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Expression and Characterization of Recombinant Human Cytochrome *c* in *E. coli*

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Cytochrome *c* is a heme protein involved in electron transfer, cell apoptosis, and diseases associated with oxidative stress. Here we expressed human cytochrome *c* in *E. coli* and purified it to homogeneity with a yield of 10–15 mg/L. The redox potential of recombinant human cytochrome *c* was 0.246 V which was measured by cyclic voltammetry. This is similar to that of horse cytochrome *c* with a value of 0.249 V. The sequential assignment and structural analysis of recombinant human ferrocytochrome *c* were obtained using multidimensional NMR spectroscopy. On the basis of our NMR studies, the recombinant human cytochrome *c* produced in *E. coli* exhibits the same tertiary fold as horse cytochrome *c*. These results provide evidence that human cytochrome *c* expressed in *E. coli* possesses a similar function and structure to that of the horse protein. It is known that cytochrome *c* plays a role in many human diseases. This study serves as the basis for gaining insight into human diseases by exploring structure and function relationships of cytochrome *c* to its interacting proteins.

KEY WORDS: Assignment; folding; NMR; heme proteins; heteronuclear; protein expression.

INTRODUCTION

Cytochrome *c* is an electron carrier protein that is responsible for accepting an electron from cytochrome *c* reductase and for transferring an electron to cytochrome *c* oxidase (Moore and Pettigrew, 1990; Pettigrew and Moore, 1987; Scott and Mauk, 1996). In addition to functioning as an electron carrier, cytochrome *c* promotes the assembly of a caspase-activating complex to induce cell apoptosis and stimulates the oxidative stress-induced diseases (Adrain and Martin, 2001; Hashimoto *et al.*, 1999; Khan *et al.*, 2000; Kluck *et al.*, 1997). These diverse functions of cytochrome *c* are associated with the protein–protein interactions between cytochrome *c* and its interacting proteins. Many cytochrome *c*-interacting proteins were identified, including cytochrome *c* oxidase, cytochrome *c* reductase, cytochrome *c* peroxidase, cytochrome b_5, and apoptosis protease-activating factor-1 (Apaf-1) (Shi, 2001). Therefore, mapping the recognition sites between cytochrome *c* and its interacting proteins and correlating their functional roles has become a subject of interest (Adrain and Martin, 2001; Kluck *et al.*, 1997; Scott and Mauk, 1996; Shi, 2001).

To date, more than 100 cytochromes *c* in eukaryotes have been found. They contain 100–120 amino acids with sequence identity ranging from 45 to 100%. Many 3D structures of cytochromes *c* have been determined by X-ray crystallography and NMR spectroscopy (Banci *et al.*, 1999). Despite variability in the sequence of individual proteins, they exhibit similar 3D structure throughout the superfamily. Analyses of the 3D structures of cytochromes *c* showed that they are all alpha proteins and consist of 3–7 α-helices. Three most conserved helices core form a basket around the heme group with one heme edge exposed to the solvent (Banci *et al.*, 1999). It was proposed that the solvent-exposed site of the heme group is its interaction site for cytochrome *c* oxidase, cytochrome *c* reducatase, and cytochrome *c* peroxidase (Banci *et al.*, 1999). However, the interaction sites of cytochrome *c* for

Key to abbreviations: HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; TOCSY, total correlated spectroscopy.

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Apaf-1 and the oxidative stress-induced proteins are still unclear.

Although many cytochromes *c* from different species have been purified and studied, little is known about human cytochrome *c*. Despite the human system being the most wildly studied one, most experimental studies in human system used horse heart, bovine heart, or rat liver cytochrome *c* as a substitute for human cytochrome *c* (Olson and Kornbluth, 2001). It was shown that cytochrome *c* could protect the ischemic myocardium during acute coronary occlusion (Zalewski *et al.*, 1987). To characterize human cytochrome *c* and to use human cytochrome *c* as the therapeutic agent, it is essential to obtain recombinant human cytochrome *c* with the correct fold.

Heterologous expression of holocytochrome *c* in *E. coli* is problematic because covalent attachment of the heme to the apocytochrome *c* requires cytochrome *c* heme lyase (Pollock *et al.*, 1998; Szabo *et al.*, 2000; Worrall *et al.*, 2001). The heme group of cytochrome *c* is linked through thioether bonds of the α-carbon atoms of the saturated vinyl groups of two adjacent pyrrole moieties to Cys-14 and Cys-17. His-18 and Met-80 serve as the fifth and sixth ligand, respectively. Recently, Pollock *et al*. have for first time overcome this problem by coexpressing yeast iso-1-cytochrome *c* and yeast cytochrome *c* heme lyase in *E. coli* (Pollock *et al.*, 1998). Using this approach, we synthesized the gene of human cytochrome *c* with *E. coli* favorable codons and then coexpressed the structural genes for human cytochrome *c* and yeast cytochrome *c* heme lyase in *E. coli*. We report a simple purification procedure to purify recombinant cytochrome *c*, and the yields of human holocytochrome *c* obtained from *E. coli* were 10–15 mg/L. Nuclear magnetic resonance (NMR) and mass spectrometric spectroscopy were used to characterize the fold of human cytochrome *c* expressed in *E. coli*. Our results show that human cytochrome *c* expressed in *E. coli* possesses similar electrochemical function and structure as those of the horse protein.

