

NH₂-terminal Substitutions of Basic Amino Acids Induce Translocation across the Microsomal Membrane and Glycosylation of Rabbit Cytochrome P450IIC2

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Abstract. Insertion of rabbit cytochrome P450IIC2 and its modified form, [2-lys,3-arg]P450IIC2, into microsomal membranes was studied in an in vitro transcription/translation/translocation system. Cytochrome P450IIC2, synthesized in the presence of chicken oviduct microsomal membranes, was resistant to extraction by alkaline solutions, but was sensitive to proteolytic digestion. In contrast, when [2-lys,3-arg]P450IIC2 was synthesized in the presence of membranes, two new species migrating more slowly during gel electrophoresis were observed. After treatment with endoglycosidase H, the more slowly migrating species comigrated with [2-lys,3-arg]P450IIC2 synthesized in the absence of membranes, indicating that the proteins had been glycosylated. Both the glycosylated and nonglycosylated forms of [2-lys,3-arg]P450IIC2 were resistant to proteolytic digestion and to extraction from the membranes by alkaline solutions. Similar results were obtained for a truncated species, [2-lys,3-arg]P450IIC2(1-155), except that only a single glyco-

sylated species was observed, consistent with the single remaining glycosylation site. In contrast to the proteolytic processing observed previously in a hybrid [2-lys,3-arg]P450IIC2/parathyroid hormone protein, little or no cleavage of the NH₂-terminal peptide of [2-lys,3-arg]P450IIC2 was observed in the presence of membranes. Since cleavage in the hybrid protein occurred after glycine 25, which is derived from [2-lys,3-arg]P450IIC2, cytochrome P450 sequences COOH terminal to the cleavage site must decrease cleavage efficiency. These results demonstrate that cytochrome P450, which is normally localized on the cytoplasmic side of the membrane, can be entirely translocated to the luminal side when two basic amino acids precede the hydrophobic core of its NH₂-terminal insertion/stop-transfer signal. None of the several internal hydrophobic regions of cytochrome P450, previously proposed as membrane spanning, function as a stop-transfer signal.

CYTOCHROME P450 (P450)¹ is an integral membrane protein of the endoplasmic reticulum. The insertion of P450 into the membrane has been shown to be dependent on a signal recognition particle and is thus mediated by an uncleaved signal sequence in P450 (Sakaguchi et al., 1984). The topology of the rest of the protein in the membrane has not yet been clearly defined. Several internal hydrophobic regions of P450s are conserved and potentially could be membrane spanning. This has led to proposals that P450 loops through the membrane several times (Tarr et al., 1983; Ozols et al., 1985; Leighton et al., 1984). Contrary to this view, antibodies to epitopes along the P450 molecule reacted with sequences on the cytoplasmic side of the membrane, except for those specific for the NH₂-terminus (DeLemos-Chiarandini et al., 1987). These results suggest that

P450 does not loop through the membrane several times, but is largely cytoplasmic and has one membrane-spanning region.

An unusual type of topogenic sequence has been demonstrated for P450 (Sakaguchi et al., 1987; Szczesna-Skorupa et al., 1988). The sequence at the NH₂-terminus of P450 serves as an uncleaved signal sequence initiating insertion into the membrane and as a stop-transfer signal which halts the translocation of the rest of the protein across the membrane. The P450 signal, thus, has properties characteristic of each of the two types of topogenic sequences described for integral membrane proteins (Blobel, 1980; Garoff, 1985). As in class II proteins the P450 signal functions both as an uncleavable signal sequence and as a membrane anchor. However, unlike class II proteins, the COOH-terminal domain of P450 remains on the cytoplasmic side of the membrane, which is characteristic of a class I protein.

By analysis of a hybrid protein in which 25 NH₂-terminal amino acids of rabbit P450IIC2 were substituted for most of

1. *Abbreviations used in this paper:* P450, cytochrome P450; PNTH, [Endo-pro^{18a}-met^{18b}-gly^{18c}-met^{18d}-PTH(19-52)^{18e}-met^{18f}]parathyroid hormone.

the cleavable signal sequence of the "stretched" preproparathyroid hormone (preproPNTH), we have shown that the nature of the charge at the NH₂-terminus plays an instrumental role in the topogenic characteristics of the P450 signal sequence (Szczesna-Skorupa et al., 1988). Replacement of the single negatively charged amino acid with two positively charged residues at the NH₂ terminus of the hybrid protein converted the NH₂-terminal sequence from a combination insertion/stop-transfer signal to an insertion/translocation signal typical of class II proteins. It is possible, however, that the charge-dependent conversion to a translocation signal depended in part on the presence in the hybrid protein of a normally translocatable sequence from parathyroid hormone. It has been suggested that amino acid sequences flanking the stop-transfer signal can play an important role in its function (Garoff, 1985; Boyd et al., 1987; Audigier et al., 1987; Paterson and Lamb, 1987). We report that the introduction of basic amino acids at the NH₂-terminus of P450 results in the translocation of the entire protein across the membrane.

