Liebman, P. A., & Pugh, E. N., Jr. (1980) *Nature (London) 287*, 734–736.
Liebman, P. A., & Pugh, E. N., Jr. (1982) *Vision Res. 22*, 1475–1480.
Lolley, R. N., & Racz, E. (1982) *Vision Res. 22*, 1481–1486.
Nakatani, K., & Yau, K.-W. (1988) *J. Physiol. 395*, 695–729.
Nambi, P., & Sharma, R. K. (1981) *Endocrinology 108*, 2025–2027.
Nambi, P., Aiyer, N. V., & Sharma, R. K. (1982) *Arch. Biochem. Biophys. 217*, 638–646.
Nicol, G. D., & Miller, W. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5217–5220.
Penn, R. D., & Hagins, W. A. (1972) *Biophys. J. 12*, 1073–1094.
Pepe, I. M., Boero, A., Vergani, L., Fanfoli, I., & Cugnoli, C. (1986) *Biochim. Biophys. Acta. 889*, 271–276.
Pfeuffer, T., & Helmreich, E. J. M. (1975) *J. Biol. Chem. 250*, 867–876.
Pugh, E. N., Jr., & Cobbs, W. H. (1986) *Vision Res. 26*, 1613–1643.
Pugh, E. N., Jr., & Lamb, T. D. (1990) *Vision Res. 30*, 1923–1948.
Robinson, W. E., & Hagins, W. A. (1979) *Nature (London) 280*, 398–400.
Sather, W. A., & Detwiler, P. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 9290–9294.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106–12109.
Sitaramayya, A., Harkness, J., Parkes, J. H., Gonzales-Oliva, C., & Liebman, P. A. (1986) *Biochemistry 25*, 651–656.
Sitaramayya, A., Casadevall, C., Bennett, N., & Hakki, S. I. (1988) *Biochemistry 27*, 4880–4887.
Tanaka, J. C., Furman, R. E., Cobbs, W. H., & Mueller, P. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 724–728.
Vuong, T. M., & Chabre, M. (1990) *Nature (London) 346*, 71–74.
Waldman, S. A., Lewicki, J. A., Chang, L. Y., & Murad, F. (1985) *Mol. Cell. Biochem. 57*, 155–166.
Wilden, U., Hall, S. W., & Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.
Yamanaka, G., Eckstein, F., & Stryer, L. (1985) *Biochemistry 24*, 8094–8101.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 313*, 579–581.
Yoshikami, S., & Hagins, W. A. (1973) in *Biochemistry and physiology of visual pigments* (Langer, H., Ed.) pp 245–255, Springer, New York.
Zimmerman, A. L., Yamanaka, G., Eckstein, F., Baylor, D. A., & Stryer, L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8813–8817.
Zuckerman, R., & Cheasty, J. E. (1986) *FEBS Lett. 207*, 35–41.

Assignment of the Histidine Axial Ligands to the Cytochrome b_H and Cytochrome b_L Components of the bc_1 Complex from *Rhodobacter sphaeroides* by Site-Directed Mutagenesis†

Chang-Hyon Yun,*,‡ Antony R. Crofts,‡ and Robert B. Gennis*,‡,§
Department of Biochemistry and Chemistry and Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

Received September 28, 1990; Revised Manuscript Received February 27, 1991

ABSTRACT: The cytochrome *b* subunit of the bc_1 complex contains two cytochrome components, cytochrome b_H and cytochrome b_L. Sequence comparisons of this polypeptide from a number of organisms have revealed four invariant histidines which have been postulated to be the heme ligands for the two protoheme IX prosthetic groups. In *Rhodobacter sphaeroides*, these correspond to His97, His111, His198, and His212. In this paper, the results of amino acid substitutions at each of these positions are reported. Replacement of His97 by either Asp or Asn and of His198 by Asn or Tyr resulted in loss of both cytochrome components. However, His111Asn, His111Asp, and His212Asp all resulted in the selective loss of cytochrome b_H and the retention of cytochrome b_L. Furthermore, flash kinetics studies show that the myxothiazol-sensitive quinol oxidase (Q_z) site associated with cytochrome b_L is still functional. These data support the assignment of the axial ligands to cytochrome b_H (His111 and His212) and cytochrome b_L (His97 and His198). This pairing is consistent with current models of the cytochrome *b* subunit with eight transmembrane α-helices.

The ubiquinol:cytochrome *c* oxidoreductases, also known as the bc_1 complexes, are central components in the energy-conserving electron-transfer chains of mitochondria, chloroplasts (where it is known as the b_6/f complex), and bacteria (Gabellini, 1988; Hauska et al., 1988; Crofts, 1985). These complexes manifest remarkable structural and functional similarities. All the bc_1 (and b_6f) complexes contain a cytochrome *b* subunit which appears to contribute major structural elements of both a quinol oxidase site (Q_z or Q_o) and a quinone reductase site (Q_c or Q_i). Two spectroscopically distinct cytochrome *b* components with different midpoint potentials (b_L and b_H) are found in all the bc_1 and b_6f complexes.

In a proposed Q-cycle mechanism (Mitchell, 1976; Crofts, 1985; Crofts et al., 1983), the low-potential cytochrome b_L component is located close to the quinol oxidase site, and the high-potential cytochrome b_H is close to the quinone reductase

† This research was supported by grants from the National Institutes of Health (GM35438 to R.B.G. and A.R.C. and GM26305 to A.R.C.).
* To whom correspondence should be addressed.
‡ Department of Physiology and Biophysics.
§ Department of Biochemistry and Chemistry.

0006-2960/91/0430-6747$02.50/0

site. These two sites are proposed to be on opposite sides of the membrane with the quinol oxidation occurring at the periplasmic surface in bacteria or at the intermembrane space in mitochondria. Ubiquinol is initially oxidized by the Rieske 2Fe/2S cluster located on a separate subunit, thus generating a transient ubisemiquinone which is the reductant for cytochrome b_L. Cytochrome b_H is subsequently reduced by cytochrome b_L, and the electron transfer between the two cytochromes across the membrane contributes to the transmembrane voltage generated by the bc_1 complex.

Knowledge of the axial ligands of the two cytochrome *b* components is clearly necessary for devising models of the bc_1 complex. Early EPR measurements showed that both cytochromes are 6-coordinate but they have unusual g_z values (3.4–3.8) (DeVries et al., 1979; Orme-Johnson et al., 1974). This is consistent with bis(histidine) ligation with sterically hindered imidazoles (Carter et al., 1981). Recent MCD studies (Simpkin et al., 1989) suggested that histidine–lysine ligation is also plausible for one of the hemes. However, a comparative study of more than 20 amino acid sequences of the cytochrome *b* subunits from different species shows no totally conserved lysine (Hauska et al., 1988). Lysine-288, using the numbering for *Saccharomyces cerevisiae*, is very highly conserved but is replaced by a phenylalanine in *Schizosaccharomyces pombe* (Lang & Ahne, 1985). Hence, it is very likely that both cytochrome components have bis(histidine) ligation.

