Identification of Retained *N*-Formylmethionine in Bacterial Recombinant Mammalian Cytochrome P450 Proteins with the N-Terminal Sequence MALLLAVF...

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The cytochrome P450 (P450) 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). 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. 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.*, 1996). In most of these cases the N-terminus has been altered to facilitate better expression (Barnes *et al.*, 1991).

In general there is little information available regarding post-translational modification of P450s, with the exception of heme binding. There are some reports of glycosylation (Shimozawa *et al.*, 1993) and phosphorylation (Eliasson *et al.*, 1994). 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 in *E. coli* in our own laboratory (Table 1). The first of these, P450 3A4, appeared to contain a block to Edman degradation. 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 P450s 2C10#28, 1A2, and 3A5 which are used N-terminal sequence MALLLAVFL... (Table 1). This block was also seen when we express recombinant P450 1A2 in *Salmonella typhimurium* TA 1538.

Since 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* (Milligam & Koshland, 1990). When *N*-formylMet groups are present, the

formyl group may be removed with mild acid hydrolysis (LeGendre *et al.*, 1993). We treated recombinant P450 3A4 with 0.6N HCl at ambient temperature and found that the expected sequence could be recovered. The yields increased when the HCl concentration was raised to 6 N (still ambient temperature). Further studies showed that P450s 1A2, 3A5, and 2C10#28 were also deblocked by such mild acid treatment.

Table 1. Sequence of Human Recombinant P450s Expressed in *E. coli*

P450	N-terminal amino acid sequence	expression level [a]	N-terminal block
1A1	MAFPISMSATEFLLASVIFCLV	low	–
2C10#29	MARQSSGRGKLPPGPTPLPV	low	–
2D6	MARQVHSSWNLPPGPLPLPG	medium	–
2E1	MARQVHSSWNLPPGPFPLP	high	–
1A2	MALLLAVFLFCLVFWVLKGLRP	high	+
3A4	MALLLAVFLVLLYLYGTHSHGLF	high	+
3A5	MALLLAVFLVLLYLYHTRTHGLF	high	+
2C10#28	MALLLAVFVLCLSCLLLLSLWRQ	low	+

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

Because of the difficulty in recovering the N-terminal peptide of P450 1A2 by protease digestion such a trypsin, 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 (LVPRGS) 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 which, when submitted to MALDI/TOF mass spectrometry, had *m/z* values of 3512 and 3520 in duplicate determinations. Combined HPLC/ES mass spectrometry

yielded a peptide with M_r 3509.65, only 0.81 amu than expected for the N-terminal peptide bearing an *N*-formylMet moiety (± 0.02%). (HPLC/MS 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.) From the results of HPLC/ES mass spectrometry of the N-terminal peptide of P450 1A2 mutant 1, we can show the N-terminal block as retention of *N*-formylMet.

Two enzymes are involved in the initial N-terminal processing of proteins, a deformylase and a Met aminopeptidase. 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 (Table 1). It would appear that there is something specific about the sequence MALLLAVFL... that effectively prevents both of these enzymes from operating.

Consideration of other expressed sequences and Edman degradation results indicated that the residues LLLAVFL at positions 3-8 are responsible for *N*-formylMet retention in P450 proteins synthesized in bacteria. In order to further examine the sequence effects, random mutants were generated in this region of the N-terminus 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 positives were found to have the original sequence as judged by subsequent nucleotide sequence analysis. The three actual P-450 expressing mutants produced were with amino acids changed in positions 3-5 (Table 2). Although we did not do nucleotide sequence analysis of all (positive) resulting constructs in gels and spectra, the low percentage 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 position 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. Mutant P450 1A2b did not have a *N*-formyl block but retained the Met. Mutant 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 2). 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 (Jaeger *et al.*, 1989 } apparently without significant effect.

Table 2. 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	$\Delta G°$ [a] (Kcal mol^{-1})	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., 1989)

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 aminopeptidase.

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