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c* was obtained from Sigma (Type VI) and used without further purification. Samples of the ferrocytochrome *c* were obtained by the addition of sodium dithionite.

Human Cytochrome *c* Expression, Construction, and Purification

Heterologous expression of holocytochrome *c in E. coli* was achieved by coexpressing the genes of cytochrome *c* and cytochrome *c* heme lyase (CCHL) (Morar *et al.*, 1999; Patel *et al.*, 2001; Pollock *et al.*, 1998; Price *et al.*, 2000; Tomlinson and Ferguson, 2000). Similar protocol with minor modification was used to express human cytochrome *c*. *Saccharomyces cerevisiae* (ATCC 18824 strain) was obtained from the Food Industry Research and Development Institute in Taiwan. The genomic DNA was extracted and the structural genes of iso-1-cytochrome *c* and cytochrome *c* heme lyase were obtained by polymerase chain reaction (PCR). The structural gene of yeast cytochrome *c* was amplified by PCR using sense primer 5′-CATATG*CATATG*GCTGAATTCAAGG CCGG-3′ with *Nde*I recognition and antisense primer 5′-GAAGCTT*AAGCTT*CTCAGTGGCTTTTTTCA-3′ with *Hin*dIII recognition. This amplified yeast gene was converted into human cytochrome *c* (hCYC) with eight primers that were designed to use codons from highly expressed *E. coli* genes (Kane, 1995; Kurland and Gallant, 1996) (Fig. 1). The structural gene of yeast CCHL was amplified by PCR using sense primer 5′-CATATG *CATATG*GGTTGGTTTTGGGCAG-3′ with *Nde*I recognition and antisense primer 5′-GGATCC*GGATCC* TTAAGGGGCGGAGGACG-3′ with *Bam*HI recognition. No additional amino acids were added into recombinant human cytochrome *c* with these primers. PCR products of hCYC and CCHL were purified and then cloned into the *Nde*I and *Hin*dIII sites, and the *Nde*I and *Bam*HI sites of the pET-21a vector, respectively. Recombinant plasmids were termed pET-21a-hCYC and pET-21a-CCHL that were then transformed into competent cells. The plasmid pET-21a-hCYC was purified and digested by *Bgl*II and *Hin*dIII. The digested fragment contained the T7 promoter region from the pET-21a vector and hCYC gene. It was then cloned into the pET-21a-CCHL plasmid that was digested by *Bam*HI and *Hin*dIII. The resulting pET-21a-CCHL-hCYC plasmid was transformed into the *E. coli* BL-21 (DE3) strain. Therefore, human cytochrome *c* and yeast cytochrome *c* heme lyase were inducibly coexpressed under the control of strong T7 promoters. Human apocytochrome *c* was covalently attached to heme with the help of coexpressing yeast cytochrome *c* heme lyase in the cytoplasm of *E. coli*.

The recombinant human cytochrome *c* was produced as follows: 10 μL of cells stock were grown at 37°C overnight in 5 mL LB medium (1% of Bacto tryptone + 0.5% of Bacto yeast extract + 1% of NaCl). One milliliter portions of cell culture were then transferred into 500 mL LB medium and grown at 37°C until the OD600 was greater than 1.5. The protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C for 12–16 h. The cells were harvested by centrifugation and lysed by liquid shear with a French press to obtain the extract. Recombinant cytochrome *c* was purified by

