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Engineering of Cysteine Residues Leads to Improved Production of a Human Dipeptidase Enzyme in *E. coli*

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Abstract Low yields, poor folding efficiencies and improper disulfide bridge formation limit large-scale production of cysteine-rich proteins in *Escherichia coli*. Human renal dipeptidase (MDP), the only human β-lactamase known to date, is a homodimeric enzyme, which contains six cysteine residues per monomer. It hydrolyses penem and carbapenem β-lactam antibiotics and can cleave dipeptides containing amino acids in both D- and L-configurations. In this study, MDP accumulated in inactive form in high molecular weight, disulfide-linked aggregates when produced in the *E. coli* periplasm. Mutagenesis of Cys361 that mediates dimer formation and Cys93 that is unpaired in the native MDP led to production of soluble recombinant enzyme, with no change in activity compared with the wild-type enzyme. The removal of unpaired or structurally inessential cysteine residues in this manner may allow functional production of many multiply disulfide-linked recombinant proteins in *E. coli*.

Keywords Cysteine · Disulfide bridge · *E. coli* · Mammalian dipeptidase · Periplasm · Recombinant protein

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Introduction

Expression of heterologous proteins in *Escherichia coli* is a powerful tool in biomedical and biotechnological applications [1, 2]. The functional yields of many multiply disulfide-bridged heterologous proteins are, however, very low in the organism [3–7]. Improper disulfide bridging patterns have been identified in human proteins such as tissue plasminogen activator [8], proinsulin [9], and muskelin [6] and have been linked in particular to proteins with bridges between cysteine residues that are nonconsecutive in the polypeptide sequence [10–12]. Methods to promote formation of native-like disulfide bridges have the potential, therefore, to be of great benefit in recombinant protein production in *E. coli*.

Disulfide bond formation and isomerization are carried out in the *E. coli* periplasm by the *di*sulfide *b*ond metabolizing (Dsb) enzymes. Of this family, DsbA is a potent protein thiol oxidant, while DsbC acts mainly as a disulfide isomerase in vivo [13–15]. Overproduction of DsbC or other Dsb enzymes does not always enable the polypeptide to adopt the correct conformation [9, 16], however, and can increase insolubility in the target protein [17]. In such cases, in vitro refolding and oxidation or cell-free synthesis [18] may remain the only routes to producing functional protein, though these approaches are typically less convenient and more expensive than bacterial expression systems—and are not applicable to mutagenesis and library screening applications.

Mammalian dipeptidase (MDP; dehydropeptidase I, renal dipeptidase; EC 3.4.13.19) is a homodimeric ectoenzyme normally found anchored to cell membranes in the kidney, lung, small intestine, and a variety of other tissues [19]. The human variant contains six cysteines in each 42-kDa MDP monomer, of which four form two intradomain disulfide bridges, a fifth mediates formation of a dimerizing disulfide bridge, and the sixth remains unpaired in the mature enzyme [20]. MDP is the only human β-lactamase known to date and hydrolyses both penem and carbapenem β-lactam antibiotics [21]. This has led to β-lactam ring-containing molecules with structural similarity to MDP's natural substrates being used as prodrug nuclei in ADEPT (antibody-directed enzyme prodrug therapy) cancer chemotherapy [22, 23].

In order to investigate the potential usefulness of MDP as a prodrug-activating enzyme in ADEPT, we undertook its cloning and functional production in *E. coli*. The following work describes improvement of the functional expression of MDP in *E. coli* in order to allow its mutagenesis and the selection of catalytically altered variants in vivo. This protein engineering approach might be generally beneficial for multiply disulfide-bridged proteins that are intransigent to recombinant production in *E. coli*.

Materials and Methods

Strains and Plasmids

E. coli TOP10 (F′ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) φ80*lacZΔM15 ΔlacX74 deoR recA1 araD139* Δ (*ara-leu*) 7697 *galU galK rpsL* (Str^R) *endA1 nupG*; Invitrogen) was used for all cloning and protein production work. The periplasmic expression vector pIG6 [24] was used for MDP production, and *E. coli dsbC*, *skp*, *fkpA*, and *surA* genes were cloned into pIG6 for co-production analyses. Plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16 (Takara; [25]), which are compatible with pIG6, were used to co-produce *E. coli* chaperones GroESL DnaKJ GrpE, GroESL, DnaKJ GrpE, GroESL, and trigger factor, respectively, with MDP.

Materials

All reagents were sourced from Sigma-Aldrich (Dublin, Ireland), unless otherwise stated. DNA-modifying enzymes were from Takara, and HPLC-purified oligonucleotides were from MWG-Biotech (Ebersberg, Germany).

Genetic Manipulation

DNA manipulations were carried out according to Sambrook and Russell [26]. All constructs were confirmed by sequencing prior to expression analysis. The gene encoding MDP was firstly amplified from human kidney cDNA (Sigma-Aldrich), using oligonucleotides *mdp* for 5′-CCGATATCGACTTCTTTCGGGACGAGGC-3′ (*Eco*RV site underlined) and *mdp* rev 5′-CCAAGC*TTATCA*AGAGGAGTAGCCGTAATGGG-3′ (*Hind*III site underlined, stop codons in italics). This cloning strategy removed the human N-terminal signal sequence and replaced the endogenous C-terminal glycosyl-phosphatidylinositol membrane anchor with two stop codons to produce the MDP protein in a non-membrane-linked form in the *E. coli* periplasm.

