ELSEVIER

Biochimica et Biophysica Acta 1363 (1998) 35–46

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Reconstitution of cytochrome *b*-560 (QPs1) of bovine heart mitochondrial succinate–ubiquinone reductase [1]

Gyesoon Yoon Lee, Jian Zhu, Linda Yu *, Chang-An Yu

Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA

Received 16 June 1997; revised 11 September 1997; accepted 15 September 1997

Abstract

The QPs1 subunit of bovine heart mitochondrial succinate–ubiquinone reductase was overexpressed in *Escherichia coli* DH5α cells as a glutathione S-transferase fusion protein (GST–QPs1) using the expression vector, pGEX/QPs1. The yield of soluble active recombinant GST–QPs1 fusion protein depends on the IPTG concentration, induction growth time, temperature, and medium. Maximum yield of recombinant fusion protein was obtained from cells harvested 3 h postinduction of growth with 0.5 mM IPTG at 27°C in an enriched medium containing betaine and sorbitol. QPs1 is released from the fusion protein by proteolytic cleavage with thrombin. Isolated recombinant QPs1 shows one protein band in SDS-polyacrylamide gel electrophoresis corresponding to subunit III of mitochondrial succinate–ubiquinone reductase. However, partial N-terminal amino acid sequence analysis of recombinant QPs1 shows two extra amino acid residues, glycine and serine, at the N-terminus of mature QPs1, resulting from the recombinant manipulation. When isolated recombinant QPs1 is dispersed in 0.01% dodecyl maltoside, it is in a highly aggregated form with an apparent molecular mass of over 1 million. Recombinant GST–QPs1 contains little cytochrome *b*-560 heme. However, addition of hemin chloride restores the spectral characteristics of cytochrome *b*-560. Cytochrome *b*-560 restoration varies with the amount of hemin used. Maximum reconstitution is obtained when the molar ratio of heme to fusion protein used in the system is 0.6. Reconstituted cytochrome *b*-560 shows a EPR signal at $g = 2.91$ which corresponds to one of the EPR signals of cytochrome *b*-560 in a QPs preparation. When GST–QPs1 with reconstituted cytochrome *b*-560 is treated with thrombin to cleave GST from QPs1, no change in the absorption and EPR characteristics of cytochrome *b*-560 is observed, indicating that the bis-histidine ligands of reconstituted cytochrome *b*-560 are provided by QPs1. © 1998 Elsevier Science B.V.

Keywords: Succinate–ubiquinone reductase; Cytochrome *b*-560; QPs1 expression

1. Introduction

Bovine heart mitochondrial succinate–ubiquinone reductase, also known as Complex II, which catalyzes electron transfer from succinate to ubiquinone (Q), has been isolated and characterized [1–3]. The reductase is composed of two parts: soluble succinate dehydrogenase and a membrane anchoring protein fraction. Purified reductase shows five protein subunits in a high resolution sodium dodecylsulfate-

Abbreviations: bp, base pair(s); DMSO, dimethylsulfoxide; DCPIP, 2,6-dichlorophenol indophenol; FPLC, fast protein liquid chromatography; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; Q, ubiquinone; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; PMSF, phenylmethylsulfonylfloride; PBS, 20 mM Na/K phosphate buffer, pH 7.3 containing 150 mM NaCl; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

* Corresponding author. Fax: +1 405 744 7799.

[1] This work was supported in part by a grant from the NIH (GM30721).

PII S0005-2728(97)00089-3

polyacrylamide gel electrophoretic (SDS-PAGE) system with apparent molecular weights of 70, 27, 15, 13, and 11 kDa [4]. The two largest subunits are succinate dehydrogenase and the three smaller ones comprise the membrane anchoring protein fraction. The isolated complex contains six prosthetic groups: one FAD, three iron–sulfur clusters (2Fe–2S, 3Fe–4S, and 4Fe–4S), cytochrome *b*-560, and Q. The FAD is covalently bound to the 70 kDa subunit (Fp) and all three iron–sulfur clusters are in the 27 kDa subunit (Ip) of succinate dehydrogenase [5,6]. Cytochrome *b*-560 and Q are found in the membrane anchoring protein fraction [7]. While studies of succinate dehydrogenase have been extensive and a wealth of information has been obtained [5,6], knowledge of the membrane anchoring protein fraction is relatively limited [8].

The membrane anchoring protein fraction has been isolated in several laboratories under different names, such as $CII_{3,4}$ [9], cytochrome *b*-560 fraction [10], or QPs [11]. QPs provides membrane anchoring for succinate dehydrogenase and is responsible for the Q-binding in succinate–ubiquinone reductase. The ubisemiquinone radicals, which do not show power saturation, even at 200 mW, are detected in intact and reconstituted succinate–ubiquinone reductases in the presence of exogenous Q [12]. When quinone derivatives are used as the electron acceptor for succinate–ubiquinone reductase, a 5 carbon side chain is required for maximum activity [13] and the methyl group at the 5-position is less important than are the methoxy groups at the 2- and 3-positions [14]. Replacing the 5-methyl group with hydrogen causes a slight increase in activity. However, replacing one or both of the 2- and 3-methoxy groups with a methyl completely abolishes electron-acceptor activity.

When succinate–ubiquinone reductase is subjected to photoaffinity labeling with an [^{3}H]azido-Q derivative, most of the radioactivity was found in the QPs1 subunit, indicating that QPs1 is involved in Q-binding [4]. The Q-binding domain in QPs1, using a structure for QPs1 based on the deduced amino acid sequence [15,16], is located in the stretch connecting transmembrane helices 2 and 3, which extrudes from the surface of the M side of the mitochondrial inner membrane [4].

In addition to participating in Q-binding, QPs1 is believed to be cytochrome *b*-560. The involvement of cytochrome *b*-560 in catalysis by succinate–ubiquinone reductase is controversial. The presence of substoichiometric amounts of cytochrome *b*-560, with respect to FAD, in isolated beef succinate–cytochrome *c* and succinate–ubiquinone reductases, together with its non-reducibility by succinate has led some investigators to rule out a catalytic function for cytochrome *b*-560. On the other hand, it has been proposed that cytochrome *b*-560 functions as a mediator between low potential $F1/F\cdot$ and $Q/Q\cdot$ couples in a dual pathway model [17] of electron flow through cardiac Complex II. Despite its uncertain catalytic role, cytochrome *b*-560 is involved in the binding of succinate dehydrogenase to QPs as indicated by the restoration of its absorption properties, redox potential, and EPR characteristics, upon reconstitution of succinate–ubiquinone reductase from QPs and succinate dehydrogenase [7].

