The 18 kDa Cytochrome *c*553 from *Heliobacterium gestii*: Gene Sequence and Characterization of the Mature Protein‡

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ABSTRACT: The 18 kDa cytochrome *c*553 is the dominant c-type cytochrome in cell membranes of *Heliobacterium gestii*. After solubilization, this cytochrome was purified in three steps as a complex with two other proteins of 32 and 42 kDa. The redox midpoint potential of the cytochrome *c*553 was determined to be +215 mV. The EPR spectra clearly show the presence of an ascorbate-reducible low-spin heme with $g_z = 3.048$ and $g_y = 2.238$. The $g_x =$ trough could not be detected. In addition, a Cu(II) signal with $g = 2.058$ was observed, indicating that one component of the cytochrome *c*553 complex contains a bound copper ion. The gene for the 18 kDa cytochrome *c*553, *cyh*A, consists of 429 bp coding for a protein of 142 amino acids. The association of the cytochrome with the cytoplasmic membrane is mediated by two fatty acid molecules, one palmitate and one stearate, that could be identified by mass spectrometry. Both fatty acids are most likely bound to the cysteine residue of the N-terminally processed protein via a glycerol moiety. The amino acid sequence deduced from the DNA sequence exhibits partial identity to the membrane-bound cytochrome *c*551 from *Bacillus* PS3 [Fujiwara, Y., Oka, M., Hamamoto, T., and Sone, N. (1993) *Biochem. Biophys. Res. Commun. 1144*, 213−219] and to the cytochrome *c* subunit (NorC) of the nitrous reductase from *Pseudomonas stutzeri* [Zumft, W. G., Braun, C., and Cuypers, H. (1994) *Eur. J. Biochem. 219*, 481−490].

Cytochromes are a rather heterogeneous group of proteins involved in electron transfer reactions, and they are found in most prokaryotic and in all eukaryotic organisms. With respect to their function in energy transduction, they are usually associated with membrane-bound protein complexes. Detailed information is available for the electron transfer chains of Gram-negative bacteria, whereas electron transfer in Gram-positive bacteria is only poorly understood to date. Several *c*-type cytochromes of low molecular mass have been isolated from Gram-positive bacteria (*1*−*10*), but their physiological functions remain unclear.

Heliobacteria are strictly anaerobic phototrophic organisms that are classified within the Gram-positive line of bacteria according to 16S rRNA analysis. In this group, they constitute the only photosynthetic representatives. Unlike most photosynthetic bacteria, the heliobacteria do not possess highly developed intracytoplasmic membranes or chlorosomes (*11*). The whole photosynthetic apparatus and also all membrane-associated metabolic and catabolic processes are harbored in the cell membrane. Spectroscopic experiments and biochemical and molecular biology studies seem to indicate that the homodimeric photosynthetic RC[1] of heliobacteria is like that of the green sulfur bacteria of the FeS-center containing photosystem I type (*12*−*16*; for a review, see *17*). In contrast to the detailed studies of the electron transfer reactions in photosystem I from chloroplasts, there are only a few data describing the electron transfer in green sulfur bacteria and heliobacteria, that are even controversial to some extent. There is some spectroscopic evidence that a cytochrome *c*553 takes part in the reduction of the oxidized photosynthetic RC of *Heliobacillus mobilis* (*Hb. mobilis*) (*18*−*22*). Flash-induced absorption change experiments performed on whole cells suggest that a heterogeneous heme *c*553 pool donates electrons to the RC. This pool could consist either of several different cytochromes or of a multiheme cytochrome (*22*). The latter could be a tetraheme cytochrome, similar to that of the purple bacteria and *Chloroflexus*, or a monoheme cytochrome *c* like the cytochrome *c*553 that is discussed to be the electron donor in the green sulfur bacterium *Chlorobium tepidum* (*23*). SDS−PAGE analysis from *Hb. mobilis* membranes reveals at least three different heme proteins with molecular masses of approximately 20, 32, and 50 kDa (*18*, *24*, *25*). Further-

‡ The sequence *cyh*A was deposited in the EMBL databank under accession number AF 063189.

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[1] Abbreviations: CAPSO, 3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid; EPR, electron spin resonance; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; Mega 9, nonanoyl-*N*-methylglucamide; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; RC, reaction center; SDS, sodium dodecyl sulfate; TMBZ, tetramethylbenzidine; TXRF, total reflection X-ray fluorescence.

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more, five or six membrane-associated heme components seem to be present according to redox titrations of membranes by optical and EPR spectroscopy (*22*, *26*), which are virtually indistinguishable by their optical spectra. The assignment of their specific function is difficult to assess by means of optical spectroscopy. Here we present the biochemical and genetic characterization of the *c*-type cytochromes in the membranes from *Heliobacterium gestii* (*H. gestii*).

EXPERIMENTAL PROCEDURES

Protein Purification. *H. gestii* Chainat strain, ATCC 43375, was obtained from Dr. M. T. Madigan (Southern Illinois University, Carbondale). The cells were grown with continuous illumination (120 W) in 1 L bottles in LYE medium [as PYE medium (*27*) with pyruvate replaced by 20 mM DL-lactic acid] and with 2.5 mM sodium ascorbate under strictly anaerobic conditions. After 1−2 days of growth, the cells were spun down at 5000*g* and stored at −20 °C until used. All subsequent steps were carried out at +4 °C with buffers purged with nitrogen for 5 min. Thawed cells were suspended in 20 mM Tris/Cl, pH 8.0, 5 mM sodium ascorbate. The cells were disrupted by passing 3 times through a French pressure cell (120 000 psi) in the presence of DNase. Crude cell fragments were spun down by centrifugation at 5000*g*. Membrane fragments were collected by ultracentrifugation at 100000*g* for 1 h. The membranes were solubilized with 1% (w/v) Mega 9 and 1% (w/v) sodium cholate in the above-mentioned buffer. The protease inhibitor Pefabloc (Boehringer Mannheim) was added to give a concentration of 0.5 mM. The final BChl concentration was 1 mM BChl*g* ($OD_{780} = 100$). The solution was stirred for 40 min on ice in the dark and was then directly loaded onto a sucrose density gradient [20−50% (w/w)] in 20 mM Tris/Cl, pH 8.0, 5 mM sodium ascorbate, 0.5% (w/v) Mega 9, 0.1% (w/v) cholate. After centrifugation for 14 h at 42000*g*, the fraction containing the 18 kDa cytochrome *c*553 was loaded onto a hydroxyapatite column (Bio-Gel HTP, Bio-Rad, München) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 0.3% (w/v) Mega 9, 0.1% (w/v) cholate. The bound proteins were eluted with a linear gradient of 10−100 mM sodium phosphate buffer. The protein solution was concentrated (Centriprep, 30 kDa cutoff; Amicon, Witten), and the sample was loaded onto a TSK gel 3000SW column (30 cm, 7.5 mm i.d.; Tosohaas, Stuttgart) connected to an HPLC system that was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, 0.3% (w/v) Mega 9 (flow rate, 1 mL/min). The 18 kDa cytochrome eluted from the column in a homogeneous fraction with two other proteins of 32 and 42 kDa, respectively.

