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## A heme tag for in vivo synthesis of artificial cytochromes

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**Abstract** A genetic approach is described here that enables the specific covalent attachment of heme via a short C-terminal peptide tag to an otherwise non-heme-binding protein. Covalent attachment of heme to the apo-protein is catalysed by the cytochrome *c* maturation system of *Escherichia coli*. While its original enzymatic activity is retained, the resulting heme-tagged protein is red, has peroxidase activity and is redox active. The presence or absence of a C-terminal histidine tag results in low-spin heme iron with six- or high-spin heme iron with five coordinate ligands, respectively. The heme tag can be used as a tool for the rational design of artificial *c*-type cytochromes and metalloenzymes, thereby overcoming previous limitations set by chemical approaches. Moreover, the tag allows direct visualisation of the red fusion protein during purification.

### Introduction

The design of molecules that mimic the catalytic properties of naturally occurring enzymes is challenging, and the ability to design and prepare them would be useful for a variety of applications in chemistry and biology. Construction of artificial metalloenzymes, in particular ones with covalently attached metal cofactors, is desirable, because metalloenzymes catalyse chemical transformations with high selectivity and reactivity under mild conditions. Several reports on the semi-synthesis of rationally designed metalloenzymes exist. By and large, they rely on knowl-

edge of the three-dimensional structure of the template enzyme (Lu et al. 2001; Qi et al. 2001; Ohashi et al. 2003). Heme is an iron-containing cofactor that catalyses a variety of reactions, depending on its surrounding matrix (Frankenberg et al. 2003). In the cytochromes, the heme iron acts as a simple redox centre. In the globins, heme reversibly binds gases such as O<sub>2</sub>, CO or NO. In cytochrome P450, O<sub>2</sub> is activated and cleaved to form water and an O atom, which is then inserted into a substrate. Such activities are of interest for the design of enzymes with new catalytic properties. Various attempts have been made to construct artificial heme enzymes (Watanabe 2002; Frankenberg et al. 2003). Here we describe a genetic approach which results in cross-linking heme to a short fusion tag in a protein of choice. The heme tag is used as a cassette for covalent heme attachment to the C-terminus of an otherwise non-metal-binding protein. We show that the resulting heme-protein fusion is red, has intrinsic peroxidase activity and is redox active.

Development of the heme tag is based on our knowledge of maturation of *c*-type cytochromes in bacteria. These proteins carry one or more heme molecules bound covalently to the amino acid sequence motif CXXCH. The two cysteines are added to the heme vinyl groups, resulting in two covalent thioether bonds per bound heme. The histidine residue serves as an axial ligand of the heme iron. Bacterial *c*-type cytochromes are synthesised as precursor polypeptides with an N-terminal signal sequence for export of the polypeptide to the periplasm by the general protein secretion system (Sec) (Thöny-Meyer and Künzler 1997). Maturation of apo-cytochrome *c* (non-heme-bound) to holo-cytochrome *c* (heme-bound) takes place on the periplasmic side of the cytoplasmic membrane. In *Escherichia coli* eight cytochrome *c* maturation (Ccm) proteins CcmA–CcmH are required specifically for this process. The corresponding genes *ccmA–ccmH* are expressed only under anaerobic growth conditions (Grove et al. 1996; Thöny-Meyer et al. 1996) when *c*-type cytochromes are used for respiratory electron transport. Previously, we constructed plasmid pEC86 (Arslan et al. 1998), which expresses the *ccmA–ccmH* operon constitutively from a vector promoter

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even under aerobic conditions. The recently characterised minimal sequence requirements for heme insertion into a cytochrome polypeptide (Braun and Thöny-Meyer 2004) allowed the development of a short heme tag. The coding sequence of this tag was fused in frame to the *malE* gene encoding the maltose-binding protein (MBP). The recombinant fusion protein was tested for heme binding in the presence or absence of the *ccm*-plasmid pEC86.

