Expression of a biologically active plant cytochrome b_5 in *Escherichia coli*

Mark A. SMITH,* Johnathan A. NAPIER,† Sten STYMNE,‡ Arthur S. TATHAM,† Peter R. SHEWRY† and A. Keith STOBART*§

*Department of Botany, University of Bristol, Bristol BS8 1UG, U.K., †Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Bristol BS18 9AF, U.K., and ‡Department of Plant Physiology, Swedish University of Agricultural Sciences, PO Box 7047, S-750 07, Uppsala, Sweden.

Cytochrome b_5 from tobacco (*Nicotiana tabacum*) was expressed in *Escherichia coli* using a T7 polymerase/promoter system as described by Studier, Rosenberg, Dunn and Dubendorff (1990) (Methods Enzymol. **185**, 60–89). Transformed cells were red in colour and accumulated cytochrome b_5 to a level of around 30% of the total cell protein. The purified cytochrome had oxidized, reduced and low-temperature absorbance spectra characteristic of plant microsomal cytochrome b_5, and exhibited a c.d. spectrum resembling that of a mammalian cytochrome b_5. The recombinant protein appeared to be correctly assembled and biologically active, being reduced by NADH in the presence of microsomal membranes prepared from the developing seeds of sunflower (*Helianthus annuus*). Inhibition of haem synthesis in the transformed *E. coli* cells expressing cytochrome b_5, by the use of gabaculin or succinylacetone, prevented the assembly of the cytochrome b_5 holoprotein but had little effect on the accumulation of cytochrome apoprotein. The recombinant protein expressed in *E. coli* therefore has the biochemical features of the higher-plant cytochrome b_5 and can be used in studies of plant microsomal oxidation/reduction reactions.

INTRODUCTION

Cytochrome b_5 is a small haemoprotein which is an integral component of the microsomal membranes of higher plants, animals and fungi (Rich and Bendal, 1975; Ozols, 1989). The protein is composed of two functional domains: a hydrophilic N-terminal domain of some 100 amino acid residues which contains a single haem group, and a hydrophobic C-terminal domain of around 30 amino acids which anchors the protein to the cytoplasmic face of the endoplasmic reticulum membrane (Ozols, 1989). It functions as an electron carrier in a number of microsomal oxidation/reduction reactions which include cholesterol biosynthesis (Reddy et al., 1977) and the reduction of cytochrome *P*-450 (Noshiro and Omura, 1978). Its role, however, is better understood in the fatty acid desaturation reactions associated with membranes of the endoplasmic reticulum. In animals, cytochrome b_5 is reduced by NADH–cytochrome b_5 reductase and used as the electron donor in the desaturation of acyl-CoA substrates (Strittmatter et al., 1974; Lee et al., 1977). In plants the cytochrome is involved in the desaturation of acyl-complex lipids and particularly the microsomal Δ^{12}-oleoyl-phosphatidylcholine desaturase (Δ^{12} desaturase) of oil seeds (Smith et al., 1990; Kearns et al., 1991). Recently it was shown that cytochrome b_5 is involved in the Δ^{12}-hydroxylation of oleate and the formation of ricinoleate in microsomal preparations from castor bean (*Ricinus communis*) (Smith et al., 1992). It appears that this cytochrome is essential for fatty acid desaturation and polyunsaturated fatty acid production in microsomal membranes of oil-seed species. The manipulation *in vivo* of cytochrome b_5 in oil seeds could thus provide a means of regulating the fatty acid quality of the storage triacylglycerols (Stobart et al., 1992).

As part of a study on microsomal fatty acid desaturation reactions in plants we have isolated a cDNA encoding cytochrome b_5 from a tobacco leaf cDNA library. The cDNA has been modified by PCR to encode a soluble form of cytochrome b_5 which consists of the hydrophilic region of the molecule, lacking the 30 C-terminal amino acid residues which compose the hydrophobic membrane-binding domain (Smith et al., 1993, 1994). Here we describe the expression of the plant cytochrome b_5 in *Escherichia coli* and its purification and biochemical characterization.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Sigma and were of AnalaR grade or better. Gabaculin was a gift from Dr. W. T. Griffiths (Department of Biochemistry, University of Bristol). Molecular-biology reagents and enzymes were purchased from Promega Ltd. (U.K.).

