Substitutions in the C-Terminal Portion of the Catalytic Domain Partially Reverse Assembly Defects Introduced by Mutations in the N-Terminal Linker Sequence of Cytochrome P450 2C2†

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Received March 18, 1999; Revised Manuscript Received May 27, 1999

ABSTRACT: Mutations in a 7-amino acid linker segment, immediately following the N-terminal signal anchor sequence of cytochrome P450 2C2, have been shown to affect proper assembly of hemoprotein and decrease activity of the mutants expressed in COS cells. In contrast, C2pmBalC1, in which cytochrome P450 2C1 residues were substituted for those of cytochrome P450 2C2 in the C-terminal region, exhibited increased activity when expressed in COS-1 cells. To examine further the basis for the increased activity of C2pmBalC1 in COS-1 cells, the protein was expressed in insect cells and *Escherichia coli*. The amounts of the functional P450 species of C2pmBalC1 expressed in these systems and the ratios of P450 to P420 were greater than those of cytochrome P450 2C2, indicating that more efficient assembly underlies the increased activity of C2pmBalC1. To determine whether the C-terminal substitutions could compensate for the decreased assembly mediated by the N-terminal linker mutations, the linker mutations were introduced into C2pmBalC1. If all 7 amino acids in the linker were deleted, no enzymatically active cytochrome P450 2C2 or C2pmBalC1 was detected in COS-1, insect, or bacterial cells expressing the mutants. The mutant C2A2, in which two alanines were substituted for the linker, had no detectable laurate hydroxylase activity in COS-1 cells, and minor amounts of hemoprotein for this mutant were expressed in *E. coli* and insect cells. In contrast, the same mutation in C2pmBalC1 reduced activity only 50% in COS-1 cells and markedly elevated levels of P450 expression in bacteria and insect cells. The A2 mutation did not affect the enzymatic activity of either cytochrome P450 2C2 or C2pmBalC1 assayed in whole cell lysates of insect cells but reduced the activity of partially purified enzymes assayed in a reconstituted assay system. These findings indicate that mutations introduced into the C-terminal region of P450 2C2 can facilitate assembly of the proteins and partially reverse the decreased assembly resulting from the N-terminal mutations.

Cytochromes P450 (P450s)[1] comprise a superfamily of hemoproteins involved in the oxidative metabolism of various biologically occurring compounds such as steroids and fatty acids, as well as most xenobiotic substrates including drugs, carcinogens, pesticides, and pollutants (*1*,*2*). In mammalian cells, these enzymes are localized to the mitochondria or microsomes. The latter isoforms constitute a large fraction of P450s and are targeted to the ER membrane by a hydrophobic N-terminal signal anchor sequence (*3*−*6*). Following the signal anchor region of P450 2C2, prior to the catalytic domain, are about 15 amino acids which can be divided arbitrarily into a relatively nonconserved segment of 7 amino acids followed by a conserved proline-rich region of 8 amino acids.

P450 2C2, when expressed in COS-1 cells, displayed relaxed sequence and minimum length requirements with regard to the 7-amino acid linker region (residues 22−28) immediately following the N-terminal signal anchor sequence (Figure 1). Substitutions of 7 alanines in this region had little effect on laurate hydroxylase activity, whereas a mutant with 7 valines in the same position failed to exhibit any enzyme activity (*7*). Progressive deletions from 7 to 2 alanine residues gradually reduced enzyme activity to undetectable levels. The decreased activity in COS-1 cells correlated with the decreased expression of P450 in insect and bacterial cells, and the mutant proteins that were expressed in insect cells had specific activities equivalent to wild-type when assayed in whole cell extracts (*7*). These results indicated that the requirement for the 7-amino acid sequence is related to the assembly of the functional protein.

P450 2C2 and P450 2C1 are laurate hydroxylases with no detectable steroid hydroxylase activity, but chimeras of these two enzymes are both laurate and steroid hydroxylases (*8*). Among several chimeras containing the N-terminal sequence from P450 2C2 and the C-terminal sequence from P450 2C1, C2pmBalC1 was found to exhibit the highest level

† Supported by a grant from the National Institutes of Health (GM35897).

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[1] Abbreviations: P450, cytochrome P450; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography.

