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Functional and Structural Roles of Residues in the Third Extramembrane Segment of Adrenal Cytochrome b_{561}

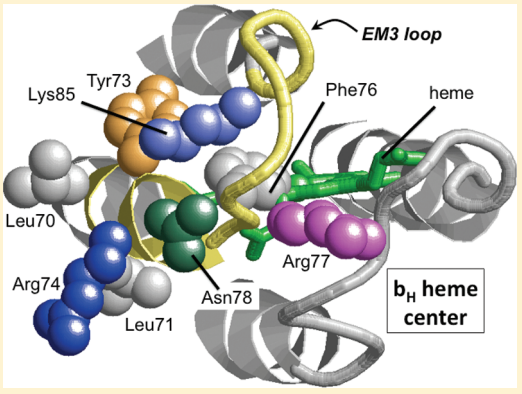
Wen Liu,[†] Giordano F. Z. da Silva,[†,‡] Gang Wu,[†] Graham Palmer,[‡] Ah-Lim Tsai,[†] and Richard J. Kulmacz*[,†]

[†]Department of Internal Medicine, University of Texas Health Science Center, Houston, Texas 77030, United States

[‡]Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251, United States

Supporting Information

ABSTRACT: Several residues in the third extramembrane segment (EM3) of adrenal cytochrome b_{561} have been proposed to be involved in this cytochrome's interaction with ascorbate, but there has been no systematic evaluation of residues in the segment. We used alanine scanning mutagenesis to assess the functional and structural roles of the EM3 residues and several adjacent residues (residues 70−85) in the bovine cytochrome. Each alanine mutant was expressed in a bacterial system, solubilized with detergent, and affinity-purified. The recombinant proteins contained approximately two hemes per monomer and, except for R74A, retained basic functionality (≥94% reduced by 20 mM ascorbate). Equilibrium spectrophotometric titrations with ascorbate were used to analyze the α-band line shape and amplitude during reduction of the high- and low-potential heme centers (b_H and b_L, respectively) and the midpoint ascorbate concentrations for the b_H and b_L transitions (C_H and C_L, respectively). Y73A and K85A markedly narrowed the b_H α-band peak; other mutants had weaker effects or no effect on b_H or b_L spectra. Relative changes in C_H for the mutants were larger than changes in C_L, with 1.5−2.9-fold increases in C_H for L70A, L71A, Y73A, R74A, N78A, and K85A. The amounts of functional b_H and b_L centers in additional Arg74 mutants, assessed by ascorbate titration and EPR spectroscopy, declined in concert in the following order: wild type > R74K > R74Q > R74T and R74Y > R74E. The results of this first comprehensive experimental test of the proposed roles of EM3 residues have identified residues with a direct or indirect impact on ascorbate interactions, on the environment of the b_H heme center, and on formation of the native b_H−b_L unit. Surprisingly, no individual EM3 residue was by itself indispensable for the interaction with ascorbate, and the role of the segment appears to be more subtle than previously thought. These results also support our topological model of the adrenal cytochrome, which positions b_H near the cytoplasmic side of the membrane.



Adrenal cytochrome b_{561} (cyt b_{561}) is an integral membrane protein that shuttles reducing equivalents from cytoplasmic ascorbate across the membrane and into the matrix of chromaffin granules to support catecholamine biosynthesis.[1] Cyt b_{561} contains two *b*-type heme centers, with redox potentials of ∼170 mV (high-potential, b_H) and ∼60 mV (low-potential, b_L).[2,3] Cyt b_{561} is the prototype of a family of diheme cytochromes that are widely distributed among animal and plant species.[4] As it is by far the best-studied member of the family, cyt b_{561} has many advantages as an experimental model for structural and mechanistic studies. The currently accepted membrane topology for the cyt b_{561} polypeptide, based originally on sequence analysis,[5] has six α-helical transmembrane segments, four extramembrane segments exposed to the cytoplasm (EM1, EM3, EM5, and EM7), and three extramembrane segments exposed to the lumenal compartment (EM2, EM4, and EM6) (Figure 1). The positions of the b_H and b_L centers in the chromaffin granule membrane have been controversial, with one model assigning b_H to the His88/His161 heme (as depicted in Figure 1)[6,7] and an earlier model assigning b_H to the His54/His122 heme.[8,9] A key issue from a mechanistic perspective is whether it is b_H or b_L that is exposed to the cytoplasm and its pool of ascorbate.

Previous efforts to map the residues important to interaction with ascorbate in the cytoplasm have focused almost entirely on residues in EM3. Several residues in and adjacent to EM3 in bovine cyt b_{561} are strongly conserved among close homologues from many organisms.[4] Residues 69−77 (ALLVYRVFR), which comprise the N-terminal part of EM3 and flanking residues in TM2, were proposed to be part of an ascorbate interaction site on the cytosolic side of the membrane, based on both sequence conservation and anticipated electrostatic interactions between strongly anionic ascorbate and the positively charged residues in the segment.[10] The C-terminal part of EM3 has three additional positively charged residues at positions 81, 82, and 85 (Figure 1). Some of the conserved residues in EM3 have turned up in chemical modification studies, and others have been targeted by

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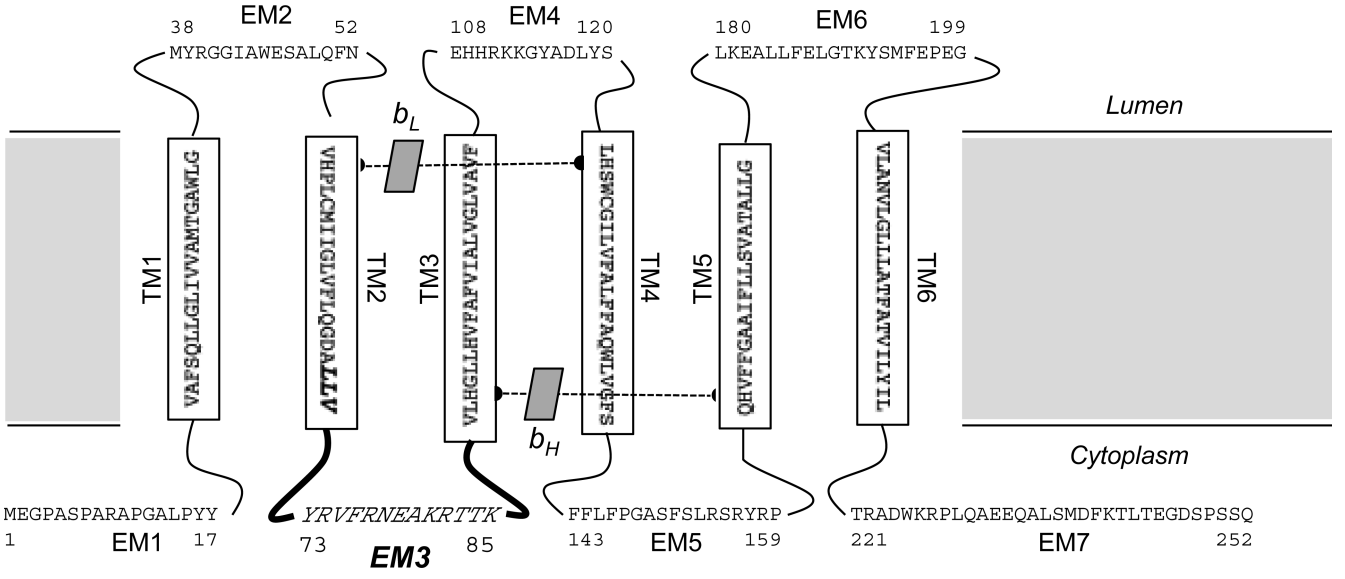

Figure 1. Model for the membrane topology of adrenal cyt b_{561} showing the predicted[39] transmembrane segments (TM1−TM6) and extramembrane segments (EM1−EM7). The orientation of the b_H and b_L heme centers is based on previous mutagenic studies.[6,7]

site-directed mutagenesis.[1,11−14] However, there has not been a systematic evaluation of the roles of individual EM3 residues in the reduction by ascorbate of the b_H and b_L heme centers. The two heme centers and the four helices carrying them (TM2−TM5 in Figure 1) have been proposed to be part of a larger structural unit, termed the kernel, whose integrity is required for proper membrane insertion and folding of the six-helix cytochrome.[6,7] The kernel in adrenal cyt b_{561} can be disrupted as a unit by even relatively conservative mutations in any one of the four histidines furnishing an axial ligand to one of the hemes.[6,7] EM3 terminates with Lys85, which is three residues from His88, the axial heme ligand in TM3 (Figure 1), raising the possibility that one or more residues in EM3 also are part of the structural kernel.

