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# *Rhodobacter capsulatus* Mutants Lacking the Rieske FeS Protein Form a Stable Cytochrome $bc_1$ Subcomplex with an Intact Quinone Reduction Site†

Edgar Davidson,‡ Tomoko Ohnishi,§ Mariko Tokito,‡ and Fevzi Daldal*,‡

*Department of Biology, Plant Science Institute, and Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

*Received November 19, 1991; Revised Manuscript Received January 22, 1992*

ABSTRACT: The ubiquinol–cytochrome *c* oxidoreductase (or $bc_1$ complex) of *Rhodobacter capsulatus* consists of three subunits: cytochrome *b*, cytochrome $c_1$, and the Rieske iron–sulfur protein, encoded by the *fbcF*, *fbcB*, and *fbcC* genes, respectively. In the preceding paper [Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E., & Daldal, F. (1992) *Biochemistry* (preceding paper in this issue)], we have observed that the apoproteins for cytochromes *b* and $c_1$ are fully present in the intracytoplasmic membrane of *R. capsulatus* mutants containing low amounts of, or no, Rieske apoprotein. Here we present evidence that the redox midpoint potentials of cytochromes *b* and $c_1$, as well as their ability to bind antimycin and stabilize a semiquinone at the $Q_i$ site, are unaffected by the absence of the Rieske subunit. This is the first report describing a mutant containing a stable $bc_1$ subcomplex with an intact $Q_i$ site in the chromatophore membranes, and provides further evidence that a functional quinone reduction site can be formed in the absence of a quinol oxidation ($Q_o$) site. Additional mutants carrying *fbc* deletions expressing the remaining subunits of the cytochrome $bc_1$ complex were constructed to investigate the relationship among these subunits for their stability in vivo. Western blot analysis of these mutants indicated that cytochromes *b* and $c_1$ protect each other against degradation, suggesting that they form a two-protein subcomplex in the absence of the Rieske protein subunit.

The ubiquinol–cytochrome *c* oxidoreductase ($bc_1$ complex)[1] of the photosynthetic bacterium *Rhodobacter capsulatus* is an important electron-transfer component involved in both photosynthetic and respiratory growth (Dutton, 1986; Cramer & Knaff, 1990). It is located in the intracytoplasmic membrane and consists of three protein subunits: cytochrome *b*, cytochrome $c_1$, amd the Rieske iron–sulfur protein, containing characteristic redox-active prosthetic groups [e.g., see Trumpower (1990)]. The primary structure of these subunits has been established through the cloning and sequencing of the *fbc* (*pet*) operon which encodes all three proteins (Gabellini

† This work was supported by NIH Grants GM 38237 (to F.D.) and NSF DMB-8819305 (to T.O.).

* Author to whom correspondence should be addressed.
‡ Department of Biology, University of Pennsylvania.
§ Department of Biochemistry and Biophysics, University of Pennsylvania.

[1] Abbreviations: bp, base pair(s); $bc_1$ complex, ubiquinol–cytochrome *c* oxidoreductase; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; cyt, cytochrome; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; $E_m$, redox midpoint potential; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; $Q_o$, quinol oxidation site; $Q_i$, quinone reduction site; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Table I: *E. coli* and *R. capsulatus* Strains Used

| strain | genotype | phenotype | reference |
|---|---|---|---|
| *E. coli* | | | |
| HB101 | F⁻ *proA2 leu hsdS20*($r_B^-m_B^-$) *recA13 ara-14, lacY galK2 mtl rpsL20 xyl-5 supE44 I*⁻ | | Ditta et al. (1985) |
| HB101(pRK2013) | $Kan^R$ helper plasmid | | Ditta et al. (1985) |
| *R. capsulatus* | | | |
| MT1131 | *crtD* | wild type | |
| MT-RBC1 | *crtD* Δ(*fbcFBC::spe*) | $Spec^R$, $FbcF^-B^-C^-$ | Atta-Asafo-Adjei & Daldal (1991) |
| plasmids | | | |
| pMT0-404 | *R. capsulatus fbcFBC* | $Tet^R$, (cyt $bc_1$) | Atta-Asafo-Adjei & Daldal (1991) |
| pMT2-404 | *R. capsulatus* $fbcF^-B^+C^+$ | $Tet^R$, Fbc $F^-$ | this work |
| pMT3-404 | *R. capsulatus* $fbcF^-B^-C^+$ | $Tet^R$, Fbc $F^-B^-$ | this work |
| pMT4-404 | *R. capsulatus* $fbcF^+B^+C^-$ | $Tet^R$, Fbc $C^-$ | this work |
| pMT5-404 | *R. capsulatus* $fbcF^+B^-C^+$ | $Tet^R$, Fbc $B^-$ | this work |
| pC133SER | *R. capsulatus fbcF* C133S | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |
| pH135LEU | *R. capsulatus fbcF* H135L | $Tet^R$, Fbc, $F^-$ | Davidson et al. (1992) |
| pC138ARG | *R. capsulatus fbcF* C138R | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |
| pC153SER | *R. capsulatus fbcF* C153S | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |
| pC155GLY | *R. capsulatus fbcF* C155G | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |
| pH156THR | *R. capsulatus fbcF* H156T | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |
| pH159SER | *R. capsulatus fbcF* H159S | $Tet^R$, Fbc $F^-$ | Davidson et al. (1992) |

& Sebald, 1986; Daldal et al., 1987; Davidson & Daldal, 1987).