10.1021/bi9906266 CCC: $18.00
Published on Web 08/19/1999

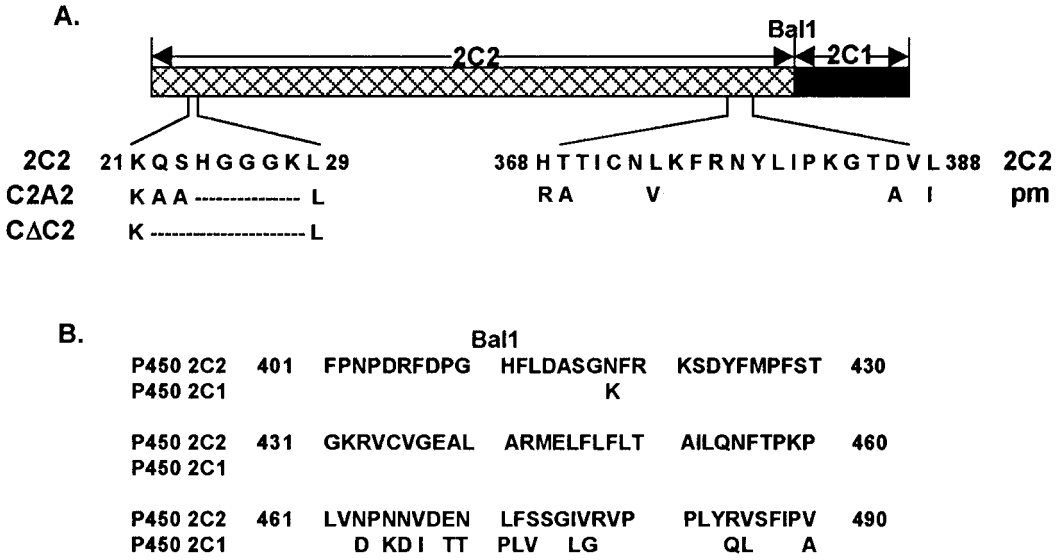

FIGURE 1: Schematic representation of 2C2pmBalC1. A. The structure of 2C2pmBalC1 is shown schematically with the P450 2C2 sequence shown as a cross-hatched bar and P450 2C1 as solid. Mutations in the N-terminal linker region between amino acids 21−29 are shown on the left, while mutations of P450 2C2 residues to P450 2C1 residues N-terminal of the *Bal*I site are shown on the right. B. Alignment of the C-terminal 90 amino acids of P450 2C2 and 2C1. The position of the *Bal*I site is indicated. For P450 2C1 only differences from P450 2C2 are shown.

of an acquired progesterone C-21 hydroxylase activity and laurate C-11 hydroxylase activity greater than that of P450 2C2 when expressed in COS-1 cells (*9*). C2pmBalC1 contains 5 substitutions of P450 2C1 residues in the region from 368 to 388 and P450 2C1 sequence from residue 410 to the C-terminus which confer steroid hydroxylase activity to the chimera (see Figure 1 and ref *18*). C2pmBalCl, thus, differs from wild-type P450 2C2 at 20 positions out of 490 amino acids in the protein, with the substituted amino acids clustered in the C-terminal portion of the protein. Since the amounts of cytochrome P450 expressed in COS-1 cells are not sufficient to detect spectrally, it was not possible to determine whether the increased activity resulted from an increase in the inherent activity of the chimera or from more efficient expression of functional protein. Sufficient P450 2C2 is expressed in *E. coli* and insect cells for spectral quantification (*7*) so that the assembly of functional protein can be determined in these model systems. In the present study, increased functional C2pmBalC1 was expressed in bacteria and insect cells which indicates that the C-terminal substitutions introduced into P450 2C2 increase the efficiency of the assembly of P450. Further, these C-terminal substitutions were able to partially reverse the decreased assembly resulting from mutations introduced into the N-terminal region so that the C-terminal substitutions behave like second-site suppressors of the assembly-defective mutations.

MATERIALS AND METHODS

Plasmid Construction. The construction of plasmids 2C2pCMV5, C2pmBalC1pCMV5, C2A2pCMV5, CΔC2pCMV5, 2C2pINIII, C2A2pINIII, CΔC2pINIII, 2C2pFB1, C2A2pFB1, and CΔC2pFB1 is described elsewhere (*7*,*9*,*10*). To construct C2pmBalC1pINIII, a DNA fragment encoding amino acids 305 to the C-terminus of the protein was made by PCR using C2pmBalC1pCMV5 as template DNA and forward and reverse primers incorporating the restriction sites *Mst*II and *Sst*I, respectively. The PCR product digested with the same enzymes was then inserted into the large fragment of *Mst*II/*Sst*I-digested 2C2pINIII to yield C2pmBalC1pINIII. The constructs C2A2pmBalC1pINIII and CΔC2pmBalC1pINIII were made by substituting the small *Mst*II/*Sst*I fragment from C2pmBalC1pINIII for the corresponding fragments in C2A2pINIII and CΔC2pINIII. C2A2pmBalC1pFB1 and CΔC2pmBalC1pFB1 were constructed by substituting the small *Mst*II/*Xba*I fragment from C2pmBalC1pINIII for the corresponding fragments in C2A2pFB1 and CΔC2pFB1.