It was recognized several years ago that there are only four invariant histidines in sequence comparisons of the cytochrome *b* subunits (Saraste, 1984; Widger et al., 1984). There are 2 each on putative transmembrane α-helices (numbered helices II and V in the 9 transmembrane helical models proposed by both groups), separated in most species by 13 amino acid residues. Assignment of these residues as heme axial ligands is consistent with experimental data showing that the hemes are arranged vertically with respect to the plane of the membrane (Erecinska et al., 1979) and are 21 Å apart (Link et al., 1979).

The models originally proposed for the heme ligation to the cytochrome *b* subunit featured nine transmembrane helices (Saraste, 1984; Widger et al., 1984). However, more recent studies are consistent with only eight transmembrane helices with helix IV (or helix *cd* in alternate nomenclature) being removed from the membrane. This is supported by further examination of the hydropathy profiles (Rao & Argos, 1986; Crofts et al., 1987) and by mapping the locations of mutations that confer resistance to inhibitors that block either the Q-reductase or Q-oxidase sites (diRago & Colson, 1988; Howell & Gilbert, 1988; Daldal et al., 1989; Brasseur, 1988) and by using gene fusions (Yun et al., 1991). Although each of these approaches has shown results consistent with the eight transmembrane helix model, none has provided an unambiguous demonstration. The goal of the present work is to use site-directed mutagenesis to test whether the four invariant histidines in the cytochrome *b* subunit of *Rhodobacter sphaeroides* are heme ligands and, if so, to assign each histidine to either cytochrome b_L or cytochrome b_H. It is concluded that all four histidines are essential for photosynthetic growth and that neither His111 nor His212 is a ligand for cytochrome b_L. If the four invariant histidines are heme ligands, then His111 and His212 are ligands to cytochrome b_H, and His97 and His198 are ligands to cytochrome b_L. This pairing is fully consistent with the current eight-span model of this subunit (Rao & Argos, 1986; Crofts et al., 1987; diRago & Colson, 1988; Howell & Gilbert, 1988; Daldal et al., 1989; Brasseur, 1988; Yun et al., 1991) but is not consistent with the original nine-span model (Saraste, 1984; Widger et al., 1984).

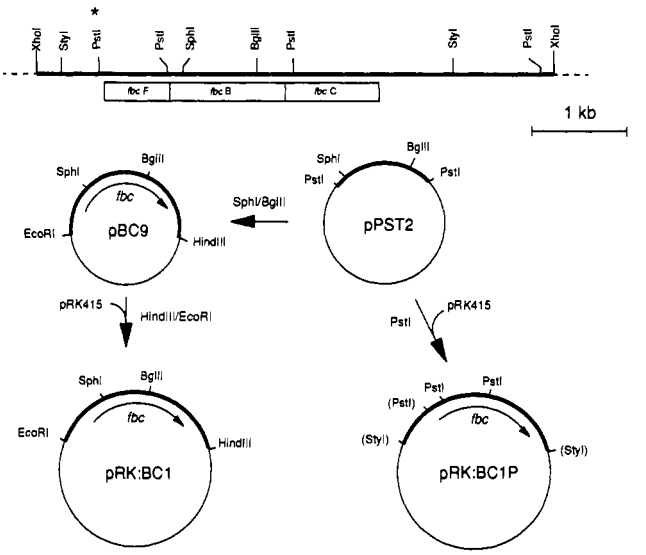

FIGURE 1: Strategy for mutagenesis of the selected histidines in the *fbc* operon of *Rb. sphaeroides*. pPST2 is an M13mp18 derivative carrying the 1.3-kb *PstI* fragment of the *fbc* operon. pBC9 is a pUC9 derivative containing a 5.6-kb *XhoI* fragment. The strategy used for the site-directed mutagenesis is described under Materials and Methods.

Materials and Methods

Materials. All restriction endonucleases and nucleic acid modifying enzymes were obtained from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., Boehringer Mannheim Biochemicals, or International Biotechnologies, Inc. Synthetic deoxyoligonucleotides used as sequencing primers within the *fbc* operon were synthesized at the Biotechnology Center of the University of Illinois at Urbana—Champaign on an Applied Biosystem Model 380A DNA synthesizer. DNA polymerase I Klenow fragment used for sequencing was the product of Boehringer Mannheim Chemicals (Indianapolis, IN).

Bacteria and Plasmids. Escherichia coli strains were grown in L broth at 38 °C. Plasmids pBC9 (Figure 1) and pRK415 (Keen et al., 1988) and their derivatives were maintained in the presence of ampicillin (50 μg/mL) or tetracycline (15 μg/mL), respectively. When appropriate, selective media were supplemented with 5-bromo-4-chloro-3-indoyl β-D-galactoside (Xgal) at 40 μg/mL and with 50 μg/mL isopropyl β-D-thiogalactoside (IPTG).

Rb. sphaeroides strains were grown photosynthetically or aerobically in Sistrom's minimal medium A (Leuking et al., 1978) by vigorous shaking. Growth medium used with strains containing pRK415 derivatives was supplemented with 1 μg/mL tetracycline. Semiaerobic growth was achieved by growing cells with vigorous shaking until the cells reached early exponential phase, after which the supply of oxygen was limited by low shaking, thereby inducing the formation of cytoplasmic membrane invaginations (Kiley & Kaplan, 1988).

Genetic Manipulations. Restriction enzyme digestions and methods for large-scale isolation or minipreparation of plasmid DNA are described elsewhere (Maniatis et al., 1982). DNA sequencing was performed by the dideoxy sequencing method of Sanger (Sanger et al., 1980). The universal 17-mer M13 primer (purchased from Amersham) as well as internal sequencing primers (synthesized in the Biotechnology Center at the University of Illinois) were used for dideoxy sequencing reactions.

Table I: Summary of the Codon Alterations of the Invariant Histidines

Mutation	Nucleotide Change	Oligonucleotide
His97 → Asp	CAT → GAT	GC TAC CTG GAT GCG AAC G
His97 → Asn	CAT → AAT	AAT
His111 → Asp	CAC → GAC	TC TAT CTG GAC ATC TTC C
His111 → Asn	CAC → AAC	AAC
His111 → Tyr	CAC → TAC	TAC
His198 → Asn	CAC → AAC	TC TCG CTG AAC TAC CTG C
His198 → Tyr	CAC → TAC	TAC
His212 → Asp	CAC → GAC	GTG GCC ATC GAC ATC TGG

Strategy for Site-Directed Mutagenesis. Figure 1 shows the strategy used for the construction of the site-directed mutations in the *fbc*B gene of *Rb. sphaeroides*. pPST2 contains the 1.3-kb *Pst*I restriction fragment from the *fbc* operon which was used as the template for the mutagenesis. Site-directed mutagenesis either was done by using the Amersham oligonucleotide-directed mutagenesis kit or was done according to Vandeyar et al. (1988). The oligonucleotides used for the mutagenesis are listed in Table I. All the mutations were characterized by DNA sequencing.

