooxygenase. However, there are several pieces of evidence that raised questions about the nature of the cofactor(s) that participate in this reaction *in vivo*. For instance, production of amidated peptides in cultured pituitary cells continued in the absence of ascorbate (May and Eipper, 1985); although, the extent of amidation was substantially increased upon inclusion of ascorbate in the medium. In vitamin C-deficient guinea pigs, amidation of gastrin was impaired but not abolished (Hilsted et al., 1986). Since ascorbate is known to maintain tetrahydrobiopterin ($BH_4$) in the reduced state (Huang et al., 2000; Heller et al., 2001) this study has also investigated whether $BH_4$ can support peptidylglycine monooxygenase activity. $BH_4$ (Kaufman, 1958) is also an essential cofactor for the aromatic amino acid hydoxylases, alkylglycerol monooxygenase, and nitric oxide synthases (Werner et al., 2011). Our earlier studies had identified bovine growth hormone (pituitary somatotropin; bGH) as having a previously unrecognised enzymatic function, i.e., peptidylglycine monooxygenase (Downey and Donlon, 1997).

GH is an extensively studied pituitary peptide hormone – with recent studies focussing on its roles in human metabolism (Vijayakumar et al.,

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2011). In humans, there are two genes, hGH-N and hGH-V, with respective proteins hGH and hGH-V (placental variant). GH is considered to be the ancestral hormone in the molecular evolution of the GH/Prolactin/Somatolactin family (Moriyama et al., 2006), at least 500 million years ago (Mya). The closely homologous primate placental lactogen (PL) is considered to have evolved from GH by gene duplication (Miller and Eberhardt, 1983). The principal monomeric form of growth hormone is a 22 kDa peptide of 191 amino acid residues. In human pituitary and circulation, oligomeric forms have also been observed; up to and including pentamers (Stolar et al., 1984; Baumann, 2009), these have diminished hormonal activity compared to monomeric hGH. Other than to acknowledge their presence (Lorenson, 1985), oligomeric forms of bGH have not been studied. Previously (Downey and Donlon, 1997), chromatographic steps and electrophoretic analyses were conducted solely under reducing conditions. Here we show that when procedures were performed under non-reducing conditions it became apparent that multiple forms of protein (bGH) are required for maximal peptidylglycine monooxygenase activity. It was also of interest to carefully prepare recombinant species of GH and test their ability to catalyse peptidylglycine monooxygenation, as well as to examine whether $BH_4$ could also function as a cofactor. The course of the evolution of the monooxygenase function of GH is also discussed, as are some aspects of the physiological significance of this function of GH.

## 2. Materials and methods

### 2.1. Materials

The following supplied the respective material requirements listed: New England BioLabs, USA, protein markers; Cambio, Cambridge, UK, PCMV-Sport plasmid with hGH; Sigma Aldrich, UK, phenyl-Sepharose, imidodiacetic-Sepharose, enterokinase, protein markers, monoclonal anti-hGH antibody secondary peroxidase-conjugated antibody and general analytical grade reagents; Fluka, Dansyl-D-Tyr-Val-Gly; monoclonal antibody to bGH and a monoclonal to cross-linked rabbit liver MTI/MT-II were obtained from Stratech, Newmarket, U.K., and tetrahydrobiopterin from Schircks Laboratories, Jona, Switzerland.

### 2.2. Chromatographic procedures

Bovine pituitaries were collected at a local abattoir, within 1 h of slaughter of animals, and homogenized in 10 volumes of cold Krebs original Ringer phosphate, pH 7.4, containing 0.5 mM benzamidine. Following homogenization the initial step in fractionations was precipitation with 45% saturation ammonium sulphate. For gel filtration over a column (2.8 × 45 cm) of Sephacryl S-100 HR, the solubilization, equilibration and elution buffer was 50 mM Tris/HCl, pH 7.0, containing 0.1 M NaCl and 0.5 mM benzamidine; a 2 mL aliquot was applied and 5 mL fractions were collected. For hydrophobic interaction chromatography, on a 10 mL column (0.9 × 16 cm) of phenyl-Sepharose, the solubilization and equilibration buffer was 50 mM Hepes, pH 7.0, also containing 0.5 mM benzamidine and 5% saturation ammonium sulphate. In order to improve the resolution both early and late in the fractionation, the elution protocol (Downey and Donlon, 1997) was modified by increasing the volume of equilibration buffer used for the initial wash of the column.