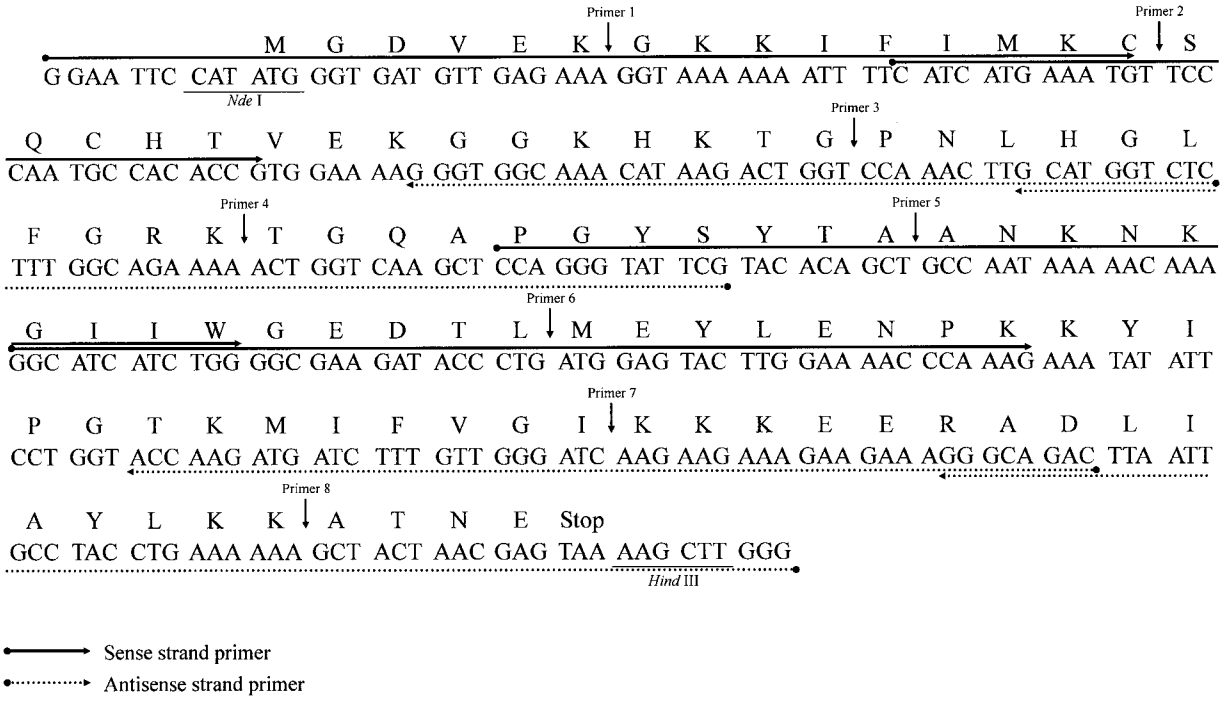

Fig. 1. Nucleotide and amino acid sequences of recombinant human cytochrome *c*. The yeast iso-1-cytochrome *c* gene was converted into human cytochrome *c* gene with eight primers that were designed to use codons from the highly expressed *E. coli* genes.

SP cation chromatography (Pharmacia) with a gradient of 0–200 mM NaCl. The solution was dialyzed four times using 3.5 kDa cutoff membrane against 4 L of ddH_2O and then lyophilized. The lyophilized protein was resuspended in 1 mL ddH_2O and then centrifuged to remove the aggregated proteins. The supernatant was further purified by Biogel P-10 (Biorad) gel filtration chromatography. The purified protein was concentrated by amicon ultrafiltration using 3.5 kDa cutoff membrane and lyophilized for storage at -70°C. The protein was further characterized by SDS-PAGE with Coomassie Brillant Blue R (Sigma) and heme stains (Merck) (Thomas *et al.*, 1976). The oxidized form of purified human cytochrome *c* had an A_{410}/A_{280} value of 4.45. The protein concentration was determined spectrophotometrically using either $\varepsilon_{410} = 106.1\ mM^{-1}cm^{-1}$ for oxidized form or $\varepsilon_{416} = 129.1\ mM^{-1}cm^{-1}$ for reduced form, respectively.

M9 minimal media was used to produce ^{15}N and $^{15}N/^{13}C$ labeled human cytochrome *c*. A total of 1 g/L $^{15}NH_4Cl$ (98% ^{15}N) and/or 2 g/L [^{13}C]-glucose (99% ^{13}C) were substituted for the unlabeled compounds in the growth media. A 500 μL of cells stock were grown at 37°C overnight in 100 mL unlabeled M9 medium. The cell culture was then transferred into 2 L unlabeled M9 medium and grown at 37°C until the OD600 was greater than 1. The cells were collected by centrifugation and suspended in 500 mL labeled M9 medium. The cell culture was grown for 1 h and then added 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to induce protein production. Additional 20 mg ferrochloride was added to increase the protein yield. The cell culture was incubated at 30°C with a speed of 60 rpm for 72 h. The same purification procedures were used to purify ^{15}N or $^{15}N/^{13}C$ labeled cytochrome *c*.

Mass Spectrometric Spectroscopy

The molecular weight of cytochrome *c* was confirmed using an API 365 triple quadrupole mass spectrometer equipped with a TurboIonSpray source (PE Sciex, Thornill, Canada). Infusion of protein solutions (1–10 μM in 50–90% methanol or acetonitrile with 0.1% formic acid) into mass spectrometer was performed using a syringe pump (Harvard Apparatus, South Natick, MA) at a flow rate of 12–20 μL/min to acquire full scan mass spectra. The electrospray voltage at the spraying needle was optimized at 5000–5300 V.

Optical Spectroscopy

The optical spectra of all samples were measured using a Beckman Model DU 640 spectrophotometer. Spectra

were recorded between 250 and 700 nm with 10 mm cell, and the samples were dissolved in 100 mM phosphate buffer at pH 7.0.

Potential Measurements

The midpoint reduction potential of cytochrome *c* was determined by cyclic voltammetry. The solution potentials were measured with a Bioanalytical System potentiostat Model BAS-100B (West Lafayette, IN) using the glassy carbon as an indicating electrode and an Ag/AgCl electrode for the reference. The latter has a potential of 200 ± 2 mV versus standard hydrogen electrode and all reported potentials have been corrected to the standard hydrogen electrode. Samples containing 1 mg/mL of human or horse cytochrome *c* in 50 mM phosphate buffer at pH 7.0 were used. The experiments were performed at least three times and the redox potentials were found reproducible within ± 2 mV.

NMR Sample Preparation

NMR samples were dissolved in 10% or 100% D_2O at a concentration of 2–3 mM in 25 mM phosphate buffer and 100 mM NaCl. The pH values were adjusted with 100 mM KOD to 6.5. The reduced form of human cytochrome *c* was obtained by adding sodium dithionite.