The mutagenesis of His111, His198, and His212 was done on the 800 bp *Sph*I–*Bgl*II fragment containing most of the *fbc*B gene (Figure 1). This was transferred from pPST2 to pBC9, to replace the wild-type DNA sequence, so the mutant allele of the *fbc*B gene could be expressed within the intact *fbc* operon. The mutant *fbc*, contained within the 5.6-kb *Eco*RI–*Hin*dIII fragment in pBC9, was then cloned into the broad host range vector pRK415 (Keen et al., 1988), yielding pRK:BC1. Mutagenesis of the His97 residue was carried out by using a different strategy, because this residue is not encoded on the *Sph*I–*Bgl*II fragment. The *Pst*I site just to the left of the *fbc*F gene (Figure 1, PstI*) was eliminated by a neutral mutation. The 4.3-kb *Sty*I fragment containing the *fbc* operon, after filling in with Klenow, was cloned into the *Hin*dIII–*Eco*RJ sites of pRK415, yielding pRK:BC1P. The 1.3-kb *Pst*I fragment from pPST2 could then be mutageneized and cloned directly into this vector. The pRK415 derivatives containing the mutant *fbc* alleles were moved into strain BC17 by biparental conjugation (Donohue et al., 1988). Strain BC17 has a chromosomal deletion of the *fbc* operon (Yun et al., 1990).

Optical Redox Titrations. Chromatophores were prepared (Bowyer et al., 1979) and chemical redox titrations were performed as previously described (Dutton, 1978). The following mediators were used (5 μM each): 2-hydroxy-1,4-naphthoquinone, pyocyanine, 1,2-naphthoquinone, 1,4-naphthoquinone, duroquinone, 2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazine methosulfate, and *N*-ethylphenazine ethosulfate. Benzyl viologen was added to a final concentration of 70 μM, and the chromatophore concentration was 4 mg of protein/mL. Anaerobically prepared dithionite and potassium ferricyanide solutions (10 mM stock solutions) were used as reductant and oxidant, respectively. Usually eight optical scans were averaged at each value of the solution potential (E_h), which was varied in increments of approximately 10–20 mV. Typically, spectra were taken from 520 to 580 nm. Data were analyzed by choosing sets of wavelength pairs appropriate for monitoring reduced *b*- and *c*-type cytochromes using a program written by Dr. E. Berry (unpublished) to find the best fits for component spectra and midpoint potentials of each independently titrating species. The program uses a nonlinear least-squares method to simultaneously fit a number of curves to a sum of Nernstian terms.

Immunochemical Analysis. Electrophoresis was carried out with 12.5% sodium dodecyl sulfate–polyacrylamide gels, and Western blotting (Burnette, 1981) was performed by using rabbit anti-bc_1 polyclonal antibodies (Yun et al., 1990). The immunoblot was developed by using goat anti-rabbit alkaline phosphatase conjugate as described for the Promega ProtoBlot AP kit (Promega Biotec, Madison, WI).

Photoinduced Kinetic Spectrophotometric Analysis. The kinetic single-beam spectrophotometer used has been described elsewhere (Crofts & Wang, 1989). The spectrophotometer was linked to an IBM PC-AT compatible computer which, through a home-built potentiostat, automatically adjusted the ambient solution potential. The suspension of chromatophores was placed in a stirred anaerobic redox cuvette and kept at constant temperature (25 ± 0.1 °C) by circulating water from a thermostated water bath. Kinetics of redox changes in the total *c*-type cytochrome (cytochromes $c_1 + c_2$), cytochrome b_H, cytochrome b_L, and the reaction center (P) were measured as previously described (Crofts et al., 1983; Meinhardt & Crofts, 1983).

Results

Site-directed mutagenesis was performed by using mixed oligonucleotides which were designed to change each of the four invariant histidines to asparagine, aspartate, and tyrosine. Not all of the mutations were obtained, but the set of mutations which was generated was sufficient to answer the questions being addressed. The following mutants were characterized (see Table I): His97 to Asn and to Asp (H97N, H97D); His111 to Asn, Asp, and Tyr (H111N, H111D, H111Y); His198 to Asn (H198N); and His212 to Asp (H212D). The function of each of these mutant bc_1 complexes was tested by expressing the *fbc* alleles in a strain of *Rb. sphaeroides* (BC17) from which the wild-type chromosomal *fbc* was deleted (Yun et al., 1990). None of the mutations conferred on strain BC17 the ability to grow photosynthetically, suggesting that each of these histidines is essential. These strains, however, grow well aerobically due to the presence of a bc_1-independent branch of the respiratory chain. At low oxygen tension, the photosynthetic components can be induced even though the cells are still growing by aerobic respiration (Kiley & Kaplan, 1988). Hence, vesicular membranes (chromatophores) can be prepared which contain the mutational variants of the bc_1 complex as a component of an otherwise functional photosynthetic apparatus. These membranes were used to evaluate the optical, electrochemical, and kinetic properties of the bc_1 mutants.

Figure 2 shows reduced minus oxidized spectra for membranes containing the various bc_1 mutants. Each panel has three spectra. The ascorbate – ferricyanide difference spectrum (lowest trace) reveals primarily the high-potential component, including the total *c*-type cytochromes ($c_1 + c_2$); the middle spectrum [dithionite (5 s) – ascorbate] includes cytochrome b_H as the main component, and other components rapidly reduced by dithionite. The upper spectrum [dithionite (10 min) – dithionite (5 s)] shows components reduced only after prolonged incubation with dithionite; the low-potential cytochrome b_L is reduced by this treatment, and is the major component when present. Although some variability in induction of the photosynthetic chain under the growth conditions used is apparent, the traces show several clear effects. Figure 2B is the spectrum of membranes from strain BC17 from which the bc_1 complex has been deleted. This strain is entirely lacking all subunits of the bc_1 complex. Note that