Biochemical studies and hydropathy profiles have provided insights into the association of the $bc_1$ complex subunits with the membrane lipid bilayer, and the relative arrangement of their prosthetic groups (Robertson & Dutton, 1988; Ohnishi et al., 1989). The Rieske protein appears to be associated with the membrane via its amino-terminal region, but the mode of interaction is unknown although recent pH extrusion experiments using thylakoid membranes suggest that Rieske has at least one membrane-spanning helix (Szczepaniak et al., 1991). Cytochrome $c_1$ is anchored to the membrane by a hydrophobic helix located at its carboxyl terminus, as proteolytic (Li et al., 1983) and genetic (Konishi et al., 1991) cleavage of this helix produces a soluble $c_1$. Cytochrome *b* appears to be an integral membrane protein with several transmembrane domains. Recent analyses of inhibitor-resistant mutants [e.g., see Daldal et al. (1986, 1989), Howell and Gilbert (1988), and di Rago and Colson (1988)] and alkaline phosphatase fusions (Yun et al., 1991) favor a model of cytochrome *b* with eight transmembrane helices over the nine transmembrane helices proposed originally (Saraste, 1984; Widger et al., 1984). It has even recently been suggested that seven transmembrane helices may be appropriate (Cramer & Trebst, 1991).

The three subunits of the $bc_1$ complex form two active domains, involved in quinol oxidation ($Q_o$) and quinone reduction ($Q_i$), located on each side of the membrane [e.g., see Robertson and Dutton (1988)]. Functional studies have indicated that cytochrome $b_L$ and the Rieske [2Fe-2S] cluster are the redox-active components of the $Q_o$ site and that the $Q_i$ site interacts with cytochrome $b_H$ [see Rich (1984)]. The locations of these sites were defined genetically by the analysis of mutants resistant to site-specific inhibitors (Howell & Gilbert, 1988; di Rago & Colson, 1988; Daldal et al., 1989). Currently, all available data are consistent with the Rieske [2Fe-2S] cluster and the $b_L$ and $c_1$ hemes being clustered on the periplasmic surface of the membrane in the vicinity of the $Q_o$ site while the $b_H$ heme is located near the center of the bilayer and the $Q_i$ site at the cytoplasmic side (Robertson & Dutton, 1988; Ohnishi et al., 1989). However, little is known about the subunit interactions which stabilize the complex in vivo.

During the work described in the preceding paper (Davidson et al., 1992) aimed at assigning the amino acid ligands of the [2Fe-2S] cluster of the Rieske protein of *R. capsulatus*, we observed that the mutagenesis of any of the six universally conserved cysteine or histidine residues of this subunit drastically decreased its level in the membrane. SDS–PAGE of membranes from these mutants indicated that the apoproteins for cytochromes *b* and $c_1$ were present in quantities comparable to the wild-type control, even in cases where the Rieske apoprotein could not be detected by Western blotting. One of these mutants was chosen for further biochemical and biophysical studies since it provided an opportunity to analyze the properties of cytochromes *b* and $c_1$ in the absence of the Rieske subunit. In this paper, we demonstrate that, in such a mutant, the absorption and redox characteristics of cytochromes *b* and $c_1$ are unchanged and that they form a quinone reduction ($Q_i$) site with characteristics similar to that of the intact $bc_1$ complex.

## Materials and Methods

*Media, Bacterial Strains, and Growth Conditions.* The growth of *R. capsulatus* and *Escherichia coli* strains was described in Daldal et al. (1989). The strains used are listed in Table I.

*Genetic and Molecular Biological Techniques.* Genetic and molecular biological techniques were as described earlier (Daldal et al., 1987; Davidson et al., 1992). Specific inactivations of the *fbc* genes, on the pBR322 derivative plasmid pPET1 (Atta-Asafo-Adjei & Daldal, 1991), were achieved as shown in Figure 6. *fbcF*, encoding the Rieske protein, was mutated by modification of the *Bst*XI site with T4 polymerase and subsequent ligation. This created a frame-shift mutation in *fbcF* at a codon corresponding to amino acid Gly23 of the Rieske protein, and resulted in the possibility of encoding a 33 amino acid unrelated peptide. *fbcB*, encoding cytochrome *b*, was mutated by deleting its 498 bp long internal *Eco*RI–*Sma*I fragment after appropriate modification and ligaton. This removed the portion of cytochrome *b* located between amino acids Ile4 and Pro170, and shifted its reading frame after Gly3 with the possibility to code for a 61 amino acid peptide. *fbcC*, encoding cytochrome $c_1$, was inactivated by deleting 600 bp from an *Apa*I fragment in *fbc*C to one 110 bp downstream from the *fbc*C stop codon, resulting in a deletion from Pro119 to the end of the gene. These mutations yielded single $FbcF^-$, $FbcB^-$, and $FbcC^-$ mutants. The double $FbcF^-B^-$ mutant was obtained by a *Bst*XI–*Sma*I deletion. The mutant pPET1 derivatives were subsequently cloned, using their unique *Hin*dIII sites, into plasmid pRK404 (Ditta et al., 1985) and conjugated into the *R. capsulatus* $bc_1$-minus strain MT-RBC1 ($FbcF^-B^-C^-$; Atta-Asafo-Adjei & Daldal, 1991).

*Biochemical Analyses.* Intracytoplasmic membranes, as chromatophores, were isolated after French pressure cell treatment as described earlier (Atta-Asafo-Adjei & Daldal, 1991). Protein concentrations were assayed by the method of Lowry et al. (1951) after extraction of pigments with acetone/methanol (7:2) (Clayton, 1963). Protein samples for gel electrophoresis were prepared as in Davidson et al. (1992). SDS–PAGE and Western blot analyses were performed as described previously using monoclonal (Daldal, 1988) or polyclonal (Davidson et al., 1987) antibodies raised against the purified subunits of *R. capsulatus* $bc_1$ complex and horseradish-conjugated secondary antibodies from Cappel (Malvern, PA) or Bio-Rad (Richmond, CA), respectively.

