Conversion of Cytochrome b_{562} to *c*-Type Cytochromes†

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ABSTRACT: Cytochrome b_{562} from the periplasm of *Escherichia coli* is the only member of a family of cytochromes sharing the 4-α-helical bundle structural motif that does not have a covalently bound heme. We have introduced cysteine residues into the amino acid sequence of cytochrome b_{562} in positions homologous to those found in the other members of the family, generating the ubiquitous heme-binding peptide (-C-X-Y-C-H-) found in virtually all *c*-type cytochromes. The resulting single-cysteine-containing mutants, R98C and Y101C, together with the double mutant combining both of these mutations have been expressed into the periplasm of *E. coli*. The apo- and holoprotein products of each mutation have been isolated, and all the mutants produce multiple species with covalently attached heme. Results from ion exchange chromatography, optical spectroscopy, SDS gel electrophoresis, and electrospray mass spectrometry identified those species that appear to be cytochrome b_{562} holoprotein with thioether covalent linkages to the heme as the only difference in chemical composition between them and the wild-type protein. Results from ^{1}H-NMR experiments prove the existence of the expected *c*-type covalent bonds in each of these proteins and show that the structure of the heme pocket is not significantly perturbed by the covalent modification(s). These proteins all have perturbed optical spectra, compared with those of the wild-type protein, that are consistent with the modifications but are still characteristic of six-coordinate, low-spin cytochromes with Met−His ligation to the heme iron in both oxidation states.

Cytochrome b_{562} from *Escherichia coli* and the cytochromes *c′* from photosynthetic species belong to a class of cytochromes sharing the same 4-α-helical structural fold but no significant sequence similarity (Mathews, 1985). This family probably also includes cytochromes c_{556} (Meyer & Kamen, 1982) which have similar electronic properties to cytochrome b_{562} (Moore et al., 1982) but have not been characterized structurally. Both cytochromes c_{556} and b_{562} are low-spin, six-coordinate cytochromes of fairly high redox potential with methionine and histidine residues providing the axial ligands to the heme iron. In contrast, the cytochromes *c′* have high-spin, five-coordinate heme centers with a histidine providing the only axial ligand. It has been suggested that this family of cytochromes has evolved from a common structure (Weber et al., 1981b). However, the diverse functions of all 4-α-helical proteins suggest that this motif may have evolved independently a number of times and that the familial relationships may be misleading (Moore, 1991). Cytochrome b_{562} is the only member of this family of cytochromes not to have a covalently bound heme and could therefore represent an evolutionary intermediate on the path to the covalent cytochromes.

Almost all *c*-type cytochromes have at the site of heme attachment two thioether linkages to the cysteine residues in the characteristic heme-binding peptide sequence -C-X-Y-C-H-[1] (Moore & Pettigrew, 1990). The thioether bonds are formed as the result of electrophilic addition of the cysteine thiols across the double bonds of the two heme vinyl groups. The histidine in the conserved attachment sequence invariably provides one of the axial ligands to the heme iron in the mature holoprotein. The stereochemistry at the chiral center produced by the addition is the same in all cytochromes *c* studied to date and is of the *S* configuration (Timkovich & Bondoc, 1990). In eukaryotes, enzymes (heme lyases) are present in mitochondria and catalyze the formation of the two thioether bonds (Baslie et al., 1980). This heme lyase activity is presumed to be present in all prokaryotic organisms that produce *c*-type cytochromes, although no direct evidence for the existence of bacterial heme lyases has been found. Heme lyase mechanisms are poorly understood, but significant attention has been focused on their role in the import of cytochrome *c* into mitochondria and a requirement for reducing cofactors, such as NADH, NADPH, and flavins, has been demonstrated (Nicholson & Neupert, 1989; Taniuchi et al., 1983). Lyase activities that differ in cytochrome substrate specificity have been identified in yeast mitochondria, and there are suggestions that the eukaryotic lyases have substrate specificity beyond the conserved -C-X-Y-C-H- sequence (Visco et al., 1985).

E. coli produces endogenous *c*-type cytochromes when grown under anaerobic or semianaerobic conditions (Bragg & Hackett, 1983; Iobbi-Nivol et al., 1994; Poole & Ingledew, 1987). Some exogenous *c*-type cytochromes can be expressed in *E. coli* and isolated as the mature holoprotein (Diaz

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[1] -C-X-Y-C-H- represents, in single-letter code, the characteristic sequence of amino acid residues which is attached to heme in *c*-type cytochromes through the two cysteines; X and Y represent any amino acid residue.

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et al., 1994; Pollock & Voordouw, 1994; Sambongi & Ferguson, 1994; Sambongi et al., 1991; Ubbink et al., 1992; Wachenfeldt & Hederstedt, 1990). Despite the obvious implication that an *E. coli* heme lyase exists, none has been specifically isolated. A mutant, mammalian, *b*-type cytochrome, in which a residue close to one of the heme vinyl groups has been replaced by a cysteine, has been expressed in *E. coli* and shown to have a single *c*-type thioether linkage involving the introduced cysteine residue (Barker et al., 1993). No clear mechanism by which heme is attached to the protein has emerged from any of these systems. Several groups have suggested (Barker et al., 1993; Pollock & Voordouw, 1994; Sambongi & Ferguson, 1994) that in cytochromes that fold to give a stable apoprotein and subsequently bind heme noncovalently, a suitably positioned cysteine thiol will spontaneously add across a heme vinyl group giving rise to the *c*-type thioether bond without enzymatic involvement. This has not been tested *in vitro* for any protein other than mitochondrial apocytochrome *c*, which does not react spontaneously with free heme to form the native holoprotein (Cohen et al., 1974; Fisher et al., 1973; Stellwagen et al., 1972). There exists the possibility that a variety of mechanisms, both enzymatic and spontaneous, lead to the correct thioether bond formation in different *c*-type cytochromes expressed in *E. coli* (Sambongi et al., 1994), but these pathways have yet to be deconvoluted.

We are interested in elucidating the protein structural requirements for obtaining *c*-type covalent linkage in cytochromes, in testing whether the attachment can occur spontaneously within a given protein scaffold, and in examining the consequences of heme attachment on properties of a cytochrome. As a prerequisite, we establish here that cytochrome b_{562} can be converted to a *c*-type cytochrome. Using *E. coli,* we have overexpressed mutants of cytochrome b_{562} with cysteine residues at positions homologous to the -C-X-Y-C-H- sequence found in cytochromes c' and related cytochromes c_{556}. The resulting variant proteins are isolated from the cells as either apo- or holoprotein. A variety of holoprotein species are observed for each of the mutant proteins studied, and it has been necessary to select, using a variety of analytical criteria, which of the major products of expression are most likely to be *c*-type cytochromes. All of the mutant proteins studied here can be isolated as holoproteins with covalently linked heme. ^{1}H-NMR analysis of the ferrocytochromes has shown that the expected *c*-type thioether bonds are present and are of unique stereochemistry.

MATERIALS AND METHODS

Cloning and Mutagenesis. The gene for *E. coli* cytochrome b_{562} was cloned from strain BL21 into a high-expression plasmid pCE820 (Lewis et al., 1993). The published sequence (Nikkila et al., 1991; Trower, 1993) was used to design forward and reverse oligonucleotide primers (P1 and P2, respectively) that would amplify the whole expressed gene, complete with signal peptide, with appropriate restriction sites (*Nco*I and *Bam*HI) at each terminus. The creation of the *Nco*I site at the initiating methionine codon required the insertion of a GGA (glycine) codon at the start of the signal peptide. The gene was amplified from a genomic DNA preparation from BL21 cells using these primers according to standard PCR techniques. *Taq* polymerase (Promega) was used in a 25-cycle amplification with annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The expected product was purified by agarose gel electrophoresis before digestion with both *Nco*I and *Bam*HI restriction endonucleases. This cut product was ligated with the gel-purified 4.1 kb fragment of *Nco*I/*Bam*HI-cut pCE820 plasmid. The ligation mix was transformed into *E. coli* strain NM554 (Stratagene) by electroporation, and after recovery the cells were plated onto TY agarose containing 100 μg of ampicillin/mL and 0.1 mg of glucose/mL. Colonies expressing cytochrome b_{562} were identified by the pink cell color after overnight growth in 2× TY media with 100 μg of ampicillin/mL. Positive clones were propagated, the plasmid (pCEB562) was isolated, and the expected sequence was confirmed by standard dideoxynucleotide-sequencing techniques.