Alanine scanning mutagenesis is a well-established approach to systematic evaluation of the structural and functional roles of individual residues in a targeted polypeptide.[15] As the first comprehensive, experimental test of the proposed roles of conserved EM3 residues in reduction of the cytochrome by ascorbate and of the disposition of EM3 with respect to the two heme centers, we applied the alanine scanning approach to residues 70−85 of bovine adrenal cyt b_{561} (except Ala80). Given the proposed importance of EM3 residues to the interaction with ascorbate, we anticipated that one or more of these alanine mutants would lack the ability to react with ascorbate yet retain native structure at the heme centers. The recombinant proteins were expressed in an *Escherichia coli* system, solubilized with detergent, purified by affinity chromatography, and characterized in spectrophotometric ascorbate titrations to evaluate the effect(s) of each mutation on the structure and function of the individual b_H and b_L heme centers. Alanine substitution at Arg74 produced major structural disruptions, and a series of additional Arg74 mutations were characterized by ascorbate titration and EPR spectroscopy to evaluate the role of side chain charge and polarity at this position on formation of the native b_H−b_L unit.

EXPERIMENTAL PROCEDURES

Materials. Hemin, ascorbic acid, sodium ascorbate, δ-aminolevulinic acid, isopropyl 1-thio-β-D-galactopyranoside, ampicillin, chloramphenicol, and egg lysozyme were from Sigma (St. Louis, MO). *n*-Dodecyl β-D-maltoside was from Anatrace (Maumee, OH). Restriction enzymes and other DNA-modifying enzymes were purchased from New England Bio-Laboratories (Beverly, MA). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Plasmid vector pET43.1a, Benzonase nuclease, and Protease Inhibitor Cocktail Set III (without EDTA) were purchased from Novagen (Madison, WI). *E. coli* strain BL21Star(DE3) was from Invitrogen (Carlsbad, CA). The chaperone plasmid, pT-groE, was generously provided by Dr. L.-H. Wang (University of Texas Health Science Center). TALON metal affinity resin was purchased from BD Biosciences Clontech (Palo Alto, CA). DNA sequencing was performed at the Microbiology and Molecular Genetics Core Facility of the University of Texas Health Science Center.

Expression and Purification of His-Tagged Recombinant Cyt b_{561} Proteins. Construction of the pET43.1a plasmid-containing sequence encoding the full-length bovine adrenal cyt b_{561}, designated pET43.1a-b_{561}C6H, was described previously.[16] Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the pET43.1a-b_{561}C6H expression plasmid as the template. Each set of mutagenic primers (sense) (Table S1 of the Supporting Information) and its complementary (antisense) strand were used in a PfuUltra polymerase-initiated reaction. The original, methylated DNA strand was digested with DpnI, and the remaining DNA was transformed into *E. coli* XL1-Blue competent cells. Each mutant construct was validated by restriction digests and DNA sequencing. Wild-type cyt b_{561} and EM3 mutants were expressed in the BL21Star(DE3)/pT-groE *E. coli* strain and purified according to published procedures.[16]

Assay of Protein, Cyt b_{561}, and Heme Content. Total protein was assayed with a modified Lowry method using bovine serum albumin as the standard.[17] Recombinant cyt b_{561} content was calculated using a reduced (dithionite-treated) minus oxidized (ferricyanide-treated) difference extinction coefficient (561−575 nm) of 34 mM^{-1} cm^{-1}.[18,19] Heme content was determined by the pyridine hemochrome method[19] using an extinction coefficient difference (556−538 nm) of 24.5 mM^{-1} cm^{-1}.[20] The homogeneity of

purified proteins was determined by densitometry (ImageJ from the National Institutes of Health, Bethesda, MD) of Coomassie blue-stained bands after separation of proteins (~1 μg/lane) by polyacrylamide gel electrophoresis under denaturing conditions.[21] The heme stoichiometry of each purified recombinant protein was calculated from its heme concentration (determined by a pyridine hemochrome assay) and its monomer concentration (determined by a total protein assay and electrophoretic analysis).

Ascorbate Titrations of Wild-Type and Mutant Cyt b_{561}. Ascorbate titrations were performed as previously described with minor changes.[7] Preoxidized cyt b_{561} samples (5−11 μM) in 100 mM potassium phosphate buffer (pH 7.2) containing 18% glycerol and 0.08% (w/v) *n*-dodecyl β-D-maltoside were placed in a 1 cm quartz cuvette. The titrations used 17−21 levels of ascorbate, typically between 0.13 μM and 20 mM, but extending to 90 mM for ascorbate-resistant samples. The system was allowed to restabilize completely (5 min) after each addition of ascorbate before the spectrum was recorded. Spectra were recorded in a Shimadzu model UV-2101PC or UV-2401PC spectrophotometer with a scan rate of 20 nm/min, with 20 data points/nm and a spectral bandwidth of 0.5 or 1 nm. All ascorbate stock solutions were kept at pH 7.2, freshly made from mixtures of ascorbic acid and sodium ascorbate, and were kept on ice and protected from light. The increases in A_{561} were corrected for dilution. In some cases, oxygen was purged with several cycles of vacuum and argon replacement and the titration was performed under anaerobic conditions; removing oxygen did not change the results significantly. For wild-type cyt b_{561} and the mutants found to be at least 94% reduced by 20 mM ascorbate, the increases in A_{561} during titration were fitted by nonlinear regression to a two-phase, four-parameter equation:

$$\Delta A_{561} = \Delta A(b_H)/(1 + C_H/[\text{Asc}]) + \Delta A(b_L)/(1 + C_L/[\text{Asc}]) \quad (1)$$

where [Asc] is the ascorbate concentration, $\Delta A(b_H)$ and $\Delta A(b_L)$ are overall absorbance changes for the high- and low-potential heme transitions, respectively, and C_H and C_L are midpoint ascorbate concentrations for the high- and low-potential heme center transitions, respectively.

Some mutants were found to require ascorbate levels of >20 mM, or even dithionite (~1 mM), for complete reduction, indicating that a portion of the recombinant cytochrome had one or more non-native forms of the heme center with an unusually low redox potential. For these mutants, the increases in A_{561} during the titration were fitted to a three-phase, six-parameter equation:

$$\Delta A_{561} = \Delta A(b_H)/(1 + C_H/[\text{Asc}]) + \Delta A(b_L)/(1 + C_L/[\text{Asc}]) + \Delta A(b_{VL})/(1 + C_{VL}/[\text{Asc}]) \quad (2)$$

The additional parameters, $\Delta A(b_{VL})$ and C_{VL}, are the overall absorbance change and midpoint ascorbate concentration, respectively, for the very low-potential heme center. Note that any heme that is unreactive with ascorbate and reduced only by dithionite is not included in this fitting.

EPR Spectroscopy. Samples of wild-type and mutant cytochromes were concentrated and oxidized before EPR spectra were recorded at 4 or 8 K as described previously.[7] The spectrometer settings were as follows: microwave frequency, 9.58 GHz; modulation frequency, 100 kHz; modulation amplitude, 10.9 G; and power, 4 mW. EPR spectra of purified N78K and H110Q mutants[7] recorded at both 4 and 8 K were used to calculate the temperature dependence of individual g = 2.95, 3.1, and 3.7 signals.

RESULTS

Expression and Purification. The level of recombinant protein expressed in the bacterial system varied considerably among the EM3 alanine mutants (Table S2 of the Supporting Information). Most mutants were expressed at levels comparable to that of the wild-type protein. R82A and K85A were expressed at somewhat higher levels, and R74A was expressed at somewhat lower levels; E79A and K81A expression levels were poor. Nevertheless, the bulk of each of the recombinant proteins was solubilized with nonionic detergent, an indication of proper folding and membrane insertion,[7] and each could be purified in sufficient yield for characterization. The electrophoretic homogeneity of the purified proteins was ~85% except for those of the few poorly expressing mutants, for which the homogeneity was ~70%.

