Identification of Quinone-binding and Heme-ligating Residues of the Smallest Membrane-anchoring Subunit (QPs3) of Bovine Heart Mitochondrial Succinate:Ubiquinone Reductase*

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The smallest membrane-anchoring subunit (QPs3) of bovine heart succinate:ubiquinone reductase was over-expressed in Escherichia coli JM109 as a glutathione S-transferase fusion protein using the expression vector pGEX2T/QPs3. The yield of soluble active recombinant glutathione S-transferase-QPs3 fusion protein was isopropyl-β-D-galactopyranoside (0.4 mM)-induction growth at 25 °C in 2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose (SOC medium) containing 440 mM sorbitol and 2.5 mM betaine. QPs3 was released from the fusion protein by proteolytic cleavage with thrombin. Isolated recombinant QPs3 shows one protein band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis that corresponds to subunit V of mitochondrial succinate:ubiquinone reductase. Although purified recombinant QPs3 is dispersed in 0.01% dodecylmaltoside, it is in a highly aggregated form, with an apparent molecular mass of more than 1 million. The recombinant QPs3 binds ubiquinone, causing a spectral blue shift. Upon titration of the recombinant protein with ubiquinone, a saturation behavior is observed, suggesting that the binding is specific and that recombinant QPs3 may be in the functionally active state. Two amino acid residues, serine 33 and tyrosine 37, in the putative ubiquinone binding domain of QPs3 are involved in ubiquinone binding because the S33A or Y37A-substituted recombinant QPs3s do not cause the spectral blue shift of ubiquinone. Although recombinant QPs3 contains little cytochrome b₅₆₀ heme, the spectral characteristics of cytochrome b₅₆₀ are reconstituted upon addition of hemin chloride. Reconstituted cytochrome b₅₆₀ in recombinant QPs3 shows a EPR signal at g = 2.92. Histidine residues at positions 46 and 60 are responsible for heme ligation because the H46N- or H60N-substituted QPs3 fail to restore cytochrome b₅₆₀ upon addition of hemin chloride.

Bovine heart mitochondrial succinate: ubiquinone (Q)₅ reductase, also known as complex II, which catalyzes electron transfer from succinate to ubiquinone, has been purified and characterized (1–3). Purified reductase shows five protein bands (4) in a high resolution sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) system, with apparent molecular masses of 70, 27, 15, 13, and 11 kDa. The reductase contains five prosthetic groups: one covalently linked FAD, three iron-sulfur clusters (2Fe-2S, 4Fe-4S, and 3Fe-4S), and cytochrome b₅₆₀. The larger two subunits (Fp & Ip) are succinate dehydrogenase and house the FAD and the three-iron sulfur clusters, respectively. The smaller three subunits (QPs1, QPs2, and QPs3) are membrane-anchoring proteins. It is still unknown which of these three membrane-anchoring subunits house cytochrome b₅₆₀ heme.

Bovine heart mitochondrial succinate:Q reductase has been resolved into two reconstitutively active fractions: soluble succinate dehydrogenase (5) and the membrane-anchoring fraction (QPs) (6). Purified succinate dehydrogenase can catalyze electron transfer from succinate to artificial electron acceptors such as phenazine methosulfate but not to its physiological electron acceptor, Q. Addition of QPs to succinate dehydrogenase reconstitutes membrane-bound succinate:Q reductase, which catalyzes TTFA-sensitive electron transfer from succinate to Q, indicating that QPs provide membrane docking for succinate dehydrogenase and Q-binding for the reductase.

The involvement of QPs in the Q-binding of succinate:Q reductase is further supported by the detection of ubisemiquinone radicals in intact or reconstituted succinate:Q reductase formed from QPs and succinate dehydrogenase but not in succinate dehydrogenase alone (7). Furthermore, when succinate:Q reductase is photofluorin-labeled with an azido-[³²H]Q derivative, radioactivity is found in the QPs subunits but not in the succinate dehydrogenase subunits (4). The radioactivity distribution is 45, 22, and 25% in QPs1, QPs2, and QPs3, respectively (4).

