Identification of Retained *N*-Formylmethionine in Bacterial Recombinant Mammalian Cytochrome P450 Proteins with the N-Terminal Sequence MALLLAVFL...: Roles of Residues 3−5 in Retention and Membrane Topology†

Mi-Sook Dong,‡ L. Chastine Bell,‡ Zuyu Guo,‡,§ Dennis R. Phillips,||,⊥ Ian A. Blair,|| and F. Peter Guengerich*,‡

Departments of Biochemistry and Pharmacology and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received April 11, 1996; Revised Manuscript Received June 4, 1996⊗

ABSTRACT: An N-terminal block to Edman degradation was observed when any of five different mammalian cytochrome P450 (P450) proteins was expressed in *Escherichia coli* using the N-terminal sequence MALLLAVFL... This block was also seen in *Salmonella typhimurium*. With all proteins examined, the block could be removed by mild acid hydrolysis (0.6−6 N HCl, 23 °C) to expose Met as the N-terminus, suggesting *N*-formylMet retention. The N-terminal peptide of a modified P450 1A2 ("mutant 1", containing a thrombin-sensitive site inserted at residue 25) was released with thrombin and analyzed by electrospray mass spectrometry and found to yield the M_r expected for the *N*-formyl derivative (±0.8 amu). The region of positions 3−5 was altered by random mutagenesis, and three P450 1A2-expressing clones were analyzed for nucleotide and amino acid sequences. The changes from LLL were to RER (P450 1A2a), VDS (P450 1A2b), and WRH (P450 1A2c); these all show slightly dissimilar hydropathy plots compared to the MALLLAVFL... sequence. Mutant P450 1A2a had the N-terminal Met removed to yield N-terminal Ala; P450 1A2b contained an unmodified Met at the N-terminus; P450 1A2c had an ∼80% block of the N-terminal Met. Experiments with bacterial membranes containing expressed P450 1A2 mutant 1 and P450 1A2 mutant 2 (thrombin-sensitive site inserted at residue 46) suggest that thrombin site 2, but not 1, is sequestered in the membrane. Spheroplasts of bacteria expressing P450 1A2 and the mutants at positions 3−5 were treated with proteinase K; amino acid analysis indicated that no cleavage occurred. These results are interpreted in a model in which most of the mammalian P450 expressed in the bacterium is located in the cytosol, the region near residue 46 is in the inner membrane, the region near residue 25 is in the cytosol, and the N-terminus is either imbedded in the membrane or free in the cytosolic space, depending upon the sequence. However, the possibility that the differences in N-terminal processing are the result of direct changes in interactions with the deformylase and Met aminopeptidase cannot be excluded.

P450[1] proteins have been studied extensively because of their prominent roles as catalysts in the oxidations of drugs, carcinogens, steroids, alkaloids, vitamins, and other important chemicals (Guengerich, 1991; Porter & Coon, 1991). In the past decade the study of these enzymes has been advanced by the cloning of cDNAs and expression of the proteins in several heterologous vector systems (Sakaki *et al.*, 1986; Zuber *et al.*, 1986b; Gonzalez *et al.*, 1991). One approach that has been employed in this and other laboratories is expression in bacteria. To date, at least 31 different mammalian P450s have been expressed in *Escherichia coli*-based systems (Guengerich *et al.*, 1996a). In most of these cases the N-terminus has been altered to facilitate better expression (Barnes *et al.*, 1991).

P450 membrane topology has been studied extensively in eukaryotic systems (Sakaguchi *et al.*, 1987). Early amino acid sequencing results were interpreted in terms of multiple membrane-spanning peptides (Tarr *et al.*, 1983), but proteolysis and antibody studies with several of the eukaryotic P450s favor a model where most of the protein is on the cytosolic face of the endoplasmic reticulum and only an N-terminal anchor crosses the membrane to the lumenal side (once) (De Lemos-Chiarandini *et al.*, 1987; Brown & Black, 1989; Vergeres *et al.*, 1989; Shimozawa *et al.*, 1993). Correct insertion is facilitated by SRP and is dependent upon halt transfer and proline signals (Sakaguchi *et al.*, 1987; Yamazaki *et al.*, 1993). Most of the attention concerning the topology of eukaryotic P450s expressed in bacteria has been given to whether the expressed P450s are membrane-bound or cytosolic (Pernecky *et al.*, 1995; Gillam *et al.*, 1995a; Kempf *et al.*, 1995; Sueyoshi *et al.*, 1995), and little is known about details of membrane insertion itelf.

In general, there is little information available regarding post-translational modification of P450s, with the exception

† This research was supported in part by U.S. Public Health Service (USPHS) Grants CA44353 and ES00267 (F.P.G.). L.C.B. and D.R.P. were supported in part by USPHS Training Grant ES07028.

* Address correspondence to this author.

‡ Department of Biochemistry and Center in Molecular Toxicology.

§ Present address: Department of Drug Disposition, Rhone-Poulenc-Rorer, 500 Arcola Rd., P.O. Box 1200, Collegeville, PA 19426.

|| Department of Pharmacology and Center in Molecular Toxicology.

⊥ Present address: Mass Spectrometry Facility, Department of Chemistry, University of Georgia, Athens, GA 30602.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: P450, cytochrome P450 [also termed "heme-thiolate protein" by the Enzyme Commission (EC 1.14.14.1)]; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ES, electrospray; PCR, polymerase chain reaction; TES buffer, 50 mM Tris-acetate buffer, pH 7.6, containing 0.25 mM EDTA and 0.25 M sucrose; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SRP, signal recognition particle.

S0006-2960(96)00873-2 CCC: $12.00

Table 1: Sequences of Recombinant P450s Expressed in *E. coli*

P450	N-terminal amino acid sequence	expression level[a]	N-terminal block	ref
2C10#29	MARQSSGRGKLPPGPTPLPV	low	−	Sandhu *et al*., 1993
2E1	MARQVHSSWNLPPGPFPLP	high	−	Gillam *et al*., 1994
2D6	MARQVHSSWNLPPGPLPLPG	medium	−	Gillam *et al*., 1995a
1A1	MAFPISMSATEFLLASVIFCLV	low	−	Guo *et al*., 1994
11A1	MVSTKTPRPYSEIPSPGDNGWLNLY	medium	−	Wada *et al*., 1991
1A2	*MALLLAVFL*FCLVFWVLKGLRP	high	+	Sandhu *et al*., 1994
3A4	*MALLLAVFL*VLLYLYGTHSHGLF...	high	+	Gillam *et al*., 1993
3A5	*MALLLAVFL*VLLYLYGTRTHGLF	high	+	Gillam *et al*., 1995b
2C10#28	*MALLLAVFVL*CLSCLLLLSLWRQ	low	+	Sandhu *et al*., 1993
17A	*MALLLAVFL*LTLAYLFWPKRRC	high	+	Barnes *et al*., 1991

[a] Typical yields, nmol of P450 (L of culture)$^{-1}$: low, 10−70; medium, 70−200; high, 200−1000.

of heme binding. There are some reports of glycosylation (Shimozawa *et al.*, 1993) and phosphorylation (Eliasson *et al.*, 1994; Jansson *et al.*, 1990). However, most of the P450s expressed in bacteria to date seem to be fully functional, and there is little evidence that any post-translational modifications are needed. We have expressed eight P450s (all humans) in *E. coli* in our own laboratory (Guengerich *et al.*, 1996a,b). The first of these expressed, P450 3A4, appeared to contain a block to Edman degradation (Gillam *et al.*, 1993). This observation with *E. coli* recombinant P450 3A4 was repeated several times and interpreted to be due to the presence of a modification. Subsequently, we also found N-terminal blocks on *E. coli* recombinant human P450s 2C10 (Sandhu *et al.*, 1993), 1A2 (Sandhu *et al.*, 1994), and 3A5 (Gillam *et al.*, 1995b) but not on other P450s (Table 1). We decided to focus our effort on P450 1A2 because of the high level of expression, which facilitated not only chemical studies but work with membrane binding because of the low background of other proteins. In this report, we characterize the block as retention of *N*-formylMet. This block was complete only when the N-terminal sequence was MALLLAVFL..., and we have investigated the mechanism and provide possible explanations.