Materials and Methods

Enzymes and Chemicals

DNA restriction and modifying enzymes were from Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), New England Biolabs (Beverly, MA), Promega Biotec (Madison, WI), and Pharmacia P-L Biochemicals (Milwaukee, WI). SP6 and T7 RNA polymerases and RNasin were from Promega Biotec, [³⁵S]methionine and L-[4,5-³H] lysine were from Amersham Corp. (Arlington Heights, IL), and endoglycosidase H was from ICN ImmunoBiologicals Inc. (Irvine, CA).

Plasmids Construction

The construction of plasmid pc2P1, coding for a [2-lys, 3-arg]P450IIIC2/PNTH fusion protein has been described (Szczesna-Skorupa et al., 1988). This plasmid was used in constructing the P450IIIC2 mutant which has aspartic acid and leucine at NH₂-terminal positions 2 and 3 replaced with lysine and arginine, respectively. From plasmid pc2, which contains full-length P450IIIC2 cDNA (Mead et al., 1986), an NcoI-BamHI fragment, containing the coding region for the amino acids 26-490, was isolated and ligated to pc2P1, from which an NcoI-BamHI fragment (coding for pro-PNTH) was deleted. The resulting plasmid (pc21) contains reconstructed full-length P450IIIC2 cDNA in an SP6 promoter plasmid, with the desired mutations in the NH₂-terminal coding region.

Transcription and In Vitro Translation

Plasmids pc2 and pc21 were linearized with Bam HI and transcribed with T7 or SP6 RNA polymerase, respectively, as described (Mead et al., 1985). Conditions of in vitro translations in reticulocyte lysate cell-free systems with or without chicken oviduct microsomal membranes, protease protection experiments, and membrane integration assays were as described (Szczesna-Skorupa et al., 1987, 1988).

Endoglycosidase H Treatment

After the translation, membranes were pelleted by centrifugation for 10 min in an airfuge (Beckman Instruments, Inc., Fullerton, CA) at 30 psi (1 psi = 6.895 × 10³ Pascal) and resuspended in 12 μl buffer containing 300 mM sodium acetate, pH 5.5, 0.2% SDS, 1% Triton X-100, 25 mM 2-mercaptoethanol. The sample was boiled 2 min and cooled. After addition of 12 μl H₂O and 1 mU (2 μl) endoglycosidase H, the sample was incubated at 37°C for 5 h. The reaction was stopped by adding SDS gel loading buffer. Samples were analyzed by SDS-PAGE on 10 or 15% acrylamide gels, and radioactive bands were visualized by fluorography using EN³HANCE (New England Nuclear, Boston, MA).

Protein Sequence Determination

The fusion protein [2-lys, 3-arg]P450IIIC2/PNTH was labeled biosynthetically with [³H]lysine, and the processed protein was eluted from the gel by overnight soaking in 0.1% SDS. The eluate was lyophilized and subjected to automated Edman degradation using a protein sequenator (Applied Biosystems, Inc., Foster City, CA).

Results

Translocation of [2-lys,3-arg]P450IIIC2 through the Microsomal Membranes

Translation of P450IIIC2 cRNA (encoded in plasmid pc2) resulted in the synthesis of a full-length protein and a number of smaller products (Fig. 1, lane 2). Initiation of translation at internal methionine codons, in addition to premature termination, probably accounted for the smaller species. The major 23-kD protein, for example, corresponded in size to a protein being initiated at the AUG codon for met(271) which is the only AUG in P450IIIC2 in a context favorable for initiation (Kozak, 1984).

Translation of P450IIIC2 cRNA in the presence of microsomal membranes did not alter the size of the translation product (Fig. 1, lane 3), which is consistent with the retention of the insertion signal in P450 (Bar-Nun et al., 1980; Sakaguchi et al., 1984). Since the substitution of two basic amino acids for an acidic amino acid converted the P450 NH₂-terminal region from a stop-transfer to a translocation signal in the P450-parathyroid hormone hybrid protein, the identical mutations were introduced into P450IIIC2 to produce [2-lys,3-arg]P450IIIC2 (encoded by plasmid pc21). Translation of [2-lys,3-arg]P450IIIC2 cRNA produced the