## Materials and methods

### Strains, plasmids and growth conditions

*E. coli* strain DH5 $\alpha$  (Hanahan 1983) was used for cloning. MBP fusions were expressed in *E. coli* EC06 ( $\Delta ccmA-H$ ) (Thöny-Meyer et al. 1995) harbouring pEC86 (Arslan et al. 1998). Plasmid pMal-p (New England BioLabs) was used for the construction of MBP fusions under the control of the *P<sub>tac</sub>* promoter, which is repressed by the *Lac* repressor. Final antibiotic concentrations were 200  $\mu\text{g ml}^{-1}$  for ampicillin and 10  $\mu\text{g ml}^{-1}$  for chloramphenicol. For expression of proteins 0.9 l of Luria–Bertani broth were inoculated with 9 ml of an overnight culture of the appropriate strain at 30°C. Cultures were grown to an optical density of  $A_{600}=0.8$  and induced with 0.1 mM IPTG. Cells were harvested 17 h after induction by centrifugation at 3300 *g*.

### Plasmid construction

Plasmids encoding MBP-heme tag fusions (Table 1) are derived from pMal-p. The *ScaI/Eco52I* DNA fragments encoding the microperoxidases of pMP126 and pMP259 (Braun and Thöny-Meyer 2004) were cloned into the corresponding sites of pMal-p, resulting in the out-of-frame pMP263 and pMP268 heme tag fusions. To obtain in-frame fusions, pMP263 and pMP268 were cut with *SacI*, blunt-ended with T4 DNA-polymerase (Roche Diagnostics) and religated, resulting in plasmids pMP292 and pMP295, respectively. This led to additional codons of the amino acid sequence NSRYPA just before the heme-binding motif CLACH. The DNA encoding the hexa-histidine (His<sub>6</sub>) tag of MP292 was removed by PCR amplification of the gene fusion, using the primer pair *malE* (5'-GGTCGTCAGACTGTCGATGAAGCC-3') that anneals within the *malE* gene and Mini-cycA4 (5'-TAAGAATTCAGCCGATCGCGTG

GCAGGCCAGG-3') encoding the C-terminal end of MP292 without a His<sub>6</sub> tag, and pMP292 as the template. The PCR-product was digested with *EcoRI* and *KpnI* and cloned into the corresponding site of pMP292, resulting in pMP301. All constructs were confirmed by DNA sequencing with primer *malE* (Microsynth, Balgach, Switzerland).

### Protein extraction and purification

Periplasmic proteins were extracted by resuspending the cell pellets derived from 0.9-l cultures in 5 ml 1 mg ml<sup>-1</sup> polymyxin B sulphate (Sigma), 500 mM sucrose and 100 mM Tris-HCl, pH 8. The suspension was stirred for 60 min at 4°C and centrifuged at 20,000 *g* for 20 min. The supernatant contained the periplasmic proteins. His<sub>6</sub>-tagged proteins were purified by Ni-NTA affinity chromatography (Qiagen). MBP (MalE) fusions were purified from periplasmic protein extracts by affinity chromatography on amylose resin (New England BioLabs). Beads were washed twice with 10 bed volumes (BV) of 50 mM Tris-HCl, pH 8, and proteins were eluted with 2 BV of the same buffer containing 10 mM maltose and used for further characterisation. Alternatively, red protein was purified from 2 ml of periplasmic extract over a 1 ml Mono-Q column HR 5/5 on an Aekta (Amersham Biosciences, Freiburg, Germany) purifier. A 20-ml gradient from 0 M to 0.8 M NaCl in 20 mM Tris, pH 8 was run and 1-ml fractions were collected.

### Analytical methods

For in-gel staining of proteins with covalently bound heme, samples were separated by SDS-PAGE as described by Laemmli (Laemmli 1970). Polyacrylamide gels were fixed in 10% TCA for 10 min and then washed twice with water for 10 min. Heme-bound proteins were detected using their intrinsic peroxidase activity by immersion of the gels in a solution of 10 mg *o*-dianisidine (one tablet, Sigma) in 10 ml 0.05 M Na-citrate, pH 4.4, and 0.7% H<sub>2</sub>O<sub>2</sub>. Molecular masses of purified peptides were determined by mass spectroscopy on an Applied Biosystems Voyager-DE Elite using 2,5-sinapinic acid as matrix. Optical spectra were recorded on a Hitachi model U-3300 spectrophotometer and exported to a Microsoft Excel table for calculations of difference spectra and determination of absorption maxima. Concentrations of purified proteins were determined with the