Since the mutations required for this study are within eight amino acids of the C-terminus, the gene was modified by reamplification of the cytochrome b_{562} insert in pCEB562 using 3′-oligonucleotide primers containing relevant mismatches and the overhanging *Bam*HI site (P3−P5). The 5′-primer used (P6) matched the plasmid sequence just upstream of the cloning site, and the resulting amplified products are 480 base pairs long. These PCR products were isolated and cloned as before, and the mutant sequence confirmed by sequencing. The oligonucleotides used were as follows with mutant codons in bold type: P1, TCCGTACCATGGGACG-TAAAAGCCTGTTAGCTATTCTTGCA; P2, CGAGGATC-CTTAACGATACTTCTGGTGATAGGCG; P3, (Y101C) CGAGGATCC TTAACGATACTTCTGGTG**ACA**GGCGT-TGCGGGTCGTTTT; P4, (R98C) AGAGGATCCTTAAC-GATACTTCTGGTGATAGGCGTT**GCA**GGTCGTTTT-CAGTTG; P5, (R98C/Y101C) AGAGGATCCTTAAC-GATACTTCTGGTG**ACA**GGCGTT**GCA**GGTC-GTTTTCAGTTG; P6, GCTGTTGACAATTAATCATCCG-GCTCG.

Protein Expression, Purification, and Analysis. Cytochromes were expressed from the same strain of *E. coli*, NM554, cultured in 2× TY media at 37 °C. Cultures were 1.5 L of medium in a 2 L flask and this was shaken at 250 rpm, conditions we consider to be microaerobic. Innoculae were grown to an optical density at 600 nm of about 1 before induction with 0.5 mM isopropyl β-D-thiogalactoside, and the cultures continued into stationary phase before harvest by centrifugation at 18 h after induction. As discussed in the results, following cell growth, cytochrome b_{562} is found in both the periplasmic fractions and the culture medium. Protein was harvested from the media by ultrafiltration of the centrifugation supernatant and then exchanged into 20 mM diethanolamine, pH 8.5, by diafiltration using 3000 molecular weight cutoff membranes in a tangential flow system (Minisette, Filtron). The material was batch adsorbed onto DEAE cellulose (Whatman DE53), and cytochrome b_{562} proteins eluted from the resin with 200 mM KCl in the same buffer. Protein was harvested from *E. coli* cells by fractionation of the pelleted cells into spheroplasts and soluble periplasm contents using the lysozyme/EDTA method (Weiss, 1976) with the modification that the cell resuspension buffer of 0.1 M Tris, pH 8.0, and 20% sucrose also contained 2 mM sodium ascorbate.

The apo- and holocytochromes in these fractions were separated on Q-Sepharose (Fast Flow or High Resolution, Pharmacia) developed with a linear gradient of 0−200 mM KCl. The apoprotein eluted at about 50 mM KCl, while the

holoproteins eluted at around 120 mM. Fractions containing apo- or holoprotein were concentrated separately to a minimum volume and purified further on Superdex 75 (Pharmacia) gel filtration columns equilibrated in either 20 mM diethanolamine, pH 8.5, and 150 mM KCl, or 20 mM MES, pH 6.0, and 150 mM KCl. Taken to this stage apoprotein was considered homogeneous. Holoproteins required further, high-resolution anion exchange chromatography on Neobar AQ resin (now sold as Resource Q, Pharmacia) packed in 4 or 8 mL columns at pH 8.5, 20 mM diethanolamine with KCl gradients. Chromatographic separations were carried out at room temperature on a Waters 625 liquid chromatography system equipped with a 996 diode array absorption detector.

Analysis of proteins in cell extracts or purified fractions was also by anion exchange chromatography using a 0.6 mL NEOBAR AQ column developed in 20 mM DEA, pH 8.5, and KCl gradients. SDS polyacrylamide gel electrophoresis (Phastgel, Pharmacia) on 20% homogeneous precast gels was also used to analyze for cytochrome b_{562}. Gels were stained for protein with Coomassie Blue or for heme with tetramethylbenzidine (Thomas et al., 1976). Free cysteine thiols were assayed using 4,4′-dithiodipyridine (Aldrich; Talgoy et al., 1979) or by reaction with β-maleimidopropionate (Fluka; Rich et al., 1975). On reaction with a cysteine thiol group, β-maleimidopropionate adds an extra negative charge to the protein, which can be detected as altered retention times by anion exchange chromatography. This method has the advantage that protein thiols can be detected on the cytochrome in crude extracts. The modification reaction, after short incubation of the sample with 2 mM dithiothreitol, was performed at 10 mM reagent concentration for at least 4 h. Pyridine hemochrome analysis was performed by the method of Antonini and Brunori (1971). The extinction coefficient for the pyridine hemochrome derived from a *c*-type cytochrome with only one thioether link was taken as halfway between that of the hemochrome from free heme and that from a regular *c*-type cytochrome (Berry & Trumpower, 1987). Butan-2-one was used to extract heme from acidified protein solutions (Reid et al., 1984). Optical spectra were recorded on a Cary 3 spectrophotometer.

Mass Spectrometry. Molecular masses were measured by electrospray ionization mass spectrometry (ESI-MS) using either a Perkin Elmer Sciex API III$^+$ or a VG BioQ (Fisons Instruments) triple-quadrupole mass spectrometer. These instruments were calibrated using horse heart myoglobin. Cytochrome b_{562} samples were exchanged by ultrafiltration into deionized water or 0.1 mM dithiothreitol prior to analysis and then diluted to concentrations of about 5 μM in 1% acetic acid in 50% aqueous acetonitrile. These solutions were introduced into the electrospray interface via a Rheodyne sample loop at a flow rate of approximately 3 μL/min.

NMR Spectroscopy. Protein was exchanged into 99% D_2O buffer by ultrafiltration. Phosphate buffers were made from deuterated phosphoric acid and sodium deuteroxide, and 0.5 M KCl was present in all samples. pH* values are uncorrected for the solvent isotope effect. Wild-type protein was exchanged in the apoprotein state, and the holoprotein was formed by adding heme (Sigma H2250) dissolved in 0.1 M sodium deuteroxide in D_2O. Protein samples, under argon in sealed 5 mm NMR tubes, were reduced by addition of aliquots of fresh 0.5 M sodium hydrosulfite (maintained under argon in 0.1 M phosphate buffer, pH* 6.6), to a final concentration of 10 mM. All ^{1}H-NMR experiments were performed on a Bruker AMX 500 spectrometer equipped with a broad-band inverse detection probe. The data set for each mutant consisted of 1D spectra and homonuclear two-dimensional NOESY and TOCSY experiments. The sweep width in all experiments was set to 10 204 Hz. The remaining water signal was irradiated by low-power presaturation. NOESY experiments (Jeener et al., 1979) were acquired as 2D matrices of 2048 × 256 complex points with a mixing time of 130 ms and 64 or 128 transients per increment. The TOCSY experiments were acquired as matrices of 2048 × 150 complex points with a mixing time of 26 ms and 32 or 64 transients per increment. Mixing was achieved via a "clean" 8.5 kHz MLEV spin−lock sequence (Griesinger et al., 1988). All experiments were processed on Silicon Graphics workstations utilizing Felix 2.30. Processing consisted of 2-fold zero filling and apodization of the data with a 75°-shifted sine-bell window function. Assignments of the heme resonances are made by simple protocols first applied using 1D NOE (nuclear Overhauser effect) difference spectroscopy (Keller & Wüthrich, 1980).

RESULTS

Cytochrome b_{562} has been cloned, and its expression has been directed to the periplasmic space of *E. coli* (strain NM554) by the protein's natural signal peptide in a system similar to that already described (Nikkila et al., 1991). In establishing the cellular location of the cytochrome b_{562} protein expressed under these conditions, we have found a significant amount of the protein in the culture medium; an observation not previously reported. We have estimated the amounts of both holo- and apocytochrome b_{562} in the culture medium and periplasmic fractions by analyzing them for holoprotein by optical spectroscopy and ion exchange chromatography before and after addition of exogenous heme to saturate any apoprotein present (data not shown). After 18 h of induction, the time of maximum total yield, 70%−80% of protein is in the culture supernatant while 20%−30% remains in the periplasm. Addition of heme to the apoproteins isolated from each of these mutants initially yielded an optical spectrum identical (in both wavelength maxima and extinction coefficient) to that of wild-type protein. Titration of isolated apoproteins with heme to saturation point allowed the extinction coefficient at 277 nm for each apoprotein to be estimated. These were 3200 M^{-1} cm^{-1} for the wild-type and R98C proteins and 1600 M^{-1} cm^{-1} for Y101C and R98C/Y101C proteins. The total yield of cytochrome b_{562} protein from this system varied from 120 to 160 mg per liter of culture. The proportion of holoprotein present was very variable, but did not exceed 25% of the total yield.