Construction of vectors for the expression of cytochrome b_5 in *E. coli*

A 677 bp cDNA encoding cytochrome b_5 was isolated from a tobacco leaf cDNA library as described elsewhere (Smith et al., 1993, 1994; EMBL data base accession number X71441). This cDNA was modified by PCR to generate a DNA sequence encoding a truncated form of the protein, lacking the 30 C-terminal amino acid residues of the full-length protein. To achieve the truncation two oligonucleotide primers were synthesized: primer TC1 (5′-GCGGAATTCTCTGAATG-TTTTATCCTG-3′) complementary to the region encoding the start of the membrane-spanning domain (-QDKTSE), and a modified M13 forward-sequencing primer. Both oligonucleotides contained an *Eco*RI restriction site at the 5′ end. PCR using these primers and a plasmid (pNTCYB5) containing cDNA encoding the full-length cytochrome b_5 [the tobacco cDNA cloned in the sense orientation into the *Eco*RI restriction site of the vector pGEM-3Zf(−)] resulted in a product that encoded the truncated protein. The product was digested with *Eco*RI to release a 363 bp fragment which was cloned into the plasmid pGEM-3Zf(−) in the sense orientation to produce the plasmid pNTCYB5-TC1. The plasmid was sequenced to verify that it encoded the correctly truncated form of cytochrome b_5.

Abbreviations used: ALA, 5-aminolaevulinic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

§ To whom correspondence should be addressed.

To enable the DNAs encoding the full-length and truncated forms of cytochrome b_5 to be cloned into the expression vector pET-3d (Studier et al., 1990) both were modified by PCR to introduce an *Nco*I restriction site into the 5′ end of the coding region. An oligonucleotide primer was synthesized (5′-GCGCCATGGGCGGTGAAACTAAG-3′) containing the *Nco*I restriction site and encoding the first six residues of the subsequent recombinant protein (MGGETK). This primer, together with an M13 reverse sequencing primer, were used in PCR with the appropriate template (pNTCYB5 or pNTCYB5-TC1). The resulting PCR products were directly cloned into the vector pET-3d by digesting the DNA with the restriction enzymes *Nco*I and *Bam*HI (this latter site is present in the polylinker of the vector 5′ to the priming site of the M13 reverse primer and 3′ to the insert and so was also present in the PCR product). Both constructs were sequenced before introduction into the *E. coli* expression strain BL21(DE3). The molecular masses of the resulting recombinant cytochrome b_5 proteins, including the haem prosthetic group, were calculated to be 15595 Da and 12244 Da for the full-length and truncated (soluble) forms of the protein respectively.

Growth of transformed *E. coli*

Transformed cells were routinely grown in 2YT medium containing 200 μg/ml ampicillin with shaking at 37 °C in aerobic conditions.

Spectrophotometric assay of cytochrome b_5

Spectrophotometric measurements were made using a Philips PU 8720 u.v./visible scanning spectrophotometer and were conducted at room temperature. Absorbance spectra were monitored by scanning the sample between 350 and 650 nm against an appropriate reference. For the determination of cytochrome b_5 content in intact *E. coli*, cells from liquid culture were collected by centrifugation for 5 min at 5000 *g* and resuspended in a small volume of sterile distilled water. Cytochrome content was determined from the α-band absorbance maximum (558 nm) of the reduced cytochrome b_5 using an absorbance coefficient of 26.5 $mM^{-1} \cdot cm^{-1}$ (Ozols, 1974). In bacterial lysates and during protein purification cytochrome content was assessed from the Soret absorbance maximum (413 nm) of the oxidized cytochrome using an absorbance coefficient of 117 $mM^{-1} \cdot cm^{-1}$ (Estabrook and Werringloer, 1978). Both absorbance coefficients were determined from mammalian microsomal cytochrome b_5. For low-temperature spectra the sample, contained in a plastic 1 cm-light-path cuvette, was plunged into liquid nitrogen and the absorbance scanned immediately between 500 and 600 nm.

Analysis of the subcellular localization of cytochrome b_5

To determine the localization of the expressed cytochrome, *E. coli* cells were collected by centrifugation and resuspended in 30 mM Tris/HCl, pH 8.0, containing 20% sucrose and 1 mM EDTA. After incubation at room temperature for 10 min the cells were harvested by centrifugation at 6000 *g* for 10 min at 4 °C and transferred to ice-cold 5 mM $MgSO_4$ for 10 min. The resulting osmotic-shock fluid contained proteins exported to the bacterial periplasmic space. To isolate cytosolic proteins, cells were lysed by two freeze–thaw cycles at −20 °C and insoluble components collected by centrifugation for 30 min at 100000 *g* in a Beckman XL-90 ultracentrifuge.

Gel electrophoresis and Western blotting

SDS/PAGE was carried out by the method of Laemmli (1970) with 12% acrylamide gels. For Western-blot analysis proteins were transferred to poly(vinylidene difluoride) membrane (Millipore) using an Atto Horizblot semidry transfer unit and 50 mM Tris/HCl buffer, pH 8.5, containing 0.04% (w/v) SDS and 20% methanol, at a constant current of 1.2 mA/cm². Goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma) was used as the second antibody. Cytochrome apoprotein content of transformed cells was estimated by densitometric scanning of proteins after separation by SDS/PAGE and Coomassie Blue staining using a Joyce–Loebl Chromoscan 3 scanning densitometer.

