Loss of Either of the Two Heme-binding Cysteines from a Class I c-Type Cytochrome Has a Surprisingly Small Effect on Physicochemical Properties

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Almost without exception, c-type cytochromes have heme covalently attached via two thioether linkages to the cysteine residues of a CXXCH motif. The reasons for the covalent attachment are not understood. Reported here is cytoplasmic expression in Escherichia coli of AXXAH variants of cytochrome c552 from Hydrogenobacter thermophilus; remarkably, the single thioether bond proteins have, apart from an altered visible absorption spectrum, almost identical properties, including thermal stability and reduction potential, to the wild-type CXXCH protein. In combination with previous work showing that an AXXAH variant of cytochrome c552 is much less stable than the CXXCH form, it can be concluded that covalent attachment of heme via either of the thioether bonds is sufficient to confer considerable stability and that these bonds contribute little to the setting of the reduction potential. The absence of AXXCH or CXXAH heme-binding motifs from bacterial cytochromes c may relate to the coexistence of the assembly pathway with that for formation of disulfide bonds in the bacterial periplasm.

Mitochondrial cytochrome c is well known to have the heme group covalently attached to two cysteine residues that occur in a sequence motif of CXXCH (1). Less well known is that in certain protozoan species the heme attachment is to an AXXCH motif (2, 3). This attachment has also been achieved by mutagenesis of the gene for human mitochondrial cytochrome c followed by expression in yeast (4). The single attachment mode has also been observed in mitochondrial cytochromes c1 from protozoa (5, 6) but has to date not been found in a bacterial c-type cytochrome.

It is still not clear what advantage accrues to a c-type cytochrome through having the heme covalently attached (7). It has been suggested that the covalent attachment is a device to prevent heme loss by dissociation (8). It has also been argued that the thioether bonds increase binding affinity of methionine to the ferrous iron and thus contribute to the relatively high reduction potentials of some c-type cytochromes (9). Recently, the effects of losing both the covalent bonds to heme have been described in studies where the cytochrome c552 from a thermophile Hydrogenobacter thermophilus was converted into a b-type cytochrome as a result of mutation of the sequence CXXCH to AXXAH (10). Expression of the b-type derivative of cytochrome c552 was achieved in the cytoplasm of Escherichia coli, which is where, most unusually, the holo form of cytochrome c552 can be expressed without the aid of the cytochrome c biogenesis apparatus that functions in the periplasm (11, 12). It is currently thought (10) that the apo polypeptide adopts a sufficient state of preorganization to bind heme and then to adopt the final fully folded structure, accompanied in the case of polypeptide with the CXXCH sequence by the spontaneous and stereo-specific formation of the two thioether bonds.

The AXXAH variant of H. thermophilus cytochrome c552 is less stable than the wild-type CXXCH protein, and the reduction potential is lowered by 75 mV (10). Understanding of the contributions of the two thioether bonds to the properties of this cytochrome c552 clearly requires comparative data for proteins with a single thioether bond. The successful formation of the b-type cytochrome derivative of the H. thermophilus cytochrome c552 prompted us to investigate whether it was possible to produce one or both of the single cysteine attached forms of cytochrome c552. A successful outcome, reported here, means that we are able to for the first time to assess the contribution of each thioether bond to the physicochemical properties of a c-type cytochrome. Surprisingly, it is found that loss of either of the thioether bonds has only a marginal effect on the stability of the cytochrome. This result raises questions as to why the AXXH motif rarely and the CXXAH never occurs in vivo. In the bacteria, as opposed to mitochondria, the AXXCH motif has never been reported, supporting the idea that the bacterial biogenesis machinery for a c-type cytochromes has co-evolved alongside the system for forming disulfide bonds (7). This implies that the two cysteines of CXXCH may form a disulfide bond before heme is attached (7).

MATERIALS AND METHODS

All experiments were carried out using E. coli strain XL1-Blue from Stratagene. Cultures for protein expression were grown in 2TY medium (16 g/liter bacteriological peptone, 10 g/liter yeast extract, and 5 g/liter sodium chloride) in 2.5-litter flasks. Cultures were grown at 37°C in an orbital incubator, shaking at 200 rpm. All other cultures were grown in LB. DNA was prepared using Promega Wizard miniprep. Oligonucleotides were synthesized by PerkinElmer Life Sciences and supplied to ReadyPure specifications.