EXPERIMENTAL PROCEDURES

Construction of P450 1A2 Mutants 1 and 2 Containing Thrombin-Sensitive Sequences. The amino acid sequence GGLVPRGSGGGG, which is sensitive to cleavage by thrombin (Nicholas *et al.*, 1993; Dong *et al.*, 1996), was inserted at the sites of modified human P450 1A2 indicated with the parenthetical inserts: MALLLAVFLFCLVFWV-LKGLRPRVP (mutant 1) KGLKSPPEPWGWPLLGHVLTL (mutant 2) GKNPHLALSRMSQRYGDVL. The construction and verification of mutant 1 has been described previously (Dong *et al.*, 1996) (termed "P4501A2+12" in that report). The method for the construction of mutant 2 was the same as for mutant 1 with the exception of using the *Nco*I−*Eco*R47 restriction site instead of the *Nde*I−*Nco*I for subcloning.

Random Mutagenesis of P450 1A2 N-Terminus (Figure 1). Oligonucleotides with randomized sequences at certain positions (designated "N" below) were generated using a standard protocol with an Applied Biosystems 381A synthesizer (Applied Biosystems, Foster City, CA). To introduce the random mutation into the N-terminal "LLLAVFL" regions of P450 1A2 by PCR, three kinds of 5′-random oligomers were designed. A 39-mer oligo (5′-GGTCATATG-GCTNNNNNNNNNGCAGTTTTTCTGTTCTGC) (N = G, A, T, or C) was used to introduce random mutations into

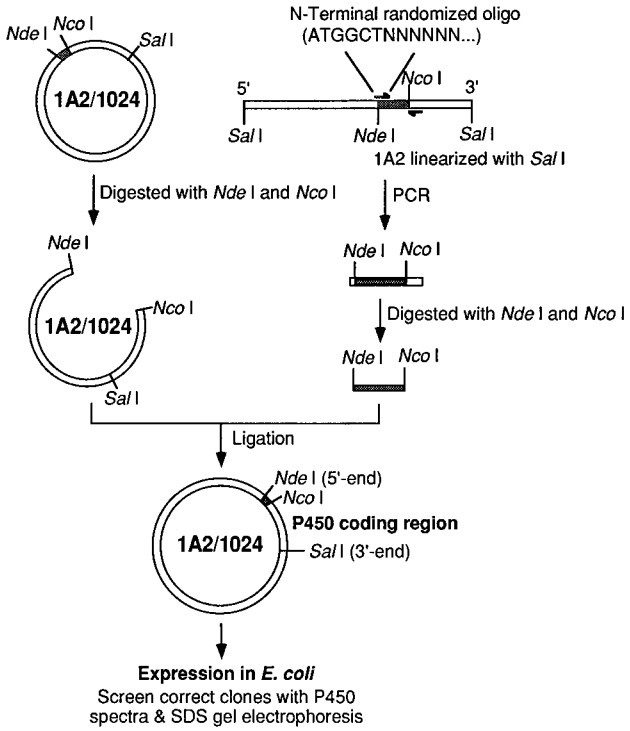

FIGURE 1: Strategy for construction of P450 1A2 variants containing random mutants in the N-terminus.

the "LLL" region (positions 3−5). Another 51-mer (5′-GGTCATATGGCTCTGTTATTANNNNNNNNNNNNTT-CTGCCTGGTATTCTGG) oligo was employed for random mutation of the "AVFL" region (positions 6−9). 5′-GGT-CATATGGCTNNNNNNNNNNNNNNNNNNNNTTCT-GCCTGGTATTCTGG-3′ was used to randomly mutate the total "LLLAVFL" region (positions 3−9). 5′-AGCCAAT-GCGGATCTGCAGGACCGT-3′ was used as the 3′-end primer to introduce a *Nco*I site. After digestion of PCR product with *Nde*I−*Nco*I, this fragment (100 bp) was ligated into the 1A2 plasmid, which had been digested with the same enzymes.

In the screening for P450 expression of N-terminal randomly-mutated constructs, each colony was expressed in 5 mL of modified TB/amp medium (Sambrook *et al.*, 1989) containing 1.0 mM IPTG and seeded with 50 μL of an overnight culture. Expression cultures were grown at 30 °C for 24 h with vigorous shaking. The cells from 1 mL of expression culture were harvested by centrifugation, resuspended in 100 μL of 1× TES buffer, and lysed by adding 100 μL of $NaDodSO_4$ solubilizing buffer (Laemmli, 1970; Guengerich, 1994). $NaDodSO_4$−polyacrylamide gel electrophoresis was used to monitor P450 bands in the cell lysis

samples, and the P450 spectra of cells from electrophoresis-positive cultures were recorded. After confirmation of P450 expression of the N-terminal mutated constructs, the 5′-nucleotide sequence of each cDNA clone was determined by the dideoxy method (Sanger *et al.*, 1977), according to the manual for SequiTherm Cycle Sequencing Kits (Epicentre Technologies, Madison, WI).

Protein Purification. *E. coli* membrane fractions were used to prepare P450 1A2 (Sandhu *et al.*, 1994), P450 3A4 (Gillam *et al.*, 1993), P450 3A5 (Gillam *et al.*, 1995b), and P450 17A (Barnes *et al.*, 1991). The construct DJ4501A2 (Josephy *et al.*, 1995) was grown in *Salmonella typhimurium* TA1538 using essentially the same growth conditions as for the P450 1A2 construct (#1024) in *E. coli* DH5α (Sandhu *et al.*, 1994). The yield was approximately 250 nmol of P450 (L of culture)$^{-1}$. P450 1A2 was prepared from *S. typhimurium* membranes using the same procedure as in the case of expression in *E. coli*.

P450 1A2 mutant 1 was purified using the same procedure described for P450 1A2 (Sandhu *et al.*, 1994).

With recombinant P450 1A2 and all of the mutants analyzed in this work, two closely migrating bands are seen using NaDodSO$_4$−polyacrylamide gel electrophoresis, with the upper band being of less intensity (Sandhu *et al.*, 1994; Dong *et al.*, 1996). Both have the same N-terminal sequence (after deblocking in mild HCl, *vide infra*) and react with antibodies raised to human or rat P450 1A2. The basis of the two bands has not yet been identified. In the work described in this article, the presence of the two bands was noted in all of the N-terminal mutants prepared and also after cleavage with thrombin (in the N-terminal region). These results suggest that the basis might be in processing of the C-terminus, but we cannot rule out the possiblity that some other post-translational modification occurs in all of these proteins.

Preparation of Spheroplasts. Cells from 10 mL of P450 expression culture were harvested by centrifugation and gently suspended in 3 mL of 2× TES buffer, to which was added 50 μL of lysozyme solution (50 mg mL^{-1}) and 3 mL of H$_2$O. The suspended cells were incubated for 30 min and recovered by centrifugation at $10^4 \times g$ for 5 min (final volume of spheroplasts was 2 mL). The spheroplasts were tested by Gram staining [for removal of the outer membrane, used according to the supplier's instructions (Fisher Scientific, Pittsburgh, PA)] and by monitoring the activity of β-galactosidase (Sambrook *et al.*, 1989) (encoded by the pCW/1A2 expression vector and used as a measure of the integrity of the inner membrane). Almost all cells were converted to spheroplasts as judged by this method, and the leakage was <1%.

Studies on Membrane Susceptibility. Membrane integration of P450 1A2 mutants was tested by a modification of the method of Scheller *et al.* (1994). Membrane fractions of P450 1A2 and mutants were resuspended in 1× TES buffer (4 mg of protein mL^{-1}) to 100 μL final volume and treated with various concentrations of thrombin for 1 h at 37 °C. The thrombin reaction was stopped by adding 1 mM PMSF and 2 μM leupetin. Then 50 μL of 0.2 M Na$_2$CO$_3$ buffer (pH 11.5) was added to 50 μL of the mixture, followed by centrifugation at $3 \times 10^5 \times g$ for 1 h. Pellet and supernatant fractions were used for NaDodSO$_4$−polyacrylamide gel electrophoresis and analyzed by immunoblotting using antibodies raised against *E. coli* recombinant P450 1A2 using the general method described earlier (Kaminsky *et al.*, 1981).