**Table 1** Heme-tagged peptides and their properties

Peptide	Heme binding	Peptide sequence <sup>b</sup>	Molecular mass (Da) holo (apo)
MP126	Yes	QDAAACLACHAIGHHHHHH	2,683 (2,066.2)
MP259	Yes	QDAAACLACHHHHHHHH	2,441.8 (1,824.9)
MP263	No	MBP'-NSSSVPGRVPLPRDRPPSPSPLNS	(42,761)
MP268	n.d. <sup>a</sup>	MBP'-NSSSVPGRVPLPPSPSPLNS	(42,324)
MP292	Yes	MBP'-NSRYPAACLACHAIGHHHHHH	43,177 (42,560)
MP295	Yes	MBP'-NSRYPAACLACHHHHHHHH	42,936 (42,319)
MP301	Yes	MBP'-NSRYPAACLACHAIG	42,354 (41,737)

<sup>a</sup>n.d. Not determined

<sup>b</sup>MBP Maltose-binding protein

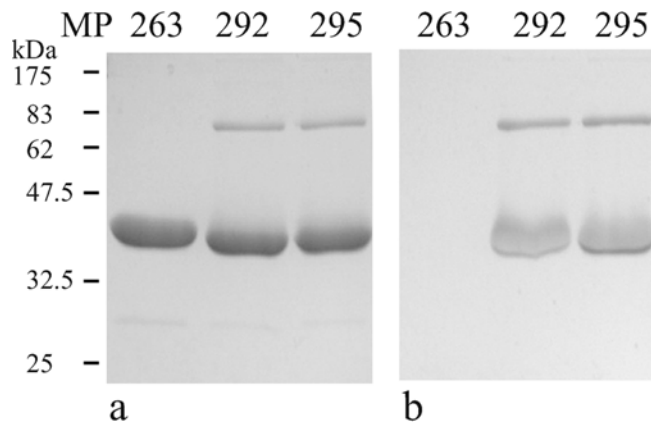
Bradford assay. Molar extinction coefficients at 278 nm and molecular weights were calculated using programs on the ExPASy Molecular Biology Server at <http://www.expasy.ch>.

For EPR measurements, a Bruker E500 spectrometer was used working in X-band (microwave frequency 9.5 GHz). The spectrometer was equipped with a Bruker ER 4122 super-high  $Q$  cavity and an Oxford continuous helium flow cryostat. The spectra were taken at 15 K. A microwave power of 0.06 mW and a modulation amplitude of 0.5 mT were used. Samples prepared for EPR spectroscopy were purified by amylose affinity chromatography. They were concentrated and prepared in a 50 mM Tris buffer, pH 8.5, containing 25% glycerol in order to obtain a good glass upon freezing the sample. The final concentration of the samples was 122  $\mu$ M for holo-MP292 and 36  $\mu$ M for holo-MP301.

## Results

The MBP–AAACLACHHHHHH fusion binds heme in the presence of the Ccm factors

The finding that the peptide QDAAACLACHHHHHH (MP251) can bind heme in the presence of the Ccm system (Braun and Thöny-Meyer 2004) raised the question of whether covalent heme attachment can also occur in larger proteins that normally do not contain this cofactor. We thus fused the majority of the coding sequences of MP126 and MP259 (Table 1) to the periplasmic MBP, resulting in MP292 and MP295, respectively. Both proteins have a proline residue preceding the AAACLACH motif that might provide a kink in the protein structure and facilitate maturation. These tagged polypeptides were expressed in *E. coli* EC06 ( $\Delta ccmA$ –*ccmH*) in the presence and absence of pEC86 (*ccmA*–*ccmH*). Periplasmic extracts were analysed spectroscopically for the presence of *c*-type cytochromes. None of the proteins bound heme in the absence of pEC86. In the presence of pEC86, periplasmic extracts of both MP292 and MP295, but not of MP263 (out-of-frame fusion) were red. His<sub>6</sub>-tagged proteins expressed in the presence of pEC86 were purified from periplasmic extracts via Ni-NTA affinity chromatography and resulted in red fractions with spectroscopic characteristics of *c*-type cytochromes. Alternatively, the proteins were purified by affinity chromatography over amylose resin, resulting in red fractions for both MP292 and MP295. This purification indicates the native folding of the MBP derivatives that retain the sugar-binding characteristics of the protein. Proteins were further analysed for covalent heme binding by heme staining after SDS-PAGE. In contrast to MP263, both MP292 and MP295 had heme-dependent peroxidase activity. For both, an additional heme-staining band with about a twofold molecular weight was found (Fig. 1), as it is often seen for *c*-type cytochromes. The molecular weight of MP292 was further determined by mass spectroscopy. The main peak detected was 42558.6  $m/z$ , which corresponds very well with the calculated mass of apo-MP292



