Converting a *c*-type to a *b*-type Cytochrome: Met61 to His61 Mutant of *Pseudomonas* Cytochrome *c*-551

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ABSTRACT: The gene *nirM*, coding for cytochrome *c*-551 in *Pseudomonas stutzeri* substrain ZoBell, was engineered to mutate Met61, the sixth ligand to the heme *c*, into His61, thereby converting the typical Met-His coordination of a *c*-type cytochrome into His-His, typical of *b*-type cytochromes. The mutant protein was expressed heterologously in *Escherichia coli* at levels 3-fold higher than in *Pseudomonas* and purified to homogeneity. The mutant retained low-spin visible spectral characteristics, indicating that the strong field ligand His 61 was coordinated to the iron. The physiochemical properties of the mutant were measured and compared to the wild-type properties. These included visible spectra, ligand binding reactions, stability to temperature and chemical denaturant, oxidation−reduction potentials, and electron-transfer kinetics to the physiological nitrite reductase of *Pseudomonas.* Despite a change in potential from the normal 260 mV to 55 mV, the mutant retained many of the properties of the *c*-551 family.

Several key characteristics distinguish the *c*-type from the *b*-type family of cytochromes. In the *c*-type the heme is covalently attached to two cysteine residues found in the signature peptide sequence Cys-X-Y-Cys-His, where the imidazole nitrogen of the histidine residue coordinates to the heme iron. In *b*-type cytochromes the fifth and sixth ligands are commonly histidines, while in *c*-types the sixth ligand is commonly a methionine. This study investigated the effects of replacing the invariant sixth ligand methionine in a *c*-type cytochrome with a histidine, thereby converting it into a hybrid *b*/*c*-type cytochrome. Another sort of hybrid has been reported in which cyt *b*562 of *Escherichia coli* was mutated to contain cysteine residues capable of forming new covalent attachments to heme b (*1*). In the present case the protein mutated into M61H was *c*-551 from *Pseudomonas stutzeri* substrain *ZoBell* (formerly *Pseudomonas perfectomarina*, hereafter referred to as *P. stutzeri* ZoBell). The *c*-551 family of bacterial cytochromes is composed of homologous proteins of ca. 82 residues in diverse genera such as *Pseudomonas*, *Nitrosomonas*, or *Alcaligenes*. Sequence homology can vary from 50 to 90%, but there is a core of invariant residues that includes the heme ligands. Cyt *c*-551 is the prototype cytochrome *c* since it is the smallest basic member of the family capable of electron transport with high specificity at a potential of ca. +260 mV. Genes have been isolated and sequenced encoding *c*-551 from *P. stutzeri* ZoBell (*2*), *Pseudomonas aeruginosa* (*3*), and *Nitrosomonas europae* (*4*).

While engineering the mutant, protein expression problems were encountered that reflect trends seen in other instances. Expression, and especially overexpression, of recombinant *c*-type proteins has been more difficult than for many other hemoproteins. Site-specific mutants of mitochondrial cyt *c* have been expressed but only at wild-type levels in yeast devoid of the wild-type gene (refs *5* and *6*, with references to extensive prior literature). Mutants of cyt c_2 have been produced (*7*) but again in the natural host *Rhodobacter capsulatus* devoid of the endogenous wild-type gene, as has the membrane complex bc_1 (*8*). Recombinant *c*-551 has been expressed in *P. aeruginosa* devoid of the wild-type gene (*9*) and in *Pseudomonas putida* (*10*), where it can be purified from other endogenous cytochromes. Heterologous expressions of *c*-type cytochromes in bacterial hosts have left conflicting records in the literature, including that it is not expressed and presumed to be toxic (*11*), expressed as apoprotein (*12*) or as insoluble inclusion bodies (*13*), expressed as cytoplasmic or periplasmic mature haloprotein (*14*−*16*), or expressed in a precursor form as a cytoplasmic membrane component (*13*). For the common host *E. coli* there has been concern whether this host could complete the covalent thioether linkages between the polypeptide of the apoprotein and the iron protoporphyrin IX precursor to produce a mature heme c (*17*). *E. coli* does not contain large amounts of *c*-type hemoproteins; however, it can make heme c. An assimilatory nitrite reductase containing heme *c* is produced when the organism is grown anaerobically on nitrate (*18*). Additional heme *c*-containing proteins have been inferred from translation of DNA sequences that show the presence of the signature heme c binding motif, for example in *nrfA,B* (*19*). That *E. coli* can heterologously express mature *c*-type cytochromes has been observed previously. Three studies have reported expression levels in *E. coli* of ca. 0.1 μmol/L of culture for *Thiobacillus versutus* cyt *c*-550 (*20*), *P. putida p*-cresol methylhydroxylase *c*-type subunit (*21*), and *Chromatium vinosum* cyt *c*′ (*22*). While useful for further studies, these expression levels are well below what has been achieved for non-cytochrome proteins. In the

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present study *c*-551 has been heterologously expressed in *E. coli* at higher levels but only under stringent culture conditions. Results to be discussed may shed some insight into the general problem of overexpressing c-type cytochromes.