In the second step, the amplification product was linked by overlap PCR to an N-terminal *E. coli ompA* signal sequence and a shortened, 4-amino acid FLAG tag (DYKD) for immunodetection and purification of the recombinant polypeptide, using oligonucleotides *pig* for 5′-GTGAGCGGATAACAATTTCAC-3′, *pig* rev 5′-GACGCAGTAGCGGT AAACG-3′, *mdp* olfor 5′-*CTACAAAGAT***GAC**TTCTTTCGGGACGAGGC-3′, and *mdp* olrev 5′-CCGAAAGAAGTC*ATCTTTGTAGTC*GGCCTGC-3′ (overlap underlined, start of mature MDP in bold and FLAG tag italicized). As immunodetection of the FLAG tag using the M1 antibody requires the FLAG epitope to be N-terminal, polypeptides that fail to traverse the cytoplasmic membrane are undetectable using this system. Therefore, a C-terminal hexahistidine tag was also added to the MDP polypeptide for subcellular localization studies. This involved removal of the stop codons using *pig* for and *mdp* hisrev 5′-GGGAATTCAGAGGAGTAGCCGTAATGG-3′ (*Eco*RI site underlined), followed by cloning into a modified pIG6 vector [16].

E. coli dsbC, *skp*, *fkpA*, and *surA* genes, encoding disulfide isomerase DsbC, chaperone Skp, and peptidyl prolyl *cis–trans* isomerases/chaperones FkpA and SurA, respectively (reviewed in [27]), were amplified from *E. coli* genomic DNA and cloned individually downstream of the MDP stop codon. The *dsbC* gene was initially cloned using an overlap PCR approach, with a new *Xho*I site inserted between the MDP and *dsbC* genes for ease of substitution of the other chaperone genes. This generated P_{lac}-*ompA*-FLAG-MDP:: chaperone constructs in which the chaperone and MDP genes were under the joint control of a P_{lac} promoter. Oligonucleotides used for these amplification reactions were *dsbC* for 5′-CTTGATAAGCTCGAGCTTTGAAAAGAACGGGAAG-3′ and *dsbC* rev 5′-CGAAGCTT ATTTACCGCTGGTCATTTTTTGG-3′, *skp* for 5′-CCGCTCGAGCAGGTTAAATA AAAATGGGATG-3′ and *skp* rev 5′-GGGAAGCTTATTTAACCTGTTTCAGTAC-3′, *fkpA* for 5′-CCGCTCGAGATGCCCCGATCCTGGAG-3′ and *fkpA* rev 5′-GGGAAGCTT ATTTTTTAGCAGAATCTGC-3′, and *surA* for 5′-CCGCTCGAGATCCGCAGTGCGGT TAATTG-3′ and *surA* rev 5′-GGGAAGCTTAGTTGCTCAGGATTTTAACG-3′ (*Xho*I sites underlined in forward primers, *Hind*III sites in reverse primers).

Mutagenesis of cysteine residues in MDP was carried out by overlap PCR, using oligonucleotides *pig*for and *pig*rev in combination with *mdp* C93Afor 5′-CCACCG CATG**GCA**CGGATGTACCCGGAGAC-3′, *mdp* C93Arev 5′-GTACATCCG**TGC**CATGC GGTGGACCACGTC-3′, *mdp* C361Afor 5′-GGTGGCTCC**GC**CAGGACCCATTACGGC TAC-3′, and *mdp* C361Arev 5′-GGGTCCTG**GC**GGAGCCACCCAGCTGGTCCAG-3′, as

appropriate (mismatched nucleotides in bold). Products were cloned into the pIG6 vector, as previously described, and sequenced to confirm introduction of the mutations prior to expression analysis.

Protein Production

E. coli clones containing a plasmid of interest were grown for 16 h at 37 °C and 250 rpm in 5 ml LB broth cultures containing 100 μg/ml ampicillin. Cultures were diluted 1:100 into 30 ml of the same medium and grown at 37 °C and 250 rpm until an OD_{600} of approximately 0.5 was reached. Cultures were then induced using 0.5 mM isopropyl-β-thiogalactopyranoside, and protein production was allowed to continue for 2–5 h at 25 °C and 250 rpm, following which a culture volume corresponding to 1 OD unit of cells was harvested by centrifugation. Blocking of free thiol groups was carried out where indicated by incubating the cells in 100 μl of 100 mM iodoacetamide at room temperature for 30 min, followed by three washes with 100 μl Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.4).

Cell Fractionation

Fractionation of cells was based on a published procedure [16]. Briefly, the cell pellet was resuspended in 100 μl of TSL buffer (0.2 mM Tris, 20% (*w*/*v*) sucrose, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Following gentle agitation on ice for 20 min, the suspension was centrifuged at 14,000 rpm for 15 min, and the supernatant was removed as the soluble periplasmic fraction. The remaining pellet was resuspended in 100 μl TBS and subjected to three freeze/thaw cycles in liquid nitrogen. After further centrifugation at 14,000 rpm for 15 min, the supernatant was stored as the soluble cytoplasmic fraction, and the pellet was resuspended in 100 μl of 1.5% (*v*/*v*) Triton X-100 in TBS. Following gentle agitation at 4 °C for 1 h, the solution was centrifuged at 14,000 rpm for 15 min, and the supernatant was removed as the membrane fraction. Resuspension of the pellet in 100 μl of 8 M urea in TBS and gentle agitation for 1 h at 4 °C was followed by centrifugation at 14,000 rpm for 15 min, after which the supernatant was removed and used as the inclusion body fraction. SDS-PAGE, with or without the addition of 4% β-mercaptoethanol to reduce disulfide bonds, and immunoblotting were carried out according to standard procedures [28]. Immunoblotting was based on a published procedure [16], and blots were developed using an M1 anti-FLAG monoclonal antibody and monoclonal goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) or a monoclonal anti-polyhistidine peroxidase-conjugated antibody (Sigma-Aldrich).