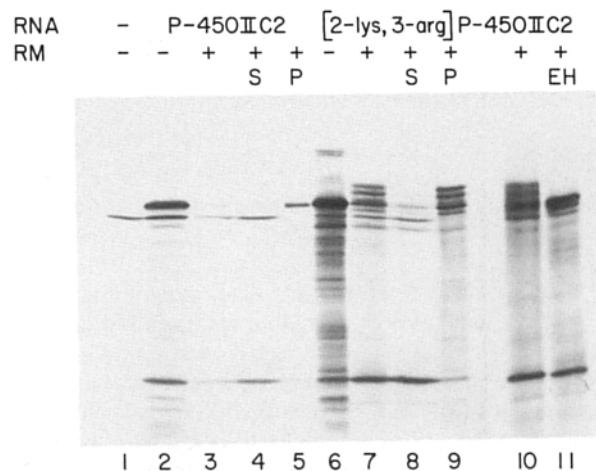


Figure 1. Glycosylation and membrane integration of P450IIIC2 and [2-lys,3-arg]P450IIIC2. RNAs coding for native P450IIIC2 and [2-lys,3-arg]P450IIIC2 were translated in a reticulocyte lysate system in the presence or absence of chicken oviduct microsomal membranes (RM). After translation was completed, membrane-containing reactions were treated with alkaline buffer and fractionated into supernatants (S) and pellets (P) as described in Materials and Methods. Samples in the supernatant and pellet lanes contain an amount of reaction mixture equivalent to twice that analyzed for the unfractionated samples. Shown are the products of [2-lys,3-arg]P450IIIC2 translated in the presence of microsomes, before (lane 10) and after (lane 11) treatment with endoglycosidase H. Samples were analyzed by SDS-PAGE on 10% acrylamide gels.

same sized products as those of P450IIC2 cRNA (Fig. 1, lane 6). When [2-lys,3-arg]P450IIC2 was translated in the presence of microsomal membranes, two new protein bands with lower mobility were observed (Fig. 1, lane 7). This surprising observation raised the possibility that [2-lys,3-arg]P450IIC2 had been glycosylated and, since glycosylation occurs in the endoplasmic reticulum lumen, that [2-lys,3-arg]P450IIC2 had been translocated across the membrane. Although P450s are not normally glycosylated *in vivo*, P450IIC2 contains three potential N-glycosylation sites at amino acid positions 56, 160, and 455 (see Fig. 5, *top*).

To examine directly whether the altered mobility of [2-lys,3-arg]P450IIC2 synthesized in the presence of membranes was due to the addition of carbohydrate residues, the protein was incubated with endoglycosidase H. After treatment with this enzyme the slower migrating bands disappeared and a major band was observed that comigrated with the protein synthesized in the absence of membranes, establishing conclusively that [2-lys,3-arg]P450IIC2 had been glycosylated (Fig. 1, lane 11). The presence of two more slowly migrating glycosylated forms might result from mono- and diglycosylation, suggesting that only two of the three potential N-glycosylation sites were used. The synthesis of truncated [2-lys,3-arg]P450IIC2(1-337), in which the last COOH-terminal glycosylation site was deleted, resulted in two more slowly migrating species (not shown), while synthesis of truncated [2-lys,3-arg]P450IIC2(1-155), which retained a single glycosylation site resulted in a single glycosylated species (see below). These results indicate that glycosylation occurred at amino acid residues 56 and 160. Since glycosylation is confined to proteins that reach the lumen of the endoplasmic reticulum (Lennarz, 1987), at least the first 160 amino acids of [2-lys,3-arg]P450IIC2 must be translocated through the microsomal membrane.

Membrane Binding of [2-lys,3-arg]P450IIC2

To determine whether [2-lys,3-arg]P450IIC2 is entirely translocated and released into the lumen of the microsomes or remains integrated in the membrane, membranes were treated with alkaline buffer (Mostov et al., 1981). P450IIC2 was recovered in the pellet after alkaline extraction of the membranes (Fig. 1, lane 5) and thus behaved as an integral membrane protein. At the same time, the 23-kD protein was extracted into the supernatant, as expected if this protein resulted from initiation at an internal methionine and did not contain the hydrophobic NH₂-terminus required for integration into the membrane. When membranes containing [2-lys,3-arg]P450IIC2 were treated with alkaline buffer, both the glycosylated and unglycosylated forms were associated with the membrane (Fig. 1, lane 9). In contrast, the 23 kD protein and a reticulocyte endogenous protein were present in the supernatant. The results suggested that the NH₂-terminal region of [2-lys,3-arg]P450IIC2 continued to function as a membrane anchor even though it now facilitated translocation and was presumably in an opposite orientation.

On the basis of the previous experiments we could not exclude the possibility that internal hydrophobic domains contributed to anchoring the protein to the membrane. To test this possibility as well as to further define the glycosylation sites, cRNA was transcribed from pc21 that had been digested with Dde I. This truncated cRNA codes for the first 155 amino acids of [2-lys,3-arg]P450IIC2. As diagrammed

in Fig. 5, in [2-lys,3-arg]P450IIC2(1-155) only the NH₂-terminal region is predicted to be a membrane-spanning helix by the Rao and Argos (1986) method and it retains only one glycosylation site. Translation of this cRNA produced a protein of the expected size and, in the presence of microsomal membranes, a single new more slowly migrating species was observed (Fig. 2, lane 2), consistent with glycosylation at a single site. This was established by treatment with endoglycosidase H which altered the gel mobility of the new species to that of unglycosylated [2-lys,3-arg]P450IIC2(1-155) (Fig. 2, lane 5). When membranes containing [2-lys,3-arg]P450IIC2(1-155) were extracted with alkaline buffer, both the glycosylated and unglycosylated forms were retained in the membrane-containing pellet (Fig. 2, lane 4). These results indicated that even in the absence of internal hydrophobic domains, P450 is anchored to the membrane.

