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Transmutation of a heme protein

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ABSTRACT **Residue Asn^{57} of bovine liver cytochrome b_5 has been replaced with a cysteine residue, and the resulting variant has been isolated from recombinant *Escherichia coli* as a mixture of four major species: A, B_I, B_{II}, and C. A combination of electronic spectroscopy, ^{1}H NMR spectroscopy, resonance Raman spectroscopy, electrospray mass spectrometry, and direct electrochemistry has been used to characterize these four major cytochrome derivatives. The red form A ($E_m = -19$ mV) is found to possess a heme group bound covalently through a thioether linkage involving Cys^{57} and the α carbon of the heme 4-vinyl group. Form B_I has a covalently bound heme group coupled through a thioether linkage involving the β carbon of the heme 4-vinyl group. Form B_{II} is similar to B_I except that the sulfur involved in the thioether linkage is oxidized to a sulfoxide. The green form C (E_m = 175 mV) possesses a noncovalently bound prosthetic group with spectroscopic properties characteristic of a chlorin. A mechanism is proposed for the generation of these derivatives, and the implications of these observations for the biosynthesis of cytochrome *c* and naturally occurring chlorin prosthetic groups are discussed.**

While iron-protoporphyrin IX is ubiquitous as a noncovalently bound prosthetic group in a wide variety of electron-transfer proteins and enzymes, other metalloporphyrins that vary in the type and degree of substitution in the macrocycle are known to be important in biological systems (1). These include hemes *a*, *c*, *o*, and *d* (2), the last of which represents the general class of metallochlorins—i.e., metalloporphyrins in which one pyrrole ring is reduced.

Site-directed mutagenesis is an established method for generation of metalloprotein variants as a means of gaining mechanistic insight into electron-transfer processes (3). We now report a cytochrome b_5 variant that is representative of a class of heme protein mutants in which the native prosthetic group undergoes specific chemical modifications. As these modifications are related to the classical designations of heme proteins, we refer to this phenomenon in which transformation between heme types results from site-specific protein mutation as *transmutation*. Characterization of the present cytochrome b_5 variant and its properties, combined with similar studies of subsequent variants based on related principles, promises to provide useful insight into the mechanisms involved in the covalent attachment of the heme prosthetic group to cytochromes *c* and in chlorin biosynthesis. The principles to be learned from this variant should lead to the design of metalloproteins with specifically modified prosthetic groups possessing useful enzymatic and/or electron-transfer properties.

Cytochrome b_5 possesses a single noncovalently bound protoheme IX with bis-histidine coordination to the iron and is one of the simplest members of the cytochrome *b* family (4). In the current study, using a synthetic gene coding for a soluble, heme-containing fragment of this membrane-bound protein (5), we have replaced the surface residue Asn^{57} with cysteine (N57C variant). This substitution places the thiol group 3–3.5 Å from the heme 4-vinyl when the heme is bound to the protein in the "major" orientation as defined by La Mar and colleagues (6, 7). The resulting protein can be isolated in four major forms, all of which represent distinct classes of heme proteins, and multiple modes of reaction of the cysteine thiol with the prosthetic group must be involved.

MATERIALS AND METHODS

Mutagenesis, Protein Preparation, and Related Methods. The synthetic gene coding for the lipase-solubilized form of bovine liver microsomal cytochrome b_5 (5) was mutated to the N57C variant with a mutagenic oligonucleotide and the polymerase chain reaction technique (8). The N57C variant was expressed in *Escherichia coli* and purified as described for the wild-type protein (5) except for the final ion-exchange chromatography fractionation [Pharmacia Mono Q, in 20 mM triethanolamine buffer (pH 7.3) with NaCl as eluent]. The major components of the mixture were coeluted (0.19–0.20 M NaCl) when developed under oxidizing conditions. Chromatography under weakly reducing conditions (5 mM 2-mercaptoethanol or 1 mM dithiothreitol added to the eluent) separated reduced (ferrous) component C ($A_{439.5}/A_{586} > 4.9$) from oxidized (ferric) components A and B. More strongly reducing conditions (2 mM sodium ascorbate) resolved component A (Fe(II): $A_{555.5}/A_{570} > 11$) from fraction B (Fe(II): $A_{557.5}/A_{567.2} < 1.06$).

