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Production of the catalytic core of human peptidylglycine α -hydroxylating monooxygenase (hPHMcc) in *Escherichia coli*

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Abstract

Most mammalian bioactive peptides possess a C-terminal amino acid amide moiety. The presence of the C-terminal amide is a significant impediment to the recombinant production of α -amidated peptides. α -Amidated peptides are produced *in vivo* by the enzymatic cleavage of a precursor with a C-terminal glycine residue. Peptidylglycine α -hydroxylating monooxygenase catalyzes the key step in the oxidation of the glycine-extended precursors to the α -amidated peptide. Herein, we detail the production of the catalytic core of human peptidylglycine α -hydroxylating monooxygenase (hPHMcc) in *Escherichia coli* possessing a N-terminal fusion to thioredoxin (Trx). Trx was fused to hPHMcc to enhance the yield of the resulting 52 kDa protein as a soluble and catalytically active enzyme. The Trx-hPHMcc-His₆ fusion was purified to homogeneity and exhibited steady-state kinetic parameters that were similar to purified rat PHMcc. The bacterial production of recombinant hPHMcc will foster efforts to generate α -amidated peptides by the co-expression of hPHMcc and the α -amidated peptide precursors in *E. coli* or the *in vitro* amidation of recombinantly expressed α -amidated peptide precursors.

Keywords

Peptidylglycine α -hydroxylating; monooxygenase; α -Amidated peptide hormones; *E. coli* expression

Introduction

Approximately 50% of all mammalian peptide hormones possess an α -amidated C-terminus, which is required for bioactivity [1,2]. The α -amidated peptides are produced by the oxidative cleavage of C-terminal glycine-extended precursors to generate the α -amidated peptide and glyoxylate [3,4]. The enzyme catalyzing this reaction is bifunctional peptidylglycine α -amidating monooxygenase (PAM)¹. Bifunctional PAM is comprised of two separate catalytic units, peptidylglycine α -hydroxylating monooxygenase (PHM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PAL, EC 4.3.2.5) (Fig. 1) [5–7]. The PAM/PHM/PAL enzyme system represents a significant impediment to the manufacture of therapeutically important α -amidated peptides. The large-scale, inexpensive production of recombinant α -amidated peptides has been thwarted by difficulties in the bacterial expression of PAM, PHM, or PAL. The only reported success in bacteria has been the co-

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¹Abbreviations used: hPHMcc, human peptidylglycine α -hydroxylating monooxygenase; Trx, thioredoxin; PAM, peptidylglycine α -amidating monooxygenase; GST, glutathione-S-transferase; NusA N utilization substance A; MBP, maltose binding protein; CHO, Chinese hamster ovary.

expression of rat PAM with the glycine-extended calcitonin or glucagon in *Streptomyces* to yield the desired α -amidated peptide [8–10]. Methods to circumvent this limitation for α -amidated peptide production include the use of PAM *in vitro* to amidate recombinantly produced glycine-extended peptides [11–15], the overexpression of a glycine-extended peptide in mammalian cells that express PAM [16–19], the expression of a suitable peptide precursor that is chemically converted to the α -amidated peptide *in vitro* [20–23], transacylation/transpeptide methods [24–27], production in the milk of transgenic rabbits [28], and an intein based method [29].

The expression of mammalian proteins in *Escherichia coli* is often limited by (a) large differences in codon frequencies between mammalian and *E. coli* proteins, (b) the inability of *E. coli* to properly carry out the post-translational modifications found in many mammalian proteins, and (c) the formation of insoluble inclusion bodies. In order to overcome such problems for the *E. coli* expression of human PHM, we utilized a synthetic gene coding for the catalytic core of human PHM (hPHMcc), which was optimized for *E. coli* expression. Kolehkar et al. [30] have shown that amino acids 42–356 of rat PHM retained the catalytic properties of the full length enzyme. We evaluated a series of fusion partners, glutathione-S-transferase (GST), thioredoxin (Trx), N utilization substance A (NusA), maltose binding protein (MBP), and a His₆ tag, for the expression of active, soluble hPHMcc. We report herein on the successful expression of a Trx-hPHMcc-His₆ fusion protein that exhibits steady-state kinetic parameters similar to that of wildtype rat PHMcc.

