The Involvement of Serine 175 and Alanine 185 of Cytochrome b of Rhodobacter sphaeroides Cytochrome bc$_1$ Complex in Interaction with Iron-Sulfur Protein

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An approach involving cysteine replacement of potentially noncritical amino acid residues, followed by chemical modification studies, was used to investigate structure-function of the “cd helix” of cytochrome b from Rhodobacter sphaeroides. Three amino acid residues, Ser-155, Ser-175, and Ala-185, which span this region of cytochrome b, were selected for this study. The S155C substitution yields cells unable to support photosynthetic growth, indicating that Ser-155 is a critical amino acid residue. Further mutational studies of Ser-155 indicate that the size of the amino acid side chain at this position is critical for photosynthetic growth of R. sphaeroides. On the other hand, the S175C and A185C substitutions yield cells with photosynthetic growth rates and enzyme kinetics of the bc$_1$ complexes very similar to those of the unmutated complex, indicating that Ser-175 and Ala-185 are noncritical residues. Thus, engineered cysteines at these two positions of cytochrome b are suitable for membrane topology and domain/subunit interaction studies. Cys-175 does not react with a sulfhydryl-modifying reagent, N-ethylmaleimide (NEM), either in sealed, inside-out chromatophores or in detergent-disrupted chromatophores, indicating that position 175 of cytochrome b is inaccessible from both sides of the membrane and is probably buried within the protein complex. Cys-185 reacts with NEM only after detergent disruption of the sealed, inside-out chromatophores, indicating that this position of cytochrome b is accessible on the outer (periplasmic) surface of the membrane. These results place the cd helix of cytochrome b on the periplasmic side of the chromatophore membrane. When purified A185C-substituted bc$_1$ complex was treated with NEM, about 87% of the activity was abolished due to NEM modification of Cys-185. The signature of the Rieske iron-sulfur center is broadened upon NEM modification of A185C, with the $g_s$ signal shifting from $g = 1.80$ to $g = 1.75$, suggesting that Alai185 of cytochrome b interacts with the iron-sulfur protein. When purified S175C-substituted bc$_1$ complex is treated with NEM, no change in the activity is observed, since Cys-175 is inaccessible to NEM. However, when the iron-sulfur protein is removed from the S175C-substituted bc$_1$ complex, Cys-175 becomes accessible to NEM, indicating that Ser-175 of cytochrome b is shielded by the iron-sulfur protein in the bc$_1$ complex.

The cytochrome bc$_1$ complex from the photosynthetic bacterium Rhodobacter sphaeroides has been purified and characterized in several laboratories (1–6). This complex catalyzes electron transfer from ubiquinol to cytochrome c$_2$ in the photosynthetic cyclic electron transfer system and concomitantly translocates protons across the membrane to generate a membrane potential and pH gradient for ATP synthesis. The purified complex contains four protein subunits and five redox-active centers. Subunit I houses cytochromes b$_565$ and b$_562$, subunit II houses cytochrome c$_1$, and subunit III houses the iron-sulfur cluster. Subunits I and IV have been identified as the Q$^1$-binding proteins in the complex by photoaffinity labeling using an azido-Q derivative (7).

The R. sphaeroides cytochrome bc$_1$ complex is functionally analogous to the mitochondrial ubiquinol-cytochrome c reductase, and the three largest subunits are homologous to their mammalian counterparts. Biophysical, biochemical, and genetic (8, 9) studies of this bacterial complex have contributed greatly to our present knowledge of its electron and proton transfer mechanisms. It is generally believed that electron and proton transfer in this complex follows the Q-cycle mechanism (10–12), which hypothesizes two Q-binding sites, a ubiquinol oxidation site (Q$_o$) and a ubiquinone reduction site (Q$_i$ site). The two Q-binding sites are thought to be on opposite sides of the membrane, with quinol oxidation occurring on the periplasmic side and quinone reduction occurring on the cytoplasmic side.