Protection of [2-lys,3-arg]P450IIC2 from Proteolytic Digestion

P450IIC2 contains several internal hydrophobic sequences, at least three of which (amino acids 165-182, 206-226, and 445-458) are potential membrane-spanning helices by several criteria (see Fig. 5). Since the second glycosylation site is at position 159, at least the first 160 amino acids must be translocated across the membrane to the location of the glycosylation enzymes. The apparent lack of glycosylation at position 454 could result if one or more of the hydrophobic regions acted as a stop-transfer sequence. In this case, the COOH-terminal domain would remain on the cytoplasmic side of the membrane. To test this possibility, the susceptibility to protease of [2-lys,3-arg]P450IIC2 was examined. When microsomes from the *in vitro* translation reactions were treated with protease, P450IIC2 protein was completely

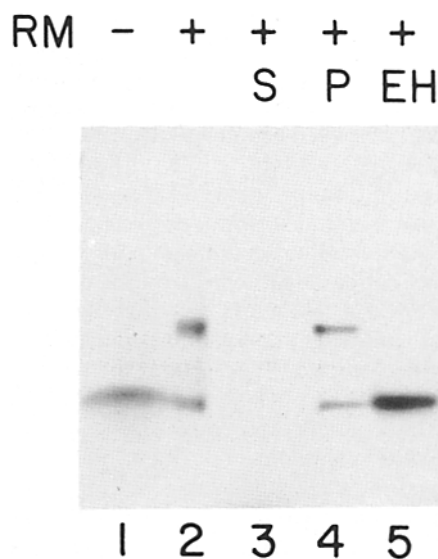


Figure 2. Membrane integration of [2-lys,3-arg]P450IIC2(1-155). Plasmid pc21 was digested with DdeI and transcribed with SP6 RNA polymerase. The truncated cRNA, which codes for the first 155 amino acids of [2-lys,3-arg]P450IIC2, was translated in a reticulocyte lysate system without or with chicken oviduct microsomal membranes (*RM*). Membrane-containing reactions were treated with endoglycosidase H (*EH*) or fractionated into supernatants (*S*) and pellets (*P*) in alkaline buffer.

