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The Axial Ligation and Stoichiometry of Heme Centers in Adrenal Cytochrome b_{561}[†]

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Abstract

Cytochrome (cyt) b_{561} transports electrons across the membrane of chromaffin granules (CG) present in the adrenal medulla, supporting the biosynthesis of norepinephrine in the CG matrix. We have conducted a detailed characterization of cyt b_{561} using electron paramagnetic resonance (EPR) and optical spectroscopy on the wild type and mutant forms of the cytochrome expressed in insect cells. The $g_z = 3.7$ (low-potential heme) and $g_z = 3.1$ (high-potential heme) signals were found to represent the only two authentic hemes of cyt b_{561}; models that propose smaller or greater amounts of heme can be ruled out. We identified the axial ligands to hemes in cyt b_{561} by mutating four conserved histidines (His54 and His122 at the matrix-side heme center and His88 and His161 at the cytoplasmic-side heme center), thus confirming earlier structural models. Single mutations of any of these histidines produced a constellation of spectroscopic changes that involve not one but both heme centers. We hypothesize that the two hemes and their axial ligands in cyt b_{561} are integral parts of a structural unit that we term the "kernel". Histidine to glutamine substitutions in the cytoplasmic-side heme center, but not in the matrix-side heme center, led to the retention of a small fraction of the low-potential heme with $g_z = 3.7$. We provisionally assign the low-potential heme to the matrix side of the membrane; this arrangement suggests that the membrane potential modulates electron transport across the CG membrane.

Most, if not all, eukaryotic organisms carry a gene for at least one member of the newly discovered protein family of cytochrome b_{561} (cyt b_{561})[1] (1-4). The oldest known member of the family, adrenal-type cyt b_{561}, is essentially an ascorbate-regeneration machine (5) that plays a key role in supplying electrons for the biosynthesis of norepinephrine by dopamine-β-hydroxylase (6) and of several important peptide hormones by peptidylglycine β-monooxygenase (6). Several other cyt b_{561} family members are either suggested to participate in ascorbate metabolism or to depend on ascorbate for ferric-reductase activity. Among them

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[3]Our earlier publication (Kamensky, Y.A. and Palmer, G. (2001) Chromaffin granule membranes contain at least three heme centers, *FEBS Lett. 491*, 119-22) considered a third heme as either a cyt b_{561} component or another heme-containing protein present in the CG membrane. That this third heme is not a part of cyt b_{561} itself is now confirmed, as neither purified cyt b_{561} from CG nor those expressed in insect, yeast or bacterial cells contain a heme with g factor of 3.62 that is not reduced by ascorbate. The origin and function of the third heme in CG remain unresolved, although Ponting speculated that it may be the heme of a cyt *b*-type iron reductase (Ponting, C. P. (2001) Domain homologues of dopamine β-hydroxylase and ferric reductase: roles for iron metabolism in neurodegenerative disorders? *Hum. Mol. Genet. 10*, 1853-1858).

are: stromal-cell derived receptor 2, which is an iron reductase expressed in major iron storage organs (7,8); a newly discovered analog with ferric-reductase activity, Lcyt b_{561}, which was found in macrophage lysosomes (9); Tcyt b_{561}, a cytochrome recently reported as a constituent of tonoplast membrane in *Arabidopsis* (10); mammalian duodenal b_{561} (Dcyt b_{561}), which participates in the uptake of dietary iron in the brush border membrane of the duodenum (8, 11) (but see (12,13)), iron uptake in airway epithelial cells (14) and possibly in extracellular ascorbate recycling in human erythrocytes (15). Another interesting member of cyt b_{561} family is the 101F6 protein, a putative tumor suppressor with an unknown mechanism of action (16, 17). Many species have multiple cyt b_{561} family members, with six known in humans and as many as sixteen in *Arabidopsis thaliana.* Thus, the cyt b_{561} family appears to be important for the biology of eukaryotes (1).

With most of the proteins constituting this family still waiting for physico-chemical characterization, the development of a structural and functional model of a prototypic representative would be valuable as a point of comparison. Adrenal cyt b_{561} is undoubtedly the current best choice for such a prototype because: (a) a significant database already exists for this protein; (b) it is highly abundant, comprising 17% of the total protein in its natural source, the membrane of chromaffin granules (CG) located in the medulla of adrenal glands (18); and (c) it has been expressed in fully functional form in insect (19,20), yeast (20,21) and recently in bacterial (22) cells.

According to the current paradigm (5), the synthesis of each molecule of norepinephrine by dopamine-β-hydroxylase within CG requires two electrons provided by intra-granular ascorbic acid (6). In this process, two ascorbic acid molecules inside the granule undergo one-electron oxidation to monodehydroascorbate (23) and must be replenished. Neither fully reduced

[1]Abbreviations:

cyt cytochrome

CG chromaffin granules

M matrix

C cytoplasmic

MDA monodehydroascorbate

PAL putative axial ligand

EPR electron paramagnetic resonance spectroscopy

HALS highly axial low spin

ALA δ-aminolevulinic acid

EDTA ethylenediamine tetraacetic acid

DM n-dodecyl-β-D-maltoside

SVD singular value decomposition

ascorbate nor matrix monodehydroascorbate can penetrate the granule membrane (24-26), and cyt b_{561} regenerates intra-granular ascorbate by transferring electrons acquired from cytoplasmic ascorbate (5,27-32).

Adrenal cyt b_{561} consists of 252 amino acid residues with an M_r of 28 kDa (33,34). Sequence analysis of adrenal cyt b_{561} predicts six transmembrane helices (33) with the central four helices forming a bundle (3) carrying the hemes (35). Although all models of cyt b_{561} beginning with that of Degli Esposti *et al.* (35) consider cyt b_{561} to contain two hemes, the heme stoichiometry has remained an unsettled issue. Based on an apparent heme-to-protein ratio of one, early literature considered cyt b_{561} to contain a single heme (27,36-39). Tsubaki *et al.* (40) developed a mild purification procedure and reported the isolation from CG of cyt b_{561} containing two hemes. Subsequent expression and purification of a recombinant, his-tagged bovine cyt b_{561} from yeast and insect cells also yielded proteins with two hemes per polypeptide (20). The two hemes were reported to have different low-spin signals in electron paramagnetic resonance (EPR) spectra, with low-field g-values of 3.1 and 3.7 (40-42), though recently Wanduragala *et al.* (43) raised doubts regarding the authenticity of the $g_z = 3.7$ signal in their sample of purified cyt b_{561}. Kipp *et al.* (44) suggested that the second EPR signal is not a manifestation of an independent heme center but represents heme-heme interaction between two different mono-heme protein molecules. On the other hand, Burbaev *et al.* (41) and later Kamensky *et al.* (19) observed heterogeneity in the EPR signal of one of the hemes and noted the possibility that membranous cyt b_{561} contained 3 hemes (41), perhaps in the framework of a dimeric cyt b_{561} structure (19).

Bis-histidine ligation is very likely for all the heme of cyt b_{561} (41,45). Kamensky *et al.* (45) observed that cyt b_{561} lacks the characteristic charge transfer band near 700 nm that is typical for methionine coordination in cytochromes *c* (46) and in cyt b_{562} from *E. coli* (47). In addition, the marked similarity of the magnetic circular dichroism in the beta-band of reduced cyt b_{561} with that of cyt b_5 (45) and the bis-imidazole complex of protoheme makes coordination by lysine unlikely (48), leaving the imidazole side-chain of histidines as the most credible candidates for axial ligands to heme in cyt b_{561} (45). The EPR spectral characteristics of cyt b_{561} (20,21,40-42) are consistent with histidine axial ligands, as are the near-IR magnetic circular dichroism spectra of cyt b_{561} (E. Duin, Y. Kamensky, M. Johnson, G. Palmer, unpublished).

Six fully conserved histidines (His54, His88, His92, His110, His122 and His161 in adrenal cyt b_{561}) have been identified in cyt b_{561} from different species (1). Degli Esposti *et al.* (35), and later other investigators (1-3,49), suggested that His54 and His122 are the axial ligands to the heme on the matrix (M) side of the CG membrane, and that His88 and His161 provide axial ligation to the heme on the cytoplasmic (C) side. However, this hypothesis has been waiting for experimental confirmation. An important step in this direction was recently taken by Bérczi *et al.* (21). Their results suggested that His110 could be excluded from the list of potential axial ligands to heme and were consistent with the idea of His88, His161, His54 and His122 being the axial ligands. In the absence of an X-ray structure, the assignment of individual axial ligands to a particular heme center can be examined by EPR analysis of recombinant cyt b_{561} in which putative axial ligands (PALs) are replaced systematically by mutagenesis. EPR is very sensitive to the properties and environment of the oxidized (paramagnetic) heme centers of cytochromes (50) and, importantly, the two heme EPR signals in cyt b_{561} are well resolved (40-42).

We present here a comparative EPR and optical study of natural and recombinant wild type cyt b_{561}, as well as mutants of bovine adrenal cytochrome, with the goal of resolving remaining questions regarding the fundamental characterization of cyt b_{561}, namely the quantity of its hemes and identification of their axial ligands.

EXPERIMENTAL PROCEDURES

Materials

Hemin, DTT, EDTA, cholate, desoxycholate, ALA, and horse heart cytochrome *c* were from Sigma (St. Louis, MO). Frozen bovine adrenal glands were purchased from Pel-Freez (Rogers, AR), while fresh glands were obtained from a slaughterhouse. Protease inhibitor cocktail Set 1 and Set III (without EDTA) were from Calbiochem (San Diego, CA). Ascorbic acid sodium salt and sodium dithionite were obtained from Fluka (Germany). n-Octyl-β-D-glucoside and n-dodecyl-β-D-maltoside, CHAPS, PMAL-c12, Triton X-100 were from Anatrace (Maumee, OH).