Heme Stoichiometry and Reactivity with Ascorbate. As expected from previous results,[7,16] the heme content of wild-type cyt b_{561} was almost exactly two per monomer (Table S2 of the Supporting Information), showing that most modifications of EM3 side chains did not prevent incorporation of a full heme complement into the protein. The basic functionality of each recombinant protein was assessed by comparison of the intensity of the α-band absorbance from cytochrome reduction upon addition of 20 mM ascorbate to that upon complete reduction by dithionite (Table S2 of the Supporting Information; full spectra of wild-type cytochrome and selected mutants are shown in Figure S5 of the Supporting Information). Wild-type cyt b_{561} was 97% reduced, and the EM3 alanine mutants were also quantitatively reduced (i.e., 94−98%), except for R74A, which only was 45% reduced. These results indicate that only the R74A mutation resulted in formation of a considerable fraction of cytochrome that did not react with ascorbate. Ascorbate reducibility values of 75−85% were reported in other studies of native and recombinant cyt b_{561} (e.g., refs 7−9). These instances of lower reducibility probably reflect the use of lower concentrations of ascorbate, less purified cytochrome preparations, or differences in buffer composition. Additional Arg74 mutations are described below.

Reaction of High- and Low-Potential Heme Centers with Ascorbate. Analyses of α-band absorbance changes during titration of cyt b_{561} family members with ascorbate have proven to be quite informative for assessing the impact of mutations on the individual heme centers.[6,7,22] Accordingly, ascorbate titrations were performed on wild-type cyt b_{561} and each of the purified EM3 alanine mutants. Examples of the resulting sets of absorbance spectra are shown for the wild-type cytochrome and the Y73A and R74A mutants in Figure 2 (spectral data for the other mutants are included in Figure S1 of the Supporting Information). In each case, the α-band absorbance increased with increases in the amount of ascorbate added. For the wild-type cytochrome and most of the EM3 alanine mutants, a shoulder near 554 nm was apparent in addition to the main peak near 561 nm (Figure 2), as observed previously.[7] The shoulder near 554 nm was not apparent in the Y73A titration (Figure 2), and the overall absorbance increase during the titration was markedly smaller for the R74A mutant (Figure 2), as expected from the lower reactivity with ascorbate in this

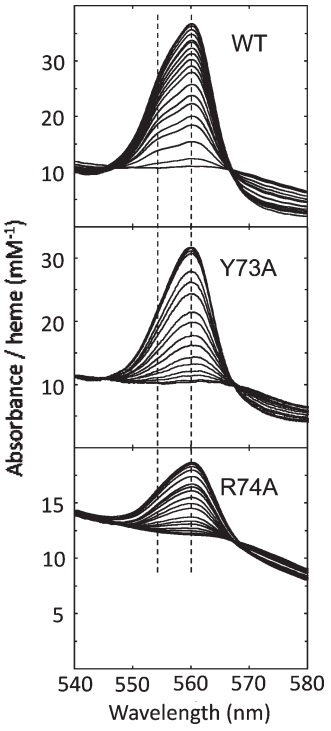

Figure 2. Increases in α-band absorbance during ascorbate titrations of the wild type (0−30 mM Asc) (top), Y73A (0−20 mM Asc) (middle), and R74A (0−90 mM Asc) (bottom).

mutant (Table S2 of the Supporting Information). The progression of each titration was monitored by plotting the ΔA at 560−561 nm as a function of the added ascorbate concentration. Examples are shown in the top panel of Figure 3 for the wild type and the Y73A and R74A mutants. The data for the wild-type cytochrome and all of the alanine mutants except R74A fit well to eq 1, which describes two independent and saturable phases of absorbance transition. The first transition, that of a high-potential heme center (b_H), had a midpoint ascorbate concentration (C_H) in the range of 2−15 μM (Figure 3, top and bottom). The second transition, that of a low-potential heme center (b_L), had a midpoint ascorbate concentration (C_L) between 170 and 810 μM. The proportions of ΔA_{561} in the first and second transitions varied considerably, as exemplified by the results for the wild type (0.60:0.40) and Y73A (0.40:0.60) (Figure 3, top panel). The titration data for R74A were found to fit rather poorly to eq 1 for the two-transition model (Figure S2 of the Supporting Information). This appeared to be due to the considerable portion of recombinant hemoprotein that was reduced only at very high ascorbate levels. Accordingly, the equation for a three-transition model (eq 2) was tried instead. Equation 2 was found to fit the R74A data quite well (Figure 3, top panel), and the fitted amplitude parameters indicated that ∼30% of the absorbance change was associated with b_H ($C_H \sim 20$ μM), ∼45% with b_L ($C_L \sim 300$ μM), and ∼25% with very low-potential heme or "b_{VL}" ($C_{VL} \sim 8$ mM). The purified R74A protein thus contained smaller but distinct portions of cytochrome with midpoint ascorbate concentrations comparable to those of the native b_H and b_L heme centers, in addition to a non-native population that was reduced only at high ascorbate levels (b_{VL}) and yet another population that was refractory to ascorbate and required dithionite for reduction [termed b_X (not shown in Figure 3)].

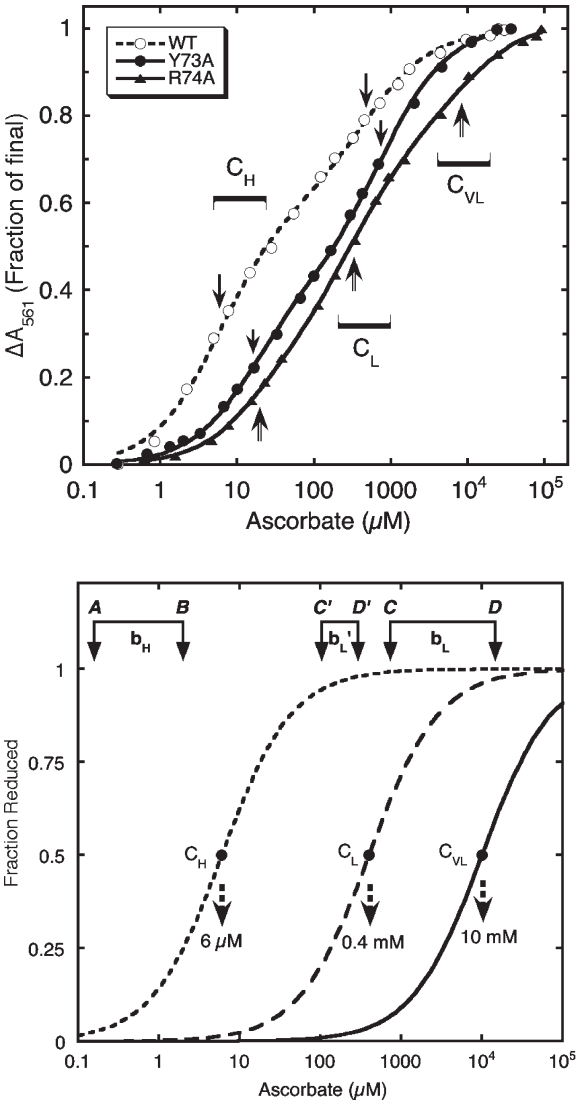

Figure 3. Ascorbate titration curves. The top panel shows titrations of wild-type (WT) cyt b_{561} and the Y73A and R74A mutants. ΔA values are normalized to the ΔA at the highest ascorbate concentration. The bottom panel shows the predicted reductive transitions (eq 2) for heme centers with midpoint ascorbate concentrations typical for b_H, b_L, and b_{VL}. Brackets indicate the titration stages at which difference spectra were obtained for b_H (spectrum B − spectrum A), b_L (spectrum D − spectrum C), and b_L in the presence of b_{VL} ($b_{L'}$, spectrum D′ − spectrum C′).