Mutagenesis—Mutagenesis was carried out by polymerase chain reaction, first using pKHC12, which contains the cytochrome c552 gene without a periplasmic targeting sequence (11), as a template and oligonucleotides EJT3 (5′-GCATGATCTGATTGAGCGTGCTGTCGTCATTTTGC-3′) and EJT4 (5′-GCATGATCTGATTGAGCGTGCTGTCGTCATTTTGCACG-3′). The resulting 250-base pair fragment was digested with EcoRI and HindIII and cloned into the Amersham Pharmacia Biotech expression vector pKK223-3 to yield pEST201, which has an AXXCH heme-binding motif and an introduced NcoI site at the first mutation site. Further polymerase chain reaction using pEST201 as the template and oligonucleotides EJT4

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1 P. D. Barker, unpublished results.
(5'-GCCGATGGGCTGACAGCTGAAAGCTAAGAGG-3') and EJT5 gave a fragment that was digested with NcoI and HindIII and cloned into pEST201. This gave pEST202, which contained mutations of both wild type cytochrome to alamines. A final round of polymerase chain reaction used pEST202 as the template and oligonucleotides EJT-10 (5'-GCATGATTCATGATGACGACTCTCGCCAAAGACGCGTAATGGCAGCAGCTGCTG-3') and EJT5. The resulting fragment was digested with EcoRI and HindIII and cloned into pKK223-3 to yield pEST203, which has a CXXAH heme-binding motif. All plasmids were sequenced to ensure that only the required mutations were present.

Expression and Purification of Cytochromes—6 ml of an overnight culture of E. coli XL1-Blue containing a cytochrome expression plasmid in 2TY with 100 mg ml−1 ampicillin was used to inoculate 600 ml of 2TY with 100 mg ml−1 ampicillin in a 2.5-liter flask. When the optical density of the culture at 550 nm had reached 0.8, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the culture was incubated for a further 6 h. Cells were harvested by centrifugation. Expressed cytochrome was purified as described previously (13) using ion exchange chromatography on CM2 Sepharose followed by gel filtration using G50 Sephadex. The N-terminal amino acid sequences up to residue 16 were determined for each protein and shown to be correct. Protein concentrations were determined using the method of Bradford, and the theoretical extinction coefficient was at 280 nm.

Determination of Reduction Potentials—These were measured by cyclic voltammetry as described by Dutton (14). Circular dichroism spectra were recorded using a Jasco J720 spectropolarimeter with a fused quartz cuvette of 1-mm pathlength and protein concentrations of 25–30 μM in 20 mM sodium phosphate, pH 7.3. All spectra were recorded at 25 °C unless otherwise stated. Acid butanone extraction of heme was carried out according to the method of Teale (16).

Unfolding of Proteins in Guanidine Hydrochloride—Unfolding of proteins in guanidine hydrochloride was carried out by appropriate dilution of an 8 M stock solution before addition to concentrated protein solution, yielding a final protein concentration of 30 μM in 20 mM sodium phosphate, pH 7.3. Samples were equilibrated at 25 °C for 2 h before spectra were measured. Wild type protein unfolding was monitored by the change in 419 nm for the oxidized form and 417 nm for the reduced form. Unfolding of the AXXCH and CXXAH proteins was monitored by the change in absorbance at 412 nm for the oxidized forms and 422 nm for the reduced forms. This facilitates following the unfolding of c-type cytochromes as, because of the covalent attachment of the heme to the polypeptide, only the methionine ligand is lost, resulting in a red shifted Soret band of increased intensity (17) rather than a loss of absorption spectra of the reduced forms of the two single cysteine mutants. All solutions for unfolding studies under reduced conditions were degassed and, together with the cuvettes, flushed with argon.

