Cloning, Gene Sequencing, and Expression of the Small Molecular Mass Ubiquinone-binding Protein of Mitochondrial Ubiquinol-Cytochrome c Reductase

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The cDNA encoding QPc-9.5 kDa (subunit VII) of bovine heart mitochondrial ubiquinol-cytochrome c reductase was cloned and sequenced. This cDNA is 665 base pairs long with an open reading frame of 246 base pairs that encodes an 81-amino acid mature QPc-9.5 kDa. The insert contains 395 base pairs of a 3'-noncoding sequence with a poly(A) tail. The amino acid sequence of QPc-9.5 kDa deduced from this nucleotide sequence is the same as that obtained by protein sequencing except that residue 61 is tryptophan instead of cysteine. The QPc-9.5 kDa was overexpressed in Escherichia coli J M109 cells as a glutathione S-transferase fusion protein (GST-QPc) using the expression vector, pGEX/QPc. The yield of soluble active recombinant GST-QPc fusion protein depends on the induction growth time, temperature, and medium. Maximum yield of recombinant fusion protein was obtained from cells harvested 3 h postinduction of growth at 27 °C on LB medium containing betaine and sorbitol. QPc-9.5 kDa was released from the fusion protein by proteolytic cleavage with thrombin. Isolated recombinant QPc-9.5 kDa showed one protein band in SDS-polyacrylamide gel electrophoresis corresponding to subunit VII of mitochondrial ubiquinol-cytochrome c reductase. Although the isolated recombinant QPc-9.5 kDa is soluble in aqueous solution, it is in a highly aggregated form, with an apparent molecular mass of over 1 million. Addition of detergent destabilizes the isolated protein to the monomeric state, suggesting that the recombinant protein exists as a hydrophobic aggregation in aqueous solution. The recombinant QPc-9.5 kDa binds ubiquinone and shows 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 the recombinant protein may be in the functionally active state.

Bovine heart mitochondrial ubiquinol-cytochrome c reductase, also known as complex III, or the cytochrome b-c1 complex, catalyzes electron transfer from ubiquinol to cytochrome c1, with concomitant transfer of protons across the membrane to generate a proton gradient and membrane potential for ATP synthesis (1). This complex has recently been crystallized (2–4) and shown to contain ten protein subunits (2) with five redox centers (1). This complex has recently been crystallized (2–4) and shown to contain ten protein subunits (2) with five redox centers (1). The functional role of subunit VI is involved in redox-linked proton pumping (11). Subunit VII (QPc-9.5 kDa), together with subunit III (cytochrome b), are identified as Q-binding proteins by protein affinity labeling using azido-Q derivatives (12). Subunit VII (also known as hinge protein) is tightly associated with cytochrome c1 and may facilitate the binding of cytochrome c to cytochrome c1 (13). Subunit IX has been shown to bind DCCD and may be involved in proton translocation (14). The functional role of subunit X is unknown.

The electron transfer mechanism of ubiquinol-cytochrome c reductase is consistent with the Q-cycle scheme originally proposed by Mitchell (15) and subsequently refined by Berry and Trumper (16). In the Q-cycle, ubiquinol is oxidized at center o. One electron from ubiquinol is transferred to iron-sulfur protein, which then reduces cytochrome c. The second electron is transferred from ubisemiquinone to cytochrome b966. The reduced cytochrome b966 gives an electron to cytochrome b562, which then reduces ubiquinone at center i. The identification of two Q-binding proteins in ubiquinol-cytochrome c reductase is consistent with the two Q-binding sites (Q1 and Q2) proposed in the Q-cycle. Whether the two Q-binding proteins form two binding sites corresponding to Qi and Qo, individually or in combination, remains to be elucidated.

To better understand the Q-mediated electron transfer mechanism in mitochondrial ubiquinol-cytochrome c reductase requires knowledge of the molecular structure of the Q-binding site(s). The Q-binding domains in cytochrome b and QPc-9.5 kDa have recently been identified by isolating and sequencing Q-peptides from [3H]azido-Q-labeled proteins. They are located at amino acid residues 142–155 and 326–336 of cytochrome b17 (17) and residues 48–57 of QPc-9.5 kDa (18).

More detailed knowledge of the amino acid residues involved in Q-binding is needed if we are to understand the molecular structure of the Q-binding site. Herein we report the cloning and nucleotide sequencing of a cDNA encoding QPc-9.5 kDa.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L06665.