Materials and methods

Materials

*Bam*HI, *Hind*III, Antarctic Phosphatase, pMAL-c2X, and T4 DNA ligase were purchased from New England Biolab. Goat polyclonal anti-PAM monoclonal antibody (sc-17393), sheep anti-goat IgGs, and horseradish peroxidase were purchased from Santa Cruz Biotechnology. Oligonucleotides were obtained from Integrated DNA Technologies. Ni-NTA resin, pET21a(+), pET32a(+), pET42b(+), pET43.1a(+) vectors, and the *E. coli* strains BL 21 (DE3) and Origami B(DE3) were purchased from Novagen (Table S1, Supplementary material). Protease inhibitor cocktail and N-dansyl-Tyr-Val-Gly were purchased from Sigma–Aldrich. Ampicillin sodium salt, carbenicillin, IPTG was purchased from Fisher Scientific. All other reagents were of the highest quality available from commercial suppliers.

Molecular cloning

The DNA sequence for the hPHMcc gene was codon optimized for expression in *E. coli* and commercially synthesized by the GeneArt (www.geneart.com). Based on the sequence of human PAM (Accession No. P19021), the coding region from amino acid 40 to 454 was supplied in the pGA4 vector. This coding sequence was amplified by PCR with pGA4 as a template (primers, forward 5'-CATGGGATCCATGAACGAATGCCTGGGTAC-3', reverse 5'-ATGAA GCTTTTAAATCGGAATATTCGCTTCCG-3') and cloned into the pET vectors using the *Bam*HI and *Hind*III restrictions site. Sequences of the cloned vectors were confirmed by DNA sequencing from MWG Operon. The recombinant plasmid was transformed into the different *E. coli* strains (Novagen) for expression. Co-expression experiments were performed using the chaperonin Plasmid Set (Takara Bio USA) containing the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16. *E. coli* Origami B(DE3) cells were transformed with pET32a-hPHMcc vector, made competent chemically, and the competent *E. coli* Origami B(DE3)-hPHMcc cells were transformed with each plasmid possessing a chaperonin gene.

Culture cultivation

An overnight culture of *E. coli* bearing pET32a-hPHMcc was diluted 100-fold in LB medium supplemented with 100 µg/ml carbenicillin and 1% glucose and cultured at 37 °C. The culture was cooled down to 10 °C, was induced with IPTG (final concentration = 100 µM) when the OD₆₀₀ reached 0.6, and incubated for another 4 h. The cells were collected by centrifugation at 10,000 rpm for 10 min and stored at –80 °C.

Protein purification

All the purification steps were performed at 4 °C. The cell pellet was resuspended in buffer A (20 mM Tris, 500 mM NaCl, and 20 mM imidazole, pH 7.8) at a concentration of 0.1 g/ml, with 1 mg/ml lysozyme and protease inhibitor cocktail added according to the manufacture's instruction. Resuspended cells were sonicated and the cell lysate collected by centrifugation at 16,000g for 20 min. The pelleted cell lysate was resuspended with Ni-NTA resin equilibrated in buffer A for 2 h. Resin was then loaded into a column and washed with 10 column volumes of buffer A. Next, the Ni-NTA column was washed with 10 column volumes of buffer B (20 mM Tris, 500 mM NaCl, 1 M guanidine hydrochloride, and 60 mM imidazole, pH 7.8), and then with 20 column volumes of buffer C (20 mM Tris, 500 mM NaCl, and 60 mM imidazole, pH 7.8). Contaminating proteins were best removed by first washing the Ni-NTA column with a partially denaturing buffer containing 1 M guanidine hydrochloride. The column was next washed with buffer C, lacking guanidine hydrochloride, to remove the denaturant from Trx-hPHMcc-His₆. Recombinant Trx-hPHMcc-His₆ was eluted from the Ni-NTA column using a linear gradient of 60–140 mM imidazole in buffer C. The fractions containing hPHMcc activity were pooled together and concentrated using YM-10 centriprep ultrafilter. The active fractions were concentrated to 0.5 mg/ml and stored at –20 °C.