Proteolysis of Spheroplast Preparations. Various amounts (1−10 μL) of a solution of proteinase K (75 μg mL^{-1}, Boehringer-Mannheim, Indianapolis, IN) or thrombin (10^3 units mL^{-1}, Sigma, St. Louis, MO) were added to 100 μL of spheroplasts. Reactions were done at 37 °C for 60 min and stopped by adding 100 μL of 2× lysis buffer (Laemmli, 1970; Guengerich, 1994) and heating at 100 °C for 2 min prior to NaDodSO$_4$−polyacrylamide gel electrophoresis.

Edman Degradation. P450s were separated by NaDodSO$_4$−polyacrylamide gel electrophoresis and electrophoretically transferred to Problot poly(vinylidene fluoride) membranes (Applied Biosystems, Foster City, CA) using the general protocols presented elsewhere (LeGendre *et al.*, 1993). Sheets were stained with Coomassie Brilliant Blue R-250 to locate polypeptides and then destained (LeGendre *et al.*, 1993; LeGendre & Matsudaira, 1988). Appropriate regions were cut and subjected to analysis in either an updated Applied Biosystems 470A or a Beckman Porton LF3000 (Beckman, Fullerton, CA) instrument. In some cases, the polypeptides were treated with 0.6, 3, or 6 N HCl for 24 h at ambient temperature before sequence analysis. Phenylthiohydantoin derivatives resulting from each cycle were analyzed by HPLC, and yields are based upon comparisons with external standards.

Mass Spectrometry. ES mass spectrometry involved introduction of peptides or proteins into a Finnigan TSQ 7000 instrument (Finnigan, Sunnyvale, CA), either infused directly using a syringe pump or injected into an on-line HPLC system. Fragments of thrombin-cleaved P450 1A2 mutant 1 were purified on a 4.6 × 250 mm Whatman Partisil 5 ODS-3 octadecylsilane column (Whatman, Clifton, NJ) using a gradient of 4−80% CH_3CN in H_2O containing 0.10% CF_3CO_2H (v/v) over 60 min and a flow rate of 1.0 mL min^{-1}. The column was heated at 40 °C. A UV detector (214 nm, SpectroMonitor 3200, Thermo-Separation Products, Piscataway, NJ) was used to monitor the effluent, followed by a splitter to direct 50−100 μL min^{-1} to the mass spectrometer. The remainder of the effluent was recovered in a fraction collector for later direct infusion of samples. Data were acquired over the *m/z* range 300−1900 with a 4 s scan time. A potential of 4 kV was applied to the source capillary to produce the spray. Calibrations were performed with apomyoglobin, and peaks for ions having *m/z* between 452 and 1696 were used for mass calibration. Masses were averaged from spectra display windows 100−200 U wide, and data were smoothed 14 times.

MALDI/TOF mass spectrometry was done using a Kratos Kompact MALDI III instrument (Kratos Analytical Ltd., Manchester, U.K.). The matrix was α-cyano-4-hydroxycinnamic acid, and insulin and mellitin were routinely used to calibrate the instrument.

Spectroscopy and Other Procedures. P450 spectra were recorded using a modified Aminco DW2a/OLIS instrument (On-Line Instrument Systems, Bogart, GA). NaDodSO$_4$−polyacrylamide gel electrophoresis (for monitoring the purification and cleavage) was generally done using the basic procedure of Laemmli (Laemmli, 1970; Guengerich, 1994) with 7.5% acrylamide (w/v) and staining with ammoniacal silver according to Wray *et al.* (1981) or with Coomassie Brilliant Blue R-250 (Guengerich, 1977).

Protein concentrations were estimated using a bicinchoninic acid procedure according to the supplier's instructions (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard.

RESULTS

Preliminary Results and MALDI/TOF Mass Spectrometry. In the course of our investigations we have already reported that attempted Edman degradation of recombinant P450s 1A2 (Sandhu *et al.*, 1994), 2C10 [#1028 (Sandhu *et al.*, 1993)], 3A4 (Gillam *et al.*, 1993), and 3A5 (Gillam *et al.*, 1995b) did not result in the detection of amino acids at >5% the level of protein applied. These proteins were all constructed with the N-terminal sequence MALLLAVFL... (Table 1). Recombinant bovine P450 17A (provided by C. Jenkins and Prof. M. R. Waterman of this Center) also has the same N-terminal sequence and was found to be blocked. However, P450 3A4 isolated from human liver microsomes yielded the expected sequence (with removal of the Met to yield the second residue, Ala, as the N-terminus) (Bork *et al.*, 1989; Meinnel *et al.*, 1993; Flinta *et al.*, 1986). However, not all P450s expressed in *E. coli* were blocked. Recombinant human P450s 1A1 (Guo *et al.*, 1994), 2C10 [#1029 (Sandhu *et al.*, 1993)], 2D6 (Gillam *et al.*, 1995a), 2E1 (Gillam *et al.*, 1994), and 11A1 (Wada *et al.*, 1991) (also from Prof. Waterman) all gave the expected sequence (after loss of Met to expose Ala) in Edman degradation. All of these proteins except P450 11A1 have Ala at the second codon but none have the N-terminal sequence MALLLAVFL....

Preliminary MALDI/TOF mass spectrometry studies were done under a variety of conditions with both recombinant P450s 1A2 and 3A4. An M_r of 57 280 ± 300 was obtained for P450 1A2 (compared to a value of 57 236 expected from the cDNA prediction) and a value of 56 490 ± 164 for P450 3A4 (compared to 56 309 from cDNA prediction). These results are not definitive enough to draw conclusions about the identity of the N-terminal block, but they do argue that a relatively small modification is present, barring the removal of a sizable fragment and subsequent modification.

A crude mixture of tryptic peptides of P450 1A2 was analyzed by MALDI/TOF mass spectrometry. A total of 25 expected peptides were identified, covering 70% of the total sequence (results not presented). None of these were modified, and no peptide corresponding to the N-terminal or a derivative was identified in these experiments.[2]

Mild Acid Hydrolysis and Edman Degradation. Since the modification was considered to be rather small in size (preliminary MALDI/TOF mass spectrometry results, *vide supra*) and bacteria tend not to do post-translational modification of proteins, we considered the possibility that the *N*-formylMet residue might be retained. Such a retention has some precedent in *E. coli* (Hauschild-Rogat, 1968; Tsunasawa *et al.*, 1983; Milligan & Koshland, 1990).[3] When *N*-acyl amino acids are present in proteins, they can sometimes be cleaved by acyl amino acid-releasing enzyme (LeGendre *et al.*, 1993). However, attempts to use this enzyme on intact P450 17A and on chymotryptic digests did not result in the recovery of peptides that could be identified as arising from the N-terminus.

When *N*-formylMet groups are present, the formyl group may be removed with mild acid hydrolysis (LeGendre *et al.*, 1993) (milder than for acetyl and other acyl groups). We treated recombinant P450 3A4 (which has repeatedly been found to be blocked in Edman degradation) with 0.6 N HCl at ambient temperature and found that the expected sequence could be recovered (Table 2). The yields increased when the HCl concentration was raised to 6 N (still ambient temperature). Further studies showed that P450s 3A5 and 17A were also deblocked by such mild acid treatment (Table 2); the same finding was observed with P450 1A2 (Dong *et al.*, 1996).

Analysis of P450 1A2 Expressed in S. typhimurium. The question of whether N-terminal blocking was specific to *E. coli* or more generalized among bacteria was considered. Recently, we have reported the expression of P450 1A2 from the pCW vector in *S. typhimurium* TA1538, a standard genotoxicity tester strain (Josephy *et al.*, 1995). Expression occurred at levels high enough to permit facile purification. The purified protein was subjected to Edman degradation and found to be blocked. After mild acid hydrolysis, the expected sequence was obtained (Table 2).