MATERIALS AND METHODS

The gene encoding *c*-551, *nir*M, is part of the *nir* locus in *P. stutzeri* ZoBell responsible for dissimilatory nitrite reduction. The gene and others contiguous to it have been cloned and sequenced as the *nirSTBMC* region by Zumft and coworkers (*2*). Professor Zumft graciously provided a 1.8 kb fragment that contained part of *nirB* and the entire *nirMC* transcriptional unit on a Bluscript cloning vector (Strategene). The *Bgl*II−*Kpn*I subfragment (*nirMC*) was subcloned into the *Bgl*II−*Kpn*I sites of ptacTrpA (ampicillin resistant) used previously for overexpression of the α-subunit of Trp synthase (*23*−*25*). A *Bsp*EI site between *nirM* and *C* was engineered into a *Kpn*I site by use of a synthetic linker, and the *Kpn*I−*Kpn*I fragment containing *nirC* was removed from the vector to give a plasmid carrying only *nirM* (ptacnirM). The tac promoter on this plasmid was also exchanged for the T7 promoter (*26*). The commercially available plasmid pALTER-EX2 (Promega) was engineered to change an *Nco*I site downstream of the T7 promoter into a *Bgl*II site. The T7 promoter was then isolated as a 630 bp *Hin*dIII−*Bgl*II fragment and used to replace the 300 bp *Hin*dIII−*Bgl*II fragment of ptacnirM that contained the tac promoter, yielding pT7nirM. Chloramphenicol resistance was engineered into ptacnirM; a 950 bp *Nci*I−*Nci*I fragment containing the *CAT* gene was removed from the commercially available plasmid pBR329 (New England Biolabs) and the ends were converted by synthetic linkers into *Bgl*II sites. These were then inserted into a vector *Bam*HI site to generate pCATnirM. *nirM* was also subcloned into a broad host vector pMMB67, capable of replication in both *E. coli* and *Pseudomonas* (*27*). A *Bgl*II−*Pst*I 461 bp fragment containing *NIR*M was inserted into a *Bam*HI−*Pst*I polycloning site on the vector yielding pMMB67nirM.

In vitro site-directed mutagenesis was performed according to the method of Kunkel et al. (*28*) with modifications reported previously (*23*, *29*). The mutation was introduced through the oligonucleotide primer:

TGG-GGT-CCG-ATC-CCG-CAT-CCG-CCA-AAC-CCA

W56 G57 P58 I59 P60 H61 P62 P63 N64 P65

whereby the normal ATG of M61 was changed to CAT. A silent mutation GGC (Gly) → GGT (Gly) was also introduced to destroy an *Apa*I restriction enzyme site in the wild-type gene to provide a convenient restriction analysis screen for mutant transformants. The isolated mutant was further confirmed by DNA sequencing (Biotechnology Facility, Iowa State University).

E. coli host strains investigated for expression (from New England Biolabs) included DH5α, JM109, UT54600, and BL21(DE3). Plasmid-transformed cells were cultured in the presence of a selection antibiotic (ampicillin or chloramphenicol) on rich YT or TB medium or minimal medium M9 (*30*). Expression of mutant protein was found to be optimal under the following conditions, where all bacterial growth was done at 37 °C. Plasmid DNA (pCATnirM$_{M61H}$) was used to transform competent cells of *E. coli* strain UT5600. Transformants were grown in TYS medium containing 300 μg/mL chloroamphenicol for 1 h and then plated by serial dilution on agar plates containing the same medium. After overnight growth, a single colony was selected from a plate and grown for 24 h in 3 mL of TYS/chloroamphenicol. It was important to use a freshly transformed colony. Stored transformants showed diminished yields of expressed protein. Also, the relatively high concentration of chloroamphenicol maintained selective pressure on the transformants to retain and express plasmid-encoded proteins. A portion of the liquid culture was removed and the plasmid was checked by a DNA minipreparation. A portion of the rest was used as a 1% inoculum to prepare a preculture that in turn was used as a 1% inoculum for the main batch run, typically 10.2 L. The growth medium was TB supplemented with 10 mM KNO_3 and chloramphenicol added to a final concentration of 300 μg/mL. Sterile medium (1.7 L) plus a 17 mL inoculum was placed in 2 L Erlenmeyer flasks and shaken at 150 rpm in a New Brunswick Model 25 Shaker. This physical arrangement did not allow air to maintain saturation in the medium and has been termed semianaerobic growth. As will be discussed later, the presence of IPTG did not have a major effect on yields, but it was typically added anyway to a final concentration of 1 mM 8 h after the inoculum. Cells were harvested by centrifugation after 24 h. The highest yields were 0.48 μmol of *c*-551/L of culture, compared to the endogenous yield in *Pseudomonas* of 0.14 μmol/L. The yield in M9 minimal medium was less, 0.09 μmol/L of culture. The amount of NH_4Cl in M9 (1 g/L) could be decreased 50% and cell growth was still 80% of the former case.