Reduced-minus-oxidized absorption difference spectra of chromatophores were taken with a Hitachi U3210 spectrophotometer, using ascorbate or dithionite as reducing and ferricyanide as oxidizing agents as described earlier (Atta-Asafo-Adjei & Daldal, 1991). Antimycin-induced shifts of the cytochrome *b* absorption spectrum were recorded on a University of Pennsylvania Instrumentation Group double-beam spectrophotometer (Courtesy of Dr. P. L. Dutton). For this measurement, chromatophores (660 $\mu$g of protein $mL^{-1}$) were reduced with sodium dithionite, a base line was recorded, antimycin dissolved in DMSO was added to a final concentration of 10 $\mu$M, and the absorption spectrum was recorded between 500 and 600 nm. Optical redox titrations of chromatophore membranes were carried out on the same spectrophotometer according to Dutton (1978). Chromatophores were resuspended to 50 $\mu$M bacteriochlorophyll in 20 mM MOPS/100 mM KCl, pH 7.0, in the presence of the following redox mediators: *N*-methylphenazonium methosulfate (PMS), *N*-ethylphenazonium ethosulfate (EMS), pyocyanine, and phenazine at 20 $\mu$M each; benzoquinone, 1,4-naphthoquinone, and 1,2-naphthoquinone at 30 $\mu$M each; 50 $\mu$M duroquinone; 70 $\mu$M 2,3,5,6-tetramethylphenylenediamine (DAD); 15 $\mu$M 2-hydroxyl-1,4-naphthoquinone and ferric EDTA.

EPR spectra were recorded using a Varian E-109 X-band spectrometer, and the potentiometric titrations were performed as in Dutton (1978). The $g = 2$ semiquinone radical titrations were carried out in the presence of the redox-mediating dyes (40 $\mu$M each) 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-naphthoquinone-4-sulfonate, duroquinone, indigo disulfonate, indigo trisulfonate, and indigo tetrasulfonate. EPR conditions for each measurement are given in the appropriate figure legend.

## Results

*Cytochromes b and $c_1$ Are Present in Full Amounts in the Chromatophores of Mutants Lacking the Rieske Protein.* Mutagenesis of the putative ligands of the [2Fe-2S] cluster of the Rieske protein of *R. capsulatus* indicated that the substitution of any of cysteines-133, -138, -153, and -155 and histidines-135 and -156 by a variety of other amino acids specifically eliminated the Rieske apoprotein from the chromatophores (Davidson et al., 1992). SDS–PAGE and Western blot analyses of the chromatophores of these Rieske-less mutants clearly demonstrated that the cytochrome *b* and $c_1$ apoproteins were present in amounts comparable to those of their parental strain MT0-404, overproducing the $bc_1$ complex [Davidson et al., 1992; an example (H135LEU) is shown in Figure 1A,B]. Optical spectroscopy was used to determine if these apoproteins contained their corresponding *b*- and *c*-type heme groups. Reduced-minus-oxidized absorption difference spectra of chromatophores derived from the mutants C133SER, H135LEU, C138ARG, C153SER, C155GLY, and H156THR (all in the MT-RBC1, $bc_1^-$ background) are shown in Figure 1C (other mutants not shown). Comparison of the dithionite-reduced-minus-ferricyanide-oxidized (i.e., total *b*- and *c*-type cytochromes) and ascorbate-reduced-minus-ferricyanide-oxidized (i.e., total *c*-type cytochromes) difference spectra of chromatophores clearly indicates that the *b* (absorbing maximally around 560 nm, mostly dithionite-reducible) and $c_1$ (550-nm absorption, ascorbate-reducible) hemes of the $bc_1$ complex are present in these mutants. A comparison of their amounts to those present in the strains MT-RBC1 (a $bc_1^-$ mutant), MT1131 (a single-copy wild-type $bc_1$ complex), and MT0-404 (a multicopy wild-type $bc_1$ complex overproducer) indicated that these cytochromes are overproduced. Therefore, the elimination of the Rieske protein from the $bc_1$ complex does not affect either the presence of the cytochrome *b* and $c_1$ apoproteins in the membrane or the incorporation of their appropriate heme groups. As expected, these mutants did not exhibit any DBH–cytochrome *c* reductase activity, nor could they grow photosynthetically. Since the mutants were apparently similar by these criteria, only one of them, H135LEU, was investigated further.

*Determination of the Redox Midpoint Potentials of Cytochromes b and $c_1$ of Mutant H135LEU.* The effects of the absence of the Rieske subunit on the spectral and thermodynamic properties of cytochromes *b* and $c_1$ were determined by comparison of H135LEU with its parent MT0-404. The reduced-minus-oxidized spectra of cytochromes $b_H$, $b_L$, and $c_1$ and their redox titrations (performed at pH 7.0) are presented in Figures 2–4. The absorption spectra of cytochromes $b_H$ and $b_L$ are characteristic (Robertson et al., 1986): $b_H$ absorbs maximally at 560 nm, while $b_L$ has the typical split $\alpha$-peak spectrum, and the absorption maxima of the *b* and *c* cytochromes from H135LEU and MT0-404 are very similar (Figure 2). The titration data are best fit by assuming three, $n = 1$, components: $b_H$, $b_L$, and a small amount of a high-potential *b*, $E_m$ around 160 mV (560–540 nm), which has been observed previously in *R. capsulatus* chromatophores [e.g., see Zannoni et al., (1980)]. The 160-mV cytochrome *b* is not associated with the $bc_1$ complex (D. Robertson, personal communication) since it is also present in the *R. capsulatus* mutant MTRK2 (Daldal, 1987) which lacks the cytochrome $b_L$ and $b_H$ hemes. There is essentially no difference in the $E_m$'s of cytochromes $b_H$ (60 and 61 mV, 560–540 nm) and $b_L$ (−125 and −118 mV, 565.2–540 nm) between MT0-404 and H135LEU, respectively (Figure 3A,B). These agree well with previously obtained values of 60 and −115 mV for *R. capsulatus* $b_H$ and $b_L$, respectively (Robertson et al., 1986). The accurate titration of cytochrome $c_1$ is more complicated due to the presence of several other *c*-type cytochromes such as cytochromes $c_2$ and $c_x$ [e.g., see Prince et al. (1986) and Jones et al. (1990)]. The titrations of total *c*-type cytochromes (551.2–536 nm) were very similar for these two strains (Figure 4) and were fitted to two, $n = 1$, components, giving midpoints of 280 and 340 mV (MT0-404) and 268 and 337 mV (H135LEU). These spectroscopic analyses indicated that the redox properties of cytochromes *b* and $c_1$ have not been significantly altered by the absence of the Rieske subunit.