Analysis of Holoproteins Produced in Vivo. Figure 1 shows the analytical separation by anion exchange chromatography of cytochrome b_{562} holoproteins from the periplasmic extracts of cultures of R98C, Y101C, and R98C/Y101C mutants. The wild-type protein eluted as a single peak at the time indicated. Essentially all of the 427 nm absorbing material eluted from this column was associated with cytochrome b_{562} protein in these samples, as judged by results (not shown) from SDS−PAGE gels. On these gels, heme-staining material was observed at a position coincident with

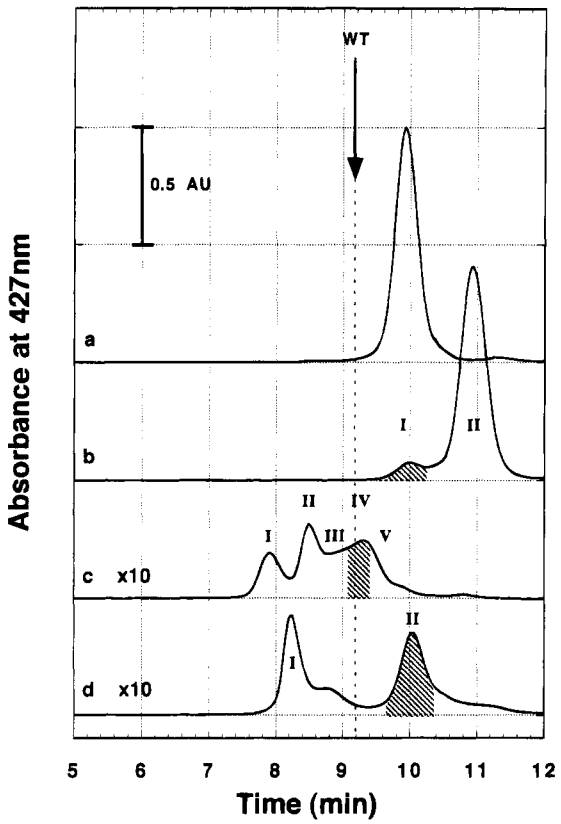

FIGURE 1: Chromatograms of periplasmic extracts of *E. coli* expressing the cytochrome b_{562} mutants as follows: a, R98C; b, R98C after complete reaction with β-maleimidopropionic acid; c, Y101C; d, R98C,Y101C. The absorbance scale for c and d is 10 times more sensitive than for a and b. Column was a 0.6 mL Neobar AQ column equilibrated with 20 mM diethanolamine, pH 8.5, containing 1 mM dithiothreitol and developed over 10 min at 1 mL min^{-1} with a linear salt gradient of 0−300 mM KCl in the same buffer. Periplasm extracts were diluted 5× in starting buffer, and 0.5 mL samples were injected. The spectrum of the eluent was monitored between 250 and 750 nm at a resolution of 1.2 nm and at a rate of 1 spectrum per second. As judged by their optical spectrum, all proteins studied here eluted in the reduced (ferrous) state. For all proteins studied >95% of the total absorbance at 427 nm eluted within the region shown. The arrow and dotted line indicate the retention time of the wild-type holoprotein. Relative to this, the retention time of the R98C variant was consistant with the change in net charge of the protein due to the mutation. The shaded areas represent the proteins isolated and subsequently shown in this communication to contain *c*-type heme.

that of the cytochrome b_{562} protein, which indicated the presence of covalently attached heme, and also close to the electrophoretic front due to the presence of free heme dissociated from protein. Only the stain due to free heme is observed following analysis of the wild-type protein. Once purified to spectral homogeneity, each covalent fraction was analyzed for cysteine thiols and the extractability of the heme group was tested. The pyridine hemochrome method was used to estimate the extinction coefficients (Antonini & Brunori, 1971; Berry & Trumpower, 1987). Properties of the optical spectra of the main species characterized here are given in Table 1. The molecular mass of each of these components was determined by ESI-MS, and these masses are given in Table 2. Under the conditions of ESI-MS analysis, noncovalently attached heme dissociates from the protein and the mass measured is that of the apoprotein. Properties peculiar to each mutant are described below. The objective of these analyses was to identify components which might contain thioether bonds between heme and the cysteine residues as the only modification to the protein. The components identified with those properties are shaded in Figure 1 and were subsequently analyzed by NMR.

Arg98Cys. The yield of holoprotein following the expression of this mutant was similar to that from expression of wild-type protein, as judged by the total absorbance in the visible region of the periplasm extract. Heme staining of SDS−PAGE gels detected cytochrome b_{562} species with covalently attached heme as well as with noncovalently attached heme, despite the fact that only one chromatographic peak was observed (Figure 1a). The optical spectrum was invariant throughout this peak and was the same as that of the wild-type holoprotein, within the resolution of the diode array detector (1.2 nm). Modification of this material with β-maleimidopropionate and reanalysis by anion exchange (Figure 1b) revealed a component (**I**) which had not reacted with the reagent, had a significantly blue-shifted optical spectrum and accounted for all of the heme stain associated with cytochrome b_{562} protein on SDS gels. The maleimidopropionate-modified and unmodified proteins were separated on a preparative scale and this was the only method by which the two species initially present could be separated. Neither component reacted with 4,4′-dithiodipyridine, and heme could only be extracted from component **II**, the mass of which was measured as that of apoprotein plus maleimidopropionate.[2]

Preparative scale anion exchange purification of component **I** revealed a subtle change in the spectrum throughout the chromatographic peak. This was not apparent on an analytical scale. Attempts to obtain significant quantities of the two spectrally distinct species observed have proved unsuccessful so far. From separate growths and preparations we have obtained material varying in the proportions of each of these coeluting species. Analysis by ESI-MS of different preparations of this component clearly indicated the presence of two species, both with covalently attached heme, differing in mass by 32 Da (Table 2). Subsequent NMR analysis of these R98C mixtures confirms that two very closely related species were present (*vide infra*). Correlation of data obtained from the three spectral methods allowed us to assign the optical (Table 1) and NMR (Tables 3 and 4) properties of each of the species of different mass. We have not observed any conditions under which these species interconvert. The composition of the mixture, as determined by NMR, has only been perturbed by the partial chromatographic separation mentioned above. Mixtures of these species have been enriched as high as 10:1 in favor of the lower-mass component but only 2:3 in favor of the higher-mass component. Consequently we cannot quantitatively define the optical properties of the higher-mass species (Table 1). The pyridine hemochrome of the lower mass species has an α-band at 552 nm, consistent with that observed for

[2] It should be noted that if the periplasm extract of this mutant is left for 12 h under aerobic conditions with only a weak reductant present (e.g., dithiothreitol or β-mercaptoethanol), a new species is observed which coelutes with component **I** and has a covalently attached prosthetic group. This blue/green species has a significantly red-shifted optical spectrum no longer resembling that of a normal heme protein and has a strong absorption band at 605 nm. The presence of this species can be minimized if the solution conditions during extraction and purification are kept strongly reducing with at least 2 mM ascorbate and exposure to oxygen is kept to a minimum by maintaining solutions under argon.

Table 1: Properties of the Optical Spectrum of Cytochrome b_{562} Proteins Isolated as Descibed in the Text[a]

protein	reduced λ_{max} [nm] (ϵ [mM^{-1} cm^{-1}])	oxidized λ_{max} [nm] (ϵ [mM^{-1} cm^{-1}])	pyridine hemochrome α-band λ_{max} [nm]
wild-type	318.5 (65)	*280*[b] (19.0)	556
	426.5 (181)	*374* (34)	
	531.0 (17.5)	418.0 (117.0)	
	561.5 (32.0)	531.0 (11.0)	
R98C **I**	319.0 (33.2)	*280* (18.0)	552
	421.5 (158)	*361* (31.2)	
	527.5 (16.0)	415.5 (127)	
	556 (24.5)	529.5 (15)	
R98C **I** + 32 Da[c]	423	nd	nd
	529		
	559		
Y101C **IV**	318.0 (40.8)	*280* (22)	552
	424.5 (178)	360.0 (38.8)	
	529.5 (19)	417.5 (135)	
	560.5 (28)	532.5 (9.9)	
R98C/Y101C **I**	317 (41)	*280* (21)	552
	423.5 (150)	355 (36)	
	530.5 (17.2)	417.0 (130)	
	560.0 (25.8)	533.0 (12.5)	
R98C/Y101C **II**	316.5 (37.2)	*280* (16.5)	549.5
	420.0 (146)	354.5 (30.7)	
	525.5 (16.4)	414.5 (136)	
	555.5 (22.9)	529.0 (10.7)	

[a] Spectra recorded at pH 7.1, 25 mM triethanolamine, 100 mM KCl, 20 °C. [b] Italicized wavelengths are shoulders not apexes. [c] Species observed to be 32 mass units greater than expected for heme attached to the apoprotein (Table 2). This material has not been obtained in isolation, and therefore we have not observed the oxidized spectrum nor can we estimate any extinction coefficients.

Table 2: Molecular Masses (Da) of Various Cytochrome b_{562} Proteins Measured by Electrospray Mass Spectrometry

protein	modification	measured mass (SD)[a]	species/expected mass	
holo wild-type	none	11 780.2 (0.6)[b]	apoprotein	11 779
			holoprotein[c]	12 395
apo wild-type	none	11 780.8 (0.8)	apoprotein	11 779
R98C **I**	none	12 345.9 (1.9)	holoprotein[c]	12 344
		12 379.7 (1.1)		
R98C **II**	βMP[d]	11 897.0 (1.3)	apoprotein	11 727
			modified apoprotein	11 896[e]
Y101C **IV**	none	12 336.5 (1.3)	apoprotein	11 719
			holoprotein[c]	12 337
apo Y101C **V**	none	11 719.7 (0.8)	apoprotein	11 719
R98C/Y101C **I**	βMP[d]	12 451.2 (0.6)	holoprotein[c]	12 284
			modified holoprotein[c]	12 453[e]
R98C/Y101C **II**	none	12 281.6 (0.9)	apoprotein	11 667
			holoprotein[c]	12 284

[a] The standard deviation in the mean is given in parentheses. [b] Free heme was detected at 616 Da. [c] Expected holoprotein masses are the sum of the apoprotein and free heme, which would be the case if the addition of cysteine thiols to heme yielded a *c*-type linkage. [d] β-Maleimidopropionate. [e] Expected mass of protein modified with a single maleimidopropionate.

cytochromes with one thioether link (Barker et al., 1993; Lin et al., 1973; Pettigrew et al., 1975).