The pyridine hemochrome spectra of the N57C derivatives were collected as described (9). The sulfhydryl reactivity of Cys^{57} was evaluated by reaction with 4,4′-dipyridyl disulfide (Aldrithiol-4; Aldrich) in 20 mM triethanolamine at pH 7.3 (10). HPLC tryptic peptide mapping was performed as described (11).

Electrochemistry. Reduction potentials were measured by cylic voltammetry through direct electrochemistry of cytochrome b_5 at a gold electrode modified with the peptide Lys-Cys-Thr-Cys-Cys-Ala (Sigma) (ref. 12; S. Bagby, P.D.B., and H. A. O. Hill, unpublished results) (20 mM Hepes, pH 7.0/97 mM KCl; 25°C; 100–400 μM cytochrome). The peak currents obtained from a solution of each component of known absorbance were compared with those obtained from a solution of wild-type cytochrome b_5 of known concentration. Because a current that is obtained while the electrochemistry is essentially reversible (i.e., at low sweep

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Abbreviation: NOE, nuclear Overhauser effect.

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rates) is proportional to the protein concentration, the extinction coefficients for each of the components A, B, and C could be estimated. Care was taken to ensure that the electrode surface used for measurements of the standard wild-type protein was the same as that used for the unknown protein. From several independent measurements, the extinction coefficients given in Table 1 were obtained with an uncertainty of ±10%.

Mass Spectrometry. Electrospray mass spectrometry of the purified N57C derivatives was performed with a triple quadrupole mass spectrometer (API III MS/MS system; Sciex, Thornhill, ON, Canada) fitted with a pneumatically assisted electrospray interface (13). The A and C components were diluted into 10% acetic acid, and the B component into 20% acetic acid, immediately prior to injection into the spectrometer. For MS/MS analysis, the charge-selected ions from the first mass spectrometer were dissociated by collision with argon gas, and the fragments were analyzed by a second mass spectrometer.

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 25°C on a Bruker WH-400 or MSL-200 spectrometer. The protein samples were exchanged into 0.2 M sodium nitrate/0.05 M sodium phosphate deuterated buffer, p^{2}H 6.8 (uncorrected pH-meter reading). Solutions of reduced proteins were obtained by addition of solid sodium dithionite. Truncated-driven nuclear Overhauser effect (NOE) difference spectra were obtained by subtracting spectra with Overhauser effects from reference spectra as described (14). Spin-decoupled spectra were obtained with the application of a selective decoupling pulse during data acquisition.

Resonance Raman Spectroscopy. Resonance Raman spectra were obtained with a computer-controlled Jarrell-Ash (Waltham, MA) spectrometer (15). Sample excitation was achieved at 457.9 nm with a Spectra-Physics krypton ion laser.

RESULTS

N57C Component A. Of the various forms of the N57C variant, the spectrum of N57C-A (Table 1) is the most similar to that of the wild-type protein. Notably, however, the α band (λ_{max}, 555 nm) of reduced A is not split. Several lines of evidence indicate that the prosthetic group of this derivative is covalently attached to the protein. (*i*) The heme is not removed from N57C-A by acidified 2-butanone (16), which quantitatively removes heme from the wild-type protein. (*ii*) This component gives no reaction with thiol-modifying reagents. (*iii*) The tryptic peptide map of N57C-A lacks the peptide containing residue 57 (11), while a new peptide with a typical heme spectrum appears late in the chromatogram. (*iv*) Electrospray mass spectrometry reveals that the mass of N57C-A is 11,238 ± 1 Da. This value corresponds to the mass of the N57C apoprotein plus the mass of unmodified heme. Similar analysis of the wild-type protein reveals a single component corresponding to the apoprotein (10,635.3 ± 1.5 Da). (*v*) MS/MS of the 11,238-Da species gives rise to two fragment peaks with masses of 617.5 and 649.5 Da, which correspond to the mass of heme and heme plus sulfur, respectively. Under similar conditions, cytochrome *c* undergoes specific cleavage of the thioether linkages that couple the heme to the apoprotein (R.F. and Y.K., unpublished data). (*vi*) The reduced pyridine hemochrome of N57C-A exhibits an α band with λ_{max} 553 nm. This spectrum is intermediate between that of free heme *b* (556 nm) and that of cytochrome *c* (551 nm), in which the prosthetic group is bound to the protein through two thioether links. This blue shift is consistent with the loss of one electron-withdrawing vinyl group in N57C-A. The pyridine hemochromes prepared from two naturally occurring species of *c*-type cytochrome known to have one thioether link (*Euglena gracilis* and *Crithidia oncopelti*) also exhibit an α band at 553 nm (17, 18). (*vii*) The reduction potential of N57C-A is −19 mV (vs. saturated hydrogen electrode, 25°C, μ = 0.1 M). This value is approximately midway between that of the wild-type protein (+4 mV) (19) and that of deuteroheme-substituted cytochrome b_5 (−40 mV) (20).