The ligands of cytochrome *b*-560 in *E. coli* and beef succinate–ubiquinone reductases have recently been identified as bis-histidine [18,19]. H42 and H98 of QPs1 are thought to be involved in heme ligation of cytochrome *b*-560 in the beef enzyme [15] because these two histidines are conserved in *sdh* C peptides of *E. coli* and *Bacillus subtilis* and are located on the matrix side of the membrane. However, the recent report of Nakamura et al. [20], using a gene deletion approach, indicates that both *sdh*C and *sdh*D peptides of the *E. coli* enzyme are required for heme ligation of cytochrome b_{556}. Precise localization of these bis-histidine ligands will facilitate our understanding of electron transfer in this segment of the mitochondrial respiratory chain.

To better understand the structure function relationship of QPs1 requires knowledge of the amino acid residues responsible for Q-binding, succinate dehydrogenase docking, and ligation of cytochrome *b*-560. This can be obtained by using expressed mutated QPs1 for in vitro reconstitution. Therefore toward this end, we have developed a method to over-express QPs1 in *E. coli* as a glutathione S-transferase (GST) fusion protein, using the pGEX expression vector system [21]. We have used this system, which allows one-step affinity purification of the recombinant fusion protein with glutathione–agarose gel, to over-express functionally active subunit IV of the *R. sphaeroides* cytochrome bc_1 complex [22] and the QPc-9.5 kDa of beef ubiquinol–cytochrome *c*

reductase [23]. Herein we report construction of a QPs1 expression vector, pGEX/QPs1, conditions for high expression of the active soluble form of GST–QPs1 in *E. coli* DH5α, and isolation and characterization of pure recombinant QPs1. Reconstitution of recombinant QPs1 with heme to form cytochrome *b*-560 and the properties of reconstituted cytochrome *b*-560 are also discussed.

2. Experimental procedures

2.1. Materials

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and T4 polynucleotide kinase were obtained from either Promega Corporation or Bethesda Research Laboratories. DNase I (Type IV), RNase A, isopropyl-β-D-thiogalactopyranoside (IPTG), gelatin, ampicillin, tetracycline, D-aminolevulinic acid, ferrous sulfate, hemin chloride, sorbitol, betaine, glutathione (reduced form), glutathione–agarose beads, thrombin, leupeptin, 2,6-dichlorophenol indophenol (DCPIP) and phenylmethylsulfonylfloride (PMSF) were from Sigma. Nitrocellulose membrane was from MSI. Agarose, acrylamide, bis-acrylamide, HRP color development reagents 4CN, and protein A horseradish peroxidase conjugate were from Bio-Rad. LB agar, LB broth base, Select Peptone-140, and Select Yeast Extract were from GIBCO BRL. Oligonucleotides were synthesized by the DNA/Protein Core Facility at Oklahoma State University. Antibodies against QPs1 were generated in rabbits and purified by the method previously reported [15]. Other chemicals were obtained commercially in the highest purity available.

2.2. Bacterial strains and plasmids

E. coli JM109 {*rec*A1, *end*A1, *gyr*A96, *thi*-, *hsd*R17 (r_k^-, m_k^-), supE44, relA1, λ$^-$, Δ (*lac*–*pro*AB), [F′, *tra*D36, *pro*AB *lac*I^{q}Z ΔM15]} was used as host for pGEM-3Zf(+) (Promega), pSelect (Promega), and pGEX-2TH (Pharmacia) plasmids. *E. coli* DH5α{F$^-$, ϕ80d, *lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, d$^-$, *gyr*A96, Δ(*lac*ZYA-*arg*F), U169}was used as host for pGEX-2TH/QPs1. *E. coli* strains were grown in LB medium [24]. When necessary, ampicillin (100 μg/ml) or tetracycline (15 μg/ml) were added.

2.3. DNA manipulation and DNA sequencing

Restriction enzyme digestion, large-scale isolation and minipreparation of plasmid DNA, were performed according to reported methods [24]. Site-directed mutagenesis was performed using the Altered Site in vitro Mutagenesis system from Promega. DNA sequencing was performed by the DNA/Protein Core Facility at Oklahoma State University.

2.4. Isolation of recombinant GST–QPs1 fusion protein

2.4.1. From soluble cell-free extract

Recombinant GST–QPs1 fusion protein was isolated from *E. coli* DH5α/pGEX/QPs1 cell extracts essentially as described previously [23]. 250 ml of an overnight grown *E. coli* DH5α/pGEX/QPs1 cell culture was used to inoculate 12 l of enriched medium containing 2.5 mM betaine, 440 mM sorbitol, and 100 μg/ml ampicillin, and incubated at 37°C with vigorous shaking until the $OD_{660\,nm}$ reached 0.7. The culture was cooled to 27°C and IPTG was added to a final concentration of 0.5 mM to induce the synthesis of the GST–QPs1 fusion protein. Cells were grown at 27°C for 3 h before they were harvested by centrifugation at 8000 × *g* for 15 min. Forty three grams of cell paste were obtained. Freshly harvested cells were suspended in 80 ml of 20 mM sodium phosphate buffer, pH 7.3, containing 150 mM NaCl (PBS) and broken with a SLM-Aminco French pressure cell at 1000 p.s.i. PMSF (100 mM in absolute alcohol) was added to the broken cell suspension in several small portions during the pressing process to a final concentration of 1 mM, to inhibit protease activity. Triton X-100 was then added to a final concentration of 1% (w/v). This mixture was stirred for 1 h at 0°C and centrifuged at 30 000 × *g* for 20 min. The supernatant obtained was mixed with an equal volume of glutathione–agarose gel equilibrated with PBS. The precipitate, which contains inclusion body QPs1, was saved for the isolation of QPs1 by urea solubilization as described in the next section. The gel mixture was gently shaken for 2 h at 0°C and packed into a

2.5 × 13 cm column. After the column was washed with 800 ml of 50 mM Tris–Cl buffer, pH 7.5, followed with 100 ml of 50 mM Tris–HCl, pH 8.0, containing 0.1 mM glutathione and 0.25 M sucrose, the GST–QPs1 fusion protein was eluted with 50 mM Tris–HCl, pH 8.0, containing 5 mM glutathione and 0.25 M sucrose and dialyzed against 50 mM Tris–Cl, pH 8.0, containing 0.25 M sucrose, overnight, with two changes of buffer. The dialyzed preparation was concentrated to 10 mg/ml with Centricon-10, mixed with glycerol to a final concentration of 10%, and frozen at −80°C until use.