Protein purification

For the purification of milligram quantities of the soluble form of cytochrome b_5, flasks containing 500 ml of 2YT medium and 200 μg/ml ampicillin were inoculated with a single colony of *E. coli* and grown at 37 °C for 24 h. Cells were harvested by centrifugation at 5000 *g* for 10 min at 4 °C and the resulting pellet was resuspended in a small volume of 100 mM Tris/HCl, pH 8.0, containing the protease inhibitor phenylmethanesulphonyl fluoride (1.0 mM). Cells were lysed by two cycles of freeze–thawing (−20 °C) and insoluble components were collected by centrifugation for 30 min at 100000 *g*. The supernatant was applied to a DEAE-cellulose anion-exchange column (typically 25 ml bed volume), equilibrated with 100 mM Tris/HCl, pH 8.0. The column was then washed with 200 ml of 20 mM potassium phosphate buffer, pH 8.0, to remove unbound proteins. Cytochrome b_5 was eluted with 150 mM potassium phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/min. Fractions were collected at 5 min intervals (2.5 ml) and monitored on the basis of their red colour and absorbance at 280 nm. Purity was further assessed by SDS/PAGE. Fractions containing pure cytochrome b_5 were pooled and either loaded on to a Sephadex G-25M column (Pharmacia PD-10) and eluted with distilled water or dialysed against a large volume of distilled water. The cytochrome was concentrated by freeze-drying and stored at −70 °C. All column chromatography was carried out at 4 °C.

Protein determinations

For total cellular protein determinations, *E. coli* cells were lysed by freeze–thawing and proteins precipitated with acetone. For all analyses protein content was determined using the Bradford protein assay (Bradford, 1976) using BSA as a standard.

C.d. spectroscopy

The c.d. spectrum of the purified cytochrome b_5 (soluble form) was recorded on a Jobin-Yvon CD6 spectrometer. The protein was dissolved in 0.01 M Tris/HCl, pH 8.0, to a concentration of 0.5 mg/ml (determined by weight) and spectra were recorded at 20 °C. Deconvolution of the spectra was carried out using the Contin method (Provencher and Glöckner, 1981).

Preparation of microsomal membranes

Microsomal membranes were prepared from the developing cotyledons of safflower (*Carthamus tinctorius*) as described previously (Stobart and Stymne, 1990).

RESULTS

Characterization of recombinant cytochrome b_5

E. coli transformed with cDNA encoding either the full-length or the soluble form of cytochrome b_5 were grown overnight in liquid culture containing 200 μg/ml ampicillin. After 16 h the cells

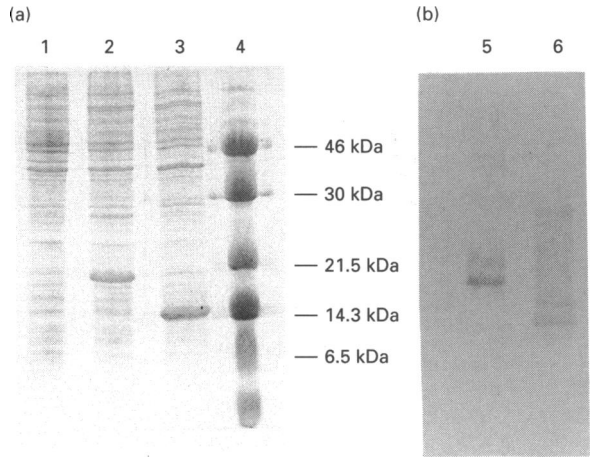

Figure 1 SDS/PAGE separation and Western-blot analysis of *E. coli* total proteins

Total protein extracts from *E. coli* cells were separated by SDS/PAGE and either (**a**) stained with Coomassie Blue or (**b**) analysed by immunoblotting using antibodies raised against cauliflower microsomal cytochrome b_5. Lane 1, control cells transformed with pET-3d; lanes 2 and 5, cells transformed with pET-3d containing cDNA encoding full-length tobacco cytochrome b_5; lanes 3 and 6, cells transformed with pET-3d containing cDNA encoding the soluble form of tobacco cytochrome b_5. Each lane contained approximately 12 μg of protein. Lane 4, molecular-mass marker proteins (Amersham Rainbow marker proteins).

appeared red in colour and exhibited an absorbance spectrum typical of reduced cytochrome b_5 with absorbance maxima at 423, 527 and 558 nm. SDS/PAGE analysis of total *E. coli* proteins from these cultures revealed prominently stained bands corresponding to molecular masses of either 14.5 or 20 kDa (Figure 1a). For both forms of cytochrome these protein bands constituted some 30 % of the total cellular protein, as determined by densitometric scanning of Coomassie Blue-stained proteins after separation by SDS/PAGE. Cells containing the pET-3d expression vector without a cytochrome b_5 coding sequence did not contain either band. Polyclonal antibodies raised against trypsin-solubilized microsomal cytochrome b_5 from cauliflower (*Brassica oleracea*) cross-reacted with both protein bands on Western-blot analysis (Figure 1b).

