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ORIGINAL PAPER

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High-level production of heme-containing holoproteins in *Escherichia coli*

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Abstract The expression of recombinant protein is essential for the investigation of the functions and properties of heme-containing protein as an electron carrier. For the expression of fully active recombinant protein, conversion of the expressed apoprotein into holoprotein is the most important and difficult problem. In this study, a system was developed for the production of heme-containing protein in a pure, recombinant holoprotein form, using the bovine cytochrome b5 tryptic fragment and *Escherichia coli* bacterioferritin as heterologous and homologous heme-containing model proteins, respectively. This system is based on the slow synthesis of recombinant apoprotein, which can maintain the balanced consumption of amino acids between protein synthesis and heme synthesis, so that the synthesized apoprotein continues to act as a heme sink. From a 1-l culture, 15 mg of cytochrome b5 and 40 mg of bacterioferritin were purified as pure holoprotein forms. Our expression system provides a rapid and simple method for obtaining large quantities of the active holo-form of heme-containing proteins.

Introduction

Heme-containing proteins, which are the most abundant electron-transferring proteins in all eukaryotes and prokaryotes, participate in many biological metabolisms. For the overproduction of these proteins, which is essential for examining their structure and function, the bacterial expression system is the best choice. A number of recombinant heme-containing proteins have been expressed in *Escherichia coli*. However, the low ratio of holo-/apoprotein was the most critical limit for the overexpression of heme-containing proteins in *E. coli* in all cases (Neeti and Ferguson 1998; Nishimoto et al. 1993; Smith et al. 1994). Therefore, it is clear that incorporation of the heme-prosthetic group is a key step for the fully active protein. It is known that the regulation of heme biosynthesis in *E. coli* is the most dominant factor for the high-level expression of the holoprotein form during the production of heme-containing protein. Although δ-aminolevulinic acid, the product of the first committed step of the heme-biosynthetic pathway, was added to the bacterial culture for enhancing the ability to supply heme in *E. coli*, this did not lead to expression of the pure holoproteins (Woodard and Dailey 1995).

In this study, we developed a system for the production of heme-containing holoprotein in *E. coli*, using a bovine cytochrome b5 tryptic fragment and *E. coli* bacterioferritin as heterologous and homologous heme-containing model proteins, respectively. Cytochrome b5 is a well-known, heme-containing protein and is an integral component of the microsomal membranes of many mammalian cells (Rich and Bendal 1975). This protein is composed of two domains: a hydrophilic, heme-containing catalytic domain of about 100 amino acids and a short, hydrophobic domain of around 30 amino acids, which anchors the protein to the cytosol (Spatz and Strittmatter 1971). A tryptic fragment (Ala7–Lys90) of cytochrome b5 is soluble in aqueous solution and is suitable for characterizing the functions of the native protein (Cristiano and Steggles 1989). In contrast, *E. coli* bacterioferritin is an iron-storage protein consisting of 24 subunits of 17–20 kDa and it contains 12 b-type heme groups between the subunit pairs (Frolow et al. 1994; Stiefel and Watt 1979). While cytochrome b5 is an electron carrier, the role of the heme group in bacterioferritin is unclear. The 24-mer bacterioferritin is also soluble in aqueous solution and can be overexpressed in *E. coli* (Guest et al. 1989). We cloned the genes encoding the bovine brain cytochrome B5 tryptic fragment and *E. coli* bacterioferritin into the vector system, under the control of a regulable T5 promoter and *lac*-operator system; and we investigated the overexpression of the recombinant

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proteins. The ratio of the expressed apoprotein to holoprotein decreased with the amounts of isopropyl β-D-thiogalactopyranoside (IPTG) added to the *E. coli* culture as an inducer for expressing the recombinant proteins. We also determined the optimum conditions under which sufficient heme was incorporated into the apoproteins to generate the holoproteins.

Materials and methods

Bacterial strains and plasmids

The strain JM109 [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (rK^-, mK^+)), *rel*A1, *sup*E44, Δ(*lac-pro*AB), (F′, *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$)] was used for plasmid-mediated transformations during the cloning of cDNA and for the isolation and purification of plasmid DNA. The host strains for recombinant protein expression were JM109 or M15/pREP4 (Qiagen). Plasmid pQE30 (Qiagen), which contains an optimized 6-His affinity tag-coding sequence at the N-terminus position, was used as the expression vector.

