C-terminal propeptide of the *Caldariomyces fumago* chloroperoxidase: an intramolecular chaperone?

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Abstract The *Caldariomyces fumago* chloroperoxidase (CPO) is synthesised as a 372-aa precursor which undergoes two proteolytic processing events: removal of a 21-aa N-terminal signal peptide and of a 52-aa C-terminal propeptide. The *Aspergillus niger* expression system developed for CPO was used to get insight into the function of this C-terminal propeptide. *A. niger* transformants expressing a CPO protein from which the C-terminal propeptide was deleted failed in producing any extracellular CPO activity, although the CPO polypeptide was synthesised. Expression of the full-length gene in an *A. niger* strain lacking the KEX2-like protease PclA also resulted in the production of CPO cross-reactive material into the culture medium, but no CPO activity. Based on these results, a function of the C-terminal propeptide in CPO maturation is indicated.

Key words: Chloroperoxidase; Chaperone; Processing; C-terminal propeptide; KEX2; Filamentous fungi; Haem

1. Introduction

Chloroperoxidase (CPO) is a glycosylated haemoprotein secreted by the fungus *Caldariomyces fumago* [1,2]. In vivo, this enzyme is involved in the synthesis of caldariomycin, a halogenated compound with antimicrobial function [3]. In vitro, CPO catalyses a number of useful chemical reactions such as enantioselective oxygen-transfer reactions, H_2O_2 disproportionations and oxidative halogenations [4].

The molecular genetics of the *C. fumago* CPO has been extensively characterised by Hager and co-workers. The protein is encoded by a single-copy gene [5] and CPO synthesis is controlled at the mRNA level, fructose being an inducing and glucose a repressing carbon-source [6]. The gene product undergoes a number of post-translational modifications [7]. A 21-aa signal sequence is removed from the CPO precursor and the resulting N-terminal glutamic acid is converted into a pyroglutamic acid. Carbohydrate moieties are incorporated at *N*- and *O*-glycosylation sites. Different isozymes are found in the extracellular medium of *C. fumago* cultures [8,9], which are indicated to be glycosylation variants of the same polypeptide [2,7]. In the major CPO isozyme, the most heavily glycosylated, three *N*- and 11 *O*-glycosylation sites are occupied with a total of 21 sugar groups [10]. Other modifications include deamidation of three amidic residues to the corresponding acids [7], one disulfide bond formation and the incorporation of a protoporphyrin XI (haem b) molecule [10]. Resolution of the crystal structure of the mature protein has shown that the haem pocket in CPO shares features of both classical peroxidases and cytochrome P450s. At the proximal site of the haem pocket, the haem is coordinated via a thiolate ligand to a cysteine, resembling the cytochrome P450, whereas at the distal site, polar residues are found similar to other haem peroxidases [10]. The crystal structure also revealed that the last 52 aa of the primary translation product are lacking in the mature protein, suggesting that CPO also undergoes proteolytic processing at the C-terminus. This processing apparently occurs downstream of a dibasic (KR) processing site [10].

C-terminal propeptides are found in intracellular and secreted proteins and, although in many cases no function has been ascribed to these sequences, they have been suggested to participate in processes such as protein folding, targeting and secretion (for examples see [11–17]). The function of the C-terminal propeptide of CPO is completely unknown. Here, we describe approaches to get more insight into the function of the CPO C-terminal propeptide using the *Aspergillus niger* expression system recently developed for CPO [18].

2. Materials and methods

2.1. Expression cassettes

Fig. 1 shows the expression cassettes used in this study. Construction of pCPO3.I-AmdS is described previously [18]. Expression vector pCPOΔ-AmdS which contains the *cpo* translation product lacking most of the C-terminal propeptide, was constructed by amplifying the corresponding sequence in the *cpo* gene present in pCf6 (kindly provided by Dr. Hager, University of Illinois at Urbana-Champaign) with primers CLP15E/A (5′-GGAATTCACATGTTCTCCAAGG-TCC-3′) and CLP3B/H (5′-CGGGATCCAAGCTTCCCATGGAG-GTGGTGG-3′). The amplification product was digested with *Afl*III/*Hin*dIII and cloned into the pAN52-10Not *Aspergillus* expression vector (carrying the *A. niger glaA* promoter; Punt, unpublished) at the *Nco*I/*Hin*dIII cloning sites. After confirming the amplified fragment by sequencing, the *Aspergillus nidulans AmdS* selection marker [19] was introduced as a 4.5-kb *Not*I fragment at the unique *Not*I site of the resulting vector.