Tyr101Cys. The number and type of holoproteins present in the periplasm of *E. coli* expressing this single variant of cytochrome b_{562} were significantly more complex than for the R98C mutant, and five major components were identified by ion exchange (Figure 1c). The total yield of 427 nm absorbing material in the periplasm was about 25% that of the wild-type protein. The 427 nm absorbing material that eluted from the anion exchange column did not account for all of the material loaded onto the column because a significant amount of heme was removed from the protein by the resin. However, protein containing noncovalently attached heme could still be detected by SDS−PAGE analysis of component **V**. The other four major components all stained for covalently attached heme on SDS−PAGE gels. The two components (**I** and **II**) that eluted ahead of the expected retention time (i.e., that of the wild-type protein) were spectrally distinct from each other and from wild-type protein. Only components **I** and **II** reacted with β-maleimidopropionate, and after modification these species eluted with **III**−**V**. Components **I** and **II** are not stable even when the thiol is modified, and we have not been able to purify them to homogeneity. These species will not be considered further here except to note that component **I** contains a species that has a single modifiable thiol yet also has a covalently bound heme.

Components **III**−**V** are also spectrally distinct from each other, and one (**V**) has the same spectrum as the wild-type protein. The spectrum of component **III** was not that of a normal ferrous cytochrome (it is a green protein), and this species is not considered further here. Components **IV** and **V** could not be completely resolved by ion exchange chromatography, but, aided by the spectral analysis afforded by diode array detection and preparative heme extraction followed by separation of the apoprotein that formed, all

Table 3: ^{1}H-NMR Chemical Shifts[a] of Heme Group Proton Resonances for Various Ferrocytochrome b_{562} Proteins

heme group[b]	wild-type major[c]	wild-type minor[c]	R98C **I**	R98C **I** + 32 Da	Y101C **IV**	R98C/Y101C **II**
1-methyl (2^1)	3.68	3.42	3.41	3.40	3.68	3.67
2α-vinyl (3^1)	8.45	7.35	np[d]	np	8.35	np
cis-2β-vinyl (3^2)	5.94	4.71	np	np	5.62	np
trans-2β-vinyl (3^2)	5.63	4.06	np	np	5.97	np
2-methine (3^1)	np	np	5.97	6.04	np	6.14
2-methyl (3^2)	np	np	2.28	2.43	np	2.32
α-*meso* (5)	9.40	9.36	9.14	9.64	9.38	9.26
3-methyl (7^1)	2.69	3.47	2.50	1.53	3.02	2.91
4α-vinyl (8^1)	8.29	8.63	8.30	8.27	np	np
cis-4β-vinyl (8^2)	5.68	6.02	5.68	5.64	np	np
trans-4β-vinyl (8^2)	4.30	5.87	4.48	4.43	np	np
4-methine (8^1)	np	np	np	np	5.77	5.73
4-methyl (8^2)	np	np	np	np	1.26	1.24
β-*meso* (10)	9.98	9.90	9.93	9.91	10.09	10.00
5-methyl (12^1)	3.78	3.86	3.81	3.81	3.70	3.75
γ-*meso* (15)	9.70	9.69	9.74	9.72	9.62	9.71
8-methyl (18^1)	3.44	3.75	3.67	3.74	3.42	3.41
δ-*meso* (20)	9.91	9.92	9.83	9.80	9.88	9.83

[a] From spectra obtained in 0.1 M phosphate, 0.5 M KCl, pH* 6.6 at 300 K. [b] Heme substituents are numbered according to the Fischer notation of the carbon atoms, with the IUPAC numbering given in parentheses. [c] Major and minor orientations as described by Wu et al. (1991) and in the text. [d] np, not present in this protein.

Table 4: ^{1}H-NMR Chemical Shifts (ppm) of Some Proton Resonances for Selected Amino Acid Residues in Ferrocytochrome b_{562} Proteins

amino acid residue proton	wild-type major[a]	wild-type minor[a]	R98C **I**	R98C **I** + 32 Da	Y101C **IV**	R98C/Y101C **II**
Met7 Me	−3.02	−3.00	−2.90	−2.98	−2.91	−2.81
Met7 γ′[b]	−3.27	−3.34	−3.18	−3.18	−3.17	−3.08
Met7 γ	−1.54	−1.54	−1.40	−1.50	−1.53	−1.40
Met7 β′[b]	−2.50	−2.46	−2.52	−2.56	−2.43	−2.42
Met7 β	0.24	0.24	0.22	0.22	0.25	0.26
Met7 α	1.63	1.63	1.63	1.63	1.67	1.67
Phe61 (2,6)	7.49	7.49	7.48	7.48	7.61	7.60
Phe61 (3,5)	8.04	8.04	8.02	8.02	8.22	8.22
Phe61 (4)	7.27	7.27	7.23	7.23	7.44	7.49
Phe65 (2,6)	6.80	6.91	6.82	6.87	6.97	6.99
Phe65 (3,5)	5.76	5.70	5.71	5.76	5.91	5.93
Phe65 (4)	5.50	5.70	5.54	5.55	5.91	5.93
Tyr101 (2,6)	7.67/7.62[c]	7.67/7.62[c]	na[d]	na	np[e]	np
Tyr101 (3,5)	7.14	7.14	na	na	np	np
Tyr105 (2,6)	7.67/7.62[c]	7.67/7.62[c]	na	na	7.69	7.68
Tyr105 (3,5)	7.14	7.14	na	na	7.70	6.99

[a] Major and minor orientations as described by Wu et al. (1991) and in the text. [b] For the Cβ and Cγ methylene protons the higher-field signals were arbitrarily labeled β′ and γ′, respectively. [c] Two signals are present and not in an equal ratio. We are unable to assign them to the specific tyrosine residues or to either isomer. [d] na, not assigned due to resonance overlap. [e] np, not present in this protein.

traces of noncovalent holoprotein **V** could be removed from species **IV**. The mass of component **IV** corresponds to the mass of apoprotein plus heme (Table 2), and the mass of the apoprotein isolated from component **V** after heme extraction corresponds to the expected mass of the apoprotein alone. This suggests that the failure of component **V** to react with β-maleimidopropionate is not due to a modification of the free cysteine thiol. The pyridine hemochrome of Y101C component **IV** also has an α-band at 552 nm, which is again consistent with the replacement of a heme vinyl group with a *c*-type thioether linkage. Component **IV** was the main subject for further analysis by NMR.

Arg98Cys Tyr101Cys. The yield of holoproteins upon expression of this mutant is at least 10-fold less than wild-type as judged by the optical spectrum (not shown) of the periplasm extract. This spectrum is remarkable because the wavelength maxima observed are significantly blue-shifted from the wild-type (noncovalent) spectrum, in contrast to those observed from the two single mutants. Two major components (**I** and **II**) were observed following the anion exchange analysis, with other minor species in evidence (Figure 1d). At least 95% of the 427 nm absorbing material applied to the column was recovered. Spectral analysis across these two major peaks suggested each was spectrally homogeneous, and cytochromes with wild-type-like spectra could not be detected. This evidence, together with results from SDS−PAGE and heme staining (not shown), showed that all the cytochrome present in the periplasm at harvest had covalently-bound heme. Following reaction with β-maleimidopropionate, component **I** coeluted with component **II**, and the relative retention times before and after modification suggested that the species that were originally present differed by one charge only. The mass of modified component **I** corresponded to the mass of protein plus heme plus a single β-maleimidopropionate group (Table 2), suggesting that one cysteine thiol was originally unreacted. The mass of component **II** corresponded to that of apoprotein plus heme with no additional modifications.