For protein sequencing and for the determination of the fatty acids, the 18 kDa cytochrome was further purified by molecular sieve chromatography in the presence of SDS. The sample was incubated with 5% (w/v) SDS at 80 °C for 5 min. The material was then subjected to HPLC on the column mentioned above that was equilibrated with 50 mM Tris/Cl, pH 7.0, 0.3% (w/v) SDS.

SDS−PAGE was performed on 12% acrylamide gels (*29*). The gels were stained with tetramethylbenzidine (TMBZ) as described by (*30*).

Protein Sequencing. Internal peptide sequences were obtained after endoproteolytic cleavage with LysC and AspN. Proteins were sequenced with an Applied Biosystems gas phase sequencer 477A connected to an on-line 120 APTH analyzer.

C-Terminal protein sequencing was carried out by Hewlett-Packard (Waldbronn) on a G1001A C-terminal sequencing system.

Alkaline Extraction of Membranes. To remove peripheral membrane proteins, membranes were incubated with 5 mM CAPSO/Na, pH 10.0, 10.5, 11.0, 11.5, and 12.0, for 30 min on ice (*30*). After centrifugation (100000*g*, 90 min, 4 °C), the supernatants and the sediments were analyzed for the presence of *c*-type cytochromes on SDS−PAGE gels that were stained with TMBZ.

DNA Isolation and Manipulation. Standard recombinant DNA techniques were used according to (*31*), if not indicated otherwise. Genomic DNA from *H. gestii* was prepared from 1 day old liquid cultures as described by (*32*). Degenerate oligonucleotides were derived from the internal amino acid sequences of the 18 kDa cytochrome *c*553. The 250 bp PCR product that was obtained with the primers P1 (5′-GAG CAG GCD CCVGCN CCN GC-3′) and P5 (5′-GCN GGN GGC ATN GTN GCY TG-3′) was used to screen a genomic library of *Eco*RI-cut genomic DNA from *H. gestii* in the Bluescript SK(+) vector. Positive clones obtained from the colony hybridization were further examined by dot blots and Southern blots. All hybridization techniques were carried out with DIG-labeled DNA probes (Boehringer Mannheim). Primer-directed sequencing was used to determine the entire sequence of the gene from the 18 kDa cytochrome *c*553. To eliminate band compressions, we used deazanucleotides in combination with T7 DNA polymerase. Nucleotide sequences were aligned and analyzed using the GCG Analysis software package.

Spectroscopy. Optical absorption spectra at room temperature were recorded with a Perkin-Elmer Lambda 15 UV−Vis spectrophotometer, at low temperatures with a microspectrophotometer (*33*) at 93 K.

Equilibrium redox titrations were carried out essentially as described by (*34*) with the purified cytochrome *c*553 complex using a DW-2000 UV−Vis spectrophotometer (SLM Aminco). The following mediators were present at 10 μM concentrations: benzoquinone, neutral red, methyl viologen, variamine blue, naphthoquinone, duroquinone. Titrations were carried out at 20 °C with the addition of small amounts of potassium ferricyanide and sodium dithionite. The amount of reduced cytochrome *c*553 was monitored by the absorption difference of the α-band at 536−553 nm.

EPR spectra were recorded with a 300 X-band spectrometer (Bruker) fitted with an Oxford Instruments temperature control system at 15 K and the following instrument settings: microwave power 6.4 mW, microwave frequency 9.415 GHz, modulation amplitude 20 G.

Analysis of Fatty Acid Methyl Esters. Fatty acid methyl esters were prepared by treating the isolated, acetone precipitated cytochrome *c*553 with 1M HCl/methanol for 20 h at 85 °C (*37*). The fatty acid methyl esters were extracted into hexane, and the solvent was evaporated with a stream of nitrogen. For the measurement, the fatty acid methyl esters were redissolved in hexane. Mass spectra were recorded on a Shimadzu QP2000 GCMS (Shimadzu Europe, Duisburg) equipped with a S. G. E. (Ringwood, Vic., Australia) BP1 nonpolar quartz capillary column (50 m, 0.15

mm i.d.). Electron spray mass spectra of the entire molecule were obtained using a Finnigan Ion Trap 700 mass spectrometer (San Jose, CA). Both spectrometers were operated in the electron impact mode at 70 eV.

Materials. Mega 9 was purchased from Oxyl (Bobingen). All restriction endonucleases and nucleic acid modifying enzymes were obtained from New England BioLabs (Beverly, MA) or GIBCO BRL (Eggenstein). Other chemicals were purchased in the highest purity from local distributors.

RESULTS

Membrane-Bound Cytochromes in H. gestii. SDS−PAGE gels of membrane preparations from *H. gestii*, that were stained with TMBZ, reveal three major heme-containing bands corresponding to molecular masses of 42, 32, and 18 kDa, respectively. As judged from the staining intensity, the small cytochrome is the dominant cytochrome in membranes of *H. gestii*. All three cytochromes are tightly bound to the membrane. The 18 and 45 kDa cytochromes could not be released from the membranes by washing isolated membranes with alkaline buffers (*30*), as determined by SDS−PAGE (data not shown). Only the 30 kDa cytochrome was extracted to about 70% above pH 11.0.