NMR Spectroscopy

NMR experiments were performed at 27°C on a Bruker Avance 600 spectrometer equipped with pulse field gradients and *xyz*-gradient triple resonance probes. Experiments of HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HBHA(CBCA)NH, and HBHA(CBCACO)NH, ^{15}N-edited and ^{13}C-edited TOCSY-HSQC, HCCH-TOCSY, and HCCH-COSY were carried out for the purpose of proton, carbon, and nitrogen resonance assignments (Bax *et al.*, 1990; Cavanagh *et al.*, 1996; Clore and Gronenborn, 1998; Fesik and Zuiderweg, 1998; Grzesick *et al.*, 1993; Grzesiek and Bax, 1992a,b; Ikura *et al.*, 1991; Marion *et al.*, 1989; Muhandiram and Kay, 1994; Wang *et al.*, 1994; Wittekind and Mueller, 1993). 2D COSY, TOCSY, and NOESY experiments with an unlabeled sample in D_2O provided the basis for the aromatic proton assignments and hydrogen bond determination (Bax, 1989; Wuthrich, 1986). Data were processed and analyzed using XWINNMR and Aurelia programs on an O2 Silicon Graphics workstation. The center frequencies of double resonance experiments were 4.75 ppm (^{1}H) and 118 ppm (^{15}N). The observed ^{1}H chemical shifts were referenced with respect to H_2O or HOD signal, which was taken as 4.754 ppm downfield from external sodium 3-trimethylsilylpropionate-2,2,3,3-d4 (TSP) in D_2O (0.0 ppm) at 300°K. The nitrogen chemical shift was referenced to external $^{15}NH_4Cl$ (3 mM in 1M HCl) at 300°K, which is at 24.93 ppm downfield from liquid NH_3.

RESULTS

Expression, Purification, and Characterization of Human Cytochrome *c*

To express human cytochrome *c* in *E. coli*, the yeast iso-1-cytochrome *c* gene was converted into human cytochrome *c* with eight primers which were designed to use the codons from *E. coli* for increasing the yield of cytochrome *c* expressed in *E. coli* (Kane, 1995; Kurland and Gallant, 1996). The sequences of the synthetic gene of human cytochrome *c* and eight primers are shown in Fig. 1. The expression vector contains the genes of human cytochrome *c* and yeast cytochrome *c* heme lyase. Although yeast and human cytochrome *c* heme lyases only share 32% sequence identity, yeast cytochrome *c* heme lyase can also efficiently attach the heme group to human recombinant apopcytochrome *c* in the cytoplasm of *E. coli*. A variety of expression conditions including the cell density, optimal temperature, and induction period were used to optimize the yield of human cytochrome *c* produced in *E. coli*. The highest yield of human cytochrome produced in LB medium was obtained with the conditions that the OD600 is greater than 1.5, the induction temperature is at 30°C, and optimal induction period is about 12–16 h. In addition, it was shown that the additions of δ-aminolevulinic acid and trace element solution are critical for expressing cytochrome *c* in minimal medium (Morar *et al.*, 1999). In contrast, we found that the addition of 20 mg/L of ferrochloride is critical and sufficient for expressing human cytochrome *c* in minimal medium. Using these approaches, the yields of unlabeled and ^{15}N/^{13}C labeled human cytochrome *c* were 10–15 and 5–10 mg/L, respectively.

The recombinant human cytochrome *c* expressed in *E. coli* was purified to homogeneity by using SP-Sepharose cation and P-10 gel filtration chromatography. Compared to other purification protocols, this is a simple purification procedure (Morar *et al.*, 1999; Patel *et al.*, 2001; Pollock *et al.*, 1998; Price *et al.*, 2000; Tomlinson and Ferguson, 2000). On the basis of SDS-polyacrylamide

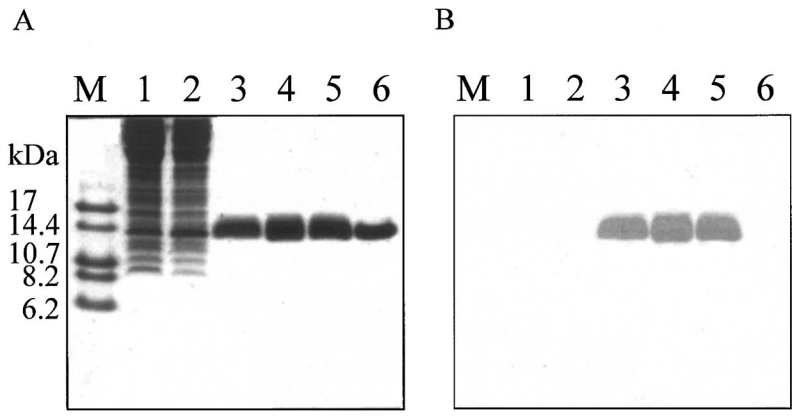

Fig. 2. SDS-PAGE analysis of cytochrome *c* expressed in *E. coli* with Coomassie-Brilliant Blue (A) and heme (B) stains. Lane M, M. W. marker (16.9, 14.4, 10.7, 8.2, and 6.2 kDa); Lane 1, cytochrome *c* expression without induction; Lane 2, cytochrome *c* expression after IPTG induction; Lane 3, recombinant human cytochrome *c* after purification by SP-sepharose cation chromatography; Lane 4, fractions purified by SP-sepharose cation chromatography after dialysis; Lane 5, recombinant human cytochrome *c* after purification by P-10 gel filtration chromatography; Lane 6, recombinant human cytochrome *c* under reducing condition.