2.4.2. From Inclusion Body Precipitate

The precipitate was suspended in an equal volume of 8 M urea in 50 mM Tris–Cl buffer, pH 8.0 and stirred at room temperature for 30 min. After the mixture was centrifuged at 30 000 × *g* for 30 min, the supernatant was dialyzed against 50 mM Tris–Cl, pH 8.0, overnight, with two changes of buffer, to remove urea. The dialyzed sample was centrifuged at 30 000 × *g* for 30 min to remove precipitates. Fusion protein was isolated from the clear supernatant using the procedure described above for the soluble cell-free extract.

2.4.3. Enzyme preparations and assays

Succinate dehydrogenase [25] and QPs [7] were prepared and assayed as previously reported. The reconstitutive activity of QPs1 was assayed by its ability to form succinate–ubiquinone reductase with succinate dehydrogenase. Succinate–ubiquinone reductase was assayed for its ability to catalyze the Q-mediated DCPIP reduction by succinate. One ml assay mixture contains 100 mmoles of sodium/potassium phosphate buffer, pH 7.4, 50 nmoles of DCPIP, 20 mmoles of succinate, 10 nmoles of EDTA, 16 nmoles of Q_2 and 0.1 mg of Triton X-100. The reduction of DCPIP was followed by an absorption decrease at 600 nm using a millimolar extinction coefficient of 21 cm^{-1}.

Absorption spectra and spectrophotometric activity assays were performed at room temperature in a Shimadzu UV-2101PC spectrometer. Protein concentration was determined by the Lowry method [26] using bovine serum albumin as standard. The heme content was determined from the reduced pyridine hemochromogen spectra using a millimolar extinction coefficient of 34.6 for the absorbance at 557 nm minus 600 nm [27]. Analytical SDS-PAGE was performed in a Bio-Rad Mini-Protein dual slab cell using the gel system of Schägger et al. [28], with modifications [4]. Western blots were performed as previously described [23]. The EPR measurements were made with a Bruker ER-200D equipped with an Air Product Heli-Tran System. EPR settings and temperatures are shown in the figure legends.

3. Results and discussion

3.1. Construction of the expression vector for QPs1 (pGEX/QPs1)

Fig. 1 summarizes the protocol used for construction of the QPs1 expression vector, pGEX/QPs1. The QPs1 gene was in-frame fused into the glutathione S-transferase (GST) gene in pGEX-2TH plasmid by generating a *Bam*HI–*Hin*dIII fragment encoding mature QPs1 and subsequently ligating it into the *Bam*HI and *Hin*dIII sites of the pGEX-2TH plasmid. The 432 bp *Bam*HI–*Hin*dIII fragment encoding mature QPs1 was obtained by first eliminating the internal *Bam*HI recognition sequence (GGATCC), located at 304 bp down stream from the N-terminus coding sequence (TTG) and 110 bp upstream from the stop codon (TGA), followed by creating a *Bam*HI site immediately before the N-terminus coding sequence and a *Hin*dIII site (AAGCTT) 7 bp down stream from the stop codon. These constructions were achieved by site-directed mutagenesis techniques.

To eliminate the internal *Bam*HI site in QPs1 sequence, a 1.7 kbp *Sac*I fragment from pGEM/QPs1 [15] was cloned into the *Sac*I site of pSelect-1 plasmid to generate $pSelect_{amp^r}$/QPs1 for the use as a template in the mutagenesis reaction. The mutant oligonucleotide, CTGGAATGGGATTCGACACTTGAT, along with an ampicillin repair oligonucleotide were included in the mutagenesis system. Although this construction eliminates the *Bam*HI site from the QPs1 sequence, the resulting plasmid, $pSelect_{amp^r}/QPs1_{\Delta BamHI}$, encodes the identical QPs1 sequence as that of intact QPs1.

To create a *Bam*HI site immediately upstream from the N-terminus coding sequence and a *Hin*dIII site 7 bp down stream from the stop codon of QPs1