Analysis of the N-Terminal Peptide of P450 1A2 Mutant 1 by HPLC/ES Mass Spectrometry. Because of the difficulty in recovering the N-terminal peptide of P450 1A2 (*vide supra*),[2] we considered an alternate strategy, in which only the peptide of interest was released. P450 1A2 mutant 1 was constructed with a thrombin-sensitive site situated just beyond the most hydrophobic segment, such that proteolysis should release only a single peptide of 3510.46 Da (adjusted for presence of *N*-formylMet). P450 1A2 mutant 1 was expressed, purified, and readily cleaved with thrombin. HPLC yielded a hydrophobic fraction (Figure 2A) which, when submitted to MALDI/TOF mass spectrometry, had *m/z* values of 3512 and 3520 in duplicate determinations. Combined HPLC/ES mass spectrometry (Figure 2) yielded

[2] Attempts were made to recover the N-terminal P450 1A2 peptide following cleavage of the carboxymethylated protein with trypsin or endolys C and HPLC of the peptides, with monitoring of the effluent at both 214 nm (amide bonds) and 290 nm (Trp). However, we were unsuccessful in recovering the N-terminal peptide. In addition, efforts to utilize [2-^{14}C]ICH_2CO_2H labeling of the Cys residues did not yield the N-terminal peptide.

[3] We searched a recent SwissProt database for the 7 residues LLLAVFL and found three complete matches. One is a eukaryotic NADH−ubiquinone oxidoreductase (Cummings & Domenico, 1988; Cummings *et al.*, 1990), one is a hypothetical protein based on a cDNA (Cummings & Domenico, 1988), and one is bovine P450 17A (Zuber *et al.*, 1986a), which contains the sequence at positions 3−9. It is interesting to note that this sequence is not expressed in *E. coli* unless the second codon is changed to Ala (GCT) (Barnes *et al.*, 1991). Whether the N-terminus is blocked in any of these cases is unknown. With the non-P450 proteins, the sequence is found internally. Nineteen proteins in the database matched 6 of the 7 residues, including human flavin-containing monooxygenase 2 (Lomri *et al.*, 1992), although this site is very near the C-terminus, considered to be the membrane anchor. A reindeer papilloma viral protein does have this sequence (6 of 7) near its N-terminus, but blockage has not been examined. Many (277) proteins had 5 of the 7 residues matched, including the lysis proteins for the colicin E_2, E_6, and E_8 precursors.[4] The list also included P450s 3A4 and 3A5 (Figure 1).

[4] One possibility was considered regarding bacterial post-translation modification. A search of sequence databases revealed that *E. coli* colicin lysis proteins showed some similarity to the N-terminal MALLLAV sequence of the P450s, although two Lys residues always follow the Met. These lysis proteins are processed by transfer of a glyceryl moiety from phosphatidylglycerol to a Cys, cleavage of the peptide band at this Cys, and fatty acylation of the resulting *S*-(glyceryl)-Cys amino group (Wu & Tokunaga, 1986). However, no ^{3}H-labeled glycerol or palmitate was incorporated into P450 1A2 in *E. coli*.

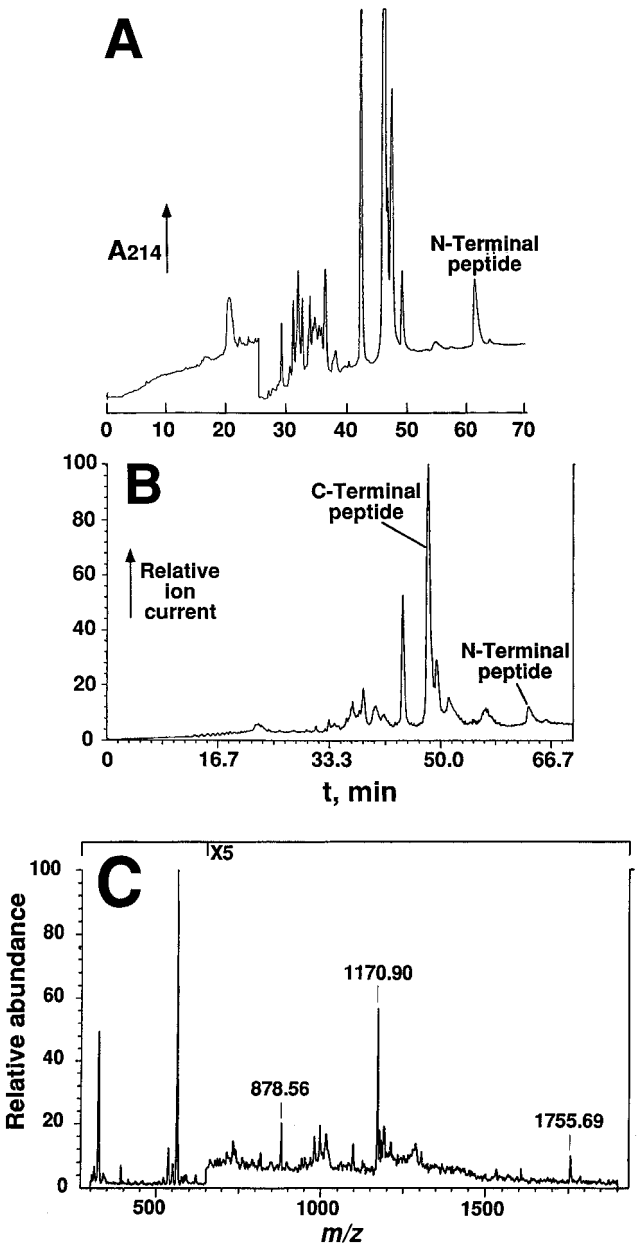

FIGURE 2: M_r analysis of the N-terminal peptide released from P450 1A2 mutant 1 by thrombin treatment. The digest was applied to an HPLC column as described and eluted with an increasing gradient of CH_3CN, with the effluent passing through a UV detector into the ES mass spectrometer. The UV trace (part A, 214 nm) and total ion current (part B) are shown, with the fragments labeled "C-terminal" and "N-terminal" from P450 1A2 mutant 1 indicated. (The UV base line was adjusted after 25 min in part A.) The ES mass spectrum of the N-terminal peptide is shown in part C, and the three indicated *m/z* values (from two injections) were used to calculate an M_r of 3509.65 (theoretical M_r 3510.46 with retention of *N*-formylMet).

a peptide with M_r 3509.65, only 0.81 amu less than expected for the N-terminal peptide bearing an *N*-formylMet moiety (±0.02%). (HPLC/ES mass spectrometry of the intact P450 1A2 mutant yielded M_r 58 325.72, 28.16 amu greater than expected for the *N*-formylMet derivative, or ±0.05%. In our experience, the error for ES mass spectrometry increases with M_r.)

Random Mutagenesis of N-Terminus of P450 1A2. Consideration of Table 1 and the results presented in Table 2 indicates that the amino acid sequence LLLAVFL in position 3−9 might be sufficient to cause nearly complete retention of the *N*-formylMet in P450 proteins synthesized in bacteria. In order to further examine the sequence effects, three sets of random mutants were generated in this region of the N-terminus (residues 3−5, 6−9, and 3−9; see Experimental Procedures), and the resulting colonies were screened for P450 holoprotein production.

Only 104 of 1112 colonies produced spectrally detectable P450 when whole cells were analyzed. However, most of the postitives were found to have the original sequence because of incomplete digestion in the construction, as judged by subsequent nucleotide sequence analysis. The three actual P450-expressing mutants produced were with amino acids changed in positions 3−5 (Table 3). Although we did not do nucleotide sequence analysis of all resulting mutants, the low percentage of those expressing P450 suggests that the sequence requirements for efficient P450 production are not very permissive, at least with this vector system. With mutant P450 1A2b, the Val at position 3 can be considered to resemble the original Leu, so the major change is the LL to DS in positions 4 and 5. Mutants P450 1A2a and 1A2c contain basic residues in this region (3−5). Mutant P450 1A2a was not blocked and also had the Met removed (Table 4). Mutant P450 1A2b did not have a formyl block but retained the Met. Mutant P450 1A2c was partially blocked (∼80%), as judged by the Met recoveries results from duplicate experiments.