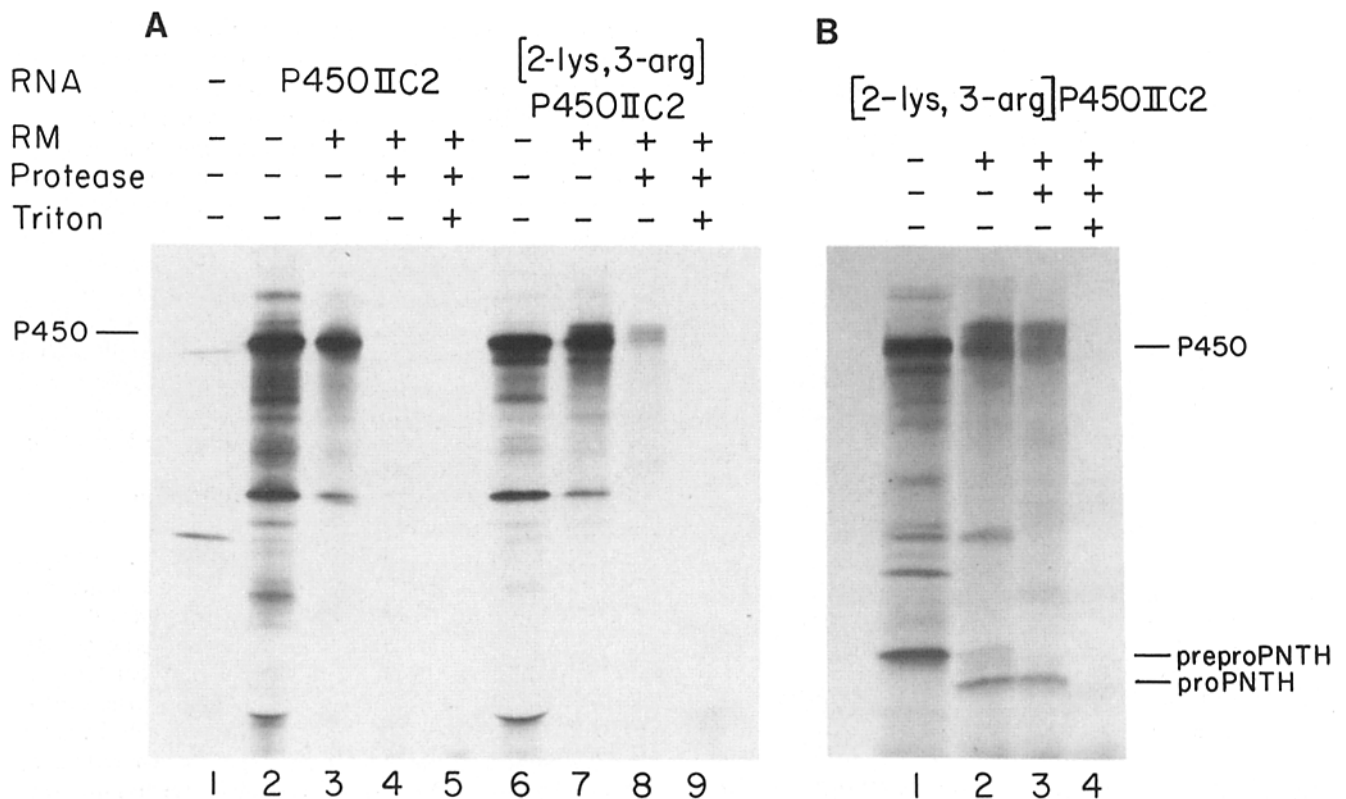


Figure 3. Translocation of [2-lys,3-arg]P450IIC2. RNAs were translated in a reticulocyte lysate system in the absence or presence of chicken oviduct microsomal membranes (RM). Samples were incubated with 0.4 mg/ml proteinase K with or without addition of 1% Triton X-100 as indicated. (B) PreproPNTH cRNA was cotranslated with [2-lys,3-arg]P450IIC2 RNA. PreproPNTH, which is not translocated and the translocated proPNTH (Szczesna-Skorupa et al., 1987) are indicated and serve as controls for protection from proteolytic digestion.

digested, indicating that it remained exposed on the cytoplasmic surface (Fig. 3 A, lane 4). In contrast, the full-length glycosylated [2-lys,3-arg]P450IIC2 species were protected from digestion (Fig. 3 A, lane 8, and B, lane 3). Although occasional protection of shorter proteins was also observed, typically full-length glycosylated and nonglycosylated P450 were the main protected species (Fig. 3). The major 23-kD protein was not protected from protease which is consistent with the hypothesis that this protein is derived from initiation at an internal AUG. As a further control preproPNTH, a secreted protein, was cotranslated with [2-lys,3-arg]P450IIC2. Under conditions that resulted in digestion of unprocessed preproPNTH, full-length [2-lys,3-arg]P450IIC2 remained protected from protease (Fig. 3 B). If membranes were disrupted with detergent before protease treatment (Fig. 3 B, lane 4), the protein was completely digested. Therefore, no COOH-terminal domain of detectable length was left on the cytoplasmic side of the membrane. These experiments demonstrated that none of the internal hydrophobic regions of P450IIC2 can function as a stop-transfer sequence or, as shown in the previous section, are necessary for membrane integration.

NH₂-Terminal Cleavage of the P450-PNTH Hybrid Protein

We have previously reported that synthesis of the hybrid protein [2-lys,3-arg]P450IIC2/PNTH in the presence of micro-

somal membranes resulted in the apparent cleavage of the NH₂-terminal region (Szczesna-Skorupa et al., 1988). Since in this construction 25 NH₂-terminal amino acids of P450IIC2 were followed by 4 COOH-terminal residues of the PNTH signal sequence, the natural cleavage site was retained and assumed to be recognized by signal peptidase during translocation. To establish the site of cleavage, we have isolated the hybrid cleavage product, which was labeled with [³H]lysine, and determined the location of the lysines by sequence analysis. As shown in Fig. 4, disregarding the wash-out radioactivity in the first two cycles, radioactivity was released in cycles 5, 8, 9, and 23. This pattern of release established that the cleavage took place after glycine at position 25, which represents the last residue of P450IIC2 in the hybrid protein, rather than after the glycine at position 29, the normal cleavage site for preproparathyroid hormone (Habener et al., 1978). An analysis based on the probability of occurrence of amino acids in a window -13+2 relative to the cleavage site (von Heijne, 1986) predicted that the observed cleavage location in the hybrid protein was the optimal site instead of the PNTH cleavage site (Fig. 4 B). A similar analysis of P450IIC2 and [2-lys,3-arg]P450IIC2 predicts several potential cleavage sites but cleavage after the same glycine at position 25 is preferred. This raised the possibility that the translocation-competent form of P450 (i.e., [2-lys,3-arg]P450IIC2) could also be proteolytically processed. However, synthesis of [2-lys,3-arg]P450IIC2 or its truncated 1-155 form resulted in little or no NH₂-terminal