For affinity chromatography on Cibracon Blue, selected pooled fractions from phenyl-Sepharose were applied to a column of Affi-Gel blue gel (Blue Sepharose CL-6B) that had been equilibrated with 50 mM Hepes, pH 7.0 containing 0.5 mM benzamidine. The column was washed with equilibration buffer, 50 mM NaCl in same buffer and, finally buffered 0.5 M NaCl. Fractions of 1.5 mL each were collected. Where metal-chelating chromatography was employed, imidodiacetic-Sepharose was charged with $ZnCl_2$ in a buffer containing 25 mM TES, pH 7.3, 0.5 M NaCl and 0.5 mM benzamidine. The column was equilibrated using same buffer. The salt concentration in samples from Affi-Gel step was adjusted, where necessary, to 0.5 M NaCl. After washing with equilibration buffer, the activity of interest was eluted from the column by the sequential addition of 10 mM and 20 mM imidazole, respectively, in equilibration buffer; 1.5 mL fractions of eluate were collected. Ideally. All fractionations were conducted at 4 °C. The generation of preparations and the analyses generally took at least 6 weeks from date of tissue isolation.

### 2.3. Assay, electrophoresis and western blotting

The assay for peptidylglycine monooxygenase used in this study was as described (Downey and Donlon, 1997), at pH 7.75, but with 20 μM Dansyl-D-Tyr-L-Val-Gly as substrate, and in a final volume of 250 μL. Ascorbate was at a final concentration of 40 μM and $BH_4$ was 20 μM, in the presence of 2.5 mM β-mercaptoethanol to maintain $BH_4$ in the reduced state. Student's t test was used to evaluate all data statistically. In order of their elution, the hydroxyglycine intermediate product, the substrate and the amidated peptide, respectively, were resolved by reversed-phase chromatography (C18) using 50% acetonitrile in 50 mM ammonium acetate buffer, pH 6.5, at a flow rate of 0.5 mL/min. Fluorescence was detected at excitation wavelength of 336 nm and emission wavelength of 510 nm (Consalvo et al., 1992). Using this assay it is possible to determine both Dansyl-Tyr-Val-hydroxyglycine and Dansyl--Tyr-Val-$NH_2$ with an order of magnitude greater sensitivity than former procedure. The identities of the reaction products were also confirmed by mass spectrometry using an Agilent 6510 QTOF HPLC Mass Spectrophotometer with electrospray ionisation. Blank reactions having heat denatured GH samples were <20% of experimental values. Protein concentrations were determined using dye (Coomassie Blue) binding assays, with bovine serum albumin as standard. Absorption spectra were recorded using a Shimadzu UV160U UV-VIS Spectrophotometer.

SDS PAGE and western blotting experiments were conducted as before (Downey and Donlon, 1997), with the exception that, unless stated, β-mercaptoethanol was not present in samples or buffers. Samples were subjected to native gel electrophoresis (10% acrylamide) with 4% stacking gel and with pH 8.3 running buffer. The separated proteins were then subjected to wet transfer onto nitrocellulose membrane. The membrane was blocked by 5% casein in TBS, pH 7.5, overnight with constant rocking in cold room; incubated with appropriate dilution of appropriate primary antibody for 3 h; and washed 5 times in TBS buffer containing 0.1% Tween-20. The target proteins were located by chemiluminescence in dark room using a peroxidase conjugated secondary antibody, after 10–15 min incubation with substrate mix at 37 °C. Film development was manual or by scanner.

### 2.4. Molecular cloning of GH, expression and purification of recombinant GH

A PCMV-Sport vector, containing respective GH cDNA was subjected to amplification by Vent polymerase chain reaction with respective GH-specific forward and reverse primers and ligated into pET32(c) expression vector. The ligated pET32(c) + GH was transformed into competent *E. coli* Top 10 cells, which were cultured by the spread plate technique on Luria-Bertani (LB) agar plates with ampicillin (50 μg/mL and X-gal (20 μg/mL)). PCR positive colonies were inoculated into LB Broth with ampicillin, cultured overnight at 37 °C followed by plasmid preparation using a plasmid maxi-prep kit. The pET32(c) + GH was sequenced to confirm that the GH cDNA was in frame and without mutation. PCR colonies positive for pET32c + GH were used to transform expression competent *E. coli* BL21 Ril cells. GH expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation shaken for 6 h pET32(c) without GH was also used to transform *E. coli* BL21 Ril cells, as a null vector control.

The culture was centrifuged at 20,000 g for 10 min. The pellet was suspended in 20 mM Tris-HCl, pH7.2, and lysozyme (2,000 units) was added, followed by incubation at 25 °C for 30 min. The suspension was sonicated (Branson Digital Sonifier) three times at amplitude 25% for 15 s on ice. DNase (10 units) was added and the lysate incubated on ice for