CG and CG membrane vesicles preparation

Fresh and pre-frozen adrenal glands were used for preparation of CG and different types of membrane vesicles as described previously (20,42).

Cyt b_{561} from CG

Purification of cyt b_{561} from natural membranes was performed according to the protocol developed by Tsubaki *et al*. (40)

Recombinant bovine cyt b_{561}

Expression of his-tagged bovine cyt b_{561} in *Sf9* insect cells and purification of membranous and isolated recombinant protein were performed as described previously (20). The membranous preparations of mutated cyt b_{561} were prepared by the method developed in (20) for the wild type, where the membranes were collected at 150,000 g with or without prior removal of the 500 g and 15,000 g fractions. Site-directed mutagenesis of bovine cyt b_{561} was performed on the b561/pBluescript II KS (+) plasmid using QuikChange site-directed mutagenesis kit (Stratagene). The primer sequences containing the mutation (the bases changed are underlined) were:

His54Gln sense (5′-GCAGTTCAACGTGCAACCCCTCTGCATGATCATAG-3′)

His54Gln antisense (5′-CTATGATCATGCAGAGGGGTTGCACGTTGAACTGC-3′)

His54Met sense (5′-CTGCAGTTCAACGTGATGCCCCTCTGCATGATC-3′)

His54Met antisense (5′-GATCATGCAGAGGGGCATCACGTTGAACTGCAG-3′)

His54Tyr sense (5′-CTGCAGTTCAACGTGTACCCCCTCTGCATGATC-3′)

His54Tyr antisense (5′-GATCATGCAGAGGGGGTACACGTTGAACTGCAG-3′)

His88Gln sense (5′-ACCAAAGTCCTGCAAGGGCTGCTGCACGTCTTC-3′)

His88Gln antisense (5′-GAAGACGTGCAGCAGCCCTTGCAGGACTTTGGT-3′)

His122Gln sense (5′-ACCTGTACAGCCTGCAAAGCTGGTGCGGCATCC-3′)

His122Gln antisense (5′-GGATGCCGCACCAGCTTTGCAGGCTGTACAGGT-3′)

His161Gln sense (5′-GCTACCGCCCGCAGCAAGTCTTCTTCGGCG-3′)

His161Gln antisense (5′-CGCCGAAGAAGACTTGCTGCGGGCGGTAGC-3′)

His161Met sense (5′-CGCTACCGCCCGCAGATGGTCTTCTTCCGGCGC-3′)

His161Met antisense (5′-GCGCCGAAGAAGACCATCTGCGGGCGGTAGCG-3′)

His161Tyr sense (5′-CGCTACCGCCCGCAGTACGTCTTCTTCGGCG-3′)

His161Tyr antisense (5′-CGCCGAAGAAGACGTACTGCGGGCGGTAGCG-3′)

E. coli strain XL-10 (Stratagene) competent cells were heat transformed with the mutagenesis reaction, plated on LB agar containing 100 μg/ml ampicillin, and grown overnight. Ampicillin-resistant colonies were grown in LB-ampicillin broth at 37 °C overnight, and plasmid DNA was isolated. Clones were screened for the presence of the desired mutation by DNA sequencing, digested with BamHI/XbaI and separated on an agarose gel. The purified b_{561} fragments containing the desired mutations were inserted into BamHI/XbaI digested pVL1393 vector (PharMingen, San Diego, CA). The final constructs were verified by restriction enzyme digestion and DNA sequencing. Construction of baculoviral vectors for expression of mutant cyt b_{561} in insect cells followed published procedures (51).

Assay of protein, cyt b_{561} and heme content

Total protein was assayed with a BioRad DC kit, using bovine albumin as the standard. Recombinant cyt b_{561} content was calculated using a difference extinction coefficient of 34 $mM^{-1}cm^{-1}$ (561 nm- 575 nm, reduced minus oxidized spectrum (20)). The concentration of membranous and purified CG cyt b_{561} was calculated using a difference extinction coefficient 37.3 $mM^{-1}cm^{-1}$ (561 nm - 575 nm, reduced minus oxidized spectrum (40)). Heme content was determined by the pyridine hemochrome assay (52), using a difference extinction coefficient (556 nm - 538 nm) of 24.5 mM^{-1} cm^{-1} (53).

EPR sample preparation

Samples were oxidized with an excess of ferricyanide. Membranous samples were then washed twice by centrifugation (1h at 120,000 g) to remove the oxidant, whereas detergent extracts and purified cytochrome were desalted using 10DG columns (BioRad). Concentrated samples were supplemented with 18% glycerol (50% glycerol for CG samples), transferred into EPR tubes, frozen in ~ 1 s in a dry ice-ethanol mixture, and stored in liquid nitrogen. Typical cyt b_{561} monomer concentrations were 25-40 μM monomer for membranous preparations and 50-70 μM for isolated cytochrome.

Spectroscopy

Electronic absorption spectra were recorded with a Shimadzu Model 2101PC or a Jasco Model V-560 spectrophotometer at room temperature. EPR spectra were collected at 5-40 K with a Bruker EMX or a Varian E-6 spectrometer equipped with a liquid helium cryostat.

Computer analysis

Experimental data were analyzed using fitting routines (including the SVD algorithm) of IgorPro 5.04 (Wavemetrics, Portland, OR). Molecular graphics of the cyt b_{561} structural model (3) were prepared using the software Ribbons 3.22 (54).

RESULTS

The stoichiometry and EPR signals of the hemes in cyt b_{561}

Cyt b_{561} manifests two distinct low-spin ferric heme signals, with g_z at 3.7 and 3.1 (Fig. 1). We found the same two low-spin signals in all samples of cyt b_{561} examined: in intact CG, which is a preparation intermediate between the parent chromaffin cells and purified membrane (Fig. 1, trace 1), in purified CG membranes (Fig. 1 traces 2, 3) and in purified recombinant, wild type, his-tagged cyt b_{561} expressed in insect cells (Fig. 1, trace 4).

In addition to the g_z ~3.7 and 3.1 signals, there is a signal from non-heme iron at g = 4.3 that is prominent in membranous samples (Fig. 1, traces 1-3) but rather insignificant in the sample of purified cytochrome (Fig. 1, trace 4). The small size of the signal at g ~ 6 establishes that

only a very small fraction of the purified cytochrome is in the high-spin form (Fig. 1, trace 4). CG membranes prepared according to a traditional protocol (42) also exhibit a low level of high-spin species (Fig. 1, trace 3). Intact CG and Tsubaki membranes (20) have a somewhat higher content of the high-spin signal (Fig. 1, traces 1 and 2). The small signal at $g_z = 2.84$, which is present in some of our experiments (e.g. Fig. 1, trace 4) was previously shown to derive reversibly from the $g_z = 3.1$ signal in a pH-dependent manner, possibly reflecting deprotonation of an axial histidine (40).

The $g_z = 3.7$ and $g_z = 3.1$ signals have been assigned to the low-potential and high-potential heme centers, respectively, based on the order in which they were reduced in titrations with ascorbate (40,41). Fig 1 (inset) compares EPR spectra of fully oxidized and semi-reduced CG membranes. It is important to note that the location of the low-potential heme's EPR signal remains unchanged while its amplitudes diminishes slightly; at the same time the amplitude of the high potential heme approaches zero and no additional signals appears. This rules out a heme-heme electronic interaction in cyt b_{561} (see Discussion).

Lowering the temperature of cyt b_{561} from 45 K to 10 K was observed to shift the position of the high-potential peak from g = 3.11 to 3.14 without changing the position of the low-potential heme signal (41). The new signal was hypothesized to have slightly different g values and significantly different relaxation properties (41). However, we suggest that the shift in peak position represents a "passage artifact"[2] (55). This phenomenon is illustrated in Fig. 2, which compares the affects of microwave power on the high potential heme of cyt b_{561} (Fig. 2A) and on cytochrome *c*, an undoubtedly homogeneous species at pH 7 (Fig. 2B). In both proteins, increasing the microwave power shifted the position of the peak to lower fields and caused a baseline "overshoot" at higher fields. Passage artifacts may be encountered with any low-spin hemeprotein when the spin-lattice relaxation process is overwhelmed, as occurs at high powers or very low temperatures, and is not evidence for the presence of multiple heme centers in cyt b_{561} as was originally suggested (41).

Global analysis of cyt b_{561} absorption spectra

We previously demonstrated that the high- and low-potential heme centers of cyt b_{561} in CG membranes have unique spectral features (42). In the current experiments, we utilized purified bovine adrenal cyt b_{561} expressed in yeast (20), thus improving the quality of the spectra by reducing the noise caused by light scattering and by eliminating optical interference from a minor component present in the CG membranes, a separate heme-containing protein with a low redox potential[2]. Fig. 3A presents absolute absorption spectra of cyt b_{561} recorded during titration with ascorbate. Because the midpoint potentials of the two hemes differ by ~100 mV (36,56), spectral changes during the earlier stages of reduction reflect reduction of the high-potential heme exclusively. This heme has a "split" alpha band, with a pronounced maximum and a shoulder to shorter wavelength (Fig. 3B). As the titration progresses, the low-potential heme begins to contribute to the total absorbance and the spectra become somewhat more symmetric (Fig. 3A) because the alpha-band of the low-potential heme is not split and consequently has a much less contribution at shorter wavelengths (Fig. 3B).