The Q-binding domains in QPs1 and QPs3 have been identified as residues 113–140 and 29–37, respectively, by matching the sequences of azido-Q-linked peptides to their respective protein sequences. The amino acid sequences of QPs1 and QPs3 are obtained from cloning and nucleotide sequencing of the cDNAs (4, 8, 9) encoding these two proteins. The Q-binding domain in the proposed model of QPs1 is located at the connecting loop between transmembrane helices II and III toward the matrix side (4). The Q-binding domain in the proposed model of QPs3 is located at the end of transmembrane helix I toward the C side of the mitochondrial inner membrane (9). Location of Q-binding domains of QPs1 and QPs3 on opposite acrylamide gel electrophoresis; SOC, 2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose; TTFA, thionyltrifluoroacetone azido-[³²H]Q, 3-azido-2-methyl-5-methoxy-[³²H]-6-decyl-1,4-benzoquinone.

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The abbreviations used are: Q, ubiquinone; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; SOC, 2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose; TTFA, thionyltrifluoroacetone azido-[³²H]Q, 3-azido-2-methyl-5-methoxy-[³²H]-6-decyl-1,4-benzoquinone.

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sides of the membrane is in line with a two-Q-binding site hypothesis formulated from inhibitor binding studies of this enzyme complex (10).

Isolated QPs contains 27 nmol of cytochrome b$_{560}$/mg of protein. The role of this cytochrome in beef succinate:Q reductase is controversial. Because cytochrome b$_{560}$ in succinate:Q reductase is not reduced by succinate and because it is present in a subs- totiomeric amount with respect to FAD, its direct involvement in succinate:Q reductase catalysis has been ruled out by some investigators (6). On the other hand, it has been proposed (11) that cytochrome b$_{560}$ functions as a mediator between low potential F/F$^+$ and Q/Q$^+$ couples in a dual pathway model of electron flow through cardiac succinate:Q reductase. Despite its rather unclear catalytic role, the involvement of cytochrome b$_{560}$ in the binding of succinate dehydrogenase to QPs is clearly indicated by restoration of the absorption properties, redox potential, and EPR characteristics of cytochrome b$_{560}$ in QPs during formation of TTF-A-sensitive succinate:ubiquinone reductase from isolated QPs and succinate dehydrogenase (6).

The ligand for cytochrome b in succinate:Q reductase from beef heart mitochondria (b$_{560}$) and Escherichia coli (b$_{560}$) has been identified as bishistidine (12, 13). Both the membrane-anchoring subunits (SdhC and SdhD) in the E. coli enzyme are involved in heme ligation of cytochrome b$_{556}$ (14). His-84 of the SdhC and His-71 of the SdhD were identified as ligands for cytochrome b$_{560}$ (15). However, information about amino acid residues involved in the bishistidine ligand of bovine cytochrome b$_{560}$ is lacking.

A better understanding of the structure-function relationship of succinate:Q reductase, especially of the amino acid residues involved in Q-binding, heme b$_{560}$ ligation, and succinate dehydrogenase docking, requires functionally active QPs subunits. There are two ways to purify purified QPs subunits: one is by biochemical resolution of QPs into their individual subunits; the other is by gene expression to generate recombinant QPs proteins. The availability of the cDNA for QPs3 (9) in our laboratory together with our past experience in overexpression of Rhodobacter sphaeroides (18) in E. coli encouraged us to obtain purified QPs3 by the gene expression approach. The pGEX expression system was used because it allows one-step purification of recombinant fusion protein with glutathione-agarose gel. Herein we report the construction of the expression vector, pGEX/QPs3, growth conditions for overexpression of the active soluble form of GST-QPs3 fusion protein in E. coli JM109 and properties of recombinant QPs3. The Q-binding function of recombinant QPs3 is established by its ability to cause a spectral blue shift of ubiquinone. The heme b$_{560}$ ligation property of recombinant QPs3 is established by its ability to restore the spectral properties of cytochrome b$_{560}$ upon addition of heme chloride. The amino acid residues of QPs3 involved in Q-binding and heme ligation were identified by site-directed mutagenesis using the Altered Sites™ Mutagenesis system from Promega. A 342-base pair EcoRI fragment was excised from pCR2.1/QPs3 plasmid (9) and cloned into the EcoRI site of pSelect-1 vector to generate pSELECT/QPs3. The single-stranded pSELECT/QPs3 was used as the template in the mutagenesis reactions. The mutagenic oligonucleotides used were as follows: H89D, 5'-CTCACGTGAAGCCCTGATC; H9D, 5'-CTCACGTGTGC-3'; H9N, 5'-CTCACCTGAGGGAC-3'; H9Y, 5'-CTCACCTCTGGGAC-3'; H46N, 5'-CTCACAGTAACTGGGGCATT; H48N, 5'-CTTACAGTAACTGGGGCATT; H48Y, 5'-CTTACAGTAACTGGGGCATT; H60N, 5'-CTCACGTGAGGGAC-3'.