15 min. The lysate was then centrifuged at 30,000 g for 12 min. The resulting pellet, containing inclusion bodies, was stored at -20 °C. The pellet was thawed on ice and washed with 1% Triton X-100, and centrifuged at 20,000 x g for 10 min. The pellet obtained was dissolved in 7 M guanidine hydrochloride, 0.1 M NaCl and 10 mMβ-mercaptoethanol to a final protein concentration of 5 mg/mL. This sample was diluted 1:50 (with slow addition) in 50 mM HEPES, pH 8.0, containing 0.5 M NaCl, 10 mM KCl, 9 mM glutathione, 1 mM oxidised glutathione, 0.5 mM benzamidine and stirred overnight at 4 °C. The refolded, renatured recombinant GH was applied to a column of immobilised nickel (5 mL), that had been equilibrated with 25 mM TES, pH 7.3, containing 0.5 M NaCl, and 0.5 mM benzamidine. The column was then washed with 4 column volumes (CV) of equilibration buffer. Protein was eluted from the column by the sequential addition of 4 CV of 50 mM, 100 mM and 250 mM buffered imidazole. For these experiments, the protein content of each fraction (1.5 mL) was monitored by absorbance at 280 nm. Selected fractions were pooled; desalted by dialysis against 20 mM TES, pH7.8, overnight at 4 °C; freeze dried and re-dissolved in 500 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$ and 1% Tween 20 to a concentration of 1.5 mg/mL. Enterokinase (0.02 units/mg of protein) was added and the sample incubated at 25 °C for 16 h. Thioredoxin- and His-tags were removed from the digest by hydrophobic interaction chromatography on a (10 mL) column of phenyl-Sepharose, that had been equilibrated using buffer containing 50 mM HEPES, pH 7.3, 0.5 mM benzamidine and 5% saturation ammonium sulphate. Ammonium sulphate was added to adjust to 5% saturation prior to application to the column, to facilitate the adsorption of hydrophobic protein. Column was then washed with 4 CV of equilibration buffer; followed by 4 CV of buffer without ammonium sulphate. The GH was eluted with 50 mM HEPES, pH 7.3, 0.5 mM benzamidine and 50% ethylene glycol at 4 °C.

## 3. Results

### 3.1. Bovine GH fractionations and outcomes

Gel filtration of the solubilized ammonium sulphate fraction (170 pmol/min/mg), prepared from fresh bovine pituitaries, using Sephacryl S-100 HR resulted in a 25% recovery of peptidylglycine monooxygenase activity. Previously (Downey and Donlon, 1997), excellent recovery (150%) was obtained using S-200 HR, in the presence of 0.2 mM β-mercaptoethanol (Downey and Donlon, 1997), with a broad profile suggestive of multiple activities with a peak at 32 kDa and shoulders at approx. 44 and 25 kDa, respectively. Recombining fractions isolated using Sephacryl S-100 HR restored activity. Addition of aliquots of proteins of low molecular mass (*circa* 20 kDa, having 80% of the residual activity) to aliquots of earlier fractions (ranging from 100 to approx. 400 kDa) resulted in a linear (concentration dependent) increase in activity resulting in full recovery of activity - indicating a requirement for a combination of proteins of low and high molecular mass for peptidylglycine monooxygenase activity.

With the revised phenyl-Sepharose fractionation the recovery of activity was 34% of that achieved previously (Downey and Donlon, 1997) due to the resolution of the proteins eluted by ethylene glycol into two peaks (3 and 4), as shown in Fig. 1. However, the loss of activity (Fig. 1) was reversible by recombination of fractions from individual peaks. Four parts of the profile (accounting for about 80% of applied 88 mg of protein) contained differential activity values. Peaks 3 and 4 (80 mL onwards) contained 97% of the recovered activity (0.65 and 0.8 nmol/min, respectively; specific activities of 34 and 44 pmol/min/mg, respectively). Peaks 1 and 2 also displayed some activity (25 and 15 pmol/min, respectively; with specific activities of 3.6 and 1.6 pmol/min/mg, respectively). When aliquots of peaks 1 or 2, were combined with aliquots of peaks 3 or 4 there was a concentration dependent stimulation of peptidylglycine monooxygenase activity such that the combined activity was (2- to 3-fold) greater than the sum of the two components – and accounting for the loss of activity due to fractionation. These observations prompted further chromatographic analyses of peaks 1–3. Peak 4 contained monomeric bGH together with some dimeric bGH (44 kDa) and some small proteins (Fig. 2, gel B).

Peak 1fractions (9–24 mL; Fig. 1) were combined and purified by metal-chelation chromatography using immobilised zinc. To characterise peaks 2 (43–54 mL) and 3 (63–80 mL), they were subjected to affinity chromatography (Affi-Gel blue gel) followed by metal-chelation chromatography. The stimulatory activity of peaks 1–3 did not bind to Affi-Gel blue. Following chelation chromatography, fractions having peptidylglycine monooxygenase stimulatory activity were located by addition of an aliquot of bGH fraction (peak 4; 81 - end) from phenyl-Sepharose step. Peak 3 lost 99% of the activity associated with it upon separation from monomeric bGH, consistent with outcome from size exclusion chromatography using Sephacryl S-100 HR. The stimulatory proteins