The cytochrome b polypeptide is major structural element of both Q-binding sites. Two Q-binding regions were identified in the cytochrome b subunit of bovine heart mitochondrial cytochrome bc$_1$ complex (13) by isolating and sequencing azido-Q-linked peptides from azido-Q-labeled cytochrome b. These two regions correspond to amino acid residues 158–171 and 369–379 of the R. sphaeroides cytochrome b$_5$ sequence (14). According to the 8-helix structural model of cytochrome b (15, 16), the first Q-labeled peptide is located in the cd helix, an amphipathic helix in the amino-terminal portion of the connecting loop between transmembrane helices C and D. The second labeled peptide is in the transmembrane helix G. Mutational studies of the cd helix region of cytochrome b have been extensive; substitutions for Gly-158, Ile-162, and Thr-163 have been reported to confer resistance to Q$_o$ center inhibitors (17, 18). These results are consistent with participation of the first labeled peptide in the formation of the Q$_o$ site. The participation of the second labeled peptide in a Q-binding site has not been well established, since no mutation studies have been reported.

To further understand the Q$_o$ site in the cytochrome bc$_1$

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The abbreviations used are: Q, ubiquinone; Q$_{H_2}$, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; DTT, dithiothreitol; DM, dodecylmaltoside; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; high pressure liquid chromatography.
complex, we have replaced a number of relatively conserved amino acid residues in the cd helix region of the R. sphaeroides cytochrome b. Herein, we report generation and characterization of R. sphaeroides mutants carrying the S155C, S175C, or A185C amino acid substitution in cytochrome b, the topology of the cd helix region of cytochrome b in the chromatophore membrane, and involvement of S175 and A185 of cytochrome b complex in the interaction with the iron-sulfur protein of the cytochrome bc$_1$ complex. The involvement of Cys-167 of cytochrome c$_1$ in interaction with the iron-sulfur protein in the bc$_1$ complex was also observed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dodecylmaltoside (DM) was purchased from Anacrace. $^3$H-Labeled N-ethylmaleimide (NEM) was from Du Pont. Dithiothreitol (DTT) was from Sigma. All other chemicals were of the highest purity commercially available. pSELNB3503, which was used as the template for mutagenesis, and pRKDNB35KmBP, which were used for the transfer and expression of wild type and engineered $\text{fbc}$ genes, were constructed in our laboratory (19). Restriction endonucleases and DNA-modifying enzymes were purchased from Promega, Life Technologies, Inc., New England Biolabs, U.S. Biochemical Corp., Perkin-Elmer, and Pharmacia Biotech Inc. Escherichia coli S17–1 (20) and R. sphaeroides BC17 (14) were generously provided by Dr. R. B. Gennis of the University of Illinois.

**Growth of Bacteria**—E. coli was grown at 37 °C on LB medium. Extra-rich media, e.g. TYP, were used in procedures for the rescue of single-stranded DNA or the purification of low copy number plasmids (21). R. sphaeroides cells were grown at 30 °C on an enriched Sistrom's medium (22) essentially as described (19). Antibiotics were added at the following concentrations: ampicillin, 100–125 mg/liter; tetracycline, 5–10 mg/liter; trimethoprim, 85–100 mg/liter for E. coli, and 25–30 mg/liter for R. sphaeroides; kanamycin sulfate, 30–50 mg/liter for E. coli and 20–25 mg/liter for R. sphaeroides; trimethoprim, 85–100 mg/liter for E. coli and 25–30 mg/liter for R. sphaeroides.

**Construction of Mutation(s) and Expression of Mutated Complexes in R. sphaeroides**—Mutants were constructed by site-directed mutagenesis using the Altered Sites system from Promega Corp. (23), and oligonucleotides were synthesized at the OSU Recombinant DNA/Protein Facility using the Altered Sites system from Promega Corp. (23). Mutants were constructed by site-directed mutagenesis of R. sphaeroides with the following choices: ampicillin, 100–125 mg/liter; tetracycline, 5–10 mg/liter for E. coli, and 20–25 mg/liter for R. sphaeroides; trimethoprim, 85–100 mg/liter for E. coli and 25–30 mg/liter for R. sphaeroides.