The levels of production of these three mutants were nearly as high as for the unmodified sequence (Table 3). It is also of interest to note that the $\Delta G°$ values for formation of secondary structure in the RNA are unfavorable in some of these cases (Zuker, 1989; Jaeger *et al.*, 1989a; Jaeger *et al.*, 1989b; Gillam *et al.*, 1993), apparently without significant effect.

Proteolysis of Spheroplast Preparations. The possibility was considered that the difference in retention of *N*-formylMet in the proteins might be related to alteration of membrane localization, particularly since the presence of basic amino acids in the early N-terminal sequence is known to retard proteins from crossing membranes, at least in eukaryotes (Sakaguchi *et al.*, 1992). It is possible to remove the outer membranes from bacteria to expose proteins and portions of proteins in the periplasmic space. We analyzed P450 1A2 and mutants 1A2a, 1A2b, and 1A2c for susceptibility to proteolysis in freshly prepared spheroplast preparations (Figure 3). No significant change in the size of the P450 band was seen with proteinase K until high concentrations were used, which destroyed all of the proteins (presumably by penetrating into the spheroplasts). The samples treated with 2 μL of the proteinase K preparation (Figure 3) were recovered, and the P450s were analyzed for N-terminal amino acid sequence. The N-terminus of P450 1A2 was still completely blocked, and P450 1A2b had its intact sequence (results not presented).

Sensitivity of P450 1A2 Mutants 1 and 2 to Thrombin. Another approach to analysis of localization of P450s in bacterial membranes is examination of the ability of a protease to cut at specific sites in membrane preparations. P450 1A2 mutants 1 and 2 were constructed with a 12-residue thrombin-sensitive insert at positions 25 and 46, respectively. These proteins were expressed at levels similar to those of P450 1A2. We have previously demonstrated that purified P450 1A2 mutant 1 has catalytic activity similar

Table 2: N-Terminal Amino Acid Analysis of Recombinant P450 Proteins Isolated from Bacteria

	E. coli P450 3A4			*E. coli* P450 17A			*E. coli* P450 3A5			*S. typhimurium* P450 1A2		
		found (pmol)[a]			found (pmol)[c]			found (pmol)[b]			found (pmol)[a]	
codon or cycle	predicted residue	0.6 N HCl	6 N HCl	predicted residue	no treatment	6 N HCl	predicted residue	no treatment	6 N HCl	predicted residue	no treatment	6 N HCl
1	M	1.9	11.7	M	0.2	18.1	M	2.0	11.4	M	0.4	10.5
2	A	3.8	10.6	A	0.3	30.5	A	1.2	15.9	A	0.9	38.5
3	L	21.6	40.2	L	1.6	58.6	L		91.8	L	2.2	33.6
4	L	24.1	41.8	L		63.5	L		67.1	L		35.9
5	L	29.4	67.5	L		74.7	L		83.0	L		34.8
6	A	6.2	13.2	A		33.3	A		26.8	A		21.4
7	V	21.3	38.6	V		27.4	V		30.6	V		23.5
8	F	19.8	33.8	F		16.3	F		36.6	F		17.3
9	L	44.9	73.2	L		67.9	L		59.7	L		31.1
10	V	27.3	51.5	L		77.8	V		35.2	F		16.5
11	L	49.6	76.7	T		7.3	L		55.9	C		
12	L	50.6	78.1	L		69.5	L		49.1	L		31.8
13	Y	10.8	17.8	A		33.4	Y		17.4	V		18.6
14	L	54.9	83.8	Y		7.7	L		58.5	F		13.2
15	Y	11.3	21.1	L		52.8	Y		13.9	W		<0.1
16	G	25.5	30.0	F		26.5	G		21.6	V		18.2
17	T	17.3	22.7	W		6.8	T		11.2	L		25.3
18	H	0.4		P		10.4	R		5.0	K		5.1
19	S	8.9	11.7	K		45.9	T		13.2	G		
20	H	0.4		T		5.9	H		0.4	L		

[a] Nominally 100 pmol applied. [b] Nominally 80 pmol applied. [c] Nominally 150 pmol applied.

Table 3: Characterization of N-Terminal Variants Isolated from Random Mutagenesis of Residues 3−9 of Human Cytochrome P450 1A2 Expressed in *E. coli*

P450	N-terminal amino acid sequence	N-terminal block	5′-nucleotide sequence	$\Delta G°$ (kcal mol^{-1})[a]	expression level, nmol of P450 (L of culture)$^{-1}$
1A2	MALLLAVFL	+	atg gct ctg tta tta gca gtt ttt ctg ttc	−6.1	950
1A2a	ARERAVFL	−	atg gct cga gag cgg gca gtt ttt ctg ttc	−14.7	250
1A2b	MAVDSAVFL	−	atg gct gta gat tca gca gtt ttt ctg ttc	−6.7	850
1A2c	MAWRHAVFL	±	atg gct tgg cga cat gca gtt ttt ctg ttc	−13.6	540

[a] For formation of secondary structure in RNA (Zuker, 1989; Jaeger *et al.*, 1989a,b).

Table 4: N-Terminal Amino Acid Analysis of P450 1A2 N-Terminal Random Mutants Expressed in *E. coli*

	P450 1A2		P450 1A2a			P450 1A2b			P450 1A2c		
		found (pmol)		found (pmol)			found (pmol)			found (pmol)	
codon or cycle	predicted residue	3 N HCl[a]	predicted residue	no treatment[b]	3 N HCl[b]	predicted residue	no treatment[c]	3 N HCl[b]	predicted residue	no treatment[c]	3 N HCl[c]
1	M	9.9	M			M	36.0	11.6	M	4.4	27.8
2	A	23.4	A	6.3	19.2	A	28.1	18.5	A	2.1	23.8
3	L	28.4	R	2.9	4.7	V	26.3	13.5	W	4.1	25.1
4	L	29.6	E	2.9	7.0	D	27.7	17.0	R	0.6	9.3
5	L	29.5	R	2.6	3.4	S	9.4	6.4	H	0.3	4.9
6	A	18.4	A	7.3	13.6	A	29.1	12.4	A	3.2	13.7
7	V	19.6	V	10.2	17.0	V	27.1	15.3	V	4.3	17.7
8	F	17.8	F	8.3	8.1	F	24.8	17.4	F	4.2	17.3
9	L	32.7	L	8.2	4.1	L	35.7	13.6	L	7.8	26.0
10	F	17.6	F	7.5	9.5	F	23.6	9.5	F	4.6	17.8

[a] Nominally 50 pmol applied. [b] Nominally 20 pmol applied. [c] Nominally 40 pmol applied.

to that of P450 1A2, even after removal of the N-terminus (Dong *et al.*, 1996).

Purified P450 1A2 mutant 1 and P450 1A2 mutant 2 in detergent-solubilized membranes (cholate plus Triton N-101) were cleaved by thrombin. However, in *E. coli* membranes, P450 mutant 1 was cleaved with a low concentration of thrombin (1−2 units), but no cleavage of P450 1A2 mutant 2 was observed even with 10 units of thrombin (results not shown). P450 1A2 mutant 2 was found to be quite resistant to thrombin (in the membranes) (Figure 4), indicating that thrombin-sensitive site 2 is sequestered in the membrane. In order to examine the location of the thrombin-sensitive site (mutant 1), spheroplasts were prepared and treated with varying concentrations of thrombin (Figure 5). No cleavage of P450 1A2 mutant 1 was detected even at the highest concentration of thrombin used. These results indicate that thrombin-sensitive site 1 is exposed in the cytosol but site 2 is sequestered in the membrane.