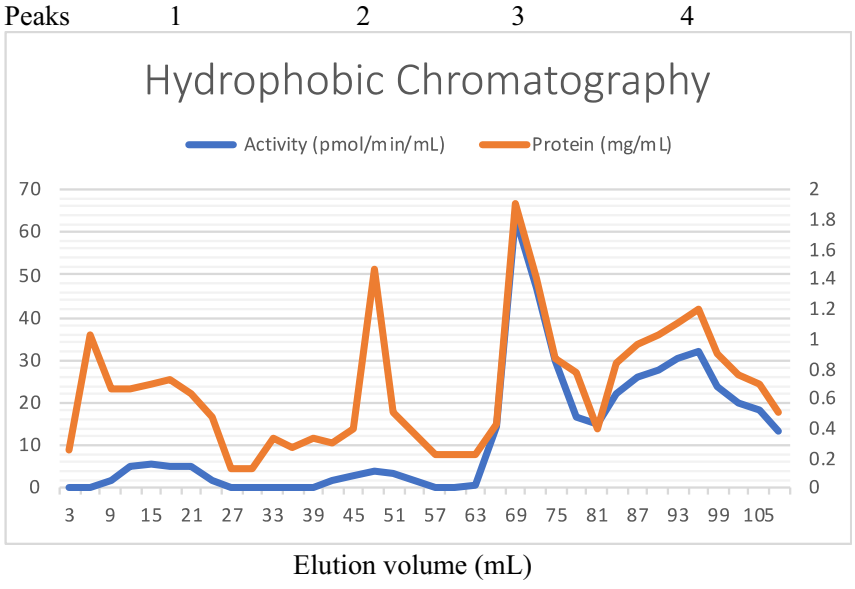


**Fig. 1.** Solubilized ammonium sulphate fraction was applied to a column of phenyl-Sepharose. The column was washed with 20 mL of equilibration buffer and then 1.3 mL fractions were collected. Peak 1 was eluted before washing with equilibration buffer not containing ammonium sulphate commencing at 26 mL. Elution of peak 2 was followed by buffer containing 50% ethylene glycol (50 mL onwards) to elute peak 3 followed by peak 4 (bGH).

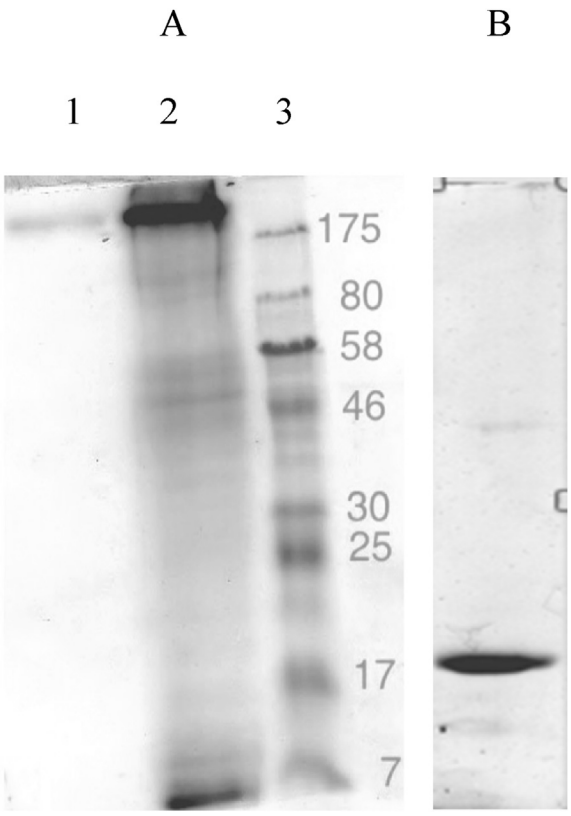


**Fig. 2.** SDS PAGE. Gel A, lane 1, oligomer *a* post metal-chelation step; lane 2, peak 1 of phenyl-Sepharose fractionation; lane 3, molecular mass markers (kDa). Gel B, bGH from phenyl-Sepharose chromatography. Coomassie Brilliant Blue G-250 staining used.

were found to be oligomers of bGH (Fig. 2, gel A, lane 1; and Fig. 3, lanes 1–3); hereafter, they are labelled oligomer forms ***a*** – ***c,*** to distinguish them from their parent peaks 1–3. Monomeric bGH (which migrated with an apparent molecular rmass of 18 kDa upon non-reducing SDS PAGE (Fig. 2, gel B) isolated by hydrophobic chromatography (peak 4) was purified to homogeneity by either affinity or metal-chelation chromatography, where it bound in both cases.