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**Isolation of $^{[3H]}$NEM-labeled Peptides from Cytochrome c$_1$**—The $[^{3}\text{H}]$NEM-labeled cytochrome c$_1$ band was excised from the SDS-PAGE gel, and the protein was eluted using an electroeluter from Bio-Rad. The electroelutically eluted protein was concentrated with a Centricon-10 and precipitated with 50% cold acetone (−20 °C). The precipitate was resuspended in 50 mM Tris-Cl buffer, pH 7.4, and digested with endoprotease Arg-C at 37 °C for 12 h. $100\mu$m aliquots of the Arg-C-digested cytochrome c$_1$ protein were then separated by HPLC on a Synchro-pak RP-18 column (0.46 × 25 cm) using a gradient from 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid containing 90% acetonitrile, with a flow rate of 0.8 ml/min. Samples were collected in 0.8-ml fractions. The absorbance at 214 nm and the radioactivity of each fraction were measured. Peaks with high specific radioactivity were collected, dried, and subjected to peptide sequence analysis.

**Other Biochemical Methods**—Protein was determined by the Lowry method (24) with the inclusion of 1% SDS and 1% mercaptoethanol in the sample and standards. For accurate measurement of the protein content of chromatophores, interfering pigments were removed by acetone/methanol extraction as described (30). Cytochrome b$_{3}$ (31) and cytochrome c$_{3}$ (32) were determined according to published methods.

**SDS-PAGE** was performed according to Laemmli (33) using a Bio-Rad Mini-Protean dual slab vertical cell.

Low temperature EPR spectra were obtained with Bruker ER200D spectrometer.
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A185C—

and Ala-185 of cytochrome chromatophore membranes and in the purified state, Ser-175 of those of the complex from the complement strain, both in other subunits. On the other hand, the substitution of Ser-155 be used to study topology of the cd helix and its interaction with other subunits. On the other hand, the substitution of Ser-155 with cysteine yields cells unable to grow photosynthetically at position 155 of cytochrome b. This peptide is located in an extra-membrane amphipathic cd helix in the eight-transmembrane helix model of cytochrome b. Certain mutations at Gly-158, Ile-162, and Thr-163 of cytochrome b confer resistance to Qo center inhibitors, indicating that the cd helix may be involved in the Qo site (17, 18). If this cd helix is indeed a part of the Qo site, it would have to be located on the periplasmic side of the chromatophore membrane according to the Q-cycle mechanism. An approach involving cysteine substitution at noncritical amino acid residues, followed by chemical modification of engineered cysteines, was adopted for a study of the topology of the cd helix.

Three amino acid residues, Ser-155, Ser-175, and Ala-185, which are located before, within, and after the putative cd helix of cytochrome b, were chosen to be mutated to cysteines. Table I summarizes the photosynthetic growth behavior of cells expressing the wild type cytochrome b complex (complement cells) and the three mutated complexes were virtually the same as that of the complement bc1 complex (−0.9 µM), indicating that the structural requirement for a small amino acid residue at position 155 of cytochrome b may not be simply to accommodate a Q molecule at this position. This speculation is consistent with the crystallographic structural data from the bovine heart cytochrome b1 complex, which shows that the corresponding amino acid residue does not contribute to the so-called Qo cavity (34).

Since serine and alanine residues occupy virtually the same volume in proteins, the 2.5-fold reduction in activity observed when alanine replaces serine at position 155 of cytochrome b suggests that the hydroxyl moiety of this serine plays some role in maintaining the optimal protein structure and/or reactivity, perhaps by participating in an important hydrogen bond. In the case of the replacement of Ser-155 by glycine, which displayed full retention of activity, the hydroxyl group could be supplied perhaps by participating in an important hydrogen bond. In the case of the replacement of Ser-155 by glycine, which displayed full retention of activity, the hydroxyl group could be supplied by a cavity-filling water molecule, a situation thought to occur in several well-studied proteins upon substitution of glycine for a serine (35–37).