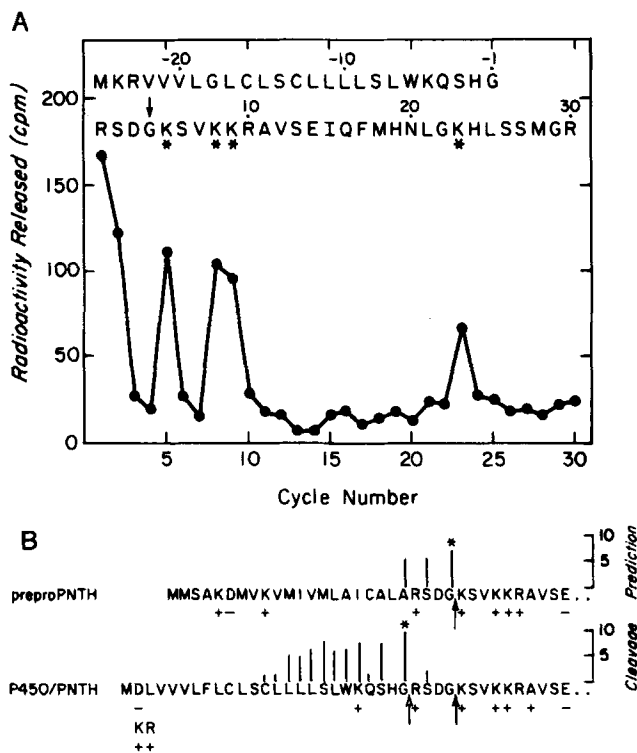


Figure 4. Cleavage site of [2-lys,3-arg]P450IIC2/PNTH by microsomal signal peptidase. (A) RNA coding for the fusion protein was translated in a reticulocyte lysate system in the presence of microsomal membranes with [³H]lysine. The processed protein was isolated and subjected to automated Edman degradation. Radioactivity released at each cycle was monitored by scintillation counting. The NH₂-terminal sequence of [2-lys,3-arg]P450IIC2/PNTH is shown at the top of the figure and asterisks indicate the positions of the lysine residues in the protein sequence. Cleavage occurred at the glycine numbered as -1 and the arrow indicates the glycine at the normal cleavage site of preproPNTH. (B) The NH₂-terminal sequences of preproPNTH and P450IIC2/PNTH are shown. The two substitutions in [2-lys,3-arg]P450IIC2 are shown below the sequence of P450IIC2. The presence of charged amino acids is indicated below the sequences. Above the sequences, values calculated as described (von Heijne, 1986) which predict the most probable cleavage site are plotted. Only scores with a positive value are presented. Asterisks indicate the predicted cleavage sites. The closed arrows indicate the normal site of cleavage for preproPNTH and the open arrow indicates the observed site in the hybrid protein.

processing. Although a protein that migrated slightly faster than [2-lys,3-arg]P450IIC2 was observed in reactions containing membranes (Fig. 1, lanes 7, 9, and 11), the amount of the protein produced was insufficient for definitive identification of the putative cleaved form by sequence analysis. These observations suggest that the amino acids COOH-terminal to the cleavage site affect cleavage efficiency.

Discussion

We have shown that the substitution of two basic amino acids for the single amino acid near the NH₂-terminus of P450IIC2 converts the NH₂-terminal region from a combination insertion/stop-transfer to an insertion/translocation signal. The glycosylation of [2-lys,3-arg]P450IIC2 by microsomal

membrane enzymes and its protection from proteolysis provide unequivocal evidence that the modified P450 is translocated across the membrane. The same modification in the P450IIC2 NH₂-terminal region fused to parathyroid hormone, a secreted protein, also led to the translocation of the hybrid protein across microsomal membranes (Szczesna-Skorupa et al., 1988). Since similar results were observed with the modified NH₂-terminal region in both full-length P450IIC2 and the hybrid protein, the basic amino acids introduced are both necessary and sufficient for its conversion to a translocation signal. Thus, a relatively small change of only two NH₂-terminal amino acids of the 490 amino acids in P450IIC2, which is normally localized on the cytoplasmic side of the endoplasmic reticulum (DeLemos-Chiarandini et al., 1987; Sakaguchi et al., 1987), is sufficient to cause its complete transfer to the luminal side.

The present results obtained with intact P450IIC2 are consistent with our model (based on studies of a P450IIC2/PNTH hybrid) which proposes that the negatively charged NH₂ terminus is inserted "head inward" into the membrane, which halts further transfer, whereas the positively charged NH₂ terminus may interact with the cytoplasmic anionic surface of the membrane, resulting in a loop structure and allowing for further translocation (Szczesna-Skorupa et al., 1988). Alternatively, the charged amino acids in the modified P450IIC2, independent of a change in orientation, may alter interaction of the NH₂ terminus with membrane components that mediate either translocation or stop-transfer functions. Since protease protection of both intact P450IIC2 and the P450IIC2/PNTH hybrid indicates that the COOH-terminal sequences of the native and mutated forms are on opposite sides of the microsomal membrane, it seems likely that the NH₂-terminal regions are also in opposite orientations. Consistent with this, partial cleavage of the NH₂-terminal sequence of [2-lys,3-arg]P450IIC2/PNTH, but not P450IIC2/PNTH (Szczesna-Skorupa et al., 1988), indicates that the cleavage sites are on opposite sides of the membrane. Audigier et al. (1987) also suggested that expression of the stop-transfer function in mutants of opsin may be dependent on the orientation of a transmembrane segment.