Expression and analysis by mass spectrometry

We evaluated the *E. coli* constructs for Trx-hPHMcc-His₆ expression by Western blot analysis. After induction, 5 ml was withdrawn from the culture at 1 h interval, centrifuged to collect the cells, lysed using lysozyme, and evaluated using SDS-PAGE. Expression of Trx-hPHMcc-His₆ was confirmed by Western blot using an anti-PAM antibody and compared it to an uninduced control.

After SDS-PAGE, the Coomassie stained protein band corresponding to the correct mass for hPHMcc or the Trx-hPHMcc-His₆ fusion protein was extracted from the gel and the protein digested using restriction grade trypsin. A hybrid linear ion trap-Orbitrap (LTQ Orbitrap XL, Thermo) mass spectrometer equipped with an Eksigent NanoLC-Ultra 2D Plus HPLC system was used for the analysis of the tryptic fragments.

Activity assay

Trx-hPHMcc-His₆ activity was assayed using a modification of a published procedures [31,32]. Briefly, the reactions at 37 °C were performed in 100 mM MES pH 6.5, 5 mM ascorbate, 2 µM CuSO₄, 5000 U bovine catalase, 0.01 (v/v) % Tween 20, 1% (v/v) ethanol, and 20 µM N-dansyl-Tyr-Val-Gly. The reaction was quenched at desired time with addition of trifluoroacetic acid to a final concentration of 2% (v/v). The product of the reaction N-dansyl-Tyr-Val-α-hydroxy-Gly was converted to N-dansyl-Tyr-Val-NH₂ by incubating with potassium hydroxide and 50 mM EDTA to a final pH = 8.5. Kinetic studies were carried out in triplicate. The product was analyzed on HPLC and the initial velocity kinetic data analyzed using SigmaPlot.

Results and discussion

The hPHMcc gene codon optimized for *E. coli* expression (55% GC content with a Codon Adaptation Index value of 0.98) was inserted into the pET vectors using *Bam*HI and *Hind*III restriction site (Table S2, Supplementary material) to generate tagged hPHMcc with a C-terminal His₆-tag. Soluble hPHMcc-His₆ was not achieved by using the GST, Nus A and DscB tag in BL 21(DE3), C-41(DE3), and C-43(DE3) *E. coli* strains even after varying parameters like induction temperature, induction time, and addition of Cu(II) and/or PHMcc substrates to the growth media. In all cases (except the Trx and MBP tag) hPHMcc was produced as inactive and insoluble inclusion bodies. We were completely unsuccessful in generating active PHMcc-His₆ after solubilizing and refolding the protein, which had been purified from the inclusion bodies.

Trx-hPHMcc-His₆ was expressed by inserting the hPHMcc gene into pET32a(+) vector to yield hPHMcc with a N-terminal Trx fusion and a C-terminal His₆-tag. Recombinant Trx-hPHMcc-His₆ was produced as insoluble inclusion bodies after transformation into *E. coli* BL 21(DE3) with the pET32a(+)-derived vector despite many experiments to optimize growth conditions for the generation of soluble protein. Our attempts to solubilize and refold Trx-hPHMcc-His₆ or hPHMcc after proteolytic separation from Trx and the His₆ tag were unsuccessful.

One likely reason for our inability to express active hPHMcc-His₆ or Trx-hPHMcc-His₆ in the *E. coli* BL 21(DE3) cells is relatively high reducing environment of cytoplasm. The high cytoplasmic reducing environment inhibits disulfide bond formation, a critical factor for the proper folding and activity of PHMcc. It has been found that PHMcc contains three disulfide linkages in the N-terminal domain and two disulfide linkages in C-terminal domain, all which are essential to enzymatic activity [30]. We overcame this problem with the expression of Trx-hPHMcc-His₆ in the Origami B(DE3) strain of *E. coli*. The cytoplasmic environment of the Origami B(DE3) cells is more oxidizing than that of the BL-21 cells due to mutations which inactivate glutathione reductase and thioredoxin reductase [33,34]. After optimization (Fig. S1, Supplementary material), we found that soluble Trx-hPHMcc-His₆ was expressed at low temperature (10 °C) and a relatively low concentration of 100 μM IPTG (Fig. 2). Optimal expression required a short period of IPTG induction (4 h) to avoid Trx-hPHMcc-His₆ degradation to lower molecular weight Western positive fragments of low activity (data not shown).