To determine the number of spectral species necessary to explain all features of the absorption spectra at all stages of reduction, we subjected the set of 15 spectra shown in Fig. 3A to SVD analysis. Fig. 4A shows the relative amplitudes of the principal components derived from this analysis. It is clear that only SVD components 1 and 2 have significant amplitude and that the

[2]Passage artifacts arise when the rate of change of the total magnetic field incident on the sample is greater than the inverse of the spin-lattice relaxation time. Under these conditions, the EPR signal has contributions from both absorption and dispersion modes, and distortions in the EPR spectrum result (Mailer, C., and Taylor, C. P. (1973) Rapid adiabatic passage EPR of ferricytochrome *c*: signal enhancement and determination of the spin-lattice relaxation time. *Biochim. Biophys. Acta 322*, 195-203).

other components can be ignored. Note that the two major SVD components (Fig. 4B) are not identical to either the spectrum of cytochrome itself or the spectra of the two heme centers shown in Fig 3B. However, using only these two components, the SVD decomposition can be reversed to reconstruct the important features of the spectra shown in Fig 3A. This is illustrated in Fig 4C, which shows experimental and calculated spectra at approximately 7, 50 and 100% reduction (corresponding to traces 1, 7 and 15 of Fig. 3A). The major features of the experimental data are reproduced so closely that the discrepancies between experimental and synthetic curves are difficult to detect (Fig. 4C). It is important to emphasize that just two SVD components are sufficient to reproduce all essential features of the cyt b_{561} absorption spectra and, hence, only two spectral species contribute to the data of Fig. 3A.

Expression of PAL mutant cytochromes in insect cells

We used our established insect cell system (20) to express recombinant adrenal cyt b_{561} with mutations in individual PAL residues (histidines 54, 88, 122, and 161) for EPR analysis. The insect cell expression system produced the four PAL mutants in comparable amounts, as assessed spectrophotometrically (Fig. 5). However, whereas more than 90% of the wild type cyt b_{561} was extracted by detergent from insect cell membranes (20), less than 15% of the PAL mutant cytochromes were solubilized by detergent (dodecyl maltoside (with or without PMAL-c12), octyl glucoside, Triton X-100, CHAPS, cholate and deoxycholate were tried at a variety of detergent-to-protein ratios, with and without up to 8 M urea). Such detergent resistance is not unique to the insect cell system or to cyt b_{561}. PAL mutants of cyt b_{561} expressed in yeast also could not be solubilized with dodecyl maltoside (W. Liu, D. Cao, unpublished). Similar effects of PAL mutations on the extractability of the mutated protein were observed in a study of the heme *b* subunit of succinate:quinone reductase from *Bacillus subtilis* (57). As the PAL mutants of cyt b_{561} resisted solubilization, we proceeded with their characterization in the membrane fraction.

Absorption spectra of membranous PAL mutant cytochromes

Fig. 5A presents the reduced minus oxidized difference absorption spectrum of wild type cyt b_{561} expressed in insect cell membranes. The spectrum is typical for low-spin heme *b*-containing cytochromes, with pronounced alpha, beta and gamma bands; its characteristics are quite similar to those of the CG cytochrome (40,42) as well as the recombinant protein expressed in yeast (20,21). Figs. 5B and 5C present the absorption spectra of PAL mutants with individual histidines substituted by glutamine, methionine or tyrosine. In addition to low-spin heme features, the spectrum of each of the mutants exhibits some high-spin characteristics (maxima at ~ 440-450, 480, 625 and 660 nm), indicating that the mutations perturbed the spin state of the heme center(s).

The difference spectra from control *Sf9* cells not infected with baculovirus or expressing an unrelated, heme-free protein, XylE, and grown with or without supplementation with ALA and heme lacked significant optical absorbance (Fig. 5B traces 5-8), showing that the host cell membranes themselves had negligible level of b-type cytochrome.

Reaction of membranous PAL mutants of cyt b_{561} with ascorbic acid

Purified wild type cyt b_{561} was reduced almost fully by ascorbate, the physiological reductant (Fig. 3), whereas its membranous counterpart was only ~70% reduced (Fig. 6); this difference presumably reflects either changes in the cytochrome's redox potentials or decreased access to the reductant because of membrane components.

The membranous PAL mutants were much less reactive with ascorbate than the wild type cytochrome. Addition of 40 mM ascorbate reduced the mutants at His161 and His88 to ~30% of the extent achieved with dithionite, as illustrated for H88Q (Fig. 6). Similar results were

reported by Berczi *et al*. for His88 and His161 (bovine cytochrome numbering) mutants of murine cyt b_{561} expressed in yeast (21). Mutants of His54 and His122 were completely unreactive with ascorbate, as shown in Fig. 6 for H122Q. This difference in ascorbate reactivity between the His54/His122 and His88/His161 mutant pairs is further evidence that the optical spectra originate from recombinant cyt b_{561} proteins rather than some endogenous *Sf*9 cytochrome. The different effects on ascorbate reactivity of mutations at the two heme centers perhaps reflects changes in the centers' redox potential, so that mutation of the center ligated by His54 and His122 causes a larger decrease in redox potential than does mutation involving the heme center ligated by His161 and His88.

Effects of PAL mutations on the EPR spectrum

EPR spectra of membranous cyt b_{561} with mutated PAL are presented on Fig. 7 along with the spectra of wild type cytochrome and control membranes. In the low spin area, the familiar wild type signals with g_z at 3.1 and 3.7 are replaced in the mutants with two new low-spin signals. The first signal has $g_z = 2.96$, $g_y = 2.26$; the second signal is characterized by $g_z = 2.46$ and $g_y = 2.26$. Similar EPR changes were observed when the targeted PAL histidine was substituted with methionine or tyrosine, which are potential axial ligands (Fig. 7A) or with glutamine, which does not ligate heme (Fig. 7B). In the broader magnetic field interval (shown in Fig. 7C for H88Q), a prominent high-spin signal at g = 6.0 and non-heme iron g = 4.3 signal are observed. A small signal at g = 1.90 is associated with $g_z = 2.46$. It is notable that all PAL mutations in either heme center resulted in the appearance of the same set of heme spectral species. Control membranes from cells expressing no cyt b_{561} (with or without addition of heme and ALA during culture), showed no significant EPR signals (Fig. 7B), indicating that the host cell membranes have negligible endogenous EPR-active species.

It is clear from the EPR spectra in Fig. 7A that tyrosine substitution at His54 or His161 did not result in ligation of either heme (58); methionine substituted at those positions may have become a ligand (much as reported in (57)), producing a complicated situation in which multiple new low-spin signals with slightly different g-values can be observed at high microwave power (not shown). These new signals could reflect species with slightly different relative orientations of the remaining histidine and methionine (59). In any case, the similarity of the spectra for tyrosine, methionine, and glutamine substitutions at His54 and His122 (Fig. 7A and B) indicate that the size and the nature of the substituted residue had little effect on the overall outcome; it was the loss of the histidine functionality as the axial ligand that was responsible for the observed effects.

With one set of PAL mutants in which glutamine was substituted for individual histidines, raising the microwave power from 1 mW (used for the spectra in Fig. 7) to higher levels more favorable for HALS observation (see Supporting Information) elicited a signal at $g_z = 3.7$ in the H88Q and H161Q mutants (Fig. 8). This indicates that a portion of the low-potential heme center survived the general perturbation of the heme environments caused by these mutations. Importantly, HALS signals were not observed in the His54Q and His122Q mutants (Fig. 8). The HALS signals are rather small and under other circumstances might have gone unnoticed because the baselines are far from ideal. To minimize the latter problem, we subtracted the spectrum of the H54Q or the H122Q mutant from that of the H161Q mutant at each microwave power. The results (Fig. 8, lower two panels), show the appearance of the HALS signal at high powers more convincingly. The presence of the negative peak in the H161Q minus H54Q difference spectrum (Fig. 8, lower left) reflects the remarkable differences in saturation properties of the signal at g ~2.95 between the H54Q and H161Q mutants. These EPR results connect the low-potential (HALS) heme center with His54 and His122.

DISCUSSION

EPR signals of heme in wild type cyt b_{561}

The experimental data in this study refute arguments that cyt b_{561} is other than a two heme-containing protein. Burbaev *et al*. (41) found the $g_z = 3.1$ signal shifts to lower field with the lowering of temperature and interpreted this as evidence for a third heme in cyt b_{561}. We reproduced the earlier data in the present experiments by varying microwave power but also found that similar spectral changes could be demonstrated with cytochrome *c* (Fig. 2B). Thus, we believe that the pseudo-heterogeneity of $g_z = 3.1$ signal is best explained as an artifact that can occur at high power and/or low temperatures[2].

Wanduragala *et al.* (43) observed that their preparation exhibited only a nominal quantity of the $g_z = 3.7$ signal and raised doubts that this signal originates from a heme center in purified cyt b_{561}. From the power and temperature dependencies of $g_z = 3.1$ and 3.7 signals (see Supporting Information) one can conclude that the quantity of low-potential heme was substantially underestimated by Wanduragala *et al.* (43) because the EPR conditions they employed (20 mW and 15 K) are far from optimal for observing the $g_z = 3.7$ species. To address concerns similar to those raised by Wanduragala *et al*. (43) that isolation of cyt b_{561} or even preparation of membranes from CG could cause partial denaturation and the appearance of an additional, non-native EPR signal, we recorded EPR spectra of freshly prepared CG. The resulting EPR spectrum (Fig. 1, trace 1) shows the same EPR signals of cyt b_{561} that are characteristic of the purified and membranous samples of endogenous and recombinant cyt b_{561}, with or without a his tag (10,20,21,40-42).

Kipp *et al*. (44) discussed the possibility that an EPR signal may be caused by electronic interactions between hemes situated on separate molecules of a cytochrome containing just one heme, thus generating the additional EPR signal which was, in their view, misinterpreted as the signal of a second heme center in cyt b_{561}. There are several difficulties with this proposal with the most telling being that the $g_z = 3.7$ resonance should shift to a position approximately midway between the $g_z = 3.7$ and $g_z = 3.1$ signals in partially-reduced cytochrome, in which most of the high-potential heme is in the diamagnetic, ferrous state. There is no shift in $g_z = 3.7$ position in the spectrum of semi-reduced protein published by Burbaev *et al*. (41), which we now confirm (Fig 1, inset).