COS-1 Cell Culture and Assay of Laurate Hydroxylase Activity. COS-1 cells were cultured and transfected with the pCMV5-based expression plasmids as described (*11*). Laurate hydroxylase activities of wild-type and mutant proteins were assayed with whole cell lysates of transfected COS-1 cells, and lauric acid metabolites were separated by reverse-phase HPLC as described (*11*).

Heterologous Expression of P450 in Insect Cells and Bacteria and Spectral Quantitation of Recombinant Protein. The wild-type and mutant P450 cDNAs cloned into the pFASTBACT1 and pINIII vectors were used for protein expression in *Trichoplusia ni* insect cells and *E. coli*, respectively, as described (*7*). P450s expressed in insect cells were spectrophotometrically quantitated by measuring the reduced CO difference spectrum of whole cell lysates following reduction with sodium hydrosulfite and binding of CO. The amount of recombinant P450 was calculated using a millimolar extinction coefficient of 91 for the absorbance difference between 450 and 490 nm of the CO complex of the reduced hemoprotein (*12*). For bacterially expressed enzymes, difference spectra were recorded by essentially the same procedure, except that preparations of proteins partially purified on a hydroxylapatite column were used for spectral measurements instead of whole cells (*13*).

Determination of Laurate Hydroxylase Activity of Recombinant Proteins. Rat NADPH-P450 reductase was expressed in *E. coli* from the vector pOR262 (*14*) and purified by 2′,5′-ADP agarose affinity chromatography (*15*). The specific activity of the P450 reductase preparation was determined

by the cytochrome *c* reductase assay as described (*16*). Laurate hydroxylase activity was assayed using whole cell lysates of recombinant baculovirus-infected insect cells containing 40 pmol of P450 as described (*11*), except that the reaction was incubated for 15 min and 8 units of P450 reductase were added so that P450 was limiting. The reaction under these conditions was linear with time for 15 min and with the amount of lysate added (data not shown).

For the bacterially expressed proteins, the activity was assayed in a reconstituted reaction as described (*13*), except that 0.2 unit of P450 reductase/pmol of P450 was used.

Isolation and Solubilization of the Membrane Fraction from Insect Cells. T. ni cells expressing wild-type and mutant proteins were harvested from 20 10-cm tissue culture dishes 72 h postinfection and resuspended in 20 mL of glycerol buffer (10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 20% (v/v) glycerol). Following lysis by sonication, the lysate was centrifuged for 1 min at 1500*g*, and the membrane fraction was isolated from the resulting supernatant by centrifugation in a Beckman Ti70 rotor at 150000*g* for 90 min. The membranous pellet was resuspended by homogenization in glycerol buffer. An aliquot of the resuspended membranes was saved, and the rest was solubilized by addition of the detergent NP-40 to a final concentration of 0.3%. The proteins were partially purified by hydroxylapatite chromatography as described for bacteria (*7*). The CO difference spectrum was determined for both the membrane and soluble fractions of wild-type and mutant proteins, and activity assays were carried out as described (*11*,*13*).

RESULTS

Expression of C2pmBalC1 in Bacteria and Insect Cells. Laurate hydroxylase activities of several chimeras of P450 2C1 and P450 2C2 with a junction at a convenient *Bal*I restriction site in the cDNAs were greater than that of P450 2C2. Of these, C2pmBalC1 (Figure 1) expressed in COS-1 cells also was the most active progesterone 21-hydroxylase (*9*). The increase in laurate hydroxylase activity for C2pmBalC1 was confirmed by the present studies (Figure 2). The increased activity could be the result of increased expression of functional protein or an increase in inherent activity of the enzyme. Insufficient amounts of P450 are expressed in COS-1 cells to permit spectral quantitation of functional hemoprotein which is needed to distinguish between these possibilities. Hence, the mutant proteins were expressed either in *E. coli* or in insect cells infected with recombinant baculovirus to obtain larger amounts of protein. Mutations which decrease the specific activity of the P450 are interpreted to affect inherent catalytic activity, and mutations which alter the amount of P450 produced and/or change the ratio of P450 to P420 are interpreted to affect the efficiency of the assembly of the protein. In *E. coli*, the amount of P450 for C2pmBalC1 was 140% of that of P450 2C2 and the ratio of P450 to P420 on average for C2pmBalC1 was 67 compared to 28 for P450 2C2 (see Figure 3 and Table 1). With the insect cell system, the yield of C2pmBalC1 was also 140% of that of P450 2C2, while the P450/P420 ratio for C2pmBalC1 was 10 compared to 2.8 for wild-type enzyme (see Figure 4 and Table 2). In addition, the specific activity for C2pmBalC1 was about 10% greater than that of P450 2C2 when assayed in microsomes in whole cell extracts from insect cells or as a solubilized form from bacteria in reconstituted systems (see Tables 1 and 2). These results indicate that the increased activity of the chimera probably results from both an increase in the inherent enzymatic activity and more efficient assembly of functional P450.