Protein Purification

For purification of the wild-type MDP and mutant MDP C93A/C361A proteins, cells containing the relevant construct were cultured and induced to produce protein in 1–3 l cultures, as outlined above. The periplasmic protein fraction was prepared as described, with proportional increases in all reagent volumes. After dialysis against 5 l of TBS at 4 °C overnight, $CaCl_2$ was added to a final concentration of 20 mM for initial FLAG-based analyses of the two proteins. Samples were passed through a 0.45 μm filter to remove particulate material, following which they were applied to a 5-ml M1 FLAG immunoaffinity column, and target protein was eluted using five column volumes of 100 μg/ml FLAG peptide, according to the manufacturer's instructions. Eluted fractions were analyzed by SDS-PAGE and immunoblotting.

Purified samples of MDP C93A/C361A protein were applied to a Superdex 200 size exclusion column (GE Healthcare, Buckinghamshire, UK) that had been equilibrated with TBS buffer. Samples and standards (Sigma-Aldrich) were applied at a flow rate of 0.4 ml/min. Elution fractions of 1 ml were analyzed by SDS-PAGE and immunodetection to determine the multimeric state of the protein.

As the wild-type enzyme was undetectable in FLAG-based eluted fractions by SDS-PAGE or activity assay, a purification procedure was developed using Talon cobalt immobilized metal affinity chromatography (IMAC; Clontech, CA, USA) according to the manufacturer's instructions. Briefly, 200 ml of periplasmic protein fraction, prepared from a 3-l bacterial culture, was passed through a 10-kDa MW cut-off filter to change the buffer to 50 mM Na_3PO_4, 300 mM NaCl, pH 7.4. This was incubated with 1 ml of cobalt affinity resin for 2–3 h at 4 °C with gentle shaking. Following centrifugation to pellet the resin, it was washed twice in the same buffer (pH 7.4), and the resin with bound protein was poured onto the column and allowed to settle under gravity. After washing with five bed volumes of the same equilibration/wash buffer, the protein was eluted in equilibration/wash buffer containing 150 mM imidazole. Eluate fractions of 500 μl in Tris-buffer (pH 8.0) were collected, and the imidazole was removed immediately using a Sephadex G-25 column (Roche Biochemicals) or 10-kDa cut-off dialysis cassettes (Pierce). The protein concentration was determined by Bradford assay (BioRad) using bovine serum albumin as standard, and fractions were analyzed by SDS-PAGE and immunoblotting.

Activity Assay and In Vitro Characterization

The ability of wild-type and mutated MDP enzymes to hydrolyze Gly-D-Phe dipeptide was determined using purified protein or cellular periplasmic fractions according to a previously described procedure [29]. For kinetic analysis of purified protein, FLAG peptide was removed prior to assay, where appropriate, using a PD-10 column (Amersham Biosciences).

Aliquots (50 μl) of the protein-containing solution were added to 50 μl of 1 mM Gly-D-Phe in the wells of a black 96-well microplate (Greiner Bio-One). The plate was covered and incubated at 37 °C. When required, the reaction was stopped by the addition of 10 μl of 1 mM cilastatin. After 60 min, 50 μl of assay mix (15 U/ml horse radish peroxidase, 0.52 U/ml D-amino acid oxidase, 0.04 mg/ml flavin adenine dinucleotide, 2.1 mg/ml *p*-hydroxyphenylacetic acid in 0.1 M Tris–HCl, pH 8.0) was added. As high Gly-D-Phe concentrations disturbed the release of fluorescence, samples were diluted prior to addition of the assay mix. The plate was sealed and incubated for 40 min in the dark at 37 °C to develop the fluorescent signal. After the final incubation, the relative fluorescence units of each well were measured using a Genios fluorimeter (Tecan, Reading, UK) with an excitation wavelength of 320 nm and an emission wavelength of 410 nm. Reaction rates were measured in triplicate over a range of Gly-D-Phe concentrations for the first 3 min of the reaction, and data were fitted using Sigmaplot 9.0 (SYSTAT, London, UK), assuming Michaelis–Menten kinetics.

Results

Periplasmic Production of Wild-Type MDP

Initial expression of wild-type MDP in the *E. coli* periplasm led to accumulation of the recombinant polypeptide almost exclusively in insoluble form. Immunoblot analysis of total

cell extracts using an anti-polyhistidine antibody led to detection of two distinct bands in insoluble cellular fractions, with molecular weights of approximately 42 kDa (not shown). Only the lower band was detected using an M1 anti-FLAG reporter antibody, however, indicating that the larger product represented MDP polypeptide with an intact signal peptide prior to cytoplasmic membrane translocation. As the smaller processed product was the more intense of the two, it was clear that the majority of the target polypeptide successfully reached the periplasm after translation but subsequently became incorrectly folded. Furthermore, MDP production placed a very significant stress on the host cell, with host bacterial cultures entering stationary phase 2–3 h after induction was initiated (not shown).

As the majority of recombinant protein was insoluble and in the periplasm, the periplasmic chaperones *skp*, *fkpA*, and *surA* were co-expressed during MDP production, but no increase in the proportion of soluble protein resulted (not shown). Co-expression of cytoplasmic chaperones has also previously been demonstrated to improve periplasmic expression of recombinant proteins. Therefore, the DnaKJ GrpE and GroEL/ES cytoplasmic chaperone systems and trigger factor were investigated, but no improvement in MDP solubility was observed (data not shown).