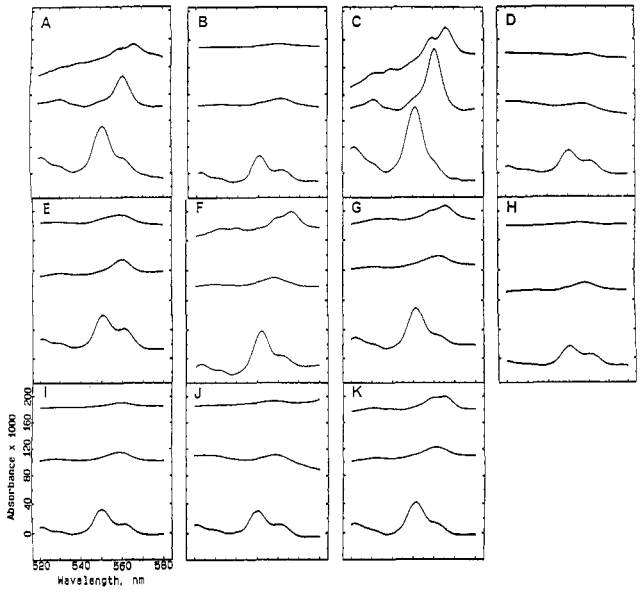

FIGURE 2: Optical difference spectra of membranes with approximately 3 mg/mL protein from the parent strain (Ga), BC17 (*fbc* deletion), BC17C (BC17 with plasmid-borne *fbc*), and the strains expressing histidine mutations. Chromatophores were prepared as described in the text. Samples were fully oxidized with potassium ferricyanide as an oxidant. High-potential components including *c*-type cytochromes were reduced by adding a small amount of solid sodium ascorbate. The midpotential components, including cytochrome b_H, were reduced by adding a few grains of dithionite, and spectra were taken within 5 s. Further incubation (typically 10 min) with dithionite reduced the low-potential cytochrome b_L. The bottom spectrum in each frame is the difference spectrum showing predominantly total *c*-type ($c_1 + c_2$) cytochromes. The middle and upper spectra represent mainly high- and low-potential *b*-type cytochromes, respectively. Strains shown are (A) Ga, (B) BC17, (C) BC17c, (D) H97D, (E) H97N, (F) H111D, (G) H111N, (H) H111Y, (I) H198N, (J) H198Y, and (K) H212D.

there are other *b*-type cytochromes in the membrane which, in particular, contribute to the two lower spectra which are where cytochrome b_H would be observed. The same strain which has the *fbc* deletion complemented by a plasmid-borne copy of the *fbc* operon is analyzed in Figure 2C. Note the double peak in the upper spectrum which is diagnostic of the split α-band of cytochrome b_L. Also, the middle spectrum has a substantially higher amplitude due to the presence of cytochrome b_H. The same features are apparent in strain Ga (Figure 2A), which contains only the chromosomal copy of the *fbc* operon.

The spectra of the bc_1 mutants reveal that both cytochromes b_H and b_L are absent from five strains: H97N (Figure 2E), H97D (Figure 2D), H198Y (Figure 2J), H198N (Figure 2I), and H111Y (Figure 2H). In each of these strains, the cytochrome *b* and c_1 subunits were undetectable. The major *b*-type cytochrome present in these strains, which is revealed in the middle spectrum, has features similar to that which is in BC17, the *fbc* deletion control (Figure 2B). In particular, the amplitude is low, the α absorption band (ca. 560 nm) is broad, and the electrochemical midpoint potential is about 140 mV (not shown). In addition, careful examination of the spectrum of the high-potential *c*-type cytochromes (lower spectrum) shows that all five of these mutants as well as the deletion strain (BC17) show a peak at 550 nm, which indicates a lack of cytochrome c_1. When present, cytochrome c_1 (λ_{max} for α-band peak at 552–553 nm) results in an apparent red-shift in the spectroscopic peak to 551.5 nm. Hence, five mutants lack all three of the heme components associated with the bc_1 complex.

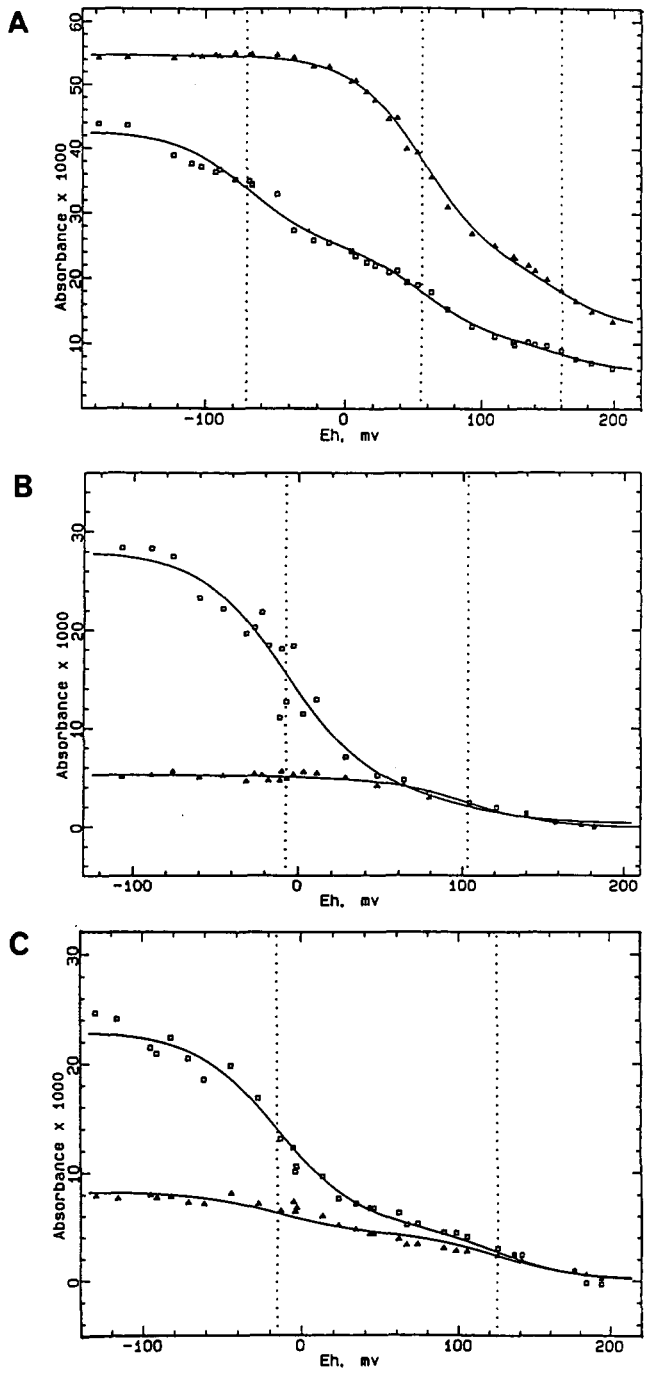

FIGURE 3: Redox titrations at pH 7.0 of the *b*-type cytochromes in membranes prepared from strains expressing the wild-type (Ga) and mutant bc_1 complexes. (A) Ga; (B) H111N; and (C) H212D. Analysis of the *b*-type cytochromes was done at two wavelength pairs, 561–569 nm (△) and 566–575 nm (□). The curves drawn through the points are best Nernstian fits using a program written by Dr. E. Berry. The dotted vertical lines indicate the midpoint potentials of each component.