Protein Purification. The 18 kDa cytochrome was efficiently solubilized from the membranes of *H. gestii* using a detergent mixture of 1% Mega 9 and 1% sodium cholate. Two differently colored bands were visible after centrifugation of the solubilized material on a sucrose density gradient at about 20% and 45% sucrose. The upper brownish red band contained the enriched 18 kDa cytochrome together with traces of the 30 kDa heme protein. The lower green band primarily contained aggregates of the photosynthetic reaction center as well as the 30 and 45 kDa cytochromes. In addition, a cytochrome *b*562 was detected spectroscopically in this green fraction. Solubilization with other detergents did not result in a good separation on the sucrose gradients (dodecyl maltoside, Mega 9) or led to the oxidative conversion of bacteriochlorophyll *g* to 8-hydroxychlorophyll *a* (LDAO). The fraction containing the 18 kDa cytochrome was loaded onto a hydroxyapatite column equilibrated with 10 mM sodium phosphate, pH 7.0, 0.3% Mega 9, 0.1% sodium cholate. During the washing step, about 10% of the 18 kDa cytochrome eluted from the column. This material could not be distinguished spectroscopically from the main fraction, but it contained several additional proteins as indicated by SDS−PAGE analysis. The 18 kDa cytochrome eluted at approximately 60 mM phosphate together with two other proteins of 32 and 42 kDa, respectively. Usually traces of proteins of high molecular weights were present in this fraction. Having been concentrated, this fraction was subjected to molecular sieve chromatography. Again the 18 kDa cytochrome and the 32 and 42 kDa proteins eluted in a homogeneous fraction with a molecular mass of approximately 200 kDa. All three proteins were present in a constant stoichiometry when rechromatographed (see Figure 1).

Attempts to further purify the 18 kDa cytochrome by IEF or different chromatographic techniques failed. The copurification of the cytochrome with the 42 and the 32 kDa proteins is therefore most likely not accidental but rather indicates the existence of a specific complex. Although it is difficult at present to assign to this complex a specific function in the electron transport chain of *H. gestii*, it will henceforth be referred to as the cytochrome *c*553 complex.

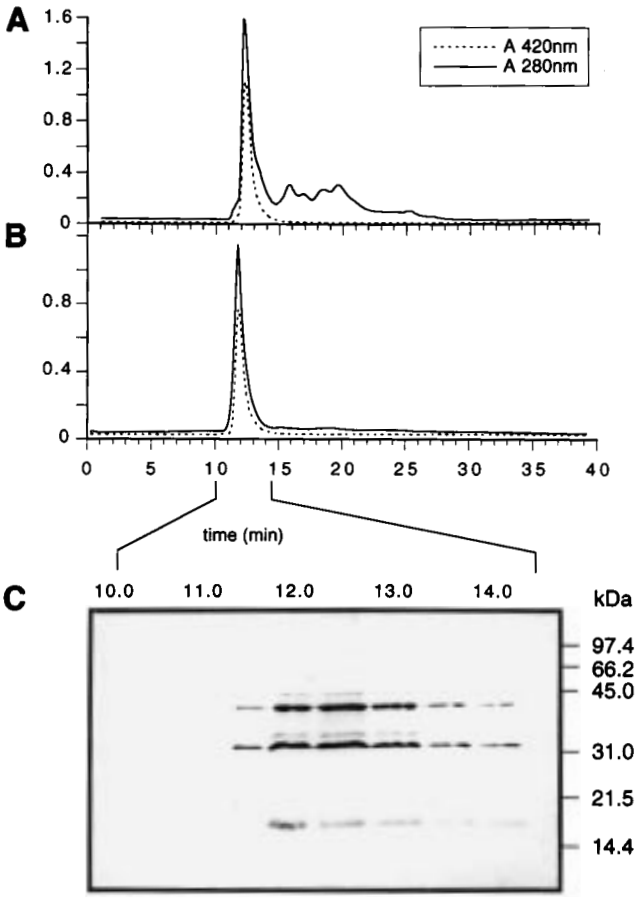

FIGURE 1: Purification of the cytochrome *c*553 complex by HPLC molecular sieve chromatography. (A) Elution profile of an enriched cytochrome *c*553 preparation after hydroxyapatite chromatography. (B) A sample of the fraction containing the cytochrome *c*553 complex, rechromatographed under the same conditions as above. (C) Aliquots from each fraction of (B) subjected to SDS−PAGE. The gel was stained with Coomassie Brilliant Blue.

All three proteins of the cytochrome *c*553 complex were found to possess blocked N-termini. Internal peptide sequences obtained after endoproteolytic cleavage with LysC and AspN did not reveal any significant homology to known proteins (not shown).

Spectroscopic Properties. Optical absorption spectra of the reduced and the oxidized 18 kDa cytochrome are presented in Figure 2. The reduced cytochrome is characterized by a rather asymmetric α-band at 553 nm. The Soret band is located at 412 nm in the oxidized state and at 417 nm in the reduced state. At low temperatures, the α-band splits, and the whole absorption range is shifted about 3 nm to shorter wavelengths. Optical equilibrium redox titrations of the purified cytochrome *c*553 complex at pH 7.0 can be fitted with a Nernst equation for a single electron transition with $E_m = +215$ mV (Figure 3).

The EPR spectrum of the purified cytochrome *c*553 complex in Figure 4a shows the characteristic bands of a low-spin *c*-type cytochrome with $g_z = 3.048$ and $g_y = 2.238$. The g_x trough could not be detected. In addition, a Cu(II) signal at $g = 2.058$ was observed. The EPR spectrum recorded from membrane fragments (Figure 4b) shows

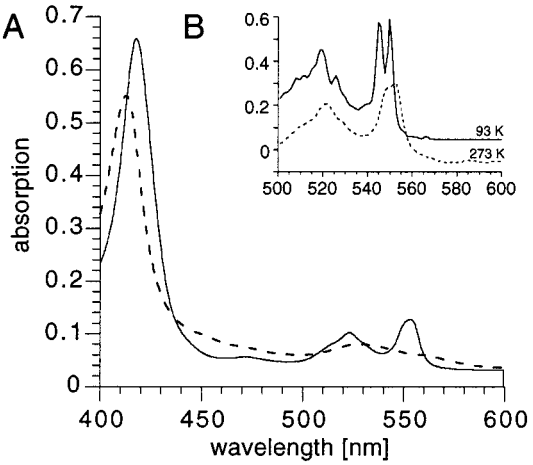

FIGURE 2: Optical absorption spectra of the 18 kDa cytochrome *c*553 from *H. gestii*. (A) The spectra were obtained using a scan rate of 120 nm/min for samples in 20 mM Tris-HCl, pH 8.0, 0.3% cholate, at 24 °C. The concentration of cytochrome was 200 μM. The protein was oxidized with $K_3Fe(CN)_6$ (dotted line) and reduced by the addition of $Na_2S_2O_4$ (solid line). (B) The α- and β-bands of the reduced cytochrome at 293 and 93 K.