It is important to note that no more than two hemes were present per monomer in any of the purified recombinant proteins (Table S2 of the Supporting Information). Thus, b_{VL} and b_X do not represent a third or fourth heme center but rather altered forms of b_H and/or b_L produced by structural or folding perturbations in the recombinant proteins. There is at least one well-characterized precedent for expression of multiple forms of a recombinant cytochrome other than cyt b_{561} in heterologous systems.[23]

Absorbance Spectra of the b_H and b_L Heme Centers. Before the quantitative differences in the C_H and C_L values observed in the ascorbate titrations of the mutants were analyzed (such as those shown in the top panel of Figure 3), it was important to determine whether individual mutations affected

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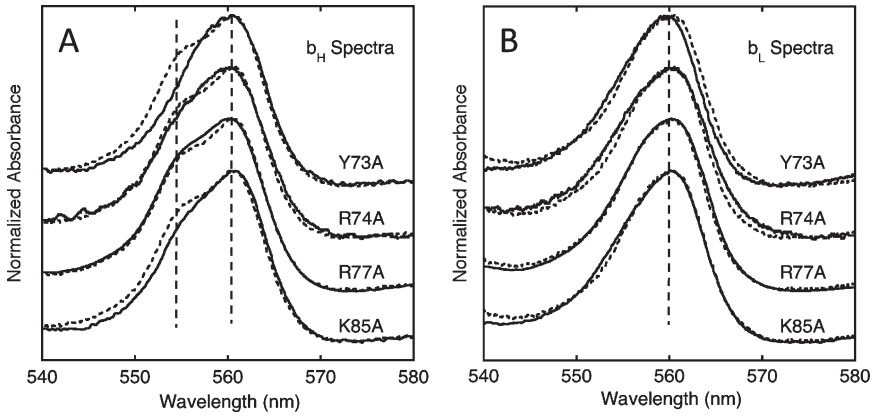

Figure 4. Difference spectra for b_H (A) and b_L (B) in selected EM3 alanine mutants determined from ascorbate titration data as indicated in Figure 3. The spectra shown are averages of difference spectra from two separate titrations, with each mutant spectrum (—) superimposed on the wild-type spectrum (---).

the structure of either heme center. Because of the ~110 mV separation of midpoint potential between the two heme centers, α-band difference spectra from early (ascorbate < C_H) and late (ascorbate > C_L) phases in an ascorbate titration provide a way of separately analyzing the spectra of the b_H and b_L centers, respectively.[7] The strategy is outlined in the bottom panel of Figure 3, with the difference spectra for b_H obtained by subtraction of the spectrum at point A from that at point B reflecting contributions almost exclusively from b_H and the spectrum at point C from that at point D reflecting contributions almost exclusively from b_L. The resulting difference spectra for the b_H and b_L centers of the Y73A, R74A, R77A, and K85A mutants are shown in Figure 4, superimposed on the corresponding wild-type spectra. It is important to note that these difference spectra arise specifically from recombinant protein containing b_H and b_L, thus providing a way to selectively characterize these species even for a mutant such as R74A for which most of the recombinant protein produced has non-native cytochrome that is resistant to, or unreactive with, ascorbate.

The b_H spectrum of the Y73A mutant lacked the shoulder at ~554 nm seen with the wild-type cytochrome, making the mutant b_H spectrum narrower and more symmetric. The b_H spectrum of the K85A mutant was also more narrow, though to a slightly lesser degree than that of Y73A (Figure 4A). In contrast, the b_H spectra of R74A and R77A had essentially the same peak width as the wild type, though the shoulder on the shorter wavelength side was less pronounced in R74A. None of the other alanine mutants in EM3 produced significant changes in the b_H spectrum (Figure S3 of the Supporting Information). The b_L spectra for the four mutants shown in Figure 4B had only subtle differences from the corresponding wild-type spectrum. The b_L peak was shifted to a slightly shorter wavelength in Y73A and was slightly broadened in R74A, but the b_L spectra for R77A and K85A were almost superimposable on the wild-type spectrum. The b_L spectra for the other EM3 alanine mutants were also little different from the wild-type spectrum, except for L70A and T83A, which were slightly blue-shifted (Figure S3 of the Supporting Information). The b_H α-bands for wild-type cytochrome and the Y73A and K85A mutants were also analyzed by deconvolution into Gaussian components (Figure S6 of the Supporting Information). The Y73A mutant exhibited a large decrease in amplitude of the component near 555 nm compared to the wild type; the amplitude of the component at 555 nm was also slightly decreased in the K85A mutant.

Effects of EM3 Alanine Mutations on Ascorbate Titration Parameters. Between two and six independent ascorbate titrations were performed for each of the EM3 alanine mutants. Data from each titration were fitted to eq 1 (eq 2 for R74A) to quantify the amplitude and midpoint ascorbate concentration for the b_H, b_L, and b_{VL} (if present) transitions, as described above. To simplify evaluation of mutational effects, we combined the two amplitudes as a ratio, $\Delta A_H/\Delta A_L$. One notable overall observation from the results, shown in Figure 5, is that each of the EM3 mutants except E79A and T84A had a statistically significant ($p < 0.05$) effect on at least one of the three ascorbate titration parameters examined. This suggests that the functional interaction of cyt b_{561} with ascorbate directly or indirectly involves many EM3 residues, not just one or two. None of the mutations of EM3 residues completely blocked reduction of either heme center, indicating that none of these residues by itself is essential to redox interaction with ascorbate. A further overall observation is that the effects on the value of C_H tended to be considerably larger than those on the value of C_L. The simplest interpretation of this outcome is that EM3 is positioned closer to b_H than to b_L. As EM3 is currently accepted as being exposed to the cytoplasm,[24] it follows that the b_H heme center is oriented toward the cytoplasmic face of the chromaffin granule membrane. This gives further support to the assignment of b_H to the heme ligated by the His88/His161 ligand pair,[6,7] as depicted in Figure 1.

To focus on EM3 residues whose side chain structures have a larger functional impact, thresholds are drawn in Figure 5 to indicate the parameter increases by a factor of 1.5 and decreases by a factor of 1/1.5, each relative to the wild-type value. Alterations in C_L exceeded the threshold factor of 1.5 in only five mutants, with an increased C_L in Y73A and decreased C_L values in V72A, F76A, R77A, R82A, and T83A (Figure 5). All of these residues are a considerable distance (~2 nm) from the b_L heme in the model, suggesting that the effects of these mutations on C_L are indirect and reflect longer-range structural perturbations.

Only Y73A, R74A, and K85A exhibited alterations in the $\Delta A_H/\Delta A_L$ ratio beyond the threshold; all exhibited a decreased ratio (Figure 5). At present, there are no crystallographic data for cyt b_{561} or for a related protein suitable for homology modeling. However, Bashtovyy et al.[25] have developed a computational model of cyt b_{561} that is useful for tentative interpretation of

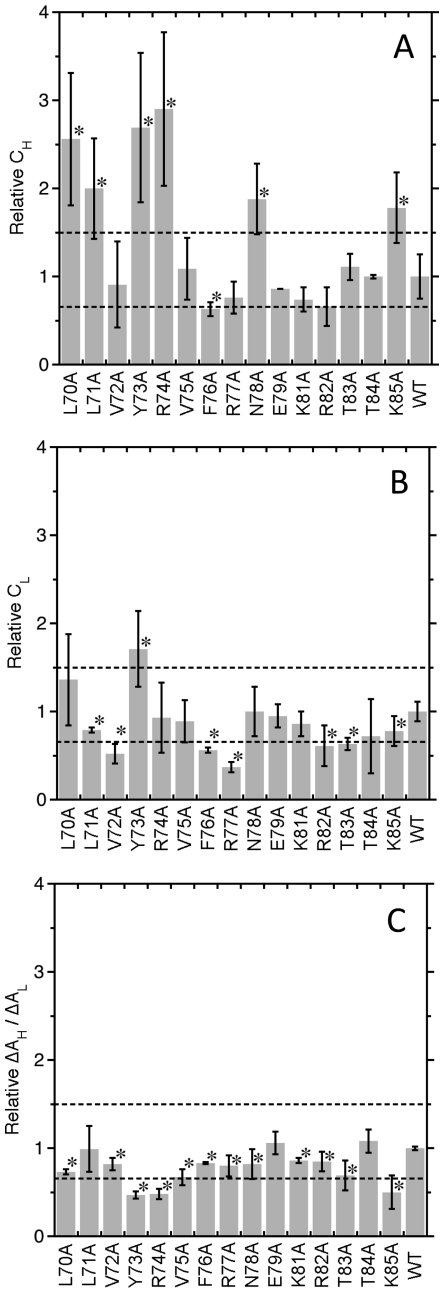

Figure 5. Ascorbate titration parameters for EM3 alanine mutants, Average values for C_H (A), C_L (B), and $\Delta A_H/\Delta A_L$ (C) were normalized to the corresponding wild-type (WT) values (5.3 μM for C_H, 394 μM for C_L, and 1.50 for $\Delta A_H/\Delta A_L$). Error bars indicate the standard deviation for two to six titrations (n = 7 for WT). Asterisks indicate a statistically significant difference from the WT value ($p < 0.05$). Horizontal dashed lines represent an increase or attenuation by a factor of 1.5.