Thus, our data provides persuasive evidence for the presence of only two types of heme in cyt b_{561}. This is consistent with the demonstration that optical changes during reduction are well reproduced using only two components (Fig 4C) and with published stoichiometry of heme-to protein close to two in the endogenous (40) and recombinant (20,22) cytochrome. There appears to be no further need to develop models that require other than two hemes per polypeptide of cyt b_{561}.

Low-spin heme EPR signals in PAL mutants

The mutations of PAL of cyt b_{561} led to loss of the characteristic low-spin signals from the native high- and low-potential hemes and appearance of an intense high-spin signal at g ~6, a low-spin signal with $g_z = 2.96$, $g_y = 2.25$ and a second low-spin signal with $g_z = 2.46$, $g_y = 2.26$, g_x ~1.90 (Fig. 7). Similar low-spin signals are known from studies of other cytochromes. An EPR signal with $g_z = 2.96$ was noted as a minor species in some samples of cyt b_{561} (20, 21,43,60) and in unrelated systems with a mutated cytochrome *b* component (61,62). This signal has been identified as “relaxed” conformation of *b*-type cytochrome in which the strain on heme ligation imposed by the native protein is removed (63). The signal with $g_z = 2.46$, $g_y = 2.26$, $g_x \sim 1.90$ resembles those of cyt P-450 (64) and P-450 mimics (65-68). These are hemeproteins where one of the axial ligands is cysteine and the other one is water, OH^-,

histidine or proline. Cyt b_{561} contains only two cysteines; in the model of Bashtovyy *et al.* (3) these cysteines are located near the M-side heme center. If this model is accurate, Cys57 might replace His54, or Cys125 might replace His122 under the disruption caused by a PAL mutation[4].

Identification of axial ligands for cyt b_{561} hemes

In a simple scenario, the absorption spectra of PAL mutants should be sufficient to determine whether particular amino acid residues furnish axial ligands to the cyt b_{561} hemes. With one putative axial ligand eliminated by mutation to glutamine, the affected heme should be converted to a 5- or 6-coordinated high-spin species with one axial ligand provided by the unaffected histidine and the second ligand site being either vacant or occupied by water or hydroxide. In this scenario, the second heme of cyt b_{561} is unaffected by mutation and remains low-spin; the resulting absorption spectrum of the PAL mutant exhibits a combination of low-spin and high-spin features in the Soret and retains roughly half of the intensity in the alpha-band where high-spin species have little contribution. The absorption spectra of PAL mutants (Fig. 5B and C) indirectly confirm the role of the targeted histidines as axial ligands because in addition to the typical low-spin peaks there are some high-spin features in the Soret region and in the near infrared that are absent in the wild type spectrum (Fig. 5A). Although the spectra of four different types of control membranes (Fig. 5B) convincingly demonstrate that endogenous hemeproteins did not interfere, it was not possible to make a firm estimate of the heme-to-protein stoichiometry for the PAL mutants in the *Sf9* membranes[5] and thus a definitive judgment on axial ligation of cyt b_{561} could not be made using optical spectra alone.

Further insights into the consequences of PAL mutations were obtained from the EPR spectra of *Sf9* membranes expressing cyt b_{561} (Fig. 7). The initial expectation was that mutation of either axial ligand to a given heme would perturb only the EPR signal of the affected heme, leaving the signal of the second heme unchanged. Instead, any substitution at any one of the four PAL histidines resulted in a similar pattern of changes in the EPR spectrum described above. The observation of global changes in the EPR spectrum of each of the mutants with affected PALs demonstrates that modification of any of them disrupts the heme environment and thus validates the models of molecular organization of cyt b_{561} introduced by Esposti *et al.* and Okuyama *et al.* (35,49). The global effects of the PAL mutations in cyt b_{561} are reminiscent of results with the cytochrome *b* subunit of quinol:fumarate reductase from *Wolinella succinogenes*, where mutation of any of the four histidines predicted to be axial ligands to the hemes (69) resulted in little or no expression of the protein (70). Assignment of those histidines as axial ligands was later confirmed by crystallography (71).

Mutations of the remaining histidine residues in bovine adrenal cyt b_{561} (His92, His109 and His110) produce recombinant proteins with EPR spectra generally similar to that of the wild type cytochrome (Liu, W., Rogge, C., Shinkarev, V., Tsai, A.-L, Kamensky, Y., Palmer, G., Kulmacz, R.J., in preparation). In murine cyt b_{561}, mutation to alanine of the histidine corresponding to His110 did not affect its absorption spectrum (21). Taken together with the

[4]The appearance of a P-450-like form of cyt b_{561} under denaturing conditions was noted by Wanduragala *et al.* (Wanduragala, S., Wimalasena, D. S., Haines, D. C., Kahol, P. K., and Wimalasena, K. (2003) pH-induced alteration and oxidative destruction of heme in purified chromaffin granule cytochrome b_{561}: implications for the oxidative stress in catecholaminergic neurons. *Biochemistry 42*, 3617-3626) but they did not definitively connect the observed signal with a P-450-like center because they observed a variation in the relative amplitudes of the two components at g = 2.46 and g = 2.26, supposedly the g_z and g_y of the same signal. We suggest that this variation occurs because the g_y of all three minor forms of cyt b_{561} discussed in this paper (the pH-dependent form with g_z = 2.84, the species with a "relaxed" conformation (g_z = 2.96) and the P-450-like species with g_z = 2.46) overlap around g = 2.26 and thus make the EPR amplitude at g_y sensitive to the relative amounts of these three minor forms.

[5]Attempts to estimate the quantity of PAL mutant proteins in *Sf9* cell membranes by immunoblotting did not produce satisfactory results because the PAL mutants were refractory to extraction with SDS at room temperature; heating cyt b_{561} in SDS causes aggregation of the protein (L. T. Duong and Fleming, P. J. (1982) Isolation and properties of cytochrome b561 from bovine adrenal chromaffin granules. *J. Biol. Chem. 257*, 8561-8564).

results of site-directed mutagenesis of PALs presented here, the accumulated evidence bring the earlier predictions (35,49) of axial ligation of hemes in cyt b_{561} past the tipping point and leaves little doubt that the heme ligation scheme in these models is correct.

The concept of a structural unit containing both heme centers of cyt b_{561}

The EPR spectra of mutants in axial ligand residues (Figs. 7 and 8) demonstrate that signals from both heme centers in cyt b_{561} are changed dramatically in response to alteration of just one, and any one, of the four axial ligands to the two hemes. To account for this behavior, we hypothesize that the two hemes and their axial ligands are linked parts of a major structural unit in cyt b_{561} that we term the "kernel." Perturbation of any of the axial ligands leads to structural changes in the whole kernel, including coordination changes at both heme centers.

Three observations illustrate the global response of the kernel to modification of any of its key elements. First, the original high-potential heme signal at $g_z = 3.1$ was not observed in any of four PAL mutants, suggesting that the high-potential heme center was fully disrupted even though its own axial ligands were not mutated. Second, although the P-450-like signal most likely originates from the M-side heme (3), with the nearby cysteine residues (Fig. 9), the signal with $g_z = 2.46$ appears not only in mutants of His54 and His122 at the M-side center but also in mutants of His88 and His161 at the C-side center (Fig. 7). Third, in the framework of the model of Bashtovyy *et al* (3) it is conceivable that when His88 is mutated it can be replaced by His 92. However, the model does not have any histidine residues that could replace His54, His122 and His161 as axial ligands. The signal of a bis-histidine ligated hemoprotein at g ~2.96 observed when those histidines were replaced by glutamine (Fig. 7B) must originate from the opposite heme center, which was not directly affected by mutation and yet was partially transformed into a "relaxed" conformation (63).

The mechanism for transmitting changes in the vicinity of one heme center to the other center is unclear, but as some other cases suggesting "cooperative" behavior involving the heme centers of bis-heme *b*-type cytochromes have been reported (70,72), this interesting general phenomenon awaits an explanation.

Arrangement of cyt b_{561} hemes in the CG membrane

Earlier topological analyses (3,35,49) have convincingly positioned the His54/His122 pair near the matrix (M) surface of the CG membrane and the His88/His161 pair near the membrane's cytoplasmic (C) surface (Fig. 9). The question of which heme center is high-potential and which is low-potential has not received much attention apparently because the answer was assumed to be self evident (3,60). Physiological electron transfer was presumed to occur down the gradient of ~100 mV between the redox potentials of the two hemes, i.e., from a low-potential heme on the C-face of the membrane to a high-potential heme on the M-face. However, our EPR data support the opposite topological arrangement of the two heme centers, with the low-potential ($g_z = 3.7$) heme center on the M-side, ligated by the His54/His122 pair and the high-potential ($g_z = 3.1$) heme center on the C-side, ligated by the His88/His161 pair (Fig. 9). Although the issue of the "sidedness" of the hemes of cyt b_{561} remains to be resolved conclusively, one interesting implication of our topological model (Fig. 9) is that physiological transmembrane electron transport, from the cytoplasm to the CG matrix, occurs *against* the ~100 mV gradient of the redox potentials of the two heme centers. We hypothesize that it is the inside-positive membrane potential of up to 90 mV (Fig. 9) created by the CG membrane H-ATPase (73) that drives electrons "uphill". The role of $\Delta\Psi$ as a driving force of electron transport was discussed when cyt b_{561} was thought to contain only one heme (32); in the context of our current hypothesis the suggested role of $\Delta\Psi$ becomes even more important. It is noteworthy that although transmembrane electron transport via adrenal cyt b_{561} has been demonstrated in CG ghosts (74) and in reconstituted liposome systems (31,75-77), electron