Availability of [2-lys,3-arg]P450IIC2 with a translocation signal allowed us to test for the presence of other topogenic sequences. Complete translocation of modified P450IIC2 across the membrane was observed indicating the absence of internal stop-transfer sequences. In contrast, Monier et al. (1988) demonstrated a stop-transfer activity in the region 165-186 of rat cytochrome P450IIB1. The hydrophobicity of this region in P450IIB1 and P450IIC2 is not detectably different. Presumably a difference in the amino acid context of the hydrophobic region or the *in vitro* systems used (canine pancreatic vs. chicken oviduct microsomal membranes) may explain the results. Regardless of the observation of stop-transfer activity of an internal region *in vitro*, the halting of translocation by the NH₂-terminal region and the lack of membrane insertion of P450s in which the NH₂-terminal region has been deleted (Monier et al., 1988; Browne, N., and B. Kemper, unpublished results) indicate that no internal stop-transfer regions are required *in vivo*. These results together with the immunological studies showing all segments of P450, except the NH₂-terminal region, on the cytoplasmic side of the membrane (DeLemos-Chiarandini et al., 1987) indicate that P450 is anchored to the membrane only by the

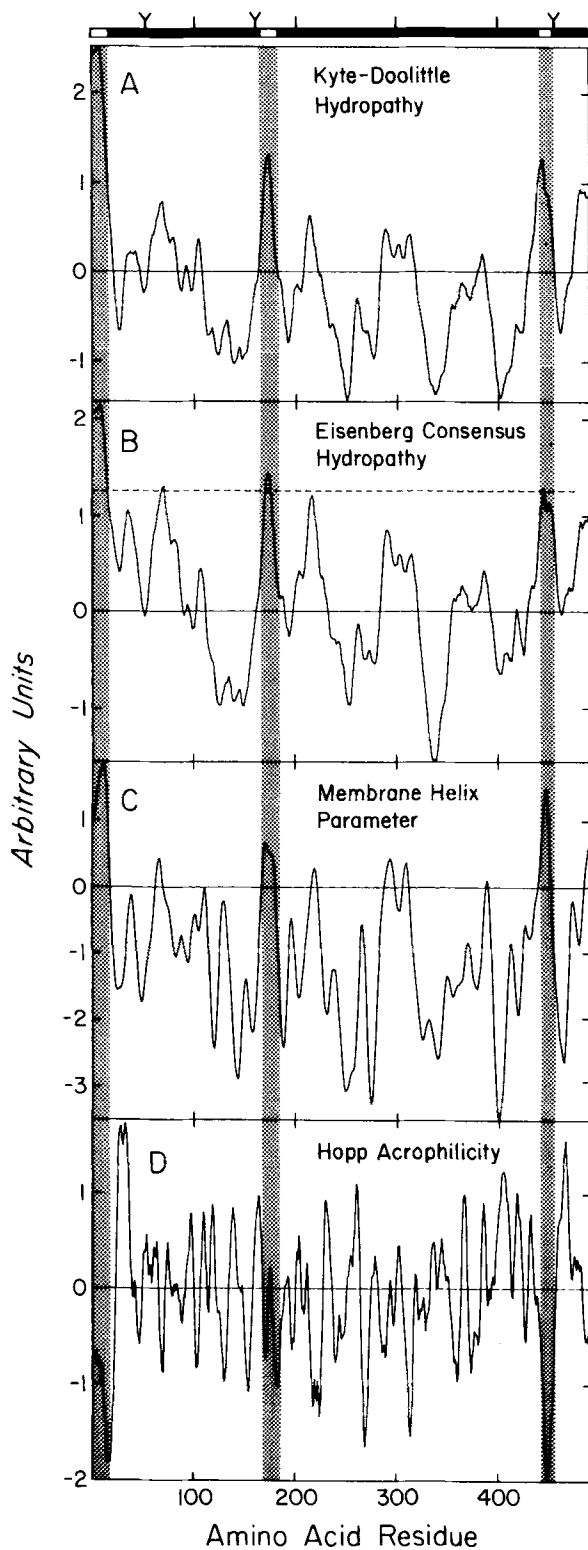


Figure 5. Prediction of potential membrane-spanning regions of P450IIC2. The bar at the top is a schematic representation of P450IIC2. The open bars indicate three regions that are potential membrane-spanning α -helices predicted with minor modifications as described by Rao and Argos (1986). For selection, peak heights >1.128 were required and membrane helices were terminated when the membrane turn potential exceeded the helix potential. (A) Hydropathy is estimated using values of hydrophobicity of Kyte and Doolittle (1982) over a sliding window of 19. The curve was

NH₂-terminal region. The other hydrophobic regions are probably involved either in protein folding or in interactions with the membrane from the cytoplasmic side.

