NMR Analysis Shows That a b-Type Variant of Hydrogenobacter thermophilus Cytochrome c_{552} Retains Its Native Structure*

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Conversion of Hydrogenobacter thermophilus cytochrome c_{552} into a b-type cytochrome by mutagenesis of both heme-binding cysteines to alanines significantly reduces the stability of the protein (Tomlinson, E. J., and Ferguson, S. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5156–5160). To understand the effects of this change on the structure and dynamics of the protein, heteronuclear 15N-edited NMR techniques have been used to characterize this b-type variant. The backbone 15N, 1H, and 1H H resonance of the protein have been assigned. Analysis of J_{HH} coupling constants, nuclear Overhauser enhancement intensities, and chemical shift index data demonstrates that the four 3-H helices present in the wild-type protein are retained in the b-type variant. Comparison of the chemical shifts for the b-type and wild-type proteins indicates that the tertiary structures of the two proteins are closely similar. Some subtle differences are, however, observed for residues in the N-terminal region and in the vicinity of the heme-binding pocket. Hydrogen exchange studies show that there are 25 backbone amide protons that exchange very slowly in the b-type variant and confirm that the fluctuations within the b-type protein are of a similar extent to those in the wild-type protein. These data demonstrate the notable retention of the native secondary structure and tertiary fold despite the absence of covalent linkages between the heme group and the protein.

A c-type cytochrome has one or more heme groups attached to a polypeptide chain via thioether bonds formed between the vinyl groups of heme and the two thiol groups of a character-

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§ The abbreviations used are: C10A/C13A HT-c552, b-type variant of cytochrome c_{552} from H. thermophilus; AAP, aminopeptidase from A. proteolytica; HSQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; WT HT-c552, wild-type cytochrome c_{552} from H. thermophilus.
with sweep widths of 11363.6, 9009.0, and 2500.0 Hz in the F3 (1H), F1 HSQC and TOCSY-HSQC experiments (10, 11). Data were collected the best yields of 15N-labeled C10A/C13A HT-c552 as deter-

maxima at 560, 529, and 425 nm, characteristic of C10A/C13A The UV-visible spectrum of a suspension of the cells gave

of the heme precursor, during expression. This hypothesis was tested by the addition of C10A/C13A HT-c552 on an SDS gel could be seen after sonication of the

showed signs of correct expression from their color, no band for the cells from the minimal media. The addition of 0.1 m M

—the sole nitrogen source and carbon dioxide as the sole carbon

point for the guanidine hydrochloride-induced unfolding of the wild-type protein is reduced from 4.5 to 4.1 m if the N-terminal methionine is present (9). Proteolysis of the N-terminal methionine from WT HT-c552 was achieved by the reaction of the protein with the leucine aminopeptidase from A. proteolytica (AAP) (9). The same method was therefore employed for C10A/

C13A HT-c552. 100% cleavage of the N-terminal methionine occurred after 1 h of incubation with AAP, whereas prior to cleavage the protein consisted of 92% methionylated protein as determined by N-terminal sequencing.

The stability of cleaved HT-c552 was compared with that of the methionylated protein by thermal denaturation studies using CD spectroscopy (Fig. 1). For both proteins a sharp decrease in the ellipticity at 222 nm was observed between 40 and 60 °C, indicating a significant reduction in secondary structure throughout this range. The transition mid-point (melting temperature) of reduced C10A/C13A HT-c552 is 58.3 °C with the N-terminal methionine cleaved. This value is significantly higher than that of the reduced methionylated protein, 51.4 °C. The cleaved protein was used in the subsequent NMR studies.