Topology of the cd Helix of Cytochrome b—The topology of the cd helix of cytochrome b was studied by comparing the reaction of NEM with the cysteines of wild type and mutated bc1 complexes contained within the membranes of sealed (inside-out) versus detergent-disrupted chromatophores. The intactness of sealed chromatophores preparations was confirmed by measuring the increase of the bc1 complex activity upon the addition of detergent; this increase averaged 6-fold after treatment with 0.2% potassium deoxycholate. Three parallel experiments were performed on each sealed chromatophore preparation (see Fig. 1) (1). The sealed chromatophores were treated with 1 mM NEM followed with 2 mM DTT to remove any unreacted NEM. The chromatophores were then treated with 0.2% deoxycholate to break the membrane. This reaction sequence will label -SH groups accessible on the outside of the chromatophore membrane (cytoplasmic surface) but not those exposed on the inside of the vesicles or buried within the membrane or protein interior (2). The sealed chromatophores were disrupted with 0.2% deoxycholate and then treated with 1 mM NEM. The excess NEM was removed by the addition of 2 mM DTT. This reaction sequence should label all of the externally accessible cysteines from either side of the chromatophore membrane (cytoplasmic and periplasmic surfaces) (3). The sealed chromatophores were broken with 0.2% deoxycholate and then treated with 2 mM DTT prior to reaction with 1 mM NEM. This control reaction

### Table I

| Mutations       | Photosynthetic growth | Enzymatic activitya | Chromatophores | Purified bc1 |
|-----------------|-----------------------|---------------------|----------------|-------------|
|                 |                       | µmol cytochrome c reduced/min/µmol cytochrome b |                |             |
| Complement (no mutation) | ++b                  | 1.9                 | 2.2            |
| S155C           | ++                    | 1.7                 | 2.0            |
| S175C           | ++                    | 1.8                 | 2.3            |
| A185C           | ++                    | 1.7                 | 2.0            |

a The Km values of S155C and A185C are almost the same as that of the bc1 from the complement strain, Km−0.9 µM Q2H2. b +; the photosynthetic growth phenotype is essentially the same as the “wild type.” c −, no photosynthetic growth within 7 days. d NA, not available.

Results and Discussion

Characterization of R. sphaeroides Mutants Expressing the S155C, S175C, and A185C Alterations in Cytochrome b—Previous studies of the cytochrome bc1 complex from beef heart mitochondria have identified Q-binding peptides within cytochrome b, one of which corresponds to residues 158–171 of cytochrome b of R. sphaeroides. This speculation is consistent with the crystallographic structural data from the bovine heart cytochrome b1 complex, which shows that the corresponding amino acid residue does not contribute to the so-called Qo cavity (34).

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sequence provides unlabelled membranes that have gone through the experimental procedure, but with sulphhydryl modification blocked by the addition of DTT before NEM.

When sealed chromatophores prepared from complement, S175C, and A185C-expressing cells were treated with NEM, no change in cytochrome bc₁ complex activity was observed. When deoxycholate-disrupted chromatophores were treated with NEM, about 85% of the cytochrome bc₁ complex activity in the cytochrome b A185C substitution was abolished, while no change in activity was observed with complement or the cytochrome b S175C substitution. Since no change in the cytochrome bc₁ activity was observed after NEM treatment of either sealed or broken chromatophores from complement cells, the endogenous cysteine residues contained in the cytochrome bc₁ complex are probably inaccessible to NEM treatment (see below). This result greatly simplifies our assessment of the location of the engineered cysteines in the chromatophore membrane. Ala-185 of cytochrome b is located on the inside surface of the chromatophore membrane (periplasmic side) because NEM did not react with the cysteine residue engineered at cytochrome b position Ala-185 in the sealed inside-out chromatophore preparation, but it did react with this engineered cysteine in disrupted chromatophores. Since Ala-185 is contained in the cd loop connecting the C and D transmembrane helices of cytochrome b, the cd loop must also be on the periplasmic side of the chromatophore membrane. The placement of the cd loop on the periplasmic side of the chromatophore membrane is consistent with the current cytochrome bc₁ crystal structure from bovine heart, which describes the cytochrome b protein as a membrane-spanning polypeptide having eight transmembrane helices (named A–H) and several transversal helices on both sides of the membrane, including a cd helix comprising the amino-terminal portion of the cd loop located on the periplasmic side. The observation of no activity loss in both sealed and disrupted chromatophore preparations from the cytochrome b S175C mutant cells upon NEM treatment indicates that the Ser-175 position of the cd helix is inaccessible to the aqueous phase, either facing the interior of cytochrome b or covered by another subunit of the complex.