Effect of Disulfide Bond Formation on MDP Production in *E. coli*

Immunoblot analysis of periplasmic protein preparations in the presence and absence of reducing agent revealed a smear of high molecular weight disulfide-linked recombinant polypeptide under non-reduced conditions (Fig. 1). The broad molecular weight range of the smear suggested the presence of multimers containing varying numbers of MDP monomers, arising from numerous different disulfide bridge arrangements. This observation that the recombinant protein was produced exclusively in multimeric complexes that reduced to a single band in the presence of reducing agents strongly indicated that disulfide bond formation is a critical, limiting step in the functional production of MDP in *E. coli*.

Co-production of *E. coli* disulfide isomerase DsbC was carried out to prevent formation of multimers or to promote their rearrangement into correctly folded monomers. This proved unsuccessful, as most of the target protein produced remained in insoluble form, and no change in disulfide patterns was detectable (not shown). DsbC co-production improved

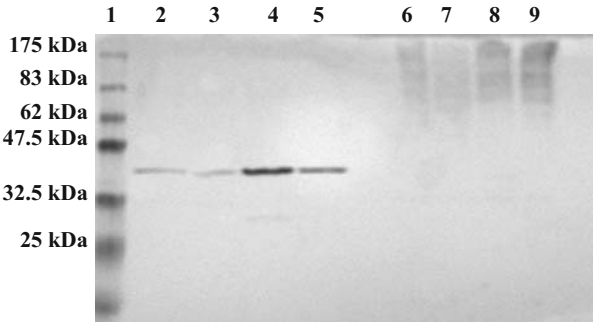

Fig. 1 SDS-PAGE and immunoblot analysis of MDP polypeptide produced in the *E. coli* periplasm, prepared in the presence and absence of reducing agent. *Lane 1* molecular weight markers, *lanes 2–5* MDP from soluble periplasmic protein preparations after 2, 3, 4, and 5 h of induction in the presence of reducing agent, *lanes 6–9* as for *2–5*, samples prepared in the absence of reducing agent. Protein was detected using an M1 anti-FLAG antibody

the growth of the expression culture, nevertheless, by significantly delaying the onset of the stationary phase (not shown). This allowed for an increase of approximately 50% in cell numbers to be achieved, with a proportional increase in the yield of target protein.

Production of MDP was also investigated in *E. coli* Origami (DE3) strain (Novagen), which allows stable disulfide bond formation in its cytoplasm due to its *trxB*$^-$ *gor*$^-$ genotype. In order to avoid blocking of membrane translocation by partially folded, disulfide-linked recombinant polypeptides destined for the periplasm, a signal sequence-less MDP gene was constructed for expression in the cytoplasm. Immunoblot analysis revealed, however, that all detectable recombinant product was insoluble (data not shown).

According to the structure of the human MDP homodimer [20], four of the six cysteine residues present in each monomer are involved in intramolecular (Cys226–Cys258 and Cys71–Cys154) disulfide bridges, while a Cys361–Cys361 intermolecular disulfide mediates dimerization. The remaining Cys93 thiol is not involved in disulfide bonding in the native molecule (Fig. 2). In order to reduce the possibility of formation of aberrant disulfide bridges in the MDP polypeptide during its production in *E. coli*, we exchanged Cys361 and Cys93, individually and in combination, for alanine residues. The Cys226–Cys258 and Cys71–Cys154 disulfide bridges that appear to have a role in stabilizing the walls of the active site [20] were left unchanged.

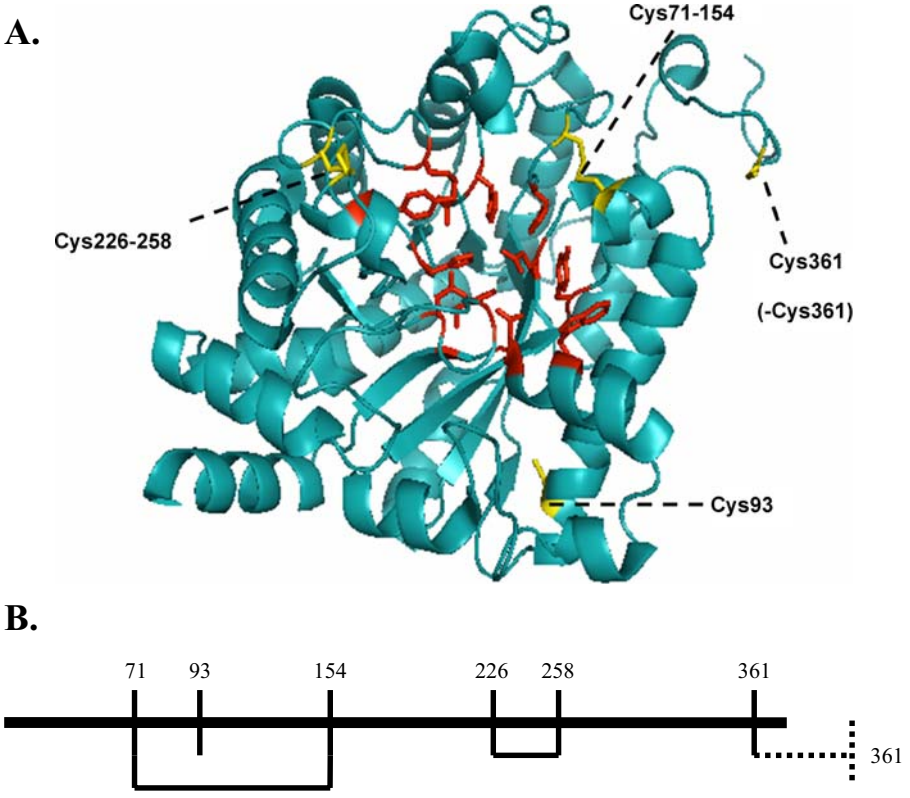

Fig. 2 **a** Structure of MDP monomer showing main chain residues in *cyan*, cysteine residues and disulfide bonds in *yellow*, and amino acid residues flanking the substrate-binding pocket in *red* (PDB: 1ITQ). The figure was generated using PyMOL (Delano Scientific). **b** Disulfide connectivity of MDP monomer