these results. This model includes transmembrane helices 2−5, which carry the four histidines that have been shown to provide axial ligands for the two hemes. The main constraints in the modeling were the His−heme−His cross-linking between helices 2 and 4 and helices 3 and 5, and the disposition of hydrophobic side chains in the transmembrane segments toward the outside of the four-helix bundle.[25] The predicted proximity of Tyr73 and Lys85 to one heme in this computational model (Figure 6) would be consistent with the observed change in the absorbance ratio for the Y73A and K85A mutants. On the other hand, the Arg74 side chain is predicted to be oriented away from the heme (Figure 6A). If this aspect of the model is correct, it appears to be inconsistent with invoking a direct effect to explain the altered absorbance ratio in R74A.

The alanine mutants of several charged and polar residues in EM3 exhibited little or no difference from the wild-type cytochrome in the ascorbate titration parameters (Figure 5). The predicted dispositions of the side chains of these residues are depicted in Figure 6B. Four of these side chains (Lys81, Arg82, Thr83, and Thr84) are clustered near the C-terminal end of EM3 and radiate outward from the four-helix bundle. This suggests that this part of EM3 does not have important interactions with ascorbate or with other parts of the protein. The side chain of Glu79 is predicted to be some distance from the b_H heme, projecting away (Figure 6B); this orientation seems quite consistent with the lack of effects seen with E79A (Figure 5).

Mutational Analysis of Arg74. As noted above, the R74A mutation was the only EM3 alanine mutation that markedly impaired reduction of cyt b_{561} by ascorbate (Table S2 of the Supporting Information). The role of Arg74 was further evaluated with additional mutations to vary the side chain charge and polarity at this position (R74K, R74Q, R74T, R74Y, and R74E). Each of these recombinant proteins (His-tagged at the C-terminus) was expressed in the bacterial system, solubilized with detergent, and purified by affinity chromatography, as described for the alanine mutants.

The basic characteristics of the Arg74 mutants are presented in Table S3 of the Supporting Information. Each was expressed in at least moderate yield, except for R74E. The heme stoichiometry ranged from 1.9 to 1.7, indicating that the disruptive effects on heme incorporation of the Arg74 mutants varied from very mild (e.g., R74K) to quite significant (e.g., R74E). The general functionality, assessed as the fraction of cytochrome that can be reduced by 20 mM ascorbate, was 91% for R74K, only slightly lower than the wild-type value. The ascorbate reducibility decreased to 81% in R74Q and R74T and was precipitously lower in the R74A (45%), R74Y (35%), and R74E (9%) mutants. The proportion of functional cytochrome in the purified recombinant protein thus decreased progressively as the side chain at position 74 went from positively charged to neutral polar to aromatic to negatively charged.

Ascorbate titrations were used for detailed characterization of each of the Arg74 mutants except for R74E, whose very low reducibility by ascorbate made titration impractical. Two reductive transitions were observed for R74K (b_H and b_L, as with wild-type cytochrome in Figure 3), whereas all of the other Arg74 mutants exhibited three reductive transitions (b_H, b_L, and b_{VL}, as for R74A in Figure 3). The b_H and b_L spectra obtained from titrations of the Arg74 mutants, using the difference approach outlined in the bottom panel of Figure 3, are shown in Figure S4 of the Supporting Information. The b_H spectra of R74K, R74Q, and R74T were little different from that of the wild type, but the shoulder was clearly shifted from 554 nm to longer wavelengths in the R74A and R74Y mutants. The b_L spectrum of R74K was indistinguishable from that of the wild type, but the spectra of each of the other Arg74 mutants were noticeably wider than that of the wild type, with R74Y presenting the largest changes. Thus, modifications at Arg74 that removed the positive charge altered the b_L spectrum, and the b_H spectrum as well for the alanine and tyrosine substitutions. Note that the patterns of spectral perturbation in R74Q and R74T represent unusual instances in which

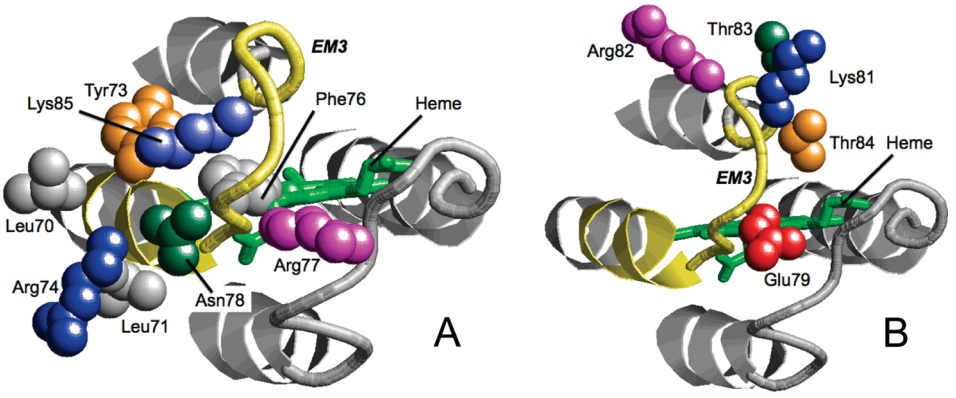

Figure 6. Computational structural model of the b_H heme region of cyt b_{561}[25] showing the positions of targeted residues in EM3 and flanking residues (backbone colored yellow). Panel A highlights residues at which mutation significantly altered one or more ascorbate titration parameters. Panel B highlights residues at which mutation had little or no impact.

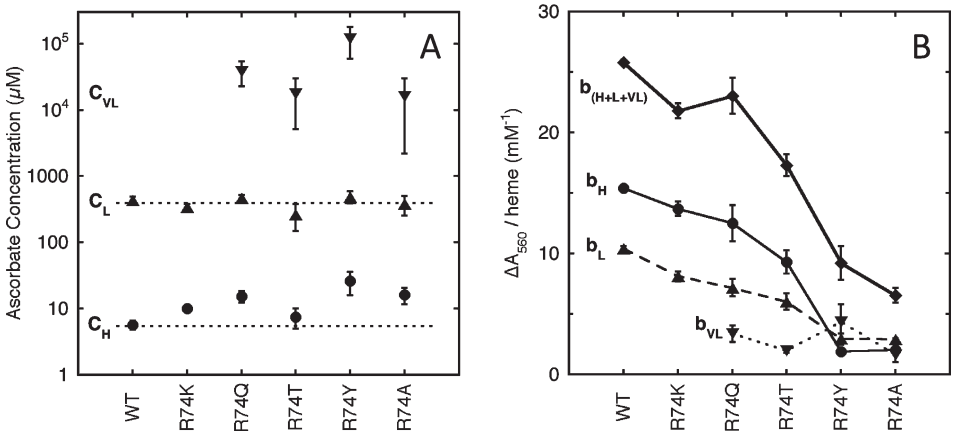

Figure 7. Ascorbate titration parameters for wild-type (WT) cyt b_{561} and the Arg74 mutants. (A) Midpoint ascorbate concentrations for the high-potential (C_H), low-potential (C_L), and very low-potential (C_{VL}) types of heme center transition. (B) Absorbance change amplitudes for the individual transitions and their sum. The third transition at high ascorbate levels was negligible for the wild type and the R74K mutant.

mutation of an EM3 residue affects a property of the b_L heme center more than that of the b_H heme center.