transport was only observed in the non-physiological direction, i.e., from the M-side to the C-side. We suspect that the membrane potential, absent in these reconstitution experiments, is an important factor modulating the directionality of electron transport.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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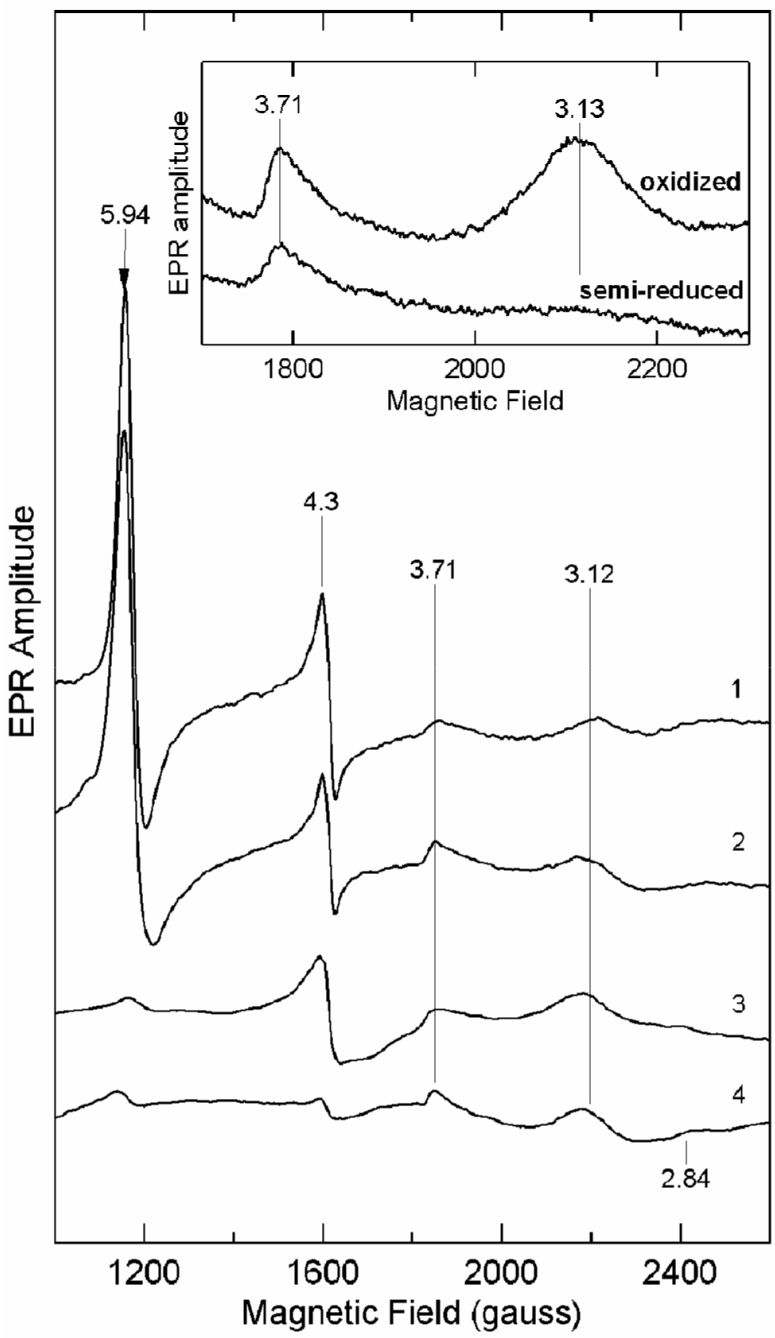

Figure 1.

EPR signals of cyt b_{561} hemes in native CG, CG membranes and the purified cytochrome. EPR spectra were recorded for freshly prepared CG (trace 1), "Tsubaki membranes" (trace 2), traditionally prepared CG membranes (trace 3), and purified His-tagged cyt b_{561} expressed in *Sf9* cells (trace 4). Signal amplitudes were normalized to the heme concentration. Membranous samples (traces 1-3) were suspended in 20 mM Tris, pH 7.2, with 18% glycerol, except for freshly prepared CG, where the glycerol concentration was 50%. Purified cytochrome sample (trace 4) was in 100 mM sodium phosphate, pH 7.2, containing 18% glycerol and 1% octyl glucoside. The microwave power and temperatures were: 1, 15 mW/11 K; 2, 32 mW/11 K; 3, 50 mW/11 K and 4, 15 mW/8 K. Other spectrometer settings were: microwave frequency, 9.60

GHz; modulation frequency, 100 KHz; modulation amplitude, 10 G except for trace 3 (20 G). **Inset**: EPR signals for oxidized and semi-reduced cyt b_{561} hemes in CG membranes. Traditionally prepared CG membranes were suspended in 50 mM Hepes, pH 7.2, 0.1 mM EDTA with 18% glycerol and either oxidized with ferricyanide (upper) or 50% reduced with ascorbate (lower) before spectra were recorded at 11.5 K with a microwave power of 50 mW. Other spectrometer settings were: microwave frequency, 9.27 GHz; modulation frequency, 100 KHz; modulation amplitude, 20 G.

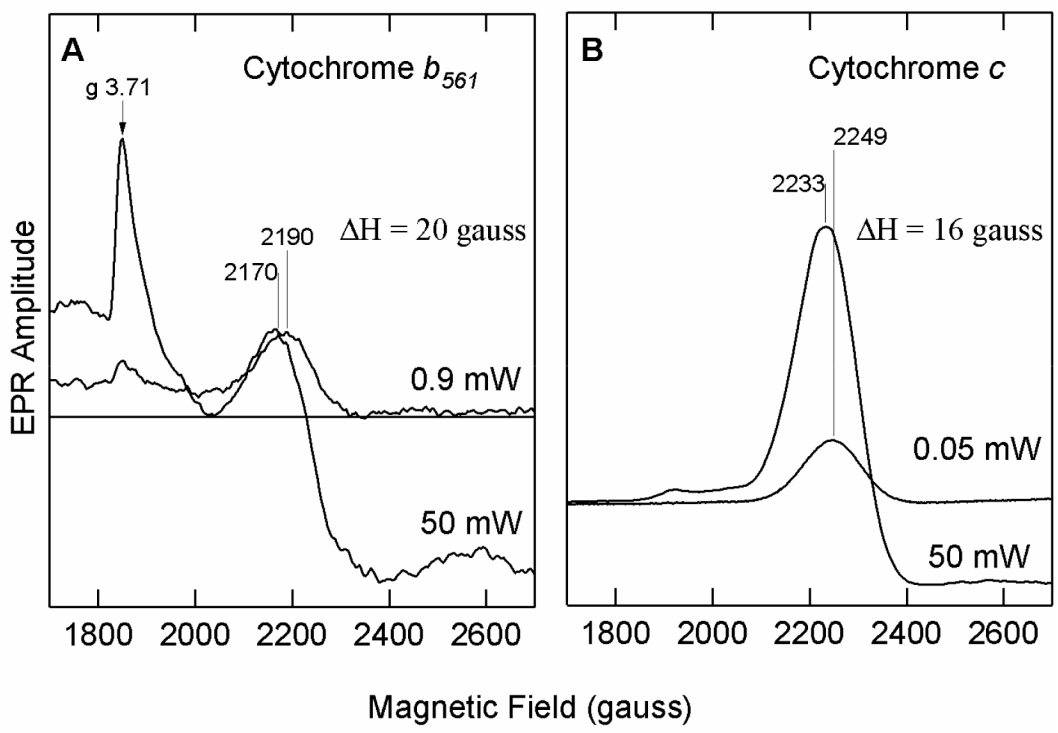

Figure 2.

Effects of microwave power on the EPR signal of the high-potential heme of cyt b_{561}. **A**: The EPR spectrum of 31 μM purified cyt b_{561} from CG membranes in 50 mM sodium phosphate, pH 7.3, containing 18% glycerol and 1% octyl glucoside was recorded at 0.9 and 50 mW. **B**: the EPR spectrum of 200 μM horse heart cytochrome c in 60 mM sodium phosphate, pH 6.8, containing 20% glycerol was recorded at 0.05 and 128 mW. Spectrometer conditions: microwave frequency, 9.60 GHz; modulation frequency, 100 KHz; modulation amplitude, 10 G; and temperature, 8.0 K.