Accounting for all the forms of purified bGH recovered (22.3 mg), forms ***a***, ***b***, ***c*** and monomeric bGH contained 5.8, 0.7, 3.6 and 90%, respectively; of the protein in the initial extract, these constituted 0.7,

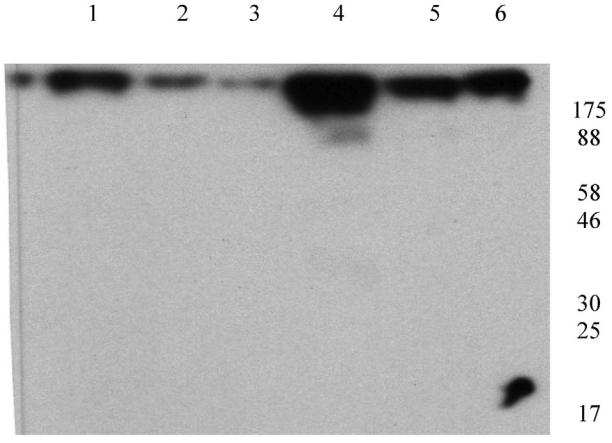


**Fig. 3.** Western blotting with monoclonal bGH antibody. Lanes 1–3, purified oligomers *a* – *c,* post respective metal-chelation step; lanes 4–6, hydrophobic chromatography fractionations, peaks 1–3, respectively. Migrations of molecular mass markers (kDa) are indicated on right.

0.1, 0.4 and 10%, respectively. Upon SDS PAGE under non-reducing conditions, the stimulatory oligomeric forms ***a*** - ***c*** had a molecular mass of approx. 320 kDa (Fig. 2, gel A; and Fig. 3, lanes 1–3), and upon western blotting with monoclonal antibody to bGH all were immunoreactive (Fig. 3). Following hydrophobic chromatography, peak 2 also contained a *circa* 250-kDa oligomer along with a trace of 88 kDa (Fig. 3, lane 4); peak 3 also contained a larger oligomer of approx. 400 kDa and some monomeric bGH (Fig. 3, lane 6). Upon SDS PAGE with 0.2M β-mercaptoethanol in samples, purified form ***a*** showed two components of 48 and 24 kDa, respectively - whereas purified form ***b*** contained dimers of 48 and 40 kDa, respectively. Unfractionated ammonium preparation also contained some dimeric and tetrameric species. Earlier studies found that plasma hGH size isomers contained the same monomeric building blocks, although in somewhat different proportions and of smaller mass than bGH (Stolar et al., 1984). Hydrophobic chromatography (as described here) provides a convenient method for fractionation of the multiple forms of bGH.

### 3.2. Metallothionein

In the course of these fractionations it was noticed that relevant fractions from peaks 1 and 4 had atypical UV spectra. Purified oligomer ***a*** protein displayed an absorbance peak at 236 nm in addition to a broad peak with maximum at 277 nm (an absorbance/mg/mL of 0.2 and 0.6, respectively), in a difference spectrum relative to respective buffer system. The absorption peak at 236 nm is consistent with Zn-metallothionein (MT) complex (Kägi et al., 1984). bGH monomer contained another unidentified variant absorbance peak at 245 nm in addition to normal protein absorbance (with both having a value of 0.37/mg/mL) and a shallow valley (absorbance of 0.25/mg/mL) centred at 253 nm. Human GH is known to bind $Zn^{2+}$ (Cunningham et al., 1991), utilizing a 2-histidine-1 one carboxylate binding motif (His-18, His-21 and Glu-174), which is widely represented in the active site of a wide variety of metalloenzymes (Amrein et al., 2012), including the iron-dependent aromatic amino acid hydroxylases (Werner et al., 2011).

MTs are ubiquitous, low molecular mass (7.0 kDa), cysteine- and metal-rich proteins containing sulphur-based metal clusters formed with $Zn^{2+}$, $Cd^{2+}$, or $Cu^{+}$ ions. All MTs characteristically lack aromatic amino acids (Vašák and Meloni, 2011). Immunoreactivity to MT-I/II had been observed in bovine pituitary (Zatt et al., 2001). In the current study, immunoreactivity to a monoclonal antibody to MT-I/II was observed in peak 1 and oligomer ***a*** (Fig. S1). Further studies are required to characterize the role, if any, of the MT moiety of this hetero-oligomer in the mechanism of the peptidylglycine monooxygenase reaction.