More interesting results were obtained with H111D, H111N, and H212D. These all have clearly retained cytochrome b_L whereas cytochrome b_H is much reduced or absent (see Figure 2F,G,K). The upper spectrum in each has the split peak (559 and 566 nm) indicative of cytochrome b_L, although the shapes of the spectra appear somewhat altered compared to that of the native enzyme (Figure 2A,C). Potentiometric titrations of the *b*-type cytochromes in the membranes of mutants H111N and H212D are shown in Figure 3 along with the wild-type control (strain Ga). Full spectra were recorded at each solution potential, and selected wavelength pairs were

Table II: Summary of Some Characteristics of Strains Containing Histidine Mutations

strain	cytochrome b_L			cytochrome b_H			cytochrome c_1			photosynthetic
	$E_{m,7}$	peak	concn[a]	$E_{m,7}$	peak	concn[a]	$E_{m,7}$	peak[d]	subunits[b]	growth
G_a (wild-type complex)	−87	559, 566	0.41	52	560.5	0.47	265	551.5	b,c_1, FeS	+++
BC17C (complemented deletion)	−92	559, 566	0.61	56	560.5	0.68	267	551.5	b,c_1, FeS	+++
BC17(bc_1-) (bc_1 deletion)								550	none	−
H111N	−8	559, 556	0.55				268	551.5	b,c_1, FeS	−
H111D	ND[c]	559, 565	0.45				ND[c]	551.5	b,c_1, FeS	−
H111Y									FeS	−
H212D	−14	559, 565	0.64				272	551.5	b,c_1, FeS	−
H097D								550	FeS	−
H097N								550	FeS	−
H198N								550	FeS	−
H198Y								550	FeS	−

[a] Concentration relative to the amount to cytochromes $c_1 + c_2$. The extinction coefficients used (mM^{-1} cm^{-1}) were 13 at 566–575 nm for cyt b_L, 25 at 561–569 nm for b_H, and 19.5 at 551–542 nm for cyt $c_1 + c_2$ (Boyer et al., 1981; Gabellini & Hauska, 1983; Meinhardt & Crofts, 1983). [b] Subunits detectable by Western blotting using polyclonal antibody generated against the purified bc_1 complex from *Rb. sphaeroides*. [c] Not determined. [d] Peak of cytochromes $c_1 + c_2$.

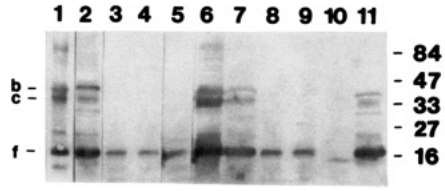

FIGURE 4: Western immunoblot analysis of membranes (chromatophores) using antibodies against the bc_1 complex from *Rb. sphaeroides*. Chromatophore proteins (60 μg) from the indicated mutants were resolved on a 12.5% SDS–PAGE gel. Lanes 1–11 contain the following: (1) purified bc_1 complex from *Rb. sphaeroides*; (2) H212D; (3) H198Y; (4) H198N; (5) H111Y; (6) H111N; (7) H111D; (8) H97N; (9) H97D; (10) BC17 (*fbc* deletion); (11) Ga (wild-type complex). The positions of the cytochrome *b*, cytochrome c_1, and the Rieske iron–sulfur subunit are indicated by the b, c, and f notation.

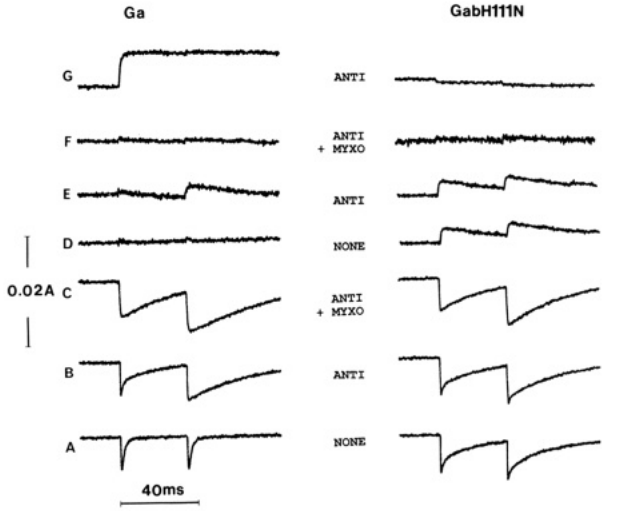

FIGURE 5: Flash-induced kinetics of cytochrome b_H ($\Delta A_{561} - \Delta A_{569}$), cytochrome b_L [$(\Delta A_{566} - \Delta A_{575}) - {}^1/_2(\Delta A_{561} - \Delta A_{569}) - 0.2\Delta A_{542}$], and cytochromes $c_1 + c_2$ ($\Delta A_{551} - \Delta A_{542}$) using chromatophores containing either wild-type (Ga) or mutant (H111N) bc_1 complex. Chromatophores were suspended to a concentration of 0.5 μM reaction center in 50 mM MOPS, pH 7.0, and 100 mM KCl. The solution was poised at 100 mV using mediators as described in the text. Also present were 2 μM valinomycin and 2 μM nigericin. Antimycin A (10 μM) (anti) and myxothiazol (5 μM) (myxo) were added where indicated.

used for the data analysis to monitor cytochrome b_L (566–575 nm) or cytochrome b_H (561–569 nm). In the control strain Ga (Meinhardt & Crofts, 1983), and in strain BC17C (complemented bc_1 deletion), cytochrome b_L titrates at about −90 mV (Table II) whereas in the mutants cytochrome b_L titrates at considerably higher potentials, −8 mV (H111N) and −14 mV (H212D). Both mutants retain cytochrome c_1 with a midpoint potential ($E_{m,7}$) at about 265 mV (Table II). The α-band of the ascorbate-reduced minus ferricyanide-oxidized spectra (lower spectrum, Figure 2) is at 551.5 nm for each of the mutants H111N, H111D, and H212D, consistent with the presence of cytochrome c_1.

Western immunoblotting of the membranes from each of these strains shows that the cytochrome *b* subunit and the cytochrome c_1 subunit are present in each of the strains which has retained cytochrome b_L (H111D, H111N, and H212D) (Figure 4, lanes 7, 6, and 2). These subunits are either absent or present at much reduced levels in each of the other mutants. Western blots also show that the Rieske Fe/S subunit is present in the membranes from all mutants.

The H111N mutant was selected for more complete characterization by flash kinetics, in order to see if cytochrome b_L was still functional despite the altered midpoint potential and despite the absence of cytochrome b_H. These data are shown in Figure 5. The membranes were poised at 100 mV so that cytochromes b_L and b_H (if present) would be nearly fully oxidized, but the high-potential components, including cytochrome c_1, cytochrome c_2, the Rieske Fe/S cluster, and the reaction center, would be completely reduced. The light flash oxidizes the bacteriochlorophyll dimer in the reaction center, and this leads to the oxidation of cytochromes c_1 and c_2, which are then re-reduced via the Rieske Fe/S center. By appropriate wavelength selection, the redox states of cytochrome b_L, cytochrome b_H, or cytochrome $c_1 + c_2$ can be monitored on the millisecond time scale following the initiation of the photooxidation. The essential features can be summarized as follows:

(*1*) *Cytochrome* b_H (*Figure 5G*). In the presence of antimycin A, the quinol oxidase site (Q_z) in the wild-type complex is functional, and in strains with a wild-type bc_1 complex (such as Ga), one of the two electrons from the quinol reduces cytochrome b_H following the first flash. This remains reduced due to the presence of the inhibitor, and subsequent flashes have little effect. In the H111N mutant, no reduction of cytochrome b_H is observed, consistent with the spectroscopic analyses which show that cytochrome b_H is missing in this mutant.