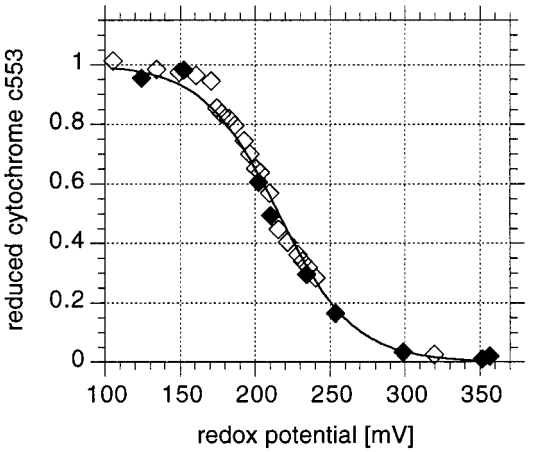

FIGURE 3: Redox titration of the 18 kDa cytochrome *c*553. The following mediators were present at 10 μM concentration each: 1,4-benzoquinone, 5-OH-1,4-naphthoquinone, duroquinone, variamine blue, benzyl viologen, methyl viologen, neutral red. Titrations were carried out at 20 °C, in 50 mM MOPS/Na, pH 7.5, 0.3% Mega 9, with ferricyanide for the oxidizing direction and with dithionite for the reducing direction. The amount of reduced cytochrome *c*553 was measured by monitoring the absorption difference $A_{536} - A_{553}$. The data obtained were fitted with a Nernst curve for a one-electron transition with $E_m = +215 \pm 10$ mV.

signals with almost the same *g*-values as the purified cytochrome *c*553 complex with $g_z = 3.036$ and $g_y = 2.263$. The copper signal did not appear in the membrane spectra. However, the presence of copper in the cytochrome *c*553 complex was confirmed by total reflection X-ray fluorescence (TXRF) analysis, where copper was detected in a 1:1 stoichiometry to the heme iron. The same ratio was obtained for different preparations of the cytochrome *c*553 complex.

EPR spectra recorded from the purified cytochrome *c*553 complex at a range of redox potentials are shown in Figure 5. The cytochrome signal disappears completely upon reduction with 30 mM ascorbate. The Cu(II) $g = 2.058$ signal diminishes stepwise when going to more reducing conditions. As judged from the very broad titration behavior, the residual copper signal seen in spectrum 5d is probably due to some cavity contamination. This result would indicate

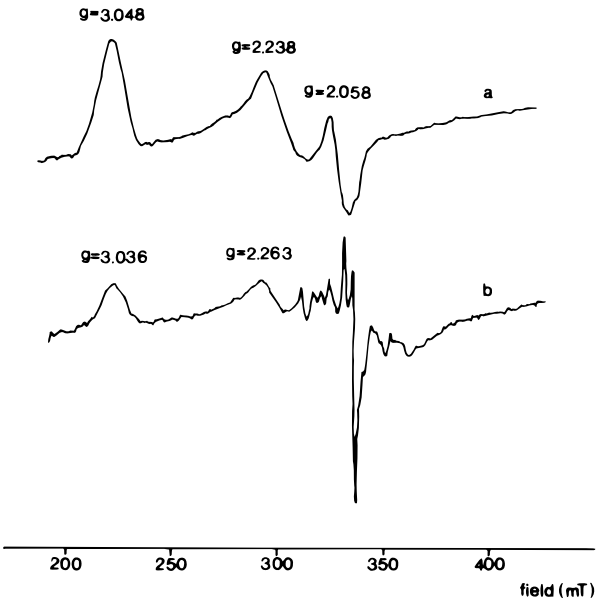

FIGURE 4: EPR spectrum of the purified cytochrome *c*553 complex (a) and of cytoplasmic membranes of *H. gestii* (b). The protein was in (a) 20 mM MOPS, pH 7.0, 0.4% cholate and in (b) 20 mM MOPS, pH 7.0, 2 mM EDTA, 10% glycerol. Instrument settings: microwave frequency, 9.415 GHz; microwave power, 6.4 mW; modulation amplitude, 20 G; temperature, 15 K. The concentration of cytochrome *c*553 was 200 μM (a), the concentration of heme *c* in (b) was 70 μM, and the concentration of BChl*g* in (b) was 250 μM.

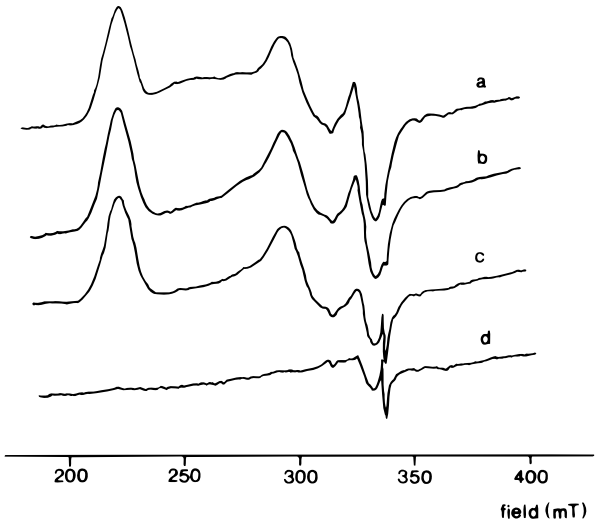

FIGURE 5: EPR spectra recorded from membranes from *H. gestii* at different redox potentials: (a) 150 μM potassium ferricyanide, (b) no addition, (c) 1 mM sodium ascorbate, (d) 30 mM sodium ascorbate. All other conditions were as in Figure 4.

that the copper in the sample has a higher redox potential than the heme *c*553.

Comparing these results to the EPR spectra of membranes from *Hb. mobilis* (*26*), it is interesting to note that the spectra under oxidizing conditions are almost identical for both organisms.