1 The abbreviations used are: Q, ubiquinone; QH2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; QcBr, 2,3-dimethoxy-5-methyl-6-[10’ bromodecyl]-1,4-benzoquinone; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
construction of a QPC expression vector, pGEX/QPC, and development of optimal conditions for high expression of an active soluble form of the GST-QPC fusion protein in Escherichia coli JM109. Isolation and characterization of pure recombinant QPC-9.5 kDa are also reported.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction endonucleases were obtained from either Promega or Life Technologies, Inc. The beef heart CDNA library in λgt11 was from Clontech. Screening primers, Labeled Phage adsorbent, T4 DNA ligase, Klenow DNA polymerase, T4 polynucleotide kinase, and E. coli lysate, used for immunological screening of the λgt11 library, were from Promega, Al−ase I (type IV), RNase A, lysozyme, 5′-chloro-3-indoly-β-D-galactopyranoside, gelatin, 5′-bromo-4-chloro-3-indoly-phosphate toluidine salt, p-nitroblue tetrazolium chloride, sodium cholate, ampicillin, tetracycline, glutathione, glutathione agarose beads, p-aminobenzamidine agarose gel, betaine, and sorbitol were from Sigma. D-α-DATP was from DuPont NEN. Centrifor-10 and centrifor-3 were from Amicon. Superose-12 FPLC column was from Pharmacia. T7 DNA polymerase DNA sequencing kit was from U.S. Biochemical Co. Dodecylmalto side was from Calbiochem. LB agar and LB broth base were from Life Technologies, Inc. Nicotinellulose discs were from Schleicher & Schuell. Oligonucleotides were synthesized by the DNA Protein Core Facility at Oklahoma State University. Antibodies against QPc-9.5 kDa were generated in rabbits and purified by the method previously reported (18). Other chemicals were obtained commercially in the highest purity available.

Bacterial Strains, Phages, and Plasmids—E. coli JM109 (araD139 mcrA supE44 ΔlacIqZ M15) was used as host for plasmid and phage DNAs. E. coli JM109/pGEX-QPc were used to inoculate 400 ml of LB broth at 20°C until use. 10 g of cell paste were suspended in 20 ml of 50 mM Tris-Cl buffer, pH 7.5, packed into a column (1.5 cm × 10 cm). The GST/QPc fusion protein was eluted from the column with 50 mM Tris-Cl, pH 7.5, and packed into a column (1.5 cm × 20 cm). The GST/QPc fusion protein was eluted from the column with 20 mM Na/Naphosphate buffer, pH 7.3, containing 150 mM NaCl (PBS). The GST/QPc fusion protein was eluted from the column with 20 mM Na/Naphosphate buffer, pH 7.3, containing 150 mM NaCl (PBS).

**RESULTS AND DISCUSSION**

Isolating and Sequencing of a cDNA Encoding QPC-9.5 kDa from a Bovine Heart CDNA Expression Library in λgt11—The availability of antibodies against QPC-9.5 kDa (18) permits immunological screening of the bovine heart CDNA expression library in λgt11 to obtain a cDNA clone for QPC-9.5 kDa. When 2 × 10⁶ plaques of λgt11 were screened, the positive clone with the highest signal intensity was isolated and purified. The cDNA insert was excised from recombinant phage DNA by EcoRI digestion and cloned into the EcoRI site of pGEM-3Zf (+) to generate pGEM/QPC. The cDNA clone for QPC-9.5 kDa is 665 bp long with an open reading frame of 246 bp that encodes 82 amino acid residues, 81 of which, starting with glycine, belong to mature QPC-9.5 kDa. The initial methionine is apparently removed post-translationally. The insert has 395 bp of 3′-noncoding sequence with a poly(A) tail, indicating that a complete clone was obtained. The nucleotide sequence and the deduced amino acid sequence of QPC-9.5 kDa are shown in Fig. 1. This amino acid sequence of QPC-9.5 kDa is the same as that obtained by protein sequencing of QPC-9.5 kDa reported by Borchart et al. (10), except that residue 61 is tryptophan instead of cysteine. The proposed structure for QPC-9.5 kDa in the membrane, based on the hydrophathy profile (26), and the predicted tendencies to form α-helices and β-sheets, are shown in Fig. 2. It contains only one transmembrane helix located at residues 41–63. Based on the observation that more polycyclonal antibodies against QPC-9.5 kDa are bound to the electron transfer particles than to the mitoplast preparations (18), we speculate that the N-terminal part of QPC-9.5 kDa is extruding from the matrix side of the inner mitochondrial membrane and the C-terminal portion is on the cytoplasmic side of the membrane. This topological arrangement, however, remains to be confirmed. The Q-binding domain in this protein is located at residues 48–57 (18).