Heterologously expressed *c*-551 was determined to be >94% localized in the periplasm by the method of Wood (*31*). Protein purification was designed to take advantage of the periplasmic localization in that initial *c*-551 release was accomplished by freeze−thaw cycles that leave the inner cytoplasmic membrane mostly intact and hence minimize contamination by cytoplasmic soluble proteins. It was subsequently determined that the freeze−thaw cycles solubilzed peptidoglycan fragments from the cell wall that persistently copurified with *c*-551. While innocuous for most purposes, they were nevertheless removed by incorporation of a lysozyme digestion step. Cytochrome purifications are traditionally monitored by determining the purity ratio from UV−visible spectrophotometry, defined as the ratio of the 550 nm absorbance of the α-band in the dithionite-reduced ferrocytochrome (reflecting the cytochrome content) minus the baseline absorbance at 570 nm divided by the absorbance at 280 nm (reflecting cytochrome plus nonchromophoric contaminant proteins) of the ferricytochrome. For homogeneous wild-type *P. stutzeri* ZoBell *c*-551 the purity ratio had previously been determined to be 1.70 (*32*). This value was expected to be lower for M61H for two reasons; the extra histidine raises the 280 nm absorbance of the cytochrome, and the altered ligand has lowered the intensity of the α-band, as will be subsequently shown.

Cell paste was resuspended in 10 mM ammonium bicarbonate and 0.1 M sodium chloride, pH 8.0. Three times the suspension was frozen at −25 °C and then allowed to melt and warm to 4 °C. The suspension was centrifuged at 20000*g* and the red supernatant was saved. Lysozyme (Sigma) was added to a final concentration of 10 μM and the mixture

was incubated at 37 °C for 48 h. It was dialyzed for 24 h in the cold against 50 mM acetic acid neutralized to pH 4.2 with NH_4OH (aqueous), and the whitish precipitate that formed was removed by centrifugation. This and the subsequent ion-exchange chromatography at acidic pH are the classic steps introduced by Ambler (*33*) for *c*-551 purification and are very efficient because many proteins denature under the acidic conditions while *c*-551 is stable. Wild-type *c*-551 is stable to pH 3 (soluble and retains low-spin ferricytochrome-type visible spectrum), while the mutant precipitates below pH 4.2. The mutant at pH 4.2 was loaded onto a CM-cellulose column (1.5 × 15 cm, equilbrated against the same pH 4.2 buffer) and the column was washed with 500 mL each of acetate buffer at pH values 4.5, 4.8, 5.1, 5.4, 5.7, and 6.0, which finally eluted the red protein. Pooled red fractions were dialyzed against 10 mM Tris-HCl (pH 8.0) and loaded onto a DEAE-cellulose column (1.5 × 15 cm). The column was washed with 500 mL of 10 mM Tris-HCl (pH 8.0) and then the cytochrome was eluted by a linear gradient from 0 to 0.1 M KCl in the same Tris buffer, where the red cytochrome came off between 0.05 and 0.06 M KCl. Cytochrome was concentrated by Amicon pressure dialysis and further purified by size-exclusion chromatography (Sephadex G-50 superfine, 2 × 65 cm column, eluted with 0.1 M ammonium bicarbonate buffer, pH 8.0). Cytochrome was again concentrated by pressure dialysis, lyophilized, and redissolved in 0.02 M ammonium bicarbonate (pH 8.0). The last step was purification by HPLC employing a Rainin PureGel SAX ion-exchange column (1 × 10 cm, eluted at 2 mL/min, 0.1−0.3 μmol of cytochrome/injection) and a linear gradient over 30 min from 0.02 to 0.2 M ammonium bicarbonate. Samples were dialyzed against a common buffer (50 mM phosphate, Na form) at indicated pH values for further experiments or lyophilized and stored at −25 °C. The final purity ratio was 1.51 and the percent yield over crude extract was 55%. A purification table is given in Supporitng Information along with an HPLC chromatogram from the final step and a figure of silver-stained SDS−PAGE demonstrating final homogeneity.

Extinction coefficients were determined by the pyridine hemochromogen method (*34*). Nitrite reductase was purified (*35*) and electron-transfer kinetics were measured (*36*) as described previously. Initial rates of reaction were determined from the integrated rate law or progression curve of Blatt and Pecht (*37*) by curve-fitting the entire time course to obtain their parameters A_1 and τ and then substituting these values into the time derivative at time = 0. This approach was believed to be more accurate than estimating rates from slopes near the beginning, because the reaction is never zero-order and the rate changes rapidly with time near the beginning. Oxidation−reduction potentials were determined by modification of the standard method of mixtures. A Thunberg-type cuvette was fitted with a Pt wire electrode and a Ag/AgCl reference electrode. In a final volume of 2−3 mL of 5 mM buffer, the cuvette contained 5−10 μM protein, 4 μM photoreductant deazaflavin (*36*), 10 mM EDTA, and 50 mM ferric mediator. For proteins where the potential was above 250 mV, this was potassium ferricyanide ($\Delta E_0' \sim +400$ mV), while for lower potentials it was ferric EDTA ($\Delta E_0' \sim +100$ mV), to match approximately the midpoint potentials of protein and mediator. Acetate was used to buffer pH values from 5 to 6, phosphate from 6 to 7.5, and Tris from 7.5 to 9. The solution was made anaerobic by cycling vacuum and argon flushing. The mediator was then reduced in steps by illuminating the solution with UV light in a Rayonet photochemical reactor for brief periods of time. The photoreductant deazaflavin uses EDTA as the ultimate source of electrons and reduces the mediator (present in excess), which then redox-equilibrates with the protein. The solution potential was read directly from the electrodes and the fraction of protein reduced at this potential was determined by visible spectrophotometry. The procedure was checked against myoglobin and cytochromes of known potentials. A representative determination is shown in Supporting Information.