(*2*) *Cytochrome* b_L (*Figure 5D–F*). In the wild-type complex (Figure 5D), the first flash does not leave any cytochrome b_L reduced. Although electron transfer proceeds via cytochrome b_L, the kinetics of oxidation of cytochrome b_L by cytochrome b_H are so rapid that no significant transient reduction of cytochrome b_L is observed. Despite the presence of excess quinol and oxidant in the high-potential chain, no marked reduction of cytochrome b_L occurs following the first flash because partial reduction of the high-potential compo-

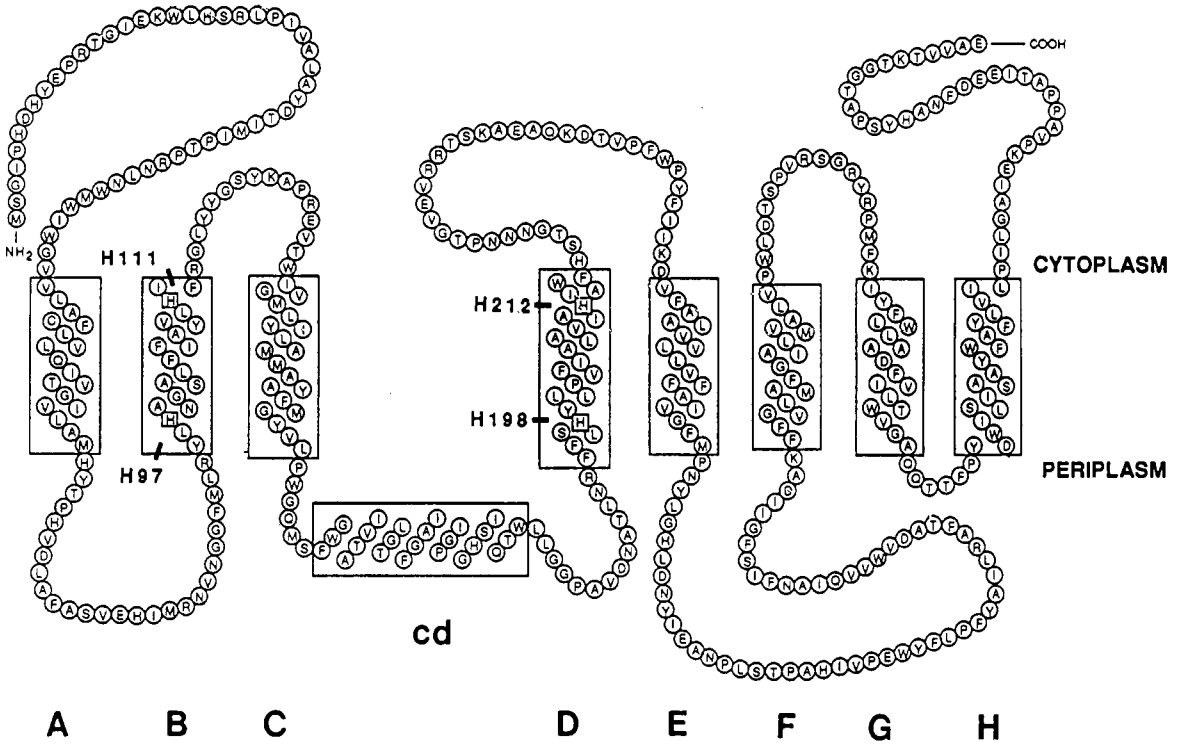

FIGURE 6: Model of the cytochrome *b* subunit of the bc_1 complex from *Rb. sphaeroides*. The four invariant histidines in helices B and D (formerly II and V) are indicated.

nents by the quinol used to reduce cytochrome b_H leaves the complex with insufficient redox drop to overcome the low equilibrium constant required to reduce cytochrome b_L (Crofts et al., 1983). However, with antimycin A, the second flash reduces cytochrome b_L, since cytochrome b_H is already trapped in the reduced form and the flash raises the potential in the high-potential chain sufficiently to overcome the barrier to cytochrome b_L reduction (Figure 5E). In contrast, with the H111N mutation, since there is no cytochrome b_H, the first flash does reduce cytochrome b_L with kinetics similar to the reduction in the wild type after flash 2 ($t_{1/2} < 1$ ms) (Figure 5D,E), which slowly reoxidizes before the second flash. In both strains, the presence of myxothiazol eliminates the reduction of cytochrome b_L. This demonstrates that in the H111N mutant, the quinol oxidase (Q_z) site is fully functional and is still myxothiazol-sensitive.

(*3*) *Cytochromes* c_1 + c_2 (*Figure 5A–C*). The myxothiazol-sensitive quinol oxidation can also be observed by monitoring cytochrome *c* status. In the presence of both antimycin and myxothiazol (Figure 5C), the rapid oxidation of cytochromes c_1 + c_2 is observed followed by a slow re-reduction. This re-reduction in the presence of the inhibitors is a leakage due to the mediators in this experiment. Comparison with the traces in Figure 5B, however, clearly shows that in the absence of myxothiazol, both the wild-type and mutant bc_1 complexes have a rapid phase of cytochrome re-reduction that is due to quinol oxidation at the Q_z site. Hence, the myxothiazol-sensitive site is still functional in the H111N mutant, as previously concluded. Comparison of the traces in Figure 5A with those in Figure 5B, however, shows that antimycin A has no influence on the kinetics observed with the H111N mutant. Antimycin A clearly influences the wild-type bc_1 complex by trapping an electron in cytochrome b_H.

These data verify that the H111N mutant lacks a functional cytochrome b_H and antimycin-sensitive quinol reductase (Q_c) site but has a functional, though perturbed, cytochrome b_L and a myxothiazol-sensitive quinol oxidase (Q_z) site.

During the physiological analysis of the above mutants, strains containing the H97N, H111N, or H198Y mutants were observed to grow photosynthetically, if aerobically grown cells were incubated under photosynthetic conditions for extended periods of time (i.e., 10–20 days), well past the time required for the growth of wild-type or other photosynthetically competent mutants. The frequency of reversion was not accurately determined, but it is estimated to be on the order of 10^{-11}–10^{-10}. DNA sequencing showed that Asn97, Asn111, and Tyr198 in the mutants had reverted to histidine, thus restoring photosynthetic growth.

Discussion

Early models of the cytochrome *b* subunit recognized that there are only four invariant histidine residues and that these are likely candidates as heme ligands for the cytochromes b_L and b_H components of the bc_1 complex (Saraste, 1984; Widger et al., 1984). Subsequent sequences of cytochrome *b* subunits all have maintained these four histidines (Gabellini, 1988; Hauska et al., 1988; Thöny-Meyer et al., 1989; Verbist et al., 1989). Spectroscopic data are consistent with both hemes having bis(histidine) ligation, but other ligands remain possible, if unlikely (Simpkin et al., 1989).