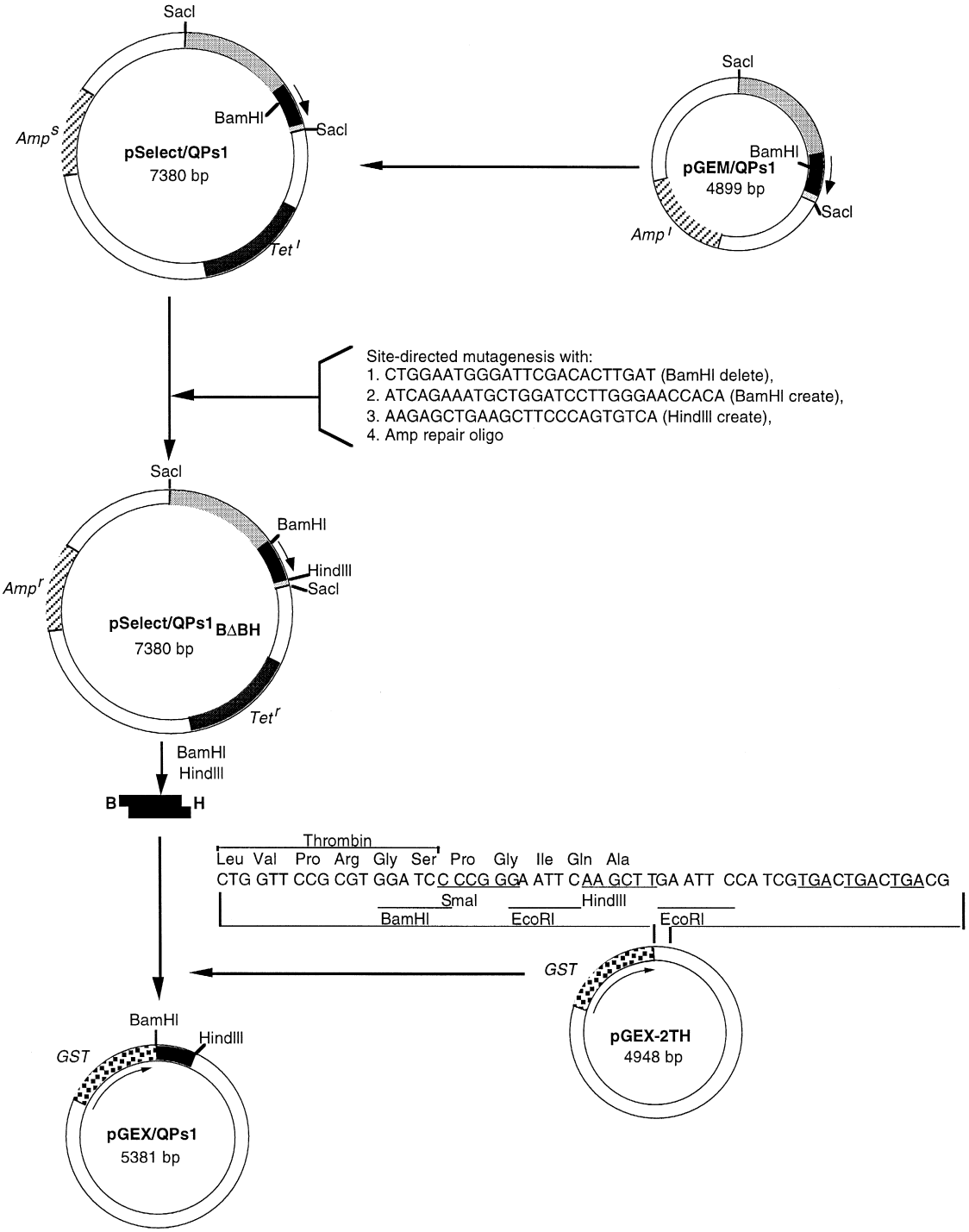

Fig. 1. Construction of the QPs1 expression vector, pGEX/QPs1.

sequence, a 1.7 kb *Sac*I fragment from pSelect$_{amp^r}$/QPs1$_{\Delta BamHI}$ was cloned into the *Sac*I site of pSelect-1 plasmid to generate pSelect$_{amp^s}$/QPs1$_{\Delta BamHI}$ for use as a template. The two mutant oligonucleotides, ATCAGAAATGCTGGATCCTTGGGAACCACA (*Bam*HI recognition sequence), and AAGAGCTGA-

AGCTTCCCAGTGTCA (*Hin*dIII recognition sequence), along with an ampicillin repair oligonucleotide were included in the mutagenesis system to generate $pSelect_{amp^r}/QPs1_{B\Delta BH}$.

A 432 bp *Bam*HI–*Hin*dIII fragment from $pSelect_{amp^r}/QPs1_{B\Delta BH}$ was cloned into *Bam*HI and *Hin*dIII sites of pGEX-2TH to generate pGEX/QPs1 expression plasmid.

3.2. Effect of IPTG concentration, induction temperature, medium, and length on generation of soluble recombinant fusion protein in E. coli DH5α

When the pGEX/QPs1 plasmid was transformed into *E. coli* JM109, DH5α, or KS1000 cells, the transformed DH5α cells required 2.5 h to reach the midpoint of their logarithmic growth phase while JM109 and KS1000 required 3.5 h. Furthermore, when cells were harvested after three hours of IPTG induction growth at 37°C, the amounts of GST–QPs1 fusion protein produced by these *E. coli* strains were in the decreasing order of DH5α > JM109 > KS1000. Therefore, DH5α cells were used as the host for expression of QPs1.

Production of recombinant GST–QPs1 fusion protein was found to be IPTG concentration and induction growth time dependent (data not shown). The yield increased as the IPTG concentration and induction growth time were increased, reaching a maximum when cells were harvested 3 h after 0.5 mM IPTG growth induction. When cells were grown for more than 3 h, the total yield decreased and degradative product increased, as determined by Western Blotting using anti-QPs1 antibodies.

The production of recombinant GST–QPs1 fusion protein by *E. coli* DH5α /pGEX/QPs1 in LB medium at 37°C is high; it accounts for about 8% of the total cellular protein (see Table 1). However, when the cells were broken by sonication in the presence of 1% Triton X-100, only 5% of the fusion protein produced was recovered in the crude extract (see Table 1), indicating that the majority of recombinant fusion protein was in insoluble aggregates (inclusion bodies). The insolubility of recombinant GST–QPs1 limits the usefulness of the one-step purification scheme of the pGEX vector system. Only when the recombinant protein is soluble (or detergent soluble) and possesses a properly folded GST-active site, the binding of fusion protein to glutathione–agarose is possible. There are two ways to obtain active soluble recombinant fusion protein: one is to change the cellular environment to make cells produce active soluble recombinant protein; the other is to develop methods for converting insoluble denatured recombinant GST–QPs1 fusion protein to a soluble active form.

It is known that cellular environments are affected by growth media, induction temperature, and length of induction. Table 1 compares the soluble yield of GST–QPs1 fusion protein from cells grown under seven conditions: (i) LB broth medium at 37°C; (ii) LB broth medium at 27°C; (iii) LB broth medium containing 2.5 mM betaine and 440 mM sorbitol at

Table 1
The yield of recombinant GST–QPs1 fusion protein in the soluble and inclusion body fractions of *E. coli* DH5α/pGEX-QPs1 cells grown under different conditions

Growth medium	Temperature °C	Length of induction growth h	% Yield of GST–QPs1 [a]		
			Total	Soluble	Inclusion body
LB	37	3	8.1	0.4	7.7
LB	27	3	4.4	1.1	3.3
LB + betaine + sorbitol	27	3	8.3	2.6	5.7
Enriched	27	3	9.4	1.4	8.0
Enriched + betaine	27	3	9.4	2.3	7.1
Enriched + sorbitol	27	3	9.4	2.3	7.2
Enriched + betaine + sorbitol	27	3	10.2	4.2	6.0

[a] % yield of recombinant GST–QPs1 fusion protein was calculated by comparing the color density at the $M_r = 41$ kDa molecular weight protein band with the total intensities of cellular protein bands in SDS-PAGE.

27°C; (iv) enriched medium (2% Select peptone-140, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.8% NaCl, 1.5% Select yeast extract, and 0.2% glucose) at 27°C; (v) enriched medium containing 2.5 mM betaine at 27°C; (vi) enriched medium containing 440 mM sorbitol at 27°C; and (vii) enriched medium containing 2.5 mM betaine and 440 mM sorbitol at 27°C.

Although induction at 27°C in LB medium (Table 1) improved the soluble yield over induction at 37°C by 3-fold, the soluble recombinant GST–QPs1 is only 1% of the total cellular protein. The total amount of recombinant GST–QP1 fusion protein produced by cells induced at 27°C was only 55% that produced by cells induced at 37°C (see Table 1). Increased yield of soluble recombinant polypeptide in *E. coli* at a low growth temperature has been reported [23,29,30].