The Gene of the 18 kDa Cytochrome c553. A 250 bp PCR product was obtained from genomic DNA of *H. gestii*. The amino acid sequence deduced from this PCR product was identical to that of two internal peptide sequences. In addition, the same reading frame contained the sequence CITCH with the characteristic heme binding motive CXYCH

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-16   AGGAGGTGAAAACACAATGAAAAACCTGAAGCTTATCGGTATC  30
                M  K  N  L  K  L  I  G  I

 31   GCCGCCGTCATGGGCCTCAGCATGGTCGCTTTGACTGCGTGCAAC  75
      A  A  V  M  G  L  S  M  V  A  L  T  A  C  N

 76   AGCGGCAGCAAGGCTCCTGACGCTGCGAAACCGGCTCCGGCTCCC 120
      S  G  S  K  A  P  D  A  A  K  P  A  P  A  P

121   TCTTCGGCTCCTGCCCCGGCTCCCGCTCCGGCAGACAAGCCGACC 165
      S  S  A  P  A  P  A  P  A  P  A  D  K  P  T

166   GCTGCTCCGGCTGCCGCTGGCGCAGACGCCAAAGCCCTCTTCACC 210
      A  A  P  A  A  A  G  A  D  A  K  A  L  F  T

211   GGCAAAGGCGCTTGCATCACCTGCCACAAGCTGGGCACCGAAGGC 255
      G  K  G  A  C  I  T  C  H  K  L  G  T  E  G

256   GCTCTGGAAGTCGGCCCCAACCTGGGCGAAGTCGGCAAGAAGTAC 300
      A  L  E  V  G  P  N  L  G  E  V  G  K  K  Y

301   AACGAGGAAAAGATCTACAAGATCCTCGTGAACCCCTCCGGCGAA 345
      N  E  E  K  I  Y  K  I  L  V  N  P  S  G  E

346   GGTCTGCAAGCGACCATGCCCGCCGCTACCACTCTCTCCGACGAC 390
      G  L  Q  A  T  M  P  A  A  T  T  L  S  D  D

391   GAGAAAAAGGCCGTTGCCAAGTTCCTGGCCGAAAAGAAATAA    429
      E  K  K  A  V  A  K  F  L  A  E  K  K  *
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FIGURE 6: Nucleotide and deduced amino acid sequences of the *cyh*A gene from *H. gestii*. The amino acid sequences that were known from the internal peptide sequencing are underlined. The heme binding motif and the methionine that is the potential candidate for the sixth iron ligand are shown in boldface letters. The Shine−Dalgarno sequence preceding the structural gene is boxed.

of c-type cytochromes. The PCR product, therefore, was unambiguously derived from a gene coding for a *c*-type cytochrome.

The gene *cyh*A of the 18 kDa cytochrome *c*553 was cloned using a partial genomic bank prepared from *Eco*RI restriction fragments. A clone was identified that contained the complete gene within a 4.3 kbp genomic fragment. The gene consists of 429 bp with a Shine-Dalgarno sequence (AGGAGGT) preceding the gene 10 bp upstream of a methionine start codon (see Figure 6). The nucleotide sequence can be translated into a protein sequence of 142 amino acids. In the center, a single heme binding motif, CXYCH (here CITCH, amino acid residues +74 to +78), is located. (This numbering already takes into account the existence of a signal peptide and refers to the mature protein; see below.) Methionine +98, located 42 residues from the heme binding site in the C-terminal part of the protein, is the only candidate for the sixth ligand of the heme iron. This finding is consistent with the results of EPR spectroscopy. The molecular mass as calculated from the primary structure is 14.169 kDa. The sequence of the ORF following *cyh*A exhibits strong homology to Rieske-type iron−sulfur proteins and will be presented and discussed elsewhere.

Determination of the N-Terminal Modifications. The molecular mass calculated from the amino acid composition is 14.169 kDa, whereas a significantly lower value of 13.171 kDa was obtained from electron spray ionization mass spectrometry (ESI MS). This discrepancy could be explained by some posttranslational modification. C-Terminal protein sequencing of five residues yielded the same result as the translated protein sequence, confirming that the C-terminus is intact. Therefore, the protein most likely is modified at the N-terminus, which, however, was found to be blocked.

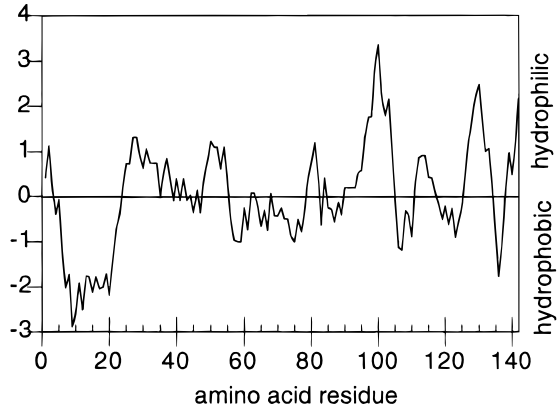

FIGURE 7: Hydropathy blot according to Kyte and Doolittle (*47*) of the *cyh*A gene product for a window of 18 amino acids.

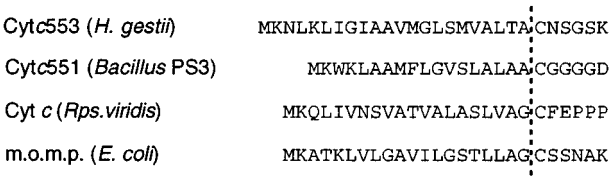

FIGURE 8: Comparison of the N-terminal amino acid sequence from the 18 kDa cytochrome *c*553 with the N-terminal signal peptides from bacterial lipoproteins: cytochrome *c*551 from *Bacillus* PS3 (*10*), the tetraheme cytochrome *c* from *Rhodopseudomonas viridis* (*37*), "major outer membrane protein" from *E. coli* (m.o.m.p.) (*48*). The putative signal peptidase cleavage sites are indicated as a dotted line.

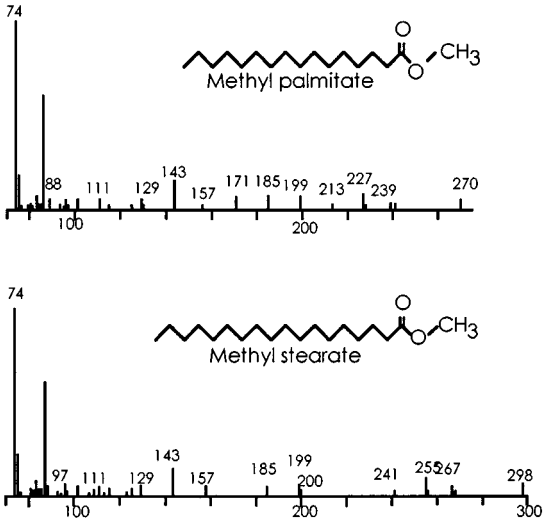

FIGURE 9: Electron impact mass spectra of the fatty acid methyl esters prepared from the isolated 18 kDa cytochrome *c*553. The upper fragmentation pattern is attributed to methyl palmitate, the lower to methyl stearate.