Spectrophotometric determination of cytochrome content indicated that cells expressing the full-length form of cytochrome b_5 contained approx. 35 μg of cytochrome b_5 per mg of total cell protein whereas those expressing the soluble form contained over 60 μg/mg of total cell protein. This method of cytochrome determination gave values for cytochrome content considerably lower than those estimated by densitometric scanning of SDS/PAGE separations of total cell protein. The spectrophotometric method, however, only detects the cytochrome b_5 holoprotein (the cytochrome apoprotein containing the haem group) whereas the densitometric method (although not strictly quantitative) will measure total cytochrome apoprotein. These results therefore indicate that only a proportion of the recombinant cytochrome b_5 produced in the cells is assembled into the holoprotein form and are consistent with observations made during the purification of the soluble recombinant protein (see below). Generally the holoprotein form of the full-length and soluble cytochrome b_5 proteins accounted for some 11 % and 20 % respectively of the total cytochrome apoprotein accumulated in the cells.

Subcellular fractionation studies indicated that the soluble form of cytochrome b_5 was accumulated in the cytosol of transformed *E. coli* and could be released simply by lysing the cells by freezing and thawing. The full-length cytochrome precipitated during the centrifugation of lysed cells and remained insoluble after treatment with 1 % Triton X-100, suggesting that the protein was located in inclusion bodies.

Effect of isopropyl β-D-thiogalactopyranoside (IPTG) on cytochrome b_5 expression

The vector pET-3d is designed to allow the expression of a cloned gene in the *E. coli* strain BL21(DE3) using an IPTG-inducible bacteriophage T7 RNA polymerase/promoter system (Studier et al., 1990). In these studies, however recombinant cytochrome b_5 accumulated in the *E. coli* cells without the need for induction by IPTG and no increase in cytochrome content was observed when IPTG was present. IPTG was therefore omitted from the culture medium in all experiments.

Purification and characterization of the soluble form of cytochrome b_5

Purification of the soluble form of cytochrome b_5 was carried out as described above with typical yields of over 60 % (Table 1). The cytochrome b_5 eluted from the anion-exchange column appeared to have been purified to homogeneity, as demonstrated by SDS/PAGE (Figure 2a). Determination of cytochrome b_5 content by spectrophotometric methods and by the Bradford protein assay indicated that the holoprotein form of the cytochrome accounted for around 30 % of the total. The purified cytochrome therefore consisted of a mixture of holoprotein and apoprotein. Reduced and oxidized difference spectra (Figure 2b) determined for the purified protein showed absorbance maxima typical of plant cytochrome b_5 (Smith et al., 1990). The protein also demonstrated splitting to the α and β absorbance bands at temperatures close to 77K (Figure 2b, inset), a feature of cytochrome b_5 (Rich and Bendal, 1975; Smith et al., 1992).

The far-u.v. c.d. spectrum of the purified cytochrome b_5 (Figure 3) shows two negative maxima around 207–208 nm and 222–224 nm; these are characteristic of α-helix. Deconvolution of the spectrum gave an α-helical content of around 35 %.

Table 1 Purification of the soluble form of cytochrome b_5 expressed in *E. coli*

Cytochrome b_5 was determined from the absorbance of the Soret band (A_{413}) of the oxidized protein.

	Total protein (mg)	Total cytochrome b_5 (μmol)	Specific content (μmol of b_5/mg of protein)	Purification	Yield (%)
Cells	267.0	1.779	0.007	1.0	100.0
100000 *g* supernatant	150.0	1.739	0.012	1.7	97.8
Eluted protein	59.3	1.116	0.019	2.7	62.7