On a preparative scale, components **I** and **II** were first separated by anion exchange and then component **I** was

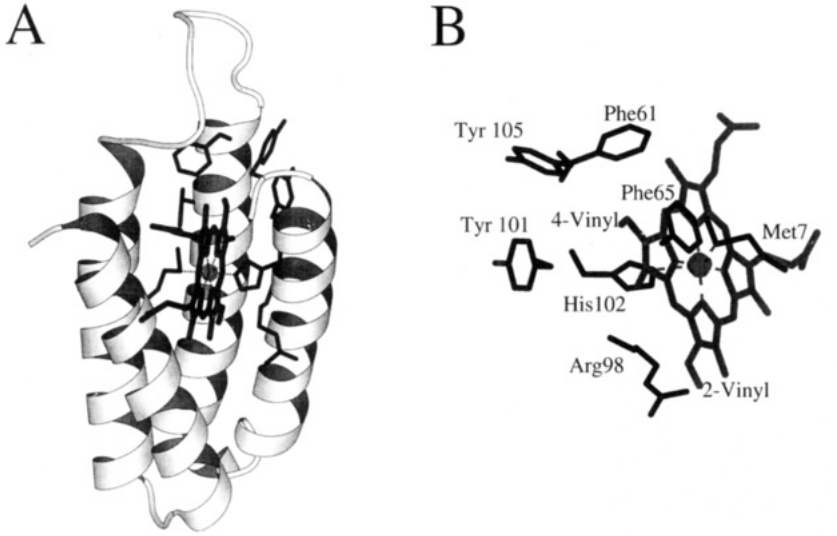

FIGURE 2: (a) Ribbon structure of cytochrome b_{562} holoprotein drawn with MOLSCRIPT (Kraulis, 1991) with the heme and residues relevant to this work highlighted as viewed from the solvent-exposed heme edge. These are (clockwise from) Met7, Phe65, Phe61, Tyr105, Tyr101, His102, and Arg98; coordinates from Brookhaven file 256b (Hamada et al., 1995). (b) Exploded view, almost 180° opposite to that in A, of the heme-binding pocket, highlighting the proximity of the 4-vinyl group to the four aromatic residues in the protein. Only side-chain and Cα atoms of the residues indicated are shown for clarity. The Cα atom is omitted from Phe65.

quantitatively modified with β-maleimidopropionate before further purification. Component **I** is not stable unless the thiol is protected by modification, in contrast to component **II** which is stable as isolated. The spectral properties of both these species are given in Table 1. Component **I** has a pyridine hemochrome spectrum similar to that from the two single-mutant proteins described above, whereas the spectrum of component **II** is identical to that of all *c*-type cytochromes with two thioether bonds. Both species have been analyzed by NMR.

^{1}H-NMR Studies of the Ferrocytochromes. To obtain proof for the existence of *c*-type thioether bonds between heme and protein, the ^{1}H-NMR spectra of the wild-type protein and all of the stable mutant proteins containing covalently bound heme were investigated with the heme in the diamagnetic, ferrous state in D_2O at pH* 6.6. Principally, the evidence sought was that the three single-proton resonances of each heme vinyl group had been replaced by a single methine proton and a new methyl group. Previous ^{1}H-NMR studies of the ferrous wild-type protein (Moore et al., 1985) did not resolve many of the resonances relevant to this question; therefore we assigned most of the resonances of the heme substituents, the methionine ligand protons and the protons from the four aromatic residues using 2D homonuclear (^{1}H) techniques. The assignment of the heme protons was based on NOESY and TOCSY experiments. TOCSY experiments establish the characteristic spin systems of the vinyl and/or thioether methine groups, and NOESY experiments connect these and the heme methyl groups to adjacent *meso* protons. The key to the strategy is the asymmetrical distribution of substituents in the heme.

Since the wild-type protein shows structural isomerism due to the heme binding in two possible orientations about the α−γ axis (Wu et al., 1991), there was always the possibility that covalent attachment of the heme could occur in one or other or both heme orientations. Figure 2 shows some simplified views of the holoprotein structure with the heme in the major orientation and highlights the proximity of certain heme substituents to the aromatic residues. The spectra of the ferrous, wild-type protein consist of two overlapping sets of resonances from heme substituents which we have assigned to the different heme orientations. This observation is in agreement with results obtained for the oxidized protein (Wu et al., 1991). Samples of the wild-type holoprotein contained either the equilibrium composition of the heme orientation isomers or a 1:1 mixture of the isomers kinetically trapped by reconstitution of holoprotein under strongly reducing and anaerobic conditions. A 1D spectrum recorded 30 min after such a reconstitution was a 1:1 mix of two closely related spectra and was indistinguishable from that obtained 24 h later. The 1D spectra of protein either isolated as holoprotein or freshly reconstituted from apoprotein and left oxidized for at least 1 week were a 6:1 mixture of spectra from the two isomers. This value was independent of oxidation state and is different from the 2:1 ratio obtained by La Mar and colleagues (Wu et al., 1991) in the oxidized state under identical solution conditions, even though our spectra are otherwise the same as those previously published. The 6:1 ratio is assumed to be the equilibrium mixture for the oxidized protein. Given the very slow reorientation rate in the reduced protein, we have no evidence that this position of equilibrium is the same in the reduced protein.

Relevant regions of the TOCSY and NOESY spectra of the 1:1 kinetically trapped mixture in the wild-type protein are shown in Figures 3 and 4, respectively. TOCSY experiments unequivocally establish the assignments of the vinyl group spin systems in both heme orientations, as shown in Figure 3. Strong scalar coupling (17 Hz) is observed between the α-vinyl and *trans*-β-vinyl protons; weaker coupling (9 Hz) between the α-vinyl and *cis*-β-vinyl protons results in each of the α-vinyl protons being observed as a doublet of doublets in the 1D spectra (not shown). TOCSY experiments also aided the assignment of the methylene protons of the methionine ligand (Met7) (data not shown, but similar to that in Figure 7), which are shifted strongly upfield due their proximity to the heme as observed previously (Moore et al., 1985; Xavier et al., 1978). Figure 4 shows the downfield region of the NOESY spectrum in which cross-peaks between all of the heme resonances can

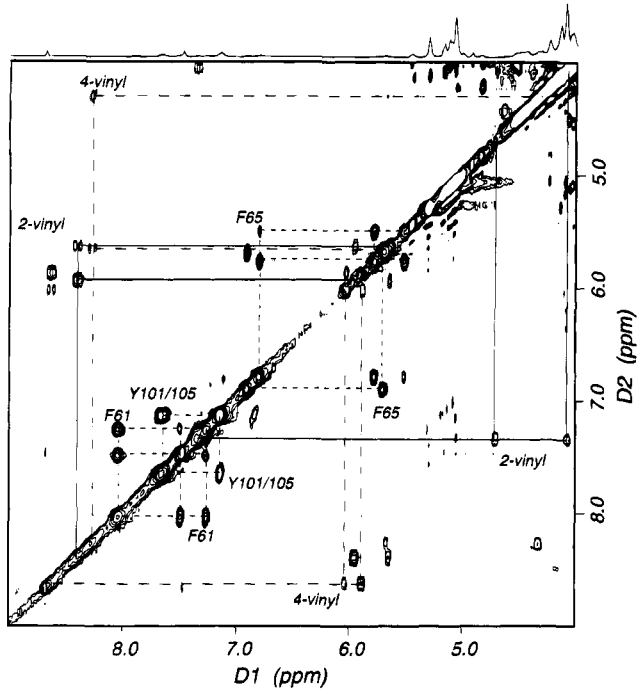

FIGURE 3: Section of a 500 MHz ^{1}H-TOCSY spectrum at 300 K of wild-type cytochrome b_{562}, freshly reconsitituted, reduced, and therefore a kinetically trapped 1:1 mixture of the two heme orientation isomers. The apoprotein concentration was 4 mM in 0.1 M phosphate, 0.5 M KCl, pH* 6.6, in the presence of 10 mM sodium hydrosulfite as reductant. Heme was added to 3.5 mM final concentration, and protein was equilibrated for 45 min before recording spectra. The sample was maintained under argon in sealed 5mm NMR sample tubes during preparation. Cross-peaks connected are from resonances of the protons in the groups indicated. Solid lines, 2-vinyl protons; large dashes, 4-vinyl protons; small dashes, aromatic group protons. Those from the major heme orientation isomer are joined above the diagonal, and those from the minor isomer are joined below the diagonal.

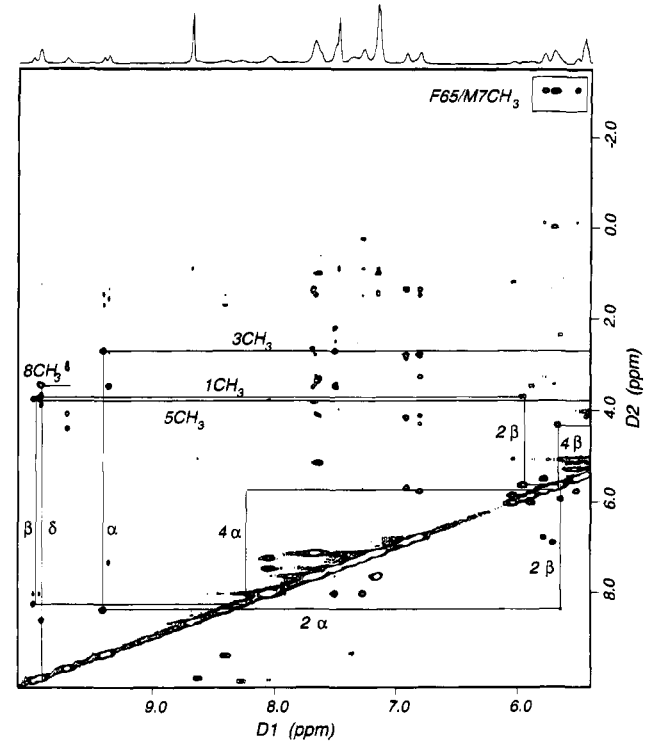

FIGURE 4: Section of the ^{1}H-NOESY spectrum of the same sample as in Figure 3. The lines join cross-peaks resulting from the through-space connectivity expected from the protons of the heme substituents in the following sequence: 8-methyl, *δ-meso*, 1-methyl, *trans*-2*β*-vinyl, *cis*-2*β*-vinyl, 2α-vinyl, α-*meso*, 3-methyl, *trans*-4*β*-vinyl, *cis*-4*β*-vinyl, 4α-vinyl, *β-meso*, 5-methyl.

be observed. The NOESY experiments connect heme substituent spin systems to the adjacent *meso* proton usually starting at the *δ-meso* proton which uniquely gives strong NOE's to two methyl groups, 1 and 8. The lines in Figure 4 connect resonances from the 8-methyl group to the 5-methyl group according to the through-space connectivities expected between the heme substituents and the *meso* proteins. Heme proton chemical shift assignments are given in Table 3, while amino acid residue assignments are given in Table 4.