Table 1. Absorbance maxima and extinction coefficients for N57C derivatives

Oxidation state	λ_{max}, nm (extinction coefficient, mM^{-1}·cm^{-1})		
		Visible bands	
	Soret band	β	α
Component A			
Oxidized	410.5 (117)	532 (9.8)	560 (8.3)
Reduced	420 (166)	527 (13.8)	555.5 (29)
Component B			
Oxidized	414 (93.4)	536 (10.4)	565 (10.4)
Reduced	429.5 (132)	530 (12.6)	557.5 (22.2)
			567.2 (21.7)
Component C			
Oxidized	424 (110)	542 (11)	584 (10.2)
Reduced	439.5 (110)	507 (8.6)	538 (8.5)
			586 (22.4)

NMR experiments provide information concerning the covalent interaction between the heme group and the apoprotein in N57C-A. Individual assignments of the heme resonances were obtained from truncated-driven NOE difference spectra of the reduced protein. Fig. 1 shows the NMR spectrum of the reduced N57C-A (spectrum A) and the truncated-driven NOE difference spectra (spectra B–D) obtained with preirradiation of three of the four heme meso-proton resonances in the low-field spectrum. The pattern of NOEs observed by preirradiating the α meso proton is essentially identical to that observed for the wild-type protein (14) and arises from the 2α-vinyl proton, one 2β-vinyl proton,

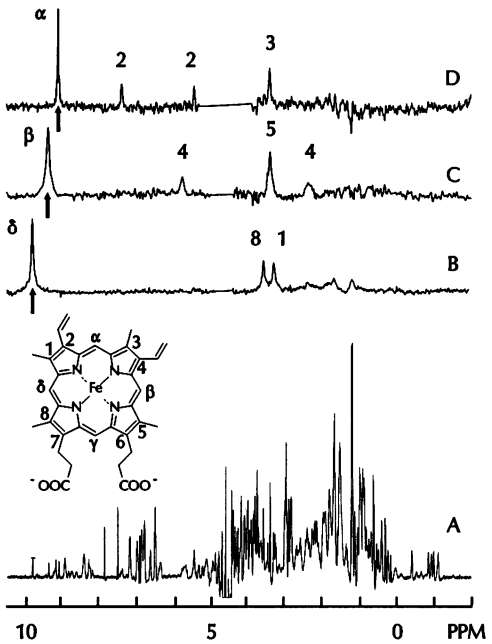

FIG. 1. Spectrum A is the 400-MHz ^{1}H NMR spectrum of reduced N57C-A. Spectral resolution was enhanced by multiplication of the free induction decay with a phase-shifted sine bell. Spectra B–D are truncated-driven NOE difference spectra obtained with preirradiation at the positions indicated by the arrows. Assignments of the heme resonances obtained from these experiments are indicated; the substituents of the porphyrin ring are labeled with the number of the β-carbon atom to which they are attached (see *Inset*).

and the 3-methyl protons. The pattern observed on preirradiation of the β meso proton, however, differs from that observed for the wild-type protein. Instead, this pattern is essentially identical to that observed for NOEs to the 5-methyl and the methine and methyl protons in the thioether link of cytochromes *c* (21). Experiments involving preirradiation of these methine and methyl resonances reveal NOEs to each other and to the β meso proton (data not shown). These experiments confirm that the 4-vinyl group has been replaced by a cytochrome *c*-type thioether moiety. The NMR spectrum of the paramagnetic, ferric N57C-A exhibits hyperfine shifted resonances characteristic of cytochrome b_5 with a heme in the major orientation (data not shown) (6, 7).