*The $bc_1$ Subcomplex of the Rieske-less Mutant H135LEU Has an Intact Quinone Reduction ($Q_i$) Site.* The presence of a quinone reduction site in H135LEU, and therefore not involving the Rieske [2Fe-2S] cluster, was tested using antimycin. This specific inhibitor of the $bc_1$ complex acts at the $Q_i$ site, presumably by displacing a semiquinone from its binding site (Ohnishi & Trumpower, 1980). The addition of antimycin to the $bc_1$ complex imposes a spectral change on the *b*-type cytochromes (van den Berg et al., 1979), in par-

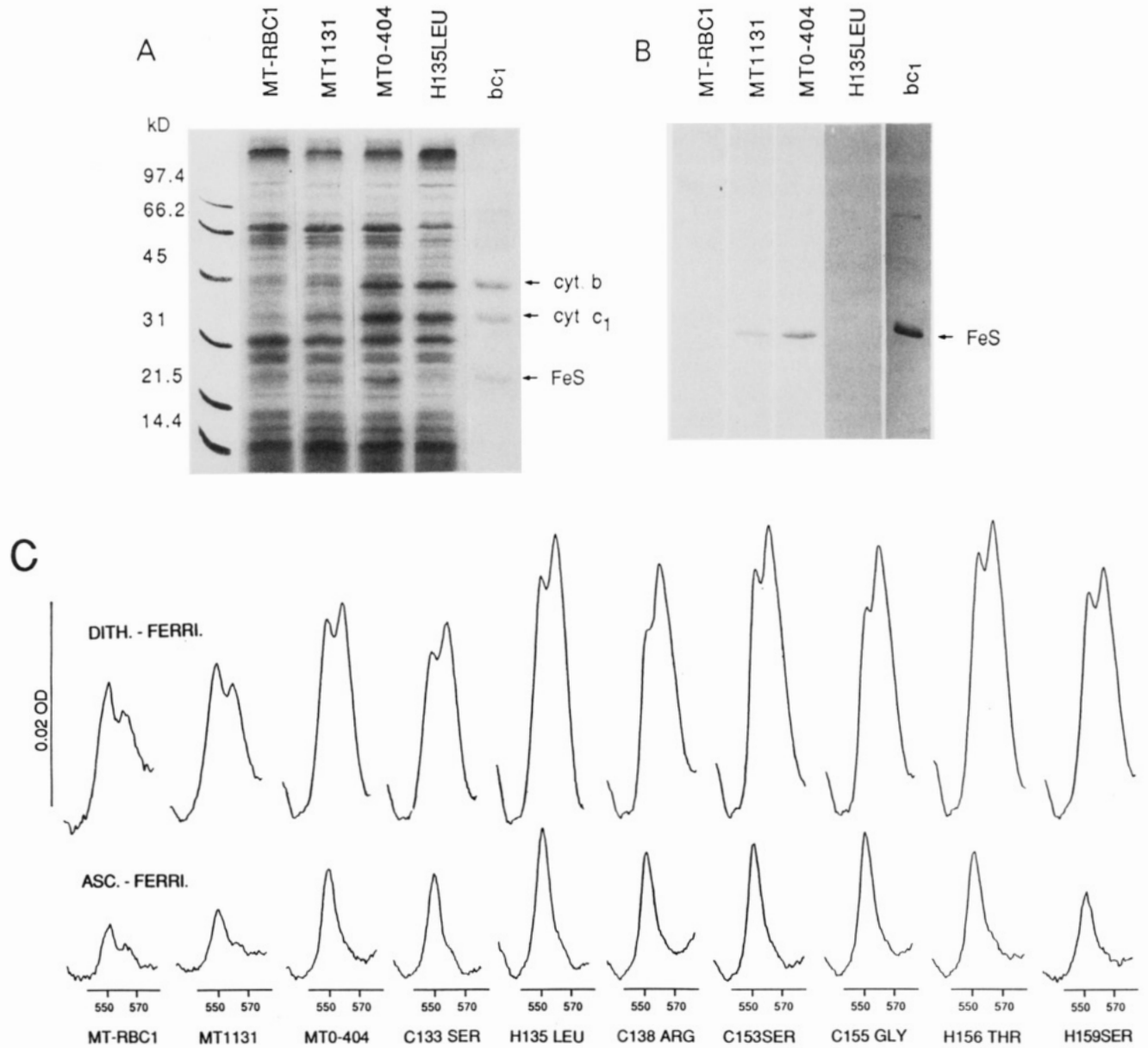


FIGURE 1: (A) SDS–PAGE (15%) of chromatophores (40 μg of protein per lane) stained for protein with Coomassie blue. MT-RBC1, $bc_1^-$; MT1131, wild type; MT0-404, MT-RBC1 containing pMT0-404, a wild-type $bc_1$ overproducer plasmid; H135LEU, MT-RBC1 containing pH135LEU. $bc_1$ corresponds to purified $bc_1$ complex; cyt *b*, cyt $c_1$, and FeS indicate its subunits. (B) Western blot of the strains in panel A, probed with a polyclonal antibody recognizing the Rieske protein (Davidson et al., 1987). (C) Reduced minus oxidized difference spectra of total *b*-type (absorbance around 560 nm) and *c*-type (absorbance around 550 nm) cytochromes in chromatophore membranes from various Rieske-less mutants of *R. capsulatus*. Upper and lower panels correspond to (dithionite + PMS)-minus-ferricyanide and (ascorbate + PMS)-minus-ferricyanide spectra, respectively.

ticular on $b_H$ which has an absorption peak at 560 nm (see Figure 2). An antimycin-induced red shift can then be detected in (reduced + antimycin)-minus-reduced difference spectra around 560 nm. Antimycin-induced red shifts for two Rieske-less mutants, H135LEU and H156LEU, are similar to that seen with the wild-type $bc_1$ complex overproducer strain MT0-404 (Figure 5A). These data clearly suggest that the binding site of the antimycin in these mutants is largely unaffected by the absence of the Rieske protein.