During the optimization of expression and purification of Trx-hPHMcc-His₆, we observed a 65 kDa protein that co-purified with the 52 kDa Trx-hPHMcc-His₆ fusion. The specific components of the Trx-hPHMcc-His₆ fusion protein are as follows: 11.8 kDa for Trx, 5.9 kDa peptide between Trx and hPHMcc, 35.2 kDa for hPMHcc, a 1.5 kDa peptide on the C-terminal side of hPHMcc, and 0.6 kDa for the His₆ tag. The sum of the individual masses are 55 kDa, in reasonable agreement with the 52 kDa we measure by SDS-PAGE (Fig. 3).

The contaminating 65 kDa protein represented 25–30% of the total protein in our sample and remained constant using a number of different purification strategies, including size exclusion chromatography, ion-exchange chromatography, and affinity chromatography. The 65 kDa protein was extracted from the SDS-PAGE gel, digested with trypsin, and the tryptic peptides analyzed by LC-MS/MS on a LTQ-Orbitrap. The 65 kDa protein was identified as the GroEL–GroES complex, an ATP-dependent molecular chaperonin which assists protein folding in bacteria [35]. It has been proposed that GroEL/GroES/ADP complex binds with non-native protein through hydrophobic contacts, followed by protein folding within the GroEL/GroES/ATP *cis* folding chamber [35]. In addition to GroEL–GroES, other chaperonins, such as dnaK, dnaJ, grpE, and tig, have been reported to increase

the solubility and yield of proteins which are difficult to express in bacteria [36]. In order to determine if coexpression of Trx-hPHMcc-His₆ with these chaperonins increased the yield of soluble, active enzyme, plasmids containing the gene for these chaperonins were inserted in Origami B(DE3) cells which already possess the pET32a(+)-hPHMcc-His₆ plasmid. Unfortunately, we found was no appreciable increase in the amount of soluble Trx-hPHMcc-His₆ upon coexpression with a chaperonin.

The GroEL–GroES complex was removed from Trx-hPHMcc-His₆ using a nickel affinity resin. The partially purified Trx-hPHMcc-His₆ was loaded onto a column packed with Ni-NTA resin, the column was washed with buffer containing 1 M guanidine hydrochloride to overcome the interactions between Trx-hPHMcc-His₆ and the GroEL–GroES complex. The bound Trx-hPHMcc-His₆ was then eluted from the Ni-NTA resin using a linear, increasing gradient of [imidazole]. Trx-hPHMcc-His₆ was 90% homogeneous by SDS-PAGE (Fig. 3) and Western analysis using an anti-His₆ antibody confirmed the presence of the His₆ on the 52 kDa Trx-hPHMcc-His₆. The final yield of purified Trx-hPHMcc-His₆ was 0.1–0.2 mg/L of culture and the purified Trx-hPHMcc-His₆ was active. The most efficient reported expression systems for recombinant PAM or PAL are in Chinese hamster ovary (CHO) cells. CHO cell expression and *E. coli* expression are different, making it difficult to compare expression yields of hPHMcc between the two systems. Our yield of 0.1–0.2 mg/L if Trx-hPHMcc-His₆ is roughly comparable to the yields per day reported by Miller et al. [37] for rat bifunctional PAM and Satani et al. [38] for hPHM. However, our yield of Trx-hPHMcc-His₆ is lower than the optimized CHO expression systems described by Bauman et al. [39] for rat PHM of 5–10 mg/day depending on the bioreactor used to grow the PHM-expressing CHO cells. Despite the lower enzyme yield from *E. coli* as reported here relative to that of Bauman et al. [39], the production of hPHMcc in *E. coli* should prove more cost-efficient than CHO cell production given the costs associated with the growth of the CHO cells and more laborious strategy required to purify PAM or PHM from the CHO conditioned media.