### EXPERIMENTAL PROCEDURES

#### Materials

- Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were obtained either from Promega or Life Technologies Inc. Plasmid and fragment isolation kits were obtained from Qiagen. Nitrocellulose membranes were from Schleicher & Schuell. Bovine serum albumin, isopropyl-$eta$-D-thiogalactopyranoside (IPTG), $\beta$-glucose, ampicillin, tetracycline, 6-aminoenolic acid, ferrous sulfate, gelatin, hemin chloride, sorbitol, betaine, glutathione (reduced form), glutathione-agarose beads, thrombin, leupeptin, phenylmethylsulfonyl fluoride, and 2,6-dichlorophenol indophenol were from Sigma. Agarose, acrylamide, bisacrylamide, horseradish peroxidase, antipeptide QPs3 antibodies (9). Both protein were identified by immunological screening of colonies with rabbit and purified as previously reported (9). Oligonucleotides were synthesized in the Recombinant DNA/protein resource facility at Oklahoma State University.

#### Bacterial Strains and Plasmids

- E. coli strain INVaF was used as host for pCR2.1 vector (Invitrogen); E. coli JM109 or DH5a was used as the host for pSelect (Promega) and pGEX2T (Amersham Pharmacia Biotech).

#### DNA Manipulation and DNA Sequencing

- General molecular genetic techniques were performed according to procedures described in Sambrook et al. (19). DNA sequencing was performed with an Applied Biosystems model 373 automatic DNA sequencer at the recombinant DNA/protein resource facility at Oklahoma State University.

#### Construction of E. coli Strains Expressing Wild-type and Mutant QPs3

- The 331-base pair BamHI-EcoRI cDNA fragment encoding mature QPs3 was amplified from a bovine heart cDNA library by polymerase chain reaction using two synthetic primers, 5'-GGAATCTCTGGTTCGATGAAGGATGGTGGGGCTTACAGTAACTGGGGCATT-3' (the sense primer) and 5'-GAATTCTTCTCTAAAGAGTCTAGAAGCCCTGATCACCAGGTCG-3' (the antisense primer). This fragment was cloned into pCR2.1 vector and confirmed by sequence analysis before being subcloned into the BamHI and EcoRI site of pGEX2T vector to generate pGEX/QPs3. E. coli transformants producing the GST-QPs3 fusion protein were identified by immunological screening of colonies with antipeptide QPs3 antibodies (9). Both E. coli strains JM109 and DH5a were found to be suitable hosts for pGEX/QPs3.

#### QPs3 DNA mutations were generated by site-directed mutagenesis using the Altered Sites™ Mutagenesis system from Promega. A 342-base pair EcoRI fragment was excised from pCR2.1/QPs3 plasmid (9) and cloned into the EcoRI site of pSELECT-1 vector to generate pSELECT/QPs3. The single-stranded pSELECT/QPs3 was used as the template in the mutagenesis reactions. The mutagenic oligonucleotides used were as follows: H89D, 5'-CACAAGCTTCCTGGAGATGCA; H9D, 5'-CACAAGCTTCCTGGAGATGCA; H9N, 5'-CACAAGCTTCCTGGAGATGCA; H9Y, 5'-CACAAGCTTCCTGGAGATGCA; H46N, 5'-CACAAGCTTCCTGGAGATGCA; H48N, 5'-CACAAGCTTCCTGGAGATGCA; H48Y, 5'-CACAAGCTTCCTGGAGATGCA; H60N, 5'-CACAAGCTTCCTGGAGATGCA; H60Y, 5'-CACAAGCTTCCTGGAGATGCA; H89D-5'-GCTCGGTGATGCAAGATGCA; H9D, 5'-GCTCGGTGATGCAAGATGCA; H9N, 5'-GCTCGGTGATGCAAGATGCA; H9Y, 5'-GCTCGGTGATGCAAGATGCA; H46N, 5'-GCTCGGTGATGCAAGATGCA; H48N, 5'-GCTCGGTGATGCAAGATGCA; H48Y, 5'-GCTCGGTGATGCAAGATGCA; H60N, 5'-GCTCGGTGATGCAAGATGCA; H60Y, 5'-GCTCGGTGATGCAAGATGCA; H89D-5'-GCTCGGTGATGCAAGATGCA; S33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA; H33A, 5'-AAACGAGTTCGATGCGTGCGAGATGCA.