N57C Component B. N57C-B is a mixture of two heme-containing proteins, N57C-B_I and N57C-B_{II}, as shown by NMR and electrospray mass spectroscopy. The prosthetic groups of these derivatives are not extracted by acidified 2-butanone. N57C-B_I exhibits an unusual stability toward acid denaturation that prevents efficient tryptic digestion of the mixture and impedes peptide mapping. The molecular mass determined for N57C-B_I by electrospray mass spectroscopy is 11,237 ± 0.5 Da, a value that corresponds to the mass of the apoprotein plus the mass of heme minus ≈2 Da. The comparable value found for N57C-B_{II} (11,254 ± 0.5 Da) corresponds to apoprotein plus heme plus ≈14 Da, suggesting the incorporation of an oxygen atom in the molecule. MS/MS experiments on N57C-B_{II} reveal the presence of a fragment peak with a mass of 663.5 Da that corresponds to heme plus sulfur and oxygen, thereby confirming the covalent attachment of the prosthetic group to the apoprotein through the cysteinyl residue.

The NMR spectrum of the reduced N57C-B mixture exhibits two sets of meso-proton resonances (Fig. 2, spectrum A and enlargement B) indicating the presence of a 3:1

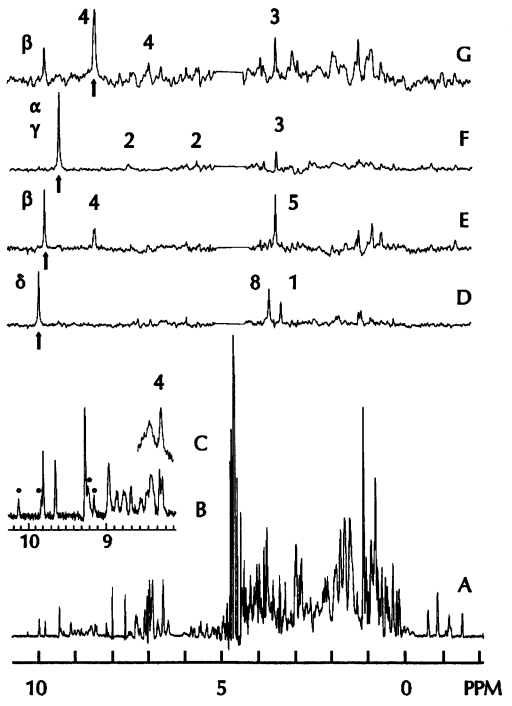

FIG. 2. Spectrum A is the 400-MHz ^{1}H NMR spectrum of reduced N57C-B (same conditions as in Fig. 1). Enlargement B shows the spectral region from 8.1 to 10.2 ppm. The four meso-proton resonances attributed to N57C-B_{II} (see text) are labeled (●). Spectrum C shows spin decoupling of the resonance at 8.31 ppm upon irradiation at 6.83 ppm. Spectra D–G are truncated-driven NOE difference spectra obtained with preirradiation at the positions indicated by the arrows. Assignments of the heme resonances for N57C-B_I are indicated.

mixture. As in the case of component A, a NOE pattern similar to that of the wild-type protein is observed on preirradiation of the α meso-proton resonance of N57C-B_I (Fig. 2, spectrum F), suggesting that the 2-vinyl group is intact in this component.

NOE experiments involving the preirradiation of the β meso proton of N57C-B_I (Fig. 2, spectrum E) allow the assignment of the doublet at 8.31 ppm to the 4α-vinyl proton. The appearance of this proton as a doublet and the large value of the coupling constant (17 Hz) (22) indicate that the 4β-vinyl carbon atom bears just a single proton trans to the 4α proton. This interpretation is confirmed through a spin-decoupling experiment (Fig. 2, spectrum C) in which this signal collapses to a singlet upon irradiation at 6.83 ppm, the chemical shift of the 4β proton.