DISCUSSION

Several of the mammalian P450s expressed in *E. coli* or *S. typhimurium* have N-terminal blocks. In general, de-

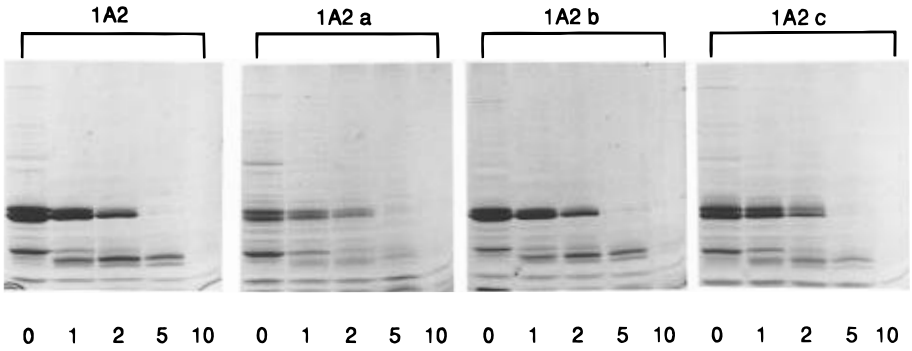

FIGURE 3: Sensitivity of P450 1A2 and mutants in spheroplasts to proteases. Equivalent amounts of spheroplasts were treated with the indicated amounts (0, 1, 2, 5, or 10 μL) of a solution of proteinase K (75 μg mL^{-1}) for 60 min at 37 °C, and aliquots were used for electrophoresis, with ammoniacal silver staining.

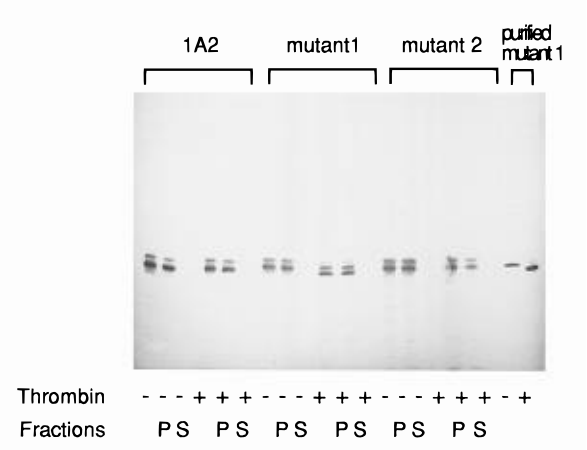

FIGURE 4: Cleavage of P450 1A2, P450 1A2 mutant 1, and P450 1A2 mutant 2 in membranes with thrombin. Experiments were done as described under Experimental Procedures. Membranes containing P450 1A2 (lanes 1−6), P450 1A2 mutant 1 (lanes 7−12), or P450 1A2 mutant 2 (lanes 13−18) and purified P450 1A2 (lanes 19 and 20) were treated with ("thrombin +") or without ("thrombin −") 2 units of thrombin per reaction mixture. The mixtures were treated with Na_2CO_3 buffer (pH 11.5) and then separated into pellet (P) and supernatant (S) fractions, which were analyzed by immunoelectrophoresis as described.

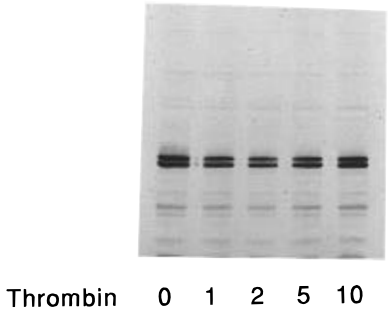

FIGURE 5: Susceptibility of P450 mutant 1 in spheroplasts to thrombin. Spheroplasts were treated with 0, 1, 2, 5, or 10 μL of thrombin (10^3 units mL^{-1}). The mixtures were analyzed by $NaDodSO_4$−polyacrylamide gel electrophoresis followed by ammoniacal silver staining.

formylation of the N-terminal Met is considered to occur during translation (Hall & Kaesberg, 1973; Housman *et al.*, 1972). Evidence that the block in these P450s is a retained *N*-formyl group was obtained by mild acid hydrolysis with all of the blocked proteins examined (Table 2). We constructed modified versions of P450 1A2 to facilitate proteolytic cleavage of the N-terminal peptides. HPLC/ES mass spectrometry of the N-terminal peptide yielded a molecular ion consistent with the presence of a retained *N*-formylMet (Figure 2).

Retention of *N*-formylMet in bacterial proteins is rare but not unprecedented. Hauschild-Roget (1968) reported that several *E. coli* ribosomal proteins contain *N*-formylMet, although this conclusion has not been addressed with newer methods. Tsunasawa *et al.* (1983) reported that 30% of Trp synthase contained *N*-formylMet in an overproducing strain. Milligan and Koshland (1990) found that *E. coli* Asp receptor contained *N*-formylMet, identified by mass spectrometry. This is an inner membrane protein, and the authors postulate that the N-terminus might be buried in the interior of the protein. This would not seem to be the situation with P450, since cleavage of the N-terminal fragment of P450 mutant 1 could be readily done with the purified protein and left a C-terminus that is catalytically active but requires a higher concentration of NADPH−P450 reductase for optimal activity (Dong *et al.*, 1996). The *E. coli* Asp receptor also has an *N*-formylMet group. The *E. coli* and *S. typhimurium* Asp receptors have the N-terminal sequences MINRIRV... and MFNRIRV.... When amino acid residue 3 of the *S. typhimurium* Asp receptor was mutated from Asn to Cys, the receptor was also blocked (to Edman degradation). These N-terminal amino sequences (of the Asp receptor) do not appear to resemble the MALLLAVFL... associated with retention of the *N*-formylMet in the P450 expressions. Milligan and Koshland (1990) also refer to a meeting abstract indicating that bacterial F_0F_1 ATPase is blocked, but further reports have not been found in our literature searches.

Two enzymes are involved in the initial N-terminal processing of proteins, a deformylase and a Met aminopeptidase. The former enzyme might be associated with ribosomes, although this hypothesis has not been proven (Meinnel *et al.*, 1993). Deformylase cleaves the *N*-formyl group from the *N*-formylMet at the N-terminus, prior to excision of Met by aminopeptidase. When Ala is the second codon, Met is usually cleaved (Meinnel *et al.*, 1993), as is the case with P450s when the formyl group is not retained. Deformylation and Met cleavage are both considered to be cotranslational processes and are limited by unshielding of the nascent peptide from the ribosome (Meinnel *et al.*, 1993). It would appear that there is something specific about the sequence MALLLAVFL... that effectively prevents both of these enzymes from operating.

The positions 3−9 of the N-terminal sequence appear to be the minimum set necessary to allow the inhibition of the deformylase and aminopeptidase. The first two residues, M

and A, are present in other P450s that are not blocked (Table 1). Also, among the P450s that are blocked, there is considerable departure of the sequences at codon 10 (Table 1). Thus ...LLLAVFL... at positions 3−9 seems to be able to impart the enzyme block. Random mutagenesis experiments with the N-terminal sequence indicated that expression of P450-holoprotein in this bacterial system is sensitive to N-terminal sequence changes but has some flexibility, not only with regard to the possible nucleotides but also their potential to hydrogen-bond to form secondary structures (Table 3). The results also indicate that the basis of the *N*-formylMet retention can be reduced to amino acid residues 3−5.

There are three major possibilities as to why these small changes in amino acid sequence signal for the retention of *N*-formylMet. One is that the Leu triplet at positions 3−5 directly inhibits the catalytic activities of both the bacterial deformylase and aminopeptidase enzymes. This is a distinct possibility but it cannot be directly addressed at this time without the availability of purified deformylase.

The second possiblity is that P450 might also be dependent upon the *secA* and *secB* gene products for its membrane localization (Wickner *et al.*, 1991). The SecA protein has been suggested to be a signal-sequence recognition protein in *E. coli* and to mediate post-translational targeting. Binding of the SecB protein to the amino terminus of the P450s during translation, due to the sequence MALLLAVFL..., could prevent deformylation until proteins are inserted into the membrane. The general significance of the Sec pathway, however, is unclear, since it does not seem to be important for the model membrane protein LacY (MacFarlane & Müller, 1995).