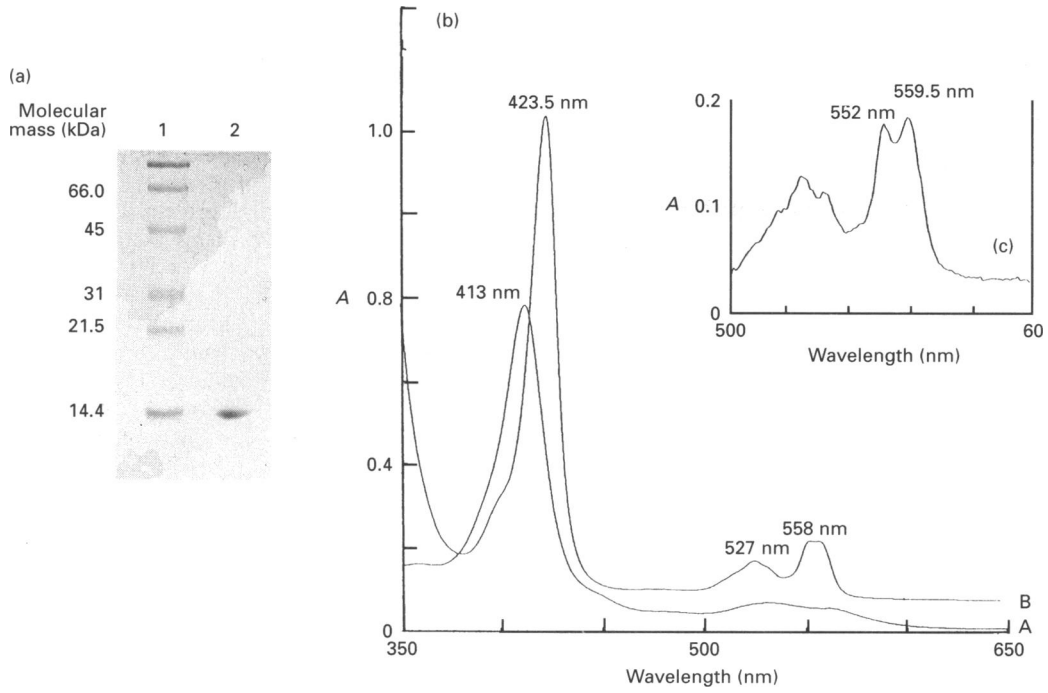

Figure 2 SDS/PAGE separation and absorbance spectra of recombinant cytochrome b_5

(**a**) The soluble form of cytochrome b_5 was purified as described above and analysed by SDS/PAGE (15% separating gel). Proteins were visualized by staining with Coomassie Blue. Lane 1, molecular-mass marker proteins; lane 2, purified cytochrome b_5 (2 μg). (**b**) Purified cytochrome b_5 (equivalent to 4 nmol of the soluble form of the protein) was suspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.2. Absorbance spectra were recorded for the air-oxidized sample (trace A) and after the addition of a few crystals of sodium dithionite (trace B). Trace C (inset) shows the low-temperature (77K) absorbance spectrum of the α- and β-bands of the dithionite-reduced cytochrome.

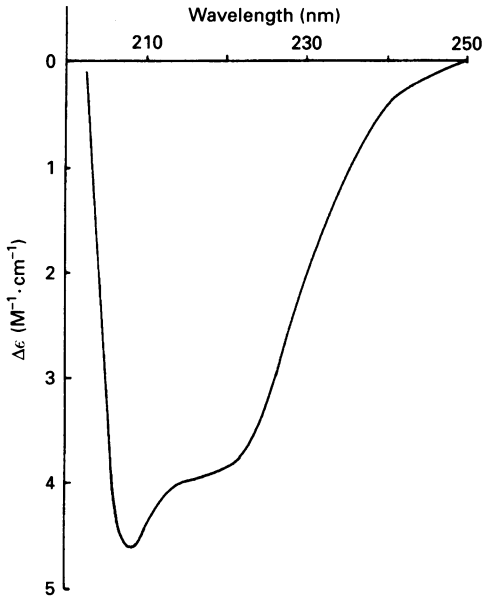

Figure 3 C.d. spectrum of cytochrome b_5

The c.d. spectrum of the purified recombinant protein (soluble form) was determined as described in the Materials and methods section. The spectrum represents the average of two independent spectra, each an average of five scans, and is corrected for baseline and solvent.

Although this spectrum represents a mixture of the apo- and holo- forms of cytochrome b_5, it is similar to that reported for calf liver microsomal cytochrome b_5 (Huntley and Strittmatter, 1972).

To assess the biological activity of the recombinant cytochrome the purified protein was incubated with a small quantity of microsomal membranes prepared from developing sunflower cotyledons. Addition of NADH resulted in the rapid reduction of the cytochrome (Figure 4). This reduction could be prevented by preincubation of the microsomal membranes with the thiol inhibitor *p*-chloromercuribenzoate, which has previously been shown to largely inhibit NADH–cytochrome b_5 reductase activity in microsomal membrane preparations (Smith et al., 1990). The cytochrome was not reduced by NADH in the absence of microsomal membranes or by microsomal membranes alone. In these assays the incubation of about 1 nmol of cytochrome b_5 holoprotein (determined by spectroscopy) with 34 μg of microsomal protein resulted in the cytochrome reaching a steady state where some 60% of the total protein was in the reduced form after the addition of NADH. This steady state could be increased to over 75% by doubling the amount of microsomal protein in the incubation mixture. The recombinant cytochrome b_5 could also be reduced by the flavoprotein NADPH–ferredoxin reductase in the presence of NADPH (results not given).