Construction of the Expression Vector for QPC-9.5 kDa—It...
has been reported (27) that a polypeptide expressed in E. coli as a fusion protein with GST, using the pGEX system, can be isolated from cell extracts by a one-step purification with glutathione agarose gel followed by thrombin cleavage. The simplicity of this procedure prompted us to use it to express mitochondrial QPc-9.5 kDa in E. coli. Fig. 3 summarizes the protocol used for construction of the QPc-9.5 kDa expression vector, pGEX-QPc. Since BamHI and HindIII sites are unique sites in the pGEX-2TH vector and are missing in QPc-9.5 kDa cDNA, an in-frame fusion of the QPc-9.5 kDa gene with the GST gene in pGEX-2TH plasmid was achieved by generating a BamHI-HindIII fragment encoding mature QPc and subsequently ligating it into the BamHI and HindIII sites of the pGEX-2TH plasmid. To obtain a BamHI-HindIII DNA fragment encoding mature QPc, a BamHI recognition sequence (GGATCC) immediately upstream from the start codon (ATG), and a HindIII recognition sequence (AAGCTT) 15-bp downstream from the stop codon (TAA) of QPc were created by site-directed mutagenesis. A 665-bp EcoRI fragment cloned into the pSelect plasmid generated pSelect/QPcE, which was used as a template for mutagenesis. Two mutant oligonucleotides, CTCGAGGCTGCGGGATCCATGGGCCGCCA and CTCACTGATAAGCTTTCCTTGTCTCT, along with an ampicillin repair oligonucleotide were included in the mutagenesis system. This mutagenesis procedure produced greater than 50% mutants. The resulting plasmid, pSelect/QPcEBHE, was digested with BamHI and HindIII to produce a 270-bp fragment containing the QPc-9.5 kDa structural gene. This 267-bp BamHI-HindIII fragment was ligated into pGEX-2TH to generate pGEX-QPc. pGEX-QPc was transformed into E. coli JM109. Transformants producing the GST/QPc fusion protein were identified by immunological screening of colonies with antibodies against QPc-9.5 kDa.

Effect of Induction Temperature, Medium, and Length on Generation of Recombinant Fusion Protein in E. coli JM109—When E. coli JM109 containing pGEX-QPc was treated with IPTG to induce synthesis of the GST-QPc fusion protein at 37 °C for 3 h, more than 10% of the total cellular protein produced was recombinant GST-QPc fusion protein, indicating a high level of expression. The level of expression was estimated by comparing the color intensity of the 35.5-kDa protein band (GST-QPc fusion protein), which reacted with antibodies against QPc-9.5 kDa, with that of total cellular protein bands in SDS-PAGE. However, when the cells were broken by sonication in the presence of detergents, such as 1% Triton X-100, 1% of dodecylmaltoside, or 2% sodium cholate, only about 10% of the GST-QPc fusion protein produced was recovered in the crude extract, indicating that the majority of recombinant fusion protein are synthesized in insoluble aggregates (inclusion bodies) (28, 29). The insolubility of recombinant GST-QPc fusion protein reduces its affinity for glutathione agarose and thus limits the usefulness of the one-step purification scheme of the pGEX vector system. In order to overcome this obstacle, conditions for the expression of recombinant protein must be selected such that the recombinant protein is 1) soluble (or detergent soluble), and 2) possesses a properly folded GST-active site capable of binding glutathione agarose. There are two ways to achieve these goals: one is to develop methods for converting insoluble denatured recombinant GST-QPc fusion protein to a soluble active form; the other is to change growth conditions to make cells produce active soluble recombinant protein.

Recovery of active recombinant protein from the inclusion body complexes has long been regarded as a formidable task due to the denaturation of the protein and the heterogeneous nature of inclusion bodies (29). Although general techniques for obtaining active recombinant protein from inclusion bodies have not been developed, some success has been reported (30–32). In these cases, recombinant protein aggregates were solubilized with a high concentration of urea, or other chaotropic
reagents, and followed by dialysis, to allow proper refolding of the protein structure (32). When insoluble aggregates of recombinant GST-QPc were treated with 8 M urea, about 75% of the fusion protein was solubilized. However, when the urea was removed by dialysis, more than 95% of the protein precipitated. When the remaining soluble protein was applied to a glutathione agarose gel, no protein was bound to the gel, indicating that the GST-active structure had not properly re-folded. The failure to regenerate active soluble recombinant GST-QPc from inclusion bodies by the urea-dialysis method suggests that this approach is difficult or impossible for GST-QPc fusion protein. Therefore, development of an environment conducive to production of active, soluble GST-QPc recombinant protein by E. coli was necessary if the pGEX/glutathione agarose system was to work for this investigation.

Changes in cellular environments are known to increase the yield of active recombinant protein either by encouraging the cells to adopt the active conformation or by increasing the stability of recombinant proteins. Variation of media, induction conditions, and length of induction are factors that affect the cellular environments of E. coli. We systematically examined the effect of these factors on the yield of soluble recombinant GST-QPc fusion protein. Fig. 4 compares the yield of recombinant GST-QPc fusion proteins eluted from glutathione agarose gel, after incubation with soluble fractions of lysate from E. coli culture induced at 37 °C (lane 3) and 27 °C (lane 4). Induction at 27 °C improved the yield over induction at 37 °C, by 4-fold, although the total amount of recombinant GST-QPc produced by the E. coli cultures incubated at these temperatures was about the same. In other words, when the cell culture was induced at 27 °C, about 40% of the GST-QPc fusion protein produced was in the soluble form, compared to 10% with the culture induced at 37 °C. This result is consistent with previous reports (32, 33) of increased soluble yield of recombinant polypeptide in E. coli using low growth temperature.