The data presented here demonstrate that substitution of residues other than histidine at each of the four positions (97, 111, 198, and 212 in *Rb. sphaeroides*) in the cytochrome *b* subunit results in the loss of one or both of the protoheme IX prosthetic groups. In three instances (H97N, H111N, and H198Y), restoration of the wild-type phenotype was shown to result from a low-frequency reversion back to histidine. These data are consistent with the contention that each of the four invariant histidines is a heme ligand and that the two hemes are ligated by pairs of histidines in between two transmembrane α-helices.

The early models of the cytochrome *b* subunit postulated nine transmembrane α-helical spans (Saraste, 1984; Widger

et al., 1984) with the hemes suspended between helix II and helix V, which ran antiparallel across the bilayer. This model places both His111 and His198 (*Rb. sphaeroides* numbering) on the same side of the bilayer, with His97 and His212 paired on the opposite side. The analysis of the H111N, H111D, and H212D mutations, however, clearly shows that His111 and His212 must be the ligands for cytochrome b_H. This pairing is inconsistent with the early nine-span model (Saraste, 1984; Widger et al., 1984), since the data require that the two helices containing these invariant histidines traverse the bilayer in a parallel manner. The results are consistent with a more recent eight-span model of the cytochrome *b* subunit in which the span assigned to helix IV is postulated not to cross the membrane (Rao & Argos, 1986; Crofts et al., 1987; diRago & Colson, 1988; Howell & Gilbert, 1988; Daldal et al., 1989; Brasseur, 1988; Yun et al., 1991). These models, supported by recent gene fusion experiments (Yun et al., 1991) and analysis of the hydrophobic and mutability moment at the helical repeat (Crofts et al., 1990a,b), place the His111–His212 pair on the cytoplasmic side of the membrane, which is also consistent with these two histidines being ligands for cytochrome b_H. The Q-cycle mechanism (Crofts, 1985; Crofts et al., 1983) places the quinol reductase site, containing cytochrome b_H, at or near the cytoplasmic surface where protons are taken up during reduction of ubiquinone. The model shown in Figure 6 is consistent with these data.

Recent analyses of missense mutants in yeast are also consistent with this model (Meunier-Lemesne et al., 1988). A change of Cys133 (equivalents to Val148 in *Rb. sphaeroides*) to tyrosine results in the loss of cytochrome b_L. Substitution of Ser206 (equivalent to Asn221 in *Rb. sphaeroides*) to leucine results in lower expression of cytochrome b_H. In the schematic in Figure 6, these mutations map in the appropriate sides of the membrane, and could be rationalized as influencing the heme binding sites.

It is of interest that the loss of cytochrome b_H does not necessarily destroy the quinol oxidase (Q_z) site. The flash kinetics of the H111N mutant show that the myxothiazol-sensitive quinol oxidase site is functional. Evidently, the steady-state turnover of this bc_1 complex in vivo is insufficient to support photosynthetic growth. None of our mutants retained cytochrome b_H in the absence of cytochrome b_L. Quite possibly, other amino acid substitutions at positions 97 or 198 would yield such a result, since Yu and his colleagues have biochemically isolated a form of the bc_1 complex from *Rb. sphaeroides* from which cytochrome b_L has been almost completely lost while cytochrome b_H has been retained (Yu & Yu, 1984; Salerno et al., 1986). The specific reason why some substitutions have more global deleterious effects than others is not at all clear. Tyrosine in position 111, for example, eliminates both cytochrome b_L and b_H whereas an aspartate in the same location retains cytochrome b_L. Possibly, Asn and Asp replacements allow a bond (H-bond or electrostatic bond) to form with the unmodified His which stabilized the structure in the absence of the heme.

Finally, it is worth emphasizing that although all the data resulting from analysis of these mutants are consistent with the assignment of the heme ligands as described, it does not constitute definitive proof. For this, a high-resolution structural determination will eventually be required.

Registry No. His, 71-00-1; protoheme IX, 14875-96-8; ubiquinol–cytochrome *c* reductase, 9027-03-6.

References

Bowyer, J. R., Tierney, G. V., & Crofts, A. R. (1979) *FEBS Lett. 101*, 201–206.

Bowyer, J. R., Meinhardt, S. W., Tierney, G. V., & Crofts, A. R. (1981) *Biochim. Biophys. Acta 635*, 167–186.

Brasseur, R. (1988) *J. Biol. Chem. 25*, 12571–12575.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Carter, K. R., Tsai, A.-L., & Palmer, G. (1981) *FEBS Lett. 132*, 243–246.

Crofts, A. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) pp 347–382, Plenum Press, New York.

Crofts, A. R., & Wang, Z. (1989) *Photosynth. Res. 22*, 69–87.

Crofts, A., Meinhardt, S., Jones, K., & Snozzi, M. (1983) *Biochim. Biophys. Acta 723*, 202–218.

Crofts, A., Robinson, H., Andrews, K., Van Doren, S., & Berry, E. (1987) in *Cytochrome Systems* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 617–624, Plenum Publishing Corp., New York.

Crofts, A. R., Wang, Z., Chen, Y., Mahalingham, S., Yun, C.-H., & Gennis, R. B. (1990a) in *Highlights in Ubiquinone Research* (Lenaz, G., Barnabei, O., Rabbi, A., & Battino, M., Eds.) pp 98–103, Taylor & Francis, Inc., London.

Crofts, A. R., Yun, C.-H., Gennis, R. B., & Mahalingham, S. (1990b) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. III, pp 263–266, Kluwer Academic Publishers, Dordrecht, Boston, and London.

Daldal, F., Tokito, M., Davidson, E., & Faham, M. (1989) *EMBO J. 8*, 3951–3961.

De Vries, S., Albrecht, S. P. J., & Leeuwerik, F. J. (1979) *Biochim. Biophys. Acta 546*, 316–333.

di Rago, J.-P., & Colson, A.-M. (1988) *J. Biol. Chem. 263*, 12564–12570.

Donohue, T. J., McEwan, A. G., Van Doren, S., Crofts, A. R., & Kaplan, S. (1988) *Biochemistry 27*, 1918–1925.

Dutton, P. L. (1978) *Methods Enzymol 54*, 80–91.

Erecinska, M., Wilson, D. F., & Blasie, J. K. (1979) *Biochim. Biophys. Acta 501*, 63–71.

Gabellini, N. (1988) *J. Bioenerg. Biomembr. 20*, 59–83.

Gabellini, N., & Hauska, G. (1983) *FEBS Lett. 153*, 146–150.

Hauska, G., Nitscheke, W., & Herrmann, R. (1988) *J. Bioenerg. Biomembr. 20*, 211–228.