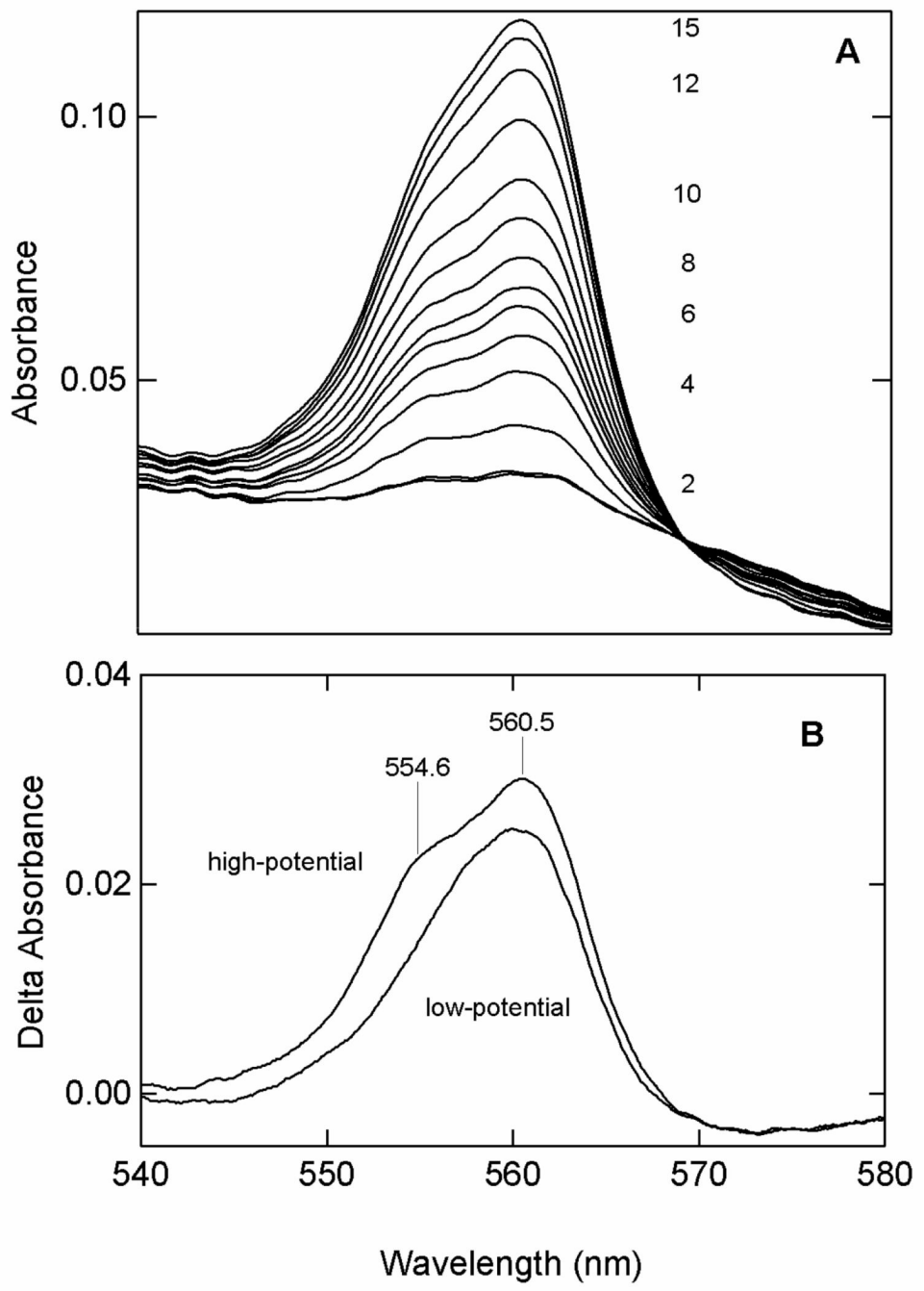

Figure 3.

Absorption spectra of the high- and low-potential hemes of cyt b_{561}. **A**: Absolute spectra of cyt b_{561} recorded during reductive titration with ascorbate. The purified cytochrome expressed in yeast (20) was diluted to 2.6 μM in 100 mM potassium phosphate, pH 7.2, containing 10% glycerol and 0.08% DM. Spectra were recorded after addition of the following concentrations of ascorbate: 1, 0.3 μM; 2, 1.2 μM; 3, 5.2 μM; 4, 12.3 μM; 5, 22.4 μM; 6, 35.5 μM; 7, 52 μM; 8, 71 μM; 9, 105 μM; 10, 170 μM; 11, 0.53 mM; 12, 1.2 mM; 13, 8.5 mM; and 14 (not shown for clarity), 19.6 mM. Trace 15 was recorded after addition of a few crystals of dithionite. Spectra were collected at 0.1 nm intervals with a 1.0 nm bandwidth and a scanning speed of 20 nm/min. **B**: Difference spectra of individual heme centers calculated from spectra in panel

A. High potential heme, trace 6 minus trace 2; low potential heme, trace 12 minus trace 9. Note that the spectra in 3B represent the shape of each component, not their relative amplitudes.

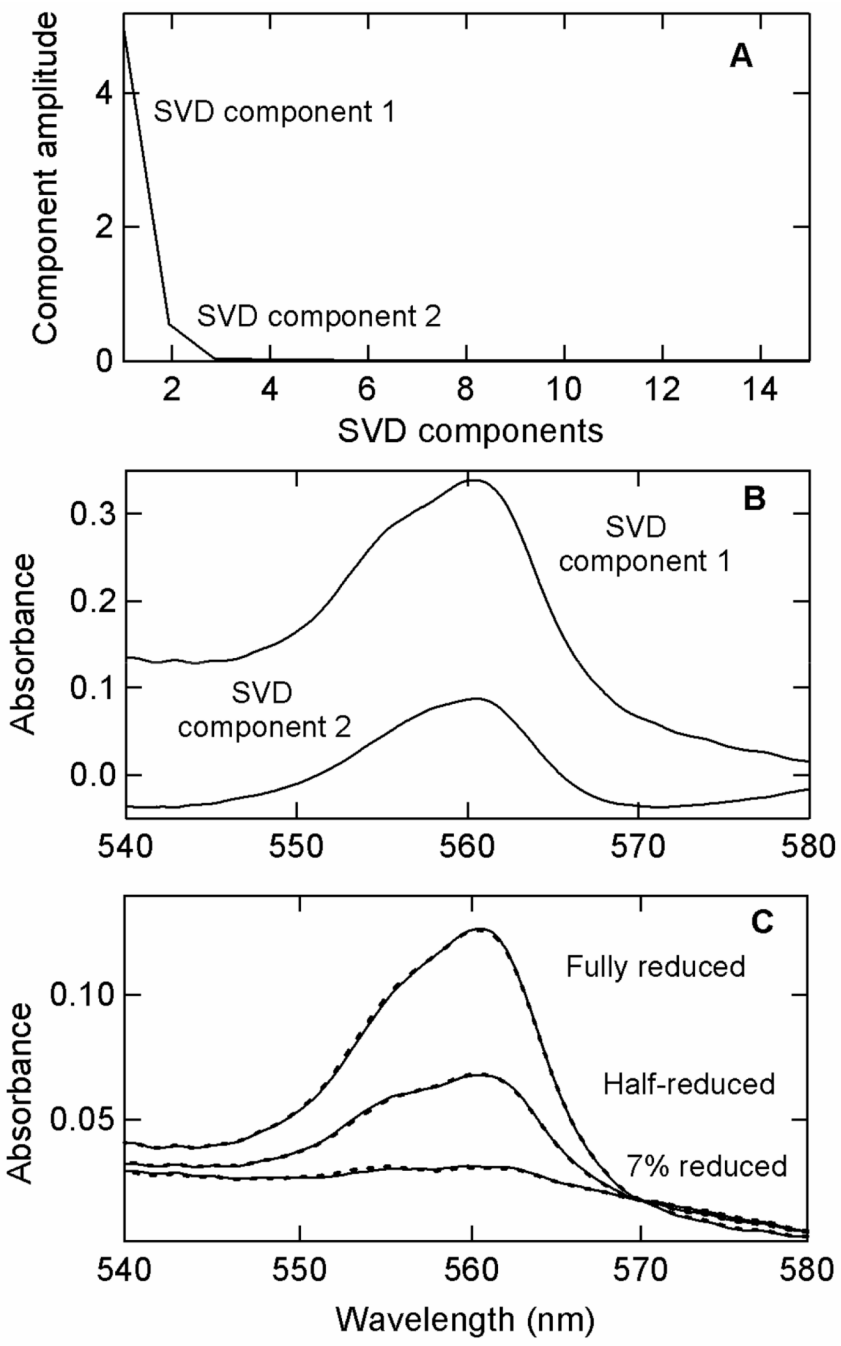

Figure 4.

SVD analysis of absorption spectra of wild type cyt b_{561} during reductive titration with ascorbate. **A**: Relative amplitudes of the principal components derived from SVD analysis of the spectra shown in Fig. 3A. **B**: Spectra of the two major components in panel A. **C**: Reconstruction of the 7 % reduced, half-reduced and fully reduced spectra from Fig. 3A (traces 1, 7 and 15, respectively). The solid lines represent the original experimental data and the dashed lines represent the reconstructed spectra using only principal components 1 and 2 from panel B.