Although lowering the induction temperature increased the yield of soluble active GST-QPc fusion protein, about 60% of the recombinant fusion protein still remained in inclusion body complexes. To further increase the soluble yield, a method involving the use of osmotic stress to facilitate the uptake of the "compatible solute" glycyl betaine (34) was adopted. When E. coli J M109/pGEX-QPc cells were grown at 27 °C on LB medium containing 2.5 mM betaine and 440 mM sorbitol, the yield of soluble recombinant protein was twice as high as that for cells grown in the absence of these compounds (see Fig. 4, lanes 4 and 5). This result is similar to that reported by Blackwell and Horgan (35) in which the inclusion of betaine and sorbitol in the growth medium converts a highly expressed Agrobacterium DMAPP-AMP transferase, which normally accumulates as inclusion complexes in E. coli, into an active soluble form. Although the reason for the increased yield of active soluble recombinant protein is unknown, it has been suggested (36) that increasing internal concentrations of compatible osmolytes, such as betaine, which are believed to be excluded from the immediate domains of proteins, causes a thermodynamically unfavorable "preferential hydration" and thus minimization of solvent-protein contact and stabilization of protein structure.

Production of active soluble recombinant GST/QPc fusion protein was found to be IPTG-induction growth temperature dependent (data not shown). The yield increased as the induction growth temperature was increased, to a maximum yield which was obtained when cells were harvested three hours after growth induction. When cells were grown for 5 h, there was a 20% decrease in yield, suggesting that the recombinant protein is unstable and susceptible to protease digestion. In summary, the optimal conditions for the production of soluble active recombinant GST/QPc fusion protein are as follows: induction of E. coli J M109/pGEX-QPc cells with IPTG at 27 °C for three hours on LB medium containing 2.5 mM betaine and 440 mM sorbitol.

Our routine cell extract procedure included treatment with 1% Triton X-100. If this treatment was omitted, the yield of soluble recombinant GST-QPc fusion protein decreased 30%. This suggests that some GST-QPc fusion protein is either in a membrane fraction or an inclusion body aggregate that can be solubilized by Triton X-100 while maintaining the GST-active site recognizable by glutathione agarose gel. Recombinant GST-QPc fusion protein obtained from cell extracts prepared with Triton X-100 has the same molecular size and Q-binding properties as that obtained without Triton X-100 treatment.

Purification, Molecular Mass, and Functional Activity of Recombinant QPc-9.5 kDa Protein—When purified GST-QPc is incubated with thrombin, 0.005 unit/μg of protein, at room temperature, QPc-9.5 kDa is progressively released from GST, with about 85% being cleaved in a 1-h incubation (data not shown). However, the cleavage of QPc from GST by thrombin never reaches completion, even after prolonged incubation with increasing amounts of thrombin. The reason for this is unknown. The released GST in the digested mixture was removed with glutathione agarose gel. The thrombin present in the GST-free sample was removed by p-aminobenzamidine agarose gel. About 4 mg of pure QPc-9.5 kDa was recovered from 18 mg of fusion protein.

Although isolated recombinant QPc-9.5 kDa is soluble in aqueous solution, it has a molecular mass of over 1 million as determined by FPLC gel filtration with Sepharose 12 in 50 mM Tris·Cl buffer, pH 8.0. Aggregation is apparently due to the hydrophobic transmembrane segment of the peptide chain. The
containing 10% ethanol, a blue spectral shift was observed (see Fig. 6). The spectral blue shift of Q was not detected when a Pronase-treated recombinant QPc-9.5 kDa was added. Titration of recombinant QPc-9.5 kDa with Q showed a saturation point at around 2 moles of Q per mole protein. These results suggest that the binding of Q to recombinant protein is specific and the recombinant QPc-9.5 kDa may be functionally active. To confirm the specific binding between recombinant QPc-9.5 kDa and Q, two other proteins, bovine serum albumin and ribonuclease A, were used as controls in the titration studies. Ribonuclease A had absolutely no effect on absorption spectral properties of Q, suggesting no interaction between them. Since bovine serum albumin is known to bind hydrophobic compounds such as fatty acid, a nonspecific binding of this protein with Q is expected. As shown in Fig. 6, the effect of bovine serum albumin on spectral properties of Q is very much different from that observed with recombinant QPc-9.5 kDa.

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