The Kyte−Doolittle hydrophobicity plot in Figure 7 demonstrates that the cytochrome primary structure is divided into a hydrophobic part of 18 amino acids at the N-terminus and a hydrophilic domain binding the heme prosthetic group. The hydrophobic stretch is preceded by the N-terminal methionine and a lysine. This feature is characteristic for N-terminal signal peptides. Figure 9 shows a comparison of the N-terminal sequence of the gene product of *cyh*A from *H. gestii* with the amino acid sequences of other bacterial membrane proteins. The stretch of 18 nonpolar residues is followed by alanine and cysteine in positions −1 and +1 (see Figure 8) forming a potential cleavage site for the signal

peptidase II. The molecular mass of the processed cytochrome *c*553 without the 22 N-terminal amino acid residues is 11.941 kDa. This value is lower than the molecular mass determined by ESI MS, suggesting that the cytochrome is posttranslationally modified at the N-terminus. After removal of the signal peptide, which is the only hydrophobic domain of the cytochrome, the membrane contact may be mediated by covalently bound fatty acid molecules. This kind of structure has been described for several bacterial lipoproteins (*36*, *37*, *10*). In these proteins, a signal peptide is cleaved off, leaving a cysteine at the N-terminus. This cysteine is modified by forming a thioether linkage with a glycerol moiety that contains two fatty acid molecules, which are bound as esters. If the N-terminal cysteine of cytochrome *c*553 carries a diacylglycerol, the molecular mass for the resulting protein would be in good agreement with the measured molecular mass (see Discussion). Two different fatty acid molecules can be identified as methyl esters in hydrolysates from the isolated cytochrome *c*553. The fragmentation patterns of the fatty acid methyl esters are given in Figure 9. They can clearly be attributed to the methyl esters of palmitate (C16) and stearate (C18). This result suggests that one stearate and one palmitate are bound to cytochrome *c*553. Since the cytochrome is blocked at the N-terminus, the cysteine must carry an additional modification, for example, an acylation or acetylation.

DISCUSSION

As proposed by Wood (*38*), all c-type cytochromes in Gram-positive bacteria should either be periplasmic or be bound to the periplasmic side of the cell membrane. Gram-positive bacteria do not possess a periplasmic compartment and therefore, with some exceptions, no soluble *c*-type cytochromes (*39*). For the association of *c*-type cytochromes with the membrane, two general strategies are found. In the case of the 13 kDa cytochrome *c*550 from *Bacillus subtilis* and the cytochrome c_y from *Rhodobacter capsulatus*, the membrane contact is established by an N-terminal hydrophobic sequence which was shown to be an unprocessed signal peptide (*7*, *40*). The protein sequence of the 18 kDa cytochrome *c*553 from *H. gestii* also contains an N-terminal signal peptide, but the results of the mass spectrometry together with the discovery of the two fatty acids strongly suggest that this cytochrome is attached to the membrane by two covalently bound fatty acid molecules. The mass of amino acid residues +1 to +122, a protoheme, an acetyl residue, a glycerol residue, one palmitate, and one stearate is 13.176 kDa = 11.941 + 0.618 + 0.043 + 0.073 + 0.239 + 0.267 − 6 (the mass of six hydrogen atoms has to be subtracted for the establishment of covalent bonds) which is very close to the mass determined by ESI MS. A model for the structure of the mature, processed cytochrome *c*553 is presented in Figure 10. The acylation of the N-terminus and also the existence of the glycerol and the relative position of the fatty acid residues on the glycerol have not been confirmed. These details of the proposed structure are therefore to be proven experimentally.

In this report, we show that the 18 kDa cytochrome *c*553 which is the most abundant cytochrome in the membranes of *H. gestii* can be purified in three steps as a complex with two other proteins of 32 and 42 kDa. The three proteins could not be separated under native conditions. Only

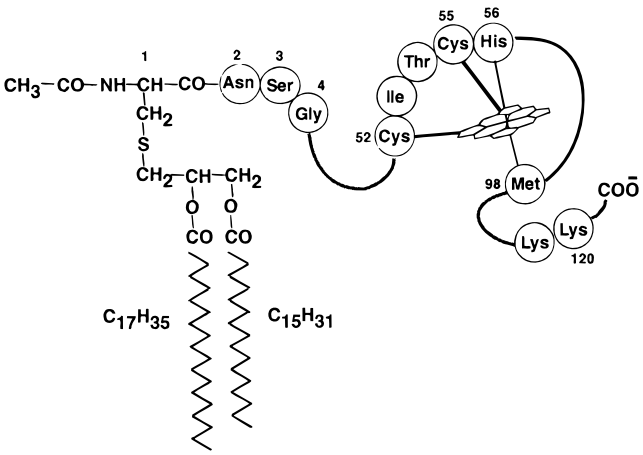

FIGURE 10: Model for the proposed structure of the mature, processed 18 kDa cytochrome *c*553. The existence of an acetyl residue at the N-terminus as well as the relative position of the two fatty acid residues on the glycerol component still have to be proven.

strongly denaturing conditions such as the incubation at 80 °C in the presence of 5% SDS or chromatography with an organic solvent mixture (*41*) led to the separation of the three components. Furthermore, the three proteins eluted in a homogeneous fraction from molecular sieve HPLC. All these observations suggest that the association of the three proteins is not due to incidental copurification, but that they constitute a complex.

Nitschke et al. (*26*) describe the partial purification of an 18 kDa cytochrome *c*553 from *Hb. mobilis* that appears in the upper part of a 15−50% sucrose density gradient. This cytochrome is characterized by an optical absorption spectrum similar to that observed for the 18 kDa cytochrome *c*553 from *H. gestii*, an EPR g_z peak at 3.03, and a redox midpoint potential of +160 mV. Apart from the redox potential, these two cytochromes seem to be comparable. Since the large discrepancy of the redox potentials (about 50 mV) cannot exclusively be explained by minor alterations in the protein conformation due to different purification protocols using different detergents, we rather attribute this discrepancy to species differences.

While Nitschke et al. (*26*) could not rule out that the 18 kDa cytochrome *c*553 purified from *Hb. mobilis* contained more than one heme with indistinguishable redox potentials, we are able to determine unambiguously that there is only a single heme *c* binding site. Despite the biochemical data obtained, it is still difficult to give precise details about the function of the cytochrome *c*553 complex. In the following section, we discuss different approaches to relate the biochemical information to the possible role of the cytochrome *c*553 complex in the electron-transfer chain of *H. gestii*.