RESULTS Expression of 15N-Labeled HT-c552—Small scale trials were used to optimize conditions for the production of 15N-labeled C10A/C13A HT-c552. The media used for the trials were the named minimal media, minimal Oregon, minimal +, Celtone, Silantes OD5, and Bioexpress (Cambridge Isotopes Inc.). These media are produced from algae using ammonium chloride as the sole nitrogen source and carbon dioxide as the sole carbon source. The cells expressed in minimal Oregon and in minimal + media were of a pale pink color, whereas the cells from the minimal, Celtone, Silantes OD5, and Bioexpress media were white and showed no signs of expression of HT-c552. Although the cells from the minimal + and minimal Oregon media showed signs of correct expression from their color, no band for HT-c552 on an SDS gel could be seen after sonication of the cells.

Correct synthesis of cytochromes requires the incorporation of a heme moiety into the apoprotein. One explanation for the lack of observable expression of C10A/C13A HT-c552 in the initial trials is that it could be due to the poor supply of heme during expression. This hypothesis was tested by the addition of the heme precursor, δ-aminolevulinic acid, to the growth media. The addition of 0.1 m M δ-aminolevulinic acid in the minimal + and minimal Oregon growth media resulted in a noticeable increase in the intensity of the red color of the cells. The UV-visible spectrum of a suspension of the cells gave maxima at 560, 529, and 425 nm, characteristic of C10A/C13A HT-c552. Expression using the minimal Oregon medium gave the best yields of 15N-labeled C10A/C13A HT-c552 as determined by the absorbance at 560 nm, and this was employed for the production of the protein for the NMR studies.

Cleavage of N-terminal Methionine—It has been found previously for WT HT-c552 that the presence of an N-terminal methionine decreases the stability of the protein. The mid-

FIG. 1. Thermal denaturation of reduced C10A/C13A HT-c552 at pH 7.4 as followed by the ellipticity in the CD spectrum at 222 nm. Open symbols represent the methionylated protein, and closed symbols represent the protein with the N-terminal methionine cleaved.

as in b-type cytochromes, there are two possible orientations with which the heme group can insert into C10A/C13A HT-
c552. These differ by a rotation of 180° of the heme about an axis connecting the α-meso and γ-meso positions. For C10A/
C13A HT-c552 there is one major set of NMR resonances for the heme group showing that there is one predominant heme orientation. However, in the NMR spectra of some samples another minor set of much weaker heme resonances could be resolved reflecting a variable low population of conformers with the alternative heme orientation (approximate relative intensities of the resonances major:minor is ≈70:≤30).

Secondary Structure Identification—The H° chemical shifts of C10A/C13A HT-c552 were used to predict the secondary structure present in the protein using the chemical shift index

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method (14) (Fig. 3). C10A/C13A HT-c552 is predicted by this method to contain five α-helices (residues 1–7, 12–16, 19–33, 38–49, and 66–80) and a region of β-sheet (residues 55–57). The short region assigned by the chemical shift index as β-sheet is found in close proximity to the heme group in the wild-type protein structure. Ring current shifts from this group rather than β-sheet secondary structure are likely to be responsible for the downfield H^N chemical shifts in this region.

To confirm the regions of secondary structure predicted using the chemical shift index method, J_HH coupling constant measurements have been made, and the intensities of sequential H^N-H^N NOEs have been analyzed. These data are summarized in Fig. 3. J_HH coupling constant values of less than 5 Hz are seen for residues 2–7, 28–31, 38–44, 46–47, and 66–77 within four of the regions identified as helical from the chemical shift index. These regions also have medium to strong intensity H^N-H^N NOEs and weaker or absent H^N-H^N sequential NOEs. Taken together these data therefore identify four main α-helical regions in C10A/C13A HT-c552 (residues 2–7, 28–31, 38–47, and 66–77). These are in closely similar positions to the helices seen in the wild-type protein (residues 3–7, 27–32, 38–47, and 67–78) (6). The fifth potential helical region for C10A/C13A HT-c552 from the chemical shift index (residues 12–16) only contains one residue with a J_HH coupling constant value of less than 5 Hz. Therefore, there is no evidence at this stage for a regular persistent helical conformation in this region. These residues do not adopt a helix in the wild-type structure.