#### TABLE I

| Growth media | Total GST- QPs3 (%) | Soluble (%) | Inclusion body (%) |
|--------------|---------------------|-------------|--------------------|
| SOC          | 7.5                 | 1.0         | 6.5                |
| SOC + betaine + sorbitol | 9.2              | 3.5         | 5.5                |
| Enriched medium | 0.3             | 0.3         |                    |
| Enriched medium + Mg$^{2+}$ | 7.5          | 1.5         | 6.0                |
| Enriched medium + Mg$^{2+}$ + betaine + sorbitol | 15       | 5.5         | 9.8                |

against the QPs3-connecting peptide and GST-QP3 were released in rabbits and purified as previously reported (9). Oligonucleotides were synthesized in the Recombinant DNA/protein resource facility at Oklahoma State University.

#### Effect of induction growth medium on the yield of soluble recombinant GST-QP3 by E. coli JM109/pGEX-QP3 cells

The % yield of recombinant GST-QP3 fusion protein was estimated by comparing the color intensity of the 37-kDa GST-QP3 fusion protein with that of the total cellular protein bands in SDS-PAGE.
H89N substitutions. The failure to obtain QPs3 mutants H46D, H48D, H60D, and H89Y was because of their production as inclusion body precipitates in E. coli cells, which were insoluble after treatment with 6 M urea and subsequent dialysis.

Isolation of Recombinant GST-QPs3 Fusion Protein—400 ml of an overnight culture of E. coli JM109/pGEX/QPs3 was used to inoculate 12 liters of SOC medium (2.9% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose) containing 440 mM sorbitol, 2.5 mM betaine, and 60 mg/liter ampicillin. The culture was grown in a fermentor chamber at 37 °C with aeration until the cell paste (48 g) was suspended in 144 ml of PBS buffer (20 mM sodium/potassium phosphate, pH 7.3, containing 150 mM NaCl) and sonicated at 30 milliwatts at 0 °C with four 20-s pulses at 3 to 4 min intervals. During sonication, protease inhibitor, phenylmethylsulfonyl fluoride, was added to a final concentration of 1 mM. Triton X-100 was added to the broken cell suspension to a final concentration of 1% (w/v). This mixture was stirred gently on ice for 1 h before being centrifuged at 30,000 × g. The supernatant was mixed with an equal volume of glutathione-agarose gel equilibrated with PBS. The mixture was gently shaken on a Varimix (Thermolyne) at 4 °C for 1 h and packed into a column. The column was washed extensively with the equilibrating buffer and then eluted with 50 mM Tris-Cl, pH 8.0, containing 5 mM reduced glutathione and 0.25 M sucrose. Fractions containing the fusion protein were pooled and dialyzed against 50 mM Tris-Cl, pH 8.0, containing 0.25 M sucrose for 8 h with 2 changes of buffer to remove glutathione. The dialyzed sample was concentrated with a Centriprep-30 (Amicon) to a protein concentration of 10 mg/ml, mixed with glycerol to a final concentration of 10%, and frozen at −80 °C until use. QPs3 protein was released from GST-QPs3 fusion protein by thrombin digestion (1 μg/500 μg of protein) and recovered by gel filtration using a fast protein liquid chromatography Superose-12 column. Recombinant QPs3 mutants were obtained in the same manner as the wild type.

