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# Mitochondrial cytochrome *c* synthase: CP motifs are not necessary for heme attachment to apocytochrome *c*

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ABSTRACT

**The function of holocytochrome *c* synthase (HCCS, also called heme lyase) is to attach covalently the heme cofactor to cytochromes *c* in the mitochondria of animals, fungi and protozoa. Little is known about how the protein functions but CP motifs, commonly found in heme-binding proteins, are present. Here we examine holocytochrome *c* production by *Saccharomyces cerevisiae* HCCS in the *Escherichia coli* cytoplasm with emphasis on the conserved CP motifs long implicated in heme transfer by this enzyme. Unexpectedly, the two motifs, both towards the N-terminus, were not required for activity. Mutations in HCCS on the C-terminal side of the CP motifs, known to cause disease states in humans (microphthalmia with linear skin defects) abolished or drastically attenuated holocytochrome *c* production.**



## 1. Introduction

The biosynthesis of cytochrome *c* is a catalysed post-translational modification performed by one of several known protein systems that vary between organisms [1–3]. Holocytochrome *c* is produced when two thioether bonds are formed between the thiols of two cysteines in a characteristic CXXCH motif in the apocytochrome and the vinyl groups of heme. In animal, fungal and protozoan mitochondria cytochrome *c* production relies on the protein holocytochrome *c* synthase, HCCS (also called heme lyase). HCCS has a well documented role in the import of the apocytochrome into the mitochondrion [4,5], but its role in the covalent attachment reaction of heme to the protein is poorly understood.

A heme-binding role for HCCS might be expected as heme is one of the two substrates. Such binding has been suggested in a study indicating heme binding to a CPV motif in *Saccharomyces cerevisiae* $HCC_1S$ [6], the protein required for heme attachment to cytochrome $c_1$ in fungi. CP sequences have been described as heme-regulatory motifs (HRM) in a variety of proteins. By binding to a HRM, heme regulates the activity of transcription factors (for example, in the transcriptional repressor Bach1 [7]) as well as mitochondrial protein import. As part of the regulation of heme biosynthesis, δ-aminolevulinate synthase import into the mitochondrion is inhibited by heme [8]. Direct binding of the heme by the cysteines in these motifs has been indicated; heme-binding studies with HRM peptides have shown that the cysteines are essential for interaction with heme [9]. Replacement of the proline in the motif also appeared to change the affinity of heme binding [9]. Mutations in the HCCS gene, elsewhere than in the sequences coding for CP motifs, have been identified in patients suffering from MLS (microphthalmia with linear skin defects), a condition causing abnormal development of the eyes and skin [10]. The amino acid replacements are thought to affect cytochrome *c* production such that apoptosis signalling is altered; cytochrome *c* release from the mitochondrion is key to initiating apoptosis [11]. The CP motifs and the amino acids associated with MLS are shown in the sequence alignment of HCCS proteins from a range of organisms in Fig. 1. Residues replaced in this work are highlighted.

Here we utilise an assay system for HCCS by coexpression of the protein with a cytochrome *c*, from a single plasmid, in the *Escherichia coli* cytoplasm [12]. The advantage of this approach, over studying HCCS activity in yeast, is that the combination of HCCS functions, including apocytochrome *c* import, heme acquisition and thioether bond formation, can be separated. In the mitochondrial intermembrane space heme has to be imported from the matrix, where it is synthesised, and apocytochrome polypeptides have to be imported from the cytosol. In *E. coli* the situation is simplified as all components are produced in the same compartment, allowing a focussing on the heme attachment reaction.

*Abbreviations:* HCCS, holocytochrome *c* synthase; $HCC_1S$, holocytochrome $c_1$ synthase; HRM, heme regulatory motif; MLS, microphthalmia with linear skin defects

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**Fig. 1.** Multiple sequence alignment of holocytochrome *c* synthase proteins. Amino acids replaced in this work are underlined, highlighted and in bold. Sc is *S. cerevisiae* GI number 595545; Nc is *Neurospora Crassa* GI number 168804; Ca is *Candida albicans* GI number 1438967; Hs is *Homo sapiens* GI number 285002259; Mm is *Mus musculus* GI number 225543251; Ce is *Caenorhabditis elegans* GI number 3879529.