Comparison with the Chemical Shifts of Wild-type HT-c552—Fig. 4 shows a comparison of the chemical shifts for the H^N, H^D, and H^H resonances of each residue in WT (6) and C10A/C13A HT-c552. Chemical shift values are very dependent on steric and electronic effects including those arising from both protein secondary and tertiary structure (15, 16). The detailed comparison of the chemical shift data for the wild-type and mutant proteins therefore provides a sensitive method for establishing the level of similarity between the structures adopted by WT and C10A/C13A HT-c552. Overall, the chemical shifts for C10A/C13A HT-c552 are almost universally 0.12 ppm greater than those determined for the authentic protein. This difference is very similar to that found in the H^1 NMR characterization of recombinant WT HT-c552 (9). The discrepancy has been attributed to variations in chemical shift referencing and solution conditions in the different NMR studies. Therefore, in this work the chemical shifts of Hasegawa et al. (6) for the wild-type protein have been corrected by 0.12 ppm prior to the comparison. As shown in Fig. 4, a very good agreement is observed between the H^N, H^D, and H^H chemical shifts of WT and C10A/C13A HT-c552 (correlation coefficients of 0.999 for H^N, 0.984 for H^D, and 0.992 for H^H) despite a difference of solution pH in the two studies (WT pH 4.8, C10A/C13A, pH 7.4). The higher pH conditions were required for the NMR study of C10A/C13A HT-c552, as this variant is not stable at low pH (7). The agreement between the chemical shifts of WT and C10A/C13A HT-c552 provides clear evidence for the close similarity of the overall tertiary structure as well as the secondary structure of the two proteins. The similarity is particularly striking for residues that have chemical shift values perturbed by heme ring current effects. This is the case, for example, for Gly-49 H^D (WT 2.98, 1.75 ppm; C10A/C13A 2.97, 1.75 ppm), Val-53 H^N (WT 10.26 ppm; C10A/C13A 10.32 ppm), and Ile-76 H^N (WT 2.62 ppm; C10A/C13A 2.62 ppm). Some differences do occur, however, between the chemical shifts of the two proteins. Differences of greater than 0.3 ppm are seen for the H^N chemical shifts of seven residues, the H^D chemical shifts of six residues, and the H^H chemical shifts of
nine residues. Nine of the 22 deviations greater than 0.3 ppm come from either the residues that are mutated (Ala-10 and Ala-13), those that are directly adjacent to the mutations in the sequence (Gly-9, Met-11, Ala-12, and His-14), or those that are very close in space to the mutated residues in the wild-type structure (Gly-22). Six of the other differences involve residues that are located in the N-terminal region of the protein (Ser-8, Ala-18, Asp-27, Tyr-32, Lys-36, and Ser-51) and suggest that there are some slight changes to the structure or dynamics of this region in C10A/C13A HT-c552. These changes could result, at least in part, from the differing pH conditions of the two NMR studies. In particular, it is possible that salt bridge formation between the side chains of Glu-2 and Lys-5 under the higher pH conditions used for the C10A/C13A HT-c552 NMR work could increase the stability of the N-terminal helix. The other chemical shift deviations greater than 0.3 ppm are seen for Lys-8, Ala-18, Asp-27, Tyr-32, Lys-36, and Ser-51. Asp-27, Tyr-32, and Ser-51 are adjacent to the heme-binding pocket. Small variations in this binding pocket may account for these chemical shift changes. The largest chemical shift difference is seen for the HN of Lys-36. The reason for this difference is not clear. However, as this residue is very exposed, the chemical shift may have been affected by the differences in solution conditions used for the wild-type and mutant NMR studies.