2.2. Fungal strains and transformation procedures

A. niger MGG029 (*prtT, gla::fleo*^{r}*, pyrG*; [20]) and *A. niger* AB1.13-ΔpclA (*pclA::pyrG*^{mut*}*, prtT, pyrG*; Punt et al., unpublished) were used a recipient strains in transformation experiments. *AmdS*/*pyrG*-based fungal transformations were carried out as described [18].

2.3. Plate assays

Transformants were analysed for CPO production by means of the

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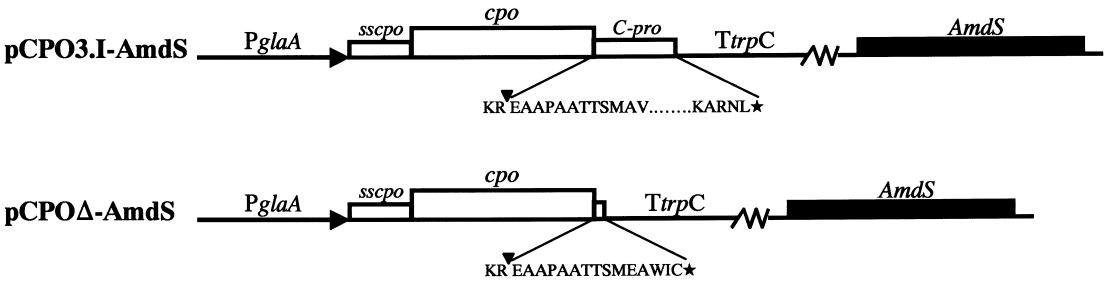

Fig. 1. Expression cassettes. P*glaA*: *A. niger* glucoamylase promoter; T*trp*C: *A. nidulans* tryptophan terminator, *cpo*: CPO, *sscpo*: signal sequence CPO (21 aa); C-pro: C-terminal propeptide (52 aa); KR: splicing site for the C-terminal propeptide; *AmdS*: *A. nidulans* acetamidase selection marker; ★: stop codon.

o-anisidine plate assay [18], and in situ immunodetection using sandwich cultures as described [21]; strains were grown between two polycarbonate filters on plates containing *Aspergillus* minimal medium [22]. After 24-h incubation at 30°C, a polyvinylidene fluoride (PVDF) membrane, previously activated with methanol and rinsed with sterile distilled water, was placed between the agar surface and the polycarbonate sandwich and the plates were incubated for another 24 h. The PVDF membrane, harbouring secretion proteins, was subsequently removed, rinsed with water and subjected to immunodetection.

2.4. Shake-flask experiments

Strains were grown from conidial inocula in 300 ml shake-flasks containing 50 ml AMM-maltose [20]. Medium samples were taken at 24, 48 and 72 h after inoculation and analysed for CPO activity using the standard monochlorodimedon (MCD)-halogenating reaction [3] and for CPO protein production by Western blotting [23] using a CPO polyclonal antiserum [18].

3. Results and discussion

To study the production of the *C. fumago* CPO in *A. niger*, we first constructed an expression vector based on the sequence information present in GenBank (accession number X04486). Later sequencing data revealed a mistake in the published *cpo* sequence (an extra C at position 1030, close to the putative stop codon). Removal of the extra nucleotide caused a shift in the reading frame, resulting in a translational product 52 aa longer than previously deduced (accession number AJ300448). Comparison of the newly deduced sequence with that available from the determination of the CPO crystal structure [10] showed that the last 52 aa were not present in the mature protein, indicating that these form a C-terminal propeptide which is removed somewhere in the secretion pathway. As a consequence of this sequence error, our CPO expression vector, now termed pCPOΔ-AmdS, lacked most of the C-terminal propeptide (41 aa), and a new CPO expression vector, pCPO3.I-AmdS, containing the full-length *cpo* sequence, was generated for our expression studies [18]. However, pCPOΔ-AmdS was used to study the function of this prosequence using the *A. niger* expression system. Interestingly, analysis of the C-terminal propeptide sequence showed the presence of two basic amino acid residues (KR) directly upstream of the processing site. As this motif resembles the recognition sequence for cleavage by Golgi-located protein convertases such as the KEX2 protease [24], we also studied the production of CPO in an *A. niger* strain in which the gene encoding this type of protease, termed *pclA*, was deleted. This strain, *A. niger* AB1-13-ΔpclA, although it shows a compact growth phenotype, retains the wild-type secretion capacity (Punt et al., unpublished) and was shown to secrete active *Phanerochaete chrysosporium* manganese peroxidase (MnP) when transformed with the MnP expression vector pMnP1.I ([20] and results not shown).