### 3.3. Peptidylglycine monooxygenase activities

Monomeric GH had a Km for D-Tyr-L-Val-Gly of 10 μM and Km for ascorbate of 30 μM (with inhibition above 100 μM), and a pH optimum of 7.7. The purified oligomeric forms ***a*** and **b** had no detectable peptidylglycine monooxygenase activity (<1.7 pmol/min/mg), but when combined with monomeric bGH (15 pmol/min/mg) they almost trebled ($P < 0.005$) the sum of the products (41.7 pmol/min/mg) in the presence of ascorbate (Table 1), which is consistent with a requirement for a combination of small and large protein(s) for maximum peptidylglycine monooxygenase activity seen earlier. This value equates to a rate of 167 pmol/min per pituitary, based on an average value of 4 mg of monomeric bGH recovered (Fig. 1). The specific activity of bGH with $BH_4$ (53.3 pmol/min/mg) was 3.6-fold greater than that with ascorbate ($P < 0.005$), in the presence of monomeric bGH alone; the added oligomeric forms of bGH did not affect the sum of the products of the $BH_4$-dependent reaction (Table 1). Addition of 2.5 mM β-mercaptoethanol to assays containing ascorbate led to inhibition; β-mercaptoethanol alone did not support activity. Monomeric bGH was subjected to scanning electron microscopy and there was insufficient metal to warrant investigation as to whether it can be (or be contained in) a prosthetic group for the

**Table 1**
Comparison of the peptidylglycine monooxygenase cofactor function of tetrahydrobiopterin (20 μM) or ascorbate (40 μM) in reaction catalysed by monomeric bGH or r-hGH, without or in combination with bovine oligomeric forms.

| | $BH_4$ | Ascorbate | P value |
|---|---|---|---|
| bGH | 53.3 ± 2.3 (75) | 15.0 ± 0.8 (84) | <0.005 |
| bGH+ | 48.3 ± 2.2 (6)[a] | 41.7 ± 1.8[b] (68)[a] | >0.05 |
| r-hGH | 58.8 ± 2.8 (6)[a] | 21.7 ± 1.3[c] (9)[a] | <0.005 |
| r-hGH+ | 53.3 ± 2.7 (0)[a] | 21.3 ± 1.2[c] (39)[d] | <0.005 |

bGH: bGH post phenyl-Sepharose (peak 4); bGH+ and r-hGH + also contained added purified oligomers ***a*** and ***b***. Product data (N = 3) are pmol/min/mg ± standard error of the mean, for the sum of hydroxyglycine intermediate and amidated peptide. Bracketed values indicate percentage amidated peptide formed.
[a] $P < 0.001$ versus bGH value; [b] $P < 0.025$ versus bGH value; [c] $P < 0.005$ versus bGH value; [d] $P < 0.001$ vs bGH+.

monooxygenase.

In addition, recombinant forms of hGH (Fig. S2; Table 1), bovine and chicken GH have been expressed and purified to homogeneity (all 22 kDa). Recombinant hGH stimulated the growth of K562 cells 2-fold at 20 ng/ml in 48 h. The specific *a*ctivity of r-hGH with $BH_4$ (58.8 pmol/min/mg) was almost treble that with ascorbate (21.7 pmol/min/mg) and neither value was affected by the inclusion of bGH oligomers – possibly due to 30% difference in sequence homology. The fact that the ascorbate-dependent activity of r-hGH is not altered by inclusion of oligomeric forms suggests that the activation of bGH is not due to additional copper or ascorbate. The specific activities for r-bGH and r-cGH with ascorbate were 24.0 and 21.4 pmol/min/mg, respectively. Another novel feature is that a differential distribution of products was observed, as indicated by the data for percentage amidated product formed, under the varied conditions (Table 1). That these two cofactors can support peptidylglycine monooxygenase of GH could explain the earlier reports of significant residual peptide amidation under conditions of ascorbate deficiency (May and Eipper, 1985; Hilsted et al., 1986).