To further improve the soluble yield, a method using osmotic stress to facilitate the uptake of the ''compatible solute'' glycyl betaine was adopted [31] in conjunction with the low growth temperature. When *E. coli* DH5α/pGEX/QPs1 cells were grown at 27°C in LB medium containing 2.5 mM betaine and 440 mM sorbitol, the total GST–QPs1 protein produced was double (8.3%) that produced without betaine and sorbitol (4.4%) with about 31% in the soluble form (Table 1). Thus at low temperature, inclusion of betaine and sorbitol causes increased production of soluble GST–QPs1 and inclusion body fusion protein. Although the reason for the increase is unknown, it has been suggested [32] that increased internal concentrations of compatible osmolytes, such as betaine, cause a thermodynamically unfavorable ''preferential hydration'' which minimizes solvent–protein contact and stabilizes protein structure.

The yield of the soluble form of a recombinant protein can be increased in *E. coli* by using an enriched medium [33]. Thus a peptone–phosphate enriched medium (2% Select peptone-140, 0.2% % Na_2HPO_4, 0.1% KH_2PO_4, 0.8% NaCl, 1.5% Select yeast extract, and 0.2% glucose), in the presence of betaine, or sorbitol, or both, was used for induction growth of *E. coli* DH5α/pGEX/QPs1 cells at 27°C. Results are presented in Table 1. It will be noted that addition of both 2.5 mM betaine and 400 mM sorbitol to enriched medium resulted in highest yield of total (10.2%) and soluble (41% of total GST–QPs1) recombinant protein. Therefore, induction growth using peptone–phosphate medium containing 2.5 mM betaine and 440 mM sorbitol at 27°C for 3 h was optimal for the production of soluble recombinant GST–QPs1 fusion protein.

It should be mentioned that our routine cell extract procedure included treatment with 1% Triton X-100. If this treatment was omitted, the yield of soluble recombinant GST–QP1 fusion protein from cells grown in enriched medium containing betaine and sorbitol decreased 15%. This suggests that some GST–QPs1 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.

3.3. Recovery of the recombinant GST–QPs1 fusion protein from inclusion body precipitates

Despite the increased yield of soluble active GST–QPs1 fusion protein (Table 1), about 60% of the fusion protein remains in inclusion body complexes. Recovery of the active soluble GST–QPs1 fusion protein from inclusion precipitates was attempted in order to increase the yields. Although recovery of active recombinant protein from inclusion bodies has been regarded as a formidable task, some successes have been reported [29,34,35]. In these cases, recombinant protein aggregates were solubilized with a high concentration of urea, or other chaotropic reagents, and dialyzed to allow proper refolding of the protein. When the urea-dialysis method was adopted, only 8.5% of the recombinant GST–QPs1 protein in inclusion bodies was recovered in the soluble form. This low recovery results from re-precipitation of the urea-solubilized fusion protein during dialysis and not from ineffective solubilization from the inclusion body precipitates. More than 85% of the recombinant fusion protein in inclusion precipitates is solubilized in 5 M urea, but about 90% of the urea-solubilized protein precipitates upon removal of urea. Inclusion of 0.2% sodium cholate in the dialysis buffer slightly increases the yield of soluble GST–QPs1 fusion protein (12%) after dialysis. About 60% of the soluble GST–QPs1 obtained from the inclusion body complex by urea-dialysis is recovered from the glutathione–agarose gel. Thus, only about 5% of the recombinant GST–QPs1 in the inclusion body

complex can be properly renatured and purified by glutathione–agarose gel.

3.4. Purity and properties of purified recombinant QPs1

About 86 mg of purified recombinant GST–QPs1 fusion protein, 78 mg from the cell extract and 8 mg from inclusion body precipitates, is recovered from a 12 l cell culture. When this purified preparation is subjected to SDS-PAGE (lane 3 of Fig. 2(A)), three major protein bands are observed: one with an apparent molecular weight of 41 kDa which accounts for 65% of the total protein, one with a molecular weight of about 68 kDa that accounts for 5% of the total protein and one smear band with the molecular weight span from 26.5 to 27 kDa that accounts for 30% of the total protein. The 41 kDa protein is GST–QPs1 fusion protein, the 26.5–27 kDa smear band contains incomplete GST–QPs1 fusion proteins, and the 68 kDa band is a contaminant protein. These assignments are based on the observation that both 41 kDa and 26.5–27 kDa proteins react with antibodies against QPs1 (lane 3 of Fig. 2(B)) and produce GST ($M_r = 26\,000$ Da) upon thrombin digestion (see lane 4 of Fig. 2(A)). To date, we have been unable to obtain an GST–QPs1 preparation free of incomplete GST–QPs1 proteins, with a reasonable yield. However, the introduction of a 0.1 mM glutathione washing of the GST–QPs1 bound glutathione–agarose gel prior to elution of GST–QPs1 with 5 mM glutathione decreases the amount of incomplete GST–QPs1 proteins in the final preparation.

QPs1 is progressively released from GST–QPs1 fusion protein by treatment with thrombin (0.5 unit/mg protein) at room temperature. The reaction is complete after 30 min of incubation at room temperature. Recombinant QPs1 released from GST becomes highly aggregated and is recovered as a precipitate. The aggregation of QPs1 in aqueous solution is apparently due to the presence of hydrophobic domains, the three transmembrane helices, in the protein [4]. Inclusion of 0.01% of dodecyl maltoside during thrombin digestion does not affect thrombin activity and permits recovery of QPs1 in a soluble (detergent dispersed) form. When this thrombin digested fusion protein is applied to a Superose-12 FPLC column equilibrated with 50 mM Tris–Cl, pH 7.8, containing 0.01% dodecyl maltoside, purified recombinant QPs1 is recovered in the void volume, indicating that purified QPs1, in 0.01% dodecylmaltoside, is still highly aggregated, with apparent molecular mass of over 1 million. The Superose-12 column chromatography separates uncleaved GST–QPs1 fusion protein, released GST, and thrombin, from QPs1. When the QPs1 containing fraction recovered from the Superose-12 column is subjected to