The four aromatic residues (Phe61, Phe65, Tyr101, and Tyr105) in cytochrome b_{562} are structurally clustered into one region of the protein (Figure 2b) and form a pocket into which the 4-vinyl group of the heme inserts (Hamada et al., 1995). Some but not all of the aromatic resonances are resolved in the spectrum of the wild-type protein (Figures 3 and 4). Observation of cross-peaks between the 4-position substituent or *β-meso* proton resonances and these aromatic residue proton resonances in the NOESY spectrum of wild-type protein confirms the major orientation as that observed in the X-ray crystal structure (Figure 2b). The upfield chemical shift position of the 3-methyl and *trans*-4*β*-vinyl protons in the major orientation and both the *cis* and *trans*-2*β*-vinyl protons in the minor orientation suggest that these protons experience the ring-current anisotropy of Phe65 that packs against the heme. In the mutants described here, NOE's to these aromatic groups in addition to some of the chemical shift information establish the absolute orientation of each of the covalently linked hemes.

Figure 5 shows the low-field region of the NOESY spectrum of component **II** of the double mutant R98C/Y101C.[3] Two singlets at *δ* 6.14 and 5.73 ppm show through-space connectivities to signals at *δ* 9.26 and 10.00 ppm, respectively (assigned as α- and *β-meso* protons, respectively), and to methyl group signals at *δ* 2.32 and 1.24 ppm, respectively. A TOCSY spectrum (not shown) revealed through-bond connectivities between these two singlets and the above mentioned methyl groups. Both their chemical shift positions and their coupling identified these signals as the resonances of the methine protons of the two thioether linkages of *c*-type cytochromes (Senn & Wüthrich, 1983). Cross-peaks in the NOESY spectrum (joined by the dashed lines below the diagonal in Figure 5) from the 4-methine to the Phe61 (2,6), Phe61 (3,5), and Phe65 (3,5) protons, between the 4-methyl and Phe65 (3,5), and between the *β-meso* and Phe61 (3,5) protons establish that the heme in the protein pocket is in the major orientation relative to the α−*γ* heme axis (*vide supra*).

A strong NOE between the methionine ligand methyl group (*δ* −2.81ppm) and the Phe65 (3,5) protons (*δ* 5.93 ppm) is observed as in the wild-type spectrum. Weaker NOE's between this methionine methyl group and both the α- and *β-meso* protons suggest that the methionine ligand side-chain conformation, and therefore the stereochemistry

[3] Some of the spectra of the protein containing covalently linked heme show signals from slow exchanging amide protons even after 1 month in D_2O at pH* 6.6. This is in contrast to the observation for the wild-type protein where amide protons were completely exchanged after 3 days in the apoprotein state and after 1 week in the holoprotein state.

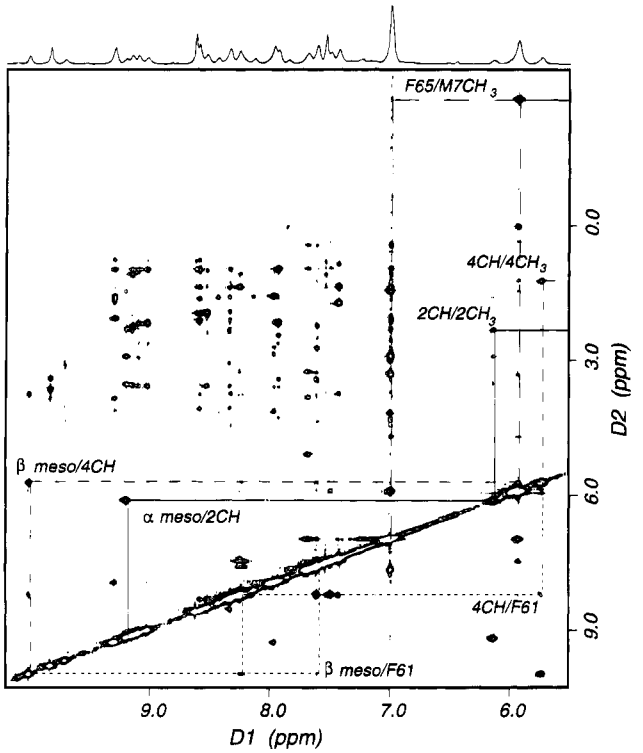

FIGURE 5: Section of the ^{1}H-NOESY spectrum of R98C/Y101C component **II**. The protein concentration was 2.4 mM in conditions given in Figure 3. The solid lines connect the signals of the α-*meso* proton and 2-position heme substituents. The large dashed lines connect the Met7 methyl group and the Phe65 aromatic protons. The intermediate dashed lines connect the signals of the *β-meso* proton and 4-position heme substituents. The small dashed lines connect the protons of Phe61 with the 4-methyl and *β-meso* resonances.

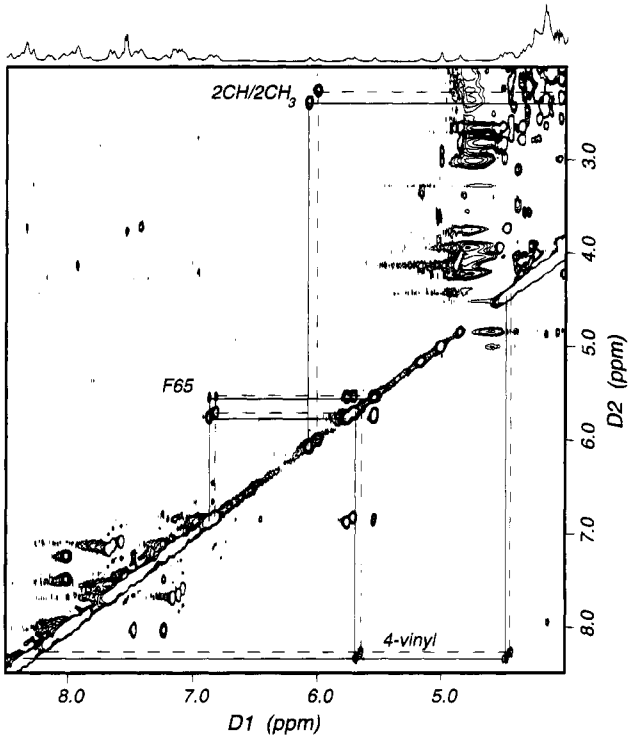

FIGURE 6: Aromatic region of the ^{1}H-TOCSY spectrum of R98C component **I**. The protein concentration was 2.5 mM in conditions given in Figure 3. The lines connect resonances from protons (of the 2-methine, 4-vinyl, and Phe65 groups) which have different chemical shifts in the two species present. The dashed lines above the diagonal connect those arising from the species of lower mass, while the solid lines connect signals from the species that has a mass with an additional 32 Da (see Table 2 and text). The ratio of the two species in this sample was 2:3, lower to higher mass, respectively.

at the sulfur atom, is the same as in the wild-type protein. We have no evidence from any of our NMR data to suggest that the stereochemical configuration at the methionine sulfur is any different from the wild-type protein in any of these covalently modified cytochromes.

The addition of the thiol sulfur to a heme α-vinyl carbon generates a new chiral center. In the R98C/Y101C protein, the 2-methine proton gives strong NOE's to the α-*meso* and the 3-methyl resonances as well as to the 2-methyl resonance. In addition, we observe a weak NOE between the 2-methine proton and the Met7 methyl group. The 2-methyl resonance shows strong NOE cross-peaks to the 1-methyl resonance and a resonance at δ −0.84 ppm, which in turn gives an NOE to the Met7 methyl group. This signal at δ −0.84 ppm almost certainly originates from a methyl group of Leu10. This pattern of NOE's to the 2-position substituents is preliminary evidence that the methine carbon is in the *S* configuration. The upfield-shifted position of the 4-methyl signal suggests it is in close proximity to Phe65. The methine proton contacts with the *β-meso*, Met7 methyl, and Phe61 (3,5) resonances together with the 4-methyl contacts with the 3-methyl and all of the Phe65 signals suggest that this methine carbon is also in the *S* configuration.