Construction of vectors for the expression of target proteins in *E. coli*

The cytochrome b5 tryptic fragment cDNA was produced from a bovine-brain mRNA pool by reverse transcriptase-polymerase chain reaction (RT-PCR); and bacterioferritin cDNA was amplified with *E. coli* chromosomal DNA. The amplification of the DNA fragments encoding target proteins involved the use of the following primers. In the case of cytochrome b5, the forward primer was 5′-CGC GGA TCC GCC GTT AAG TAC TAC ACC C-3′ and the reverse primer was 5′-CGG GGT ACC TCA CTT TGA TCT GTC ATC CG-3′. In bacterioferritin, the forward primer was 5′ CGC GGA TCC ATG AAA GGT GAT ACT AAA GTT 3′ and the reverse primer was 5′ CGG GGT ACC TCA ACC TTC TTC GCG 3′. These primers carried the *Bam*HI and *Kpn*I site for cloning into the expression vector pQE30. The *Bam*HI/*Kpn*I-digested PCR products were then ligated with *Bam*HI/*Kpn*I-digested pQE30 vector to give the expression plasmids, pQcyb and pQbfr.

Production and purification of cytochrome b5 and bacterioferritin

E. coli JM109 or M15(pREP4) cells containing the relevant plasmids were grown overnight in LB medium containing 100 mg ampicillin l^{-1} and 25 mg kanamycin l^{-1}. This overnight culture was diluted 300-fold into the same medium. The flasks were shaken at 250 rpm and 37 °C until the absorbance at 600 nm (A_{600}) of the culture was 0.4–0.6. IPTG was then added and the cells were grown for about 18 h. For the detection of the ratio of holo-/apoprotein according to IPTG concentration, IPTG was added at concentrations of 0.001, 0.002, 0.004, 0.01, and 1 mM into individual sample cultures grown under the same conditions. The cells were pelleted and washed with buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 8.0) at 5 °C. The pelleted cells were resuspended in buffer A. Lysozyme was sequentially added at a concentration of 1 mg ml^{-1} and the cells were then sonicated on ice. This suspension was centrifuged at 10,000 *g* for 30 min and the supernatant that was colored red by the hemoproteins was applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography resin (Qiagen), equilibrated with buffer A at a flow rate of 0.5 ml min^{-1}. The column was then washed with about 15 column-volumes of buffer A. The bound protein was eluted with buffer B (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0) at 5 °C. In the case of bacterioferritin, the red-colored solution was heated to 65–70 °C for 15 min and centrifuged before affinity column chromatography, because heat treatment is the most effective purification step for bacterioferritin (Andrews et al. 1993). Purified hemoprotein fractions were readily detected, because of the solution's red color. The salts in protein solution were removed by dialysis against distilled, deionized water and the protein solution was concentrated by freeze-drying.

Spectrophotometric analysis and determination of hemoproteins

UV-visible spectra of the proteins were obtained using a Genesys Spectronic system. The heme content of each recombinant protein was determined from the Soret absorbance maximum (A_{413}) for oxidized cytochrome b5, using an absorbance coefficient of 117 mM^{-1} cm^{-1} (Estabrook and Werringloer 1978), and for bacterioferritin (A_{418}), using 107 mM^{-1} cm^{-1} (Deeb and Hager 1964). All protein contents were determined by bicinchoninic acid (BCA) assay, using bovine serum albumin as the standard.

Cyclic voltammetry experiment

Electrochemical experiments were performed at room temperature using the Bioanalytical Systems electrochemical system. The cell was composed of three-electrode systems as described previously (Song et al. 1993). The working electrode was a gold film electrode, prepared by sputter deposition on glass. The reference electrode was a mercury sulfate electrode (Hg/Hg_2SO_4, 0.6158 V) vs the normal hydrogen electrode (NHE), with an internal filling solution of 3 M K_2SO_4. The metal electrode was immersed in absolute ethanol containing 1 mM 3-mercaptopropionic acid for 18–24 h. The modified working electrode was immersed into a 100 μM cytochrome b5 solution containing an 8.8 mM phosphate buffer, pH 7.0, as an electrolyte. For titration of the protein solution, poly-L-lysine was used as described previously (Rivera et al. 1994).