The above model of P450 topology, based on experimental evidence, differs from those proposed earlier based on the protein hydrophobicity (Tarr et al., 1983; Ozols et al., 1985; Leighton et al., 1984). Hydrophobic profiles for P450IIC2, generated by using three different indices, are shown in Fig. 5. In each case three or more membrane-spanning domains are predicted: 8 major hydrophobic regions by the Kyte and Doolittle (1982) method; 4 regions that exceed a value of 1.26, recommended as the criteria for membrane-spanning regions (Eisenberg, 1984); and 8 regions above 0 for the membrane helix parameter. The number of potential spanning regions is reduced to 3 (indicated by the stippling) if a minimum of 12 amino acids in the hydrophobic sequence is required. Since only the NH₂-terminal region of P450IIC2 functions as a membrane stop-anchor sequence, the limitations of the prediction value of hydrophobicity profiles alone is illustrated. The prediction may be refined further by considering acrophilicity which is based on the frequency of amino acids in turns at the cytoplasmic interface of membrane-spanning regions (Hopp, 1985). Only the sequence after the NH₂-terminal hydrophobic region is strongly predicted to be adjacent to a membrane-spanning domain. Therefore, consideration of acrophilicity and the fact that the NH₂-terminal sequence is by far the most hydrophobic region suggest that only the NH₂-terminal region spans the membrane, in accordance with experimental observations.

Recently, Nelson and Strobel (1988) proposed a model for the membrane topology of P450 based on the comparative analysis of the sequences of 31 microsomal P450s. This model also predicts that, except for the NH₂-terminal region, P450 resides on the cytoplasmic side of the membrane. However, since the NH₂-terminal amino acid is postulated to be on the cytoplasmic side of the membrane, a second transmembrane region (residues 29–45 in P450IIC2) is needed to form a hairpin loop. The evidence that the NH₂-terminal amino acid is on the cytoplasmic side of the membrane is that the NH₂-terminus of P450 was blocked if isolated by SDS-PAGE from microsomal membranes that had been treated with a membrane-impermeant reagent that

smoothed once by averaging a sliding window of 7. (B) Hydropathy is estimated using the normalized consensus hydrophobicity values of Eisenberg (1984) which have been multiplied by three. Peaks that exceed the value indicated by the dashed line are predicted to be membrane spanning. (C) Potential for a membrane-spanning helix is estimated from the frequency of occurrence of individual amino acids in known membrane-spanning helices as described by Rao and Argos (1986). Values have been scaled by subtracting 1.05 and multiplying by 6.73 so that the zero line corresponds to the value recommended by Rao and Argos as indicating the probability of a membrane-spanning helix. Mean values were calculated for a sliding window of 7 with two rounds of smoothing by averaging over sliding windows of 7. (D) Acrophilicity is estimated as described by Hopp (1985) based on the frequency of amino acids in turns adjacent to membrane-spanning regions. Mean values were determined for a sliding window of 5 with one round of smoothing by averaging over a sliding window of 5. The computer programs AMPHI and MCF, developed by Antony Crofts, University of Illinois at Urbana-Champaign, were used for all the analyses.

reacts with α -amino groups (Bernhardt et al., 1983). As noted (Nelson and Strobel, 1988), some data conflict with this hairpin model. Antibodies presumably specific for segments within the proposed second membrane-spanning region react with cytoplasmic determinants in the endoplasmic reticulum (DeLemos-Chiarandini et al., 1987) and 20–29 NH₂-terminal amino acids of P450 are sufficient to anchor hybrid proteins to the membrane (Sakaguchi et al., 1987; Szczesna-Skorupa et al., 1988; Monier et al., 1988). Moreover, it is difficult to explain the conversion of the NH₂-terminal peptide to a translocation signal by substitution of positive charges at residues 2 and 3, if the native P450 is already in a loop form, although conformational changes or direct effects on the translocation apparatus could be invoked. In P450IIC2 much of the region proposed as the second transmembrane segment is highly acrophilic (Fig. 5) suggesting that it is more likely involved in a turn out of the membrane rather than membrane spanning. Finally, in contrast to the NH₂-terminal hydrophobic region, which is one of the least conserved regions of P450s in primary sequence, the second region is highly conserved including the sequence PPGP which is almost perfectly conserved in all P450I and P450II members. Such conservation suggests a more specific structural/functional requirement than spanning the membrane.

After the initial submission of this manuscript, Monier et al. (1988) proposed a model of P450 insertion into the membranes which predicts luminal localization of the extreme NH₂ terminus of its insertion/stop-transfer signal. However, these authors postulate formation of a transient loop as the signal enters the membrane and subsequent reorientation. The basis for this proposal is that partial cleavage of the P450 NH₂-terminal region occurred in a P450/growth hormone hybrid, indicating that the COOH-terminal portion of the signal sequence reached the luminal side of the membrane. In contrast, little or no cleavage occurred with similar hybrids of P450 with parathyroid hormone (Szczesna-Skorupa et al., 1988) or interleukin (Sakaguchi et al., 1987). Additional studies will be required to define the initial conformation of the P450 NH₂-terminal region in the membrane.

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