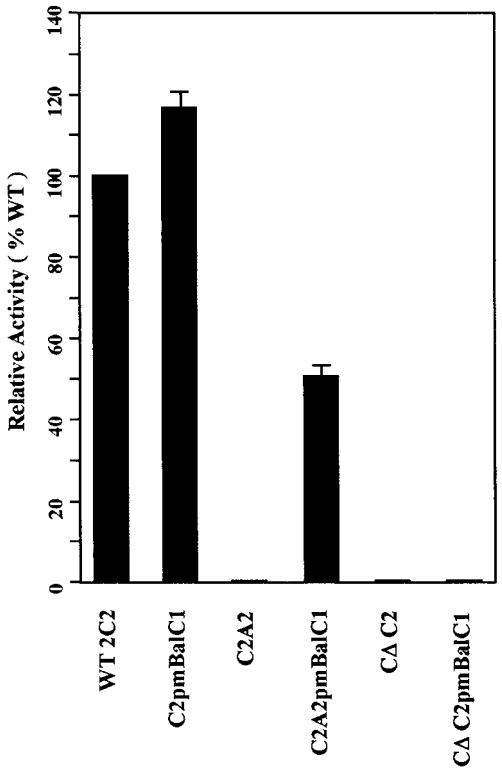

FIGURE 2: Laurate C11-hydroxylase activity of mutants in the N-terminal linker region of P450 2C2 and C2pmBalC1 expressed in COS-1 cells. Cell lysates from COS-1 cells transfected with P450 2C2 and mutant cDNAs in pCMV5 were assayed for laurate hydroxylase activity as described under Materials and Methods. Activity is expressed relative to the wild-type activity which was assigned as 100%. The means of four independent transfections and the standard errors of the mean are shown.

Laurate (ω-1) Hydroxylase Activity of N-Terminal Deletion Mutants in the Context of C2pmBalC1 Expressed in COS-1 Cells. Since the C-terminal substitutions introduced in the chimera might have increased the efficiency of P450 assembly, we considered the possibility that these mutations might compensate for the decreased assembly observed with mutations in the N-terminal region linker sequence (*7*). To examine this possibility, the most severe N-terminal mutations examined in P450 2C2, C2A2, and CΔC2 (Figure 1) were introduced into C2pmBalC1. The mutant proteins were expressed in COS-1 cells, and (ω-1) laurate hydroxylase activity was determined in whole cell extracts (Figure 2). While C2A2 had no detectable enzyme activity, the same mutant in C2pmBalC1 displayed 50% of wild-type activity (43% of C2pmBalC1). The CΔC2pmBalC1 mutant, like CΔC2 however, exhibited no laurate hydroxylase activity which suggests that one or more amino acids in this region are critical for the assembly and/or activity of the P450 in COS-1 cells irrespective of the nature of the catalytic domain.

Effects of Mutations in the N-Terminal Linker Region on the Expression of Wild-Type and Chimeric Proteins in E. coli and Insect Cells. To determine whether the dramatic difference of the A2 mutation on P450 2C2 and C2pmBalC1 activity in COS-1 cells was the result of effects on inherent