Results

Expression of the recombinant hemoproteins

To construct the expression system in *E. coli* for the bovine cytochrome b5 tryptic fragment as a heterologous hemoprotein and *E. coli* bacterioferritin as a homologous hemoprotein, we prepared the DNA fragments containing their coding sequences by PCR. The expression vector pQE30 carrying the T5 promoter and *lac*-operator system was used to construct plasmids pQcyb and pQbfr1, which generated the recombinant cytochrome b5 fragment and bacterioferritin, respectively. The *E. coli* strain JM109 containing the plasmids was examined for the synthesis of hemoproteins upon induction with IPTG. Following the induction with 0.001–1 mM IPTG for 1 h, the cells accumulated a large amount of recombinant protein, but did not show a red color in the culture. Since the red color is an indication of high levels of heme in the culture, we expected a low level of heme content in the recombinant protein. As expected, the recombinant protein had a very low level of heme content (data not shown). The low heme content of the recombinant protein could be ascribed to the high expression of recombinant apoprotein during such a short period of time, because the burst of apoprotein expression might not be able to activate heme synthesis in the cell. To control the level of expression of the recombinant apoproteins more precisely, the host strain was changed to *E. coli* M15/pREP4, where multiple copies of plasmid pREP4 overexpressed the *lac* repressor, which interacts with the

lac operator in the expression vector. In this system, the expression level of the recombinant proteins was more precisely controlled by the amount of IPTG (Fig. 1). The amount of overexpressed recombinant protein in the presence of 1 mM IPTG was approximately 60% of the total *E. coli* protein. When the IPTG concentration was lowered to 0.01 mM, the level of induced protein did not significantly decrease. As the IPTG concentration was further brought down to 0.001 mM, the expression level decreased to about 8% of the total *E. coli* protein. It was also noticeable that approximately 90% of the recombinant protein was synthesized within 2–4 h under induction with 0.01 mM or 1 mM. Obviously slow synthesis was observed after induction with 0.004 mM or 0.001 mM IPTG, together with low level expression (Fig. 1). However, high expression of the holoproteins was directly seen (red coloration) with cell cultures induced with IPTG at these low concentrations. The recombinant proteins were purified from the cultures induced with different concentrations of IPTG through Ni-NTA affinity chromatography, because they were expressed as his-tag fusions. The purified proteins were observed as single bands at the positions of expected molecular weight in a sodium dodecyl sulfate-polyacrylamide gel (data not shown). Proportions of the holoproteins in the purified protein were monitored by native polyacrylamide gel electrophoresis (Fig. 2). There were two bands migrating with different electrophoretic mobility, which should represent the apoprotein and holoprotein, respectively. Spectroscopic analysis showed that the lower band of cytochrome b5 and the upper band of bacterioferritin corresponded to the holoproteins. As the concentration of IPTG decreased, the ratio of holoprotein to apoprotein increased. The level of the recombinant proteins and holoproteins in *E. coli* total protein was calculated from the cultures induced with different concentrations of IPTG (Fig. 3). The amount of recombinant protein increased when the IPTG concentration was increased, but the proportion of holoprotein decreased. At 0.002 mM IPTG, the recombinant proteins were expressed as pure holoprotein forms, to approximately 15 mg of cytochrome b5 and 40 mg of bacterioferritin.