Effect of haem synthesis inhibitors

The ability of the transformed cells to synthesize the relatively large amounts of haem required for the production of the

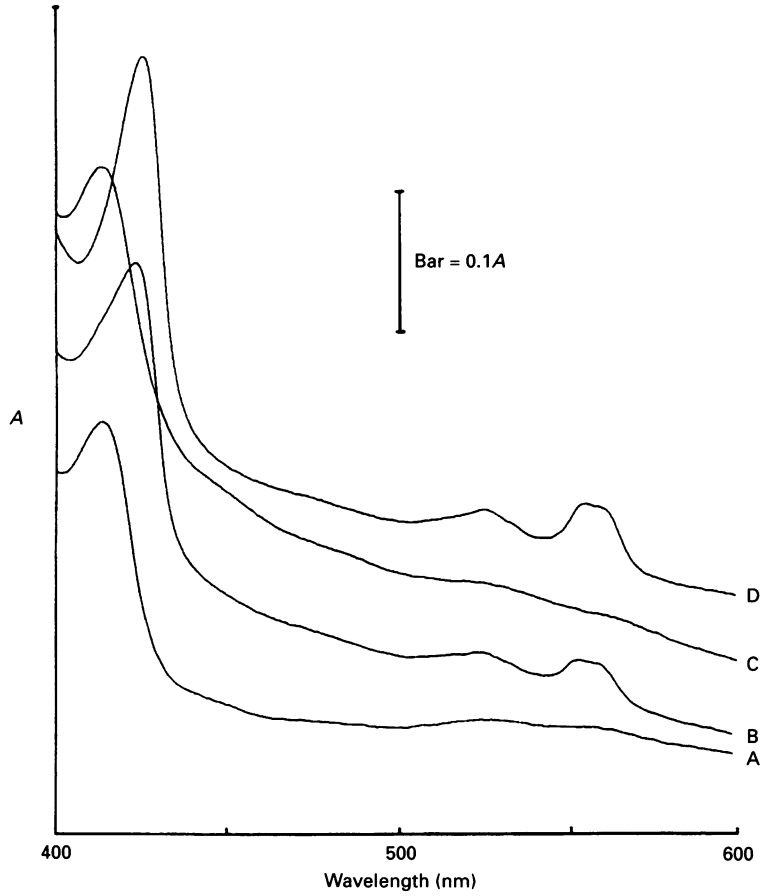

Figure 4 Reduction of recombinant cytochrome b_5 by NADH and microsomal membranes

Recombinant cytochrome b_5 (equivalent to approx. 1 nmol of the purified soluble form of the protein) was suspended in 0.1 M Tris/HCl buffer, pH 7.6, containing NADH (500 nmol) in a final volume of 0.6 ml. Absorbance spectra were then recorded (trace A) after 6 min, and for separate samples, 6 min after the addition of the following: trace B, microsomal membranes (prepared from the developing cotyledons of sunflower seeds and equivalent to 34 μg of protein); trace C, microsomal membranes (equivalent to 34 μg of protein) preincubated for 10 min with 1 mM *p*-chloromercuribenzoate; trace D, a few crystals of sodium dithionite.

Table 2 Effect of inhibitors of haem synthesis on cytochrome b_5 assembly in *E. coli*

For each set of experiments cells from a single colony of *E. coli* expressing the soluble form of cytochrome b_5 were grown for 1.5 h at 37 °C in 10 ml of 2YT medium containing 200 μg/ml ampicillin. This culture was then used to inoculate 15 ml volumes of 2YT containing 200 μg/ml ampicillin and inhibitor at the concentration given above. Cells were subsequently grown at 37 °C for 16 h before samples were removed for the spectrophotometric determination of culture density (A_{600}) and cytochrome b_5 content. The effect of each inhibitor was determined by separate experiments which were repeated three times.

Inhibitor	Inhibitor concentration (mM)	Average culture density (A_{600})	Relative cytochrome b_5 content (nmol/A_{600})	Percentage Inhibition (%)
Succinyl-acetone	0.00	1.95	1.27	0
	1.00	1.60	0.07	95
	0.10	2.00	0.59	55
	0.01	2.07	1.07	16
Gabaculin	0.00	2.24	1.19	0
	2.00	1.64	Trace	100
	1.00	1.80	0.16	87
	0.10	1.82	0.42	65

cytochrome b_5 holoprotein prompted a study of the initial steps of haem synthesis.