The final possibility for *N*-formylMet retention is related to membrane trafficking. Membrane insertion of protein in *E. coli* is also considered to be concerted with protein synthesis by SRP, as in the case of eukaryotic systems (Briggs & Gierasch, 1986). Since the discovery of sequence homology between the *E. coli* 4.5S RNA (Ffs) and 7SL RNA with the *E. coli* P48 (Fth) and 54 kDa SRP receptor, more evidence has been obtained that *E. coli* also possesses an SRP-type pathway (Poritz *et al.*, 1988; Römisch *et al.*, 1989; Bernstein *et al.*, 1989). MacFarlane and Müller (1995) suggested that this pathway could possibly be involved in cotranslational integration of hydrophobic membrane proteins that cannot be effectivly targeted post-translationally due to folding and aggregation. Some proteins may be inserted into membranes in such a way as to shield them from deformylation and Met cleavage, and we have considered this possibility. The hydrophobicity plots of *E. coli*-expressed P450s are presented in Figure 6. There is a small but distinct difference in the initial shape of the first hydrophobic segment between the MALLLAVFL... sequences and the MARQ... sequences (Table 1, Figure 6), P450 1A1, and the P450 1A2 (random) mutants 1A2a, 1A2b, and 1A2c (Figure 7). When the N-terminal amino acid sequence of a protein is very hydrophobic, e.g., MALLLAVFL..., the protein might be quickly inserted into the membrane by SRP before the deformylase or aminopeptidase can act. The rate of protein insertion into the membrane could depend upon the N-terminal hydrophobicity, and as a result the N-terminus of the protein migh be completely or partially processed. P450 1A2a has a hydrophilic amino acid region (3−5) before the start of the hydrophobic domain. The protein was deformylated and the N-terminal Met was cleaved. P450 1A2b is hydrophilic compared to P450 1A2 but not much more hydrophobic than P450 1A2a. P450 1A2b was deformylated but retained Met. When the second amino acid is Ala, the N-terminal Met is usually cleaved (Meinnel *et al.*, 1993). The possibility can be considered that the Val in position 3 is responsible for blocking the Met aminopeptidase, in the absence of a membrane effect. However, a survey of prokaryotic proteins (Flinta et al., 1986) indicates that Val in position 2 is associated with N-terminal Met cleavage, so a direct blocking effect of the Val in position 3 seems unlikely. N-Terminal amino acid analysis of the N-terminal mutants indicates that not only the second amino acid but also the third (or third to fifth) amino acids might affect the activity of the aminopeptidase. However, P450 1A2c has Trp in the third position with a larger side chain. It might inhibit the deformylase, even if the second amino acid is Ala and the hydrophobicity of positions 3−5 is hydrophilic enough for the deformylase to act. Thus, deformylation of the *N*-formylMet of P450 1A2c was partially blocked.

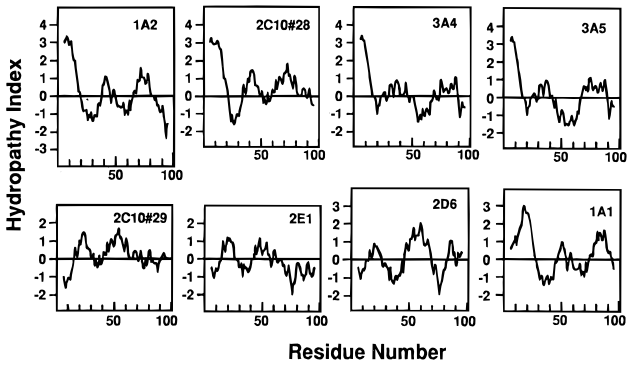

FIGURE 6: Hydropathy plots of N-terminal regions of recombinant human P450s expressed in *E. coli*. A 6-residue weighting was used (Kyte & Doolittle, 1982).

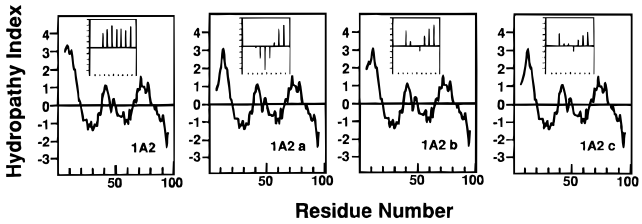

FIGURE 7: Hydropathy plots of N-terminal regions of P450 1A2 (with MALLLAVFL... N-terminus) and P450 1A2 random mutants 1A2a, 1A2b, and 1A2c. A 6-residue weighting was used (Kyte & Doolittle, 1982). The insets show plots with each line corresponding to a residue, using the method of Kyte and Doolittle (1982), with 3-residue weighting.

A possible model for the membrane topology of human recombinant P450 1A2 (and possibly, by inference, other mammalian P450s expressed in bacteria) is presented (Figure 8) on the basis of these experiments. When we digested spheroplasts of P450 1A2 and the P450 1A2 N-terminal mutants with proteinase K, they were not cleaved, indicating that no portion of P450 1A2 is in the periplasmic space. In the membrane preparation, thrombin-sensitive site 1 but not site 2 was very sensitive to thrombin cleavage; in spheroplasts, however, thrombin-sensitive site 1 was not cleaved. These results indicate that thrombin-sensitive site 1 (centered at residue 25) is in the cytosol and site 2 (centered at residue 46) is sequestered from thrombin and might be imbedded in the membrane. P450 1A2 N-terminal (random) mutants have

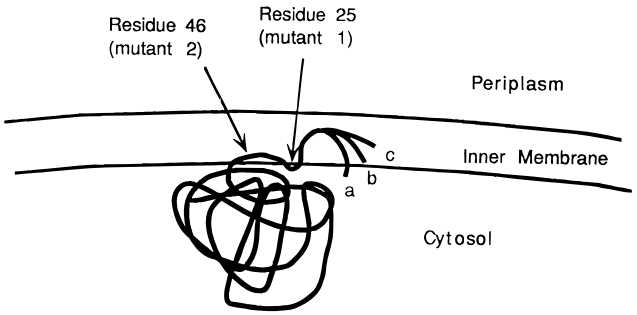

FIGURE 8: Schematic description of postulated localization of recombinant P450s in bacterial membranes.

different hydrophobicity plots (Figure 7) and processing of the N-terminus (Tables 3 and 4). The possible N-terminal locations of the N-terminal mutants are shown in Figure 8. We do not feel that the blocks and postulated changes in localization are artifacts due to high level expression, because (i) the retention of *N*-formylMet was observed in cases of high and low level expression (Table 1) and (ii) at high levels of expression (Table 3) both blocked and unblocked P450s were present, depending upon the N-terminal sequence.

In conclusion, we have characterized the retention of *N*-formylMet in a series of P450 proteins expressed in bacteria. Analyses of sequences and random mutagenesis experiments indicate that the presence of a Leu triplet at positions 3−5 is sufficient to block the action of both the deformylase and N-terminal Met aminopeptidase. Direct inhibition of action of these two enzymes is a possibility. Alternatively, differences in sequestration of the N-terminus in the inner bacterial membrane may lead to differences in access of the hydrolases to the proteins.

ACKNOWLEDGMENT

We thank Prof. M. R. Waterman for providing expressed P450s 11A1 and 17A, E. Howard for amino acid sequence analysis, Dr. T. Baba for his original observations on N-terminal blocks in P450 3A4, Dr. E. M. J. Gillam for examining the possibility of modifications resembling those for *E. coli* colicin lysis proteins, Prof. T. Omura for discussions of membrane localization and his helpful suggestions, and E. Rochelle for assistance in preparation of the manuscript.