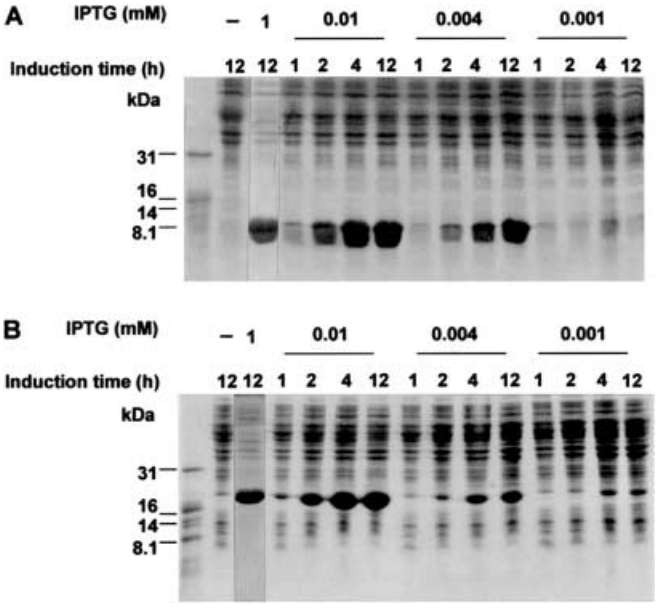

Fig. 1A, B Expression of recombinant holoproteins. *Escherichia coli* M15(pREP4) cells containing plasmids pQcyb (**A**) and pQbfr(**B**) were grown for the times indicated, after induction with the indicated concentrations of isopropyl β-D-thiogalactopyranoside (*IPTG*). Whole cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) on 15% gels and visualized with Coomassie blue

Characterization of the recombinant holoproteins

Since the recombinant hemoproteins were exclusively expressed as holo-forms under induction with IPTG at 0.002 mM or lower, they were purified from cultures induced with 0.002 mM IPTG and were used to determine their spectroscopic properties or electrochemical properties. The recombinant cytochrome b5 showed a UV-visible spectrum (Fig. 4) with an A_{max} typical of mammalian cytochrome b5 (Bodman et al. 1986). It also showed a cyclic voltammogram on a gold electrode with a peak separation of ΔEp=60 mV, which was almost identical to the value of 59 mV theoretically calculated for a one-electron process in the solution (Fig. 5). The

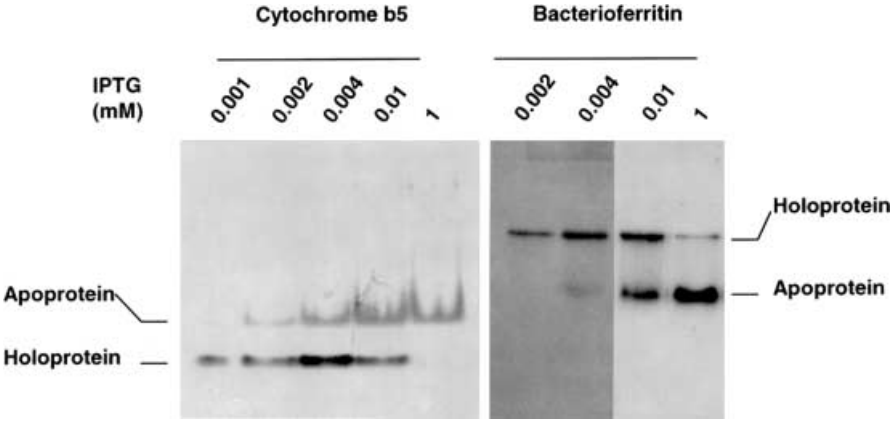

Fig. 2 Non-denaturing PAGE analysis of the purified proteins. The recombinant holoproteins were expressed as His-tag fusions and purified by nickel-nitrilotriacetic acid chromatography. The purified proteins overexpressed at various concentrations of IPTG were analyzed on 12% PAGE gels. The positions of *holo-* and *apoproteins* are indicated