gel electrophoresis, human cytochrome *c* produced in *E. coli* was homogenous (Fig. 2(A)). In addition, SDS-PAGE using heme stain showed that heme was synthesized and attached to apopcytochrome *c* (Thomas *et al.*, 1976) (Fig. 2(B)). Mass spectrometry was used to identify the formation of holocytochrome *c*. The experimental molecular weight of human cytochrome *c* produced in *E. coli* is 12233.5 that is in excellent agreement with the calculated value, 12233.9. The value is calculated by adding the molecular weight of heme, 616.4, indicating that the heme group is covalently attached to apocytochrome *c*. Mass analysis also indicated that no additional amino acids were added into recombinant human cytochrome *c* which has the same amino acid sequence as authentic cytochrome *c*.

Optical Spectra of Human Cytochrome *c*

The recombinant human cytochrome *c* exhibited absorption maxima at 415, 520, and 550 nm in reduced form, and at 410 and 529 nm in oxidized form, respectively (Fig. 3). The optical spectra of oxidized and reduced human cytochrome *c* were similar to those of horse cytochrome *c* (Korszun *et al.*, 1982).

Electrochemical Characterization

A single voltammetric signal was observed for both human and horse ferri/ferro-cytochrome *c* equilibrium that was reversible, monoelectronic and diffusion controlled. The redox potential of recombinant human cytochrome *c* was 0.246 ± 0.02 V that was measured by cyclic voltammetry measurement (Fig. 4). This is similar to that of horse cytochrome *c* with a value of 0.249 ± 0.02 V (Fig. 4). This result indicates that the difference in E^0 between human and horse cytochromes *c* is small.

NMR Analysis of Human Ferrocytochrome *c*

NMR spectroscopy was used to examine the folding of recombinant human cytochrome *c*. NMR spectra

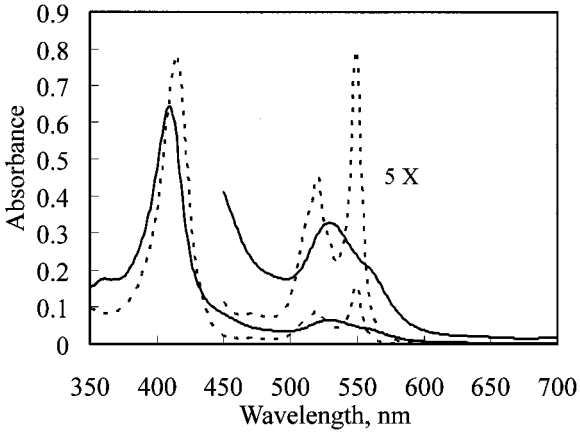

Fig. 3. Absorption spectra of reduced and oxidized forms of human cytochrome *c*. Sample containing 1 mg/mL of reduced (------) and oxidized (——) forms of human cytochrome in 50 mM potassium phosphate buffer at pH 7.0 was used.

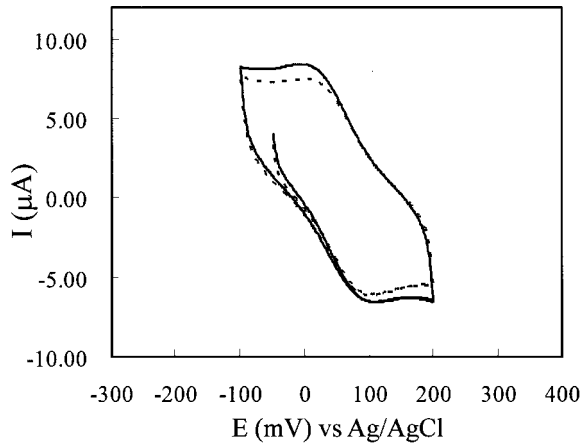

Fig. 4. Cyclic voltammogram of human and horse cytochrome *c*. Sample containing 1 mg/mL of human (– – – –) or horse (———) cytochrome in 50 mM potassium phosphate buffer at pH 7.0 was used.

of human ferrocytochrome *c* are not available and 92 of 104 residues in human cytochrome *c* are identical to horse heart cytochrome *c*. Since ^{1}H and ^{15}N chemical shifts of horse heart cytochrome *c* have been reported (Morar *et al.*, 1999; Qi *et al.*, 1994, 1996), we compared 2D NOESY spectra of human and horse heart ferrocytochrome *c* (Fig. 5). They have similar NOE pattern, suggesting that they have similar fold. To obtain the sequential assignments of human cytochrome *c*, we performed heteronuclear 3D and homonuclear 2D NMR experiments of recombinant human cytochrome *c*. Strip plots of G1–V20 from the HNCACB spectrum of human cytochrome *c* clearly showed the Cα and Cβ connectivities (Fig. 6). ^{1}H, ^{15}N, and ^{13}C chemical shifts of human cytochrome *c* could be assigned without any ambiguity. Their chemical shifts were similar to those of horse heart cytochrome *c*. Only NH chemical shifts of D2 ($\Delta\delta = 0.19$ ppm), Q16 ($\Delta\delta = 0.16$ ppm), and T19 ($\Delta\delta = -0.18$ ppm) exhibit the largest shifts, suggesting that human and horse cytochrome *c* have similar fold and minor difference in the heme environment.