The first committed step in tetrapyrrole biosynthesis is the formation of 5-aminolaevulinic acid (ALA), either by the condensation of succinyl-CoA and glycine or by the C_5 pathway from glutamate (Jordan, 1990; Beale and Weinstein, 1990). In many eubacteria, as in plants, it is considered that ALA is derived mainly from glutamate via the C_5 pathway (Jahn et al., 1992). Cells expressing the soluble form of cytochrome b_5 were therefore incubated as described in Table 2 with gabaculin,

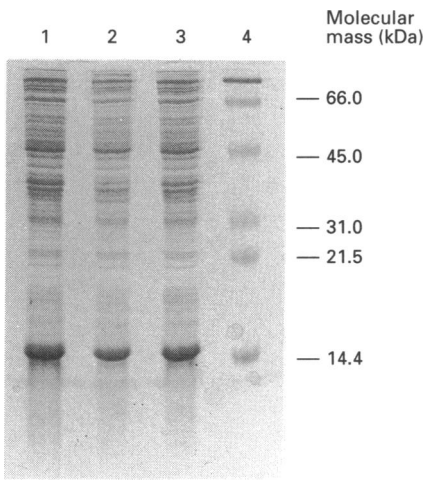

Figure 5 SDS/PAGE analysis of total proteins from *E. coli* cells grown in the presence of haem-synthesis inhibitors

Total protein extracts from *E. coli* cells grown as described in Table 2 were separated by SDS/PAGE and stained with Coomassie Blue. Lane 1, cells grown in the presence of 1 mM succinylacetone; lane 2, cells grown in the presence of 2 mM gabaculin; lane 3, control cells grown in the absence of inhibitor. Each lane contained approximately 15 μg of protein. Lane 4, molecular-mass marker proteins (Bio-Rad prestained standard proteins).

which inhibits the synthesis of ALA by the C_5 pathway (Flint, 1984), or with succinylacetone which inhibits the conversion of ALA into porphobilinogen (Shioi et al., 1985).

Both compounds inhibited the synthesis of the cytochrome b_5 holoprotein, as shown by spectrophotometric measurement (Table 2), with a 2 mM concentration of gabaculin reducing the cytochrome to barely detectable levels. However, neither of the inhibitors appeared to have any effect on the synthesis of the cytochrome apoprotein (Figure 5). For both inhibitors there was a slight decrease in the average density of the cultures at the highest inhibitor concentration used, as determined by the A_{600}.

DISCUSSION

The present study demonstrates that large quantities of plant cytochrome b_5 can be obtained by expressing a cDNA encoding this protein in *E. coli*. The recombinant protein exhibits absorbance spectra identical with those of cytochrome b_5 from plant microsomal preparations and is recognized by antibodies raised against a cauliflower cytochrome b_5 in Western blots. Reduction of the purified cytochrome b_5 by NADH and microsomal membranes indicates that the protein has biological activity and is able to interact with the NADH–cytochrome b_5 reductase in the microsomal membranes. The c.d. spectrum of the soluble form of cytochrome b_5 is very similar to that reported for lipase-extracted calf liver microsomal cytochrome b_5, which also lacks the membrane-binding region of the full-length protein (Huntley and Strittmatter, 1972). These results indicate that the recombinant protein has a structure resembling that of a correctly folded native cytochrome b_5 and further demonstrates the similarity between the plant protein and its animal counterparts. *E. coli* therefore appears to synthesize the cytochrome apoprotein and haem prosthetic group and correctly assemble the mature plant haemoprotein.

The use of the pET-3d expression system for the production of recombinant cytochrome b_5 has the advantage that the protein is produced in its native state and not as a fusion protein. For the soluble form of the cytochrome, purification is therefore possible using a relatively rapid method developed for the purification of trypsin-solubilized microsomal cytochrome b_5. Although the expression of the cytochrome b_5 cDNAs cloned into the pET-3d vector was expected to be inducible by IPTG, transformed cells expressed the proteins to high levels in the absence of IPTG. No increase in cytochrome production was observed when IPTG was present. In this study it appeared that the pET-3d expression system did not require induction by IPTG for cytochrome b_5 synthesis to occur.

Both forms of recombinant cytochrome b_5 accumulated predominantly as the apo- form of the protein lacking the haem prosthetic group. This observation is in agreement with previous studies involving expression of animal cytochrome b_5 in *E. coli* where it was suggested that the rate-limiting step in cytochrome b_5 holoprotein assembly was at an early stage (before the production of ALA) in the *de novo* synthesis of haem (Gallagher et al., 1992). The synthesis and stability of the cytochrome b_5 apoprotein in the present study appears to be unaffected by the rate of its subsequent conversion into the holoprotein. Cells in which haem synthesis had been severely inhibited still accumulated the apoprotein to the level of control cells and there was little obvious breakdown of the protein even after 4 weeks storage of bacterial cultures at 4 °C (results not shown). Although not investigated here, the clear stimulation of haem synthesis induced by the expression of cytochrome b_5 in these cells offers an opportunity to investigate the factors regulating haem synthesis in *E. coli*.