RESULTS AND DISCUSSION

Protein Expression. Edman analysis of *c*-551 from *E. coli* and electrospray mass spectrometry (obs 9184 ± 2 Da; calc 9185.25) indicated that the heterologously expressed protein had the same N-terminal sequence (Gln-Asp-Gly...) as mature native protein (*32*). Therefore the hydrophobic 22 residue signal sequence (*2*) was removed, presumably in concert with periplasmic translocation. The proteolytic site might be signaled by length or by the occurrence of the first hydrophilic residues. In *P. aeruginosa* a 22 residue signal is removed to generate the N-terminus Glu-Asp-Pro... (*3*), while in *N. europaea* a 22 residue signal is removed to generate Asp-Ala-Asp (*4*).

While greater than endogenous levels, the expression level of *c*-551 was disappointing. This same vector and promoter when used for Trp synthase α-subunit led to 4 μmol/L of culture with IPTG induction (*23*). General heme biosynthesis in *E. coli* is unlikely to be limiting, since other hemoproteins such as cyt *d* (*38*) and cyt *b*562 (*1*) have been overexpressed by inserting only the apoprotein structural genes. Special biosynthetic requirements could be limiting, such as when siroheme-containing protein was engineered into *E. coli* (*39*). For *c*-551, special processing might require heme covalent attachment to polypeptide. This could be catalyzed by a so-far uncharacterized *E. coli* heme *c* lyase (also called heme *c* synthase), well-known in yeast (*40*), or by spontaneous reaction of active Cys side chains across the vinyl groups of protoporphyrin IX (*1*). If heme attachment was limiting *c*-551 expression in *E. coli*, then there should be an excess of apoprotein. By comparing heme *c* expressed by visible difference spectroscopy with 9 kDa protein expressed by SDS−PAGE, we showed that the amounts were the same.

Higher levels of heterologous expression (1.2 μmol/L) have been reported for *Saccharomyces cerevisiae* iso-1-cytochrome *c* in *E. coli* achieved by coexpressing the cyt *c* gene and the *S. cerevisiae* gene for heme lyase. However, both of these gene products are cytoplasmic proteins, whereas the heme lyase is expected to be periplasmic (*17*). As discussed previously, mature *c*-551 is found in the periplasm after removal of a signal sequence.

There are some different codon usage preferences between *E. coli* and *Pseudomonas* (*41*). Still, *Pseudomonas* genes have been highly overexpressed in *E. coli* and an excellent example is the flavoprotein subunit of *p*-cresol methylhydroxylase (*21*). Every codon in *nirM* is also found in the flavoprotein gene except for CTA (Leu), but, that codon is not especially rare in *E. coli* (*42*). It is also used in the overexpressed trp synthase α-subunit.

The broad host plasmid pMMB67nirM was engineered in *E. coli*, isolated, and then used to transform wild type *P. stutzeri* ZoBell by electroporation. The chromosomal copy of *nirM* would be present, but an increase in *c*-551 content was expected due to the extra plasmid-encoded copy. At most a 5% increase in *c*-551 content was observed, indicating expression limitation even in the endogenous producer.

The T7 promoter has been successfully used for high heterologous overexpression (*26*). The host must contain the T7 RNA polymerase under control of another promoter, usually *lac*. Induction by IPTG expresses the RNA polymerase, which then has a very high affinity for the T7 promoter, leading to large amounts of mRNA and high protein expression. When used with *nirM* the T7 system gave an informative result. In the absence of IPTG, *E. coli* transformed with pT7nirM grew; in its presence it would not. Aliquots (10 μL) of 1 mM IPTG were spotted at discrete points onto a nutrient agar plate, which was then overlaid with uninduced *E. coli* carrying pT7nirM. A confluent lawn grew everywhere except where IPTG had been spotted (shown in Supporting Information). This suggests that high levels of *c*-551 are toxic to the host.

For pCATnirM (*tac* promoter) the lack of an IPTG induction effect suggests expression occurs without IPTG due to the known leaky nature of this promoter, while with IPTG, only those cells producing sublethal amounts of *c*-551 are surviving in batch culture. The observed lack of an IPTG effect is not unique. The heme *c*-containing subunit of *p*-cresol methylhydroxylase of *Pseudomonas* was not overexpressed when placed on the same vector in the same host that did overexpress the flavo subunit, and furthermore, the limited expression was the same with or without IPTG (*21*).