Although the chemical shifts and secondary structures of recombinant human cytochrome *c* were similar to those of horse heart cytochrome *c* (Fig. 7), it was still necessary to identify the covalent attachment of heme to apopcytochrome *c*. We performed NOESY experiments of recombinant human cytochrome *c* in 100% D_2O to determine whether the thioether bonds between the vinyl groups of heme and Cys-14 and Cys-17 residues were formed. The formation of thioether bonds was determined by searching the NOE patterns between CβH of the cysteine residues and the meso and vinyl protons

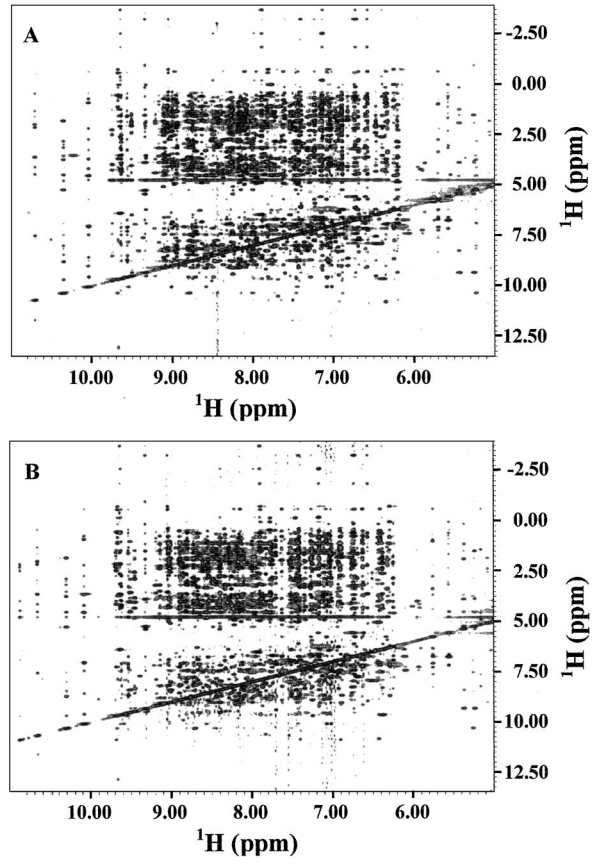

Fig. 5. 2D NOESY spectra of human (A) and horse (B) cytochrome *c*. Sample was dissolved in 10% D_2O/90% H_2O at pH 6.5 and 300 K.

of the heme group. As shown in Fig. 8, the protons of the heme group were identified by using 2D homonuclear NOESY spectra. NOEs between the α-meso proton of porphyrin to CαH and CβH of Cys-14, and the β-meso proton of porphyrin to CαH and CβH of Cys-17 were found, indicating that the heme group is covalently attached to apopcytochrome *c*. In addition, NOEs between the protons of His-18 to the protons of the heme group, and the protons of Met-80 to the protons of the heme group were identified, suggesting that the His-18 and Met-80 residues serve as the ligands for the heme group (Fig. 8).

DISCUSSION

Heterologous expression of holocytochrome *c* in *E. coli* is problematic because covalent attachment of the heme to the apocytochrome *c* requires cytochrome *c* heme lyase (Pollock *et al.*, 1998; Szabo *et al.*, 2000; Worrall *et al.*, 2001). In this study, we synthesized the gene