FIG. 1. Identification of recombinant QPs3 by Western blot. A, SDS–PAGE of succinate:ubiquinone reductase (lane 2), recombinant GST-QPs3 fusion protein (lane 3), thrombin-treated fusion protein (lane 4), purified recombinant QPs3 (lane 5). The molecular mass standard containing phosphorylase B (108 kDa), bovine serum albumin (84 kDa), ovalbumin (53 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (28 kDa), and lysozyme (20 kDa) is in the lane 1. The proteins in A were electrophoretically transferred to nitrocellulose membrane and reacted with anti-QPs3 peptide antibodies (B) and anti-GST-QPs3 antibodies (C). Protein A horseradish peroxidase conjugate was used as a second antibody.

FIG. 2. Effect of recombinant wild-type and mutant QPs3s on the absorption maximum of Q. To 1.8-ml aliquots of 20 mM sodium phosphate buffer, pH 7.3, containing 150 mM NaCl, 2.5 mM CaCl₂, 10% ethanol, and 0.01% dodecyl maltoside was added 100 μl of 50 mM Tris–Cl buffer, pH 8.0, containing recombinant GST ( ), recombinant wild-type QPs3 ( ), S33A ( ), D56A ( ), or Y37A ( ). The final protein concentration was 300 μM. The mixtures were placed equally in a pair of identical cuvettes (1-cm light path). Q0C10Br in 95% alcohol was added to one sample cuvette in 1-ml increments to obtain the indicated concentrations. At the same time, alcohol (95%) was added to the second sample (reference) cuvette in an identical manner. A difference spectra was calculated between the Q0C10Br-added and alcohol-added samples recorded from 320 to 250 nm after 5 min incubation.

FIG. 3. Absorption spectra of reconstituted cytochrome b₅₆₅ in recombinant GST-QPs3 fusion protein. Three-μl aliquots of hemin chloride (6 mM) in Me₆SO were added to 1 ml of purified recombinant GST-QPs3 (1.5 mg) (A) and 1 ml of recombinant GST (1.5 mg) (B) in Tris-Cl buffer, pH 8.0, containing 0.25 M sucrose. The mixtures were incubated at room temperature, and absorption spectra were recorded from time to time during the incubation period. When the oxidized Soret absorption peak no longer changed (solid line), a small amount of dithionite was added and spectra recorded (dashed line). The inset on A displays the difference spectra of the dithionite-reduced versus oxidized form in the α and β absorption regions.
Enzyme Preparations and General Biochemical Techniques—Succinate-Q reductase (1) and QPs (6) were prepared as reported previously. Absorption spectra and enzyme assays were performed at room temperature in a Shimadzu UV-2101PC. Protein concentration was determined by the Lowry method (20) or by Bradford assay (21) using a kit from Bio-Rad. The heme content was determined from the pyridine hemochromogen spectra using a millimolar extinction coefficient of 34.6 for the absorbance at 557 nm minus that at 600 nm (22). SDS-PAGE was done according to Laemmli (23) or for high resolution, according to Schägger et al. (24). The EPR measurements were made with a Bruker ER-200D equipped with an Air Product Heli-Tran System. EPR instrument settings are given in the figure legends.

RESULTS AND DISCUSSION

Effect of Induction Growth Conditions on Production of Recombinant GST-QPs3 Fusion Protein—The successful overexpression of functionally active subunit IV of the R. sphaeroides cytochrome bc₁ complex (18), the QPc-9.5 kDa of beef ubiquinol-cytochrome c reductase (16), and QPs1 of beef succinate:Q reductase (17) in E. coli, using the pGEX system, encouraged us to use this system to obtain recombinant QPs3.

Production of recombinant GST-QPs3 fusion protein depends on IPTG concentration, induction growth time, and induction growth medium. The yield increases as the IPTG concentration and induction growth time are increased, reaching a maximum at 0.4 mM IPTG and 3.5 h post-induction growth (data not shown). When IPTG concentration is increased to 1 mM, no further increase in expression yield is observed. When cells are grown for more than 3.5 h, the total yield decreases and degradative products increase, as determined by Western blotting using anti-QPs3 peptide antibodies.