## 2. Materials and methods

### 2.1. Plasmids and strains

Mutations were introduced by site-directed mutagenesis into the *S. cerevisiae* holocytochrome *c* synthase gene in the plasmid pWT cytochrome *c* [12], producing plasmids pRM01-09. Quikchange PCR mutagenesis (Stratagene) with KOD DNA polymerase (Stratagene) was used. A plasmid expressing a His-tagged form of HCCS (pRM15) was produced by insertional mutagenesis introducing codons for 6 C-terminal histidine residues into the HCCS gene in pWT cytochrome *c*. The MLS mutations were also performed in pRM15 producing pRM17-20. The plasmids are listed in Table 1; all DNA constructs were sequenced before use (Geneservice, Oxford). *E. coli* strain DH5α was used for routine DNA manipulations. The activity assays were performed in *E. coli* strain BL21(DE3).

### 2.2. Culture conditions and bacterial fractionation

HCCS activity was determined in the *E. coli* cytoplasm using a plasmid that coexpresses the *S. cerevisiae* HCCS (and variants) and *Equus caballus* cytochrome *c* [12], transformed into *E. coli* BL21(DE3). Bacteria were cultured in Luria Bertani medium supplemented with 100 μg/ml ampicillin and grown for 18–20 h at 37 °C. For some variants small scale cultures were sufficient for analysis; 20 ml cultures in a 50 ml tube were inoculated directly with single colonies from agar plates. When more cell extracts were required for analysis, 1 l cultures in 2 l flasks were inoculated with 2 ml of overnight cultures and grown for 18 h at 37 °C with shaking at 200 rpm. All cultures were harvested and the periplasmic fractions removed; spheroplasts were produced as described [13]. The periplasms were removed because of the possibility of endogenous heme-containing proteins in this compartment. For the small scale cultures the cytoplasmic extracts were prepared using BugBuster (Novagen), followed by centrifugation (according to the manufacturer's instructions). For the large scale cultures cytoplasmic extracts were produced by sonicating the spheroplasts, followed by centrifugation at 16000×*g* at 4 °C for 20 min.

### 2.3. Analysis of holocytochrome c content

Cytoplasmic extracts were analysed by SDS–PAGE on precast 10% Nu–PAGE gels (Invitrogen). The gels were stained for proteins containing covalently attached heme according to the method of Goodhew [14]. UV–visible spectroscopy was performed on a Perkin Elmer UV–visible spectrophotometer Lambda 2 instrument. Extracts were reduced by the addition of a few grains of sodium dithionite. Pyridine hemochrome spectra were obtained following treatment of the extracts with 19% (vol/vol) pyridine in 0.15 M NaOH. Results were normalised according the mass of the wet cell pellet after harvesting. At least six replicates were performed for each experiment. Quantitations were performed by measuring absorbances of cytoplasmic extracts at 550 nm (with baseline correction for extracts of cells not expressing a cytochrome) following reduction. Standard deviations were calculated for these values and are shown in the bar charts. The SDS–PAGE gels show a representative extract for each variant.

### 2.4. Western blots

HCCS protein expression was examined by performing Western blots following SDS–PAGE analysis of cytoplasmic extracts expressing pRM15 and the 4 MLS variants. A penta-His antibody (Qiagen) was used as the primary antibody and the secondary antibody was anti-mouse alkaline phosphatase-conjugated antibody (Sigma). Detection was performed using SIGMAFAST BCIP/NBT tablets (Sigma) according to the manufacturer's instructions.

## 3. Results

### 3.1. Functional importance of the CP motifs in S. cerevisiae holocytochrome c synthase

In order to study the function of the CP motifs, mutations were introduced into both CP motifs present in *S. cerevisiae* HCCS