Possible Involvement of the 18 kDa Cytochrome c553 in Photosynthetic Electron Transfer. In membranes of *Hb. chlorum*, the time dependence of cytochrome *c*553 oxidation was observed that corresponds to the reduction of the photooxidized primary donor $P798^+$ of the photosynthetic RC (*18*−*21*). Since the different heme proteins are optically indistinguishable, it is difficult to assign the observed kinetics to a particular protein. Both the 50 kDa and the 18 kDa cytochromes are discussed as candidates for being the immediate donor to $P798^+$ (*26*). In whole cells of *Hb.*

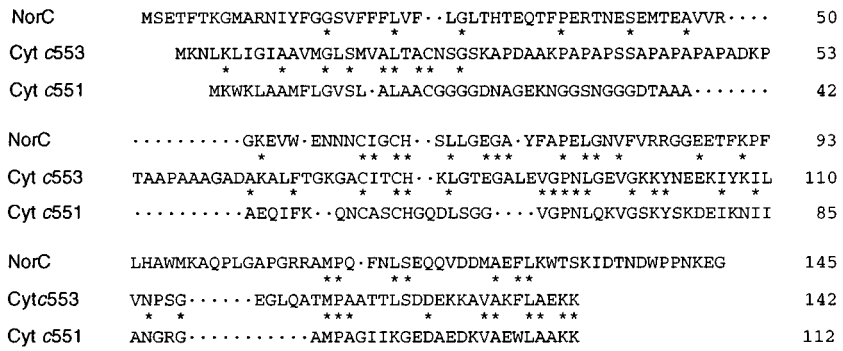

FIGURE 11: Comparison of the amino acid sequences of the 18 kDa cytochrome *c*553 from *H. gestii* with the 17 kDa cytochrome *c* subunit NorC of the nitrous reductase from *Pseudomonas stutzeri* (*43*) and the membrane-bound cytochrome *c*551 from *Bacillus* PS3 (*10*). Identical residues are marked with an asterisk.

mobilis, the redox midpoint potentials of the different heme components were determined with $E_m = +170, +120, +90$, and -60 mV (*22*, *27*). The photooxidized primary donor $P798^+$ in *Hb. mobilis* has a redox potential of $+240$ mV (*22*). In most photosynthetic organisms, the difference of the redox potentials between the photooxidized primary donor and the associated cytochrome *c* is bigger than 100 mV. Despite the above-discussed species differences between *Hb. mobilis* and *H. gestii*, we would assume that the redox properties of P798 and the c-type cytochromes are at least of the same order of magnitude. The 18 kDa cytochrome *c*553 can therefore still be considered as a possible donor to $P798^+$. The function of the 32 and 42 kDa proteins, however remains entirely unclear in this context.

Possible Function of the Cytochrome c553 Complex on the Basis of Protein Sequences. The peptide sequences obtained from the 32 and 42 kDa subunits from the cytochrome *c*553 complex do not display any homology to known protein sequences that could hint at a possible function of the complex. However, the genes coding for these proteins have not been cloned, and complete sequence information is missing. Similarly, no homologies to known *c*-type cytochromes could be found for the 18 kDa cytochrome *c*553. We therefore compared the protein sequence of cyhA with protein sequences of cytochromes which are still without a counterpart among the known *c*-type cytochromes. In *Chlorobiom vibrioforme*, an 18 kDa cytochrome *c*551 was purified with the RC (*23*) that was identified as a monoheme cytochrome by the protein sequence derived from the gene. This cytochrome is suggested to be the electron donor to $P840^+$ although this role could not be proved unambiguously. Another small c-type cytochrome, cytochrome c_y, that is involved in an alternative electron-transfer pathway to the RC, was observed in *Rhodobacter capsulatus* (*42*). The protein sequences of both cytochromes do not display any significant homology to the protein sequence of cyhA.

In contrast to the large number of genes of *c*-type cytochromes of Gram-negative bacteria, only a few sequences of *c*-type cytochromes from Gram-positive bacteria have been reported (*7*). Figure 11 shows the alignment of cyhA of *H. gestii* with the membrane-bound cytochrome *c*551 from *Bacillus* PS3 (*10*). Although the sequence similarity between these two proteins is not strong, the position of the heme binding motif as well as the position of the methionine ligand are superimposed. The *Bacillus* cytochrome is reported to be expressed abundantly under low oxygen conditions and to mediate the electron flow between the cytochrome *bc* complex and the alternative oxidase cytochrome *o* (*6*).

Copper is a common cofactor in oxidative enzymes such as the heme copper oxidases. The analogies that are found between terminal oxidases and denitrifying enzymes suggest that both sets of enzymes originate from common ancestors (*44*, *45*). The 17 kDa cytochrome *c* subunit NorC of nitrous reductase from *Pseudomonas stutzeri* (*43*) is compared to cyhA in Figure 11. Both proteins share the position of the ligands and some sequence similarities. The sequence homology of cyhA to the *Bacillus* cytochrome *c*551 and to NorC as well as the presence of copper indicate that the cytochrome *c*553 complex has some features in common with the members of the heme copper oxidase superfamily. However, due to the unusual cofactor composition (heme copper oxidases usually contain additional heme groups, like heme A, B, or O), this relation must be treated as very hypothetical. Taking into account the versatility of bacterial energy-converting systems, the cytochrome *c*553 complex can be described as a novel redox complex.

Small *c*-type cytochromes serve as mobile electron transport proteins in several energy-transducing membranes. These cytochromes are often water-soluble and diffuse in the aqueous phase. But there is also some evidence that membrane-bound cytochromes may fulfill the same function as cytochrome c_y in *Rhodobacter capsulatus* (*42*), cytochrome *c*551 in *Bacillus* PS3 (*10*), and cytochrome *c*552 in *Paracoccus denitrificans* (*46*). Obviously, diffusion in the aqueous phase is not a prerequisite for electron transport. The above-described sequence similarities of the 18 kDa cytochrome *c*553 with cytochromes involved in electron transfer to terminal oxidases may suggest that this protein is a component of a respiratory electron transfer reaction. The neighborhood of the cytochrome *c*553 gene to a Rieske-type iron−sulfur protein also points in this direction. However, since energy-generating mechanisms of the chemotrophically growing heliobacteria appear to depend primarily on fermentation (*49*), it is difficult to link the cytochrome *c*553 complex to a respiratory pathway. Therefore, further studies are necessary to determine precisely whether this complex may be related to the photosynthetic or the respiratory energy transfer.