Hydrogen Exchange Study of C10A/C13A HT-c552—Studies of the hydrogen exchange properties of C10A/C13A HT-c552 provide a method for probing the dynamics and the extent of backbone fluctuations in the mutant protein (17, 18). Slowly exchanging amide protons in reduced C10A/C13A HT-c552 were identified by recording an HSQC spectrum immediately after the lyophilized protein was dissolved in D2O and then another spectrum 3 days after the initiation of the reaction. Each HSQC experiment was recorded in 2 h. The results of these experiments are summarized in Fig. 3. For mitochondrial cytochrome c, a conformational change in the iron coordination has been found to occur on hydration of lyophilized protein (19). It is possible that a similar change could also occur in HT-c552 and may reduce the hydrogen exchange protection for some residues. This work concentrates, in comparison with the wild-type protein, on the amides that show a high level of protection and therefore have not been significantly affected by this possible change. In C10A/C13A HT-c552 there are 36 slowly exchanging amides whose HSQC peaks are present after 2 h and 25 amides whose HSQC peaks are present after 3 days. 20 of the 25 slowly exchanging amides that are present after 3 days are located within the α-helical regions of the protein. The other five residues are in the loop region between helices 3 and 4 and are found in close proximity to the heme group (Fig. 5b).

The level of hydrogen exchange protection seen for C10A/C13A HT-c552 is very similar to that found in the wild-type protein, where 36 slowly exchanging amides were found to be present after 20 h (6). As in the mutant the majority of these residues are located in the four α-helices (23 residues) and in the loop region connecting helices 3 and 4 (13 residues; Fig. 5a).

The different pH conditions of the two NMR studies will have a significant effect on the hydrogen exchange rates, the intrinsic rates at pH 7.4 (C10A/C13A HT-c552 study) being expected to be of the order of 500 times faster than those at pH 4.8 (WT HT-c552 study) (20). Overall, the large number of protected amides for C10A/C13A HT-c552 at pH 7.4 indicates that the protein retains a stable core despite loss of the thioether linkages to the heme group.

The largest differences between the hydrogen exchange data for the wild-type and mutant proteins are seen for Leu-4, Ala-5, Cys/Ala-13, His-14, Gly-22, Lys-31, Ser-51, and Ile-79. Cys-13, His-14, Gly-22, Lys-31, and Ile-79 all have an increased protection in the wild-type protein, their amide protons exchanging within 2 h in C10A/C13A HT-c552 but not exchanging within 20 h in WT HT-c552. These differences presumably relate both to the higher pH conditions of the C10A/C13A HT-c552 study and also to the mutation at Cys-13. As discussed above Gly-22 is in close proximity to Cys-13 in the wild-type structure. For Leu-4 and Ala-5 in the N-terminal region and
Ser-51 in the vicinity of the heme-binding site, there is a significantly higher level of protection in the mutant. The amide protons of these residues persist after 72 h in C10A/C13A HT-c552 but exchange within 20 h in WT HT-c552. These are all residues that also show a change in $\text{H}^b$ or $\text{H}^c$ chemical shift of greater than 0.3 ppm between the wild-type and mutant proteins. The higher protection for Leu-4 and Ala-5 may reflect the greater fluctuation in these regions.

**FIG. 4.** Differences in $\text{H}^a$ (a), $\text{H}^b$ (b), and $\text{H}^c$ (c) chemical shifts observed for reduced WT HT-c552, pH 4.8 at 25 °C (6), and reduced C10A/C13A HT-c552 at pH 7.4 and 25 °C.

**DISCUSSION**

The present work shows for the first time that the cytochrome $c$ fold can be attained without the formation of the thioether bonds that are characteristic of this class of molecules. There is recent evidence (21) that this fold can be at least approached in the absence of such bonds for mitochondrial cytochrome $c$, contrary to previous views. Despite the absence of the two covalent thioether linkages to the heme group present in wild-type HT-c552, all the main features of the wild-type tertiary structure, including the characteristics of the heme-binding site, are retained in C10A/C13A HT-c552. This is consistent with the observation that there is only a slight lowering in reduction potential (by 75 mV) of C10A/C13A HT-c552 compared with that of the WT HT-c552 (7). In addition the high level of protection from hydrogen exchange observed for 25 backbone amides in C10A/C13A HT-c552 indicates that the folded mutant protein is not in equilibrium with the partially folded apo state or the unfolded state to any significant extent.