**Fig. 1** Heme binding of MP292 and MP295. Samples were separated by SDS-15% PAGE and stained for the presence of proteins with Coomassie (a) or for covalently bound heme by peroxidase activity stain (heme stain) (b). Each lane was loaded with 5  $\mu$ g protein

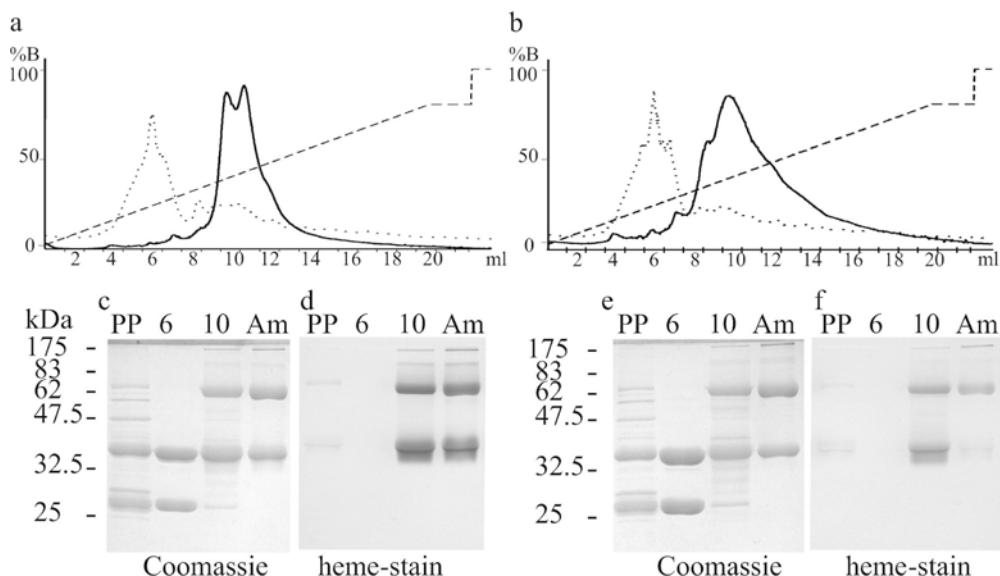
(42560 Da). A peak corresponding to the expected mass of holo-MP292 (43177 Da) was also detected.

The His<sub>6</sub> tag is not required for heme binding

Our previous work on heme peptides had shown that a His<sub>6</sub> tag is important for heme insertion (Braun and Thöny-Meyer 2004). Hence, we wondered whether the His<sub>6</sub> tag is also required for heme attachment in the MBP fusion. We thus constructed plasmid pMP301, which encodes MP292 lacking the His<sub>6</sub> tag (Table 1). MP301 was expressed in the presence of the Ccm system, purified by amylose affinity chromatography and analysed for heme binding by heme stain. The results were identical to those obtained for MP292 and MP295 (Fig. 2d, f). We conclude that the His<sub>6</sub> tag is not required for heme binding.

Heme-tagged maltose-binding protein has spectral characteristics of a *c*-type cytochrome

Cytochromes of the *c*-type such as horse heart cytochrome *c* have typical  $\alpha$  bands at 550 nm in the reduced state. To analyse the spectral characteristics of the fusion proteins, ascorbate (5-mM and 10-mM final concentrations) and dithionite (5-mM final concentration) were used as reducing agents and added to samples of amylose-purified MP292, MP295 and MP301. As previously described for microperoxidases MP126 and MP259 (Braun and Thöny-Meyer 2004), none of the fusion proteins was reduced with the weak reducing agent ascorbate ( $E^0 = +58$  mV). Dithionite-reduced *minus* air-oxidised difference spectra of both MP292 and MP295 showed maxima in the range of 417–418 nm for the Soret band, 522–523 nm for the  $\beta$  band and 550 nm for the  $\alpha$  band, which are typical for *c*-type cytochromes (Fig. 3). While the Soret peak of MP301 also shifted from 408 nm to 415 nm when dithionite was added,