Similar NOESY and TOCSY analyses were applied to the proteins isolated from the two single mutants R98C component **I** and Y101C component **IV**. Each has only one set of vinyl group resonances and one proton with the through-space and through-bond connectivities characteristic of the methine proton at a *c*-type covalent link. However, analysis of R98C **I** is complicated by the fact that all samples analyzed were mixtures of the two species shown above to differ in mass by 32 Da. The low-field region of a NOESY map obtained from a 3:2 mixture of the two R98C species is shown in Figure 6. The two overlapping spectra are very similar, and the only heme proton resonances which differ significantly in chemical shift between the two forms are those from the 2-methine, 2-methyl, and the α-*meso* (Table 3). The 1-methyl, 4α-vinyl, and *cis*-4*β*-vinyl proton chemical shifts also differ slightly. Two sets of resonances are also observed from the methionine ligand protons (Figure 7 and Table 4) and the ring-current-shifted methyl resonances from unidentified amino acid residues (probably Leu10). NOE cross-peaks assigned so far suggest that the contacts between the equivalent protons in each form are virtually identical, the notable exceptions being contacts involving 2-methine and 2-methyl protons. This suggests that any structural difference between the two forms is restricted to this region of the heme pocket. The small chemical shift differences could be primarily due to differences in the electronic structure of the heme in each form, which is already indicated by the difference in the optical spectra between the two species (*vide supra*). The chemical shifts of these resonances and that of the α-*meso* proton are also significantly different in either species from those assigned to the same protons in the double mutant R98C/Y101C. However, the 4-vinyl group protons show a similar set of cross-peaks to the aromatic protons in this mutant as those observed in the wild-type protein.

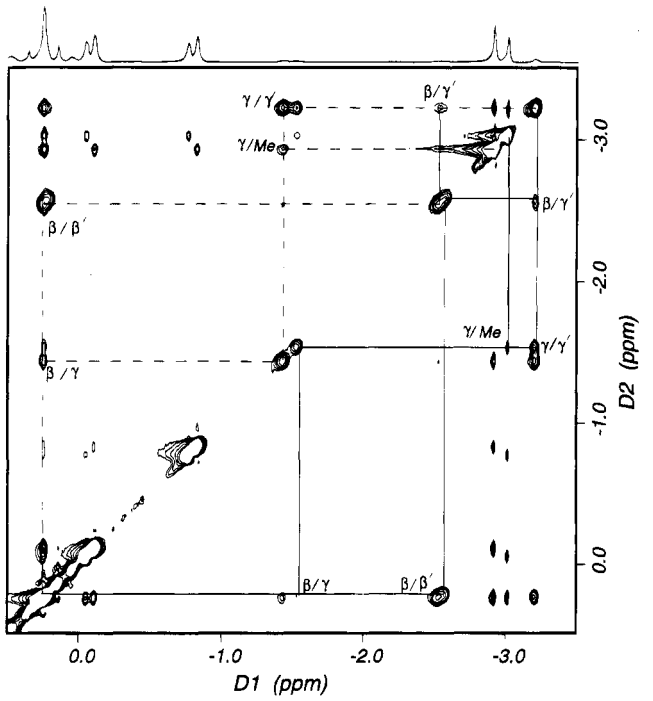

FIGURE 7: High-field region of the ^{1}H-NOESY spectrum of R98C component **I**. The sample and conditions are as in Figure 6. The lines connect cross-peaks between protons of the side chain (methyl group, γ-, γ'-, β-, and β'-methylene) of Met7 in each of the two species in this sample. The dashed lines above the diagonal connect those arising from the species of lower mass, while the solid lines connect signals from the species that has a mass with an additional 32 Da (see Table 2 and text).

Compared to those of the wild-type protein, the spectra (not shown) of Y101C component **IV** are missing the 4-vinyl group resonances, which are replaced by signals from a methine proton and a methyl group. They give strong NOE contacts to protons already assigned to phenylalanines 61 and 65, which again places the heme in the major orientation in this protein. Only one set of resonances attributable to a tyrosine residue is observed (Table 4). The NOE cross-peaks involving these resonances also indicate that the chiral methine carbon is again in the *S* configuration. The chemical shifts and NOE contacts of most of the protons in common between Y101C and the double mutant R98C/Y101C are very similar indicating that the electronic and structural environment of these protons is similar in both proteins. The 2-vinyl protons show through space connectivities qualitatively similar to those observed in the spectra of the wild-type protein.

NOESY and TOCSY data (not shown) obtained from component **I** of the double mutant R98C/Y101C were also consistent with the presence of a single *c*-type covalent bond at the 2-substituent and indicated that the 4-vinyl group is still present in this protein. NOE's observed between the 2-substituent protons and the aromatic side-chain protons clearly indicated that the heme is in an alternative orientation about the $\alpha-\gamma$ heme axis within this protein. However, the NMR spectra revealed the presence of two very closely related species, the origin of which we cannot explain at present, and we have therefore not presented assignments or further data.

DISCUSSION

We have mutated the cytochrome b_{562} gene to produce sequences with cysteine residues at either or both of the two positions sequentially homologous to those found in naturally occurring *c*-type cytochromes. After overexpression of each of the resulting three mutants into the periplasmic space of *E. coli*, we have isolated the stable holoproteins and identified those with properties in common with *c*-type cytochromes using the following analytical criteria. The proteins stain for heme on SDS polyacrylamide gels; their prosthetic group cannot be extracted by acidified butanone; they yield blue-shifted pyridine hemochrome spectra; their mass, as measured by ESI-MS, is that of apoprotein plus heme with no other modifications; and they elute from the anion exchange resin at a time, relative to the wild-type protein, expected from their predicted net charge. We have used two-dimensional ^{1}H-NMR methods to probe the exact nature of the covalent linkages and to answer the following questions. Do these proteins have the expected *c*-type thioether covalent linkage(s) between heme and protein? Are the mode and orientation of heme binding in the protein similar to that of the wild-type? Is the stereochemistry unique at the new chiral centers generated by covalent attachment and, if so, what is their configuration?

Assignment of the heme proton resonances in the ^{1}H-NMR spectrum of wild-type and mutant proteins was made on the basis of the connectivity between the heme resonances of the ferrous cytochromes observed in ^{1}H-NOESY and ^{1}H-TOCSY experiments compared to the well-established connectivity patterns observed for *b*- and *c*-type cytochromes (Keller & Wüthrich, 1980; Senn & Wüthrich, 1983). Spectra of wild-type protein show two sets of heme resonances due to the heme orientation isomerism already described for the oxidized protein (Wu et al., 1991). In the spectra of each of the three mutants only one set of heme resonances is observed, with each of the 2- and/or 4-vinyl group resonances replaced by signals from a single methine and new methyl protons. This established the presence of thioether bonds to the heme.

The orientation of the heme about its $\alpha-\gamma$ axis in the mutant proteins is the same as that of the major orientation in the wild-type protein. This is evident from the observation that the 2-position substituent is altered in the R98C variant while for the Y101C variant the 4-position substituent becomes the methine bridge. These results would be predicted if the respective cysteine thiols reacted with the heme in the major orientation. NOE contacts between heme substituents and the aromatic residues confirm this analysis. This heme orientation relative to the -C-X-Y-C-H- sequence is the same as observed in the three cytochromes *c'* that have been structurally characterized to date. NOE's observed between protons of the Met7 ligand sidechain and heme protons suggest that this sidechain occupies a similar position relative to the heme in all the proteins studied here. Additionally, contacts between the Met7 methyl protons and Phe65 side-chain protons together with inter-residue NOE's between the aromatic side chains suggest that the structure in this region of the heme pocket is largely unperturbed, even when the 4-vinyl group (which is observed to insert into a pocket formed by the four aromatic side chains in the wild-type structure, Figure 2b) is replaced by a thioether linkage to Cys101.

The close proximity of certain heme substituents to the aromatic side chains results in very different chemical shift values for similar heme protons (Table 3) due to the ring-current anisotropy they experience. For example, in the

major orientation of the wild-type protein the *trans*-4-β-vinyl proton is shifted significantly upfield due to its proximity to the Phe65 ring-current. In the minor orientation, it is the 2-β-vinyl protons that are shifted. Similarly, in the double mutant the 4-methyl group resonance is shifted significantly upfield by the same Phe65 aromatic group anisotropy when compared to the 2-methyl signal.