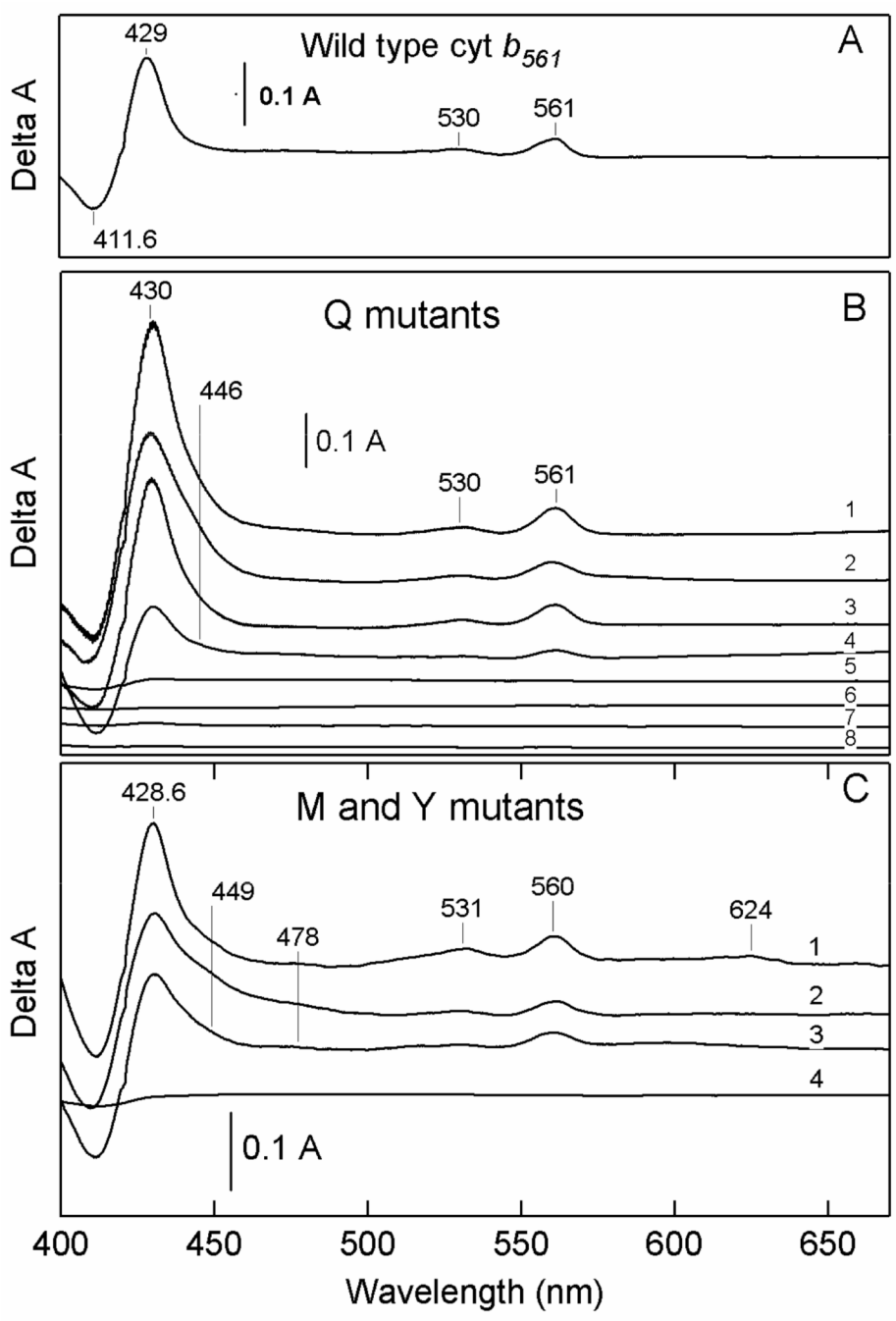

Figure 5.

Reduced minus oxidized difference absorption spectra of wild type and mutant cyt b_{561} in *Sf*9 cell membranes. **A**: Wild type cyt b_{561}. **B**: 1, H161Q; 2, H122Q; 3, H88Q; 4, H54Q; 5, membranes from cells expressing XylE and supplemented with ALA and hemin; 6, membranes from unsupplemented cells expressing XylE; 7, membranes from uninfected cells supplemented with ALA and hemin; and 8, membranes from unsupplemented, uninfected cells. **C**: 1, H161Y; 2, H161M; 3, H54Y; and 4, membranes from cells expressing XylE and supplemented with ALA and hemin. All membrane samples were suspended in 60 mM Tris, pH 7.4, containing 20% glycerol and were reduced with a few grains of dithionite. The absolute absorption spectra were collected at 0.2 nm intervals with a 0.1 nm bandwidth and a scanning

speed of 200 nm/min; difference spectra were produced by subtraction of oxidized from reduced spectra and their amplitudes normalized for total membrane protein and for the relative level of wild type cytochrome expression in the corresponding batch of *Sf9* cells.

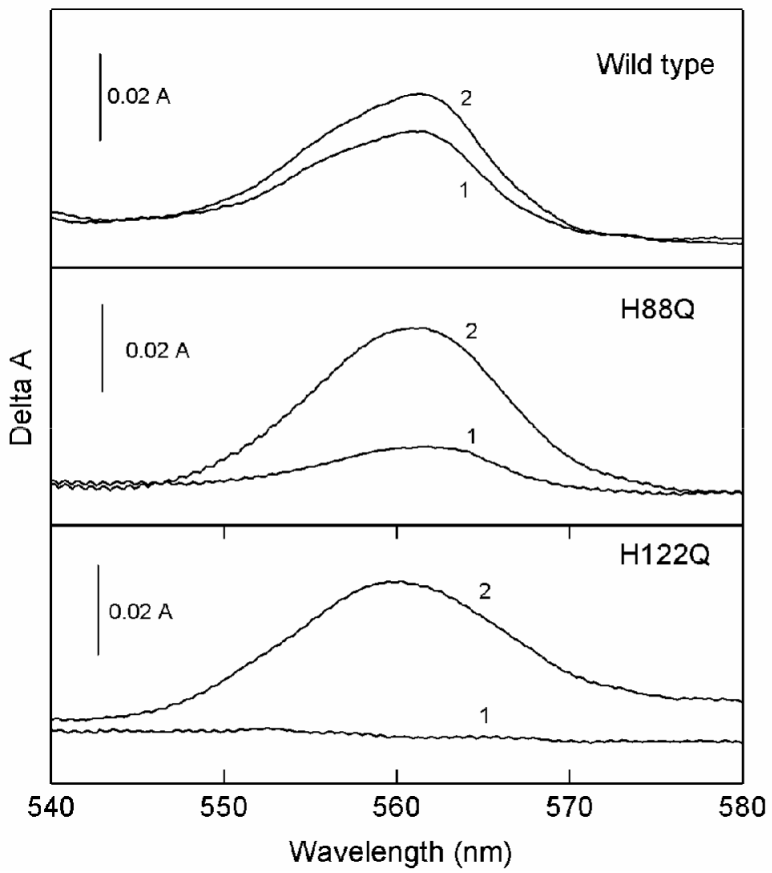

Figure 6.

Difference optical spectra of *Sf9* cell membranes containing recombinant wild type cyt b_{561} and two PAL mutants after reaction with ascorbate (1) and dithionite (2). Membrane suspensions in 60 mM Tris, pH 7.4, containing 20% glycerol were reacted first with 40 mM ascorbate and subsequently with a few grains of dithionite. Absolute absorption spectra were collected for oxidized and reductant-treated samples at 0.1 nm intervals with a 0.05 nm bandwidth and a scanning speed of 40 nm/min. Reduced minus oxidized difference absorption spectra were calculated and normalized to a protein concentration of 1 mg/ml.

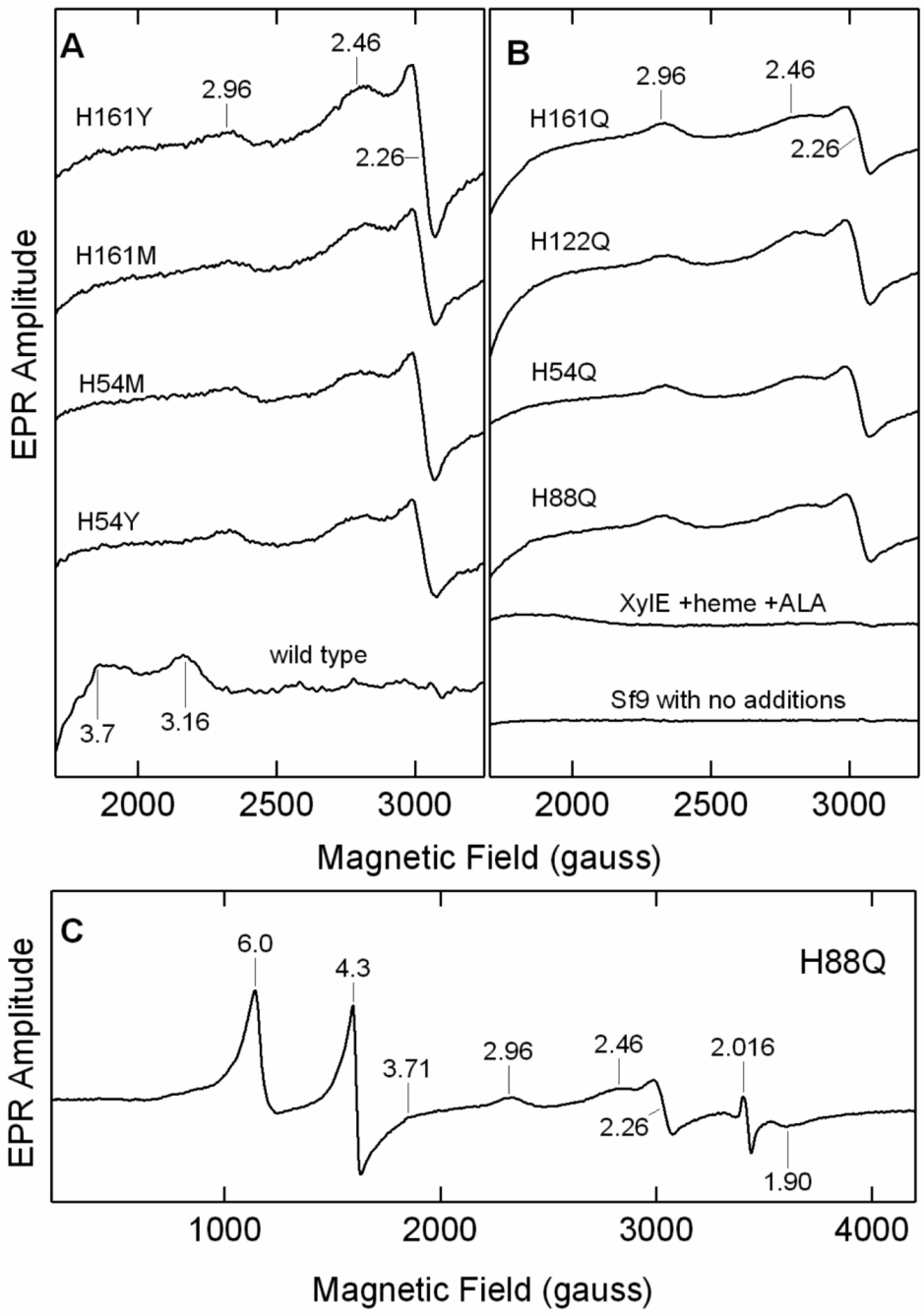

Figure 7.