Mutant Characteristics. The ferric visible spectrum of M61H was indistinguishable in its main features from that of wild type. It corresponded to a low-spin ($S = {}^1/_2$) ferric hemoprotein, establishing that the strong field ligand His61 was coordinated to the heme iron. As expected, it was missing the weak 695 nm charge-transfer band of wild type characteristic of methionine−iron ligation (*43* and references therein). The ferrous spectrum was also typical of a low-spin ($S = {}^1/_2$) ferrous hemoprotein, indicating that His 61 remained coordinated in both redox states. The ferrous spectrum was altered from wild type as shown in Figure 1. The normally sharp ferrous α-band was split, broadened, and reduced in intensity. Splitting is not common but has been observed for *c*-type cytochromes [the split α cytochromes (*44*), e.g., cyt cd_1 (*45*)]. The α-band of metalloporphyrins is degenerate (*46*, *47*), but the precise structural reasons for lifting the degeneracy in some cytochromes are not well understood. The extinction coefficient for M61H was 25.6 mM^{-1} compared to 27.8 mM^{-1} for wild type. Cyanide displaces the normal methionine ligand in ferric *c*-type cytochromes (*48*) and similar reactivity was observed for M61H. First noted in the 1930s, it has been long known that CO does not bind to ferrous cytochromes *c* at neutral pH, and this behavior became the basis for a classical test of the integrity of protein preparations (*49*). Ferrous wild-type *c*-551 does not bind CO, but the mutant does within seconds. The Soret band shifts from 414 to 410 nm and the α-band from 551 to 548 nm.

Because of its role as the penultimate reductant in respiratory electron transport just prior to cytochrome oxidase, an important characteristic of cytochromes *c* is their lack of autoxidizability, a historical term describing the ferrous protein's resistance to reoxidation by other cellular components, especially O_2. In air-saturated buffer at pH 7, wild-type *c*-551 reoxidizes with a pseudo-first-order rate constant of 3.28×10^{-6} s^{-1} (half-time of ca. 60 h) while for M61H the rate constant is 7.04×10^{-3} s^{-1} (half-time ca. 100 s).

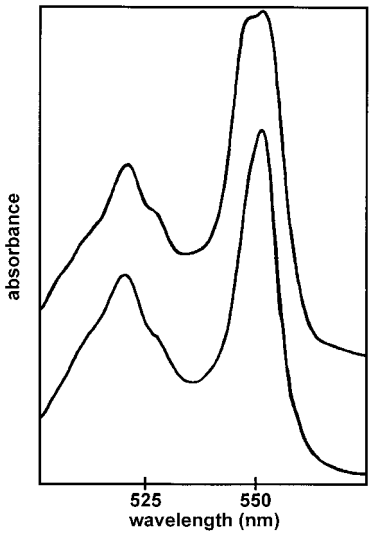

FIGURE 1: Visible spectra of sodium dithionite-reduced M61H (upper trace) and wild-type *c*-551 (lower trace) demonstrating the split mutant α-band.

CO binding and autoxidizability for M61H suggest its ligand structure may not be as stable as in wild type. This was further investigated by perturbing structure with heat and a chemical denaturant while monitoring effects by visible spectroscopy. When *c*-type cytochromes are subjected to harsh denaturing conditions, major polypeptide disruptions, up to unfolding to random coil, are accompanied by a drastic change in the visible spectrum. When normal ligand bonds are broken, Fe goes from low-spin (ferric $S = {}^1/_2$ or ferrous $S = 0$) to high-spin (ferric $S = {}^5/_2$ or ferrous $S = 2$) (for example, see ref *50*). The spin state change is accompanied by large wavelength and intensity changes as the protein goes from a characteristic cytochrome-type spectrum to a globin-type spectrum. These optical changes have been used to follow the unfolding of mitochondrial cyt c (ref *51* is an early example of many papers employing this strategy). The visible spectra of ferrous and ferric wild-type and M61H were constant from room temperature up to 100 °C as illustrated for the ferric Soret band in Figure 2. The slight hyperchromic shift for the Soret of M61H above 70 °C was a reproducible feature of unknown significance, but it is much too small an effect to denote a ligand dissociation-induced spin state change. Figure 3 compares the stability of wild type and M61H toward increasing concentrations of the common denaturant guanidine hydrochloride. The midpoint for unfolding wild type was 4.2 M, while for M61H it was 3.4 M, thus indicating a decrease in stability for the mutant. It should be stressed, however, that many proteins are denatured at concentrations of 2 M or less (*52*), and thus if one also considers the thermal stability, the mutant is still remarkably stable. The unfolding process was fitted to the two-state linear denaturant binding model ofTanford as implemented by Matthews and Crisanti (*53*). The fitting parameter Δn was

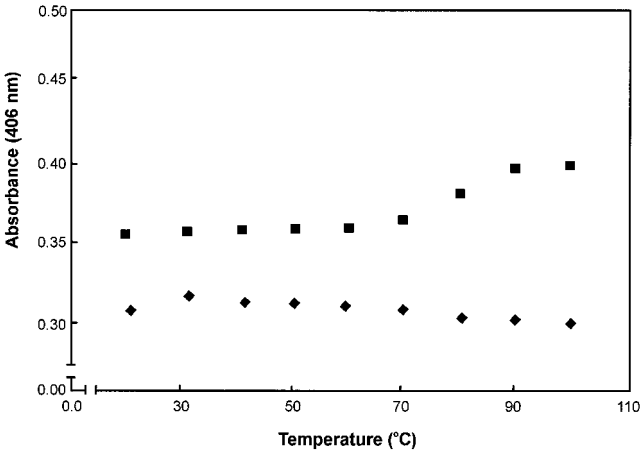

FIGURE 2: Thermal stability of wild-type *c*-551 (◆) and M61H (■) as monitored by the Soret band absorbance versus temperature.