REFERENCES

Barnes, H. J., Arlotto, M. P., & Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5597−5601.
Bernstein, H. D., Portiz, M. A., Strub, K., Hoben, P. J., Brenner, S., & Walter, P. (1989) *Nature 340*, 482−486.
Bork, R. W., Muto, T., Beaune, P. H., Srivastava, P. K., Lloyd, R. S., & Guengerich, F. P. (1989) *J. Biol. Chem. 264*, 910−919.
Briggs, M. S., & Gierasch, L. M. (1986) *Adv. Protein Chem. 38*, 109−180.
Brown, C. A., & Black, S. D. (1989) *J. Biol. Chem. 264*, 4442−4449.
Cummings, D. J., & Domenico, J. M. (1988) *J. Mol. Biol. 204*, 815−839.
Cummings, D. J., McNally, K. L., Domenico, J. M., & Matsuura, E. T. (1990) *Curr. Genet. 17*, 375−402.
De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D., & Kreibich, G. (1987) *J. Cell Biol. 104*, 209−219.
Dong, M.-S., Yamazaki, H., Guo, Z., & Guengerich, F. P. (1996) *Arch. Biochem. Biophys. 327*, 11−19.
Eliasson, E., Mkrtchian, S., Halpert, J. R., & Ingelman-Sundberg, M. (1994) *J. Biol. Chem. 269*, 18378−18383.
Flinta, C., Persson, B., Jörnvall, H., & von Heijne, G. (1986) *Eur. J. Biochem. 154*, 193−196.
Gillam, E. M. J., Baba, T., Kim, B.-R., Ohmori, S., & Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 305*, 123−131.
Gillam, E. M. J., Guo, Z., & Guengerich, F. P. (1994) *Arch. Biochem. Biophys. 312*, 59−66.
Gillam, E. M. J., Guo, Z., Martin, M. V., Jenkins, C. M., & Guengerich, F. P. (1995a) *Arch. Biochem. Biophys. 319*, 540−550.
Gillam, E. M. J., Guo, Z., Ueng, Y.-F., Yamazaki, H., Cock, I., Reilly, P. E. B., Hooper, W. D., & Guengerich, F. P. (1995b) *Arch. Biochem. Biophys. 317*, 374−384.
Gonzalez, F. J., Aoyama, T., & Gelboin, H. V. (1991) *Methods Enzymol. 206*, 85−92.
Guengerich, F. P. (1977) *J. Biol. Chem. 252*, 3970−3979.
Guengerich, F. P. (1991) *J. Biol. Chem. 266*, 10019−10022.
Guengerich, F. P. (1994) in *Principles and Methods of Toxicology* (Hayes, A. W., Ed.) pp 1259−1313, Raven Press, New York.
Guengerich, F. P., Gillam, E. M. J., & Shimada, T. (1996a) *Crit. Rev. Toxicol.* (in press).
Guengerich, F. P., Martin, M. V., Guo, Z., & Chun, Y.-J. (1996a) *Methods Enzymol.* (in press).
Guo, Z., Gillam, E. M. J., Ohmori, S., Tukey, R. H., & Guengerich, F. P. (1994) *Arch. Biochem. Biophys. 312*, 436−446.
Hall, L. A., & Kaesberg, P. (1973) *J. Mol. Biol. 79*, 531−537.
Hauschild-Rogat, P. (1968) *Mol. Gen. Genet. 102*, 95−101.
Housman, D., Gillespie, D., & Lodish, H. F. (1972) *J. Mol. Biol. 65*, 163−166.
Jaeger, J. A., Turner, D. H., & Zuker, M. (1989a) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7706−7710.
Jaeger, J. A., Turner, D. H., & Zuker, M. (1989b) *Methods Enzymol. 183*, 281−306.
Jansson, I., Curti, M., Epstein, P. M., Peterson, J. A., & Schenkman, J. B. (1990) *Arch. Biochem. Biophys. 283*, 285−292.
Josephy, P. D., DeBruin, L. S., Lord, H. L., Oak, J., Evans, D. H., Guo, Z., Dong, M.-S., & Guengerich, F. P. (1995) *Cancer Res. 55*, 799−802.
Kaminsky, L. S., Fasco, M. J., & Guengerich, F. P. (1981) *Methods Enzymol. 74*, 262−272.
Kempf, A., Zanger, U. M., & Meyer, U. A. (1995) *Arch. Biochem. Biophys. 321*, 277−288.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105−132.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
LeGendre, N., & Matsudaira, P. (1988) *BioTechniques 6*, 154−159.
LeGendre, N., Manfield, M., Weiss, A., & Matsudaira, P. (1993) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., Ed.) pp 71−101, Academic Press, San Diego.
Lomri, N., Gu, Q., & Cashman, J. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1685−1689.
MacFarlane, J., & Müller, M. (1995) *Eur. J. Biochem.* 233, 766−771.
Meinnel, T., Mechulam, Y., & Blanquet, S. (1993) *Biochimie 75*, 1061−1075.
Milligan, D. L., & Koshland, D. E., Jr. (1990) *J. Biol. Chem. 265*, 4455−4460.
Nicholas, R. A., Lamson, D. R., & Schultz, D. E. (1993) *J. Biol. Chem. 268*, 5632−5641.
Pernecky, S. J., Olken, N. M., Bestervelt, L. L., & Coon, M. J. (1995) *Arch. Biochem. Biophys. 318*, 446−456.
Poritz, M. A., Strub, K., & Walter, P. (1988) *Cell 55*, 4−6.
Porter, T. D., & Coon, M. J. (1991) *J. Biol. Chem. 266*, 13469−13472.
Römisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingron, M., & Dobberstein, B. (1989) *Nature 340*, 478−482.
Sakaguchi, M., Mihara, K., & Sato, R. (1987) *EMBO J. 6*, 2425−2431.
Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K., & Omura, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 16−19.
Sakaki, T., Oeda, K., Yabusaki, Y., & Ohkawa, H. (1986) *J. Biochem. 99*, 741−749.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sandhu, P., Baba, T., & Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 306*, 443−450.
Sandhu, P., Guo, Z., Baba, T., Martin, M. V., Tukey, R. H., & Guengerich, F. P. (1994) *Arch. Biochem. Biophys. 309*, 168−177.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Scheller, U., Kraft, R., Schröder, K.-L., & Schunck, W.-H. (1994) *J. Biol. Chem. 269*, 12779−12783.
Shimozawa, O., Sakaguchi, M., Ogawa, H., Harada, N., Mihara, K., & Omura, T. (1993) *J. Biol. Chem. 268*, 21399−21402.
Sueyoshi, T., Park, L. J., Moore, R., Juvonen, R. O., & Negishi, M. (1995) *Arch. Biochem. Biophys. 322*, 265−271.
Tarr, G. E., Black, S. D., Fujita, V. S., & Coon, M. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6552−6556.
Tsunasawa, S., Yutani, K., Ogasahara, K., Taketani, M., Yasuoka, N., Kakudo, M., & Sugino, Y. (1983) *Agric. Biol. Chem. 47*, 1393−1395.
Vergeres, G., Winterhalter, K. H., & Richter, C. (1989) *Biochemistry 28*, 3650−3655.
Wada, A., Mathew, P. A., Barnes, H. J., Sanders, D., Estabrook, R. W., & Waterman, M. R. (1991) *Arch. Biochem. Biophys. 290*, 376−380.
Wickner, W., Driessen, A. J. M., & Hartl, F. U. (1991) *Annu. Rev. Biochem. 60*, 101−124.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197−203.
Wu, H. C., & Tokunaga, M. (1986) *Curr. Top. Microbiol. Immunol. 125*, 127−157.
Yamazaki, S., Kato, S., Suhara, K., Sakaguchi, M., Mihara, K., & Omura, T. (1993) *J. Biochem. (Tokyo) 114*, 652−657.
Zuber, M. X., John, M. E., Okamura, T., Simpson, E. R., & Waterman, M. R. (1986a) *J. Biol. Chem. 261*, 2475−2482.
Zuber, M. X., Simpson, E. R., & Waterman, M. R. (1986b) *Science 234*, 1258−1261.
Zuker, M. (1989) *Science 244*, 48−52.

BI960873Z