## 4. Discussion

In these studies using 5 fresh bovine pituitaries, we show that the peptidylglycine monooxygenase activity of monomeric bovine pituitary GH, in the presence of ascorbate, is stimulated by combination with oligomeric forms of bGH one of which is a hetero-oligomer with metallothionein. This novel finding is in contrast with the outcome of studies conducted using commercial frozen bovine pituitaries (Murthy et al., 1986). It is notable that the extraction buffer used and the initial steps of that purification scheme were performed at pH 8.5. The peptidylglycine monooxygenase activity per pituitary, in initial extracts, was 2.8 nmol/min from fresh tissues and 43 pmol/min from frozen samples. In that study (Murthy et al., 1986), two active forms were identified - PAM-A (54 kDa) and PAM-B (38 kDa), with a total of 9 μg being recovered from 300 glands. The data show the total amount of PAM in a pituitary to be 0.66 μg (0.44 μg of PAM-A and 0.22 μg of PAM-B). Together those amounts have activities of 17 and 26 pmol/min due to PAM-A (38 pmol/min/μg) and PAM-B (116.5 pmol/min/μg), respectively, at pH 8.5, with ascorbate as cofactor; according to the pH plots shown that sum equates to 28 pmol/min at pH 7.8. An average of 4 mg of monomeric bGH was recovered per pituitary (Fig. 1), i.e. 22% of protein content. Monomeric bGH (Peak 4) in conjunction with oligomers ***a*** and ***b,*** had an activity of 167 pmol/min, with ascorbate as cofactor – or 213 pmol/min with $BH_4$ as cofactor (Table 1). The combined recovery of the peptidylglycine monooxygenase activities (Fig. 1) was 11% due to the resolution of the proteins eluted by ethylene glycol into two peaks (3 and 4), as shown in Fig. 1. As in the instance of gel filtration upon Sephacryl S-100 HR, this sharp decline in activity was reversible by recombination of fractions from individual peaks. However, almost all of the activity associated with peak 3 was unrecoverable after fractionation by affinity chromatography. Peak 3 contained some monomeric bGH (Fig. 3, lane 6). Thus, the oligomeric forms of peak 3 are essential for optimization of its activity of 130 pmol/min per pituitary. In our former study (Downey and Donlon, 1997) we showed that 96% of peptidylglycine monooxygenase activity in the ammonium sulphate fraction was inhibited by a polyclonal antibody to bGH. Also, during those studies we noted that an anti-PAM antibody (Ab 100) cross reacted with purified monomeric GH upon western blotting. This might be due to two sets of tripeptide sequences being common to the selected 23-mer sequence of PAM and to bGH, i.e. val-phe-thr and gly-thr-ser in the loop between α-helix 2 and α-helix 3. Overall, the peptidylglycine monooxygenase activity of monomeric bovine pituitary GH (stimulated by its oligomeric forms, with ascorbate as cofactor) is very significantly greater than what can be associated with PAM at physiological pH. Also, the activity of monomeric bGH with $BH_4$ as cofactor was found to be very significantly greater than that with ascorbate.

Recombinant forms of rat peptidyl α-hydroxylating monooxygenase (Kolhekar et al., 1997; Bell et al., 2003) have been prepared and purified. However, a feature of those preparations has been that they have acidic pH optima with little activity above pH 6.5 (Bell et al., 2003). In the latter study, two preparations had specific activities of 1.2 and 1.5 μmol/mg/h, respectively, at pH 5.5, but the activity generated by those recombinants derived from PAM-B declined rapidly with increasing pH. So that at pH 7.0 the activities were 60 and 70 pmol/μg/h, respectively. Therefore, at pH 7.0, only a peptidylglycine monooxygenation rate of <1 pmol/min can be generated by either of those with the amount of PAM proteins found in an average bovine pituitary. PAL has good activity at that pH (Katopodis et al., 1991). A bifunctional alpha-amidating enzyme (α-AE, having hydroxylating and dealkylating functions), derived from rat thyroid medullary carcinomas and expressed in CHO cells, had a specific activity of 7 pmol/μg/min (Kulathila et al., 1994). Mean circulating hGH ranges from 0.7 μg/L in obese males (Veldhuis et al., 1991) to 13.8 μg/L in adolescent males (Nieves-Rivera et al., 1993), which suggests that the average peptidylglycine monooxygenase activity of hGH, in bloodstream, can range from at least 1 to at least 20 pmol/h/L with ascorbate as cofactor; and from about 2.5 to 50 pmol/h/L with $BH_4$ as cofactor. Some stimulation of ascorbate-dependent activity by circulating oligomeric forms of hGH cannot be excluded.

This evidence for the hormonal/enzymatic bifunctionality of GH gives rise to a number of considerations. First, as shown above, the peptidylglycine monooxygenase activity of GH is significant and the activity with $BH_4$ as cofactor is more than treble the value observed with ascorbate as cofactor. An interaction of GH with a metabolite of $BH_4$ was noted during early investigations on rat liver phenylalanine hydroxylase (Kaufman, 1970). In that study, GH was seen to behave in the same manner as carbinolamine dehydratase, i.e. it stimulated the conversion of pterin-4a-carbinolamine to quinonoid dihydrobiopterin (Lazarus et al., 1981). Further credence for $BH_4$-dependent peptidylglycine monooxygenase activity of bifunctional GH arises from a consideration of the pattern of its molecular evolution. The metal-binding triad has been present in GHs from ancestral vertebrate onwards. With regard to the $BH_4$-dependent aromatic amino acid hydroxylases (Erlandsen et al., 2000), two conserved residues (glu-286 and phe-254) have been shown to be required for the interaction of $BH_4$. In addition to the conserved glu-174 of the metal-binding triad, a number of other strongly conserved glutamate residues (32, 56, 66, 74, 118, 119 and 161) occur in GH - as well as an invariant phe-166 [Protein – NCBI AIA66930]. However, of those glutamate residues only residues 118, 119 and 161 appear to be suitably located to participate in $BH_4$ binding to GH to support the peptidylglycine monooxygenase reaction. Glu-118 and glu-119, in α-helix 3 of GH, evolved in amphibia about 300 million years ago. These residues are located close to the bound metal (Cunningham et al., 1991) on α-helix 1 and phe-166 residue in α-helix 4 (Protein Data Bank ID:

1HGU). GH from jawed fishes (dating from circa 400 Mya (Kawauchi and Sower, 2006)) has a strongly conserved glu-161 (α-helix 4). The crystal structures of those molecules are not available, but by analogy with the structure of human GH (1HGU) this residue could be located sufficiently close to the bound metal. Hence, GH molecules having a copper-binding site (metal triad or otherwise (Shimazaki et al., 2015)) together with glu-118 or -119 or -161 and phe-166 can be considered to have evolved to having hormonal activity and the capacity to utilise both ascorbate and $BH_4$ for peptidylglycine monooxygenase activity.