EPR spectra of PAL mutants of cyt b_{561} in *Sf9* cell membranes. **A** and **B**: Low-spin region of the EPR spectra of *Sf9* cell membranes containing recombinant wild type cyt b_{561} and mutants with PAL histidines replaced by methionine or tyrosine (A) or glutamine (B). The spectra of membranes from *Sf9* cells expressing XylE and supplemented with ALA and hemin, and membranes from unsupplemented, uninfected *Sf9* cells are presented in panel B as controls. **C**: Wide-scan EPR spectrum of *Sf9* cell membranes containing the H88Q mutant. Membranes were suspended in 20 mM Tris, pH 7.2, and containing 20% glycerol. Signal intensities are normalized for total protein concentration and for the relative level of wild type cytochrome expression in the corresponding batch of *Sf9* cells. Spectrometer conditions: microwave

frequency, 9.60 GHz; microwave power, 1 mW; modulation frequency, 100 KHz; modulation amplitude, 10 G; and temperature, 8 K.

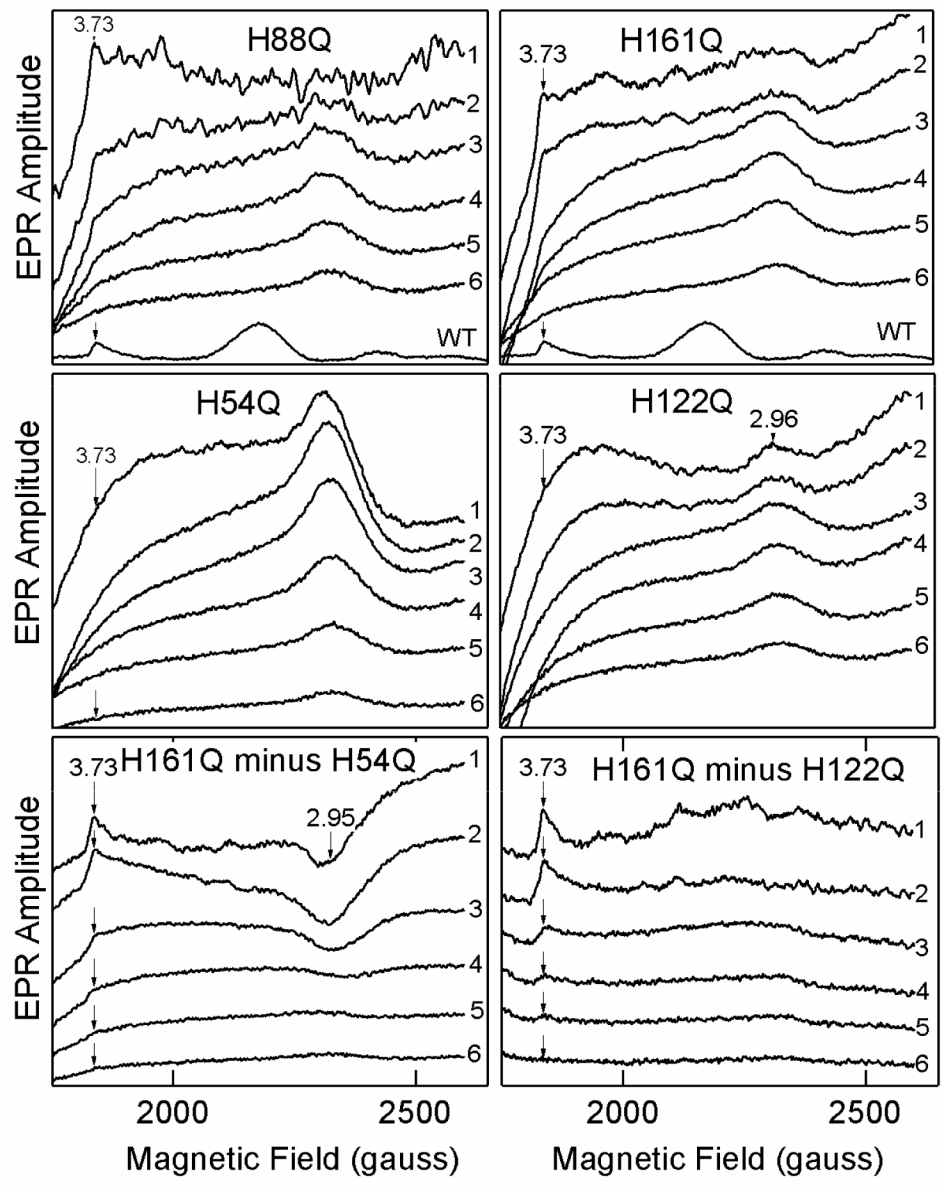

Figure 8.

Effects of microwave power on EPR spectra of *Sf9* cell membranes containing glutamine-substituted PAL mutants of cyt b_{561}. The spectra were recorded at 200 (1), 50 (2), 12.8 (3) 3.2 (4), 0.8 (5) or 0.2 mW (6). Signal intensities were normalized for total protein concentration in the individual samples. Spectrometer conditions were: microwave frequency, 9.60 GHz; modulation frequency, 100 KHz; modulation amplitude, 10 G; and the temperature, 8K. The two lowest panels represent difference EPR spectra derived from the upper panels as indicated. The spectrum of purified wild type (WT) cytochrome expressed in *Sf9* cells (collected at 4 mW) is shown in the two upper panels for comparison.

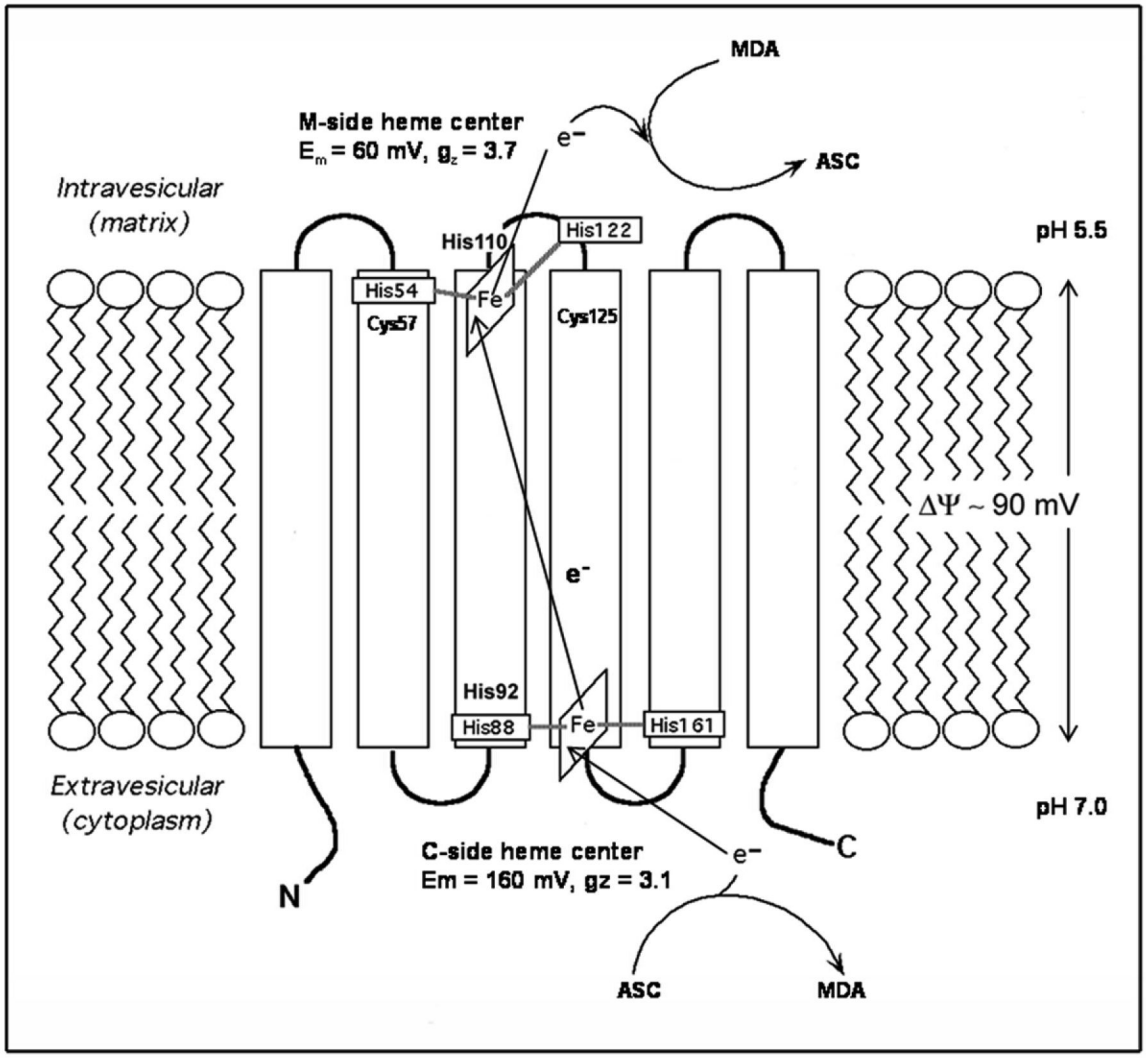

Figure 9.

Topological model of adrenal cyt b_{561} in the CG membrane. The axial ligation of heme centers is based on proposals by Degli Esposti *et al.* (35) and Okuyama *et al.* (49) and on the results of the present study. The disposition of the low-potential heme on the M-side and the high-potential heme on the C-side of the membrane is justified in the text. According to the current paradigm, cytoplasmic ascorbate is oxidized to MDA by cyt b_{561}, which shuttles electrons across the membrane and reduces MDA in the matrix. The V_1V_0 type H^+-ATPase (not shown) maintains a transmembrane H^+-electrochemical gradient. Its membrane potential component, ΔΨ may be important for driving electrons against the gradient of the redox potentials of two hemes of the cytochrome, as discussed in the text. Histidines 122, 54, 161, 88, marked with boxes, are putative axial ligands to the hemes. ASC, ascorbate; MDA, monodehydroascorbate; ΔΨ, membrane potential.