We have not undertaken quantitative NOESY experiments, and without complete assignment of the protein resonances or detailed distance measurements we cannot assess further the details of the differences, due to the covalent linkages, of the heme−protein interactions. However, the presence of only one set of resonances for each bridging methine group suggests that the stereochemical configurations are unique, and the NOE's observed have allowed us to suggest that the stereochemistry at each of the new chiral methine carbon atoms is in the *S* configuration. This is most clear in the double mutant, R98C/Y101C, and relies on the available NOESY data to establish on which side of the heme plane the 2-/4-methine and 2-/4-methyl protons lie. For example, strong NOE's observed between the 2-methine proton and both the α-*meso* proton and the 3-methyl protons indicate that the 2-methine proton is close to the plane of the heme. The 2-methyl protons show a strong NOE to an unassigned side-chain methyl group, which in turn shows a strong contact to the Met7 methyl group. This places the 2-methyl on the Met7 side of the heme plane. The 2-methyl resonance also gives a very strong NOE with the 1-methyl resonance. The Cys98 sulfur atom must reside on the opposite side of the heme plane to the 2-methyl group, and hence we can establish the topology around the 2-methine carbon atom as being in the *S* configuration. Similar analysis indicates that the same stereochemistry exists at the 4-methine carbon atom.

When applied to the R98C variant, this qualitative analysis is complicated by the overlap of the relevant resonances from the two species present. We have no reason to believe that the stereochemistry at the 2-methine bridge in either of these species is different to that observed in the double mutant. The origin of extra 32 Da in one of the R98C species observed in all preparations is unknown. Since the differences in the NMR spectra of the two species are limited to the 2-substituent region, we are tempted to speculate that the difference is due to the oxidation of the sulfur at the thioether bridge to a sulfone. If this is the case, then it must have occurred after the covalent attachment, since oxidation of the cysteine thiol would be expected to proceed to the sulfonic acid, which could not then add to the vinyl group. Additionally, if the 32 Da are from two oxygen atoms bonded to this sulfur, one might expect to observe a species with the mass of an intermediate suphoxide in which one oxygen was added to the sulfur. No intermediate species with an additional mass of 16 Da has been detected. Alternatively, other residues could have been oxidized, and the most likely candidates to gain two oxygens are the methionines Met33 and Met68. Oxidation of the ligand Met7 can be ruled out since it would not then be able to ligate to the heme iron, as observed in cytochrome c_3 mutants (Dolla et al., 1994). The site of oxidation may be answered by tandem MS experiments which have previously identified oxidation at a bridging sulfur atom (Barker et al., 1993) albeit in a different type of covalent linkage between heme and cysteine residue.

We have shown here that cytochrome b_{562} can be engineered to have a single *c*-type covalent linkage or two covalent linkages as found in the cytochromes c' or c_{556}. The introduction of the two covalent linkages alters the optical spectrum of cytochrome b_{562} such that it is indistinguishable from that of cytochrome c_{556} (Van den Branden, 1975). The detailed effects of the introduction of covalent linkage on the protein stability, reduction potential, and electronic properties of the oxidized protein are currently under study. However, the optical properties of each of these proteins are consistent with the premise that the covalent modifications are the only differences in chemical composition when compared to the wild-type protein. Specifically, the loss of the vinyl group(s) causes the shift to higher energy of all the absorbance bands in the spectrum of the holoproteins. The shift is not the same in the two single mutants, with the Y101C covalent protein having spectral properties much closer to the wild-type protein than the R98C protein. Taken together with the difference between the optical spectra of the two R98C species this shows that there are subtle effects of heme−protein interaction upon the electronic properties of the heme. This will be examined further when we present the NMR properties of the paramagnetic, oxidized proteins (P. D. Barker, E. P. Nerou, and S. M. V. Freund, unpublished results). At this stage our data show that all of these proteins maintain the same Met−His ligation to the heme iron in both oxidation states and that the methionine ligand in the ferrous proteins is in the same chiral configuration about the iron−sulfur coordination bond as observed in the wild-type protein.

The identification, isolation, and characterization of these "*c*-type" cytochromes b_{562} have been necessary pretexts to experiments designed to probe the mechanisms by which the covalent bonds are formed. Comparison of the solution structure of the apoprotein (Feng et al., 1994) with the crystal structure of the holoprotein (Hamada et al., 1995) shows that C-terminal helix observed in the holoprotein is not folded into any secondary structure in the absence of heme while the remainder of the apoprotein adopts similar secondary and tertiary structure to that with heme bound. It is this C-terminal helix which contains the heme binding sequence in the structurally homologous *c*-type cytochromes. The covalent linkage to cysteines and ligation of the histidine to the heme iron must place conformational restrictions upon the -C-X-Y-C-H- peptide (Finzel et al., 1985). However, Hamada et al. (1995) have superimposed their high-resolution structure of holocytochrome b_{562} on that of cytochrome c' from *Rhodospirulum molicianum* (Finzel et al., 1985; Weber et al., 1981a) and found that the helices of these two proteins are congruent at this heme-binding site. Therefore, the protein fold in our cysteine-containing noncovalent holoproteins is expected to bring the cysteine thiols into close proximity with the heme vinyl groups (Hamada et al., 1995) resulting in the possibility of spontaneous reaction.

Heme binds noncovalently to all the apoproteins studied in this work, albeit with reduced affinity (P. D. Barker and E. P. Nerou, unpublished results). Our *in vitro* reconstitution experiments are still in progress, and we defer description of them for future publication. However, the number and complexity of species observed in experiments analyzed to date are even greater than observed after expression in *E. coli*. A number of holoproteins with covalently bound heme can be detected, but we have yet to identify conditions under which the species identified in this communication as *c*-type cytochromes will form spontaneously by reaction of heme with the apoproteins. So far all of the *c*-type cytochrome

b_{562} proteins described in detail here have been isolated exclusively from extracts of *E. coli* cultures. Therefore we consider the possibility that in the folded structure of the noncovalent holoproteins, the cysteine thiols are not optimally placed for electrophilic addition to the heme vinyl groups, a reaction which is expected to have strict stereochemical requirements. In this case we suggest that enzymatic assistance is required to make the observed thioether linkages.

While the proximity of the cysteine thiols to the heme vinyl groups in the noncovalent holoproteins may not be optimal for spontaneous electrophilic addition, other reaction mechanisms may be operative. In particular, the thiol can be easily oxidized to a thiyl radical by the ferric heme iron initiating free radical reactions which could yield a number of products, as has been observed for a different protein (Barker et al., 1993). In particular a green protein predominates if, during preparation of the R98C mutant from *E. coli*, the heme iron is not maintained fully reduced.[2] Similar species can be observed during the preparation of the other mutants described here, though few are stable. Whatever the mechanistic origin of these side reactions, our initial results suggest that they occur in the periplasm of *E. coli* containing the overexpressed proteins and are competing with the mechanism, enzymatic or not, which yields the product of electrophilic addition of the cysteine thiols to heme. As stated before (Barker et al., 1993), the side reactions between oxidized heme and cysteine thiols may be one reason why cytochrome *c* synthesis *in vivo* requires reducing equivalents (Nicholson & Neupert, 1989; Taniuchi et al., 1983).

The two variants containing the Y101C mutation yield an increased number of covalently attached heme proteins when compared to the R98C single mutant. This may result from the perturbation, due to the loss of an aromatic group which contacts the heme (Hamada et al., 1995), of the major heme-binding mode observed in the wild-type protein. The affinity of these Y101C-containing proteins for heme is significantly lower than that of the wild-type and R98C proteins (P. D. Barker and E. P. Nerou, unpublished results). Consequently, different orientations of heme binding may have similar energies and result in different mechanisms of reaction of the thiol with the iron and/or heme. A stable example of this may be component **I** from the double mutant R98C/Y101C. A 2D NMR analysis reveals that this protein is also a mixture of two very closely related species, but our ESI-MS measurements do not detect species of differing mass. The results from NOESY experiments (not shown) clearly indicate that the heme in this protein is covalently locked into an alternative heme orientation compared to all the other proteins we have studied in detail. Therefore, this species does not represent an intermediate on the path to the doubly covalently linked species R98C/Y101C component **II**. A protein with identical spectral and chromatographic properties to component **I** is the major product following addition of heme to the R98C/Y101C apoprotein *in vitro* under anaerobic and reducing conditions. We defer a full description of this protein until we present results from our *in vitro* reconstitution experiments, since we have yet to establish the origin of either the heterogeneity in the NMR spectra or the change in net charge of the protein. It does, however, suggest that under some circumstances a single *c*-type thioether bond can form spontaneously.

The simple structural fold (Figure 2) of cytochrome b_{562} represents a suitable scaffold for protein engineering of cytochromes with novel properties. There is significant interest in synthetic helical peptides that can be designed to assemble into bundles with significant affinity for heme. These may be useful for assembling larger multiredox center complexes with novel electronic properties (Choma et al., 1994; Robertson et al., 1994; Sligar & Salemme, 1992). The ability to introduce covalent linkages between heme and the 4-helical bundle motif is a useful additional tool available to the cytochrome engineer. While the *c*-type bond is a ubiquitous covalent linkage between heme and protein observed in naturally evolved cytochromes, our results suggest that it is not necessarily easily transposed for use in other proteins. We are currently investigating different sequences and different biochemical conditions (especially *in vitro*) in order to increase the efficiency of this *b*- to *c*-type cytochrome transmutation so that stable heme sites can be built into non-heme proteins.

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