The $Q_i$ site of the $bc_1$ complex can be probed more directly by the detection of an antimycin-sensitive semiquinone radical $Q_i^{\bullet -}$ ($g$ = 2.0) by electron paramagnetic resonance (EPR) spectroscopy. In *R. capsulatus*, the size of this semiquinone radical signal is pH-dependent and is stabilized at pH values above 7.0 (Robertson et al., 1986). Its redox midpoint increases with pH and reaches a maximum around 0 mV at pH 9.3. In chromatophores of H135LEU (and H156LEU and MT0-404, data not shown), the antimycin-sensitive $Q_i^{\bullet -}$ signal (Figure 5B) titrated as a bell-shaped curve (Figure 5C), as has previously been established for chromatophores from a wild-type strain of *R. capsulatus* (Robertson et al., 1986). This antimycin-sensitive $Q_i^{\bullet -}$ radical was absent from chromatophores of the $bc_1^-$ mutant MT-RBC1 (Figure 5B), indicating that it is correlated with the presence of a $bc_1$ complex in the membrane. The antimycin-sensitive semiquinone signal in H135LEU indicates that, without the Rieske protein, the $Q_i$ site of these mutants behaves like that of an intact $bc_1$ complex. All together, these data show that the spectral, thermodynamic, and quinone binding properties of the cytochrome *b* and $c_1$ subunits of H135LEU are similar to those of a wild-type $bc_1$ complex. The stable presence of cytochromes *b* and $c_1$ in chromatophores is independent of the presence of the Rieske protein; however, these results do not indicate that these subunits are physically associated.

Table II: Summary of Western Blot and Absorption Data for pMT Mutants

| strain | genotype | Rieske | $A_{560}$[a] (nm) | cyt *b* | $A_{550}$[a] (nm) | cyt $c_1$ |
|---|---|---|---|---|---|---|
| MT-RBC1 | Δ*fbcFBC* | – | 0 | – | 0 | – |
| MT2 | $F^-B^+C^+$ | – | 53 | ++ | 58 | ++ |
| MT3 | $F^-B^-C^+$ | – | 0 | – | 32 | ++[b] |
| MT4 | $F^+B^+C^-$ | +[b] | 20 | ++[b] | 0 | – |
| MT5 | $F^+B^-C^+$ | – | 0 | – | 21 | +[b] |
| C133S | $F^-B^+C^+$ | – | 75 | +++ | 95 | +++ |
| MT0-404 | $F^+B^+C^+$ | +++ | 100 | +++ | 100 | +++ |

[a] Values are calculated using MT-RBC1 as a base line and MT0-404 as 100% absorption. [b] This subunit was present in degraded as well as in intact forms.

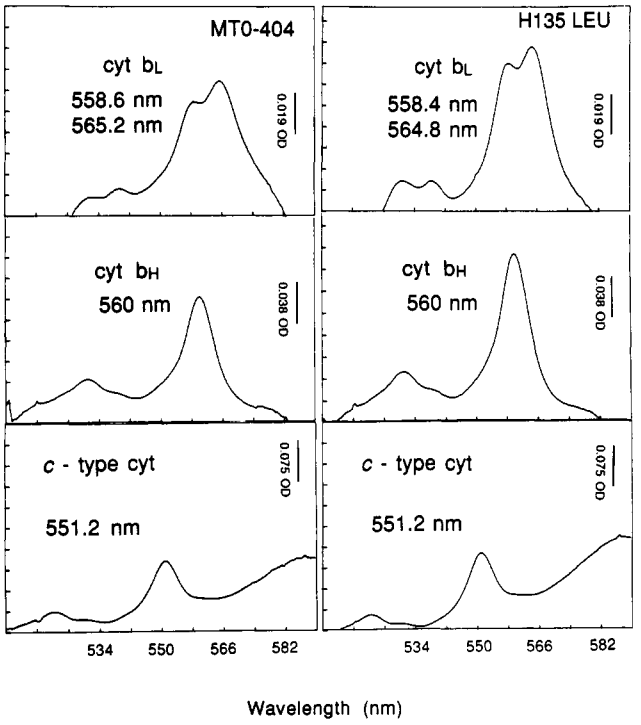


FIGURE 2: Absorption spectra of cytochromes $b_H$ and $b_L$ and *c*-type cytochromes of H135LEU and MT0-404. Redox titrations of the *b*- and *c*-type cytochromes in chromatophores were performed by recording an absorption spectrum at each $E_h$ (see Figures 3 and 4). The spectra shown were obtained by subtracting spectra recorded at the following $E_h$ values for MT0-404 and H135LEU, respectively; cytochrome $b_L$, −180 – −40 mV and −180 – −39 mV; cytochrome $b_H$, 28 – 206 mV and 29 – 206 mV; *c*-type cytochromes, 207 – 403 mV and 206 – 408 mV.

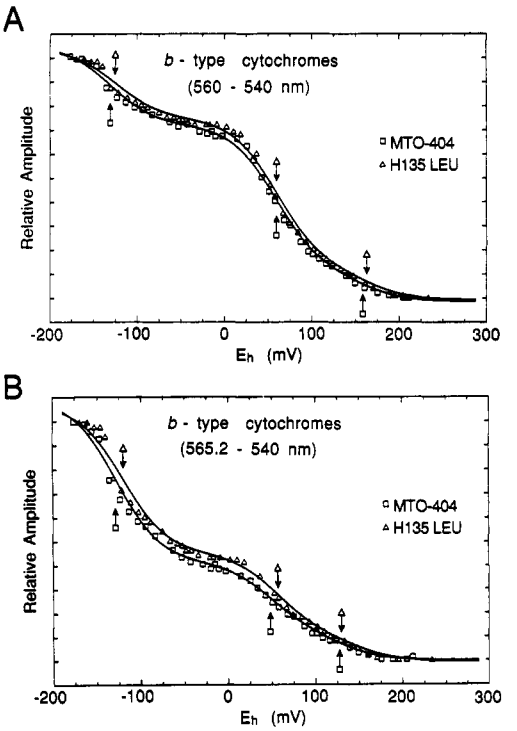


FIGURE 3: Redox titrations of total *b* cytochromes of chromatophores. Reductive titrations of chromatophores (50 μM bacteriochorophyll) were performed as described under Materials and Methods. The data obtained were fit to three, $n = 1$, components, whose redox midpoints are indicated by arrows. (A) Redox titrations of total *b* cytochromes in chromatophores monitored at 560–540 nm. (B) Redox titrations of total *b* cytochromes in chromatophores monitored at 565–540 nm.