Furthermore, it appears that in C10A/C13A HT-c552, one of the two possible heme orientations predominates. This correlates in a satisfying fashion with several recent observations on the in vitro formation of thioether bonds in HT-552 and derivatives that have only one thioether bond. In the case of the wild-type polypeptide, carrying the CXXCH motif, the correct product forms via a non-covalent intermediate state (22). This implies that the heme must be in one orientation in order for bond formation to occur. This contrasts with certain other examples of thioether bond formation in vitro where incorrect products are formed (23, 24). Additionally, when derivatives of heme with just one vinyl group were allowed to react in vitro with variants of HT-c552 carrying either AXXCH or CXXAH motifs, only two combinations of protein and vinyl heme gave a smooth product formation, again implying that the non-covalent complex that preceded covalent bond formation was stereoselective (25). Thus the apoprotein and heme together generate interactions that favor formation of a stereoselective complex. It is possible that, as with cytochrome $b_5$ (22, 26), this is not formed immediately but rather is preceded by a mixture of two orientations of heme binding. The kinetics of in vitro thioether bond formation was interpreted in this fashion although there was no independent evidence for this (22).

Some small variations have, however, been identified between the NMR data for WT and C10A/C13A HT-c552, both in chemical shift values and hydrogen exchange protection. Most of these differences involve the residues at which the mutations have been made or those adjacent to them, either in the sequence or in the tertiary structure. These differences could merely reflect the local electronic or steric changes from swapping cysteine to alanine. However, there may also be some subtle changes in structure, particularly as Ala-13, His-14, and Gly-22 all lose the hydrogen exchange protection that they have in the wild-type protein. Most interesting, some differences between the NMR data for WT and C10A/C13A HT-c552 are seen in other parts of the structure away from the mutation sites. These differences are particularly concentrated in the N-terminal region of the protein and for a few residues that are adjacent to the heme-binding pocket. In both of these areas there are also residues that show a higher level of hydrogen exchange protection in the mutant than in the wild-type protein, suggesting that there are small alterations in the dynamic fluctuations in these regions.

It is interesting to compare the results from the $c$-type to $b$-type conversion of HT-c552 to those reported for the $b$-type to $c$-type conversion of E. coli cytochrome $b_{562}$ (27). The addition of a covalent linkage to the heme in cytochrome $b_{562}$ via a cysteine...
residue introduced from an Arg-98 → Cys mutation resulted in a structure that was essentially identical to that of wild-type cytochrome \( b_{562} \). However, studies of NOE intensity ratios suggested that there might be an increased rigidity in Arg-98 → Cys cytochrome \( b_{562} \) particularly in the region of a long external loop and the start of helix \( a_3 \) (27). In a similar fashion chromatographic and spectropolarimetric studies of a Cys-14 → Ser variant of mitochondrial cytochrome \( c \) have identified that this variant is more disordered than the wild-type protein (28). This variant has lost just one thioether bond to the heme present in wild-type mitochondrial cytochrome \( c \) and the resistance of the protein to denaturation (29). For cytochrome \( c \), there are four typographical errors in the chemical shift table of Hasegawa et al. (16) for residues 8(H9251–H9252, 27(H9253–H9254), 28(H9255), and 51(H9256) in WT-c552. The changes to these chemical shifts do not have any significant effects on the conclusions of this paper.

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Note Added in Proof—It has been brought to our attention that there are four typographical errors in the chemical shift table of Hasegawa et al. (16) for residues 8(H9251–H9252, 27(H9253–H9254), 28(H9255), and 51(H9256) in WT-c552. The changes to these chemical shifts do not have any significant effects on the conclusions of this paper.

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