Effect of NEM on Purified Cytochrome bc₁ Complexes from S175C and A185C Mutant Cells—The cytochrome bc₁ complex contains nine cysteine residues: one in cytochrome b, four in cytochrome c₁, and four in the iron-sulfur protein. It has been established that two cysteines in the iron-sulfur protein are ligands to the [2Fe-2S] cluster (38), and two cysteines in cytochrome c₁ are covalently bonded to heme c (39). Thus, there are five free cysteines that are potential candidates for NEM modification. When the cytochrome bc₁ complex from complement strain was treated with NEM, no change in enzymatic activity was observed (Table II), indicating that cysteine residues contained in the cytochrome bc₁ complex are either inaccessible to NEM or the reaction product is functionally active. Radioactive NEM was used to distinguish these two possibilities. Since no radioactivity was found in any of the four subunits of the complement bc₁ complex treated with [³H]NEM (see Fig. 2), the lack of inhibition by NEM treatment must be due to the inaccessibility of the free cysteines rather than to formation of active cysteine-NEM products.

When the cytochrome bc₁ complex containing the cytochrome b S175C replacement was treated with NEM, no loss of activity was observed (see Table II), suggesting that Ser-175 of cytochrome b is shielded by other subunits or other parts of cytochrome b in the bc₁ complex. The inaccessibility of the engineered cysteine of cytochrome b to NEM treatment is further confirmed by the absence of radioactive labeling of the cytochrome b subunit of the cytochrome b S175C cytochrome bc₁ complex treated with [³H]NEM (see Fig. 2). These results are consistent with the three-dimensional structure analysis of the bovine heart bc₁ complex, which shows that the amino acid residue corresponding to Ser-175 is not exposed on the surface of the molecule.

When the cytochrome b A185C-cytochrome bc₁ complex was treated with various concentrations of NEM, about 87% of the bc₁ activity was lost (Table II) when 2.0 mol of NEM/mol of cytochrome b heme was used, consistent with the results observed in chromatophores (see above). The loss of activity correlates with the incorporation of NEM into the cytochrome b subunit; when [³H]NEM-treated cytochrome b A185C-cytochrome bc₁ complex, which had lost 87% of its activity, was subjected to SDS-PAGE, all of the radioactivity was located in the b subunit (Fig. 2). About 1 mol of NEM was incorporated into one mol of cytochrome b protein.

EPR Characteristics of Cytochrome b, Cytochrome c₁, and Iron-Sulfur Cluster in NEM-treated, A185C Cytochrome bc₁ Complex—EPR spectra properties of cytochrome b, cytochrome c₁, and the Rieske iron-sulfur cluster in the cytochrome b A185C-cytochrome bc₁ with and without NEM treatment, were examined in an attempt to identify which active centers of the complex are perturbed by the modification at position 185 and thus may interact with this region of cytochrome b.
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Fig. 2. Radioactivity distribution among subunits of the cytochrome bc1 complexes from complement, S175C, and A185C treated with [3H]-NEM. Purified bc1 complexes from complement, S175C, and A185C were incubated with [3H]NEM as described under "Experimental Procedures." ISP, iron-sulfur protein.