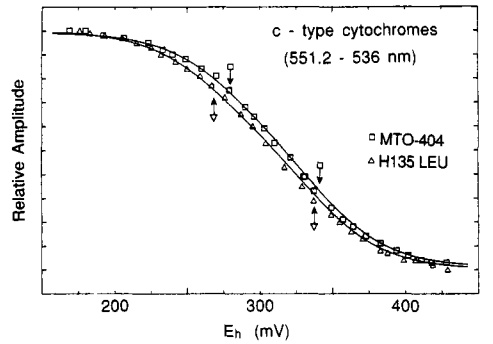


FIGURE 4: Redox titrations of total *c*-type cytochromes. Chromatophores, 50 μM bacteriochlorophyll, were titrated as described under Materials and Methods. The data obtained at 551.2–536 nm were fit to two, $n = 1$, components, whose redox midpoints are indicated by arrows.

*Interactions among the Subunits of the $bc_1$ Complex in Chromatophores.* In order to examine the possible stabilizing interactions among the three subunits of the $bc_1$ complex in chromatophores, four strains (pMT2-404/MT-RBC1 to pMT5-404/MT-RBC1, named here MT2–MT5) were constructed to contain deletions of the *fbcFBC* genes in several combinations [*fbc*($F^-B^+C^+$,$F^+B^-C^+$,$F^+B^+C^-$,$F^-B^-C^+$] (Figure 6 and Materials and Methods), and chromatophores were isolated from these merodiploids. The presence of a given subunit in the membranes was determined by Western blot analyses using antibodies recognizing the Rieske, cytochrome *b*, and cytochrome $c_1$ subunits specifically (Figure 7). In each case, ascorbate- or dithionite-reduced-minus-ferricyanide-oxidized absorption difference spectra were also recorded to determine the respective amounts of total *b* and $c_1$ hemes in the membranes (spectra not shown). The overall data obtained by Western blot analyses and by spectroscopy are summarized in Table II.

As would be expected from our previous observations on the Rieske-less mutants containing point mutations in *fbcF* (i.e., C133SER, or H235LEU), cytochrome *b* and $c_1$ were also present and stable in higher amounts in chromatophore membranes of MT2 containing the *fbcF*Δ(*BstXI*) deletion (Figure 7). A comparison of MT2 to C133SER indicated that although these mutants have the same phenotype (i.e., FbcF$^-$), the latter yielded higher amounts of cytochrome *b* and $c_1$

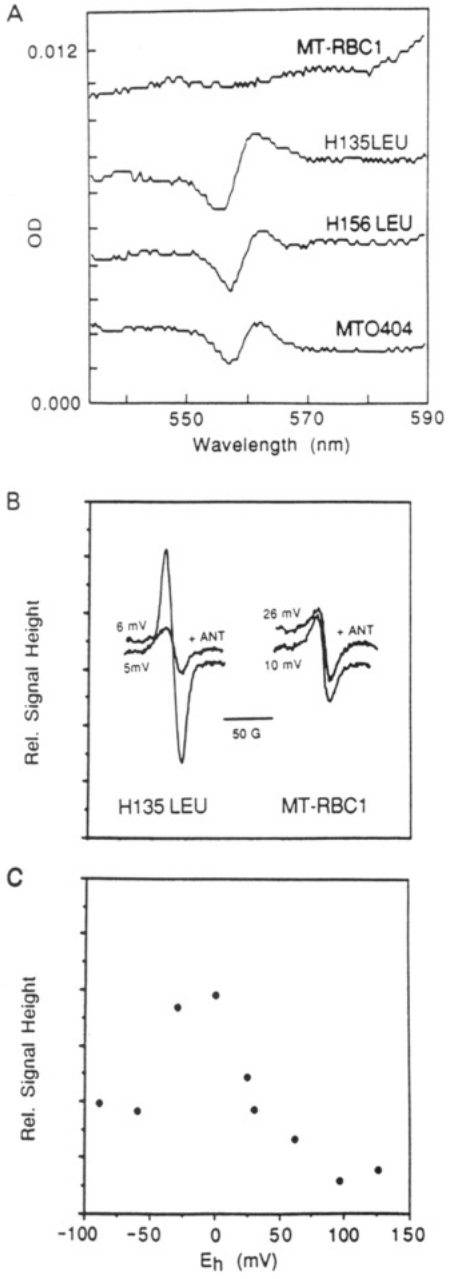


FIGURE 5: (A) Antimycin-induced absorption shifts in *R. capsulatus*. Chromatophore membranes (660 $\mu$g of protein/mL) were reduced with dithionite, and the absorption spectrum was recorded as a base line. Antimycin (10 mM in DMSO) was then added to a final concentration of 10 $\mu$M, and the absorption spectrum was recorded. (B) Effect of antimycin (+ANT) on the EPR-detectable $g = 2$ radical signal in chromatophores. Samples were prepared as for panel C; after a titration, antimycin was added (to 20 $\mu$M) and a spectrum recorded at the appropriate redox potential ($E_h$) as indicated. (C) Redox titration of the stable, antimycin-sensitive, semiquinone $Q_i^-$ at the quinone reduction site of the $bc_1$ complex. Chromatophores were resuspended in 50 mM glycine to a final pH of 9.3 with redox-mediating dyes as described under Materials and Methods. The EPR conditions were 20 $\mu$W of power and $T = 140$ K.