The steady-state kinetic parameters for the Trx-PHMcc-His₆ catalyzed oxidation of N-dansyl-Tyr-Val-Gly to N-dansyl-Tyr-Val- α -hydroxyglycine were as follows: $K_M = 7.7 \pm 0.8 \mu\text{M}$, $V_{\text{MAX}} = 0.40 \pm 0.02 \mu\text{moles/min/mg}$, $k_{\text{cat}} = 0.35 \pm 0.02 \text{ s}^{-1}$, and $k_{\text{cat}}/K_M = 4.68 \pm 0.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4). Normalizing the V_{MAX} we obtained in using the Trx-PHMcc-His₆ fusion to the mass of hPHMcc, results in a normalized V_{MAX} of 0.6 $\mu\text{moles/min/mg}$ ($=0.4 \text{ l moles/min/mg} \times 55 \text{ kDa}/35.2 \text{ kDa}$). The K_M and V_{MAX} values of N-dansyl-Tyr-Val-Gly for rat PHMcc, under similar experimental conditions, are reported as 7 μM and 0.5–6 $\mu\text{moles/min/mg}$ [30], which are similar to the values we measured for Trx-hPHMcc-His₆.

In other experiments (not shown), we found that both hPHMcc and MBP-PHMcc-His₆ were active when expressed in the *E. coli* Orgami B (DE3). The specific activity of MBP-PHMcc-His₆ was low, (0.12 $\mu\text{moles/min/mg}$), and we did no further work with this hPHMcc fusion. We had engineered in a thrombin cleavage site C-terminal to Trx and N-terminal to the His₆ tag to enable the thrombinolytic generation of hPHMcc from Trx-hPHMcc-His₆. Unfortunately, thrombin cleavage of the Trx-hPHMcc-His₆ unexpectedly cleaved within hPHMcc generating smaller PHMc-derived fragments. Experiments varying the conditions for the thrombin cleavage of Trx-hPHMcc-His₆ always lead to the cleavage of hPHMcc and we were unable to completely purify full length hPHMcc away from the thrombin-derived fragments. Because the specific activity of the Trx-hPHMcc-His₆ fusion was in agreement with wildtype rat PHMcc for N-dansyl-Tyr-Val-Gly substrate, we did not pursue the expression of hPHMcc in *E. coli* any further.

Conclusion

We report here on the expression of a soluble, active form of the catalytic core of hPHMcc in *E. coli* [30]. The successful production of hPHMcc in *E. coli* will foster future mutagenesis studies to elaborate mechanistic details of PHM catalysis and new strategies for the inexpensive production of α -amidated peptides in *E. coli*. Co-expression of glycine-extended peptides and hPHMcc in *E. coli*, in combination with the methods to incorporate non-natural amino acids into α -amidated peptides [40], could rapidly lead to novel peptide analogs useful as therapeutics and research tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pep.2012.04.012>.

Fig. 1.

The peptide amidation reaction. Glycine hydroxylation requires O₂, Cu(II), and a reductant and is catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM). Dealkylation of the α-hydroxyglycine-extended peptide requires Zn(II) and Ca(II) and is catalyzed by peptidylamidoglycolate lyase (PAL).

Fig. 2. Effect of induction temperature on expression of Trx-hPHMcc-His₆ (A = 24 °C and B = 10 °C). Cells were collected at different time interval (0–5 h) after induction and the cell lysate was used for the western blot analysis with the anti-PAM antibody. Lane 1 – Marker (50 kDa), lane 2 – control (uninduced), lane 3–7 – induced sample after 1–5 h respectively.

Fig. 3. SDS-PAGE analysis hPHMcc expression. Lane-1, molecular weight markers (with the molecular weight highlighted inside the bands); lane-2, purified hPHMcc inclusion bodies from affinity column; lane-3 purified Trx-hPHMcc-His₆ with the GroEL–GroES complex; and lane-4, the purified Trx-PHMcc-His₆.

Fig. 4.
Steady-state kinetic analysis of N-dansyl-Tyr-Val-Gly hydroxylation.