The yield of recombinant GST-QPs3 fusion protein in E. coli is high using SOC medium at 37 °C, about 90% expressed protein is in the inclusion body precipitate. Purification of fusion protein from inclusion bodies is not practical, at least in our hands, because the recovery of soluble, active GST-QPs3 from dialyzed, urea-solubilized inclusion body is very low (less than 1%). Because it has been reported that including betaine and sorbitol in the induction growth medium and lowering the induction growth temperature greatly increases the soluble yield of GST fusion proteins (16, 17, 25), these induction conditions were adopted. About 40% of the expressed GST-QPs3 fusion protein in E. coli is in the soluble form when IPTG induction growth is in SOC medium, containing 0.44 M sorbitol and 2.5 mM betaine at 23–25 °C for 3.5 h. About 4 mg of purified fusion protein is obtained from a liter of cell culture. When the purified fusion protein is subjected to SDS-PAGE, a protein band with apparent molecular mass of 37 kDa is obtained. This protein band is confirmed to be GST-QPs3 fusion protein by Western blotting with antibodies against QPs3 and GST-QPs3.

Ubiquinone-binding Property of Recombinant QPs3—Purified recombinant QPs3 disperses in 0.01% dodecylmaltoside with an apparent molecular mass of more than 1 million. This is expected because QPs is a very hydrophobic protein containing three transmembrane helices. Upon SDS-PAGE, purified enzymes...
recombinant QPs3 shows only one band, corresponding to the fifth subunit in succinate:Q reductase (Fig. 1, panel A, lane 5).

Because QPs3 has been identified as one of the Q-binding proteins in succinate:Q reductase (4), it is important to know whether or not recombinant QPs3 binds Q. The Q-binding function of recombinant QPs3 is indicated by its ability to cause the blue spectral shift of Q. This method has been used to establish the Q-binding function of recombinant QPc-9.5 kDa (a small molecular mass Q-binding protein in ubiquinol-cytochrome c reductase) (16). When Q$_{c10}$ is added to recombinant QPs3, a blue spectral shift of Q is observed (Fig. 2, the curve with solid circles). This spectral blue shift of Q is not observed with Pronase-treated recombinant QPs3 or with recombinant GST (Fig. 2, the curve with open circles). Titration of recombinant QPs3 with Q$_{C10}$ shows saturation at around 1.1 mol of Q/mol of protein (see Fig. 2, the curve with solid circles), suggesting that the binding is specific and that the recombinant QPs3 is in the functionally active form for Q-binding.

Identification of Amino Acid Residues Involved in Q-binding of QPs3—Previous photoaffinity labeling studies indicate that the Q-binding domain in QPs3 (residues 29–37) is at the end of transmembrane helix 1 toward the C side of the mitochondrial membrane (9). Once the Q-binding property of recombinant QPs3 was established, site-directed mutagenesis coupled with Q-binding spectral analysis was used to identify the amino acid residues in the putative Q-binding domain responsible for Q-binding. Serine 33 and tyrosine 37 were selected for mutagenesis because they can form hydrogen bonds with the carbonyl group of the benzoquinone ring of Q similar to those found in the photosynthetic bacterial reaction center (26). Aspartic acid 36 was selected because it is a conserved residue in this region of QPs3 proteins from bovine mitochondria, Ascaris suum (adult) and yeast (9). Replacing Ser-33 or Tyr-37 with alanine results in recombinant mutant QPs3 (S33A or Y37A) unable to bind Q, as no spectral blue shift of Q is observed upon its addition because they can form hydrogen bonds with the carbonyl group of the benzoquinone ring of Q similar to those found in the photosynthetic bacterial reaction center (26). Aspartic acid 36 was selected because it is a conserved residue in this region of QPs3 proteins from bovine mitochondria, Ascaris suum (adult) and yeast (9). Replacing Ser-33 or Tyr-37 with alanine results in recombinant mutant QPs3 (S33A or Y37A) unable to bind Q, as no spectral blue shift of Q$_{C10}$ is observed upon its addition (see Fig. 2, the curve with open triangles or with solid diamonds), indicating that these two amino acid residues are involved in Q-binding. Replacing Asp-36 with alanine results in recombinant mutant QPs3 (D36A) having the same Q-binding activity as the recombinant wild-type protein; added Q$_{C10}$ shows a spectral blue shift (see Fig. 2, the curve with open squares), indicating that Asp-36 is not involved in Q-binding.