Determination of Stability to pH—Determination of stability to pH was carried out by diluting a concentrated protein stock solution with 10 mM each: acetate, HEPES, MES,2 CAPS, CHES, and Tris-HCl. The pH was adjusted with hydrochloric acid or sodium hydroxide or sodium phosphate, pH 7. All protein solutions were 20 mM in 20 mM sodium phosphate, pH 7. Comparison of reduction potentials and visible absorption spectra for oxidized, reduced, and pyridine hemochrome forms of cytochrome c_552 and the heme binding site mutants.

| Protein | Amax/nm oxidized | Amax/nm reduced | Amax/num pyridine hemochrome | Reduction potential E°/mV |
|---------|------------------|-----------------|----------------------------|--------------------------|
| Wild type |                   |                 |                            |                          |
| CXXCH   | 410              | 417             | 550                        | 245                      |
| AXXCH   | 411              | 418             | 553                        | 225                      |
| AXXAH   | 416              | 425             | 556.5                      | 170                      |
| CXXAH   | 412              | 420             | 553                        | 250                      |

The abbreviations used are: MES, 4-morpholineethanesulfonic acid; CAPS, 3-cyclohexylamino)propanesulfonic acid; CHES, 2-cyclohexylamino)ethanesulfonic acid.

Results

Expression from either plasmid pEST201 or plasmid pEST203 gave red cytoplasmic proteins, which after purification proved to have covalently attached heme and thus could be regarded as c-type cytochromes. A lower yield was obtained for the CXXAH protein (Table I). Also shown in Table I, for comparison, are the yields of the AXXCH and AXXAH proteins. Direct protein sequencing confirmed the presence of the AXXCH and CXXAH sequence motifs, respectively. Visible absorption spectra of the reduced forms of the two single cysteine proteins, with the wild type for comparison, are shown in Fig. 1. Table II shows that the o-band maxima were at 558 and 556 nm for the AXXCH and CXXAH proteins, respectively, but the pyridine hemochrome was at 553 nm for both proteins in contrast to 550 nm for the CXXCH protein (Table II). The 695 nm charge transfer band in the wild type, reflecting the methionine ligation of the iron, is also red shifted in both single alanine mutants (Table II). By comparison, the band is shifted to 720 nm in the AXXAH protein (Table II), similar to the value
Loss of a Thioether Bond from a c-Type Cytochrome

Previously reported for cytochrome \( b_{562} \), which also has histidine/methionine coordination of the heme iron (18). These spectroscopic characteristics for the single alanine derivatives of cytochrome \( c_{552} \) are very similar to those observed with forms of mitochondrial cytochrome \( c \) that have the AXXCH-binding motif (19). The far UV CD spectra of the two single alanine mutants are essentially identical to that of the wild type protein as shown in Fig. 2. Measurement of CD spectra at 400–450 nm indicated that the heme had attached to the proteins in essentially one conformation. In neither case was the reduction potential affected more than 20 mV by the loss of one thioether bond (Table II).

Samples of purified AXXCH protein analyzed by SDS-polyacrylamide gel electrophoresis and stained for heme showed small amounts of heme on the dye front. This could have been due to either degradation of the protein yielding a small heme-containing peptide or the presence of some noncovalently bound heme in the sample. A small percentage of noncovalently bound heme would not be readily detectable in the pyridine hemochrome spectrum. Acidified butanone was used to extract any \( b \)-type heme from purified samples of the holo proteins and the ratio of the reduced Soret maximum:absorbance at 280 nm compared before and after such treatment. Only the ratio of \( A_{422\text{red}}/A_{280\text{ox}} \) in the AXXCH mutant was changed, falling from 3.85 to 3.31. This result suggests that approximately 14% of the holo AXXCH cytochrome contained noncovalently attached heme. No heme could be extracted from the CXXAH mutant, demonstrating that the heme is 100% covalently attached to the polypeptide with this binding motif.

Stability of Single Cysteine Variant Proteins—It has previously been observed that the loss of two thioether bonds so as to generate a \( b \)-type cytochrome from cytochrome \( c_{552} \) resulted in a substantial destabilization with respect to chemical and thermal denaturation (10). Remarkably, both the proteins incorporating the single alanine substitutions were only slightly less stable than the native protein (see Fig. 4). In the case of thermal denaturation no change in visible absorption of the oxidized AXXCH or CXXAH proteins could be observed up to 70 °C, whereas in CD spectroscopy only at 90–95 °C was there evidence for partial unfolding (data not shown). These observations are identical to those for the wild type. The midpoints for guanidine hydrochloride induced unfolding are shown in Table III; the AXXCH protein was less stable in both the oxidized and reduced forms than the wild type or CXXAH protein. In contrast, the oxidized form of the CXXAH protein had similar stability to the wild type, whereas the reduced form was somewhat less stable. The CXXAH protein is also the only one of the four proteins (Table I) to show no alteration in the far UV CD spectrum on reduction; the wild type, AXXCH, and AXXAH proteins all showed increased \( \alpha \)-helical content on reduction (data not shown).