Production and Analysis of Cysteine-Mutated MDP Variants

Production and analysis of wild-type MDP and the MDP C93A, MDP C361A, and MDP C93A/C361A variants was carried out as described for the wild-type enzyme. SDS-PAGE and immunoblotting analysis of periplasmic fractions prepared in the absence of reducing agent revealed, in addition to the multimers observed with the wildtype, a product in cells producing the double mutant that migrated with the size expected of monomeric protein (not shown). This monomeric protein product was not detectable in cells producing either single mutant or, as outlined above, in the wild-type culture.

Comparison of the dipeptidase activity of periplasmic fractions prepared from equal numbers of cells producing the wild-type and all mutated proteins indicated that both single mutant proteins exhibited activities similar to the wildtype (Fig. 3). A four- to fivefold increase in activity was noted for the double mutant, however, which was thought likely to be due to an increased proportion of the polypeptide assuming the disulfide pattern of the native enzyme as a result of the reduced number of thiol groups present. The growth behavior of cells producing the wild-type and all mutated enzymes was similar (not shown).

In order to investigate the activity of the MDP C93A/C361A enzyme variant, it was produced and purified by affinity chromatography using its N-terminal FLAG tag (Fig. 4a). The dipeptidase activity of eluate fractions correlated with the appearance of the major protein product in SDS-PAGE gels (not shown). Investigation of the eluted protein in the presence and absence of reducing agent confirmed that it appeared to exist in a single disulfide state with a molecular weight that corresponded to monomeric MDP (Fig. 4b). Size exclusion chromatography was used to confirm that the purified MDP C93A/C361A variant corresponded in size to monomeric enzyme. Finally, kinetic characterization revealed that the enzyme followed Michaelis–Menten behavior closely, with a K_m and V_{max} for Gly-D-Phe of 2.51 mM and 149.6 μmol D-Phe/min/mg enzyme, respectively. A cobalt-based IMAC purification procedure was developed for purification of the wild-type enzyme, exploiting its C-terminal hexahistidine tag. This confirmed that there was no significant change in the activity of the cysteine-deficient enzyme variant relative to the wild-type enzyme: The K_m and V_{max} for Gly-D-Phe of the latter were calculated as 2.38 mM and 147.5 μmol D-Phe/min/mg enzyme, respectively. Comparative values for this

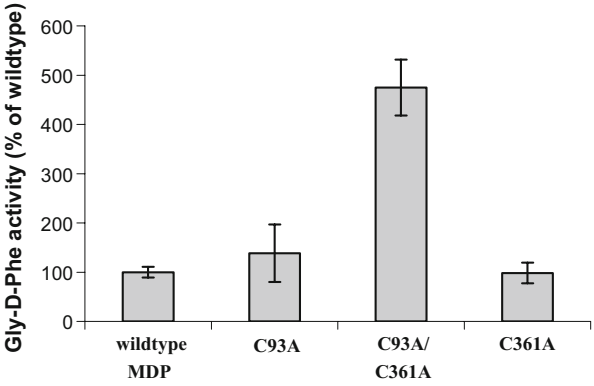

Fig. 3 Analysis of activity of wild-type MDP and cysteine-mutated MDP variants. Dipeptidase activity was measured in periplasmic protein fractions, prepared from MDP-, C93A-, C93A/C361A- and C361A-producing *E. coli* clones. Activity is expressed as a percentage of activity in the wild-type sample, and values are the mean of three independent measurements±the standard deviation. Free thiol groups were not blocked prior to fractionation

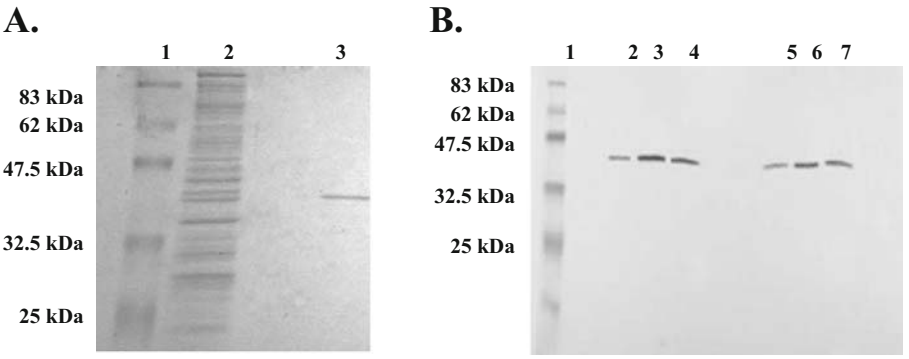

Fig. 4 Purification and analysis of MDP C93A/C361A enzyme variant. **a** Coomassie-stained SDS-PAGE gel showing M1 anti-FLAG-based purification of MDP C93A/C361A enzyme variant. *Lane 1* molecular weight markers, *2* column pre-binding fraction, *3* eluted sample. **b** Investigation of disulfide state of eluted MDP C93A/C361A protein by immunoblotting. *Lane 1* molecular weight marker, *lanes 2–4* MDP C93A/C361A elution fractions in the presence of reducing agent, *5–7* as for *2–4*, samples in the absence of reducing agent. Protein was detected using an M1 anti-FLAG antibody

substrate and reporting method are unavailable for the human enzyme purified from its natural source.