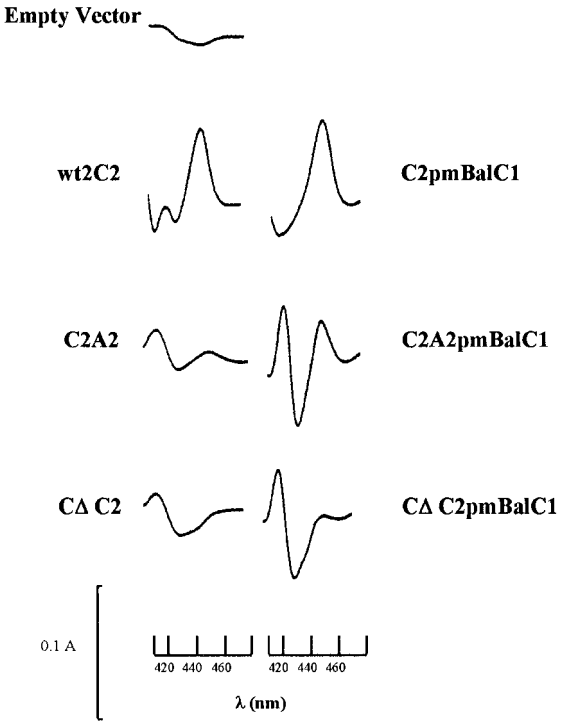

FIGURE 3: Reduced CO difference spectra of P450 2C2 and C2pmBalC1 and the N-terminal linker region mutants expressed in *E. coli*. P450 2C2 and C2pmBalC1 and the N-terminal region mutants were expressed in *E. coli* as described under Materials and Methods. Difference spectra were determined using protein partially purified from detergent-solubilized membranes by hydroxylapatite chromatography.

activity or on assembly, the proteins were expressed in *E. coli* and insect cells. Representative reduced CO difference spectra of P450 2C2 and the N-terminal region mutants, C2A2 and CΔC2, in both the wild-type and chimeric context expressed in bacteria are shown in Figure 3. A Soret absorption peak at 450 nm is characteristic of a proper conformation of the ferrous protein in the environment of the heme and its ability to bind CO, an analogue of the substrate O_2. The 420-nm Soret peak represents a stable but catalytically inactive species of P450, otherwise known as P420, which is believed to lack enzyme activity due to its inability to bind substrate and to retain the proper proximal thiolate−heme interaction in both the ferric and ferrous oxidation states (*17*). Little or no P450 and P420 was observed in insect cell lysates or partially purified preparations from *E. coli* when the cells were transfected with vectors lacking P450 cDNAs (Figures 3 and 4, empty vector).

In *E. coli*, the expression of total hemoprotein for C2A2 was 20% that of P450 2C2, whereas the total hemoprotein for this mutation in C2pmBalC1 was 76% relative to P450 2C2 (55% that of C2pmBalC1) (Table 1). More dramatically, the amount of P450 for C2A2 was only 7% of P450 2C2, while the amount of P450 was 5-fold higher for the A2 mutation in C2pmBalC1. The mutant CΔC2, in which residues 22−28 were deleted, was expressed entirely as the P420 species with no detectable P450 in bacteria. CΔC2pmBalC1, however, yielded a small amount of P450 (6% of wt) while large amounts of P420 were produced so that total hemoprotein was only slightly decreased. These results indicate that the C-terminal substitutions are able partially to correct or suppress assembly defects arising from mutations in the N-terminal region such that a larger fraction of the molecules are now able to achieve the correct functional conformation.

When expressed in recombinant baculovirus-infected insect cells, C2A2pmBalC1 also displayed an increase in the level of P450 and a more dramatic increase in the ratio of P450 to P420 compared to C2A2 (Table 2). Although no detectable hemoprotein was expressed for CΔC2, hemoprotein of CΔC2pmBalC1 was expressed to about 20% the level of P450 2C2, albeit only in the P420 form. These results, like the bacterial expression studies, suggest that the C-terminal substitutions suppress the effects of the N-terminal mutations on the assembly of hemoprotein.

Specific Activity of the N-Terminal Linker Region Mutants of 2C2 and C2pmBalC1. On the basis of spectral analysis, mutations in the N-terminal linker region of P450 2C2 affect the assembly of the protein, but it is also possible that the mutations might have effects on the inherent activity of the P450. Since the bacterial and insect cell expression systems yield measurable quantities of P450, it is possible to determine the specific enzymatic activity of the various mutants. The enzyme activity of mutants expressed in insect cells was assayed using whole cell lysates containing intact microsomal membranes. As expected, no activity was detectable for the Δ22−28 mutants since no P450 was detected for these proteins (Table 2). As shown previously (*7*), the A2 mutation did not substantially decrease the specific activity of P450 2C2, even though this mutant is essentially inactive when expressed in COS-1 cells (Table 2). Likewise, the specific activity of C2A2pmBalC1 was similar to that of C2pmBalC1 (Table 2). These results indicate that this mutation in the 22−28 region does not affect the conformation of the folded catalytic domain of P450 nor its interaction with the membrane in a way that has a substantial effect on its activity in microsomal membranes.

In contrast, the specific activity of partially purified bacterially expressed C2A2pmBalC1 in a reconstituted assay system was 22% of the specific activity of P450 2C2 (Table 1). Likewise the activity of C2A2 was 21% of that of P450 2C2. Therefore, under these conditions, the linker mutations decrease the inherent activity of the enzyme. In contrast to the compensation of the C-terminal substitutions on N-terminal linker mutation assembly defects, there is no compensation for the effects on activity in the reconstituted system. Interestingly, there was no detectable activity for the Δ22−28 mutation in C2pmBalC1 even though a small amount of P450 was detected spectrally for this mutant. This observation further supports the conclusion that changes in the N-terminal linker region can influence the activity of the catalytic domain in the reconstituted assay system.