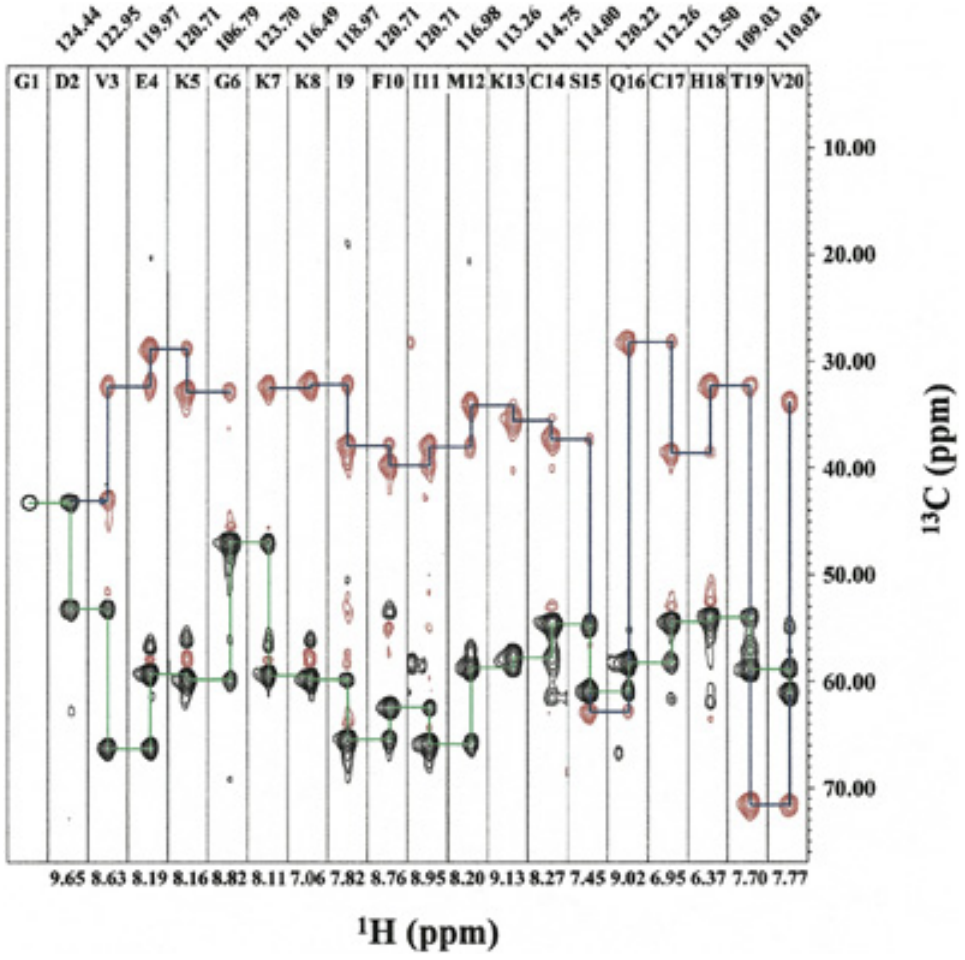

Fig. 6. Strip plots of residues G1 to V20 taken from NHCACB. The NHCACB experiment correlates the Cα and Cβ resonances of the i and $i-1$ residues with the NH resonance of i residue.

of human cytochrome *c* using *E. coli* favorable codons and then coexpressed the structural genes for human cytochrome *c* and yeast cytochrome *c* heme lyase in *E. coli*. Although human and yeast cytochromes *c* share 56% sequence identity and 73% sequence similarity, yeast cytochrome *c* heme lyase can efficiently couple the heme to human recombinant apopcytochrome *c*. This expression system produced recombinant human cytochrome *c* with the correct fold and good yield. The yields of unlabeled and ^{13}C, ^{15}N-labeled human holocytochrome *c* produced in *E. coli* were 10–15 and 5–10 mg/L, respectively. The availability of ^{13}C, ^{15}N-labeled cytochrome *c* allows the use of heteronuclear NMR to resolve resonances and to study the protein dynamics and protein–protein interaction (Wand, 1996). It is known that cytochrome *c* interacts with many different proteins, such as cytochrome *c* oxidase, cytochrome *c* reductase, cytochrome *c* peroxidase, cytochorome b_5, and apoptosis protease-activating factor-1 (Apaf-1) (Shi, 2001). The 3D structures of the cytochrome *c*-protein complexes can be determined using intra- and inter-molecular NOE correlations that are observed by a series of isotope-filtered and -edited NMR experiments (Wang, 1996). The expression and labeling of relatively large amounts of recombinant human cytochrome *c* will be feasible for these experiments.

Many cytochromes *c* from different species are well characterized (Moore and Pettigrew, 1990; Pettigrew and Moore, 1987; Scott and Mauk, 1996). Little is known about human cytochrome *c* since it is practically impossible to obtain human cytochrome *c* from human organism (Tanaka *et al.*, 1988). Horse cytochrome *c* shares 89% sequence identity to human cytochrome *c* and is well characterized (Morar *et al.*, 1999; Qi *et al.*, 1994, 1996). Therefore, we compared the results of recombinant human cytochrome *c* with those of horse cytochrome. The redox potential and optical spectrum of recombinant human