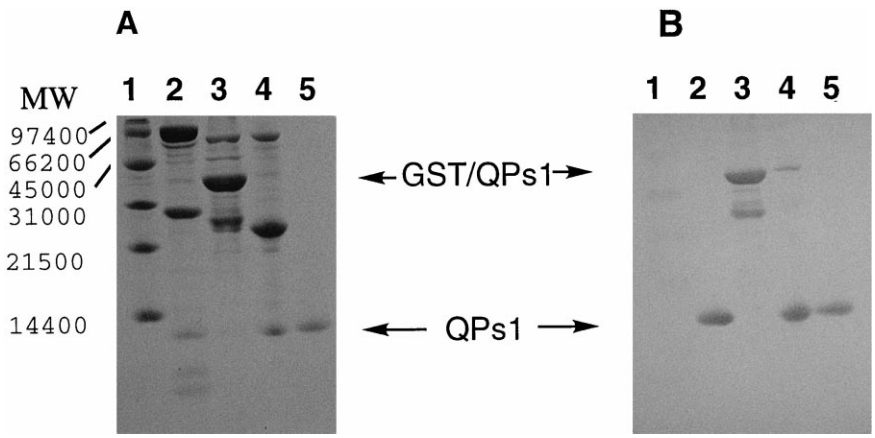

Fig. 2. Identity of recombinant QPs1. (A) SDS-PAGE of isolated recombinant QPs1. Lane 1, molecular weight standard; lane 2, bovine heart mitochondrial succinate–ubiquinone reductase; lane 3, glutathione–agarose gel purified recombinant GST–QPs1; lane 4, thrombin digested recombinant GST–QPs1; and lane 5, purified recombinant QPs1. The high resolution SDS-PAGE gel of Schägger et al. [28] was used. (B) The proteins on the gel of (A) were electrophoretically transferred to a nitrocellulose membrane, without staining, and reacted with anti-QPs1 antibodies. Protein A horseradish peroxidase conjugate was used as the second antibody.

SDS-PAGE, a single band, corresponding to the third subunit (QPs1) of purified succinate–ubiquinone reductase, is observed (lane 5 of Fig. 2(A)). However, when this purified recombinant QPs1 is subjected to partial N-terminal amino acid sequencing, two extra residues, glycine and threonine, are observed. The addition of these two extra residues is due to the recombinant manipulation.

It should be noted that purified recombinant QPs1 obtained from soluble cell extracts, and from the soluble fraction recovered from inclusion body precipitates, have the same purity and hydrodynamic properties.

3.5. Reconstitution and properties of cytochrome b-560 formed from GST–QPs1 fusion protein and heme

Although QPs1 is believed to be cytochrome *b*-560 with bis-histidine ligands [19], isolated recombinant QPs1, obtained from cultures with or without aminolevulinic acid, contains little cytochrome *b*-560 heme. Probably the lack of cytochrome *b*-560 heme in recombinant QPs1 results from the inability of *E. coli* DH5α/pGEX-QPs1 cells to either incorporate heme into apoprotein or to produce enough heme. In other words, the expressed GST–QPs1 fusion protein has the correct structure for heme ligation and cytochrome *b*-560 can be formed if heme is available. One way to test these possibilities is to reconstitute cytochrome *b*-560 to recombinant QPs1 with heme. Since recombinant GST–QPs1 is soluble, reconstitution of cytochrome *b*-560 was first attempted with the fusion protein.

Fig. 3 shows generation of cytochrome *b*-560 from GST–QPs1 fusion protein after addition of hemin chloride. The maximum absorption peak (Soret band) of the oxidized form of heme progressively shifts from 398 nm to 411 nm, with increasing absorption intensity, during the incubation (data not shown). It takes 2 h to complete the spectral red shift and to reach maximum absorption. When the reaction was complete, dithionite was added to reduce the sample which now showed a symmetrical α-absorption at 559.5 nm, a broad β-absorption peak at 530 nm, and Soret absorption at 425 nm. These absorption characteristics are identical to those of cytochrome *b*-560 in an isolated, reconstitutively active QPs preparation

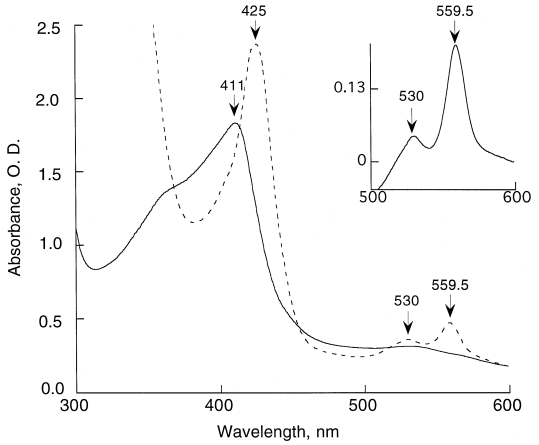

Fig. 3. Restoration of cytochrome *b*-560 to isolated recombinant GST–QPs1 fusion protein by heme. 3 μl aliquots of hemin chloride (6.18 mM) in DMSO were added to 1 ml of purified recombinant GST–QPs1 fusion protein, 3.1 mg/ml, in 50 mM Tris–Cl buffer, pH 8.0, containing 0.25 M sucrose. The mixtures 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 (————), a small amount of dithionite was added and spectra recorded (---). The inserts are the difference spectra of the dithionite-reduced vs. oxidized form at the α and β absorption regions.

[7]. Thus, cytochrome *b*-560 is restored to GST–QPs1 fusion protein by heme addition.

Since GST from *Scistosoma japonicum* in recombinant GST–QPs1 contains 6 histidines and is reported to have heme binding activity [36], it is important to establish that the bis-histidine ligands of reconstituted cytochrome *b*-560 in GST–QPs1 are provided by the QPs1, and not by the GST moiety. This is achieved by comparing the absorption spectra and EPR characteristics of heme-ligated GST with those of heme-ligated GST–QPs1, and by isolating reconstituted cytochrome *b*-560 in QPs1 from heme-ligated GST–QPs1 after thrombin digestion. Recombinant GST is produced in DH5α/pGEX cells and purified by glutathione–agarose gel. Since, after addition of heme to purified recombinant GST, under conditions identical with those for GST–QPs1, no cytochrome *b*-560 spectra is produced (data not shown), the bis-histidine ligands of reconstituted cytochrome *b*-560 are provided by the QPs1 moiety of the fusion protein. The heme-ligated GST shows a very broad α-absorption at 540 nm in the dithionite-reduced form.