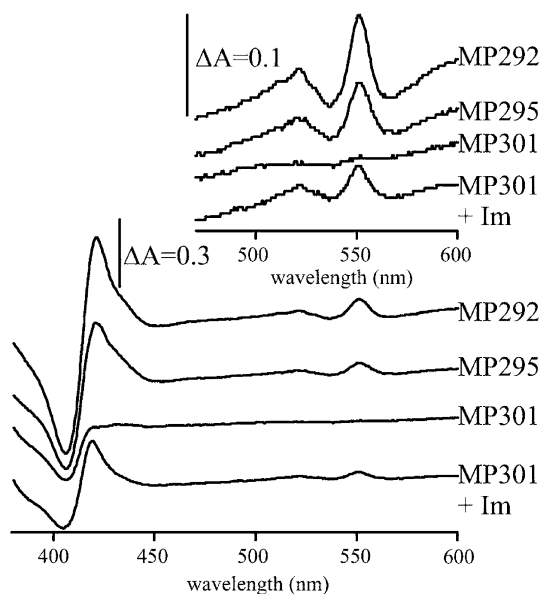
**Fig. 2** Purification of MP292 and MP301 from periplasmic extracts. Periplasmic extracts of MP292 (a) and MP301 (b) were separated by anion exchange chromatography and absorption was monitored at 280 nm (..) and 410 nm (—). The gradient was from 0M to 0.8 M NaCl indicated as %B (---). Fractions of MP292 (c, d) and of MP301 (e, f) eluting after 6 ml (6) and 10 ml (10) were compared

with the periplasmic extracts (PP) and amylose affinity purified protein (Am) by SDS-12% PAGE. Gels were stained for peroxidase activity, showing the presence of covalently bound heme (d, f) and for the presence of proteins with Coomassie (c, e). Each lane was loaded with 5  $\mu$ g protein

its amplitude diminished, so that the difference spectrum mainly reflects the absorption of the oxidised form (Fig. 3) with a minimum at 406 nm. Furthermore, no peaks were detected at the position of the  $\beta$  or  $\alpha$  band (523 nm or 550 nm, respectively). Heme ligands have previously been shown to influence the spectral behaviour in the 550-nm range (Arcovito et al. 2001; Lee et al. 2001). Upon addition of 300 mM imidazole to MP301, the dithionite-reduced

spectrum resembled that of the other heme His<sub>6</sub>-tagged fusion proteins (Fig. 3), suggesting that imidazole can function as sixth ligand in the MBP-heme tag protein lacking the His<sub>6</sub> tag. In summary, the oxidised and reduced states of all three fusion proteins have spectral characteristics typical for *c*-type cytochromes.

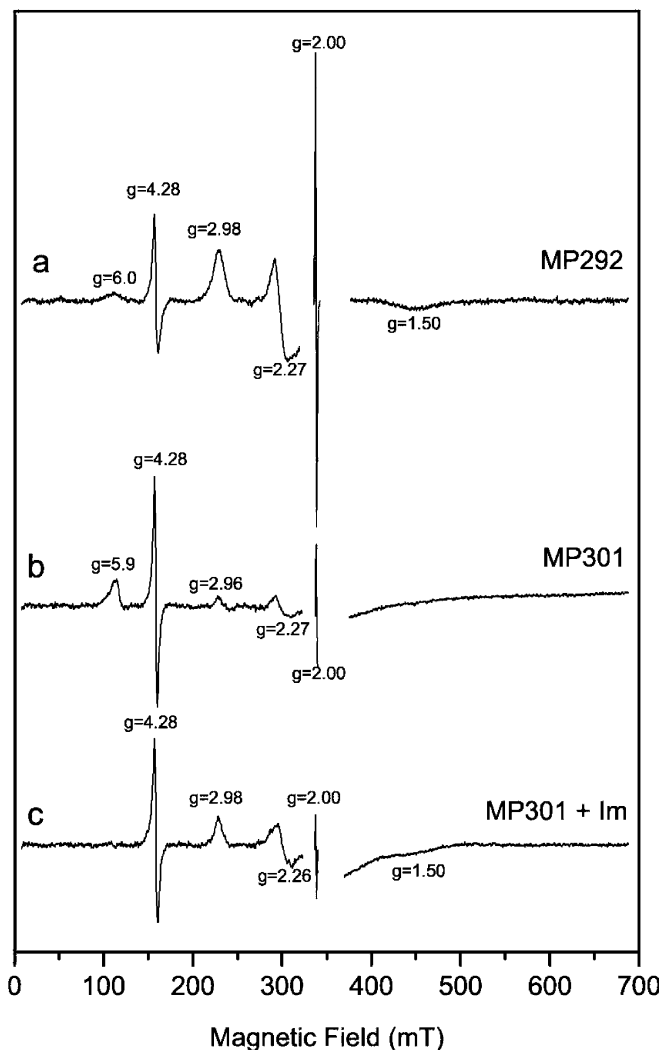
The His<sub>6</sub> tag serves as sixth ligand