Fig. 3 shows the EPR characteristics of cytochromes b in the cytochrome bc1 complexes of complement and cytochrome b A185C with and without NEM treatment. The cytochrome b A185C-cytochrome bc1 complex exhibits two EPR signals at g = 3.5 and g = 3.76, corresponding to b565 and b562, respectively. These two b signals are identical to those observed in cytochrome b of the complement bc1 complex, indicating that the cytochrome b A185C substitution probably has little effect on the cytochrome b heme environments. When the cytochrome b A185C-cytochrome bc1 complex was treated with NEM to inactive the complex, the EPR characteristics of cytochrome b (Fig. 3) and cytochrome c1 (data not shown) in the treated complex were the same as those in the untreated complex, suggesting that Ala-185 is not involved in interaction with the hemes of the cytochrome b molecule or with cytochrome c1.

Fig. 4 compares EPR characteristics of the Rieske iron-sulfur clusters in the cytochrome bc1 complexes of complement and A185C, with and without NEM treatment. When complement and A185C-substituted bc1 complexes were reduced by a small excess of ascorbate, the EPR signals of the Rieske iron-sulfur cluster in these two cytochrome bc1 complexes were essentially the same, with resonances at g\(_x\) = 2.02, g\(_y\) = 1.89, and g\(_z\) = 1.80. However, when the A185C bc1 was treated with NEM, the g\(_x\) = 1.80 signal broadened and shifted to 1.75, while no change in the iron-sulfur spectrum was observed in NEM-treated complement bc1 complex. Upon complete reduction of the NEM-treated complement and A185C mutant bc1 complexes with dithionite, the spectrum of the bc1 complement complex is broadened, with g\(_x\) shifting to 1.75, as previously reported for the wild type bc1 complex under fully reduced conditions (4, 5), whereas the dithionite-reduced spectrum of the NEM-treated A185C complex remains unchanged with g\(_x\) = 1.75. The NEM-treated A185C complex thus has a spectrum closely resembling the "reduced state" spectrum of the complement, regardless of the redox state of the ubiquinone pool.

The iron-sulfur subunit is thought to bind in the general vicinity of b565 on the positive side of the membrane to form part of the quinol-oxidizing center, because the iron-sulfur cluster is a primary electron acceptor from the quinol. The particular line shape observed for the [2Fe-2S] cluster is thought to be mediated by the oxidation state of the ubiquinone present in the Q\(_o\) site (4, 5, 40–42). When oxidized quinone is present, the g\(_x\) signal is sharper than that observed when quinol is present. The g\(_x\) of the bc1 from R. sphaeroides is found at g = 1.80 when ubiquinone is present, but shifts to 1.75 and becomes much broader when ubiquinol is present. NEM modification of the engineered cysteine at position 185 of cytochrome b resulted in broadened [2Fe-2S] EPR signals with g\(_x\) = 1.75, independent of the redox potential. There was no detectable difference between the EPR spectrum of the NEM-treated A185C complex and the full reduction spectrum of the NEM-
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A185C, cytochrome b, complex. This idea is consistent with the current x-ray crystal structure of the [2Fe-2S] cluster or is located in the close vicinity of the cluster. This density gradient serves two purposes: to separate the iron-sulfur protein from the gradient mainly consisting of cytochrome b, cytochrome c1, and subunit IV (the pH of these middle fractions is neutral); lane 9, the bottom-most fraction, which contains no significant protein bands.

The effect of NEM treatment on the iron-sulfur cluster of the A185C complex is also reminiscent of the change observed for the substitution of Leu for Phe-144 (F144L) in the cytochrome b from R. capsulatus (41). The F144L complex in R. capsulatus chromatophores was reported to have a very low turnover rate with a broadened, redox state-insensitive, extremely broad line shape reported for the quinone-depleted state, they are probably not due to a complete absence for quinone and quinol binding to the Qo center.

In a subsequent study of the effect of extraction of ubiquinone from chromatophore membranes on the iron-sulfur cluster, Ding et al. (42) found that the g6 signal of the “depleted state” at approximately g = 1.765 was broadened considerably beyond that seen in the presence of either ubiquinone or ubiquinol. Since the changes in the g6 signal of iron-sulfur clusters resulting from the NEM modification of Cys-185 of R. sphaeroides cytochrome b in the complex do not exhibit the extremely broad line shape reported for the quinone-depleted state, this is not due to a complete absence for quinone and quinol binding to the Qo center.