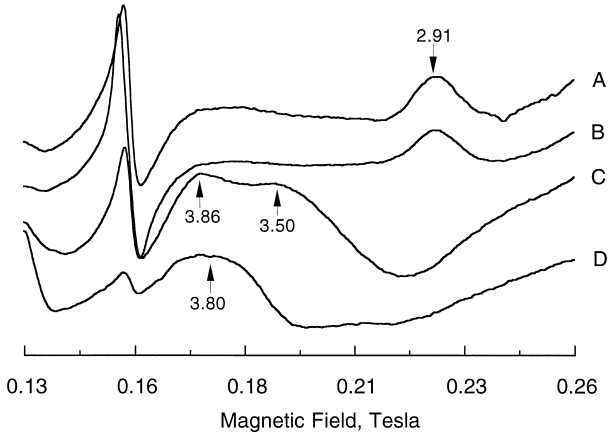

Fig. 4. Epr spectra of reconstituted cytochrome *b*-560, thrombin-treated reconstituted cytochrome *b*-560, heme-added GST, and free heme. 18 μl aliquots of hemin chloride, 8.0 mM, in DMSO were added to 0.9 ml of 50 mM Tris–Cl buffer, pH 8.0, containing (A) GST–QPs1 fusion protein, 10 mg/ml; (C) purified recombinant GST, 10 mg/ml; (D) none. The mixtures were incubated at room temperature, with stirring, for 2 h prior to EPR measurement. Trace (B) was taken after treatment of (A) with thrombin (0.01 unit/μg protein) for 1 h at room temperature. The EPR instrument settings were: modulation frequency, 100 kHz; modulation amplitude, 20 G; time constant, 0.5 s; microwave frequency, 9.42 GHz; microwave power, 20 mW; scan rate, 200 G/s; and temperature, 11 K.

Fig. 4 shows EPR characteristics of heme-ligated GST–QPs1, heme-ligated GST, and free heme. Reconstituted cytochrome *b*-560 in GST–QPs1 shows an EPR peak at $g = 2.91$ (trace A of Fig. 4), which differs completely from the EPR characteristics of heme-treated GST ($g = 3.50$ and $g = 3.86$, trace C of Fig. 4) and of free heme (a broad peak with $g = 3.80$, trace D of Fig. 4). When reconstituted cytochrome *b*-560 in GST–QPs1 is treated with thrombin, no change in EPR characteristics is observed (see trace B of Fig. 4). These support the results obtained from absorption spectra which indicate that the bis-histidine ligands of cytochrome *b*-560 are in the QPs1 moiety. The $g = 2.91$ EPR signal observed for reconstituted cytochrome *b*-560 in recombinant QPs1 corresponds to one of the two EPR signals ($g = 2.91$ and $g = 3.07$) observed for cytochrome *b*-560 in isolated QPs. This $g = 2.91$ signal of cytochrome *b*-560 in isolated QPs is not affected by the addition of succinate dehydrogenase to QPs to form succinate–ubiquinone reductase [7].

To further confirm that QPs1 provides bis-histidine ligands for cytochrome *b*-560 reconstituted with GST–QPs1, the heme-ligated fusion protein was treated with thrombin and subjected to a Superose-12 FPLC column chromatography to separate QPs1 from GST. Each column eluate fraction was analyzed by spectra, SDS-PAGE, and Western blotting. About 80% of the reconstituted cytochrome *b*-560 was recovered in fractions containing only QPs1. No cytochrome *b*-560 was found in the GST-containing fractions. Thus, QPs1 provides both histidine ligands of cytochrome *b*-560. This is in contrast to the report by Nakamura et al. [20] which says that one of the bis-histidine ligands of cytochrome *b*-556 of *E. coli* succinate–ubiquinone reductase is contributed by SdhC and other by Sdh D. We are currently trying to identify, by site-directed mutagenesis, which two histidines in QPs1 are involved in heme ligation.

Reconstitution of cytochrome *b*-560 into GST–QPs1 is heme concentration dependent. When GST–QPs1 is incubated with various amounts of hemin chloride, the peak height at 559.5 nm increases as the amount of heme added is increased (Fig. 5). Maximum reconstitution is reached when the molar ratio

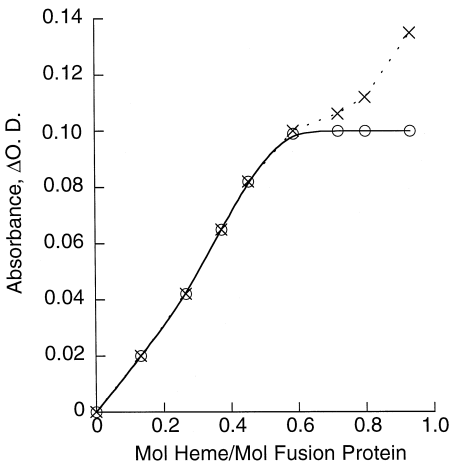

Fig. 5. Effect of heme concentration on the reconstitution of cytochrome *b*-560 in recombinant GST–QPs1 fusion protein. One ml aliquots of purified recombinant GST–QPs1 fusion protein, 0.7 mg/ml, in 50 mM Tris–Cl, pH 8.0, were added to 3 μl of DMSO containing indicated concentrations of hemin chloride. Mixtures were incubated at room temperature for 2 h and difference spectra between the oxidized and reduced forms of each sample were measured before (×) and after thrombin digestion (○). The thrombin digestion was carried out with 0.01 unit/μg protein at room temperature for 1 h.

of heme to fusion protein used in the system is 0.6. When the heme to protein ratio is higher than 0.6, the sample shows an increased absorbance at 559.5 nm compared to that reconstituted with a 0.6 molar ratio of heme, but with a discernible broad shoulder at around 580 nm (data not shown). This increased 559.5 nm absorbance is diminished when the sample is treated with thrombin (see Fig. 5), indicating that excess heme cross ligated to a histidine residue in the GST moiety to form a cytochrome *b*-560-like spectra. No change in 559.5 nm absorbance is observed with reconstituted cytochrome *b*-560, formed with less than 0.6 molar ratio of heme, after thrombin digestion. A millimolar coefficient for reconstituted cytochrome *b*-560 is calculated to be $9.9\,cm^{-1}$ for $A_{559.5\,nm} - A_{575\,nm}$ of the difference spectra of the dithionite-reduced minus the oxidized forms. This value is about half that determined for cytochrome *b*-560 in isolated QPs.

The less than unit reconstitution can be explained by (1) the presence of some incomplete GST–QPs1 proteins in the isolated fusion protein preparation; (2) partial availability of one of the histidine ligands in the QPs1 moiety resulting from the aggregation of isolated fusion protein in aqueous solution. When this partially available histidine residue is completely ligated by heme, excess heme is ligated to a histidine residue in the GST moiety to form a cytochrome *b*-560-like spectra which is diminished by thrombin digestion; (3) the two histidine ligands are from different QPs1 molecules. In this case, only one histidine residue of each QPs1 is involved. Results described in the next section seem to support the first possibility.