The stabilities of the two single alanine proteins to variations in pH were very similar to the wild type protein, with no change at high pH and significant unfolding only below pH 3. The CXXAH protein showed some decrease in the Soret absorbance between pH 4 and pH 3 (Fig. 3), thus distinguishing it from the other forms of the protein.

Guanidine Hydrochloride-induced Unfolding—The guanidine hydrochloride induced unfolding profiles for oxidized and reduced wild type and single alanine mutant proteins are shown in Fig. 4 and the midpoints given in Table III. Both the wild type and CXXAH protein gave smooth unfolding profiles with good agreement between data obtained by visible absorption spectroscopy, using the change in the Soret or \( \alpha \)-bands, and by CD spectroscopy, monitoring changes at 222 nm. This was not the case for the AXXCH protein, which is discussed below.

Variability of the Heme Environment on Unfolding of the Holo AXXCH Mutant Protein—At low guanidine hydrochloride concentrations (0–1.5 M) and before the start of the main unfolding transition, the oxidized AXXCH protein showed a 15% decrease in the intensity of the Soret band with no concomitant change in the \( \alpha \)-band region. The unfolding of oxidized AXXCH monitored by CD spectroscopy showed no change over this range of guanidine hydrochloride concentration. The different unfolding profiles are illustrated in Fig. 5. Guanidine hydrochloride induced unfolding of the reduced AXXCH protein showed similar profiles when Soret and \( \alpha \)-band changes were monitored, but these profiles indicated complex behavior. The \( \alpha \)-band maxima of the reduced protein moved from 556 to 558 nm and increased in intensity, and the peak became symmetrical over the range of 0–2.6 M guanidine hydrochloride (Fig. 6). The Soret band intensity also increased over the same range.

The small changes in the oxidized visible spectra, which are not mirrored by changes in the CD spectra, may indicate subtle alterations in the heme environment at low denaturant concentrations. They were not seen for the wild type CXXCH or mutant AXXAH and CXXAH proteins and may be related to the small percentage of noncovalently attached heme. To date it has not been possible to prepare the AXXCH protein free of noncovalently bound heme.

**Discussion**

We have argued previously that the unusual formation of holo \( H. \) thermophilus cytochrome \( c_{552} \) in the cytoplasm of \( E. \) coli is a consequence of the apo form of the protein being able to adopt sufficient structure that noncovalent binding of heme is followed by spontaneous thioether bond formation. Recently, this view has been supported by the finding that a \( b \)-type cytochrome derivative of cytochrome \( c_{552} \) can be formed in the \( E. \) coli cytoplasm if the CXXCH heme-binding motif is changed to AXXAH (10). It ought, therefore, to be possible to form the corresponding single cysteine variants in the cytoplasm, and this is confirmed in the present work. Just as in the case of the

**Table III**

Midpoints of guanidine hydrochloride induced unfolding

|         | Oxidized \( G_{u/h^1} \) | Reduced \( G_{u/h^1} \) |
|---------|-------------------------|------------------------|
| AXXCH   | 4.1                     | Not fully unfolded in 7 M |
| AXXAH   | 6.5                     | 1.4                    |
| CXXAH   | 3.4                     | 4.5                    |

Values for the wild type and double alanine mutants of cytochrome \( c_{552} \) are included for comparison.
wild type CXXCH protein, it has not strictly been proved that the single cysteine proteins are formed completely spontaneously. For example, either the cysteines or the heme might in principle be modified in some way before the covalent attachment can occur. However, the possibility of a transient disulfide bond formation, in the cytoplasm and independent of the periplasmic cytochrome c biogenesis apparatus, between the two cysteines of the CXXCH motif can now be largely disregarded, given the successful formation of the single cysteine derivatives. The fact that for the monocysteine proteins the heme is found in only one of the two possible orientations suggests that as for the wild type protein, any initial heme binding in the wrong orientation must be followed by dissociation and reassociation before covalent bond formation can occur. The present work also shows that in principle either of the thioether bonds can be formed first in the assembly of a c-type cytochrome.