Second, it is notable that in human placental lactogen (PL), which has 84% homology with GH, all of the key residues [Protein – NCBI AAA98747] and the structure (PDB: 1Z7C) required for ascorbate- and $BH_4$-dependent peptidylglycine monooxygenase activity are conserved. Despite 95% homology with hGH, hGH-V (placental variant) is devoid of the metal-binding triad (Seeburg, 1982). The levels of serum hGH-V increase steadily during pregnancy while hGH values decrease - whereas PL is elevated during the third semester (Daughaday et al., 1990). This suggests that the postulated peptidylglycine monooxygenase activity of PL may be associated with the increased plasma oxytocin occurring during the third trimester of human pregnancy (Prevost et al., 2014). PL and oxytocin are secreted by the syncytiotrophoblast of placenta during pregnancy. The peptidylglycine monooxygenase function of GH may also contribute to the reproductive actions of GH in vertebrates (Hull and Harvey, 2014).

Third, other physiologically significant amidated peptides are GLP-1 and PYY. GLP-1 stimulates insulin release, plus beta cell proliferation and regeneration, and inhibits glucagon secretion (Cho et al., 2014; Lee and Jun, 2014). Almost all of human GLP-1 is in the amidated form (Holst, 2007) and it has become of major interest with regard to glucose-lowering medications, as well as having multiple other effects (Nauck and Meier, 2018). $PYY_{3\text{-}36}$ plays a key role in appetite regulation and obesity (Karra et al., 2009). GLP-1 is derived from proglucagon in the enteroendocrine L cells of the small intestine and (and to a lesser extent) in pancreatic alpha cells (Mojsov et al., 1990; Fava et al., 2016). $PYY_{3\text{-}36}$ is also synthesised in the gut and (also to a lesser extent) in alpha, delta and PP cells (Persaud and Bewick, 2014). Neither PHM nor PAL immunoreactivities were detected in rat enteroendocrine cells (Martínez et al., 1993a); PAM and PAL immunoreactivities were found in human alpha cells (Martínez et al., 1993b) - but both were absent in pancreatic PP cells (Martínez et al., 1993b; Garmendia et al., 2002). As there is doubt about the role of PAM in tissues not known to produce significant levels of amidated peptides (Garmendia et al., 2002; Braas et al., 1992), the peptidylglycine monooxygenase activity of GH (in facilitating the generation of active GLP-1 and $PYY_{3\text{-}36}$) could have a role in the previously unexplained effects of GH on glucose metabolism and in obesity (Vijayakumar et al., 2011). Interestingly, an intravenous injection of hGHRH increased insulin concentrations in rat hepatic portal vein (Bailey et al., 1989). The obverse of that is that the somatostatin (growth hormone release-inhibiting factor) analogue octreotide has been observed to suppress GLP-1 and insulin secretion in humans (Plöckinger et al., 1999). Reduced levels of both GLP-1 and $PYY_{3\text{-}36}$ are linked with obesity (Holst, 2007; Anandhakrishnan and Korbonits, 2016; Grundell and Camilleri, 2007), as are decreased levels of basal serum hGH (Veldhuis et al., 1991; Iranmanesh et al., 1991).

In summary, this study has expanded on our earlier identification (Downey and Donlon, 1997) of the peptidylglycine monooxygenase activity of bGH. Monomeric and the oligomeric forms of bGH have been isolated and purified, and their respective roles in peptide amidation have been explored. In the presence of ascorbate, peptidylglycine monooxygenase of monomeric bGH is greatly stimulated by combination with very large oligomeric forms of bGH - with one of the latter forms being a hetero-oligomer of GH and metallothionein. Further studies on the mechanism(s) of these synergistic collaborations are required. The other significant finding here (Table 1) is that with tetrahydrobiopterin ($BH_4$) bGH and r-hGH are about 3-fold more active than with ascorbate. These current findings provide further support for the role of GH in initiating peptide amidation *in vivo*. The peptidylglycine monooxygenase activity of GH presents some novel considerations of its roles in physiology, and reveals a previously unknown physiological mechanism for the regulation of peptide amidation.

## Declarations

### *Author contribution statement*

John Donlon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Patrick Ryan: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

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