Loss of Activity in the A2 Mutants Is a Result of Solubilization and Reconstitution. The differences in the activities of the A2 mutants expressed in bacteria or insect cells could result either from the differences in the assay procedures or from the cellular source of the protein. To distinguish between these possibilities, P450 2C2, C2pmBalC1, C2A2, and C2A2pmBalC1 were expressed in insect cells and assayed in microsomal membrane preparations or as partially purified reconstituted preparations. Compared to the whole lysates, the activities of the enzymes were reduced in the isolated microsomal membrane preparations (compare

Table 1: Expression of P450 and P420 in *E. coli* and Laurate Hydroxylase Activities of Proteins Partially Purified from Detergent-Solubilized Membranes by Hydroxylapatite Chromatography

	P450			P420				laurate hydroxylase		
	nmol[a]			nmol[a]			P450 + P420	s.a.[c]		
P450 variant	I	II	wt %[b]	I	II	wt %[b]	wt %[b]	I	II	wt %[b]
2C2 (wt)	6.24	7.92	100	0.13	0.38	100	100	0.67	0.59	100
C2pmBalC1	8.0	12.6	140	nd	0.15	60	136	0.71	0.67	109
C2A2	0.44	0.56	7	1.08	0.96	408	21	0.12	0.12	21
C2A2pmBalC1	2.60	2.40	36	2.60	3.70	1240	76	0.13	0.14	22
[Δ22−28]C2	nd	nd	0	0.84	1.30	428	15	nd	nd	0
[Δ22−28]C2pmBalC1	0.35	0.44	6	4.0	8.0	2400	87	nd	nd	0

[a] Total yield as determined by reduced CO difference spectra of P450 or P420/500 mL of cultured cells following partial purification as described under Materials and Methods. [b] Values are averages of the two independent experiments shown (I, II). [c] Specific activity (nmol of 11-OH laurate/min/nmol of P450) was determined by assaying the amount of hydroxylated [^{14}C]lauric acid formed in 15 min in a reconstituted reaction. nd, not detectable.

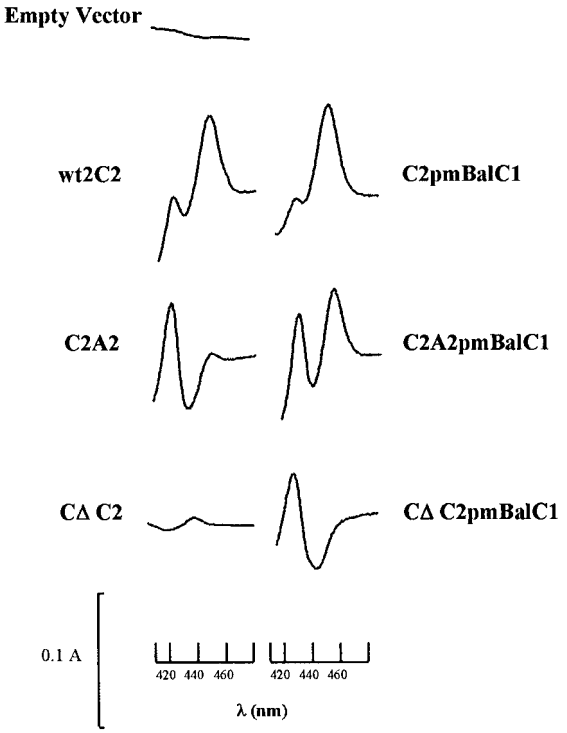

FIGURE 4: Reduced CO difference spectra of P450 2C2 and C2pmBalC1 and the N-terminal linker region mutants expressed in insect cells. P450 2C2 and C2pmBalC1 and the N-terminal linker region mutants were expressed in *T. ni* cells infected with recombinant baculovirus vectors as described under Materials and Methods. Difference spectra were determined using whole cell lysates prepared by sonication.