The incomplete GST–QPs1 (C-terminal truncated QPs1) proteins account for about 30% of the total protein in the isolated fusion protein preparation (see line 3 of Fig. 2(A)). These C-terminal truncated QPs1 proteins are probably missing one or both of the two highly conserved histidine residues (H42 and H98) in QPs1 since the apparent molecular masses of these proteins are 500–1000 Da. If the bis-histidine ligands of reconstituted cytochrome *b*-560 in GST–QPs1 are provided by the intact QPs1 moiety, 1 mol of heme ligated into 1 mol of intact QPs1–GST in the preparation will give maximum reconstitution with a 0.6 molar ratio of heme added. This idea is supported by the recovery of reconstituted cytochrome *b*-560 in QPs1, with a heme to protein ratio of 0.9, by a Sepharose-12 column chromatography, from thrombin treated, heme-ligated GST–QPs1 having a heme to protein ratio of 0.57. Furthermore, the amount of cytochrome *b*-560 restoration into GST–QPs1 correlates with the amount of intact QPs1 present in the fusion protein preparation. Addition of heme to a GST–QPs1 preparation enriched with incomplete QPs1–GST proteins (to about 80%), gives maximum reconstitution (2 nmol/mg) when the heme to protein ratio in the system is 0.18. The incomplete GST–QPs1 enriched preparation is obtained in a 0.1 mM glutathione washing of GST–QPs1 adsorbed on glutathione–agarose gel during purification.

To date we have been unable to reconstitute recombinant QPs1 with succinate dehydrogenase to form succinate-Q reductase. We observe no change in the absorption spectra, EPR characteristics, and carbon monoxide reactivity of reconstituted cytochrome *b*-560 in GST–QPs1 or in QPs1 upon addition of succinate dehydrogenase. This indicates that no specific interaction occurs between succinate dehydrogenase and recombinant QPs1. The failure of reconstituted cytochrome *b*-560 in GST–QPs1 or in QPs1 to interact with succinate dehydrogenase to form succinate–ubiquinone reductase may be due to a requirement of QPs2 or QPs3 for this interaction. Reconstitution studies including all the recombinant QPs subunits are currently in progress in our laboratory.

Acknowledgements

We thank Dr. Roger Koeppe for his critical reading of this manuscript.

References

[1] D.M. Ziegler, K.A. Doeg, Arch. Biochem. Biophys. 97 (1962) 41–45.
[2] R.A. Capaldi, J. Sweetland, A. Merli, Biochemistry 10 (1977) 2509–2514.
[3] L. Yu, C.A. Yu, J. Biol. Chem. 257 (1982) 2016–2021.
[4] G.Y. Lee, D.-Y. He, L. Yu, C.A. Yu, J. Biol. Chem. 270 (1995) 6193–6198.
[5] L. Hederstedt, T. Ohnishi, in: L. Ernster (Ed.), Molecular Mechanisms in Bioenergetics, Elsevier Science, New York, 1992, pp. 163–198.

[6] B.A.C. Ackrell, M. Johnson, R.P. Gunsalus, G. Cecchini, in: F. Muller (Ed.), Chemistry and Biochemistry of Flavoenzymes, vol. III, CRC Press, London, 1992, pp. 229–297.
[7] L. Yu, J.-X. Xu, P.E. Haley, C.A. Yu, J. Biol. Chem. 262 (1987) 1137–1143.
[8] C. Hägerhäll, L. Hederstedt, FEBS Lett. 389 (1996) 25–31.
[9] B.A.C. Ackrell, M.B. Ball, E.B. Kearney, J. Biol. Chem. 255 (1980) 2761–2769.
[10] Y. Hatefi, Y.M. Galante, J. Biol. Chem. 255 (1980) 5530–5537.
[11] C.A. Yu, L. Yu, Biochemistry 19 (1980) 3579–3585.
[12] T. Miki, L. Yu, C.A. Yu, Arch. Biochem. Biophys. 299 (1992) 61–66.
[13] C.A. Yu, L.-Q. Gu, Y. Lin, L. Yu, Biochemistry 24 (1985) 3897–3902.
[14] L.-Q. Gu, L. Yu, C.A. Yu, Biochim. Biophys. Acta 1015 (1990) 482–492.
[15] L. Yu, Y.Y. Wei, S. Usui, C.A. Yu, J. Biol. Chem. 267 (1992) 24508–24515.
[16] B. Cochran, R.A. Capaldi, B.A.C. Ackrell, Biochim. Biophys. Acta 1188 (1994) 162–166.
[17] R. Cammack, in: H. Matsubara, T. Katsube, K. Wada (Eds.), Iron–Sulfur Protein Research, Japan Sci. Soc. Press, Tokyo, 1986, pp. 40–55.
[18] J. Peterson, C. Vibat, R.B. Gennis, FEBS Lett. 355 (1994) 155–156.
[19] B.R. Crouse, C.A. Yu, L. Yu, M.K. Johnson, FEBS Lett. 367 (1995) 1–4.
[20] K. Nakamura, M. Yamaki, M. Sarada, S. Nakayama, C.R.T. Vibat, R.B. Gennis, T. Nakayashiki, H. Inokuchi, S. Kojima, K. Kita, J. Biol. Chem. 271 (1996) 521–527.
[21] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31–40.
[22] Y.-R. Chen, C.A. Yu, L. Yu, J. Biol. Chem. 271 (1996) 2057–2062.
[23] L. Yu, K.-P. Deng, C.A. Yu, J. Biol. Chem. 270 (1995) 25634–25638.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Appendix 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
[25] C.A. Yu, L. Yu, Biochim. Biophys. Acta 591 (1980) 409–420.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[27] K.G. Paul, H. Theorell, A. Akesson, Acta Chem. Scand. 7 (1955) 1248–1251.
[28] H. Schägger, T.A. Link, W.D. Engel, G. von Jagow, Methods Enzymol. 126 (1986) 224–237.
[29] J. Hartman, P. Daram, R.A. Frizzell, T. Rado, D.J. Benos, E.J. Sorscher, Biotechnol. Bioeng. 39 (1992) 828–832.
[30] C.H. Schein, Biotechnology 6 (1988) 291–294.
[31] J.R. Blackwell, R. Horgan, FEBS Lett. 295 (1991) 10–12.
[32] T. Arakawa, S.N. Timasheff, Biophys. J. 47 (1985) 411–414.
[33] J.T. Moore, A.U. Uppal, F. Maley, G.F. Maley, Protein Expr. Purif. 4 (1993) 160–163.
[34] N.J. Derby, T.E. Creighton, Nature 334 (1990) 715–716.
[35] R.A. Hart, J.E. Bailey, Biotechnol. Bioeng. 39 (1992) 1112–1120.
[36] D.B. Smith, K.M. Davern, P.G. Board, W.U. Tiu, E.G. Garcia, G.F. Mitchell, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 8703–8708.