A. niger MGG029, the production strain used for expression of the full-length CPO [18], was transformed with pCPOΔ-AmdS, encoding the C-terminal deletion form of CPO, and *A. niger* AB1.13-Δ-pclA was transformed with the full-length CPO construct pCPO3.I-AmdS. Transformants were selected by their ability to grow and sporulate on acetamide-containing agar plates and were screened for secretion of active CPO using the *o*-anisidine plate assay. *A. niger* MGG029 transformants expressing the full-length CPO construct developed a purple halo in this assay [18]. No colour formation was visible for any of the analysed MGG029[pCPOΔ-AmdS] and AB1.13-ΔpclA[pCPO3.I-AmdS] transformants, indicating the absence of extracellular peroxidase activity in these strains. To study whether these transformants secreted any CPO protein at all, we used the sandwich mode of culture, as described [21], to monitor protein secretion in situ. This method has shown to be useful in detecting secretion of heterologous proteins that are rapidly degraded in the extracellular medium [25]. Using this technique, αCPO cross-reactive material could be detected at the place of colony growth of MGG029[pCPO3.I-AmdS]-, MGG029[pCPOΔ-AmdS]- and AB1.13-ΔpclA- [pCPO3.I-AmdS]-containing transformants. No cross-reactivity was observed for the parental strains MGG029 and AB1.13-ΔpclA. The results of both activity and secretion analysis are summarised in Table 1. From this result, we concluded that the CPO was synthesised and secreted in the three types of transformants and we selected the representative strains

Table 1
Analysis of CPO production in *A. niger* strains MGG029 and AB1.13-ΔpclA[a]

Expression cassette	MGG029		AB1.13-ΔpclA	
	secretion[b]	activity	secretion	activity
pCPO3.I-AmdS	+	+	+	-
pCPOΔ-AmdS	+	-	nd[c]	nd
pMnP1.I	+	+	+	+

[a]CPO production was determined for at least four transformants per expression cassette. In all cases co-transformation with pAB4-1, carrying the pyrG selection marker, was carried out.
[b]Secretion and activity of CPO was determined on agar plates by in situ immunodetection and *o*-anisidine test respectively (for details see Section 2).
[c]nd, not determined.

MGG029[pCPO-AmdS]#3 and AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 together with the previously characterised MGG029-[pCPO3.I-AmdS]#5 [18] to further analyse CPO production in liquid medium.

Under inducing conditions, strain MGG029[pCPO3.I-AmdS]#5 secretes active recombinant CPO (rCPO) into the extracellular medium [18]. Accordingly, when grown in shake-flasks containing maltose as the carbon source, CPO activity (MCD halogenation, [3]) was observed in the culture broth of strain MGG029[pCPO3.I-AmdS]#5 and rCPO was readily detected by Western blotting as a strong band, migrating slightly slower than native CPO (nCPO; Fig. 2, lanes 1 and 6). Confirming the results of the plate assays, no MDC-halogenating activity was present in the extracellular medium of strains MGG029[pCPOΔ-AmdS]#3 or AB1.13-ΔpclA[pCPO3.I-AmdS]#A3. However, both strains showed multiple αCPO cross-reactive bands when analysed by SDS–PAGE (Fig. 2, lanes 2 and 4). In particular, in strain AB1.13-ΔpclA-[pCPO3.I-AmdS]#A3, the CPO cross-reactive material appeared as an intense smear (Fig. 2, lane 4). As the CPO protein synthesised in MGG029[pCPOΔ-AmdS]#3 lacks most of the C-terminal propeptide, we concluded from these results that this sequence is required to ensure the production of active rCPO in *A. niger*.

