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Aberrant Attachment of Heme to Cytochrome by the Ccm System Results in a Cysteine Persulfide Linkage

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The system I heme attachment apparatus in the periplasm of Gram-negative bacteria, exemplified by the cytochrome *c* maturation (Ccm) proteins of *Escherichia coli*, is rather promiscuous in its cytochrome substrate specificity but highly specific in terms of the local sequence motif that it recognizes.[1,2] The peptide sequence to which heme is covalently attached through two thioether bonds is CXXCH, where in natural sequences X can be any amino acid except cysteine. The product of heme attachment to this sequence is highly stereo- and regiospecific.[3] Since the observation of an unusual heme−protein covalent bond in CcmE,[4] the chemical mechanism of the attachment reaction of heme to apocytochrome *c* has been the subject of a great deal of speculation, but despite considerable experimental effort it remains a mystery.[5] Every bacterial system I apparatus involves disulfide isomerase activity, and it is commonly assumed that the substrate cysteines are oxidized to a disulfide during the transport of the apocytochrome into the periplasm.[6,7] The oxidation state of the cysteines when they are presented to the heme is not known, but they are assumed to be free thiols.

E. coli cytochrome b_{562} can be a substrate for Ccm catalysis by incorporation of the CXXCH motif in a sequence location homologous to that of the well-known cytochromes *c′* and c_{556} from other bacteria.[8,9] The product (cb_{562}) is that expected for a classical *c*-type cytochrome.[8,10] Variants containing different lengths of intervening sequence between the cysteines (CX_nCH, where n = 3, 4, 5, or 6) have recently been generated.[11] Sequences with X_3 and X_4 are known in natural cytochromes,[12] but there are no natural examples with X_1, X_5, or X_6.[13] cb_{562} sequences with X_2, X_3, or X_4 are clearly substrates of Ccm and yield *c*-type cytochromes with the expected heme attachment.[11] Expression of sequences with X_5 or X_6 yields mature *c*-type cytochromes with heme attached and spectral properties consistent with the normal mode of heme attachment.[11] However, electrospray ionization (ESI) mass spectrometry (MS) of these purified proteins shows that they are actually mixtures containing, in addition to the protein of expected mass, very closely related species with extra mass relative to the expected mass of the apoproteins plus attached iron protoporphyrin IX. In the cases of X_1 and X_6, some of these species are more abundant than the correctly matured protein. In particular, all samples of X_1, X_5, and X_6 contain a species that is 32 Da heavier than the expected mass. For X_5 and X_6, these proteins of higher mass have been shown to be products of the Ccm machinery.[11] Given their abundance, we reasoned that understanding their origin may shed light upon the mysterious mechanism of the Ccm enzymatic apparatus. In this work, we have identified the location and elemental origin of the extra 32 Da in each protein using high-resolution accurate ion-trap MS combined with peptide fragmentation.

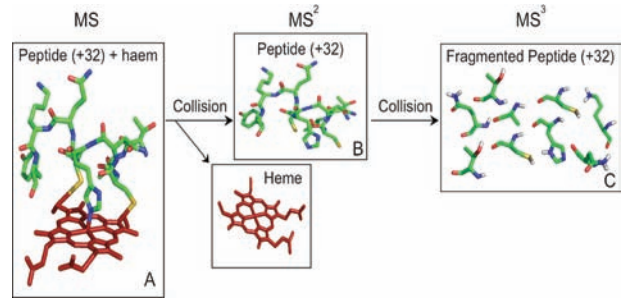

Figure 1. Fragmentation analysis of mass spectra of cb_{562} variants. The peptide mixture is introduced to the spectrometer by nano-ESI. The peptide + heme species (A) is selected in the ion trap and fragmented so the heme dissociates (B). Further fragmentation of the peptide yields species with and without the extra 32 Da mass (C) in addition to the y and b ions that are used for sequence identification. (Identifying letters here also correlate with the spectra in Figure 2.)

The additional mass could be due to oxidation of a sulfur (two oxygen atoms at 16 Da each) or to an extra sulfur atom (32 Da). For each protein, we analyzed tryptic digests of the combined species using high-accuracy MS. In all cases, peptides with heme attached could be identified and subjected to rounds of MS/MS (Figure 1) at different collision energies, resulting in fragmentation of different bonds.

Detailed explanations and specimen data (Figure 2) are shown only for the X_5 protein; however, the data for the other two proteins (X_1 and X_6) were obtained and analyzed in the same way. The X_2 protein, for which a species with extra mass has never been observed,[8,10] served as a control. Mass and assignment data are given in Table S1 in the Supporting Information. For protein X_5, the tryptic peptide containing the variant heme attachment motif, with heme covalently bound, can be found as two species [TTCNAAGSCHQK + heme, *m*/*z* 918.87; same peptide + 32 Da, *m*/*z* 934.83 (see Figure 2A)]. Isotope patterns consistent with the fact that these peptides contain iron were observed. These ions were subjected to 17−20 V of collision energy to cleave the heme from the peptide. Comparison of the accurate masses (*m*/*z* 1220.5110 and *m*/*z* 1252.4831) from $FTMS^2$ spectra (Figure 2B) revealed that the mass difference between the two peptides without heme is accurately 31.9721 Da (error <8 ppm). These data confirmed that the extra mass observed, remarkably, is due to sulfur (mass 31.97207 Da) and not two oxygens (mass 31.9898 Da) *and* that it is located on the peptide and not a modified heme.