The components of the N57C-B mixture cannot be resolved by ion-exchange chromatography under a variety of reducing conditions, and direct electrochemistry of the mixture shows only one voltammetric wave, suggesting that N57C-B_I and N57C-B_{II} have very similar reduction potentials. The midpoint potential of N57C-B (+12 mV vs. saturated hydrogen electrode, 25°C, μ = 0.1 M) is 8 mV higher than that of the wild-type protein. This result is consistent with the observation that this mixture exhibits a pyridine hemochrome with a reduced α band (λ_{max}, 558 nm) at lower energy than that of the wild-type protein (λ_{max}, 556 nm) and indicates that the heme substituents in N57C-B have slightly more electron-withdrawing character than those of heme *b*. The structures proposed for the prosthetic groups of N57C-B_I and N57C-B_{II} (see Fig. 4) are compatible with all experimental evidence and mechanistic considerations (23) currently available.

N57C Component C. The electronic spectra of both oxidized and reduced component C are completely different from those of components A and B (Table 1). The intense α band at 586 nm in the spectrum of the ferroprotein is reminiscent of similar bands exhibited by chlorins (24), though it occurs at higher energy than that of known chlorins. The Soret absorbance bands of ferro- and ferri-C are significantly red-shifted from their positions in the heme-containing proteins. The prosthetic group in component C can be extracted by acidified 2-butanone, though the stability of this prosthetic group to the conditions of extraction has not been evaluated. Mass measurement from electrospray mass spectrometry gives a value of 10,623 ± 0.9 Da, which corresponds to the molecular mass of the apoprotein (10,623.8 Da) and suggests a noncovalent association between the prosthetic group and the polypeptide chain. The reduced pyridine hemochrome produced from component C has an α band (λ_{max}, 581.5 nm) that is consistent with that expected for either a heme substituted with a strong electron-withdrawing group (25) or a chlorin.

Fig. 3 shows the resonance Raman spectra of reduced N57C-C and reduced wild-type cytochrome b_5. Several features of the N57C-C spectrum are consistent with the conclusion that the prosthetic group of this derivative is a chlorin. First, the great increase in the number of bands relative to the number observed for the wild-type protein is expected for a chlorin owing to the decrease in symmetry on conversion of a heme to a chlorin. Second, the intense band at 746 cm^{-1} is a characteristic feature of resonance Raman spectra of chlorins (26). Finally, the unusual resonance Raman bands around 1240 cm^{-1} are similar to features recently reported for the spectrum of cytochrome *d* (27), which is known to possess a chlorin prosthetic group.

The reduction potential of component C is +175 mV (vs. saturated hydrogen electrode 25°C, μ = 0.1 M), a value 170 mV higher than that of the wild-type protein. The reduction of one pyrrole ring of a heme is expected to result in a *lower* reduction potential of the corresponding chlorin. The much

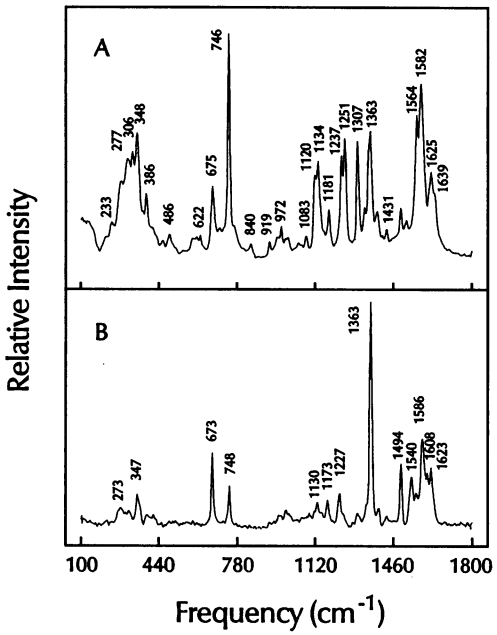

FIG. 3. Resonance Raman spectra of reduced N57C-C (*A*) and wild-type ferrocytochrome b_5 (*B*).

greater oxidizing potential of N57C-C suggests that additional modification of the prosthetic group has resulted in further substitution on the macrocycle. On the other hand, the tryptic peptide map of this protein differs from that of the wild-type protein only in the position of the peptide containing residue 57. This result, combined with (*a*) the observation of one thiol group in N57C-C as determined with sulfhydryl titration and (*b*) the mass spectrometry of N57C-C, indicates that the apoprotein is not modified during conversion of the heme to a chlorin.