The ability of gabaculin to inhibit almost completely the synthesis of the haem required for cytochrome assembly in these cells supports the work of Li and co-workers (1989). They demonstrated that in *E. coli*, ALA is synthesized by the C_5 pathway from glutamate and not by the activity of classical ALA synthase (see Jordan, 1991). Succinylacetone also inhibited haem synthesis in *E. coli*, but this had little effect on the growth of the transformed cells. This suggests that the partitioning of haem prosthetic groups between the cytochrome b_5 apoprotein pool and the normal pathways of bacterial cytochrome synthesis was adequate for the maintenance of bacterial growth.

Both forms of cytochrome b_5 were present in the cells in their reduced states which probably reflects the highly reduced intracellular environment of *E. coli* (Pollitt and Zalkin, 1983). Cell lysis resulted in a rapid oxidation of the cytochrome, the disappearance of the reduced form offering a simple means of monitoring cell lysis. The different intracellular locations of the two forms of cytochrome b_5 are likely to be functions of the hydrophobic domain of the protein.

The recombinant protein therefore offers, in quantity, an active form of plant cytochrome b_5 suitable for use in studies into the role of the haemoprotein in plant fatty acid desaturation reactions and electron-transport processes.

We thank the Science and Engineering Research Council (U.K.) for financial support and Dr. A. R. Clarke, University of Bristol, for the use of the c.d. spectrometer.

REFERENCES

Beale, S. I. and Weinstein, J. D. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 287–391, McGraw-Hill, New York
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Estabrook, R. W. and Werringloer, J. (1978) Methods Enzymol. **52**, 212–220
Flint, D. H. (1984) Plant Physiol. **75** Suppl., 170
Gallagher, J., Kaderbhai, N. and Kaderbhai, M. A. (1992) Appl. Microbiol. Biotechnol. **38**, 77–83
Huntley, T. E. and Strittmatter, P. (1972) J. Biol. Chem. **247**, 4641–4647

Jahn, D., Verkamp, E. and Söll, D. (1992) Trends Biochem. Sci. **17**, 215–218
Jordan, P. M. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 55–122, McGraw-Hill, New York
Jordan, P. M. (1991) in New Comprehensive Biochemistry, vol. **19**, Biosynthesis of Tetrapyrroles (Jordan, P. M., ed.), pp. 1–42, Elsevier, Amsterdam
Kearns, E. V., Hugly, S. and Somerville, C. R. (1991) Arch. Biochem. Biophys. **284**, 431–436
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lee, T. C., Baker, R. C., Stephens, N. and Snyder, F. (1977) Biochim. Biophys. Acta **489**, 25–31
Li, J.-M., Brathwaite, O., Cosloy, S. D. and Russell, C. S. (1989) J. Bacteriol. **171**, 2547–2552
Noshiro, M. and Omura, T. (1978) J. Biochem. (Tokyo) **83**, 61–77
Ozols, J. (1974) Biochemistry **13**, 426–434
Ozols, J. (1989) Biochim. Biophys. Acta **997**, 121–130
Pollitt, S. and Zalkin, H. (1983) J. Bacteriol. **153**, 27–32
Provencher, S. W. and Glöckner, J. (1981) Biochemistry **20**, 33–37
Reddy, V. V. R., Kupfer, D. and Caspi, E. (1977) J. Biol. Chem. **252**, 2797–2801
Rich, P. R. and Bendal, D. S. (1975) Eur. J. Biochem. **55**, 333–341
Shioi, Y., Doi, M. and Sasa, T. (1985) Plant Cell Physiol. **26**, 379–382
Smith, M. A., Cross, A. R., Jones, O. T. G., Griffiths, W. T., Stymne, S. and Stobart, A. K. (1990) Biochem. J. **272**, 23–29
Smith, M. A., Jonsson, L., Stymne, S. and Stobart, K. (1992) Biochem. J. **287**, 141–144
Smith, M. A., Stobart, A. K., Shewry, P. R. and Napier, J. A. (1993) J. Exp. Bot. **44S**, abstr. P6.5
Smith, M. A., Stobart, A. K., Shewry, P. R. and Napier, J. A. (1994) Plant Mol. Biol. **25**, 525–537
Stobart, A. K. and Stymne, S. (1990) Methods Plant Biochem. **4**, 19–46
Stobart, A. K., Stymne, S. and Shewry, P. R. (1992) in Control of Plant Gene Expression (Verma, D. S., ed.), pp. 499–533, CRC Press, Boca Raton, FL
Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4565–4569
Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Methods Enzymol. **185**, 60–89

Received 6 October 1993/25 March 1994; accepted 14 April 1994