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REFERENCES

1. Miki, K., and Okunuki, K. (1969) *J. Biochem. 66*, 831−843.
2. Miki, K., and Okunuki, K. (1969) *J. Biochem. 66*, 845−854.
3. Jakobs, A. J., Kalra, V. K., Cavari, B., and Brodie, A. F. (1979) *Arch. Biochem. Biophys. 194*, 531−541.
4. Woolley, K. J. (1987) *Arch. Biochem. Biophys. 254*, 376−379.
5. Davidson, M. W., Gray, K. A., Knaff, D. B., and Krulwich, T. A. (1988) *Biochim. Biophys. Acta 933*, 470−477.
6. Sone, N., Kutho, E., and Yanagita, Y (1989) *Biochim. Biophys. Acta 977*, 329−334.
7. von Wachenfeldt, C., and Hederstedt, L. (1990) *J. Biol. Chem. 265*, 13939−13948.
8. Hreggvidsson, G. O. (1991) *Biochim. Biophys. Acta 1058*, 52−55.
9. Yumoto, L., Fukumori, Y., and Yamanaka, T. (1991) *J. Biochem. (Tokyo) 110*, 267−273.
10. Fujiwara, Y., Oka, M., Hamamoto, T., and Sone, N. (1993) *Biochim. Biophys. Acta 1141*, 213−219.
11. Beer-Romero, P. (1986) M.A. Thesis, Indiana University, Bloomington.
12. Trost, J. T., Brune, D. C., and Blankenship, R. E. (1992) *Photosynth. Res. 32*, 11−22.
13. Fischer, M. (1990) *Biochim. Biophys. Acta 1015*, 471−481.
14. Nitschke, W., Sétif, P., Liebl, U., Feiler, U., and Rutherford, A. W. (1990) *Biochemistry 29*, 11079−11088.
15. van de Meent, E. J., Kleinherenbrink, F. A. M., and Amesz, J. (1990) *Biochim. Biophys. Acta 1015*, 223−230.
16. Liebl, U., Mockensturm-Wilson, M., Trost, J., Brune, J., Blankenship, R. E., and Vermaas, W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7124−7128.
17. Nitschke, W., and Rutherford, A. W. (1991) *Trends Biochem. Sci. 16*, 241−245.
18. Fuller, R. C., Sprague, S. G., Gest, H., and Blankenship, R. E. (1985) *FEBS Lett. 182*, 345−349.
19. Prince, R. C., Gest, H., and Blankenship, R. E. (1985) *Biochim. Biophys. Acta 810*, 377−384.
20. Vos, M. H., Klaassen, H. E., and van Gorkom, H. J. (1989) *Biochim. Biophys. Acta 973*, 163−169.
21. Kleinherenbrink, F. A. M., and Amesz, L. J. (1993) *Biochim. Biophys. Acta 1143*, 77−83.
22. Nitschke, W., Liebl, U., Matsuura, K., and Kramer, D. M. (1995) *Biochemistry 34*, 11831−11839.
23. Okkels, J. S., Kjær, B., Hansson, Ö., Svendsen, I., Møller, B. L., and Scheller, H. V. (1992) *J. Biol. Chem. 267*, 21139−21145.
24. Trost, J. T., and Blankenship, R. E. (1989) *Biochemistry 28*, 9898−9904.
25. Nitschke, W., and Liebl, U. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. III, pp 507−510, Kluwer Academic Publishers, Dordrecht, The Netherlands.
26. Nitschke, W., Schoepp, B., Flo*β*, B., Schricker, A., Rutherford, A. W., and Liebl, U. (1996) *Eur. J. Biochem. 242*, 695−702.
27. Madigan, M. T. (1992) in *The Prokaryotes* (Balows, A., Trüper, H. G., Dworkin, M., and Schleifer, K.-H., Eds.) 2nd ed., pp 1982−1992, Springer, New York.
28. Fling, S. P., and Gregerson, D. S. (1986) *Anal. Biochem. 155*, 83−88.
29. Thomas, P. E., Ryan, D., and Levin, W. (1976) *Anal. Biochem. 75*, 168−176.
30. Singer, S. J. (1971) in *Structure and Function of Biological Membranes* (Rothfield, I. L., Ed.) pp 145−222, Academic Press, New York.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboatory Press, Cold Spring Harbor, NY.
32. Laussermair, E., and Oesterhelt, D. (1992) *EMBO J. 11*, 777−783.
33. Salvestrin, H. (1995) Thesis, Johann Wolfgang Goethe-Universität, Frankfurt am Main.
34. Dutton, P. L. (1978) *Methods Enzymol. 54*, 411−435.
35. Aase, B., Jantzen, E., Bryn, K., and Ormerod, J. (1994) *Photosynth. Res. 41*, 67−74.
36. Hantke, K., and Braun, V. (1973) *Eur. J. Biochem. 34*, 284−296.
37. Weyer, K. A., Schäfer, W., Lottspeich, F., and Michel, H. (1987) *Biochemistry 26*, 2909−2914.
38. Wood, P. M. (1983) *FEBS Lett. 164*, 223−226.
39. Jacobs A. J., Kalra, V. K., Cavri, B., and Brodie, A. F. (1979) *Arch. Biochem. Biophys. 194*, 531−541.
40. Myllykallio, H., Jenney, F. E., Moomaw, C. R., Slaughter, C. A., and Daldal, F. (1997) *J. Bacteriol. 179*, 2623−2631.
41. Feick, R. G., and Shiozawa, J. A. (1990) *Anal. Biochem. 187*, 205−211.
42. Jenney, F. E., Jr., and Daldal, F. (1993) *EMBO J.*, 1283−1293.
43. Zumft, W. G., Braun, C., and Cuypers, H. (1994) *Eur. J. Biochem. 219*, 481−490.
44. García-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R. B. (1994) *J. Bacteriol. 176*, 5587−5600.
45. Saraste, M., and Castresana, J. (1994) *FEBS Lett. 341*, 1−4.
46. Turba, A., Jetzek, M., and Ludwig, B. (1995) *Eur. J. Biochem. 231*, 259−265.
47. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105−132.
48. Nakamura, K., and Inouye, M. (1979) *Cell 18*, 1109−1117.
49. Kimble, L. K., Stevenson, A. K., and Madigan, M. T. (1994) *FEMS Microbiol. Lett. 115*, 51−56.

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