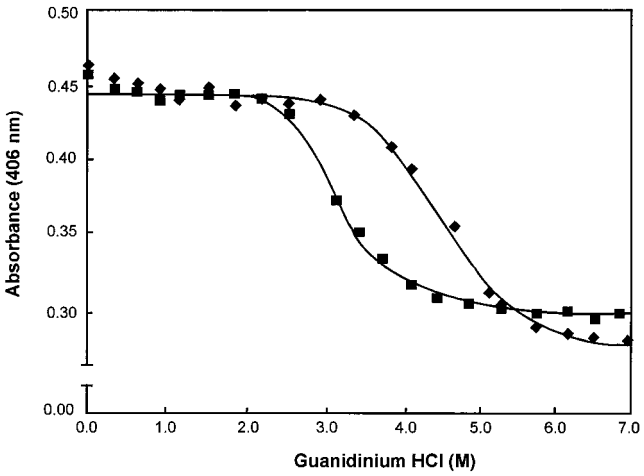

FIGURE 3: Stability of wild-type *c*-551 (◆) and M61H (■) as monitored by the Soret band absorbance versus increasing concentrations of guanidine hydrochloride. The solid curves represent the fit to a two-state model (native and unfolded) as described in the text.

30 for both wild type and mutant, while ΔG^{H2O} was +6.4 kcal for wild type and +5.0 kcal for M61H.

Members of the *c*-551 family typically have oxidation−reduction potentials around +260 mV and thus are very similar to mitochondrial cyt *c*. The two families differ in one significant way. The potential of mitochondrial cyt *c* is pH invariant away from denaturing extremes, while the potential of *c*-551 varies with pH over the range of 5−9. Dependence of potential on pH was measured for both wild-type *P. stutzeri* ZoBell *c*-551 and M61H as shown in Figure 4. The behavior of *P. stutzeri* ZoBell *c*-551 is very similar to that reported for *c*-551 from *P. aeruginosa*, *P. stutzeri*, *and P. mendocina* (*54*). In all cases the potential falls over the range of 5−9 with a midpoint potential of 230−260 mV around pH 7.4−7.6. Substitution of Met 61 by histidine results in a 200 mV drop to 55 mV at pH 7. This value is in the typical range for *b*-type cytochromes, e.g., cyt *b*5 (*55*), and demonstrates that in the *c*-551 family sulfur ligation is the dominant factor in raising the oxidation−reduction potential. On the basis of earlier work on model systems (*50*, *56*), Moore and Pettigrew (*57*) estimated sulfur versus histidine ligation should account for a redox potential difference of 160−168 mV, which is close to the observed value. Experimental verification of the prediction is interesting, but it is not an obvious point and one that may not be followed in other proteins. The model systems were not embedded in a protein interior. Bacterial *c*-type cytochromes have potentials that vary from −200 to +500 mV, and *b*-type cytochromes can range in potential from −100 to −400 mV (*57*, *58*). The theoretical basis for the redox tuning has been and continues to be a subject of investigation (*59*, *60*).

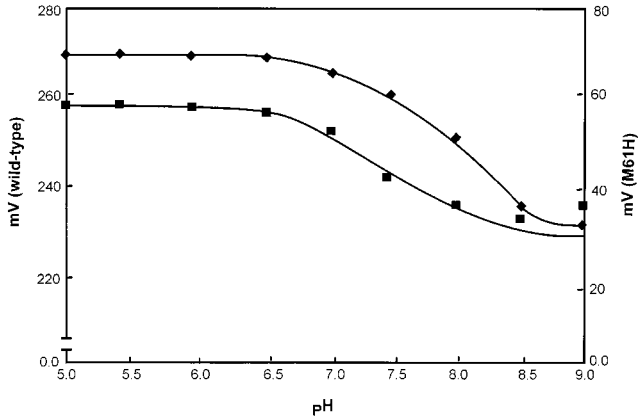

FIGURE 4: Variation of redox potential versus pH for wild-type *c*-551 (◆) and M61H (■).