Tables 2 and 3). The activities of the A2 mutants compared to P450 2C2 and C2pmBalC1 were 70% and 89%, respectively, in the isolated microsome assays so that the mutants had relatively minor effects on the activities in intact membranes (Table 3) which is consistent with the 10% decrease in activity observed with the A2 mutants in the whole cell extracts (Table 2). Compared to the activity in isolated microsomal membranes, the specific activities of P450 2C2 and C2pmBalC1 were reduced only slightly by solubilization followed by assay in a reconstituted system (Table 3). In contrast, the activities of C2A2 and C2A2pmBalC1 were substantially reduced to 35% and 39%, respectively, of that of P450 2C2 in the solubilized preparations (Table 3) which is consistent with the decreases in activity observed with the solubilized bacterially expressed proteins (Table 1). These results establish that the processes of solubilization and reconstitution underlie the decreased activity of the mutants expressed in bacteria and not the cellular source of the enzyme.

DISCUSSION

Decreasing the length of a 7-amino acid N-terminal linker region of P450 2C2 has been shown to affect both the assembly of functional P450 2C2 and the inherent activity of the enzyme (*7*). The effects on activity, however, were revealed only in assays with partially purified enzyme expressed in bacteria and not in assays in which enzyme expressed in insect cells was assayed in microsomal membranes in whole cell extracts. The present studies demonstrate that mutations near the C-terminal region of P450 2C2, which slightly increase the efficiency of assembly, can substantially compensate for the assembly defects introduced by the N-terminal mutations. Both the amounts of P450 produced and the ratio of the functional P450 to nonfunctional P420 are increased. Even for the mutants with the entire linker region deleted, a substantial fraction of total hemoprotein, including some P450 in bacteria, compared to wild-type was expressed in the C-terminal mutant context, while these mutants in P450 2C2 eliminate hemoprotein expression in insect cells and P450 expression in bacteria. In contrast to the effects on assembly, the C-terminal substitutions did not compensate for the loss in inherent activity of the N-terminal linker mutations in the reconstituted assays using partially purified enzymes from either bacteria or insect cells. The mutational analysis of the N-terminal region suggested that it has the properties of a simple spacer or linker sequence connecting the N-terminal transmembrane signal anchor sequence and the cytoplasmic catalytic domain. However, the compensatory effects of C-terminal substitutions on N-terminal mutation assembly defects and the decreased inherent activity of the N-terminal mutants suggest a more intimate relationship with the catalytic domain than would be expected of a simple spacer sequence.

The mechanism by which the C-terminal substitutions compensate for N-terminal mutations is not immediately apparent, but the compensation is reminiscent of second-site suppressor mutations which function as global suppressors of mutations that affect the folding or stability of proteins. Experimental verification of the mechanism of second-site suppression has been elusive (*19*). A global suppressor of folding or stability defects which was identified in *E. coli* β-lactamase could not be rationalized in terms of

Table 2: Expression of P450 and P420 in *T. ni* Cells and Laurate Hydroxylase Activities of Whole Cell Lysates

	P450			P420				Laurate Hydroxylase		
	nmol[a]			nmol[a]			P450 + P420	s.a.[c]		
P450 variant	I	II	wt %[b]	I	II	wt %[b]	wt %[b]	I	II	wt %[b]
2C2 (wt)	1.10	1.47	100	0.15	0.77	100	100	0.95	0.91	100
C2pmBalC1	1.50	2.10	140	0.06	0.33	50	136	1.02	1.03	110
C2A2	0.12	0.42	22	0.50	1.29	289	92	0.88	0.84	92
C2A2pmBalC1	0.74	0.83	61	0.75	0.81	170	90	0.99	0.93	103
[Δ22−28]C2	nd	nd	0	nd	nd	0	0	nd	nd	0
[Δ22−28]C2pmBalC1	nd	nd	0	0.31	0.41	78	21	nd	nd	0

[a] Total yield as determined by reduced CO difference spectra of P450 or P420/10-cm tissue culture plate following preparation of whole cell lysates from *T. ni* cells infected with recombinant baculovirus as described under Materials and Methods. [b] Values are averages of the two independent experiments shown (I, II). [c] Specific activity (nmol of 11-OH laurate/min/nmol of P450) was determined by assaying the amount of hydroxylated [^{14}C]lauric acid formed in 15 min using whole cell lysates. nd, not detectable.

Table 3: Specific Activity[a] of Microsomal Membrane-Bound P450 Compared with Detergent-Solubilized Enzyme

	microsomal membrane[b]			reconstituted soluble[c]		
P450	I	II	wt %[d]	I	II	wt %[d]
wt2C2	0.43	0.52	100	0.40	0.41	100
C2A2	0.35	0.31	70	0.14	0.14	35
C2pmBalC1	0.46	0.51	102	0.42	0.44	106
C2A2pmBalC1	0.42	0.42	89	0.15	0.16	39