Reconstitution of Cytochrome b$_{560}$ from Recombinant QPs3 and Hemin Chloride—Isolated QPs contains three protein subunits (QPs1, QPs2, and QPs3) with a heme b$_{560}$ content of 27 nmol/mg of protein (6). The ligand for this cytochrome has been identified as bishistidine (12). However, unknown is which QPs subunit is involved in heme b$_{560}$ ligation, and whether the bishistidine ligands are provided by a single subunit or by two different subunits, as reported for cytochrome b$_{560}$ of E. coli succinate-Q reductase (14, 15). Because recombinant QPs3 contains little cytochrome b$_{560}$, heme, the involvement of QPs3 in heme ligation was investigated by testing the ability of recombinant QPs3 to reconstitute in vitro with heme chloride to form cytochrome b$_{560}$. Reconstitution was first attempted with the fusion protein because it is soluble in aqueous solution.

When hemin chloride in Me$_2$SO was added to GST-QPs3, the maximum absorption peak (Soret band) of the oxidized form of heme progressively shifts from 398 to 411 nm, with increasing absorption intensity during the incubation. It takes 1 h to complete the spectral red shift and to reach maximum absorbance. When sample is reduced with dithionite, it shows symmetrical $\alpha$-absorption at 560 nm, a broad $\beta$-absorption between 526 and 528 nm, and Soret absorption at 424 nm (see Fig. 3A). These spectral characteristics are identical to those of cytochrome b$_{560}$ in an isolated, reconstitutively active QPs preparation (6), indicating that b$_{560}$ is restored in GST-QPs3 fusion protein by heme addition. Because no cytochrome b$_{560}$ spectral properties are observed with heme-treated GST (see Fig. 3B), the cytochrome b$_{560}$ restored in GST-QPs3 is in the QPs3 moiety of the fusion protein.

Reconstituted cytochrome b$_{560}$ in GST-QPs3 shows an EPR peak at $g = 2.92$ (see Fig. 5). This signal differs with the EPR characteristics of heme-treated GST ($g = 3.50$ and $g = 3.86$) and of free heme (a broad peak with $g = 3.80$) (17). It resembles the one in the isolated QPs ($g = 2.92$ and $g = 3.07$), which does not respond to interaction with succinate dehydrogenase to form succinate:Q reductase (6). The EPR signal of cytochrome b$_{560}$ in intact succinate:Q reductase is at $g = 3.46$. When succinate dehydrogenase is removed from the reductase, the EPR signals of cytochrome b$_{560}$ in the resulting QPs preparations are at $g = 3.07$ and 2.92. The $g = 3.07$ signal converts to $g = 3.46$, whereas the $g = 2.92$ signal remains unchanged upon reconstitution with succinate dehydrogenase to form succinate:Q reductase. The inability of the b$_{560}$ with $g = 2.92$ to convert to $g = 3.46$ upon reconstitution with succinate dehydrogenase suggests that this cytochrome b$_{560}$ has been somewhat modified during the isolation of QPs.

The observation that absorption and EPR spectral properties of reconstituted cytochrome b$_{560}$ in GST-QPs3 fusion protein remain unchanged upon thrombin digestion together with the fact that heme-treated GST does not have the absorption or EPR spectral properties of cytochrome b$_{560}$ indicates that the ligands of reconstituted b$_{560}$ heme are from QPs3. Moreover, the spectral properties of cytochrome b$_{560}$ are associated with the recombinant QPs3-containing fractions when thrombin-treated, heme-reconstituted fusion protein is subjected to gel filtration chromatography (Superose 12, Amersham Pharmacia Biotech) to separate GST from QPs3. The QPs3-containing
fraction has a heme $b_{560}$ to protein ratio of 0.75. This stoichiometry may result from part of the recombinant protein not being in the right orientation for ligation, from part of reconstituted heme $b_{560}$ being released during gel filtration, or from dimerization of recombinant protein. Perhaps part of the observed $b_{560}$ spectral properties result from heme-ligated with two molecules of QPs3.