A time course production of the MDP C93A/C361A enzyme variant revealed that the mutated polypeptide also accumulated in high molecular weight protein aggregates in the *E. coli* periplasm, as the length of the induction period was increased (not shown). Co-production of DsbC did not increase the solubility of the double mutant enzyme or prevent its sequestering into non-native multimers (Fig. 5). The growth of host *E. coli* cells was again improved upon DsbC overproduction, however, which allowed for accumulation of greater amounts of target polypeptide (not shown). Interestingly, yields of the monomeric protein continued to increase for at least 5 h upon co-production of DsbC (Fig. 5), while the amount of soluble target protein showed a clear decrease after only 3 h of induction of the MDP C93A/C361A mutant alone (not shown).

Discussion

Initial production of MDP in the *E. coli* periplasm led to accumulation of the polypeptide in insoluble form, with the majority adjudged to occur in the periplasm, based on removal of

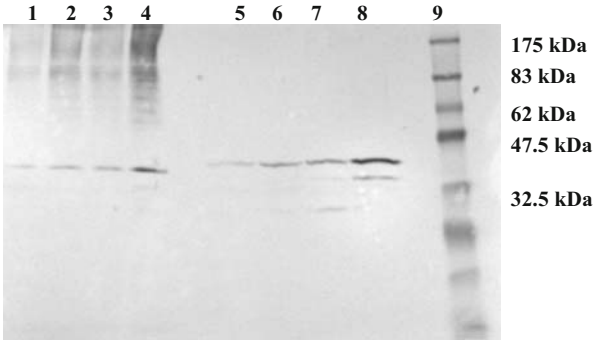

Fig. 5 Immunoblot-based detection of the MDP C93A/C361A mutant produced during overproduction of DsbC in *E. coli*. *Lanes 1–4* soluble periplasmic protein harvested after 2, 3, 4, and 5 h of induction, respectively, prepared without reducing agent, *lanes 5–8* as for *lanes 1–4*, but with reducing agent, *lane 9* molecular weight markers. Protein was detected using an M1 anti-FLAG antibody

the OmpA leader peptide. The low yield of functional protein due to insolubility of the target polypeptide was exacerbated by the stress that production of MDP placed on the host cell: Host *E. coli* cultures entered stationary phase 2–3 h after induction, which further limited the ability to accumulate functional protein through extended induction periods.

Co-expression of periplasmic chaperones *skp*, *fkpA*, and *surA*, that have previously been found to overcome bottlenecks in heterologous production in *E. coli*, led to no increase in the solubility of the MDP protein in this work. As both *fkpA* and *surA* demonstrate peptidyl *cis*/*trans* isomerase activity in addition to their chaperone capabilities [27], the orientation of proline residues in the polypeptide is therefore not the primary bottleneck in the folding process. Co-expression of cytoplasmic chaperones has also been demonstrated to improve periplasmic expression of recombinant proteins, possibly through maintaining precursor polypeptides in a soluble state and releasing them to the membrane translocation machinery in a more controlled manner [30, 31], and so, the DnaKJ GrpE and GroEL/ES cytoplasmic chaperone systems and trigger factor were also investigated. Again, however, this conventional approach of chaperone overproduction was unable to increase the proportion of MDP polypeptide produced in soluble form.

As the occurrence of aberrant disulfides in multiply disulfide-bridged recombinant targets is well documented [8, 11, 12, 32–34], the potential role of disulfide bridges in the misfolding of MDP was investigated. This revealed that the target protein was produced in the form of high molecular weight, disulfide-linked complexes that reduced to a single detectable band in the presence of reducing agent, but that no monomeric or Cys361-linked dimeric products were detectable by immunoblotting in non-reduced samples. The broad molecular weight range of the aggregates suggested the occurrence of numerous different arrangements of disulfide bridges, including bonds that are not found in the native MDP molecule, and strongly indicated that disulfide bond formation is a critical, limiting step in the functional production of MDP in *E. coli*.

Somewhat surprisingly, given the apparent prominence of non-native disulfide bridges within the target polypeptide population, overproduction of *E. coli* disulfide isomerase DsbC did not prevent the sequestering of polypeptide into non-native multimers or promote rearrangement of protein multimers into correctly folded molecules in the periplasm. This is in spite of the previous report that DsbC is required for correct folding in *E. coli* of many eukaryotic proteins with nonconsecutive disulfide bridges [10, 12]—such as that between Cys71 and Cys154 in MDP in this work. A benefit of DsbC overproduction, nevertheless, was a significant delay in the onset of the stationary phase in the producing culture, as reported previously with other heterologous proteins [16, 35], which allowed for an increase in accumulation of target protein due to the increase in cell density in the culture.