Extraction of the prosthetic group from N57C-C allows the resulting apoprotein to be reconstituted with "fresh" ferriprotoporphyrin IX. Preliminary experiments of this type indicate that reconstitution of component C with ferriprotoheme IX yields a mixture of components B and C. This observation is the strongest evidence currently available that heme modification is not attributable to expression of the protein in *E. coli* or to the isolation procedures. Moreover, this observation suggests that replacement of Asn^{57} by a cysteine residue in cytochrome b_5 converts the cytochrome to a heme-modifying enzyme.

DISCUSSION

Mechanism of Reaction of Cys^{57} with Protoporphyrin IX. Thiols can add to activated double bonds through an acid-catalyzed mechanism to generate thioethers. The addition products follow the Markovnikov rule, with the sulfur atom being attached to the carbon with fewer hydrogens. This simple mechanism can explain the formation of N57C-A (Fig. 4), although the stereochemistry of the 4α-vinyl carbon atom is unknown at present. On the other hand, it is also known that ferricytochrome b_5 can be reduced to the ferrous form by free thiols to generate a thiyl radical that usually recombines with another molecule of thiol to form a disulfide. In the case of N57C, the thiyl radical first generated by electron transfer from the sulfur atom of Cys^{57} to the iron atom would be located adjacent to the heme 4-vinyl group when the heme is bound in the major orientation. Thiyl radicals can also add to double bonds, typically following a chain-reaction mechanism to produce anti-Markovnikov addition products. The first step of this mechanism is the addition of the thiyl radical to the double bond to form a carbon-based radical, which, in

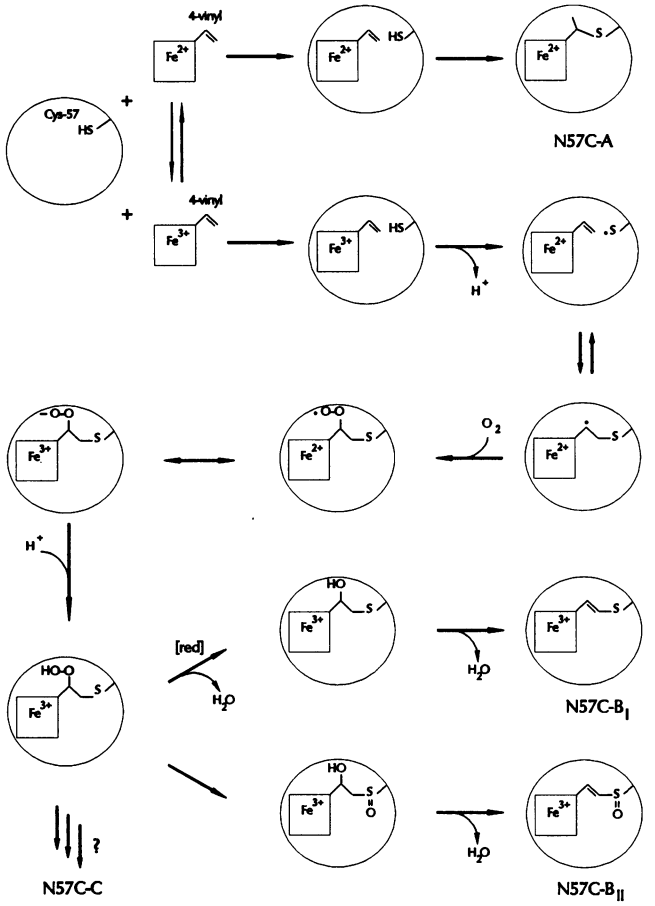

FIG. 4. Proposed mechanism for the formation of N57C-A, N57C-B_I, and N57C-B_{II}. The heme prosthetic group is represented by a square, and the circle represents the apoprotein of the N57C cytochrome b_5 variant.

the presence of oxygen, combines spontaneously with the O_2 diradical to form a peroxy radical (23). This intermediate can undergo electron transfer with the heme iron to generate a peroxy anion and an oxidized heme iron moiety (Fig. 4). The hydroperoxides initially formed in this kind of reaction have been isolated in several cases, but they are usually transformed into the isomeric hydroxy sulfoxides or, in the presence of a reductant, they are converted to the corresponding thioalcohols (23). In the present case, the first type of reaction would lead, after dehydration of the intermediate hydroxy sulfoxide, to N57C-B_{II}. N57C-B_I would arise from reduction of the hydroperoxide with subsequent dehydration.