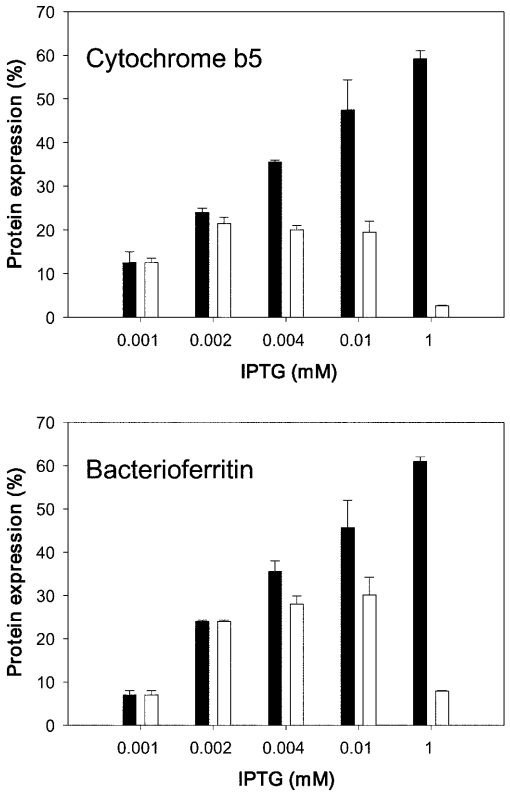

Fig. 3 The ratios of expressed holo-/apoprotein. The expression levels of the recombinant proteins in total cellular protein were determined by a densitometric scanning of the protein gels. Holoprotein levels in the total recombinant protein were calculated by the non-denaturing PAGE analysis of purified recombinant protein (Fig. 2) and the spectrophotometric analysis of the heme content in the protein. *Solid bars* total expressed recombinant protein (%) in total bacterial protein, *open bars* expressed recombinant holoprotein (%) in total bacterial protein

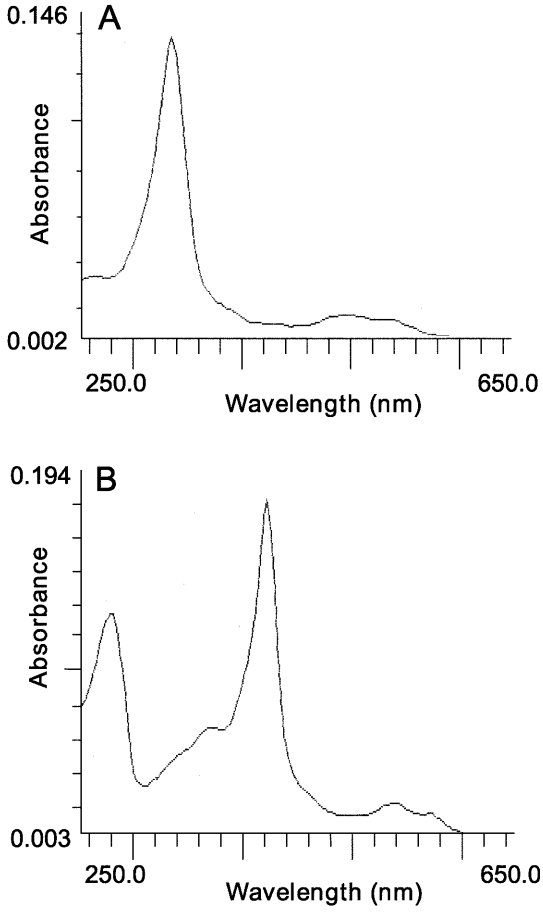

Fig. 4 UV-visible spectra of purified recombinant proteins: (**A**) cytochrome b5, (**B**) bacterioferritin

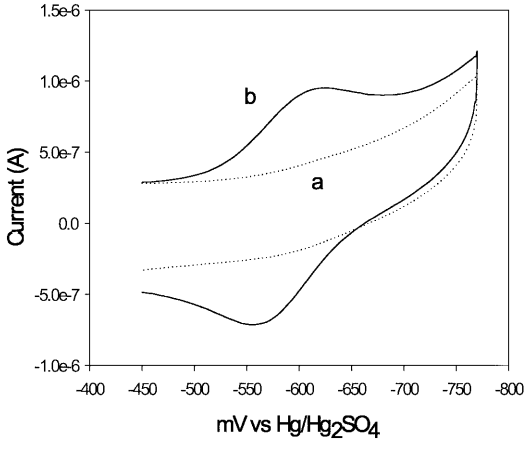

Fig. 5 Cyclic voltammogram of 0.1 mM cytochrome b5 at a gold electrode modified by 1 mM of cysteine (*a*) in the absence of poly-L-lysine and (*b*) in the presence of 0.3 mM poly-L-lysine. Experimental conditions: pH 7.0, 8.8 mM phosphate buffer; scan rate: 100 mV s–1