**Fig. 3** Spectral analysis of purified maltose-binding protein-heme tag fusions. Dithionite-reduced *minus* air-oxidised optical difference spectra are shown. +Im indicates the presence of 300 mM imidazole. The *inset* shows the  $\alpha$  and  $\beta$  bands at higher magnification

As the presence of imidazole significantly changed the optical spectra of MP301, we decided to characterise the heme ligands in more detail. EPR spectra of the two heme-binding fusion proteins, with and without exogenous imidazole, were recorded. MP292 (Fig. 4a) displays a characteristic low-spin ferric heme spectrum (spin, 1/2). The three features associated with the principal values of the anisotropic *g*-tensor are resolved ( $g_z=2.98$ ,  $g_y=2.27$ ,  $g_x=1.50$ ). These values correspond typically to a bis-imidazole-coordinated heme centre, with approximately parallel axial ligands (Palmer 1983; Walker 1999). In addition to the heme iron signal, two other lines were observed in the spectrum. The one at an effective *g* value of 4.28 is attributed to a non-heme ferric iron impurity in a highly rhombic environment (Pilbrow 1990). The region of the spectrum around  $g \sim 2$  was omitted because of a strong signal from the cavity. Nevertheless, a signal at exactly  $g=2.00$  was detected and assigned to a radical species. In spite of their high intensity in the spectra, the signals at  $g=4.28$  and 2.00 are minority centres.

The EPR spectrum of MP301 (Fig. 4b) shows two different heme iron signals corresponding to two different species. The majority species has a signal at  $g \sim 6$  that corresponds to a typical high-spin ferric-heme iron (spin,



**Fig. 4** EPR spectroscopy. EPR spectra of MP292 (a), MP301 (b) and MP301 after the addition of an excess of imidazole (c) are shown. The region around  $g \sim 2$  was omitted due to a strong baseline. All spectra were taken at 15 K

5/2). This means that the axial coordination of the heme iron is weaker than in MP292, i.e. the iron is either five-coordinated or six-coordinated with a weak sixth ligand such as water (Palmer 1983). Since the difference between the two proteins is the absence of the His<sub>6</sub> tag in MP301, we conclude that the sixth ligand of iron in MP292 is probably a side chain from the His<sub>6</sub> tag.

The second species in Fig. 4b also seems to correspond to a low-spin bis-histidine coordinated heme ( $g_z=2.96$ ,  $g_y=2.27$ ,  $g_x$  not resolved). Here, the sixth ligand could either be of intra- or inter-molecular origin. If the latter were the case this signal would be related to dimers.

Figure 4c displays the spectrum of sample MP301 after the addition of imidazole to the protein solution. The high-spin species is missing and has been transformed into a low-spin species with  $g$  values ( $g_z=2.98$ ,  $g_y=2.26$ ,  $g_x=1.50$ ). The exogenous imidazole is probably coordinating the heme iron as the sixth ligand.

As can be seen in Fig. 4a, there is also a small amount of high-spin signal in the spectrum of MP292 (small hump at  $g \sim 6$ ). This high-spin signal was completely removed upon addition of imidazole to the sample.