The values of C_H, C_L, and C_{VL} calculated from the ascorbate titrations of the Arg74 mutations are shown in Figure 7A. Each of the mutants except R74T had a C_H value significantly higher than that for wild-type cytochrome, and there was a trend toward increasing C_H values in the following order: WT < R74T < R74K < R74Q < R74A < R74Y. In contrast, the values of C_L for the mutants were similar to the wild-type value (Figure 7A). R74Y had a higher C_{VL} value than the other three Arg74 mutants exhibiting a significant b_{VL} transition; however, the uncertainties in estimating C_{VL} were large, and there was no obvious trend in the data.

The ascorbate titrations of the Arg74 mutants were also analyzed to quantitate the amplitudes of ΔA_{560} associated with the b_H, b_L, and b_{VL} transitions (Figure 7B). The ΔA_{560} amplitudes were normalized to the heme concentration to estimate the contribution of individual ascorbate-reducible heme centers in each purified hemoprotein sample. In wild-type cyt b_{561}, the b_H and b_L transitions produced ΔA_{560}/heme values of ~16 and ~11 $(mM)^{-1}$, respectively, for an overall ascorbate-driven transition of ~27 $(mM\ heme)^{-1}$ (Figure 7B). Both b_H and b_L transitions had smaller amplitudes in the R74K mutants, at ~14 and ~8 $(mM\ heme)^{-1}$, respectively, for a total of ~22 $(mM\ heme)^{-1}$. The b_{VL} component was too small to be analyzed in the wild type or R74K. The R74Q, R74T, and R74Y mutants had increasingly smaller b_H and b_L transition amplitudes, decreasing from ~13 and ~8 to ~2 and ~3 $(mM\ heme)^{-1}$, respectively, with no further decrease observed in R74A (Figure 7B). The amplitude of the b_{VL} transition, observed in R74Q, R74T, R74Y, and R74A, did not increase as the b_H and b_L transitions became smaller. As a result, the total amplitude of ascorbate-driven transitions ($b_H + b_L + b_{VL}$) decreased in the R74K → R74A series of mutants.

The heme centers in the purified Arg74 mutants were also characterized by EPR spectroscopy. The low-spin heme region of the EPR is shown in Figure 8; the variation in the signal-to-noise ratio among samples reflects the differences in heme concentration due to different expression efficiencies (Table S3 of the Supporting Information). Each of the Arg74 mutants exhibited a distinct $g = 3.7$ signal, reflecting the presence of the b_L heme center. The proportion of intact b_L center, assessed by the normalized amplitude of the $g = 3.7$ signal, declined in the following order: WT > R74K > R74Q > R74T ~ R74Y ~ R74A (Figure S7 of the Supporting Information). A $g = 3.13$ EPR signal, reflecting the b_H heme center, was discernible in R74K and R74T, and perhaps in R74Q and R74Y; this signal is clearly

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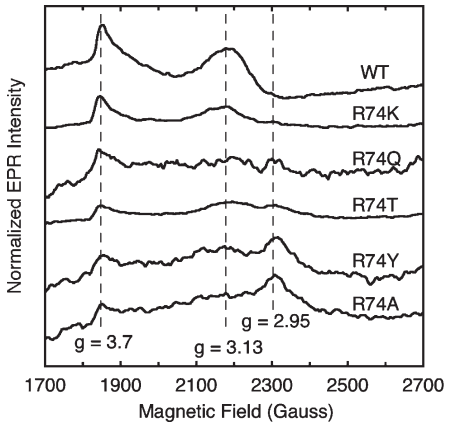

Figure 8. Low-spin heme region of the EPR spectra of wild-type (WT) cyt b_{561} and the Arg74 mutants in the ferric state. Amplitudes are normalized to total heme concentration: WT, 143 μM; R74K, 150 μM; R74Q, 32 μM; R74T, 102 μM; R74Y, 45 μM; and R74A, 50 μM. The temperature was 4 K except for WT (8 K).

Table 1. Comparison of Ascorbate Titration Parameters for Wild-Type Cyt b_{561} and Dcytb

protein	C_H (μM)	C_L (μM)	ref
bovine cyt b_{561} (endogenous) purified	11 ± 1	360 ± 50	11
bovine cyt b_{561} (recombinant) purified	5.4 ± 1.5	369 ± 119	7
bovine cyt b_{561} (recombinant) purified	5.3 ± 2.0	394 ± 79	this study
murine cyt b_{561} (yeast) membranous	16 ± 5	1240 ± 190	11
murine cyt b_{561} (yeast) purified	21 ± 6	1500 ± 500	29
murine Dcytb (yeast) membranous	70 ± 20	1820 ± 140	26
human Dcytb (endogenous) membranous	10 ± 0	770 ± 230	26
Arabidopsis cyt b_{561} (yeast) membranous	<100 (?)	~1500	27

absent in R74A (Figure 8). The $g \sim 3.13$ EPR signal amplitude declined in the following order: WT > R74K > R74Q ~ R74T > R74Y > R74A (Figure 8 and Figure S7 of the Supporting Information). A $g = 2.95$ EPR signal, ascribed to recombinant cyt b_{561} with a *b*-type heme center in a relaxed conformation,[6] was evident in each of the Arg74 mutants but not in the wild-type cytochrome (Figure 8). The intensity of the $g = 2.95$ signal increased in the following order: WT < R74K < R74Q ~ R74T < R74Y < R74A (Figure S7 of the Supporting Information). Finally, EPR signals from multiple species of high-spin ferric heme were present near $g = 6$ in all the samples; with the exception of R74T, the intensity of the $g = 6$ signals increased monotonically in the R74K → R74A series of mutants (data not shown).

Overall, the pattern of decreases in the native b_H and b_L EPR signals ($g = 3.1$ and $g = 3.7$, respectively) for the series of Arg74 mutants (Figure S7 of the Supporting Information) generally paralleled the pattern of decreasing amplitudes of the b_H and b_L transitions observed in the ascorbate titrations (Figure 7B). In addition, the trend in the $g = 2.95$ non-native EPR signal was opposite that of the native EPR signals (Figure 8 and Figure S7 of the Supporting Information). This consistent pattern of native versus non-native components in the titration and EPR analyses indicates that replacing the positively charged side chain at Arg74 with progressively less conservative substitutions decreased the fraction of recombinant hemoprotein carrying functional b_H and b_L centers.

DISCUSSION

Characterization of Ascorbate Interactions of Cyt b_{561} by Spectrophotometric Titration. Physiological functioning of cyt b_{561} requires its reduction by cytoplasmic ascorbate,[1] and the efficiency of reduction of the two heme centers can be quantitated as the midpoint ascorbate concentrations of the two redox transitions. The C_H and C_L values found here for purified wild-type bovine cyt b_{561} agree well with those reported in earlier studies (Table 1), supporting the reproducibility of the titration approach. Ascorbate titration of murine cyt b_{561} gave a C_H value 45−90% higher than that of the bovine cytochrome (Table 1). As the cyt b_{561} forms from the two species have similar b_H redox potentials,[3,11] the difference in C_H values suggests that murine cyt b_{561} has a slightly less efficient interaction with ascorbate. The difference between murine and bovine cytochromes is more dramatic for C_L, being ~4-fold higher in the murine cytochrome, presumably at least partially because of its lower b_H redox potential (19 mV for murine cyt b_{561} vs 60 mV for bovine cyt b_{561}[3,11]). Ascorbate titration has also been used to characterize the human and murine forms of duodenal cyt b_{561} (Dcytb), another member of the cyt b_{561} family;[26] examination of the C_H and C_L values (Table 1) indicates that the ascorbate reduction efficiencies of the heme centers in these cytochromes are comparable to those of murine adrenal cyt b_{561}. The pattern of distinct ascorbate reduction efficiencies of the two heme centers also has been observed in the tonoplast cyt b_{561} from *Arabidopsis* (Table 1),[27] suggesting that it may be a general property of the cyt b_{561} family. Interestingly, an alanine mutant of the tonoplast cytochrome in the residue corresponding to Lys85 in adrenal cyt b_{561} markedly impaired reduction by ascorbate.[27] This was ascribed to a large increase in the value of C_L, but the generation of ascorbate-resistant or refractory species similar to b_{VL} and b_X encountered in the Arg74 mutants (Figures 7 and 8) should also be considered.