Also, a non-active CPO polypeptide is secreted in transformant AB1.13-ΔpclA[pCPO3.I-AmdS]#A3. Strain AB1.13-ΔpclA lacks the KEX2-like protease PclA, which in *A. niger* is known to be responsible for cleavage of the glucoamylase propeptide (Punt et al., unpublished). Expression of the full-length CPO construct in this strain resulted in secretion of an inactive CPO, suggesting that the PclA protease is also involved in the maturation of the CPO polypeptide. As a dibasic site (KR), similar to the PclA recognition motif, is present at the site of processing of the C-terminal propeptide, we postulate that PclA is directly involved in the cleavage of this sequence. A possible explanation for the results obtained in these experiments is that the C-terminal propeptide in CPO is required to ensure correct protein maturation, whereas its removal at a later stage during secretion is required to yield an active CPO protein. The C-terminal sequence may thus be considered to have a chaperone-like function for the CPO protein. The SDS–PAGE migrating behaviour of CPO in MGG029[pCPOΔ-AmdS]#3 and AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 as multiple or diffuse bands is not exactly understood. These multiple bands could originate from aggregation, heterogeneous glycosylation and/or incorrect processing of the incorrectly maturated CPO polypeptide. Addition of haem in the culture medium, which increases the yield of CPO in wild-type strains producing full-length CPO [18], did not result in secretion of an active CPO in either MGG029[pCPOΔ-AmdS]#3 or AB1.13-ΔpclA[pCPO3.I-AmdS]#A3 (results not shown), suggesting that these strains do not secrete CPO molecules capable of activation by exogenous haem.

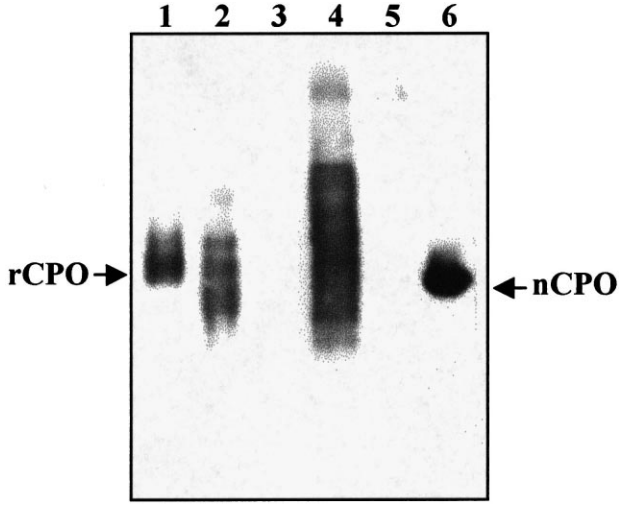

Fig. 2. Western blot analysis of CPO production. Medium samples of an AMM-maltose culture 48 h after inoculation. Lane 1: MGG029[pCPO3.I-AmdS]#5; lane 2: MGG029[pCPOΔ-AmdS]#3; lane 3: MGG029 transformed with selection markers *pyrG* and *AmdS*; lane 4: AB1.13-ΔpclA[pCPO3.I-AmdS]#A3; lane 5: AB1.13-ΔpclA transformed with selection markers *pyrG* and *AmdS*; lane 6: *C. fumago* CPO. Migrating positions of rCPO and native CPO (nCPO) are indicated.

C-terminal propeptides have been identified for a number of secretory proteins. For example, bacterial proteases such as the *Serratia marcescens* serine protease (SSP) and the *Thermus thermophilus* aqualysin I are synthesised as large precursors with removeable of sequences at both N- and C-termini. The C-terminal pro-region in the SSP has shown to have different functional domains that function in protein folding and secretion [12], whereas the aqualysin I C-terminal propeptide may have a role in stabilising an unfolded conformation to facilitate translocation across the cell membrane [14]. These C-terminal sequences, however, are much longer than that of the *C. fumago* CPO and no structural relation is evident from sequence comparison. Interestingly, C-terminal propeptides have been reported for the horseradish peroxidase [26,27] and several fungal laccases ([28,29] and references therein), and the existence of a C-terminal propeptide in the *Curvularia inaequalis* vanadium CPO has been also postulated [30]. The biological function of these sequences has not been investigated at an experimental level, although for the vanadium chloroperoxide propeptide, a role in secretion has been suggested [30]. The CPO C-terminal propeptide also shows no sequence homology with any of these peptides. Our results, however, indicate a role of this C-terminal sequence in providing CPO with its active conformation. To establish the mechanism in which this would operate and whether there is any interaction with the incorporation of the haem prosthetic group needs further research.

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