redox potential of cytochrome b5 is 36 mV vs NHE; and this value is 27 mV more positive than that of the bovine liver microsomal cytochrome b5 (Qian et al. 1998). The UV-visible spectrum of the purified bacterioferritin (Fig. 4) was the same as that of the authentic 24-mer holoenzyme (Andrews et al. 1993). The heme content of the recombinant bacterioferritin calculated by the spectrophotometric method as described (Andrews et al. 1993) were about ten hemes per 24-meric holoprotein.

Discussion

In this paper, we describe the high-level expression of holoprotein forms of cytochrome b5 and bacterioferritin in *E. coli*. Although the expression of bovine cytochrome b5 and bacterioferritin as recombinant proteins in *E. coli* has previously been studied, only a portion of the expressed proteins were assembled into holoproteins (Andrews et al. 1993; Hewson et al. 1993; Holmans et al. 1994). Therefore, the former view was that the high expression of hemoproteins in *E. coli* would not be a suitable way to obtain pure holoprotein. To overcome this basic obstacle, many factors must be considered, such as heme synthesis, stability of apoprotein, and covalent-bond formation between heme and protein in bacteria. Fortunately, the maturation of the cytochrome b5 and bacterio-

ferritin we used, in this study, as heterologous and homologous model proteins, respectively, is relatively simple because heme can be directly inserted into the apo-hemoproteins without any covalent bond formation in the cell. Therefore, these model proteins are appropriate subjects for studying the high expression of pure holo-hemoprotein in relation to the regulation of heme synthesis.

Through the tight regulation of apoprotein expression from the T5 promoter with the *lac*-operator system, we obtained about 15 mg of cytochrome b5 and 40 mg of bacterioferritin as pure holoprotein forms from a 1-l culture. These amounts are higher (at least 4.5-fold for cytochrome b5 and 20-fold for bacterioferritin) than those previously reported (Andrews et al. 1993; Rivera et al. 1994). Furthermore, these proteins were expressed only as holoprotein forms. We ascribe the high expression of the holoproteins mainly to the slow synthesis of the apoproteins, which can provide the sufficient time and nutrient for enough synthesis of heme in the cell. The overexpressed apoproteins serve as heme sinks by binding to any free heme present in the cell. Theoretically, as more apoprotein is expressed, the heme content in the cell increases through a feedback regulation in heme biosynthesis (Woodard and Dailey 1995). However, the level of heme content decreased under the condition of high protein-expression (Fig. 2). This can be explained by the fact that the synthesis of heme and apoprotein can compete with each other for the amino acid pool. Therefore, appropriate adjustments of apoprotein expression are necessary for the activation of heme synthesis, which is essential for high production of holoenzyme in the cell. Here, we achieved the adjustment of the apoprotein synthesis by using *E. coli* host cells containing multiple copies of plasmid pREP4, which carries the gene encoding the *lac* repressor (Farabaugh 1978), to ensure the tight regulation of protein expression from the T5 promoter under the control of the *lac*-operator system.

Our recombinant holoproteins were expressed as His-tag fusions at the amino termini and were purified through Ni-NTA resin. The N-terminus of cytochrome b5 is distant from its active site, as shown in the three-dimensional structure of the protein (Whitford 1992). The recombinant cytochrome b5 exhibited a UV-absorption spectrum identical with that of typical cytochrome b5; and it showed a cyclic voltammogram very similar to that theoretically calculated for a one-electron process. These findings suggest that it is electrochemically and biologically active. Since the UV-absorption spectrum of the recombinant bacterioferritin was the same as the native protein (Andrews et al. 1993), we believe that the recombinant bacterioferritin is also electrochemically and biologically active. Therefore, the expression of recombinant hemoprotein by controlling the balance of protein synthesis and heme synthesis, as described in this paper, offers a rapid and simple method for obtaining large quantities of the active holo-form of the hemoprotein, which are essential for investigating the functions of hemoproteins.

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