A second example of a c-type cytochrome that can form in the cytoplasm of E. coli is cytochrome c\textsubscript{552} from Thermus thermophilus. In this case a mixture of products is found. In the present context it is of interest that in one study (20) but not in the other (21), there was evidence for attachment to only one cysteine of the CXXCH motif. It is difficult to understand why the second thioether bond did not form. It has never before been possible to study the physicochemical effects of losing one thioether bond from a c-type cytochrome. Pettigrew (2) measured the reduction potentials for the AXXCH species from Euglena gracilis and Crithidia oncopelti but could only compare them with horse heart cytochrome c. Thus, it could not be concluded with certainty that the relatively small changes in reduction potential seen for the AXXCH proteins reflected the loss of a thioether bond and/or compensations from differences in overall structure and heme environment between the protozoan AXXCH and horse heart cytochrome c proteins. In the present work we find that the loss of one thioether bond on going from CXXCH to AXXCH or CXXAH has only a small effect on the reduction potential. Although this could mean that the consequence of loss of the thioether bond is coincidentally balanced by other changes in the heme environment, the fact that the stability toward thermal and chemical denaturation is very similar for the CXXCH, AXXCH, and CXXAH proteins suggests, as do far UV CD spectra, that there is little change in overall structure. In turn this suggests that the larger change in reduction potential previously observed on going from the CXXCH to the AXXAH protein (10) may be substantially correlated with a general change in heme environment, correlating with the loss of stability toward thermal and chemical denaturation, rather than...
with the loss of the thioether bonds. Thus overall the results of our work suggest that the formation of thioether bonds in c-type cytochromes is not for the purpose of tuning reduction potential, as previously discussed by Barker and Ferguson (7).

There seems to be no particular major disadvantage of the AXXCH species relative to the CXXCH. Broadly the same conclusion was drawn by Pettigrew et al. (19) in respect of the naturally occurring AXXCH protein in *Euglena*. We are left with the questions of why do some protozoa have the single cysteine attachment mode and why it apparently does not occur *in vivo* in bacteria? The answer to the latter may relate to the biogenesis mechanism in the latter, which seems to have evolved to involve the participation of a disulfide (22).

In the present work the CXXCH variant of *H. thermophilus* cytochrome *c*$_{552}$ differs little from the wild type CXXCH protein. On the other hand, the AXXCH variant does show some differences, not only in being slightly less stable but also in having some tendency to distortion in the heme region. Thus, contrary to what has been observed in protozoan species, the present work suggests that CXXCH rather than AXXCH might occur naturally. However, the single cysteine variants of the cytochrome *c*$_{552}$ studied here are artifacts, and no evolutionary selection elsewhere in the polypeptide has occurred to give enhanced stability. In this context it is notable that the known c$_1$ proteins with a single cysteine attachment all have the heme-binding motif FAPCH (6), and the protozoan cytochromes c have AXQCH (1), suggesting some structural compensation for the loss of one heme-binding cysteine. Nevertheless, the *Euglena* cytochrome *c*$_{558}$ was noted (19) to be subjected to a high spin transition on heating that is not normally observed under comparable conditions with the double cysteine proteins, indicative of a correlation between loss of a thioether bond and some decrease in overall stability.

It is instructive to compare the present work in which the properties of CXXCH, AXXCH, and XAXXH variants of *H. thermophilus* cytochrome *c*$_{552}$ are described with that of Arnesano et al. (23), in which the consequences of introducing a thioether bond into the heme-binding site of cytochrome *b*$_{562}$ were studied. The latter workers also found that introduction of the thioether bond conferred a significant extra stability toward denaturation by both heat and guanidine hydrochloride. Arguably, the widespread occurrence of heme attachment to two cysteines in a CXXCH motif in c-type cytochromes reflects an enhanced stability toward extreme conditions of pH and temperature that are not identified in the present work but that were present at an important stage in the evolution of c-type cytochromes.

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