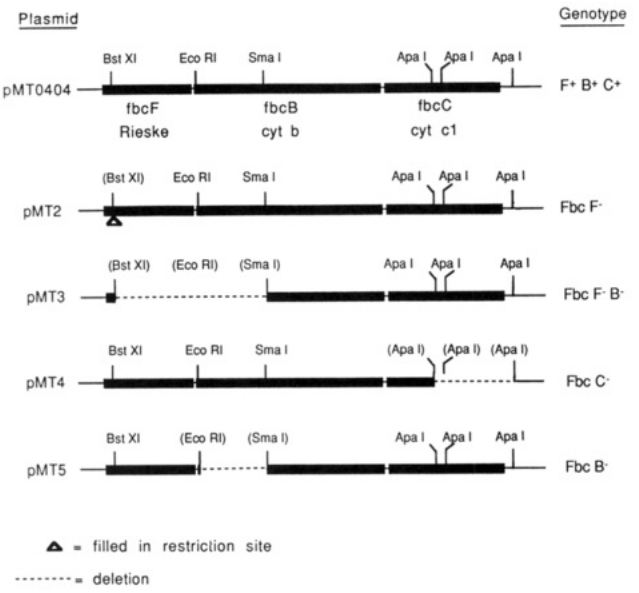


FIGURE 6: Plasmids containing various combinations of frameshift mutations in the *R. capsulatus fbcFBC* genes, encoding the Rieske protein, cytochrome *b*, and cytochrome $c_1$ subunits of the $bc_1$ complex. They are listed in Table I and were obtained as described under Materials and Methods.

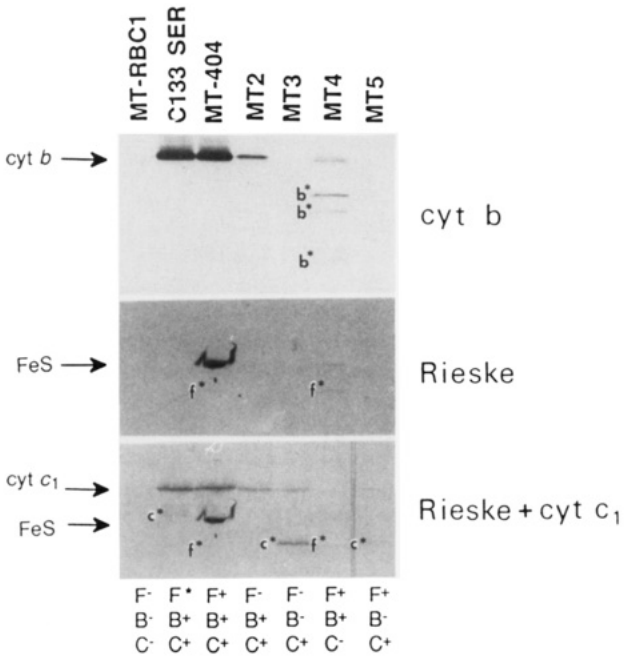


FIGURE 7: Western blot analyses of chromatophore membranes from merodiploid strains carrying the mutant plasmids described in Figure 6. Rieske protein was detected by polyclonal antibodies (Davidson et al., 1987) and the cytochromes *b* and $c_1$ by the monoclonal antibodies D50 and D42, respectively (Daldal, 1988). The filter shown in the middle panel was probed first with the antibodies against the Rieske protein, photographed, and then subsequently probed with antibodies against cytochrome $c_1$ (lowest panel). The twice-probed filter is shown in the lowest panel, and f*, b*, and c* indicate the degradation products of the Rieske protein, cytochrome *b*, and cytochrome $c_1$, respectively.

apoproteins per total amount of membrane proteins than the former, possibly due to some polar effects of this deletion (Figure 7 and Table II). In the absence of cytochrome *b* (MT5, FbcB$^-$), cytochrome $c_1$ was present and degraded. Rieske protein was not detected in MT5, but it could be poorly stabilized by cytochrome *b* as seen in MT4 (FbcC$^-$). The low amount of Rieske protein in MT4 was also reflected in a low amount of its EPR signal (not shown). In the absence of cytochrome $c_1$ (MT4, FbcC$^-$), the Rieske protein and cytochrome *b* were detectable but highly degraded. In the absence of cytochrome *b* and the Rieske protein (MT3, FbcF$^-$B$^-$), cytochrome $c_1$ was present but showed a major low molecular weight degradation product which did not stain for the presence of heme (data not shown). These degradation products were not detected in MT2 or C133SER (FbcF$^-$), suggesting that the cytochromes *b* and $c_1$ observed in the membranes of the Rieske-less mutants (Figure 1) stabilize each other to form a subcomplex. When these experiments were repeated with

whole cells, no qualitative differences in the results described above were observed.

## Discussion

This work clearly indicates that, in *R. capsulatus*, the presence of a Rieske protein is not required for the incorporation or maintenance of cytochromes *b* and $c_1$ in the cytoplasmic membrane nor does it affect the redox midpoints of cytochromes $c_1$, $b_L$, and $b_H$. In the absence of Rieske, the ability of antimycin to shift the cytochrome *b* spectrum and the presence of a stable, antimycin-sensitive, semiquinone radical at the $Q_i$ site strongly suggest that cytochrome *b* has a conformation very similar to its normal conformation in an intact $bc_1$ complex. These results further confirm that the $Q_i$ site is structurally and functionally distinct from the $Q_o$ site as has already been indicated by the study of *R. capsulatus* mutant R126 (Robertson et al., 1986). In the mutant studied here (H135LEU), the $Q_o$ site is destroyed by the elimination of the Rieske subunit while R126 is unable to catalyze quinol oxidation due to a point mutation in cytochrome *b* (Daldal et al., 1989) although its $Q_i$ site is fully functional (Robertson et al., 1986). Examination of *R. capsulatus* mutants which have the ability to synthesize only some of the subunits of the $bc_1$ complex indicated that, independently of the presence of Rieske, cytochromes *b* and $c_1$ protect each other from degradation.