It is significant that, despite the potential drop, the mutant retains the pH dependence. Using Clark formalism, Moore and co-workers (*54*, *61*, *62*) pointed out that this pH dependence for a cytochrome Fe^{3+}/Fe^{2+} couple that does not explicitly contain a proton or hydride must be due to some site in the molecule having a different proton affinity (acid dissociation constant, K_a or pK_a in log form) in the oxidized state (pK_a^{ox}) and in the reduced state (pK_a^{red}). The data of Figure 4 were fit by their method. For wild type the values were pK_a^{ox} = 7.5 and pK_a^{red} = 8.1; for the mutant the respective values were 7.4 and 7.9. Moore et al. suggested one of the heme propionate groups, relatively buried in the protein interior, as the site with an abnormally high and redox-dependent pK_a. Subsequent studies proposed that the site of protonation/deprotonation was not a single specific functional group but was the cluster of ionizable groups including the buried propionate involved in an interlocking network of hydrogen bonds at the bottom of the heme crevice (*63*). This network stabilizes the hydrophilic carboxylate groups of the heme that are buried in an otherwise hydrophobic interior. Moratal et al. (*64*) have presented data suggesting that the other propionate is the ionization site, and this alternative has been considered by Costa and Moore (*65*). In *P. aeruginosa c*-551, Cutruzzola et al. (*10*) mutated Trp 56, which participates in the inner propionate network, into tyrosine and phenylalanine. The tyrosine mutant had a similar pH profile to wild type except it was displaced by about 20 mV to lower values. The phenylalanyl mutant showed more dramatic effects with a shifting of the midpoint transition to pH 9.5 and a lowering of the midpoint potential by 60 mV. Results for *P. stutzeri* ZoBell M61H show that methionine ligation is not an integral contributor to this phenomenon and that it has little if any impact on the propionate hydrogen-bonding network.

There is a long record of observations linking *c*-551 to both aerobic and anaerobic respiration in facultative anaerobes, dating back to Yamanaka's observation (*66*) of *c*-551 reduction in whole cells linked to lactate and succinate

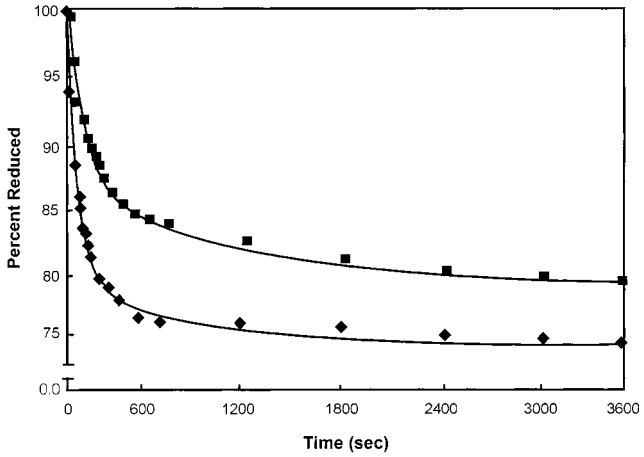

FIGURE 5: Time course of the reoxidation of wild-type *c*-551 (◆) and M61H (■) by cytochrome cd_1 with sodium nitrite as ultimate electron acceptor. The solid lines were fit to the data by use of the integrated rate law of Blatt and Pecht.

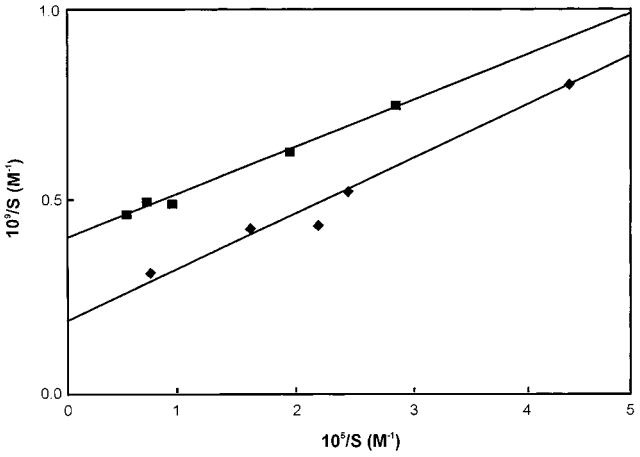

FIGURE 6: Lineweaver−Burk double-reciprocal plot of initial velocity versus initial reduced cytochrome concentration for wild-type *c*-551 (◆) and M61H (■).

dehydrogenases and oxidation linked to O_2 or nitrate consumption. The discovery that the structural gene for *c*-551 is in the same gene cluster as the nitrite reductase, cyt cd_1, in *Pseudomonas aeruginosa* and *P. stutzeri* ZoBell is compelling evidence, strongly supported by in vitro assays, that *c*-551 is a physiological donor for the nitrite reductase (reviewed in ref *67*). However, in *Paracoccous denitrificans*, the analogous *c*-550 gene is located adjacent to the gene for subunit I of the aerobic terminal oxidase (*68*). The gene location in *Pseudomonas* does not fully explain the participation of *c*-551 in aerobic respiration. It is significant that *c*-551 is expressed at about the same level in aerobically or anaerobically grown *Pseudomonas*, while the nitrite reductase is only expressed in anerobically grown cells (*69*). It has been readily shown that membrane fractions of *Pseudomonas* rapidly oxidize reduced *c*-551 in aerated buffer, while if cyanide is added as a terminal oxidase inhibitor, then the same membrane fractions rapidly reduce *c*-551 in the presence of succinate (*70*, *71*). Members of the *c*-551 family have been shown in vitro to be effcient electron donors to purified aerobic terminal oxidases from *Nitrosomonas* (*72*) and *Pseudomonas* AM 1 (*73*).