Howell, N., & Gilbert, K. (1988) *J. Mol. Biol. 203*, 607–618.

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988) *Gene 70*, 191–197.

Kiley, P. J., & Kaplan, S. (1988) *Microbiol. Rev. 52*, 50–69.

Lang, B. F., & Ahne, F. (1985) *J. Mol. Biol. 184*, 353–366.

Leuking, D. R., Fraley, R. T., & Kaplan, S. (1978) *J. Biol. Chem. 253*, 451–457.

Link, T. A., Schagger, H., & von Jagow, G. (1986) *FEBS Lett. 204*, 9–15.

Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Meinhardt, S. W., & Crofts, A. R. (1983) *Biochim. Biophys. Acta 723*, 219–230.

Meunier-Lemesne, D., Di Rago, J. P., & Chevillotte-Brivet, P. (1988) *ICSU Short Rep. 5*, 76.

Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.

Orme-Johnson, N. R., Hanse, R. E., & Beinert, H. (1974) *J. Biol. Chem. 249*, 1928–1939.

Rao, J., & Argos, P. (1986) *Biochim. Biophys. Acta 869*, 197–214.

Salerno, J. C., McCurley, J. P., Dong, J. H., Doyle, M. F., & Yu, C. A. (1986) *Biochem. Biophys. Res. Commun. 136*, 616–621.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Saraste, M. (1984) *FEBS Lett. 166*, 367-372.
Simpkin, D., Palmer, G., Devlin, F. J., McKenna, M. C., Jensen, G. M., & Stephen, P. J. (1989) *Biochemistry 28*, 8033-8039.
Thöny-Meyer, L., Stax, D., & Hennecke, H. (1989) *Cell 57*, 683-697.
Vandeyar, M. A., Weiner, M. P., Hutton, C. J., & Batt, C. A. (1988) *Gene 65*, 129-133.
Verbist, J., Lang, F., Gabellini, N., & Oesterhelt, D. (1989) *Mol. Gen. Genet. 219*, 445-452.
Widger, W. R., Cramer, W. A., Herrmann, R. G., & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 674-678.
Yu, L., & Yu, C. A. (1984) *Biochem. Biophys. Res. Commun. 123*, 1234-1239.
Yun, C.-H., Beci, R., Crofts, A. R., Kaplan, S., & Gennis, R. B. (1990) *Eur. J. Biochem. 194*, 399-411.
Yun, C.-H., Van Doren, S. R., Crofts, A. R., & Gennis, R. B. (1991) *J. Biol. Chem.* (in press).

Evidence for Essential Histidine and Cysteine Residues in Calcium/Calmodulin-Sensitive Cyclic Nucleotide Phosphodiesterase†

Ho-Sam Ahn,*,‡ Michael Foster,‡ Carolyn Foster,‡ Edmund Sybertz,‡ and Jack N. Wells§
Department of Pharmacology, Schering-Plough Research, Bloomfield, New Jersey 07003, and Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

Received January 2, 1991; Revised Manuscript Received March 27, 1991

ABSTRACT: Ca/calmodulin-sensitive cyclic nucleotide phosphodiesterase (CaM-PDE) is an important enzyme regulating cGMP levels and relaxation of vascular smooth muscle. This modification study was conducted mostly with bovine brain CaM-PDE to identify essential functional groups involved in catalysis. The effect of pH on V_{max}/K_m indicates two essential residues with pK_a values of 6.4 and 8.2. Diethyl pyrocarbonate (DEP), a histidine-modifying agent, inhibits CaM-PDE with a second-order rate constant of 130 M^{-1} min^{-1} at pH 7.0 and 30 °C. Activity is restored by NH_2OH. The pH dependence of inactivation reveals that the essential residue modified by DEP has an apparent pK_a of 6.5. The difference spectrum of the intact and DEP-treated enzyme shows a maximum between 230 and 240 nm, suggesting formation of carbethoxy derivatives of histidine. The enzyme is also inactivated by *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-(2-nitrobenzoic acid), both sulfhydryl-modifying agents, with the latter effect reversed by dithiothreitol, which suggests inactivation resulting from modification of cysteine residue(s). Partial inactivation of the enzyme by DEP or NEM results in an apparent decrease in the V_{max} without a change in the K_m or the extent of CaM stimulation. The rate of inactivation by DEP is greater in the presence than in the absence of Ca/CaM. A substrate analogue, Br-cGMP, and the competitive inhibitor 3-isobutyl-1-methylxanthine partially protect the enzyme against inactivation by DEP or NEM, suggesting that the modification of histidine and cysteine residues occurs at or near the active site. DEP also inactivated porcine brain CaM-PDE. Free carboxyl-, amino- or hydroxyl-modifying agents such as N^2-(bromoacetyl)-cGMP, 4-nitro-2,1,3-benzoxadiazole chloride, and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine had no effect on the enzyme activity. The present study indicates for the first time the presence of catalytically essential histidine and cysteine residues at the active site of CaM-PDE.

Guanosine cyclic 3′,5′-phosphate (cGMP)[1] plays an essential role in the modulation of vascular smooth muscle tone [for reviews, see Murad et al. (1985) and Ignarro and Kadowitz (1985)]. Although there are five known families of cyclic nucleotide phosphodiesterase (PDE) (Beavo, 1988), CaM-PDE is one of the most important enzymes for the regulation of vascular cGMP levels and vasorelaxation (Lorenz & Wells, 1983; Hagiwara et al., 1984; Lugnier et al., 1986; Ahn et al., 1989; Weishaar et al., 1990).

CaM-PDE preferentially hydrolyzes cGMP in the vascular tissues and is detected in most tissues including vascular smooth muscle cells (Lorenz & Wells, 1983; Hagiwara et al., 1984; Lugnier et al., 1986; Ahn et al., 1991; Beavo & Reifsnyder, 1990; Nicholson et al., 1991). CaM-PDE isozymes exhibit a very high overall homology with the homologous (catalytic) domain being nearly identical and the regulatory (CaM binding) domain heterologous (Novack et al., 1990).

Despite the importance of CaM-PDE, little is known about its active site and catalytic mechanism. On the basis of the

†This work was supported in part by National Institutes of Health Grant GM 21220 (to J.N.W.).
‡Schering-Plough Research.
§Vanderbilt University.

[1] Abbreviations: cGMP, guanosine cyclic 3′,5′-phosphate; CaM, calmodulin; PDE, cyclic nucleotide phosphodiesterase; CaM-PDE, Ca/CaM-sensitive PDE; DEP or DEPC, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*CMB, *p*-(chloromercuri)benzoic acid; DTT, dithiothreitol; Br-cGMP, 8-bromoguanosine cyclic 3′,5′-phosphate; MIX, 3-isobutyl-1-methylxanthine; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PDP-CaM, [3-(2-pyridyldithio)propionyl]calmodulin; *p*CMS, *p*-(chloromercuri)benzenesulfonic acid.

0006-2960/91/0430-6754$02.50/0