Interestingly, whenever a "+32 Da peptide" was identified, it was accompanied in the MS^2 spectra by singly charged heme fragment ion with one additional hydrogen atom (*m*/*z* 617.1830),

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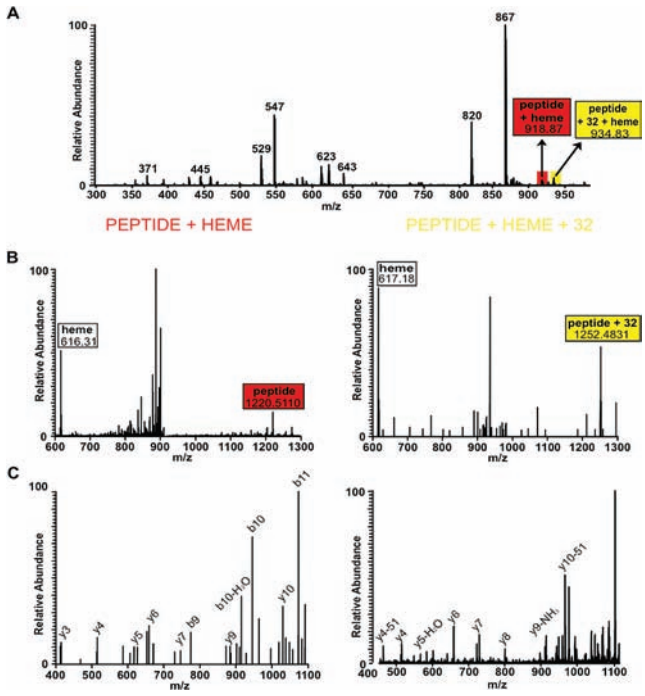

Figure 2. Orbitrap mass spectra of X_5 cb_{562}. (A) FTMS spectrum showing a close-up of the region containing the tryptic peptides of interest, at high mass accuracy (~2 ppm). These peaks at *m*/*z* 918 and 934 correspond to doubly charged peptides containing the heme attachment motif plus heme with and without an extra 32 Da, respectively. (B) $FTMS^2$ high-accuracy spectra of the fragments resulting from collision of these ions at 17 V. Heme and both peptides are observed as singly charged ions in these spectra. (C) $FTMS^3$ spectra indicating the y and b ions observed. Of particular interest are the peaks corresponding to losses of 51 Da from the y4 and y10 ions.

which seems to be a signature for these sulfur-modified proteins (normal-mass heme peptides fragmented to give a heme ion at *m*/*z* 616.3077; see Table S1). This confirms the presence of different chemistry at the heme peptide linkage (see the further discussion in the Supporting Information).

The heme-cleaved peptide daughter ions were then selected for further fragmentation (see Figure 2C and Figure S3 in the Supporting Information). Assignment of the resulting MS^3 spectrum (see Figure S2) revealed that the extra sulfur atom resides on the first cysteine in the CX_nCH motif, since in the case of X_5, for example, the y9 to y4 ions do not carry the extra mass. Also of interest in the MS^3 spectrum of the +32 Da species was the observation that peptide ions with a cysteine residue at the N-terminus (y10 and y4 in the case of X_5) are 51 Da smaller than expected (i.e., 83 Da smaller than the species with expected mass plus 32 Da). This was not observed for any of the correct-mass peptides and is consistent with a modified-cysteine-specific internal fragmentation involving formation of a propenamide (Figure S4).[14] *Both* the y10 and y4 ions show this behavior, while only the y10 ion is observed to carry an extra sulfur atom. This suggests that the two cysteines can cyclize in the gas phase (Figure S5). Figure 2C shows that some correct-mass y4 ions can be detected, while correct-mass y10 ions cannot but instead appear at a mass of y10 minus 51 Da.

Two lines of evidence suggest that both cysteines are linked to the heme in the intact protein, even with an extra sulfur atom present in one linkage. First, the pyridine hemochromes are consistent with the loss of both heme vinyl groups.[11] Second, analysis of X_1 fragmentation by MALDI−TOF/TOF identified both y5 and y6 ions with heme still attached.

Several lines of evidence strongly suggest that our observations are relevant to the mechanism of Ccm-catalyzed heme attachment. The expression levels of these proteins, both those with correct mass and those with aberrant mass, are linked to coexpression of the *ccm* gene products.[11] The electronic spectra of the intact proteins and their pyridine hemochromes are consistent with two thioether linkages to the α-carbons of the heme vinyl groups when expressed in the presence of the Ccm apparatus but not when expressed in its absence.[11] The extra 32 Da mass was not observed on the apoproteins, suggesting that it is a consequence of heme attachment by the Ccm apparatus. Reactions in vitro between heme and purified cb_{562} apoproteins have never yielded any of the products observed here (see the further discussion in the Supporting Information).

We therefore propose that the species with an extra 32 Da all have a persulfide bond between the heme 2-vinyl group and the first cysteine in the CX_nCH sequence for $n = 1$, 5, and 6. This requires that the apoprotein be presented to heme in the Ccm catalytic site with at least the first cysteine chemically modified, possibly as a mixed disulfide. Misalignment of this modified apoprotein substrate due to the constraints imposed by the altered, intervening (i.e., X_n) sequence then results in reaction between heme and the wrong sulfur atom. The origin of the mixed disulfide is unknown, but it seems unlikely that it would be a protein component of the catalytic units (e.g., CcmH or CcmG). It is more likely that it originates from a small-molecule thiol. This is the first evidence to suggest that the substrate of the Ccm apparatus may not be the CXXCH peptide present in the free thiol state,[5] and given recent evidence for new redox roles for CCM proteins,[15] it also suggests new avenues for exploring this perplexing mechanism.

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Supporting Information Available: Full details of proteins and the experimental methods as well as data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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