In order to reduce the potential for formation of aberrant disulfide bridges during MDP production in *E. coli*, Cys361, which is responsible for dimerization through a Cys361–Cys361 intermolecular bond, and Cys93, which is not involved in disulfide bonding in native MDP, were mutated individually and in combination to alanine residues. Mono- or dimeric protein products were not detectable in cultures producing either single mutant, in common with the wild-type culture. Cellular fractions from clones producing the singly mutated enzymes also exhibited similar activities to wild-type cultures, in contrast with a previous mutagenic analysis of recombinant porcine MDP, which reported a 52% loss in activity in a Cys93-mutated enzyme, while a Cys361-deficient enzyme retained full activity as a monomeric enzyme [36]. Of most interest in the present study, however, was that expression of the double cysteine-mutated enzyme in *E. coli* led to the appearance of a polypeptide product of the size of monomeric protein. The purified protein was confirmed by size exclusion chromatography to occur exclusively in a monomeric state, while analysis

in the presence and absence of reducing agent revealed that it existed in the form of a single, presumably native-like, disulfide state. An almost fivefold increase in activity was detected in the periplasm of cultures producing the MDP double mutant. It was initially unclear whether this was due to improved folding and production in *E. coli*, due to a greater probability of the polypeptide assuming the disulfide pattern of the native enzyme as a result of the reduced number of thiol groups present or a change in activity of the mutated protein.

Therefore, purification and kinetic analysis of the wild-type and double mutant enzymes in vitro was carried out. This confirmed that the hydrolytic activity of the cysteine-deficient enzyme variant against Gly-D-Phe dipeptide was unchanged. While direct comparison with the native human dipeptidase is not possible due to lack of comparative data for this assay method, this in vitro analysis nevertheless confirms the applicability of the approach to optimize expression of MDP in *E. coli* and its potential usefulness in producing heterologous proteins in the bacterium in general. The improved functional yield of the modified enzyme also makes it possible to carry out mutagenesis and screening for modified activity in *E. coli*.

A cysteine-removal approach has also been successfully applied to engineer human tissue factor for improved recombinant production [37]. In this case, the sole cytoplasmic cysteine residue of the membrane-bound protein was mutated, leading to reduced formation of disulfide-linked multimers during its production in *E. coli* and a twofold increase in specific activity, the latter most likely due to production of a greater proportion of the purified protein in a correctly folded active form. Similarly, in a detailed analysis of overproduction of a native phytase enzyme in *E. coli*, removal of single cysteine residues led to significantly diminished activity in the mutated enzymes, whereas further mutagenesis of the newly unpaired cysteines restored significant activity to the enzymes by reducing the formation of incorrect disulfide bonds during their production [12]. These reports, together with the present study, indicate that the mutagenesis of cysteine residues not involved in disulfide bridge formation—or residues involved in structurally inessential disulfide bridges—may be broadly useful in improving the expression of multiply disulfide-linked recombinant proteins in *E. coli* by reducing the formation of disulfide-linked, non-native multimers during production.

A more detailed investigation of MDP production in *E. coli* revealed that the double cysteine mutant also accumulated in high molecular weight protein aggregates in the periplasm as the induction time was extended and an increased polypeptide concentration resulted. The ratio of non-native, multi- to monomeric protein molecules increased with longer induction periods, indicating that the double mutant enzyme appeared to be subject to the same aggregation phenomenon as the wild-type enzyme, albeit at higher polypeptide concentrations.

Overproduction of DsbC was investigated as a tool to isomerize the reduced number of non-native disulfide combinations possible in the double cysteine mutant also—even though the removal of Cys93 rendered consecutive the Cys71–Cys154 bridge, which was previously the only nonconsecutive bridge in the wild-type enzyme. The major consequence of DsbC overproduction was to extend the time period during which the MDP double mutant accumulated in soluble, monomeric form from 3 to over 5 h, while the ratio between mono- and multimeric polypeptides also remained unchanged in the periplasm throughout this 5-h analysis period. This suggests that further extension of this production period would be possible in order to accumulate more target protein, though the effect of the isomerase ultimately is likely be to delay rather than prevent the appearance of disulfide-mediated aggregates of the mutated MDP in *E. coli*. Finally, the growth of the

host *E. coli* cells was again improved by DsbC overproduction, which allowed for accumulation of greater amounts of target polypeptide during extended induction periods, as previously observed with the wild-type protein.

In summary, mutagenesis of cysteine residues that are not involved in disulfide bridge formation greatly improved the production of a human dipeptidase in this work. This approach may be broadly useful in improving expression and, thus, increasing the functional yields of many multiply disulfide-linked recombinant proteins in *E. coli*, such as receptors and transporters that are notoriously cysteine rich and difficult to produce in the bacterium.