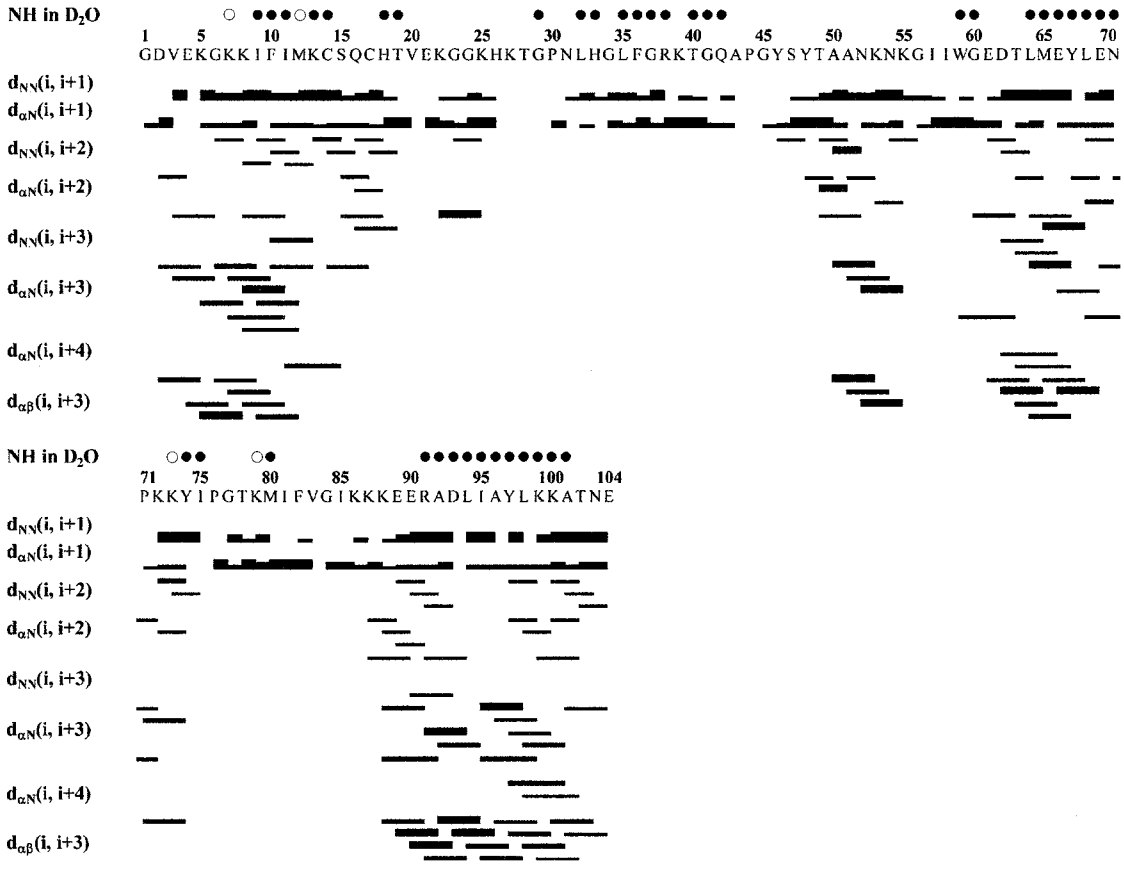

Fig. 7. The secondary structures for human cytochrome *c* are deduced from NOE data. The intensities of NOEs are represented by the thickness of the blocks.

cytochrome *c* was similar to those of horse cytochrome *c*. Our NMR analysis showed that the structure of recombinant human cytochrome *c* is also similar to that of horse heart cytochrome *c*. NOE patterns between porphyrin and two cysteine residues were found, indicating that the heme *c* group is linked through thioether bonds of the α-carbon atoms of the saturated vinyl groups of two adjacent pyrrole moieties to Cys-14 and Cys-17. In addition, NOE patterns between the protons of porphyrin to the protons of His-18 and Met-80 were found. These observations suggest that both residues serve as the ligands for the heme. These results provide the direct evidence that human cytochrome *c* expressed in *E. coli* exhibits the correct fold. The surface electrostatic features of cytochromes *c* from various organisms are quite different and the electrostatic profile of surface-exposed residues in cytochromes *c* plays the most important role in recognizing its interacting proteins (Banci *et al.*, 1999). Therefore, it is of interest to compare the surface electrostatic features of human cytochrome *c* to those of cytochromes *c* from different species (Banci *et al.*, 1999). The expression of human cytochrome *c* in *E. coli* with the correct fold allows us to engineer the cytochrome *c* gene by site-directed mutagenesis. Determination of the interaction sites of human cytochrome *c* and its interacting proteins will become feasible. This study serves as the basis for exploring the structure and function relationships of cytochrome *c* and its interacting proteins in human system.

In conclusions, we expressed human cytochrome *c* in *E. coli* expression system. We found that human cytochrome *c* produced in *E. coli* possesses similar function and structure as those of horse protein. The feasibility to obtain $^{15}N/^{13}C$-labeled human cytochrome *c* provides the chance to study the structure–activity relationships and protein–protein interactions of human cytochrome *c*. It is known that cytochrome *c* is involved in human diseases. This study will serve as the basis for gaining insight into human diseases by exploring the structure and function relationships of cytochrome *c* to its interacting proteins.

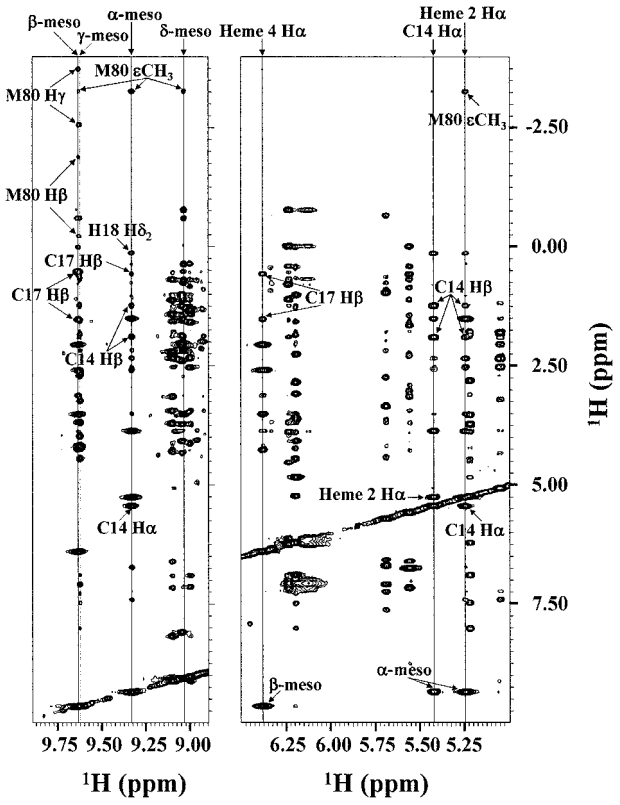

Fig. 8. NOE patterns between two cysteine residues and the meso protons of heme. The sample was dissolved in 100% D_2O at pH 6.5. Four meso protons of the heme group are represented by the vertical lines.

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