It is tempting to consider the C_H and C_L values from ascorbate titrations as affinity constants for ascorbate binding, but this is almost certainly mistaken. For one thing, the interaction of ascorbate with the b_H and b_L centers involves both a redox step and a binding step. In addition, ascorbate is converted to semidehydroascorbate, which undergoes several further reactions, some irreversible,[28] thereby making true equilibration impossible. The fact that the C_H values were generally comparable to the concentration of cyt b_{561} used for titration raises the possibility that the C_H value might be sensitive to the cytochrome level used. However, replicate titrations of several wild-type and mutant samples at cytochrome concentrations of 6−14 μM gave no indication of a systematic variation in C_H with cytochrome level (data not shown). The accumulated experience with ascorbate titrations of cyt b_{561} (refs 7, 11, and 29; and Figures 3, 5, and 7) indicates that C_H and C_L values are useful and reproducible as empirical indicators of the efficiency of reduction of b_H and b_L by ascorbate. It is worth noting that ascorbate titrations, which combine effects on ascorbate binding and on subsequent redox steps, can be more informative than potentiometric titrations with a nonselective reductant (dithionite) that reflect only the redox properties of the heme centers. For example, the midpoint potentials of the H92Q mutant were indistinguishable from the wild-type values, whereas the C_H value was markedly elevated in the mutant.[7] Further, the results for the Arg74 mutant (Figures 7 and 8) show that ascorbate titration can assess the proportions of native and non-native protein species

and thus evaluate the roles of individual residues in the formation of native cytochrome.

EM3 Structure and Ascorbate Titration Parameters. The b_H and b_L spectra are known to be sensitive to structural changes in the heme centers.[7] Thus, the lack of a major effect on the b_H and b_L spectra with all but two of the EM3 alanine mutations indicates that the EM3 structure is largely decoupled from the heme centers' structure. Further, with the exception of R74A, none of the EM3 alanine mutants resulted in substantial amounts of dysfunctional protein (Table S2 of the Supporting Information), arguing against longer-range protein structural disruptions. This gives some reassurance that the observed alterations in ascorbate titration parameters in the EM3 mutants can reasonably be attributed to local structural changes.

Many of the alanine mutants in EM3 had alterations in C_H or C_L (Figure 5), supporting the earlier general hypothesis that some EM3 residues make important contributions to the redox interactions of cyt b_{561} with ascorbate.[4,10] It is important to note that none of the EM3 alanine mutants completely blocks reduction of the b_H center (Figure 5). This result implies that no individual EM3 residue is absolutely required for reduction of the b_H heme by ascorbate. This outcome is surprising given the longstanding expectation that one or more conserved EM3 residues would prove to be crucial to interaction with ascorbate.[10] The role of the EM3 segment thus appears more subtle and complex than previously thought. A mutation of Arg72 of murine cyt b_{561} to alanine (corresponds to Arg74 in the bovine cytochrome) was reported to lack the b_H transition but retain the b_L transition,[11,29] suggesting selective blockage of ascorbate reduction of b_H. However, the murine R72A titration was incomplete even at ~100 mM ascorbate, indicating the presence of considerable b_{VL} and/or b_X, just as we find with the bovine R74A mutant (Figure 3). Thus, it remains to be seen whether murine Arg72 mutants actually have coordinated loss of both b_H and b_L.

Several of the mutants yielded large increases in the C_H value, namely, L70A, L71A, Y73A, R74A, N78A, and K85A (Figure 5). The effects of the latter four mutants might be ascribed to the loss of electrostatic or polar interactions with the negatively charged and very polar ascorbate. Such charge−polar interactions with substrate are specific to particular positions in EM3, because an alanine substitution at Glu79, Lys81, Arg82, Thr83, or Thr84 had little effect on the C_H value (Figure 5). Leu70 and Leu71 are predicted to be on the outer face of the TM2 helix (Figure 6A), so the increases in C_H seen with alanine substitutions at these positions may reflect disrupted interactions of TM2 with TM1 or TM6 (not included in the model). In this context, C_H was unchanged in the V72A mutant (Figure 5), and the Val72 side chain is predicted to be oriented toward the inside of the four-helix bundle.[25] Only one substitution, F76A, resulted in a decrease in C_H past the threshold. Phe76 lies above the b_H heme in the structural model (Figure 6A), and insertion of the much smaller alanine side chain at that position might improve the access of ascorbate to the heme.

EM3 Structure and the b_H and b_L Heme Centers. None of the alanine mutants in EM3 prevented incorporation of close to the full complement of two hemes (Table S2 of the Supporting Information), and most of the mutations had very modest effects on the absorbance spectra of the b_H and b_L heme centers (Figure 4). Thus, none of the EM3 residues appears to be essential to the "kernel", the structural core of cyt b_{561} whose modification (e.g., by mutation of any one axial heme ligand) results in major structural disruption at both heme centers.[6,7] Two EM3 alanine mutations, Y73A and K85A, did markedly narrow the b_H spectrum (Figure 4). The distinctive and remarkably similar absence of the shoulder at 554 nm in these two mutants located near opposite ends of EM3 suggested they are structurally linked to each other and to the b_H heme center, a concept supported by the proximity of Tyr73 and Lys85 predicted by the model of Bashtovyy et al.[25] (Figure 6). The proposed interaction between Lys85 and Tyr73 may be of the "cation−π" type, observed in several transmembrane proteins.[30]

Position of EM3 in Relation to the b_H and b_L Heme Centers. There has been considerable controversy over the disposition of the b_H and b_L centers in adrenal cyt b_{561} with respect to the cytoplasmic and lumenal sides of the chromaffin granule membrane.[6,7,31] As noted in Results, the effects of EM3 mutations are associated more with b_H than with b_L, indicating that EM3 is physically closer to b_H. Such proximity of EM3 to b_H is consistent with the recent conclusion that the b_H heme ligands are His88 and His161, while the b_L heme ligands are His54 and His122.[6,7] An earlier topological model for cyt b_{561} puts b_L on the cytoplasmic side, near EM3, largely on the basis of chemical modification studies with DEPC and 4,4′-dithiopyridine[8,9] and the presumption that the center with the more negative redox potential (b_L) is exposed to the more reducing compartment, the cytosol.[32,33] Results of chemical modification by DEPC are difficult to interpret conclusively because the reagent produced varying degrees of derivatization of many residues.[3,12] Reaction with dithiopyridine (targeting Cys57 and Cys125 near the heme ligated by His54 and His122, on the matrix side) did decrease the magnitude of the b_H EPR signal (g = 3.1), but the lack of a significant b_L EPR signal (g = 3.7) in the same sample[9] left open the possibility that the sulfhydryl reagent actually perturbed both heme centers. In this context, disruption of both heme centers is seen with even relatively conservative point mutations at His54 or His122.[6] As for the presumption that the electron from cytoplasmic ascorbate enters cyt b_{561} at b_L, flows to b_H, and exits to an acceptor on the matrix side,[31] electron flow in the opposite direction, from b_H to b_L, can be demonstrated in the diheme cytochromes of bacterial and mammalian bc_1 complexes and bacterial quinol fumarate reductase.[34−36] Thus, the accumulating evidence indicates that adrenal cyt b_{561} does indeed have its b_H heme center near the cytoplasmic face of the membrane, and any cyt b_{561} mechanism needs to account for the consequent thermodynamically "uphill" electron transfer. While this paper was being reviewed, Desmet et al. reported a cytoplasmic topology for the high-potential heme of a cyt b_{561} analogue in *Arabidopsis* tonoplasts.[37] With two examples of the cytoplasmic orientation of b_H now in hand, it remains to be seen how general this topology is among other members of the cyt b_{561} family.