#### Enrichment of the heme-tagged protein by MonoQ ion exchange chromatography

One goal of this work was to create an optically detectable tag that facilitates protein purification. Periplasmic extracts containing heme-tagged proteins were separated over a MonoQ column, and absorption of fractions was monitored at 410 nm in addition to 280 nm. The absorption at 410 nm corresponds to heme absorption in the Soret region. While the main absorption at 280 nm for both analysed fusion proteins MP292 and MP301 was in fraction no. 6, the absorption at 410 nm was maximal in fraction no. 10 (Fig. 2a, b). Optical spectra of these fractions were recorded to determine the ratio of  $A(410)/A(278)$ . For MP292, fraction no. 6 had a ratio of 0.016 and fraction no. 10 of 0.228. This corresponds to a 3.7-fold enrichment of holo-protein from the initial periplasmic extracts (ratio = 0.061). In MP301 the enrichment of holo-protein in fraction no. 10 was 3.27-fold and the final ratio 0.17. For both MP292 and MP301 the ratios in fraction number 10 corresponded well with the ratios obtained after purification via amylose affinity chromatography ( $A(408)/A(278)=0.39$  for MP292 and 0.16 for MP301). Under the same buffer conditions horse heart cytochrome *c* showed a ratio of 3.9. The calculated extinction coefficient at 280 nm for MP292 is 4.3-fold higher than that of cytochrome *c*. Taking these factors into consideration, purified MP292 contained approximately 40% and purified MP301 about 16% holo-protein. While fraction 10 still contains minor amounts of other contaminating proteins (Fig. 2c, e), amylose affinity-purified protein was pure. Additionally, Western blot analysis with anti-MBP antibodies confirmed that the two major bands visible in fraction number 10 of MonoQ-purified samples indeed are MBP-fusions (data not shown).

#### Discussion

A C-terminal AACLACHAIG tag is recognised by the Ccm system for covalent attachment of heme to the MBP fusion, which leads to an artificial *c*-type cytochrome with peroxidase and redox activities. Besides introducing these new characteristics, the initial feature of the MBP, i.e. the binding of amylose, is maintained.

The red colour of the fusion protein helps to fractionate the proteins during the initial steps of purification. In addition, protein-bound heme allows easy and sensitive detection by peroxidase activity. Transfer of MP292 to nitrocellulose membranes and subsequent detection by enhanced chemiluminescence (Feissner et al. 2003) resulted in detection of  $\geq 0.4$  pmol of holo-protein (data not shown). This method is normally used as the last step in immunodetection protocols when antibodies linked to horse-

radish peroxidase are used. The covalent modification of a peptide with heme thus allows a one-step peroxidase detection in which antibodies are not needed. Recently a haemoglobin tag based on the bacterial haemoglobin of *Vitreoscilla stercoraria* has been developed (Park et al. 2003). This tag consists of 146 amino acids and binds heme non-covalently. By contrast, covalent binding as found in our heme tag enables detection of the tagged protein even under denaturing conditions during purification or after SDS-PAGE. The shortness of the tag also allows cloning by PCR, using primers that contain the coding sequence of the heme tag. In contrast to the haemoglobin tag, our system requires periplasmic targeting for heme attachment. This can be advantageous, as proteins over-produced in the bacterial periplasm have already a high degree of purity after the first steps of cell fractionation.

We have produced an artificial *c*-type cytochrome by the fusion of a heme tag to a periplasmic, non-cytochrome polypeptide that was produced in cells expressing the cytochrome *c* maturation system Ccm. The target protein obtained a new function, while the initial, maltose-binding characteristic was retained. The heme iron of many cytochromes in nature is either five- or six-coordinated, whereas heme-based sensors and enzymes are mostly five-coordinated with an open site to allow binding of small molecules such as O<sub>2</sub> or CO (Lu et al. 2001). We found that in the presence of a His<sub>6</sub> tag, the heme iron of the tagged fusion protein was six-coordinated, but in the absence of the His<sub>6</sub> tag it was five-coordinated. Previously, we could only speculate on the role of the His<sub>6</sub> tag in maturation of heme peptides (Braun and Thöny-Meyer 2004). We now show that the His<sub>6</sub> tag is not a general prerequisite for heme incorporation when linked to CXXCH-tagged maltose-binding protein. However, when the tag is present it acts as sixth ligand to the heme iron.

In summary, covalent attachment of heme to a heme tag (with or without an additional His<sub>6</sub> tag) fused to the C-terminus of any periplasmically expressed protein can be a valuable tool for protein purification. The resulting red colour and the peroxidase activity are two characteristics worth considering when choosing an appropriate purification scheme for any protein of interest (Terpe 2003). Analogous to other chemically linked microperoxidase conjugates (Padeste et al. 2003), our tool also has the potential for a broad application, for example as biosensor or specific biocatalyst.

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