The effect of removal of the Rieske protein from $bc_1$-type complexes in vivo varies among the type of complex. Although many chloroplast mutants, as yet undefined, lack the entire cytochrome $b_6f$ complex (Metz et al., 1983; Bendall et al., 1986; Lemaire et al., 1986), a mutant of *Chlamydomonas reinhardtii*, *ac*21, has been described as missing the Rieske protein but containing cytochromes *f* and $b_6$ and subunit IV proteins and showing 50% of the $b_6f$ cytochrome absorption (Bendall et al., 1986; Lemaire et al., 1986). This is analogous to our observations in *R. capsulatus*. Mitochondrial $bc_1$ complexes contain a larger number of subunits than their bacterial or chloroplast counterparts (Teintze et al., 1982), and consequently the subunit interactions are more complicated. In early in vitro experiments, the Rieske protein was extracted from purified yeast $bc_1$ complex and then reconstituted with an associated restoration of activity. The absorption maxima of cytochromes $b_H$, $b_L$, and $c_1$ were unaffected by extraction of Rieske (Trumpower et al., 1980) as was the antimycin red shift of cytochrome $b_H$ (Trumpower, 1981). Analyses of yeast strains with mutations in the subunits of the $bc_1$ complex suggested that deletion of the Rieske protein generally decreased cytochrome absorption, especially *b* (Crivellone et al., 1988; Ljungdahl et al., 1989; Shanker & Beattie, 1989), but had almost no effect on the levels of the other subunits (Crivellone et al., 1988), or decreased levels of the lower molecular weight subunits (Shanker & Beattie, 1989).

The necessity of an intact cytochrome $c_1$ for the stability of the Rieske and cytochrome *b* is consistent with several previous observations in bacterial $bc_1$ and chloroplast $b_6f$ complexes, both of which contain only three or four subunits [subunit IV in the $b_6f$ complex is analogous to the carboxyl terminus of cytochrome *b* (Widger et al., 1984)]. First, the *R. capsulatus* mutant MT113, which is defective in the synthesis of all *c*-type cytochromes, also lacks the *b*-type hemes and [2Fe-2S] cluster of the $bc_1$ complex, and contains low amounts of the corresponding apoproteins in the chromatophore membranes (Davidson et al., 1987). Deletion of the putative membrane-spanning region of cytochrome $c_1$ from *R. sphaeroides* to produce a truncated, soluble form disrupted the assembly of the other components: cytochrome $b_L$ was lost, and the amount of Rieske protein was reduced (Konishi et al., 1991). In *Paracoccus denitrificans*, the chromosomal deletion of the *fbcC* gene also resulted in the loss of cytochrome *b* and the Rieske protein from membranes (Gerhus et al., 1990). In *R. capsulatus*, a similar strain (MT4) contained some cytochrome *b* and Rieske protein, possibly due to the overexpression of these subunits. There has been no biochemical cross-linking study of the *R. capsulatus* $bc_1$ complex which would complement the mutant studies described here, but such studies have been performed on $b_6f$ complexes. For example, very recent experiments using cleavable reagents 10–15 Å long demonstrated cross-links between cytochrome *f* and subunit IV, cytochrome *f* and cytochrome $b_6$, and cytochrome $b_6$ and subunit IV (Shallan et al., 1991).

In conclusion, this study indicates that in *R. capsulatus* chromatophores, cytochromes *b* and $c_1$ form a stable $bc_1$ subcomplex in the absence of the Rieske protein. This complex has properties similar to those of a wild-type complex in terms of its $Q_i$ site and the spectral and thermodynamic properties of its cytochrome $b_H$, $b_L$, and $c_1$ heme groups. While cytochromes *b* and $c_1$ can form a stable subcomplex, cytochrome *b* and Rieske are present but highly degraded in the absence of cytochrome $c_1$. These mutants will undoubtedly be useful for probing the ability of inhibitors to bind to the $Q_o$ site in the absence of the Rieske protein.

## Acknowledgments

We thank Drs. P. L. Dutton and D. E. Robertson for their help in teaching us how to perform the dark redox titrations of the cytochromes, and for letting us use their equipment (supported by NIH Grant GM 27302).

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# Expression and Characterization of Recombinant Hepatitis A Virus 3C Proteinase

Bruce A. Malcolm,* Sherman M. Chin, David A. Jewell, Jennifer R. Stratton-Thomas, Kent B. Thudium, Robert Ralston, and Steven Rosenberg

*Chiron Corporation, 4560 Horton Street, Emeryville, California 94608*

*Received June 13, 1991; Revised Manuscript Received December 31, 1991*

ABSTRACT: The 3C proteinase from the hepatitis A virus (HAV) was cloned into a multicopy expression vector in *Escherichia coli* under control of the *tac* promoter. The resulting plasmid construction produced 3C proteinase as a soluble and active enzyme constituting approximately 10% of total cellular proteins. The enzyme was purified to apparent homogeneity as judged by SDS gel electrophoresis and HPLC reversed-phase and FPLC ion-exchange chromatography. A colorimetric assay was developed, and synthetic peptides derived from the predicted cleavage sites of the HAV polyprotein were tested for proteolysis of the enzyme. The peptide representing the 2B/2C cleavage site was cleaved most efficiently with a $K_m$ and $k_{cat}$ of 2.1 ± 0.5 mM and 1.8 ± 0.1 $s^{-1}$, respectively. Site-directed mutagenesis was then used to identify the cysteine at position 172 as the active site nucleophile. Finally, the purified enzyme showed the expected endoproteinase activity on the P1 precursor protein generated by in vitro transcription/translation.

Hepatitis A virus is a member of the picornavirus family which appears to represent a distinct new genus (Jia et al., 1991). Picornaviridae, which include poliovirus and rhinovirus, possess a small (7-8-kb) positive strand RNA genome and a nonenveloped icosahedral capsid. The HAV[1] genome has been cloned and found to possess an organization characteristic of these viruses (Linemeyer et al., 1985; Najarian et al., 1985). Translation of this genome yields a polyprotein of approximately 251 kDa. Analysis of the picornaviral family suggests

* To whom correspondence should be addressed.

[1] Abbreviations: HIV, human immunodeficiency virus; HAV, hepatitis A virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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