[a] Specific activity (nmol of 11-OH laurate/min/nmol of P450) was determined by assaying the amount of hydroxylated [^{14}C]lauric acid formed in 15 min using either homogenized membranes or a reconstituted reaction. [b] Microsomal membrane-bound P450 was prepared from whole cell lysates by ultracentrifugation as described under Materials and Methods. [c] Microsomes were solubilized with 0.3% NP-40 and the proteins partially purified on a hydroxylapatite column. [d] Values are averages of the two independent experiments shown (I, II).

the three-dimensional structure of the molecule (*20*), and understanding these effects is even more difficult in P450, a protein for which the structure is not known, which requires a heme prosthetic group and which is assembled while inserted in a membrane. The results do indicate that the effects of the N-terminal mutations are not restricted to an early step in biosynthesis of the protein since mutations in the C-terminal portion can still partially reverse the defect. This would suggest that the conformation of the nascent chain, which is presumably altered by the linker mutations, remains somewhat plastic until the final folding of the protein or the incorporation of heme, so that mutations near the C-terminal are able to reverse the linker mutation effects at a final stage of protein assembly.

The effects of the N-terminal mutations on the specific activity of the solubilized enzyme are somewhat surprising given its spacer-like characteristics and the fact that this region does not have a corresponding sequence in soluble bacterial P450s (reviewed in ref *21*) and presumably is not part of the folded catalytic domain. It is clear that the effects on activity are distinct from those on assembly because the C-terminal substitutions did not compensate for the activity defects as they did for the assembly ones. In previous studies, we were not able to distinguish whether the difference in activity of these mutations when assayed in intact membranes compared to soluble forms was due to the assay conditions or to the source of the enzyme, insect cells versus bacteria. The former is clearly the case, since solubilization of the proteins expressed in insect cells and assayed in a reconstituted system also reveals the decreased activity of the N-terminal mutants. In purified P450 2B4, in which the signal anchor and the linker region were removed, circular dichroism spectral analysis and measurement of tryptophan fluorescence indicated that the conformation of the protein was significantly altered in the truncated P450 (*22*). If similar long range effects result from only deletion of 22−28 amino acids in P450 2C2, then the decreased activity could be correlated with conformational changes. Interaction with the membrane of these truncated forms might provide stabilization of the enzyme and allow it to maintain its normal conformation. Alternative explanations cannot be ruled out at present, such as an effect of the linker mutations on the interaction of P450 with P450 reductase, via the hydrophobic membrane anchors, for example, in the solubilized assay systems, while membrane interaction may predominate in this role in the microsomal membrane assay systems.

Deletion mutations within the linker region have not been reported for other P450s, but deletions of the N-terminal signal anchor plus the linker region have been characterized. The effects of these deletions were P450-dependent. Deletion of residues 2−29 in P450 2E1 had little effect on the specific activity of P450 2E1 in reconstituted systems (*23*). In contrast, a yeast-expressed P450 1A1 with the first 30 amino acids deleted was expressed at low levels and had low activity (*24*). Deletion of residues 2−27 of P450 2B4 reduced specific activity in a substrate-dependent manner by up to 65% in a reconstituted assay (*25*). The K_m, relative to P450 reductase, of the truncated P450 2B4 was reduced 2.3-fold compared to wild-type, and impaired interactions between the reductase and truncated P450 2B4 were postulated to affect the mechanism of electron transfer between these redox partners (*22*). Interactions between these redox partners detected by optical biosensing were also consistent with this hypothesis (*26*). The present studies which demonstrate that deletion of the linker region, residues 22−28, in P450 2C2 reduces activity in reconstituted assays, but does so minimally in intact membrane assays, raise the questions of whether the decreased activity of the truncated P450 2B4 is due to deletion of the hydrophobic signal anchor, the following linker sequence, or both and whether the decrease in activity is dependent on assay in a reconstituted system. With regard to the first, deletion of part or all of the signal anchor sequence, but not the linker sequence, in P450 2C3 (*27*) and P450 2C11 (*28*) did not impair enzymatic activity suggesting the importance of the linker sequence. Since P450-specific differences, rather than the absence or presence of the linker sequence, could underlie the observed different

effects on activity, a systematic analysis in a single P450 will be required to distinguish between the relative importance of the signal anchor sequence and the linker sequence in maintaining the catalytic activity of the enzymes in reconstituted systems.

The sequence linking the membrane signal anchor sequence and the proline-rich region at the beginning of the catalytic domain is a rather unauspicious sequence. Its sequence is not conserved in P450s although all microsomal P450s have sequences of more than 3 residues in this region which have a composition characteristic of spacer sequences (*29*). Nevertheless, this sequence facilitates the assembly of the protein, is required for full activity of the solubilized activity, and, thus, is fundamentally important for the production of functional P450.

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BI9906266