[3H]NEM Distribution among Subunits of the Iron-Sulfur Protein (ISP)-Depleted bc1 Complex—Since we have shown that the Ala-185 residue of cytochrome b is near the iron-sulfur protein, the inaccessibility of the engineered cysteine at position 175 could be the result of close interaction between the iron-sulfur protein and the cd helix of cytochrome b. In that case, removal of iron-sulfur protein from the bc1 complex might expose Ser-175 and thus make it accessible to NEM. The bc1 complex was dissociated into Rieske iron-sulfur protein and the iron-sulfur protein-depleted bc1 subcomplex by incubation with Na2CO3 (pH 10.5) under reducing conditions (43). The addition of urea (0.6 M) to the solution helps the dissociation process. Since NEM is not stable at alkaline pH, modification of dissociated subcomplex cannot be carried out without neutralization. To prevent the reassociation of the iron-sulfur protein to the complex upon neutralization, the dissociated iron-sulfur protein was removed by pH sucrose density gradient centrifugation. This density gradient serves two purposes: to separate the iron-sulfur protein from the bc1 subcomplex and to restore neutral pH to avoid further destruction of subcomplex. Under the centrifugation conditions used, the subcomplex and iron-sulfur protein fractions are well separated. A typical distribution of the two components obtained after centrifugation is shown in Fig. 5. The fractions at the top of the gradient contained iron-sulfur protein, whereas the fractions at the bottom contained the larger, faster sedimenting bc1 subcomplex. The bc1 subcomplex was modified with [3H]NEM after removal of DTT by repeated dilution and concentration using Centricon-30. The incorporation of NEM into wild type bc1 subcomplex...
was 1.1 NEM/bc1, while 2.2 molecules of NEM were incorporated into subcomplex with the S175C replacement. Fig. 6 shows the \(^3\)H radioactivity distribution among subunits of complex and S175C-substituted cytochrome bc1 subcomplexes. When \(^3\)H-NEM-treated complement cytochrome bc1 subcomplex was subjected to SDS-PAGE, radioactivity was found in cytochrome c subunit (see Fig. 6A), indicating that one of the cysteines in cytochrome c is shielded by the iron-sulfur protein in the intact bc1 complex and became accessible to NEM after its removal. When the cytochrome bc1 subcomplex containing cytochrome b with the S175C mutation was treated with \(^3\)H-NEM, both cytochrome b and cytochrome c subunits became labeled (see Fig. 6B), indicating that Ser-175 of cytochrome b is also shielded by the iron-sulfur protein in the intact bc1 complex.

Isolation of a \(^3\)H-NEM-labeled Peptide from Cytochrome c—The fact that one of the cysteines from cytochrome c became labeled in bc1 subcomplex indicates that this cysteine may be located in the interface between cytochrome c1 and the iron-sulfur protein. To identify which one of the cysteines reacts with NEM, \(^3\)H-NEM-labeled cytochrome bc1 was eluted from SDS-PAGE gels and digested with arginine-specific protease (Arg-C). Fig. 7 shows the radioactivity distribution among the Arg-C-digested peptides of cytochrome c1 separated by HPLC. The majority of the radioactivity was found in fractions 14, 22, and 47. Since very few amino acids were detected in fractions 14 and 22, it is likely that they contained decomposed \(^3\)H-NEM. Some radioactivity was also found in fractions 60–70, due to the incomplete digestion of cytochrome c1.

The partial N-terminal amino acid sequence of the labeled peptide in fraction 47 was determined to be AGFHPGMGT. From the primary sequence of cytochrome c1, this can be seen to be the initial portion of the expected Arg-C proteolytic fragment encompassing residues 130–180. The only cysteine in this peptide is Cys-167, which is thought to be located in the soluble domain of cytochrome c1. Our results suggest that the region containing Cys-167 forms part of the interface of cytochrome c1 with the Rieske iron-sulfur protein. Elucidation of the exact docking surfaces of these two subunits, as well as of cytochrome b and the iron-sulfur protein, requires refinement of the crystal structure and/or other detailed protein characterizations.

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