Measuring the electron transfer activity of the mutant required some special considerations. The drop in potential of M61H rendered it thermodynamically unsuitable to act as an acceptor from *Pseudomonas* cytochrome reductases. Its rapid autooxidizability short-circuits its potential ability to act as a donor to an O_2-linked terminal oxidase. The turnover kinetics were therefore measured for reduced M61H as donor to cyt cd_1 with nitrite as the ultimate electron acceptor and compared to rates for wild type. This assay was complicated by the fact that while wild-type reduced *c*-551 does not react with nitrite, reduced M61H was oxidized directly by nitrite. At 10 mM nitrite, the pseudo-first-order rate was 2.7×10^3 s^{-1}. Fortunately, at catalytic concentrations of cyt cd_1, the enzymatic rate was higher and could be measured by subtracting out the nonenzymatic transfer.

Figure 5 displays the time courses for the enzymatic oxidation of wild type and M61H by cyt cd_1. It was early noted that this reaction was subject to complicated product inhibition, evidenced by the dramatic decrease in oxidation rate during progression of the reaction (*36*, *74*). Blatt and Pecht (*37*) proposed a mechanism to account for this inhibition; the key elements were that both the ferrous and ferric forms of *c*-551 had equal reaction affinities to cyt cd_1 and that a low thermodynamic driving force led to substantial backward electron flow from cyt cd_1 to ferric *c*-551 as its concentration rose during the time course of transfer to nitrite. This is an extremely reasonable hypothesis given that the potential of the receiving site within cyt cd_1 is 271 mV, or only ca. 15 mV higher than a donor like wild-type *P. stutzeri* ZoBell *c*-551. On the basis of their mechanism, Blatt and Pecht proposed an integrated rate law that fit the observed time course very well for the *P. aeruginosa* system. Figure 5 demonstrates that this rate law also provided an excellent match for both *P. stutzeri* ZoBell *c*-551 and M61H. Rate constants for the net forward electron transfer (*c*-551 to cd_1), termed k_1, and the reverse reaction, termed k_{-1}, were calculated from the integrated rate law as given by Blatt and Pecht. These were 6.7×10^4 s^{-1} (k_1) and 8.1×10^4 s^{-1} (k_{-1}) for wild type and 9.6×10^3 s^{-1} and 1.0×10^4 s^{-1} for M61H. On the basis of the forward or reverse rates, one might therefore conclude that the mutant was 12−15% as "active" as wild type. This conclusion must be tempered by the realization that the underlying tenet for the Blatt−Pecht mechanism, low thermodynamic driving force, cannot apply to the mutant. Since its potential at neutral pH is 55 mV, the forward driving force is substantial and the thermodynamic barrier to backward electron flow is 21 kJ. It does not seem reasonable to postulate that the mutant, despite its altered potential, happenstantially follows a different mechanism from wild type but with the same form for the integrated rate law. The rate law itself fits well. The key Blatt−Pecht mechanistic step to explain product inhibition was back transfer from reduced cd_1 to ferric *c*-551. Different mechanisms can produce the same form of the integrated rate law. For example, ferric *c*-551 may bind to the reductase, forming a dead-end complex. Unfortunately, the current data do not prove any other alternative mechanism.

Since the interpretation of the constants k_1 and k_{-1} derived from the time course of reaction is ambiguous, turnover kinetics were also assessed by initial rates and Lineweaver−Burk formalism as shown in Figure 6. This approach must be considered cautiously since it has not at all been shown that *c*-551 and cd_1 form a true enzyme−substrate complex

in the classical sense. Apparent values of maximal velocity (all measured at 80 nM cyt cd_1) and the Michaelis−Menten constant for wild type were 6.5×10^{-9} M s^{-1} and 9.3×10^{-6} M respectively, which are comparable for the values reported for *P. aeruginosa c*-551 by Blatt and Pecht of 2.3×10^{-9} M s^{-1} and 2.2×10^{-6} M. Values for M61H were 2.4×10^{-9} M s^{-1} and 2.7×10^{-6} M, respectively. The decrease in maximal velocity can be interpreted as suggesting that the mutant is ca. 3-fold less efficient as an electron donor to the nitrite reductase despite a greater thermodynamic driving force, but at the same time there is a slight decrease in apparent K_M.

When viewed in terms of either Blatt−Pecht parameters or initial rates, the mutant does show a lower rate of transfer. The mutation did not change net charge and, as shown by the stability studies, was unlikely to change global folding. The rate decrease could be due to any of several factors, including a subtle change of a possible docking interface (if a discrete, relatively long-lived binary complex is important in electron transfer); an electronic rearrangement causing mismatch of donor−acceptor orbitals; or an alteration in a discrete pathway for electron transmission in a mainly outer-sphere-type reaction. The present data do not illuminate a clear choice among such alternatives.

SUPPORTING INFORMATION AVAILABLE

A table of *c*-551 purification and four figures showing an HPLC chromatogram, silver-stained SDS−PAGE, and redox potential determination for M61H and an illustration of toxicity of heterologously expressed *c*-551. This information is available free of charge via the Internet at http://pubs.acs.org.

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