Arg74 and Structural Integrity of the b_H−b_L Unit. R74A was the only alanine mutant that exhibited a major decrease in the ascorbate reducibility of the cytochrome (Table S2 of the Supporting Information). Alteration of side chain structure at position 74 affected both heme centers, with the b_H and b_L content decreasing in concert as progressively more radical substitutions were made (Figures 7 and 8 and Figure S7 and Table S2 of the Supporting Information). Coordinated effects on both heme centers in adrenal cyt b_{561} are a hallmark of single mutations in any one of the four axial heme ligands.[6,7] These similar, pleiotropic effects of mutations at sites that are widely separated in the polypeptide sequence prompted our proposal that the four-helix bundle cross-linked by bis-histidine ligation

		70 75 80 85	
conserved >85% =>		G***L*YR*FR***K***K*LH	
kernel defect =>		****R***** *****	
altered bH spectrum =>		***Y****** ****K	
altered CH value =>		LL*YR*F*N* ****K	
IDENTIFIER	***ORGANISM***		
P10897	B. taurus	67	GDALLVYRVFRNEAKRTTKVLH
NP001125175	P. abelii	66	GDALLVYRVFRNEAKRTTKVLH
XP001495680	E. caballus	62	GDALLVYRVFRNEAKRTTKVLH
EFB24487	A. melanoleuca	60	GDALLVYRVFRNEAKRTTKVLH
XP853258	C. familiaris	67	GDALLVYRVFRNEAKRTTKVLH
NP001087259	O. aries	67	GDALLVYRVFRNEAKRTTKVLH
NP001906	H. sapiens	66	GNALLVYRVFRNEAKRTTKVLH
NP001090991	S. scrofa	67	GDALLVYRVFRNEAKRTTKILH
NP001100526	R. norvegicus	65	GDALLVYRVFRKEAKRTTKILH
AAA65643	M. musculus	65	GDALLVYRVFRREAKRTTKILH
NP001089047	X. laevis	66	GEALLVYRVFRHETKRSTKILH
NP001005633	X. tropicalis	65	GEALLVYRVFRHETKRATKILH
NP001122216	D. rerio	61	GDAVLVYRVFRNESKRSVKILH
CAG05955	T. nigroviridis	53	GDAIVVYRVFHNESKRTIKMLH
NP499095	C. elegans	76	GEALLVYRVFRNERKKFSKTLH
XP002641730	C. briggsae	76	GESILVYRVFRNERKKFSKTLH
XP001894183	B. malayi	71	GDGILVYRIFRHERKRFSKLLH
XP002574073	S. mansoni	71	GDAILVYRVFRSFRKLPIKILH
XP001635650	N. vectensis	69	SEAMIVYRVFRNETKYTVKLVH
ACO10642	C. rogercresseyi	66	GNGMLVYRNFRGEAKKRLKILH
ACO15392	C. clemensi	66	GNGMLVYRNFRGEAKKRLKLLH
ACI90364	P. roseola	73	GDAILAYRVFRDVKKIRVKILH
BAB32556	D. japonica	76	GNAIMVYRVFRNTKKIRAKWLH
XP002635501	C. briggsae	85	GEALLAYRVYRHDAKMVSKLLH
XP002595114	B. floridae	83	GNAILTYRVFRDQVKLGVKILH
NP001041449	C. intestinalis	70	GEAALVYRVFRQTEKLKAKIIH
XP002160113	H. magnipapillata	81	GNAAIAYRVFRNQNKLKIKILH
CAX70071	S. japonicum	71	GDSILVFRVFRSYRKLPVKILH
NP001023900	C. elegans	80	GEALLAYRLYRYDAKIISKLLH
XP002426701	P. humanus	64	ANGILVYRSFRNSRKRKLKIAH
XP001656283	A. aegypti	69	GNSILVYRGFRYARKKPLKVTH

Figure 9. Alignment of EM3 segments and flanking residues in bovine adrenal cyt b_{561} homologues from several organisms. The numbering of the bovine cyt b_{561} residues under study is shown at the top, along with conserved residues and residues for which alanine substitution affected the kernel structure, the b_H spectrum, or the C_H value.

of the two hemes is part of a larger structural unit or kernel.[6,7] (A recent report[37] indicates that the kernel may be less rigid in a plant tonoplast b_{561} analogue.) The coordinated effects of Arg74 on b_H and b_L indicate that the side chain structure at residue 74 impacts the functional integrity of the kernel. Unlike mutants of the heme axial ligands, even R74A retains two hemes, implying that modifications at Arg74 lead to a more subtle modification of the kernel than does loss of a heme cross-link.

The observed link between the nature of the substitution at Arg74 and the extent of loss of the kernel indicated by coordinated decreases in b_H and b_L signal intensities (Figures 7 and 8 and Figure S7 of the Supporting Information) indicates that a positively charged side chain at position 74 is critical to formation of the native kernel structure. One possibility is that EM3 forms a key electrostatic interaction via Arg74 to an anionic residue elsewhere on the cytoplasmic side of the cytochrome. An alternative explanation is that the Arg74 side chain has a charge interaction with a propionate group on the b_H heme. However, disruption of heme propionate−arginine interactions can perturb visible absorption bands,[38] and the b_H spectrum is only modestly affected in the Arg74 mutants of cyt b_{561} (Figure S4 of the Supporting Information). Further study will be needed to clarify the exact role(s) of Arg74.

As depicted in the scheme in Figure S8 of the Supporting Information, some of the "lost" b_H-b_L unit in the Arg74 mutants can be ascribed to protein with weakly functional b_{VL} centers (Figure 7B), but the majority of the lost b_H-b_L unit appears to reflect recombinant protein carrying heme that is completely refractory to ascorbate (b_X). This ascorbate-refractory fraction is not simply grossly misfolded protein because the ascorbate-refractory species can be extracted by mild detergent, and it retains *b*-type heme that can be reduced by dithionite. Rather, it appears that either the ascorbate binding site is lost in the ascorbate-refractory fraction or the redox potential of its heme is shifted significantly below that of ascorbate.

EM3 Residue Functions in Other Cyt b_{561} Family Members. Seven of the 15 residues in the segment of bovine cyt b_{561} examined by alanine scanning are well-conserved among the homologous proteins (Figure 9). Arg74, mutation of which removes the positive charge-disrupted formation of the kernel (Figures 7 and 8 and Figure S8 of the Supporting Information), is completely conserved, suggesting that Arg74 is an important shared structural determinant. Tyr73 and Lys85, whose alanine mutants exhibited similar narrowing in the b_H spectrum (Figure 4), are both conserved in all but one of the homologues, suggesting that the proposed interaction between Tyr73 and Lys85 (Figure 6) is another shared structural feature. Among the positions at which alanine mutants exhibited increases in C_H values (Figure 5), all but Leu70 and Asn78 are well-conserved; Phe76, at which mutation to alanine decreased C_H, is also strongly conserved. Only one mutant in a strongly conserved residue, K81A, did not exhibit a perturbed C_H value (Figure 5). This general conservation of residues linked by the alanine scanning results to reduction of b_H suggests that the cyt b_{561} homologues share a common mechanism for ascorbate interaction on the cytoplasmic face of the cytochrome.

In summary, the cytoplasmic EM3 segment of adrenal cyt b_{561} has often been implicated in the interaction with ascorbate. This alanine scanning study provides the first systematic evaluation of the roles of individual residues in this segment. The results identify several EM3 residues linked to the efficiency of cyt b_{561} reduction by ascorbate, two residues that influence the α-band spectrum of the b_H heme center, and one residue that is important to the integrity of the structural unit carrying both heme centers. These findings substantially improve our understanding of the structural and functional relationships between EM3 residues and the heme centers in this important cytochrome.

■ ASSOCIATED CONTENT

Supporting Information. Sequence information for the mutagenic primers, absorbance spectra observed during ascorbate titrations of additional cyt b_{561} mutants, difference spectra of b_H and b_L heme centers for additional cyt b_{561} mutants, deconvolution of b_H heme spectral components, EPR signal amplitudes in Arg74 mutants, and tabulated data for the various mutants studied. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Internal Medicine, University of Texas Health Science Center, 6431 Fannin St., MSB 5.016, Houston, TX 77030. Phone: (713) 500-6772. Fax: (713) 500-6812. E-mail: richard.j.kulmacz@uth.tmc.edu.

Author Contributions

W.L. and G.F.Z.d.S. contributed equally to this work.

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■ ABBREVIATIONS

cyt b_{561}, adrenal cytochrome b_{561}; EM, extramembrane segment; TM, transmembrane segment; b_H and b_L, high- and low-potential heme centers, respectively; C_H and C_L, midpoint ascorbate concentrations for the b_H and b_L ferric−ferrous transitions, respectively; EPR, electron paramagnetic resonance spectroscopy.

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