Because recombinant QP3 has also been reported to restore cytochrome $b_{560}$ spectral properties upon addition of hemin chloride, it is of interest to see whether or not restoration of cytochrome $b_{560}$ by recombinant QP3 is affected by the presence of recombinant QP3. When hemin chloride was added to a 1:1 mixture of recombinant QP3 and QP3, the amount of cytochrome $b_{560}$ restoration equals the sum of cytochrome $b_{560}$ restored by the individual recombinant proteins, suggesting that each of these proteins can provide bis-histidine ligands for cytochrome $b_{560}$ in bovine heart mitochondrial succinate:Q reductase. This differs from the report that cytochrome $b_{560}$ in E. coli succinate:Q reductase is ligated to two histidine residues located, respectively, at SdhC and SdhD (14).

Identification of Amino Acid Residues of QP3 Involved in Ligation of Heme $b_{560}$—QP3 contains histidine residues at positions 9, 46, 48, 60, and 89. To locate the heme-ligating residues, we altered each of these residues to asparagine, tyrosine, or aspartate by site-directed mutagenesis followed by spectral (absorption and EPR) characterizations of heme-reconstituted recombinant QP3 mutants.

Fig. 4 shows absorption spectra of heme-reconstituted recombinant QP3 mutants. The absorption spectra of heme-reconstituted wild-type QP3 and GST are included for comparison. The addition of hemin chloride to the H9D, H9N, H9Y, H48N, H48Y, or H89N mutant yields absorption spectra similar to substituted wild-type QPs3 and GST are included for comparison.

Identification of Amino Acid Residues of QPs3 Involved in Ligation of Heme $b_{560}$—QP3 contains histidine residues at positions 9, 46, 48, 60, and 89. To locate the heme-ligating residues, we altered each of these residues to asparagine, tyrosine, or aspartate by site-directed mutagenesis followed by spectral (absorption and EPR) characterizations of heme-reconstituted recombinant QP3 mutants.

Fig. 4 shows absorption spectra of heme-reconstituted recombinant QP3 mutants. The absorption spectra of heme-reconstituted wild-type QP3 and GST are included for comparison. The addition of hemin chloride to the H9D, H9N, H9Y, H48N, H48Y, or H89N mutant yields absorption spectra similar to those of reconstituted wild type, indicating that H9, H48, and H89 of QPs3 are not involved in heme $b_{560}$ ligation. The Soret absorption peaks of heme-reconstituted mutants H46Y (Fig. 4F) and H60N (Fig. 4I) are very different, with a 14-nm red shift of the peak maximum and a drastic decrease in absorbance. Thus His-46 and His-60 of QPs3 are involved in heme $b_{560}$ ligation. The involvement of His-46 is further supported by the diminishing of the $a$-absorption peak in the heme-reconstituted H46N mutant QPs3 (see Fig. 4E). As expected, when a double mutant, H46N,H60N was reconstituted with heme chloride, no cytochrome $b_{560}$ spectral characteristics were obtained (see Fig. 4K).

Fig. 5 compares the EPR characteristics of heme-reconstituted wild-type and mutants QP3. The heme-reconstituted H9D, H9N, H9Y, H48N, and H89N mutants of QP3 have EPR spectra similar to reconstituted wild type, indicating that histidines at positions 9, 46, 48, and 89 of QPs3 are not involved in heme $b_{560}$ ligation. Mutants H46N, H46Y, H60N, and H46N,H60N did not produce a $g = 2.92$ signal upon treatment with hemin chloride, indicating that cytochrome $b_{560}$ is not formed in these mutants and consistent with the lack of cytochrome $b_{560}$ absorption characteristics when these mutants are treated with heme chloride. Therefore, histidines 46 and 60 provide ligands for reconstituted cytochrome $b_{560}$ in recombinant QP3.

Sequence alignments of the smallest membrane-anchoring subunit of succinate:Q reductases (9) reveal that His-46 of mitochondrial QP3 corresponds to histidine 71 of the E. coli SdhD subunit, which has been identified as one of the bis-histidine ligands for cytochrome (15). The involvement of this histidine residue in cytochrome $b_{560}$ heme-ligating is also supported by the observation that yeast succinate:Q reductase and E. coli fumarate-Q reductase contain no cytochrome $b_{560}$ and the residues corresponding to His-46 of beef QPs3 in the yeast.