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References

1. Rai, M., & Padh, H. (2001). *Current Science*, *80*, 1121–1128.
2. Spada, S., & Walsh, G. (2004). *Directory of approved biopharmaceutical products*. Boca Raton: CRC.
3. Ito, M., Yamagoe, S., Tomizawa, K., Mizuno, S., Tanokura, M., & Suzuki, K. (1997). *Cytotechnology*, *25*, 235–238. doi:10.1023/A:1007976103088.
4. Kurokawa, Y., Yanagi, H., & Yura, T. J. (2001). *The Journal of Biological Chemistry*, *276*, 14393–14399. doi:10.1074/jbc.M104341200.
5. Kolade, O. O., Bamford, V. A., Ancillo Anton, G., Jones, J. D., Vera, P., & Hemmings, A. M. (2006). *Biochimica et Biophysica Acta*, *1764*, 1043–1053.
6. Kiedzierska, A., Czepvzynska, H., Smietana, K., & Otlewski, J. (2008). *Protein Expression and Purification*, *60*, 82–88. doi:10.1016/j.pep.2008.03.019.
7. Xu, C. G., Fan, X. J., Fu, Y. J., & Liang, A. H. (2008). *Protein Expression and Purification*, *59*, 103–109. doi:10.1016/j.pep.2008.01.009.
8. Qiu, J., Swartz, J. R., & Georgiou, G. (1998). *Applied and Environmental Microbiology*, *64*, 4891–4896.
9. Winter, J., Neubauer, P., Glockshuber, R., & Rudolph, R. (2001). *Journal of Biotechnology*, *84*, 175–185. doi:10.1016/S0168-1656(00)00356-4.
10. Joly, J. C., & Swartz, J. R. (1997). *Biochemistry*, *36*, 10067–10072. doi:10.1021/bi9707739.
11. Ito, M., Nagata, K., Kato, Y., Oda, Y., Yamagoe, S., Suzuki, K., & Tanokura, M. (2003). *Protein Expression and Purification*, *27*, 272–278. doi:10.1016/S1046-5928(02)00634-4.
12. Berkmen, M., Boyd, D., & Beckwith, J. (2005). *The Journal of Biological Chemistry*, *280*, 11387–11394. doi:10.1074/jbc.M411774200.
13. Bardwell, J. C. (1994). *Molecular Microbiology*, *14*, 199–205. doi:10.1111/j.1365-2958.1994.tb01281.x.
14. Sone, M., Akiyama, Y., & Ito, K. (1997). *The Journal of Biological Chemistry*, *272*, 10349–10352. doi:10.1074/jbc.272.16.10349.
15. Beckwith, J. (2007). *Genetics*, *176*, 733–740. doi:10.1534/genetics.107.076240.
16. Hu, X., O'Dwyer, R., & Wall, J. G. (2005). *Journal of Biotechnology*, *120*, 38–45. doi:10.1016/j.jbiotec.2005.05.018.
17. Joly, J. C., Leung, W. S., & Swartz, J. R. (1998). *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 2773–2777. doi:10.1073/pnas.95.6.2773.
18. Kang, S. H., Kim, D. M., Kim, H. J., Jun, S. Y., Lee, K. Y., & Kim, H. J. (2005). *Biotechnology Progress*, *21*, 1412–1419. doi:10.1021/bp050087y.
19. Kera, Y., Liu, Z., Matsumoto, T., Sorimachi, Y., Nagasaki, H., & Yamada, R. H. (1999). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *123*, 53–58. doi:10.1016/S0305-0491(99)00039-5.
20. Nitanai, Y., Satow, Y., Adachi, H., & Tsujimoto, M. (2002). *Journal of Molecular Biology*, *321*, 177–184. doi:10.1016/S0022-2836(02)00632-0.
21. Kropp, H., Sundelof, J. G., Hajdu, R., & Kahan, F. M. (1982). *Antimicrobial Agents and Chemotherapy*, *22*, 62–70.
22. Bagshawe, K. D., Sharma, S. K., & Begent, R. H. (2004). *Expert Opinion on Biological Therapy*, *4*, 1777–1789. doi:10.1517/14712598.4.11.1777.

23. Grant, J. W., & Smyth, T. P. (2004). *The Journal of Organic Chemistry*, *69*, 7965–7970. doi:10.1021/jo048970i.
24. Ge, L., Knappik, A., Pack, P., Freund, C., & Plückthun, A. (1995). In C. A. K. Borrebaeck (Ed.), *Antibody engineering* (2nd ed., pp. 229–266). London: Oxford University Press.
25. Nishihara, K., Kanemori, M., Yanagi, H., & Yura, T. (2000). *Applied and Environmental Microbiology*, *66*, 884–889. doi:10.1128/AEM.66.3.884-889.2000.
26. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York: Cold Spring Harbor Laboratory.
27. Baneyx, F., & Mujacic, M. (2004). *Nature Biotechnology*, *22*, 1399–1408. doi:10.1038/nbt1029.
28. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.
29. Heywood, S. P., & Hooper, N. M. (1995). *Analytical Biochemistry*, *226*, 10–14. doi:10.1006/abio.1995.1184.
30. Pérez-Pérez, J., Martínez-Caja, C., Barbero, J. L., & Gutiérrez, J. (1995). *Biochemical and Biophysical Research Communications*, *210*, 524–529. doi:10.1006/bbrc.1995.1691.
31. Hu, X., O'Hara, L., White, S., Magner, E., Kane, M., & Wall, J. G. (2007). *Protein Expression and Purification*, *52*, 194–201. doi:10.1016/j.pep.2006.08.009.
32. Zhang, Z., Li, Z. H., Wang, F., Fang, M., Yin, C. C., Zhou, Z. Y., et al. (2002). *Protein Expression and Purification*, *26*, 218–228. doi:10.1016/S1046-5928(02)00502-8.
33. Maskos, K., Huber-Wunderlich, M., & Glockshuber, R. (2003). *Journal of Molecular Biology*, *325*, 495–513. doi:10.1016/S0022-2836(02)01248-2.
34. Leinweber, B., Barofsky, E., Barofsky, D. F., Ermilov, V., Nylin, K., & Beckman, J. S. (2004). *Free Radical Biology & Medicine*, *36*, 911–918. doi:10.1016/j.freeradbiomed.2003.12.021.
35. Kondo, A., Kohda, J., Endo, Y., Shiromizu, T., Kurokawa, Y., Nishihara, K., et al. (2000). *Journal of Bioscience and Bioengineering*, *90*, 600–606. doi:10.1263/jbb.90.600.
36. Keynan, S., Habgood, N. T., Hooper, N. M., & Turner, A. J. (1996). *Biochemistry*, *35*, 12511–12517. doi:10.1021/bi961193z.
37. Paborsky, L. R., Tate, K. M., Harris, R. J., Yansura, D. G., Band, L., McCray, G., et al. (1989). *Biochemistry*, *28*, 8072–8077. doi:10.1021/bi00446a016.