As indicated above, N57C-B_I exhibits unusual stability toward acid denaturation, which could be explained by oxidative crosslinking of the apoprotein. The reduction of the intermediate hydroperoxide to the thioalcohol requires two reducing equivalents presumably provided by the apoprotein to generate protein-based radicals that can recombine to form a covalent bond. A radical-based oxidative mechanism has also been proposed for the crosslinking reactions observed following addition of hydrogen peroxide to myoglobin (28) or cytochrome-*c* peroxidase (29) in the absence of exogenous electron donors. Proteins other than cytochromes *c* and the examples discussed here that have been reported to have covalently bound heme groups include a bacterial oxygen-binding protein (30), lactoperoxidase (31), and ferromyoglobin reacted with $BrCCl_3$ (32); interestingly, the linear tetrapyrrole present in phytochrome is also bound through a thioether bond (33).

In contrast to the mechanism proposed for formation of the A and B forms of N57C, the likely route by which a chlorin prosthetic group for the C derivative could be generated is less apparent. On an interim basis, however, we propose a working hypothesis involving an oxidative mechanism that branches from one of the putative intermediates leading to the B derivatives as indicated in Fig. 4. Until the structure of this prosthetic group has been established unequivocally, more extensive discussion of this mechanism is premature.

Implications of the N57C Cytochrome b_5 Derivatives. The initial oxidation state of the heme iron when it is presented to the apoprotein is a critical determinant of the distribution of products formed by the N57C variant (Fig. 4). Thus, when the iron is initially reduced, the dominant process is the simple addition of the thiol across the vinyl double bond to generate N57C-A, the product with a single cytochrome *c*-type thioether linkage. When the iron is initially oxidized, the radical-based mechanism dominates and leads to the formation of the alternative products N57C-B_I and -B_{II}, and probably N57C-C. We conclude that these considerations are critical in the biosynthesis of cytochrome *c*. Reduced heme has been shown to be required for covalent attachment of heme to apocytochrome *c* by the enzyme cytochrome-*c* heme lyase (34). The mechanism shown in Fig. 4 dictates that insertion of oxidized heme into apocytochrome *c* would initiate a radical-based sequence of reactions and lead to the formation of several alternative products.

Based on this analysis, it is possible to speculate on the function of the cytochrome *c* heme lyase *in vivo*. It is known that apocytochrome *c* is a random coil structure (35) whereas apomyoglobin, apocytochrome b_{562}, and apocytochrome b_5, which bind heme noncovalently without enzymatic assistance, have tertiary structures similar to those of the corresponding holoproteins (36–38). In light of the present results, it is apparent that the addition of the cysteine thiol group to a heme vinyl group can proceed spontaneously without enzymatic assistance as long as the sulfhydryl group is located near the heme vinyl group. Apart from its possible involvement in translocation of the protein into mitochondria (39), the proposed role of cytochrome-*c* heme lyase would be then twofold. First, it would selectively bind reduced heme or facilitate reduction of oxidized heme. Second, it would bind apocytochrome *c*, possibly through the consensus sequence Cys-Xaa-Xaa-Cys-His, so that the two cysteine residues involved in the covalent attachment are positioned close to the two vinyl groups of the reduced heme moiety and with the correct geometry for the addition reaction to proceed spontaneously.

The current findings also imply that it may be possible to create cytochrome b_5 variants that can facilitate the otherwise synthetically challenging preparation of chlorins from heme precursors. Several mechanistic issues are pertinent to the design of such variants. The most basic consideration, of course, is definition of the mechanism by which N57C-C forms and the reaction conditions that are critical. One such factor to include in this mechanism will undoubtedly be the manner in which orientation of heme binding to the apoprotein is regulated, as this binding orientation must be critical to regulating the regiospecificity of heme modification. Presumably, insight provided by studies of the type reported by La Mar, Sligar, and their colleagues (6, 7) will permit rational design of variant cytochromes b_5 that exhibit predetermined heme-binding preferences. In addition, we note that use of recombinant, chlorin-producing cytochromes can be complemented by use of suitable synthetic heme precursors to provide significant flexibility in the range of chlorins that might be produced by this strategy.

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